Title

Electronic Carotenoid-Chlorophyll Interactions Regulating Photosynthetic Light Harvesting of Higher Plants and Green Algae

Authors

Walla, Peter Jomo
Holleboom, Christoph-Peter
Fleming, Graham R

Publication Date

2014

DOI

10.1007/978-94-017-9032-1_9

Peer reviewed

Advances in Photosynthesis and Respiration 40
Including Bioenergy and Related Processes

Barbara Demmig-Adams
Győző Garab
William Adams III
Govindjee *Editors*

Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria

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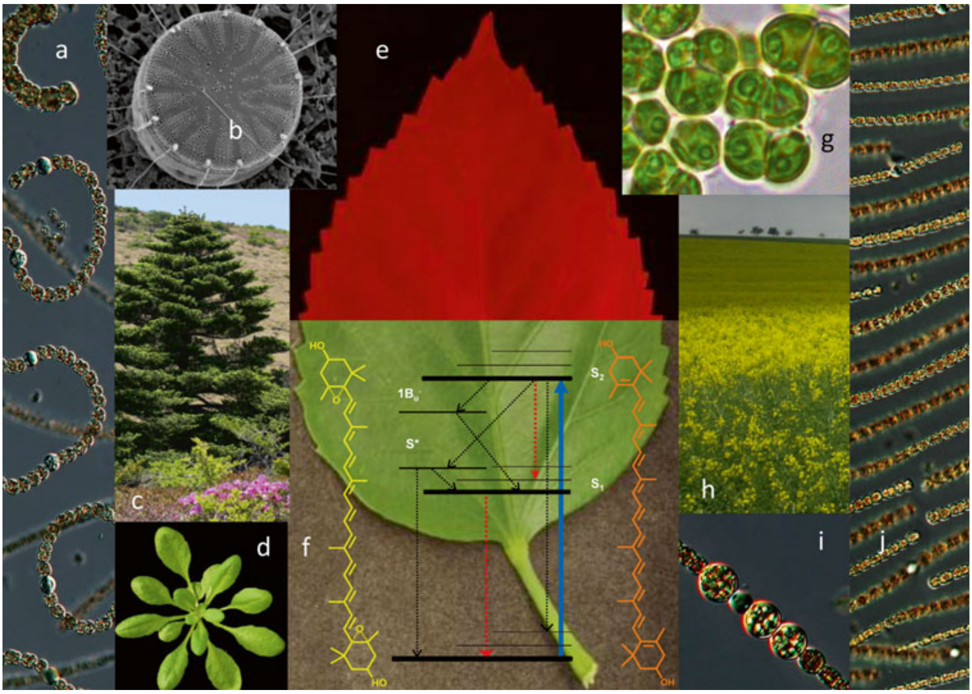

Figures showing species and properties relevant to non-photochemical quenching studies. (**a**) Filamentous planktonic cyanobacteria *Dolichospermum crassum* and *D. flos-aquae* by Petr Znachor; (**b**) SEM image of the diatom *Cyclotella meneghiniana* by Claudia Büchel; (**c**) *Abies koreana* (Korean fir) on Mount Halla, Korea, by Seok Chan Koh; (**d**) *Arabidopsis* Col-0 by Jared Stewart; (**e**) Combination of two photographs, by Wolfgang Bilger and Hartmut Kaiser, of a *Hibiscus rosa-sinensis* leaf in room light (*lower part*), and of the leaf's chlorophyll fluorescence (*upper part*). Fluorescence was excited by *blue* LED of an Imaging-PAM fluorometer (IMAG-MAX/L, Walz, Effeltrich, Germany) at 350 μmol photons m^{-2} s^{-1}, and the objective had a far-red filter to collect chlorophyll fluorescence; (**f**) energy-level scheme and relaxation dynamics of carotenoids and molecular structure of violaxanthin (*yellow*) and zeaxanthin (*orange*) by Tomas Polivka; (**g**) green alga *Scenedesmus* by Nicoletta La Rocca and Tomas Morosinotto; (**h**) canola field in Germany by Melanie Adams; (**i**) Spheroid akinets and a heterocyst of cyanobacterium *Anabaena aphanizomenoide* by Petr Znachor; (**j**) Straight filaments of cyanobacterium *Dolichospermum planctonicum* by Petr Znachor.

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Edited by

Barbara Demmig-Adams
Department of Ecology and Evolutionary Biology
University of Colorado
Boulder, CO
USA

Gyozo Garab
Institute of Plant Biology, Biological Research Center
Hungarian Academy of Sciences
Szeged
Hungary

William Adams III
Department of Ecology and Evolutionary Biology
University of Colorado
Boulder, CO
USA

and

Govindjee
Department of Plant Biology, Department of Biochemistry,
and Center of Biophysics and Quantitative Biology
University of Illinois at Urbana-Champaign
Urbana, IL
USA

Editors
Barbara Demmig-Adams
Ecology and Evolutionary Biology
University of Colorado
Boulder, CO
USA

William Adams III
Ecology and Evolutionary Biology
University of Colorado
Boulder, CO
USA

Gyozo Garab
Biological Research Center
Institute of Plant Biology
Hungarian Academy of Sciences
Szeged
Hungary

Govindjee
Department of Plant Biology
University of Illinois at Urbana-Champaign
Urbana, IL
USA

ISBN 978-94-017-9031-4 ISBN 978-94-017-9032-1 (eBook)
DOI 10.1007/978-94-017-9032-1
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2014955362

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From the Series Editors

Advances in Photosynthesis and Respiration
Including Bioenergy and Related Processes
Volume 40: Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria

We are delighted to announce the publication of Volume 40 in this series. The series publisher, Springer, now makes the table of contents of all of the volumes freely available online. Links to each volume are given below. The increased color and web presence of these books since Volume 35 makes the books more accessible and allows bibliographic tracking. We hope that these updates will maintain the importance of these edited volumes in the dissemination of the science of photosynthesis and bioenergy. We believe these books provide a forum for discussion of important developments in the field in a more in-depth and complete way than can be achieved in individual papers or even in extended reviews.

This Book: Volume 40

Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria was conceived and edited by Barbara Demmig-Adams, Győző Garab, William W. Adams III, and Govindjee. Govindjee, in his role as both Series Editor and Editor, and Tom Sharkey are grateful to the editors and the authors who have contributed to this important volume.

The overall topic of this book is the regulation of solar-energy collection (i.e., light harvesting) by plants, algae and cyanobacteria via processes functioning to (i) optimize the efficiency of light collection and (ii) safely deal with excess absorbed light when the rate of excitation energy use for productive photochemistry falls behind the rate of light absorption. The major focus of the book is the safe disposal of excess excitation energy via thermal dissipation as conveniently monitored through the decrease (or quenching) of chlorophyll a fluorescence by processes other than photochemistry (i.e., non-photochemical quenching, NPQ).

Several chapters include cautions against some assumptions about the meaning of NPQ. Normally, chlorophyll fluorescence decreases due to an increase in photochemistry (via photochemical fluorescence quenching) since energy used in photochemistry is energy not available for fluorescence. However, what is described in this volume is the discovery that restrictions in the rate of photochemistry actually result in less chlorophyll fluorescence emission (hence termed non-photochemical quenching of chlorophyll fluorescence), opposite to what would be observed in a simpler system. This realization had a profound effect on studies of photosynthesis. In hindsight, it makes sense to dissipate the flow of energy as close to its absorption as possible whenever the downstream reactions are unable to use all of the incoming energy.

The discovery of the role of the xanthophyll cycle is described in an engaging way, especially in Chap. 2. Barbara Demmig-Adams' singular role in making the connection between the xanthophyll cycle (specifically the VAZ, i.e., violaxanthin antheraxanthin zeaxanthin, cycle) and NPQ should be highlighted and for this reason it is especially relevant that she is lead Editor of this volume. It is important to distinguish between the essential nature of the cycling of the xanthophylls and the mechanism of NPQ. Further, the cycling between violaxanthin and zeaxanthin is not the mechanism by which excess light is dissipated.

Among the authors are many of the scientists who have made seminal discoveries that led to new insights. The book is a comprehensive – and in many cases personal – look at NPQ. At the same time, the book has educational aspects, with clear recommendations for strict use of terms and methods. This guidance can be invaluable to help a field speak the same language. A case in point is NPQ itself. It originally meant, and the book recommends should continue to mean, quenching of fluorescence. The actual quenching (or dissipation) of incoming photons is a distinct phenomenon even if closely linked.

Nearly everyone who studies photosynthesis needs to understand NPQ. This book provides a single source to learn the history of important discoveries, the role and relevance of NPQ today and how to use the concepts of NPQ in developing explanations of the phenomena we observe. This volume is a must-read for photosynthesis researchers.

Authors

The book contains 28 chapters written by 54 authors from 15 countries [Canada (3); The Czech Republic (3); France (4); Germany (7); Greece (1); Hungary (1); Italy (3); Japan (1); Korea (1); The Netherlands (5); South Africa (1); Spain (5); Sweden (1); UK (5); and USA (13)]. We thank all the authors for their valuable contribution to this book; their names (arranged alphabetically) are: Anunciación **A**badía (Spain; Chap. 27); Javier Abadía (Spain; Chap. 27); William W. Adams III (USA; Chaps. 2, 7, 23, 24 and 28); Maxime Alexandre (The Netherlands; Chap. 6); Roberto **B**assi (Italy; Chap. 14); Wolfgang Bilger (Germany; Chaps. 7 and 19); Matthew D. Brooks (USA; Chap. 13); Claudia Büchel (Germany; Chap. 11); Christopher M. **C**ohu (USA; Chaps. 23 and 24); Barbara **D**emmig-Adams (USA; Chaps. 2, 7, 23, 24 and 28); Raquel **E**steban (Spain; Chap. 12); Giovanni **F**inazzi (Italy; Chap. 21); Graham R. Fleming (USA; Chap. 9); Harry A. Frank (USA; Chap. 8); Győző **G**arab (Hungary; Chap. 16); José I. García-Plazaola (Spain; Chaps. 12 and 26); Reimund Goss (Germany; Chap. 20); Govindjee (USA; Chaps. 1 and 4); Jeremy **H**arbinson (The Netherlands; Chap. 25); Michel Havaux (France; Chap. 26); Christoph-Peter Holleboom (Germany; Chap. 9); Alfred R. Holzwarth (Germany; Chap. 5); Peter Horton (UK; Chaps. 3 and 6); Cristian **I**lioaia (The Netherlands; Chap. 6); Peter **J**ahns (Germany; Chap. 5); Stefan Jansson (Sweden; Chap. 13); Radek **K**aňa (The Czech Republic; Chap. 22); Yaser R. Khan (Canada; Chap. 4); Diana Kirilovsky (France; Chap. 22); Seok-Chan Koh (Korea; Chap. 24); David M. Kramer (USA; Chap. 18); Tjaart Krüger (South Africa and The Netherlands; Chap. 6); Johann Lavaud (France; Chap. 20); Barry Logan (USA; Chap. 7); Jun **M**inagawa (Japan; Chap. 21); Fermín Morales (Spain; Chap. 27); Tomas Morosinotto (Italy; Chap. 14); Onno Muller (USA; Chaps. 23 and 24); Conrad W. Mullineaux (UK; Chap. 17); Erik H. Murchie (UK; Chap. 25); Krishna K. **N**iyogi (USA; Chap. 13); Evgeny E. **O**stroumov (Canada; Chap. 4); George **P**apageorgiou (Greece; Chap. 1); Andrew K. Pascal (UK; Chap. 10); Tomáš Polivka (The Czech Republic; Chap. 8); Ondřej Prášil (The Czech Republic; Chap. 22); Bruno **R**obert (France; Chap. 10); Alexander Ruban (UK; Chaps. 10 and 17); Greg **S**choles (Canada; Chap. 4); Jared J. Stewart (USA; Chaps. 24 and 28); Deserah D. Strand (USA; Chap. 18); Herbert **v**an Amerongen (The Netherlands; Chap. 15); Rienk van Grondelle (The Netherlands; Chap. 6); Peter J. **W**alla (Germany; Chap. 9).

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- Cyanobacteria II
- Ecophysiology
- Evolution of Photosynthesis
- Global Aspects of Photosynthesis
- Green Bacteria and Heliobacteria
- Interactions between Photosynthesis and other Metabolic Processes
- Limits of Photosynthesis: Where do we go from here?
- Photosynthesis, Biomass and Bioenergy
- Photosynthesis under Abiotic and Biotic Stress
- Plant Respiration II

If you have any interest in editing/co-editing any of the above listed books, or being an author, please send an e-mail to Tom Sharkey (tsharkey@msu.edu) and/ or to Govindjee at gov@illinois.edu. Suggestions for additional topics are also welcome.

In view of the interdisciplinary character of research in photosynthesis and respiration, it is our earnest hope that this series of books will be used in educating students and researchers not only in Plant Sciences, Molecular and Cell Biology, Integrative Biology, Biotechnology, Agricultural Sciences, Microbiology, Biochemistry, Chemical Biology, Biological Physics, and Biophysics, but also in Bioengineering, Chemistry, and Physics.

We take this opportunity to thank and congratulate Barbara Demmig-Adams and her co-editors Győző Garab, William W. Adams III, and Govindjee for their outstanding editorial work; they have done a fantastic job, not only in editing, but also in organizing this book for all of us, and for their highly professional dealing with the reviewing process. We thank all 54 authors of this book (see the contributor list); without their authoritative chapters, there would be no such volume. We give special thanks to Srinath Raju of SPi Global, India, for directing the typesetting of this book; his expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Andre Tournois, and Ineke Ravesloot (of Springer) thanks for their friendly working relation with us that led to the production of this book.

August 25, 2014

Thomas D. Sharkey
Department of Biochemistry
and Molecular Biology,
Michigan State University,
East Lansing, MI, 48824, USA
email: tsharkey@msu.edu

Govindjee
Department of Plant Biology
Department of Biochemistry and
Center of Biophysics &
Quantitative Biology
University of Illinois at
Urbana-Champaign
Urbana, IL 61801, USA
email: gov@illinois.edu

Series Editors

A 2012 photo of Govindjee with Neelam Sodha, of the School of LifeSciences, Jawaharlal Nehru University, New Delhi, India. Photo Credit: Ashwani Pareek.

Govindjee, who uses one name only, was born on October 24, 1932, in Allahabad, India. Since 1999, he has been Professor Emeritus of Biochemistry, Biophysics and Plant Biology at the University of Illinois at Urbana-Champaign (UIUC), Urbana, IL, USA. He obtained his B.Sc. (Chemistry, Botany and Zoology) and M.Sc. (Botany; Plant Physiology) in 1952 and 1954, from the University of Allahabad. He studied 'Photosynthesis' at the UIUC under two pioneers of photosynthesis, Robert Emerson and Eugene Rabinowitch, obtaining his Ph.D. in 1960 in Biophysics. He is best known for his research on excitation energy transfer, light emission (prompt and delayed fluorescence, and thermoluminescence), primary photochemistry and electron transfer in "Photosystem II" (PS II, water-plastoquinone oxido-reductase).

His research, with many collaborators, has included the discovery of a short-wavelength form of chlorophyll (Chl) *a* functioning in what is now called PS II; of the two-light effect in Chl *a* fluorescence; and, with his wife Rajni Govindjee, of the two-light effect (Emerson Enhancement) in NADP reduction in chloroplasts. His major achievements, together with several other researchers, include an understanding of the basic relationship between Chl *a* fluorescence and photosynthetic reactions; a unique role of bicarbonate/carbonate on the electron acceptor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants; the first picosecond measurements on the primary photochemistry of PS II; and the use of fluorescence lifetime imaging microscopy (FLIM) of Chl *a* fluorescence in understanding *photoprotection* by plants against excess light.

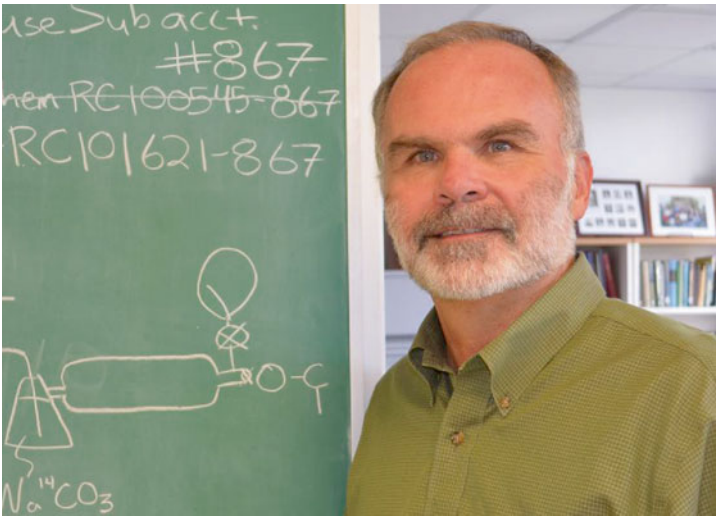
His current focus is on the "History of Photosynthesis Research", in "Photosynthesis Education", as well as in the "Possible Existence of Extraterrestrial Life". He has served on the faculty of the UIUC for ~40 years.

Govindjee's honors include: Fellow of the American Association of Advancement of Science (AAAS); Distinguished Lecturer of the School of Life Sciences, UIUC; Fellow and Lifetime member of the National Academy of Sciences (India); President of the American Society for Photobiology (1980–1981); Fulbright Scholar (1956), Fulbright Senior Lecturer (1997), and Fulbright Specialist (2012); Honorary President of the 2004 International Photosynthesis Congress (Montréal, Canada); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology, 2006; Recipient of the Communication Award of the International Society of Photosynthesis Research, 2007; and the Liberal Arts & Sciences Lifetime Achievement Award of the UIUC, 2008.

Further, Govindjee was honored (**1**) in 2007, through two special volumes of Photosynthesis Research, celebrating his 75th birthday and for his 50-year dedicated research in "Photosynthesis" (Guest Editor: Julian Eaton-Rye); (**2**) in 2008, through a special International Symposium on "Photosynthesis in a Global Perspective", held in November, 2008, at the University of Indore, India; (**3**) Volume 34 of this Series "*Photosynthesis: Plastid Biology*, *Energy Conversion and Carbon Assimilation*", edited by Julian Eaton-Rye, Baishnab C. Tripathy, and one of us (TDS), was dedicated to Govindjee, celebrating his academic career; and (**4**) in 2013, through a special issue of *Photosynthesis Research* (volumes 117 and 118) edited by Suleyman Allakhverdiev, Gerald Edwards and Jian-Ren Shen celebrating his 80th (or rather 81st) birthday. An additional honor was the celebration of his birthday (during October 23–25, 2013) in Trebon, The Czech Republic (see O. Prasil [2014] Govindjee, an institution, at his 80th [or rather 81st] birthday in Trebon in October, 2013: A pictorial essay. *Photosynth Res*. doi:10.1007/s11120-014-9972-0).

Govindjee is coauthor of *Photosynthesis* (John Wiley, 1969) and editor of many books, published by several publishers including Academic Press and Kluwer Academic Publishers (now Springer).

Since 2007, each year a Govindjee and Rajni Govindjee Award (http://sib.illinois.edu/grants_Govindjee.htm) is being given to graduate students, by the Department of Plant Biology (odd years) and by the Department of Biochemistry (even years), at the UIUC, to recognize Excellence in Biological Sciences. For further information on Govindjee, see his web site at http://www.life.illinois.edu/govindjee.



Thomas D. (Tom) Sharkey obtained his Bachelor's degree in Biology in 1974 from Lyman Briggs College, a residential science college at Michigan State University, East Lansing, Michigan. After 2 years as a research technician, Tom entered a Ph.D. program in the Department of Energy Plant Research Laboratory at Michigan State University under the mentorship of Klaus Raschke and finished in 1979. Post-doctoral research was carried out with Graham Farquhar at the Australian National University, in Canberra, where he coauthored a landmark review on photosynthesis and stomatal conductance. For 5 years he worked at the Desert Research Institute, Reno, Nevada. After Reno, Tom spent 20 years as Professor of Botany at the University of Wisconsin in Madison. In 2008, Tom became Professor and Chair of the Department of Biochemistry and Molecular Biology at Michigan State University.

Tom's research interests center on the exchange of gases between plants and the atmosphere. The biochemistry and biophysics underlying carbon dioxide uptake and isoprene emission from plants form the two major research topics in his laboratory. Among his contributions are measurement of the carbon dioxide concentration inside leaves, an exhaustive study of short-term feedback effects in carbon metabolism, and a significant contribution to elucidation of the pathway by which leaf starch breaks down at night. In the isoprene research field, Tom is recognized as the leading advocate for thermotolerance of photosynthesis as the explanation for why plants emit isoprene. In addition, his laboratory has cloned many of the genes that underlie isoprene synthesis and published many papers on the biochemical regulation of isoprene synthesis. Tom has co-edited three books, the first on trace gas emissions from plants in 1991 (with Elizabeth Holland and Hal Mooney) and then volume 9 of this series (with Richard Leegood and Susanne von Caemmerer) on the physiology of carbon metabolism of photosynthesis in 2000 and volume 34 (with Julian Eaton-Rye and Baishnab C. Tripathy) entitled *Photosynthesis*: *Plastid Biology*, *Energy Conversion and Carbon Assimilation*. Tom has been co-series editor of this series since volume 31.

Contents

Preface

While few would disagree that interdisciplinary studies are important, bringing together researchers from vastly different fields remains quite a challenge. All authors and editors contributing to the present book have made a genuine effort to integrate different views, and we are proud of the remarkable outcome. This book brings together viewpoints from disciplines as diverse as photo-physics, chemistry/biochemistry, physiology, molecular genetics, and comparative ecophysiology (covering much of the diversity of photosynthetic life that evolved to inhabit many environments). Authors and editors have endeavored to contribute the best each discipline had to offer towards presenting an updated view of the current understanding of our field and to outline a vision of what is needed next.

This book focuses on the harvesting of solar energy by plants and photosynthetic microbes like algae and cyanobacteria, and the regulation of light harvesting via the photoprotective removal of excess absorbed light. Why is studying the regulation of light harvesting important? Natural photosynthesis provides virtually all food and fuel (fossil fuels from past photosynthesis and "biofuels" from current photosynthesis), as well as many materials (from, e.g., fiber and building materials to vitamins and medicines). While sunlight harnessed in photosynthesis is the basis of virtually all food chains on this planet, too much of a good thing – more light being absorbed than can be utilized in photosynthesis – presents a potentially deadly threat. An excess of excitation energy can lead to the formation of potentially damaging oxidants, which is why photosynthetic organisms universally employ powerful mechanisms to safely remove excess excitation energy in a process (thermal energy dissipation) that can be monitored by its impact on chlorophyll fluorescence (quantified as non-photochemical quenching of chlorophyll *a* fluorescence, NPQ), the topic of this book.

Future opportunities to manipulate photosynthesis by engineering will depend on an improved understanding of all processes involved in its operation and regulation. The ability to mimic natural photosynthesis, and potentially increase the portion of sunlight that goes to the accumulation of energy carriers as opposed to supporting the photosynthetic organisms' own growth and reproduction – via synthetic systems or "biohybrids" – will depend on further improvements in the mechanistic understanding of *how* natural light harvesting works. This understanding of *how* it works depends critically on contributions from, e.g., photophysical and molecular genetic studies, as outlined in this book. In turn, an improved understanding is needed of *why* all known photosynthetic organisms fall behind in the utilization of absorbed light under full sun exposure, a question to which integrative studies can contribute, as also outlined in this book.

Furthermore, it is becoming increasingly clear that the photosynthetic light-harvesting system provides essential input into the signaling networks that control the photosynthetic organism's rate of growth, cell division, reproduction, and, eventually, an organism's demise via aging (senescence; see Volume 36 [2013] Plastid Development in Leaves during Growth and Senescence, edited by Basanti Biswal, Karin Krupinska and Udaya Biswal). Any excitation energy that is not utilized for energy-carrier production or safely diverted via thermal energy dissipation produces potentially destructive oxidants that shift the cellular redox state (balance of oxidants and anti-oxidants; see Volume 21 [2006] Photoprotection, Photoinhibition, Gene Regulation, and Environment, edited by Barbara Demmig-

Adams, William W. Adams III and Autar K. Mattoo). Cellular redox state, in turn, orchestrates growth, development, and multiple defenses of the organism. The state of the light-harvesting system (see Volume 13 [2003] Light-Harvesting Antennas in Photosynthesis, edited by Beverley R. Green and William W. Parson) thus exerts far-reaching control over virtually all aspects of the structure and the function of the organism. In plants, signals derived from the leaf's light-harvesting system are integrated with signals carrying information about the leaf's carbon-export capacity and whole-plant demand for the products of photosynthesis. An understanding of the impact of light harvesting on whole-organism function in particular environments thus requires integrating studies of whole organisms in different environments. Doing so will help to understand, and predict, the responses of different species in communities to the impacts of climate change. Moreover, such an understanding is needed to allow applications in agriculture to improve crop productivity and defenses against physical (e.g., unfavorable temperature or water shortage) and/or biological factors (pathogens and pests) that currently cause staggering losses in crop yields. This book brings together studies addressing all of these aspects.

In addition to addressing the mechanisms underlying photoprotective thermal dissipation, and placing these into the context of the whole organism, this book identifies challenges in the measurement, interpretation, and nomenclature of non-photochemical fluorescence quenching and remaining unresolved questions. For example, while much agreement exists that non-photochemical quenching involves xanthophylls and proteins, the roles of specific xanthophylls and proteins continues to be debated. Another area of debate is the nature of the relationship between plant productivity and non-photochemical quenching.

This volume on *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria* includes 28 chapters and is authored by 54 researchers from 15 countries. The book begins with three chapters that provide personal perspectives on the history of contributions to this research field: George Papageorgiou (Greece) and Govindjee (USA) present definitions, timelines, viewpoints, and open questions surrounding the non-photochemical quenching of the excited state of chlorophyll a in plants (Chap. 1); William W. Adams III (USA) and Barbara Demmig-Adams (USA) provide their personal perspective on lessons from nature as obtained via comparative ecophysiology, involving fieldwork in many different habitats and controlled environment studies (Chap. 2); Peter Horton (UK) discusses the history of developments in NPQ research, especially the emergence of key ideas, theories and experimental approaches (Chap. 3).

These three historical perspectives are followed by 25 additional chapters. In Chap. 4, Evgeny E. Ostroumov, Yaser R. Khan, Gregory Scholes (all from Canada) and Govindjee describe the photophysics of photosynthetic pigment-protein complexes. Alfred R. Holzwarth and Peter Jahns (both from Germany) address how ultrafast-fluorescence-kinetics measurements have been used to study mechanisms of NPQ in intact organisms in Chap. 5. Tjaart Krüger (South Africa), Cristian Ilioaia (The Netherlands), Maxime Alexandre (The Netherlands), Peter Horton (UK) and Rienk van Grondelle (The Netherlands) discuss how inherent protein disorder in light-harvesting complexes controls NPQ (Chap. 6). In Chap. 7, Barry Logan (USA), Wolfgang Bilger (Germany), William W. Adams III (USA), and Barbara Demmig-Adams (USA) place NPQ into the context of other photoprotective mechanisms, and provide a guide for the measurement and quantification of NPQ. Tomáš Polivka (The Czech Republic) and Harry A. Frank (USA) summarize spectroscopic investigations of carotenoids involved in NPQ in Chap. 8. In Chap. 9, Peter Jomo Walla (Germany), Christoph-Peter Holleboom (Germany), and Graham Richard Fleming (USA) present a summary of electronic carotenoid-chlorophyll interactions regulating photosynthetic light harvesting of higher plants and green algae. Andrew

A. Pascal (France), Alexander Ruban (UK), and Bruno Robert (France) present how resonance Raman spectroscopy can reveal conformational changes in antenna proteins (Chap. 10). In Chap. 11, Claudia Büchel (Germany) provides an overview of fucoxanthin-chlorophyll-proteins and NPQ of diatoms. In Chap. 12, Raquel Esteban and José I. García-Plazaola (both from Spain) discuss an involvement in NPQ of the lutein epoxide cycle, as a second xanthophyll cycle in plants. In Chap. 13, Matthew D. Brooks (USA), Stefan Jansson (Sweden), and Krishna K. Niyogi (USA) review PsbS-dependent NPQ. Tomas Morosinotto and Roberto Bassi (both from Italy) discuss molecular mechanisms for the activation of NPQ in organisms from unicellular algae to mosses and higher plants in Chap. 14.

The question of whether chlorophyll-carotenoid interactions are responsible for rapidly reversible NPQ is discussed by Herbert van Amerongen (The Netherlands) in Chap. 15. Győző Garab (Hungary) describes structural changes and NPQ in oxygenic photosynthetic organisms in Chap. 16. In Chap. 17, Alexander V. Ruban and Conrad W. Mullineaux (both from UK) discuss NPQ and the dynamics of photosystem II structure. Deserah Strand and David Kramer (both from USA) describe how the proton circuit of photosynthesis controls non-photochemical quenching (Chap. 18). In Chap. 19, Wolfgang Bilger (Germany) summarizes what is known about the desiccation-induced quenching of chlorophyll fluorescence in cryptogams, such as lichens and mosses. Johann Lavaud (France) and Reimund Goss (Germany) describe the features of NPQ in diatoms and brown algae in Chap. 20. A review by Giovanni Finazzi (Italy) and Jun Minagawa (Japan) on the high-light acclimation of green microalgae is available in Chap. 21. Diana Kirilovsky (France), Radek Kaňa and Ondřej Prášil (both from The Czech Republic) review mechanisms that modulate energy arriving at the reaction centers in cyanobacteria in Chap. 22. In Chap. 23, William W. Adams III, Onno Muller, Christopher M. Cohu, and Barbara Demmig-Adams (all from USA) discuss links among whole-plant demand for the products of photosynthesis, leaf carbohydrate status, photosystem II efficiency and photoinhibition, and NPQ. Chap. 24, by Barbara Demmig-Adams (USA), Seok-Chan Koh (Korea), Christopher M. Cohu (USA), Onno Muller (Germany), Jared J. Stewart (USA), and William W. Adams III (USA), provides an overview of differences in the capacity for NPQ as dependent on plant species and the environment. Erik H. Murchie (UK) and Jeremy Harbinson (The Netherlands) discuss measurements and the diverse manifestations of NPQ across scales in Chap. 25. In Chap. 26, Michel Havaux (France) and José I. García-Plazaola (Spain) discuss the overlapping antioxidant functions of zeaxanthin and tocopherols ("vitamin E"). Fermín Morales, Javier Abadía, and Anunciación Abadía (all from Spain) summarize findings about thermal energy dissipation in plants growing under unfavorable soil conditions in Chap. 27. The final Chap. 28, by Barbara Demmig-Adams, Jared J. Stewart, and William W. Adams III (all from USA), places chloroplast photoprotection into the context of the control of cellular redox state, and outlines possible trade-offs between the abiotic and biotic defenses of plants.

By bringing together chapters describing approaches from different disciplines, such as physics/chemistry and biology, this book also offers directions for future progress via an even closer integration of these disciplines. For example, while physics/chemistry offers powerful, highly exact spectroscopic measurements, biology offers rigorously defined contrasting states of the plant/algal system for analysis. Moreover, mutant analysis has contributed to the important conclusion that elimination of one of the steps in the cascade of photoprotective processes leads to augmentation of others. One promising future direction is thus to complement existing studies with the employment of spectroscopic approaches to the analysis of biological wildtype systems carefully defined as having high versus low electron transport capacities (photochemical quenching capacities) in all possible combinations with high versus low thermal dissipation capacities (non-photochemical quenching capacities).

We are grateful for the dedication and patience of the authors in making this volume possible. We, the editors, learned much from our communication with the authors and hope that the authors and readers of this volume share this sentiment and the sense of excitement and inspiration that surrounded much of the work on this book. We are also most grateful to series editor Tom Sharkey with his sure sense of direction and great insight. Furthermore, three of us (B.D-A., G.G. and W.W.A.III) thank the indomitable Govindjee who wore multiple hats as series editor, editor of this volume, and co-author of two chapters.

Barbara Demmig-Adams
Department of Ecology
and Evolutionary Biology
University of Colorado
Boulder, CO, USA

Győző Garab
Institute of Plant Biology,
Biological Research Center,
Hungarian Academy of Sciences
Szeged, Hungary

William W. Adams III
Department of Ecology and
Evolutionary Biology
University of Colorado
Boulder, CO, USA

Govindjee
Department of Plant Biology,
Department of Biochemistry and
Center of Biophysics and
Quantitative Biology University of Illinois at
Urbana-Champaign
Urbana, IL, USA

The Editors

Barbara Demmig-Adams (*second from left*) and William W. Adams III (*far right*) with their children Robert Adams (*first from left*) and Melanie Adams (*third from left*)

Barbara Demmig-Adams and **William W. Adams III** are Professors in the Department of Ecology and Evolutionary Biology at the University of Colorado at Boulder, USA. **Barbara Demmig** received her undergraduate degree (1979) in biology and chemistry and her graduate degree (1984) in plant physiology (with the late Prof. Hartmut Gimmler) from the Universität Würzburg. She subsequently spent two eventful years (1984–1986) as a postdoctoral fellow in the laboratory of Prof. Olle Björkman (Department of Plant Biology, Carnegie Institution of Washington) in Stanford, California. Barbara and Olle characterized the photoprotective thermal energy dissipation that occurs in the antenna pigments of photosystem II, including the fact that this process can become sustained in leaves of evergreens thereby lowering the photon yield of photosystem II for prolonged periods.

William attended the University of Kansas, receiving degrees in biology (1981) and atmospheric sciences (1983) as well as a Master's degree (1984) in botany (investigating adaptations of epiphytic bromeliads from Mexico under the guidance of Prof. Craig E. Martin). This was followed by 18 months (1984–1985) of research in Reno, Nevada and Death Valley, California, for the first half of his Ph.D. work.

During this time, William and Barbara began to collaborate personally and professionally across the Sierra Nevada divide that separated Reno and Stanford. Barbara returned to Würzburg in 1986 and provided the first evidence that the xanthophyll zeaxanthin, formed from violaxanthin under excess light, is involved in thermal energy dissipation. While Barbara was in Germany pursuing the role of zeaxanthin, William completed another 17 months of research in Canberra and at three field sites in Australia, resulting in a Ph.D. (1987) from the Australian National University under Prof. C. Barry Osmond's mentorship. This work centered on photoinhibition in crasulacean acid metabolism (CAM) plants, and included the first reports of photoinhibition and photoprotection under natural conditions in the field. With the support of a North Atlantic Treaty Organization (NATO) postdoctoral fellowship and a fellowship from the Alexander von Humboldt Foundation, William then spent 2 years at the Universität Würzburg, where the personal and professional collaboration with Barbara became more firmly established. In the spring of 1988, Barbara completed her habilitation in plant biology (at the Lehrstuhl II of Prof. Otto L. Lange in Würzburg) and Barbara and William made their union official. They moved to the University of Colorado in 1989, saw their personal collaboration come to fruition with two children (Robert and Melanie), and continued their collaborative scientific efforts on various aspects of the ecology and physiology of zeaxanthin-dependent thermal dissipation.

One focus of their work has been the study of unique modifications to photoprotective energy dissipation in evergreen species. They have used tropical evergreens as models for the role of sustained thermal dissipation during shade-sun acclimation, and conifers and other evergreen species to study its importance in acclimation during Colorado winters. These studies have included ecological, comparative, and mechanistic approaches to integrate photoprotective energy dissipation and photoinhibition into whole plant functioning. This has also led them to evaluate the influence of foliar carbon-export pathways on the acclimation of photosynthesis. In addition, Barbara has had a long-standing interest in the role of zeaxanthin and other plant protective compounds in human health. Their research has been cited frequently by colleagues, leading to their recognition as highly cited researchers in the Plant & Animal Science category by the Institute for Scientific Information (http://isihighlycited.com/). Furthermore, William has been honored for his efforts in teaching by University of Colorado students (Mortar Board Certificate of Recognition for Exceptional Teaching, 2000), and both have been recognized for their teaching by faculty peers (Boulder Faculty Assembly Excellence in Teaching Award, to William in 2004 and to Barbara in 2010). Barbara was elected to *Leopoldina* (National Academy of Sciences of Germany, Austria, and Switzerland) in 2011, and appointed Professor of Distinction at the University of Colorado in 2013.

Győző Garab, born in 1948, is the Head of the Laboratory for Photosynthetic Membranes (since 1987), at the Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged. After obtaining his *Diplom* (equivalent to M.Sc. degree) in Physics in 1971 from the University of Szeged, he switched from solid-state physics to biology, and joined the newly formed Photosynthesis Group led by Ágnes Faludi-Dániel (1929-1986) in the Biological Research Center (BRC), a modern basic biology institute that is now a Center of Excellence of the European Union. His Ph.D, in 1974, was on the "*Band-structure of the 77K fluorescence of thylakoid membranes*", which he showed to originate from six different chlorophyll forms. During his Ph.D. studies and as a young postdoc, he had several 2–4 month-long fellowships to foreign laboratories. In the Service de Biophysique, Commissariat à l'Ènergie Atomique (CEA) Saclay (France), hosted by Jacques Breton, he received an excellent introduction to pioneering research on the orientation of pigment molecules.

Systematic studies in the 1970s and 1980s in several laboratories, including the BRC, led to the recognition of what is known today as a universal property, i.e., that the pigment molecules in all mature photosynthetic membranes and pigment-protein complexes are non-randomly oriented with respect to the membrane plane and the protein axes. In other visits to Saclay, he worked with Jack Farineau on the slow (ms) rise of the electrochromic absorbance transients related to proton pumping. In the Institut de Biologie Physico-Chimique, working with Pierre Joliot and also in collaboration with Guy Paillotin (CEA, Saclay), he provided an understanding of the nature of light-induced fast and slow light scattering transients in intact algal cells. Also in the 1970s, he visited the Moscow State University, where, in the laboratory of Andrey B. Rubin and Vladimir Z. Paschenko, he performed picosecond fluorescence spectroscopy experiments. Later, in Szeged, his attention turned to the interaction between the respiratory and the photosynthetic electron transport chains, and to chlororespiration. His studies provided the first experimental evidence for the existence of chlororespiration in higher plants – a subject that was later explored more systematically in collaboration with Claudia Büchel.

During his visits to USA, between 1985 and 1990, his circular dichroism spectroscopy and microscopy experiments revealed what has been termed the macrodomain (long-range order) organization of the complexes in the thylakoid membranes – ideas stemming from the Szeged laboratory with key experiments performed at the Brookhaven National Laboratory, in the laboratory of Geoffrey Hind, and in Albuquerque, with Carlos Bustmante. These highly organized macroassemblies, extended ordered arrays of light-harvesting and core complexes of photosystem II (LHCII:PS II), have turned out to be most interesting for their ability to undergo light-induced reversible structural reorganizations that are also observed in lamellar aggregates of isolated LHCII and are largely independent of the photochemical activity of thylakoid membranes.

These experiments, conducted in Szeged together with his Ph.D. student, Virginijus Barzda (now at the University of Toronto Mississauga), led to the discovery of what they termed the thermo-optic mechanism in photosynthesis, i.e., elementary structural changes induced by ultrafast thermal transients due to the dissipation of excess excitation energy in light harvesting antenna complexes. The mechanisms and effects of excess-excitation energy dissipation remains the focus of his research group. The formation of LHCII:PS II macrodomains has also offerred a mechanism to sort the two photosystems between the stacked and unstacked thylakoid membrane regions, and thus aimed at the understanding of the self-assembly and 3D ultrastructure of granal thylakoid membranes, which he studied together with László Mustárdy, using electron tomography in the laboratory of Carmen Mannella (Wadsworth Center, Albany, NY). In order to monitor the structural-functional plasticity of these and other highly organized multilamellar systems, he turned to small-angle neutron scattering. This non-invasive technique revealed a much greater-than-expected structural flexibility in isolated thylakoid membranes as well as in intact cyanobacteria, algal cells and higher plant leaves. Another form of structural plasticity of high interest to his group is the (enigmatic) role of non-bilayer lipids in the bilayer thylakoid membranes. Phosphorus-31 NMR experiments, conducted in collaboration with Herbert van Amerongen, have shown the presence of non-bilayer lipid phases in functional thylakoid membranes.

Győző has served as secretary general of the XI International Biophysics Congress (Budapest, 1993), chairman of the XI International Congress on Photosynthesis (Budapest, 1998), and has organized several international schools, conferences and workshops on photosynthesis and biophysics. He has also served(s) in different international and Hungarian committees and societies, including the Biophysics of Photosynthesis Programme of the European Science Foundation, the International Society for Photosynthesis Research, the Hungarian Biophysical Society, and the Photosynthesis – Life from Light – Foundation (Hungary). He is co-owner and manager of the spin-off company Biofotonika R&D Ltd. He has been awarded the Straub medal (from the BRC), the Ernst Jenő medal (from the Hungarian Biophysical Society), the Farkas Gábor medal (from Scientia Amabilis Foundation for the Hungarian Plant Biology), the title of Knight (from the International Order of Inventors) and the Széchenyi Professor fellowship (from the Ministry of Education of Hungary).

Contributors

Anunciación Abadía
Department of Plant Nutrition, Aula Dei Experimental Station-CSIC, Zaragoza, Spain

Javier Abadía
Department of Plant Nutrition, Aula Dei Experimental Station-CSIC, Zaragoza, Spain

William W. Adams III
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Maxime T.A. Alexandre
Department of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, Amsterdam, The Netherlands

Roberto Bassi
Dipartimento di Biotecnologie, Università di Verona, Verona, Italy

Wolfgang Bilger
Ökophysiologie der Pflanzen, Botanisches Institut der Christian-Albrechts-Universität zu Kiel, Kiel, Germany

Matthew D. Brooks
Department of Plant and Microbial Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA, USA

Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Claudia Büchel
Department of Biosciences, Institute of Molecular Biosciences, Goethe University Frankfurt, Frankfurt, Germany

Christopher M. Cohu
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Dow Agro Sciences, Portland, OR, USA

Barbara Demmig-Adams
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Raquel Esteban
Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Bilbao, Spain

Agrobiotechnology Institute, IdAB-CSIC-UPNA-Government of Navarre, Pamplona, Spain

Giovanni Finazzi
Unité Mixte Recherche 5168, Laboratoire Physiologie Cellulaire et Végétale, Centre National Recherche Scientifique, Grenoble, France

Institut National Recherche Agronomique, Grenoble, France

Commissariat à l'Energie Atomique et Energies Alternatives, l'Institut de Recherches en Technologies et Sciences pour le Vivant, Grenoble, France

Université Grenoble 1, Grenoble, France

Graham R. Fleming
Department of Chemistry, University of California, Berkeley, CA, USA

Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Harry A. Frank
Department of Chemistry, University of Connecticut, Storrs, CT, USA

Győző Garab
Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

José I. García-Plazaola
Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Bilbao, Spain

Reimund Goss
Department of Plant Physiology, Institute of Biology, University of Leipzig, Leipzig, Germany

Govindjee
Department of Biochemistry, Department of Plant Biology, and Center of Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Jeremy Harbinson
Horticultural Supply Chains Group, Department of Plant Sciences, Wageningen University, Wageningen, The Netherlands

Michel Havaux
Department of CEA, DSV, IBEB, Laboratoire d'Ecophysiologie Moléculaire des Plantes, Saint-Paul-lez-Durance, France

CNRS, UMR 7265 Biologie Végétale et Microbiologie Environnementales, Saint-Paul-lez-Durance, France

Aix-Marseille Université, Saint-Paul-lez-Durance, France

Christoph-Peter Holleboom
Department for Biophysical Chemistry, Institute for Physical and Theoretical Chemistry, Technische Universität Braunschweig, Braunschweig, Germany

Alfred R. Holzwarth
Max-Planck-Institute for Chemical Energy Conversion (MPI-CEC), Mülheim an der Ruhr, Germany

Peter Horton
Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK

Cristian Ilioaia
Department of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, Amsterdam, The Netherlands

Commisariat à l'Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay and CNRS URA 2096, Gif-sur-Yvette, France

Peter Jahns
Plant Biochemistry, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

Stefan Jansson
Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, Umeå, Sweden

Radek Kaňa
Laboratory of Photosynthesis, Institute of Microbiology, Center Algatech, Czech Academy of Sciences, Třeboň, Czech Republic

Yaser R. Khan
Department of Chemistry, University of Toronto, Toronto, ON, Canada

Diana Kirilovsky
Commissariat à l'Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay (iBiTec-S), Gif sur Yvette, France

Centre National de la Recherche Scientifique (CNRS), UMR 8221, Gif sur Yvette, France

Seok-Chan Koh
Department of Biology, Jeju National University, Jeju, South Korea

David M. Kramer
Department of Plant Biology, Department of Biochemistry and Molecular Biology, and MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI, USA

Tjaart P.J. Krüger
Department of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, Amsterdam, The Netherlands

Department of Physics, Faculty of Natural and Agricultural Sciences, University of Pretoria, Hatfield, South Africa

Johann Lavaud
UMR 7266 'LIENSs', CNRS/University of La Rochelle, Institute for Coastal Research and Environment (ILE), La Rochelle cedex, France

Barry A. Logan
Biology Department, Bowdoin College, Brunswick, ME, USA

Jun Minagawa
Division of Environmental Photobiology, National Institute for Basic Biology, Okazaki, Aichi, Japan

Fermín Morales
Department of Plant Nutrition, Aula Dei Experimental Station-CSIC, Zaragoza, Spain

Tomas Morosinotto
Dipartimento di Biologia, Università di Padova, Padova, Italy

Onno Muller
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Institute of Bio- and Geosciences, IBG-2: Plant Sciences, Forschungszentrum Jülich GmbH, Jülich, Germany

Conrad W. Mullineaux
School of Biological and Chemical Sciences, Queen Mary University of London, London, UK

Erik H. Murchie
Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Nottingham, UK

Krishna K. Niyogi
Department of Plant and Microbial Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA, USA

Physical Biosciences Division Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Evgeny E. Ostroumov
Department of Chemistry, University of Toronto, Toronto, ON, Canada

George C. Papageorgiou
Institute of Biosciences and Applications, National Center of Scientific Research Demokritos, Athens, Greece

Andrew A. Pascal
Institut de Biologie et de Technologies de Saclay (iBiTec-S), CEA Saclay, Gif-sur-Yvette, France

Tomáš Polivka
Institute of Physics and Biophysics, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Ondřej Prášil
Laboratory of Photosynthesis, Institute of Microbiology, Center Algatech, Czech Academy of Sciences, Třeboň, Czech Republic

Bruno Robert
Institut de Biologie et de Technologies de Saclay (iBiTec-S), CEA Saclay, Gif-sur-Yvette, France

Alexander V. Ruban
School of Biological and Chemical Sciences, Queen Mary University of London, London, UK

Gregory D. Scholes
Department of Chemistry, Princeton University, Princeton, NJ, USA

Jared J. Stewart
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA

Deserah D. Strand
Department of Plant Biology, and MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI, USA

Herbert van Amerongen
Laboratory of Biophysics, Wageningen University, Wageningen, The Netherlands

Rienk van Grondelle
Department of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, Amsterdam, The Netherlands

Peter Jomo Walla
Biomolecular Spectroscopy and Single-Molecule Detection Group, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany

Department of Biophysical Chemistry, Institute for Physical and Theoretical Chemistry, Technische Universität Braunschweig, Braunschweig, Germany

Chapter 1

The Non-Photochemical Quenching of the Electronically Excited State of Chlorophyll a in Plants: Definitions, Timelines, Viewpoints, Open Questions

George C. Papageorgiou*
Institute of Biosciences and Applications, National Center of Scientific Research Demokritos, Athens 15310, Greece

and

Govindjee
Department of Biochemistry, Department of Plant Biology, and Center of Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, 265 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801, USA

Summary

Chlorophyll *a*-containing plants, algae and cyanobacteria absorb sunlight in order to perform oxygenic photosynthesis using two sequential photoreactions: Light Reaction II, which takes place in Photosystem II (PS II), oxidizes water to molecular oxygen (O_2) and reduces plastoquinone to plastoquinol; Light Reaction I, which takes place in Photosystem I (PS I),

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*Author for Correspondence, e-mail: gcpap@bio.demokritos.gr; 533gcp@gmail.com

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_1,

oxidizes plastoquinol to plastoquinone, via cytochrome *b_6f* complex, and reduces NADP$^+$ (nicotinamide adenine dinucleotide phosphate) to NADPH. In most cases, a large fraction of the electronic excitation acquired by absorbing sunlight is used for running the photoreactions of photosynthesis, a small fraction is emitted as chlorophyll fluorescence, and the remainder is degraded to heat and dissipated to the surroundings. These electronic excitation degradation processes encompass both spontaneous (i.e., "unprovoked") de-excitations (internal conversion) as well as de-excitations triggered and regulated by various physical and chemical signals. These signals involve photosynthetic electron transport (PSET) and are generated within and across the thylakoid membranes. Only the regulated dissipation of electronic excitation is assessed as *non-photochemical quenching* (NPQ) of chlorophyll fluorescence. Signals triggering NPQ include redox potential shifts of intramembranous electron transport intermediates, electrostatic potential shifts at membrane surfaces, and formation of trans-membrane ion concentration gradients, such as a proton concentration difference (ΔpH). Oxygenic photosynthetic organisms employ various processes to relieve the sensitive PS II from destructive effects of excess electronic excitation (excess excitation energy). The latter goal is achieved either by directly quenching excited states of pigments in the peripheral and the core antenna pigment-protein complexes of PS II, or by moving peripheral antenna complexes from the vicinity of PS II to PS I. In this chapter, we shall outline the remarkable and unprecedented

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Abbreviations: A – antheraxanthin; ATM – atmosphere; ATP – adenosine triphosphate; CAC – Core antenna light harvesting Chl *a*-protein complexes; Chl – chlorophyll; CP22 – see PsbS below; CP24, CP26, CP29 – chlorophyll *a*-, *b*-, and xanthophyll-binding proteins of photosystem II with molecular mass of 24, 26 or 29 kDa, respectively; CP43, CP47 – Chl a binding protein of 43kDA, 47 kDa molecular mass; Cyt – cytochrome; D1, D2 – two major proteins of Photosystem II reaction center complex; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Dd – diadinoxanthin; DDE – diadinoxanthin de-epoxidase; Dd-Dt – diadinoxanthin-diatoxanthin cycle; DGDG – digalactosyldiacylglycerol; Dt – diatoxanthin; ΔpH – trans-thylakoid proton concentration gradient; Fd – ferredoxin; FI – fluorescence induction; FNR – ferredoxin NADP$^+$ reductase; fs, ps, ns – femtosecond, picosecond, nanosecond; Ga – billions of years before present; hv – represents a photon of light (h = Planck's constant, and v is frequency of light); L – lutein; LHC – light harvesting chlorophyll protein complex; Lhcb – light harvesting protein complex of photosystem II; Lhcbm – m subunit of light harvesting protein complex of photosystem II; LhcbM5 – a specific subunit « M5 » of light harvesting protein complex of photosystem II; LHCSR – stress-related (SR) light-harvesting chlorophyll protein complex (LHC); Lhcx – a light harvesting protein complex found in diatoms equivalent to LHCSR of *Chlamydomonas*; Lhcx1-6 – different forms of Lhcx; LHCX6 – equivalent term for Lhcx6; LHCII – light harvesting complex of photosystem II; LHCIIb – specific light harvesting complex of photosystem II containing chlorophyll *b*; Lx – lutein epoxide; Lx-L – lutein epoxide-lutein cycle; MGDG – monogalactosyldiacylglycerol; NADP$^+$, NADPH – oxidized, reduced nicotinamide adenine dinucleotide phosphate; NPQ – non-photochemical quenching (lowering) of the singlet electronic excitation energy of Chl *a* in photosystem II; N – Neoxanthin; OEC – oxygen evolving complex; OCP, OCP^o, OCP^r – orange carotenoid protein, its orange (o) form, and its red (r) form; PAC – peripheral antenna light harvesting Chl *a*/*b*-protein complex(es); PAL – present atmospheric level; PBS – phycobilisome(s); PC – Plastocyanin; PGR5 – proton gradient regulator; Pheo – pheophytin; PMF – proton motive force; PQ, PQH$_2$ – plastoquinone, plastoquinol; PS I, PS II – Photosystem I, Photosystem II; PsbS or CP22 – A 22 kDa pigmentless protein of photosystem II, involved in NPQ; PSET – photosynthetic electron transport; Q$_A$, Q$_B$ – first and second plastoquinone electron acceptors of Photosystem II, the former being a one-electron acceptor, and the latter being a two-electron acceptor; qE – NPQ dependent upon trans-thylakoid proton gradient (ΔpH); qT – related to state changes (see text); RC – reaction center; ROS – Reactive oxygen species; STT7 – LHCIIb specific kinase in *Arabidopsis* related to state changes; stt7 – LHCIIb specific kinase in *Chlamydomonas* related to state changes; V – violaxanthin; VAZ – violaxanthin-antheraxanthin-zeaxanthin cycle; VDE – violaxanthin de-epoxidase; WWC – Water-water cycle; Y$_Z$, Y$_D$ – tyrosine-161 and tyrosine-160 on D1 and D2 proteins, respectively, Y$_Z$ donates electrons to the oxidized reaction center P680$^+$ but Y$_D$ is a very slow electron donor; Z – zeaxanthin; ZEP – zeaxanthin epoxidase discoveries of the last 60 years that have led to the current understanding of processes leading to thermal dissipation of excess excitation energy by photosynthetic organisms.

I Introduction

With the rare exception of chemolithoautotrophs (organisms producing energy-rich molecules via oxidation of inorganic compounds; Pfannschmidt and Yang 2012), all life on Earth depends on *Photosynthesis*, a complex process by which plants, algae and cyanobacteria, as well as anoxygenic photosynthetic bacteria, convert the fleeting energy of sunlight into storable and transportable chemical energy on a massive scale. For the basics of photosynthesis and its potential for practical use, see Rabinowitch and Govindjee (1969) and Blankenship (2014). For an overview of the molecular mechanism of light harvesting, see Ruban (2013). Although the annually available energy from sunlight far exceeds the annual energy demand of our world, improvements in natural as well as in artificial photosynthesis must be vigorously pursued in order to meet the energy needs of the increasing human population (Blankenship et al. 2011; Najafpour et al. 2012).

The inner sanctum of plants, which oxidizes water to oxygen, and produces ATP and the reducing power for the reduction of CO_2 to sugars, is embedded in the thylakoid membranes of the chloroplasts (Fig. 1.1). Thylakoids are closed vesicles, which define an outer aqueous phase called the *stroma* and an inner aqueous phase called the *lumen*. The photosynthetic apparatus in the thylakoid membrane includes four protein supercomplexes: Photosystem II (PS II), Cytochrome (Cyt) b_6f, Photosystem I (PS I), and the ATP synthase. Both PS I and PS II collect sunlight and facilitate two energetically uphill steps at the reaction center chlorophylls; PS II decomposes H_2O photochemically to O_2 and protons (H^+s), and reduces plastoquinone; PS I oxidizes plastoquinol via Cyt b_6f, and produces the reductant NADPH that reduces CO_2 to carbohydrate. See Wydrzynski and Satoh (2005) for a discussion of PS II, and Golbeck (2006) for a discussion of PS I. Two facts are critical: (1) only the reaction-center chlorophylls a (Chls a) of PS II (P680) and PS I (P700) convert light energy into chemical energy, with all other reactions following from there; (2) during electron transfer a proton motive force is built up that leads to the synthesis of ATP (at the ATP synthase), which is essential for the conversion of CO_2 to carbohydrate. The latter occurs in the stroma using the enzymes of the Calvin-Benson cycle (details in the Fig. 1.1 legend; see Strand and Kramer, Chap. 18).

Each photosystem carries a reaction center complex (PS I_{RC}, PS II_{RC}) and ensembles of light-harvesting Chl-protein complexes, which are characterized as *Core Antenna*

Glossary of Chemical Terms: **Alkene** – hydrocarbon containing a single carbon-carbon double bond: >**C**=**C**<; **Allene** – hydrocarbon containing two adjacent carbon-carbon double bonds: >**C**=**C**=**C**< with planes of the double bonds normal to each other; **Conjugated double bonds** – two carbon-carbon double bonds separated by a carbon-carbon single bond: >**C**=**C**–**C**=**C**< with the bond-forming p electrons of the carbon atoms delocalized over the conjugate structure and often referred to as *π-electrons* or *pi-electrons*; the two double bonds are coplanar; **Alkyne** – hydrocarbon containing one carbon-carbon triple bond: –**C**≡**C**–; **Diol** – an organic compound containing two hydroxyl groups (a di-alcohol); **Epoxide** – a ring formed by two carbon atoms and an oxygen atom, the anhydritic product of a 1, 2 diol; Epoxides are strained structures; **Di-epoxide** – organic molecule with two epoxide groups; **Epoxidic** – property of an organic compound of being an epoxide; **Epoxidation** – addition of an oxygen atom to a double bond to form an epoxide; **De-epoxidation** – removal of an oxygen atom from an epoxide and reformation of the double bond; **Lipocalins** – family of multifunctional proteins that bind small lipophilic molecules

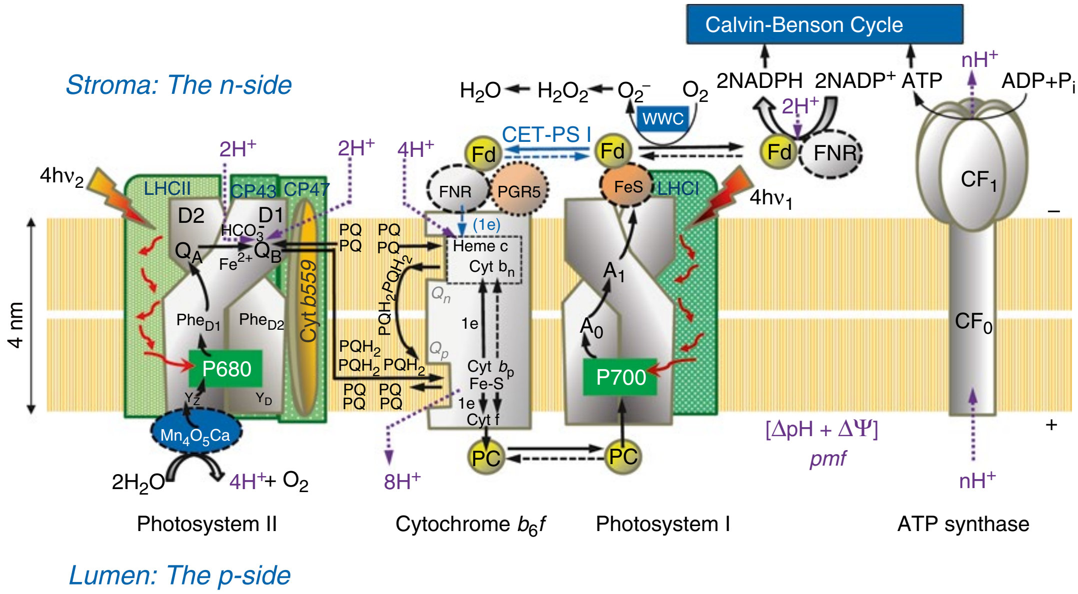

Fig. 1.1. A diagrammatic scheme of the thylakoid membrane of oxygenic photosynthetic organisms. Four major intramembranous protein complexes are shown: *From left to right*: Photosystem II (PS II; water-plastoquinone oxido-reductase); cytochrome (Cyt) b_6f (plastoquinol-plastocyanin-oxidoreductase); Photosystem I (PS I; plastocyanin-ferredoxin-oxido-reductase); and ATP synthase. Absorption of photons by each of the two photosystems by their respective light harvesting complexes (LHC) and other antenna subunits, and after excitation energy transfer, ultimately leads to charge separations within the PS I and the PS II reaction center complexes. hν stands for a photon of light (h = Planck's constant; and ν = frequency of light). **Photosystem II**: Primary radical pair in PS II is $[P680^+Phe_{D1}^-]$, where P680 (represents the participation of a few Chl *a* molecules in the D1/D2 complex, not shown) and Phe_{D1} (pheophytin on the D1 protein) are the primary electron donor and acceptor of PS II, respectively. The electron is transferred from Phe_{D1}^- to Q_A (a one-electron accepting plastoquinone, tightly bound to a site on the D2 protein). The oxidized primary donor $P680^+$ receives an electron via Y_Z (tyrosine -161 on the D1 protein), which, in turn, receives an electron from the tetra-nuclear manganese–oxygen–calcium cluster (Mn_4O_5Ca) in the O_2-evolving complex (OEC). Further, the electron from Q_A^- is transferred to Q_B (a two-electron accepting plastoquinone, docking on the D1 protein); this plastoquinone is bound weakly to its protein site in its oxidized state, but tightly when it is reduced to Q_B^-. A bicarbonate anion (HCO_3^-) is bound to a non-heme iron (Fe^{2+}) that sits between Q_A and Q_B, which is suggested to participate in Q_B^{2-} protonation. The formed PQH_2 (plastoquinol) at the Q_B-site is released, and then replaced by a new PQ (plastoquinone) molecule from a mobile PQ-pool in the thylakoid membrane. **Cyt b_6f**: The intermediary electron transport, from PS II to PS I, takes place via the Cyt b_6f complex that contains the following intersystem components of the electron transport chain: an iron-sulfur (Fe–S) protein, known as the Rieske FeS protein, one cytochrome *f* (Cyt *f*), two cytochromes b_6 (i.e., Cyt b_p and Cyt b_n) and a heme c (the subscripts "p" and "n" refer to the electrically positive (*inner*) and negative (*outer*) sides of the thylakoid membrane). At the Cyt b_6f complex, PQH_2 is re-oxidized at the Q_p-site (toward the lumen, close to the electrically positive side of the membrane, the p-side), while PQ is reduced, during what has been termed the Q-cycle, at the Q_n-site (toward the stroma, close to the electrically negative n-side of the membrane). Associated with the Cyt b_6f complex there is also a ferredoxin $NADP^+$ reductase (FNR), as well as what has been termed the proton-gradient regulator (PGR5), which is involved in cyclic electron transport around PS I (CET-PS I) via ferredoxin (Fd). **Photosystem I**: The primary charge separation in PS I generates the primary radical pair $[P700^+A_0^-]$, where P700 (a special Chl *a* pair) and A_0 (a special Chl *a* molecule) are the primary electron donor and acceptor of PS I, respectively. Plastocyanin (PC; there are more than one PC molecules per PS I), a mobile, water-soluble, copper protein situated on the lumen side of the membrane, transfers electrons from Cyt *f* to $P700^+$, while on the electron acceptor part of PS I the electron from A_0^- is transferred successively to: A_1 (vitamin K_1); three non-heme iron–sulfur centers (shown as *FeS*); and the mobile water-soluble non-heme iron protein ferredoxin (*Fd*), situated on the stroma (or the n) side of the membrane (there are more than one Fd molecules per PS I). The reduced Fd transfers electrons mainly to $NADP^+$ (nicotinamide–adenine dinucleotide phosphate), which is reduced to NADPH via FNR. However, the electrons on reduced ferredoxin may also go to Cyt b_6f leading to a cyclic electron transfer (*CET*). In addition, there is the possibility of other electron acceptors that may receive electrons from reduced Fd; one example is the water-water cycle (WWC), in which O_2 is reduced to

Complexes (CAC) when they sit next to the reaction centers (PS I_{CAC}, PS II_{CAC}) and as Peripheral Antenna Complexes (PAC) when they sit at a distance from it (PS I_{PAC}, PS II_{PAC}). In the current literature, CACs are often called "inner antenna", and PACs "outer antenna". Electronic excitation transfer occurs from PACs to CACs in the two photosystems (Scheme 1; see, e.g., Ke 2001). We note that the terminology of PAC and CAC, used here, has not been in use in much of the current literature in photosynthesis, but we recommend that it be used because it clearly distinguishes between the three major components of the antenna system without having to constantly spell out their full forms.

$$\begin{gathered}\text{PS I}_{\text{PAC}} \rightarrow \text{PS I}_{\text{CAC}} \rightarrow \text{PS I}_{\text{RC}} \\ \text{and} \\ \text{PS II}_{\text{PAC}} \rightarrow \text{PS II}_{\text{CAC}} \rightarrow \text{PS II}_{\text{RC}}\end{gathered} \tag{1}$$

Energetically uphill (implying energy storage) photosynthetic electron transport (PSET) from H_2O to CO_2 occurs in a linear manner (Scheme 2), using the energy of photons absorbed by light-harvesting PACs and CACs. The end products are carbohydrates or sugars (from the reduction of CO_2) and O_2 (from the photochemical decomposition of H_2O). In addition to the uphill linear electron transport from H_2O to CO_2, using PS II and PS I, electrons on reduced ferredoxin and other post-PS I electron accepting intermediates move energetically downhill (implying energy release as heat) to plastoquinone or to Cyt b_6f. The redox energy made available in this way is used for the synthesis of energy-rich ATP in what is known as "cyclic electron flow". In fact, three such pathways exist (see, e.g., Bukhov and Carpentier 2004).

$$\begin{gathered}\text{H}_2\text{O} \rightarrow \cdots \rightarrow \text{PS II}_{\text{RC}} \rightarrow \left(\text{intersystem PSET}\right) \\ \rightarrow \text{PS I}_{\text{RC}} \rightarrow \cdots \rightarrow \text{CO}_2\end{gathered} \tag{2}$$

Ideally, in order to use its machinery safely and efficiently, a photosynthetic organism must absorb the exact amount of light needed to drive the PS II and PS I photoreactions (see, e.g., Barber and Andersson 1992; Ort 2001; Demmig-Adams et al. 2012). What is the right amount of light cannot be defined on an absolute energy scale, and instead depends on the organism, on its physiological state, as well as on environmental parameters (e.g., temperature). Furthermore, the photon flux falling on the system is subject to fast and slow, as well as

Fig. 1.1. (continued)

O_2^- by Fd^-; subsequently, the H_2O_2 formed can be converted to water. **ATP Synthase**: The enzyme ATP synthase, which is made up of intramembranous (but lumen accessible) CF_0 and stroma-exposed CF_1, lumen-exposed CF_1, synthesizes ATP from adenosine diphosphate (*ADP*) and inorganic phosphate (P_i) using the proton motive force (*pmf*) made up of the transmembrane electrical potential difference ($\Delta\Psi$) and the transmembrane proton concentration difference (ΔpH). ΔpH is built across the thylakoid membrane by protons originating from water "splitting" at the OEC of PS II, and by the translocation of protons from the stroma to the lumen during electron transfer from PS II to plastoquinone and from plastoquinol to Cyt b_6f. The number of protons transferred to the lumen (the *p*-side) from the stroma (the *n*-side) of the thylakoid membrane are represented in a stoichiometric relation with the number of electrons transferred after the absorption of 4 quanta of light by each photosystem (necessary to evolve one O_2 molecule and to reduce 2 $NADP^+$ molecules). We note that the above statement needs modification because the cyclic electron transferred envisaged in the scheme would increase the quantum requirement in PS I. Further, the scheme is not meant to show the details of the number of protons taken up from the "n" side, and released on the "p" side. However, the ATP and NADPH, produced during the process, are finally used, via the Calvin–Benson cycle, to fix CO_2 to produce sugars. The entry of protons into PS II may involve a role of bicarbonate (HCO_3^-; for a review see Shevela et al. 2012). (Source of the figure: Stirbet and Govindjee (2012), as modified by A. Stirbet and Govindjee (unpublished); it also includes information from Stirbet and Govindjee (2011), Cramer and Zhang (2006), Baniulis et al. (2008), and Hasan et al. (2013).

periodic and aperiodic, fluctuations. A photosynthetic organism must therefore be able to first assess the momentary excitation energy level generated by incoming radiation, and then to mobilize its machinery to deal with it.

In 1992, Barber and Andersson stated "Too much of a good thing: light can be bad for photosynthesis". Later, Ort (2001) discussed what plants do when there is too much light. We know that there can be either *too little* or *too much light* for a plant. At suboptimal illumination, a plant underperforms photosynthetically, while superoptimal light may trigger various photo-oxidative, and potentially damaging, processes. Particularly sensitive to inactivation is PS II because it generates a very strong oxidant and is relatively slower (compared to PS I) in using its electronic excitation. In contrast, PS I generates weak oxidant(s) and uses its excitation a bit faster. In other words, excited Chl *a* lives longer and is more prone to photo-oxidative inactivation in PS II than in PS I (see discussion in the context of photochemical damage in Renger 2008; for an interpretation of PS II inactivation as feedback downregulation in whole plants, see Adams and Demmig-Adams, Chap. 2; Adams et al. 2006, Chap. 23; Demmig-Adams et al., Chap. 24, who report an invariable association of photosynthetic inactivation in leaves with accumulation of sugar or starch produced in photosynthesis).

Experiments of Emerson and Arnold (1932a, b) on the green alga *Chlorella*, using repetitive brief and strong light flashes, interspersed by optimal dark periods, led to the conclusion that ~2400 chlorophylls co-operate in the evolution of one O_2 molecule. Soon thereafter, Gaffron and Wohl (1936) explained this result by suggesting that under these conditions, there is transfer of excitation energy, absorbed by many molecules, to a photoenzyme where oxygen evolution occurs. The concept of "antenna" and "reaction center" was born, without those particular terms being used then. We now know that, in addition to Chl *a* as present in all oxygenic photosynthetic organisms, excitation energy transfer (or migration) involves several other pigments, e.g., Chl *b* (in green algae and plants), Chl *c* (in diatoms), and Chl *d* (marine red algae), carotenoids (many organisms) and phycobilins (red algae and cyanobacteria); all these pigments serve as photon harvesters for antenna associated with PS II_{RC} and PS I_{RC} (see Ostroumouv et al., Chap. 4, and references therein; also see Govindjee 1999 for role of carotenoids).

Absorbed photons (in the form of excitation energy) that cannot be used for photochemistry represent excess excitation energy, which has the potential to cause damage to the system. Several mechanisms are known that deal with this excess excitation energy in PS II. In general terms, these are assessed and labeled as NPQ (Non-Photochemical Quenching) processes of the excited state of Chl *a*, the most prominent of which (under conditions conducive for cell division and growth of photosynthetic organisms) are: (a) qE quenching of the excess excitation energy in Chl *a*, and (b) the state 1 (high fluorescent) to state 2 (low fluorescent) transition ($qT_{1\rightarrow 2}$). qE encompasses both non-enzymatic (i.e., physicochemical) and enzymatic processes that lead to the dissipation of excess excitation energy as heat; it is triggered by a high proton concentration in the lumen, which is a consequence of water splitting and proton transfer from the stromal space to the lumen space during electron transport (see Fig. 1.1); qE operates via enzymatic processes sensing the level of lumen acidity and ultimately leading to the dissipation of excess excitation energy as heat. Several xanthophyll molecules (see chemical structures in Fig. 1.2) have been shown to play a role in this process; there are several cycles involving different epoxidized and de-epoxidized forms of xanthophylls, each one characteristic of a particular class of photosynthetic organisms (see, e.g., Bungard et al. 1999; and Goss and Jakob 2010).

One of the most studied xanthophyll cycles is termed the VAZ cycle in this book, where V stands for violaxanthin, A for antheraxanthin, and Z for zeaxanthin (see chemical structures in Fig. 1.2). The VAZ cycle is typical of plants and green algae. Diatoms

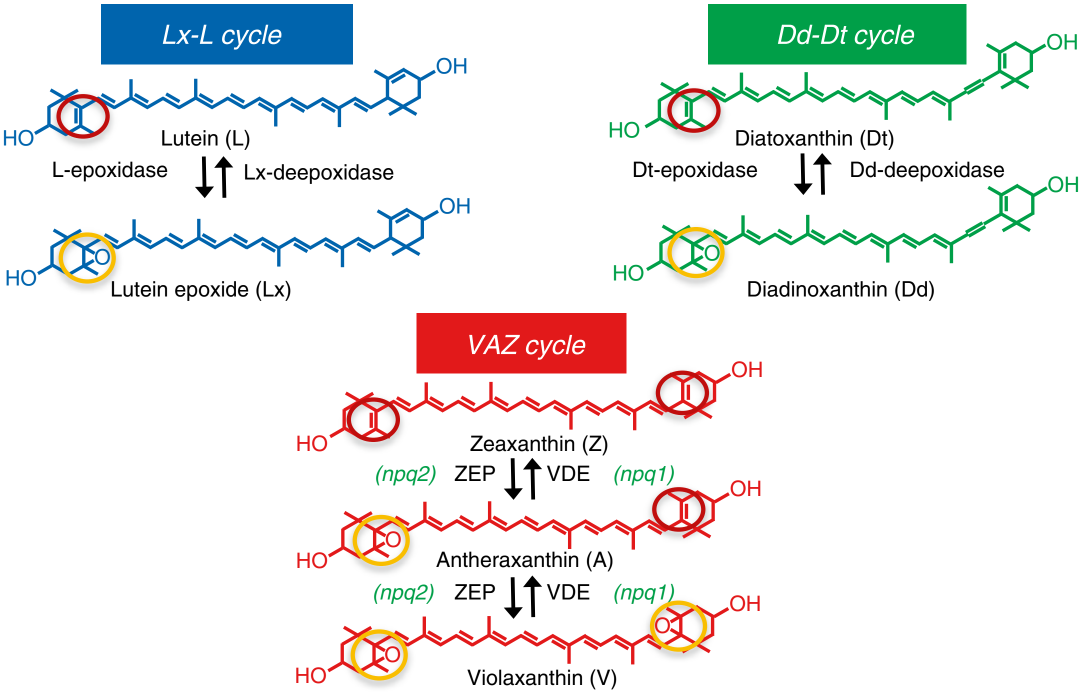

Fig. 1.2. The xanthophyll cycles, which serve to dissipate excess of electronic excitation, that momentarily resides on the Chls *a* of the peripheral antenna complexes of plants and algae, involve enzyme-catalyzed de-epoxidation of epoxidic xanthophylls in strong light and enzyme-catalyzed re-epoxidation in dim light or darkness. The figure illustrates the mechanism of the lutein epoxide-lutein (Lx-L) cycle of plants and green algae (*upper left*), the diadinoxanthin-diatoxanthin (Dd-Dt) cycle of Xanthophyceae, Euglenophyceae, Chrysophyceae, Bacillariophyceae, and diatoms (*upper right*) and the violaxanthin-antheraxanthin-zeaxanthin (*VAZ*) cycle of plants, green algae, brown algae and diatoms (*lower middle*).

possess the diadinoxanthin (Dd) – diatoxanthin (Dt) cycle (dinoxanthin in dinoflagellates) or, for short, Dd-Dt cycle. Finally, there is the lutein epoxide (Lx) - lutein (L) cycle, or Lx-L cycle. On the other hand, the state 1 to state 2 change (or state 1 to state 2 shift), $qT_{1\rightarrow 2}$, is a phenomenon where specific (mobile) subunits of the peripheral antenna complex of PS II (or PS II_{PAC}) migrate and attach to PS I. In state 1, there is more antenna in PS II, whereas in state 2, there is more antenna in PS I. These state changes are triggered by the redox state of intersystem intermediates (plastoquinones and Cyt b_6f) in the membrane phase (reviewed by Allen and Mullineaux 2004), and changes in NADPH/ATP ratio in the stroma space (reviewed by Cardol et al. 2011). In plants and green algae, the $qT_{1\rightarrow 2}$ process involves activation of kinases in the thylakoid membrane that phosphorylate specific light-harvesting complexes (LHCs) in the PS II-rich regions of the thylakoid membrane and enable their translocation, together with Cyt b_6f, to PS I-rich regions. In PS I, the extra excitation energy is used to generate ATP by driving cyclic electron transport around its reaction center (PS II_{RC}), or is dissipated non-photochemically as heat.

II The Reign of Photochemical Quenching

As late as 1960, it was firmly believed that Chl *a* in plants dissipates a constant fraction of its excitation energy as heat, and what remains is subject to a competition between photosynthetic utilization and Chl *a* fluorescence emission. The complementarity of photosynthesis and Chl *a* fluorescence was in apparent agreement with Warburg's photolyte model (1Chl *a*: $1CO_2$: $1O_2$; Nickelsen 2012; Nickelsen and Govindjee 2012), and thus measuring fluorescence, by the then newly designed photoelectric methods, was a convenient way of measuring photosynthesis (Papageorgiou and Govindjee 2011). Experimental evidence for the photosynthesis – Chl *a* fluorescence complementarity model was obtained by McAlister and Myers (1940a,b). Characteristic of the prevalence and the longevity of this model are the following two statements. As late as 1971, the two of us wrote: "*. . . both Chl a fluorescence and photosynthesis draw on the excited Chl a population, and thus a change in the photosynthetic rate is reflected as a change in the yield of Chl a fluorescence . . .*" (Govindjee and Papageorgiou 1971). In the same spirit, a few years later, Myers (1974) asserted: "*. . . a Chl a molecule cannot use the same quantum of energy for both fluorescence and photochemistry . . .*".

The first evidence that PS II and PS I interact antagonistically was reported by Govindjee et al. (1960). These authors discovered that, when *Chlorella* cells are illuminated with Light 2 (light preferentially absorbed by PS II) and with Light 1 (light preferentially absorbed by PS I), the Chl *a* fluorescence emitted is lower than when they are illuminated with Light 2 alone, suggesting that Light 1 exerts a quenching effect on the Chl *a* fluorescence that Light 2 excites. Subsequently, Duysens et al. (1961) reported that illumination of algal cells in suspension with Light 2 causes photoreduction of an intersystem cytochrome (see Scheme 2), whereas illumination with Light 1 causes its photo-oxidation. Finally, Duysens and Sweers (1963) demonstrated that only the fluorescence emitted by Chls *a* of PS II upon Light 2 excitation competes with photochemical utilization of excitation energy at physiological temperatures, and confirmed that Light 1 causes fluorescence decline (or quenching). On the basis of these observations, Duysens and Sweers concluded that, when oxidized, PS II_{RC} (which they denoted as Q) quenches Chl *a* fluorescence of PS II, while, when reduced (denoted as QH), it does not (i.e., fluorescence is higher). In this newer concept, the complementarity between photosynthetic O_2 evolution and Chl *a* fluorescence of the McAlister and Myers (1940a, b) era was narrowed down to a complementarity between the rate of reduction of Q (now known as Q_A), i.e., photochemistry of PS II, and the yield of Chl *a* fluorescence of PS II.

III The Emergence of the Non-Photochemical Quenching (NPQ) Concept

Although the complementarity between photosynthesis and Chl *a* fluorescence was tenaciously upheld in the 1960s and beyond, its erosion had started much earlier. Already in the early 1950s, it became known that assimilation of CO_2 requires not only energy-rich reductant, reduced nicotinamide adenine dinucleotide phosphate NADPH, but also energy-rich adenosine triphosphate ATP that provides energy upon its hydrolysis. Frenkel (1954) demonstrated that chromatophores of photosynthetic bacteria produce ATP in the light, and Arnon et al. (1954) showed that chloroplasts of higher plants make ATP in the light, i.e., they photophosphorylate (also see Strehler 1953). How the light absorbed by photosynthetic cells is converted to the free energy stored in the ATP molecule was discovered by Jagendorf and his co-workers (Hind and Jagendorf 1963; Jagendorf and Uribe 1966; reviewed by Jagendorf 2002) and was explained on the basis of the chemiosmotic theory of Mitchell (1961) for mitochondria. According to Mitchell's theory, respiratory electron transport is coupled to the translocation of protons across the inner mitochondrial membrane. As hinted earlier, this creates a pH difference (ΔpH), whose free energy is subsequently conserved in the enzymatically synthesized ATP molecule as phosphate bond energy. Jagendorf and coworkers (Hind and Jagendorf 1963; Jagendorf and Uribe 1966; reviewed by Jagendorf 2002) succeeded in demonstrating that an artificially created ΔpH across the thylakoid membrane is capable of driving ATP synthesis in the absence of light. Therefore, photosynthetic phosphorylation occurs in two sequential stages: **(i)** a light stage that produces ΔpH (acidic inside) via "splitting" of water and proton translocation-coupled PSET; and **(ii)** an enzymatic stage, during which relaxation of ΔpH is coupled to conversion of ADP to ATP. For a discussion of the involvement of the electrochemical potential gradient in ATP production, see Junge and Jackson (1982).

Since PSET is coupled to proton translocation across the thylakoid membrane, any interference with ΔpH would be expected to change the rate of PSET and, through it, to modify *photochemically* the yield of Chl *a* fluorescence. Accumulation of ΔpH (e.g., in the absence of ADP and/or of phosphate) will slow down PSET and lead to a rise of Chl *a* fluorescence from PS II. On the other hand, release of ΔpH by a protonophoric uncoupler will accelerate PSET and cause the Chl *a* fluorescence to drop. In addition to this photochemical quenching, PSET also affects the excited state of Chl *a* non-photochemically by two mechanisms: a faster one triggered by light-induced formation of trans-thylakoid ΔpH (known as "high energy" quenching, or X_E- or qE-quenching, see section "*High Energy Quenching of Chlorophyll a Excitation*") and a slower one triggered by the redox level of intersystem intermediates, e.g., plastoquinone (that leads to state 1 to state 2 transitions, section "*State Transitions*"; also see Krause and Jahns 2004).

In addition, a form of NPQ that is continuously maintained for days or weeks is seen in plants in response to stress (for recent reviews, see Adams et al. 2006, Chap. 23; Demmig-Adams et al. Chap. 24). Many plants experience days, weeks, or entire seasons, during which their green leaves or needles are exposed to intense sunlight while plant growth may be arrested and CO_2 fixation is either minimal or completely absent. Under such conditions, excitation energy is excessive and thermal dissipation of excess energy might be expected to remain high for as long as CO_2 fixation remains low or absent. Such a continuous maintenance of very high NPQ levels in whole leaves or plants is exactly what is observed under both natural and experimental conditions. Two forms of continuously high NPQ have been reported. One form involves continuous maintenance of trans-thylakoid ΔpH in low light and/or in darkness (over entire 24-h day/night periods in nature) and is observed, e.g., during intermittent freezing days in the fall. Using uncoupler treatments, this continuously maintained NPQ was demonstrated to be ΔpH-dependent under experimental (Gilmore and Björkman 1994, 1995) and natural (Verhoeven et al. 1998) conditions. A second form of continuously (24-h per day) maintained high levels of NPQ was reported, e.g., for leaves of the drought-tolerant shrub *Nerium oleander* under long-term drought stress (Demmig et al. 1988), for leaves of the highly salt-tolerant mangroves under a combination of high salinity and high light (Björkman et al. 1988), and for leaves or needles of evergreen shrubs and trees overwintering in areas where soil water remains frozen for months (see Adams and Demmig-Adams, Chap. 2). This continuously maintained strong NPQ in leaves or needles was shown (i) to be accounted for by increases in the rate constant of thermal energy dissipation (Björkman et al. 1988; Demmig et al. 1988), (ii) to correspond to a pronounced shortening of chlorophyll fluorescence lifetime (Gilmore et al. 1995; Gilmore and Ball 2000), and (iii) to be closely and positively correlated with long-term maintenance of high levels of zeaxanthin and arrest of the operation of the VAZ cycle (Adams and Demmig-Adams, Chap. 2; Demmig-Adams et al., Chap. 24). This strong continuously maintained NPQ in plants in nature is associated with strong continuously maintained decreases in F_v/F_m, and thus also corresponds to what is termed photoinhibition in whole plants.

Not all plants and/or natural conditions that inhibit plant growth induce strong continuously maintained NPQ; for a host of mechanisms that lower light interception by leaves, see Logan et al., Chap. 7; for environmental conditions inducing a lowering of leaf chlorophyll content, see Morales et al., Chap. 27. Lastly, drought-deciduous and or winter-deciduous plant species simply drop their leaves altogether for entire seasons with severe drought or freezing, respectively.

A High Energy Quenching (qE) of Chlorophyll a Excitation

According to Krause and Jahns (2004), "high energy" quenching was recognized first by Papageorgiou and Govindjee (1968a, b) in studies with cyanobacteria and green algae, and by Murata and Sugahara (1969) and Wraight and Crofts (1970) with isolated thylakoids, via demonstratation that Chl *a* fluorescence can also be quenched non-photochemically, namely by the build-up of a "high-energy state" in thylakoids, consisting of a trans-thylakoid proton gradient and related structural alterations of the photosynthetic apparatus.

Demonstration of qE quenching would generally rely on evidence of a light-induced change of Chl *a* fluorescence level, while keeping the primary electron acceptor of PS $II_{RC,}$ Q_A, stably reduced by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Papageorgiou and Govindjee (1968a,b) explained the light-induced changes of Chl *a* fluorescence in DCMU-treated green algae and cyanobacteria, and their reversal by protonophoric uncouplers, in terms of ΔpH-related changes in thylakoid membrane conformation (cf. Packer 1963; Packer et al. 1965). On the other hand, Murata and Sugahara (1969) ascribed the light-induced Chl *a* fluorescence decrease in DCMU-treated chloroplasts to photophosphorylation as a whole. Subsequent evidence, however, consolidated the concept that local changes in thylakoid membrane conformation mediate the effect of trans-membrane ΔpH on Chl *a* fluorescence. This evidence includes the following: **(i)** results from the comparison by Krause (1973) of the kinetics of Chl a fluorescence and of the light-induced absorbance change at 535 nm (ΔA535; a measure of chloroplast shrinkage); **(ii)** the early interpretation by Crofts and Yerkes (1994) of the then existing experimental evidence in terms of a protonation of lumen-exposed glutamate residues of the minor Chl *a*/Chl *b* light harvesting complexes (minor LHCs) resulting in pigment dimers (for a more definitive experiment on the role of glutamate using specific mutants, see Li et al. 2004 and Niyogi and Jansson, Chap. 13); and **(iii)** the demonstration by Horton et al. (2000) that the double de-epoxidation of violaxanthin (V) to zeaxanthin (Z) can cause allosteric changes that convert peripheral PS II antenna (LHCII) from an emissive state to a quenched state.

B State Transitions

Oxygenic photosynthesis uses two pigment systems, PS II and PS I. The absorption spectrum of PS II does not overlap with the absorption spectrum of PS I, particularly at the longer wavelengths beyond, e.g., 690 nm. This is the cause of the "Red Drop" in the action spectrum of the quantum yield of photosynthesis, and the Emerson Enhancement Effect (Emerson and Lewis 1943; Emerson et al. 1957; see Govindjee and Björn 2012). Therefore, depending on the wavelength of the absorbed light, one photosystem may be overstimulated relative to the other. To rectify such an imbalance, oxygenic photosynthetic organisms evolved a light-adaptation mechanism, known as *state transitions*, with which they optimize photosynthesis by adjusting antenna sizes of PS II and PS I, and thereby the amount of excitation energy delivered to PS II_{RC} and PS I_{RC}. When a photosynthetic organism absorbs more PS II light (Light 2), it shifts to *state 2*; in this state, the excitation energy share of PS II decreases and that of the PS I rises. Conversely, when a plant absorbs more PS I light (Light 1) it shifts to *state 1*; in this state, the excitation energy share of PS I decreases and that of PS II rises.

Diagrammatically, these events can be represented as follows (Scheme 3):

$$\begin{gathered}
\textit{State 1 – to – State 2 transition}\\
\textit{State 1}\left[\textit{more}\ \text{excitation}\ \textit{to PS II},\ \textit{less}\ \textbf{to}\ \textit{PS I}\right]+\textbf{Light 2}\\
\rightarrow \textit{State 2}\left[\textit{more}\ \text{excitation}\ \textbf{to PS I, less to PS II}\right]\\
\textit{State 2 – to – State 1 transition}\\
\textit{State 2}\left[\textit{more}\ \text{excitation}\ \textit{to PS I},\ \textit{less to PS II}\right]+\textbf{Light 1}\\
\rightarrow \textit{State 1}\left(\textbf{more}\ \text{excitation}\ \textbf{to PS II, less to PS I}\right)
\end{gathered}\tag{3}$$

Light-adaptive redistribution of excitation energy between PS II and PS I in cyanobacteria and green algae was first reported in detail by the two of us (see Papageorgiou and Govindjee 1967, 1968a, b; reviewed by Govindjee and Papageorgiou 1971; Papageorgiou 1975). However, this redistribution was then attributed to light-induced conformational changes such as those that had been shown to occur in isolated chloroplasts (Packer 1963; Packer et al. 1965). The breakthrough interpretation of this phenomenon, in terms of the state transitions concept, and its terminology, is due to Murata (1969) and to Bonaventura (1969) and Bonaventura and Myers (1969). This interpretation was based on fluorescence spectra of the red alga *Porphyridium cruentum* in the first case (Murata), and on fluorescence spectra and oxygen evolution by the green alga *Chlorella pyrenoidosa* in the second (Bonaventura and Myers 1969).

In all oxygenic photosynthetic organisms, the chemical signal that triggers the state 1–to–2, and the state 2–to–1 transitions is the redox (oxido-reduction) state of a set of plastoquinone molecules involved in transporting electrons from PS II to PS I (the plastoquinone pool, or PQ pooi, see Fig. 1.1). We know that PS II (water-plastoquinone oxidoreductase) reduces the PQ pool, whereas PS I (plastocyanin-ferredoxin oxidoreductase) oxidizes the PQ pool by receiving electrons from it via the Cyt b_6f complex (Ke 2001; Wydrzynski and Satoh 2005; Golbeck 2006). Thus, there is an antagonistic regulation (feedback regulation, so-to-say) of the PQ pool by the photochemical activities of PS II and PS I. In addition to this regulation, transitions to state 1 or to state 2 can be realized in the absence of light by means of chemical manipulation of the oxido-reduction state of the PQ-pool (Vener et al. 1995).

Beyond these two first steps, however, the detailed molecular mechanism of the state transitions in green algae and plants, which contain only intrinsic Chl *a*/Chl *b*-binding antenna proteins (LHC proteins), is quite different from that in cyanobacteria and red algae that contain no intrinsic Chl *a*/Chl *b*-binding proteins. In plants and green algae, state transitions involve lateral displacement of the intramembranous specific mobile LHCII subunits and of the Cyt b_6f complex from PS II to PS I and back. In cyanobacteria, however, light harvesting is by phycobilisomes (PBS) that are extrinsic to the thylakoid membrane (i.e., they are extramembranous antenna) and can associate with PS II and with PS I. The PBS have three types of chromophores: phycoerythrins, phycocyanins and allophycocyanins. It has long been known that excitation energy is efficiently transferred from phycoerythrin to phycocyanin and from phycocyanin to allophycocyanin, and then from allophycocyanin to Chls *a* in PS II or PS I (see, e.g., Glazer 1989; Mimuro 2004; Adir 2005; and citations therein). Translational and/or rotational movements of the PBS brings them closer to one or the other photosystem and facilitate excitation energy transfer from the PBS to that respective photosystem. Since at room temperature, PS II emits more Chl *a* fluorescence than PS I, transition from *state 1* → *state 2* leads to a decrease in Chl *a* fluorescence intensity, and conversely, transition from *state 2* → *state 1* leads to an increase in fluorescence. At 77 K, state 1 (also referred to as state I) and state 2 (also referred to as state II) can be recognized by the characteristic emission spectra: in state 1, one observes higher PS II (F686 and F696) emission bands, and a lower PS I (F730) emission band. For further background on these emission bands, see chapters in Govindjee et al. (1986) and Papageorgiou and Govindjee (2004); for LHCII subunits, see Kargul and Barber (2008), Iwai et al. (2008, 2010a, b), and Minagawa (2011); for the Cyt b_6f complex, see Cramer and Zhang (2006), Baniulis et al. (2008), Kallas (2012), and Hasan et al. (2013); for the PBS, see Allen and Mullineaux (2004).

Judging from the number of published research papers and reviews, we may say that the state transition concept has proven to be highly fertile in the area of photosynthesis research. Here we list only few selected post-2001 reviews: Allen and Forsberg (2001), Haldrup et al. (2001), Kruse (2001), Wollman (2001), Allen (2002), Zer and Ohad (2003), Allen and Mullineaux (2004), Bruce and Vasil'ev (2004), Finazzi and Forti (2004), Rochaix (2007), Murata (2009), Lemeille and Rochaix (2010), Minagawa (2011), Tikkanen et al. (2011), Papageorgiou and Govindjee (2011), Mohanty et al. (2012), Papageorgiou (2012), and Puthiyaveetil et al. (2012).

Quite often state transitions are listed as NPQ processes and are symbolized as qT, as we have often done ourselves. In our opinion, this designation, however, and its symbol are inaccurate and misleading. Here is the reason: State transitions are two processes, with different impact on the electronic excitation energy that momentarily resides on Chl *a* molecules of PS II. The state 2 →1 process provides excitation to PS II and therefore it cannot be a true qT or NPQ process. Since the state 1 → 2 transition depletes excitation energy from PS II and transfers it to PS I, it may be counted as an NPQ or $qT_{1\rightarrow 2}$ process. However, the excitation energy that is transferred to PS I is conserved, for the most part, as ATP via the cyclic PSET pathway (see section on "Terminology and Semantics" for relevant citations).

Several reviews (see, e.g., Tikkanen and Aro 2012; Tikkanen et al. 2012) have discussed connections between (i) mechanisms serving to re-distribute excitation energy between PS II and PS I under limiting light and (ii) mechanisms serving in *net* energy dissipation under excess-light conditions and also involving interactions among PS II, LHCII, and PS I.

C *Terminology and Semantics*

As applied to photosynthesis, *non-photochemical quenching* (NPQ) is a scientific term whose semantics do not coincide sensu stricto with its literal content. Thus, *non-photochemical* excludes only the exciton trapping act (or the primary charge separation) at the PS II and PS I reaction centers but includes all other possible photochemical reactions. Secondly, the *quenching* part of the term does not necessarily pertain only to a stable population of Chl *a* molecules but may also involve population shifts, as for example in the state 1 to state 2 transition ($qT_{1\rightarrow 2}$; see section on "State Transitions").

In addition, most groups researching NPQ processes focus on short-term physicochemical events (up to a few minutes; although see section "The Emergence of the Non-Photochemical Quenching (NPQ) Concept" on a form of NPQ continuously maintained for days, weeks, or months in certain plant species and certain growth-inhibiting environments; see also Adams and Demmig-Adams, Chap. 2 and Demmig-Adams et al., Chap. 24). The NPQ processes may **(a)** be triggered and regulated antagonistically by chemical or physical signals that PSET generates (e.g., low lumen pH, electrical polarization of the thylakoid membrane, oxidoreduction level of the intersystem intermediates), or **(b)** by PSET-independent signals, such as strong blue-green light as in the case of the PBS-containing cyanobacteria (reviewed by Kirilovsky and Kerfeld 2012; see also Kirilovsky et al., Chap. 22). In the latter case, blue-green light activates a stroma soluble carotenoid protein, known as the Orange Carotenoid Protein (OCP) that, after light activation ($\mathrm{OCP}^0 + h\nu \rightarrow \mathrm{OCP}^r$, the superscripts “o” and “r” refer to orange and red forms) attaches to the terminal allophycocyanin emitter of the PBS and dissipates its (excess) excitation energy before it is transferred to the intra-membranous Chls *a* of the cyanobacterium. According to Gorbunov et al. (2011), the light activation leads first to an intermediate form, OCP^i, which in a subsequent dark step transforms to the active form OCP^r. The inactivation of OCP^r is effected by a stroma protein, known as the *fluorescence recovery protein*.

OCP quenching is not antagonistically regulated by PS II and PS I, but it is triggered by strong excitation energy and not necessarily by excess excitation energy. Characteristically, whereas in plants and algae, the excess excitation energy threshold depends on the physiological state of the cell, there is no physiologically set excitation energy threshold for OCP quenching. In fact, OCP-dependent quenching has been demonstrated to occur in a reconstituted system in vitro (Gwizdala et al. 2011) and in a S*ynechocystis* mutant that lacked both photosystems (Rakhimberdieva et al. 2011).

IV NPQ Mechanisms and Atmospheric Oxygen Content

The principal biological role of the qE mechanism (see section on “High Energy Quenching of Chlorophyll *a* Excitation”) is protection from photo-oxidative damage. We first present a basic background on the energy levels of molecules: Molecules have different electronic excitonic energy levels: ground state, first singlet excited state, higher singlet-excited states, and corresponding triplet states. The spin multiplicity of the system equals $S = 2J + 1$, where J stands for the sum of electron spins in the molecule. The electron spin has a value either +1/2 or -1/2, and since all electrons in a molecule are paired, $J = 0$ and $S = 1$. This is the singlet state. However, if the spin of one electron is flipped by some means, then J becomes 1 and the multiplicity ($S = 2J + 1$) becomes 3. This is the triplet state (see Clayton 1970; Rabinowitch and Govindjee 1969). Excitation of ^{1}Chl *a* (in the ground state, the lowest singlet state) leads to excited Chl *a* singlet state (^{1}Chl *a**); and if by some means, the spin of one electron in an electron pair is flipped, it would give rise to longer-living excited triplet state (^{3}Chl *a**); the process that leads to the transition of excited singlet to a triplet state is called intersystem crossing. This triplet state can react with $^3O_2$ to produce excited O_2 singlets ($^1O_2^*$) and ground-state reactive oxygen species (ROS), all potent oxidants. Triplet Chl *a* may also arise by charge recombination reactions, such as between a Chl *a* cation (Chl^+a) and a pheophytin anion (Pheo^-a) in the PS II reaction center (Pospisil 2012). We refer the reader to Fig. 1.1 and its detailed legend where the photosynthetic reactions are described; the primary photochemistry of PS II leads to the formation of Chl^+a and Pheo^- *a*, and it is recombination of these charges that is suggested to lead to the formation of ^{3}Chl *a** among other reactions.

Were such photo-oxidative reactions a problem for the earliest cyanobacteria on Earth? As conjectured from geochemical evidence, cyanobacteria must have appeared some time between 3.85 and 2.7 Ga (billions of years before the present time; Falkowski 2006; Buick 2008; Blankenship 2010; Hohmann-Marriott and Blankenship 2011). At that time, the Earth was warmer and covered by seawater, while its atmosphere was a mixture of methane, carbon dioxide, nitrogen and hydrogen. Free oxygen was essentially absent, well below 10^{-5} of its Present Atmospheric Level (PAL; see Holland 2006; Buick 2008, and citations therein), although it may have risen to $\sim 3 \times 10^{-4}$ PAL at ~3 Ga (Crowe et al. 2013). Nevertheless, the answer to the question posed above is probably yes. In view of the fact that there was always plenty of light and that cyanobacteria have survived, it seems logical that they must have evolved some sort of photoprotective mechanism (Blankenship 2010), essentially the Orange Carotenoid Protein (OCP) mechanism that dissipates excitation

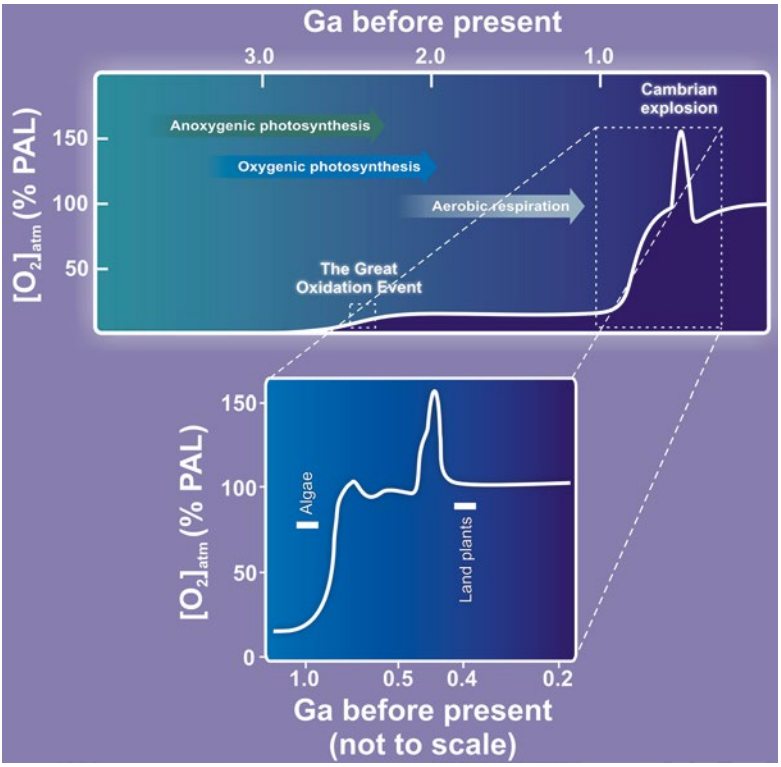

Fig. 1.3. A schematic view of the changing concentration of atmospheric O_2 as a function of geological time in billions of years (*Ga*) before the present time. Correlations between the estimated changes of O_2 concentration in the atmosphere (in % of present atmospheric level, PAL of O_2) and the evolution of metabolic pathways are based on numerous data (see, e.g., Falkowski 2006; Tomitani et al. 2006; Kump 2008; Blankenship 2002, 2010; Björn and Govindjee 2007, 2009; Hohmann-Marriott and Blankenship 2011 and references therein). The dates in the figure and in the zoomed insert are not to scale and are approximations. Uncertainties of the selected evolutionary events in the zoomed insert are depicted with bars. This figure was prepared by D. Shevela and Govindjee by modifying and adapting the figures published by Govindjee and Shevela (2011) and Shevela et al. (2013).

energy of the PBS before this energy is transferred to the core antenna complexes (Kirilovsky and Kerfeld 2012; Kirilovsky et al., Chap. 22).

As a result of oxygenic photosynthesis by cyanobacteria, the O_2 level in the atmosphere rose and by the time of the primary endosymbiosis event, estimated to have occurred at ~0.9 Ga (Shih and Matzke 2013), when a heterotrophic eukaryotic cell engulfed a cyanobacterium, the O_2 level rose to about 0.2 PAL (see Fig. 1.3). The engulfed cyanobacterium eventually became a chloroplast, having transferred most of its genome to the nucleus of the host, and after having replaced the extramembranous PBS and OCP complexes with intramembranous, Chl *a*/*b*/xanthophyll-binding complexes, the LHC proteins (see, e.g., Hohmann-Marriott and Blankenship 2011; Niyogi and Truong 2013).

Were the intra-membranous light-harvesting antenna of the oxygenic photosynthetic eukaryote an advantage, or a disadvantage, compared to the extra-membranous light harvesting antenna of the oxygenic prokaryote? First of all, internal antenna were structurally more stable than external PBS whose structural integrity is known to be temperature- and ionic strength-dependent (Gantt et al. 1979). Considering the structural integrity, therefore, the internalization of the peripheral light harvesting antenna in the thylakoid membranes can definitely be viewed as an advantage.

On the other hand, since ROS primarily attack lipids, having ROS formed outside the thylakoid membrane by the excited PBS would be far less dangerous for the sensitive intra-membranous complexes. A simple mechanism, based on one and the same protein, the OCP, for sensing and dissipating the excitation energy of the PBS, would suffice for both early and present-day cyanobacteria. In contrast, in the case of the membrane-internal LHC proteins, the chance for photo-oxidative damage is more serious, taking into account the rise in atmospheric O_2 to 1 PAL (Fig. 1.3). We may speculate that, to meet the increased danger of photo-oxidative damage, LHC proteins evolved to serve not only for light-harvesting function, but also for sensing and dissipating excess excitation energy, e.g., the LHCSR (stress-related light-harvesting complex) proteins in green algae and the equivalent LHCX6 protein in diatoms; see section on "Xanthophyll-dependent "High Energy" Quenching of Chlorophyll *a* Excitation" for further information and citations.

Not much is known about the early evolution of NPQ, especially as oxygen concentration changed over time; in fact, lateral transfer of genes makes this exercise difficult. However, based on their studies with the moss *Phycomitrella patens*, Alboresi et al. (2010) have discussed the evolution of photoprotection mechanisms as land plants evolved. Gerotto et al. (2012) showed the co-existence of algal as well as higher-plant mechanisms of photoprotection in this moss. Since light-harvesting antennas are involved in photoprotection, their evolution is of interest to us (see Koziol et al. 2007). For a discussion of the evolution of photosynthesis, see, e.g., Blankenship (2002, 2010) and Björn and Govindjee (2007, 2009). Further research and insight is needed to deal with the important question about the evolution of NPQ. We must indeed wait.

Concerning the evolution of rapidly reversible NPQ versus continuously maintained NPQ, one may speculate that the ability to perform strong and continuous NPQ may have preceded the need to disengage NPQ as quickly as possible to facilitate rapid return to maximal photochemical efficiency whenever light was limiting photosynthesis of fast-growing land plants with very high maximal photosynthesis rates. The evolution of several xanthophyll cycles may, likewise, also have been driven by the need to quickly *remove* "dissipaters" rather than the need to *form* dissipaters (see also Adams and Demmig-Adams, Chap. 2).

V Timeline of Discoveries Relating to the Major NPQ Processes

There is a message in all timelines: they trace the evolution of ideas and are instructive in themselves. We can call this idea «*scaling from the past*». For us, these timelines are fascinating and useful in teaching as well as in research. We need to learn to weave in and weave out to grasp the insights of the process as we read through the timeline discoveries that follow. We recognize that a novice to the field may not always see connections upon first reading, but timelines will help highlight the evolution of ideas and thoughts over time. We refer the readers to the timeline of photosynthesis research by Govindjee and Krogmann (2004).

Going back to the topic of this chapter, the major NPQ process that dissipates most of the excess excitation energy of PS II as heat is triggered by physicochemical signals generated by intersystem PSET, and is subject to antagonistic regulation by PS II and PS I activities (feedback regulation). The "high-energy" state of the thylakoid membrane also encompasses an electrostatic potential difference ($\Delta\Psi$) across the membrane (lumen side positive), as well as concentration differences of ionic and non-ionic solutes ($\Delta\mu_i$). Two light-induced processes lead to accumulation of protons in the lumen: **(i)** decomposition of H_2O into electrons, O_2 and protons; and **(ii)** the translocation of stroma protons to the lumen by non-cyclic and cyclic PSET. The high-energy state of the membrane is known to somehow quench singlet excited Chl *a* (^{1}Chl *a**). This excitation quenching process is usually described as "high-energy quenching" and is symbolized as qE (see section on "High Energy Quenching (qE) of Chlorophyll *a* Excitation").

On the other hand, during state 1-to-state 2 transition, Lhcb subunits of peripheral PS II antenna shift (move) to the vicinity of PS I; this happens when intersystem PSET intermediates (e.g., plastoquinones) are reduced when PS II activity outpaces PS I. The Lhcb subunit is shifted (moved) back to the PS II vicinity from PS I during the state 2 to state 1 transition, which occurs when PSET intermediates become oxidized as a result of PS II being outpaced by PS I. The important process for relieving PS II from excess excitation energy is the state 1-to-state 2 transition.

If we define NPQ to be a process that facilitates dissipation of excess excitation energy, state transitions can hardly be termed as such, as mentioned earlier in this chapter. The extra excitation energy that PS I receives upon going from state 1 to state 2 is used primarily to make ATP via cyclic electron transport around PS I. On the other hand, the reverse process, that of state 2-to-state 1 transition, supplies additional excitation energy to PS II.

A High-Energy Quenching of Chlorophyll a Excitation

1 Xanthophyll Cycles: Biochemistry and Occurrence

1957

Discovery that light lowers, and that darkness restores, the violaxanthin content of plant leaves

Sapozhnikov et al. (1957) reported that, during a period of high light illumination, the violaxanthin (V) content of leaves decreased and that of lutein (L) increased. A subsequent dark period reversed these changes, i.e., L content was decreased and V content increased. These reciprocal changes in the oxidation state of plant xanthophylls were interpreted as indicating a light-induced, single step, de-epoxidation of di-epoxide V to the 1, 2-diol L. See reviews by Sapozhnikov (1973), Pfündel and Bilger (1994), and Yamamoto (2006b).

1959

Light-induced de-epoxidation of violaxanthin in green algae is quantitatively reversed in darkness

Working with the green algae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*, and using ^{14}C-labeling for quantitatively tracking xanthophyll changes, Blass et al. (1959) determined that light-induced de-epoxidation of V and its dark-induced re-epoxidation are fully reversible. Further, they postulated an enzymatic dark resynthesis of V from Z via A.

1962

Discovery of the xanthophyll cycle

Yamamoto et al. (1962) had well-founded doubts about the light-induced conversion of V to L proposed by Sapozhnikov et al. (1957): first because of the implied double de-epoxidation in a single-step, and second, because of the implied isomerization of a β-carotene-type xanthophyll (V) to the α-carotene-type xanthophyll (L). Experimenting with spinach and bean leaves and using two-stage column chromatography, Yamamoto et al. (1962) succeeded in isolating the mono-epoxide antheraxanthin (A) and diepoxide zeaxanthin (Z), both β-carotene-type xanthophylls, as the products of light-induced de-epoxidation of V, while L remained unchanged; to interpret these light-induced/dark-reversed changes, they proposed the following mechanism:

$$\text{Light} \Rightarrow \quad V \Leftrightarrow A \Leftrightarrow Z \quad \Leftarrow \text{Dark}$$

Since additional light-dark induced de-epoxidation/epoxidation cycles, involving other xanthophylls (e.g., diadinoxanthin and lutein epoxide; see below Fig. 1.2) were subsequently discovered, the first xanthophyll cycle of Yamamoto et al. (1962) is now widely designated as the *violaxanthin cycle* (VAZ cycle, where V stands for violaxanthin, A for antheraxanthin and Z for zeaxanthin, as noted above). For a review, see Yamamoto (2006b).

1967

The VAZ cycle is associated with light-induced acidification of the thylakoid lumen

Hager (1967a, 1969) demonstrated that the VAZ cycle occurs in plant leaves (e.g., spinach) as well as green algal cells (e.g., *Chlorella pyrenoidosa*) and showed that V de-epoxidation required an acidic lumen and the presence of ascorbate.

In spinach chloroplasts, de-epoxidation of V can be triggered (a) in the light, by acidification of the lumen via H^+-translocating PSET (both non-cyclic and cyclic; see Fig. 1.1), (b) in the dark, by hydrolysis of exogenous ATP, and (c) in the dark and in the absence of ATP, by lowering pH of the suspension to ~5.0 (after the experiment of Hind and Jagendorf 1963). In *Chlorella* cells, V-to-Z conversion occurs upon lowering suspension pH to below 7.0. Protonophoric uncouplers, eliminating trans-membrane ΔpH, abolish both photophosphorylation and light-induced Z formation. Sonicated chloroplasts that cannot maintain a trans-membrane ΔpH are incapable of photophosphorylation as well as V-to-Z de-epoxidation. This suggests that a closed thylakoid membrane, capable of maintaining a proton concentration difference between the external and the internal aqueous phases, is a necessary condition for light-induced V de-epoxidation.

Both the forward (V → A → Z) and the backward (V ← A ← Z) reactions are enzymatic

Heating *Chlorella* cells for a short time stops the de-epoxidation reaction (V → A → Z), implicating the involvement of an enzyme (Hager 1967a); a Cu-containing enzyme was invoked to catalyze the epoxidation reaction (V ← A ← Z; Hager 1967b).

1970

Isolation of the enzyme violaxanthin de-epoxidase (VDE)

Hager and Perz (1970) isolated a VDE complex from spinach chloroplasts that, in the presence of ascorbate, de-epoxidizes V to Z. Using lettuce chloroplasts, Siefermann and Yamamoto (1974) established that de-epoxidation of V is a first order reaction, suggesting diffusion control for the process. Observing that VDE is isolated together with one monogalactosyl diacylglycerol (MGDG) molecule, Yamamoto and Higashi (1978) proposed that MGDG molecules in the vicinity of VDE are necessary for solubilizing the substrates (carotenoids being water-insoluble) and that the MGDG molecules act as functional components at the enzyme's active center. Since VDE de-epoxidizes only straight-chain all-*trans* carotenoids, such as V and A, but not bent-chain carotenoids, such as the 9-*cis* neoxanthin (N), Yamamoto and Higashi (1978) visualized the active center of VDE to be situated within a hollow well-like structure into which only straight chain carotenoids can fit. Lastly, Hager and Holocher (1994) found that VDE moves freely in the lumen at near-neutral pH and binds maximally to the lumen-exposed side of the membrane at pH 5.0. Thus, the two enzymes of the VAZ cycle are located on the opposite sides of the membrane, VDE in the lumen-exposed side and ZEP (zeaxanthin epoxidase) in the stroma-exposed side. See a review by Hager (1980).

Discovery and occurrence of the diadinoxanthin-diatoxanthin (Dd-Dt) cycle

The alkyne (one triple bond) mono-epoxidic carotenoid diadinoxanthin (Dd) is dominant in yellow-green algae, the *Xanthophyceae*. Stransky and Hager (1970) discovered that, during illumination, the epoxy oxygen of Dd is cleaved in a single step and a –C = C– double bond is formed in its place, yielding diatoxanthin (Dt) (also see Fig. 1.2). The de-epoxidation reaction is reversed in the dark. The Dd-Dt cycle was also identified in *Euglenophyceae*, *Chrysophyceae* and *Bacillariophyceae* (Stransky and Hager 1970; Demers et al. 1991), and in diatoms (Arsalane et al. 1994). Later, however, Lohr and Wilhelm (1999, 2001) discovered that both the VAZ cycle and the Dd-Dt cycle operate in diatoms. In fact, it appears that V (double bond at carbons 7′,8′) is the precursor of both Dd (triple bond at carbons 7′,8′) and fucoxanthin (allenic double bonds at carbons 6′,7′ and 7′, 8′). For further information, see Goss and Jakob (2010); and Büchel, Chap. 11 and Lavaud and Goss, Chap. 20.

1974

Enzyme kinetics of V de-epoxidation by VDE

Siefermann and Yamamoto (1974) established that light-induced de-epoxidation of V in lettuce chloroplasts, in the presence of ascorbate, obeys 1st-order kinetics. The extent of de-epoxidation depends on availability of the substrate, which was also shown to be light-dependent, and, indirectly, on PSET (Siefermann and Yamamoto 1975a; Bilger and Björkman 1990; Demmig-Adams and Adams 1990).

1975

Characterization of zeaxanthin epoxidase (ZEP)

ZEP, the enzyme that epoxidizes Z to A and A to V, was characterized by Hager (1975) and Siefermann and Yamamoto (1975b, c) in chloroplasts. ZEP is located on the stroma-facing side of the thylakoid membrane, requires O_2 and NADPH (and flavin-adenine dinucleotide (FAD) as shown later by Büch et al. 1995) as co-substrates, and exhibits optimal activity at pH ~7.3; it has no activity below pH 5.5. Further, ZEP was characterized as a mono-oxygenase because it donates one O-atom of the O_2 molecule to substrates (Z and A) and forms H_2O with the other. The ZEP-catalyzed epoxidation was found to be much slower than VDE-catalyzed de-epoxidation; further, it did not require any light if supplied with the required co-substrates. However, of the two consecutive back reactions, Z → A was shown to be faster than the A → V reaction (Goss et al. 2006).

1978

The two consecutive de-epoxidations V → A and A → Z occur at different rates

Both de-epoxidations are catalyzed by VDE that, however, has a higher affinity for A than for V (Yamamoto and Higashi 1978; Grotz et al. 1999; Goss 2003).

1994

At neutral pH VDE is freely mobile in the lumen; it binds to the thylakoid membrane at ~pH 5

Hager and Holocher (1994) detected VDE in the supernatant of pelleted chloroplasts isolated from dark-pretreated spinach leaves. VDE, that must have been released from the pellet to go into the supernatant, decreased in quantity at pH < 6.5 and was absent at pH ~5 (near the optimum of enzyme activity). These findings support the view that the two enzymes of the VAZ cycle are located on the opposite sides of the thylakoid membrane, i.e., the ascorbate-dependent VDE on the lumen side and the NADPH-dependent ZEP on the stroma side, while V is located within the lipid phase of the membrane. See also Bratt et al. (1995) for supporting evidence.

VDE activity is controlled by the pH in sequestered membrane domains

Using high- and low-salt suspensions of broken pea chloroplasts, and conditions with and without ATP synthesis, Pfündel et al.

(1994) succeeded in differentiating between lumen pH and the pH in sequestered membrane domains. It is the latter pH that determines the activity of VDE that can be active even when bulk lumen pH is above 6.0.

1996

Purification and characterization of VDE

Rockholm and Yamamoto (1996) purified and characterized VDE from lettuce. Its apparent molecular mass was determined to be 43 kD, its optimal pH at ~5.2 and its isoelectric point at 5.4. Purified VDE could be precipitated by centrifugation only after addition of MGDG which is a Hex-II lipid containing mostly unsaturated fatty acids, and therefore forms tubular structures, not bilayers, with the hydrophobic fatty acid tails pointing outwards. The affinity of VDE to MGDG is specific; other thylakoid membrane lipids do not precipitate VDE. The molecular mass of VDE purified from spinach was determined to be 41 kD (Kuwabara et al. 1999).

1998

The VAZ cycle enzymes are members of the lipocalin family of proteins

Amino-acid-sequence analyses of VDE and ZEP, using cDNA libraries from several plants, enabled Bugos et al. (1998; see the review by Hieber et al. 2000) to classify these two VAZ cycle enzymes as lipocalins. Lipocalins are multifunctional proteins capable of carrying small lipophilic substrates (see, e.g., Boca et al. 2013). In the case of the VAZ cycle enzymes, the lipophilic substrates V and Z enter into hydrophobic, barrel-like cavities of the enzymes VDE and ZEP where they are processed by the active centers. Only straight chain (*all-trans*) carotenoids can fit into these cavities. A, the intermediate in the reversible V → Z transformation, fits into the active-center cavities of both VDE and ZEP. A hollow, well-like structure, such as the active center of VDE, was anticipated earlier by Yamamoto and Higashi (1978; see above) in view of VDE's inability to de-epoxidize the 9-cis carotenoid neoxanthin.

Interestingly, isolated LHCII, the major light-harvesting complex of PS II, also epoxidizes Z to V (Gruczecki and Krupa 1993a, b); this observation needs further confirmation.

1999

The majority of the violaxanthin available for de-epoxidation binds to LHCII

Isolated trimeric LHCII complexes from the annual plant spinach contain the majority of the VAZ cycle carotenoids (estimated at 15–19 molecules per reaction center; see Ruban et al. 1999 for detailed experimental conditions). With a mild detergent extraction, it was further established that V binds more loosely than the other xanthophylls (2 L and 1 neoxanthin, N) of the LHCII monomer. The latter result anticipated the subsequent elucidation of the crystal structure of LHCII monomers (Liu et al. 2004) according to which the VAZ cycle carotenoids attach to the periphery of the complex, making nearly no contact with the protein backbone, in contrast to the two L, which do; N is an intermediate between the two. Also see Fig. 1.2.

2000

Monogalactosyldiacylglycerol (MGDG) domains of the thylakoid membrane are the likely sites for VDE– and DDE–catalyzed de-epoxidation

To be de-epoxidized, all-*trans* V must fit into the barrel-like cavity, where the active center of the VDE is located (Bugos et al. 1998; also see Bugos and Yamamoto 1996). This requires V to dissociate from its site on an Lhcb antenna protein and to move to the surrounding lipid phase for an encounter with a VDE molecule. According to this picture, the fluidity of the lipid phase and the solubility of the V in it are expected to be important determinants for the rate and extent of VDE-catalyzed de-epoxidation. A number of important in vitro studies with V-containing liposomes of known lipid composition substantiate the following expectation: An increase in MGDG content will increase both fluidity of the liposome lipid bilayer (Latowski et al. 2000, 2002) and solubility of V and of Dd in it (Goss et al. 2005; reviewed by Goss and Jakob 2010).

The phase structure of membrane lipids determines the de-epoxidizing activity of VDE

In vitro studies with liposomes and micelles made from thylakoid membrane lipids showed higher V solubility and VDE activity in micelle-forming lipids like MGDG than in bilayer-forming lipids like digalactosyldiacylglycerol (DGDG; Latowski et al. 2004; Goss et al. 2005; Yamamoto 2006a; Vieler et al. 2008). MGDG and DGDG are the major lipids of thylakoid membranes.

2001

VDE activity in primitive green algae (Prasinophyceae)

Frommolt et al. (2001) discovered that the prasinophycean alga *Mantoniella squamata* accumulates A upon illumination instead of Z that is accumulated by green algae and plants. The reason is that in this alga, the reaction V → A runs much faster, in either way, compared to the rate of Z formation in Z-accumulating plants and algae. Compared to spinach, the A → Z rate in *Mantoniella* is 20-times slower. In *Mantoniella* VDE has a reduced affinity for A, as well as for other mono-epoxides (e.g., Dd, Lx, and N) compared to plants (Goss 2003).

2 Xanthophyll-Dependent “High-Energy” Quenching of Chlorophyll a Excitation

1987–1989

Zeaxanthin is correlated with qE in plant leaves

Demmig-Adams et al. (1989) demonstrated that the extent of qE (at the time quantified as the rate constant for thermal energy dissipation, k_D), upon illuminating plant leaves with excess actinic light, is linearly related to the Z content of leaves. On this basis, they proposed that Z, derived from V in the VAZ cycle, is a link between the “high-energy” state of thylakoids and the dissipation of excess excitation energy of Chl *a* as heat. This demonstration of a correlation between Z and qE followed the initial establishment of a correlation between Z and a dark-sustained form of NPQ (Demmig et al. 1987).

1991

qE has two distinct kinetic components in isolated chloroplasts

Using isolated chloroplasts, Gilmore and Yamamoto (1991) obtained evidence for two kinetically distinct components of ΔpH-dependent quenching of Chl *a* fluorescence: a faster (~1 min) Z-independent, and a slower (~10 min) Z-dependent quenching component. The faster quenching component was suggested to reflect quenching by the mono-epoxidic antheraxanthin (Gilmore and Yamamoto 1993) and by non-epoxidic xanthophylls (Gilmore et al. 1994). See also Niyogi et al. (1997) for evidence supporting dependence of the rapidly reversible ΔpH-dependent quenching on lutein of the LHC proteins.

qE quenches singlet excited Chl a in the LHCII antenna of PS II

Quenching of singlet-excited Chl *a* can be monitored by the effect of qE on Chl *a* fluorescence in isolated LHCII complexes as reduction in its fluorescence intensity. In fact, one can look at the entire emission spectrum either at room temperature or after cooling to 77 K; further, since qE appears in the light, evidence for this quenching can be obtained by comparing 77 K fluorescence emission spectra of a dark treated (minus qE) and a light-treated leaf (plus qE; Ruban et al. 1991; Ruban and Horton 1994). Results obtained by these authors support this expectation. Another technique that directly monitors the loss of energy as heat is photoacoustic spectroscopy (see its use, e.g., by Carpentier et al. 1985). Mullineaux et al. (1994), using laser light–induced photoacoustic spectroscopy, showed that qE dissipates Chl *a* excitation within 1.4 μs, much more slowly than expected for excess excitation energy dissipation by reaction center complexes. Thus, the qE effect was interpreted to occur in the antenna complexes of PS II.

1992

Zeaxanthin and lumen acidity are sufficient for quenching Chl a fluorescence in darkness

Schreiber and Avron (1979) had already shown that hydrolysis of exogenous ATP in isolated chloroplasts induces a back-flow of electrons that reduces the primary quinone acceptor (Q_A) of the PS II_{RC}, and thus causes an increase in Chl *a* fluorescence within ~1 min. However, it was Gilmore and Yamamoto (1992) who demonstrated, in isolated chloroplasts, that hydrolysis of exogenous ATP leads to a slower (~10 min) ΔpH-dependent NPQ of Chl *a* fluorescence that includes both Z-dependent and Z-independent components. Further, the light-independent Z-quenching lasted for longer periods (under conditions of little or no reverse electron flow). This shows that actinic light is only indirectly involved in qE (i.e., by driving acidification of the lumen).

1993

qE can reflect aggregation of isolated LHCII proteins due to trans-thylakoid membrane ΔpH

By comparing 77 K fluorescence emission spectra of dark-treated (−qE) and light-treated (+qE) leaves, Ruban et al. (1993) established that the difference emission spectrum had a maximum at 700 nm, suggesting that it may originate from an aggregated form of LHCII complex (see chapters in Govindjee et al. 1986 for location of emission peaks in vivo). Furthermore, it was determined that the extent of qE, upon illumination of dark-pretreated leaves, correlates linearly with selective light scattering at 535 nm (ΔA535), which was shown earlier to reflect LHCII aggregation (Ruban et al. 1992). These results are consistent with the hypothesis formulated by Horton et al. (1991), according to which aggregation of isolated LHCII complexes in PS II can enable dissipation of excess excitation energy by qE.

qE in the monomeric peripheral minor antenna complexes CP29 and CP26

On the other hand, Bassi et al. (1993) determined that 80 % of V in dark-adapted maize leaves is contained in the minor monomeric LHC proteins CP24, CP26, and CP29. On the basis of this result, they proposed that these proteins are the sites of qE. Gilmore et al. (1996a) showed that both intensity and lifetime of Chl *a* fluorescence of PS II (the fluorescence lifetime is a direct measure of the quantum yield of fluorescence) were independent of antenna size differences between wild type barley and the chlorina mutant that lacked LHCIIb. [Note that change in fluorescence intensity can simply be due to change in the concentration of Chl; thus, measurement of lifetime of fluorescence is crucial in reaching firm conclusions; see, e.g., Holub et al. 2000, 2007]. This suggested involvement of the innermost peripheral antenna complexes (i.e., CP24, CP26, and CP29) in qE. This proposal was further supported by the lowering of Chl *a* fluorescence lifetimes upon binding of Z to recombinant CP26 (~10 % quenching; Frank et al. 2001) and CP29 (~30 % quenching; Crimi et al. 2001) proteins.

1995

qE lowers both intensity and lifetime of Chl a fluorescence

Gilmore et al. (1995) established that the extent of qE quenching and of the lifetime of Chl *a* fluorescence change in parallel, thus demonstrating that qE quenching is a true quenching in the physico-chemical sense. In the unquenched state, lifetime values centered at ~2 ns, but in the quenched state lifetime dropped to ~0.4 ns (~80 % quenching). In the authors' own words, "the fluorescence quenching [was] interpreted as the combined effect of the pH gradient and xanthophyll concentration, resulting in the formation of a quenching complex with a short (approximate to 0.4 ns) fluorescence lifetime." Upon lowering chloroplast Z level with dithiothreitol, the Chl *a* fluorescence fraction with lifetime ~0.5 ns decreased and that with lifetime ~1.6–1.8 ns increased. It was concluded that binding of Z to LHC proteins (most likely to CP26) acts like a "*dimmer switch*" for excitation energy on Chl *a* (Gilmore et al. 1996a, b, 1998, 2000; Holub et al. 2000, 2007; for reviews, see Gilmore 1997; Gilmore and Govindjee 1999; and Govindjee and Seufferheld 2002).

PsbS (*or PS II-S*), *an LHC-like protein of PS II*, *does not bind chlorophylls and xanthophylls in vivo*, *and does not harvest light*

PsbS (or PS II-S) is a 22 kDa nucleus-encoded protein of PS II that is homologous to the nucleus-encoded Lhcb1-6 peripheral antenna complexes, although it has four α-helices instead of three (see citations in Funk et al. 1994, 1995). The PsbS protein, unlike the Lhcb proteins, is present in etiolated spinach leaves and it does not require any pigments in order to be a stable molecule (see, e.g., Dominici et al. 2002). However, it was shown that, in vitro, PsbS binds Z (Aspinall-O'Dea et al. 2002). Funk et al. (1994) showed that PsbS binds Chl *a* and Chl *b*, although not stably; however, this concept was not supported by recombinant protein experiments (Dominici et al. 2002). Thus, although PsbS had been named CP22 (Funk et al., 1994), in analogy to the minor antenna Chl-protein complexes CP29, CP26 and CP24, it seems unlikely that PsbS has any light-harvesting role. It was only later that Li et al. (2000, 2002, 2004) demonstrated that PsbS is a key participant in qE, the ΔpH-dependent component of NPQ. For reviews, see Niyogi (1999), Govindjee (2002), Golan et al. (2004), Bonente et al. (2008a, b), Kiss et al. (2008), and Kereïche et al. (2010).

1997

Mutants come into the picture for unraveling qE: *for violaxanthin and zeaxanthin*

Niyogi et al. (1997, 1998), using video imaging of Chl *a* fluorescence in *Chlamydomonas reinhardtii* cells and *Arabidopsis thaliana* plants exposed to excess light, succeeded in isolating and characterizing VDE-deficient (*npq1*) and ZEP-deficient (*npq2*) mutants, which proved to be useful in analyzing the biochemical pathway of qE. The mutant *npq1* lacks Z, as it is unable to de-epoxidize V, while the mutant *npq2* accumulates Z as it is unable to epoxidize it to A and V. See reviews by Baroli and Niyogi (2000) and Müller et al. (2001).

Significantly, Pogson et al. (1998) established, in the *Arabidopsis npq1* mutant, that the absence of Z does not fully suppress the fast-relaxing qE, indicating the presence of a Z-independent component in this mutant. Pogson and Rissler (2000) and Niyogi et al. (2001) reported that, in L-deficient *C. reinhardtii* and *A. thaliana* mutants, both extent and induction rate of qE were depressed. A full suppression of qE was achieved in a double mutant *npq1 lut2* that lacks both Z and lutein, implicating, thereby, a possible involvement of lutein in qE. Li et al. (2009) concluded that, in a Z-deficient mutant, lutein can substitute for the role Z plays in the wildtype.

Further, Matsubara et al. (2011), using fluorescence lifetimes for estimating qE, established that in leaves of the tropical plant avocado (*Persea americana*), both A and photoconverted L, formed from lutein epoxide, enhance the qE associated with the 0.5-ns lifetime component of Chl *a* fluorescence. Unlike Z, however, retention of A and photoconverted L were not involved in continuously maintained NPQ in avocado leaves. See reviews, e.g., by Niyogi (1999, 2000); Müller et al. (2010); and Jahns and Holzwarth (2012); Esteban and García-Plazaola, Chap. 12.

1999

Discovery of a lutein epoxide-lutein (Lx-L) cycle that runs in parallel with the VAZ cycle

An Lx-L cycle that operates in parallel with the VAZ cycle was identified by Bungard et al. (1999) and Matsubara et al. (2011) in two parasitic plants. Lx is a mono-epoxide of the α-branch (L 5,6-epoxide), while V is a di-epoxide of the β-branch. Lx is de-epoxidized under strong irradiance to L, and L is epoxidized to Lx under weak irradiance or in darkness. This epoxidation is much slower compared to the epoxidation of Z to V. While direct evidence for an involvement of the Lx-L cycle in qE is not available to date, Esteban and Garcia-Plazaola (Chap. 12) postulate such a function.

2000

PsbS, a PS II protein, is a crucial component of qE in plants

PsbS, a 22 kDa membrane protein of PS II (CP22; four trans-membrane helices; pigment free in vivo) was described earlier (see above) by Funk et al. (1994, 1995) but its role in photosynthesis had remained unknown. Analysis of PsbS-minus (Li et al. 2000, 2002) and PsbS-defective (Peterson and Havir 2001) *Arabidopsis* mutants led to the recognition that PsbS participates quantitatively in qE, and that availability of PsbS determines the extent of qE. In view of the fact that in vitro PsbS is capable of binding Chls and xanthophylls, it was proposed that it is a potential site for the dissipation of the excess excitation energy of Chl *a* by qE; see Brooks et al., Chap. 13.

Protonation of VDE at acidic pH enables its attachment to the thylakoid membrane

Using anion-exchange chromatography, Kawano and Kuwabara (2000) obtained evidence that protonation of VDE at acidic pH changes not only its surface charge but also its conformation.

2001

Peripheral antenna complexes of PS II are the sites of qE

Andersson et al. (2001) showed that antisense *Arabidopsis* plants lacking CP26 or CP29 have normal qE, suggesting that these complexes are unnecessary for photoprotection. By modifying the Chl *b* and LHCIIb content of *Phaseolus vulgaris* plants by subjecting etiolated seedlings to a regime of intermittent and continuous illumination, Chow et al. (2000) established a positive correlation between increasing Chl *b* and LHCII content and qE levels, suggesting a role for LHCII in the thermal dissipation of excitation energy from ^{1}Chl *a**.

In contrast, Moya et al. (2001) reported that Chl *a* fluorescence is quenched more in purified monomeric CP29, CP26 and CP24 antenna complexes than in purified LHCII, and Morosinotto et al. (2002) reported that newly synthesized Z appears primarily in CP24 and CP26.

2002

Mutants come into the picture for unraveling qE: for Lhcb in Chlamydomonas

Elrad et al. (2002) identified and characterized a *Chlamydomonas reinhardtii* mutant (*npq5*) lacking Lhcbm1, a light-harvesting polypeptide present in the LHCII trimers. The *npq5* mutant showed less than one-third reversible qE compared to the wild-type cells, and most of qE was not reversed by the addition of nigericin (a protonophore that dissipates trans-membrane ΔpH), suggesting that a defect in thermal dissipation is the cause of diminished qE. This mutant of *Chlamydomonas* was normal for the phenomenon of state transitions (see, e.g., Papageorgiou and Govindjee 2011), for high light–induced de-epoxidation of V, and for growth in low light, but was more prone to photoinhibition than wild-type *Chlamydomonas*. The PS II antenna in the *npq5* mutant has one-third fewer LHCII trimers compared to the wild-type cells. Elrad et al. (2002) interpreted these results to suggest that a large part of thermal dissipation within PS II antenna of *Chlamydomonas* occurs in LHCII. For reviews, see Govindjee (2002) and Golan et al. (2004).

Proton efflux rate through the ATP synthase of the thylakoid membrane conductivity regulates qE

Protons are transferred from the stroma to the lumen by non-cyclic electron transport through PS II and PS I and by cyclic electron transport through PS I (see Ke 2001) and are exported from the lumen to the stroma through the ATP synthase of the thylakoid membrane during ATP synthesis. Kramer and co-workers (Kanazawa and Kramer 2002; Takizawa et al. 2008) analyzed the observation that lumen acidification is enhanced when the level of inorganic phosphate in the stroma is lowered, and established that this is caused by a decrease in proton conductivity through the ATP synthase. Govindjee and Spilotro (2002) showed that an *Arabidopsis thaliana* mutant, altered in the γ subunit of the ATP synthase, had a different pattern of intensity dependent changes in NPQ, confirming the relation of the functioning of ATP synthase with NPQ (see also Strand and Kramer, Chap. 18).

2004

Plants use PsbS, a pigment-free LHC protein of PS II, to sense lumen pH and initiate the qE process

A double *Arabidopsis* mutant (*npq4*), lacking two lumen-exposed glutamates (E122, E226) of the PsbS protein, was found by Li et al. (2004) to be defective in qE quenching and in the absorption change at 535 nm (ΔA535), which monitors a conformational change within PS II, while it resembled the qE–defective mutant *npq1* with respect to Chl *a* fluorescence lifetimes. On the basis of this result, Li et al. (2004) proposed PsbS to be the sensor that perceives lumen acidity level (and thereby the level of excess Chl *a* excitation energy) and initiates membrane protein conformation changes, which, together with Z, enable dissipation of the excess excitation as heat. For further information, see Brooks et al., Chap. 13.

For earlier evidence for the involvement of lumen-exposed carboxylates in acidic pH-induced quenching of Chl a fluorescence, see Walters et al. (1996).

Excitation-energy transfer from bulk Chls a to a Chl a-zeaxanthin heterodimer followed by charge transfer within the latter

The excited heterodimer (Chl *a*-Z)* may also relax to Chl *a*-Z by way of reversible charge transfer (Holt et al. 2004). Supporting evidence has been obtained from observations of qE in LHCII crystals (Pascal et al. 2005) and from LHCII complexes embedded in solid gel (Ilioaia et al. 2008). Both systems preclude displacement and the aggregation of LHCII trimers. For further discussion of photo-physical mechanisms of thermal dissipation involving energy transfer, charge transfer states, and/or excitonic coupling, see Ostroumov et al. (Chap. 4), Polivka and Frank (Chap. 8) and Walla et al. (Chap. 9).

2005

In which antenna complex and how may Z quench singlet excited Chl a (^{1}Chl a)?*

The quenching of the excited *^{1}Chl a** (the superscript “1” on the left of “Chl” means that we are talking about a singlet state, and the “*” means that it is in an excited state) by Z may not only occur by different mechanisms, but it may also involve different antenna complexes. Holt et al. (2005) showed that, in monomeric LHC proteins of PS II, quenching of Chl fluorescence can occur by means of excitation-energy transfer from excited Chl *a* dimer of the reaction center to the radical cation of Z (Z^+). The latter was suggested to act as a direct quencher of singlet Chl *a* excitation (see Ahn et al. 2008).

Another suggested mechanism involves conformational changes in the PS II antenna that cause de-excitation of ^{1}Chl a*, and this is suggested to be triggered by the protonation of the PsbS protein (see Ahn et al. 2008; Horton et al. 2008; Avenson et al. 2009). Still another proposed mechanism involves Z acting as an allosteric regulator, with Z altering the conformation of the LHC protein, enabling excitation energy transfer from ^{1}Chl* to L and leading to a loss of energy from ^{1}L* as heat (Pascal et al. 2005). For further discussion, see, e.g., Walla et al., Chap. 9, Pascal et al., Chap. 10, and Morosinotto and Bassi, Chap. 14.

The excitation-dissipative state of isolated LHCIIb crystals involves changes in the orientation of specific pigment molecules

Pascal et al. (2005) presented spectroscopic data on isolated LHCIIb crystals, and concluded that specific changes occur in the configuration of its pigment population that allow LHCII to regulate the flow of excitation energy in the system. For example, one of the possible quenching sites may be a pair of specific Chl a molecules (Chl a 611 and Chl a 612) along with lutein 620 (where the numbers indicate their positions in the crystal structure of LHCIIb; see also Pascal et al., Chap. 10).

Excitation energy dissipation by qE can occur via the transient formation of a Chl$^-$Z$^+$ biradical

Detecting ultrafast absorption changes, generated upon selective excitation at 664 nm of light-adapted (+qE) and of dark adapted (−qE) spinach thylakoids, Holt et al. (2005) established that the + qE state is associated with the transient appearance of a Z cation radical ($Z^{+\cdot}$) whose absorbance at 1000 nm ($\Delta A_{\sim 1000\ \mathrm{nm}}$) rises within ~10 ps (picoseconds, 10^{-12} s) and decays in ~150 ps. [This means that $Z^{+\cdot}$ is formed within 10 ps and then disappears since it must transfer its "+" charge to another intermediate within 150 ps.] A correlation of qE with formation of $Z^{+\cdot}$ was supported by the presence of ΔA at ~1000 nm in thylakoid preparations from wild-type *A. thaliana* and in qE-capable and Z-containing mutants (*npq2; npq2-Lutein2*) thereof; further, this interpretation is consistent with the absence of the $Z^{+\cdot}$ absorption difference signal in Z–minus (*npq1*) and PsbS–minus (*npq4-1*) *A. thaliana* mutants. In addition, charge transfer from Chl a^- to Z^+ (and/or L^+) has been shown to occur in minor antenna complexes (Lhcb4-6), but not in the major LHCII antenna complexes; see also Polivka and Frank, Chap. 8 and Walla et al., Chap. 9.

2006

qE is more pronounced in plants than in green algae

Studies on a green alga *Chlamydomonas reinhardtii* (Finazzi et al. 2006) revealed that its ability to generate NPQ is less pronounced than that of higher plants, and that $qT_{1\rightarrow 2}$, but not qE, contributes predominantly to the quenching in the alga. The diminished role of qE is due both to an intrinsically smaller ΔpH and to a limited capacity to translate it into a quenching response in this green alga. However, when photosynthesis is impaired, e.g., at low temperature (0 °C) or in mutants lacking Rubisco activity, the qE contribution increases, but its nature is different from that observed in higher plants, being characterized by a long-lived reaction center-based quenching that is inefficiently converted into an antenna-based quenching.

2007

Lutein 620 (Lut 1) may be the site of qE in isolated Lhcb antenna complexes

On the basis of the 2.72 Å resolution structure of the LHCII monomer (Liu et al. 2004), and of 77 K fluorescence emission spectra of LHCII in crystal and in solution, Yan et al. (2007) identified L620 (L at position 620 in the structure) as the likely site of qE. This conclusion assumes an intra-subunit conformational change in the LHCII monomer that leads to the formation of a heterotrimer consisting of Chl *a* 612, Chl *a* 611 (specific chlorophyll molecules as numbered in the atomic resolution structure) and L620 (also see Pascal et al. 2005). The close proximity of the π-electron clouds in the trimer enables transfer of excitation energy from Chls *a* to L where it is downgraded to heat. [We note that π (pi) electrons are those that are involved in π (pi) bonds; these are double bonds; see the *glossary*.

That intra-Lhcb conformational changes accompany the establishment of qE was also concluded by Ruban et al. (2007) from the application of optical spectroscopic methods. Resonance Raman spectra of light-treated (+qE) and dark-treated (−qE) LHCII preparations isolated from spinach enabled detection of light-induced conformational changes in the vicinity of the lutein binding site of LHCII monomers. By measuring femtosecond (fs, 10^{-15} s) transient absorption changes at selected wavelengths, it was established that Chl *a* transfers singlet excitation energy to lutein in isolated LHCII. These results led Ruban et al. (2007) to propose that conformational changes occurring under qE conditions bring Chl *a* 610, Chl *a* 611, and Chl *a* 612 closer to lutein and facilitate dissipation of excitation energy as heat.

Furthermore, using absorption, fluorescence excitation and fluorescence emission spectra, Johnson and Ruban (2009) observed red shifts in the spectral bands of Chl *a* and a corresponding shift in the L band in the excitation spectrum when LHCII complexes (both isolated and in-situ) were placed in the +qE state. These spectral shifts were interpreted to indicate a closer approach of the trimeric cluster pigments that enables the emitting Chl *a* to transfer excitation energy to *Lut 1* where it is downgraded to heat.

Using absorption and circular dichroism spectroscopy (differential absorption by left and right circularly polarized light, a measure of the structure of proteins) to examine isolated wild-type and mutagenized Lhcb complexes, Mozzo et al. (2008) established that identical interactions occur in the Chl *a* 611/Chl *a* 612/L620 pigment cluster of the LHCII, CP29 and CP26 antenna proteins of PS II (but not in CP24), indicating that qE may occur indiscriminately in nearly all the light-harvesting subunits.

qE in isolated LHCII cannot be accounted for solely by replacement of violaxanthin by zeaxanthin

Amarie et al. (2007) observed that replacing V by Z in isolated LHCII samples has no effect on the lifetime of excited Chl *a*, although both $V^{\cdot+}$ and the $Z^{\cdot+}$ carotenoid cation radicals are detectable by means of resonance two-color, two-photon spectroscopy.

Zeaxanthin may have other functions besides that in qE: it is also an antioxidant

Havaux et al. (2007) examined the antioxidative activity of Z and other xanthophylls with a Chl *b*-less and LHCII-deficient *Arabidopsis* mutant (*ch1*) that has a very low qE capacity. Double mutants with different xanthophyll compositions were obtained by crossing *ch1* with xanthophyll mutants. This study showed that, under high light, suppression of Z (in the *ch1 npq1* double mutant) increased photo-oxidative stress relative to that in the mutant *ch1*, while constitutive accumulation of Z (in the *ch1 npq2* mutant) increased phototolerance. Further, the protection of polyunsaturated lipids by Z was enhanced when L was present. Suppression of other xanthophylls, or of PsbS-dependent qE, did not increase photo-oxidative stress. Havaux et al. (2007) concluded that the antioxidant activity of Z (distinct from its function in qE) is higher than that of all other xanthophylls in *Arabidopsis*, and that it occurs even in the absence of LHCII. For further information, see Havaux and García-Plazaola, Chap. 26.

The qE mechanism in brown algae differs from that of green algae and plants

Comparing the brown alga *Macrocystis pyrifera* with the plant *Ficus* sp., García-Mendoza and co-workers (see, e.g., García-Mendoza et al. 2011; Ocampo-Alvarez et al. 2013) concluded that this brown alga displays only a slower (presumably Z-dependent) component of qE and lacks a faster ΔpH-dependent (and presumably Z independent) component. In this alga, the strong light-induced ΔpH serves only to activate VDE and to convert V to Z and has no other effect on the excitation energy of Chl *a*. Several other brown algae are known to behave in a similar manner (Rodrigues et al. 2002; Fernandez-Marin et al. 2011). A comparison of these results with those of green algae and plants suggest that the VAZ cycle may have preceded the evolutionary appearance of brown algae. On the other hand, Chl *c*-containing diatoms display both xanthophyll-independent and xanthophyll-dependent components of qE (Goss and Jakob 2010). Further experiments and analysis is needed before any picture of evolution of qE mechanism will be available.

2009

Lutein can replace zeaxanthin as the quencher of singlet excited Chl a (^{1}Chl a) in qE of a Z-deficient Arabidopsis mutant*

Using a Chl *a* fluorescence video imaging system, Li et al. (2009) isolated suppressors of the *npq1* mutant of *Arabidopsis* (lacking Z) that shows a much higher qE capacity compared to the *npq1* parent. One of these suppressors (named suppressor of Z–less1, *szl1*) had a very small xanthophyll cycle pool size, and instead accumulated twice as much L. Wild-type, *npq1*, *szl1*, and *szl1npq1* plants showed that L, in higher amount, was able to substitute for Z in qE in this Z-deficient mutant. Li et al. (2009) interpreted these results by suggesting that L cation radicals also play a direct role in the dissipation of Chl *a* excitation by qE in Z-deficient mutants.

A common mechanism may underly zeaxanthin-dependent and zeaxanthin-independent components of qE in various mutants

To address the question whether both Z and L quench singlet excited Chl *a* independently, operating at separate sites, or whether L is the quencher and Z only an allosteric regulator that modifies only the conformation of the of Lhcb protein, Johnson et al. (2009) compared qE-related conformational changes (ΔA535) in anti-sense *Arabidopsis* plants with altered contents of the minor and the major antenna complexes. Data on conformational changes within an Lhcb protein, on Lhcb-Lhcb interactions, and on the effects of externally and internally bound xanthophylls suggested that both Z-dependent and the Z-independent components of qE may arise from a common quenching mechanism based on intra-Lhcb conformational changes, inter Lhcb-Lhcb interactions, and on internally-bound xanthophylls.

Algae employ different LHC proteins as NPQ effectors than plants

Peers et al. (2009) constructed the qE-deficient mutant *npq4* of *Chlamydomonas reinhardtii* that could grow normally under weak light, but was inhibited under strong light. The *npq4* mutant lacked a specific *Light-Harvesting Complex Stress-Related* (LHCSR) protein, known also as the LI818 protein. According to Bonente et al. (2010), LHCSR protein serves both as lumen-acidity sensor and as Chl *a* excitation energy dissipator in *Chlamydomonas*. Unlike the PsbS protein, the pigment-free lumen acidity sensor of plants (Li et al. 2004), the LHCSR protein binds Chl *a*/Chl *b* and xanthophylls. The very short Chl *a* fluorescence lifetime ($t < 100$ ps) of LHCSR strongly implies that it may function as an excitation-energy quenching center. Quenching was proposed to proceed via transient formation of an L radical cation, instead of the Z radical cation formed in plants, suggesting that L, not Z, is important for qE in this green alga. In addition, Niyogi and Truong (2013) have shown that, as a result of phosphorylation by the STT7 kinase, LHCSR moves from PS II-rich regions to PS I-rich regions of the thylakoid membrane, leading to state 1-to-state 2 transition.

In the marine diatom *Thalassiosira pseudonana*, two light stress-related proteins have been described, Lhcx1 and Lhcx6, both closely related to the LHCSR protein of *Chlamydomonas* (Zhu and Green 2010). The Lhcx1 protein accumulates in both light-stressed and unstressed cells, and its amount is unrelated to the Dd content of the cell. The Lhcx1 protein is presumed to have a role in assembly and stability of thylakoid membranes. The Lhcx6 protein accumulates only in strong light, and in parallel to a rise in the Dd content, and is thus suggested to have a role in the Dd-Dt cycle of diatoms. An Lhcx1 protein, with the same properties, was described in the diatom *Pheodactylum tricornutum* by Bailleul et al. (2010).

2010

An alternative suggestion is that qE occurs via Chl: Chl interactions and does not involve xanthophylls

Using an extremely fast (femtosecond) absorption spectrophotometer, Müller et al. (2010) studied ultrafast dynamics in isolated LHCII oligomers obtained from the *Arabidopsis thaliana* mutants *npq1* and *npq2*. The data were described kinetically by a two-state compartment model assuming that only Chl-excited states (^{1}Chl a^*) are involved in the quenching process. These results challenge earlier proposals, in which excitation-energy dissipation in Chl *a* in LHCII was assumed to involve direct energy transfer to carotenoids (e.g., ^{1}Chl $a^* \rightarrow$ Z, Frank et al. 2001; or ^{1}Chl $a^* \rightarrow$ L, Ruban et al. 2007). A Chl – Chl charge-transfer state is suggested in this paper to play an important role in the quenching process. For further information, see Holzwarth and Jahns, Chap. 5. However, further research is needed to reach a firm conclusion, especially to find if different mechanisms may operate in different systems, such as mutants with different pigment composition versus wildtype, isolated complexes versus intact leaves, different species with different NPQ features, and plants grown in the absence or presence of regular exposure to clearly excessive light.

May lutein epoxide (Lx) be involved in qE?

Leaves of the tropical plant species avocado (*Persea americana*) contain high concentrations of Lx in addition to V, A, and Z. Like V, Lx is also de-epoxidized in the light. In contrast, however, to Z and A that are rapidly epoxidized in darkness, L is epoxidized only slowly, such that illumination of avocado leaves gives rise to a persistent pool of photoconverted L (ΔL). Matsubara et al. (2011) showed in avocado leaves that qE is correlated with the sum of L, Z and A, while Förster et al. (2011) analyzed the qE development in avocado in three phases: **(i)** before Lx de-epoxidation, **(ii)** after Lx de-epoxidation, and **(iii)** after A and Z epoxidation but in the presence of the persistent ΔL pool. For further details, see the reviews by Jahns and Holzwarth (2012), Holzwarth and Jahns, Chap. 5, and Esteban and García-Plazaola, Chap. 12.

B State Transitions

1967

Pre-illumination of algae and cyanobacteria changes the spectrum and yield of Chl a fluorescence

Papageorgiou and Govindjee (1967, 1968a, b) reported that pre-illumination of a cyanobacterium, *Anacystis nidulans*, and of a green alga, *Chlorella pyrenoidosa*, with light mainly absorbed by PS II or by PS I causes characteristic changes in the yield and spectrum of Chl *a* fluorescence of these organisms (also see Mohanty et al. 1970). These changes were attributed to conformational modifications of the pigment-protein complexes in the thylakoid membrane.

1968

Algae adapt to the color of ambient light by shifting excitation energy to go to the less favored photosystem

As discussed earlier, the regulatory adaptation of photosynthetic organisms to the quality of ambient light was independently discovered by Murata (1969, 1970) and by Bonaventura and Myers (1969; also see Bonaventura 1969) who named this phenomenon *light state transition* or, simply, *state transition*. At 77 K, photosynthetic samples give rise to three major fluorescence emission bands: at 685 nm (F685, which originates from CP43 of PS II), at 696 nm (F696, which originates from CP47 of PS II) and at 720–740 nm (F720-740, which originate mostly in PS I) (see Fig. 1.1 and chapters in Govindjee et al. 1986, Papageorgiou and Govindjee 2004 and the review by Papageorgiou 2012, and citations therein). When a photosynthetic sample is excited with light absorbed in PS I, one observes a high ratio of F720-740/F685-F696; this ratio is lower when one excites with PS II light. From these ratios, we can thus estimate the fraction of excitation energy going to PS II or PS I. Murata (1969) observed that pre-illumination of the red alga *Porphyridium cruentum* with Light 2 shifts excitation energy from going to PS II to PS I (labeled as state 1 to state 2 transition). Conversely, upon pre-illumination with Light 1, *P. cruentum* shifts excitation energy from going to PS I to PS II (state 2 to state 1 transition). Measuring photosynthetic O_2 evolution and Chl *a* fluorescence in the green alga *Chlorella pyrenoidosa*, Bonaventura and Myers (1969) estimated that after illumination with Light 2 less excitation energy is delivered to PS II (state 2) than after illumination with Light 1 (state 1). Both research groups suggested that these light quality adaptations improve the efficiency of photosynthesis in weak light.

1977

In plants, state transitions relate to reversible light-induced phosphorylation of LHCII

Bennett (1977) was the first to report light-dependent and dark-reversed phosphorylation of thylakoid membrane proteins. When isolated pea chloroplasts are illuminated, several thylakoid membrane proteins become phosphorylated, with the most conspicuous being the Chl*a*/Chl*b*-binding LHCII complex. Light-induced phosphorylation of LHCII was subsequently found by Bennett et al. (1980) to decrease the amount of excitation energy that goes to PS II and to increase that going to PS I (as inferred from fluorescence spectra at 77 K), to be inhibited by DCMU and to be insensitive to protonophoric uncouplers. Bennett et al. (1980) concluded that phosphorylated LHCII alters the properties and the interactions of PS II and PS I, such as to redistribute excitation energy in favor of PS I.

1981

The redox state of the PQ-pool regulates the light-induced phosphorylation of LHCII

The reduction of the PQ pool by PS II activates a membrane-bound kinase that phosphorylates LHCII and, thereby, causes a redistribution of excitation energy in favor of PS I (state 2). Oxidation of PQH_2 to PQ by PS I inactivates the kinase, phosphorylated-LHCII is de-phosphorylated by a phosphatase, and excitation energy is redistributed in favor of PS II (state 1; Allen and Bennett 1981; Allen et al. 1981). The critical role of the PQ-pool in state transitions was also inferred from the fact that electron donors to PS I do not reverse LHCII phosporylation-dependent quenching of chloroplast fluorescence (Horton and Black 1981). Similar to plants, light-induced phosphorylation of LHCII was shown to accompany the transition from state 1 to state 2 in the green alga *Chlorella vulgaris* (Saito et al. 1983).

1983

Lateral mobility of phosphorylated LHCII complexes

Using freeze-fracture electron microscopy, Kyle et al. (1983) detected that the light-induced phosphorylation of LHCII causes a partial de-stacking of the grana accompanied by a redistribution of LHCII particles from the PS II-rich grana to the PS I-rich stroma lamellae.

1984

Is protein photophosphorylation required for state 1-to-2 transition of red algae?

According to Biggins et al. (1984), the light-induced protein phosphorylation patterns of thylakoid membrane proteins in state 1 and state 2 *Porphyridium cruentum* were the same. Thus, in this alga, protein phosphorylation was not implicated in state 1-to-state 2 transition. Only energy *spillover* seems to regulate the redistribution of excitation energy between PS II and PS I.

1988

Cyt b_6f is indispensible for state 1 to state 2 transition in Chlamydomonas reinhardtii

Wollman and Lemaire (1988) obtained evidence that, whereas a *Chlamydomonas reinhardtii* mutant lacking the proteins of the core complex of PS II was fully capable of state 2 to state 1 transition, a mutant lacking the Cyt b_6f complex was locked in state 1.

1991

The state 1-to-state 2 transition shifts not only LHCII complexes but also the Cyt b_6f complex from grana thylakoids to stroma thylakoids

Experimenting with maize chloroplasts and *Chlamydomonas* cells, Vallon et al. (1991) found that a transition to state 2 shifts not only LHCII complexes but also the Cyt b_6f complex from PS II-rich grana thylakoids to PS I-rich stroma thylakoids. Thus, state 1-to-state 2 transition should not be viewed simply as a light-adaptation mechanism for optimizing non-cyclic PSET; it should also be regarded as a re-routing of PSET from the non-cyclic to the cyclic path to enable cells to meet changes in ATP demand. Importantly, the state 1-to-2 transition in *Chlamydomonas* also involves a shift of the pH-sensing and excitation-energy dissipating LHCSR protein to PS I, suggesting the likelihood of qE operating in PS I-associated LHC antenna (Niyogi and Truong 2013).

Compelling evidence that state 1-to-state 2 transition involves a shift from non-cyclic PSET to cyclic PSET was obtained by Finazzi et al. (1999, 2002), who showed that DCMU inhibits the turnover of Cyt b_6f in state-1 *Chlamydomonas* cells, but not in state-2 cells. Further, the shift of LHCII from PS II to PS I is indeed linked to a shift from non-cyclic PSET to cyclic PSET since an LHCII-minus mutant of *Chlamydomonas* was unable to perform it (see reviews by Finazzi and Forti 2004 and Minagawa 2011).

1997

A reduced PQ-pool is not sufficient to activate the LHCII kinase; it must be sensed and transduced by Cyt b_6f: Studies with Chlamydomonas reinhardtii

According to experiments with *C. reinhardtii* by Vener et al. (1997), the kinase that phosphorylates LHCII is activated by the binding of one plastoquinol (PQH_2) molecule to the quinol-binding site of Cyt b_6f (i.e., Q_0). [We remind the reader that PS II reduces PQ to PQH_2 that is oxidized back to PQ by Cyt b_6f (see Fig. 1.1).] When PQH_2 is replaced with 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, a reduced PQ-pool fails to activate the kinase. The kinase is also inactive after oxidation of bound PQH_2 by a single, light flash-induced turnover of PS I. It was further established that binding of PQH_2 at the Q_0 site of Cyt b_6f causes a structural change in the Rieske Fe-S protein of the complex (Carrell et al. 1997; Zhang et al. 1998; Breyton 2000; reviewed by Vener et al. 1998; Wollman 2001; Finazzi and Forti 2004).

What forces are behind the diffusion of LHCII from PS II to PS I

Allen and Nilsson (1997) used a synthetic polypeptide with identical sequence to the N-terminus of LHCIIb (where threonine at position 5 is phosphorylated) and found (by circular dichroism spectroscopy) that phosphorylation entails a structural change in the synthetic peptide. From this result, Allen and Nilsson (1997) proposed that a photophosphorylation induced structural change reduces the affinity of phosphorylated-LHCII for PS II, causing it to diffuse away toward PS I.

2000

In the state 1-to-state 2 transition, phosphorylated LHCII subunit docks directly on the H subunit of the core PS I complex

Using transgenic *Arabidopsis* mutants lacking specific subunits of PS I, Lunde et al. (2000) established that, when subunit PS I-H and, to a lesser extent, subunit PS I-L were missing, state 1 to state 2 transition was impaired. Further, Lunde et al. (2000) proposed that migrating phospho-LHCII complex docks on the PS I-H on the side of the PS I core complex, opposite to the side where the LHCI proteins are attached. For a review, see Haldrup et al. (2001).

2002

Identification of LHCII-specific kinases in Chlamydomonas and Arabidopsis

LHCII-specific kinases have been identified in the green alga *Chlamydomonas* (stt7; Depège et al. 2002) as well as in *Arabidopsis* (STN7; Bellafiore et al. 2005). Mutants deficient in these kinases were locked in state 1, unable to phosphorylate the LHCII proteins, and unable to drive their translocation or the translocation of the Cyt b_6f complex from the PS II-rich region of the thylakoid membrane to the PS I-rich region.

2006

Isolation and characterization of state 1- and state 2-specific PS II and PS I super-complexes from Chlamydomonas

Employing centrifugal and chromatographic methods for protein isolation, immunoblotting for composition analyses, and spectroscopy for activity assays, Jun Minagawa and co-workers (see below) demonstrated that the molecular mass and the composition of PS II and PS I super-complexes, isolated from *Chlamydomonas*, depend on whether they had been derived from state 1-adapted or state 2-adapted cells. These differences relate to migration of light-harvesting proteins CP29, CP26, LhcbM5 as well as of trimeric LHCIIs from PS II-rich appressed lamellae to PS I-rich unappressed lamellae as a result of a state 1 to-state 2 transition. State 2 cells thus have a smaller PS II super-complex than state 1 cells, whereas the same cells have a larger PS I super-complex than state-1 cells (Takahashi et al. 2006; Iwai et al. 2008). Further, using fluorescence lifetime microscopy to identify PS II-bound

LHCII from its characteristic lifetime of Chl *a* fluorescence (170 ps) and free phospho-LHCII from its characteristic fluorescence lifetime (270 ps), Iwai et al. (2010a) succeeded in visualizing dissociation of phosphorylated-LHCII from PS II in live *Chlamydomonas* cells. Further, Iwai at al. (2010b) isolated a 1.4–1.6 MDa PS I supercomplex from state-2 *Chlamydomonas*, which consisted of PS I, LHCI, LHCII, Cyt b_6f, Ferredoxin NADP$^+$ Reductase (FNR) and the integral membrane protein PGRL1. This super-complex was shown to perform cyclic PSET upon illumination; electrons on the electron-acceptor end of PS I were transferred to Cyt b_6f, and from Cyt b_6f back to PS I. For recent reviews, see Minagawa et al. (2011), Papageorgiou and Govindjee (2011), and Mohanty et al. (2012); also, Finazzi and Minagawa, Chap. 21.

VI Concluding Remarks

Until about the middle of the twentieth century, scientific research had focused on how plants optimize the use of absorbed light for photosynthesis. Starting in the 1960s, it became evident that how plants dispose of the excess of absorbed light was also an intellectually challenging and practically relevant pursuit. In the course of the following 50 years, intensive research led to formulation and refinement of new ideas and concepts on how oxygenic photosynthetic organisms perceive and assess any absorbed light in excess of their needs, and on how they remove it non-photochemically to avoid any photo-oxidative damage. In fact, the existence of multiple regulatory mechanisms, at the individual cell level as well as at the level of the whole plant (see, e.g., Logan et al., Chap. 7), that control the allocation of absorbed light to photosynthesis versus non-photochemical removal, is one of the key targets of photosynthesis research today.

The most widely applied technique that allows quantitative estimation of the non-photochemically disposed fraction of absorbed light has been the *pulse amplitude modulation* method (PAM) of Ulrich Schreiber and co-workers (see Schreiber 2004; Logan et al., Chap. 7, and citations therein). Another widely applied method to detect non-photochemical dissipation of the excitation energy of Chl *a* is fluorescence induction (FI), namely the kinetic trace of the Chl *a* fluorescence of a dark-adapted sample after transfer to continuous exciting illumination. The FI trace responds to both the qE and qT variants of NPQ and, historically, has been the method that led to the discovery of the regulated non-photochemical quenching of Chl *a* fluorescence (Papageorgiou et al. 2007; Papageorgiou and Govindjee 2011). For a relationship of slow fluorescence changes (termed P-to-S decay) with ΔpH, which is related to NPQ, see Briantais et al. (1979). For recent applications and theoretical treatments of this method, see Kaňa et al. (2012), and Stirbet and Govindjee (2011, 2012).

Of the many revelations from NPQ research (and which are detailed in this volume), perhaps the most central is the discovery of the multifunctionality of the LHC proteins and of the xanthophylls they bind. While formerly, LHC proteins were assigned only photon harvesting and excitation-energy supplying roles, it has now become apparent that they also sense, assess and degrade excess excitation energy to heat that is dissipated to the surroundings before it can be transferred to core complexes and reaction centers. Likewise, while formerly LHC xanthophylls were assigned only anti-oxidant and structural roles, and with no particular distinction for the epoxidic xanthophylls, it is now apparent that the latter are involved in enzyme-catalyzed, light-induced and dark-reversed transformations that convert an LHC protein from an excitation-energy supplying conformation to an excitation energy-dissipating conformation (see, e.g., Ruban and Mullineaux, Chap. 17).

We also note also that a qE-dependent, but xanthophyll-independent, dissipation of the excitation energy of Chl *a* has been suggested to occur in the core PS II complexes of plants (Finazzi et al. 2004, 2006; Ivanov et al. 2007). In contrast to the qE effects in peripheral LHC antenna, which materialize only under strong light, the pure qE effects in the PS II core complexes occur in weak light. Recently, qE of Chl a fluorescence has been shown to occur in the PS II core complexes of cyanobateria (Stamatakis and Papagerogiou 2014). The difficulty in detecting qE in cyanobacteria arises from the state 2-to-state 1 transition (and the resulting fluorescence rise), which masks qE effects (Papageorgiou et al. 2007).

Acknowledgments

We thank Sandra Stirbet for providing Figs. 1.1 and 1.2 and their legends, and Dima Shevela for providing Fig. 1.3 and its legend. In addition, Sandra Stirbet has generously checked our references with reference list and vice versa. We thank Barry Logan for helping us improve our chapter, particularly by suggesting addition of explanations of technical terms, and Beverly Green for providing evolutionary insights for the green, red and brown algae. We are thankful to William W. Adams III for his help during proof-reading of this chapter.

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Chapter 2

Lessons from Nature: A Personal Perspective

William W. Adams III* and Barbara Demmig-Adams
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

Summary

This chapter highlights selected contributions to photosynthesis research made from an evolutionary and ecological perspective and, specifically, to the characterization of zeaxanthin-associated thermal energy dissipation. First, contributions of comparative ecophysiology to the discovery of different CO_2 fixation pathways are examined, followed by a summary of the historical developments leading to documentation of the relationship between zeaxanthin and photoprotective energy dissipation. Evergreen species exhibit exceptionally strong non-photochemical quenching of chlorophyll fluorescence (NPQ) and

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*Author for Correspondence, e-mail: william.adams@colorado.edu; barbara.demmig-adams@colorado.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_2,

very high levels of zeaxanthin formation. This enabled an unveiling of the correlation between zeaxanthin versus NPQ and/or photosystem II quantum efficiency (as inferred from the ratio of variable to maximal fluorescence, F_v/F_m), even prior to development of technology currently used in the assessment of these features. Results from characterization of the wide variety of different manifestations (with respect to extent and/or kinetics) of the conversion of xanthophylls, and changes in NPQ and/or F_v/F_m in different plant species and diverse environments are placed in an evolutionary and ecological context. Lastly, themes emerging from the international research community on NPQ and photoprotective thermal dissipation are summarized, and suggestions presented for how utilization of plants genetically adapted and acclimated to high levels of light stress may aid in addressing open questions.

I Introduction

The placement of photoprotective energy dissipation into a context of the ecology and evolution of photosynthetic organisms has made contributions to the understanding and relevance of the non-photochemical quenching of chlorophyll fluorescence (NPQ), and continues to do so today as reflected in many of the chapters that follow. An ecological and evolutionary perspective examines the employment of thermal dissipation in different organisms under diverse environmental conditions and addresses question such as: *What is the variation on the theme of photoprotective energy dissipation available to an organism and to different organisms? What parts of the system are flexible? For which of a myriad of different environmental niches is a particular variation of the theme most suited? What are the different ways of surviving in a challenging environment and how does this influence the employment of energy dissipation by different organisms? What are the costs and benefits (trade-offs) of the different variations?*

II Standing on the Shoulders of Giants

A Comparative Ecophysiology

1 Adaptation and Acclimation to the Environment

The field of comparative plant ecophysiology has provided insights into the genetic adaptations and individual flexibility (phenotypic plasticity or acclimation potential) crucial to plant survival, productivity and reproductive success. Such a comparative approach identifies features underlying the success of different species in the same habitat, multiple species across different habitats, and the same species in different habitats (Osmond et al. 1980; Lange et al. 1981a, b, c, d; Baker and Long 1986; Mooney et al. 1987; Mooney 1991; Schulze and Caldwell

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Abbreviations: CAM – Crassulacean acid metabolism; Car – Carotenoid; Chl – Chlorophyll; ELIP – Early light inducible protein; F_m, F_m' – Maximal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_o, F_o' – Minimal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_v, F_v' – Variable chlorophyll fluorescence in the dark- ($F_m - F_o$) and light-adapted ($F_m' - F_o'$) state, respectively; F_v/F_m, F_v'/F_m' – Interpreted to be intrinsic efficiency (or quantum yield) of photosystem II in the dark and light-adapted state, respectively; hECN – 3-hydroxy-echinenone as a xanthophyll bound to the orange carotenoid protein of cyanobacteria; HLIP – High light-inducible protein; HPLC – High-pressure liquid chromatography; LHC – Light-harvesting complex; LHCII – Light-harvesting complex of photosystem II; LHCSR – Light-harvesting complex stress-related; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OCP – Orange carotenoid protein of cyanobacteria; PAM – Pulse-amplitude-modulated (chlorophyll fluorometry); PS II – Photosystem II; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin (V) antheraxanthin (A), and zeaxanthin (Z)

1994; Larcher 2003). Some of the pioneering work in this area involved characterization of plants subjected to reciprocal transplants between contrasting habitats as well as the growth and characterization of species from different habitats under "common garden" conditions (Clausen et al. 1941, 1947; Hiesey et al. 1942; Clausen and Hiesey 1960; Mooney and Billings 1961; Mooney and Johnson 1965). Such experiments allow for identification of adaptations among plant species and varieties as well as the individual's acclimation potential to a changing environment (for a study on the model species *Arabidopsis thaliana*, see Ågren and Schemske 2012). Comparative plant ecophysiology, furthermore, links the fields of plant molecular biology and physiology to whole-plant function and its role in communities, ecosystems, and the biosphere (Feder 2002; Beyschlag and Ryel 2007).

The evolutionary history of different plant species is reflected in their genetic *adaptations* as having provided advantages for survival and reproduction in the habitats where the respective species successfully evolved. Such traits improved plant performance with respect to the physical environment (e.g., soil conditions, climatic factors, etc.) as well as biological (e.g., herbivory, infection by viruses and fungi, etc.) challenges faced over multiple generations. In addition, the individual *acclimation* potential allows those genetic traits to be adjusted to a certain extent. For instance, as a winter annual adapted to germinate in the fall and form a rosette of overwintering leaves, followed by bolting, flowering, and seed set in the spring, *A. thaliana* grown under controlled cool versus warm temperatures develops thicker leaves with more layers of chloroplast-packed palisade mesophyll cells (Gorsuch et al. 2010; Cohu et al. 2014), higher rates of photosynthesis (Gorsuch et al. 2010; Cohu et al. 2013b, 2014), and leaf veins with more (phloem) cells for exporting sugars produced in photosynthesis (Cohu et al. 2013a, b, 2014). Such morphological and physiological plasticity within individuals represents *acclimation*.

There are, however, limits to the acclimatory range of the individual. For instance, leaf veins develop with a greater number of sugar-exporting (phloem) cells in support of higher rates of photosynthesis in *A. thaliana* plants *adapted* to the Swedish climate (Swedish ecotype) compared to plants adapted to the Italian climate (Italian ecotype) irrespective of growth temperature (Cohu et al 2013a, b). In contrast, leaf veins develop more water-transporting (xylem) cells in response to growth under warmer versus cooler temperatures in the Italian but not the Swedish ecotype (Cohu et al. 2013b). The Italian ecotype, based on its evolutionary history in the warmer and drier climate of Italy, is thus adapted to respond to growth at warm temperature through an acclimatory response that can increase water delivery to the leaves. On the other hand, such a response of water-transporting cells was presumably not advantageous in the moister and colder climate of Sweden, while the adaptation underlying the ability to upregulate the capacity to export photosynthetically generated sugars in the face of lower temperatures (and greater phloem sap viscosity) is important for plants growing in Sweden compared to Italy.

2 *Pioneers of Comparative Ecophysiology*

The mentors of the authors (B.D.-A. and W.W.A.) were Olle Björkman and C. Barry Osmond, respectively; they both conducted pioneering comparative plant ecophysiological work on features important to the adaptation and acclimation of plants to different environmental conditions. This included leaf and photosynthetic acclimation to the light environment among plants adapted to shaded versus sun-exposed habitats (Björkman and Holmgren 1963, 1966; Björkman 1968; Björkman et al. 1973; Osmond 1983), photosynthetic adaptation and acclimation to temperature (Björkman et al. 1972; Armond et al. 1978; Mooney et al. 1978; Berry and Björkman 1980; Badger et al. 1982), characterization of plants with different photosynthetic pathways (Osmond 1967, 1970, 1971, 1974, 1978;

Björkman and Gauhl 1969; Woo et al. 1970; Kluge and Osmond 1971, 1972; Osmond and Harris 1971; Björkman et al. 1973; Osmond et al. 1973, 1980; Ehleringer and Björkman 1977), responses of plants to salinity stress (Osmond 1966; Greenway and Osmond 1972; Osmond and Greenway 1972), and responses of plants to excess light (Powles and Osmond 1978; Powles et al. 1979, 1983; Osmond 1981; Powles and Björkman 1982; Björkman and Powles 1984).

We will use the discovery and characterization of three different photosynthetic pathways, C3, C4, and crassulacean acid metabolism (CAM) to illustrate the evolution of adaptations allowing plants to diversify and colonize multiple niches in the natural world (for historical and basic aspects of C3 photosynthesis, see Benson 2005 and Bassham 2005; for C4, see Hatch 2005, and for CAM, see Black and Osmond 2005.) In a nutshell, the latter photosynthetic pathways offer distinct advantages in some environments at the cost of disadvantages in other environments, resulting in unique benefits and costs (trade-offs). Remarkably, all three pathways share the ancestral feature of fixing carbon into sugars in much the same way, while C4 and CAM plants employ an additional cycle of fixing atmospheric CO_2 and then concentrating it for use by the C3 pathway of photosynthesis in the chloroplast. In the case of C4, this additional cycle operates during the day in the leaf mesophyll cells and shuttles the CO_2 to specialized cells surrounding the veins where only the C3 pathway operates. In the case of CAM, the additional cycle operates during the night, storing the fixed CO_2 in the vacuoles for utilization in the C3 pathway during the subsequent day (largely behind stomata that remain closed because of the high internal levels of CO_2 that arise as it is released for use by the chloroplasts in the light).

The evolution of variation in the pathway for CO_2 acquisition presumably aided in the exploitation of many diverse habitats. Compared to C3 plants, plants utilizing the C4 and CAM pathways of photosynthesis typically require less nitrogen, exhibit little to no photorespiration, are more efficient at capturing CO_2, and consequently typically lose less water to the atmosphere for a given level of CO_2 fixation compared to plants utilizing the C3 pathway of photosynthesis (Osmond 1978; Winter 1985; Larcher 2003; Raghavendra and Sage 2011). On the other hand, plants using C4 and CAM have increased energetic costs associated with the use of an additional carboxylation pathway and of specialized anatomical features (modified leaf venation in C4 plants and succulent cells with large vacuoles in CAM; Björkman et al. 1973; Osmond et al. 1980; Edwards and Walker 1983; Winter 1985; Hatch 1992; Larcher 2003; Silvera et al. 2010; Raghavendra and Sage 2011; Sage and Zhu 2011). Because of their highly efficient fixation of CO_2, some CAM species are able to thrive in aquatic habitats with very low CO_2 levels (Keeley 1998) and to fix CO_2 through tissues without stomatal leaf pores (Keeley et al. 1984; Cockburn et al. 1985; Winter et al. 1985). Furthermore, there are species with intermediate levels of C4 or CAM features (Holaday et al. 1981; Ku et al. 1983; Harris and Martin 1991a, b; Leegood and von Caemmerer 1994; Lüttge 2006), as well as others exploiting a greater water availability during spring as a C3 plant and transitioning to CAM developmentally and, most importantly, during the hot and dry summer (Winter et al. 1978, 2008; Winter 1985). Such variation in plant form and function among species, and even within a species, has allowed plants to thrive in an expanded range of ecological niches (specific environments) with, e.g., low levels of soil nitrogen and/or water availability. Each set of photosynthetic adaptations has its own trade-offs, or costs versus benefits, for a particular growing condition.

In addition to the three major photosynthetic pathways, many other anatomical, morphological, physiological, and developmental plant features impact plant success. For instance, in the hot deserts of North America (Mojave, Sonoran, and Chihuahuan) all three pathways (C3, C4, CAM) are found. In locations or during seasons with continuous access to water, C4 plants thrive under very hot conditions, with extremely high rates of photosynthesis (Björkman et al. 1972; Larcher 2003; Sage and Kubien 2007). In deserts where water is periodically available, water-storing CAM plants (e.g., succulent cacti, agaves, yuccas, and euphorbs) are found (Kluge and Ting 1978; Osmond 1978; Winter 1985). C3 plants are also remarkably well represented in these deserts, as (i) extremely fast developing "ephemerals" (with high rates of growth and photosynthesis, and rapid seed set following, e.g., intense rainfall events; Patten 1978), (ii) perennials leafing out during the respective moister seasons (Mooney and Strain 1964; Nedoff et al. 1985; Yoder and Nowak 1999), (iii) as plants like palms or mesquite with deep roots tapping into the permanent water table (Nilsen et al. 1983, 1984; Sala et al. 1996; Hultine et al. 2003, 2005), and (iv) as slow-growing evergreens with low rates of photosynthetic water loss, such as the Joshua tree (*Yucca brevifolia*) and creosote bush (dominant shrub of all three North American hot deserts; Armond et al. 1978; Mooney et al. 1978; Smith et al. 1983). All species are similarly well adapted to these hot deserts, yet each by virtue of its own particular adaptation and advantage (that might be a disadvantage in other habitats, particularly in competition with other species adapted to the other habitats). Regardless of the particular photosynthetic pathway employed, all of these species share common features of photosynthetic electron transport and carbon fixation in the Calvin-Benson cycle (C3 photosynthetic pathway in the chloroplast), as well as zeaxanthin-associated photoprotective energy dissipation.

B Prerequisite Contributions to Linking Thermal Dissipation and Zeaxanthin from Biochemistry and Physics

1 Pioneers in the Discovery of Rapid Xanthophyll Conversions in Plants

In addition to possessing chlorophyll, photosynthetically competent chloroplasts typically contain several carotenoids, including oxygen-containing xanthophylls (neoxanthin, violaxanthin, lutein) and oxygen-free carotenes (β-carotene and, for some species, α-carotene). The levels of most of these chloroplast pigments are relatively static over short periods of time. However, in the late 1950s it was discovered that several xanthophylls are inter-converted over short time spans (minutes) in response to changes in light level (reviewed in Sapozhnikov 1973). This observation was followed by intensive investigation of the biochemical factors controlling de-epoxidation of violaxanthin (with two epoxide groups), via the intermediate antheraxanthin (with one epoxide group) to zeaxanthin (epoxide-free), as well as factors responsible for re-epoxidation of zeaxanthin to violaxanthin (reviewed in Yamamoto 1979, 2006; Hager 1980; Yamamoto et al. 1999). This cyclical interconversion of the latter three xanthophylls became known as the violaxanthin cycle or xanthophyll cycle (here referred to as the VAZ cycle, where V, A and Z stand for violaxanthin, antheraxanthin and zeaxanthin, respectively, for distinction between the VAZ cycle and two other xanthophyll cycles present in some plants and algal groups; see, e.g., Esteban and García-Plazaola, Chap. 12 and Lavaud and Goss, Chap. 20). While it was known (i) that these xanthophyll conversions occurred in the chloroplast, (ii) that zeaxanthin levels increased under high light conditions, and (iii) that zeaxanthin was converted back to violaxanthin under low light, a specific function for the cycle and these carotenoids remained elusive until the late 1980s.

2 History of Chlorophyll Fluorescence in Photosynthesis

The reader is referred to other chapters (Papageorgiou and Govindjee, Chap. 1, Horton, Chap. 3, and Logan et al., Chap. 7) in this volume for detailed accounts of the historical developments in the measurement and understanding of chlorophyll fluorescence emission and factors influencing the level of fluorescence emission. For further information on the relation of chlorophyll fluorescence to photosynthesis, we refer the reader to books edited by Govindjee et al.

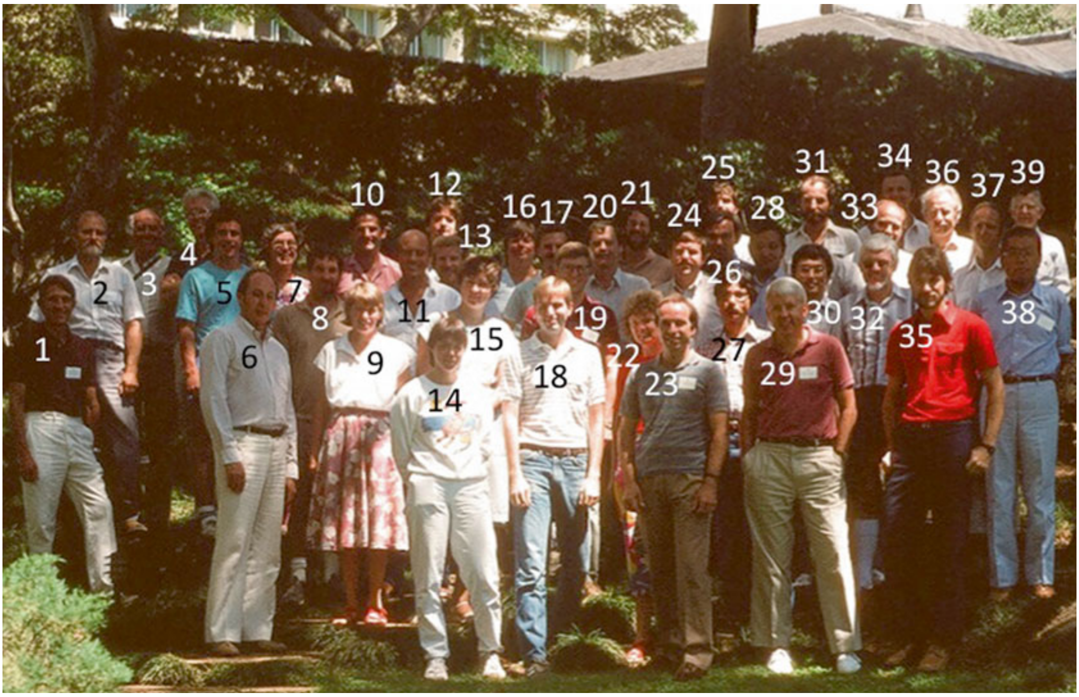

Fig. 2.1 Participants in a workshop on "structure, function and photoinhibition of photosystem II in relation to plant stress" held at the East-West Center, University of Hawaii, in September of 1985. Identified individuals pictured include: (*1*) David Fork, (*2*) Robert Smillie, (*3*) George Cheniae, (*4*) Dick Dilley, (*5*) unidentified, (*6*) C. Barry Osmond, (*7*) Jan Anderson, (*8*) Neil Baker, (*9*) Sue Hetherington, (*10*) Steve Powles, (*11*) Jim Barber, (*12*) Bob Sharp, (*13*) Dennis Greer, (*14*) Robyn Cleland, (*15*) Barbara Demmig, (*16*) Kit Steinback, (*17*) Peter Horton, (*18*) William Adams, (*19*) Gunnar Öquist, (*20*) Olle Björkman, (*21*) unidentified, (*22*) Christa Chritchley, (*23*) David Kyle, (*24*) unidentified, (*25*) unidentified, (*26*) Gabriel Cornic, (*27*) Salil Bose, (*28*) Norio Murata, (*29*) Charlie Arntzen, (*30*) M.-A. Takahashi, (*31*) Paul Armond, (*32*) Merv Ludlow, (*33*) Don Ort, (*34*) unidentified, (*35*) Patrick Neale, (*36*) G. Heinrich Krause, (*37*) John Boyer, (*38*) Kozi Asada, and (*39*) Aubrey Naylor. Although the authors (W. W. A. and B. D.-A.) had met during the summer of 1984, this conference marked the beginning of their professional and personal relationship (see Fig. 2.4, less than 1 month later). Note: if you know the identity of #5, #21, #24, #25, or #34, please send an email to william.adams@colorado.edu.

(1986) and Papageorgiou and Govindjee (2004). As we attempted to interpret chlorophyll fluorescence measurements in various plant species in 1984 (B.D.-A. in O. Björkman's lab and W.W.A. in C.B. Osmond's lab), we found the work of Kitajima and Butler (1975) particularly enlightening. Using empirical data, Butler and Kitajima had developed a model for the fates of excitation energy absorbed by photosystem II (PS II) and its antennae, including non-radiative, thermal dissipation in what he termed the chlorophyll pigment bed that offered a theoretical explanation for decreases in the level of initial or instantaneous fluorescence (F_o) and maximal fluorescence (F_m). Each of us shared our findings of strong (non-photochemical) quenching of both F_m and F_o in various plant species exposed to excess light under controlled experimental conditions or in the field at a small gathering of photosynthesis investigators in 1985 (Fig. 2.1) before publishing our findings a couple of years later (Adams et al. 1987, 1988; Demmig and Björkman 1987; Adams and Osmond 1988; Demmig and Winter 1988). Upon completing a postdoctoral appointment with O. Björkman, Barbara returned to Germany, where she began exploring the relationship between chlorophyll fluorescence quenching and the VAZ cycle.

III Contributions of Comparative Ecophysiology to the Initial Linking of Non-Photochemical Quenching of Chlorophyll Fluorescence and Zeaxanthin

A The Vantage Point of Ecology and Evolution

As stated in the beginning of this chapter, multiple questions lend themselves to exploration in a comparative ecophysiological framework, including: *What variation on the theme of photoprotective energy dissipation is available (i) to an organism under different environmental conditions and (ii) to different organisms? What parts of the system are flexible? For which of a myriad of different environmental niches is a particular variation of the theme most suited? What ways of surviving in a challenging environment exist and how does this influence the employment of energy dissipation by different organisms? What are the costs and benefits (trade-offs) of the different variations of thermal dissipation?*

Comparing and contrasting the features of zeaxanthin-associated energy dissipation in plants with different growth rates and life spans in response to a variety of environmental conditions unveiled a multitude of facets of the employment of photoprotective energy dissipation differing widely in, e.g., extent and kinetics. This comparative approach included different types of plants predicted to experience different demands on the extent and kinetics of their thermal dissipation, i.e., soft-leafed annuals (completing their life cycle in one growing season), soft-leafed biennials (experiencing multiple seasons over 1.5 years before reproducing and dying), deciduous species (persisting over multiple years but shedding their leaves during the harshest season every year), and evergreen species (maintaining green leaves or needles through many years). Comparing and contrasting annual and evergreen lifeforms provided examples of the predicted pronounced differences in the magnitude of employment of zeaxanthin-associated energy dissipation as well as different modes of employment with vastly different kinetics. Many of these differences parallel, and are expected to result from, differences inherent in the annual versus evergreen lifestyle.

Annuals grow rapidly and complete their life cycle over few weeks to several months (typically before climatic conditions become unfavorable for rapid growth via, e.g., reduced water availability or growth-precluding temperatures). Since annuals are adapted for inherent high rates of growth and photosynthesis, they thrive in high-light environments under conditions favorable for rapid growth and, conversely, possess a low shade tolerance. Leaves of high light-acclimated annual species, unless subjected to other environmental stresses, utilize a relatively large fraction of the light they absorb in photosynthesis, and exhibit low maximal levels of photoprotective energy dissipation (typically ranging between 1 and 2 when quantified as NPQ = $F_m/F_m'-1$) as well as, typically, incomplete conversion of their VAZ pool to zeaxanthin (Demmig-Adams and Adams 1994; Demmig-Adams et al. 1995, 1996a, 2006; Demmig-Adams 1998; see also Demmig-Adams et al., Chap. 24).

Evergreens receive their name from the fact that they keep green leaves, with a high light-harvesting capacity, throughout both favorable and unfavorable seasons. Over their lifetime, evergreen species persist throughout multiple unfavorable seasons (e.g., summer heat in hot, dry climates or winter frost in temperate climates). Evergreens have genetically fixed low maximal rates of photosynthesis even under conditions ideal for growth. Evergreen species, therefore, successfully grow under conditions with limiting resources, but are unable to compete with rapidly growing species in resource-rich environments. Many evergreen species are highly shade tolerant, but also thrive in high-light environments, where their reproductive success is, in fact, enhanced (Adams 1988). When acclimated to high

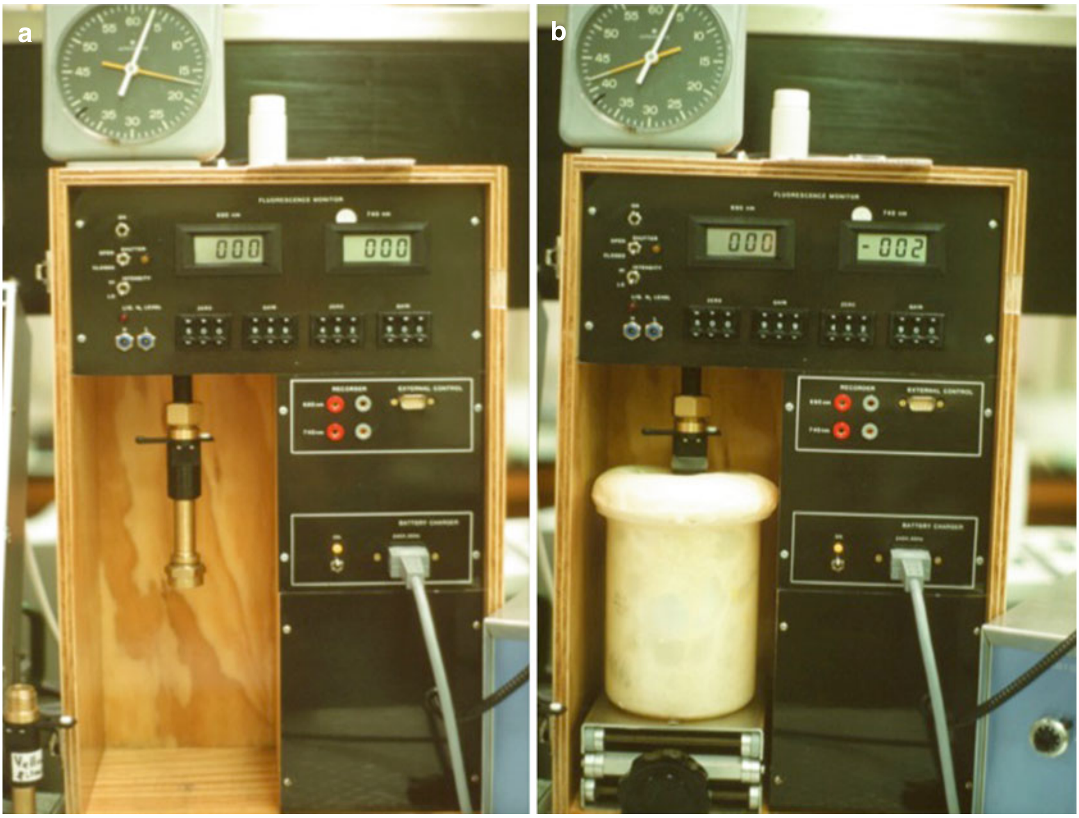

Fig. 2.2 Custom-built instrument for measuring chlorophyll fluorescence from photosystem II (leaf sample illuminated with blue light and fluorescence detected at 690 nm) and photosystem I (detection at 740 nm) from samples that were darkened for several minutes (**a**) then frozen to 77 K (**b**) before illumination. Tissue (leaf or cladode) samples of 1 cm diameter were appressed against one end of a quartz rod enclosed in a brass housing and allowed to equilibrate in darkness for several minutes (see *lower left corner* of **a**). The brass holder was screwed into the instrument and a shutter slid to the side (from *right* to *left*; compare brass holder in *lower left* of **a** to that in the *center* of **a**) to allow the other end of the quartz rod to become appressed to the fiber-optic cable that both delivered the blue light to the sample and returned the chlorophyll fluorescence to the photodiode detector (employed in this instrument) or photomultiplier (employed in other instruments). After darkening, the sample and lower portion of the brass holder and quartz rod were submerged in liquid nitrogen (**b**) for 6 min to ensure complete freezing before opening of the shutter that permitted a low level of blue excitation energy (1.3 μmol photons m^{-2} s^{-1}) to illuminate the sample, allowing the assessment of F_o and F_m. The signal from the photodiode or photomultiplier was captured on a strip-chart recorder (see Figs. 2.5 and 2.6). This particular instrument was built by Dr. S. Chin Wong, Win Coupland, and Peter Groeneveld at the Research School of Biological Sciences (now Research School of Biology), Australian National University. Photographs by W.W. Adams.

light, evergreen leaves are able to quickly and fully (90 % and above) convert their VAZ cycle carotenoids to antheraxanthin and zeaxanthin and reach a high maximal capacity for photoprotective energy dissipation (high NPQ levels of 4–8; Demmig-Adams and Adams 1994, 1996a; Demmig-Adams et al. 1995, 1996a, 2006; Barker and Adams 1997; Demmig-Adams 1998).

B Quenching of Chlorophyll Fluorescence in Evergreens

During the mid-1980s, both authors of this chapter conducted chlorophyll fluorescence measurements on excised leaf (or cactus pad, or cladode) segments (from experimental or field-grown plants) darkened for 5 or 10 min (Fig. 2.2a) and frozen to 77 K

Fig. 2.3 (**a**) Barbara Demmig motoring along a waterway near the Pacific ocean on the eastern coast of Australia (just south of Brisbane) with the evergreen species *Rhizophora stylosa* (the small-stilted mangrove) growing in the background (March 1987; photograph by W.W. Adams) and (**b**) *Monstera deliciosa* (Swiss cheese plant), adjacent to W.W.A. and B.D.-A., growing in a glasshouse at the University of Colorado in July 2008; photograph courtesy of The Daily Camera.

(Fig. 2.2b) before determining the levels of F_o and F_m fluorescence. By virtue of the fact that evergreen species exhibit much greater decreases than annuals in the levels of both F_m and F_o fluorescence in response to exposure to excess light (e.g., much higher levels of NPQ), strongly decreased levels of F_m and F_o fluorescence were still detectable from high-light-acclimated evergreens exposed to excess light even after darkening of leaves for several minutes. Employment of evergreens was thus instrumental in the recognition of strong concomitant decreases in F_o and F_m. It is worth mentioning that the numerous data collected at 77 K invariably revealed a concomitant strong quenching of F_o and F_m (with concomitant strong decreases in F_v/F_m) in both PS II and photosystem I (Demmig and Björkman 1987). B.D.-A. worked predominantly with two evergreen species, Australian salt-tolerant mangroves (Fig. 2.3a) Olle Björkman had brought back to Stanford and the climbing rainforest vine *Monstera deliciosa* (Fig. 2.3b; Demmig and Björkman 1987), and W.W.A. worked with evergreen cacti (Fig. 2.4) and other CAM plants (Figs. 2.5 and 2.6; Adams et al. 1987, 1988; Adams 1988; Adams and Osmond 1988), all of which were both *adapted* and *acclimated* to highly excessive light levels and exhibited strong and sustained (non-photochemical) quenching of the F_m and F_o levels of chlorophyll fluorescence.

Shortly thereafter, the pulse-amplitude-modulated (PAM) system for room-

Fig. 2.4 (**a**) View overlooking the Laboratory of Desert Biology (and luxury accommodations) in the northern reaches of Death Valley, California. The green vegetation is largely *Larrea tridentata* (creosote bush), and Stanley D. Smith is visible on the right side of the image. This trailer/laboratory was established by Frits Went (pioneer in the discovery of the growth hormone auxin; Went 1926) as part of the Desert Research Institute associated with the University of Nevada (see Went 1968); Went was still in Reno when W. W. A. began his PhD work there in 1984. (**b**) C. Barry Osmond, Cornelia Büchen-Osmond, Barbara Demmig, and William Adams at the Laboratory for Desert Biology in early October 1985 (prior to Barry Osmond becoming afflicted with heatstroke later that day). The laboratory was not equipped with air conditioning, and the swamp coolers were non-functional. (**c**) *Opuntia basilaris* (the beavertail cactus) growing nearby (see Adams et al. 1987). Photographs by W.W. Adams.

temperature fluorescence measurement from intact, attached leaves, permitting assessment of chlorophyll fluorescence quenching during illumination independently of the level of direct sunlight or artificial light, became available (Schreiber 1986; Schreiber et al. 1986). Our first use of this new system still involved darkening of leaf samples for several minutes for comparison with those fluorescence measurements obtained prior to the development of the PAM fluorometer (Demmig et al. 1987a, 1988; Adams et al. 1988, 1989, 1990a; Demmig and Winter 1988; Demmig-Adams et al. 1989d). However, it soon became clear that ascertaining the level of F_o and F_m quenching directly under (actinic) illumination or in the field (as F_o' and F_m') was preferable unless one was interested in characterizing only the portion of photoprotection that remained engaged for extended periods, e.g., nocturnally-sustained "photoinhibitory" depressions in PS II efficiency (Adams et al. 1995a, 2006, 2013a; see Adams et al., Chap. 23 and Demmig-Adams et al., Chap. 24).

Fig. 2.5 (**a**) William Adams in the midst of measuring chlorophyll fluorescence from leaf punches (darkened and frozen to 77 K) of (**b**) the CAM fern *Pyrrosia confluens* growing epiphytically in Dorrigo National Park (a subtropical rainforest approximately 15 km from the coast, southwest of Coffs Harbour, New South Wales, Australia). William (bundled up because the predawn temperature reached a low of −5 °C on this particular field trip [1 August 1986]) is sitting beside the strip-chart recorder and the low temperature chlorophyll fluorometer, with a dewar of liquid nitrogen on the ground and the power cords (lower right hand corner) receiving electricity from a gasoline-powered generator (off to the right, out of view). See Adams (1988). Photographs by E. Brugnoli (**a**) and W. W. Adams (**b**).

C Zeaxanthin in Evergreens

Following the completion of her postdoctoral appointment in Olle Björkman's laboratory, B.D.-A. returned to Germany to pursue research at the Lehrstuhl für Botanik II in Würzburg. After coming across papers on VAZ cycle operation, she was intrigued by the similarity of the features of VAZ cycle conversions and her measurements of fluorescence quenching at Stanford, expressed (after Kitajima and Butler 1975) as changes in the rate constant for thermal dissipation in the pigment bed. The laboratory of professor Franz-Christian Czygan, where separation, identification, and quantification of plant pigments was routinely carried out by experienced technician Frau Almuth Krüger, was in the neighboring Lehrstuhl for Pharmaceutical Biology. When sought out by Barbara, Franz-Christian Czygan kindly offered Frau Krüger's assistance, which led to the first concomitant analyses (in the late 1980s) of chlorophyll fluorescence quenching and leaf xanthophyll content, the latter laboriously conducted using thin layer chromatography before the development of faster and more sensitive high-pressure liquid chromatography (HPLC) methods for the separation of the close structural

Fig. 2.6 (**a**) Enrico Brugnoli and the 77 K chlorophyll fluorescence equipment on the beach in the shade of Middle Head (south of Scotts Head and north of Grassy Head, New South Wales, Australia) where both *P. confluens* (see Fig. 2.5b) and (**b**) *Hoya australis* (common waxflower) were characterized where they both grew as lithophytes on Middle Head. Photographs by W.W. Adams; see Adams 1988 and Adams et al. 1988.

isomers zeaxanthin and lutein. Once again, evergreens with their low rates of photosynthesis (and resulting high need for photoprotection) proved invaluable since thin layer chromatography requires large amounts of pigment. The evergreens conveniently generated the required large amounts of zeaxanthin and antheraxanthin, by virtue of de-epoxidizing their VAZ pool to over 90 % (versus typically only about 60 % in annuals). With the support of the German academic system that did not require young faculty members to seek their own grants for highly exploratory ideas, many hundreds of leaf carotenoid analyses (expertly performed by Frau Krüger) helped establish close relationships between VAZ pool conversion and chlorophyll fluorescence quenching indicative of photoprotective thermal energy dissipation.

Initial studies established close correlations between zeaxanthin level and sustained (for 5 or 10 min in darkness), "photoinhibitory" decreases in chlorophyll fluorescence (Demmig et al. 1987a, 1988). Subsequently, very similar correlations were established between zeaxanthin level and the rapidly reversible (high-energy state) quenching of chlorophyll fluorescence for intact evergreens and annuals under experimental treatments (Demmig-Adams et al. 1989a, b, c) or in the field (Demmig-Adams et al. 1989d) as well as for plants and green-algal lichens treated with an inhibitor of violaxanthin conversion to zeaxanthin (Demmig-Adams and Adams 1990; Bilger and Björkman 1991) and for lichens accumulating zeaxanthin either rapidly (via the VAZ cycle) or slowly (from β-carotene; Demmig-Adams et al. 1990b, c).

Table 2.1 Major topics touched upon in this volume, major emerging conclusions, and the chapters in which the topics and conclusions are discussed. ELIPs and HLIPs have not been included under proteins in plants because it remains to be determined whether the correlative evidence for their involvement in sustained thermal energy dissipation in evergreen plants following sudden transfer from low to high light (Demmig-Adams et al. 2006) and during winter stress (Zarter et al. 2006a, b; Wang et al. 2009; Demmig-Adams et al., Chap. 28) can be supported by mechanistic studies

Topic	Emerging conclusions	Chapters
Identification of factors involved in NPQ	**LHC family proteins**	
	Plants	
	PsbS	3, 5, 13, 14
	Various algal groups	
	LHCSR	14, 21
	LHCSR-related proteins	11, 14, 20
	Cyanobacteria	
	OCP	14, 22
	Xanthophylls	
	Products of xanthophyll cycles	
	Zeaxanthin & Antheraxanthin (from Violaxanthin)	3, 4, 5, 6, 13, 14, 23, 24
	Diatoxanthin (from Diadinoxanthin)	11, 14, 20
	Lutein (from Lutein epoxide)	12
	Other xanthophylls	
	Hydroxy-echinenone	22
	Lutein (not from Lutein epoxide)	6, 15
Photophysical mechanism	Fate of excitation energy strongly affected by local environment of pigment-binding complexes; much support for Chl-Car exciton coupling	4, 6, 8, 9, 10, 16
Possible sites of quenching	Minor LHC proteins	14
	LHCII	3, 6, 10, 15
	Both minor LHCs and LHCII	5
Ecological studies	Multiple genetic adaptations to different specific environments plus strong acclimation of individuals to growth light environment	2, 12, 20, 23, 24, 25, 26, 27

IV Additional Contributions of Ecophysiology and Evolutionary Biology to the Understanding of Photoprotection via Thermal Energy Dissipation

A Further Contributions Since the Initial Linking

1 Evolutionary Relationships: Emerging Themes and Their Variations

Table 2.1 includes a list of some of the major topics covered in this volume, some of the conclusions with support from multiple investigators, and a list of the chapters in which those topics and conclusions are discussed. In most of the large diversity of photosynthetic organisms examined, engagement of photoprotective energy dissipation (reflected in increased NPQ and/or decreased PS II efficiency) is thought to involve both (i) a xanthophyll and (ii) a member of the light-harvesting family of proteins. Evidence for the involvement of multiple light-harvesting complex (LHC) family proteins and multiple different xanthophylls in different taxa of photosynthetic organisms is summarized throughout this book (see Table 2.1 for specific chapters). Xanthophyll-associated thermal energy dissipation is thought to be a mechanism to harmlessly dissipate excess excitation energy absorbed by chlorophyll and, thereby, prevent passing of excitation energy on to oxygen (see Demmig-Adams et al., Chap. 28). Many additional photoprotective responses of plants and algae prevent or minimize the absorption of excess light or detoxify reactive oxygen species and other radicals that may result from exposure to excess light energy (see Logan et al., Chap. 7, for an overview of the entire suite of photoprotective mechanisms available to plants, and Havaux and García-Plazaola, Chap. 26, for differences among taxa in the employment of energy dissipation versus anti-oxidation).

2 Ecological Relationships

During the 1990s, we characterized (using HPLC analysis of chloroplast pigments and laboratory and portable PAM chlorophyll fluorometers) multiple plant species (including annuals, biennials, deciduous species, and evergreens) growing naturally either in open sunny locations or in the shade of tree canopies or other structures, where they typically naturally received occasional sunflecks every day. Remarkably, in all of these situations with extremely different levels of NPQ and NPQ relaxation kinetics, the same increase in NPQ and decrease in PS II light-harvesting efficiency (ascertained as the ratio of variable to maximal chlorophyll fluorescence, i.e., F_v'/F_m' determined during illumination or F_v/F_m determined in darkness) was seen for a given VAZ cycle conversion state to zeaxanthin + antheraxanthin (Demmig-Adams et al. 1995; Demmig-Adams and Adams 1996a). We note that these inter-species comparisons apply to leaves exposed to peak light levels in clear excess of that which can be utilized in photosynthesis, thus presumably minimizing involvement of mechanisms that optimize excitation energy distribution and instead focusing on mechanisms serving to thermally dissipate excess excitation energy. From an evolutionary standpoint, the observation of uniform responses to excess light among species suggests that the underlying mechanism of quenching may be conserved, while additional, species- and growth condition-dependent factors provide diversification to meet the demands of contrasting environments. For summaries of other ecophysiological work on photoprotective thermal dissipation and/or xanthophylls, see, e.g., Esteban and García-Plazaola (Chap. 12; see also García-Plazaola et al. 2007, 2012), Lavaud and Goss (Chap. 20), Adams et al. (Chap. 23), Demmig-Adams et al. (Chap. 24), Murchie and Harbinson (Chap. 25), Havaux and García-Plazaola (Chap. 26), and Morales et al. (Chap. 27) as well as other papers (Königer et al. 1995; Krause et al. 2004; Savage et al. 2009; Matsubara et al. 2012).

How could it be possible that the same NPQ (and F_v/F_m) level is attained for a given VAZ cycle conversion state in a leaf performing pronounced changes in NPQ (and F_v/F_m) over many minutes versus over only a single second – a time scale known to be too fast for VAZ cycle conversions? Leaves can contain zeaxanthin that is apparently not engaged in energy dissipation as long as leaves are not experiencing actual excessive light. This was demonstrated experimentally by inducing intact leaves to retain zeaxanthin in darkness, which was followed by rapid engagement of energy dissipation upon exposure to excess light over one to a few second(s) in zeaxanthin-preloaded leaves but not in zeaxanthin-free leaves (Demmig-Adams et al. 1989b). Likewise, leaves of two *Yucca* species experiencing the summer heat of the Mojave desert (Fig. 2.7a) were found to nocturnally retain 60 % or more of the VAZ cycle as zeaxanthin + antheraxanthin that apparently only became engaged in thermal dissipation as the sun rose (Barker et al. 2002), presumably in response to proton accumulation in the thylakoid lumen as photosynthetic electron transport was activated (see Strand and Kramer, Chap. 18). Similarly, plants under either a dense rainforest canopy (Fig. 2.7b; Logan et al. 1997) or a more open *Eucalyptus* forest (Fig. 2.7c, d; Adams et al. 1999) retain zeaxanthin + antheraxanthin overnight and throughout the day, which, during sunflecks, apparently becomes instantly engaged in thermal energy dissipation to provide photoprotection and is instantly disengaged upon return to low light limiting to photosynthesis. For plants in nature, no increases in NPQ or

Fig. 2.7 (**a**) *Yucca schidigera* (Mojave yucca, on the left and right side in the foreground, with CAM photosynthesis) and *Yucca brevifolia* (the Joshua tree, center foreground, with C3 photosynthesis) growing near Red Rocks Conservation Area in the Mojave Desert of Nevada (Photograph by S.D. Smith; see Barker et al. 2002), (**b**) *Alocasia brisbanensis* growing in the understory of a subtropical rainforest (Dorrigo National Park) during exposure to a sunfleck (Photograph by W.W. Adams; see Logan et al. 1997), and two vines from which chlorophyll fluorescence was measured and samples for pigments and antioxidants were removed during exposure to sunflecks in the understory of an open *Eucalyptus* forest south of Middle Head along the eastern coast of New South Wales, Australia, (**c**) *Stephania japonica* and (**d**) *Smilax australis* (*left side*) and *Stephania japonica* (*lower right corner*. Photographs in **c** and **d** by W.W. Adams; see Adams et al. 1999.

decreases in PS II efficiency in response to excess light have thus been observed in the absence of a corresponding level of zeaxanthin + antheraxanthin.

It is attractive to assume that evolution of *dual control* of thermal dissipation by *not only* (i) removal of quenching xanthophyll(s) via a xanthophyll cycle *but also* (ii) instantaneous disengagement of NPQ upon dissipation of the trans-thylakoid pH gradient allowed a swift return to a high light-harvesting capacity whenever light is limiting to photosynthesis. A central prerequisite for the evolution of land plants may thus have been the *removal* of zeaxanthin by the VAZ cycle (with *formation* of zeaxanthin from β-carotene having evolved long before in the earliest oxygenic photosynthetic organisms; Hager 1980; see also Esteban et al. 2009). By this token, it would be unattractive to assume that land plants would use any xanthophyll as a quencher that is not tightly controlled by a xanthophyll cycle (see also Esteban and Garcia-Plazaola, Chap. 12; Demmig-Adams et al., Chap. 24). Similar considerations also apply to certain groups of algae employing xanthophyll cycles (see Lavaud and Goss, Chap. 20).

Walla et al. (Chap. 9) state that, “it has been suggested that even [carotenoids] other than those involved in the VAZ cycle dissipate excess energy as a result of, for example, conformational changes within the pigment-protein complexes. However, zeaxanthin-free intact leaves never show quenching as deep as plants under excess light in nature. … the role of [carotenoid-chlorophyll] interactions in various proposed structural rearrangements that may contribute to NPQ, such as LHCII aggregation, state transitions and other membrane restructuring, is not yet clear." While some groups propose that zeaxanthin exerts control over putative quenching by lutein (see, e.g., Horton, Chap. 3; Krüger et al., Chap. 6), others have argued against "a direct involvement" of constitutively present lutein in thermal dissipation and instead proposed roles in optimizing antenna structure for light harvesting (Lokstein et al. 2002) and quenching of triplet chlorophyll (Dall'Osto et al. 2006; Mozzo et al. 2008). Horton (Chap. 3) concludes that, "there is little doubt about the importance of the VAZ cycle in NPQ. No theory for NPQ can be complete unless it explains the remarkable link between the extent of quenching and DES [de-epoxidation state] … However, exactly how this link operates is still a matter of debate and disagreement."

The results of fluorescence lifetime analyses also remain to be reconciled. Gilmore et al. (1995), in Govindjee's laboratory, associated NPQ onset with a shift from a fluorescence component with a longer (2 ns) to one with a shorter (0.4 ns) lifetime, and concluded that this shift was responsible for a pH- and zeaxanthin-associated major component of the flexible, rapidly reversible form of NPQ. A similar conclusion was reached for the major component of flexible NPQ in diatoms: this NPQ component was proposed to be both pH- and diatoxanthin-dependent (see Holzwarth and Jahns, Chap. 5; Lavaud and Goss, Chap. 20). Gilmore and Ball (2000) extended fluorescence lifetime analysis to overwintering evergreens in the continuously dissipative state (see below) and concluded that the latter involved a zeaxanthin-dependent, but pH-independent (and thus continuously engaged) form of NPQ. Jahns and Holzwarth (2012; see also Holzwarth and Jahns, Chap. 5), using the PsbS-deficient *npq4* mutant of *Arabidopsis*, on the other hand, proposed that zeaxanthin-dependent NPQ is not pH-dependent even in wild type exhibiting flexible NPQ. The possibility should be evaluated that zeaxanthin-dependent NPQ may revert to a pH-independent form (possibly involving a pH-independent protein factor to engage quenching) in PsbS-deficient mutants, while wild type may employ PsbS for flexible quenching involving both pH-control and zeaxanthin as a quencher as envisioned by Gilmore et al. (1995).

Although all plant species exhibit the same relationship between the level of zeaxanthin + antheraxanthin and the level of thermal energy dissipation (assessed as NPQ and decreases in PS II efficiency), different species do exhibit different absolute levels of both responses (of both of the latter parameters in concert) when growing side-by-side under the same environmental conditions. For instance, an annual species, utilizing a relatively higher proportion of absorbed light energy in photosynthesis, showed a peak level of NPQ of 2 and a decrease in PS II efficiency to 0.5 at midday, whereas an evergreen groundcover, with a lower capacity for photosynthesis, exhibited a peak NPQ of over 4 and a reduction in PS II efficiency to 0.25 at midday (Demmig-Adams et al. 1996a; see also Demmig-Adams et al. 2012 and Chap. 24).

Annuals and evergreens also differ in their response to prolonged environmental stress. While many annuals and deciduous species exhibit reduced leaf chlorophyll content, resulting in reduced levels of light absorption, in response to prolonged environmental stresses (Verhoeven et al. 1997; Logan et al. 1999; see Morales et al., Chap. 27), such a response is less common in evergreens. Instead, evergreen species arrest their VAZ cycle and maintain large amounts of zeaxanthin that either apparently become engaged in energy dissipation when light is excessive and disengaged when it is not (e.g., in response to drought and high temperature; Barker et al. 2002) or enter into a locked-in dissipative state continuously maintained regardless of the presence of light (e.g., during winter; see Adams et al. 1995a, 2001, 2002, 2006, 2013a; Gilmore and Ball 2000; Öquist and Huner 2003; Demmig-Adams and Adams 2006; Adams et al., Chap. 23; Demmig-Adams et al., Chap. 24).

Plants respond to winter stress in different ways depending on species and environmental conditions. Soft-leafed annuals or biennials (herbaceous species) persisting through winter exhibit an upregulation of photosynthesis in response to lower versus warmer temperature (Adams et al. 1995b, 2001, 2002, 2013b; Verhoeven et al. 1999; Cohu et al. 2013b, 2014). The latter species retain zeaxanthin on colder nights in response to nocturnal maintenance of the trans-thylakoid pH gradient, exhibit an increased ATP to ADP ratio, and a continuously maintained apparent engagement of zeaxanthin in energy dissipation (reflected in continuously maintained low levels of F_v/F_m), all of which is rapidly reversed upon warming of the leaves (Demmig-Adams et al. 1996b; Verhoeven et al. 1999). Evergreen species (conifers, bushes, and groundcovers) exhibit similar responses during the autumn-to-winter transition as temperatures drop below freezing on some days and rise above freezing on others (Adams and Demmig-Adams 1994, 1995), and throughout winters in environments with soil temperatures that intermittently reach above-freezing levels conducive to allowing plants to resume photosynthetic activity (Verhoeven et al. 1998). However, in the most extreme habitats where soil water remains frozen for long periods of time, thus precluding any intermittent photosynthetic activity, zeaxanthin (and antheraxanthin)-associated energy dissipation is not pH-dependent and is continuously maintained (does not reverse upon warming of leaves or needles; Fig. 2.8) as winter progresses, and this continuously maintained dissipative state constitutes all of the nocturnally-maintained decreases in F_v/F_m (Adams et al. 1995a, 2002, 2006; Verhoeven et al. 1996, 1998; Adams and Barker 1998; Zarter et al. 2006a,b,c). Although modulation of the retention of zeaxanthin and its engagement in energy dissipation differs as dependent on species and environmental conditions, the relationship between the VAZ conversion state and PS II efficiency is the same irrespective of whether the two metrics are rapidly reversible and determined under midday exposure to sunlight in the summer or are continuously maintained in their dissipative state in winter (Adams et al. 1995a, 2006). In other words, maximal NPQ in leaves can be predicted from foliar zeaxanthin content in a range of different natural environments, while NPQ *kinetics* vary from seconds to minutes or days.

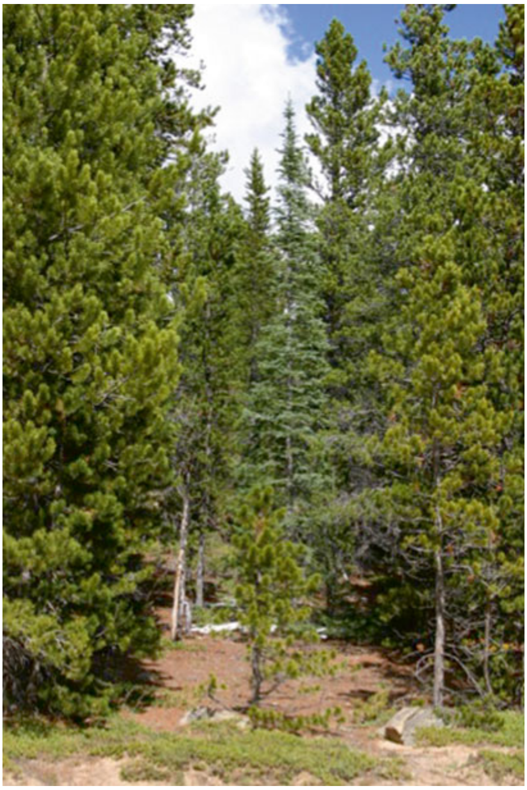

Fig. 2.8 The evergreen groundcover *Arctostaphylos uva-ursi* (bearberry, or kinnikinnick; see Zarter et al. 2006a) in the foreground with the conifers *Pinus contorta* (lodgepole pine, *left* and *right sides*) and *Picea engelmannii* (Engelmann spruce) growing in Roosevelt National Forest, Colorado (see Zarter et al. 2006b,c). Photograph by W.W. Adams.

The latter features show that modulation of zeaxanthin-associated thermal energy dissipation is fine-tuned to closely respond to the opportunity (or lack thereof) for photosynthetic activity present in each specific environment. For instance, a continuously maintained dissipative state is seen only in environments where conditions (such as frozen soils preventing plant water uptake) continuously preclude plant carbon uptake. Algae likewise exhibit adaptation and acclimation to multiple niches with respect to the intensity and distribution of light, as well as other features of the aquatic habitats in which they are found (see Lavaud and Goss, Chap. 20). While there is no evidence that the widely variable relaxation kinetics of NPQ in nature must be due to different quenchers, there clearly are different manifestations of quenching. Evolution of multiple different manifestations of one fundamental mechanism under the selective pressures of different environments is a common theme in biology. The many different time scales of VAZ cycle operation and the several different protein and other factors correlating with these different manifestations suggest that zeaxanthin-associated NPQ may be another example for this theme.

Fig. 2.9 The evergreen groundcover *Arctostaphylos uva-ursi* (bearberry, or kinnikinnick) growing (**a**) in full sunlight in Gregory Canyon (City of Boulder Open Space and Mountain Parks) and (**b**) in a shaded site in Roosevelt National Forest, Colorado. Photographs by W.W. Adams; see Zarter et al. 2006a.

Acclimation of plants to the degree of light stress in their growth environment involves adjustments in both xanthophylls and specific thylakoid proteins, as the two major chloroplast constituents implicated in thermal energy dissipation leading to NPQ (Table 2.1). The total VAZ pool is larger in leaves (and lichens) acclimated to higher versus lower light (Adams et al. 1992, 1993, 1996, 1999; Demmig-Adams and Adams 1992a, b, 1994, 1996b; Demmig-Adams et al. 1995; Logan et al. 1996; Demmig-Adams 1998), in winter compared to summer (Adams and Demmig-Adams 1994; Adams et al. 1995a, 2002), and in plants subjected to less than optimal soil conditions (Morales et al., Chap. 27). The level of the PS II protein PsbS (as a member of the light-stress-related subfamily of LHC proteins), involved in the flexible, pH-dependent engagement and disengagement of NPQ (Li et al. 2000, 2002a, b, 2004), is likewise upregulated in leaves of evergreen species acclimated to high versus low light (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006). On the other hand, different members of the sub-family of light-stress-related LHC proteins, such as early light-inducible proteins (ELIPs) and/or high light-inducible proteins (HLIPs), were upregulated in evergreen species upon sudden experimental transfer from a low to a high light growth environment (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006) and upon the natural seasonal shift into winter (Zarter et al. 2006a, b; Wang et al. 2009), both of which transitions also caused the leaves to enter a continuously dissipative state not requiring a trans-thylakoid pH gradient (Verhoeven et al. 1998; Demmig-Adams et al. 2006). While an evergreen groundcover overwintering in full sun exhibited no upregulation of the PsbS protein (Fig. 2.9a), leaves of the same species overwintering in the shade did so (Fig. 2.9b; Zarter et al. 2006a; see also Ottander et al. 1995). There is also evidence for a possible involvement of thylakoid protein phosphorylation in the regulation of thermal energy dissipation (Demmig et al. 1987b; Cleland et al. 1990; Adams et al. 2001; Ebbert et al. 2001, 2005; Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006), which should receive further attention (see below).

Leaves grown in the absence of excess light show very different responses than leaves grown in the presence of regular, intermittent exposure to excess light (see below). Leaves grown without excess light include leaves grown indoors under low, non-fluctuating light levels as well as plants growing outside in the extremely rare shade environment without sunflecks (e.g., leaves heavily shaded by environmental features and/or the rest of the plant). The very first exposure to excess light of such a leaf grown in the absence of excess light results in only a very low initial level of NPQ – accompanied by initial conversion of only a small fraction of the existing VAZ pool (Demmig-Adams et al. 1995, 1998; see also Demmig-Adams et al., Chap. 24). Over the course of continued exposure to excess light, NPQ levels subsequently increase very slowly and gradually – over the course of many hours – to similarly high NPQ levels as seen in plants grown under conditions including daily exposure to excess light (peak sunlight in open locations and sunflecks in otherwise shaded locations). The latter slow increase in NPQ is closely correlated with a slow increase in VAZ pool conversion to zeaxanthin and antheraxanthin, and eventually an increase in total VAZ pool size as additional zeaxanthin is synthesized from β-carotene (Demmig-Adams et al. 1998; see also Demmig-Adams et al. 1989e; Adams and Demmig-Adams 1992; Adams et al. 1999; Demmig-Adams et al., Chap. 24).

While the use of model species grown under controlled conditions presents advantages with regard to understanding the underlying genetics, this approach does have its limitations. Model plant species used thus far are typically short-lived, annual mesophytes, which, among plants, are the least tolerant of environmental stress. To understand the full range of adaptation and acclimation available to plants with regard to photosynthesis and photoprotection, it is necessary to take a broader, ecophysiological approach that includes multiple species and their myriad of responses to different genetic and environmental constraints to growth (Demmig-Adams et al. 2012; García-Plazaola et al. 2012).

B How Ecophysiology May Aid in Addressing Open Questions

Both adaptation and acclimation to the growth light environment determines the kinetics and capacity for NPQ (as a measure of energy dissipation). Techniques capable of exploring photo-physical mechanisms in intact leaves (Walla et al., Chap. 9) and/or the involvement of one or more quenching sites in intact leaves (via time-resolved fluorescence; Holzwarth and Jahns, Chap. 5) should be applied to leaves from plants (i) grown in environments with regular exposure to excess light (compared with plants grown in the absence of excess light) and (ii) plants genetically adapted to high versus low levels of excess light.

The tremendous utility of the many mutant studies conducted on this topic has been (i) in pinpointing factors (such as various LHC family proteins and various xanthophylls) that can be involved in NPQ and (ii) in demonstrating the astonishing flexibility of plants and other photosynthetic organisms in being able to employ apparent compensatory adjustments to tolerate full sunlight despite a missing component. Yet, the ability to compensate for missing components is, at the same time, a limitation of mutant studies that should be acknowledged. In consideration of the compensatory mechanisms employed by mutants, as evidenced by their astounding tolerance of full sunlight, a mutant lacking, e.g., the PsbS protein may be recruiting an alternative factor to replace PsbS, albeit at some cost to the flexibility of thermal dissipation modulation (Demmig-Adams and Adams 2006; Zarter et al. 2006a, b; Wang et al. 2009).

It therefore remains to be elucidated whether or not the quenching component termed qZ, assigned to zeaxanthin and described to be pH-independent and relaxing over 20 min (rather than over a few seconds) in the PsbS-deficient *npq4* mutant (Holzwarth and Jahns, Chap. 5), is also employed by wild type. It cannot be ruled out that the wild type may still be employing PsbS to modulate zeaxanthin-dependent quenching in a pH-dependent, rapidly reversible manner, while the PsbS-deficient mutant may be forced to replace flexible modulation with a less flexible modulation of a zeaxanthin-dependent quenching, possibly via one or more other members of the stress-inducible LHC sub-family and including pH-independent manifestations. Future studies should, therefore, also address costs and benefits of the employment of different possible mechanisms and sites of thermal dissipation as well as of different extent of thermal dissipation (see Demmig-Adams et al. 2013, Chap. 28). For further insight, existing mutant studies should be complemented by studies of plant species genetically adapted to high versus low light stress (e.g., annuals versus evergreens) and leaves grown in the absence versus presence of excess light.

In the various chapters in this volume, different views are expressed as to the mechanism underlying thermal energy dissipation. Many authors argue that a xanthophyll is involved in thermal dissipation, and that this xanthophyll is formed by a xanthophyll cycle (Table 2.1). Likewise, much discussion of the photo-physical mechanisms involved in thermal dissipation centers on the ability of the local protein environment to modulate energy levels of pigments involved and on the involvement of exciton coupling between chlorophyll and carotenoids (see, e.g., Ostroumov et al., Chap. 4, Krüger et al., Chap. 6 and Walla et al., Chap. 9). It should prove insightful to apply these techniques, wherever possible, to plant systems both adapted and acclimated to excess light. As part of such an approach, and as proposed by Walla et al. (Chap. 9), "a quantitative study is needed that directly assesses the spectroscopic signatures of all suggested electronic interactions in a single system."

V Concluding Remarks

Comparative eco-physiology facilitated the uncovering of a close correlation between zeaxanthin level and non-photochemical fluorescence quenching (as an indicator for the thermal dissipation of excess excitation energy) in intact leaves. Comparison of annual and evergreen lifeforms with different inherent rates of growth and photosynthesis, furthermore, revealed stark differences in the magnitude and kinetics of zeaxanthin-associated energy dissipation. An evolutionary vantage point suggests that evolution of dual control over thermal energy dissipation, by *removal* of xanthophyll(s) via a xanthophyll cycle plus rapid *disengagement* of NPQ via dissipation of trans-thylakoid ΔpH in environments favorable for plant growth, may have helped to maximize photosynthesis' photon yield by allowing swift return to high light-harvesting capacity during every transition from excess to limiting light over the course of a day in naturally fluctuating sunlight. Conversely, under environmental conditions less favorable for, or altogether preventing, plant growth, a cascade of different manifestations of zeaxanthin-associated thermal energy dissipation is evident especially in evergreens: each manifestation appears fine-tuned to closely match the level of opportunity for growth and photosynthesis present in each unique environment. A dissipative state continuously maintained over the entire winter season is seen only in environments where conditions (such as frozen soils) preclude plant carbon uptake for extended time periods. Likewise, both genetic adaptation and individual acclimation to growth light environment strongly influences a plant's kinetics and maximal capacity for thermal energy dissipation (as assessed via chlorophyll fluorescence). Since leaves grown in the absence of fluctuating (intermittently limiting and excessive) light show very different responses than leaves grown in the presence of regular exposure to excess light, future research on a wide range of mechanistic questions will benefit from a comparison of plant systems adapted and acclimated to different levels of excessive light and corresponding different demands for thermal dissipation of excess excitation energy, as well as combining multiple approaches in a single system with a high capacity for NPQ.

Acknowledgments

We wish to thank our mentors, Olle Björkman and C. Barry Osmond, and the many colleagues and students who have contributed to the development of our work over three decades. We are grateful to J. I. García-Plazaola and Barry A. Logan for their thorough evaluation and suggested changes that have improved the chapter. We also acknowledge funding from various sources for three decades, including the University of Colorado at Boulder, the Australian National University, the Universität Würzburg, the Carnegie Institution of Washington, the National Science Foundation (including our current award, DEB-1022236), the David and Lucile Packard Foundation, the US Department of Agriculture, the Deutsche Forschungsgemeinschaft, the Andrew W. Mellon Foundation, the Alexander von Humboldt Stiftung, and a NATO Postdoctoral Fellowship to W.W.A.

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Chapter 3

Developments in Research on Non-Photochemical Fluorescence Quenching: Emergence of Key Ideas, Theories and Experimental Approaches

Peter Horton
*Department of Molecular Biology and Biotechnology, University of Sheffield,
Firth Court, Western Bank, Sheffield S10 2TN, UK*

Summary

Research into non-photochemical quenching of chlorophyll fluorescence (NPQ), as an indicator of the thermal dissipation of excess excitation energy, has involved a number of different of approaches, drawing technology and intellect from a wide range of disciplines, from physics and chemistry through to ecology and agronomy. The timing of these approaches owed much to developments and advances occurring in these disparate areas of science, but

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*Author for Correspondence, e-mail: p.horton@sheffield.ac.uk

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_3, © Springer Science+Business Media Dordrecht 2014

also depended on the gradual evolution of our thinking about what NPQ was, what function it reflects, how its measurement could be exploited and ultimately what its molecular mechanism could be. The foundations of our current knowledge were laid 40 years ago, following intensive investigation of the bioenergetics of the thylakoid membrane, and the seminal papers on which this was based are described here. As our understanding of the biochemistry and biophysics of the thylakoid membrane increased, new ideas about NPQ emerged. The light-harvesting complexes of photosystem II and the xanthophyll cycle carotenoids bound to them have been the central features of NPQ research during the last 20 years. Outstanding advances in molecular genetics, in structural biology, in time-resolved spectroscopy and in the extraction and purification of membrane proteins all had major influence. The development of two main NPQ theories was the hallmark of this period. First, the idea that the process underlying NPQ was intrinsic to the main LHCII trimers, that these complexes can switch between unquenched to quenched states, that the equilibrium between these states is dependent on protonation and de-epoxidation of the xanthophyll cycle carotenoids and that the process is ultimately governed by the dynamic macrostructure of the grana membranes. Second, the notion of zeaxanthin as the specific obligatory quencher, bound to internal sites in the monomeric minor complexes (the gateways between the “bulk” LHCII antenna and the PS II cores), the activation of which is triggered by protonation of the PsbS protein. The emergence of these ideas is described, critical experiments highlighted and prospects for future progress assessed. The article concludes with a note on the ecological and agricultural implications of NPQ.

I Introduction

Research into the phenomenon that became known as non-photochemical quenching of chlorophyll fluorescence (NPQ) has taken place during an era of huge advances in our understanding of photosynthesis in particular and biology in general. The experimental approaches taken to investigate NPQ, the aims involved and the concepts developed reflected the scientific pre-occupations and limits to knowledge of the time. The study of NPQ had its origins in the earliest studies of chlorophyll fluorescence emission in photosynthetic systems by Kautsky, Franck and others. Although essentially phenomeno-

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Abbreviations: ΔA_{535} – The absorbance change with a maximum at 535 nm that accompanies light-induced NPQ; ΔpH – pH gradient across the photosynthetic (thylakoid) membrane; A, V, Z, VAZ – Antheraxanthin, violaxanthin and zeaxanthin, collectively the xanthophyll cycle carotenoids. CP26, CP29 – The minor light harvesting complexes of PS II; DES – De-epoxidation state of the VAZ cycle pool=(½A + Z)/VAZ; ELIP – Early-light-inducible protein; EM – Electron microscopy; Lhca1-6 – The proteins that comprise the light-harvesting proteins of PS I; Lhcb1-6 – The proteins that comprise the light-harvesting proteins of PS II; LHCII – The major light harvesting complex of PS II; NPQ – Non-photochemical quenching of chlorophyll fluorescence; pK_E – The negative logarithm of the dissociation constant for the induction of qE in response to acidification of the thylakoid lumen. pK_E is estimated by titrating the extent of qE as a function of increasing ΔpH; PMF – Proton motive force; PS I – Photosystem I; PS II – Photosystem II; Q – The notional primary electron acceptor of photosystem II discovered by Duysens arising from the idea that chlorophyll fluorescence is quenched when Q is oxidised but unquenched when it is reduced to QH; qE – Quenching coefficient and abbreviation for energy-dependent (ΔpH-dependent) quenching of chlorophyll fluorescence superseding the earlier term Q_E; qI, qT – Quenching coefficients and abbreviations for energy dissipation associated with photoinhibition (I), and the increase in distribution of excitation energy to PS I at the expense of PS II, the State Transition (T) quenching of chlorophyll fluorescence, respectively; qN – Quenching coefficient and abbreviation for non-photochemical quenching superseded by the later term NPQ; qP – Quenching coefficient and abbreviation for photochemical quenching superseding the earlier term Q_Q

logical, these studies nevertheless uncovered fundamental characteristics of photosynthesis, notably the complex kinetics of the changes in fluorescence yield that occur when photosynthesis commenced, and the idea that in part this somehow reflected an adaptation of the system to the presence of light rather than the mechanics of photosynthesis itself. The pioneering studies of these changes in fluorescence by Duysens revealed the fundamental details of the photosystems, the identification of a photochemical fluorescence quencher Q that was reduced to a non-quenching state QH by photosystem II light and re-oxidised by photosystem I light (Duysens and Sweers 1969). Duysens proposed a second quenched state Q′ that was slowly formed and "*traps excitation energy that might otherwise cause photobleaching and other harmful effects*". This statement encapsulates the essence of NPQ – a quenching process not directly connected to the primary photochemistry of photosynthesis, indicating a process with a photoprotective function, preventing damage to the photosynthetic apparatus by trapping (dissipating) unwanted (excess) excitation energy. These early studies are described in more detail elsewhere in this volume (Papageorgiou and Govindjee, Chap. 1). In the present chapter, I will start the account of the development of NPQ research from the mid-1960s, the dawn of the modern era of photosynthesis research.

II The Bioenergetics Era

In the 1960s, the quest to discover the mechanism by which redox free energy was used to drive ATP synthesis in respiratory and photosynthetic systems had intensified. Previous hypotheses had invoked the existence of chemical "high energy" intermediates, but none were found. Then, Peter Mitchell introduced a radical idea (the Chemiosmotic Hypothesis) that was to revolutionize the field of bioenergetics – rather than a chemical intermediate, a trans-membrane electrochemical gradient of protons, the proton motive force (PMF), coupled electron transport and ATP synthesis (Mitchell 1961). There followed intensive investigations of proton transport and counter-ion movements in isolated organelles. Isolated chloroplast thylakoids were particularly amenable to such experiments, principally because electron transport could be initiated by switching on the light. Compelling evidence from ever more sophisticated experiments was obtained, which supported the central tenets of Mitchell's hypothesis. Proton translocation into the thylakoid lumen was demonstrated, the PMF measured and the sites of proton uptake and release determined. The importance of the organization of the electron transport chain components in the thylakoid membrane was realized and the first evidence for the membrane asymmetry implicit in the notion of a trans-membrane PMF was obtained.

The basic features of the PMF were established – in chloroplasts, it was mostly a pH gradient (ΔpH). The latter was formed in the light, had a magnitude determined by the movement of counter ions that neutralise the electrical potential, a capacity resulting from the buffering of protons in the lumen, and decayed in darkness. Hence, the ΔpH was considered to confer upon the thylakoid a "high-energy state", formed by electron transport and consumed in ATP synthesis. Instrumental in such discoveries was the use of a variety of chemical reagents that inhibited and enhanced electron transport and ATP synthesis in isolated plant thylakoid membranes and cells of green algae; particularly significant were reagents that uncoupled electron transport from ATP synthesis, which worked by dissipating the ΔpH.

Investigations using these reagents led to the discovery of NPQ. I want to pick out three landmark papers that, together, first suggested not only the mechanism of NPQ, but its function. Firstly, Papageorgiou and Govindjee (1968) studied the second wave of chlorophyll fluorescence induction in *Chlorella*, discussing it in terms of "*non-photochemical components*" and concluded that it does not directly depend on the rate of electron transport. Using inhibitors of ATP synthesis, they linked this quenching to the high-energy state. Moreover, they suggested a mechanism – to quote "*A modification of the arrangement of the Chl a molecules imposed by the variable conformation of the supporting lamella, may in principle alter the fluorescence yield. For example, shrinkage or distension of the quantasome may form or dissociate nonfluorescing Chl a aggregates.*" Murata and Sugihara (1969) supported these conclusions, stating "*It will be assumed, therefore, that a high energy state of photophosphorylation may influence in some way the state of chlorophyll a molecules in the chloroplasts, and, in turn, modify the fate of excited state to result in a decrease in fluorescence yield*" and "*It may be that the mechanism perhaps reduces the inactivation of the chloroplasts under too strong light.*" Finally, Wraight and Crofts (1970) firmly established that the ΔpH was responsible for NPQ, for example showing in isolated thylakoids that NPQ decayed rapidly when uncoupling agents were used. The latter authors also emphasized a photoprotective role of NPQ and were the first to describe explicitly that NPQ is a non-radiative thermal degradation. Again, quoting "*It is evident, therefore that in the absence of a protective mechanism, coupled chloroplasts could be severely damaged by intense light*", "*a quenching mechanism of the uncoupler-sensitive type described here for the higher pH values, is operative to prevent just this occurrence. The energy-dependent quenching, as with other quenching phenomena, must occur by an increase in the rate of non-radiative dissipation of the chlorophyll excited singlet state. It is most probable that the actual pathway is that of thermal degradation*".

Thus, by 1970 there was experimental evidence that (a) NPQ was dependent on the trans-thylakoid pH gradient, (b) it was caused by non-radiative decay, (c) this non-radiative decay had a photoprotective function and (d) it might arise by alteration in the organization of pigments in the thylakoid membrane. It is remarkable that all of these assertions have been validated and now form the core knowledge about NPQ. Equally remarkable is that it has taken 40 years to reach this validation and, even now, considerable knowledge gaps remain.

III The Importance of the Thylakoid Membrane

Mitchell's chemiosmotic hypothesis emphasized the importance of the bioenergetic membrane in energy transduction. It was soon discovered that the presence of the ΔpH was associated with changes in thylakoid structure. Most notably, Murakami and Packer (1970) measured a reduction in membrane thickness upon light-induced proton uptake. They suggested "*the temporal sequence of events involved in the hierarchy of changes in structure in chloroplast membranes following illumination probably is: (a) protonation, (b) change in the environment within the membrane, (c) change in membrane thickness*". A landmark, making the link between the change in membrane conformation and NPQ, came from the work of Heinrich Krause and co-workers, who studied the absorbance increase at 535 nm (ΔA_{535}), an indicator of a structural change that resulted from light-induced proton uptake (Krause 1973). As will be discussed below, discovering the origin of this change was later to prove fundamental to understanding NPQ. In 1973, Krause stated "*that the light-induced proton translocation may cause certain structural changes of the thylakoid membrane system, which in turn are responsible for both absorbance increase and fluorescence quenching*". Movement of magnesium ions from the lumen into the stroma was implicated as a key event in the structural change (Krause 1974; Mills and Barber 1975). Later, Briantais et al. (1979) established the quantitative relationship between lumen acidification and NPQ, and re-affirmed the involvement of magnesium ions – "*as Mg^{2+} is displaced there by protons, a non-photochemical quenching of Photosystem II fluorescence is induced*". The conformational state of the thylakoid was referred to as the "high energy state", and therefore Krause introduced the term qE (initially called Q_E) to describe the NPQ

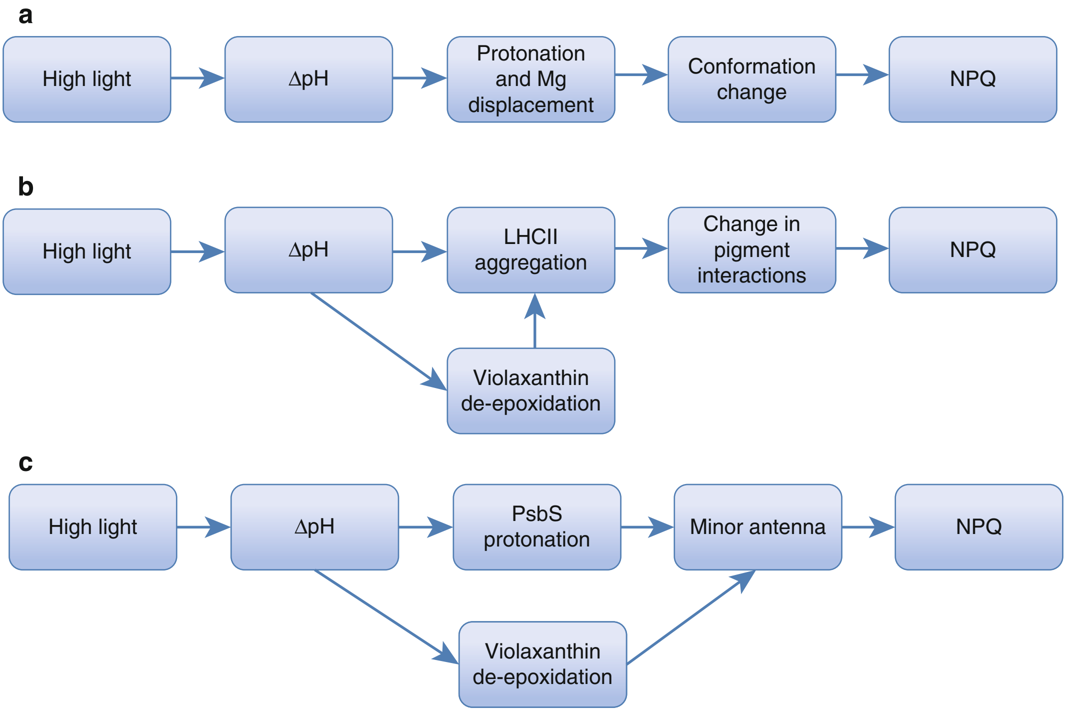

Fig. 3.1. Schematic models of NPQ. (**a**) Early ideas from the 1970s. (**b**) Model from 1991 following the discovery of the role of the VAZ cycle and the LHCII aggregation hypothesis. (**c**) Model depicting the direct involvement of PsbS as the proton sensor, controlling zeaxanthin-dependent quenching in the minor antenna.

associated with this state (Krause et al. 1982). In part, this was recognition that NPQ had a number of causes, not only the ΔpH, an idea that was developed analytically several years later (e.g., Horton and Hague 1988). Moreover, there was an implied separation of the formation of ΔpH from the molecular transformations causing qE, which was subsequently proven by the use of the inhibitor antimycin A that blocked qE (and ΔA_{535}) but not ΔpH (Horton et al. 1991). Figure 3.1a is a schematic model for NPQ based on the state of knowledge at circa 1980.

IV A Return to Phenomenology: Probing the Physiology of Leaf Photosynthesis

With NPQ firmly established as reflecting the major component of the thylakoid PMF, and photochemical quenching reflecting the redox state of photosystem II (PS II) according to Duysen's Q theory (Duysens and Sweers 1963), chlorophyll fluorescence quenching had the potential to be an incisive probe of photosynthesis; the ΔpH and the redox state of PS II estimate ATP/ADP and NADPH/NADP, respectively. It was surmised that the complex kinetics of chlorophyll fluorescence yield observed upon illumination of intact (i.e., metabolically active) photosynthetic systems, whether it be algal cells, intact chloroplasts or leaves, reflected the equally complex induction of the metabolic reactions of carbon assimilation occurring in the stroma and cytoplasm and consuming NADPH and ATP produced by the thylakoids (Horton 1985). How to separate photochemical (initially termed Q_Q, later qP) and non-photochemical components of fluorescence quenching (initially called qN, later NPQ) was first shown using the biphasic collapse of quenching after addition of DCMU to isolated chloroplasts (Krause et al. 1982). Such an approach could not be applied to leaves and a major breakthrough came in the paper by Bradbury and

Baker (1981) who used a second saturating light source to probe steady state qP. This principle was used by Quick and Horton (1984) who were able to monitor qP more easily using a modulated measuring beam and a saturating light pulse given repetitively at regular intervals. The equivalence between this latter method and the measurement of qP and NPQ using DCMU was demonstrated. The saturation pulse method became the standard for fluorescence quenching analysis as the basis of PAM fluorometry developed by Schreiber and colleagues (Schreiber et al. 1986). Application of this methodology led to an explosion of studies on NPQ in leaves, and also allowed the rapidly relaxing, ΔpH-dependent component of NPQ (qE) to be separated from another component that relaxed very slowly or not at all (at first called qR). Later, qR was resolved into components arising from relaxation of State Transitions (qT) and from sustained or photoinhibitory quenching (qI), respectively (Horton and Hague 1988). It should be noted that various problems have since arisen with this kind of distinction based solely on relaxation kinetics, since the relaxation time of qE has been shown to vary and the distinction between qI and qE is often semantic rather than mechanistic (e.g., see recent discussion in Horton 2012; see also Demmig-Adams et al., Chap. 24).

Most remarkable in these studies was the observation of the largely antiparallel relationship between qP and NPQ, which explained both the complex and highly variable kinetics of fluorescence induction and the fact that steady state fluorescence yields were mostly independent of light intensity (Quick and Horton 1984). This latter observation suggested that "*qQ and qE as well as qR in some way act to regulate the excitation density in PS II and so provide protection from the damaging effects of over-excitation*". This regulatory role was confirmed by Weis and Berry (1987), who elegantly demonstrated that the redox state of PS II is controlled both by the rate of electron transport and the extent of NPQ. An algebraic correction of the value of qP based on the value of NPQ allowed the electron transport rate in leaves to be estimated from observation of chlorophyll fluorescence quenching. Later, the same approach was refined by Genty et al. (1989) who introduced a parameter φPS II (defined as the quantum efficiency of PS II), which was not only simple to measure but was also both empirically and theoretically a measure of the quantum yield of photosynthetic electron transport. Combined with PAM fluorometry, this approach became hugely important in plant biology, enabling non-invasive estimates of photosynthetic rates in leaves, contributing knowledge in physiological, ecological and agricultural investigations. These studies similarly cemented the idea that NPQ was an indicator of a regulatory mechanism with an important function in photoprotection, experimentally demonstrated first by Krause and Behrend (1986). This prompted resurgence in research aimed at discovering its mechanism.

V Biochemical Approaches to Discovering the Mechanism of Quenching

A *The Xanthophyll Cycle*

One of the landmarks in NPQ research was the establishment of the link between the xanthophyll cycle (specifically the VAZ cycle that converts violaxanthin to zeaxanthin) and the induction of NPQ. The enzymatic interconversion of the xanthophylls violaxanthin (containing two epoxide groups) and zeaxanthin (without epoxide groups) in thylakoids had been known since the 1960s, but a physiological role had not been proven. In 1987, a correlation was found between the appearance of zeaxanthin in leaves and the extent of NPQ (Demmig et al. 1987). Although initially shown for the sustained qI component of NPQ found under stress conditions, it was later found that the ratio of zeaxanthin/violaxanthin, or more precisely the de-epoxidation state of the VAZ cycle carotenoids (DES), also correlated with rapidly relaxing qE. This discovery prompted an intensive period of research, which consolidated this correlation under a range of condition in many plant species (cf. Demmig-Adams et al., Chap. 24). The fact that de-epoxidation is activated by acidification of the thylakoid lumen leads to a simple model for qE, i.e., that qE was directly caused by zeaxanthin synthesis. Later, it was found that qE relaxed more slowly than the epoxidation of zeaxanthin, showing that both the ΔpH and zeaxanthin need to be present for quenching to occur.

The idea that zeaxanthin was the quencher of the excited state of chlorophyll a in NPQ gained credence from studies on isolated carotenoids, and a satisfyingly simple model was put forward based on the chemistry of carotenoids – the lengthening of the chain of conjugated carbon double-bonds to 12 in zeaxanthin from 10 in violaxanthin would be sufficient to change the direction of energy transfer between chlorophyll and carotenoid (Owens et al. 1992). The "molecular gear shift" model for NPQ (Frank et al. 1994) caused great excitement, but unfortunately was inconsistent with a number of observations, some of which will be outlined below (cf. also Polívka and Frank, Chap. 8).

As discussed in more detail elsewhere in this volume, there is little doubt about the importance of the VAZ cycle in NPQ. No theory for NPQ can be complete unless it explains the remarkable link between the extent of quenching and DES (see, e.g., Demmig-Adams et al., Chap. 24). However, exactly how this link operates is still a matter of debate and disagreement.

B Lessons from Enzymology

The involvement of lumen acidification (and by implication protonation of thylakoid membrane proteins) and the requirement for zeaxanthin prompted application of the experimental and conceptual approaches developed for the study of enzyme-catalyzed reactions. Following on from the work of Briantais et al. (1979), it was now found that the pH-dependency of qE, measured as the estimated pK (negative logarithm of dissociation constant) for proton-induced activation of NPQ (pK_E), was not fixed but varied according to experimental conditions, such as heat treatment or the presence of chemical activators or inhibitors (Krause et al. 1988). Such variability in pK_E is similar to that shown for K_m, the Michaelis-Menten constant, in many regulatory enzymes. The effect of VAZ on pH dependency of qE was investigated (Rees et al. 1989; Gilmore and Yamamoto 1992). A major advance in NPQ research was the finding that DES controls the pK_E: in chloroplasts containing only violaxanthin, the pK_E was estimated to be around 4.5 but shifted to around 5.5 with a DES of about 0.6 (Rees et al. 1989; Noctor et al. 1991). This result not only showed that zeaxanthin was not obligatory for qE, but also that qE could be considered as similar to a regulatory allosteric enzyme whose activity is dependent on endogenous effectors and inhibitors. In retrospect, this was not an unexpected result – qE is a feedback regulatory mechanism conceptually similar to those occurring throughout biology. Subsequent experiments verified this concept, showing how pK_E continuously responds to DES as well as to a variety of chemical agents and to magnesium concentration (Noctor et al. 1991, 1993). Formally, a 4-state model was set out to describe the effect of de-epoxidation (Horton et al. 1991; Fig. 3.2). This model was to form the foundation for three decades of research into NPQ, and was later amended by the demonstration that pK_E was tuned not only by the de-epoxidation state but by the macro-organization and composition of the thylakoid membrane itself (Horton et al. 2008; Horton 2012).

Right at the onset the physiological meanings of this model were pointed out, which are again reminiscent of other biological phenomena. NPQ indicates a feedback control to limit the excitation and redox pressures on PS II. Modulation of pK_E by DES allows regulation by ΔpH within physiological limits: lumen pH should not, and does not, fall below around 5.5 even in saturating light, so avoiding inhibition of electron transport;

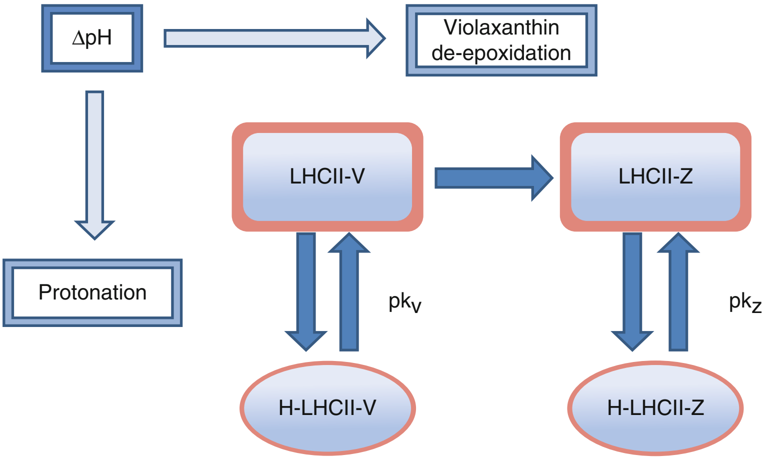

Fig. 3.2. The allosteric model for NPQ. This model formally depicts four states of LHCII depending on their protonation and de-epoxidation states Adapted from Horton et al. (1991).

in limiting light, ΔpH drives ATP synthesis without unwanted NPQ (see further discussion of the regulatory role of ΔpH in Strand and Kramer, Chap. 18). Thus, activation of NPQ by violaxanthin de-epoxidation is part of the chloroplast features allowing simultaneous photoprotection (high rates of excitation energy dissipation) and photosynthesis (rapid electron transport) in high light, and yet efficient light utilization in low light. Moreover, involvement of DES as the modulator of NPQ confers two other physiological benefits. First, it acts as a stabilizer, preventing rapid and possibly chaotic fluctuations in PS II function. Second, it provides a signal enabling the state of the light harvesting system to adjust to the average prevailing light conditions, making it better able to react to future changes based on the "memory" of past conditions. Such features of the control of NPQ are fundamental and predictable attributes of biological regulatory mechanisms.

C The Light-Harvesting Proteins of the Thylakoid Membrane

When NPQ was being investigated in the 1970s, there was little information about the thylakoid proteins involved in photosynthesis or even proof whether chlorophyll was bound to them. Subsequently, the pioneering work of several laboratories, in particular those of Thornber, Anderson and Green, demonstrated the biochemical basis of the photosystems, identified the light-harvesting proteins and proved beyond doubt that all the thylakoid chlorophyll was bound to them (Anderson et al. 1978; Markwell et al. 1979; Green 1988). Methods were developed to purify these proteins and their organization in the granal membranes was elucidated. The complexity of the light-harvesting system was uncovered: refined biochemical analysis, together with the application of rapid advances in genetics, characterized a family of related proteins, Lhcb1-6 associated with PS II, and Lhca1-4 associated with photosystem I (PS I) (Jansson 1994). These proteins bind chlorophyll a and chlorophyll b in varying ratios and a number of xanthophylls, including those of the above-mentioned VAZ cycle. For PS II, trimeric LHCII (the major light-harvesting complex of PS II), comprising the Lhcb1-3 proteins was the dominant constituent, and the ease with which it could be purified in large quantities made it the object of intense scrutiny, eventually, some 20 years later, leading to its analysis by 2-D electron crystallography (Kühlbrandt and Wang 1991; Kühlbrandt et al. 1994) and structural analysis of 3-D crystals by X-ray crystallography (Liu et al. 2004). Accompanying this structural knowledge was the elucidation of energy transfer patterns in the complex using advanced spectroscopic techniques and modelling (van Amerongen and van Grondelle 2001).

In terms of NPQ research, LHCII became important for several reasons. A number of observations, summarized by Horton et al. (1996), had indicated that NPQ is a process occurring in the antenna of PS II rather than the reaction centers as proposed earlier (Weis and Berry 1987). This was a crucial advance because it focused attention on what properties of the antenna might be important for NPQ. It was also the first step in deconstructing NPQ, to ask the question could it (NPQ) be reduced experimentally to the properties of a particular component of the PS II antenna?

The first key fact was that the VAZ cycle carotenoids had long been thought to be associated with the Lhcb proteins, and this was confirmed using highly refined analytical approaches (Bassi et al. 1993; Ruban et al. 1994). All antenna complexes bind xanthophylls, but in different ratios. Violaxanthin and zeaxanthin were also bound to them all. There remains some controversy of how many violaxanthin molecules are bound, where exactly they are bound, and how accessible they are to de-epoxidation in conditions when NPQ is induced. This uncertainty stemmed from the weakness of violaxanthin and zeaxanthin binding and the probable incomplete occupancy of binding sites (Ruban et al. 1999).

One startling feature to emerge from structural studies of LHCII was confirmation of the close packing of chlorophyll molecules in these complexes. It was estimated that the concentration was equivalent to at least 0.6 M. Years earlier, Beddard and Porter (1976) had studied a phenomenon known as concentration quenching, the observation that excitation energy is always quenched when the concentration of a pigment exceeds a certain threshold; they remarked that it was extraordinary that photosynthesis worked at all given the density of chlorophyll in the chloroplast. Thus, it was argued that it was easy to imagine how quenching could occur in LHCII; the difficulty was understanding how quenching is prevented in the first place (Horton and Ruban 1995)! Logically, the architecture of the Lhcb proteins can be assumed to keep pigments in just the right configuration and with just the right spatial separation to allow (useful) energy transfer, while preventing quenching interactions (see Ostroumov et al., Chap. 4 and Krüger et al., Chap. 6 for discussion of the principles underpinning this statement). In corollary, NPQ would thus require only a subtle conformational change, sufficient to alter one or more of those pigment interactions to allow quenching. This may be a chlorophyll-chlorophyll or a chlorophyll-xanthophyll interaction or a mixture of both.

In fact, evidence for such a view already existed in the literature. One of the most widely used LHCII purification procedures in the 1980s involved magnesium-induced aggregation of soluble extracts taken from a sucrose gradient. It was noticed that the chlorophyll fluorescence yield of aggregated LHCII was extremely low and red-shifted compared to detergent-solubilized samples (Burke et al. 1978). In 1991, it was hypothesized that, since NPQ was a property of the PS II antenna, NPQ perhaps occurred by a process similar to that of LHCII aggregation (Horton et al. 1991). This model was a synthesis of the knowledge on LHCII, the postulated role of the VAZ cycle, the allosteric behavior of NPQ, and its dependence on the changes in thylakoid membrane structure described above. Though controversial, evidence accumulated over the subsequent 5 years that the two processes were indeed very similar (Horton et al. 1996). Crucial was the fact that the numerical extent of quenching upon LHCII aggregation was more than sufficient to account for NPQ in vivo. Other key facts included that violaxanthin inhibited and zeaxanthin stimulated the process of aggregation and the chlorophyll fluorescence quenching associated with it. Such indirect action of these carotenoids was entirely consistent with the allosteric model for qE, but not with the idea that zeaxanthin was the obligatory quencher. Thus, the four kinetic states of LHCII depicted in the allosteric model shown in Fig. 3.2 became different states of LHCII aggregation. Figure 3.1b shows how the general scheme for NPQ was modified to take into account this new approach.

Controversy surrounded the term “aggregation” because it may signify an unspecific, random process. There was naturally some reluctance to accept that a highly regulated in vivo process such as NPQ could so easily be reproduced in the test tube. Certainly, the process occurring when solubilized LHCII aggregates in an aqueous environment as a result of decreased detergent concentration cannot be exactly the same as when these complexes come closer together in the thylakoid membrane. Rather, as will be elaborated later, it may have been better to refer to “changes in association between complexes” or “macromolecular re-organization”. There is little doubt in the author’s mind that NPQ research in the decades following 1991 was held back because of confusion about what was meant by LHCII aggregation, as well as by the failure (until very recently, see below) to directly demonstrate via electron microscopy changes in associations between LHCII resembling aggregation when NPQ is induced.

Central to the controversy about this new NPQ hypothesis was the emergence of two opposing views of how the VAZ cycle acts. First, that zeaxanthin acts photo-physically, at the internal sites on the minor antenna complexes. Second, that zeaxanthin has an indirect role, modulating transition of LHCII between intrinsically different (non-quenching and quenching) states. Related to this controversy was the question of whether only LHCII, or also the minor complexes, play(s) any part in NPQ. This remains a hotly debated issue and will be discussed further below. In vitro, all Lhcb-containing complexes exhibit rather similar aggregation-induced quenching, and are similarly stimulated by exogenous zeaxanthin presumed to be acting at *peripheral* binding sites on the complexes. On the other hand, using the powerful approach of reconstituting the minor antenna complexes in vitro (Sandona et al. 1998), the extent of quenching was shown to depend on which xanthophylls were bound to the *internal* Lutein 1 and Lutein 2 sites. However, it should be remembered that the extent of quenching in these latter experiments is much smaller than expected if it was the major component of NPQ in vivo.

More recently, the ability of internally bound xanthophylls to modulate chlorophyll fluorescence quenching was confirmed by study of complexes prepared from *Arabidopsis* mutants lacking specific xanthophylls (Johnson et al. 2010). These latter workers have developed a new theory that unifies the two contrasting ideas about how NPQ is controlled by the VAZ cycle. Here, the complete complement of xanthophylls bound to each complex is suggested to confer a particular degree of hydrophobicity. It is this property of the complexes that controls the switch between unquenched and quenched states (Ruban et al. 2012). In this context, the VAZ cycle is the variable component of this xanthophyll complement. Biophysical approaches later shed light on these controversies.

VI Biophysical Approaches to Discovering the Mechanism of Quenching

A Spectroscopy of Light-Harvesting Complexes In Vitro

The biophysical laboratories were less sceptical about the possible relevance of LHCII aggregation, and a number of studies applied spectroscopic methods to compare quenched and unquenched states of LHCII. These studies were very productive. During the 1980s and 1990s, the power of spectroscopy in terms of spectral and temporal resolution had increased hugely, in part driven by the quest to unravel the primary processes of photosynthesis first in bacteria and then in plants. Centers of excellence were built in the USA, the Netherlands, Germany and

France. These centers were mobilized in the investigation of NPQ, initially examining purified light harvesting complexes including LHCII aggregates. Every type of spectroscopy employed (absorption, fluorescence, linear dichroism, circular dichroism, resonance Raman, transient absorption, Stark) revealed differences between the quenched and unquenched state of LHCII brought about by aggregation.

Mullineaux et al. (1993) were the first to apply fluorescence lifetime analysis to LHCII aggregates. Later, Moya et al (2001) carried out a particularly incisive study. Here, analysis of chlorophyll fluorescence lifetimes showed how the xanthophyll content of the Lhcb proteins determined the ratio between two lifetime components, strong evidence for the idea that quenching resulted from a change in ratio of two different conformations. Further, increasing the protein-protein interaction in liposomes led to a stronger quenching associated with a new short lifetime component. Thus, both intramolecular and intermolecular processes involving Lhcb proteins could contribute to NPQ, and xanthophylls are involved in both.

A red shift in chlorophyll absorption and a change in configuration of bound neoxanthin detected using Raman spectroscopy both became spectroscopic signatures of the quenched state, even though neither is directly involved in the quenching mechanism. These studies have been reviewed by Horton et al. (1996) and Ruban et al. (2012) (see also Pascal et al., Chap. 10). An important step forward came when these specific spectral features were found in crystals of LHCII. With high-resolution atomic structural detail available, it became possible for the first time to look with confidence for the structural features that give rise to quenching (Pascal et al. 2005). A number of sites of pigment interactions were identified where quenching could occur. These target domains are now being mapped with refined modelling techniques, as theoretical approaches to NPQ start gaining strength, and underpin interpretation of spectroscopic data.

Femtosecond transient absorption spectroscopy revealed a possible mechanism of quenching, i.e., promotion of energy transfer from chlorophyll a to lutein bound to the Lutein 1 site of LHCII (Ruban et al. 2007). As described elsewhere in this volume (Walla et al., Chap. 9; van Amerongen, Chap. 15), these types of studies are ongoing, not only searching for the energy transfer event or events that quench, but also seeking to identify the change in pigment configuration that brings this about. A recent innovation (Krüger et al., Chap. 6) uses spectroscopic investigation of single molecules of LHCII. These studies confirm the intrinsic capacity of LHCII to become a quencher and moreover prove how each molecule can reversibly visit the quenched state as first suggested from observations of LHCII ensembles. Most significantly, the dwell time in each state depends upon external conditions (Krüger et al. 2012). This revealed the essence of the NPQ process – factors such as ΔpH and DES change the dynamic equilibrium between different conformational states of LHCII in which energy can either be available for photosynthesis or dissipated as heat. As discussed above, this validates previous conclusions that qE may be no more and no less than the typical allosteric regulation exhibited by many proteins in biology.

B Spectroscopy of NPQ In Vivo

NPQ requires an intact system – at the very minimum isolated thylakoid membranes with the ability to generate a ΔpH. Maximum values of NPQ are only found in intact chloroplasts or whole leaves. Therefore, the results from in vitro systems needed validation to ensure they were physiologically relevant. However, spectroscopy of such systems, particularly looking for changes in “bulk” chlorophyll, remains a challenge, irrespective of the sophistication of the hardware – looking for a needle in a haystack. Resonance Raman spectroscopy proved particularly useful – this technique turned out to an incisive probe of the molecular configuration of carotenoids, even in complex systems (Robert et al. 2004). Thus, the same signal, attributed to the twisted conformation of neoxanthin found in aggregates of LHCII, was seen in leaves when NPQ was induced, providing compelling evidence for the LHCII aggregation hypothesis (Ruban et al. 2007; see also Pascal et al., Chap. 10).

Prior to this, the absorption change with a principal maximum at 535 nm (ΔA_{535}), referred to above as "light scattering", was subject to renewed investigation (Bilger and Björkman 1989). Raman absorption resonated at 535 m only in samples with NPQ and so proved that this was a true electronic absorption change, arising from zeaxanthin (Ruban et al. 2002). Better spectral analysis revealed a series of sub-bands not detected previously, showing that ΔA_{535} was a difference spectrum for a red-shifted zeaxanthin. Further, in the absence of zeaxanthin, NPQ caused a similar spectral change, but at different wavelengths (Johnson et al. 2009). Thus, rather than indicating a macroscopic conformational change as assumed before, the ΔA_{535} is due to a very subtle change in the local environment of a bound xanthophyll. Recent studies have shown that shifts in the chlorophyll Soret absorption contribute to the spectral changes in this region, and correlate also with red shifts in the Qy band (Johnson and Ruban 2009). These results confirm that NPQ is indeed associated with changes in the configuration of pigments, most probably in LHCII. This is ongoing research, as the search continues to identify precisely which pigment domains are participating (see Pascal et al., Chap. 10).

The studies of circular dichroism (CD) made on intact systems should also be mentioned here. It has been known for a long time that the CD signal of thylakoid membranes is anomalously larger than for isolated solubilised complexes. This "big CD" was attributed to some feature of the long-range order of the grana membranes. What was interesting was that this signal diminished under illumination conditions, in a manner that resembled NPQ (Garab et al. 1988). This was some of the first direct evidence that illumination perturbed the organization of pigments in the thylakoid membrane, although it is still not yet understood precisely how this phenomenon relates to NPQ (see discussion of this by Garab, Chap. 16).

Chlorophyll fluorescence measurements were also applied with ever-increasing sophistication, not to capture NPQ as described above, but in an effort to uncover aspects of mechanism. Simple models of energy distribution in the antenna and reactions centers devised in the 1970s by Butler (Butler and Kitajima 1975) allowed the broad location of quenching processes to be distinguished. Analysis of NPQ showed it to be consistent with an antenna quenching (Rees et al. 1990). Moreover, application of more complex models allowed the conclusion that NPQ is a static process, in which antenna units were functionally detached from PS II (Walters and Horton 1993). Fluorescence spectroscopy at 77 K showed how NPQ selectively acted on the LHCII antenna, one of the first pieces of evidence for their involvement (Ruban and Horton 1994). Gilmore et al. (1998) were the first to apply time-resolved fluorescence measurement to the study of NPQ, confirming its static nature by showing that NPQ involved changing the proportions of two different lifetime components, as strong support for a switch between two discrete antenna states.

Transient absorption spectroscopy has also been applied to intact systems. Here evidence for charge transfer states of chlorophyll and carotenoid were identified, suggesting an entirely different mechanism of quenching to that associated with LHCII aggregation (Holt et al. 2005; see also Walla et al., Chap. 9). These events were duplicated in isolated minor complexes CP29 and CP26 but most importantly not LHCII (Ahn et al. 2008). This dichotomy gave rise to the idea that there might be two sites of NPQ with two different mechanisms occurring in parallel. Modelling of time-resolved fluorescence data lends support to this idea – the data would fit a model in which NPQ caused dissociation (and aggregation) of LHCII from the PS II core (causing one type of NPQ), leaving a quenching mechanism in the core, possibly in the minor antenna, the second type of NPQ (Holzwarth et al. 2009). This idea is gaining broad acceptance and is reminiscent of that proposed earlier by Moya et al. (2001), and reconciles different ideas about the role of the VAZ cycle and the different components of the PS II antenna (see detailed discussions in Holzwarth and Jahns, Chap. 5 and Morosinotto and Bassi, Chap. 14). Another model, in which the "two sites" of quenching merely result from heterogeneity could explain the data equally well – not all LHCII would be detached, and trimers associated with PS II would still quench. This kind of polarization of opinion is to be expected. Proving anything in science is difficult, but here, dealing with complex heterogeneous intact systems, the measurement techniques and the methods used to analyze the data are at the limits of feasibility.

VII Molecular Genetics: The Rise of Arabidopsis

The advances in plant molecular genetics arising from the development of *Arabidopsis thaliana* as the model organism finally impacted on photosynthesis research in the early part of the twenty-first century. Application of the two classical approaches used to link gene to function, reverse and forward genetics, led to major discoveries in NPQ research. Firstly, use of antisense expression and, later, isolation of knockout mutations in which specific known genes were targeted allowed exploration of possible roles of each Lhcb gene in NPQ (Andersson et al. 2001, 2003). Secondly, screening techniques using imaging of chlorophyll fluorescence quenching from large populations of mutant collections of *Arabidopsis* plants allowed the isolation of NPQ-deficient mutants (Niyogi et al. 1998). Combined with the capability to over-express the target genes, huge possibilities emerged for intricate experiments in which all factors involved in NPQ could be varied in whole plants, in a multitude of double or triple mutants.

The work of Jansson pioneered the genetic approach to understanding the function of the Lhcb proteins. In terms of NPQ, the results were in some respects disappointing. Elimination of CP26 had no effect on NPQ, removing CP29 only affected the kinetics of NPQ formation and complete depletion of Lhcb1 and 2, the main components of LHCII, caused only a small decrease in NPQ capacity. The latter findings exposed the dangers of this approach – the eliminated LHCII trimers were replaced by CP26 trimers that maintained many aspects of both structure and function (Ruban et al. 2003). Removing CP24 had the most pronounced effect, reducing the amplitude of NPQ and altering its kinetics (Kovacs et al. 2006). A surprising result was the restoration of wild type phenotype in a double mutant lacking both CP26 and CP24 (de Bianchi et al. 2008). Such results were baffling at first sight, but only because we do not fully understand the organization of the complexes in the membrane or how exactly this determines their function. However, the inescapable conclusion was that NPQ was not a property in any one pigment complex, but somehow depended on their collective presence in the functional grana membrane.

The most dramatic outcome of the genetic approach to NPQ research came from the types of NPQ-deficient mutants selected. Predictably, an *npq1* mutant was defective in violaxanthin de-epoxidase (Niyogi et al. 1998). Similarly, the *pgr* mutants were unable to build up a large ΔpH under high light (Shikanai et al. 1999). However, it was the *npq4* mutant that threw the NPQ research community into a frenzy in 2000 (Li et al. 2000; see also Brooks et al., Chap. 13). This mutant was found to be deficient in a then obscure thylakoid protein known as PsbS, previously thought to have some undefined role in pigment binding similar to ELIPs (early light-inducible proteins, with both PsbS and ELIPs now being recognized as light-stress-associated members of the light-harvesting complex gene family). What role could this protein possibly have in NPQ? Initially, PsbS was proposed to be the site of NPQ, based on various studies suggesting that PsbS had the capacity to bind both chlorophylls and carotenoids. However, the results of investigations of pigment binding proved to be prone to artefact, due to PsbS' extremely hydrophobic nature. More intriguing was the possibility of PsbS being the site where ΔpH was sensed through protonation. Site-directed mutations of lumen-facing acidic residues in PsbS abolished NPQ (Li et al. 2004) and the protein was shown to bind DCCD (dicyclohexylcarbodiimide), a reagent that had been used to identify acidic residues of functional significance in other systems. New NPQ models were proposed, in which protonation and zeaxanthin binding by PsbS were the triggers for NPQ (Fig. 3.1c, see also Morosinotto and Bassi, Chap. 14).

From the start, however, it was realized that this picture was not entirely correct. NPQ was not eliminated in the *npq4* mutant, but only suppressed. In thylakoid membranes isolated from the *npq4* mutant, ΔpH-dependent NPQ was detected (Horton et al. 2000). Indeed, it was subsequently found that, if ΔpH is high enough, the amplitude of NPQ in *npq4* is close to that of the wild type (Johnson and Ruban 2011). Large NPQ amplitudes were later detected in leaves of *npq4* under extended illumination (Johnson and Ruban 2010). Thus, PsbS determines the kinetics of NPQ induction and its pH-sensitivity and, in effect, is an NPQ catalyst. Understanding how this worked came from another strand of research, the renewed investigation of the macromolecular organization of the grana membranes.

VIII The Key to NPQ: Understanding the Organization of the Thylakoid Membrane

A *Electron Microscopy and Image Analysis*

After the 1970s, the focus of research on the thylakoid membrane shifted to the isolation and purification of its constituent complexes, with the aim of obtaining high-resolution structural data using crystallographic methods. Following on from pioneering studies into the organization of complexes in the membranes of photosynthetic bacteria, increasing success resulted from efforts to do the same for PS II and PS I complexes. Using electron microscopy (EM) and image analysis, it was discovered that PS II existed as a macromolecular super-complex that could be detected in various forms following partial solubilization of thylakoids with detergent (see the seminal review by Dekker and Boekema 2005). The basic PS II core was dimeric, and bound the light-harvesting complexes, both LHCII trimers and the minor complexes CP24, CP26 and CP29. The structure of these super-complexes could be determined, albeit at low resolution. Different sizes of particles were classified according to how many LHCII trimers were bound and which of the minor complexes were present, e.g., C2S2 and C2S2M2, which refer to numbers of the (C)ores and (S)trongly or (M)oderately bound LHCII trimers. As this technique developed further, it was used to investigate the structural differences in the various *Arabidopsis* Lhcb mutants described above. Indeed, it even became possible to do such analyses on fragments of intact membrane because they contained areas of ordered semi-crystalline arrays of LHCII-PS II super-complexes, allowing structural analysis of these complexes in situ in grana membranes.

The CP24 mutant, which had the strongest NPQ phenotype, also showed the largest difference in LHCII-PS II organization – rows of C2S2 super-complexes were found in the mutant rather than crystalline arrays of C2S2M2 in the wild-type (Kovacs et al. 2006). In the *npq4* mutant and its counterpart, the L17 PsbS over-expresser, no difference in super-complex structure was detected. However, something was found that was at first dismissed as just curious but was later seen to be fundamental: in the presence of excess PsbS, no crystalline arrays of PS II were detected, whereas in the absence of PsbS, their frequency doubled (Kereiche et al. 2010). Could this be the basis of the catalytic effect of PsbS in NPQ? Were the crystalline areas somehow a reflection of a state or organization inactive in NPQ because it prevented a re-organization leading to LHCII aggregation? Was PsbS in effect a regulator of membrane organization, a kind of dynamic assembly factor for the super-complexes? This suggestion brought into focus ideas increasingly voiced during the previous 10 years that, even though the quenching mechanism may be intrinsic to an individual LHCII subunit or an individual minor antenna complex, dynamic changes in the organization of the grana membrane were at the heart of NPQ (e.g., Horton 1999; Moya et al. 2001; Horton et al. 2008). Such changes were inextricably linked to the stabilization of the quenched state and its promotion by protonation and by violaxanthin de-epoxidation. In this scenario, PsbS would be the controller and/or initiator of the process of re-organization. This suggestion was about to receive validation from two very different approaches to studying membrane dynamics.

B Analysis of Associations Between Complexes

The Bassi laboratory had used biochemical approaches to study membrane organization for many years – such as gentle solubilization followed by separation of the resulting supra-molecular complexes on sucrose gradients or isoelectric focusing gels (e.g., Bassi et al. 1991). This powerful approach had been successful, for example, in accurately mapping the organization of LHCII-PS II super-complexes long before application of the EM approaches discussed above (Bassi and Dainese 1992). In 2009, they found important differences when comparing samples dark-adapted and illuminated to induce NPQ. In contrast to the analysis of membranes from dark adapted plants, after exposure to NPQ conditions, the B4 super-complex comprising CP24, CP29 and LHCII (M) trimers was not detected (Betterle et al. 2009). This was the first direct evidence of an NPQ-related structural change within PS II and it was accompanied by EM evidence of a re-distribution of PS II core complexes within grana membranes when NPQ is formed. The controlling influence of VAZ and PsbS on this structural change was emphasized (see also Morosinotto and Bassi, Chap. 14).

C Freeze Fracture Electron Microscopy

In the 1970s, the principal new tool for determining the structure of thylakoid membranes was EM of membranes frozen and then sheared to reveal both membrane surfaces and inter-membrane fracture planes (Staehelin et al. 1977). This freeze-fracture EM showed the presence of numerous particles, arranged in domains corresponding to the appressed membranes of the grana and those unappressed in the stroma (Armond et al. 1977). These particles were later identified and, together with other measurements, allowed the locations of LHCII-PS II and PS I to be determined, a landmark in photosynthesis research (Andersson and Anderson 1980). However, in time, this approach went out of favor with the shift in focus to analysis of the structure of these complexes, and the number of experts and working facilities dwindled. But, in 2011, a landmark paper in NPQ research was published, which built upon the findings of Betterle et al. (2009) and Kereiche et al. (2010) described above, and showed the results of freeze-fracture EM analysis of samples in each of the four states depicted in the LHCII aggregation model (Johnson et al. 2011; see also Ruban and Mullineaux, Chap. 17). The results were dramatic – clustering of LHCII was observed, which correlated with the extent of NPQ. In further experiments, the changes in organization in the *npq4* mutant and the CP24-deficient mutant deduced from previous results of EM and image analysis were confirmed (Goral et al. 2012). Most interesting was the observed reversion to a wild type thylakoid organisation in the double CP24/CP29 mutant in contrast to that of the

CP24-deficient mutant, correlating with the differences in NPQ between these mutants discussed above (Sect. VII).

IX Integration: The State of the Art

Although many details remain to be resolved, by the start of 2103 we have a coherent hypothesis of how NPQ works, supported by a wealth of different kinds of experimental evidence (Horton 2012). When viewing the process as a hierarchy, we can see how the intrinsic disorder of the LHCII molecules, perhaps due to fluctuations in the Chl-lutein domain that determine the direction of energy transfer, are harnessed for physiological control (Fig. 3.3). This property is vividly seen in single-molecule experiments. The factors controlling the equilibrium between unquenched and quenched states are exemplified in observations of in vitro LHCII aggregation. Finally, the aggregation of LHCII in vivo relies on the dynamic organization of LHCII in the grana membrane. Either (unquenched or quenched) state is stabilized by forces acting at the membrane level – the protonation state of the lumen surface and the hydrophobicity of the bound xanthophylls (Fig. 3.4). We have a new understanding of how the pK_E for the protonations (of PsbS and/or Lhcb) that drive NPQ is controlled through the sum of forces linked to aggregation of LHCII that, in turn, depends on the composition of the grana membrane. In particular, the transition between these states is wholly dependent on the presence of PsbS. In its absence, the organization is locked into one that stabilizes the unquenched state. We can also speculate that all NPQ in a range of different organisms can be explained in similar

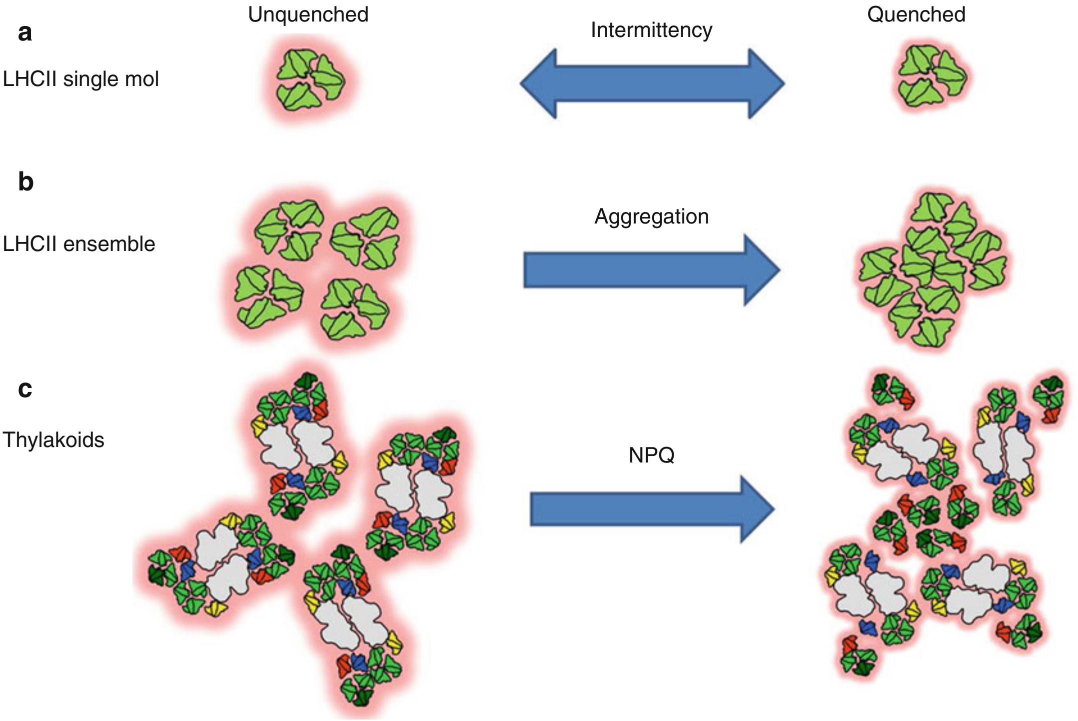

Fig. 3.3. Hierarchy in the experimental observation of the behaviour of LHCII as a quencher. (**a**) Intrinsic quenching via the controlled disorder as observed in single molecule experiments. (**b**) Expression of single molecule behavior in ensemble experiments, where aggregation causes quenching. (**c**) Observation of clustering of LHCII-PS II complexes giving rise to quenching in thylakoid membranes. *Green* denotes the Lhcb monomers of the LHCII trimers; *Red*, *blue*, *yellow*, minor antenna complexes; *grey*, reaction center cores.

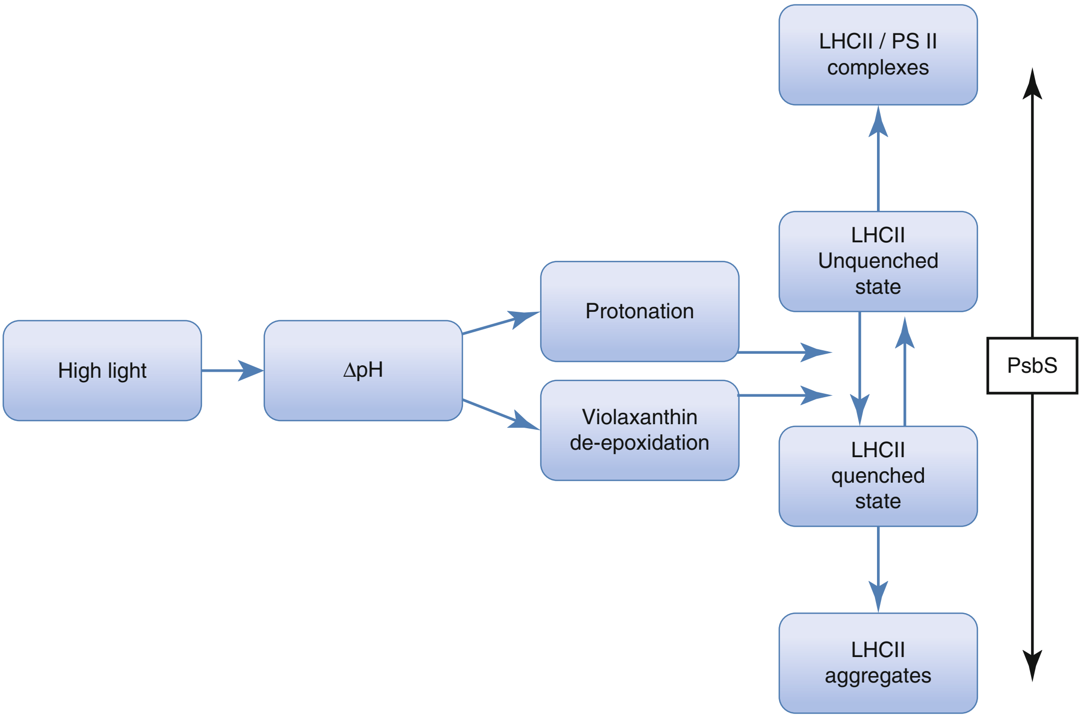

Fig. 3.4. Development of the model depicted in Fig. 3.1. This model depicts the further development of the theory shown in Fig. 3.2, that protonation and de-epoxidation set the equilibrium between two intrinsic states of LHCII. Here, the organization of the membrane is crucial, clustering (aggregation) of LHCII and weakening of the LHCII-PS II supercomplexes stabilizing the quenched state, whereas the "normal" supercomplex structure favors the unquenched state. The transition between these states depends upon the dynamic flexibility conferred upon the thylakoid membrane by PsbS.

terms – other proteins, such as ELIPs, covalent modifications by protein phosphorylation, specific lipid composition, turnover of PS II cores all tune the membrane environment to give NPQ a range of time-constants for formation and relaxation, of temperature dependency, of pH dependency, and so on (Horton et al. 2008). Indeed, it is when these other factors come into play that we see the NPQ state highly stabilized, for example, in some cold-adapted leaves where, over the winter, strongly red-shifted LHCII aggregates predominate.

The evidence supporting the LHCII aggregation model for NPQ is strong. This model encompasses all the data, whether spectroscopic, physiological or structural. However, this need not exclude another process operating alongside it, in parallel, taking place by an entirely different mechanism in the minor antenna complexes. Could processes depicted in Fig. 3.1b, c be working together, co-ordinated together according to particular membrane composition and physiological conditions? Only time (and further experiments) will tell if this is the case.

X Addendum: Ecology and Agriculture

NPQ is ubiquitous in higher plants. However, its amplitude and kinetics are highly variable across species. Within a species, these properties of NPQ are variable according to growth conditions. Finally, the extent and character of NPQ depends on the illumination conditions to which the plant has been recently exposed. All of this variability has been exposed by detailed analyses carried out over a number of years, from the 1980s until the present day. Following the visionary lead of Björkman, Osmond and others, the laboratory of Demmig-Adams and Adams has been the major player in this area, and they have established beyond doubt the beautiful way in which NPQ is tuned to the physiology of the whole plant (Demmig-Adams and Adams 2006; see also Demmig-Adams et al., Chap. 24). Now that the main factors determining the properties of NPQ are known (ΔpH, VAZ cycle pool size, the concentration of PsbS and the composition of the PS II antenna), this variability has been explained in mechanistic terms. Whether this involves changes in relative dominance of two different NPQ mechanisms or just increasing strength of a single mechanism remains to be decided. Moreover, other properties of the thylakoid membrane have also been shown to be involved in modulating the character of NPQ – phosphorylation of PS II core proteins seems to stabilize NPQ formed under low temperature conditions, for example as mentioned above. Variation in xanthophyll cycles in the form of the lutein epoxide cycle modulates NPQ in some shade plants (Garcia-Plazoala et al. 2007; see also Esteban and Garcia-Plazaola Chap. 12). How NPQ is integrated into other aspects of whole plant physiology is only just beginning to be understood (see Adams et al. 2013, Chap. 23). The dependency of NPQ on thylakoid dynamics is particularly important – only recently have there been glimpses of the huge extent to which the membrane rapidly changes its organization in response to environmental change (Anderson et al. 2012). Continued exploration promises to give a new picture of how the thylakoid membrane functions in nature where the environment continuously fluctuates and the metabolic demands placed on the chloroplast are rarely constant (Adams et al. 1999).

The relationship between NPQ and the agricultural yield of plants is also only just beginning to be explored experimentally. Whilst the photoprotective role of NPQ is clear, suggesting that crop plants may be improved by having a greater capacity for energy dissipation to resist extreme conditions, the converse was also proposed: perhaps NPQ is unnecessary in some agricultural scenarios and there could be gains by eliminating or reducing its capacity (Horton 2000; cf. also Demmig-Adams et al. 2013). In more general terms, the plant's ability to survive in fluctuating conditions requires dynamic regulatory mechanisms such as NPQ, but could these restrict maximal photosynthesis and growth rates? As first suggested by Horton (1985), the trade-off between maximum throughput and survival (stability), a fundamental property of all natural and man-made production systems, could determine the yield potential of crops. There is proof of genetic variability in where the balance is set, and perhaps agricultural improvement could result from adjusting this (Murchie et al. 2008). In the specific case of NPQ, this latter train of thought received some support from Long and co-workers, who calculated the lost productivity arising from the slow recovery from NPQ when conditions of excess light were relieved (Zhu et al. 2004). Whilst this gives an estimate of the potential losses that could occur, it can be argued that the development of sustained NPQ arises from downstream restrictions on photosynthesis and it is only by relief of these that NPQ relaxes (see evidence in Adams et al. 2013, and Chap. 23). Murchie and co-workers studied the effect of manipulating the extent of NPQ on photosynthesis and growth in rice, via genetically changing PsbS content (Hubbart et al. 2012 see also Murchie and Harbinson, Chap. 25); evidence was obtained that rapidly relaxing NPQ could impose a restriction on photosynthetic but whether this affects total photosynthesis and productivity is not yet known. The issue may be complicated by other types of potential trade-offs such as between PsbS content and plant defense, uncovered by Jansson – reviewed in Demmig-Adams et al. (2013). Whether manipulation of the factors that control NPQ capacity and dynamics proves to be a fruitful pursuit in terms of agricultural gain is unknown, but the fact remains that the processes plants use to harness as much sunlight as possible for their growth and reproduction are finely balanced against the potential damage that can result.

XI Concluding Remarks

The state of knowledge about NPQ that we find now, in 2013, is in some ways only an elaboration of what was suggested 40 years earlier. But this should not diminish the merit of this elaboration. NPQ is firmly embedded in plant physiology, but its mechanism touches on processes of energy transfer, protein dynamics and membrane function that are not yet fully understood or described. However, we are close to understanding NPQ at the very finest detail, and this has involved a truly multidisciplinary effort, from physical chemistry to ecology. Approaches drawn from the forefront of technical and intellectual advances in science have been used. Integrating and communicating knowledge across such disciplinary boundaries has been a great challenge.

So, what were the reasons for the convoluted journey between 1970s and the present day? In part, this is due to the limitation of scientific capabilities at the time. Progress in biology has been immense in the last 40 years and this progress has allowed the key steps to be taken. However, there are other factors, probably many, but I could suggest a few. Firstly, the discovery of LHCII phosphorylation and its role in state transitions dominated thinking about thylakoid dynamics because it was so beautifully clear and specific. In contrast, NPQ was vague and even its function was constantly questioned. During the 1980s and early 1990s hardly anyone considered the processes underlying NPQ important. Secondly, the unfortunate proliferation of terms and parameters that continues to this day did not help in the communication. Even the term "non-photochemical quenching" conveys very little to anyone outside the immediately involved researcher. Thirdly, one could point to a number of great ideas that turned out to be wrong, such as the molecular gearshift model that provided such a simple and attractive NPQ mechanism and the suggestion that PsbS is the unique quenching site, or oversimplified, such as the belief that zeaxanthin is the obligatory and only quenching molecule. Finally, there were the battles between opposing viewpoints, which were sometimes destructive rather than constructive. As we look forward, what optimism is there that the mistakes of the past will not be repeated? Almost all of the main players in NPQ research over the last 20 years are represented in this volume. That in itself is a good omen. Undoubtedly, new developments not only in biology but in physics and chemistry will drive progress, particularly in unravelling exactly how energy transfer in antenna complexes is controlled. Understanding precisely how proteins such as PsbS, together with small molecules, lipids and carotenoids, regulate the structure and function of membrane protein complexes will have implications beyond NPQ research. Equally, the impetus provided by the huge challenges facing humankind to sustainably produce enough food and energy will stimulate further intellectual and technological evolution in photosynthesis research that undoubtedly will drive progress in understanding NPQ. The possibility that the remarkable molecular transitions that underlie NPQ can be exploited in the design of biomimetic or hybrid nanoscale devices is a particularly exciting prospect for the future.

Acknowledgments

I wish to thank all those who have been my collaborators and/or my competitors over the last 30 years of researching into NPQ. I am indebted to all the excellent students and postdocs who have worked in my group and contributed to the story depicted in this chapter, in particular Alexander Ruban whose contribution to the development and exploration of the LHCII aggregation model was pivotal. Finally, I acknowledge the inspirational work of Jean Marie Briantais and Heinrich Krause that was the starting point for my quest.

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Chapter 4

Photophysics of Photosynthetic Pigment-Protein Complexes

Evgeny E. Ostroumov and Yaser R. Khan
Department of Chemistry, University of Toronto,
80 St. George Street, Toronto, ON M5S 3H6, Canada

Gregory D. Scholes*
Department of Chemistry, Princeton University,
Washington Rd, Princeton, NJ 08544, USA

and

Govindjee
Department of Plant Biology,
Department of Biochemistry and Center of Biophysics and Quantitative Biology,
University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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*Author for correspondence, e-mail: gscholes@princeton.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_4, © Springer Science+Business Media Dordrecht 2014

Dedication This chapter is dedicated to the late Professor Robert M. Clegg (July 18, 1945 — October 15, 2012) of the University of Illinois at Urbana-Champaign (UIUC). He was a pioneer of the physics of living cells, and a world leader in fluorescence lifetime-resolved imaging microscopy (FLIM) of biological systems. He was an authority on fluorescence spectroscopy and rapid kinetic methods as applied to biological systems. Bob was a great human being, an intellectual, a “living library” to his students and his colleagues, a “connoisseur” of creative literature (not only in English, but in French and German as well), a historian of science, a great teacher, a poet, a storyteller, a great chef of diverse cuisine for friends and family, and a friend to all with whom he associated. One of us (Govindjee) was fortunate to have worked with him since he came to UIUC; they used FLIM in studying photoprotection against excess light in the green alga *Chlamydomonas reinhardtii*, and in avocado, a higher plant.

Summary

Photosynthesis converts solar energy into energy of chemical bonds. This process is initiated when a photon of sunlight is absorbed by a photosynthetic pigment molecule, followed by a highly efficient transfer of the excitation energy, excitation trapping, and charge separation at the reaction center. The excited state dynamics initiated by light absorption are central to the primary reactions of photosynthesis. Unless successfully transferred away from the excited chromophore within the excitation lifetime, the excitation energy relaxes back to the electronic ground state, either via emission of a photon (radiative decay) or through various nonradiative processes. The photosynthetic machinery can control the nonradiative relaxation rate: it can increase it under stress conditions (e.g., high light) by adjusting electronic properties of chromophores as well as their interaction, or decrease it under optimal conditions reaching >90 % efficiency of energy transfer. Some background in photophysics is, therefore, needed to understand the mechanistic aspects of the initial events following photoexcitation of photosynthetic complexes. The goal of this chapter is to describe the excited states involved in photoreactions and to outline the physical basis of photophysical processes involved in photosynthesis. We introduce the principles of light absorption and the nature of electronic excited states and light-initiated dynamics in photosynthetic complexes. De-excitation pathways, rate constants, quantum yields and lifetimes of fluorescence, excitation energy transfer and related photophysics are discussed. In the concluding section, we present an overview of the mechanisms of non-photochemical quenching (NPQ) of chlorophyll fluorescence in terms of photophysics of the excited states of photosynthetic pigments.

Abbreviations A_g, B_u, E_u – Excited state symmetry; a_u, b_g, e_g – Molecular orbital symmetry; B, Q_x, Q_y – Chlorophyll singlet states; C_n, S_n, σ_h, *I*, *e* – Molecular symmetry operators; Chl – Chlorophyll; EET – Excitation energy transfer; EM – Electromagnetic; FRET – Förster resonance energy transfer; HOMO – Highest occupied molecular orbital; IC – Internal conversion; ISC – Intersystem crossing; LUMO – Lowest unoccupied molecular orbital; LH2 – Light-harvesting complex 2 of purple bacteria; LHC – Light-harvesting complex; LHCII – Light-harvesting complex II, a major antenna of PS II in plants and green algae; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PS I, PS II – Photosystem I, photosystem II; PSU – Photosynthetic unit; RC – Reaction center; S_0, S_1, and S_2 – Ground, first excited and second excited singlet states of carotenoids, not to be confused with similar names for the states of the oxygen evolving complex, the 4Mn-Ca (water) complex; TDC – Transition density cube; Φ_{fl}, Φ_{ET}, Φ_{tr}, Φ_{PQ}, Φ_{NPQ} – Quantum efficiencies of fluorescence (fl), energy transfer (ET), trapping (tr), photochemical (PQ) and non-photochemical quenching (NPQ) respectively; note that Φ_{PQ} is equivalent to Φ_p used by other authors

I Introduction

Photosynthesis starts with absorption of light by a pigment molecule that, in most photosynthetic organisms, is embedded in pigment-protein complexes. The processes following light absorption occur on remarkably fast timescales by vastly distinctive mechanisms. The aim of this chapter is to present an overview of photophysical background relevant for the light reactions of photosynthesis. For background on physical aspects of the interaction of light with living matter, see Clayton (1970); for basic background knowledge on photosynthesis, see Rabinowitch and Govindjee (1969) and Blankenship (2014); for details on the entire process of photosynthesis, see Eaton-Rye et al. (2012).

An array of different pigments is used for the harvesting of light. Among them are members of the porphyrin family – chlorophylls or bacteriochlorophylls – found in all photosynthetic membranes (see Grimm et al. 2006). Light absorption promotes the light-absorbing molecule (chromophore) from the ground state to an excited electronic state, thereby storing much of the energy of the photon in the molecule. This energy, however, is only stored transiently because excited electronic states decay back to the ground state by de-excitation processes involving either emission of light (fluorescence or phosphorescence) or "rapid cooling" by nonradiative processes, e.g., heat emission. Isolated chlorophylls, for example, have an excited-state lifetime on the order of 5 ns (Brody and Rabinowitch 1957; Kaplanová and Parma 1984). For fluorescence properties of photosynthetic pigments in vitro, see Seely and Connolly (1986). When chlorophyll *a* molecules are embedded in a protein, nonradiative quenching of their excited states increases due to interactions between the pigments and their environment, decreasing their lifetime to ~4 ns (Mullineaux et al. 1993; Connelly et al. 1997). The deactivation of excited states is determined by the intrinsic properties of the chromophore and how it interacts with the environment. It is through such interactions that the protein can tune, sometimes quite remarkably, the properties of the chromophores in light-harvesting complexes, either directly or by arrangement of several chromophores into aggregates with a strong inter-chromophore interaction.

The primary event in photosynthesis, after light is absorbed by chromophores in a light-harvesting complex, is transfer of that excitation energy to reaction centers (RC) where the energy is stored by charge separation. This *excitation energy transfer* (EET) process must be faster than the excited-state lifetime of chlorophyll in order for the excitation energy to reach the RC rather than be lost via fluorescence or nonradiative decay. We know that nature does this successfully because the efficiency of energy transfer from arrays of light-harvesting complexes to reaction centers is very high (typically >90 %, see e.g., Krause and Weis 1991; Wientjes et al. 2013). This efficiency, which in some cases requires rapid jumps (~300 fs on average) of excitation from molecule to molecule, is facilitated by chlorophyll molecules being present in thylakoid membranes at a typical concentration of >0.2 M. In solution, isolated chlorophylls are efficiently quenched by concentration quenching at concentrations as low as ~0.1 M (Beddard and Porter 1976). The remarkably high efficiency of energy transfer achieved in natural photosynthetic complexes is due partly to the arrangement of chlorophyll and other chromophores in a way that prevents concentration quenching. Other physical principles, such as a dense, ordered arrangement of bacteriochlorophylls in a ring of the light-harvesting complexes, LH2, of purple photosynthetic bacteria, allow the chromophores to work cooperatively. States termed *exciton states* are formed (Monshouwer and van Grondelle 1996; van Amerongen et al. 2000a) leading to a shift of the absorption spectrum, delocalization of excitation energy, and efficient intra- and inter-protein energy transfer (Sundström et al. 1999; Scholes and Fleming 2000; Robert 2008). Another example is the exceptionally rapid energy transfer from carotenoids to (bacterio-)chlorophylls in light-harvesting complexes (LHCs) despite the ultrashort lifetimes of carotenoid excited states (Gradinaru et al. 2000; Croce et al. 2003; Frank and Polívka 2008). For overviews on the photochemistry of carotenoids, see Frank et al. (1999).

Efficient harvesting of solar energy is achieved by using more than one type of chromophore in antenna complexes. For a discussion of various light-harvesting antenna systems of plants, see chapters in Green and Parson (2003). Accessory pigments used by plants and photosynthetic microbes include, e.g., carotenoids that absorb blue-to-green light and phycobilins that absorb green-to-orange light. These pigments transfer absorbed energy to chlorophyll *a* with high efficiency (see, e.g., Duysens 1952; Govindjee 1999; Clegg et al. 2010). Despite their simple molecular structure, carotenoids have unusual electronic properties, e.g., an optically forbidden lowest singlet state with an ultrashort (~10 ps) lifetime (Christensen 2004). Because of their short excited-state lifetime, carotenoids are efficient quenchers of both singlet and triplet excited states (Telfer et al. 2008). Moreover, carotenoids can form charge-transfer complexes with chlorophylls, creating efficient traps for excitation energy (Gradinaru et al. 2000; Hsu et al. 2001).

A significant feature of LHCs is their ability to respond to ambient light conditions, and modify chromophores and/or interactions among chromophores such that excess electronic excitation is dissipated by heat if the amount of the absorbed light exceeds the capacity of the photochemical machinery (i.e., charge separation in RCs). This process, accessed via non-photochemical quenching (NPQ) of chlorophyll fluorescence, can decrease the efficiency of energy transfer by a factor of 2–10 (as judged by fluorescence lifetime) on a time scale of several seconds to minutes (see, e.g., Weis and Berry 1987; Krause and Weis 1991; Horton et al. 1996; Gilmore 1997; Gilmore and Govindjee 1999). The mechanisms of photoreactions in photosynthesis remain incompletely understood due to the complexity of the electronic structure of chromophores (chlorophylls and carotenoids in algae and higher plants), the sophisticated schemes of chromophore interaction (excitonic coupling, charge transfer states, forbidden transitions), and the significant role of the protein environment in tuning the properties of embedded chromophores (heterogeneity of electronic properties, polar environment, alteration of chromophore geometry). In this chapter, we focus on electronic properties of chlorophylls and carotenoids, the interaction of their excited states, and excitation energy pathways relevant to NPQ of fluorescence of chlorophyll *a* molecules.

II Chromophores in Photosynthesis and Their Electronic Properties

A Chlorophylls

Chlorophyll molecules serve as the primary photoreceptors in most photosynthetic organisms (Scheer 2006). The latter molecules belong to the cyclic tetrapyrrole family (porphyrins) with four pyrrole residues in the macrocycle (Fig. 4.1). In different chlorophylls, the peripheral pyrrole carbons bear various side chains. The nitrogen atoms of the porphyrin ring bind magnesium ion (Mg^{2+}).

The porphyrin ring is near-planar, and the electron density of its π-electrons is strongly delocalized over the entire ring. In accordance with its square symmetry, porphyrin belongs to the D_{4h} point group (Rubio et al. 1999; Liao and Scheiner 2002). Defining symmetry of a molecule provides a convenient way to label molecular orbitals and thus electronic transitions. Well established rules then allow prediction of the spectroscopic properties of the transitions, for example, whether they are allowed (a bright absorption band) or forbidden (a dark state – not seen in the absorption spectrum, but nevertheless present in the ladder of electronic states). In this context, symmetry indicates operations on the molecule leading to a state indistinguishable from the original state (see Fig. 4.2), e.g., rotation of the molecule by $180^0/n$ (C_n), reflection in a given plane (σ_h), identity operation (e), center of inversion (I), and rotary reflection S_n (Willock 2009). The symmetry operations and the corresponding irreducible representations (a symmetry

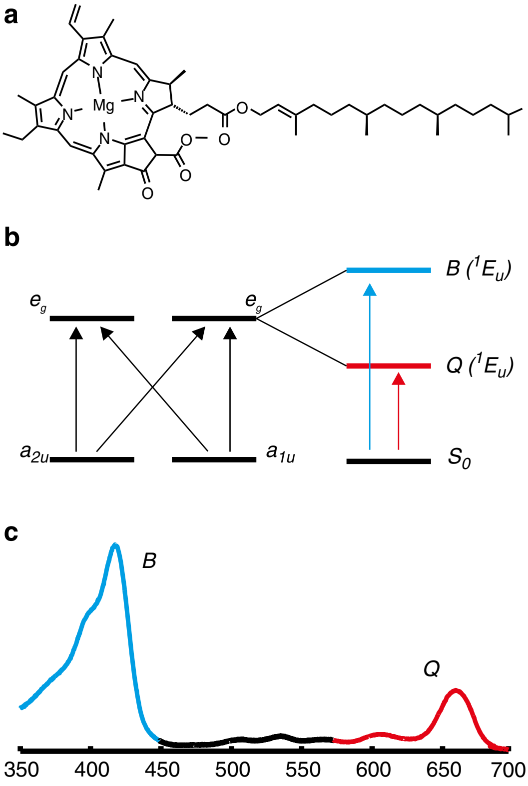

Fig. 4.1. Chlorophyll *a* molecule. (**a**) Molecular structure, (**b**) molecular orbitals and electronic level structure, (**c**) absorption spectrum. The *capital letters* indicate states, *lower case letters* indicate orbitals.

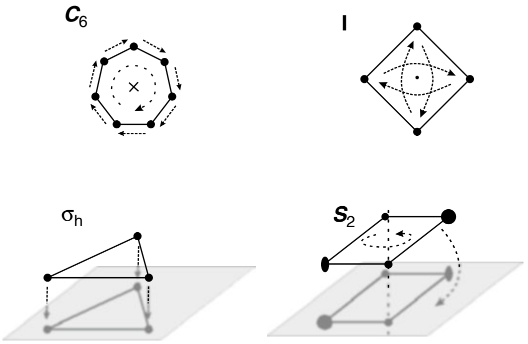

Fig. 4.2. Molecular symmetry operations. C_6 – rotation by 360°/6=60°, i – center of inversion, σ_h – plane reflection, S_2 – rotary reflection by 360°/2=180°.

property of an excited state) for D_{4h} point group are shown in Table 4.1. Here, A and B designate singly degenerate symmetric and anti-symmetric representations with respect to rotation (C_n), E designates the doubly degenerate representation with respect to rotation (C_n), the g and u subscripts designate symmetric (gerade: even) and anti-symmetric (ungerade: odd) representation with respect to the center of inversion (I), and $'$ and $''$ designate symmetric and anti-symmetric representation, respectively, with respect to plane reflection (σ_h).

In chlorophylls, the lowest-energy optical absorption is due to two electronic π-π^* transitions between the two a_{1u} and a_{2u} highest occupied molecular orbitals (HOMO) and two e_g lowest unoccupied molecular orbitals (LUMO) (Rubio et al. 1999). The relative energies corresponding to these two transitions depend on the central metal ion and the ring substituents. These two configurations mix quantum-mechanically to yield electronic excited states that can be measured by spectroscopy. Owing to that mixing, the electronic states in the absorption spectrum are split into two excited states of ^1E_u symmetry. The higher energy state is known as the Soret band (B band) and the lower energy state is known as the Q band (Gouterman 1961; Nemykin and Hadt 2010). Each band is further decomposed into two sub-bands corresponding to x-polarized and y-polarized transitions relative to the square symmetry of the macrocycle (e.g., Q_x and Q_y; Gouterman 1961; Weiss 1978; Shipman 1982; Scheer 2006). The intense Soret band with an absorption maximum at ~400 nm is associated with the symmetric nitrogen atoms of the macrocycle (Britton 1983). The weaker Q band is more strongly perturbed by the peripheral groups of the macrocycle. For instance, the elongation of the π-system in bacteriochlorophylls causes a shift of the Q_y band to 770 nm (in solution). Notably, the Q_x state (Q band is polarized along the x-axis of the macrocycle) is weaker and less sensitive to the peripheral groups, showing no red-shift. Distortions of the chlorophyll ring from ideal planar geometry serve as an additional perturbation factor (Zucchelli et al. 2007).

When bound to protein, chlorophyll's electronic properties change and both the total absorption spectrum and the excitation

Table 4.1. Symmetry characters and irreducible representations of porphyrins (D_{4h} point group). Numbers designate the level of degeneracy (1 or 2), the sign designates symmetric/antisymmetric representation (+/−)

D_{4h}	E	$2C_4$	C_2	$2C_2'$	$2C_2''$	I	$2S_4$	σ_h	$2\sigma_v'$	$2\sigma_d''$
a_{1g}	1	1	1	1	1	1	1	1	1	1
a_{2g}	1	1	1	−1	−1	1	1	1	−1	−1
b_{1g}	1	−1	1	1	−1	1	−1	1	1	−1
b_{2g}	1	−1	1	−1	1	1	−1	1	−1	1
e_g	2	0	−2	0	0	2	0	−2	0	0
a_{1u}	1	1	1	1	1	−1	−1	−1	−1	1
a_{2u}	1	1	1	−1	−1	−1	−1	−1	1	1
b_{1u}	1	−1	1	1	−1	−1	1	−1	−1	1
b_{2u}	1	−1	1	−1	1	−1	1	−1	1	−1
e_u	2	0	−2	0	0	−2	0	2	0	0

lifetime are affected. For a discussion of the lifetime of fluorescence in vivo, see Moya et al. (1986), and for basics on lifetimes measured by fluorescence, see Noomnarm and Clegg (2009). There are two common kinds of excited states of molecules, designated as singlet or triplet states, that differ in the arrangement of electron spins among the orbitals involved in the transition. Further details can be found elsewhere (see, for example, Zahlan 1967; Angerhofer 1991). The ground electronic state of photosynthetic pigments is singlet, such that the transitions seen as absorption bands (and those that transfer energy for light harvesting) are the singlet states. Singlet-to-triplet transitions are forbidden at first order of theory, but can occur in relaxation pathways, as we describe later in this chapter. The triplet states of chlorophyll can activate a highly reactive and potentially destructive excited state of oxygen called *singlet oxygen*. Triplet states of carotenoid molecules play an important role by quenching singlet oxygen (Ke 2001a; Telfer et al. 2008).

Chlorophylls not only absorb light, but also function as efficient electronic energy donors and acceptors, mediating ultrafast energy transfer across the photosynthetic unit. For a discussion of which properties allow Chl a to have different functions, see, e.g., Björn et al. (2009). After light absorption, the excitation often has to travel up to tens of nanometers in order to reach the RC. A large number of chlorophyll-chlorophyll energy-transfer events are required in sequence and in competition with the excited-state lifetime. Many details of this process are optimized, as summarized by Scholes et al. (2011). One interesting example of optimization involves the central element of the chlorophyll molecule, Mg^{2+}, that maximizes the lifetime of the excited states, lengthening the time for the excitation to reach the reaction center (Kobayashi et al. 2006).

B Carotenoids

As mentioned above, photosynthetic cells also contain carotenoids that serve as accessory pigments (Govindjee 1999; Ke 2001b; Telfer et al. 2008). Carotenoids consist of long conjugated alternating single and double carbon bonds, and hydrocarbon side chains. The molecular structure of one of the most ubiquitous carotenoids, β-carotene, is shown in Fig. 4.3a. Owing to their special electronic structure, carotenoids have remarkable electronic and spectroscopic properties (Christensen 2004).

The electronic properties of carotenoids, as polyenes, are dictated mostly by the π-electronic structure of conjugated double carbon bonds. Polyenes have thus been extensively used as a model system of carotenoids both experimentally and in theoretical

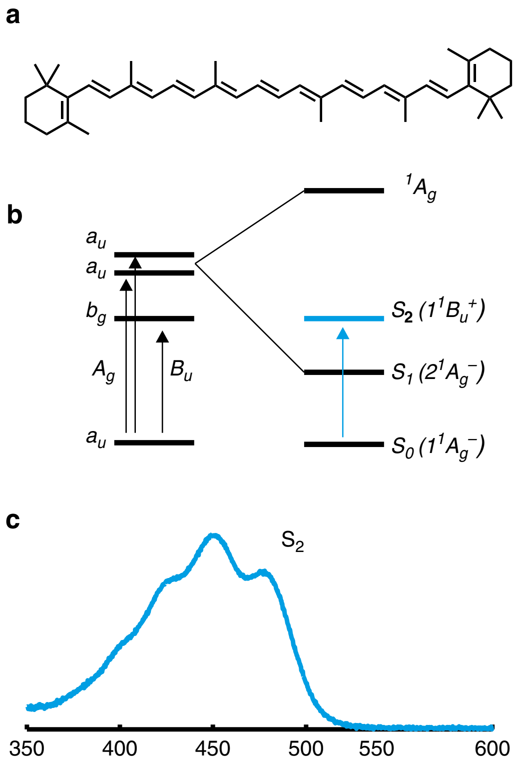

Fig. 4.3. β-carotene molecule. (**a**) Molecular structure, (**b**) molecular orbitals and electronic level structure, (**c**) absorption spectrum. The *capital letters* indicate states, *lower case letters* indicate orbitals.

Table 4.2. Symmetry table of linear polyenes (C_{2h} point group). All orbitals have single degeneracy level; the sign designates symmetric/antisymmetric representation (+/−)

C_{2h}	E	C_2	I	σ_h
a_g	1	1	1	1
b_g	1	−1	1	−1
a_u	1	1	−1	−1
b_u	1	−1	−1	1

calculations. Polyenes belong to the C_{2h} point group. The symmetry operations and irreducible representations for this point group are summarized in Table 4.2. The excited states of polyenes are of A_g and B_u symmetries only (see Fig. 4.3b) because all π-orbitals (a_u and b_g) are anti-symmetric with respect to reflection operation (σ_h in Table 4.2). According to the simple free-electron model, where no correlation effects are taken into account, the lowest excited state is predicted to have B_u symmetry. However, numerous experiments have shown that the first excited state is a different state and has A_g symmetry. The latter low-energy $2A_g^-$ (S_1) state is, similarly to porphyrin Q and B states, due to configuration interaction. It results from mixing of two different transition configurations involving the a_u orbitals as shown in Fig. 4.3b.

The S_1 excited state has the same symmetry as the ground state S_0 ($1A_g^-$), and the corresponding transition is thus forbidden by selection rules. The S_1 state is often referred to as the "dark" state. However in some cases, e.g., when the conjugated chain of the molecule is short, the S_1 state couples to the higher lying S_2 ($1B_u^+$) state and gains weak transition dipole moment. Several studies have shown that in these cases weak fluorescence is emitted from the S_1 state of carotenoids (Mimuro et al. 1992; DeCoster et al. 1992). An exceptional feature of the S_1 state of carotenoids, as compared to that of other pigment molecules, is an ultrafast relaxation time on the order of ~10 ps (Polívka and Sundström 2004). This efficient thermal relaxation to the ground state makes carotenoids efficient quenchers of excited electronic states and points to their possible role in NPQ (Frank et al. 1994; see also, e.g., Polivka and Frank, Chap. 8; Walla et al., Chap. 9; van Amerongen, Chap. 15). Carotenoids are well known for their photoprotective functions, including quenching of chlorophyll triplet states as well as singlet oxygen via triplet-triplet excitation energy transfer (Truscott and Edge 2004; Telfer et al. 2008). Quenching of excess chlorophyll excitation via singlet-singlet EET to the carotenoid S_1 state would augment carotenoid photoprotective function by preventing formation of singlet oxygen and chlorophyll triplet states. For a more complete picture of the photochemistry of carotenoids, see chapters in Frank et al. (1999).

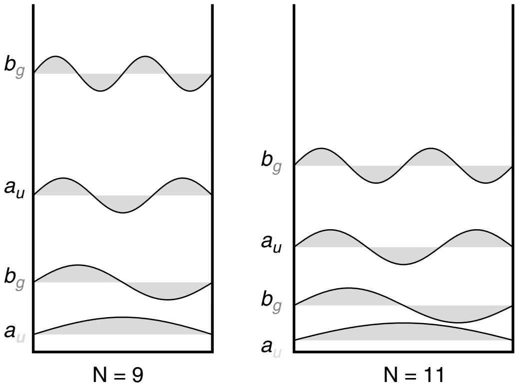

Fig. 4.4. Free electron (particle-in-a-box) model calculation showing the effect of an increase in conjugation length, N, from nine to eleven double carbon bonds on the lowest orbital energies. The orbitals are labeled by their respective symmetry (see Table 4.2).

The transition from the ground state to the S_2 ($1B_u^+$) state is strongly allowed and is responsible for the absorption band with notable vibrational structure in the 400–500 nm spectral range (Fig. 4.3c). The S_2 state has a very short lifetime (100–200 fs) owing to fast relaxation to the lower S_1 state by internal conversion. Despite such ultrafast internal conversion, the S_2 state contributes significantly to carotenoid-to-chlorophyll energy transfer in several light-harvesting complexes, and especially in LH2 complexes of purple bacteria (MacPherson et al. 2001; Cong et al. 2008).

The properties of the electronic states depend strongly on the structure of the carotenoid molecule, and in particular on the length of the conjugation chain. The $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transition energies are decreased as conjugation length of the molecule increases. The qualitative trend in transition energies can be predicted by a calculation using the free-electron model ("particle-in-a-box"; Christensen 2004; Bittner 2009; Scherer and Fischer 2010), as shown in Fig. 4.4. The dependence of the excited-state relaxation rate on the conjugation length corresponds to the changes of the transition energies and can usually be described by the energy-gap law (Englman and Jortner 1970). The energy-gap law allows satisfactory prediction of the energies of the S_1 state (Andersson et al. 1995; Chynwat and Frank 1995), whereas substantial deviation from the law is found for the S_2 state (Frank et al. 1997; Kosumi et al. 2006). One of the explanations of this disagreement with the energy-gap law is the presence of an intermediate "dark" state below the S_2 state. The early theoretical work by Tavan and Schulten (1986) predicted that two dark states, of A_g^- and B_u^- symmetry, should be found below the S_2 state for carotenoids with a conjugation system longer than 10 (see also Schmidt and Tavan 2012). However, experimental observations of these states have been controversial, and the existence of such states has been hotly debated during the last decade (Wohlleben et al. 2004; Yoshizawa et al. 2006; Polívka and Sundström 2009; Christensson et al. 2010).

III Radiative Transitions

Optically allowed electronic states of the photosynthetic pigments may be excited and de-excited radiatively, i.e., by absorption and emission of a photon (Fig. 4.5a). With the notable exception of the dark S_1 state of the carotenoids, the lowest excited singlet states of other photosynthetic pigments (e.g., chlorophylls) absorb light and subsequently emit fluorescence.

Light absorption can be modeled by describing a pigment molecule as a classical oscillator with a natural frequency and oscillation direction. A harmonic electromagnetic (EM) field can transfer energy to such a molecule only if the electric field (i) has a non-zero component in the direction of the pigment's natural oscillation (Fig. 4.5b), and (ii) drives the pigment's electrons at a frequency close to resonance with the pigment's natural frequency (in other words, if the energy of a photon, $h\nu$, is close to the transition energy $\varepsilon(S_1) - \varepsilon(S_0)$ of the pigment molecule, Fig. 4.5a). These principles can be described quantitatively in a quantum-mechanical context. Interaction between the radiation field (E) and the pigment is

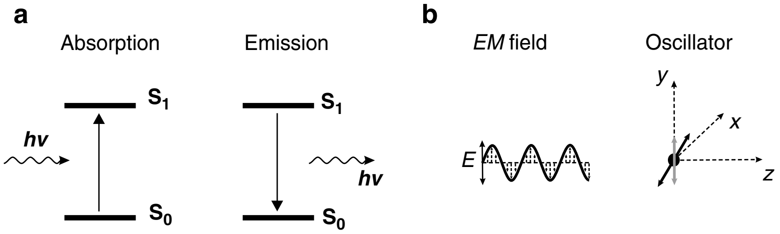

Fig. 4.5. (**a**) Radiative transitions and (**b**) interaction of y-polarized electromagnetic (*EM*) wave with an oscillator. Projection of the oscillator polarization vector on the y-axis is shown in *grey*.

described in terms of a coupling (V_{nm}), which mediates an electronic transition from state $|m\rangle$ to state $|n\rangle$, and is given by

$$V_{nm} = -\langle n|\vec{E}\cdot\hat{\mu}\quad|m = -E\cdot\langle n|\hat{\mu}\quad|m\rangle = -E\cdot\mu_{nm} \tag{4.1}$$

where $\hat{\mu}$ is a transition dipole moment vector – the operator behind the optical transition between all of the electronic states of the molecule, and μ_{nm} is the transition dipole moment between states $|m\rangle$ and $|n\rangle$.

Equation 4.1 is a simplification of a more general expression, where we have assumed that the electric field strength $E(r)$, as a function of coordinate "r", is constant over the spatial extent of the pigment. For radiation in the infrared, visible and UV regions of the spectrum, this simplification is an excellent approximation because the wavelength of the light (>100 nm) is two orders of magnitude larger than the size of a pigment molecule ($r \sim 1$ nm). Thus, the phase of the EM wave and, therefore, the EM-field amplitude do not vary appreciably over the molecule. The result of such an analysis is that the electronic transitions of a molecule can be described as a transition-point dipole. The point-dipole approximation does not account for spatial inhomogeneity of electronic features of the molecule, i.e., the extent of delocalization or shape of the transition density; this approximation retains only a specific magnitude and direction in analogy to the classical oscillator model discussed above (van Amerongen et al. 2000a; Renger and Holzwarth 2008). Despite its simple form, the point-dipole approximation is often sufficient to describe a field-matter interaction with good precision.

For the discussion of dark states, the model of purely electronic transition dipoles needs modification. Formally, this involves making corrections beyond the Born-Oppenheimer approximation that considers changes in electronic and nuclear properties induced by excitation of a molecule to be independent. The corrections to the Born-Oppenheimer approximation open up mechanisms whereby dark states can "borrow" transition strength from a bright state if vibrations appropriately distort the molecule during the transition. A good example is the carotenoid S_1 state. Although in linear polyenes, the optical transition to this state is strictly forbidden by selection rules and the coupling element V_{nm} ($\sim \mu_{nm}$) = 0, the S_1 state of carotenoids can gain weak transition dipole moment μ_{nm} by various mechanisms, e.g., due to deviation of carotenoid geometry from linear polyene (side groups, protein environment) or by coupling to an optically allowed state. The latter mechanism is known as Herzberg-Teller vibronic coupling, where a dark excited state gains weak transition dipole moment μ_{nm} by coupling to an optically allowed excited state lying in the vicinity of the dark state (Herzberg and Teller 1933; Zgierski 1974). In accordance with this mechanism in short-chain carotenoids, where S_2 and S_1 states are close in energy, the dark S_1 state shows fluorescence due to vibronic coupling to S_2 state (Mimuro et al. 1992; DeCoster et al. 1992). However, the yield of this fluorescence is very low due to extremely fast (~10 ps) internal conversion to the ground state.

IV Nonradiative Transitions

After light absorption, the energy stored in the excited state of a chromophore can decay by competing pathways that are summarized by the Jabłoński diagram, shown in Fig. 4.6 (Lakowicz 1999). These pathways can be separated into radiative transitions, such as fluorescence and phosphorescence, and nonradiative transitions, such as internal conversion and intersystem crossing. According to the Kasha rule (Kasha 1950), vibrational relaxation and radiationless processes from higher excited states are much faster than fluorescence. Therefore, steady-state radiative emission (e.g., fluorescence) is observed predominantly from the lowest vibrational level of the lowest excited electronic state of a certain spin multiplicity (Lakowicz 1999). In isolated molecules, the three main processes competing with each other during singlet electronic state de-excitation are fluorescence, internal conversion (IC), and intersystem crossing (ISC). Fluorescence is a convenient probe of the efficiencies of de-excitation processes, because any increase in efficiency (rate) of IC or ISC will immediately suppress fluorescence yield and decrease overall excited-state lifetime, while the rate corresponding to the fluorescence (also known as radiative) pathway will remain unchanged.

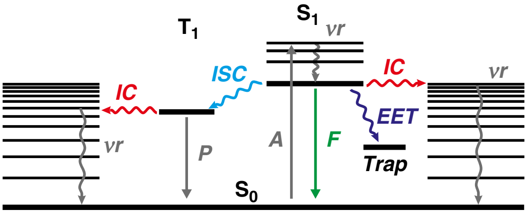

Fig. 4.6. Simplified Jabłoński diagram showing energy pathways in a molecule. *S_0*: ground state; *S_1*: excited singlet state; *T_1*: excited triplet state; *A*: absorption of a photon; *F*: fluorescence; *P*: phosphorescence; *IC*: internal conversion; *ISC*: intersystem crossing; *EET*: excitation energy transfer; *vr*: vibrational relaxation.

The two main non-radiative transitions in photosynthetic pigments, IC and ISC, differ from each other by the spin multiplicity of the acceptor state. IC refers to the redistribution of electronic excitation energy into vibrational motions of a lower-lying electronic state of the same spin multiplicity as the donor state (see Fig. 4.6), and is highly efficient when a dense manifold of vibronic states of the acceptor state is resonant with the vibrationally relaxed level of the donor state. Typically, the higher-energy excited states have very high IC rates and energy relaxes to the lowest excited state within <1 picosecond (ps). This is the case for both carotenoid and chlorophyll molecules. In contrast, the lowest excited state usually shows slower relaxation, on a timescale of several nanoseconds (ns). The Q_y state of chlorophylls is a good example of that rule, showing a lifetime of 5 ns (Brody and Rabinowitch 1957; Livingston 1960; Kaplanová and Parma 1984). IC does not contribute to the relaxation of the Q_y state of chlorophyll in solution (Livingston 1960; Bowers and Porter 1967), and it only slightly increases the overall relaxation rate in chlorophylls embedded in proteins (Mullineaux et al. 1993; Connelly et al. 1997). The lowest excited state of the carotenoid, the S_1 state, is a notable exception from the latter rule. The relaxation of the carotenoid S_1 state is very fast, due to highly efficient IC, and has a typical lifetime of several ps (Polívka and Sundström 2004). As a result of high IC rates, very little, or no fluorescence is observed from carotenoids.

Intersystem crossing is a non-radiative transition between two electronic states with different spin multiplicity. Formally, transitions with changing multiplicity are spin forbidden, and can only take place when additional processes relax the selection rules. Most of the observed singlet-triplet transitions are due to spin-orbit coupling. Spin-orbit coupling is a relativistic effect and is especially pronounced when heavy atoms are involved in the excitation process.

Coupling strength can also be influenced by a small energy gap, a change in the orbital type, strong displacement of the potential energy surfaces, and vibronic interaction of the singlet and triplet states (Marian 2012).

In chlorophyll molecules, ISC is facilitated by spin-orbit and spin-vibronic coupling between $\pi\pi^*$ triplet state and $n\pi^*$ singlet state (change of orbital type). This coupling is mostly due to nitrogen and oxygen atoms of the porphyrin ring, whereas the central Mg^{2+} is not significantly involved in ISC (Clarke et al. 1976). ISC has a substantial yield in chlorophylls (>60 % in isolated chlorophylls; Bowers and Porter 1967) and the generated chlorophyll triplet states (^{3}Chl*) can be easily quenched by oxygen, forming reactive oxygen species. Because reactive oxygen species can potentially significantly damage photosynthetic apparatus of the organism, ISC in chlorophylls is a critical process.

In carotenoids, ISC is very inefficient owing to ultrafast IC rates (<1 ps). However carotenoid triplet states play a crucial role in quenching ^{3}Chl* and in deactivating reactive oxygen species, and therefore they can protect the organism against photodamage (Truscott and Edge 2004; Telfer et al. 2008). This function of carotenoids is especially important in the RCs of photosystems I and II (PS I and PS II), where an additional pathway of ^{3}Chl* formation is activated as a result of the charge recombination (Ke 2001b).

V Radiative Versus Nonradiative Processes in Chlorophyll

In isolated chlorophyll molecules, fluorescence (radiative transition, with a rate constant k_{rad}) from the Q_y excited electronic state back to the ground state competes with other de-excitation processes, such as ISC and IC (with rate constants k_{ISC} and k_{IC}). The total lifetime $\tau = 5$ ns of the excited state is the inverse of the total k (total transitions per unit time) of excitation quenching pathways:

$$\tau = \frac{1}{k} = \frac{1}{k_{rad} + k_{IC} + k_{ISC}} \tag{4.2}$$

The quantum yield of each quenching process can be calculated as a ratio of the rate of the process of interest to the total rate of excitation quenching (inverse of lifetime). The quantum yield of fluorescence, for instance, can be calculated as follows:

$$\Phi_{fl} = \frac{k_{rad}}{k_{rad} + k_{IC} + k_{ISC}} \tag{4.3}$$

For chlorophyll in solution, fluorescence competes mostly with ISC, while k_{IC} is negligible (Fig. 4.7a; Bowers and Porter 1967). However, chlorophylls embedded in isolated proteins show a decrease of the fluorescence lifetime to ~4 ns by a small amount of thermal dissipation k_{IC} (Mullineaux et al. 1993; Connelly et al. 1997).

In vivo, two more processes contribute to chlorophyll de-excitation – photochemical quenching k_{PQ} and thermal de-excitation reflected in non-photochemical quenching k_{NPQ}

$$\tau = \frac{1}{k_{rad} + k_{IC} + k_{ISC} + k_{PQ} + k_{NPQ}} \tag{4.4}$$

In photosynthesis, photochemical quenching of chlorophyll fluorescence refers to processes of de-excitation of chlorophyll excited states that lead to photochemical charge separation in the reaction center. It is important to note that in Fig. 4.7 and Table 4.3, k_{PQ} is rate constant of the first reaction of charge separation. In closed RCs, the electron transfer chain is blocked after the second charge separation step, while the first charge separation reaction is still active. Therefore, the efficiency diagrams for open and closed RCs, as shown in Fig. 4.7, do not differ significantly. The **rate** of photochemical quenching k_{PQ} is very high and, as a result, the **yield** of chlorophyll fluorescence is reduced by a factor of >10 (see Fig. 4.7b;

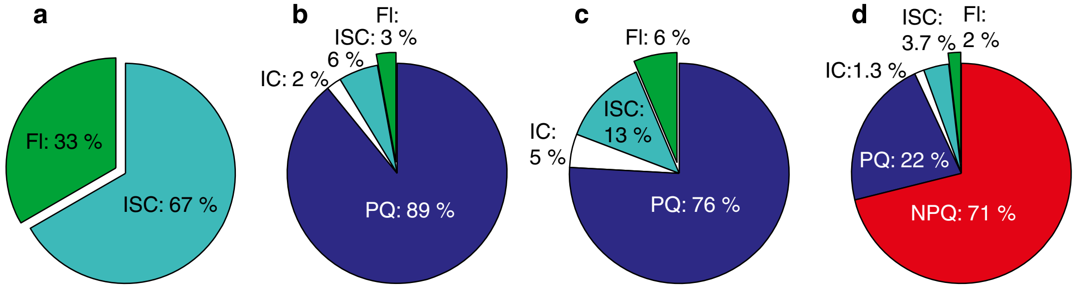

Fig. 4.7. Yields of various de-excitation mechanisms of chlorophyll *a* in (**a**) solution, (**b**) PS II with open reaction centers, (**c**) PS II with closed reaction centers, (**d**) PS II with closed reaction centers and under NPQ conditions. *Fl* fluorescence (*green*), *IC* internal conversion (*white*), *ISC* intersystem crossing (*cyan*), *PQ* photochemical quenching (trapping by RC, *dark blue*), *NPQ* non-photochemical quenching (*red*). The yields were calculated (see Eqs. 4.3 and 4.5) from kinetic rates shown in Table 4.3 and should not be confused with yields based on fluorescence intensity (Brody and Rabinowitch 1957; Barber et al. 1989).

Table 4.3. Rate constants involved in de-excitation of chlorophyll *a*.

	FI	IC	ISC	PQ (open RC)	PQ (closed RC)	NPQ
k, s^{-1}	$6.5 \cdot 10^7$	$5 \cdot 10^7$	$1.3 \cdot 10^8$	$2.0 \cdot 10^9$	$7.7 \cdot 10^8$	$2.5 \cdot 10^9$
Refs.	*1,2,3*	*4,5*	*1,2,3*	*6,7*	*6,7,8*	*9,10*

References: *1* (Shipman 1980); *2* (Renger 1999); *3* (Peterman et al. 1995); *4* (Mullineaux et al. 1993); *5* (Connelly et al. 1997); *6* (Schatz et al. 1988); *7* (Hansson and Wydrzynski 1990); *8* (Brody and Rabinowitch 1957); *9* (Miloslavina et al. 2008); *10* (Gilmore et al. 1995)

Barber et al. 1989; Krause and Weiss 1991). Non-photochemical quenching of chlorophyll fluorescence (the topic of this book) is used as a measure of all other processes of chlorophyll de-excitation that do not result in charge separation. When activated, some of these processes create additional pathways for chlorophyll de-excitation. The rate of de-excitation measured as NPQ varies depending on plant species and conditions, with $k_{NPQ}=2.5 \cdot 10^9$ s^{-1} reported for higher plants (Gilmore et al. 1995; Miloslavina et al. 2008; see Demmig-Adams et al., Chap. 24, for pronounced differences in NPQ among plants). This means that fluorescence yield is strongly diminished when the organism is exposed to high-light conditions (Fig. 4.7c). The values of the rate constants of the above-described processes are summarized in Table 4.3.

Chlorophyll fluorescence is a sensitive probe of the energy conversion processes in photosynthesis and it is broadly used for monitoring the "metabolic status" of cyanobacteria, algae and plants, as well as for studying the mechanisms of responses to the environment in vivo (Govindjee et al. 1986; Papageorgiou and Govindjee 2004; Papageorgiou 2012). A number of techniques have been developed for fast and efficient measurements of fluorescence yield of both terrestrial plants (from leaves) and algae (in the liquid phase). Responses to the environment and/or metabolic status are readily assessed by measuring fluorescence upon exposure to high light intensity, as what is termed the Kautsky effect (Govindjee 1995, 2004; Kolber et al. 1998; Schreiber 2004; Strasser et al. 2004). Moreover, photosynthetic activity can be monitored remotely using laser-based instruments, LIDARs – light detection and ranging (Gorbunov et al. 2000; Burikov et al. 2001; Ananyev et al. 2005). Typically, the chlorophyll fluorescence spectrometry techniques provide the following quantities: minimal fluorescence yield of dark- and light-exposed organism (F_o and F_o'), maximal fluorescence yield of dark and light-exposed organism (F_m and F_m'), fluorescence yield under ambient conditions

(F) and their products. The quantum yields (i.e., efficiencies) of photochemical (Φ_{PQ}) and non-photochemical quenching/thermal de-excitation (Φ_{NPQ}) can be calculated using both rate constants, measured by time-resolved spectroscopies, and fluorescence yields (F; F_m and F_m'), measured by chlorophyll fluorescence techniques, as follows:

$$\Phi_{PQ} = \frac{F}{F_m'} - \frac{F}{F_m} = \frac{k_{PQ}}{k_{rad} + k_{IC} + k_{ISC} + k_{PQ} + k_{NPQ}} \tag{4.5a}$$

$$\Phi_{NPQ} = \frac{F_m' - F}{F_m'} = \frac{k_{PQ}}{k_{rad} + k_{IC} + k_{ISC} + k_{PQ} + k_{NPQ}} \tag{4.5b}$$

Detailed information on calculation of quantum yields using chlorophyll fluorescence parameters and rates can be found in Kasajima et al. (2009; see also, e.g., Logan et al., Chap. 7).

VI Excitation Energy Transfer, Förster Theory

Light-harvesting proteins are organized into photosynthetic units (PSU), where a large number of pigments (~300) harvest light and funnel excitation energy to the RC. For the concept of the PSU, i.e., of an antenna and a reaction center and experiments supporting this concept, see Clegg et al. (2010) and Govindjee and Björn (2012). For an overview of the primary processes of photosynthesis, see Renger (2008a). While various pigments absorb light and convert it to molecular excitation, it is the chlorophyll-type molecules that are predominantly responsible for migration of the excitation in PSU – the process of accepting excitation energy from neighboring excited chromophores and forwarding it to other chlorophylls, – eventually leading to trapping of excitation by the RC. The accessory pigments (e.g., carotenoids) only transfer energy that they acquire by absorbing light themselves to chlorophyll a molecules. An exception to this rule are bilin-containing antenna complexes, where no chlorophyll is present and the excitation migration is facilitated by the phycobilin chromophores, such as phycocyanins and phycoerythrins. EET can be considered macroscopically, when the excitation dynamics in PSU are treated kinetically, and microscopically, by calculating actual energy transfer rates based on the electronic properties of interacting chlorophylls.

On a macroscopic scale, electronic properties of chlorophyll molecules are approximated by a simple two-level system (van Amerongen et al. 2000b). This approximation is qualitatively reasonable because the actual migration of excitation takes place only between the Q_y states of chlorophylls, i.e., between the thermally equilibrated lowest excited chlorophyll states. To a first degree of accuracy, excitation energy migration in the antenna complex can be described by the "random walk" model with hops (single EET event) only between nearest-neighbor pigments organized into a square lattice (Pearlstein 2005). In that case, average migration time $\tau_{mig} = 1/k_{mig}$ of the excitation to the RC can be calculated as follows:

$$\tau_{mig} = \frac{1}{\pi} N \cdot \log(N) \cdot \tau_{hop} \tag{4.6}$$

where, N is the number of chlorophyll molecules per RC (the size of the PSU) and τ_{hop} is the average hopping time of excitation between neighboring chlorophyll molecules (Montroll 1969). The size of the PSU varies among organisms, with an average of ~300 chlorophyll molecules per PSU (Malkin et al. 1981; Clegg et al. 2010; Govindjee and Björn 2012). Hopping time τ_{hop} can be estimated from Eq. 4.6 if migration time is known, e.g., from the expression for quantum efficiency of excitation trapping:

$$\Phi_{tr} = \frac{k_{tr}}{k_{loss} + k_{tr}} \tag{4.7}$$

where, $k_{loss} = k_{rad} + k_{IC} + k_{ISC}, k_{tr} = 1/\left(t_{mig} + t_{cs}\right)$ is total rate constant of excitation trapping by RC (Pearlstein 1982; van Grondelle and Gobets 2004; Broess et al. 2006), τ_{mig} is the effective time constant of excitation migration to RC, τ_{cs} is the effective time constant of charge separation in RC, and Φ_{tr} is the quantum efficiency of excitation trapping.

However, the approximation of the antenna by a regular two-dimensional square lattice of pigments in Eq. 4.7 is a significant simplification. Natural PSUs typically consist of several light-harvesting antenna proteins, where each pigment experiences a different environment, and intra-pigment EET rates are, therefore, different for each step. In addition, intra-protein EET rates can be substantially different from rates of inter-protein EET. Therefore, τ_{mig} contains several components describing inter-protein and intra-protein migration rates. Several experimental studies have addressed the problem of migration and equilibration dynamics of excitation energy in natural PSUs, and three models have emerged: (i) trap-limited, (ii) diffusion-limited and (iii) diffusion-to-trap limited (for reviews, see Barter et al. 2005; Croce and van Amerongen 2011). In each model, one of the three kinetic rates, indicated in Fig. 4.8, limits energy equilibration. In the trap-limited model, migration occurs on an ultrafast timescale, followed by slower trapping in the RC, k_{CS}. The PSUs with a small antenna (e.g., core complexes of PS II) can be described by the latter model (Miloslavina et al. 2006). The most broadly used trap-limited model is the exciton/radical pair equilibrium (ERPE) model (van Grondelle 1985; Nuijs et al. 1986; Schatz et al. 1988; Miloslavina et al. 2006), in which the primary and secondary charge separation steps are included to describe the temporal response of the system. In the ERPE model, both forward and back electron transfer processes are included; therefore, the total charge separation τ_{cs} time constant is the product of all of these processes. In the diffusion-limited model, it is migration of the excitation ($k_{Ant\text{-}Ant}$) that slows down equilibration of the excitation energy, while charge separation is relatively fast (Melkozernov et al. 2004; Miloslavina et al. 2006; Broess et al. 2008; Van Oort et al. 2010). This model was reported to be appropriate for systems with a large antenna (Caffarri et al. 2011). Furthermore, some experiments have indicated that energy equilibration can occur via an intermediate mechanism, a diffusion-to-trap limited mechanism, where the limiting step is the transfer of the excitation energy from the antenna complex to the RC, $k_{Ant\text{-}RC}$ (Visscher et al. 1989; Valkunas et al. 1995; Dekker and van Grondelle 2000; Vasil'ev et al. 2001; Raszewski and Renger 2008).

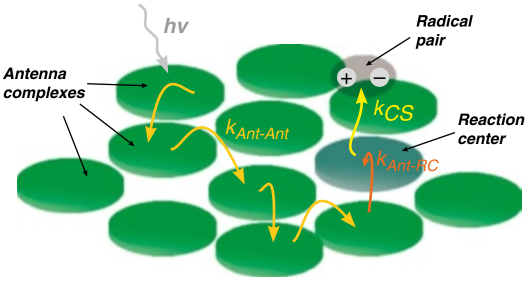

Fig. 4.8. Migration of excitons in PSU after absorption of light photon by antenna chlorophyll molecules. Three kinetic rates are shown: between neighboring antenna complexes ($k_{Ant\text{-}Ant}$), from antenna complex to reaction center ($k_{Ant\text{-}RC}$), and trapping of exciton in reaction center by charge separation (k_{CS}).

The rate constants of excitation migration and charge separation, reported in numerous experimental and theoretical studies, have shown a large range of variation. The primary charge separation time constant has been reported to be between 300 fs and 8 ps (Wasielewski et al. 1989; Greenfield et al. 1997; van Amerongen et al. 2000c; Dekker and van Grondelle 2000; Holzwarth et al. 2006; Raszewski and Renger 2008), while the total charge separation time, τ_{cs}, has been estimated to be in the range of 60–180 ps (Miloslavina et al. 2006; Broess et al. 2006). Values for excitation migration to RC have been reported between 9 and 150 ps (Miloslavina et al. 2006; Van Oort et al. 2010), while hopping time is between <1 and >10 ps (Barzda et al. 2001; Broess et al. 2006; Caffarri et al. 2011). For detailed reviews on migration and trapping of excitation in PS II-containing systems, see Croce and van Amerongen 2011 and van Amerongen and Croce 2013; for a discussion of basics of excitation-energy transfer, see Clegg 2004).

It is important to emphasize that in Eq. 4.7, the quantum efficiency does not account for the energy of excitation quanta and only reports the ratio of the number of excitation quanta that have reached the RC (and resulted in charge separation) to the number of photons absorbed in the antenna. The quantum efficiency of excitation trapping in systems without regulated thermal de-excitation (NPQ) and open RCs typically ranges between 85 % and 95 % (Wraight and Clayton 1974; Clayton and Yamamoto 1976; Rijgersberg et al. 1980; Vredenberg 2004; Wientjes et al. 2013). It is important to distinguish *quantum efficiency* from the absolute efficiency of EET that is often referred to as *storage efficiency* (Ross and Calvin 1967; Jursinic and Govindjee 1977; Dau and Zaharieva 2009; Shevela et al. 2013). Storage of the absorbed energy in a primary charge-separated state is calculated in terms of *free energy* (see Boeker and van Grondelle 2011). In these calculations, the decrease of energy of an excitation quantum during its migration to RC, followed by charge separation, is accounted for. The resulting values of storage efficiency have been estimated to be 68–73 % (Duysens 1958; Ross and Calvin 1967; Jursinic and Govindjee 1977). If efficiency of light absorption over the entire solar spectrum is included in the calculation (i.e., the relative area of solar spectrum covered by absorption bands of photosynthetic pigments), the efficiency drops to <34 % (Dau and Zaharieva 2009).

In the excitation-energy transfer (EET) studies on a microscopic scale, not only the electronic properties of chlorophylls are taken into account, but also their arrangement in the protein complex. Several mechanisms of EET in LHCs are found depending on the interaction of chlorophylls with each other and with their protein environment. For a review of the mechanism of excitation energy transfer, see Renger (2008b).

It is often assumed that the electronic interaction between two chromophores is weak in comparison to chromophore interaction with the environment. In that case, the key parameter of the EET is the Coulombic interaction (coupling V_{DA}) between donor D and acceptor A molecules. When the distance between chromophores is large (3–10 nm), coupling can often be approximated by a dipole-dipole interaction:

$$V_{DA} = \frac{1}{4\pi\varepsilon_o n^2}\frac{\breve{\mu}_D\breve{\mu}_A}{R_{DA}^3} - 3\frac{\left(\breve{\mu}_A\breve{R}_{DA}\right)\left(\breve{\mu}_D\breve{R}_{DA}\right)}{R_{DA}^5} \tag{4.8}$$

where, $\breve{\mu}_A$ and $\breve{\mu}_D$ are unit vectors of the acceptor and donor transition dipole moments, respectively, and $\breve{R}_{DA}$ is unit vector pointing from the center of the donor molecule to the center of the acceptor. This is how T. Förster (Förster 1946; for English translation, see Förster 2012) introduced the theory of energy transfer known as Förster Resonance Energy Transfer (FRET; Knox 2012). For a history of FRET, see Clegg (2006). The Förster coupling regime is often called the "weak coupling" limit and the rate of resonance energy transfer from donor D to acceptor A, k_{DA}, can be calculated using Fermi's Golden Rule (Dirac 1927):

$$k_{DA} = \frac{2\pi}{\hbar}\left|V_{DA}\right|^2 J_{DA} \tag{4.9}$$

where $\hbar = h/2\pi$ is reduced Planck's constant, V_{DA} is electronic coupling of D and A excited states from Eq. 4.8, and J_{DA} is the spectral overlap integral between the area-normalized donor fluorescence spectrum (f_D) and the area-normalized acceptor absorption spectrum (ε_A). If the spectra are plotted on a wavelength (λ) scale, the expression is (Scholes 2003):

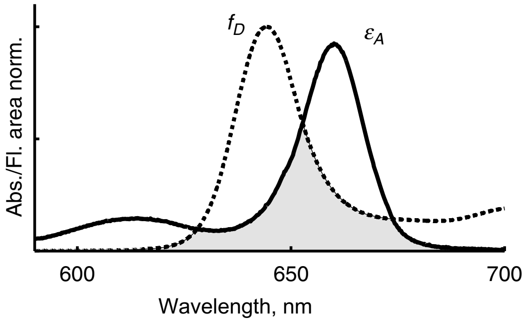

Fig. 4.9. Overlap region of area normalized chlorophyll *b* fluorescence spectrum f_D (·····) and chlorophyll *a* absorption spectrum ε_A (——).

$$J_{DA} = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4\, d\lambda \tag{4.10}$$

In simple terms, the rate of EET from the excited state of donor D^* to the ground state of acceptor A depends upon the coupling strength between the transitions $D^* \rightarrow D$ and $A \rightarrow A^*$, and their spectral properties. The example of spectral overlap between chlorophyll *b* and chlorophyll *a* molecules in solution is shown in Fig. 4.9. For basic principles of calculation of spectral overlap see Lakowicz 1999.

In experimental studies of energy transfer, the characteristic distance at which the efficiency of the energy transfer is 50 % is of special importance. Because the main competing de-excitation process is donor fluorescence, the rate of Förster energy transfer (k_{FRET}) can be rewritten as follows (Förster 1946, 1948; Braslavsky et al. 2008):

$$k_{FRET} = \frac{1}{\tau_d}\left(\frac{R_0}{R_{DA}}\right)^6 \tag{4.11}$$

where, $1/\tau_d$ is the fluorescence rate of the donor in the absence of acceptor, and R_0 is the Förster radius (Förster 1965, 1967):

$$R_0^6 = \frac{9(ln10)\kappa^2\Phi_D I}{128\pi^5 N_A n^4} \tag{4.12a}$$

$$\kappa = \frac{\vec{\mu}_D \cdot \vec{\mu}_A - 3(\vec{\mu}_D \cdot \vec{r})(\vec{\mu}_A \cdot \vec{r})}{\mu_D \mu_A} \tag{4.12b}$$

Here, κ is an orientation factor associated with donor-acceptor spatial geometry, $\vec{\mu}_D$ and $\vec{\mu}_A$ are donor and acceptor transition dipole moment vectors, r is the unit vector in the direction of R_{DA}, n is the effective refractive index of the medium surrounding donor and acceptor, N_A is Avogadro's number, and Φ_D is the fluorescence quantum yield of the donor in the absence of the acceptor, I is Förster spectral overlap integral defined like J_{DA} in Eq. 4.10 except that the acceptor absorption spectrum is not normalized, but is plotted as extinction coefficient vs wavelength λ (Braslavsky et al. 2008). The Förster radius for a pair of photosynthetic chromophores is estimated to be approximately 60–100 Å (Colbow 1973; van Grondelle 1985; Dekker et al. 1998; Berghuis et al. 2010).

Another important quantity is FRET transfer efficiency (Φ_{ET}), defined as the probability of the excitation being transferred away from the chromophore rather than being quenched by another process (e.g., IC, ISC). Transfer efficiency, expressed as a function of donor-acceptor separation, is

$$\Phi_{ET} = \frac{1}{1+(R/R_0)^6} \tag{4.13}$$

The EET efficiency is highly sensitive to donor-acceptor separation and decreases quickly at distances greater than the Förster radius R_0 of a given donor-acceptor pair (Fig. 4.10). The significance of Förster's theory is that all quantities appearing in the FRET expressions, Eqs. 4.11, 4.12a, 4.12b and 4.13, can be obtained from experimental data (van Zandvoort et al. 1995).

Owing to the strong coupling of pigments to their dissipative environment (coupling to the bath), energy transfer processes in the weak chromophore-coupling limit (when inter-chromophore coupling is weaker than chromophore-to-bath coupling) are

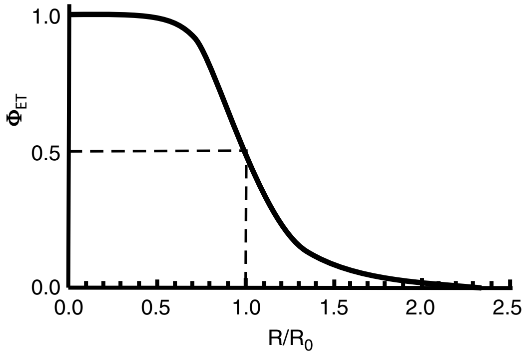

Fig. 4.10. The transfer efficiency (Φ_{ET}) as a function of the donor acceptor distance (R).

characterized by transfer from a thermally equilibrated (thermalized) donor. After the event of EET, excitation is, once more, thermalized on the excited electronic state of the acceptor on a time scale that is fast compared to energy migration rate. Rapid thermalization of excitation causes what is termed "loss-of-memory" of the excitation trajectory, meaning that each new EET process is independent of the history of the migration of the excitation energy before reaching the last chromophore. Therefore, FRET represents a diffusive hopping of energy from chlorophyll to chlorophyll through the PSU (Fig. 4.8; Jean et al. 1989; Pullerits and Freiberg 1992; Pullerits et al. 1994; Somsen et al. 1994).

VII Considerations Beyond Förster Theory

While Förster theory has proven to be extremely successful and predictive, it does have shortcomings, which can pose a challenge when chromophores are packed close together – like in LHCs. Over the past years, studies of LHCs have helped reveal and clarify processes of energy transfer by mechanisms not explained by Förster theory (Scholes and Fleming 2005; van Grondelle and Novoderezhkin 2006; Beljonne et al. 2009; Novoderezhkin and van Grondelle 2010; Scholes et al. 2011). The main problem with Förster theory is that it works well only when the distance between chromophores is large compared to the dimensions of the chromophores, r_D and r_A, for instance when donor and acceptor chromophores are located on different protein complexes. However, certain ingredients within the theory need to be modified when inter-chromophore distance R_{DA} is comparable or smaller than r_D or r_A, or when chromophore-chromophore interaction competes with chromophore-environment interaction; in the latter events, dipole-dipole electronic coupling approximation fails, and, in some cases, orbital overlap effects start to contribute to the EET.

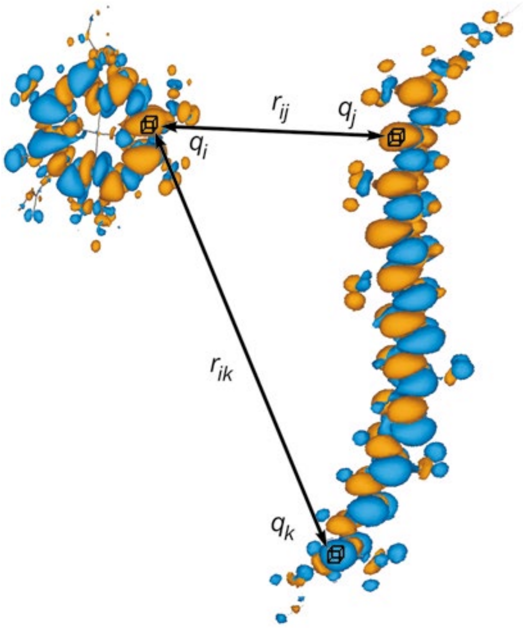

Fig. 4.11. Coulombic interaction between the transition density cubes of a bacteriocholorophyll (*left*) and a carotenoid molecule (*right*). The diagram shows a transition density cube q_i centered on the bacteriochlorophyll interacting with two cubes, q_j and q_k, centered on the carotenoid and their corresponding separation r_{ij}. The sum over all such interactions gives the total Coulombic coupling (Scholes and Fleming 2005).

A good example is the energy transfer between the carotenoid and the bacteriochlorophyll in LH2 complexes of purple bacteria, where the distance between the chromophores is much smaller than their dimensions (see Fig. 4.11). The dipolar approximation forbids energy transfer to and from optically dark electronic states since these states have negligible transition dipole moments and V_{DA} in Eq. 4.9 therefore equals zero. This prediction is contradicted by the observation of energy transfer from the optically dark carotenoid S_1 state to the chlorophyll Q_y state in LH2 complexes (Gradinaru et al. 2000; Hsu et al. 2001; Croce et al. 2003). The breakdown of the dipole approximation occurs because transition dipoles do not account for the shape or extent of the transition densities (Scholes 2003) that are highly elongated in carotenoid molecules. In such cases, size and shape of molecular transition densities should be taken into account when calculating V^{coul}, which is done by summation of Coulombic interactions between monopoles distributed at points r_i around the chromophores, i.e., using the transition density cube (TDC) method (Krueger et al. 1998):

$$V_{DA}^{Coul} = \frac{1}{4\pi\varepsilon_o n^2} \sum_{i,j} \frac{q_i q_j}{r_{ij}} \tag{4.14}$$

where, the index i (j) runs over the grid discretizing the donor (acceptor) transition density, q_i (q_j) are the discrete charges associated with the donor (acceptor) transition density at position r_i (r_j), and $r_{ij}=|r_i-r_j|$ is the distance between the points r_i and r_j (Fig. 4.11).

The accuracy of the TDC method can be improved by taking successively smaller volume elements (cubes) until, for infinitely small cubes, the calculated coupling becomes exact and limited only by the accuracy of the quantum-chemical determination of the wavefunction. In this implementation limit of the TDC method, the coupling term is calculated using the following expression:

$$V_{DA}^{Coul} = \frac{1}{4\pi\varepsilon_o n^2} \int dr_D \int dr_A \frac{\rho_D(r_D)\rho_A(r_A)}{r_{DA}} \tag{4.15}$$

Here, $\rho_D(r_D)$ and $\rho_A(r_A)$ denote the transition density associated with the donor/acceptor at position r_A and r_D, and the integration coordinates are varied over the entire three dimensional coordinate space.

VIII Delocalization of Excitation, Molecular Excitons

Insight into thermal de-excitation mechanisms (assessed via measurements of NPQ) requires an understanding of the principles of excitation-energy migration through the antenna. Excitation energy migration is, in turn, determined by interaction of the chromophores with each other as well as with their environment, the pigment-binding protein. In a number of photosynthetic complexes, the coupling between chlorophylls is substantially stronger than the coupling to the "bath" (pigment-binding protein), which determines the homogeneous broadening. In other words, electronic coupling energy is larger than the width of spectral absorption bands. Under these conditions (with what is termed a strong coupling limit), Förster's theory fails to describe EET as diffusive hopping of excitation among molecules. In particular, when the coupling between pigments, V_{DA}, is greater than the transition energy difference between pigment excited states ΔE as well as the coupling to the environment of each individual pigment V_{bath}, excitation becomes coherently delocalized over the system of interacting pigments, and it is no longer possible to identify a donor and acceptor molecule. The latter, newly formed delocalized excited state belongs to all of the coupled pigments and is called an exciton state $|M\rangle$, as compared to localized states of individual pigments $|M\rangle$ (van Grondelle and Novoderezhkin 2006; Scholes and Rumbles 2006). Each exciton state, also simply termed exciton, is given as a linear combination of the localized states:

$$|M\rangle = \sum_n c_m^{(M)} |m\rangle \tag{4.16}$$

where, $c_m^{(M)}$ are the elements of the eigenvector matrix of the system Hamiltonian H_S,

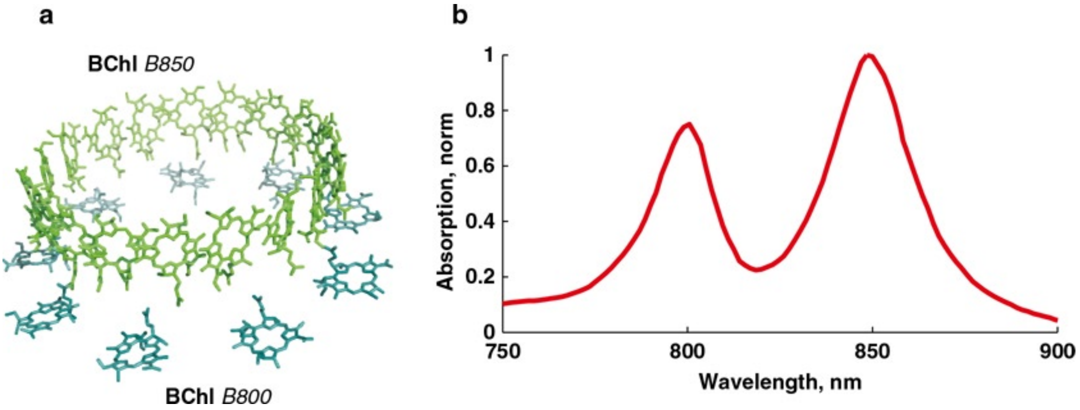

Fig. 4.12. (**a**) Molecular structure of LH2 complex from *Rhodobacter* (*Rba.*) *sphaeroides*, showing only bacteriochlorophyll (*BChl*) molecules. Strongly coupled BChls (B850 ring) are shown in *green*, weakly coupled BChls (B800 ring) are shown in *blue*. (**b**) Absorption spectrum of LH2 complex from *Rba. sphaeroides.*

consisting of localized state energies E_m and couplings V_{mn}:

$$H_S = \sum_m E_m |m\rangle\langle m| + \sum_{mn} V_{mn} |m\rangle\langle n| \tag{4.17}$$

The work of Fidder et al. (1991) provides a clear and practical example for how exciton states can be calculated. The latter approach has found numerous applications in the interpretation of excited states and ultrafast dynamics in LHCs. Delocalization of the excitation energy results in a collective, or coherent, behavior of the excited electronic states, and, as a consequence, the properties of excitons can be markedly different from those of the excited states of individual pigments. A characteristic example of exciton delocalization in photosynthetic complexes is that of bacteriochlorophyll (BChl) light-harvesting LH2 proteins of purple bacteria shown in Fig. 4.12a (Novoderezhkin et al. 1999a, b). In these complexes, 18 of the 27 BChls are arranged in a ring with an ~0.9 nm center-to-center inter-chromophore distance (McDermott et al. 1995). Because of strong electronic coupling between two nearest neighbour BChls in this B850 ring (~300 cm^{-1}), 18 exciton states are formed. The red-shift of the bright (optically allowed) exciton transitions, in combination with pigment-protein interactions, shifts the absorption spectrum from 800 to 850 nm, as shown in Fig. 4.12b (Jimenez et al. 1996; Hu and Schulten 1997; Chachisvilis et al. 1997; Kennis et al. 1997; Monshouwer et al. 1997).

Deviation of a chlorophyll aggregate's exciton properties from those of its constituent individual chlorophyll molecules can be illustrated by considering a simple dimer system like that shown in Fig. 4.13, which represents what is termed the "J" and "H" aggregates (Kirstein et al. 2000). In the absence of interaction between the individual pigments of the dimer, it has a doubly-degenerate excited state (two states of the same energy) arising from the excitation of each pigment (Fig. 4.13a). The coupling between pigment molecules results in exciton splitting (formation of two exciton states of different energy) because the light-absorbing quantum mechanical states are a linear combination of indistinguishable excitations localized on each chromophore (Scholes and Rumbles 2006). When pigments are arranged in "side-to-side" geometry (*H*-aggregate), the exciton state with antiparallel (⇆) transition dipole moments is optically forbidden ("dark") and has lower energy, while the exciton state with parallel (⇇) transition dipole moments is higher in energy and optically accessible (Fig. 4.13b).

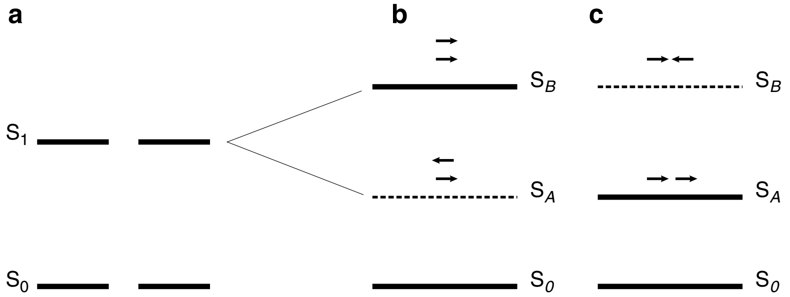

Fig. 4.13. Effect of coupling on excited states. The direction of the transition dipoles are shown by *small arrows* above the corresponding exciton states. (**a**) Non-interacting molecules; (**b**) dimer with 'sandwich' transition dipole arrangement (*H*-aggregate); (**c**) dimer with 'head-to-tail' transition dipole arrangement (*J*-aggregate). Optically accessible exciton states are shown in *solid line*, dark states are shown in *dotted line*.

When pigments are arranged with their transition dipoles in a co-linear, "end-to-end" geometry, parallel arrangement (→→) minimizes the interaction energy and is optically accessible, while antiparallel arrangement (→←) maximizes the energy and is optically forbidden (Fig. 4.13c). For the ring geometry of BChls in the LH2 complex, the lowest exciton state is formally forbidden (Hu and Schulten 1997).

The effect of delocalization and exciton formation can, however, be diminished by disorder in excited-state energies of the BChls contributing to the exciton. In most photosynthetic complexes, each pigment experiences a different protein environment, which causes a shift of its absorption band. As a result, the total absorption spectrum is defined not only by the homogeneous broadening of the absorption band, which is identical for each pigment, but also by inhomogeneous broadening – distribution of the band positions and widths among different pigments of the complex. Inhomogeneous broadening leads to decreased delocalization. The effect of disorder in photosynthetic proteins has been extensively studied (for a review, see van Grondelle and Novoderezhkin 2006; see also, e.g., Krüger et al., Chap. 6). A good example of the role of inhomogeneous broadening is observation of the formally forbidden lowest exciton state of LH2 complexes (Kennis et al. 1997; Monshouwer et al. 1997).

Excitation in the B850 band (of an LH2 complex) does not migrate as diffusive hopping, but is instead often described as a wave-like movement (Chachisvilis et al. 1997). Theoretical techniques other than FRET have to be used to describe this EET. One of the approaches, the Redfield theory, is based on the density-matrix formalism and is described in detail in May and Kühn (2011). In the Redfield theory, chromophore-bath interaction is considered to be small and is treated as a perturbation of inter-chromophore interaction. This perturbative approach is known as the Born approximation, and is an opposite limiting case to FRET, where chromophore-bath coupling is assumed to be much stronger compared to inter-chromophore coupling. Within the second approximation of the Redfield theory, Markov approximation, the time evolution of the system does not depend on the past history (previous EET steps) and no memory effects are taken into account.

As a result of interaction among different LHCs, the EET in photosynthetic organisms often has to be described by a combination of Redfield theory, accounting for strongly interacting intra-aggregate chlorophylls (here aggregate is an assembly of chlorophylls bound together by strong interaction), and Förster theory, accounting for weak inter-aggregate interaction. This combined approach leads to the *generalized* Förster theory (Mukai et al. 1999; Sumi 1999; Scholes and Fleming 2000; Yang et al. 2003; van Grondelle and Novoderezhkin 2006; Novoderezhkin and van Grondelle 2010), which allows a better microscopic description of EET in PSU.

Over the last decade, a number of researchers have proposed quantum-coherence effects to be involved in excitation-energy migration in LHCs (Engel et al. 2007; Collini et al. 2010; Calhoun and Fleming 2011; Ishizaki and Fleming 2012). The mechanism behind quantum-coherent energy transfer can be thought of as the interference of all possible EET pathways. These pathways contribute either constructively (add up) or destructively (annihilate), and result in the most efficient transfer of energy to RC. This is a formal statement and the precise physical meaning depends on the dynamics model and the basis in which the system is described (e.g., exciton *vs* localized). Coherent EET can more appropriately be thought of as transfer occurring with changes in delocalization. Coherent EET is discussed from our point of view in Fassioli et al. 2014.

IX Excited State Complexes

After photoexcitation, excited molecules or excitons can sometimes interact with, or become coupled to, neighboring non-excited molecules. The complex formed by such interaction is called an exciplex ("excited complex"; Birks 1970; Förster 1975). Because the exciplex is formed after excitation, and the pigments interact with the radiation field independently, the absorption spectrum of the system remains unchanged (McGlynn et al. 1965). The excitation is subsequently delocalized over the coupled molecules, forming the exciplex, which leads to altered fluorescence properties in the form of a red-shifted emission wavelength. Formation of an exciplex is sometimes coupled to a geometric reorganization of the pigments and their solvent environment (Scholes et al. 1991). The process of exciplex formation can be demonstrated for the example of two identical anthracene molecules, one in the excited and the other in the ground state, as shown in Fig. 4.14. When two identical molecules interact, the exciplex is often called an excimer ("excited dimer").

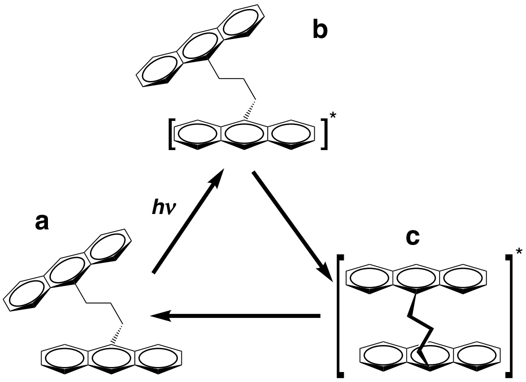

Fig. 4.14. The dynamics of an excimer formation from two anthracene molecules, 9-(9-anthryl) anthracene. A photon is absorbed by an individual chromophore (**a**), resulting in a localized excited state (**b**). The excitation is subsequently delocalized over interacting pigments to form an excimer (**c**).

Interaction of individual pigments within an exciplex can be studied by either assuming (i) that molecules comprising the exciplex are bound together by molecular exciton interactions (Förster 1963), or (ii) that the complex is formed by the coulombic attraction between an electron donor and acceptor pair (Ferguson 1958). In both limits, formation of the exciplex leads to lowering of excitation transition energy. Exciplexes can be accurately described by a superposition of exciton and charge transfer character (Murrell and Tanaka 1964).

In photosynthesis, exciplexes can be formed between chlorophyll and carotenoid molecules via a charge-transfer mechanism. Such exciplexes have been observed in minor light-harvesting complexes of PS II (Holt et al. 2005; Avenson et al. 2008), and are associated with one of the components of regulated thermal de-excitation (reflected in NPQ) activated under excess light conditions (see, e.g., Walla et al., Chap. 9; Polivka and Frank, Chap. 8). Here, excitation energy localized on a chlorophyll molecule that is placed very close to a zeaxanthin molecule is quenched by transfer of an electron from the highest occupied molecular orbital (HOMO) localized on zeaxanthin to the lowest unoccupied molecular orbital (LUMO) localized on the chlorophyll molecule. This process is followed by rapid charge recombination and relaxation to the ground state (Holt et al.

2005; Dreuw et al. 2005; Avenson et al. 2008).

An excimer can be present in photosynthetic complexes even without involvement of carotenoids, and can be formed by interaction of two chlorophyll molecules. If chlorophylls are less than 10 Å apart, they can form an excimer trap, and excitation energy absorbed by any of them will be quenched by the trap. This effect has been observed in light-harvesting proteins of PS I (Romero et al. 2009) and has been suggested to occur in LHCII complexes as well (Miloslavina et al. 2008; Müller et al. 2010; Wahadoszamen et al. 2012; see also, Holzwarth and Jahns, Chap. 5). The protein scaffold that dictates the spacing and relative orientation of pigments therefore plays an essential role in regulating the efficiency of energy transfer and photochemical charge separation (see Horton, Chap. 3; Pascal et al., Chap 10; Büchel, Chap. 11; Morosinotto and Bassi, Chap. 14; van Amerongen, Chap. 15; Garab, Chap. 16; Ruban and Mullineaux, Chap. 17).

X Basic Photophysics of Non-Photochemical Quenching of Chlorophyll Fluorescence

Intense light can create excitations in photosynthetic systems at a rate exceeding the capacity for efficient photochemical charge separation (Demmig-Adams and Adams 1992; Horton et al. 1996). The resulting increase in excited-state lifetime of chlorophylls, caused by closing of RCs, makes the photosynthetic apparatus susceptible to oxidative damage from singlet oxygen produced via the chlorophyll excited triplet state (Ke 2001b). In plants and algae, thermal energy dissipation (for which NPQ is an indicator) is the regulatory process that involves nonradiative dissipation, as heat, of singlet excitation energy (for general introduction into NPQ, see, e.g., Papageorgiou and Govindjee, Chap. 1; Horton, Chap. 3; Holzwarth and Jahns, Chap. 5; Logan et al. Chap. 7; Morosinotto and Bassi, Chap. 14; Garab, Chap. 16; Ruban and Mullineaux, Chap. 17; Murchie and Harbinson, Chap. 25). Thermal dissipation protects plants and algae from photodamage occurring under excess light absorption (see Adams and Demmig-Adams, Chap. 2; Lavaud and Goss, Chap. 20; Demmig-Adams et al., Chap. 24). While the mechanisms of NPQ can vary among pigment-protein complexes and different organisms, there are two main strategies: (i) energy transfer to an individual pigment molecule that dissipates excitation energy on an ultra-short timescale (see section IV of this Chapter) and (ii) thermal dissipation in an excitation trap, formed by interaction of several pigment molecules (see section IX of this Chapter).

Numerous experimental studies have shown that carotenoids are involved in NPQ (see, e.g., Adams and Demmig-Adams, Chap. 2; Polivka and Frank, Chap 8; Walla et al., Chap. 9; Esteban and Garcia-Plazaola, Chap. 12; van Amerongen, Chap. 15). Currently, two general NPQ mechanisms have been formulated. In the first group, carotenoids are presumably directly involved in quenching of the excessive excitation energy, whereas in the second group, carotenoids presumably create conditions for the quenching and are not involved in direct de-excitation processes.

In algae and plants, NPQ is associated with one of three different xanthophyll cycles, in each of which a polar carotenoid is chemically transformed to a less polar carotenoid under excess light conditions (see Papageorgiou and Govindjee, Chap. 1; Adams and Demmig-Adams, Chap. 2; Morosinotto and Bassi, Chap. 14), with (i) the major conversion in all plants and green algae, involving violaxanthin and zeaxanthin (see, e.g., Demmig-Adams et al., Chap 24; Finazzi and Minagawa, Chap. 21), (ii) an additional, more minor conversion in specific plant families, involving lutein epoxide and lutein (Esteban and Garcia-Plazaola, Chap. 12), and (iii) conversion of diadinoxanthin to diatoxanthin in several algal groups (Büchel, Chap. 11; Lavaud and Goss, Chap. 20). The proponents of an involvement of zeaxanthin as a quencher have long proposed that zeaxanthin is rapidly “engaged” and “disengaged” by a mechanism dependent on the trans-thylakoid pH gradient in rapidly growing plants, and that zeaxanthin is continuously engaged in quenching by a pH-independent mechanism in evergreens under growth-arresting conditions (reviewed by Adams and Demmig-Adams, Chap. 2 and Demmig-Adams et al., Chap. 24). Frank et al. (1994; see also Owens 1994) had calculated energies of violaxanthin and zeaxanthin in accordance with the energy-gap law, and had suggested that zeaxanthin can quench chlorophyll excitation by direct energy transfer from Q_y excited state to the carotenoid S_1 state. Indeed, the increase of conjugation chain length from 9 (in violaxanthin) to 11 (in zeaxanthin) leads to lowering of the singlet-transition energies in zeaxanthin (see its justification by reference to the simple particle-in-a-box model, Fig. 4.4). However, these energies have since been shown to be even lower, such that both violaxanthin and zeaxanthin fall below the lowest singlet-excited state of chlorophyll (Polivka et al. 1999; Polivka and Frank, Chap. 8). It is therefore clear that conversion of violaxanthin to zeaxanthin alone cannot account for NPQ. Another possible direct mechanism was reported to take place in minor light-harvesting complexes of higher plants (Holt et al. 2005; Dreuw et al. 2005; Avenson et al. 2008; see also Walla et al., Chap. 9). After conversion of violaxanthin to zeaxanthin, zeaxanthin binds to a light-harvesting protein, where a neighboring excited chlorophyll molecule may form an excited state charge-transfer complex (charge transfer exciplex) with the newly bound zeaxanthin molecule via transfer of an electron from zeaxanthin to chlorophyll. A chlorophyll-zeaxanthin charge transfer state would be stabilized with respect to the localized excitation on the chlorophyll by the coulombic attraction between the resulting electron and hole. The exciplex energy is thermalized along with rapid charge recombination to the ground state (see section IX of this Chapter).

Formation of carotenoid-chlorophyll exciton states was proposed to be the mechanism of NPQ in LHCII complexes (Razi Naqvi 1998; van Amerongen and van Grondelle 2001), where the S_1 state of a xanthophyll carotenoid molecule is coupled to the Q_y excited sate of chlorophyll (see, e.g., Walla et al., Chap. 9; van Amerongen, Chap. 15). Recently, an experimental conformation of this process in LHCII has been reported using two-photon excitation (Liao et al. 2010a, b). However, an independent study, using LH2 complexes, showed that the results of two-photon excitation experiments on chlorophyll-containing proteins can be misinterpreted (Krikunova et al. 2002). Therefore, a confirmation of that mechanism by alternative techniques is necessary.

Zeaxanthin has also been proposed to play an indirect role in NPQ by changing the hydrophobicity of light-harvesting proteins (see, e.g., Horton, Chap 3). Allosteric regulation, triggered by binding of the hydrophobic zeaxanthin to the surface of the LHCII, can change the conformation of the light-harvesting complex, thus switching it to a quenched state (Horton et al. 2000; Pascal et al. 2005). Alternatively, zeaxanthin can act as a stabilizer of the quenched conformation, which is an intrinsic feature of the LHCII complex (Krüger et al. 2010, 2012; see also Krüger et al., Chap. 6). In this new, quenched conformation, direct energy transfer between excited chlorophyll and a carotenoid (suggested to be neoxanthin), located within the protein, was proposed to be activated (Ruban et al. 2007; see also, e.g., Pascal et al., Chap. 10; van Amerongen, Chap. 15). While neoxanthin has been used as an indicator for a conformational change of LHCII during the onset of NPQ (Ruban et al. 2011; Ilioaia et al. 2011; Zubik et al. 2011), Dall'Osto et al. (2007) showed that neoxanthin is not involved in NPQ.

Finally, the conformational change of LHCII has also been suggested to lead to formation of charge-transfer states between two (or more) chlorophylls without direct participation of a carotenoid molecule (Miloslavina et al. 2008; Müller et al. 2010; see also Holzwarth and Jahns, Chap. 5). The resulting chlorophyll exciplex traps may be dissipating excitation energy by nonradiative internal conversion (heat).

XI Concluding Remarks

In this chapter, we have focused primarily on electronic properties of two classes of photosynthetic chromophores, chlorophylls and carotenoids, and on investigations into their role(s) in primary photoprocesses, such as: light absorption and emission, excitation-energy transfer, thermal dissipation, and trapping in RCs. As numerous examples from different pigment-protein complexes have shown, interactions between chromophores are of critical importance in the overall light-harvesting process (Scholes et al. 2011). Assembly of chromophores in a protein scaffold allows fine-tuning of their spectral properties leading to broadening and distribution of absorption bands over a wide spectral range, thereby improving total absorption cross-section. By orienting chromophores at specific distances and angles within the protein, remarkably high EET efficiencies are achieved, e.g., up to 99 % for single chromophore-chromophore EET (Duysens 1952; Song et al. 1976; Frank and Cogdell 1996; van Amerongen and van Grondelle 2001) and >90 % for total EET within PSU (Wraight and Clayton 1974; van Grondelle et al. 1994; Vredenberg 2004). Depending on the intra-chromophore distances, orientations and couplings different mechanisms dominate the EET, such as FRET between weakly coupled chromophores in biliproteins of cyanobacteria (Sharkov et al. 1992), Redfield energy transfer in LH2 antenna of purple bacteria (Novoderezhkin and van Grondelle 2010) or recently proposed coherent energy transfer in Fenna-Matthews-Olson complex of green sulfur bacteria (Ishizaki and Fleming 2009).

Of particular interest for the current book are changes of photophysical properties of chromophores induced by variation in chromophore environment, because these changes are the foundation of physiological acclimation (and genetic adaptation) of photosynthetic organisms to their natural environments. Different mechanisms of thermal dissipation of excess excitation energy can, and may, contribute to NPQ. The basis of all of these mechanisms is, as discussed above, strong interaction among chromophores. This interaction, measured in terms of coupling of excited states of the interacting molecules, is able to produce drastic increases of local internal conversion rates and formation of excitation-energy traps. Once it hops onto such a chromophore-trap, excitation is removed from the antenna and efficiently dissipated as heat. Thermal dissipation processes eliminate excess excitation energy from antenna, avoid damage to the photosynthetic apparatus, and protect the organism's ability to quickly return to a conformation with highly efficient light harvesting and photochemistry under constantly fluctuating natural environmental conditions.

Acknowledgments

The authors thank R. van Grondelle and H. van Amerongen for their suggestions and critical comments. Authors also acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Defense Advanced Research Projects Agency (DARPA, QuBE) for financial support. Govindjee acknowledges the support and friendship of the photosynthesis community at large.

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Chapter 5

Non-Photochemical Quenching Mechanisms in Intact Organisms as Derived from Ultrafast-Fluorescence Kinetic Studies

Alfred R. Holzwarth*
Max-Planck-Institute for Chemical Energy Conversion (MPI-CEC),
Stiftstrasse 34-36, Mülheim an der Ruhr D-45470, Germany

and

Peter Jahns
Plant Biochemistry, Heinrich-Heine-University Düsseldorf,
Universitätsstrasse 1, Düsseldorf D-40225, Germany

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*Author for Correspondence, e-mail: alfred.holzwarth@cec.mpg.de; pjahns@uni-duesseldorf.de

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria,* Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_5,

Summary

Chlorophyll fluorescence kinetic studies provide strong evidence for the occurrence of at least two independent quenching sites, giving rise to non-photochemical quenching of fluorescence, in plants. We discuss a "4-state 2-site quenching model" that integrates the data from those fluorescence studies as well as a large body of additional, previously contradicting, information in a consistent manner. In vitro models of the two quenching sites, named Q1 (PsbS-dependent; located in detached antenna complexes) and Q2 (zeaxanthin dependent, located in antenna proteins that remain attached to the reaction center), are also discussed, along with currently available evidence for the different quenching mechanisms in these sites. At the end of the chapter, we discuss the exceptional potential of ultrafast time-resolved fluorescence to characterize in vivo quenching situations in detail and to distinguish between a range of possible theoretical quenching models and sites.

I Introduction

The accumulation of excess light energy in thylakoids is the primary source for the formation of reactive oxygen species (ROS) in all organisms performing oxygenic photosynthesis. The harmless dissipation of excess light energy as heat, typically assessed as non-photochemical quenching (NPQ) of chlorophyll (Chl) fluorescence, represents a very powerful photoprotective regulatory mechanism. In this chapter, we address the importance of ultrafast Chl fluorescence measurements for obtaining an understanding of thermal dissipation (NPQ) processes in plants and intact microorganisms under in vivo conditions. See Papageorgiou and Govindjee (Chap. 1) for a discussion of the definitions and the history of the evolution of research of the phenomenon under discussion.

A The Requirement of Non-Invasive Measurements on Intact Organisms for Deducing the Quenching Processes

In plants, several different quenching processes, termed energy-dependent quenching, qE (Krause et al. 1982), zeaxanthin (Z)-dependent quenching, qZ (Dall'Osto et al. 2005; Nilkens et al. 2010), and photoinhibitory quenching, qI (Krause 1988), have

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Abbreviations: A – Antheraxanthin; Chl – Chlorophyll; CT (state) – Charge transfer state; DAS – Decay associated spectra; ERPE – Exciton-radical pair equilibrium; FCP – Fucoxanthin-chlorophyll protein; FR – Far-red (fluorescence); k_D – Rate constant of the constitutive non-radiative decay of the antenna; $k_{D\ total}$ – Rate constant of the total non-radiative decay of the antenna (sum of k_D and k_{NPQ}); k_{NPQ} – Rate constant of the high-light induced non-radiative decay of the antenna; k_Q – Rate constant of the non-radiative decay of the detached antenna; k_{rec} – The rate constant of recombination of the first radical pair back to the excited state; k_{tr} – The total trapping rate in the system; LHC – Light-harvesting chlorophyll binding protein; LHCII – Major light-harvesting complex of photosystem II; LHCSR – Stress-related light-harvesting chlorophyll-binding protein; NPQ – Non-photochemical quenching of excited chlorophyll *a*; OCP – Orange carotenoid protein; PAM – Pulse-amplitude-modulated; PS I – Photosystem I; PS II – Photosystem II; PsbS – PsbS protein of photosystem II involved in qE quenching; qE – Rapidly induced and relaxing energy-dependent (pH-dependent) quenching; qI – Photoinhibitory quenching; qT – State transition quenching; qZ – Zeaxanthin-dependent pH-independent quenching; RC – Reaction center; ROS – Reactive oxygen species; RP1 – First radical pair; V – Violaxanthin; VAZ cycle – Xanthophyll cycle involving violaxanthin, antheraxanthin, and zeaxanthin; Z – Zeaxanthin been defined and shown to contribute to NPQ. The originally proposed contribution to NPQ of state transitions, termed the qT component of NPQ (Quick and Stitt 1989), was later disproved in studies on the state transition deficient *Arabidopsis* mutant *stn7* (Bellafiore et al. 2005; Nilkens et al. 2010).

Since it is unclear as to what extent these different NPQ processes might interact with or compensate for each other, this complexity in and by itself generally makes it very difficult to precisely characterize single NPQ processes under in vitro conditions. For example, relaxing quenching components (such as qZ or qI) cannot be precisely studied in isolated thylakoids/chloroplasts due to the limited stability of isolated chloroplasts and/or the requirement of plastid/nucleus interaction (as, e.g., for D1 protein turnover). Moreover, the overall NPQ response is further integrated into a complex network of other photoprotective strategies (Li et al. 2009), ranging from the reduction of the absorption cross-section of an intact leaf (such as chloroplast or leaf movement) to detoxification of ROS (through a complex network of antioxidants) and the general adaptation and/or acclimation of the entire plant metabolism to varying environmental conditions (for further details, see Logan et al., Chap. 7). In view of this complexity of the photoprotective and photo-regulatory responses of plants, it is highly desirable (i) to use non-invasive methods and (ii) to apply these methods to intact leaves or microorganisms under well-defined conditions, whenever possible.

During the past decades, Chl fluorescence quenching measurement has been established as a powerful tool to study NPQ (Krause and Jahns 2003). In most studies, slow-kinetic (time resolution in the range of seconds to minutes) pulse-amplitude-modulated (PAM) fluorescence measurements have been used as a standard method for the characterization of different NPQ components and their basic properties (Quick and Stitt 1989; Walters and Horton 1991; Nilkens et al. 2010; see Logan et al., Chap. 7, for guidelines to counteract confounding influences on PAM measurements in ecophysiological studies). However, such an approach can only provide limited insight for a detailed characterization of quenching location and mechanisms. PAM measurements – even if time-resolved on the micro/milli-second time scale or longer – reflect a type of steady-state measurement, where fluorescence *intensity* is the measuring parameter. Among other limitations (see below for further discussion), this type of measurement is inherently unable to distinguish between changes in absorption cross-section versus internal changes in reaction rates, i.e., the two latter types of parameters cannot be separated or determined in an *intensity* measurement even though both changes influence the observed fluorescence *intensity*. Only real time-resolved measurements are principally able to differentiate between these two types of parameters and to determine them separately. Hence, the application of more sophisticated spectroscopic techniques is required, foremost among them is the use of ultrafast methods (Beddard et al. 1978; Karukstis and Sauer 1983; Holzwarth 1986, 1987; Beauregard et al. 1991).

Avoiding the latter basic problem is the premise of the dramatically increased capability of *inherently time-resolved* methods to differentiate between different, complex model situations as compared to steady state intensity measurements. This capability was recognized early on by Adam Gilmore and Govindjee (Gilmore et al. 1995, 1996) who introduced ultrafast fluorescence to characterize NPQ processes in intact photosynthetic organisms. For studies of NPQ mechanisms, ultrafast spectroscopic methods with time resolution down to the femto-/pico-second time range are required, since that is the time range in which the photochemical quenching reactions, competing with the photosynthetic charge separation and radical pair stabilization, take place. However, such methods – when applied to optically very complex samples such as an intact leaf – encounter many severe problems, both on the technical side of the measurement as well as on the analytical side, since time-resolved fluorescence signals from an intact leaf are highly complex and require sophisticated data analysis procedures. These problems must be overcome to allow these fundamentally powerful methods to provide unequivocal mechanistic conclusions. Over the recent years, we have established the application of ultrafast fluorescence spectroscopy for the analysis of NPQ processes in intact leaves. As expected, this method – in combination with the appropriate target analysis methods – has turned out to be very powerful in distinguishing between various components of excess energy dissipation and in determining the localization of the latter processes in the photosynthetic apparatus (Holzwarth et al. 2009; Miloslavina et al. 2009, 2011). Further, it has turned out that these methods are also very useful in providing information related to light-induced structural rearrangements in large-scale functional reorganization of the photosynthetic membrane. The latter aspect is likely to be highly important for the understanding of the wide range of NPQ processes. Structural changes at the local organization level of photosystem (PS) II super-complexes have also been shown to contribute critically to NPQ under in vivo conditions (Betterle et al. 2009; Ruban et al. 2012; see also Ruban and Mullineaux, Chap. 17).

B Ultrafast Fluorescence Spectroscopy and Use of Mutants: A Powerful Combination to Distinguish Between Various NPQ Mechanisms and Locations

The application of non-invasive methods to study NPQ in intact leaves and microorganisms has the further invaluable advantage that it can easily be combined with the use of NPQ-affecting mutants. Chl fluorescence-based screens have played a crucial role in the identification of components that are critical for NPQ, such as the violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle (Demmig-Adams et al. 1987, 1989; Niyogi et al. 1998), the PsbS protein (Li et al. 2000), and the thylakoid lumen pH (Demmig-Adams et al. 1987; Adams et al. 1990; Munekage et al. 2001). The availability of specific mutants opened the door for more detailed studies on the role of the different components and their interdependence, and provided the basis for the present understanding of NPQ in plants (Niyogi 1999; Müller et al. 2001; Jahns and Holzwarth 2012; Ruban et al. 2012). While the general impact on and relevance of PsbS, Z and the lumen pH to NPQ has been characterized, one of the most challenging remaining tasks of current investigations is characterization of the exact location of the quenching site(s) in Photosystem II (PS II), and of the underlying photophysical/photochemical quenching mechanisms. Major questions are due to uncertainties related to (i) localization and interacting partners of PsbS (see also, e.g., Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14), (ii) the exact role of Z in quenching (direct or indirect; see also, e.g., Horton, Chap. 3; Walla et al., Chap. 9), (iii) localization of the quenching site(s), and possible identification of multiple quenching sites, and (iv) characterization of the – possibly multiple – photophysical quenching mechanisms (see also, e.g., Ostroumov et al., Chap. 4; Polivka and Frank, Chap. 8; Walla et al., Chap. 9).

While PsbS localization and the question of the interacting partners of PsbS can hardly be solved by non-invasive methods, the other aspects listed above have been approached by various spectroscopic techniques under in vitro and in vivo conditions (Holzwarth et al. 2009; Ruban et al. 2012). The possible existence of multiple different quenching sites/mechanisms has been under debate for a long time, particularly in relation to the role of Z and the exact localization of the related quenching site within the antenna of PS II. Although the availability of specific mutants allowed significant progress in the understanding of NPQ, contrasting models for the molecular basis of qE quenching have developed during the past years.

Peter Horton, Alexander Ruban and co-workers favor what they term the *aggregation model* of quenching (Horton et al. 1991, 1996, 2005; Ruban et al. 2012; see also, e.g., Horton, Chap. 3; Krüger et al., Chap. 6; Pascal et al., Chap. 10; van Amerongen, Chap. 15), which assumes that qE requires conformational changes as well as structural reorganization in the major light-harvesting chlorophyll-binding protein complexes of PS II (LHCII) antenna (see also Ruban and Mullineaux, Chap. 17) controlling the switching of LHCII proteins from efficient light harvesting to the dissipative state. In this scenario, Z is proposed to act only as an allosteric modulator of qE (Johnson et al. 2008, 2009; see also Horton, Chap. 3) that enhances aggregation and structural reorganization of LHCII and, via these effects, shifts the pH-sensitivity curve of qE by about 1.5 pH units to more alkaline pH values (see Ruban et al. 2012; Horton, Chap. 3).

In contrast to the *aggregation model*, a direct role of Z in NPQ, and more specifically in qE, either based on energy transfer from Chl to Z (Owens 1994; Frank et al. 2000) or due to formation of a carotenoid cation in a $\mathrm{Chl^{-}Z^{+}}$ charge transfer (CT) state (Holt et al. 2005; Ahn et al. 2008; Avenson et al. 2008, 2009) has been proposed (see also Ostroumov et al., Chap. 4; Walla et al., Chap. 9). Such direct Z quenching is assumed, and actually highly likely, to be located in the minor antenna complexes of PS II, i.e., CP24, CP26, and in particular CP29 (Gilmore et al. 1998; Holzwarth et al. 2009; Ballotari et al. 2010; Miloslavina et al. 2011; see also Morosinotto and Bassi, Chap. 14), since no major quenching effects of Z were detected in vitro in LHCII (Ahn et al. 2008; Avenson et al. 2008; Miloslavina et al. 2008; Müller et al. 2010).

These contrasting models could be explained by the fact that the supporting data for each model have been derived mainly from in vitro measurements and might thus not reflect the in vivo situation. Alternatively, the conflicting data and interpretations might easily be explained if different, independent quenching mechanisms were active in parallel in different sites. Indeed, application of ultrafast fluorescence kinetics applied to wild type and various mutants of intact *Arabidopsis* leaves under in vivo-conditions provided strong evidence for the existence of two independent quenching sites differing in their PsbS- and Z-dependence (Holzwarth et al. 2009; Miloslavina et al. 2011).

Horton and Ruban initially suggested that qE is located, and modulated by Z action, in the trimeric major LHCII complex only (Horton et al. 1991), but later extended their model to the entire PS II antenna system (Horton et al. 2005). In contrast, ultrafast transient absorption spectroscopy applied to isolated thylakoids and recombinant antenna proteins suggested that Z-induced charge transfer (CT) quenching (Holt et al. 2005) – proposed by the latter authors to be the quenching mechanism related to qE – is limited to the minor antenna complexes only and does not occur in trimeric LHCII (Ahn et al. 2008; Avenson et al. 2008).

The application of PAM fluorescence analysis to mutants deficient in single antenna proteins also did not clarify the exact site of quenching. While the absence of CP26 had little effect on qE, knockout of CP29 resulted in a reduction of qE of about 30 % (Andersson et al. 2001). The most pronounced effect (about 50 %) was found in the absence of CP24 (Kovacs et al. 2006). However, the knockout of both CP24 and CP26, concomitant with a reduction of CP29, again did not result in significant reduction of qE (de Bianchi et al. 2008). These findings indicate a high degree of flexibility of the PS II antenna with respect to qE formation. Furthermore, the absence of trimeric LHCII was also only accompanied by a moderate reduction of qE by about 30 % (Andersson et al. 2003). This observation might be explained by the fact that CP26 is able to form trimers in these LHCII-deficient plants (Ruban et al. 2003), and supports not only the view of a highly flexible organization of the PS II antenna, but further indicates that quenching might occur in all antenna sub-complexes of PS II.

Some of the reported quenching effects may be the consequence of mutation-induced structural modification in the PS II antenna or overall thylakoid structure, and might only occur in such special mutation-induced situations. Many of these effects that were initially difficult to explain are now understood on the basis of substantial compensation effects in the relative amount and organization of PS II antenna complexes that occur in knockout mutants of the minor antenna complexes (de Bianchi et al. 2007, 2008, 2010, 2011; Miloslavina et al. 2011). Thus, great care has to be taken to ensure that overall thylakoid and PS II antenna structure is not altered by the mutation. In that sense, "neutral" mutations need to be distinguished from more or less "invasive" mutations. The latter mutants may have limited general value, and only for understanding wild type quenching location and mechanisms. In agreement with many of the above-described findings, ultrafast fluorescence data provided compelling evidence for the existence of at least two different quenching sites under in vivo conditions in wild type. The two sites are proposed to be located (i) in the major LHCII complex and (ii) in the monomeric PS II antenna proteins (Holzwarth et al. 2009; Miloslavina et al. 2011). Based on these and complementary data, we have derived what we have termed a *4-state 2-site* model of NPQ that is discussed in detail in the following section.

II The 4-State 2-Site Model of NPQ in Higher Plants

The 4-state 2-site model of NPQ (Fig. 5.1) summarizes data obtained from ultrafast fluorescence studies applied to intact leaves from *Arabidopsis* wild type and mutant plants (Holzwarth et al. 2009; Miloslavina et al. 2011) and also incorporates complementary results from conventional (steady state) NPQ studies (Lambrev et al. 2010; Nilkens et al. 2010). In the wild type, two different quenching sites were identified: A quenching site (termed Q1), not seen in

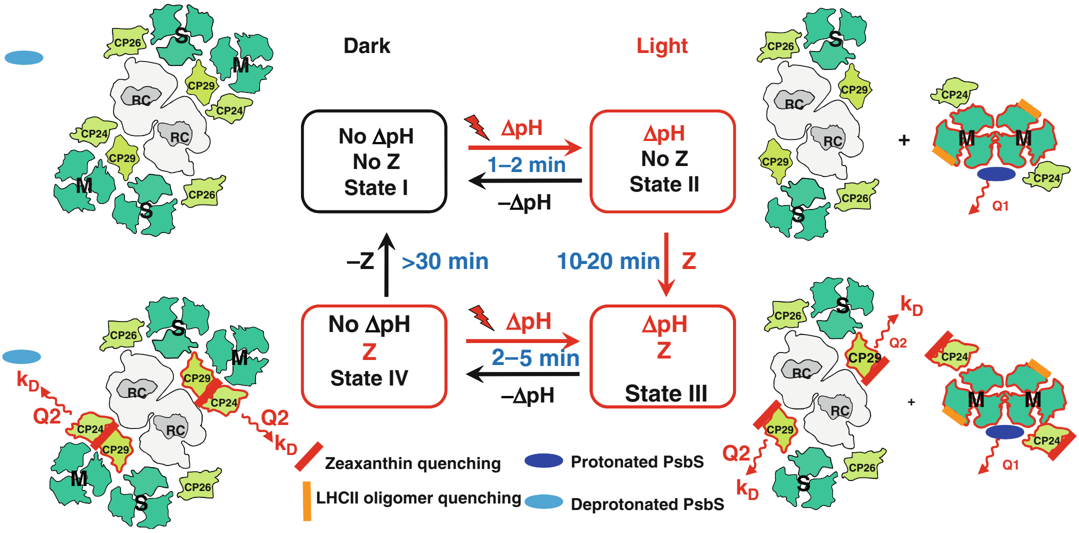


Fig. 5.1. The 4-state 2-site quenching model for in vivo quenching in the model plant *Arabidopsis. Left part*: dark-adapted state (*top*) and slowly relaxing state (*bottom*). *Right part*: states appearing upon actinic light adaptation or NPQ induction. Four states are indicated in boxes by roman numbers along with the different quenching conditions. The *wavy arrows* indicate the quenching processes at sites Q1 and Q2. The *red* and *orange bars* indicate the quenching locations in the various antenna parts. *Right-hand side*: The *blue oval* represents protonated PsbS. The model shown indicates that protonated PsbS may be associated with the detached and quenched LHCII aggregate. This is actually an unproven hypothesis since no direct association of PsbS with detached LHCII has been demonstrated thus far. The detached LHCII aggregate is also associated with CP24, which may be enhancing (or modulating) quenching in this site if Z is present (The above follows the suggestion of Betterle et al. 2009.) S and M denote the strongly and moderately, respectively, coupled LHCII trimers in the PS II supercomplex.

PsbS-deficient *Arabidopsis* lines, and a second quenching site (termed Q2), not seen in VAZ cycle mutants deficient in V to Z conversion. Consequently, we assigned the formation of Q1 to a PsbS-dependent process, while formation of Q2 was attributed to be Z-dependent (Holzwarth et al. 2009).

Identification of these two different quenching sites was based on target analyses of spectrally resolved ultrafast fluorescence kinetics covering the entire fluorescence region of PS II as well as PS I. Instrumental for the assignment of these quenching sites was the use of various mutant plants affected in PsbS or VAZ cycle (V to Z) conversion. Furthermore, this work involved the use of specific knockout mutants of minor PS II antenna complexes (Miloslavina et al. 2011). Since the dynamics of the physiological activation and relaxation of the two quenching sites could not be followed directly by ultrafast fluorescence spectroscopy, only the physiological quasi steady states of quenching could be characterized by ultrafast fluorescence. Thus, additional data from (slow, steady state) kinetic fluorescence measurements (Lambrev et al. 2010; Nilkens et al. 2010) were used to complement this information. Based on these analyses, four different NPQ states of PS II, termed state I to state IV, can be distinguished in wild type *Arabidopsis* upon illumination of dark-adapted plants (grown under 150 μmol photons m^{-2} s^{-1}) with saturating red actinic light of an intensity of 600 μmol photons m^{-2} s^{-1} (Fig. 5.1). We note that 'state I' and 'state II' are not identical with 'state 1' and 'state 2' defined almost 40 years ago for the regulation of the distribution of light energy to PS I and PS II via state transitions (Bonaventura and Myers 1969; Murata 1969; see also Papageorgiou and Govindjee, Chap. 1).

State I: Fully relaxed dark state; Q1 and Q2 sites are inactive

State II: Early (time scale of 1–2 min after actinic light on, fast phase of NPQ) light-activated NPQ state; Q1 active, Q2 inactive

State III: Fully light-activated NPQ steady state: both Q1 and Q2 are active

State IV: Early, partially relaxed, dark state: Q1 inactive, Q2 still active

A The PsbS-Dependent Quenching Site Q1

Dissection of the spectral characteristics of each lifetime component contributing to fluorescence quenching allows not only discrimination between PS I- and PS II-related components, but also the capability to monitor relative changes in functional PS II antenna size (see below). The quenching site Q1 was identified as related to the appearance of a high light-induced far-red (FR) fluorescing quenching component. Target analysis of the kinetic data showed that the antenna component related to far-red (FR) fluorescence was functionally independent of either PS II or PS I. This latter component instead originates from an antenna complex that became functionally detached from PS II. This assignment is based on a number of observations (see Holzwarth et al. 2009 for details). Specifically, functional detachment of the FR-emitting antenna complexes from the PS II super-complex was deduced from the fact that generation of the FR fluorescence component occurred at the expense of the amplitude of PS II fluorescence. This observation indicates a decreased PS II antenna cross-section, and thus functional detachment of parts of the PS II antenna proteins. The unusual spectral characteristics of this component, i.e., strongly enhanced intensity in the FR region (>700 nm) in comparison with typical PS II or LHCII fluorescence, and the lifetimes of 300–500 ps, were strongly reminiscent of the fluorescence properties of quenched oligomeric LHCII trimers (Miloslavina et al. 2008).

A comparative analysis of the PsbS-deficient *npq4* (Li et al. 2000) and *npq1* (Niyogi et al. 1998) mutants further showed that Q1-site quenching was absent in the PsbS-deficient mutant but was present in the mutant deficient in V to Z conversion. This indicates that the formation of Q1 is strictly dependent on the presence of the PsbS protein and independent of Z, although a slight modulation by the presence of Z was observed in the lifetime of this component (Holzwarth et al. 2009). Also taking into account the relatively large decrease of the

PS II cross-section (up to about 50 % in a PsbS-overexpressing mutant, the L17 mutant) and the associated large increase in the amplitude of the FR-fluorescing component (Holzwarth et al. 2009), the Q1 quenching site can thus be assigned to a fraction (or the full complement in the L17 mutant, respectively) of the major LHCII proteins detaching from PS II and being quenched in a PsbS-dependent manner (Fig. 5.1). The overall behavior of the functionally detached antenna protein, giving rise to in vivo FR-fluorescence, observed exclusively under quenching conditions, was highly reminiscent of LHCII aggregates in its spectral and kinetic properties (Mullineaux et al. 1993; Miloslavina et al. 2008).

This striking similarity, between features of LHCII aggregates and apparently PsbS-dependent Q1 quenching, allowed for a further characterization of the associated quenching mechanism in vitro (see below). Since PsbS-dependent quenching processes are controlled by thylakoid lumen pH, Q1 quenching formation is apparently controlled by lumen pH in a PsbS-dependent process. We proposed that detachment and quenching of major LHCII provides the molecular basis of qE quenching and does not require the presence of Z (Holzwarth et al. 2009). The relative contributions of the Q1 and Q2 quenching sites to total NPQ as well as their contributions to photoprotection have been discussed quantitatively in Lambrev et al. (2012).

Based on the available evidence, it is unclear whether or not minor PS II antenna complexes are also detached, along with the major LHCII, upon Q1 formation. However, our model includes CP24 as a likely candidate also involved in the Q1 quenching site (Fig. 5.1) since (i) a reversible, PsbS-dependent dissociation of PS II antenna proteins (composed of LHCII and CP24) from the PS II–LHCII super-complex under NPQ conditions was demonstrated by biochemical analyses and electron microscopy (Betterle et al. 2009) and (ii) CP24-deficient mutant plants exhibit the most pronounced reduction of qE (about 50 %) among all antenna-deficient mutants (Kovacs et al. 2006), though the latter fact might also be partly related to the reduction of electron transport and changes in the organization of the PS II antenna observed for CP24-deficient plants (de Bianchi et al. 2008). It is important to point out that the functional LHCII detachment characteristics of the Q1 quenching site cannot be clearly identified by any method other than time-resolved fluorescence. Steady-state fluorescence methods are, in principle, unable to distinguish between functionally bound and detached states. Unfortunately, the dynamics of the development of Q1-site quenching (1–2 min) could not be, thus far, followed directly by ultrafast fluorescence, due to the overall time (30–45 min) required to perform a full set of fluorescence decay measurements over the entire spectral range. Therefore, at present, additional approaches must be applied to solve this problem. Using a multi-wavelength spectrometer allowing slow, with a time-resolution in the ms range, time-dependent measurements of fluorescence spectra on intact leaves, we were able to confirm that the FR-fluorescing component possesses the same spectral and PsbS-dependent characteristics as qE (Lambrev et al. 2010). Formation/relaxation of Q1, furthermore, parallels the kinetics of the build-up (upon illumination) and dissipation (upon re-darkening) of the trans-thylakoid pH gradient over ~1–2 min (Fig. 5.1).

B The Zeaxanthin-Dependent Quenching Site Q2

The quenching site Q2 is characterized by a pronounced increase in k_D (see Fig. 5.1), as a direct measure of the effective, non-radiative, non-photochemical deactivation (and thus of NPQ) in the PS II-attached antenna (Holzwarth et al. 2009). In contrast to Q1, the Q2 site is not associated with detachment of antenna proteins from PS II. Rather, it reflects the quenching properties of antenna proteins functionally connected to the PS II super-complex and the PS II reaction center, since this quenching drastically affects all three PS II fluorescence lifetime components. The observation that light-inducible increases of k_D were similar in both PsbS-deficient (*npq4*) and PsbS-overexpressing (L17) plants indicates that Q2 activation is independent of PsbS, while the absence of a high light-induced increase of k_D in the Z-deficient *npq1* mutant supported the assignment that Q2 is strongly dependent on Z formed in the VAZ cycle (Holzwarth et al. 2009). The PsbS-independent formation of Q2 and the correlation of the time constant of induction show that this quenching site represents the more slowly inducible/relaxing NPQ-component qZ (Nilkens et al. 2010), rather than a Z-dependent modulation of the strictly pH-dependent qE component. The kinetics of Q2 induction can therefore be expected in the range of about 10–20 min along with conversion of violaxanthin (V) to Z (Nilkens et al. 2010; Jahns and Holzwarth 2012). Q2 relaxation occurs over even longer time scales (>30–60 min) accompanied by Z epoxidation (Fig. 5.1). We note, however, that VAZ cycle conversions are variable and respond strongly to plant growth light environment (see Demmig-Adams et al., Chap. 24).

Since the kinetics of Q2 formation and relaxation cannot, thus far, be followed directly with ultrafast fluorescence methods (measurements to date were invariably performed in quasi-steady state of either fully quenched or fully unquenched cases), we cannot completely rule out that Q2 may contain some (limited) contribution of qE as well (Kalituho et al. 2007). These uncertainties are related to the complexity of the roles of Z in photoprotection, as a contributor to NPQ and/or to anti-oxidation in the lipid phase of the membrane (Havaux and Niyogi 1999; Havaux et al. 2004) and, furthermore, Z's specific function in NPQ as a direct or indirect quencher (Jahns and Holzwarth 2012). It is thus essential to further develop the method of ultrafast fluorescence spectroscopy to overcome the present limitations to directly follow the dynamics of Q1 and Q2 formation/relaxation. This can be done, in the future, by extension to a multi-channel detection scheme and addition of an additional (slow) time axis in the measurement scheme, which would allow for a much faster recording of an entire time-resolved spectrum than is possible at present.

Irrespective of these remaining questions, however, two important basic conclusions can be drawn: (i) Q1 and Q2 quenching sites are located in different parts of the PS II complex and (ii) Q1 and Q2 quenching sites function largely independently of each other in PsbS- versus VAZ-cycle mutants, while still allowing for a limited cross-play between these two quenching sites. Both features have important implications for the general understanding of NPQ processes under in vivo conditions that will be discussed below in more detail.

C Q1 and Q2 are Located in Different Parts of the PS II Super-Complex

The ultrafast fluorescence data, obtained thus far, has clearly indicated that the full set of observations cannot be explained within a simple quenching model with only a single quenching site in which the quenching is modulated by both PsbS and Z (the major potential of ultrafast fluorescence measurements to distinguish clearly between such situations is discussed at the end of this chapter). Rather, the Q1 site had to be assigned to quenching processes in detached antenna proteins, while Q2 was assigned to antenna proteins remaining functionally connected to PS II. This distinction provided the clue to understand and integrate previously conflicting data on the localization of NPQ within the PS II antenna into a uniform model (see above). The two-quenching-site model can satisfactorily explain all of these seemingly contradictory data. Location of Q1 in detached and aggregated trimeric LHCII proteins is in line with the proposed pH-dependent, aggregation/structural-change-based NPQ capacity of LHCII (Andersson et al. 2003; Horton et al. 2005; Ruban et al. 2012). Similarly, activation of Q2 explains the previously described Z-dependent NPQ capacity of isolated minor LHCII proteins (Ahn et al. 2008; Avenson et al. 2008), although a direct role of Z in the quenching process cannot be derived on the basis of time-resolved fluorescence measurements alone. Our model for the localization of the quenching sites Q1 and Q2 is thus consistent with the described quenching properties of the different (major and minor) PS II antenna proteins that can also be observed in vitro. The determination of the underlying photophysical mechanisms of quenching requires other methods. Thus, the fluorescence- and mutation-based model of Fig. 5.1 per se does not allow assessment of whether quenching at Q1 is based on a Chl-carotenoid (Crimi et al. 2001; Holt et al. 2005; Ruban et al. 2007; Fuciman et al. 2012) or Chl-Chl (Miloslavina et al. 2008; Müller et al. 2010) interaction. Other methods, i.e., ultrafast transient absorption in vivo, preferentially in combination with suitable in vitro models for the two quenching sites Q1 and Q2, are required for making this assessment.

D Q1 and Q2 Can be Initiated Independently

One important and crucial feature of the *4-state 2-site model* of NPQ is that the two quenching sites, Q1 and Q2, can operate independently from each other in PsbS-deficient and VAZ-cycle-deficient mutants, respectively. Operation of the two sites is linked by the fact that activation of both sites requires the presence of a low pH in the thylakoid lumen. Since Q1 is formed more rapidly (1–2 min), due to its fast, pH-induced activation of PsbS, activation of Q1 will presumably always precede activation of Q2 in wild type plants, giving rise to the consecutive formation of states II and III (see Fig. 5.1). Once established, however, Q2 remains active for a longer time (>30 min), even after rapid (1–2 min) relaxation of Q1 along with relaxation of the trans-thylakoid proton gradient (state IV), which had already been noticed by Roberto Bassi and coworkers (Dall'Osto et al. 2005). What they called "pH-independent quenching" is, in fact, identical to the Q2-site quenching in our model. Likewise, the Q1-site quenching is, to a large part, identical to the rapidly inducing and relaxing qE quenching (Horton et al. 1996; Li et al. 2000; Müller et al. 2001).

A combined action of two independent quenching sites provides a highly flexible response to fluctuating light conditions in both the short and long-term. It is important to note that both quenching sites can be regulated differently, particularly after prolonged excess light exposure. Inactivation of the Q1 site might be delayed by slow dissipation of the trans-thylakoid pH gradient due to down-regulation of ATP synthesis or due to sustained ATP hydrolysis in the dark (Gilmore and Yamamoto 1992; Gilmore and Björkman 1995). In contrast, inactivation of Q2 is likely to be modulated by regulation of Z expoxidation in response to previous light stress experienced by a leaf (Reinhold et al. 2008; Nilkens et al. 2010). Consequently, such differential regulation of the inactivation of both quenching sites allows for a very flexible acclimation of plants to varying light intensities. In particular, gradual delay of Q2 inactivation, by down-regulation of Z epoxidation, may enable plants to regain their maximal photoprotective capacity (state III) more quickly in a rapidly fluctuating light environment (see also Demmig-Adams et al., Chap. 24). From a systems-regulation point of view, NPQ regulation according to our 4-state 2-site model has many similarities with a typical technical regulation system. The latter typically consist of two regulation loops, a fast-responding derivative branch and a slow-responding (integrating branch) regulation loop. The fast (derivative) branch is regulated in proportion to the time derivative of a signal change (in the NPQ case, light intensity or delta pH), while the slow (integrating) branch responds to long-term changes of the signal (again, light intensity or delta pH). Such a fast/slow two-loop combination as employed in most regulation systems ensures a rapid response while avoiding a deleterious overshoot of the response associated with a fast-response system alone. In this technical picture, the PsbS-dependent Q1 site is the fast loop and the Z-dependent Q2 site the slower, integrating loop.

E NPQ and Photoinhibition

We note that not all NPQ provides protection against photoinhibition, as defined by the production of triplet states, singlet oxygen and other reactive oxygen species, and inactivation of PS II reaction centers as well as other components of the photosynthetic apparatus. This entire issue is more complex than what can be briefly described here (see also Zaks et al. 2012). It may thus suffice to note that we have developed – albeit in a still fairly simple model – a first quantitative description that links the two phenomena. An important general conclusion is that there exists no direct – and certainly no linear – relationship between total NPQ on the one hand and protection against photoinhibition on the other hand (Lambrev et al. 2012).

Furthermore, Lambrev et al. (2012) have shown that the dominant contribution to NPQ in *Arabidopsis* (and assessed via conventional fluorescence intensity changes), i.e., the Q1 (or qE) mechanism, apparently provides much less photoprotection than the Q2 (or pH-independent) mechanism, although the latter contributes less to the total NPQ value in *Arabidopsis*. One might thus speculate that the Q1 site mechanism may be primarily responsible for fast regulation of photosynthesis and less for net thermal dissipation and actual photoprotection. Such an interpretation would be in line with results from *Arabidopsis* plants grown under constant high light versus rapidly changing light intensities (sunflecks), where lack of a qE response (in *npq4*) only had a noticeable effect under rapidly changing light intensity (Külheim et al. 2002). However, there is evidence for a greater apparent production of reactive oxygen in *npq4* than in the wild type plant. It was shown by Frenkel et al. (2009) that *npq4* plants, grown under natural, fluctuating field conditions, exhibited a greater production of the lipid-peroxidation-derived plant stress and defense hormone jasmonic acid as well as an increased resistance to plant herbivores (see review by Demmig-Adams et al. 2013).

III Similarity of the NPQ Quenching Sites and Mechanisms in Plants and Diatoms

The dissipation of excess light energy as heat is a photoprotective and regulatory mechanism active in all photosynthetic organisms among the plant kingdom. Although PS II is composed of different antenna systems and pigments in photosynthetic bacteria, algal classes and land plants, all organisms performing oxygenic photosynthesis are capable of inducing NPQ processes under high light conditions. In most cases, NPQ activation has been demonstrated to require a light- (or pH-) sensing domain/protein (like PsbS in plants) in combination with xanthophyll conversion (like the VAZ cycle in plants). Active xanthophyll cycles (predominantly based on either V to Z or diadinoxanthin to diatoxanthin conversion) have been found in taxa that use Chl a/b or Chl a/c binding antenna proteins, such as light-harvesting protein complexes (LHC), stress-related light-harvesting complexes (LHCSR or LI818) or fucoxanthin-Chl proteins (FCPs) (Green 2011; Pan et al. 2012), but not in those with phycobilisome-based antenna systems (as in cyanobacteria and red algae) (Stransky and Hager 1970; Jahns et al. 2009). Likewise, light-/pH-sensing proteins have been identified or proposed for most taxa, such as the orange-carotenoid protein (OCP) in cyanobacteria (Rakhimberdieva et al. 2004; Wilson et al. 2008), the FCPs in diatoms and brown algae (Dittami et al. 2010; Gundermann and Büchel 2012), and the stress-related LHCSR (or LI818) in mosses (Alboresi et al. 2010) and green algae (Peers et al. 2009).

Ultrafast fluorescence measurements on the diatoms *Cyclotella meneghiniana* and *Phaeodactylum tricornutum* (Miloslavina et al. 2009; Chukhutsina et al. 2013) have revealed a very similar picture of NPQ processes as discussed above for plants, although earlier models had assumed that NPQ processes in diatoms are organized very differently. In both the diatom species, mentioned above, two independent quenching sites have been identified: one quenching site was located in an FCP antenna system functionally detached from both the photosystems, while the second quenching site was found to be located in the PS II-attached antenna (Miloslavina et al. 2009). NPQ processes in diatoms and plants are thus apparently based on similar general principles, although NPQ induction in the two groups shows different characteristics and time scales. In particular, contribution of xanthophyll cycle-dependent NPQ is often the dominating NPQ process in certain diatoms, and pronounced pH-independent, diatoxanthin-dependent quenching has been described for a number of other diatom species (Goss et al. 2006). This xanthophyll cycle-dependent NPQ thus resembles the features of qZ quenching in plants (Nilkens et al. 2010) that is likely to be related to the quenching site Q2 (Fig. 5.1), as determined in *Arabidopsis* (Holzwarth et al. 2009). Further, a pronounced xanthophyll cycle-dependent (and apparently pH-independent) NPQ has also been described for brown algae (Ocampo-Alvarez et al. 2013) indicating that the latter type of quenching might be a common feature of FCP-containing organisms.

IV Emergence of a Third NPQ Quenching Mechanism/Site

One may ask whether the Q1 and Q2 quenching sites and associated mechanisms (see below) are the only sites activated by oxygenic photosynthetic organisms for NPQ regulation and photoprotection. The potentially important role of major thylakoid reorganization in NPQ and other regulation processes occurring at the thylakoid level has only recently received attention (see Anderson et al. 2012 for a review). The prevailing picture of the thylakoid organization of higher plants is still the lateral segregation model, with PS II located in appressed grana region and PS I located in stroma thylakoids and grana margins.

Many adaptation processes to light quality and intensity are intimately related to changes in thylakoid protein phosphorylation patterns (Ebbert et al. 2001; Dietzel et al. 2011; Tikkanen and Aro 2012; Tikkanen et al. 2012). Although it is known that long-term acclimation to different light intensities (e.g., shade versus sun leaves of the same species) influences thylakoid structure (Anderson 1986; Osmond 1994; Matsubara et al. 2012), detailed structural information is only beginning to appear (Kouril et al. 2012). Even less is known about functionally important structural changes in thylakoid organization induced by short-term exposure to high light (and resulting in NPQ, Rozak et al. 2002; Kirchhoff 2008) or to light quality changes (and resulting in, e.g., state transitions, Chuartzman et al. 2008; Dietzel et al. 2011; Nevo et al. 2012). Several studies have now suggested that pronounced membrane reorganization occurs during short-term adjustments to high light exposure (Rozak et al. 2002; Fristedt et al. 2010; Kirchhoff et al. 2011; Nevo et al. 2012).

It is important to realize that the present thylakoid organization model was developed on the basis of thylakoid structural information derived from dark-adapted plant tissues and from plants grown under constant light, rather than from plants grown under natural fluctuating light conditions. The latter growth conditions might actually require a substantially different thylakoid structure and photoprotection capacity (Alter et al. 2012). It is thus important to ask whether or not the established lateral segregation model of the photosynthetic apparatus accurately describes plants in their photosynthetically "active state", and whether important structural reorganizations may occur upon both short-term and long-term acclimation to high light (Alter et al. 2012). Certainly, the lateral segregation model established for higher plants does not apply to the thylakoid structures of many photosynthetic microorganisms that exhibit only little membrane appression. In green algae, for example, there is no pronounced thylakoid grana stacking and no strict lateral separation of PS II and PS I as opposed to what is seen in the thylakoids of higher plants (Anderson and Andersson 1982). Due to the lack of pronounced differentiation into grana stacks and stroma thylakoids, PS II and PS I are actually mixed in the same membrane areas in green algae (Krämer et al. 1988; Larkum and Barrett 1983; Larkum and Howe 1997).

We have recently observed that such mixing in the chlorolichen *Parmelia*, with a green algal photobiont, gives rise to pronounced spillover (i.e., direct PS II to PS I energy transfer process) in the normal active state (Slavov et al. 2013). Lichens are characterized by extreme PS II quenching upon drying, which provides pronounced photoprotection (Heber et al. 2001). Slavov et al. (2013) have studied this drying-induced quenching mechanism in *Parmelia* and found 70 % of the extreme PS II quenching in the dried state to occur via a novel *spillover mechanism* caused by major thylakoid and protein reorganization, while direct antenna quenching based upon similar mechanisms as described above for higher plants contributed only about 15 % to total PS II quenching. Paul (2014) has observed a similar *spillover quenching* in higher plants as well. Although too early for a final conclusion, it appears possible that this type of *spillover quenching* might emerge as a third general quenching type – in addition to the Q1 and Q2 types of quenching – that may provide extreme NPQ quenching and photoprotection capacity in a variety of photosynthetic organisms.

V In Vitro Models for the Q1 and the Q2 Quenching Sites

In order to study photophysical/photochemical quenching mechanism (s) in detail, which requires the use of ultrafast transient absorption and related techniques, the availability of good in vitro models performing similar or identical quenching reactions as in the in vivo sites Q1 and Q2 is highly desirable since such signals on intact organisms are generally too complex to measure and/or difficult to interpret. Such models would ideally be isolated pigment-protein complexes that undergo the same quenching reactions in detergents as in vivo (see also Pascal et al., Chap. 10; van Amerongen, Chap. 15).

A Q1 Site Model

It is now well accepted that the "LHCII aggregation model" (Horton et al. 1991, 1996, 2005; Ruban et al. 2012) is a good in vitro model for the Q1 site (or qE) quenching. Likewise, crystals of LHCII are also highly quenched in a similar manner (Pascal et al. 2005; van Oort et al. 2011) and can be used as a model system for studying this quenching. Quenching in LHCII aggregates is associated with some conformational changes of the carotenoid neoxanthin and some Chls, as detected by Raman spectroscopy (Ruban et al. 2007). While neoxanthin was used as an indicator for a conformational change of LHC proteins during the onset of NPQ (Ilioaia et al. 2011; Zubik et al. 2011; Ruban et al. 2012), Dall'Osto et al. (2007) concluded that neoxanthin is not involved in thermal dissipation (NPQ) (Dall'Osto et al. 2007; Zubik et al. 2011; Ruban et al. 2012).

Quenching in LHCII aggregates is, furthermore, associated, in a complex manner, with protein dynamics, which has been studied by single molecule spectroscopy (Krüger et al. 2011a, b). Likewise, the charge environment around some Chls in aggregates versus trimers of LHCII changes upon quenching induction (Pandit et al. 2011, 2013). While these phenomena associated with LHCII quenching in aggregates (and even in LHCII trimers devoid of a detergent environment; Ilioaia et al. 2008) demonstrate that aggregation per se is not the controlling factor for quenching, they shed light only on accompanying or enabling phenomena, but do not represent the actual photophysical quenching mechanism itself. Thus, the latter observations do not clarify the actual quenching mechanism, but rather provide additional information. The first mechanistic study of aggregated and quenched LHCII suggested occurrence of a rapid Chl-to-lutein excited-state energy-transfer mechanism enabled by conformational changes (Ruban et al. 2007). This interpretation has, however, been questioned since Chl excited-state quenching did not follow the proposed kinetics, nor did the intermediates in the quenching reaction exhibit signs of a carotenoid excited state (Müller et al. 2010). Rather, only decaying-Chl bleaching signals with complex kinetics, not consistent with a single energy transfer step to a carotenoid, were observed as intermediates during the quenching reaction.

Formation of a Chl-Chl charge transfer (CT) state, consistent with fluorescence kinetic measurements in vitro (Miloslavina et al. 2008) and in vivo (Holzwarth et al. 2009), was instead proposed as the quenching mechanism. This interpretation was independently confirmed by the observation of at least one strong Chl-Chl CT state detected by its Stark fluorescence (Wahadoszamen et al. 2012), in agreement with the interpretation of fluorescence kinetics (Miloslavina et al. 2008). A second observed longer-wavelength CT fluorescence band could not be clearly assigned in Stark fluorescence. The latter band may originate either from Chl-carotenoid CT state or a second Chl-Chl CT state. Remaining problems for future research concern the understanding of the complex interplays and connections among protein dynamics (Krüger et al. 2011a, b), conformational changes (Ruban et al. 2007), and switching-on and -off of the main Chl-Chl CT quenching mechanism (Müller et al. 2010). All of these observations are related to LHCII aggregates or crystals, where quenching is invoked without the presence of PsbS. However, Q1 (or qE) quenching in vivo is strictly associated with pH-induced activation of the PsbS protein (Li et al. 2000; Holzwarth et al. 2009). It must thus be concluded that the reported in vitro LHCII quenching may only be similar, but not identical, to the in vivo quenching mechanism. The following questions thus arise: Is PsbS associated with LHCII in vivo – and thus directly involved in quenching? No such direct interaction has been demonstrated thus far. Does PsbS play an active or rather a passive role (e.g., by providing the required environment at some part of the LHCII complex) in enabling LHCII quenching? All these are presently open questions that remain to be answered before a full understanding of the LHCII based Q1 (or qE) quenching mechanism has been reached.

B Q2 Site In Vitro Model

Isolated minor antenna complexes (CP24, CP26, CP29) containing Z in the L2 binding site have been proposed to be good in vitro models for Q2-site quenching (pH-independent in the *npq4* mutant) (Crimi et al. 1998, 2001; Frank et al. 2001; Ahn et al. 2008; Avenson et al. 2008, 2009; Fuciman et al. 2012). The latter notion is in agreement with the suggestion that the minor antenna complexes of PS II are the sites of Q2-site quenching in vivo (Holzwarth et al. 2009; Miloslavina et al. 2011). In vitro, ultrafast transient absorption studies of minor antenna complexes reconstituted with Z indicated formation of Chl^-Z^+ CT states as quenching intermediates (Ahn et al. 2008; Avenson et al. 2008, 2009; Fuciman et al. 2012) and the suggestion was thus made that the latter reaction may represent the Z-induced quenching mechanism in vivo. Interestingly, no formation of a carotenoid cation state – which should be easily observable in transient absorption due to its large difference extinction coefficient in the NIR region – has, however, been detected in femtosecond transient absorption measurements on intact *Arabidopsis* leaves in which strong quenching was induced by actinic light irradiation (Müller et al. 2013). This raises doubt on the validity of the proposed Chl^-Z^+ CT state mechanism as the actual in vivo mechanism for Q2 site quenching. A model for thermal dissipation based on excitonic coupling between Chl and carotenoid molecules is described by Walla et al. (Chap. 9) that claims to explain the reduction of Chl fluorescence lifetimes (van Amerongen and van Grondelle 2001; Johnson and Ruban 2009). It has not been shown, however, that these coupling effects would quantitatively explain the observed in vivo quenching, even though they might well contribute to some extent, and thus this question is still open in our view.

VI Compartment Modeling of Fluorescence Kinetics for Distinguishing Between Various Possible Mechanisms

A Choice of the Individual Compartment Models for PS II and PS I

Before entering into a discussion of the need for, and the power of, compartment modeling for testing and distinguishing among various in vivo quenching models, we briefly present here the rationale for the choice of the individual PS II and PS I kinetic compartment models. These models have a very broad experimental basis since they have been tested extensively on isolated intact PS II and PS I particles, using both ultrafast fluorescence methods as well as femtosecond transient-absorption kinetic methods (Holzwarth et al. 2006; Miloslavina et al. 2006; Holzwarth 2008a, b; Szczepaniak et al. 2009). Notably, a common important feature of these compartment models is the fact that they are able to correctly and simultaneously describe both fluorescence (i.e., Chl excited state) kinetics as well as radical pair (i.e., charge separation and stabilization) kinetics. The latter can only be deduced unequivocally from ultrafast transient absorption data and not from fluorescence data alone. However, once the principal characteristics of these kinetic schemes accurately describing PS II kinetics are established, analysis of fluorescence kinetics data alone is fully sufficient to determine all the relevant rate constants in the model. Thus fluorescence kinetics alone provide the basis for a full characterization of the rates and locations of NPQ processes.

Since the underlying studies on the isolated antenna/RC complexes have already been published and discussed in detail (see Holzwarth 2008a, b for reviews), we will limit the following section to a few general remarks and comments on common misunderstandings. It is important to note that the kinetic models used here represent compartment models of *minimal complexity* (i.e., the smallest number of kinetic components) required to accurately describe the important salient features of PS II and PS I kinetics.

B PS II Kinetics

The compartment model for PS II (Fig. 5.2, left) represent the original "exciton-radical pair equilibrium" (ERPE) model developed initially to describe fluorescence as well as transient-absorption kinetics (i.e., including the radical pair kinetics in the reaction center, RC) of PS II core particles (Schatz and Holzwarth 1986; Schatz et al. 1988) and was later extended to reflect the fact that two radical pairs, instead of only one, had to be taken into account (Holzwarth et al. 2006; Miloslavina et al. 2006; Szczepaniak et al. 2009). A key feature of the ERPE model – as implied in the name – is the description of the fact that charge separation at the RC is reversible, which puts radical pairs and excited states into a dynamic equilibrium, in contrast to models representing a purely forward reaction scheme. It is this key feature of the reversibility of the charge separation process that is required to allow kinetic models to accurately describe PS II kinetics. In turn, models neglecting reversibility are incapable of describing both fluorescence kinetics and transient absorption (radical pair) kinetics simultaneously within the same model and set of rate constants, and can thus be considered inadequate descriptions. Snellenburg et al. (2013) have shown that the reversibility of radical pair formation and the equilibrium between excited states and radical pairs applies even at low temperatures in both PS II and PS I. All additional features of particular kinetic models merely represent complexities for the description of energy transfer processes in the antenna at different levels of detail. Within that context, suitable more complex models range from appropriate (i.e., including charge recombination) coarse-grain compartment models (Broess et al. 2008) to complex full quantum-mechanical descriptions (Bennett et al. 2013).

Here, we do not concern ourselves with the question of whether the kinetics of PS II

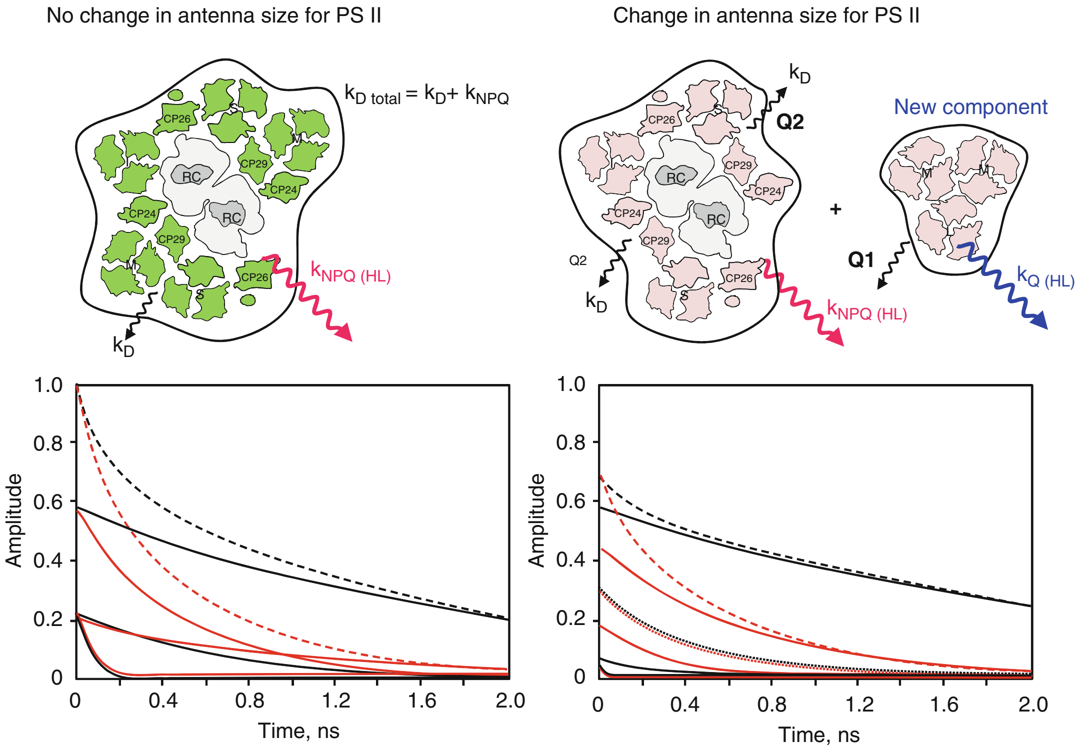


Fig. 5.2. Kinetic compartment models used for target analysis of the kinetics from Photosystem II, PS II (left) and Photosystem I, PS I (right) of higher plants. Internal reaction rates are indicated by arrows. k_{tr} *is the total trapping rate in the system, which contains contributions from energy diffusion through the antenna, transfer to the reaction center (RC), and charge separation to the first radical pair (RP1).* k_{rec} *is the rate constant of recombination of the first radical pair back to the excited state. RP2 denotes the secondary radical pair. In PS II, it has been shown that a secondary radical pair is required, the formation of which is also reversible, and is thus relevant for the description of the kinetics.* $k_{D\ total}$ *in PS II reflects the deactivation rate of the antenna that includes both the normal non-radiative decay of the antenna (*k_D*, typically in the range 0.3–0.4* ns^{-1}) as well as contributions due to NPQ processes (k_{NPQ}). PS I contains additional "red antenna" pools Red1 and Red2, which show distinctly separate kinetics and must be included as separate compartments in the model. The equation at the bottom shows the overall fluorescence kinetics given as a sum of exponentials with amplitude factor $A_i(\lambda)$ and lifetime τ_i. The *blue arrows* connecting PS II and PS I antennas reflect possible spillover forward and backward rates observed in certain systems. S, M and L denote the strongly, moderately and loosely, respectively, coupled LHCII trimers in the PS II supercomplex.

are trap- or diffusion-limited, as has been discussed recently (Miloslavina et al. 2006; Caffarri et al. 2011; Weij-de Wit et al. 2011; Tian et al. 2013). The latter question is merely of interest with regard to theoretical interpretation and understanding of the total trapping rate in the system (k_{tr}) and the rate constant of recombination of the first radical pair back to the excited state (k_{rec}) (Fig. 5.2), but is not relevant for the understanding of the overall dynamics of PS II super-complexes. In particular, this question is also not very relevant for the determination of the effective quenching rates $k_{D\ total}$ (=the deactivation rate of the antenna that includes both the constitutive non-radiative decay of the antenna, k_D, as well as contributions due to regulated NPQ processes, k_{NPQ}) (c.f. Figs. 5.1 and 5.2). It is often erroneously assumed that the ERPE model represents, or has been defined as, a trap-limited kinetic model. On the contrary, the model is fully flexible, accurately describing changes in the rate constants k_{tr} and k_{rec}, in trap-limited, intermediate, or diffusion-limited situations. The original cases to which the model was applied – i.e., small antenna size PS II core particles – indeed represent a trap-limited situation (Holzwarth et al. 2006; Miloslavina et al. 2006; Szczepaniak et al. 2009), but larger antenna PS II particles, or intact in vivo PS II super-complexes, represent an intermediate situation between a pure trap-limited and a pure diffusion-limited situation (Broess et al. 2008; Holzwarth 2008a, b). A discussion of trap- versus diffusion-limited PS II kinetics is presented in Holzwarth (2008a, b). Note that in Fig. 5.2, the rate constant for overall trapping k_{tr} is used instead of the previously used term k_{CS} for charge separation (Holzwarth et al. 2009). This change in the term does not, however, involve a change in the interpretation of this rate, but is fully consistent with the definition of the overall trapping rate as used in Holzwarth (2008a) and Miloslavina et al. (2006). Therefore, k_{tr} involves all contributions from energy migration, transfer-to-the trap, as well as primary charge separation at the RC, thus describing the *effective* rate of trapping by the primary radical pair formation (Miloslavina et al. 2006; Holzwarth 2008a).

We note that the same principal type of kinetic scheme applies to PS II with either open or closed RCs (Miloslavina et al. 2006; Szczepaniak et al. 2009). The difference between the two cases is due to the different sets of rate constants for the kinetics from PS II units with open versus closed RCs (Miloslavina et al. 2006; Szczepaniak et al. 2008a, b).

Consistent with the above discussion, the simplest ERPE model shown in Fig. 5.2 contains three compartments since there are at least three states to be described: The combined excited state of antenna/RC and the two radical pairs (RPs) (Holzwarth et al. 2006; Miloslavina et al. 2006; Szczepaniak et al. 2009). This model results in three-exponential fluorescence decays, where all decay-associated spectra (DAS) possess identical spectral shape and differ only in their amplitudes. These features are the direct consequence of the fact that the model contains only one fluorescent-excited-state compartment. Further, the three DAS differ in spectral shape once spillover processes from PS II to PS I are included in the model (c.f. Fig. 5.2, blue arrows). The three-exponential kinetic description is a consequence of the high level of simplification involved in this model. Nevertheless, despite the fact that all details of internal energy transfer processes in the antenna and from antenna to RC are ignored and included in a single effective-trapping-rate constant k_{tr}, this model describes the experimental fluorescence and transient absorption kinetics and – very importantly – also the time-course of radical pair concentrations extremely well.

Moreover, this model can be easily extended to include additional details of energy transfer processes as has been done in Broess et al. (2008). In the latter case, more kinetic components will be present, reflecting details of the antenna energy-transfer processes. However, the lifetimes of these additional components has been shown to be rather short (Caffarri et al. 2011) – most of them below the time-resolution of typical fluorescence setups used for in vivo measurements. More importantly, however, the amplitudes of these additional lifetime components are predicted to be very small (Broess et al. 2008), and can be further reduced by an appropriate choice of excitation wavelength (λ_{exc}~660 nm), which creates an initial state roughly corresponding to an equilibrated excited antenna. Thus, these energy equilibration components in the antenna can be neglected and the chosen approximation is appropriate for all practical purposes under our conditions. Under in vivo conditions, this simple model provides an excellent description of the experimental kinetics without significant deviations even at very high signal/noise ratios (Miloslavina et al. 2006).

It has often been asked whether PS II models could be simpler than one involving three-exponential kinetics. Our answer is that this is not possible since further simplification would neglect one of the essential three compartments in the model, which would lead to severe deviations in the kinetic description from the experimental data.

C PS I Kinetics

The minimal compartment model for describing PS I kinetics – containing four compartments and describing a four-exponential kinetics – is based on similar arguments as given above for discussing the PS II kinetic models. Again, the most essential feature of the model is the fact that radical pair formation is reversible and that the excited Chl core antenna and the first radical pair consequently are in equilibrium with each other (Müller et al. 2003; Holzwarth et al. 2005; Slavov et al. 2008). This fact leads to the model shown in Fig. 5.2 (right side) that has been extensively tested on intact isolated PS I particles from higher plants as well as from green algae (Müller et al. 2003, 2010; Holzwarth et al. 2005; Slavov et al. 2008). PS I in higher plants contains two special "red" antenna pools (green algae and other photosynthetic microorganisms possess only one red antenna pool) with very different spectral and kinetic properties from the rest of the antenna complex (Wientjes et al. 2011, 2012). Their different kinetics must thus be described by separate compartments. This latter feature, together with the excited state of the main antenna, requires a four-compartment model for higher plant PS I, which represents the *minimal possible* kinetic model for this case. All the four corresponding DAS of the four-exponential kinetics for PS I have different spectral shapes, in contrast to the DAS of PS II (Holzwarth et al. 2009). The simpler PS I antenna of most microorganisms (except for cyanobacteria) can be described by a three-exponential kinetic model.

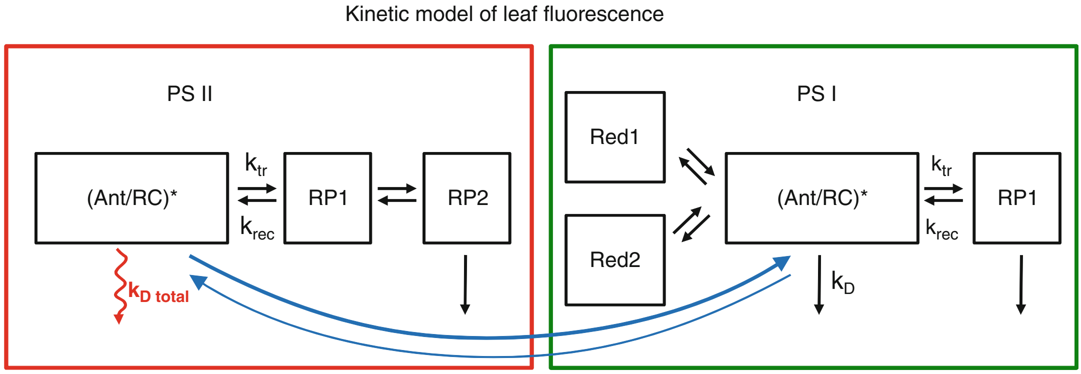
VII The Importance of Target Analysis for Dissecting and Interpreting Intact Leaf Fluorescence and Differentiating Between Quenching Models

The method of ultrafast fluorescence, combined with target analysis of kinetic data, allowed the first dissection of two different quenching sites under well-defined in vivo NPQ conditions (Holzwarth et al. 2009). It is important to discuss here why it had not been possible to identify and separate these two separate quenching sites via conventional (steady state) fluorescence experiments, PAM type NPQ induction measurements (also an **intensity** measuring steady state method) or other methods. It should first be noted that NPQ induction measures one single parameter, i.e., the development of F_m' (a **fluorescence intensity**) as a function of time after switching on the actinic light (Krause and Jahns 2003; Holzwarth et al. 2013). According to systems theory, a measurement that yields only one single parameter can only be used to support a minimal mechanistic or functional model likewise containing only one single parameter. In the case of NPQ, this means that even in an optimal scenario only the quenching behavior at a single site (and a single mechanism) can be deduced unequivocally by such methods. Thus, more complex, multi-parameter models cannot be excluded – but would be consistent with – such a minimalistic model deduced on the basis of measurements allowing determination of only a single parameter. Most importantly however, all **fluorescence intensity**-measuring techniques are principally unable to distinguish between a change of absorption cross-section of a component (in this case, e.g., PS II) and changes in internal dynamics of the system (i.e., energy transfer and quenching rates) since fluorescence intensity is a product of fluorescence amplitude and lifetime (Holzwarth 1995, 1996; van Stokkum et al. 2004; Holzwarth et al. 2013). Such distinction is, however, necessary to distinguish between different possible NPQ models and multiple quenching sites. The decisive advantage of ultrafast time-resolved fluorescence is its ability to assess changes in absorption cross-section of the fluorescing entities, as reflected in **changing amplitudes** of the fluorescence lifetime components, and changes in the internal dynamics of the system, as reflected in **changes of fluorescence lifetimes** (more appropriately the rate constants in the target analysis) (Beauregard et al. 1991;

Holzwarth 1989, 1991, 1996, 2004; van Stokkum et al. 2004). This capability allows for a clear separation of the latter two types of parameters, which is essential for being able to distinguish, e.g., between cross-section changes in a system and a change of the internal rates like, e.g., quenching, energy transfer, spillover, etc. (c.f. Figs. 5.2 and 5.3). Therefore, ultrafast time-resolved fluorescence has a much higher potential to exclude certain complex multi-parameter quenching models and to suggest or support others. Since ultrafast time-resolved fluorescence spectroscopy can resolve, particularly when combined with target analysis, many lifetime components, it is able to distinguish among several alternative complex mechanistic and organizational quenching models.

VIII A Multitude of In Vivo Quenching Situations to be Distinguished

Figure 5.3 shows a selection of the major quenching situations (only involving PS II) that could principally occur in an intact organism with two quenching sites. This – still relatively simple – model assuming only a homogeneous PS II pool and no spillover effects to PS I (Fig. 5.2) still results in six fundamentally different quenching situations



$$F(t,\lambda) = \sum_i A_i(\lambda) \cdot \exp\left(-\frac{t}{\tau_i}\right)$$

Fig. 5.3. Top: (left side) Intact super-complex of PS II and (right side) with partially detached antenna (LHCII aggregate) showing internal non-radiative deactivation k_D and additional antenna quenching rates k_{NPQ}. The sum of these rates gives the total non-radiative deactivation rate of the antenna $k_{D\ total}$. The quenching sites Q1 and Q2 are indicated in the models. The quenching rate of the detached antenna is determined by k_Q. The corresponding simulated kinetics (bottom parts of figure) show the fluorescence kinetics of the different PS II lifetime components in the unquenched dark-adapted state (black solid lines) and the quenched state (red solid lines). The total fluorescence decays (sum of the three PS II lifetime components) for the unquenched and the quenched states are shown as black and red, respectively, dashed lines. The left side illustrates the case of antenna quenching without a decrease in the antenna size of PS II. Quenching caused by an increase in k_D relative to the dark-adapted state causes a decrease in lifetimes only, while the total fluorescence amplitude (value of *dashed lines* at $t=0$) of PS II remains unchanged. The *right side* shows the case of antenna quenching involving a detachment of parts (corresponding to a decrease of 30 % of the absorption cross-section) of the antenna. Total fluorescence amplitude (amplitude of *dashed lines* at $t=0$) is thereby decreased to 70 % in this case. An additional increase in $k_{D\ total}$ in this case also causes a decrease in the overall lifetime, but NO change in total PS II amplitude (*right side*, amplitude of *dashed lines* at $t=0$). The detached antenna part (assumed here to be LHCII) shows its own fluorescence kinetic components. The corresponding lifetime component now carries the amplitude (value of amplitude at $t=0$, *dotted lines*) lost from the PS II fluorescence kinetics due to detachment of part of the antenna. The fluorescence of the detached antenna may or may not be quenched relative to that of an isolated antenna. In the particular case shown, the excited state of the detached antenna part is assumed to be quenched, with a lifetime of approximately 400 ps (*dotted lines*).

that need to be distinguished (i.e., no quenching, quenching on both sites, detachment/no detachment of antenna reflecting changes in absorption cross-section combined with quenching or no quenching in only one or both of the detached and non-detached sites). The chosen method of analyzing quenching thus needs to be capable of clearly distinguishing between these six different cases in an in vivo sample, like an intact leaf or microorganism, as well as simultaneously determining the effective quenching rate constants k_D. In fact, all of these six structurally and mechanistically very different quenching situations can be tested and distinguished by ultrafast time-resolved fluorescence (see Supplementary Information in Holzwarth et al. 2009), while **fluorescence intensity** measuring methods are unable to distinguish a single one of these situations from the others.

Comparison of the kinetic effects of the two different principal quenching schemes without (Fig. 5.3, left top) or with (Fig. 5.3, right top) change in the PS II antenna size are provided in the bottom parts of Fig. 5.3. These curves demonstrate the important distinction between **amplitudes** of lifetime components (as deduced from proper target analysis of the signals) and the **associated lifetimes**. It is important to note that in a fluorescence lifetime measurement the sum of the amplitudes of PS II-related lifetime components, for example, is proportional to total antenna size (i.e., total absorption cross-section) of PS II. This relationship allows determination of whether or not the antenna size of PS II is changed in the NPQ process.

While the situation shown in Fig. 5.3 is still relatively simple, it nevertheless results in six different cases to be distinguished. In an intact photosynthetic system (leaf or microorganism), even more complex scenarios are encountered. For example, PS II can be heterogeneous for some reason (Roelofs and Holzwarth 1990; Roelofs et al. 1992) and/or part of PS II may be involved in a spillover process to PS I (c.f. Fig. 5.2) (Slavov et al. 2013; Holzwarth et al. unpublished results 2014). These additional possibilities greatly increase the overall number of different scenarios that need to be distinguished in a real system. However, even these more complex situations can all be distinguished and analyzed on the basis of ultrafast fluorescence measurements combined with appropriate target analysis methods (Holzwarth 1996; Holzwarth et al. 2009).

Some important underlying conditions must, nevertheless, be fulfilled: The kinetic measurements must cover and resolve the whole relevant emission wavelength range, and the signal/noise ratio of the data must be very high. The required signal-to-noise ratio is best established by numerical simulations. Furthermore, the physiological state of the system must be kept stable over the entire time of the experiment, which is often difficult, considering the fact that effects of the measuring light, and further time-dependent changes or drifts must be controlled and excluded. Again, regular in situ checks are required to ensure that these conditions are fulfilled. In addition, measurements need to be performed under conditions with either fully open or fully closed PS II reaction centers. Any major mixing of the closure of PS II-RCs will lead to additional difficulties in the analysis and interpretation of the data, and may preclude firm conclusions. When all these conditions are fulfilled, analysis and interpretation largely depend on the availability and use of appropriate kinetic target models for description of the kinetics of the individual components. This is the reason why we have investigated in detail the kinetics of very well defined isolated PS II and PS I particles. Mere use of global lifetime data analysis, as has been often the case in the literature, does not result in reliable answers since the number of lifetime components required to describe the fluorescence kinetics of intact organisms, particularly in the case of NPQ induction, state transitions, and use of mutants, is generally much too high to be successfully resolved by global lifetime analysis alone. Global target analysis provides this distinction capability, since the method works much like pattern recognition. The target model for a particular system is a pattern (or filter) used to extract the relevant associated information from a background of a large number of other signals. If applied correctly, ultrafast fluorescence is capable of distinguishing a large number of extremely complex cases. We are not aware of any other method with these capabilities, in particular with the additional ability to be applicable to intact in vivo-samples in a non-intrusive manner.

IX Concluding Remarks

The application of non-invasive methods (ideally to intact leaves of wild-type plants and their specific mutants) is essential to dissect and characterize the different NPQ processes that are active in plants. Slow-kinetic measurements of the steady state Chl fluorescence (such as PAM measurements), commonly used to characterize the different NPQ components and their basic properties, are severely limited in their ability to conclusively distinguish between different quenching sites and mechanisms, and thus different quenching models. The use of time-resolved fluorescence and transient absorption is the most powerful approach to overcome nearly all of these limitations. Based on time-resolved (femto/picosecond range) fluorescence measurements applied to intact leaves of wild-type and mutant *Arabidopsis* plants, activation of at least two different quenching sites has been demonstrated: Quenching site 1 (Q1), a PsbS-dependent and pH-regulated site that is active in detached and aggregated PS II antenna proteins, and Quenching site 2 (Q2), a zeaxanthin-dependent site that is activated in PS II antenna proteins bound to the PS II reaction center. Further measurements have provided strong evidence that an efficient spillover mechanism (i.e., excitation energy transfer from PS II to PS I), enabled by a major thylakoid reorganization related to the loss of stacking, is very likely to be additionally activated in high-light-acclimated plants. While time-resolved fluorescence measurements yield information about different quenching sites, time-resolved transient absorption measurements are required to understand the underlying photo-physical quenching mechanisms. Application of this method to intact leaves has not supported the existence of a Chl-to-lutein excited-state energy-transfer mechanism or a Chl-carotenoid charge transfer, but it has favored the formation of Chl-Chl charge transfer states as the basis of the underlying NPQ mechanism at both the quenching sites **Q1** and **Q2**.

Acknowledgments

The work from our research groups reviewed in this article has been carried out by numerous PhD students, postdocs and technicians. In particular we express our gratitude to Yulia Miloslavina, Manuela Nilkens, Petar Lambrev, Marc Müller, Chavdar Slavov, Malwina Szczepaniak, Michael Reus, and Suman Paul for their relentless efforts to improve our understanding of the excited state dynamics and quenching in intact photosynthetic organisms. Work carried out in our laboratories has over the years been funded by several organizations. We acknowledge the EU Training and Research Network "Harvest" (to A.R.H.), the Deutsche Forschungsgemeinschaft for a joint research grant (HO-924/3-1 to A.R.H. and JA-665/9-1 to P.J.), as well as the Sonderforschungsbereich SFB 663, Heinrich-Heine-Universität Düsseldorf and Max-Planck-Institutes Mülheim a.d. Ruhr, and also the EU Research and Training Network "Intro2", Human Resources and Mobility Activity, contract MRTN-CT-2003-505069 (to A.R.H.). We give special thanks to the Max-Planck-Institute for Chemical Energy Conversion (previously Max-Planck-Institute for Bioinorganic Chemistry) for continuous generous support of this work.

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Chapter 6

How Protein Disorder Controls Non-Photochemical Fluorescence Quenching

Tjaart P.J. Krüger*
Department of Physics and Astronomy, Faculty of Sciences,
VU University Amsterdam, De Boelelaan 1081,
Amsterdam 1081HV, The Netherlands

Department of Physics, Faculty of Natural and Agricultural Sciences,
University of Pretoria, Private bag X20, Hatfield 0028, South Africa

Cristian Ilioaia
Department of Physics and Astronomy, Faculty of Sciences,
VU University Amsterdam, De Boelelaan 1081,
Amsterdam 1081HV, The Netherlands

Commisariat à l'Energie Atomique (CEA), Institut de Biologie
et Technologies de Saclay and CNRS URA 2096,
Gif-sur-Yvette F-91191, France

Peter Horton
Department of Molecular Biology and Biotechnology,
University of Sheffield, Firth Court, Western Bank,
Sheffield S10 2TN, UK

and

Maxime T.A. Alexandre and Rienk van Grondelle*
Department of Physics and Astronomy, Faculty of Sciences,
VU University Amsterdam, De Boelelaan 1081,
Amsterdam 1081HV, The Netherlands

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*Author for Correspondence, e-mail: tjaart.kruger@up.ac.za

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_6,

Summary

We discuss the de-excitation of electronically excited states of chlorophyll *a*, monitored via non-photochemical quenching (NPQ) of chlorophyll fluorescence, with respect to (i) involvement of the main light-harvesting complex of photosystem II (LHCII trimers) and (ii) a change in pigment properties following a change in the conformation of this protein complex. We suggest that LHCII exhibits dynamic behavior arising from a fundamental property of proteins, i.e., their intrinsic disorder. Photosynthetic pigment-protein complexes, such as LHCII, constitute complex environments. The pigments responsible for absorption and subsequent transfer of light energy are subject to multiple interactions in a highly heterogeneous protein environment. This feature gives rise to an intrinsic structural and energetic disorder of the pigment-protein complexes as well as complicated dynamics of excitation-energy transfer within the complexes. In particular, individual complexes show rapid and reversible quenching on timescales of milliseconds to minutes. We propose that plants employ this intrinsic capacity to reversibly switch between unquenched and quenched states to control the de-excitation (i.e., thermal dissipation) of potentially harmful excess excitation energy. Modulation of de-excitation by the local environment of pigment complexes will be demonstrated, with a particular focus on how this modulation manifests itself as chlorophyll fluorescence quenching of individually measured LHCII trimers. It will be shown how the results point to the concept of environmentally controlled disorder as a basis for the energy-dependent component of NPQ, i.e., that the intrinsic capacity of a pigment-protein complex to rapidly switch between light-harvesting and dissipating states can be controlled by the local environment of the complex. This can be explained by assuming that pigment-protein complexes are in an unstable equilibrium between different structural and

corresponding emissive states, where subtle perturbations in the physico-chemical environment can shift the equilibrium to favor one or more of these states. As such, regulation of a disordered conformational nanoswitch provides a satisfying explanation for NPQ.

I Introduction

Proteins are in constant motion around one or more average conformations. This structural flexibility is an important property that enables regulatory switching between active and inactive functional states of proteins. Some proteins, furthermore, possess the ability to switch among several functional states, thereby exhibiting multi-functionality. The peripheral light-harvesting (LH) complexes of plant photosystem (PS) II are examples of such proteins: under light-limiting conditions, they ensure a highly efficient transfer of absorbed solar energy to the reaction center, but when light is in excess, a portion of the absorbed energy is efficiently dissipated as heat (Horton et al. 1996; Niyogi 1999; Horton 2012; Ruban et al. 2012). In the LH state, the complex delivers excitation energy with a high efficiency to a neighboring LH complex, and eventually to the reaction center (RC); in the quenched, photoprotective state, excitation energy is rapidly and efficiently converted into heat. The latter photoprotective state is activated, as a feedback mechanism on PS II, when the rate of light absorption by the photosynthetic organism exceeds the rate of electron/proton transport, such that there is almost complete reduction of the plastoquinone pool, thus increasing the probability of charge recombination and chlorophyll (Chl) triplet formation with increasing light input. When this photoprotective state is entered, excitation energy is dissipated as heat or "quenched", a process readily observed via a resultant decrease in fluorescence (FL) yield, also termed non-photochemical quenching (NPQ) of Chl *a* FL.

A major part of NPQ in unstressed leaves, the rapidly reversible, ΔpH-dependent component of NPQ, takes place in PS II and is commonly referred to as qE (for energy-dependent quenching). This chapter will focus only on the latter component of NPQ by considering the role of disorder in the primary steps of plant photosynthesis, i.e., in the peripheral PS II-antenna complexes of plants. The latter macromolecules are composed of six homologous pigment-protein complexes from the multi-genic *Lhcb* (LH proteins of PS II) family (Green et al. 1991). The first three complexes form hetero-trimers (mixed trimers) of different compositions (Jansson 1999; Caffarri et al. 2004) and are known as the major antennae, often referred to as LHCII, while the other three complexes naturally exist as monomers (Dainese and Bassi 1991) and are known as the minor antennae CP29, CP26, and CP24. The proteins of all six complexes bind the same Chl and carotenoid pigments, but in different ratios.

The Chl concentration of ~0.5–0.6 M in these complexes is extremely high, and if dissolved in an organic solvent, this high Chl concentration would result in almost complete concentration quenching of Chl FL (Beddard and Porter 1976). In natural LH complexes, the resulting distance between nearest-neighbor Chls can be as short as

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Abbreviations: Chl – Chlorophyll; CT – Charge transfer; FL – Fluorescence; K_D – Degree of thermal energy dissipation of a single complex; $K_D = I_U/I_Q - 1$ – Where I_U and I_Q refer to the fluorescence intensity in the unquenched and quenched environments, respectively; L – Lutein; LH – Light harvesting; Lhca – Light-harvesting complex of photosystem I; Lhcb – Light-harvesting complex of photosystem II; LHCII – Major light-harvesting complex II of plants; NPQ – Non-photochemical quenching of chlorophyll *a* fluorescence; PS I – Photosystem I; PS II – Photosystem II; qE – Energy-dependent component of NPQ; RC – Reaction center; S_1 – First excited state; SMS – Single-molecule spectroscopy; ß-DM – n-dodecyl-*β*, D-maltoside; V – Violaxanthin; VAZ – Violaxanthin-antheraxanthin-zeaxanthin; Z – Zeaxanthin

9–10 Å, thus frequently giving rise to strong Chl–Chl interactions. As a result, the antenna is generally characterized by a complicated manifold of excited states, including collective electronic excitations (excitons) with a high degree of delocalization, and more localized excitations on weakly coupled pigments (Van Amerongen et al. 2000; van Grondelle and Novoderezhkin 2006a, b; 2010; see also Ostroumov et al., Chap. 4).

In antenna complexes, each Chl-binding site is characterized by its own specific protein micro-environment, giving rise to a set of "site" energies. Each of these site energies is modulated by slow conformational motion of the protein matrix, resulting in "static" disorder of site energies within a single complex. In conventional bulk spectroscopy, these conformational dynamics are obscured by the average disorder pattern of large ensembles of complexes, contributing to what is termed inhomogeneous broadening. However, by monitoring the spectroscopic properties of individual complexes, much can be learned about the complexes' conformational dynamics (Moerner 2002; Barkai et al. 2004; Krüger et al. 2010).

In this chapter, we assume that it is mainly LHCII trimers that are involved in qE (for other views, see, e.g., Holzwarth and Jahns, Chap. 5; Morosinotto and Bassi, Chap. 14). Most attention will therefore be given to the disorder in LHCII trimers, while some comparison with minor antenna complexes and PS I complexes is also made. We will demonstrate that the transition between LH and energy-dissipating states in LHCII trimers works like a switch between different conformational states naturally available to the complex. This switch, reflecting a disordered behavior on many different timescales, is controlled by the LHCII trimer's natural environment. The complex thus controls its own disordered energy landscape. In addition, combining single-molecule spectroscopy (SMS) with modeling allows differentiation between distinct origins of low-energy (long-wavelength, red-shifted) emission states and the association of some of these states with qE. The role of disorder in the formation of red-shifted states will also be discussed.

Sections "Physical Descriptions of (Excitation) Energy Transfer" and "Protein Dynamics and Functionality" serve to introduce a few basic principles, while the subsequent sections relate these principles to various aspects of qE in which disorder plays an important role. In particular, in section "Physical Descriptions of (Excitation) Energy Transfer", the exciton concept is introduced and different types of disorder are defined. These latter two aspects are then used to briefly describe different physical models for excitation energy transfer in the PS II antenna. Section "Protein Dynamics and Functionality" introduces the important property of proteins being structurally dynamic over many timescales. The concept of the protein conformational-energy landscape is briefly described and used to introduce the general, mechanistic framework of protein multi-functionality as a result of a population shift among different intrinsically accessible conformational states. Since room-temperature SMS provides a unique means to investigate spectroscopic signatures of disorder in LHCII complexes, this novel technique for the study of NPQ is covered in considerable depth.

II Physical Descriptions of (Excitation) Energy Transfer

A Excitons: Collective Electronic Excitations

Photosynthetic LH complexes possess three important physical properties that ensure effective harvesting of light: (i) relatively long intrinsic decay times (lifetimes) of the excited states of their pigments (typically in the order of nanoseconds), (ii) a large cross-section for light absorption, and (iii) a sufficiently high pigment density to give rise to a phenomenon termed "coherence" (Brixner et al. 2005; van Grondelle and Novoderezhkin 2006a, b; Read et al. 2007; Novoderezhkin and van Grondelle 2010; Scholes et al. 2011; see also Ostroumov et al., Chap. 4). The latter property can be understood in the following context. Let the wave function $|n\rangle$ denote the elementary excitation of the n-th pigment (e.g., Chl) in the antenna. Quantum mechanics allows superposition of such wave functions, i.e., $c_1|n_1\rangle + c_2|n_2\rangle + \ldots$, for constants c_1, c_2, …, where one elementary excitation is shared among a number of pigments. Such a collective excitation is known as an "exciton" and differs from the independently excited pigments n_1, n_2,… due to correlations ("coherences") between the participating pigments. Coherences arise when the Coulombic interactions between pigments are strong enough to give rise to sufficiently large values of the cross-terms $c_i{}^*c_j$ (for $i \neq j$), i.e., the product of constants related to two different pigments. The cross-terms reflect couplings that produce new, collective states delocalized over a number of pigments rather than being localized on individual pigments. This singlet excited-state "coherence" dramatically changes the spectrum of the LH pigment-protein complex as well as its energy-transfer dynamics, resulting in more efficient light absorption, faster conversion from short- to long-wavelength spectral bands, and more efficient trapping of excitations by the RC. [The main reason for the increased irreversibility of trapping is that in the excitonic model only a few of the excitonic states (i.e., the low energy states) are quasi-resonant with the excited state of the primary donor (van Grondelle and Novoderezhkin 2006b). Therefore, instead of a factor N appearing in the equilibrium between an antenna complex and RC, where N reflects the number of antenna complexes per RC, a much smaller number appears, which favors the energy to be localized in the RC. For *Rhodobacter sphaeroides* the effect is about a factor of 4].

Recently, long-lived (>1 ps) quantum coherence between electronic states has been observed in a variety of photosynthetic LH complexes (Engel et al. 2007; Schlau-Cohen et al. 2009; Collini et al. 2010; Ishizaki et al. 2010; Panitchayangkoon et al. 2010) and during charge separation in plant PS II RCs Romero E, Augulis R, Novoderezhkin VI, Ferretti M, Thieme J, Zigmantas D, van Grondelle R, unpublished. These long-lived coherences are sustained and may even be generated by non-equilibrium vibrations in resonance with the important excitonic states (Chin et al. 2013) and help in overcoming the rough energy landscape of pigment-proteins during excitation energy transfer and charge separation (Scholes et al. 2011).

B Types of Disorder

In a photosynthetic pigment-protein complex, identical pigments at different sites can have very different transition energies, also known as "site energies". This is mainly the result of interactions among different pigments and interactions between a pigment and its highly charged protein micro-environment, with the latter process including deformation of Chl's porphyrin rings (Zucchelli et al. 2007). The site energies are not constant, but fluctuate over many timescales due to the protein's large structural flexibility (see section "Protein Dynamics and Functionality"). In other words, structural disorder of the protein gives rise to energetic disorder of the pigments. Energetic disorder is commonly separated into "static" and "dynamic" disorder, where the former generally refers to spectroscopic changes on a timescale accessible by the particular experimental approach, while the latter takes place on a significantly faster timescale and thus leads to homogeneous broadening of the spectral line shapes.

Generally, energies of exciton states exhibit shifts from site energies due to splitting of the energy levels related to individual pigments. The extent of these energy shifts is determined by the combination of the coupling strength between interacting pigments and the site energies themselves. For a number of N interacting pigments, the resulting exciton band is given by a set of N discrete exciton transitions. In addition, the dipole strength associated with each of these transitions is redistributed, depending on the geometry of the interacting pigments. [For a comprehensive theoretical treatment of exciton dynamics in the context of photosynthetic LH complexes, see, e.g., van Amerongen et al. (2000) and Valkunas et al. (2013)].

Two additional types of interactions experienced by photosynthetic pigments, both of which contribute to further energetic disorder, are coupling to phonons (the fast, collective nuclear vibrational modes of the protein) and coupling to intra-pigment vibrations. Exciton-phonon coupling induces homogeneous broadening and shifting of absorption and emission spectra due to intra-molecular reorganization effects (i.e., molecular geometrical changes). Because phonons and molecular vibrations form a quasi-continuum of modes, these energies are often referred to as a bath. Phonons and molecular vibrations thus give rise to rapidly changing energies of the coupled pigments, a process generally referred to as "dynamic disorder", as opposed to the slower "static disorder". Coupling between excitons and this bath allows energy transfer between different excited states delocalized over different sets of pigments. This energy transfer is the basic mechanism of photosynthetic light harvesting, producing an ultrafast cascading from higher- to lower-energy states, an effective energy migration in the antenna, and delivery of excitation energy to the RC (Novoderezhkin and van Grondelle 2010).

C Models of Energy Transfer

Pigment interactions are frequently described in one of two limits, i.e., the weak or the strong coupling limit. In the weak-coupling case, interaction among a number of transition dipole moments is much smaller than the difference in the site energies of the corresponding pigments. As a result, coupling to the bath dominates, the excited-state wave functions are mainly localized on individual pigments, and energy transfer between pigments occurs via resonance, which constitutes a relatively slow "hopping" process. The energy dynamics in this picture is described by standard Förster theory (Förster 1965). This approach is often extended to the generalized Förster theory (Novoderezhkin and Razjivin 1994, 1996; Sumi 1999; Damjanović et al. 2000; Scholes and Fleming 2000; Jang et al. 2004), which describes energy transfer among weakly coupled pigment clusters, and allows for the excitonic interactions within each cluster to be arbitrarily strong.

In the strong-coupling limit, transition-dipole interaction between two pigments dominates their site-energy difference and bath interaction, and the excited-state wave functions are delocalized over the interacting pigments. This is generally the case for inter-pigment distances smaller than 2 nm, a property of most photosynthetic LH complexes. Redfield theory (Redfield 1957, 1965; Mukamel 1995) provides a realistic description of the energy dynamics in this limit (Novoderezhkin and van Grondelle 2010). In standard Redfield theory, all exciton couplings are explicitly taken into account, thus allowing a description of all types of exciton relaxation, including coupled dynamics of populations and coherences among exciton states. (Here, the term "exciton relaxation" reflects the description of energy dynamics in terms of the relaxation among exciton states). A modified version of Redfield theory (Meier et al. 1997; Zhang et al. 1998) is often employed to provide more realistic line shapes and relaxation rates by including the effect of multi-phonon processes.

For moderate pigment coupling, both Förster and Redfield theories give rise to artifacts, and a combined Redfield-Förster picture is often employed (Yang et al. 2003; Novoderezhkin and van Grondelle 2010). Here, relaxation dynamics within strongly coupled clusters are calculated with modified Redfield theory, while generalized Förster theory is used to model energy transfer among these clusters.

When two or more pigments are sufficiently close to one other, charge-transfer (CT) states can form. The energy of these CT states is typically close to some of the exciton states, in which case mixing between CT and exciton states occurs. The typically strong coupling of CT states to phonons gives rise to significant spectroscopic changes, such as strong homogeneous line broadening, a large Stokes shift, and pronounced vibrational tails (Renger 2004; Vaitekonis et al. 2005; Novoderezhkin et al. 2007).

III Protein Dynamics and Functionality

A Protein Conformational Energy Landscape

Crystal structures of proteins may give the impression that proteins are static structures. However, these structures are merely models representing averages of a vast ensemble of conformations. Both the polypeptide backbone, and especially the side chains, are, in fact, in constant motion, due to thermal energy and numerous intrinsic and extrinsic interactions. In addition, the close packing of a protein frequently leads to different side chains trying to occupy the same space, a property coined by the protein folding research community as "frustration" (Bryngelson and Wolynes 1987). A theoretical study of protein motion is restrained by the intrinsic complexity of even the simplest proteins. Proteins' complex structures give rise to a bewildering variety of motions. Since the average displacement of protein atoms is generally larger than those found in crystals and glasses but smaller than liquids, protein motions are often called semi-liquid.

A leading conceptual framework for protein motion has emerged from the pioneering experiments of Frauenfelder and co-workers on the well-studied myoglobin protein (Frauenfelder et al. 1979, 1991) and complementary molecular dynamics simulations (see, e.g., Elber and Karplus 1987; van Gunsteren et al. 2006). In this context, the concept of an energy-level scheme is replaced with the abstract idea of a conformational energy landscape, which describes all possible protein conformations and the associated free energy of each conformation (see Fig. 6.1). Such an energy hyper-surface exists in a high-dimensional space, where each degree of freedom of the protein adds another coordinate. The energy landscape consists of hierarchically ordered conformational sub-states (local minima), where sub-states belonging to a particular tier are often nearly isoenergetic and in dynamic equilibrium.

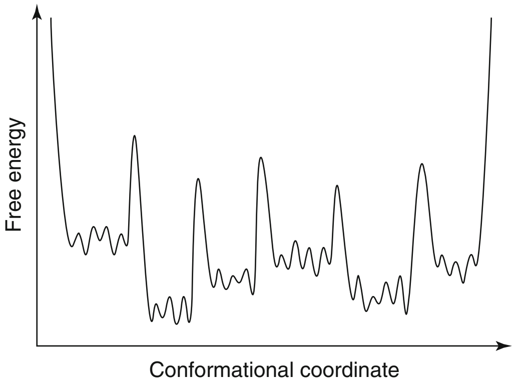

Fig. 6.1 Scheme of a highly simplified protein-conformational energy landscape, projected onto one conformational coordinate, illustrating the hierarchical arrangement of barrier heights that separate local energy minima.

Protein motion can be described as transitions between different conformational sub-states. Structural disorder, therefore, means that, as a function of time, every protein follows a unique structure-energy path on the landscape. The energy landscape itself is also dynamic, resulting from variations in free energy of certain conformational coordinates, due to processes like interaction with ligands and excitation of protein-bound pigments. The height of the free energy barriers separating different sub-states reflects the activation energy required for a protein to leave a particular sub-state and assume a different conformation. If barrier heights are comparable to, or lower than, the Boltzmann energy (kT, where k is the Boltzmann constant and T the temperature), thermally induced structural disorder can cause more than one conformational state to be populated at the same time by different proteins in the population. The populations of sub-states therefore follow statistical thermodynamic distributions, and barrier heights define the timescales over which conformational changes ensue.

B Protein Functionality Based on Conformational Selection

Proteins generally respond to changes in their environment by redistributing the populations of the above-described preexisting sub-states, an idea commonly known as "conformational selection" or "population shift" (Ma et al. 1999; Tsai et al. 1999). Recent spectroscopic studies on numerous proteins, employing mainly SMS, nuclear magnetic resonance, and computer simulations, have demonstrated that functional changes can be explained in the same framework by associating two or more protein sub-states with different functional characteristics (Boehr et al. 2009). The proteins in most of those studies were allosteric proteins, where inter-molecular interactions trigger conformational and associated functional changes. For these allosteric proteins, the "population shift" mechanistic framework is closely related to the Monod-Wyman-Changeux theory of allostery (Monod et al. 1965). Usual descriptions of allostery involve multiple subunits displaying co-operativity in their affinity to bind a ligand. Such co-operativity is positive in the presence of an allosteric activator and negative in the presence of an allosteric inhibitor. This view is in contrast to the classical "induced-fit" model of enzyme molecular recognition (Koshland 1958), a context in which the particular environment created by interactions with bound ligands would induce a new, stable conformation with a different activity.

Examination of the response of qE to thylakoid lumen pH first suggested that LHCII behaved like an allosteric enzyme (Horton et al. 1991, 2000). Positive co-operativity among LHCII subunits is found when qE is "activated" by de-epoxidation of violaxanthin (V) to zeaxanthin (Z) compared to that observed in the presence of V (the inhibitor). The associated inter- and intra-molecular changes in LHCII are thought to be tuned by the local environment of the complex, such as protonation, binding of xanthophylls, and interaction with other proteins in the grana membranes, possibly involving LHCII aggregates and PsbS. This allosteric theory for qE, which was developed on the basis of experiments on isolated thylakoids, was also applied to studies of purified, isolated, solubilized, detergent-bound LHCII complexes, in which spectral heterogeneity was associated with transitions between protein sub-states (Horton et al. 1996).

By considering qE in the framework of conformational population shifts, the dissipative states associated with qE would not be induced by particular environmental conditions, but are intrinsic to the LH complexes under any environmental conditions. In this context, the local environment would only change the probability of LHCII assuming one or more of its possible dissipative states. For example, interaction with binding ligands, such as the PsbS protein or Z, would not *induce* a conformational change but merely lead to a population shift of the pre-existing, intrinsically available conformational sub-states of the complex, hence resulting in stabilization of the latter states. In other words, instead of creating a new, previously inaccessible state, the ligand would allow easier access to the pre-existing state and increase the average time spent in this state. In the same context, according to the classical Monod-Wyman-Changeux formulation, interaction between subunits to form aggregates is an essential part of the stabilization of specific sub-states and determines the degree of subunit co-operativity. Indeed, it is possible that the effective ligands exert their principal effect by promoting or inhibiting subunit interactions, as discussed further below.

IV Spectral Heterogeneity of Bulk LHCII In Vitro

The occurrence of qE in PS II is accompanied by various spectroscopic changes. One such signature for qE is the appearance of low-energy (red-shifted) components in the absorption and FL emission spectra of PS II peripheral antenna complexes (Ruban et al.

1991; Ruban and Horton 1992; Miloslavina et al. 2008; Johnson and Ruban 2009). The low-energy emission and the amount of Chl *a* FL quenching are considerably enhanced when protein aggregates are formed, with this observation being the primary reason why LHCII aggregates were investigated extensively in the early studies of qE (Ruban and Horton 1992; Ruban et al. 1994, 1995, 1996; Vasilev et al. 1997). While the yield of the red emission is very low at room temperature, this yield increases dramatically at lower temperatures (Ruban and Horton 1992; Ruban et al. 1995; Zubik et al. 2013). In the aggregates, the level of quenching correlated with the ratio of emission peaking near 700 nm (F700) versus emission peaking near 680 nm (F680) (Ruban and Horton 1992), and the formation of aggregates was accompanied by site-energy changes of several bound pigments (Chl *a*, Chl *b* and various xanthophylls), as revealed by low-temperature absorption spectroscopy, linear and circular dichroism (Ruban and Horton 1992; Ruban et al. 1997), and resonance Raman studies (Ruban et al. 2007; Ilioaia et al. 2011).

Later, conditions were found where quenching in LHCII trimers could be induced by Z in the absence of protein aggregation (Wentworth et al. 2000, 2003). These isolated, quenched complexes showed very similar spectroscopic properties as the protein aggregates in earlier experiments (Ruban and Horton 1992; Ruban et al. 1995, 1997). More recently, the same spectroscopic features were found in samples where no protein aggregation was possible, i.e., in crystals consisting of weakly interacting LHCII trimers (Pascal et al. 2005) and trimers immobilized in a gel matrix (Ilioaia et al. 2008), indicating that the spectroscopic changes and FL quenching (as an indicator of thermal dissipation) are not caused by aggregation itself but are rather inherent to a single LHCII complex (for a discussion of the role of detergent on the emissive properties of LHCII, see Garab, Chap. 16). Furthermore, these spectroscopic signatures were also found in chloroplasts and leaves after qE induction (Ruban et al. 2007; Bode et al. 2009; Johnson and Ruban 2009; Zubik et al. 2013), which strongly suggests that the changes in pigment configuration are involved in the mechanism of qE in vivo. Indeed, red states of LHCII are found in vivo in both cold- and heat-stressed plants, when NPQ is very high (Gilmore and Ball 2000; Tang et al. 2007). The spectroscopic similarity of all these systems suggests that qE can be investigated to a reasonable extent at the level of a single LH complex.

Different lines of evidence indicated that LHCII trimers can form aggregates in the photosynthetic membrane under NPQ conditions, indicating that early aggregation studies provided important structural and spectroscopic information for qE. For example, large aggregates of LHCII were observed in overwintering evergreen plants (Öquist and Huner 2003), characterized by a FL band peaking typically at 700–715 nm at 77 K (Gilmore et al. 2003). Intact leaves of tobacco plants stressed by CO_2 starvation also displayed LHCII aggregation (Siffel and Vacha 1998). Furthermore, using electron microscopy to study detergent-solubilized grana membranes from light-treated *Arabidopsis* leaves, it was found that NPQ involves dissociation of LHCII-CP29-CP24 heteromers from PS II and a subsequent reorganization of the PS II-LHCII macrostructure (Betterle et al. 2009). A recent freeze-fracture electron-microscopy study provided further evidence for aggregation, showing that the qE-associated trans-membrane pH gradient and V de-epoxidation co-operatively drive LHCII aggregation (Johnson et al. 2011). (For an alternative view on the relationship between aggregation and NPQ, see Garab, Chap. 16, where it is suggested that it is not the presence of large protein aggregates but rather the irregular interactions between adjacent LHCII trimers in small aggregates that play a role in quenching).

The qE-related red emission of antenna protein aggregates consists of a broad band that typically peaks at 700 nm at 77 K. Temperature and FL lifetime studies have indicated that this band can be deconvoluted into at least five components, each of which showed a distinct temperature dependence (Ruban et al. 1995; Vasilev et al. 1997). An additional emission band, with peak wavelength up to 740 nm, was observed from wild-type and mutant *Arabidopsis* leaves after NPQ induction (Miloslavina et al. 2008; Holzwarth et al. 2009; Lambrev et al. 2010), but are likely the result of artifacts caused by reabsorbed FL (Johnson and Ruban 2009). In a recent Stark-FL spectroscopy study on (quenched) LHCII aggregates, two distinct emission sites were also resolved, the associated bands of which peaked at 696 nm and 713–715 nm, respectively (Wahadoszamen et al. 2012). These results indicate that qE is associated with considerable spectral heterogeneity. In sections "Spectral Heterogeneity of Individually Probed LHCII Trimers" and "Physical Model for qE: Controlled Disorder", it will be shown that the presence of disorder adds a time-dependent dimension to this property, thereby relating many of the long-wavelength components to one another.

Although the site of the long-wavelength emission within the PS II antenna and its involvement in qE are still strongly debated, some consensus is emerging about the underlying photo-physical mechanism. The involvement of CT states is the only feasible explanation for such large energy shifts and the strong response to an externally applied electrical field (Novoderezhkin et al. 2007; Wahadoszamen et al. 2012; see also Ostroumov et al., Chap. 4; Walla et al., Chap. 9). Various studies indicate that one or more CT states are accompanied by significant thermal energy dissipation in LHCII trimers. For example, LHCII aggregates exhibiting a high degree of quenching were shown, in a Stark-spectroscopy study, to display two types of CT states (Wahadoszamen et al. 2012). Formation of CT states between two closely separated pigments within the PS II antenna would be followed immediately by mixing between the CT states and one or more of the lowest exciton states (see section "Physical Descriptions of (Excitation) Energy Transfer"). In section "Spectral Heterogeneity of Individually Probed LHCII Trimers", further attention is given to the different possible origins of the red emission as assessed via studies on individual LHCII complexes combined with Redfield modeling. While large red shifts require the presence of CT states, some smaller shifts (and other related spectroscopic changes) can be explained solely by disorder of the pigment site energies.

V Spectral Heterogeneity of Individually Probed LHCII Trimers

A Single-Molecule Spectroscopy (SMS)

Unless time-dependent structural dynamics of protein systems are synchronized, information about their dynamics is averaged out, and thus undetectable, in conventional ensemble experiments (i.e., experiments where the average spectroscopic behavior of large numbers of protein complexes is assessed). A laser pulse can, for example, be used to synchronize the initial state of many molecules. However, the subsequent relaxation process depends on the particular, unique path that a protein follows on its structure-energy landscape. This is particularly relevant when considering the static disorder of proteins, i.e., disorder of site energies and pigment couplings resulting from relatively slow conformational motions of the protein. Ensemble approaches average over a large number of possible values of the static disorder (where each observed value is known as a different "realization"). The associated spectral responses and dynamics are consequently also averaged out. Moreover, in-vitro techniques examining purified complexes in solution inevitably consider all complexes to be identical to the obtained average, whereas, in reality, significant heterogeneity results from each complex's unique micro-environment. A powerful way to circumvent these problems

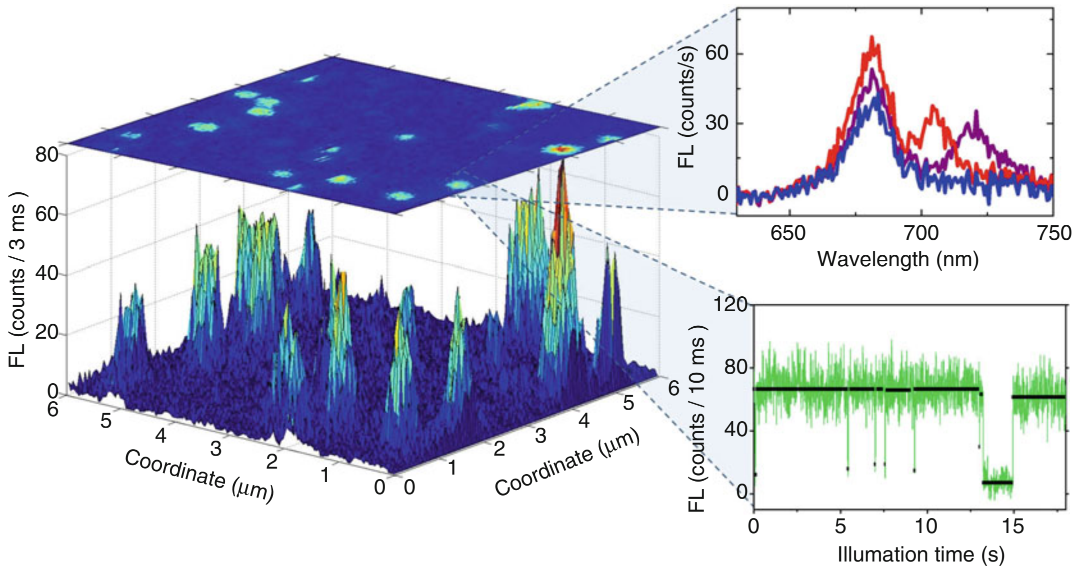

Fig. 6.2 Example of raster-scanned fluorescence (*FL*) map of a few immobilized LHCII trimers from spinach (*left*) and two types of spectroscopic switches from a single complex (*right*). Different spectral shapes (*top right*) were interconvertible and quasi-stable for periods of up to minutes. The rapidly fluctuating wavelength-integrated FL intensities (*bottom right*) signify considerable disorder. Feasible resolutions for spectra and intensity traces were obtained by typically using integration times of 1 s and 10 ms, respectively, and an excitation intensity of ~250 W cm^{-2} (corresponding to a photon flux density of 4.7×10^{7} μE m^{-2} s^{-1}, or 8.1×10^{-12} μE s^{-1} over the absorption cross-section of a single LHCII). All measurements were performed near room temperature and in an oxygen-free, aqueous environment. Raster scanning (left) was performed at 3 ms/px at an excitation intensity of 50 W cm^{-2} and shows a ~4 times higher density of excitation spots than generally used.

is to investigate one complex at a time by employing a technique known as single-molecule spectroscopy (SMS).

In SMS, FL from a single fluorescing quantum unit – one pigment-protein complex in the present context – is probed. This condition is typically fulfilled by, firstly, using a sufficiently low concentration of solubilized complexes, such that the probability to find more than one complex within the laser excitation focal volume at a given instant is very small (Moerner 2002). Secondly, the FL emitted by one complex should be sufficiently larger than the background signal for a reasonable averaging time (Moerner 2002). This latter condition obviously places severe demands on the experimental setup and the photo-physical properties of the sample. Complexes are usually immobilized to enable sufficiently long measuring times, i.e., seconds to tens of minutes per complex. Surface immobilization by means of electrostatic interactions is commonly employed for photosynthetic LH complexes studied near room temperature. When a monolayer of well-separated complexes is obtained, the surface is raster-scanned in the two lateral directions (i.e., perpendicular to the propagation direction of the incident light). The resulting FL intensity map can then be used to locate the complexes (Fig. 6.2).

B Red-Shifted Spectra Explained by Site-Energy Disorder

By employing SMS, it has been shown that various photosynthetic LH complexes exhibit considerable spectral fluctuations (van Oijen et al. 1999; Tietz et al. 2001; Loos et al. 2004; Rutkauskas et al. 2004; Liu et al. 2008; Brecht et al. 2009; Nieder et al. 2009) indicative of substantial underlying protein structural disorder.

The conformational energy-landscape model (see section "Protein Dynamics and Functionality" and Fig. 6.1) provides a feasible context to explain these spectral fluctuations. Perturbations of protein conformation increase the probability with which relatively large energy barriers separating different sub-states are crossed. If these sub-states correspond to distinct emissive energies, a spectral shift is observed. Due to generally strong exciton-phonon coupling in such complexes, the charge displacement resulting from excitation of the complex may cause such a perturbation, in which case the spectral switches are light induced. In addition, thermal energy dissipation may also perturb the protein sufficiently to cause a spectral change, in which case the spectral switches are said to be thermally induced. (For antenna protein reorganizations driven by heat packages generated upon the dissipation of photon energy, see Garab, Chap. 16). Alternatively, single LH2 bacterial complexes of *Rhodopseudomonas acidophila* were observed to exhibit spectral changes spontaneously (i.e., in the dark; Rutkauskas et al. 2006). Considering the interplay between static and dynamic disorder in LH2, it was demonstrated that a large variety of spectral changes were induced by combination of protein structural disorder and disorder of pigment site energies (Novoderezhkin et al. 2006).

Here, some spectroscopic features of LHCII trimers resolved by SMS and relevant to qE will be highlighted. Complexes were measured one at a time and under the conditions utilized survived for typically 1–2 min before being irreversibly photobleached. The relatively short survival times can be attributed to high excitation intensities of typically ~250 W cm^{-2}, corresponding to an excitation rate of ~1 μs^{-1} complex^{-1}, which leads to a significant amount of singlet-triplet annihilation (Krüger et al. 2010). All spectroscopic changes were reversible, except blue-shifts of $\gtrsim$5 nm that were a signature of degradation (Krüger et al. 2010). For a statistically significant data set, which comprises at least several hundreds of individually measured complexes, the spectroscopic behavior was highly reproducible among different sample batches.

In Fig. 6.3, the spectral peak distribution of individually measured LHCII complexes is displayed, indicating that the FL peak position fluctuates around the FL maximum of the bulk spectrum at ~682 nm. Remarkably, a large part of the observed spectral fluctuations and associated spectral line shapes can be reproduced with the same modified Redfield model used to describe bulk spectroscopic data (i.e., data from ensemble experiments; Novoderezhkin et al. 2005, 2011; Krüger et al. 2010). In the latter model, static disorder of pigment site energies was varied. As a result, each spectral shape corresponds to a different realization of static disorder. Figure 6.3 also shows the spectral peak distribution based on the model calculations. Clearly, this pure-exciton model can explain only spectral profiles that peak below ~695 nm. According to the model, these spectra originate from conformationally-induced disorder of the site energies. On the other hand, the model shows that larger red shifts (to above ~695 nm) cannot be similarly explained, but instead require the presence of special protein conformations that lead to formation of CT states. The effects of "static" and "dynamic" disorder on pigment site energies, as distinguished using this experimental approach, are reflected to a large extent by the significantly narrower widths of the peak distributions in Fig. 6.3 compared to the steady-state room-temperature spectrum.

C Red-Shifted Spectra Explained by Charge-Transfer–Exciton Mixing

Information about the origin and possible sites of the long-wavelength emission (beyond ~695 nm) of LHCII can be obtained by comparing single-molecule spectral dynamics of PS II antenna complexes (Lhcb) with those of peripheral antenna complexes of PS I (Lhca). Although protein structure and pigment composition and arrangement of Lhca and Lhcb complexes are comparable, the bulk emission maxima of all Lhcb complexes are typically found at

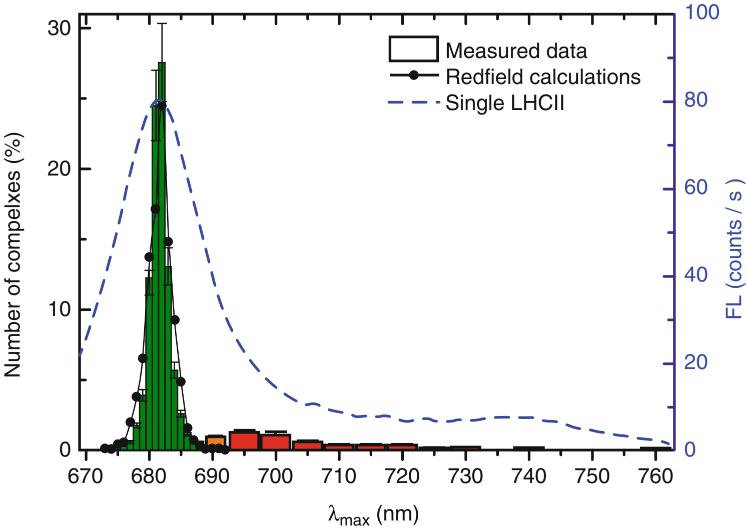

Fig. 6.3 Fluorescence spectral peak ($\lambda_{\max}$) distribution obtained from ~2,000 LHCII trimers, individually measured (*bars*) and calculated (*dots connected by lines*). *Green bars* correspond to peak positions that were within the range of calculated positions, while *red bars* refer to spectra that could not be reproduced by the calculations. The *orange bar* signifies a mixed region. Calculations are based on the modified Redfield model described in Novoderezhkin et al. (2005). A typical 1-min integrated fluorescence (FL) spectrum of a single LHCII trimer is shown for comparison (blue, dashed), where, during the full integration period, the spectral profile showed no noticeable dynamics for successive 1-s time steps.

~682 nm, while Lhca complexes are characterized by long-wavelength emission typically peaking between ~690 and ~730 nm (Schmid et al. 1997; Castelletti et al. 2003). It has been established that the red emission originates from the Chl dimer 603–609 in the L2 site of an Lhca complex (Ihalainen et al. 2003; Morosinotto et al. 2003; Croce et al. 2007; Romero et al. 2009; for nomenclature, see Liu et al. 2004). These two Chls are sufficiently close to each other to create a CT state that strongly mixes with lower exciton states of the complexes (Ihalainen et al. 2003; Morosinotto et al. 2003; Croce et al. 2007; Romero et al. 2009). Long-wavelength emission of Lhcb complexes in various respects resembles emission from Lhca complexes (Miloslavina et al. 2008; Krüger et al. 2011c). Strikingly, while single Lhcb complexes were occasionally found to behave spectroscopically like Lhca complexes, single Lhca complexes occasionally displayed the opposite time-dependent spectral behavior by reversibly switching off their CT character (Krüger et al. 2011c). This behavior was observed on a timescale of seconds to minutes, reflecting the presence of relatively slow protein-conformational dynamics.

Based on the above-described spectral behavior of single Lhca and Lhcb complexes and their structural homology, it was suggested that all of these complexes can be approximated by a single generic protein structure (Krüger et al. 2011c). Furthermore, the generic protein can exist in two conformations, corresponding to typical “blue” (680–685 nm) emission and typical “red” (690–730 nm) emission, respectively (Krüger et al. 2011c). “Blue” and “red” spectroscopic states can consequently be considered to be intrinsic to the disordered energy landscape of this protein. As a result, intrinsic structural disorder of the protein gives rise to switching between these two major states at random times and for random durations. It was, furthermore, proposed that this disorder is controlled by the immediate protein environment by shifting the population distribution between “blue” and “red” conformations, in agreement with the “population shift” model (see section “Protein Dynamics and

Functionality”), explaining why the “blue” conformation is more stable for Lhcb complexes and the “red” more stable for Lhca complexes. These results strongly point to CT-exciton coupling as the mechanism underlying the red-shifted emission from Lhcb and suggest that a major part of the latter emission originates from the same site as that of Lhca complexes, involving the Chl dimer *a*603-*b*609 in the L2 domain. However, it is not excluded that some fraction of the emission originates from the L1 domain, as suggested on the basis of Stark measurements (Wahadoszamen et al. 2012) as well as results from two-photon experiments (see Walla et al., Chap. 9 and Liao et al. 2010).

Figure 6.3 also indicates that the qE-associated low-energy emission probably consists of even more components than resolved by bulk spectroscopic techniques (section “Spectral Heterogeneity of Bulk LHCII In Vitro”). In fact, due to the high environmental sensitivity of CT states (involving charges that interact strongly with their local micro-environment), CT–exciton states have a considerably higher tendency to fluctuate spectrally than red states that do not possess a CT character, as illustrated by SMS (Krüger et al. 2011c), site-directed mutagenesis (Wientjes et al. 2012), and theoretical studies (Renger 2004; Vaitekonis et al. 2005). Energetic differences among the red components can therefore be explained by conformationally induced disorder of CT–exciton mixing, which may give rise to numerous distinct, quasi-stable sub-populations in a large ensemble. The information provided by SMS about low-energy emission of LHCII under qE conditions is described in section “Physical Model for qE: Controlled Disorder”.

VI qE: Regulation of a Conformational Nanoswitch

A Conformational Switching Between Unquenched and Quenched States

The spectroscopic differences between isolated complexes in quenched and unquenched states (see section “Spectral Heterogeneity of Bulk LHCII In Vitro”) all point to conformational changes, and the spectroscopic similarities of isolated, quenched LHCII complexes, LHCII aggregates, and NPQ-induced chloroplasts and leaves furthermore strongly suggest that associated conformational changes in all these systems are similar. The size of this conformational change must be rather small as judging from the free-energy difference between quenched and unquenched states (Wentworth et al. 2003; van Oort et al. 2007; Santabarbara et al. 2009). This is supported by studies based on resonance Raman spectroscopy, a highly sensitive technique, where it was revealed that changes are likely in the Ångström range, as indicated by, e.g., a neoxanthin twist and the formation of at least one new H-bond of Chl *b* in the quenched state (Pascal et al. 2005; Ilioaia et al. 2011). In the context of the conformational-energy landscape model (see section “Protein Dynamics and Functionality”), conformational transition between quenched and unquenched states involves crossing one or more energy barriers, and therefore constitutes a switch between two functional states. A small structural perturbation of the protein would provide free energy to facilitate or enable the switch. Such a perturbation could have various origins, e.g., light-induced (Jennings et al. 1991; Barzda et al. 1999; Zer et al. 1999), thermally induced (Cseh et al. 2005; Gulbinas et al. 2006), protonation of the protein at a low luminal pH, interactions with other proteins in the thylakoid membrane (such as PsbS), or binding of Z.

An explanation for the mechanism of thermal energy dissipation within LHCII trimers was provided based on femtosecond transient-absorption spectroscopy of solubilized aggregates of *Arabidopsis* LHCII trimers (Ruban et al. 2007). This approach identified energy transfer from a low-lying Chl exciton state to a carotenoid S_1 state and subsequent rapid dissipation of the energy as heat upon relaxation of S_1. The carotenoid was identified as lutein (L) in the L1 site of the crystal structure of LHCII (Liu et al. 2004), based on the evidence that the signal forming upon thermal dissipation had the same spectral characteristics as the one that forms upon triplet-state quenching of this particular L. Support for involvement of this L in the quenching mechanism was provided by a study of isolated LHCII trimers from wild-type *Arabidopsis* plants incorporated into a solid-state gel (Ilioaia et al. 2011). Removal of the solute detergent in this construction caused Chl *a* FL quenching in the absence of protein aggregation. By conducting resonance Raman spectroscopy on the complexes, it was found that L in the L1 site undergoes a significant configurational change when LHCII switches to the energy-dissipative state. In addition, investigation of the configuration of the two luteins in the crystal structure of LHCII led to the suggestion that L in the L1 site is in closer contact with Chl *a*612 during NPQ (Yan et al. 2007).

Since in the latter two spectroscopy studies (Ruban et al. 2007; Ilioaia et al. 2011) the isolated LHCII trimers were free of Z, the latter carotenoid could be excluded from the molecular mechanism. This, however, does not detract from the prominent role of Z in NPQ. For example, it was reported that the relationship between the decrease in PS II quantum yield of open RCs and the conversion of the VAZ cycle pool to Z and A is very similar in plants in nature, irrespective of vastly different kinetics of onset and relaxation of NPQ (Adams et al. 1995, 2006; Demmig-Adams et al. 1995; Demmig-Adams and Adams 1996). It is also important to note that the quenching capability of L is strongly affected by Z, likely via positive co-operativity. This view is in line with the allosteric model explained below, which suggests that Z behaves as an allosteric activator of qE. For example, it was found that intact, L-rich shade-grown leaves show only very low levels of NPQ, which match the low levels of Z, when first exposed to very high light levels, until they have formed much additional Z over a time period of hours (Demmig-Adams et al. 1995, 1998; Demmig-Adams 1998). The latter observation indicates that L could not be an effective quencher of LHCII trimers in intact leaves until a high fraction of V is converted into Z (or that Z may be the primary quencher of LHCII trimers in intact systems in whole leaves). On the other hand, evidence consistent with L acting as a quencher in nature has been obtained, although not through constitutive L but rather via the lutein epoxide–lutein cycle that generated relatively low levels of L-associated NPQ compared to the impact of much higher levels of Z-associated NPQ in those leaves (see Esteban and García-Plazaola, Chap. 12).

Two-photon excitation studies on isolated LHCII trimers of *Arabidopsis* plants as well as on wild-type and various mutants of *Arabidopsis* plants provided supporting evidence for energy transfer between a Car S_1 state and Chl Q_y state upon thermal energy dissipation (Bode et al. 2009; Liao et al. 2010, 2012; see also Walla et al., Chap. 9). It was concluded that Z facilitates the transformation of LHCII complexes into the quenched state but is not necessarily involved in the quenching itself (Liao et al. 2010), in agreement with the allosteric model (vide infra). However, Walla et al. (Chap. 9) did not exclude that Z could also act as the quencher, a capability that can be taken over by other carotenoids when Z is absent.

Although various other qE mechanisms have also been proposed, this chapter focuses mainly on the particular L-Chl coupling mechanism described here. The structural change of the L molecule (e.g., a change in planarity) tunes its electronic properties as well as its interaction with neighboring Chls. Similarly, the qE-associated protein-conformational change tunes the electronic properties of the Chls involved in the molecular mechanism. The switch between quenched and unquenched states can therefore be considered in a simplistic representation as a relative energy shift between the L S_1 state and the lowest Chl exciton states, as displayed in Fig. 6.4a. In the LH state, the S_1 energy level is above the lowest Chl exciton energy levels, which would require uphill energy transfer to be populated, a process of low probability. Under conditions of strong thermal energy dissipation, the energy of the L S_1 state would be shifted below that of the lowest Chl exciton states, thereby significantly

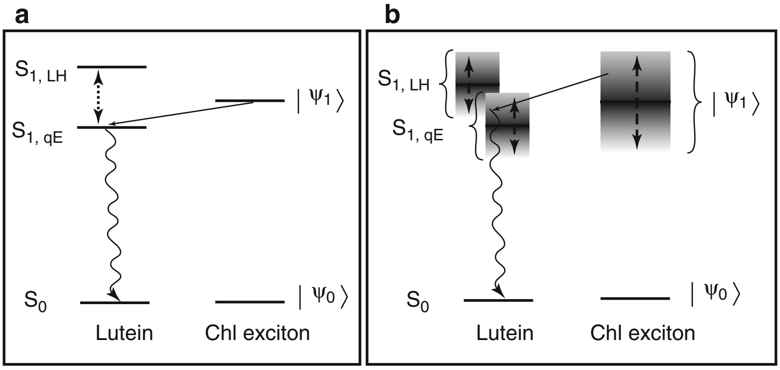

Fig. 6.4 Energy-level model of switching between energy-dissipative and light-harvesting states of an LHCII trimer, involving pigments in the terminal-emitter (L1) domain. (**a**) Relative energy change of the L S_1 state between conditions promoting efficient light harvesting (*LH*) and thermal energy dissipation (*qE*), where the switch is denoted by the *dashed arrow*, and the pathway of thermal energy dissipation depicted by the *solid arrows*. (**b**) Disordered-based switching, where *dashed arrows* signify diffusion of energy levels within shaded regions, the degree of shading qualitatively representing the probability amplitude of the level, and the *solid arrows* indicating one of the possible quenching pathways that can occur both under qE and LH conditions.

increasing the probability of Chl-to-L excitation-energy transfer. The dynamics of the switch can be expected to be determined by protein conformational disorder (see section "Physical Model for qE: Controlled Disorder"), such that the energy levels of the S_1 and exciton states will display fluctuations within an energy band. This concept is depicted in Fig. 6.4b, and suggests that transition from a LH to a qE state is not a spectral switch itself, but rather changes the switching probability between quenched and unquenched states. This idea is further elaborated in section "Physical Model for qE: Controlled Disorder".

Figure 6.4 is somewhat reminiscent of what was called the "molecular gear shift" model, where xanthophyll-cycle carotenoids were considered to be in different energy states because of the number of their carbon-carbon double bonds: V (10 double bonds) was suggested to transfer energy to Chl, and Z (12 double bonds) was regarded as a recipient of transfer from Chl and a quencher (Frank et al. 1994; Owens 1994). In the case of our model for the involvement of L as the NPQ quencher, on the other hand, the shift in energy level is not due to a covalent change but to a more subtle structural fluctuation of the local protein environment.

B Regulating the qE-related Switch: An Allosteric Model

Based on the findings that thermal energy dissipation and the accompanying reversible conformational changes are intrinsic features of each LHCII complex, an allosteric model was proposed to explain the molecular process underlying qE (Horton et al. 2000, 2008; see also Horton, Chap. 3). The latter model is a refinement of the earlier "LHCII aggregation model" proposed almost 10 years before and based on similarities between in-vitro quenching induced by protein aggregation and qE with and without Z (Horton et al. 1991). The two models proposed that in vivo, under different light conditions, the trans-membrane pH gradient and VAZ cycle de-epoxidation state determine the equilibrium between the different emissive states of LHCII, thereby establishing the extent of qE (Horton et al. 2008; Horton 2012). In the latter context, the equilibrium between LH and quenched states is regulated by the complex's local environment, and Z and V behave as classical allosteric effectors of qE. Organization and composition of LHCII in the LHCII-PS II super-complexes in the grana membranes was considered to be a key factor in enabling stabilization of the different states and the regulated transition between them (Horton et al. 2008; Horton 2012). Such an equilibrium between different functional states is consistent with the "conformational selection" model of protein functionality (see section "Protein Dynamics and Functionality"), which predicts that the quenched state can also be visited when the complex is in a LH environment, albeit with a low probability.

Even though major reorganizations in the arrays of LHCII-PS II super-complexes could hypothetically cause additional conformational changes of the LHCII protein, such changes would be restricted by the numerous local and inter-domain interactions that limit the degree of conformational freedom of the protein domains that bind pigments (Barros et al. 2009). Small structural changes would therefore be sufficient to significantly shift the equilibrium between unquenched and quenched states. In fact, the speed of the transition between unquenched and quenched states as observed using SMS (<1 ms) excludes large-scale rearrangements (Krüger et al. 2011a). We therefore propose that major reorganizations in the grana membranes such as reported in Boekema et al. (2000) would only stabilize this equilibrium shift (i.e., increase the average time a complex spends in a quenched state) but would not create new dissipative states.

The ensemble measurements on which the allosteric model is based cannot adequately test the model's hypothesis. In contrast, SMS is a powerful technique to test this hypothesis, by probing individual complexes as they switch between different emitting states, and to investigate how this switching is affected by the local environment. In other words, SMS is able to reveal unique properties of the qE-related structural switch that are generally masked by ensemble averaging in conventional spectroscopic techniques. Single-molecule studies of LHCII trimers have demonstrated that these complexes indeed visit quenched states occasionally when exposed to an environment that mimics in-vivo LH conditions (Krüger et al. 2010, 2011b). Switches to quenched states occur rapidly, at apparently random times, and are completely reversible. This confirms the idea derived from ensemble experiments that switching to quenched states is an intrinsic property of LHCII. Furthermore, the irregular switching dynamics, ranging over at least four orders of magnitude in time, reflect the presence of significant energetic disorder in these complexes. The relevance of this switching behavior to qE is investigated in the next section.

VII Physical Model for qE: Controlled Disorder

In this section, the significance of disorder to thermal dissipation of excitation energy in pigment-protein complexes is demonstrated, focusing mainly on isolated LHCII trimers. The specificity of this process is shown and contrasted with results obtained from isolated PS II minor antenna complexes. The associated spectral dynamics are investigated and compared to bulk studies of qE.

A qE Explained by Environmental Control of Conformational Disorder

The phenomenon of extensively fluctuating FL emission is not unique to LHCII or LH complexes in general, but is a common feature of intrinsically fluorescent quantum units (Kulzer and Orrit 2004) and is commonly referred to as "FL intermittency" or "FL blinking". It is of note that the photophysics involved depends strongly on the type of fluorescing system; for example, FL blinking of single chromophores is explained by formation of radicals or triplet states (Zondervan et al. 2003), while in LHCII, this phenomenon is more likely protein-assisted and related to disorder of the protein backbone (Krüger et al. 2011a, 2012; Chmeliov et al. 2013).

A series of experiments was recently conducted to determine the relevance of FL intermittency to qE (Krüger et al. 2011a, 2012, 2013, 2014; Valkunas et al. 2012;

Chmeliov et al. 2013), i.e., whether the molecular dynamics underlying FL blinking serve a functional role in LHCII trimers. Time-dependent (ms–min) FL changes of individual complexes were investigated under various experimental conditions, where the in-vitro environment to which the complexes were exposed was varied in each experiment. The average emission of many individually measured complexes was compared with that from ensemble spectroscopic experiments, considering that the blinking behavior of the large number of complexes in an ensemble experiment (typically $>10^9$) is averaged to give a certain FL level. It was assumed that this FL level was directly correlated with the average FL quantum yield of the ensemble. In other words, a FL decrease of a large ensemble of complexes indicates a decrease of the average quantum yield and is explained by a larger number of complexes being in a quenched state at any given time.

In the main study on the latter topic (Krüger et al. 2012), the physiological conditions associated with efficient light harvesting and qE were mimicked at the single-molecule level, examining the effect of three protein environmental conditions, i.e., VAZ cycle de-epoxidation state, pH, and detergent concentration. It was demonstrated that each of the three qE-related conditions affected FL blinking such that the average FL intensity decreased and average dwell time in quenched states increased, i.e., as more qE-associated conditions were added, quenched states were, on average, visited for increasingly longer times. Even when some properties, such as the fraction of complexes that were quenched for the whole duration of the experiments, were not taken into account, the observed trends were in accordance with previous ensemble measurements of LHCII quenching concerning, e.g., the large effect of detergent and the weak sensitivity to Z (Horton et al. 1996). It was argued that these findings point to a direct relationship between qE and FL blinking. In addition, it was found that both switching frequency between quenched and unquenched states and average time spent in quenched states increased with the excitation light intensity (Krüger et al. 2011a), indicating that the underlying conformational switches are strongly light- or thermally induced.

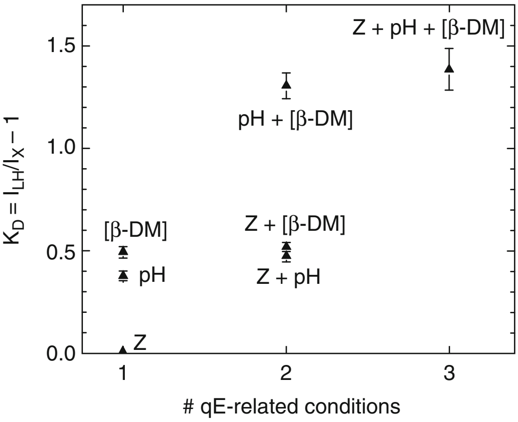

Fig. 6.5 Average amount of fluorescence quenching of single LHCII trimers induced by different combinations of qE-related environmental conditions. The quenching strength (K_D) reflects the change in the time- and population-averaged intensity (*I*) incurred by quenching environment X with respect to light-harvesting conditions (*LH*), where for environment X was used Z-enriched complexes (*Z*), a pH of 5.5 (pH), and/or a considerably low detergent concentration ([ß-DM]).

In a follow-up study (Krüger et al. 2013), the contribution of complexes that were in partially quenched states for the whole duration of the experiment (i.e., 1–2 min per complex) was explicitly taken into account, leading to an even stronger correlation between thermal energy dissipation and qE-related conditions. Figure 6.5 shows the strong increase in the degree of quenching as LHCII trimers in a single-molecule environment were exposed to more qE-related conditions. Furthermore, in the latter study, the same experiments were conducted on minor antenna complexes of PS II to determine whether the observed environmental sensitivity of protein dynamics is specific for LHCII trimers or perhaps artificially induced by the SMS environment. The former outcome was found, i.e., the minor antennae exhibited a distinctly different environmental sensitivity of their FL blinking, and therefore appear to be unable to utilize their intrinsic capability of quenching in the same way as LHCII trimers. This finding lends strong support to the proposed relationship between qE and FL blinking in LHCII trimers and indicates that the minor antennae possess an intrinsically lower degree of dynamicity than LHCII trimers.

The experimental blinking behavior of LHCII trimers was recently reproduced quantitatively by a conformational diffusion model (Valkunas et al. 2012; Chmeliov et al. 2013). Here, the protein structural motion in the terminal emitter domain (i.e., in the locus of L in the L1 site) was described to contain a relatively slow, diffusive component modulated by the local environment. Accordingly, the transition probability between unquenched and quenched states was shown to fluctuate extensively and on a similar timescale as FL blinking, thus supporting the notion that FL blinking is an intrinsic property of LHCII, and is associated with qE according to the mechanism described in section "qE: Regulation of a Conformational Nanoswitch".

These single-molecule studies and modeling results indicate that the intrinsic dynamic disorder of LHCII trimers, reflected by the phenomenon of FL blinking, can be controlled by the local environment of the complexes. On a molecular scale, the environmental control features as a subtle conformational change, shifting the population equilibrium between quenched and unquenched states. Hence, no new dissipative states are invoked; instead, as more qE-related conditions are incorporated, the probability for accessing one or more dissipative states increases, in agreement with the population-shift model of protein functionality (see section "Protein Dynamics and Functionality").

B Spectral Switches: A Four-State Conformational Model

As red-shifted FL states are often associated with qE (see section "Spectral Heterogeneity of Bulk LHCII In Vitro"), it is important to consider the spectral properties of quenched states observed under SMS conditions. In Fig. 6.6, intensity changes are related to the shift in FL peak maximum. For emissions below a peak maximum of 750 nm, there is no relationship between intensity and spectral changes shown, neither in LH- nor in qE-mimicking environments. No, or only small, spectral shifts are related to strongly quenched states, whereas large spectral

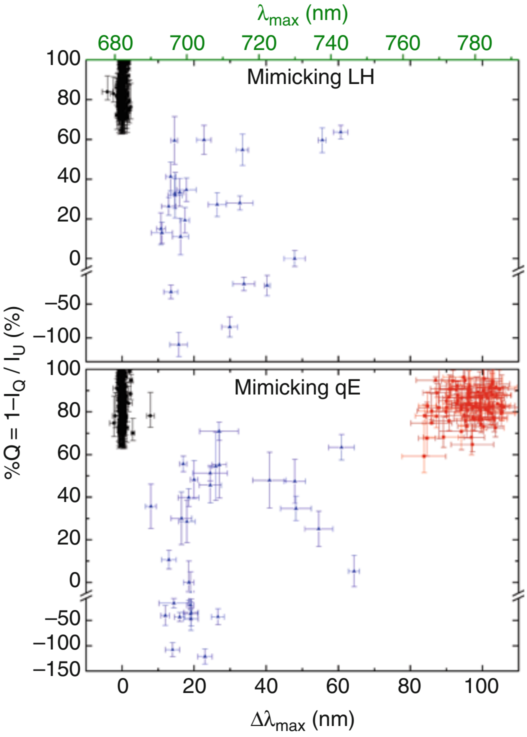

Fig. 6.6 Relationship between the two spectral switches of single LHCII trimers under conditions that mimic efficient light-harvesting (*LH*, *top*) and qE (*bottom*). The percentage of fluorescence quenching (%Q) and associated fluorescence peak shift ($\Delta\lambda_{max}$) of ~400 individually measured LHCII trimers are shown. I_Q and I_U refer to the average intensity before and after the spectral switch, respectively. The parameter %Q thus indicates the relative amount of quenching with respect to I_U, and relates to K_D through $K_D = \%Q/(1-\%Q)$. Approximate peak position, λ_{max}, of quenched states is indicated by green axis. *Black* and *red* data points correspond to spectra characterized by one resolvable spectral band, while *blue* data refer to switches into double-band spectral states, with $\Delta\lambda_{max}$ denoting the wavelength difference between the two peak positions and λ_{max} the peak position of the redder band. For *black* data, only switches corresponding to %Q ≥ 70 % are shown.

shifts, on average, correspond to a small degree of quenching. Lack of correlation between peak wavelength and the extent of quenching in this spectral window implies that the absorbed energy can be quenched to different degrees, unrelated to the different spectral shapes, indicating that this red-shifted emission likely has a different origin than FL blinking. In addition, absence of large spectral changes for the majority of switches into quenched states implies that creation of the quenched state has little impact on the excitonic states of Chls in LHCII and must thus involve an only subtle conformational change. It should be noted that the set of excitonic states depends sensitively on distances and orientations of the transition dipoles of all pigments in LHCII (van Grondelle and Novoderezhkin 2006a and 2010; Novoderezhkin et al. 2011).

Figure 6.6 also indicates that replacing the LH environment with the qE-mimicking environment did not notably affect the spectral peak populations below 750 nm. However, under the latter conditions, an excessively red-shifted band (peaking at ~760–790 nm) accompanied reasonably quenched states for a significant fraction of complexes (~10 %). Such spectra have also been observed under conditions that resemble the LH state, but for a considerably smaller fraction of complexes (Krüger et al. 2012, 2014), indicating that the population equilibrium is shifted toward the far-red states under qE conditions. It is noteworthy that far-red emission states with very similar spectral properties were observed from isolated PS II-core complexes of spinach and *Thermosynechococcus vulcanus* by employing FL line-narrowing on ultrapure samples (Morton et al. 2014). Furthermore, SMS studies of $C_2S_2M_2$ supercomplexes and small pieces of grana preparations revealed the same far-red-shifted band (Michael Gruber, personal communication). The latter observations suggest that, under physiological conditions, this state is populated and is not an artifact due to the SMS micro-environment.

The two distinct spectral signatures that accompany formation of quenched states in single, isolated LHCII trimers (i.e., emission peaking at ~682 nm and ~760–790 nm, respectively) point to at least two distinct mechanisms of energy dissipation in LHCII complexes. The strong population shift to both types of quenched states upon exposure of the complexes to qE-mimicking conditions suggests a common site of origin, which we assume to be the L1 domain (see section "qE: Regulation of a Conformational Nanoswitch" and Krüger et al. 2014).

In a very recent SMS study, it was shown that the average of a considerable number of single-molecule spectra of isolated LHCII trimers gives rise to a small, broad band with a peak maximum at ~700 nm in addition to the prominent ~682-nm emission band (Krüger et al. 2014). The amplitude of the ~700-nm band increased as more qE-related experimental conditions were added, in agreement with the characteristic F700 emission related to qE in ensemble studies. It is, therefore, very likely that the latter emission band is a superposition of many distinct low-energy emissive bands, the differing energies of which result from ample disorder in the complexes.

Different lines of evidence suggest that the major sites responsible for the ~700-nm emission and that of thermal energy dissipation are disconnected (Mullineaux et al. 1993; Vasilev et al. 1997; Krüger et al. 2014). The characteristic red emission that generally accompanies thermal dissipation may be explained by assuming that the conformational change accompanying the switch into a quenched state is not restricted to the L1 locus but also affects the L2 domain, assuming the latter to be the primary site of F700 (see section "Spectral Heterogeneity of Individually Probed LHCII Trimers"). Such an effect would not be surprising in light of the high packing density of LHCII (Barros et al. 2009). It was, for example, demonstrated for a homologous protein (Lhca4 of PS I) that a small structural change, as the result of a

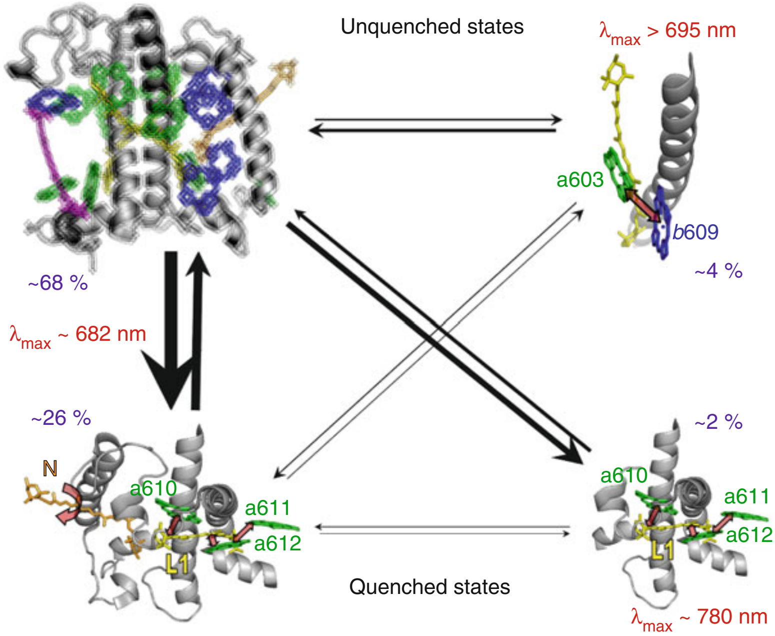

Fig. 6.7 Model illustrating the switching mechanism of LHCII to red-shifted and two types of energy-dissipating states (Krüger et al. 2012). *Top left*: LHCII monomeric structure (Liu et al. 2004) in an unquenched state (the induced uncertainty in the molecular positions indicates the subtle structural difference between the unquenched structure and the resolved quenched structure); lutein (*L*), neoxanthin (*N*), violaxanthin (*V*), Chl *a*, and Chl *b* are depicted in *yellow*, *orange*, *magenta*, *green*, and *blue*, respectively. *Top right*: key pigments involved in the establishment of the unquenched, red-shifted states (Krüger et al. 2010, 2011c), based on measurements from isolated complexes with and without zeaxanthin (*Z*). *Bottom left*: key pigments involved in the spectrally non-shifted, quenched state, based on the model in Ruban et al. (2007) for Z-free, isolated complexes. *Bottom right*: key pigments involved in the spectrally shifted, quenched state, based on measurements from isolated complexes with and without Z. L1 and L2 refer to L in the L1 and L2 sites, respectively, while *a* and *b* denote Chl *a* and Chl *b*, respectively. Straight, red arrows signify strong interactions, curved arrow indicates a configurational twist, and black arrows represent transitions between different states, with the thickness giving a qualitative indication of the frequency of occurrence. Percentages in *purple* denote the average time spent in each state under qE-mimicking conditions, according to Krüger et al. (2014).

point mutation, has a significant effect on the spectroscopic properties of its pigments, in particular when a mixed exciton–CT state is involved (Wientjes et al. 2012).

The scheme in Fig. 6.7 illustrates how LHCII exists in a dynamic equilibrium between a small number of dominant states with different quenched and/or spectral conformations. The most likely sites associated with the various spectroscopic states are indicated. In this model, protein structural disorder is responsible for frequent switching between different quasi-stable states, while the local environment of the complex determines which of these states are favored, meaning that the local environment controls the intrinsic switching capability.

VIII Concluding Remarks

A The Role of Disorder in qE

Proteins exhibit a high level of structural motion, on many different timescales, as the result of their thermal energy and numerous intrinsic and extrinsic interactions. This structural disorder is generally described as transitions between different conformational sub-states of the energy-landscape hypersurface of the protein. The free energy of this landscape also follows time-dependent disorder, due to processes such as interaction with neighboring proteins and excitation of protein-bound pigments, thereby varying the transition probability between different substates. Protein-structural disorder is therefore an intrinsic property of all proteins and has to be modulated and controlled in order for the protein to exhibit functionality and to switch between different functional states.

Disorder is not only central to the high efficiency of light harvesting and energy transfer in photosynthetic pigment-protein antenna complexes, but it is also the basis of the important photoprotective thermal energy dissipation (assessed from Chl FL quenching, such as qE). This was demonstrated in the present chapter by results obtained with single-molecule spectroscopy (SMS), a novel approach to the study of thermal energy dissipation in photosynthetic light-harvesting complexes. By probing the emission of individual complexes in real time, information could be retrieved about the conformational disorder reflected in emission fluctuations.

B Contributions of a Single-Molecule Approach to qE

The unique contributions of SMS to the study of qE, as discussed in this chapter, can be summarized as follows:

- Light-harvesting complexes have the intrinsic capability to switch to various energy-dissipating states on a ms to s timescale. This switching occurs rapidly and randomly, and among numerous distinct intensity levels.
- The molecular mechanisms underlying qE are related to the primary mechanism of FL blinking in LHCII trimers. In other words, the structural disorder, that gives rise to spectral disorder and appears as FL blinking, is used by plants to regulate light harvesting.
- The amount of thermal energy dissipation (i.e., the extent of qE) of a large ensemble of isolated LHCII trimers in solution is environmentally controlled by shifting the population distribution between quenched and unquenched states. This means that the local environment of a single LHCII trimer controls its probability (i.e., frequency) of switching into, or out of, a quenched state. Although experimental evidence of such environmental control is provided in the present chapter at the level of isolated trimers, it is very likely that this physical property of the complexes is exploited in a similar manner in vivo. It is important to note that the concept of environmental control of intrinsic protein structural disorder is related to general protein behavior, as described by the "conformational selection" model of protein functionality.
- This environmental control in relation to qE is specific for LHCII trimers and is finely tuned.
- Intensity and spectral switches are unrelated for the main mechanism of qE, i.e., most red-shifted spectral states are not connected to energy-dissipating states.
- At least two energy-dissipating mechanisms are present in LHCII trimers, each of which is related to a distinct spectral signature and FL peak position at ~682 nm and ~780 nm, respectively.
- Relatively small spectral shifts (peaking within ~15 nm of the ensemble peak) can be explained solely by the disorder of pigment site energies that originates from relatively slow protein conformational changes.

In general, the high selectivity and sensitivity of SMS, as opposed to conventional ensemble-averaging techniques, provides new information in the following aspects (Moerner 2002):

- The probability distribution of an observable parameter (such as the spectral peak position) can be obtained instead of a single mean value. This enables identification of functional sub-populations within a larger population.

- Static and dynamic heterogeneity of observable parameters can be separated on timescales of ms to minutes.
- Time-dependent processes can be elucidated without the difficulty, or often impossibility, to synchronize these processes in many molecules.
- Statistically rare events can be explored, making it possible to detect new phenomena.

C Challenges of a Single-Molecule Approach to qE

As is the case for any experimental technique, SMS is subject to specific limitations. Of high importance in the study of qE is that the employed conditions on the isolated system under SMS conditions should retain the key properties of the whole system in its native (in-vivo) environment. As SMS conditions are generally more demanding than in-vitro environments created in many conventional ensemble techniques (vide infra) and are, in certain respects, more artificial, the properties of the system should be compared with the in-vivo situation in at least two steps. First, the average behavior of thousands of individually measured complexes is compared with that of an ensemble of isolated complexes measured simultaneously (i.e., bulk in-vitro measurements) and this result is, in turn, compared to that from complexes and assemblies in their native environments.

The particular challenges of SMS involve the following:

- A highly purified sample is required to reduce ambiguous spectroscopic results. A high homogeneity is also necessary if the sample has not been well characterized. This means that novel, creative SMS approaches are needed to investigate the role of protein interactions or Z, macro-organization in the thylakoid membrane, etc.
- High-light conditions are required to detect a reasonable amount of emission. This is at the expense of relatively short survival times (1–2 min) of LHCII complexes and a systematic shift of the population equilibrium to quenched states (Krüger et al. 2011a), the latter of which can partially be explained by thermally induced switches to quenched states due to a high excitation rate (~1 μs^{-1} per complex) and a subsequent large amount of singlet-triplet annihilation.
- The isolated sample needs to be extremely diluted (~10 pM) to suppress signal interference (i.e., sources of noise).
- Surface attachment is mostly used to immobilize the complexes, a process that introduces interactions with the surface. It is of note, however, that the FL lifetimes of single, surface-bound LHCII trimers are the same as those of freely diffusing trimers in ensemble experiments, suggesting a weak influence of surface binding (Michael Gruber, personal communication).
- Because only FL is detected, interpretations depend to a large degree on changes in this emission.
- The extent of energy dissipation (in terms of the calculated K_D value) is strongly underestimated in the SMS environments utilized thus far.

The single-molecule results shown in this chapter provided a largely qualitative explanation for the spectroscopic features and related protein dynamics that underlie qE in these isolated systems. Efforts are currently underway to make the environments increasingly more physiological. For example, single-molecule FL lifetime studies on different complex assemblies in the PS II membrane will provide valuable insight into different quenching states of these systems.

Acknowledgments

This work was supported by the EU FP6 Marie Curie Early Stage Training Network via the Advanced Training in Laser Sciences project (T.P.J.K.); EU FP7 Marie Curie Reintegration Grant (ERG 224796) (C.I.); CEA-Eurotalents program (European contract PCOFUND-GA-2008-228664) (C.I.); Project Sunshine, University of Sheffield (P.H.); TOP grant (700.58.305) from the Foundation of Chemical Sciences, part of the Netherlands Organization for Scientific Research (C.I. and R.v.G.); Netherlands Organization for Scientific Research program in Fundamental Research of Matter (The Thylakoid Membrane: A Dynamic Switch; FP126; 12.0344) (M.T.A.A. and R.v.G); Advanced Investigator Grant (267333, PHOTPROT) from the European Research Council (ERC) (C.I., T.P.J.K., and R.v.G.).

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Chapter 7

Context, Quantification, and Measurement Guide for Non-Photochemical Quenching of Chlorophyll Fluorescence

Barry A. Logan*
Biology Department, Bowdoin College,
6500 College Station, Brunswick, ME 04011, USA

Barbara Demmig-Adams and William W. Adams III
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA
e-mail: barbara.demmig-adams@colorado.edu; william.adams@colorado.edu

and

Wolfgang Bilger
Ökophysiologie der Pflanzen, Botanisches Institut der Christian-Albrechts-Universität zu Kiel, Am Botanischen Garten 3-9, Kiel 24118, Germany
e-mail: wbilger@bot.uni-kiel.de

Summary

In this chapter, we (i) place photoprotective thermal dissipation of excess light into the context of the many adjustments plants employ to maximize photosynthesis and growth while minimizing the destructive potential of excess light and (ii) describe the historical development of key measures of thermal energy dissipation and related processes (e.g., common coefficients and other quantification of non-photochemical quenching, or NPQ, of chlorophyll *a* fluorescence and chlorophyll *a* fluorescence transients), emphasizing the theoretical and practical

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*Author for Correspondence, e-mail: blogan@bowdoin.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_7,

advantages and limitations surrounding the use of NPQ as a measure of dissipation of excitation energy from singlet excited state of chlorophyll *a* as "harmless" heat. Furthermore, we provide guidance on the proper measurement of NPQ and advise readers of the methodological issues that, if not avoided, can render measures of this parameter non-interpretable.

I Introduction

We will first place photoprotective dissipation of excess absorbed sunlight into the broader context of the many adjustments plants make to maximize both light utilization for photosynthesis and growth as well as the safe avoidance of potentially destructive excess light. We will then focus on the measurement of thermal energy dissipation via non-photochemical quenching (NPQ) of chlorophyll fluorescence. For a book on photoprotection in plants, in a broader context, see Demmig-Adams et al. (2006). For a historical perspective of this field, see Papageorgiou and Govindjee, Chap. 1, and for background in photophysics, see Ostroumov et al., Chap. 4.

II Thermal Energy Dissipation in Context: Many Means of Adjustment for Optimal Utilization of Sunlight While Avoiding its Hazards

Plants employ an evolutionarily conserved photosynthetic pathway to reduce atmospheric CO_2 using chemical potential energy generated via the absorption of sunlight (for a general description, see Rabinowitch and Govindjee 1969; Blankenship 2002). Components of the photosynthetic pathway are regulated, and acclimate to prevailing conditions, over time scales ranging across many orders of magnitude, so that photosynthetic production of reduced carbon compounds meets demand from the whole plant across the diverse conditions in which plants grow (Barber and Baker 1985; Baker and Long 1986; Baker 1996; Aro and Andersson 2001; Demmig-Adams et al. 2006). The dynamic nature of photosynthesis has been the subject of research for decades.

Oxygenic photosynthesis requires two light reactions and two photosystems: photosystem I (PS I; see Golbeck 2006) and photosystem II (PS II; see Wydrzynski and Satoh 2005). The evolution of the Z-scheme that runs these reactions is described by Govindjee and Björn (2012). Photosynthetic electron transport, and particularly PS II, is sensitive to imbalances between light absorption by chlorophyll (Chl) and the use of light energy in support of photosynthetic carbon fixation and other reductive processes (Demmig-Adams and Adams 2006). Absorption of light in excess of photosynthetic light utilization can potentially cause oxidative modification of PS II and associated proteins via several molecular mechanisms generally involving reactive oxygen

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Abbreviations: Chl – Chlorophyll; F – Fluorescence; F_o, F_o' – Minimal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_m, F_m' – Maximal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_s – Steady-state chlorophyll fluorescence emission during illumination; F_v, F_v' – Variable chlorophyll fluorescence in the dark-adapted (F_m-F_o) and light-adapted ($F_m'-F_o'$) state, respectively; F_v/F_m, F_v'/F_m' – Interpreted to be intrinsic efficiency (or quantum yield) of photosystem II in the dark- and light-adapted state, respectively (equivalent to $\Phi_{PS\ II}$); NPQ – Non-photochemical quenching of chlorophyll fluorescence; OJIP – One-letter names associated with phases of the chlorophyll fluorescence transient curve upon onset of illumination; O is for the initial minimum level, P for peak, and J and I are inflections between the two; PAM – Pulse-amplitude modulated (fluorescence or fluorometer); PS I – Photosystem I; PS II – Photosystem II; Φ_{NPQ} – Quantum yield of the *regulated* portion of thermal dissipation of the singlet excited state of Chl *a*; $\Phi_{PS\ II}$ – Quantum yield (intrinsic efficiency) of photosystem II photochemistry (equivalent to F_v/F_m); qE, qI, qN, qP – Quenching coefficients for energy-dependent (E), photoinhibitory (I), non-photochemical (N), and photochemical (P) quenching of chlorophyll fluorescence, respectively; VAZ cycle – the xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin, and zeaxanthin species (reviewed in Melis 1999; Takahashi and Murata 2008; Kornyeyev et al. 2010). Such modifications can render PS II ineffective at electron transfer, thus lowering PS II efficiency (i.e., decreasing the proportion of light absorbed by PS II antenna Chl used to support photosynthetic electron transport). Lasting decreases in PS II efficiency that are not associated with rapidly reversible NPQ and the trans-thylakoid pH gradient, while being associated with lasting decreases in the light- and CO_2-saturated photosynthetic capacity are commonly referred to as *photoinhibition* (reviewed in Kyle et al. 1987). While the causal relationships between photoinhibition and low plant productivity remain unelucidated (Adams et al. 2013), it is clear that photoinhibition occurs in response to lasting imbalances between the absorption and utilization of light in photosynthesis (see also Adams et al., Chap. 23).

Plants have evolved numerous means of avoiding lasting imbalances between light absorption and utilization (and the associated *excess* light stress and persistent decreases in PS II efficiency). These protective mechanisms are manifest at every scale of the organism hierarchy (Fig. 7.1), and can be clustered into mechanisms *minimizing absorption* of excess light and mechanisms *enhancing utilization* of excitation energy via photochemical and non-photochemical pathways (i.e., pathways that do, or do not, lead to the generation of reduced compounds, respectively).

When compared with shade-acclimated plants of the same species, many plants acclimated to direct sunlight grow more steeply angled leaves and/or branches, with shorter petioles, and spaced more densely along the branch (i.e., shorter internodes) (Mooney et al. 1977; Givnish 1988; Valladares and Pearcy 1998). This suite of acclimatory responses results in canopies with lower light absorption efficiencies (Valladares and Pearcy 1998). For the leaves of all plants, direct full sunlight super-saturates photosynthesis and is therefore in excess. The structural features of full-sunlight acclimated canopy architecture described above may limit the absorption of excess light by the majority of leaves without compromising photosynthetic productivity (Valladares et al. 2000). In fact, these architectural features may, in some instances, serve to enhance whole canopy photosynthesis in natural sunlight, since outer-canopy, steeply-angled leaves may intercept more sunlight in the early morning and late afternoon, when sunlight can be limiting for photosynthesis (McMillen and McClendon 1979; Givnish 1988; Marias 2010). On the other hand, certain very rapidly growing (and rapidly photosynthesizing) annual species tend to orient leaves parallel to the ground in full sunlight, and – like sunflower – may even track the sun to further maximize light absorption (Ehleringer and Forseth 1980; Forseth and Ehleringer 1983; Greer and Thorpe 2009).

Exposure to environmental stress (e.g., extreme temperatures, soil nutrient deficiency, or drought) generally lowers photosynthetic CO_2 assimilation (see also Adams et al., Chap. 23; Demmig-Adams et al., Chap. 24; Morales et al., Chap. 27). Decreased assimilation can increase excess light absorption since the level of excess light is a function of both irradiance *and* photosynthetic light utilization. In response to environmental stress, many plants will acclimate by rendering the adaxial (upper) planes of the leaf more optically opaque through various means, thus lowering the effective irradiance experienced by chlorophyllous cells below. Leaves may secrete a thicker layer of reflective cuticular waxes (Barker et al. 1997) or may develop thicker pubescence (Ehleringer and Björkman 1978). Many plants accumulate red anthocyanins in the vacuoles of epidermal or palisade parenchyma cells that absorb light otherwise absorbed preferentially by Chl *b* (Gould et al. 1995; Hughes et al. 2007; Merzlyak et al. 2008).

Leaf Chl content is also subject to acclimation. In winter, when chilling temperatures curb photosynthesis rates and exacerbate light stress, many broad-leafed

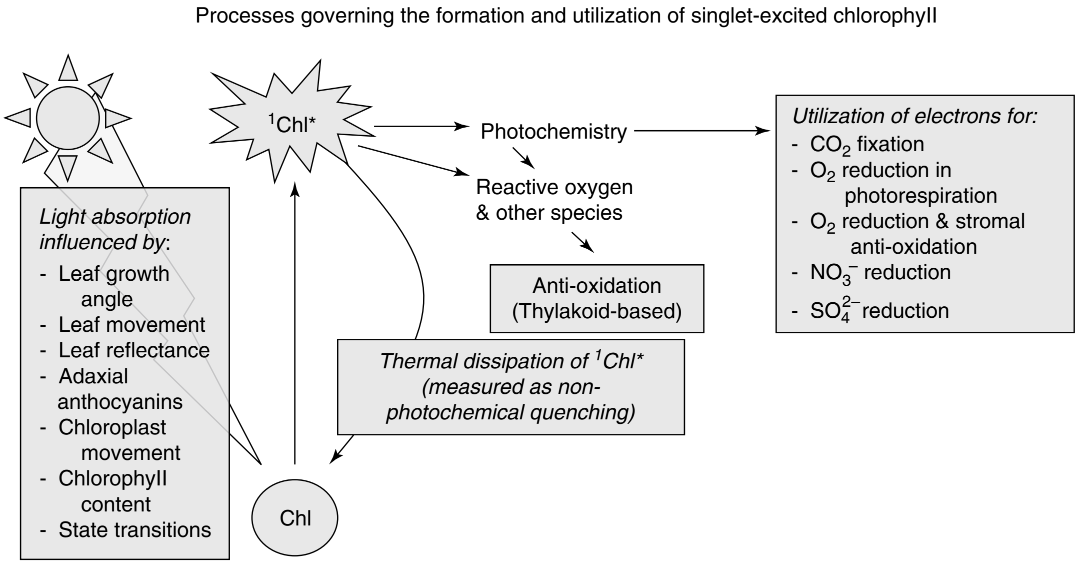

Fig. 7.1 Schematic depiction of light absorption by Chl, processes influencing light absorption by Chl, and processes influencing the utilization and harmless removal of excited-state Chl (^{1}Chl*).

evergreen plants decrease leaf Chl content (Adams and Barker 1998; Logan et al. 1998b). Furthermore, soil nutrient deficiency limits growth, which leads to sugar accumulation in the leaves, feedback inhibition of photosynthetic capacity (see Adams et al., Chap. 23), and profound reductions in leaf Chl content (Verhoeven et al. 1997; Logan et al. 1999; Morales et al., Chap. 27).

A minority of plant species is capable of leaf movements apparently enacted to either stave off light stress or to maximize photosynthetic light use (e.g., in sunflower, as mentioned above). Paraheliotropic movements (orienting the leaf lamina parallel to the sun's rays) minimize excess light absorption during drought or nutrient deficiency (Ludlow and Björkman 1984; Rosa et al. 1991; Kao and Forseth 1991, 1992a, b). Likewise, the movement of *Rhododendron* leaves from a horizontal to a more vertical orientation in response to low temperature is thought to minimize light absorption on colder winter days (Nilsen 2008; Wang et al. 2008, 2009). In contrast, diaheliotropic leaf movements (orienting the leaf lamina perpendicular to the sun's rays) have been observed in plants from xeric environments after transient rainfall, which allows higher stomatal conductance in support of photosynthesis (Ehleringer and Forseth 1980).

The intracellular position of chloroplasts is tightly controlled by the actin cytoskeleton (Takagi 2003) and apparently further balances photosynthetic light utilization align the potential for high light stress. During exposure to low light intensities, chloroplasts along the upper and lower planes of the cell (periclinal cell surfaces) to maximize light absorption, whereas during exposure to intense light, they can be found self-shading each other along the lateral (anticlinal) cell surfaces (Haupt and Scheuerlein 1990; Brugnoli and Björkman 1992; Park et al. 1996; Kasahara et al. 2002; Williams et al. 2003).

At the molecular scale, positions and binding associations of some light-harvesting complex protein subunits are also dynamic. For instance, protein phosphorylation cascades trigger migration of light-harvesting complexes between PS II and PS I to balance light inputs across photosynthetic electron transport under limiting light in a process known as state transitions (see, e.g., Williams and Allen 1987; Allen 1992; Krause and Jahns 2004; and citations therein). In addition, protein phosphorylation has recently been invoked in energy transfer from PS II to PS I, followed by net energy dissipation by P700 under excess light (Tikkanen and Aro 2012; Tikkanen et al. 2012).

Utilization of absorbed light energy in photosynthesis clearly benefits plants by (i) supporting their bioenergetic needs and (ii) acting as a sink for excitation energy, thereby reducing potential excess light stress. Herbaceous annuals and biennials employ this approach via rapid growth and high photosynthesis rates when acclimated to full sunlight (Adams and Demmig-Adams 2004; Adams et al. 1995b; Demmig-Adams et al., Chap. 24). However, even electron transport activity not leading to CO_2 fixation serves to alleviate excess light stress. Molecular oxygen (O_2) can be reduced by iron-sulfur clusters of PS I and/or other photosynthetic electron carriers (Mehler 1951; Asada 1999; Badger et al. 2000). Photoreduction of O_2 via the "Mehler reaction" (Figs. 7.1 and 1.1 in Chap. 1 of this volume) generates superoxide, a reactive and potentially cytotoxic molecule, that is further reduced to water by a series of reactions depending directly or indirectly on photosynthetic electron transport for reducing equivalents (Asada 1999). In this series of reactions, water is both the source of reducing equivalents (when oxidized by the Oxygen Evolving Complex) and the product of the final reduction-oxidation reactions, which is why this pathway has been termed the "water-water cycle" (Asada 1999). This cycle is futile insofar as it generates no reduced carbon compounds; since it potentially consumes excess absorbed light energy, it has also been considered photoprotective (Rizhsky et al. 2003). Safe functioning of the water-water cycle depends upon adequate activities for constituent antioxidant enzymes (e.g., superoxide dismutase, ascorbate peroxidase) and enzymes involved in the reduction of electron donors (e.g., glutathione reductase). Plants acclimated to high light environments can possess several-fold higher activities for such enzymes, indicating that the light energy sink represented by the water-water cycle is greater in environments with greater excess light absorption (Grace and Logan 1996; Logan et al. 1998a).

Furthermore, the oxygenation of ribulose bisphosphate, as the initiating step in the process of photorespiration, is also followed by the consumption of electrons from the photosynthetic electron transport chain. Such "wasteful" consumption of energized electrons can therefore contribute to the photoprotection of chloroplasts and leaves (Kozaki and Takeba 1996; Wingler et al. 2000). In fact, utilization of photosynthetic electron transport for the reduction of any protein, metabolite, or compound (e.g., thioredoxin, reduction of nitrate to ammonia, reduction of sulfate to sulfide) has the potential to contribute to the protection of photosynthesis and photosynthetic cells.

Regulated removal of excitation energy via its conversion to heat, harmlessly radiated to the environment as thermal energy, is a ubiquitous photoprotective mechanism (reviewed in Demmig-Adams and Adams 2006; Demmig-Adams et al. 2012; Jahns and Holzwarth 2012; Ruban et al. 2012; see also Adams and Demmig-Adams, Chap. 2, Adams et al., Chap. 23 and Demmig-Adams et al., Chap. 24). Thermal energy dissipation, as this process is commonly known, has been observed in diverse algal taxa and every Chl-containing member of the plant kingdom thus far examined.

Thermal energy dissipation appears to be the manifestation of multiple molecular/biophysical mechanisms, many of which involve xanthophylls (i.e., oxygenated carotenoids) with long conjugated carbon backbones. For a discussion of the photochemistry of carotenoids, see Frank et al. (1999). The current view is that xanthophylls facilitate direct de-excitation of singlet-excited Chl *a* (for detailed discussions on the molecular mechanisms involved, see Polívka and Frank, Chap. 8 and Walla et al., Chap. 9) and/or facilitate a conversion of light-harvesting complexes to a dissipating state (see, e.g., Horton, Chap. 3). Over time-scales of seconds to seasons, plants modulate levels of thermal energy dissipation in response to prevailing conditions (Adams et al. 1995a;

for a recent review, see Demmig-Adams et al. 2012), apparently to optimize both photoprotection and the productive photochemical use of absorbed light.

While initial inquiry into the features of thermal energy dissipation was made via other approaches, the advent of pulse-amplitude-modulated analysis of Chl fluorescence emission (described below; Schreiber et al. 1986) has been central to the development of knowledge of photosynthetic dynamics and photoprotection. With this technical advance came the ability to quantify the fate of absorbed photons (including those utilized in photosynthesis and those safely converted to heat via thermal energy dissipation) non-invasively and rapidly in situ (Bilger et al. 1995). As with the early development of most methodologies, the use of Chl fluorescence and the chosen parameters calculated on the basis of Chl fluorescence emission have evolved with the continuing development of improved avenues to exploit the phenomenon and the instruments designed to capture it (for an historical review on fluorescence and instrumentation, see Kalaji et al. 2012). The study of Chl fluorescence emission has grown into a sub-discipline unto itself within photosynthesis research (see Papageorgiou and Govindjee 2004). While important advances have resulted, the literature has unfortunately become unwelcoming to the non-specialist, thus limiting the relevance and utility of a powerful tool to the broader community of plant physiologists, ecologists, horticulturalists, silviculturalists, and plant breeders. In the following, we describe the historical development of parameters designed to capture aspects of thermal energy dissipation, with special attention to NPQ of Chl fluorescence as an estimate of the de-excitation of singlet excited Chl *a* via thermal energy dissipation. We also describe commonly encountered issues that can interfere with the proper collection of informative and interpretable Chl fluorescence signals. Lastly, we recommend experimental approaches that maximize the usefulness of the study of Chl fluorescence emission.

III Methods of Quantifying Thermal Energy Dissipation

Prior to development of the pulse-amplitude modulation approach, Chl fluorescence emission could only provide interpretable information if the leaves, algae, or other photosynthetic structures under study were pre-darkened before measurement (referred to in the literature as dark acclimation or dark adaptation). Upon abrupt transfer from darkness to illumination, Chl fluorescence emission increases and then quickly decreases to a steady-state level (Walker 1981). The fluorescence transient upon dark-to-light transfer, and the intermediate levels through which fluorescence quickly progresses over the first few seconds, was discovered by Hans Kautsky and later named the "Kautsky effect" (Govindjee 1995; Lichtenthaler 1992). Various features of the transient can be deconvoluted, yielding information about photochemical and non-photochemical processes. A principal disadvantage of this technique, as it was originally conceived, is its inability to capture dynamic processes of photosynthetic structures continuously exposed to ambient illumination.

The resolution of the rapid kinetics of fluorescence induction during exposure to saturating illumination has enabled a further analysis of the function of PS II (Schreiber and Neubauer 1987; Strasser et al. 2004). The resulting OJIP method (so termed for the one-letter names associated with phases of the transient curve) has brought examination of fluorescence transients back into use (see, e.g., Stirbet and Govindjee 2012; Schreiber et al. 2012; and literature cited therein).

Custom-built fluorometers employing tunable amplifiers to detect periodically modulated weak signals on the background of strong continuous signals became available in the mid-sixties of the last century. With the introduction of the pulse-amplitude-modulated (PAM) technique of measuring Chl fluorescence emission, combined with the saturation pulse analysis, commercial fluorescence instruments became more widely available for the study of photosynthesis both in the laboratory and the field (Schreiber et al. 1986; Schreiber 2004). PAM fluorescence analysis employs a weak monochromatic measuring beam that is switched on and off over microsecond time scales. That is, its amplitude (intensity) is modulated in a rapidly repetitive, pulsed manner – hence the name *pulse amplitude modulation.*

PAM fluorometers report to the user only fluorescence emission exhibiting modulation with the same frequency as the measuring light. Thus, fluorescence resulting from the absorption of ambient light, whose intensity can exceed modulated fluorescence by more than six orders of magnitude, is not reported. This enables the user to make meaningful comparisons of fluorescence emission between tissues exposed to darkness and those exposed to ambient illumination. The measuring light source is non-actinic, i.e., it must be set to such a low intensity that it does not alter the reduction state of PS II reaction centers, and therefore does not introduce an artifact when used to assess the status of PS II.

Saturation pulses (that differ from, and are given in addition to, pulse-amplitude modulation of the measuring light) are routinely employed to reveal various aspects of photosynthetic physiology (Bradbury and Baker 1981). Saturation pulses are user-generated exposures of the tissues under study to intense visible light, typically for between 0.7 and 1.0 s (saturation intensities generally exceed 3,000 μmol photons m^{-2} s^{-1}, or about 1.5 times direct full sunlight). Saturation pulses are intended to be of sufficient intensity to simultaneously reduce all PS II reaction centers (although Loriaux et al. 2013 report that "multiphase flashes" allow better estimation of the fully reduced state under some measurement conditions). Because the intensity of the saturation pulse is many orders of magnitude greater than the intensity of the measuring light, saturation pulses reduce to nearly zero the probability that a photon generated by the measuring light could be utilized in support of photochemistry. During the saturation pulse, there is thus no photochemical use of the measuring light. However, even saturating pulses will not eliminate non-photochemical processes serving as alternative routes for de-excitation of singlet-excited Chl (in leaves exposed to ambient light or in leaves exhibiting sustained non-photochemical quenching in darkness). Indeed, this sensitivity to other processes lies at the heart of the usefulness of the measurement of PAM Chl fluorescence emission. Pulse amplitude modulated fluorescence measured during the saturation pulse is referred to as maximal fluorescence, or F_m (Fig. 7.2). Comparison of F_m with initial minimum fluorescence measured in darkness (after PS II centers are oxidized), F_o, yields the range across which fluorescence can vary (variable fluorescence F_v, equal to $F_m - F_o$), which can be used to calculate the intrinsic efficiency (F_v/F_m) with which light absorbed by PS II and its antennae can be used in support of photochemistry (for a review of the fundamentals of Chl fluorescence, see Govindjee 2004).

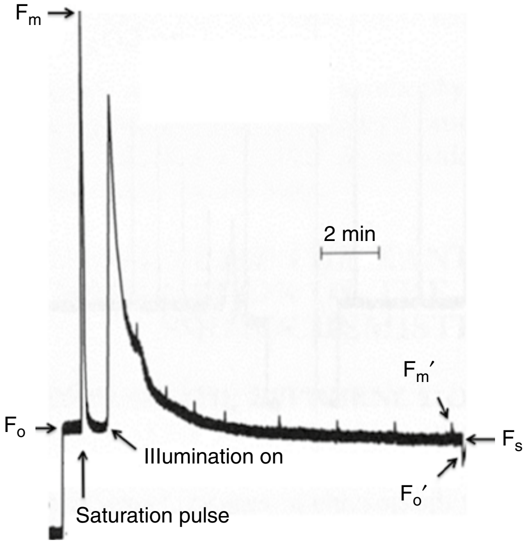

Fig. 7.2 A pulse-amplitude-modulated fluorescence trace of a dark-acclimated *Vinca major* leaf grown outdoors in full direct sunlight and exposed to illumination equivalent to full sunlight. *Arrows* indicate various fluorescence values (note that only the eighth determination of F_m' is labeled).

The first approach using PAM fluorometry to measure non-photochemical processes

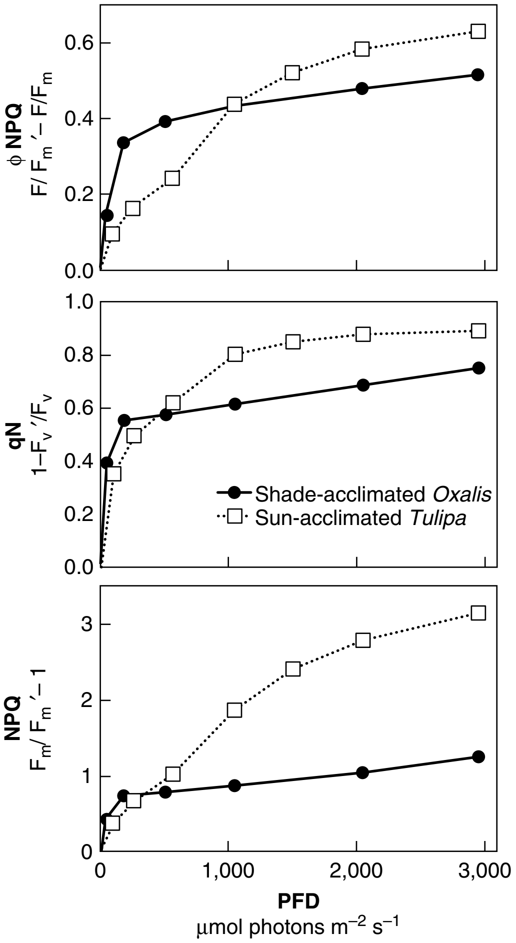

Fig. 7.3 Light response curves of the quantum yield of regulated thermal energy dissipation (ΦNPQ; *upper panel*), the non-photochemical quenching coefficient (qN; *middle panel*), and non-photochemical quenching (NPQ; *lower panel*) calculated from fluorescence data collected from shade-acclimated *Oxalis sp.* (*closed circles*) and sun-acclimated *Tulipa sp.* (*open squares*). Data were collected from previously dark-acclimated detached leaves exposed to a range of light intensities under an atmosphere of 5 % CO_2 in a temperature-controlled chamber.

was development of the parameter qN by Schreiber et al. (1986). The q is in reference to quenching, a term commonly used to refer to a decrease in the intensity of fluorescence emission. This coefficient was initially termed qE because its primary mechanism was known to depend on a proton gradient across the photosynthetic (thylakoid) membrane (termed membrane energization). Later, the use of qE was distinguished from qI, a term assigned to slowly-reversible photoinhibitory fluorescence quenching (Krause and Weis 1991). However, it should be noted that qE and qI both show the same relationship with zeaxanthin levels and decreases in F_v/F_m (see Demmig-Adams et al., Chap. 24). Schreiber et al. (1986) developed the equation for qN ($qN = 1 – F_v'/F_v$, where $F_v' = F_m' − F_o'$, and F_m' is maximal fluorescence collected during a saturation pulse applied during ambient illumination and F_o' is minimal fluorescence collected in darkness immediately following exposure to ambient illumination) in analogy to the equation for photochemical fluorescence quenching, qP. The parameter qN is a dimensionless quantity and varies between values of zero and 1.0. As such, qN exhibits poor sensitivity to changes in non-photochemical processes when they are highly engaged and exhibit a large magnitude (see Fig. 7.3). In addition, qN requires determination of F_o', which can be difficult to measure in some experimental settings (Baker and Oxborough 2004).

In a subsequent approach (Bilger and Björkman 1990), non-photochemical fluorescence quenching was calculated in a manner analogous to the Stern-Volmer principle, which relates the extent of fluorescence quenching to the concentration of a quenching molecule, Q (in a solution of the fluorescing molecule and the quencher):

$$\left(F - F'\right) / F' = F / F' - 1 = k \times [Q],$$

where F and F′ are unquenched and quenched fluorescence, respectively, and k is a proportionality constant. Although the physical conditions in a solution and a highly structured pigment protein complex are not the same, the application of the latter approach to saturation pulse analysis of Chl fluorescence resulted in a highly useful alternative parameter to quantify non-photochemical quenching:

$$NPQ = \left(F_m - F_m'\right) / F_m'$$

NPQ can be understood from a conceptual standpoint when one considers that *photochemical* processes cannot be involved in differences between F_m and F_m', since photochemical utilization of the measuring light cannot occur during exposure to the saturating pulse. Therefore, any differences between F_m and F_m' must be interpreted as due to *non-photochemical* processes. The magnitude of non-photochemical processes is reflected in the degree to which F_m' is lower than F_m. The parameter NPQ offers advantages when compared with qN; it does not require simultaneous measurement of F_o' (while there still is concurrent quenching of F_m' and F_o', the latter does not have to be measured to quantify NPQ), it is not constrained arithmetically at its upper ranges to values below 1.0, and, as such, has been shown to correlate linearly with light-induced increases in the rate constant of thermal dissipation K_D (Kitajima and Butler 1975).

Shade-acclimated leaves typically do not generate NPQ values exceeding 2.0 (Fig. 7.3), whereas sun-acclimated leaves of evergreens can display values above 4.0 (see Adams and Demmig-Adams, Chap. 2). This shade/sun difference in the capacity for NPQ is consistent with the effects of growth light environment on the conversion state of the VAZ cycle (see Demmig-Adams et al., Chap. 24). Induction of NPQ in a dark-acclimated leaf after transfer to light typically exhibits a biphasic response to irradiance, with a first phase of modest non-photochemical quenching (possibly representing state transitions) saturating at relatively low irradiance and a pronounced second phase under higher light intensities. For state transitions, see Krause and Jahns (2004) and Papageorgiou and Govindjee (2011).

Another approach used to quantify non-photochemical Chl fluorescence quenching considers the fraction of absorbed light energy lost as heat (thermal dissipation or non-radiative decay). This was first addressed by Demmig-Adams et al. (1996) and was further elaborated upon by Kramer et al. (2004) and Hendrickson et al. (2004). This approach assumes that quantum yields (proportions of photons absorbed by PS II-Chl dedicated to a given process) for each process consuming the excited state of Chl must sum to 1.0. The two predominant processes are (i) photochemistry (whose quantum yield for PS II is $\Phi_{PS\,II}$) and (ii) regulated thermal energy dissipation (Φ_{NPQ}; Fig. 7.3). In addition, the modest non-radiative, presumably thermal, dissipation occurring in all leaves, even under optimal conditions after dark-acclimation, is considered constitutive thermal dissipation, to which any energy lost by fluorescence emission is added (Kramer et al. 2004; Hendrickson et al. 2004). Different equations used by the latter authors vary only slightly, with that of Hendrickson et al. (2004) being the simplest for Φ_{NPQ}:

$$\Phi_{NPQ} = F_s / F_m' - F_s / F_m$$

where F_s represents steady-state fluorescence emission during illumination. This parameter is convenient to use since its calculation does not require determination of F_o'. F_s/F_m represents the *constitutive* non-radiative thermal energy dissipation, *total* thermal dissipation is calculated as F_s/F_m' (which equals 1- $\Phi_{PS\,II}$), and Φ_{NPQ} thus represents the *regulated* portion of thermal dissipation. Thermal dissipation can, in principle, be directly determined using photoacoustic spectroscopy or micro-calorimetry, but these techniques require extensive equipment and cannot be easily applied to leaves under natural conditions. Moreover, because the photoacoustic changes can arise from multiple sources that have nothing to do with thermal energy dissipation (Malkin and Canaani 1994), some authors have concluded that there is no relationship between zeaxanthin and energy dissipation when utilizing this approach (Havaux et al. 1991; Havaux and Tardy 1997). However, when applied and interpreted properly, good correlations between zeaxanthin and heat emission have been obtained (Buschmann and Kocsányi 1989; Eyletters and Lannoye 1992).

In an unstressed, pre-darkened, sun-grown leaf (i.e., not experiencing a stress-induced, lasting depression in F_v/F_m), $\Phi_{PS\ II}$ (estimated from F_v/F_m) is ~0.83, while regulated thermal energy dissipation Φ_{NPQ} is, by definition, zero. With increasing irradiance, $\Phi_{PS\ II}$ typically declines and both regulated, harmless thermal energy dissipation and non-regulated, non-radiative decay (largely reflecting de-excitation of singlet Chl via triplet conversion and the formation of potentially destructive singlet oxygen) increases. The relationship between Φ_{NPQ} and decay via the triplet route depends on exposure conditions (e.g., irradiance and the presence of other stresses), plant genetics, and growth light environment.

IV Proper Measurement and Interpretation of NPQ

Generating fluorescence signals is relatively easy. Generating interpretable signals, and interpreting those signals properly, is more challenging. The need for methodological guidance grows as the tools for Chl fluorescence analysis have become more widely available (Logan et al. 2007). Below, we describe common pitfalls associated with the measurement of NPQ and offer advice on the design of studies employing this parameter.

Calculation of NPQ assumes unquenched F_m values in dark-adapted leaves. Use of already quenched F_m values in the calculation of NPQ will lead to an underestimate of NPQ. Such underestimation will occur if (i) the period of dark adaptation before measurement of F_m is not long enough, (ii) overly frequent saturating pulses are applied, or (iii) there is pre-existing, lasting quenching of F_m. F_m quenching contributing to nocturnally-sustained depressions in F_v/F_m is commonly observed in plants experiencing environmental stresses such as low temperatures during the winter (see Adams et al. 1995a, 2004, 2006; Öquist and Huner 2003; Demmig-Adams and Adams 2006). Under such circumstances, even pre-dawn measurements of F_m are unlikely to provide unquenched values, in which case NPQ cannot be correctly calculated.

There are additional factors that can compromise calculation of NPQ. Inability to hold the fiberoptic probe at uniform distance and angle from the leaf (or any other sample under study) for the measurement of both F_m and F_m' results in inaccuracies in the calculation of NPQ, and so will failure to collect F_m and F_m' fluorescence data from precisely the same region of the sample. Biological processes occurring between the measurement of F_m and F_m' can also introduce measurement artifacts. Both state transitions (Krause and Jahns 2004) and chloroplast movements (Brugnoli and Björkman 1992) driven by exposure to light can reduce the effective absorption cross-section of PS II antenna Chl, decreasing PS II fluorescence emission during the determination of F_m'.

Use of insufficient, non-saturating light pulses for the measurement of F_m and F_m' renders calculations involving their use meaningless and uninterpretable. Non-saturating pulses can result from the use of inappropriate instrument settings, positioning of the instrument fiberoptic probe too far from the sample, or failure to account for the absorptive effects of leaf surface features (e.g., trichomes, cuticular waxes, etc.) or epidermal pigments such as anthocyanins. This latter consideration is especially important to keep in mind when using fluorometers with pulse-modulated measurement light sources whose wavelength falls outside the red region of the visible spectrum (Cessna et al. 2010).

Many fluorometers report weak, non-zero background signals. The influence of saturating pulses and measuring light intensity on the strength of background signals varies by instrument type (B.A. Logan and K.S. Gould unpublished). The possible effect of background signals on calculations of NPQ will thus vary from instrument to instrument, may be affected by the experimental protocol (e.g., alteration of measurement light intensity between samples), and will be greatest when fluorescence signals are weak.

Chl associated with PS I also emits fluorescence, albeit weaker than the emission from PS II-Chl and with a different wavelength distribution (see, e.g., Itoh and Sugiura 2004). The design of many commercial fluorometers allows for the collection of data across a fluorescence emission wavelength band that captures some PS I fluorescence. Under many experimental circumstances, the effect of this PS I contamination on calculations of NPQ is minimal. However, the artifact represented by PS I fluorescence can be exacerbated if wavelength bands of the measuring light and fluorescence detector are not sufficiently selective for PS II (Franck et al. 2002). PS I contamination can also be problematic in the study of plants with unusual PS II/PS I stoichiometries, such as those exhibiting C4 photosynthesis that have a higher level of PS I centers relative to PS II centers compared to C3 plants (Pfündel 1998) and deep shade-acclimated plants, whose PS II/PS I ratio can be closer to unity than is observed in plants acclimated to higher irradiances (Chow and Anderson 1987; Adams et al. 1990; Adams and Demmig-Adams 2004).

V Concluding Remarks: Avoiding Pitfalls when Measuring Fluorescence

Many contemporary fluorometers are computer-operated and automatically calculate fluorescence parameters, such as NPQ, $\Phi_{\mathrm{PS\,II}}$, etc. We urge users to begin each set of measurements on a biological system by carefully examining raw fluorescence traces by hand, to insure that fluorescence signals and instrument-derived parameters were collected at the appropriate times and under the appropriate environmental conditions. Since automated instruments deliver values for parameters irrespective of the quality of the raw fluorescence data, there is no substitute for the informed judgment of the user to insure quality of data.

As stated above, care must be taken to properly measure and interpret Chl fluorescence emission if meaningful insights are to be gained. Here we summarize our advice on how best to avoid common pitfalls when assessing thermal energy dissipation as NPQ. Pre-existing engagement of thermal energy dissipation in dark-acclimated samples generally leads to decreases in F_v/F_m. Verifying that F_v/F_m values are at/near maximum (just slightly above 0.8 for a sun-acclimated leaf and slightly below 0.8 for a shade-acclimated leaf) thus ensures that dark-adapted F_m values used in the calculation of NPQ are not quenched. Since thermal energy dissipation leads to a decrease in PS II quantum efficiency measured during illumination F_v'/F_m', collecting parallel measurements of NPQ and F_v'/F_m' under illumination offers stronger supporting evidence. One may resort to measurements of F_v'/F_m' alone, when experimental constraints or already quenched dark-adapted F_m values make it impossible to reliably measure NPQ (Adams et al. 1995a, 2006, 2013; see Adams et al., Chap. 23). Concomitant measurements of carotenoid composition, thylakoid protein levels, and photosynthesis rates (O_2 evolution or CO_2 uptake) can furthermore strengthen the interpretive utility of NPQ measurements.

Acknowledgments

Work presented in this chapter was supported by the University of Colorado. We thank Dr. Christopher Cohu and Mr. Jared Stewart for assistance with the generation of the figures and George Papageorgiou and Govindjee for constructive comments on this chapter.

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Chapter 8

Spectroscopic Investigation of Carotenoids Involved in Non-Photochemical Fluorescence Quenching

Tomáš Polívka*
Institute of Physics and Biophysics, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 37011, Czech Republic

and

Harry A. Frank
Department of Chemistry, University of Connecticut, 55 North Eagleville Road, Storrs, CT 06269-3060, USA

Summary

This chapter will review the recent results in the field of carotenoid photophysics and relate excited-state properties of carotenoids to their potential roles in the dissipation of the singlet-excited state of chlorophyll *a* resulting in non-photochemical quenching (NPQ) of chlorophyll fluorescence. Investigations into the structure and dynamics of excited states of carotenoids have revealed potential mechanisms regarding the involvement of spectroscopically

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*Author for Correspondence, e-mail: tpolivka@jcu.cz

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_8,

forbidden "dark" excited states in carotenoid relaxation pathways. The generally accepted model for deactivation of excited states of carotenoids following photo-excitation invokes three states, the ground state S_0, the first excited state S_1, considered "dark" because the $S_0 \rightarrow S_1$ transition is forbidden by symmetry, and the second excited state S_2, where the $S_0 \rightarrow S_2$ transition is strongly allowed. In addition to these states, a number of other excited states have been proposed to be involved in carotenoid de-excitation on the basis of theoretical computations and ultrafast spectroscopy. Yet, the properties of these additional states, and even their very existence, are still being questioned. We will describe the current state of knowledge regarding energetics and dynamics of carotenoid excited states and molecular factors that control these properties, which include the π-electron conjugated chain length, various attached functional groups, configuration and conformation of the molecules, and, perhaps most importantly, carotenoid interaction with the local environment. Many of these factors tune the excited-state spectra and dynamics of carotenoids, and there is accumulating evidence that they are crucial for carotenoid function in photosynthetic systems.

I Introduction

Carotenoids are inexorably linked to non-photochemical quenching (NPQ) of chlorophyll fluorescence as accompanying, and a measure of, thermal dissipation of excess excitation energy. It has long been proposed that enzymatic conversion of the carotenoid violaxanthin (V) to zeaxanthin (Z; see Fig. 8.1a for structures) via the intermediate antheraxanthin (A) in the xanthophyll (VAZ) cycle is one of the key processes triggering NPQ (Demmig et al. 1987). This observation gave rise to a number of studies aiming to elucidate spectroscopic properties of these two carotenoids, with the ultimate goal to explain the mechanism of thermal dissipation of excited states of chlorophyll and NPQ at the molecular level, i.e., in terms of interactions between carotenoids and chlorophyll a (Chl *a*). Yet, even after years of research, the precise molecular mechanism by which excess excited states of Chl *a* are harmlessly dissipated as heat, ultimately giving rise to NPQ, remains unknown. A few promising schemes have suggested a direct involvement of carotenoids in the process of quenching. These mechanisms propose quenching via energy transfer from Chl *a* to a carotenoid (Frank et al. 1994; Ruban et al. 2007), electron transfer from a carotenoid to Chl *a* (Holt et al. 2005), or excitonic interaction between carotenoids and Chl *a* (Bode et al. 2009), and are described in other chapters (see, e.g., Walla et al., Chap. 9; Ostroumov et al., Chap. 4; Van Amerongen, Chap. 15). Therefore, they will not be described here. While there is not much discussion about whether or not zeaxanthin has a role in NPQ, the key question is whether zeaxanthin is the direct quencher or is a regulator

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Abbreviations: A – Antheraxanthin; A_g, B_u, C_{2h} – Classifications of molecular symmetry; Chl – Chlorophyll; Chl *a* – Chlorophyll *a*; Chl *b* – Chlorophyll *b*; CP24, CP26, CP29 – Core light-harvesting proteins of photosystem II; D_0, D_1, D_2 – Doublet states; ESA – Excited-state absorption; GSB – Ground state bleaching; hECN – Hydroxyechinenone; HOMO – Highest occupied molecular orbital; IR – Infrared; LH – Light harvesting complex of purple bacteria; LHCII – Major light-harvesting complex of plants and algae; LUMO – Lowest unoccupied molecular orbital; *n* – Refractive index of a solvent; N – Conjugation length; N_{eff} – Effective conjugation length; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OCP – Orange carotenoid protein; Q_y – The lowest energy state of Chl *a*; $R(n)$ – Solvent polarizability; S_0, S_1 and S_2 – Singlet states; SE – Stimulated emission; V – Violaxanthin; Z – Zeaxanthin; ε – Molar extinction coefficient

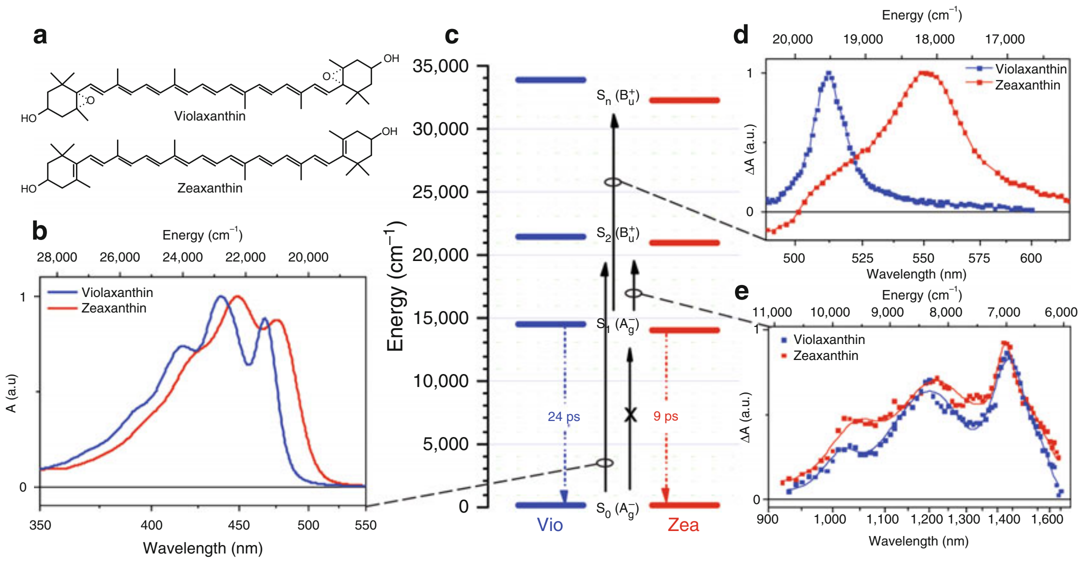


Fig. 8.1 (**a**) molecular structures of violaxanthin and zeaxanthin. (**b**) Normalized absorption spectra of violaxanthin and zeaxanthin in methanol. (**c**) Scheme of energy levels of violaxanthin (*blue*) and zexanthin (*red*). *Solid arrows* represent absorptive transitions between states, *dashed arrows* denote the S_1-S_0 internal conversion.(**d**) Normalized transient absorption spectra in the visible region showing the $S_1 \to S_n$ transitions of violaxanthin (*blue*) and zeaxanthin (*red*). (**e**) transient absorption spectra in the near-IR region showing the $S_1 \to S_2$ transitions of violaxanthin (*blue*) and zeaxanthin (*red*). All transient absorption spectra were recorded in methanol and measured at 3 ps after excitation at 480 nm (violaxanthin) and 490 nm (zeaxanthin).

that switches the system into a quenching state. Thus, schemes of thermal dissipation of Chl *a* excited states in plants have been proposed with lutein (see Esteban and Garcia-Plazaola, Chap. 12) as a quencher in the major light-harvesting complex of photosystem II (LHCII), or with an indirect modulation of LHCII quenching by zeaxanthin (see, e.g., Horton, Chap. 3; Krüger et al., Chap. 6; Van Amerongen, Chap. 15). For certain algal groups, the carotenoid diatoxanthin, as the product of another de-epoxidation/epoxidation cycle, has been proposed as the quencher in NPQ (Büchel, Chap. 11; Lavaud and Goss, Chap. 20). It should be noted that further alternate schemes suggesting involvement of zeaxanthin as a regulator promoting formation of quenching states formed solely by Chl-Chl interaction were also proposed (Müller et al. 2010) as well as schemes where NPQ is zeaxanthin-dependent in antennae functionally connected to PS II, but not in those detached from PS II (see Holzwarth and Jahns, Chap. 5).

To understand whether or not carotenoids are directly involved in thermal dissipation resulting in NPQ, it is important to elucidate their spectroscopic properties, including their excited state energies and dynamics, because these are the features that pertain to carotenoid interaction with chlorophyll excited states, and ultimately control the thermal deactivation of excess Chl *a* excitation. However, investigating the spectroscopic properties of carotenoids is complicated. It has been known for more than 40 years that a transition to the lowest excited state of carotenoids, contrary to that of many other pigments including chlorophyll (Chl), is forbidden for a one-photon transition from the ground state (Schulten and Karplus 1972). The fact that this transition is forbidden is a consequence of the polyene-like molecular structure of carotenoids, and hampers detection of their lowest excited state via the standard spectroscopic techniques of absorption and fluorescence spectroscopy.

The spectroscopic properties of carotenoids are determined in large part by their linear chain of conjugated C = C bonds, the number of which is denoted as the conjugation length, N. Carotenoid excited states are typically classified according to symmetry labels derived from the carotenoids' parent polyene analogues. Because these molecules belong to the C_{2h} symmetry point group, their excited states are classified as having either B_u or A_g symmetry. The latter states are further sub-divided according to the so-called Pariser alternancy symmetry as B_u^-, B_u^+, A_g^- and A_g^+ states (Tavan and Schulten 1987). States with "+" Pariser label are often referred to as ionic states while those with "–" label are termed covalent states (Hudson et al. 1982). According to basic selection rules of quantum mechanics, a one-photon transition between two states having the same symmetry label has no transition dipole moment and is therefore forbidden (Hudson et al. 1982). For carotenoids, the ground state is always of A_g^- symmetry. Thus, the only allowed transition is to a state with B_u^+ symmetry. However, because of electron correlation effects in the linear conjugated polyene chain, the $A_g^- \rightarrow B_u^+$ transition, that corresponds to a simple one-electron transition from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO), is not the energy transition with the lowest energy. Instead, interactions between electronic configurations push the energy of a state with A_g^- symmetry below the B_u^+ state, thus making the state with A_g^- symmetry the lowest excited state. Consequently, the lowest energy transition is that between two states of A_g^- symmetry, which is forbidden by the selection rules of quantum mechanics. This scenario applies to all polyenes and carotenoids having N > 3, which includes all naturally occurring carotenoids (Polívka and Sundström 2004).

The photophysics of carotenoids are thus often explained using a scheme of excited states depicted in Fig. 8.1c. The basic three-level scheme consists of the ground state, S_0 ($1A_g^-$), the lowest energy forbidden excited state S_1 ($2A_g^-$) and the allowed S_2 ($1B_u^+$) state. The $1B_u^+$ state is the one responsible for the strong absorption of carotenoids in the 400–550 nm spectral region that provides the characteristic visible coloration of carotenoids. A key factor determining the excited-state properties is the conjugation length, N, and this is illustrated by a plot of the energies of the S_1 and S_2 states of the two carotenoids, violaxanthin and zeaxanthin, associated with the VAZ cycle (Fig. 8.1c). While violaxanthin has a conjugation length N = 9, the conjugation in zeaxanthin is extended into the terminal ring structures, resulting in N = 11. In the following sections, we will describe the spectroscopic properties of carotenoids with a particular focus on violaxanthin and zeaxanthin, the VAZ cycle carotenoids that have been the subject of numerous investigations.

II NPQ Carotenoids in Solution

In order to reveal principal spectroscopic properties of those carotenoids that are critical for NPQ-related actions in photosynthetic organisms, it is important to study these properties for the molecules dissolved in organic solvents. Although these studies cannot disclose critical direct effects of local environment or structural distortions often present for carotenoids bound in a specific site in a protein, such studies provide basic information on how environmental factors of polarity and polarizability affect the energetic and dynamic behavior of carotenoid excited states.

A Absorption Spectra

The absorption spectra of violaxanthin and zeaxanthin dissolved in methanol are shown in Fig. 8.1b. The spectra consist of a single absorption band corresponding to the S_0-S_2 transition (Fig. 8.1c), whose position depends on the conjugation length. The transition is strongly allowed, resulting in extinction coefficients, ε, on the order of 10^5 M^{-1} cm^{-1} at the absorption maximum. For violaxanthin and zeaxanthin in methanol, the reported values are $\varepsilon = 153{,}000$ M^{-1} cm^{-1} and $\varepsilon = 144{,}300$ M^{-1} cm^{-1}, respectively (Britton 1995). The energy of the S_0-S_2 transition, which is readily obtained from an absorption spectrum, is unrelated to thermal dissipation because the carotenoid S_2 state is far above the energy of the Q_y band of Chl *a*. However, the absorption spectrum is easily measurable and provides basic information about the spectroscopic properties of a particular carotenoid. In fact, for most carotenoids knowledge of the S_0-S_2 energy may serve as a rough estimate of the S_1 energy, because the S_1-S_2 energy gap is not very dependent on the conjugation length (Christensen et al. 2013).

The absorption spectra of nearly all carotenoids exhibit a characteristic three-peak structure corresponding to the lowest three vibrational levels (0-0, 0–1 and 0–2) of the S_2 state. The resolution of these vibrational peaks in the absorption spectrum is an important spectroscopic measure, as it indicates certain structural properties of a carotenoid molecule. For carotenoids such as violaxanthin, the vibrational bands are better resolved than for zeaxanthin, whose conjugation is extended into the terminal rings that possess two C = C bonds twisted into an s-cis configuration. The loss of vibrational resolution in this configuration is attributed to the repulsion between methyl groups located on the terminal ring and hydrogen atoms on the conjugated backbone (Christensen and Kohler 1973). This situation leads to a broader distribution of torsional angles between planes of the terminal ring and the main conjugation, resulting in conformational disorder, which causes the observed reduction of vibrational structure of the $S_0 \rightarrow S_2$ transition. For carotenoids without conformational twisting, even higher vibrational levels of the S_2 state can be resolved, as is shown for violaxanthin (Fig. 8.1b) for which even the 0–4 vibrational transition can be identified as a shoulder at ~390 nm in the absorption spectrum. The resolution of the vibrational bands is sometimes quantified by the ratio of the 0–0 to 0–1 band amplitudes measured from a horizontal line drawn through the absorbance minimum between the two peaks. This ratio is denoted as III/II, and is 0.67 and 0.27, respectively, for violaxanthin and zeaxanthin (Britton 1995).

The energy gap between vibrational peaks in the absorption spectrum is approximately 1,350 cm^{-1}. This gap results from combination of two symmetric vibrational modes of the conjugated backbone with energies of ~1,150 cm^{-1} (C–C stretch) and ~1,600 cm^{-1} (C = C stretch). At low temperatures, the vibrational resolution increases further, and the 0–1 band is often split to reveal the individual C-C and C = C mode features (Cong et al. 2008), which allows an estimation of the frequencies of the C-C and C = C stretches in the S_2 state. The precise values of these stretching frequencies in the S_2 state are not known, but low-temperature absorption spectra confirm that vibrational frequencies do not differ much in the S_0 and S_2 states. Ground-state vibrational frequencies have been obtained at high precision from resonance Raman spectroscopy because carotenoids are very efficient at Raman scattering (Rimai et al. 1973; Koyama et al. 1988; Robert 1999). The ground-state vibrational frequencies in solution determined by this technique are 1,160 cm^{-1} for the C-C stretch and 1,525 cm^{-1} and 1,520 cm^{-1} for the C = C stretch of violaxanthin and zeaxanthin, respectively (Gruszecki et al. 2009; Wang et al. 2012). It is also observed that C = C stretching frequency decreases with increasing N, and this is a characteristic feature of carotenoids. As the conjugation length increases, the delocalization of the π-electrons over the conjugated system becomes greater, making the difference in bond lengths and bond orders of the C-C and C = C bonds smaller, which in turn reduces (down-shifts) the C = C stretching frequency (Robert 1999).

In addition to differences in the resolution of the vibrational bands, another major difference between the absorption spectra of violaxanthin and zeaxanthin (Fig. 8.1b) is the energy of the $S_0 \rightarrow S_2$ transition that decreases with conjugation length. In methanol, the 0-0 band of the $S_0 \rightarrow S_2$ transition for violaxanthin (N = 9) occurs at 21,400 cm^{-1} (467 nm), while for zeaxanthin (N = 11) the 0-0 peak is at 21,000 cm^{-1} (476 nm). For a series of carotenoids having the same structure but different π-electron conjugation lengths, such as β-carotene analogues measured in the same solvent (Kosumi et al. 2006), the dependence of the S_2 state energy on N can be expressed as E = A + B/N, where the parameter B is the slope of the energy dependence on the reciprocal of the conjugation length, and the parameter A is the energy of the S_2 state of a hypothetical infinitely long carotenoid (Christensen et al. 2004).

However, estimation of the S_2 state energy based on extrapolation as described above is not applicable if the structure of the conjugated chain is not identical in carotenoids whose S_2 state energies are being compared, as is the case for the violaxanthin-zeaxanthin pair. Whereas violaxanthin has an entirely linear conjugated chain, the two C = C bonds in the terminal rings of zeaxanthin, that adopt an s-cis conformation, make the structure of the conjugated chain different. This difference led to the concept of an effective conjugation length, denoted N_{eff}, which takes into account the differences in structure of the conjugated chains. For linear π-electron conjugated chains, N_{eff} = N. For carotenoids having C = C bonds twisted in an s-cis conformation or having conjugation extended by different functional groups such as carbonyl or allene groups, N_{eff} is estimated by comparison of the spectroscopic properties with those of a linear carotenoid with the same number of conjugated bonds (Chábera et al. 2009).

Thus, violaxanthin has N_{eff} = N = 9, but the two s-cis bonds of zeaxanthin make its effective conjugation shorter than expected for a carotenoid having N = 11. Comparing the S_2 energy of zeaxanthin in methanol (21,000 cm^{-1}) with that of a linear carotenoid having N = 11 (e.g., lycopene, 20,000 cm^{-1}), it is clear that for zeaxanthin, $N_{eff} < N$. A very valuable parameter to estimate N_{eff} in these situations is the lifetime of the S_1 state (section "Excited-State Dynamics"). By comparing the S_1 lifetimes of zeaxanthin and lycopene, N_{eff} of zeaxanthin is found to be ~10. Thus, although it appears at first glance that the conversion of violaxanthin to zeaxanthin should extend the π-electron conjugation by two C = C bonds, the s-cis conformation of the bonds makes the structure of zeaxanthin equivalent to a carotenoid that has its linear conjugated chain extended by only a single C = C bond.

Various functional groups that are not part of the π-electron conjugated chain have no influence on N_{eff}. For example, β-carotene that differs from zeaxanthin in lacking the two hydroxyl groups on the terminal rings, has an identical π-electron conjugated chain as zeaxanthin, which causes these two carotenoids to have indistinguishable absorption spectra (Niedzwiedzki et al. 2006). Similarly, the terminal rings of violaxanthin that, due to the presence of epoxide groups on carbons 5,6 and 5',6', are not part of the π-electron conjugation, have no effect on the spectral properties, thus making violaxanthin essentially spectroscopically identical to linear carotenoids, such as neurosporene having N = 9.

The energy of the strongly allowed $S_0 \rightarrow S_2$ transition of carotenoids depends to a large degree on the refractive index of the solvent, n, and consequently on solvent polarizability, defined as $R(n) = (n^2-1)/(n^2+2)$. In a solvent such as pyridine with high polarizability, the 0-0 band of the zeaxanthin $S_0 \rightarrow S_2$ transition is down-shifted to 19,800 cm^{-1}, which is more than 1,000 cm^{-1} lower than that observed for the same carotenoid dissolved in methanol with a lower polarizability. In contrast to the effect of

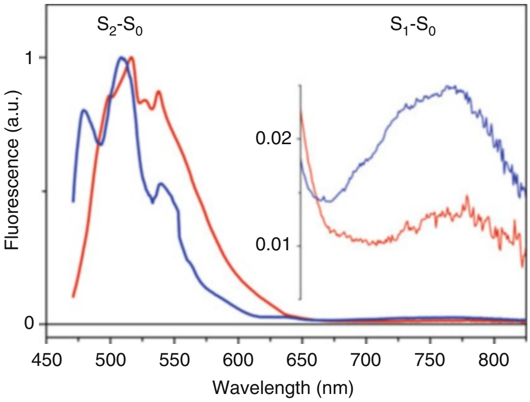


Fig. 8.2 Emission spectra of violaxanthin (*blue*) and zeaxanthin (*red*). To show the weak S_1 emission, the 650–800 nm spectral region is magnified. Fluorescence emission, in arbitrary units (a.u.), was obtained from the carotenoids in n-hexane after excitation at 454 nm using an argon laser.

solvent polarizability on the spectral properties of carotenoids, the polarity of the solvent has only a small effect on the S_2 energy and on the $S_0 \rightarrow S_2$ transition (with the exception of carotenoids possessing a carbonyl group in the conjugated π-electron system; Frank et al. 2000a; Zigmantas et al. 2004). Thus, the S_2 energies of violaxanthin and zeaxanthin are essentially the same in n-hexane compared to methanol.

B Emission Spectra

Carotenoids exhibit very weak emission with quantum yields on the order of 10^{-5} (Frank et al. 1997). Carotenoids with $N > 8$ are typically in violation of Kasha's rule stating that fluorescence originates from the lowest excited state of a π-electron conjugated molecule (Kasha 1950). Carotenoids with $N > 8$ fluoresce primarily from the S_2 state rather than the S_1 state. In fact, S_2 fluorescence dominates the emission spectra of violaxanthin and zeaxanthin (Fig. 8.2). For these molecules, the emission spectra appear almost as a mirror image of their absorption spectra, although, due to the very low emission quantum yields, it is difficult to obtain S_2 emission spectra lacking the effects of re-absorption, which diminishes the intensity of the 0-0 emission band. Recent careful measurements of the S_2 emission spectra of the carotenoids lutein and β-carotene suggested deviations from the mirror image of their S_2 absorption profiles, indicating that another state may exist below the S_2 state (Ostroumov et al. 2009). Both violaxanthin and zeaxanthin also have detectable emission from the dark S_1 state (Frank et al. 2000b) that is nearly 100 times weaker than the S_2 emission (Fig. 8.2). Determining the full spectral profile of the S_1 emission is complicated due to overlap with the low energy tail of the S_2 emission. Yet, measurements of the S_1 emission, especially for shorter ($N < 9$) carotenoids whose quantum yields of S_1 emission are higher than that of the longer carotenoids, revealed that the maximum of the S_1 emission typically corresponds to the 0–2 vibrational band, implying that the potential surfaces of the S_0 and S_1 states are significantly shifted relative to each other (Fujii et al. 1998; Frank et al. 2000b).

C Transient Absorption Spectra

Transient absorption spectroscopy provides a snapshot in time of the excited-state properties of a carotenoid by providing an absorption spectrum measured after the molecule is promoted to an excited state. In practice, transient absorption is measured as the difference between the absorption spectrum from an excited state and the ground state at a certain time after excitation of the carotenoid. Wasielewski and Kispert (1986) were the first to use pulsed lasers to obtain transient absorption spectra of carotenoids. These light sources are ideal for studying the properties of the dark S_1 state, because virtually all S_2 photo-excited carotenoids decay to the dark S_1 state in 100–200 fs (Polívka and Sundström 2004). Therefore, transient absorption spectra of carotenoids measured >1 ps after excitation are dominated by a transition from the S_1 state.

Because the transient absorption spectrum is a differential spectrum, it inevitably contains both positive and negative signals. The positive signal corresponds to excited-state absorption (ESA) and is due to absorption occurring from the excited state. Alternatively, a positive signal could arise from the absorption of a new species (e.g., radical) generated by photo-excitation. The negative signals appearing in the transient absorption spectra arise in two ways: (i) from ground state bleaching (GSB) found in the spectral region of the ground state absorption (at wavelengths where ground-state absorption occurs, the intensity is decreased upon photo-excitation, resulting in a negative signal in a transient absorption spectrum obtained from the difference between excited- and ground-state absorption) and (ii) from stimulated emission (SE). The measuring beam also actively stimulates dumping of the excited-state population to the ground state by photon emission. When this happens, the intensity of the transient absorption spectrum appears negative because more photons at the probe wavelength are detected in a transient spectrum obtained as a difference spectrum between excited- and ground-state absorption.

Transient absorption spectra of violaxanthin and zeaxanthin are shown in Fig. 8.1d, e. These spectra were measured 3 ps after excitation of the S_2 state, and therefore correspond to a signal originating from the S_1 state. In the visible spectral region (Fig. 8.1d), the dominant feature of the transient absorption spectrum is the ESA band arising from the $S_1 \rightarrow S_n$ transition (see energy level scheme in Fig. 8.1c). As observed for the $S_0 \rightarrow S_2$ transition described in the previous section, the energy of the $S_1 \rightarrow S_n$ transition depends on the conjugation length of the carotenoid. For violaxanthin and zeaxanthin in methanol, the $S_1 \rightarrow S_n$ transition peaks at 512 and 550 nm, respectively. The spectral shift induced by the change from $N_{eff}=9$ (violaxanthin) to $N_{eff}=10$ (zeaxanthin) is ~1,300 cm^{-1} for the $S_1 \rightarrow S_n$ transition. This is larger than observed for the $S_0 \rightarrow S_2$ transition where the change in molecular structure results in a shift of 400 cm^{-1}. It should be noted that this large shift observed for the $S_1 \rightarrow S_n$ transition is caused mainly by a change in the energy of the upper S_n state. This is because the difference in S_1 energies of violaxanthin and zeaxanthin in solution is apparently only ~450 cm^{-1} (see section "Excited-State Energies".).

Beside the large spectral shift induced by the change in conjugation length, the influence of conformational disorder is also enhanced in the transient absorption spectrum compared to that seen in the ground state absorption spectrum. While violaxanthin has a narrow (~500 cm^{-1}) S_1-S_n ESA band, this band is much broader for zeaxanthin (~900 cm^{-1}). Thus, the shape and energy of the S_1-S_n band is, as is the case for the ground-state absorption spectrum, an indicator of the structure of the carotenoid molecule. The dependence of the S_1-S_n maximum on conjugation length typically follows that of the $S_0 \rightarrow S_2$ transition, but the shift of the S_1-S_n band is larger. Interestingly, no vibrational structure of the S_1-S_n band has ever been reported in an excited state spectrum from carotenoids. A shoulder on the short wavelength side of the transient absorption spectrum of zeaxanthin in Fig. 8.1d – rather than being due to a 0–1 band of the $S_1 \rightarrow S_n$ transition – was instead shown to be due to a separate state denoted S*, whose precise nature remains controversial at this point (Polívka and Sundström 2009). Moreover, the lack of vibrational structure of the $S_1 \rightarrow S_n$ transition has not yet been fully explained. The search for higher vibrational bands is complicated by the fact that the short wavelength (blue) part of the S_1-S_n ESA overlaps with the GSB, resulting in the negative signal below 500 nm for zeaxanthin (Fig. 8.1d).

Figure 8.1e shows the transient absorption spectra of violaxanthin and zeaxanthin in the near-IR region from 1,000 to 1,800 nm. This part of the spectrum contains only ESA arising from a transition between the S_1 and S_2 states (Fig. 8.1c). This $S_1 \rightarrow S_2$ transition is allowed by symmetry because the states are of A_g^- and B_u^+ symmetry, respectively. The spectra of violaxanthin and zeaxanthin both resemble the three− vibrational peak structure of their respective $S_0 \rightarrow S_2$ transitions, and have vibrational spacing almost perfectly matching that observed in the ground state spectra shown in Fig. 8.1b. This observation, together with the fact that the near-IR ESA signal decays with the same lifetime as observed for the ESA measured in the visible region (Polívka et al. 1999), proves that the origin of the near-IR ESA is due to the $S_1 \rightarrow S_2$ transition. The similarity of the S_1–S_2 profiles of violaxanthin and zeaxanthin (Fig. 8.1e) demonstrates that the $S_1 \rightarrow S_2$ transition, in contrast to the $S_0 \rightarrow S_2$ and $S_1 \rightarrow S_n$ transitions, is rather insensitive to the conjugation length and structure of the carotenoid. Nevertheless, this transition has proven valuable in determining the energy of the forbidden S_1 state, because knowledge of the energies of the $S_0 \rightarrow S_2$ and $S_1 \rightarrow S_2$ transitions allows calculation of the S_1 energy by simple subtraction of these two values (Polívka et al. 1999).

D Excited-State Energies

The dynamic properties of the S_1 state described in the previous section provide information about the S_1 lifetime. Because the carotenoid S_1 state has been suggested as a possible quencher in the thermal dissipation process, the S_1 lifetime is a property relevant to NPQ, but the key factor in deciding whether the S_1 state is directly involved in thermal dissipation is the energy of the state.

While the energy of the S_2 state can be readily deduced from the absorption spectrum – it is assigned by the energy of the 0-0 vibrational band of the $S_0 \rightarrow S_2$ transition – determining the S_1 energy is much more complicated due to the forbidden $S_0 \rightarrow S_1$ transition. Yet, because the S_1 state has been suggested to play a key role in some of the proposed mechanisms of NPQ, the S_1 energy is crucial to assess the feasibility of the proposed mechanisms. Early attempts to obtain the S_1 energy of carotenoids were based on extrapolation from the S_1 energies of shorter carotenoids with weak S_1 emission, and whose S_1 energies could be determined directly from S_1 emission spectra (Frank et al. 1994). By applying the energy gap law for radiationless transitions (Englman and Jortner 1970), the S_1 energies of violaxanthin and zeaxanthin were estimated to be 15,200 and 14,200 cm^{-1}, respectively (Frank et al. 1994). Since these energies appeared to be above (violaxanthin) and below (zeaxanthin) the energy of the lowest Chl *a* state, Q_y (~14,800 cm^{-1}, Fig. 8.1c), in the LHCII pigment-protein complex from green plants, this estimation led to the proposal of what was termed a “molecular gear shift mechanism.” According to this simple model, violaxanthin could act as an efficient light-harvesting carotenoid, and its enzymatic conversion to zeaxanthin shifts the S_1 state down below that corresponding to the Q_y transition of Chl *a*, making zeaxanthin a potential quencher of the excited state energy of chlorophyll (Frank et al. 1994).

Later, more direct measurements of the S_1 energies of violaxanthin and zeaxanthin, employing either measurements of the S_1-S_2 transient absorption (Polívka et al. 1999, Fig. 8.1e) or detection the extremely weak S_1 emission (Frank et al. 2000b), showed that the difference between S_1 energies of violaxanthin and zeaxanthin was much less than predicted by the extrapolation according to the energy gap law. Furthermore, both of these measurements placed the S_1 energy of both carotenoids below (or in the case of violaxanthin nearly equal to) the lowest singlet state of Chl *a* in LHCII. Experiments based on recording S_1-S_2 spectra provided values of 14,470 cm^{-1} (violaxanthin) and 14,030 cm^{-1} (zeaxanthin) (Polívka et al. 1999); S_1 fluorescence measurements yielded S_1 energies of 14,880 cm^{-1} (violaxanthin) and 14,550 cm^{-1} (zeaxanthin) (Frank et al. 2000b). While not ruling out zeaxanthin as a possible quencher per se, these results suggest that lowering the S_1 energy due to elongation of conjugation length when switching from violaxanthin to zeaxanthin cannot be the only factor controlling quenching. Both methods demonstrated that the energy difference is 330–440 cm^{-1}, which is much less than the 1,000 cm^{-1} value predicted by the energy gap law (Frank et al. 1994). Interestingly, however, the values obtained from transient absorption spectroscopy were systematically lower by about

400 cm^{-1} than those obtained by fluorescence methods. This difference can be explained by the S_1–S_2 technique probing an allowed transition, whereas fluorescence monitors a forbidden transition ($S_1 \rightarrow S_0$). Thus, fluorescence, as a one-photon process, must be detecting predominantly those molecules in the sample that deviate from idealized C_{2h} symmetry in the forbidden S_1 state, since only these conformations acquire sufficient allowedness to be detected in the emission spectrum. The exact opposite situation holds for the allowed $S_1 \rightarrow S_2$ transition because the least distorted conformations will give rise to the dominant contribution to this ESA spectrum. The difference in S_1 energies obtained by the different techniques is consequently a result of the fact that each technique probes a different subset of S_1 state conformations in the sample (Polívka et al. 2001).

It should be noted that other techniques have also been employed to determine the energy of the S_1 state of carotenoids, although they have not been used for violaxanthin or zeaxanthin. One such technique relies on a measurement of the resonance Raman excitation profile. Carotenoids exhibit very strong Raman scattering, and when the excitation light is in resonance with an electronic transition of the molecule, the intensities of the (resonance) Raman lines are greatly enhanced. Thus, tuning detection to one of the carotenoid Raman lines (typically either a C = C or C-C stretching mode) and scanning the excitation light over a broad spectral range, a resonance Raman excitation profile can be obtained. The high sensitivity of Raman line intensities to resonance conditions enables detection of forbidden states with very low transition dipole moments (Thrash et al. 1977; Sashima et al. 1998). Another method to determine the S_1 energy of carotenoids is two-photon excitation, resulting in a two-photon transition for which $S_0 \rightarrow S_1$ transition is allowed (Tavan and Schulten 1987). The disadvantage of the two-photon method lies in the necessity to use fluorescence emission as an indicator of two-photon absorption. Because carotenoids have very low emission yields from their S_1 state, two-photon excitation has been reliably reported only for light-harvesting proteins, in which the energy absorbed by carotenoids in a two-photon experiment is transferred to Chl *a*, from where its emission can then be readily detected (Walla et al. 2000).

E Excited-State Dynamics

Excitation of a carotenoid populates the S_2 state via absorption of a photon whose energy matches that of the $S_0 \rightarrow S_2$ transition. The S_2 state then relaxes on a time scale of 100–200 fs to the S_1 state. This very fast depopulation of the S_2 state via S_2-S_1 internal conversion is the primary reason for the extremely low quantum yield of S_2-S_0 fluorescence from carotenoids. The most trustworthy measurements of S_2 lifetimes have come from fluorescence up-conversion experiments that recorded the faint S_2 emission with a time-resolution better than 100 fs (Ricci et al. 1996; Macpherson and Gillbro 1998). These measurements demonstrated that, for a series of carotenoids with similar structure but different conjugation length, S_2 lifetime decreases with conjugation length (Kosumi et al. 2006). Fluorescence up-conversion measurements of S_2 lifetimes for zeaxanthin and violaxanthin have not yet been reported. In fact, the S_2 lifetimes of these molecules have been obtained either from global analyses of transient absorption spectra or from the rise times of the S_1-S_n spectra monitored at single wavelengths and recorded in the visible spectral region (Polívka et al. 1999; Billsten et al. 2005; Niedzwiedzki et al. 2006). The resulting values are in the range of 140–180 fs for zeaxanthin. Essentially the same value, of ~160 fs, was obtained for violaxanthin that has a shorter π-electron chain length. This finding suggests that conjugation length is not the only factor determining S_2 lifetime, and that carotenoid structure may also play a role.

The precise mechanism of the very fast S_2-S_1internal conversion has not been fully explained yet. It is very likely that S_2-S_1 relaxation occurs via conical intersection, which has been proposed on the basis of quantum computations on model systems, such as hexatriene or decapentaene (Garavelli et al 1997; Fuss et al. 2000). The conical intersection model would explain the role of carotenoid structure in the dynamics of internal conversion, because the shape of multidimensional potential surfaces forming the conical intersection will depend on carotenoid structure. The S_2-S_1 internal conversion may also involve other dark states predicted to exist between S_2 and S_1 states. Such dark states were predicted by quantum calculations many years ago (Tavan and Schulten 1987), but compelling experimental evidence for them has been slow to develop (Sashima et al. 1999; Polívka and Sundström 2009; Ostroumov et al. 2009; Maiuri et al. 2012). Ultrafast experiments carried out on several carotenoids in recent years suggests that at least one dark state of B_u^- symmetry may indeed be located just below the S_2 state for carotenoids with $N > 10$. This state would be likely to be involved in promoting S_2-S_1 relaxation. The energy of the B_u^- state is expected to decrease sharply with conjugation length (Tavan and Schulten 1987), making the energy gap between the S_2 and B_u^- states strongly dependent on N, and resulting in a dependence on N of the S_2 lifetime as has been reported (Kosumi et al. 2006).

In contrast to the difficulty in obtaining the precise values of S_2 lifetimes, S_1 lifetimes are readily obtained from fitting exponential decays of S_1-S_n visible or S_1-S_2 near-IR spectral bands in the transient absorption spectra (Fig. 8.1d, e). The S_1 decays of violaxanthin and zeaxanthin are shown in Fig. 8.3. The S_1 lifetime of violaxanthin is 24 ps, while zeaxanthin's S_1 lifetime is shortened to 9 ps due to the zeaxanthin's shorter conjugation (Frank et al. 1994; Polívka et al. 1999; Niedzwiedzki et al. 2006). Thus, the S_1 lifetimes are, at least qualitatively, consistent with the energy gap law (Chynwat and Frank 1996), i.e., as S_1 energy decreases with increasing N, the S_1 lifetime becomes shorter. The shorter S_1 lifetime of zeaxanthin may indicate it is a better quencher because it dissipates energy faster. However, it should be noted that under normal, ambient, sunlight conditions the lifetime shortening has essentially no effect because the probability that the same molecule will be excited twice within a few picoseconds is essentially zero. It should be also noted that, in contrast to the S_2 lifetimes, the S_1 lifetimes of violaxanthin and zeaxanthin are independent of solvent (Frank et al. 1994; Polívka et al. 1999; Billsten et al. 2002; Niedzwiedzki et al. 2006). This can be explained by the fact that the $S_1 \to S_0$ transition is symmetry forbidden, and that S_1 depopulation thus occurs via internal conversion controlled by vibrational coupling between the S_1 and S_0 states via localized C = C stretching modes rather than by alterations in the energy of the excited states induced by solvent interactions (Nagae et al. 2000).

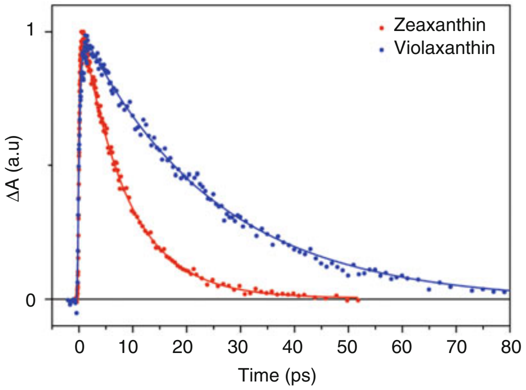
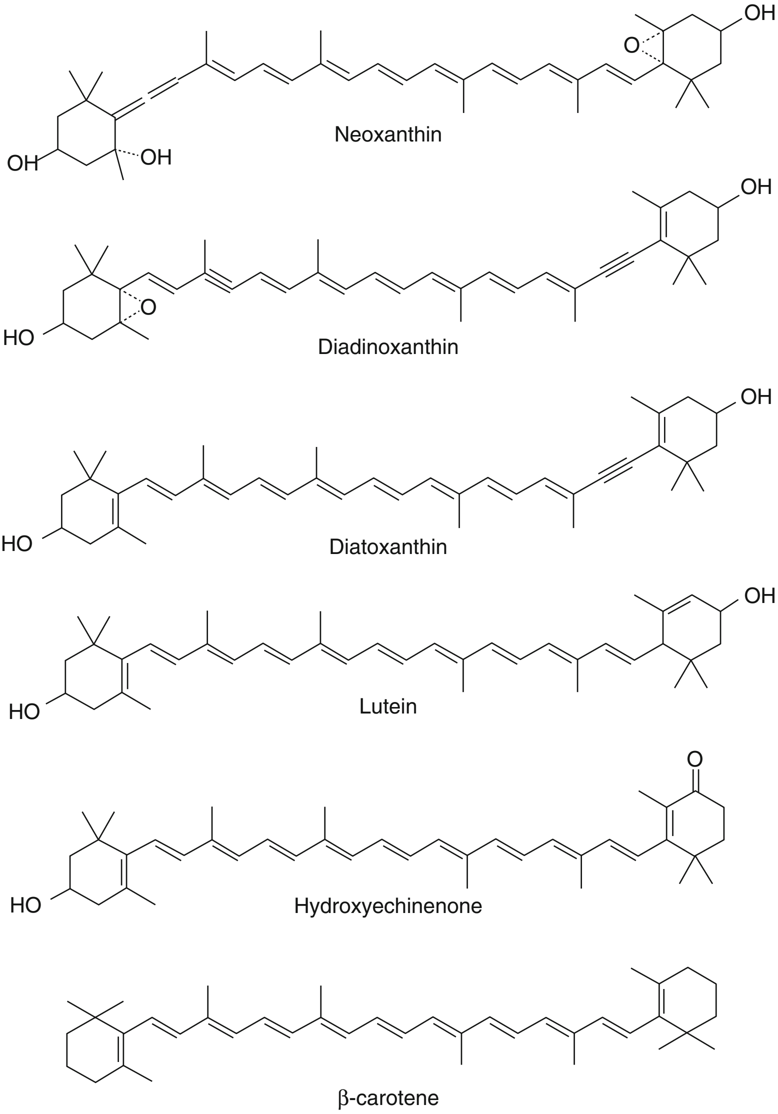


Fig. 8.3 Transient absorption kinetics of zeaxanthin (*red*) and violaxanthin (*blue*) recorded at the maximum of the $S_1 \to S_n$ transition of both carotenoids. The *solid lines* are fits resulting in the S_1 lifetimes of 9 ps (zeaxanthin) and 24 ps (violaxanthin). The change in absorbance is in arbitrary units (a.u.) normalized to the highest absorption for each.

F Other Carotenoids

Up to this point, this chapter has focused on the two VAZ cycle carotenoids, violaxanthin and zeaxanthin, in describing the basic spectroscopic properties of carotenoids. However, other carotenoids have also been proposed to play a role in NPQ in plants and algae. It has

Fig. 8.4 Molecular structures of additional carotenoids related to NPQ.



been suggested that lutein and neoxanthin, as two additional carotenoids – besides violaxanthin and zeaxanthin – bound to the major light-harvesting complex (LHCII) of plants, may be important in NPQ (Ruban et al. 2007; on the topic of lutein epoxide-derived lutein and NPQ, see Esteban and Garcia-Plazaola, Chap. 12; on the topic of the correlation between rapidly reversible NPQ and zeaxanthin in nature, see Demmig-Adams et al., Chap. 23). In addition, some marine photosynthetic microorganisms employ an alternative xanthophyll cycle involving the two carotenoids diadinoxanthin and diatoxanthin (Olaizola et al. 1994; see also Büchel, Chap. 11; Morosinotto and Bassi, Chap. 14). Moreover, in cyanobacteria, NPQ is associated with a specific orange carotenoid protein (OCP) that binds the carotenoid hydroxyechinenone (Kerfeld 2004; see also Kirilovsky et al. Chap. 22). The spectroscopic properties of these carotenoids, whose structures are shown in Fig. 8.4, are summarized in Table 8.1. The table illustrates that, besides conjugation length as a key parameter determining the excited-state properties of carotenoids, other factors, such as carotenoid structure, attached functional

Table 8.1 Conjugation length, absorption maxima, vibrational structure, S_1 and S_2 energies and lifetimes of carotenoids associated with NPQ. n.m. – not measured

Carotenoid	Solvent	N	N_{eff}	λ_{max} (nm)	III/II	S_2 energy (cm^{-1})	S_1 energy (cm^{-1})	S_1–S_n max (nm)	τ_2 (fs)	τ_1 (ps)	Reference
Neoxanthin	Pyridine	9	8.5	451	0.71	20,700	n.m.	540	110	37	Niedzwiedzki et al. (2006)
Violaxanthin	MeOH	9	9	440	0.68	21,400	14,470	512	n.m.	24	Polívka et al. (1999)
Violaxanthin	Pyridine			456	0.87	20,550	n.m.	530	165	26	Niedzwiedzki et al. (2006)
Diadinoxanthin	Acetone	9	9	450	0.56	20,880	14,120	540	140	22	Enriquez et al. (2010)
Diatoxanthin	Acetone	10	9.5	457	0.34	20,730	13,930	555	140	13	Enriquez et al. (2010)
Lutein	MeOH	10	9.5	443	0.61	21,180	n.m.[a]	528	130	15	Billsten et al. (2003)
Zeaxanthin	MeOH	11	10	450	0.27	21,000	14,030	550	130–180	9	Billsten et al. (2005)
Zeaxanthin	Pyridine			468	0.18	20,050	n.m.	575	145	10	Niedzwiedzki et al. (2006)
β-carotene	Hexane	11	10	450	0.28	21,000	14,500[b]	550	140	9	Billsten et al. (2002)
Hydroxyechinenone	Hexane	11	10.5	454	n.a.	20,800	14,300	575	160–220	6	Polívka et al. (2005)

[a] The S_1 energy of lutein in octanol was estimated by two-photon excitation spectrum, but no precise energy was given due to featureless shape of the two-photon excitation profile (Walla et al. 2002)

[b] The S_1 energy of β-carotene in n-hexane was measured by deconvoluting the weak S1 emission (Onaka et al. 1999)

groups, and solvent environment, affect some of the excited-state properties.

III Changes in Molecular Structure and Excited-State Properties

In the previous sections, the basic spectroscopic properties of violaxanthin and zeaxanthin were described, and it was shown how the structure of the conjugated chain affects transition energies and lifetimes of excited states. However, other changes in molecular structure may further affect excited-state properties. For example, it has thus far been assumed that carotenoids are in an all-trans configuration, but it is well-known that carotenoids readily isomerize to form cis-isomers, and that the latter can be taken into specific protein binding sites in green plants and photosynthetic bacteria (Koyama and Fujii 1999). Also, subtle changes in the configuration of a carotenoid may occur during transitions between excited states that can have significant consequences for the action of carotenoids as quenchers in the processes of thermal dissipation and NPQ.

A Different Molecular Configurations of Carotenoids in the S_0, S_1 and S_2 States

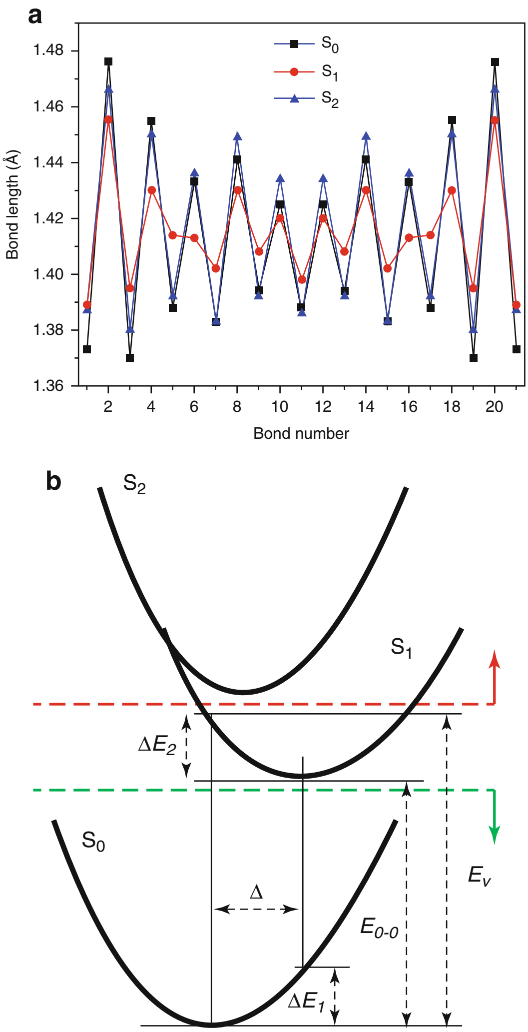


Fig. 8.5 (**a**) Calculated bond lengths in S_0, S_1 and S_2 states of zeaxanthin (Redrawn from Dreuw (2006). (**b**) Schematic picture of potential surfaces of the S_0, S_1 and S_2 states showing the shift *Δ* between the minima of S_0 and S_1 potential surfaces. See text for details.

When a carotenoid undergoes a transition from one electronic state to another, and the molecular configurations of initial and final state are the same, the transition is classified as a vertical transition. However, the molecular configuration of a molecule in a vibrationally relaxed excited state versus its relaxed ground state is typically not the same. This suggests that promotion of a carotenoid from its ground state to an excited state (or from a lower excited state to a higher excited state) causes the molecule to exist in a non-vibrationally-relaxed configuration in the final state. The past decade has seen rapid development of quantum chemical methods for quantifying such changes in molecular configurations between S_0, S_1 and S_2 states. Dreuw (2006) analyzed configurations and energies of the vertical transitions of violaxanthin, zeaxanthin and lutein, and the results obtained for zeaxanthin are summarized in Fig. 8.5.

The top panel of Fig. 8.5 shows the C-C and C = C bond lengths of zeaxanthin in the vibrationally-relaxed S_0, S_1 and S_2 states. While changes in bond lengths are minor during the $S_0 \rightarrow S_2$ transition, a transition to the relaxed S_1 state is accompanied by significant alterations in bond lengths.

Especially in the center of the conjugated chain, the individual C-C and C = C bonds for the molecule in the S_1 state do not retain the typical lengths they possess in the ground state. Instead, their lengths tend to become comparable, implying that the distinction between the carbon-carbon single and double bonds in the conjugated chain is lessened in the S_1 state.

In considering transitions between states, it is helpful to view the transitions on a potential energy-surface diagram (Fig. 8.5b), which reveals differences in molecular configuration of the individual excited states. Because molecular configuration in the S_1 state is significantly different than in the S_0 and S_2 states, the minimum in the S_1 potential surface is represented as being shifted with respect to the S_0 and S_2 surfaces (see Fig. 8.5b). The shift, denoted Δ in the diagram, indicates that the relaxed configuration of the molecule in the S_1 state does not correspond to the relaxed configuration in the S_0 state, which is consistent with experimental observations. As described above, the weak emission from the S_1 state has a characteristic spectrum with a maximum corresponding to the 0–2 vibrational band (Fujii et al. 1998; Frank et al. 2000b). This is because a transition starting at the bottom of the S_1 potential surface (the vibrationally relaxed S_1 state) will terminate at a point on the S_0 potential surface that is higher by an amount ΔE_1 than the lowest energy point on the S_0 potential curve. From emission spectra taken on numerous carotenoids, it is known that ΔE_1 can be as high as 2,500 cm^{-1}, confirming the significant difference in molecular configurations of the molecules in their S_0 and S_1 states. Also, changes in bond lengths are confirmed by reported differences in the C = C stretching modes obtained from resonance Raman studies of carotenoids. Typically, the stretching frequency is up-shifted from ~1,560 cm^{-1} in the S_0 state to ~1,750 cm^{-1} in the S_1 state supporting this idea (Hashimoto et al. 1991; McCamant et al. 2003; Marek et al. 2011).

The above-described difference in molecular configuration of S_0 and S_1 states has a significant consequence pertaining to thermal dissipation as indicated by NPQ that assumes quenching by the S_1 state of carotenoid of the Chl *a* state associated with its Q_y transition. In this dissipation/quenching mechanism, the carotenoid in its S_0 state is available to accept energy from the excited S_1 (Q_y) state of Chl *a*, suggesting that the carotenoid must retain its ground state configuration during energy transfer. This mechanism further implies that the carotenoid has to accept sufficient energy to promote it to a point on its S_1 potential surface that corresponds to the molecular configuration of the ground state. That point is higher by ΔE_2 than the minimum in the S_1 potential surface (Fig. 8.5b). Thus, as pointed out by Dreuw (2006), the 0-0 S_1 energies determined by various methods during the past decade are, in fact, not necessarily relevant to the question of whether the carotenoid S_1 state can quench the S_1 (Q_y) state of Chl *a*.

The above discussion suggests that for a carotenoid to facilitate efficient quenching of Chl *a* excited states, the energy of the Chl *a* donor should be higher than $E_{0-0}+\Delta E_2$, i.e., above the red dashed line depicted in Fig. 8.5b. On the other hand, if there were to be efficient light-harvesting via energy transfer from the S_1 state of a carotenoid to Chl *a*, the S_1 (Q_y) energy of Chl *a* should fall below that of the carotenoid in its relaxed S_1 state, i.e., below the green dashed line in Fig. 8.5b. If the energy of the Chl *a* S_1 (Q_y) state were to reside in the region between the two lines, both Q_y-S_1 and S_1-Q_y energy transfer processes would be inhibited. Experimental values for ΔE_2 are not available, but calculations suggest that it is in the range of 1,600–1,700 cm^{-1} for zeaxanthin (Dreuw 2006). Thus, given that the 0-0 energy of the S_1 state of zeaxanthin is ~14,000 cm^{-1} (Polívka et al. 1999; Frank et al. 2000b), efficient quenching would require the energy of the Chl *a* donor to be at least 16,000 cm^{-1} (625 nm), which is higher than that of the S_1 (Q_y) state of Chl *a* in LHCII that is ~14,800 cm^{-1} (676 nm). This line of reasoning suggests that zeaxanthin should not be able to function as a direct quencher via simple energy transfer from the excess excited states of Chl *a*.

However, it must be noted that, due to conformational disorder, thermal movement and molecular vibrations, there is still a possibility of quenching even if the energy of the Chl *a* donor falls below the red line in Fig. 8.5b. Occasionally, carotenoids may happen to be in a configuration that will allow a transition from the S_0 to the S_1 state even if the acceptor energy is lower than $E_{0\text{-}0}+\Delta E_2$. Again, support for this can be found in the S_1 emission spectra for the $S_1 \rightarrow S_0$ transition of carotenoids that clearly contain a contribution from photons having energy comparable to the 0-0 transition (Fujii et al. 1998; Frank et al. 2000b). In terms of the various models for energy transfer, the spectral overlap integral that sums the area occupied simultaneously by the donor emission and acceptor absorption spectral lineshapes is not zero even if the energy of the vibrationally-relaxed donor state falls below the red line in Fig. 8.5b. Yet, the efficiency of energy transfer, and consequently of the quenching mechanism, is predicted to be low for such a situation.

B Effect of Isomerization on Excited-State Properties

Carotenoids are known to form a number of cis-isomers (Koyama and Fujii 1999) identifiable by characteristic changes in their absorption spectra. One of these changes arises from the slightly higher S_2 energy of the cis isomer compared with the molecule in the all-trans configuration. For example, the S_2 energy of the central cis-isomer of zeaxanthin ($N_{eff}=10$, Fig. 8.6) is ~200 cm^{-1} higher than that of all-trans zeaxanthin (Niedzwiedzki et al. 2010), and this difference increases with decreasing conjugation length. For neoxanthin ($N_{eff}=8.5$), the difference in S_2 state energy between the all-trans and central-cis configurations is ~500 cm^{-1} (Niedzwiedzki et al. 2010). However, the most important marker in the absorption spectra of cis-isomers is what is referred to as the "cis-peak" that is due to a transition often denoted $S_0 \rightarrow S_3$. The S_3 state is of A_g^+ symmetry, and while A_g^- to

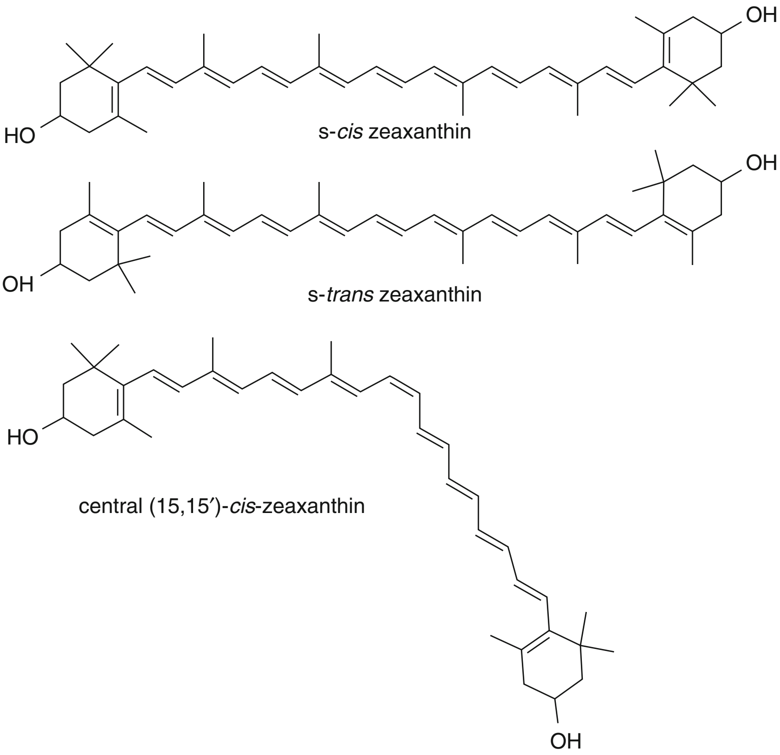


Fig. 8.6 Molecular structures of three different isomers of zeaxanthin.

A_g^+ transition is spectroscopically forbidden for all-trans carotenoids, the reduction in symmetry induced by isomerization of the molecule from an all-trans to a cis configuration renders the transition allowed. The cis-peak is readily discerned in the spectral region between 300 and 350 nm, with an intensity as high as 50 % of the maximum of the $S_0 \rightarrow S_2$ transition (Koyama and Fujii 1999; Niedzwiedzki et al. 2009, 2010). As for the major band, the position of the cis peak depends on conjugation length; the maximum of the cis-peak occurs at 340 nm for zeaxanthin ($N_{eff} = 10$) and at 320 nm for neoxanthin ($N_{eff} = 8.5$) (Niedzwiedzki et al. 2010). As is the case for the $S_0 \rightarrow S_2$ transition, a fine structure of vibrational bands in the cis-peak appears for carotenoids with linear conjugated chain, e.g., violaxanthin and neoxanthin, while no vibrational structure is present for zeaxanthin with its π-electron conjugation extended into the terminal rings.

The effect of isomerization on excited-state properties of carotenoids beyond what can be described simply on the basis of effective conjugation length is illustrated by the following observation. While $S_0 \rightarrow S_2$ transition of a cis isomer is blue-shifted versus that of the all-trans carotenoid, the $S_1 \rightarrow S_n$ transition is shifted in the opposite direction, i.e., whereas isomerization increases the energy of the $S_0 \rightarrow S_2$ transition, it decreases the energy of the S_1-S_n transition (Niedzwiedzki et al. 2009, 2010). Decreasing $S_1 \rightarrow S_n$ transition energy upon isomerization to a cis configuration can be demonstrated by measurements of the S_1 energies of cis isomers that are systematically higher than the corresponding S_1 energies of the corresponding all-trans carotenoids. This change also leads to a red-shift in the S_1-S_n ESA. Low temperature measurements revealed that S_1 energy of central-cis zeaxanthin is ~300 cm^{-1} higher than that of all-trans zeaxanthin, and that this difference is nearly independent of conjugation length (Niedzwiedzki et al. 2010). Interestingly, even though cis-isomers have a higher S_1 energy compared to all-trans carotenoids, they have a shorter S_1 lifetime, further emphasizing the unique excited-state properties of cis-isomers. Although the changes are not dramatic, with all-trans zeaxanthin having at 77K an S_1 lifetime of 14.8 ps versus 12.4 ps for the central cis-zeaxanthin (Niedzwiedzki et al. 2010), these changes may fine-tune the excited-state energies and dynamics of carotenoids, which could be important to their function in biological systems.

Carotenoids with their π-electron conjugation extended into terminal rings exhibit further changes in excited-state spectra and dynamics because of twisting of the terminal rings with respect to the rest of the conjugation. In Fig. 8.6, two configurations of the terminal rings of zeaxanthin, denoted s-cis and s-trans, are shown. It is well established that the s-cis configuration of carotenoids with terminal rings has the lowest energy, i.e., is the most stable (Dreuw 2006), which is why it is not possible to carry out measurements on carotenoids with their rings exclusively in the s-trans configuration. However, for retro-carotenoids (“retro” indicates a shift of all single and double bonds of the conjugated polyene system by one position), e.g., rhodoxanthin, the s-trans configuration is the most stable, and these molecules therefore provide an opportunity to obtain data on carotenoids with their rings in an s-trans configuration (Chábera et al. 2009). Linearization of the conjugated chain leads to extension of the effective conjugation. For example, s-trans zeaxanthin should have an $N_{eff} = 11$ versus $N_{eff} = 10$ for the “normal” s-cis zeaxanthin. Rhodoxanthin has an $N_{eff} = 12.5$ (Chábera et al. 2009). Of course, this effect of linearization has implications for the energies of the S_2 and S_1 states that will be significantly lower in the s-trans versus s-cis configuration. Linearization of the conjugated chain of s-trans zeaxanthin would make its spectroscopic properties comparable to that of a linear carotenoid with $N = 11$, with S_2 and S_1 energies of approximately 20,000 and 12,500 cm^{-1}, respectively. Thus, carotenoids with π-electron conjugation extended to the terminal rings may alter S_1 energy and lifetime significantly by switching from s-cis to s-trans configuration.

IV Spectroscopic Studies of Protein-Bound Carotenoids

The previous section showed that changes in π-electron configuration may significantly alter the spectroscopic properties of carotenoids. Although many configurations cannot be easily achieved in solution, a number of configurations that are typically unstable in solution can be locked into position when a carotenoid is bound to a protein. The influence of protein environment on the spectral properties of carotenoids is exemplified by two proteins related to NPQ, the LHCII antenna complex from plants and the OCP from cyanobacteria. Absorption spectra of these two proteins are shown in Fig. 8.7. LHCII binds various carotenoids, including lutein, neoxanthin, violaxanthin, and sometimes zeaxanthin, and also Chl *a* and Chl *b*. The individual contributions of the chlorophylls and carotenoids to the LHCII absorption spectrum can be analyzed by deconvolution of the spectral profile (Croce et al. 2000).

The deconvolution of the LHCII absorption spectrum given in Fig. 8.7 shows the separate contributions of Chls and carotenoids, and this particular absorption band includes contributions from three carotenoids, lutein, neoxanthin and violaxanthin, that bind in a 2:1:1 ratio (Liu et al. 2004). Here, lutein contributes the largest part of the carotenoid absorption profile in LHCII. Comparison of the carotenoid absorption profile in LHCII with the absorption spectrum of lutein in solution (dashed line) reveals a typical effect of the protein environment, i.e., a protein-induced red-shift of the absorption spectrum due to dispersion interactions of the carotenoid with the protein. Protein-induced red-shifts in absorption spectra are observed for virtually all carotenoids bound to proteins. The magnitude of the shift typically ranges from 500 to 1,000 cm^{-1}.

Another effect induced by the binding of carotenoids to protein is seen in OCP. This protein binds only a single carotenoid, hydroxyechinenone (hECN), and no chlorophylls. Thus, it is an ideal system to investigate carotenoid-protein interactions in the absence of interactions with chlorophyll.

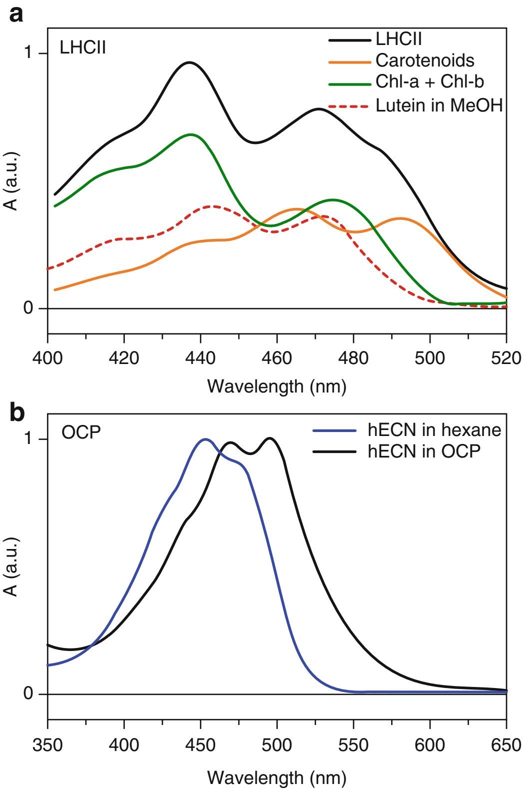


Fig. 8.7 (**a**) Absorption spectrum of LHCII (*black*) deconvoluted into the contribution from chlorophylls (*green*) and carotenoids (*orange*). The *dashed spectrum* is absorption spectrum of lutein in methanol. (**b**) Absorption spectra of the carotenoid hydroxyechinenone (hECN) in n-hexane (*blue*) and bound to OCP (*black*). Absorption spectra provided in arbitrary units (a.u.).

Besides the red-shift in absorption described above, binding to OCP enhances the resolution of the vibrational bands of the $S_0 \rightarrow S_2$ transition (Polívka et al. 2005). This enhancement of resolution of vibrational bands is due to a decrease in conformational disorder caused by the locking of the carotenoid into a specific configuration in the protein-binding site. Although this effect was observed previously in other carotenoid-binding proteins, e.g., LH2 from photosynthetic bacteria (Polívka et al. 2002a), it is further enhanced in OCP. Indeed, the high-resolution X-ray structure of OCP (Kerfeld et al. 2003) revealed that the hECN-binding site forces the carotenoid to twist its terminal ring into an s-trans configuration. The s-cis configuration is more stable in solution than the trans configuration. Thus, it is clear that binding to OCP linearizes the conjugated chain, which results in much higher resolution of the vibrational bands in its absorption spectra, as is alluded to above.

OCP exemplifies that protein binding significantly alters the spectroscopic properties of carotenoids. Experiments whereby carotenoids are reconstituted into various apoproteins, i.e., proteins lacking bound pigments, further illustrate that, even when a non-native carotenoid is incorporated into a binding site that normally binds a different carotenoid, the carotenoid does not retain the properties it has in solution. For example, when LHCII that normally binds lutein in its two central binding sites (Liu et al. 2004) is reconstituted with violaxanthin or zeaxanthin, the S_1 lifetimes of these latter two carotenoids bound to LHCII are nearly identical at 11 ps, whereas their S_1 lifetimes in solution are 24 and 9 ps, respectively (Polívka et al. 2002b). If the same experiment is done with *Arabidopsis thaliana* plants genetically engineered to contain only one specific carotenoid, the result is different from the experiments with reconstituted LHCII, but the S_1 lifetimes are still both shorter and more similar (12.5 and 4.8 ps for violaxanthin and zeaxanthin, respectively) than those of the molecules in solution (Fuciman et al. 2012). These observations, that the S_1 lifetimes of violaxanthin and zeaxanthin bound to LHCII are less different than those in solution, were also supported by calculations (Dreuw and Wormit 2008).

The above-described observations underline the tremendous importance of the protein-binding site in tuning the spectroscopic properties of carotenoids. Examples of linearization of the conjugated chain, by switching from s-cis to s-trans configurations of the terminal rings upon binding to protein (Kerfeld et al. 2003) or effective detachment of the double bonds in the terminal rings by out-of-plane twisting (Polívka et al. 2009), clearly show that proteins can alter the effective conjugation length, and consequently the energies and dynamics, of the excited states of carotenoids. Also, recent progress in quantum chemical calculations have revealed that specific carotenoid configurations not achievable in solution may be present in proteins, and that protein-bound carotenoids may thus have significantly different state energies than their configurations achievable in solution (Cerezo et al. 2012). Since many of these configurations are difficult to stabilize for studies in solution, and obtaining spectroscopic properties of individual carotenoids in proteins is complicated by spectral interferences among multiple pigments bound to a single protein, high-resolution structures in combination with quantum-chemical calculations will be required to interpret and predict spectroscopic properties of protein-bound carotenoids (Dreuw 2006; Dreuw and Wormit 2008; Duffy et al. 2010; Macernis et al. 2012). Most importantly, determination of the vertical $S_0 \rightarrow S_1$ transition energies of carotenoids bound to proteins is needed as a critical factor in determining the energetic feasibility of specific energy transfer mechanisms from Chl *a* to carotenoid.

V Spectroscopic Properties of Carotenoid Radicals

As described in the previous sections, the spectroscopic properties of neutral, non-radical carotenoids have been studied in great detail. However, much less effort has been devoted to spectroscopic studies of carotenoid radicals. From the viewpoint of understanding NPQ, redox and spectroscopic properties of carotenoid radicals are important. Indeed, it has been proposed that the Chl *a* excited state may be quenched via accepting an electron from a nearby carotenoid (Holt et al. 2005).

Carotenoid cation radicals can be readily identified by their characteristic absorption bands in the near-IR spectral region. These IR bands are well separated from the absorption bands of neutral carotenoids. The absorption spectrum of zeaxanthin cation radical is shown in Fig. 8.8. The band

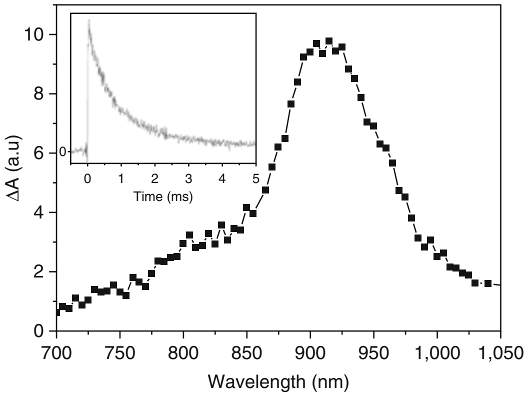


Fig. 8.8 Absorption spectrum of zeaxanthin cation radical in methanol generated by an 8 ns laser pulse at 355 nm. *Inset* shows the kinetics measured at the absorption maximum (910 nm). The lifetime of zeaxanthin cation radical in methanol is 1 ms.

peaking at 910 nm is due to the $D_0 \rightarrow D_2$ transition (both ground and excited states of carotenoid radicals are doublet states) that has an intensity comparable to the $S_0 \rightarrow S_2$ transition of a neutral carotenoid (Jeevarajan et al. 1996). The lowest energy transition, $D_0 \rightarrow D_1$, is also allowed and has a maximum in the 1,300–1,500 nm spectral region (Jeevarajan et al. 1996). While the absorption profile of the $D_0 \rightarrow D_1$ transition has been recorded for only a few carotenoids (Jeevarajan et al. 1996), the $D_0 \rightarrow D_2$ transition was extensively studied for a number of carotenoids (Galinato et al. 2007). It must be noted that the maxima reported for the $D_0 \rightarrow D_2$ transition depend on the method by which the carotenoid cation radical is generated. For example, the maximum of the $D_0 \rightarrow D_2$ transition for β-carotene varies from 790 to 1,040 nm, depending on whether the radical cation was generated via pulse radiolysis, photoexcitation, or electrochemical or chemical oxidation (Galinato et al. 2007). However, a systematic study of a series of carotenoids with different N_{eff} values, for which the carotenoid radical was generated by the same method, showed that the maximum of the $D_0 \rightarrow D_2$ transition shifts to red with increasing conjugation length (Galinato et al. 2007), following the trend established for the $S_0 \rightarrow S_2$ transition of neutral carotenoids.

These cation radical transitions in the near-IR provide a means of detecting carotenoid radicals in isolated light-harvesting complexes, e.g., the LH2 complex from purple bacteria (Polívka et al. 2002a) and LHCII complexes from genetically modified plants that contained only one specific carotenoid (Fuciman et al. 2012). Yet, in these complexes, carotenoid radicals were observed only after direct excitation of the carotenoid. No cation radicals appeared when Chl (or BChl) was directly excited, suggesting that the observed carotenoid radicals were not related to NPQ. The seminal work that gave rise to a number of studies searching for carotenoid radicals in proteins was published by Holt et al. (2005) and reported a zeaxanthin cation radical signal after exciting directly into the Chl *a* Q_y band of a preparation of thylakoid membranes isolated from *Arabidopsis thaliana*. Most importantly, the magnitude of the zeaxanthin cation radical signal was correlated with the presence of the PsbS protein previously shown to be obligatory for quenching in vivo. These observations led to the proposal that quenching (and thermal de-excitation) of excited Chl *a* occurs via reversible electron transfer from zeaxanthin to Chl *a* (Holt et al. 2005). Subsequent studies identified the minor light-harvesting complexes CP24, CP26 and CP29 as the primary quenching sites (Avenson et al. 2008) with CP29 complex playing a regulatory role in the electron transfer quenching mechanism (Ahn et al. 2008).

The proposal of excited Chl *a* being quenched via reversible electron transfer was also supported by quantum-chemical calculations showing that, for certain zeaxanthin-Chl distances and orientations, the charge-transfer state was characterized by a transfer of electron density from an excited Chl *a* to zeaxanthin significant enough to provide favorable energy for quenching the Q_y state of Chl *a* (Dreuw et al. 2003). On the other hand, the hypothesis of electron-transfer quenching is based on the assignment of the spectrum of the zeaxanthin cation radical in the minor light-harvesting complexes. In view of the strong solvent dependence of the absorption maximum of carotenoid cation radicals (Galinato et al. 2007; Amarie et al. 2009), further experiments have focused on directly generating carotenoid radicals in light-harvesting complexes and examining their effect on quenching. It is known that excitation of a carotenoid by two subsequent excitation pulses of ~100 fs duration can lead to a "ladder"-type excitation process that promotes a carotenoid to a very high excited state resulting in ionization and formation of a carotenoid radical (Papagiannakis et al. 2006). This approach was exploited by Amarie et al. (2009) who directly generated violaxanthin, lutein, and zeaxanthin radicals in isolated LHCII complexes. The results of the latter study showed that the absorption spectrum of the zeaxanthin cation radical in LHCII differs from that observed by Holt et al. (2005) in thylakoid membranes, thus challenging the assignment of the electron-transfer quenching mechanism. Therefore, additional work will be required to reveal the molecular details controlling thermal de-excitation of Chl *a* excited states in isolated complexes and in vivo (see also Walla et al., Chap. 9).

VI Conclusions

More than 20 years of systematic investigation of spectroscopic properties of carotenoids has provided a vast amount of information about the energies and lifetimes of their excited states and the dependence of these parameters on the properties of their environment. An important controlling factor thought to play a role in the dissipation of excess energy during NPQ is the S_1 energy of carotenoids. The characterization of this factor has been hampered by the forbiddenness of the $S_0 \rightarrow S_1$ transition, but this obstacle has been largely overcome by the application of sophisticated spectroscopic methods. Currently, there exist a few methods to determine the S_1 energy of carotenoids in both solution and in proteins with precision that is high enough to assess its relevance to NPQ. Similarly, the dynamics of the processes that occur after photo-excitation of a carotenoid have been reported in great detail. Yet, the precise mechanism of the S_2-S_1 internal conversion and the possible involvement of additional excited states in proximity to S_1 and S_2 remain unresolved. The knowledge gained during the past two decades has clearly shown that further research into the precise roles of carotenoids in the thermal dissipation of excess energy related to NPQ must be directed at understanding how the spectroscopic properties of carotenoids may be tuned by their protein environment. It is now clear that relatively small structural distortions of carotenoids may alter the energies and lifetimes of their excited states. Because distorted carotenoid structures cannot be experimentally isolated and stabilized for spectroscopic studies, quantum chemical computations will be required to provide key information that augments the experimental findings. The same holds true for carotenoid radicals that, so far, have been reported only in a few specific cases for carotenoids bound to proteins. Therefore, the combination of experimental work and quantum computations will be critical to understanding the role of carotenoid radicals in the protection of the photosynthetic apparatus through the process of energy dissipation reflected in NPQ.

Acknowledgments

TP thanks the Grant Agency of the Czech Republic (P501/12/G055) for financial support. Work in the laboratory of HAF was supported by grants from the National Science Foundation (MCB-0913022 and MCB-1243565) and the University of Connecticut Research Foundation.

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Chapter 9

Electronic Carotenoid-Chlorophyll Interactions Regulating Photosynthetic Light Harvesting of Higher Plants and Green Algae

Peter Jomo Walla*
Biomolecular Spectroscopy and Single-Molecule Detection Group, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Department of Biophysical Chemistry, Institute for Physical and Theoretical Chemistry, Technische Universität Braunschweig, 38106 Braunschweig, Germany

Christoph-Peter Holleboom
Department of Biophysical Chemistry, Institute for Physical and Theoretical Chemistry, Technische Universität Braunschweig, 38106 Braunschweig, Germany

and

Graham R. Fleming*
Department of Chemistry, University of California, Berkeley, CA 94720, USA

Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Summary

Electronic interactions between chlorophylls (Chl) and carotenoids (Car) play a major role in the regulation of photosynthetic light harvesting of higher plants and green algae. In this contribution, we discuss different electronic regulation models that are based on vari-

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*Author for Correspondence, e-mail: pwalla@gwdg.de; fleming@cchem.berkeley.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_9,

ous Chl-Car interactions, leading to switching between efficient light harvesting and thermal dissipation of excess energy. Most regulation models are based on three types of electronic Chl-Car interactions: direct Chl → Car S_1 energy transfer, quenching by Car radical cations and bidirectional quenching processes. We will give an overview of the observations that have been made in our as well as other laboratories supporting one or the other model and discuss possible scenarios that may provide a unified picture considering all three types of regulation models.

I Introduction

A consensus exists that interactions between chlorophylls (Chl) and carotenoids (Car) play a major role in the regulation of photosynthetic light harvesting in the antenna of photosystem II of higher plants and green algae (Demmig-Adams 1990; Young and Frank 1996; van Amerongen and van Grondelle 2001; Holt et al. 2004; Ruban et al. 2007; Holleboom and Walla 2014). However, the precise nature of these interactions, how they are triggered, which pigments are actually involved, where the site of dissipation is located and how the confluence of these factors leads to thermal dissipation of excess excitation energy are still debated. In this contribution, we will describe electronic interactions between Chls and Cars that lead to switching between efficient light harvesting and thermal dissipation of excess energy. Several different mechanisms have been proposed, and it is not unlikely that more than one mechanism contributes to the switching.

Among the first mechanisms to be proposed were those based on Coulombic energy transfer from the lowest Chl states to the optically forbidden, short lived, first excited singlet state of Cars (Demmig-Adams 1990; Frank et al. 1994). At first glance, this would seem an unlikely mechanism since the simplest resonance energy transfer models based on a point dipole approximation suggest a rate of zero for such an energy transfer. However, models based on the spatial extent of the transition density in Car and Chl molecules show that ultrafast (~10^{11} s^{-1} to 10^{12} s^{-1}) energy transfer rates via Coulombic coupling are entirely possible for such molecular pairs (Hsu et al. 2001). Many suggested mechanisms consider pure excitation-energy transfer to Cars a major thermal dissipation mechanism, and are based on different triggering processes, such as conformational changes within pigment-protein complexes (Ruban et al. 2007) or protein-protein interactions caused by membrane reorganizations (see also, e.g., Ruban and Mullineaux, Chap. 17). A second possible mechanism was revealed by the observation that formation of Car radical cations is correlated with the magnitude of the rapidly reversible component of quenching of the singlet-excited state of Chls (assessed as non-photochemical quenching of Chl fluorescence, e.g., as the coefficient of "energy-dependent" quenching, qE) in isolated intact thylakoids (Holt et al. 2005). More recently, it was observed that the onset of non-photochemical quenching of Chl fluorescence in living plants is related to increased energy transfer between Chl and Car in both directions (Bode et al. 2009; Holleboom and Walla 2014). While this finding provided further evidence that elec-

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Abbreviations: BChl – Bacteriochlorophyll; Car – Carotenoid; Chl – Chlorophyll; CP24, 26, 29 – Minor chlorophyll proteins associated with Photosystem II; DB – Double bond; ESA – Excited state absorption; F_m, F_m' – Maximal chlorophyll fluorescence in the limiting-light- and excess-light-state, respectively; F^{OPE}, F^{TPE} – Chlorophyll fluorescence upon one-photon and two-photon excitation, respectively; g – Gerade symmetry; LH2 – Light-harvesting complex 2 of purple bacteria; LHCII – Light-harvesting complex II of higher plants and algae; lut2 – Lutein-deficient mutant of *Arabidopsis thaliana*; NPQ – Non-photochemical quenching; npq1, 2, 4 – Mutants of *Arabidopsis thaliana* (1, no zeaxanthin; 2, enriched zeaxanthin; 4, no PsbS); PAM – Pulse-amplitude modulation; PS I, PS II – Photosystem I and II, respectively; PsbS – 21-kDa photosystem II protein subunit; qE – Energy-dependent chlorophyll fluorescence quenching; $S_{0, 1, 2, n}$ – Singlet energetic states (0, ground state; 1, 2, n, excited states); u – Ungerade symmetry; V – Violaxanthin; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin, and zeaxanthin; VDE – Violaxanthin de-epoxidase enzyme; wt – Wild type; Z – Zeaxanthin; ZEP – Zeaxanthin epoxidase enzyme tronic interactions between Cars and Chls play an important role, the observation of a bidirectional energy flow was puzzling. Nonetheless, this observation of two-way energy flow clearly indicates an effective connection of Chl excitation to a short-lived, dissipating Car state. In this chapter, we give an overview of the observations made in our own laboratory as well as others and discuss possible scenarios that may provide a unified picture considering all three types of regulation models.

In addressing the mechanism of the regulation of photosynthetic light-harvesting activity, two major aspects have to be discussed: (1) What are the structural changes connected to this regulation, and (2) what are the electronic processes that change during regulation. Both aspects comprise many different levels of complexity, which is one important reason why there is still no clear picture about the exact mechanism of this regulation with its vital importance for survival and fitness of photosynthetic organisms (Demmig-Adams and Adams 2002). Structural changes range from local conformations of individual atoms up to a rearrangement of the locations and interactions of pigment-protein complexes within the thylakoid membrane. These changes are most likely initiated by a trans-membrane proton gradient generated by photosynthetic electron transport (Strand and Kramer, Chap. 18). These structural changes, in turn, lead to new charge-transfer and energy pathways involving both Chl and Car molecules. While for many biological processes structural aspects are regarded as most essential, in the case of photosynthetic energy conversion as well as thermal dissipation of excess energy, an understanding of the electronic interactions between pigments is equally significant.

II Spectroscopic Observations

Significant complexity is introduced into spectroscopic observations of Cars and their interactions with Chls by the fact that the first excited singlet state of Cars (S_1) has the same approximate A_g symmetry as the Car ground state (S_0) and optical transition from S_0 to S_1 is thus forbidden by symmetry in one-photon absorption (Friedrich 1982). Neither conventional absorption nor fluorescence spectroscopic methods can therefore probe the transition from S_0 to S_1. Strong optical transitions are observed for $S_0 - S_2$, and $S_1 - S_n$ (where S_n denotes a higher lying singlet state) absorptions that involve g → u (“gerade” to “ungerade”) symmetry changes. The $S_0 \rightarrow S_1$ transition is allowed in two-photon absorption, which has been exploited in a number of studies (Shreve et al. 1990; Walla et al. 2000a, b, c, 2002; Shima et al. 2003; Wehling and Walla 2005; Bode et al. 2009). The strong $S_0 \rightarrow S_2$ transition is responsible for the intensive colors of Cars. The electronic interactions and associated dynamic processes occurring between Chls and Cars in light-harvesting systems have been probed by a wide variety of spectroscopic methods (Frank et al. 1994; van Amerongen and van Grondelle 2001; Holt et al. 2005; Ruban et al. 2007; Ahn et al. 2008; Avenson et al. 2008; Bode et al. 2009; Liao et al. 2010). These experiments have led to the proposal of at least four types of Chl-Car interactions that could be responsible for quenching Chl excitations. These interactions include, (1) energy transfer from Chl states to the short-lived first excited states of Car, Car S_1 (Frank et al. 1994; Ruban et al. 2007), (2) formation of charge-transfer states between Chls and Cars resulting in Car radical cations (Holt et al. 2005; Ahn et al. 2008; Avenson et al. 2008), (3) formation of mixed excitonic states between Chl states and the first excited states of Cars (van Amerongen and van Grondelle 2001; Bode et al. 2009; Liao et al. 2010), and (4) electronic interactions between two Chl molecules (Miloslavina et al. 2008; Holzwarth et al. 2009). All of the latter interactions have the potential to dissipate excess energy as heat, thereby preventing excess energy transfer to the reaction center Chls and/or excess excitation energy migrating throughout the antenna complexes, possibly leading to the formation of Chl triplet states that can generate damaging singlet oxygen. In the following, we will describe in more detail what spectroscopic observation can be connected to the presence or change of Chl-Car interactions during regulation of photosynthetic light harvesting, and how these interactions may result in quenching of the first excited state of Chl.

One of the most straightforward possibilities for an electronic regulation mechanism is interaction (1) above, in which energy transfer from the lowest Chl excited state (Chl Q_y) occurs to the lowest Car singlet state (Car S_1). The lowest excited singlet states of both pigments are of very similar energy (Walla et al. 2000c; Polívka et al. 2002; Polívka and Sundström 2004). Thus, subtle changes in the arrangement, environment or conformation of one or both pigments could regulate energy flow between these (Frank et al. 1994; Ruban et al. 2007). Since the transition from the electronic ground state to the first excited state of Cars is optically forbidden, one of the first questions that needed to be answered was whether or not effective energy transfer to Car S_1 from Chl S_1 (Q_y) is possible and, if so, how fast it occurs. As noted in the introduction, the latter picture seemed unlikely until a more realistic view of the spatially extended nature of the electronic transition density was available. In addition, electron exchange mechanisms (often called Dexter energy transfer; Dexter 1953) are unable to result in the observed rapid rates of transfer, partly because of the double excitation character of the Car S_1 state. Introduction of the transition-density-cube method by Krueger, Scholes and co-workers (Krueger et al. 1998a; Scholes et al. 1999) allowed quantitative employment of the spatial extent of transition densities in conjunction with structural information from x-ray crystallography and electronic structure calculations via quantum chemistry methods. The latter theoretical approach was soon supported by a wide range of experimental evidence (Krueger et al. 1998b; Walla et al. 2000b; Hsu et al. 2001). Car S_1 → Chl energy transfer time scales observed were typically a few ps and sometimes as short as in the fs-range (Walla et al. 2000b, c; Wehling and Walla 2005). Depending on the direction of energy transfer (Chl → Car S_1 or Car S_1 → Chl), two techniques provided this evidence (Fig. 9.1), i.e., (1) pump-probe spectroscopy via excitation of both the lowest (Q_y) and higher Chl states, followed by subsequent observation of the very prominent transient absorption signal Car S_1 → Car S_n ($\lambda_{ESA} \sim 550$ nm) (Ma et al. 2003; Liao et al. 2010), (2) two-photon spectroscopy allowing selective population of the Car S_1 state and subsequent observation of Chl fluorescence (Walla et al. 2000a, b, c, 2002; Wehling and Walla 2005; Bode et al. 2009; Liao et al. 2010, 2011). Chl fluo-

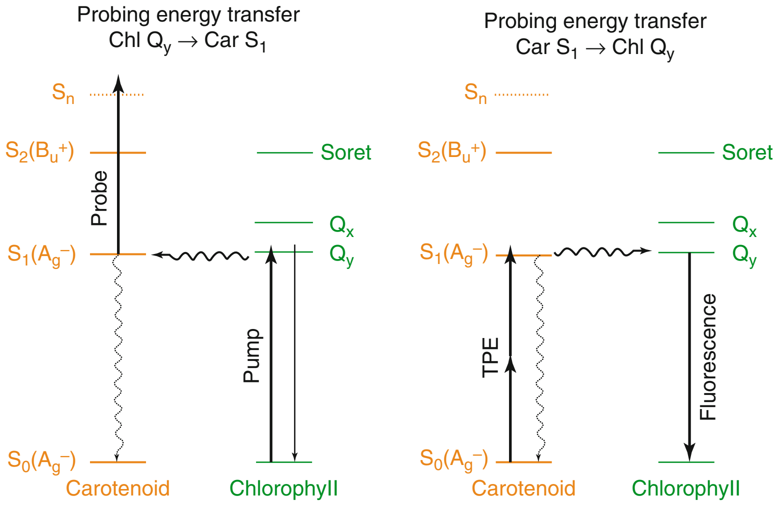

Fig. 9.1. Two experimental techniques proving energy transfers between carotenoids and chlorophylls. *Left*, Pump-Probe or transient absorption experiments measuring the excited state absorption of the dark Car S_1 state after excitation of Chls provide evidence for Chl Q_y → Car S_1 energy transfer; *right*, observed Chl fluorescence after selective two-photon excitation of the dark Car S_1 state is a measure for Car S_1 → Chl Q_y energy transfer.

rescence observable after two-photon excitation of the Car S_1, but not observable with two-photon excitation of pure Chl (Shreve et al. 1990; Walla et al. 2000c), is a very clear measure for the presence and extent of Car S_1 → Chl energy transfer in Chl-Car complexes.

Based on the latter two-photon approach, the first evidence for effective Car S_1 → Chl energy transfer was observed in isolated light harvesting complex 2 of purple bacteria (LH2) and light-harvesting complex II of higher plants (LHCII) as well as in thylakoid membranes (Shreve et al. 1990; Krueger et al. 1999; Walla et al. 2000c). Shreve and co-workers (1990) observed a two-photon excitation spectrum in thylakoid membranes showing a band with many characteristics of Car spectra. Later, two-photon spectra observed in well defined, isolated pigment-protein complexes allowed characterization of vibrational bands that fit very well with the vibrational energies known from the Cars in solution (Krueger et al. 1999; Walla et al. 2002; Shima et al. 2003; Hilbert et al. 2004). For LH2 from *Rhodobacter sphaeroides*, an energy-transfer time constant from the Car spheroidene to bacteriochlorphyll (BChl) of 1.9 ± 0.5 ps was observed (Walla et al. 2000b), and in the case of PS I, an energy-transfer time constant from β-carotene to Chl *a* of 800 fs was found (Wehling and Walla 2005). In LHCII, an extremely short time constant of 250 ± 50 fs was observed (Walla et al. 2000c), which was explained by the large number of accepting Chls in the vicinity of the Cars. These studies provide clear evidence that the optically forbidden Car S_1 state can couple effectively to the lowest Chl excited singlet states when the molecules are in close spatial proximity.

III Mechanisms of Non-Photochemical Quenching

A Direct Chl → Car S_1 Energy Transfer and the Molecular Gear Shift Mechanism

As mentioned above, one of the first models proposed to describe the non-photochemical quenching of Chl fluorescence (NPQ, as an indicator for de-excitation of the Q_y singlet-excited state of Chl) was the molecular gear-shift model based on energy transfer between the Car S_1 state and the Chl Q_y state (Frank et al. 1994). Biochemical evidence has shown that, during adaptation from low or moderate light intensities to high light intensities, foliar xanthophyll pools are converted from violaxanthin (V) through antheraxanthin to zeaxanthin (Z) by the enzyme violaxanthin de-epoxidase (VDE) as part of the VAZ cycle (Fig. 9.2a; Yamamoto 1979). During this conversion, the number of conjugated double bonds (DB) increases from 9 in violaxanthin to 11 in zeaxanthin. In limiting light, the process is reversed by the enzyme zeaxanthin epoxidase (ZEP; Yamamoto 1979). Electronic transition energies usually decrease with increasing conjugation length, leading to the very appealing idea that the Car S_1 state of violaxanthin might have an energy level just above the lowest Chl states, thus contributing to light-harvesting via Car S_1 → Chl energy transfer, whereas the Car S_1 state of zeaxanthin might have an energy level below that of Chl, resulting in effective Chl → Car S_1 energy transfer with subsequent dissipation of excess energy via very fast Car S_1 → Car S_0 internal conversion (Frank et al. 1994).

However, the latter model was soon questioned based on biochemical as well as spectroscopic arguments. Formation of zeaxanthin by VDE takes place on a significantly longer timescale than the rise time of the rapidly-developing energy dissipation (measured as qE or NPQ) observed in plants on a seconds to minutes time scale (Nilkens et al. 2010). Similarly, when leaves are returned to low light conditions from high light, zeaxanthin concentration decreases more slowly than the drop in NPQ. The time scale of VDE-dependent zeaxanthin formation indicates that the latter can only account for adaptation processes occurring at longer times. An alternative explanation would be that, even though a full transformation of all violaxanthin takes minutes, the zeaxanthin generated in the first seconds are sufficient to act as a trap for the excitation energy of the entire pigment pool in a

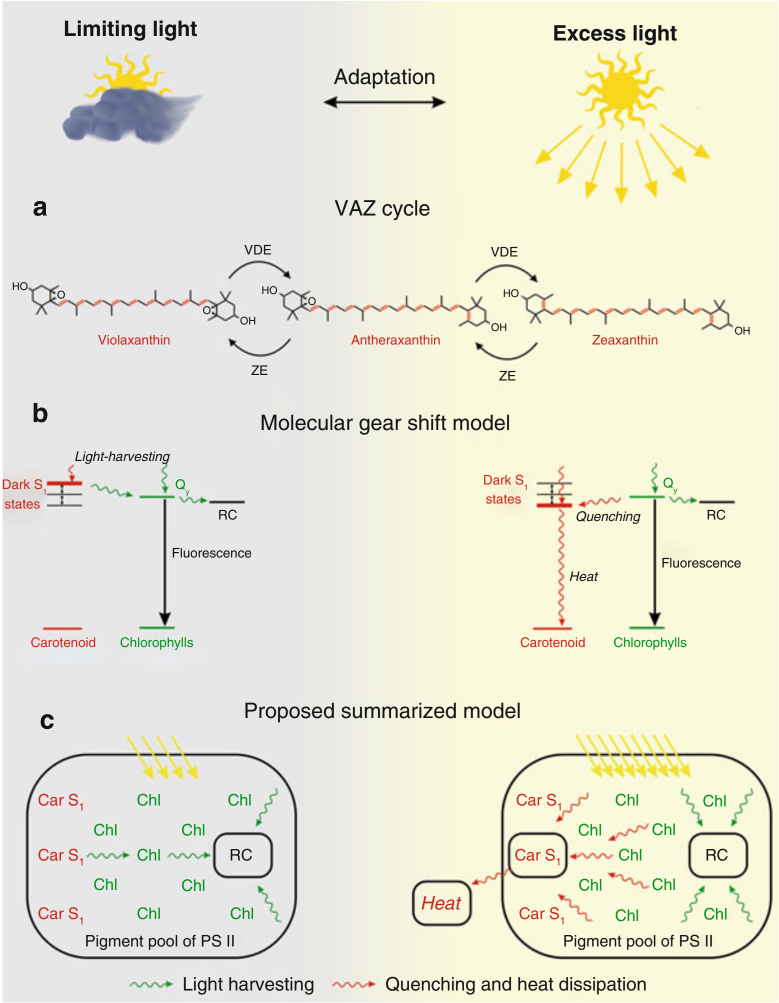

Fig. 9.2. Molecular gear-shift model. De-epoxidation of the VAZ cycle Car violaxanthin provides a possibility for plants to engage thermal energy dissipation under excess light conditions. Triggered by light intensity, the VAZ cycle Cars violaxanthin and zeaxanthin are interconverted by the enzymes violaxanthin de-epoxidase and zeaxanthin epoxidase via the intermediate xanthophyll antheraxanthin. This reversible conversion is termed the VAZ cycle (**a**). Violaxanthin contributes to light-harvesting in the limiting-light state of plants, whereas the Car S_1 state (of zeaxanthin), lowered by elongation of the conjugated double bond system, provides a quenching site for excess energy in the excess light state (Frank et al. 1994) (**b**). Summarizing, the VAZ cycle Cars, as part of the pigment pool of PS II and light-harvesting proteins, support effective light harvesting when available light is limiting to photochemistry and enable energy dissipation as harmless heat to avoid damage to photosynthesis by excess light (**c**).

similar fashion as one reaction center special pair traps the energy of ~300 pigments for photosynthesis (Fig. 9.2c). In Chap. 24 it is also described that plants in nature pre-form zeaxanthin for subsequent very rapid engagement and disengagement of NPQ, while zeaxanthin-free leaves do not show any rapid deep quenching. Chapter 24 also shows, however, that V to Z conversion cannot be the only requirement for development of energy dissipation assessed as NPQ.

Most likely, there are at least two steps involved in the regulation, e.g., a fast regulation connected to the pH gradient and a slow one connected to the VAZ cycle. Such a two-step regulation process may be necessary to provide the level of fine control that appears to be advantageous in nature to protect the high photon yield of photochemistry in intact leaves whenever light is limiting. A spectroscopic argument against the conversion from V to Z as an explanation for the whole regulation was provided by the observation that all three Cars have – at least in solution – an S_1 energy level well below that of the lowest Chl states (Polívka et al. 1999, 2002; Frank et al. 2000). On the other hand, the transition energies of the various xanthophylls in their native protein environment may be significantly different from what has been established in vitro, and it is difficult to assess which energy state is really present at a potentially sparse Car-quenching site in the intact organism (see Chap. 24 for a discussion of NPQ in natural systems). A further complication is that the excited-state potential surface for S_1 of Cars may contain more than one energy minimum, such that arguments comparing Q_y and S_1 energies may be oversimplified (Pang et al. 2009, 2010).

Even if a simple molecular gear-shift mechanism is ruled out, other very reasonable possibilities exist for a quenching mechanism based on simple Chl → Car S_1 energy transfer. The observation of Chl fluorescence after selective Car S_1 two-photon excitation (Walla et al. 2000a, b, c, 2002; Bode et al. 2009; Liao et al. 2010) provides evidence for energy transfer in the direction Car S_1 → Chl and for non-negligible levels of electronic coupling involving Car S_1. As noted earlier, energy transfer in the opposite direction, Chl → Car S_1, can be investigated by observation of the transient absorption signal Car S_1 → Car S_n at around 550 nm after one-photon excitation of the lowest Chl states. Such transient absorption was used to identify Car S_1 in quenched thylakoid preparations (Ma et al. 2003). In the latter work, changes in electronic interactions were observed during the regulation process in thylakoids. Based on such studies, it has been suggested that even Cars other than those involved in the VAZ cycle dissipate excess energy as a result of, for example, conformational changes within the pigment-protein complexes. However, zeaxanthin-free intact leaves never show quenching as deep as plants under excess light in nature. Ruban et al. (2007) used a time-resolved transient absorption approach to provide a measure of the population of Car S_1 in quenched LHCII samples compared to unquenched samples. Stronger S_1 – S_n absorption was observed in the quenched samples. The latter study also used resonance Raman experiments to show changes in the region of Car vibrations indicating a conformational change as the origin for increased energy flow from Chl to Car S_1. Based on the latter transient absorption spectra and resonance Raman data, lutein in LHCII was proposed as the key player for this dissipation channel (Ruban et al. 2007). Thus, a conformational change in the LHCII complex was proposed to be changing the electronic coupling and energy transfer between this Car and neighboring Chls in a way that switches on thermal energy dissipation (Fig. 9.3). It is still a matter of debate as to what triggers this conformational change. The overall thermal dissipation process is triggered by development of a pH gradient across the thylakoid membrane, but the details of subsequent changes in both membrane organization and internal pigment-protein structure are not yet clear. In the model by Ruban et al. (2007), zeaxanthin is not directly involved in the quenching, but serves as an allosteric regulator of the quenching by increasing the pH sensitivity of qE (Horton et al. 2005; Johnson et al. 2009). Other authors propose interactions at the periphery of trans-membrane proteins such as LHCII and the photosystem II protein PsbS [21-kDa photosystem II protein subunit] (Barros et al. 2009; Fig. 9.4).

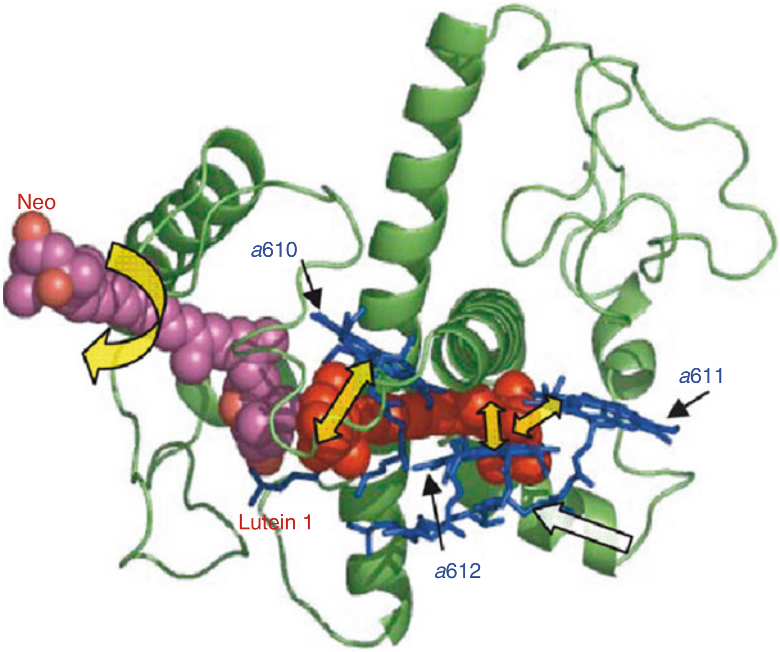

Fig. 9.3. Molecular mechanism proposal of energy quenching qE by conformational changes in the pigment-protein complex LHCII (Ruban et al. 2007). A structural model of an LHCII monomer is illustrated containing photosynthetic pigments that contribute to the proposed mechanism of qE. Neoxanthin (*pink*) and Lutein (*red*) are shown positioned very closely to the Chls *a* 610, 611 and 612 (*blue*, *black arrows*). The *broad arrows* visualize possible conformational changes supporting energy transfer to lutein followed by excess energy dissipation, i.e., a twist of neoxanthin (*curved yellow arrow*) and a movement of lutein towards the Chl cluster (*white* and *yellow arrows*) (Reprinted with permission from Macmillan Publisher Ltd: *Nature* (Ruban et al. 2007), copyright 2007).

B Quenching by Carotenoid Radical Cations

Based on theoretical work by Dreuw, Head-Gordon and Fleming, another potential electronic mechanism was identified – quenching of excess excitation energy by charge transfer between Cars and Chls (Dreuw et al. 2003a, b; Fig. 9.5). The results obtained by the latter group indicated that the pair Z-Chl has a much-improved capability for electron transfer than other possible Car-Chl combinations. Subsequently, Holt et al. (2005) found clear transient absorption signatures in quenched thylakoids that are specific for radical cations and fit the spectra of radical cations of the corresponding Cars isolated in solution. In the latter study, whole thylakoids were investigated during adaption to high as well as low-light conditions. A later inspection in different isolated pigment-protein complexes indicated that the Car zeaxanthin in minor complexes such as CP29 makes dominant contributions to the Car cation spectroscopic signal (Avenson et al. 2008; Cheng et al. 2008). One potential problem with a charge-transfer quenching mechanism is that the amount of zeaxanthin cation generated in isolated minor complexes (CP29, CP26 and CP24) is quite small. Although the signals observed in intact membranes are consistent with much larger amounts of charge transfer, quantitative estimates are more difficult in whole-membrane samples. In mutant plants that lack zeaxanthin, qE is partly restored by accumulation of larger amount of lutein than present in wild-type plants (Li et al. 2009). In this case,

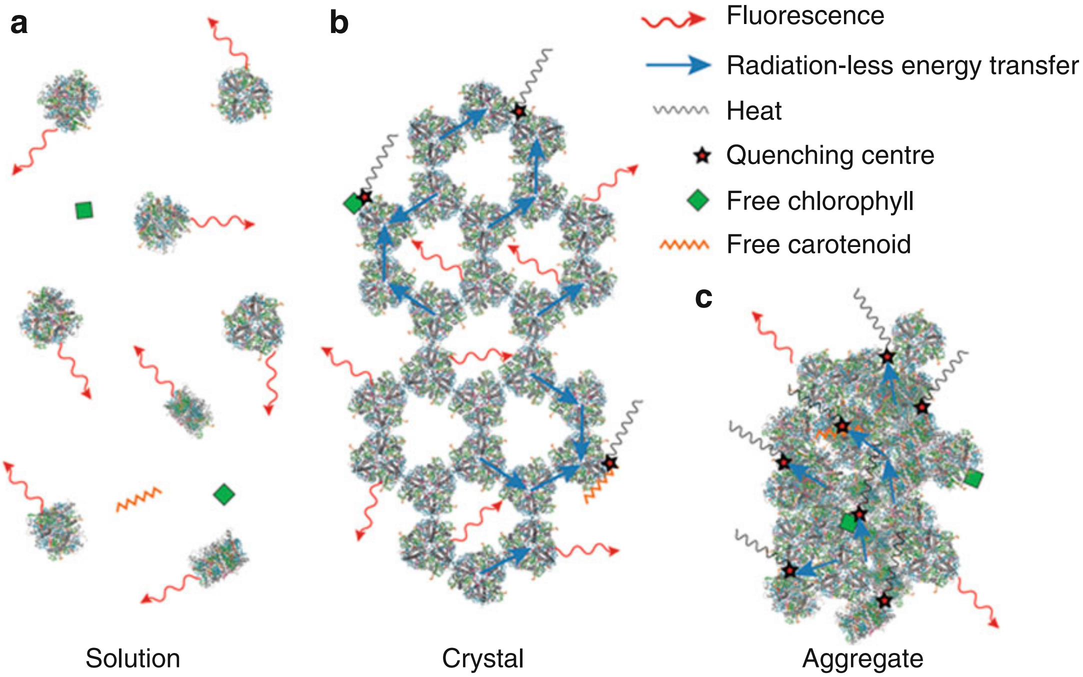

Fig. 9.4. Model of LHCII trimers in vitro, forming quenching centers at the protein periphery upon aggregation (Barros et al. 2009). In detergent solution, functionally uncoupled LHCII trimers act as single fluorescent units after excitation (**a**). Crystalized LHCII trimers generate some quenching centers with each other or contaminating free pigments, which enables partial dissipation of the excitation energy as heat (**b**). A higher amount of such quenching centers are formed in randomly aggregated LHCII trimers, causing the typically observed strong Chl fluorescence intensity and lifetime reduction (**c**) (Reprinted with permission from Macmillan Publisher Ltd: *The EMBO Journal* (Barros et al. 2009), copyright 2009).

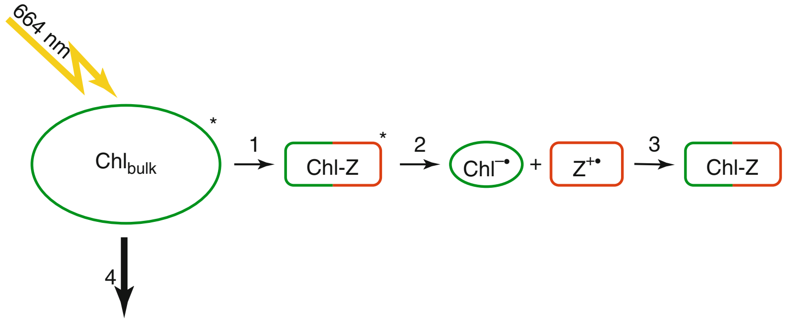

Fig. 9.5. Energy-quenching mechanism based on formation of a Chl-Car charge-transfer state and resulting Car radical cation (Holt et al. 2005). Starting with light absorption by bulk Chl in the Chl Q_y band (664 nm), energy is partly transferred from excited Chls to a quenching Chl-Z pair (*1*). Subsequent relaxation of the excited (Chl-Z)* leads to charge-separated chlorophyll anion and zeaxanthin cation (*2*). Charge recombination and corresponding energy dissipation restore the unexcited Chl-zeaxanthin pair (Adapted from Holt et al. 2005).

lutein radical cations can be detected in CP24 and CP29 containing only lutein (Li et al. 2009). Lutein is also the main source of the Car radical signal found in the pigment-binding protein LhcSR3 of algae, and lutein has thus been implicated in the regulation of light harvesting in these species (Bonente et al. 2011).

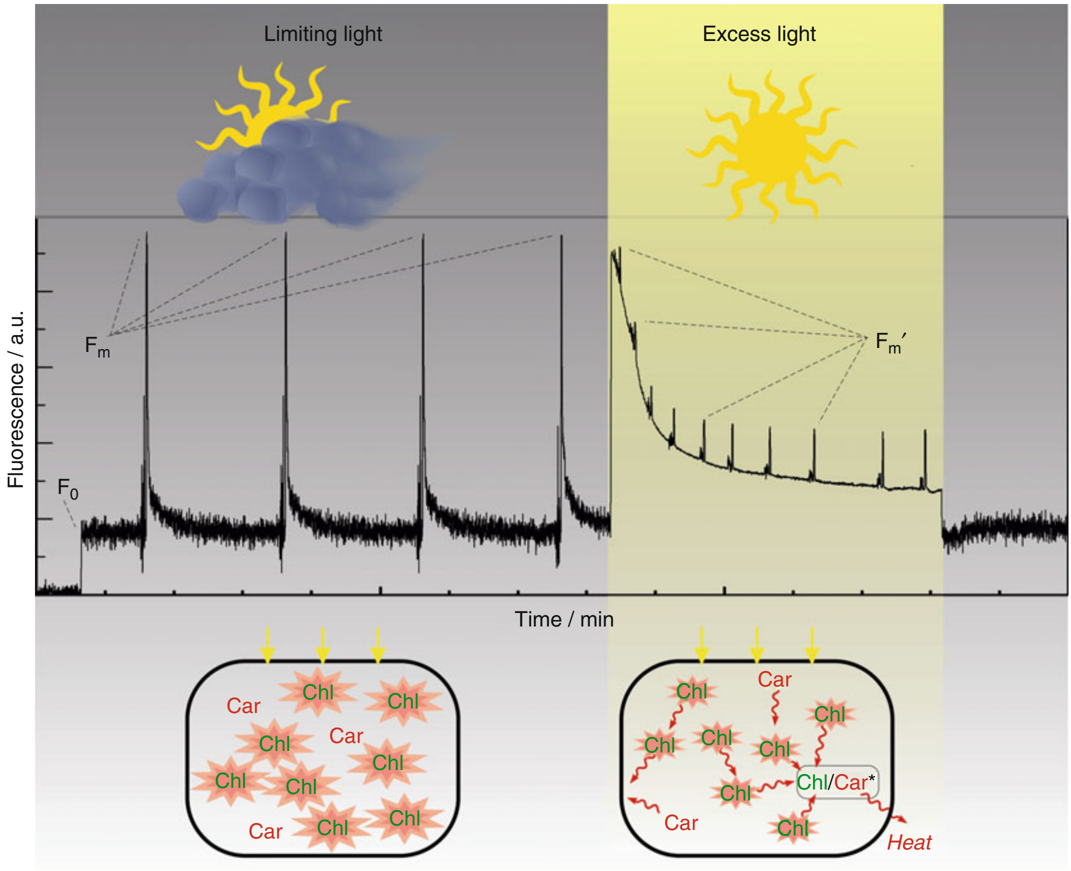

Fig. 9.6. Chl fluorescence measurements indicate the extent of remaining excitation energy not quenched via NPQ. A typical pulse-amplitude modulation (*PAM*) Chl fluorescence measurement is shown that covers the limiting-light state of a plant (*left side*) and the response to excess-light conditions (*right side*). In this technique, modulated light pulses (594 nm) are used to measure maximal fluorescence (F_m) of the investigated pigment pool. The average intensity of the measuring light is chosen to remain too weak to cause high-light responses. A second light source (*actinic light*) provides excess light, during which the plant's response is monitored. During this response to excess light, maximal fluorescence yield (F_m') of the pigment pool is significantly decreasing, as can be seen in the right part of the figure. This quenching is caused by dissipation of excitation energy as heat at a non-photochemical quenching site. The same approach can also be taken with two-photon excitation of Car S_1. Chl fluorescence observed after selective two-photon excitation of Car S_1 directly gives information about the extent of Car S_1 → Chl energy transfer, even in intact plants exposed to excess light.

C Bidirectional Energy Quenching Mechanisms

Measurement of Chl fluorescence is less sensitive to light scattering by the photosynthetic membrane than transient absorption spectroscopy. The level of Chl fluorescence (calculated as NPQ) observed from intact plants is used as an indicator of the activity of the thermal dissipation mechanisms regulating photosynthetic light harvesting (Fig. 9.6; Schreiber 1986; Lichtenthaler et al. 2005). However, this latter approach does not allow inspection of changes in energy transfer between Car S_1 and Chl as do transient absorption approaches. Therefore, single-photon excitation of Chl fluorescence as an indicator of energy dissipation has been combined with specific two-photon excitation of Car S_1 in intact plants to determine changes in electronic Car S_1 ↔ Chl interactions during the regulation of light harvesting (Bode et al. 2009).

As mentioned previously, Chl fluorescence observed after specific Car S_1 two-photon excitation is a direct indication of Car $S_1 \rightarrow$ Chl energy transfer. An increase in this Chl fluorescence signal compared to the Chl fluorescence observed after direct Chl excitation clearly indicates an increase in the Car $S_1 \leftrightarrow$ Chl interactions of the entire Car and Chl pool during thermal energy dissipation. A significant increase in Car $S_1 \rightarrow$ Chl energy transfer was indeed observed when plants were exposed to high light (Fig. 9.7). In addition, comparison of PsbS-overexpressing mutants (with more NPQ than wild-type plants) and PsbS-deficient mutants (deficient in rapidly reversible NPQ) showed a clear correlation between the extent of NPQ in plants exposed to high versus low light, and the increase in Car $S_1 \rightarrow$ Chl energy transfer. This finding provided evidence from intact plants for a clear correlation between the electronic interactions of Cars with Chls and NPQ (Bode et al. 2009).

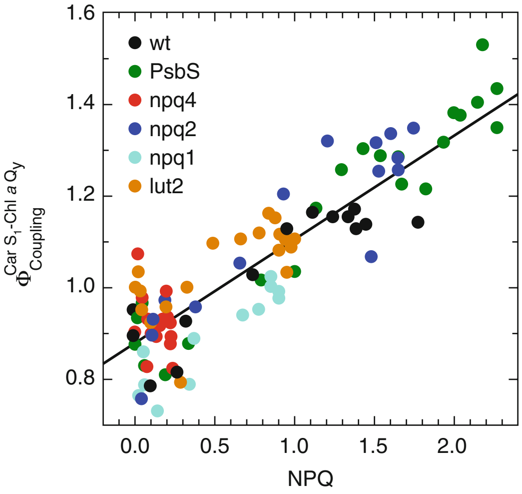

Fig. 9.7. Correlation of energy transfer Car $S_1 \rightarrow$ Chl Q_y and NPQ (as a measure of the level of thermal energy dissipation). The figure shows the results of Chl fluorescence measurements after selective Car two-photon excitation (compare right method in Fig. 9.1) in various mutants and wild type of *Arabidopsis thaliana* during exposure to various levels of excess light. Car $S_1 \rightarrow$ Chl Q_y transfer is represented by a coupling parameter Φ_{Coupling} calculated from fluorescence data after Car S_1 two-photon excitation F^{TPE} and after Chl one-photon excitation F^{OPE}($\Phi_{\text{Coupling}} = F^{\text{TPE}}/F^{\text{OPE}}$) (Bode et al. 2009). NPQ levels are determined from the F^{OPE} data. Some of the investigated NPQ mutants have a higher adaptation capability (PsbS, *green*, overexpressed PsbS; npq2, *blue*, enriched zeaxanthin). Other mutants lack important quenching factors (npq1, *cyan*, no zeaxanthin; npq4, *red*, no PsbS). Additionally, in lut2 (*orange*) lutein is replaced by other Cars. All data together provide a clear correlation between increasing Car-Chl interaction or Car $S_1 \rightarrow$ Chl Q_y energy transfer with increasing non-photochemical quenching (Reprinted with permission from Bode et al. 2009. Copyrights (2009) National Academy of Science, USA).

However, surprisingly, increased Car $S_1 \rightarrow$ Chl energy transfer was observed under high-light conditions even though dissipating Chl $\rightarrow$ Car S_1 energy transfer had been instead expected in the simplest scenario. Investigation of many different systems, from simple Car-tetrapyrrole model compounds (Kloz et al. 2011; Liao et al. 2011) to light-harvesting complexes (Liao et al. 2010) and entire photosynthetic membranes (Ma et al. 2003; Bode et al. 2009), provides evidence that increased energy transfer in *both* directions, i.e., Car $S_1 \rightarrow$ Chl as well as Chl $\rightarrow$ Car S_1, is generally observed in quenched (dissipating) samples (Holleboom and Walla 2014). This finding can be explained in two ways. A first explanation was proposed based on the additional observation of red-shifted absorption (Liao et al. 2010) and Chl fluorescence spectra (Johnson and Ruban 2009), as well as almost instantaneous Chl $\rightarrow$ Car S_1 energy transfer rates (Ma et al. 2003; Liao et al. 2010; Kloz et al. 2011) in quenched samples. These observations, together with simultaneous bidirectional energy transfer, Car $S_1 \leftrightarrow$ Chl, can be easily explained by the occurrence of excitonic interactions between the two states. Such interactions can occur when the two state energies are similar and there is significant electronic coupling. Both of the latter conditions apply to the Car S_1 state and the lowest excited singlet states of Chl *a*, Chl Q_y (Bode et al. 2009). Under these conditions, the same Coulombic coupling that enables ultrafast Car $S_1 \leftrightarrow$ Chl Q_y energy transfer will also create mixed Car/Chl exciton states. The coupling creates two new states at least partially delocalized over both pigments and sharing some characteristics of the original uncoupled states.

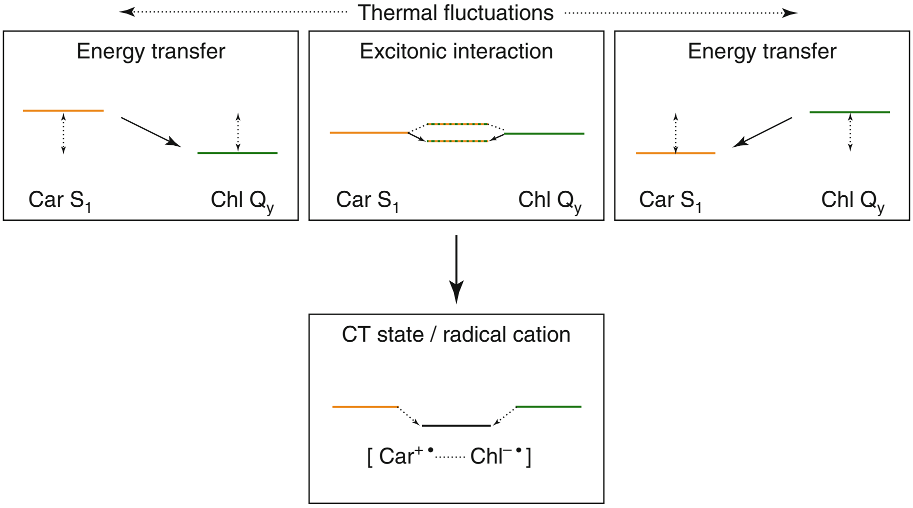

Fig. 9.8. Model suggestion for the interconversion of possible quenching Car S_1 – Chl Q_y interactions. Excitonic interactions might present an intermediate situation between the Förster-like energy transfers Car S_1 → Chl Q_y and Chl Q_y → Car S_1 during thermal energy fluctuations. If the energy states of Car S_1 and Chl Q_y are close, excitonic states can be formed. Additionally, these excitonic states might act as a precursor for Chl-Car charge-transfer states containing a Car radical cation (see text).

In the case of identical uncoupled energies, the two states are separated by two times the electronic coupling, J. The electronic-structure calculations of Dreuw et al. (2003a) suggest that the lower state of the exciton pair created from Chl Q_y and Car S_1 coupling would be dominated by the Car molecule. In this case, a ready quenching mechanism is available, even without charge transfer. The S_1 lifetime of Cars is significantly shorter than that of Chl Q_y (20 ps vs. 1–5 ns) (Ide et al. 1987; Polívka and Sundström 2004), and the excitation of the lower exciton state will thus rapidly decay to the ground state by internal conversion. Even a single Chl/Car excitonic pair, if well connected to the rest of the antenna system, could serve as an effective trap since the excitation diffusion length in photosystem II is at least as large as the size of a photosystem II super-complex (Bennett et al. 2013).

However, the picture described above is a static one, while, in reality, the relative energy levels of Chls and Cars will fluctuate as a result of the nuclear motions of the protein. Thus, all three schemes shown in Fig. 9.8 may occur at different times in the same Chl-Car pair (Liao et al. 2012; Holleboom and Walla 2014). Rapid fluctuation between Chl → Car and Car → Chl energy transfer as a result of thermal fluctuations of the protein environment, leading to an equilibration between Chl Q_y and Car S_1, will also produce quenching proportional to the fraction of time the system spends in the Car S_1 state. Indeed, the excitonic model and the kinetic model just described essentially differ only with respect to time scale.

D A Unifying Model

Extending these ideas slightly, Chl → Car energy transfer can be viewed as a necessary precursor to charge transfer, which may or may not occur depending on the specific protein environment. The scheme in Fig. 9.8 explains, at least qualitatively, many of the experimental observations. Both the observation of increased Chl → Car S_1 and of inversed Car S_1 → Chl energy transfer, when Chl fluorescence is quenched during exposure to high light, can be explained by this picture. The model also explains the reduction of Chl fluorescence life-times (van Amerongen and van Grondelle 2001; Johnson and Ruban 2009) and the occurrence of various red-shifted

Chl and Car absorptions as well as fluorescence bands (Bode et al. 2009; Johnson and Ruban 2009; Liao et al. 2010) during exposure to high light. Finally, the observation of spectroscopic signals for Car radical cations (Holt et al. 2005) is also explained. What is not yet clear, however, is how much of the quenching observed in intact plants arises from what type of Chl-Car electronic interactions, and how these interactions are modulated in response to light conditions. It is also not clear whether different triggering mechanisms may apply to different quenching mechanisms, with the latter possibility hinted at by recent studies of algal adaptation to high light (Amarnath et al. 2012). Similarly, the role of Car-Chl interactions in various proposed structural rearrangements that may contribute to NPQ, such as LHCII aggregation, state transitions and other membrane restructuring, is not yet clear. To definitively answer these questions, a quantitative study is needed that directly assesses the spectroscopic signatures of all suggested electronic interactions in a single system.

Acknowledgments

PJW acknowledges support from the German Science Foundation (DFG) and the Fonds der Chemischen Industrie. GRF's work was supported in its entirety by the Director, Office of Science, Office of Basic Energy Sciences, Chemical Sciences, Geosciences & Biosciences Division, of the U.S. Department of Energy under Contract DE-AC03-76SF00098.

We thank K. Amarnath and E. J. Sylak-Glassman for their help in preparing the manuscript.

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Chapter 10

Antenna Protein Conformational Changes Revealed by Resonance Raman Spectroscopy

Andrew A. Pascal
Institut de Biologie et de Technologies de Saclay (iBiTec-S),
CEA Saclay, 91191 Gif-sur-Yvette, France

Alexander V. Ruban
School of Biological and Chemical Sciences,
Queen Mary University of London, Mile End Road, London E1 4NS, UK

and

Bruno Robert*
Institut de Biologie et de Technologies de Saclay (iBiTec-S),
CEA Saclay, 91191 Gif-sur-Yvette, France

Summary

The aim of this chapter is to show how spectroscopic techniques, especially resonance Raman spectroscopy, have been used to characterize the structure of plant light-harvesting proteins in vitro and in vivo – revealing in particular changes in the conformation of these proteins upon induction of non-photochemical quenching of chlorophyll fluorescence (NPQ). Firstly, we will show that the major light-harvesting protein in higher plants can exist in at least two functionally different states, with a different conformation in the crystal form (as well as upon aggregation) from that in solution. Thus this protein can switch from a state where it harvests

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*Author for Correspondence, e-mail: bruno.robert@cea.fr

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_10,

and transfers light energy with high efficiency (as is the case for the protein in solution), to a quenched, energy-dissipating state (in the crystal or aggregated form). These results led us to propose a role for this protein in photoprotection. After summarizing these in vitro results, we will explain how similar measurements in chloroplasts and whole leaves confirmed that these conformational changes were also seen in vivo, correlating with NPQ. Finally, we will present a model to explain our conclusions and will, furthermore, introduce some of the more recent advances in this field involving multi-molecular photosystem II architectures.

I Principles of Resonance Raman Spectroscopy

The Raman effect is the change of frequency observed when monochromatic light is scattered by polyatomic molecules. During Raman scattering, energy is exchanged between the incoming photon and the scattering molecule. Since molecular energy levels are discrete, the energy h * $\Delta\nu$=h * (ν_o–ν_r) corresponds to the energy of a transition between energy levels of the scattering molecule, where ν_o and ν_r are the frequencies of incident and scattered light, respectively (Fig. 10.1a). Raman spectroscopy thus yields information on the energy of the vibrational levels of a given electronic state, usually the ground state – or any excited electronic state that has been populated beforehand (e.g., by a prior laser pulse). The vibrational levels of a particular molecule are determined by its structure, i.e., the nature of its constituent atoms, the bonds between these atoms, and its molecular symmetry. Raman spectroscopy can thus be used as an analytical method for determining the chemical structure of molecules. As a molecular spectroscopy, Raman may also give indications on the conformation of the scattering molecules and/or on intermolecular interactions with

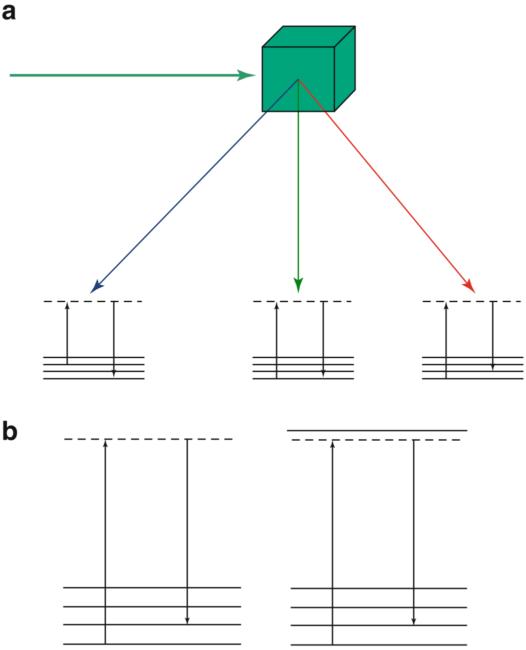

Fig. 10.1. Principle of resonance Raman spectroscopy. (**a**) A photon of light (*thick green arrow*) is scattered by a molecule (*green cube*). Most events are elastic (Rayleigh scattering, involving no energy exchange) – the molecule returns to the ground state (middle energy level diagram), and the emitted photon has the same energy and wavelength (*thin green arrow*). Energy exchange is also possible, involving changes in vibrational energy of the molecule and in energy and wavelength of the emitted photon – the Raman effect. Both Stokes (*right*; *red arrow*) and anti-Stokes (*left*; *blue arrow*) Raman scattering can occur. (**b**) Energy level diagrams for classical and resonance Raman (*left* and *right*, respectively).

Abbreviations: Chl – Chlorophyll; FLIM – Fluorescence lifetime imaging; LHC – Family of related light-harvesting trans-membrane proteins in oxygenic photosynthetic organisms; LHCII – Major LHC of photosystem II; NPQ – Non-photochemical quenching of chlorophyll fluorescence; VAZ – Enzymatic cycle of interconversions between the xanthophylls violaxanthin, antheraxanthin and zeaxanthin. Operation of the cycle correlates with changes in NPQ; ν_{1-4} – Four main groups of bands in vibrational spectra of carotenoids their immediate surroundings (e.g., H-bonds). Since the Raman effect is, however, a very low-probability process, a major drawback of Raman spectroscopy is that the signal measured is usually very weak, and it can be blurred by traces of fluorescing molecules present in the sample. On the other hand, since Raman-active molecular vibrations are those involving changes in molecular polarizability, the Raman signal of water seldom interferes with that of the biological molecules studied. This constitutes an important advantage for the Raman technique over, e.g., infrared absorption spectroscopy.

In classical Raman spectroscopy, the signal only depends on the frequency ν_o of the light used for inducing the Raman effect, i.e., signal intensity varies according to the fourth power of this frequency, ν_o^4. However, when this frequency matches an electronic transition of the irradiated molecule, an enhancement of a subset of Raman-active modes is observed that may reach six orders of magnitude (Fig. 10.1b). This increase in Raman intensity, for excitation near a given electronic transition, is termed the resonance effect. Resonance Raman spectroscopy thus allows selective observation of a pigment molecule in a complex medium by matching the energy of the exciting photons with the absorption transition of this molecule. This unique property explains why resonance Raman is widely used for studying pigment cofactors such as hemes, iron-sulfur clusters, chlorophylls (Chl) and carotenoid molecules within proteins, as it yields the contributions of these chromophores selectively – that is, without interference from the signal of the protein or the surrounding medium (for a review see, e.g., Carey 1982).

In resonance conditions, only a subset of the vibrational modes of the scattering molecule is enhanced. In the simplest case, when only one electronic state is involved in the resonance phenomenon, the resonance Raman signal arises from those vibrational modes involving nuclei motions that correspond to distortions experienced by the molecule during the transition between the ground and excited states used for inducing the resonance (Albrecht 1961). This intra-mode selection may be considered a limitation – for example, if a domain of the molecule is not involved in the electronic transition, resonance Raman will not yield any information about this domain. However, for most biological chromophores the functional part of the molecule consists of those atoms that are conjugated with the electronic transition. Resonance Raman will, therefore, yield selective information on the latter, biologically-active sub-molecular structures – the intra-mode selection may thus be considered a clear advantage of the technique. Moreover, the analysis of resonance Raman-active modes observed upon excitation with a given electronic transition will thus yield information about this transition. For instance, these modes may be used to identify the molecule responsible for a given transition (in a complex biological medium), or they may provide information on the nature of the excited state. Indeed, attribution of the carotenoid absorption bands in higher plant light-harvesting complexes was performed in this way (Ruban et al. 2000; Robert et al. 2004). In short, the position of bands in resonance Raman spectra yields information about the vibrational structure of the ground electronic state involved in the transition used for inducing the resonance, whilst the intensity of these peaks yields information about the excited electronic state involved in this transition.

II NPQ Mechanisms

Many open questions remain concerning the molecular mechanisms underlying NPQ, such as the chemical nature of the quencher. Only a few experiments have up to now provided direct information on this molecular species, and their validity is still debated. A number of cofactors could play the role of the quencher – individual Chl molecules, Chl dimers, or carotenoids – the latter either through their cation state, through charge transfer states, or via their lower singlet-excited state (see, for example, van Grondelle

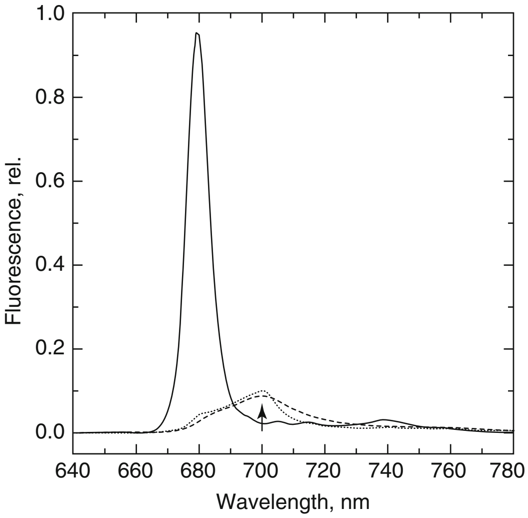

Fig. 10.2. Fluorescence properties of LHCII at 77 K. LHCII trimers in solution (*full line*) are highly fluorescent, with a principal emission peak at 680 nm. Upon formation of either aggregates (*dashed line*) or crystals (*dotted line*), the fluorescence becomes highly quenched and the peak shifts towards 700 nm (*arrow*).

and Novoderezhkin 2006; Holt et al. 2005; see also Ostroumov et al., Chap. 4; Krüger et al., Chap. 6; Walla et al., Chap. 9). Besides the chemical nature of the quencher, until recently there was no general agreement on the precise nature of the proteins involved in the NPQ mechanism, although it became increasingly obvious that at least a sizeable part of the quenching occurred in the antenna of photosystem II (see below).

In the late 1980s, it was noticed that the fluorescence yield of the major photosystem II antenna protein, LHCII, strongly depends on its level of association (see Fig. 10.2). When this protein is in its trimeric state, its fluorescence quantum yield is about 75 % that of isolated Chl *a*, with a lifetime around 4 ns (Palacios et al. 2002). However, upon detergent removal, the fluorescence yield decreases progressively to 10 % or even lower, corresponding to a lifetime of as low as 200 ps. Furthermore, the difference between the fluorescence spectra of trimeric and aggregated LHCII exhibits many similarities with the difference between the in vivo fluorescence of unquenched and quenched leaves (Horton et al. 1991). In particular, upon LHCII-association-induced quenching, the fluorescence maximum shifts to the red in precisely the same way as observed for NPQ in vivo (Fig. 10.2). These experiments, performed mainly in Peter Horton's group (see Horton, Chap. 3), showed that the association of LHCII proteins promotes formation of an excitation-energy trap (although the relevance of these results for understanding the NPQ mechanism has been questioned for many years). From these studies, it was proposed that LHCII association induces a conformational change in the protein, which in turn promotes the appearance of a quenching species. Resonance Raman spectroscopy was applied to this in vitro system in order to test the conformational change hypothesis (Ruban et al. 1995).

III Raman Studies on LHCII

Each LHCII monomer binds three types of carotenoid – two luteins, one neoxanthin, and one (weakly-bound) VAZ-cycle carotenoid (either violaxanthin or zeaxanthin). In addition, each monomer binds eight molecules of Chl *a* and six Chls *b*. For such a complex system, it is obviously difficult to get information on individual pigments, even with the most selective techniques. Carotenoid and Chl molecules do not exhibit electronic transitions in the same spectral range – Chls display two intense electronic transitions in the blue (410–450 nm) and the red (620–700 nm) regions of the spectrum (termed the Soret and Q_y regions, respectively), while carotenoids absorb mainly in the blue-green range (450–510 nm), thus acting as complementary light-harvesters. This is summarized for LHCII in Fig. 10.3. Thus it is possible to observe the signals of either Chl or carotenoid molecules selectively, by choosing appropriate excitation wavelengths. Similarly, as the Soret electronic transition of Chl *b* is somewhat red-shifted relative to Chl *a*, selectivity between these two latter classes of molecule is also

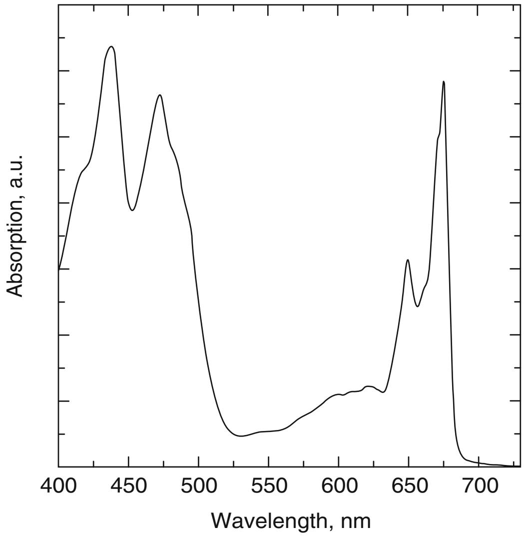

Fig. 10.3. Electronic absorption of LHCII (recorded at 77 K). In this spectrum, the main contributions to electronic transitions arise from (*left to right*): the Soret electronic transitions of Chl *a* and *b* molecules (420–450 nm), the S_0–>S_2 transitions of carotenoid molecules (460–510 nm), and the Q_y transitions of Chls *b* (650 nm) and *a* (668–680 nm).

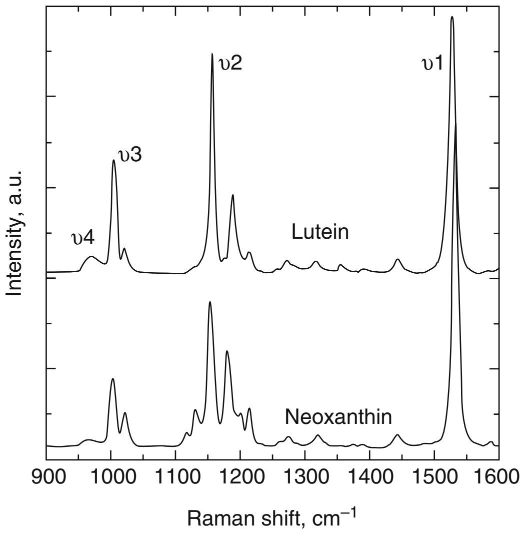

Fig. 10.4. 77 K resonance Raman spectra of isolated carotenoid molecules in pyridine, recorded at resonance (488 nm) excitation. *Top*: lutein, bottom: 9′-*cis* neoxanthin. The different spectral regions are denoted υ_1 to υ_4. In υ_2 the 9′-*cis* conformation of neoxanthin induces the presence of new bands, which are symmetry-forbidden in the *all-trans* conformation.

possible, at least to a degree (Lutz and Robert 1988; see Fig. 10.6).

Resonance Raman spectra of Chl molecules comprise more than 60 bands, most of them arising from combinations of the vibrational modes of the conjugated macrocycle. The spectral region that has up to now been used the most in biological studies corresponds to the highest frequency region (1620–1710 cm^{-1}). Bands contributing in this region arise from the stretching modes of the conjugated carbonyl groups of Chls *a* (keto) and *b* (formyl and keto). Because these modes involve an oxygen atom, their frequency is higher than that of the other modes of the molecule, and they combine only poorly with the latter modes. Since the frequency of the conjugated carbonyl depends tightly on its intermolecular interactions (H-bonds), analysis of this spectral region provides a precise diagnostic of how Chl molecules interact with their environment. For instance, the formyl group of Chl *b* vibrates at approximately 1660 cm^{-1} when free from intermolecular interactions, while it may downshift by up to 40 cm^{-1} upon H-bond formation (Lutz and Robert 1988).

Resonance Raman spectra of carotenoid molecules display four main groups of intense bands, which provide information on conformation and configuration for these molecules (see Fig. 10.4; for a review, see Robert 1996). The ν_1 band around 1530 cm^{-1} arises from stretching vibrations of the C=C bonds of the carotenoid. The frequency of this band is sensitive to the length of the conjugated system of the scattering carotenoid, and to its molecular conformation (trans or cis). The ν_2 bands mainly arise from the stretching modes of the C-C bonds. The structure of this complex cluster also depends on the molecular conformation of the scattering carotenoid. Another important region of these spectra is a band termed the ν_4 band (around 960 cm^{-1}). ν_4 arises from out-of-plane wagging motions of the C-H groups of these molecules, which, for symmetry reasons, are not coupled with the electronic transition of perfectly planar carotenoid molecules. Hence this band depends strongly on the molecular configuration of the carotenoid, gaining intensity when the molecule is distorted out of the plane.

One of the major problems in applying resonance Raman to LHCII was determining which resonance conditions induce contributions of any given molecule. Indeed, as the protein environment may influence the electronic properties of bound chromophores, it was impossible to predict where a specific carotenoid would absorb in LHCII. The carotenoids of the VAZ cycle are bound very loosely to LHCII – indeed it is quite difficult to preserve them during purification. On the other hand, when the isolation procedure is carried out carefully enough to retain the VAZ carotenoids, a further "detergent wash" can be performed afterwards to remove them. In this way, it is possible to study LHCII both with and without these carotenoids, in order to determine at which wavelength they absorb and to measure their Raman contribution (Ruban et al. 2002a). For the other LHCII-bound carotenoids, lutein and neoxanthin, a more complex approach was required to distinguish their absorption properties.

When bound to LHCII, neoxanthin adopts a 9′-*cis* conformation. This is expected to affect both its ν_1 and ν_2 resonance Raman bands (Fig. 10.4). Scanning the Raman excitation through the carotenoid electronic transition induces net frequency changes of the ν_1 band, from that expected for 9′-*cis* neoxanthin to that expected for all-trans lutein, thus indicating that the contribution of these two carotenoids dominates the resonance Raman spectra alternately (Ruban et al. 2000). Such experiments allowed deduction of the precise position of the absorption transitions for each carotenoid, as these transitions must correspond to the excitation wavelengths where that particular carotenoid dominates the resonance Raman spectrum. For instance, it was concluded that lutein 2 possesses a red-shifted electronic absorption, with a 0–0 transition peaking at 510 nm, while the other lutein (lutein 1) contributes at 495 nm. Once it is known which bound carotenoid dominates the resonance Raman spectrum at a given wavelength, precise molecular information can then be obtained selectively for each molecule. In such spectra, the intensity and structure of the ν_4 band strongly depends on the configuration of the carotenoid. Thus it is possible to evaluate to what extent these molecules are distorted from the planar by their protein binding pockets. It was shown that the red-shift in absorption transition of lutein 2 is caused by LHCII trimer formation. It could also be concluded, from analysis of the ν_4 region, that this lutein was distorted upon trimerization. Resonance Raman thus allows the selective observation of the three main carotenoids of LHCII, the blue and red lutein molecules, and neoxanthin (0–0 transition at 486 nm; Ruban et al. 2000).

Once conditions for selective observation via resonance Raman were determined for each carotenoid, the molecular processes involved in LHCII aggregation (and thus potentially underlying the quenching mechanism) were studied. It was shown that further association of trimeric LHCII into small oligomers induces a distortion of a carotenoid molecule (Fig. 10.5). This distortion is more easily seen at wavelengths where the neoxanthin contribution is expected to dominate (Ruban et al. 2000). It was thus concluded that, upon LHCII aggregation, neoxanthin becomes distorted, and that this distortion probably reflects changes in the conformation of the protein-binding site of this molecule. Using excitation wavelengths enhancing Chl *a* or *b* contributions, clear differences in the resonance Raman spectra of LHCII trimers and oligomers were also observed (Ruban et al. 1995). These changes show that the H-bonding state of the formyl carbonyl of at least one, and probably two, molecules of Chl *b* change upon LHCII association (Fig. 10.6), as well as that of at least one Chl *a* keto carbonyl (not shown). Self-association of LHCII thus induces a change in the binding sites of at least three of its pigment cofactors. However, it remained unclear at that stage whether these changes in pigment interactions were an artifact of the aggregation process itself or whether

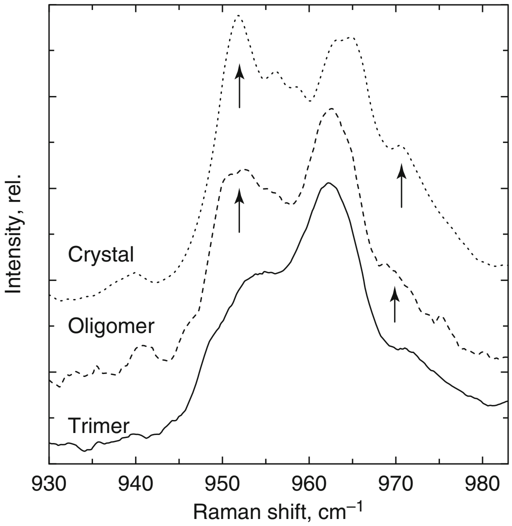

Fig. 10.5. 77 K resonance Raman spectra (488.0 nm excitation, favoring neoxanthin contributions) in the ν_4 region, sensitive to out-of plane deviations of the carotenoid molecule, for (*bottom to top*): LHCII trimers, oligomers, and crystals. The intensity of the 950 and 970 cm^{-1} Raman contributions increases upon LHCII aggregation (*arrows*), indicating a distortion of the neoxanthin molecule. In crystals, these bands are even more intense than in self-associated LHCII, revealing a net difference in structure between LHCII in solution and in crystals at the level of the neoxanthin binding site.

they were caused by conformational changes within LHCII trimers upon association. If the cofactors involved were bound to the periphery of LHCII, then the changes observed could merely represent artifactual interactions with the neighboring trimer. Only the precise study of LHCII crystals could help in answering this question.

More recently, we have also observed small changes in the lutein 1 Raman signal upon LHCII self-association consistent with a subtle twisting of this carotenoid (Ilioaia et al. 2011). These lutein 1-associated changes, being much smaller than those observed for neoxanthin, are far more difficult to observe, and disentangling them from the overlapping contributions from the latter carotenoid required extensive differential analysis. However, these changes clearly show that lutein 1 also undergoes subtler, but nevertheless significant, alterations in fine

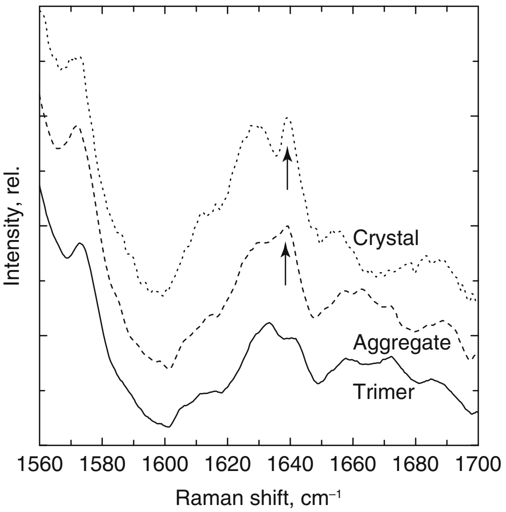

Fig. 10.6. 77 K resonance Raman spectra at 441.6 nm excitation (favoring Chl *b* contributions) – higher frequency region, where conjugated carbonyl stretching frequencies mainly contribute. From *bottom to top*: spectra of LHCII trimers, oligomers, and crystals. In oligomers (*dashed line*), new contributions at 1639 cm^{-1} are observed (*arrow*), together with loss of intensity at 1670 cm^{-1}. This indicates a change of interaction of at least one Chl *b*, upon LHCII self-association, where a free-from-interaction formyl carbonyl (vibrating at 1670 cm^{-1}) enters into H-bonding interactions, downshifting to 1639 cm^{-1}. In LHCII crystals (*dotted line*), the 1639 cm^{-1} band is even more prominent, and associated with a second new contribution at 1625 cm^{-1}. This indicates that during the crystallization process, the formyl group of one, and probably two, Chls *b* enters into H-bonding interactions, indicating structural changes in the binding pockets of these molecules or reorganization of Chl *b* pairs.

structure associated with in vitro quenching. This result is of particular interest, given that a number of models predict this carotenoid as the site of energy quenching (Ruban et al. 2007; Bode et al. 2009).

IV Crystallographic Structure of LHCII

The structure of LHCII has been the object of intense work for more than 15 years. In 1994, an initial structure of pea LHCII was obtained from electron diffraction of 2-D crystals (Kühlbrandt et al. 1994).

This structure provided a general description of the LHCII fold, containing three transmembrane α-helices (A, B and C), as well as the positioning of a large number of chromophores. However, limited resolution made it impossible to distinguish Chl *a* from Chl *b* in this structure, or to position the binding site of the neoxanthin molecule. This incomplete structure was nevertheless the only one available for nearly 10 years, and it proved extremely useful in improving our understanding of LHCII function, and in directing ongoing research. In 2004, a 3-D structure of spinach LHCII was obtained by X-ray crystallography at 2.7 Å resolution in Chang's group in Beijing (Liu et al. 2004). Since then, a second structure has been obtained in Kühlbrandt's group at slightly better resolution, which largely confirmed the LHCII description deduced from Chang's crystals (Standfuss et al. 2005). These new LHCII structures allowed the first precise determination of Chl stoichiometry for this protein, and no less than 8 Chls *a* and 6 Chls *b* were determined in the atomic model (instead of 12 Chls in the earlier structure). These Chls are organized in two layers, one on the stromal and one on the lumenal side of the protein. As predicted from the first structure, the two luteins bound to LHCII form a cross-brace in the very heart of the protein, in the groove of the supercoil formed by the two major helices (A and B). 9'-cis neoxanthin is bound near helix C, close to a Chl *b*-rich domain of LHCII, in a cleft formed by hydrophobic amino acids, and the rings and phytyl tails of Chl *b* molecules. Approximately a third of this carotenoid sticks out of the protein. The fourth carotenoid found in the structure, violaxanthin, is bound at the monomer/monomer interface, with a 1:1 violaxanthin:polypeptide stoichiometry. The overall structure of LHCII reveals an extremely complex network of pigment cofactors, with a number of strongly-interacting Chl molecules, forming Chl *a*-*a*, *a*-*b* and *b*-*b* pairs. Extensive calculations performed on this structure have given an evaluation of the interaction energies between these pairs, and suggested which of these pigments participate in the different excitation energy levels of the LHCII protein (Novoderezhkin et al. 2005).

Besides its value to the study of LHCII, the work described above also allowed description of a novel type of membrane protein crystal. In the crystals obtained by the Beijing team, LHCII is organized into icosahedral proteo-liposomes, containing 20 LHCII trimers oriented radially from the center (Liu et al. 2004). As the inner diameter of these proteoliposomes is rather small (160 Å), the angle between adjacent trimers in this unit is large, and the molecular contacts between trimers is thus limited to a small region of their lumenal surfaces mainly involving a molecule of the lipid digalactosyldiacylglycerol. The only cofactors near this region are two weakly-interacting Chl *a*-*b* pairs. Contacts in the crystal between icosahedral units are ensured by polar interactions involving the hydrophilic stromal domain of LHCII. These crystals are thus formed from the regular packing of LHCII proteoliposomes, with little protein/protein interactions between liposomes. As described above, the properties of LHCII trimers strongly depend on their association state, being highly fluorescent when isolated, and highly quenched when forming aggregates. It was thus interesting to study the properties of LHCII in these crystals, to determine whether they were fluorescent or quenched. Considering the limited nature of interactions in the crystal, they were expected to be highly fluorescent.

V Properties of LHCII in Crystals

Surprisingly, when the first fluorescence emission spectra of LHCII crystals were obtained (Pascal et al. 2005), these spectra displayed a principal emission peak dramatically shifted to the red as compared to that of LHCII trimers (peak at 700 nm, c.f. 680 nm for LHCII trimers; Fig. 10.2). Altogether, this fluorescent pattern is reminiscent of that of highly-aggregated, quenched LHCII. From this single result, it is, however, impossible to make inferences about the quenching state of LHCII in crystals. Indeed, because of the extremely high concentration of LHCII in these crystals, it is very difficult to determine its fluorescence yield. The most direct way of determining the quenching state of a fluorescent protein is to measure its fluorescence lifetime. This yields a much more accurate determination of the quenching state than does the fluorescence emission spectrum. Fluorescence lifetime imaging (FLIM) of single LHCII crystals was thus performed. FLIM is an imaging, confocal-based method that yields an estimation of the fluorescence lifetime with a resolution as high as 50 ps, with a spatial resolution of about half a micrometer. Using FLIM for fluorescence lifetime determination in a crystal is particularly important, as it allows discrimination between proteins belonging to the crystal and those eventually left in the mother liquor. FLIM can also distinguish between domains in the crystals, which is important, e.g., when some degradation is observed at the crystal edges.

FLIM performed on single crystals of LHCII trimers led to an unambiguous determination of the quenching state of the proteins. Fluorescence lifetimes were found to be homogeneous over whole crystals, and were determined at 0.89 ns, as compared to 4 ns for isolated LHCII trimers, and 0.65 ns for aggregated LHCII. From this result it was thus concluded that, although LHCII in the crystals is largely in its trimeric state, it is nevertheless highly quenched, nearly as much as in LHCII aggregates (Pascal et al. 2005). Resonance Raman spectroscopy was also performed on LHCII single crystals. We observed that, when LHCII is in its crystallized state, the conformation of neoxanthin is even more distorted than that observed in LHCII aggregates (Fig. 10.5). At the same time, Raman spectra excited at 441.6 nm (where Chl *b* principally contributes) showed that at least one (and probably two) Chls *b* are involved in new H-bonds with their protein environment upon crystal formation, as is the case for the aggregated protein (Fig. 10.6). For selective Chl *a* excitations (406.7 and 413.1 nm), the new H-bond involving a Chl *a* keto carbonyl previously observed for the aggregated protein is also seen in the crystal form (not shown). As none of the contacts between LHCII trimers can account for these changes in conformation/intermolecular interactions, it was concluded that LHCII trimers possess the intrinsic ability of undergoing conformational change, and that this conformational change was promoting the observed changes in cofactor properties – including the appearance of excitation quenchers. It was also concluded that, more generally, aggregation-induced quenching is not a direct result of aggregation, but rather that both aggregation and crystallization select, or in some way induce, the new protein conformation. Thus LHCII behaves as a molecular switch, able to shift from a state of efficient harvesting of energy to a dissipating state (see also Krüger et al., Chap. 6). The structure of LHCII in crystals thus reflects the molecular organization of this protein in its quenching form. It was further proposed that these conformational changes are responsible for the appearance of non-photochemical quenching in higher plant membranes in vivo.

VI Measurements In Vivo

Extracting the Raman signal coming from a single pigment in a mixture as complex as a whole tissue or organism (i.e., whole leaves) is significantly more complicated than for the isolated protein. However, knowing that the neoxanthin signal should dominate at 488.0 nm, resonance Raman experiments were conducted on chloroplasts and whole leaves at this wavelength to test for evidence of the same conformational changes in LHCII in vivo. Measurements were taken after induction of NPQ, and following a 5-min relaxation in the dark. In this way, the effects of the presence of zeaxanthin could be dissociated from those of NPQ itself, as this carotenoid is not reconverted to violaxanthin in such a short time period. In Fig. 10.7, it can clearly be seen that, upon

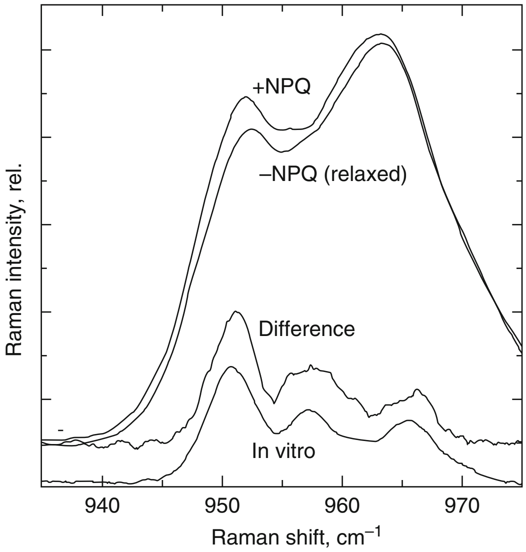

Fig. 10.7. *77* K resonance Raman spectra excited at 488.0 nm for chloroplasts upon NPQ induction (+NPQ) and after 5 min relaxation (−NPQ). The NPQ-associated changes, revealed in the difference, are almost identical to those associated with quenching in LHCII (in vitro).

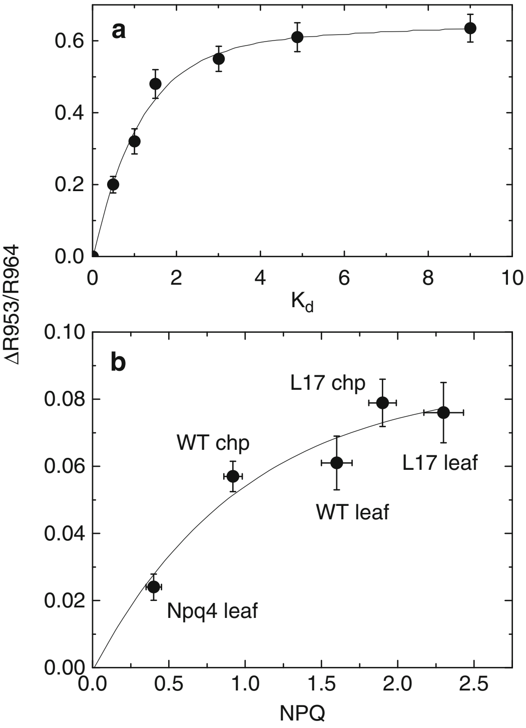

Fig. 10.8. Dependence of the "neoxanthin twist" Raman signal (see Figs. 10.5 and 10.7) on the extent of fluorescence quenching. (**a**) LHCII self-association was induced in vitro by continuous stirring with biobeads in a fluorimeter chamber. Aliquots were removed at increasing quenching levels (K_d=(unquenched-quenched)/quenched) and frozen immediately in liquid nitrogen on the Raman sample holder. (**b**) NPQ in vivo (measured as F_m–F_m'/F_m'). Leaf and chloroplast (*chp*) samples were used for wild-type *Arabidopsis* (*WT*), as well as for the PsbS deletion mutant (npq4) and the over-expresser (*L17*). This ensured a range of NPQ levels for identical sample treatments.

NPQ induction, a resonance Raman signal corresponding to the twisting of neoxanthin in LHCII is observed (Ruban et al. 2007).

Resonance Raman experiments were conducted in vitro on different-size LHCII aggregates to assess any correlation between the extent of quenching and the resonance Raman signal. As shown in Fig. 10.8a, the level of quenching in the in vitro system increases as more detergent is removed (in this case, by increasing incubation time with biobeads). In parallel, the extent of the neoxanthin Raman changes described previously also increases, and there is a clear relationship between the two parameters. A number of different in vivo samples were then tested for the same relationship. Chloroplasts and whole leaf samples were used, from wild-type *Arabidopsis* plants as well as from two mutants (npq4-1 and L17) with differing extents of NPQ (lower and higher than wild type, respectively). These experiments revealed a remarkably close relationship between NPQ and the neoxanthin twist, very similar to that observed in vitro (Fig. 10.8b). In each case, this signature of the LHCII conformational change had an intensity nearly equal to that expected for the level of quenching observed. This suggests that the conformational change observed in the crystal is probably responsible for the majority of the quenching observed in vivo (Ruban et al. 2007). It should be noted that, as most LHC-type proteins bind a neoxanthin molecule in the equivalent position as LHCII, it is possible that a proportion at least of the quenching is actually occurring in the minor LHCs by the same mechanism.

Measurements were also carried out to elucidate the structure of the VAZ pigments in vivo (Ruban et al. 2001). This was somewhat more complicated, requiring precise determination of the absorption properties of these carotenoids followed by differential analysis. For 488 nm-excitation, near the peak of violaxanthin absorption, the difference "(violaxanthin-containing samples) minus (zeaxanthin-containing samples)" was calculated, normalizing on Chl bands, to give the spectrum of violaxanthin in vivo. Similarly for 514.5 or 528.7 nm-excitation, on the red edge of zeaxanthin absorption (but further from the other pigments), "(zeaxanthin-containing samples) minus (violaxanthin-containing samples)" gave the in vivo spectrum of zeaxanthin. In this way we showed that the majority of the VAZ pigments were present in the thylakoid bound to proteins, and not free in the membrane as proposed in the literature (Ruban et al. 2001). These measurements also revealed that isolated LHC preparations (whether LHCII or the minor LHCs), while they bind the VAZ pigments, do not do so in the native configuration. It appeared that the protein-protein interactions involved in organization of the thylakoid membrane are necessary to retain the finer details of VAZ structure – and indeed, these structural signatures are also seen in more "native" preparations (photosystem II membranes; the oligomeric LHCII A-band; Ruban et al. 2002a).

VII Recent Developments and Perspectives

As mentioned above, the LHCII conformational change is sufficient to account for the majority of the fast-relaxing NPQ observed in the various *Arabidopsis* plants used in the study, assuming that the strong correlation between neoxanthin twist and energy dissipation is not a coincidence. However, other mechanisms have been proposed for NPQ, most of them involving the PsbS protein and/or the carotenoid zeaxanthin (see, e.g., Holzwarth and Jahns, Chap. 5; Brooks et al., Chap. 13). These are largely founded in the observed correlation between these two factors and NPQ in vivo (discussed in Adams and Demmig-Adams, Chap. 2; Brooks et al., Chap. 13; Demmig-Adams et al., Chap. 24). Some give a central role to putative interactions between PsbS and LHCII (possibly involving zeaxanthin). Whether these actually represent alternative mechanisms, or are rather different aspects of the same overall system, is currently difficult to assess. For instance, it is possible that both act as activators of some kind (involved in ΔpH sensing, acting allosterically on the target protein, or through other similar functions). It is also possible, as some have hypothesized, that more than one mechanism is present, allowing for a finer control of energy dissipation in a wide range of conditions (see Holzwarth and Jahns, Chap. 5). Determining whether one or more of these putative mechanisms are responsible for NPQ in a range of different conditions and species represents one of the major questions currently facing the field.

At the same time, a large body of evidence has accumulated indicating major organizational changes within the thylakoid membrane associated with NPQ (discussed in Garab, Chap. 16). Given that these changes must involve significant rearrangement of protein-protein (and probably protein-lipid) interactions, they could represent one possible way forward in assessing the relative importance of the various mechanisms in the literature. Indeed, one of the observations associating organizational changes with NPQ was the appearance of a new absorption band at 535 nm. For a long time this was interpreted in terms of specific scattering, but we showed that it was in fact a red-shifted population of zeaxanthin in a specific, distorted conformation (Ruban et al. 2002b). We also demonstrated that such an "activated" form of zeaxanthin could be reproduced by its binding in vitro to PsbS (Aspinall-O'Dea et al. 2002). It can be imagined how, by attempting in this way to relate specific spectroscopic signatures of NPQ to specific pigment-pigment and/or pigment-protein interactions, the detailed reorganizations involved in NPQ and their inter-relationships may be unraveled.

It is of particular interest, when considering the status of light-harvesting and energy dissipation in the thylakoid membrane, that the in vivo fluorescence lifetime (in conditions of no NPQ) is already 2 ns (Johnson et al. 2010) – i.e., significantly quenched relative to the isolated proteins. Experiments are currently being performed on a number of different photosystem II sub-complexes to test whether this shortening of the lifetime is also associated with similar conformational changes.

Acknowledgments

This work was supported by EU program Marie Curie (FP7 Initial Training Network HARVEST), the ERC funding agency (PHOTPROT project), the French National Research Agency (ANR, Cyanoprotect Project), and by The Royal Society and BBSRC of the UK.

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Chapter 11

Fucoxanthin-Chlorophyll-Proteins and Non-Photochemical Fluorescence Quenching of Diatoms

Claudia Büchel*
Department of Biosciences, Institute of Molecular Biosciences,
Goethe University Frankfurt, Max-von-Laue-Str. 9,
60438 Frankfurt, Germany

Summary

Diatoms possess membrane-intrinsic light-harvesting proteins, called fucoxanthin-chlorophyll-proteins (FCPs) that, based on their protein sequences, are related to higher plant light-harvesting complexes (LHCs). FCPs differ from LHCs in overall organization around the photosystems, and in that fucoxanthin and chlorophyll *c* (Chl *c*) serve as the diatoms' accessory pigments in a high carotenoid-to-chlorophyll ratio. Most FCPs assemble into trimeric complexes, but higher oligomers of specific polypeptide composition were also found. Polypeptides fall into three different groups, namely Lhcf (Lhcs named after the main carotenoid fucoxanthin, the main light harvesters), Lhcr (photosystem I-associated polypeptides closely related to red algal intrinsic Lhc), and Lhcx proteins related to LhcSR proteins (stress related Lhcs) identified in the green alga *Chlamydomonas reinhardtii* as functional homologues to PsbS (photosystem II protein S). In the genomes of diatoms published so far, sequences for four to five different Lhcx proteins can be found. All of the latter proteins

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*Author for Correspondence, e-mail: c.buechel@bio.uni-frankfurt.de

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_11,

are expressed and most of them are up-regulated under high light. On the other hand, no homologue of *psbS* is present in diatoms. The Lhcx content was shown to correlate with the ability for non-photochemical quenching (NPQ) of chlorophyll fluorescence (as a measure of thermal dissipation of singlet-excited chlorophyll) in pennate diatoms, e.g., *Phaeodactylum tricornutum*, as well as in centric diatoms, e.g., *Thalassiosira pseudonana*. Localization of Lhcx and the supra-molecular organization of FCP complexes, however, appear to differ between the latter two diatom groups. For several centric species, occurrence of Lhcx proteins within trimeric FCP complexes was shown, whereas no Lhcx proteins have been identified in FCP trimers from pennate species thus far. In centric diatoms, the content of Lhcx proteins in trimeric FCP complexes, furthermore, correlates with the level of diatoxanthin, a carotenoid of the xanthophyll cycle of diatoms synthesized under high light. Diatoxanthin content was inversely correlated with the fluorescence yield of trimeric FCP complexes, i.e., higher diatoxanthin levels were associated with a more highly quenched state. Another factor which influences NPQ in vivo is a low lumenal pH. Low pH values were also shown to exert effects on the fluorescence yield of isolated trimeric FCPs. Likewise, aggregation of FCP complexes in vitro affected their fluorescence yield as proposed for the case in vivo. A model for the involvement of FCPs in NPQ is presented.

I Introduction

A Fucoxanthin-Chlorophyll-Proteins (FCP) of Diatoms

Diatoms are unicellular, eukaryotic algae evolutionary derived from a secondary endosymbiosis event, whereby an ancient rhodophyte was engulfed by a non-photosynthetic eukaryont (Archibald and Keeling 2002). The chloroplast of diatoms is thus surrounded by four envelope membranes instead of the two usually found in higher plants and related species. The thylakoid membrane system of diatoms is comprised of bands of three thylakoids each, with no differentiation into stacked and unstacked regions, i.e., no separation into grana and stroma thylakoids. Despite these differences, the light reactions of photosynthesis of diatoms are similar to those of higher plants, except for the light-harvesting systems. Diatoms possess membrane-intrinsic antenna proteins that belong to the light-harvesting-complex (LHC) superfamily (Durnford et al. 1996). These proteins are thus predicted to have three membrane spanning α-helices, whereby sequence homology with LHCII, the major trimeric LHC of photosystem II of higher plants, is found mainly in helix 1 and 3 of the LHCs of diatoms. The latter antenna proteins are smaller and more hydrophobic, since loops and termini are shorter. The most obvious difference concerns the pigment complement: Chl *a* is accompanied by Chl *c*, a phytol-less Chl, and the main carotenoid (Car) is fucoxanthin (Fx). The Car to Chl ratio of about 1:1 is much higher than in higher-plant LHCII (Papagiannakis et al. 2005), which relates to the importance of Fx

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Abbreviations: Car – Carotenoid; Chl – Chlorophyll; Dd – Diadinoxanthin; Dt – Diatoxanthin; FCP – Fucoxanthin-chlorophyll-protein; Fx – Fucoxanthin; HL – High light; LHC – Light-harvesting complex; Lhcf – Main light harvesting proteins of diatoms, named according to their fucoxanthin content; LHCII – Light harvesting complex II; Lhcr – Photosystem I related Lhcs of diatoms most closely related to red algae intrinsic Lhcs; LhcSR – Stress related Lhc first found in green algae; Lhcx – Lhcs of diatoms working in light protection; LL – Low light; NPQ – Non-photochemical quenching of chlorophyll fluorescence (as a measure of thermal dissipation of the singlet-excited state of chlorophyll *a*); PS – Photosystem; PsbS – Photosystem II protein S; Q1 – Hypothetical quenching site being due to aggregation of pigment-protein complexes; Q2 – Hypothetical quenching site depending on xanthophyll cycle pigments; qE – Part of NPQ related to the energization of the thylakoid membrane (rapidly reversible pH-dependent NPQ); qI – Part of NPQ related to photoinhibition (slowly reversible, pH-independent NPQ) for light harvesting. Fucoxanthin is an allenic Car with a carbonyl moiety in conjugation with the polyene backbone, as is also the case for peridinin found in dinoflagellates. In acetone, the main absorbance maximum of Fx is around 450 nm. Upon binding to Lhc proteins, Fx exhibits a pronounced bathochromic, i.e., red shift. Three differently bound Fx pools could be identified in FCP complexes, absorbing more to the blue, in the green and to the red, whereby the Fx'red' absorb up to 565 nm (Premvardhan et al. 2009). Because of their Fx content, light-harvesting proteins in diatoms are often referred to as FCPs (fucoxanthin-chlorophyll-binding proteins).

FCP proteins can be divided into three groups based on gene sequence analysis: *Lhcf* encode for the main light harvesters, whereas *Lhcr* genes are closely related to those of the only membrane-intrinsic light-harvesting proteins of red algae associated with photosystem (PS) I. A presumed function of Lhcr proteins as PS I antenna in diatoms was supported biochemically (Veith and Büchel 2007; Veith et al. 2009). The third Lhc group called *Lhcx* was demonstrated to be involved in non-photochemical quenching (NPQ) (Bailleul et al. 2010), and will thus be the main topic of the present chapter. Lhcx proteins are closely related to LhcSR (Lhc-stress related; formerly known as LI818) proteins of *Chlamydomonas reinhardtii* (Eppard and Rhiel 1998) identified being involved in photoprotection (Richard et al. 2000), and later proven to be indispensable for NPQ (Peers et al. 2009). In contrast to higher plants, no genes for PsbS or a similar protein were found in diatoms. In addition, no direct homologues to the minor LHCs of higher plants (Lhcb4, 5, 6) have thus far been detected.

B Factors Relevant for NPQ in Diatoms

NPQ in diatoms was shown to be larger than in annual higher plants (Ruban et al. 2004; but see Demmig-Adams et al., Chap. 24, for a comparison of the low NPQ of fast-growing, rapidly photosynthesizing annual plants with the much higher NPQ of slow-growing evergreens with low photosynthesis rates), and consists only of an energy-dependent part (qE, as rapidly reversible, pH-dependent NPQ) and quenching due to photoinhibition (qI, as slowly reversible NPQ), since state transitions (qT, as balancing the excitation energy between the photosystems by moving LHCs) are missing in diatoms (Owens 1986). The factors influencing NPQ are similar: the qE part depends on build-up of a pH-gradient across the thylakoid membrane. Diatoms also possess a xanthophyll cycle, in which diadinoxanthin (Dd) is de-epoxidized to diatoxanthin (Dt) under strong illumination (Stransky and Hager 1970). The de-epoxidation ratio and Dt content were shown to correlate with NPQ (Lavaud et al. 2002). In addition, Lhcx proteins are required for NPQ (Bailleul et al. 2010; Zhu and Green 2010). Diatoms can be sub-divided into *Coscinodiscophyceae*, pennate *Bacillariophyceae* and centric *Mediophyceae* (Medlin et al. 1996; Bowler et al. 2008). *Phaeodactylum tricornutum* (Fig. 11.1a) has become a model organism for pennates since its genome is fully sequenced (Bowler et al. 2008), whereas *Thalassiosira pseudonana*, whose full genome became available first (Armbrust et al. 2004), and *Cyclotella meneghiniana* (Fig. 11.1a) are centric diatoms often used in NPQ research.

II Lhcx Proteins in Centric Versus Pennate Diatoms

While the term "Lhcx" for a certain group of FCP proteins was only introduced after the genomes of two diatoms, *T. pseudonana* and *P. tricornutum*, had been sequenced and annotated, Lhcx proteins had already been described before without attributing a special function to them. The first report on the existence of an Lhcx protein is from Eppard and Rhiel (1998), who identified several genes for FCP proteins in the diatom *Cyclotella cryptica* (later re-named *C. meneghiniana)*. In addition to *Fcp1-3* and *Fcp5*, coding for Lhcf proteins, and *Fcp4* (Lhcr family), the genes for the Lhcx proteins Fcp6 and, later, Fcp12 were described (Eppard and Rhiel 2000). In addition, high

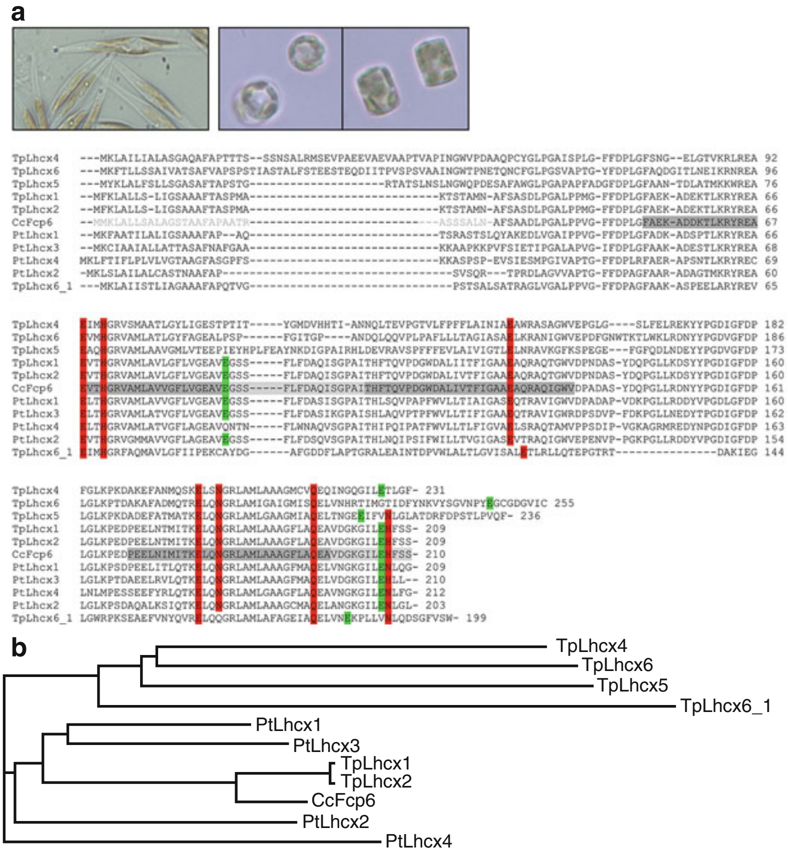

Fig. 11.1. Sequence comparison of Lhcx proteins from *T. pseudonana* (Tp), *P. tricornutum* (Pt) and Fcp6 from *C. cryptica* (=*meneghiniana;* Cc) (**a**) Exemplarily for pennate and centric diatoms, light microscopy pictures of *P. tricornutum* (*left*) and *C. meneghiniana* (*right*) are shown as well. In Fcp6, predicted α-helices (According to Eppard and Rhiel (1998)) are highlighted in *dark grey*, the signal sequence for chloroplast import is written in *grey*, and lumen exposed parts (loop and C-terminus) are shaded in *light grey*. In all sequences the Chl binding sites homologous to LHCII are depicted in *red* and lumenal glutamate residues, i.e., probable pH sensing residues within the loop or the C-terminus, are marked in *green* (Adopted from Gundermann and Büchel (2012)). Note that Lhcx1 is identical to Lhcx2 in case of *T. pseudonana*. In (**b**) all sequences are shown in a phylogram based on the alignment shown above (ClustalW phylogeny tool).

copy numbers and multiple copies of identical genes were found to be present (Eppard and Rhiel 2000), such as identical *Fcp6-8* and *Fcp12* as the first descriptions of Lhcx genes in diatoms. The sequence homology of all FCP sequences to higher plant LHC was recognized (Eppard and Rhiel 1998), and a model of the secondary structure and pigment binding was built based on the LHCII structure available at that time (Kühlbrandt and Wang 1991). Thereby, Chl-binding sites were also distinguished; five Chl-binding sites could be identified based on conserved Chl-binding amino acids in Lhcf, and also in Lhxc (Fcp6) proteins (Fig. 11.1). No hypotheses about Car-binding could be made at that time. However, these data provided the first hints for Lhcx proteins as pigment-binding proteins.

Fcp6 was later identified as one of the subunits of the major FCP complex, the trimeric FCPa complex, in the diatom *C. meneghiniana* (Beer et al. 2006). The latter complex was found to consist of no less than two polypeptides, the Lhcf protein Fcp2 and the Lhcx protein Fcp6. In high-light-grown (HL, which in diatoms is around 120–180 µmol photons m^{-2} s^{-1}) versus low-light-grown (LL, in diatoms around 10–40 µmol photons m^{-2} s^{-1}) cultures, differences in the amount of Fcp6 were detected, with FCPa from HL grown cells being enriched in Fcp6. Since it had already been known that Fcp6 is related to LhcSR proteins (Eppard and Rhiel 1998), this HL response gave rise to the hypothesis that Fcp6, and thus Lhcx, is involved in photoprotection. This hypothesis was further supported by the fact that *C. meneghiniana* shows a higher expression of Fcp6 genes at the beginning of the daily light phase as well as, generally, under high-light growth conditions (Oeltjen et al. 2002, 2004).

It is currently known that both centrics and pennates contain many *Lhcx* genes. For *P. tricornutum,* four *Lhcx* are described, and *T. pseudonana* contains sequences coding for five different Lhcx proteins (Fig. 11.1; note that in *T. pseudonana, Lhcx1* and *Lhcx2* encode identical proteins). Transcript levels depend on growth light intensity, whereby, in *P. tricornutum,* up-regulation under HL is most pronounced for *Lhcx2*, followed by *Lhcx3* and *Lhcx1* (Nymark et al. 2009, 2013; Lepetit et al. 2013). In *Chaetoceros neogracile*, a centric diatom the genome of which has not been sequenced, Park et al. (2010) found up-regulation of *Lhcx* genes under HL, which also correlated with Dt accumulation and formation of NPQ. Zhu and Green (2010) investigated *T. pseudonana*, and found that *Lhcx4* was up-regulated to the highest levels compared to a constitutive (house-keeping) gene, followed by *Lhcx1* and *Lhcx6.* However, relative changes were most prominent for *Lhcx6*. Up-regulation in response to HL exposure was also demonstrated for Lhcx1 and Lhcx6 on protein level, with Lhcx1 levels staying high during a subsequent dark period, while Lhcx6 levels decreased again. Figure 11.1 provides an alignment of the different Lhcx sequences from the pennate *P. tricornutum* and the centric diatom *T. pseudonana*, with Fcp6 from *C. meneghiniana* added. The latter protein is almost identical to Lhcx1/2 of *T. pseudonana* and Lhcx1 of *P. tricornutum*, and fairly similar to Lhcx3 of *P. tricornutum*. The other Lhcx sequences differ slightly, with most pronounced amino acid changes compared to Lhcx1/2 in *T. pseudonana* Lhcx6_1, but also in Lhcx6, Lhcx5 and Lhcx4 of this species.

At the beginning of this century, not only did genome sequences become available, but tools were also established to genetically transform diatoms. Bailleul et al. (2010) published proof for a function of Lhcx in NPQ. By using knock-down and over-expressing mutants created in several *P. tricornutum* strains, the latter authors were able to demonstrate that NPQ depends on Lhcx1. Shortly before, the same link had been established for the LhcSR proteins in *Chlamydomonas reinhardtii* (Peers et al. 2009). In addition, Zhu and Green (2010) published the first correlation between NPQ and Lhcx6 content in *T. pseudonana,* the species closely related to *C. meneghiniana*. Thus, the first evidence of an involvement of Lhcx proteins came from the pennate diatom *P. tricornutum*, whereas most of the biochemical work thus far had been done on the diatom *C. meneghiniana*.

In the pennate diatom *P. tricornutum*, FCP trimers had been isolated early on, almost exclusively using sucrose gradient centrifugation methods (Alberte et al. 1981; Friedman and Alberte 1984, 1986; Owens and Wold 1986; Guglielmi et al. 2005; Lavaud et al. 2003; Lepetit et al. 2007). In the centric diatom *C. meneghiniana*, two complexes differing in polypeptide composition were consistently reported, with only the trimeric FCPa complex containing Lhcx, while the other major complex, FCPb, consisted solely of Lhcf proteins (Beer et al. 2006; Gundermann and Büchel 2008). With the sequences available, mass spectrometric identification of polypeptides became possible, and all Lhc genes, including all Lhcx genes, were found by Lepetit et al. (2010) to be expressed in *P. tricornutum*. In the latter study, Lhcx proteins were found in the FCP fraction as well as in PS I. Grouneva et al. (2011) published a comparable study, in which two different FCP complexes were, again, isolated from the centric diatom *T. pseudonana*, this time using non-denaturing

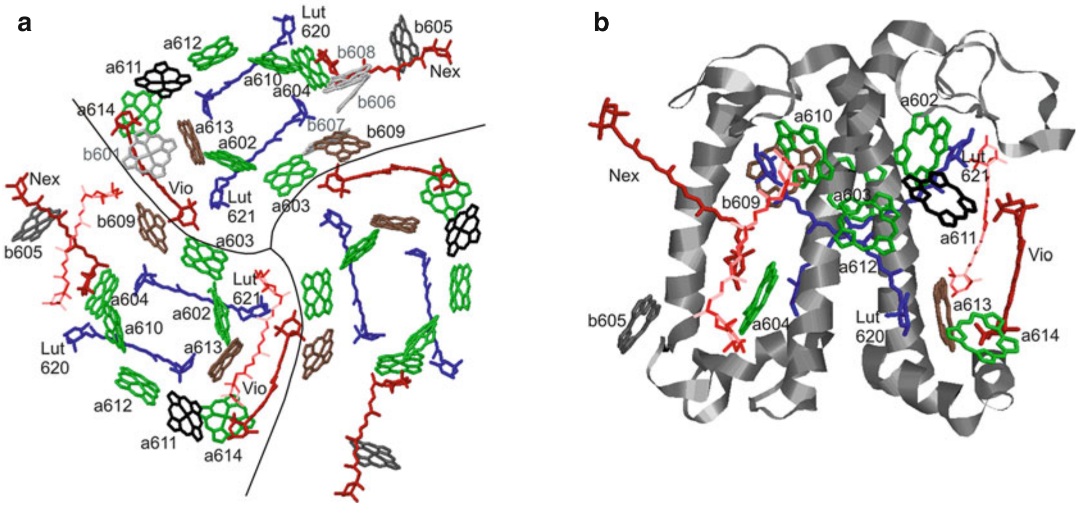

Fig. 11.2. Structural model of the trimeric FCPa consisting of Lhcf and Lhcx polypeptides, based on the structure model of LHCII (According to Liu et al. (2004)). Chl *a* is shown in *green*, Chl *c* in *brown*, Chl *a* molecules only loosely bound are demonstrated in *grey* and *black*, respectively, and the binding sites are labeled according to Liu et al. (2004). The Fx molecules proposed to absorb at shorter wavelength in the native FCP complexes localized in the lutein (Lut) sites are depicted in *blue*, the others with a larger bathochromic shift, including the one occupying the neoxanthin (Nex) site, are drawn in *red*. Dt or Dd is supposed to bind to the violaxanthin (Vio) site in Lhcx monomers, which is harboring Fx in case of Lhcf proteins. In the lumenal view of a trimer only the pigments are presented (**a**) All LHCII pigments are shown in the upper monomer, whereas the Chls missing in FCPs (*light grey*) are omitted from the other two monomers. In the *lower left* monomer two additional Fx molecules are modeled. (**b**) Represents a side view of this monomer, including the protein backbone (Adopted from Premvardhan et al. (2010)).

blue native polyacrylamide gel electrophoresis. As had already been shown for *C. meneghiniana*, the trimeric FCP contained Lhcx proteins, whereas the oligomeric complex consisted only of Lhcf proteins. In addition, Lhcx were again detected in PS I complexes. In contrast, Lhcx proteins were only found in the free-protein fraction, and associated with PS I, but not in the trimeric FCPs, in *P. tricornutum*. Subsequently, Gundermann et al. (2012) reported different sub-populations of trimers from *P. tricornutum,* and, again, could not detect Lhcx proteins by mass spectrometry. Biochemical data thus point to differences in FCP suprastructure between the two groups of diatoms; whereas Lhcx polypeptides are constituents of trimers in centric diatoms, no trimers containing these polypeptides have thus far been found in pennates. The Lhcx proteins might therefore either be monomeric in these species or only loosely assembled into higher oligomeric complexes.

Despite the latter differences between diatom groups, protein sequences are very similar in both groups and point to similar pigment-binding sites (Fig. 11.1). Figure 11.2 depicts a model of pigment binding by FCPs, developed by Premvardhan et al. (2010) built on results from resonance raman spectroscopy of FCPa and FCPb of *C. meneghiniana*. Based on its similarity to LHCII, the latter structure (Liu et al. 2004) was taken as a starting point for assigning the pigments to binding sites. The fact that neither the spectra nor the protein sequences of FCPs exhibited obvious differences points to a similar pigment configuration. The model can thus be applied to both FCPa and FCPb, and, more importantly, to both Lhcf and Lhcx proteins. According to this model, two Fx molecules are bound in the two sites that bind lutein in LHCII, whereas an additional Fx occupies the space left by the Chl molecules missing in FCPs when compared to LHCII. The violaxanthin-binding site of

LHCII was hypothesized to be the one binding Dd or Dt in Lhcx subunits and (mainly) Fx in Lhcf subunits, respectively, but the exclusive binding of Dd or Dt to Lhcx has not been proven yet. Since the Lhcx sequences of centrics and pennates are very similar (i.e., Fcp6 of *C. meneghiniana* is almost identical to Lhcx1 of *T. pseudonana*, which, in turn, is very similar to Lhcx1 of *P. tricornutum*, see Fig. 11.1), the proposed binding sites are likely the same in all species. Modeling therefore points to Lhcx proteins being pigment-binding proteins, a view supported by the fact that the related LhcSR proteins were recently shown to bind pigments (Bonente et al. 2011). However, no direct proof for pigment binding of any Lhcx is thus far available.

III Influence of Diadinoxanthin and Diatoxanthin Bound to FCP Complexes

NPQ in diatoms also depends on de-epoxidation of the xanthophyll Dd to Dt. Diatoms contain comparably large and extremely variable total levels of the latter pigments. For example, Lepetit et al. (2010) reported 0.237 mol (Dd + Dt)/mol Chl for cells grown under LL (10–15 μmol photons m^{-2} s^{-1}) versus 0.738 mol (Dd + Dt)/mol Chl for cells grown under HL (160–180 μmol photons m^{-2} s^{-1}). The de-epoxidation rate correlates with the formation of NPQ, and very similar enzymes compared to those of higher plants are active in the conversion from Dd to Dt and vice versa in diatoms. Nevertheless, some important differences exist. The pH optimum of Dd de-epoxidase is shifted to higher pH values in comparison to violaxanthin de-epoxidase of higher plants. Conversion to Dt is thus activated by a much lesser lumen acidification, i.e., under lower light intensities (Jakob et al. 1999). In addition, de novo synthesis of Dt as a function of irradiance was also demonstrated for diatoms (Lavaud et al. 2004), leading to an increased total pool of xanthophyll cycle pigments under prolonged irradiance. For a more detailed description, see Lavaud and Goss (Chap. 20).

Concerning the function of Dt in NPQ in diatoms, the question of the Dt localization needs to be addressed. Whereas it is clear that FCPs do bind Dd as well as Dt (Lavaud et al. 2003; Beer et al. 2006; Guglielmi et al. 2005; Lepetit et al. 2007), there is a debate of whether or not all Dt is bound to FCPs. It was hypothesized initially that Dd and Dt do not participate in excitation-energy-transfer to Chl *a* in FCPs (Büchel 2003; Lepetit et al. 2010), since a strong absorption is present around 490 nm, which is usually attributed to the xanthophyll cycle pigments, but excitation energy transfer cannot be seen in fluorescence-excitation spectra. Photosystem I preparations including PS I antennae were always found to contain Dd as well (Lepetit et al. 2007; Veith and Büchel 2007; Veith et al. 2009). More importantly, under HL the relative amount of Dt was increased (Lepetit et al. 2007; Juhas and Büchel 2012). This additional Dt was discussed as a scavenger for reactive oxygen species (Lepetit et al. 2010), although such a role can, principally, be fulfilled by other carotenoids as well. Moreover, the fact that Dt is associated with PS I in a light-dependent way argues for rather indiscriminate Car binding to all FCPs under conditions when Dd de-epoxidase is activated. Lepetit et al. (2010) argued that a substantial amount of Dd and Dt must also occur in the lipid phase. The latter authors isolated different FCP pools from HL- or LL-grown cells using sucrose density centrifugation as a method allowing FCPs to remain surrounded by lipids after purification. FCPs from LL- versus HL-grown cells bound exactly the same amounts of light-harvesting pigments Chl *a*, Chl *c* and Fx, but differed in Dd and Dt content. The authors concluded that all binding sites of the proteins must already be occupied under LL conditions, and that the additional Dd and Dt found in HL must thus be located in the lipid phase. This argument, however, does not take fully into account the fact that protein composition of FCP complexes also differs between HL and LL, with more Lhcx bound to FCP complexes under HL. Thus, a difference in the binding capacity of Lhcx proteins versus Lhcf proteins can account for at least some of the additional xanthophyll binding, as was indeed shown for FCPa from *C. meneghiniana* (Beer et al. 2006). On the other hand, differences in Dd and Dt content between cells grown under HL and LL are large, suggesting that some of the Dd and Dt is not located in specific binding sites of FCPs like in higher plants where zeaxanthin is bound to LHCII complexes (Liu et al. 2004; Standfuss et al. 2005) but also found dissolved in the thylakoid membrane (e.g., Dall'Osto et al. 2010, see Chap. 26 for more details). Schumann et al. (2007)) estimated that about 10 % of the HL induced Dt enrichment is not bound to FCPs. Whether this Dd and Dt is found freely dissolved in the lipid phase, or whether it is peripherally associated with FCPs, remains to be elucidated. In the latter case, interaction with pigments properly bound to the proteins would still be possible. In both centrics and pennates an absorbance change at 552 nm correlates with NPQ formation, and was attributed to a fraction of Dt which is active in NPQ, whilst another fraction of Dt, which is likely to be located in the lipid phase, does not serve this function (Ruban et al. 2004; Lavaud and Kroth 2006; Lavaud and Lepetit 2013). For further discussion of Dt produced under HL, but not involved in NPQ, the reader is referred to Chap. 20.

In 2008, Gundermann and Büchel were able to demonstrate changes in Chl fluorescence yield of FCP complexes in vitro depending on the amount of Dt bound. In FCPa, the trimeric complex from *C. meneghiniana* that contains Lhcx proteins, fluorescence yield was negatively correlated with Dt content. Later, a spectral fingerprint in fluorescence emission spectra, with a small, but significant red shift of Chl *a* emission was, furthermore, attributed to the action of increased amounts of Dt on FCPa (Gundermann and Büchel 2012). In contrast, FCPb, the complex without Lhcx, also bound Dt at different levels depending on growth conditions, but its fluorescence yield was independent of the amount of Dt bound. Since highly purified FCPa complexes were used, the amount of Dt was rather minimal, with 0.01 (LL) to 0.05 mol Dt/mol Chl *a* (HL). Possibly due to this reason, fluorescence lifetimes of HL- and LL-FCPa were quite similar (Gildenhoff et al. 2010a), and there were no significant differences in fluorescence yield between the preparations used for this study.

In addition to the direct influence of Dt on the fluorescence yield of Chl *a* in FCPa, Dd unexpectedly exerted an effect. Gundermann and Büchel (2012) reconstituted highly purified FCPa from *C. meneghiniana* into liposomes. This FCPa carried only negligible amounts of Dd. When Dd was added to liposomes with a high lipid-to-protein ratio, the fluorescence yield of the complexes decreased. This phenomenon was interpreted as Dd aiding in the formation of protein-protein interactions (see below). This feature might be another explanation for the high Dd concentrations found in the thylakoids membranes of diatoms. No such studies are available for the FCP complexes of pennate diatoms.

IV Influence of pH on the Fluorescence Yield of FCP Complexes

Little is known about the influence of pH on FCP complexes. In contrast to e.g., PsbS or LhcSR, no pH-sensitive lumenal glutamate residues have been documented thus far by e.g., DCCD binding. Lhcx proteins in diatoms, whether from pennates or centrics, do not contain any glutamate residues in the lumenal loop. Only one glutamate is found at the C-terminus (Premvardhan et al. 2010), and one at the lumenal end of helix 1 (Fig. 11.1). In contrast, in most Lhcfs a glutamate is present in the lumenal loop (Gundermann and Büchel 2012).

Despite this apparent lack of pH-sensing residues, an influence of pH on the fluorescence yield of FCPa complexes from *C. meneghiniana* was reported (Gundermann and Büchel 2008). In contrast to what might be expected, lower pH values decreased the fluorescence yield of FCPa, the Lhcx-containing complex, only after aggregation by detergent removal. No such change was detectable for FCPb, the purely Lhcf-containing complex. However, since detergent removal induces rather unspecific aggregation, this situation may not mimic the in vivo situation. To further evaluate this phenomenon under more native environment, FCPa complexes were tested at different lipid/protein ratios in liposomes (Gundermann and Büchel 2012). The amount of protein in these liposomes was chosen to be in a range where different degrees of protein-protein interactions were observable. The effect of pH on the latter complexes was most pronounced at a low lipid/protein ratio, i.e., at short protein-protein distances. The effect of pH gradually decreased towards lipid/protein ratios resembling a fully solubilized complex. A conformational change directly induced by lumenal protonation of the protein is thus unlikely, since this should work independently of the interaction with other FCP complexes. The latter findings are consistent with the lack of glutamate residues on the lumenal side. A possible explanation for the pH effect is facilitated aggregation of FCP complexes at low pH, which should reduce Chl fluorescence yield (see below). This explanation is consistent with measurements on whole cells by resonance raman and IR spectroscopy, demonstrating a conformational change of FCPs in whole cells upon formation of NPQ (Alexandre MTA, Mezzetti A, Ilioaia C, Gundermann K, Pascal AA, van Grondelle R, Büchel C, Robert B, 2014).

V Aggregation of FCP Complexes and NPQ

Miloslavina et al. (2009) were the first to analyze whole diatom cells (the pennate *P. tricornutum* as well as the centric *C. meneghiniana*) by ultrafast spectroscopy. Dark-adapted cells were compared to cells in the NPQ state, and fluorescence decays were measured. The NPQ state was characterized by faster time constants for PS II fluorescence decay, and by an additional component with a shorter lifetime but with a longer wavelength emission. This was interpreted in the model of the Holzwarth group (Holzwarth et al. 2009) for higher plant NPQ as an indication of two quenching sites, Q1 and Q2, in diatom thylakoids. In analogy, it was proposed that the additional component with a longer wavelength emission, Q1, should depend solely on aggregation of FCPs, which at the same time are detached from the photosystems. Data supplied by Nagy et al. (2011, 2012), using small-angle neutron scattering, support such a major rearrangement of the thylakoid membrane under NPQ conditions. In addition, a further quenching site, Q2, located within PS II-FCP complexes, should be Dt-dependent. This feature was identical in pennate and centric diatoms, but the fluorescence decay-associated spectra of the two species, *P. tricornutum* and *C. meneghiniana*, differed slightly with respect to maxima and amplitude, respectively. In *P. tricornutum,* the spectrum related to Q1 was more pronounced and its second maximum was found at around 710 nm, whereas in *C. meneghiniana* a broad emission up to 735 nm was reported. In view of the earlier work of Gundermann and Büchel (2008), it was hypothesized that Q1 should be found in FCPb, whereas Q2 should be due to FCPa, despite the lack of FCPb in the pennate *P. tricornutum*. Later, Lavaud and Lepetit (2013) obtained 77 K spectra of *P. tricornutum*, *Skeletonema costatum* (another centric species) and *C. meneghiniana* cells both in the dark and after illumination. In *P. tricornutum*, a long-wavelength emission was detected. The ratio between this 710 nm emission and the peak at 687 nm depended on the NPQ capacity of the cells and on pre-illumination, with the latter feature having been observed in a xanthophyte alga as well (Büchel and Wilhelm 1990). A variable 710 nm emission at room temperature had been reported earlier for *P. tricornutum* grown under red light (Fujita and Ohki 2004). Only a low level of 710 nm fluorescence at 77 K was detected for the centric species, and this emission was not variable (Lavaud and Lepetit 2013). The latter authors attributed the 710 nm emission in *P. tricornutum* to some biochemically unidentified FCP complexes that detach from PS II upon illumination and thus resemble the Q1 quenching site. Unfortunately, too little is known about FCP composition in pennates to attribute these aggregated complexes to any of the preparations thus far. Using very low detergent concentrations, FCPs in a higher oligomeric state were isolated from *P. tricornutum* but, unfortunately, the presence or absence of Lhcx was not examined. More importantly, no long-wavelength emission was detected in the 77 K emission spectra of the isolated complexes (Lepetit et al. 2007). Thus, either these relatively unstable higher oligomers are not responsible for the 710 nm emission detected in pennates or had lost this feature upon isolation.

For the centric diatom *C. meneginiana*, the hypothesis of FCPb and FCPa corresponding to Q1 and Q2, respectively, was also tested using isolated FCPa complexes reconstituted into liposomes to mimic the natural environment (Gundermann and Büchel 2012). Aggregation had been shown before to influence the Chl fluorescence yield of both complexes (Gundermann and Büchel 2008), thus confirming similar spectral features for Q1 as seen by Miloslavina et al. (2009) in whole cells for both isolated FCPa and FCPb. In contrast to the model by Miloslavina et al. (2009), that attributes Q1 and Q2 to different proteins, FCPa, the complex containing Lhcx proteins as well as Lhcf, was shown to display all features required for both quenching sites: its Chl fluorescence yield turned out to be influenced by the amount of Dt present, by aggregation state, as well as by the pH. FCPb, the complex consisting of Lhcf proteins, did not respond to either Dt content or pH, but only to aggregation, which makes it a good candidate for Q1 (Gundermann and Büchel 2008). Thus, in centric diatoms FCPa features both quenching-site mechanisms (Dt-dependent and aggregation-dependent quenching), with Q1 being either solely featured by FCPa or by both FCPa and FCPb. However, no biochemical data are available about the precise attribution of FCPa and FCPb to the two photosystems, and only limited inferences can be made based on PS I preparations (Veith and Büchel 2007) or the deconvolution of spectra of whole cells for the attribution of FCP complexes to the photosystems (Szabó et al. 2010; Chukhutsina et al. 2013). Thus, the precise molecular identity of quenching centers remains elusive.

VI Proposed Molecular Mechanisms for the Reduction of Fluorescence Yield of FCP Complexes

A key question concerns the molecular mechanism of the decreased fluorescence yield under NPQ conditions at the pigment level, but no conclusive work is published so far. The same hypotheses as for higher plants have been discussed, and the reader is referred to the appropriate Chapters (e.g., 8,9,10) for further details concerning, e.g., generation of specific Chl-Chl interactions (Müller et al. 2010), Dd and Dt acting as light harvester or heat emitter, respectively, according to the gear-shift model (Frank et al. 1996), or the opening of a new energy dissipating channel via a low-lying Car excited state (Ruban et al. 2007), whereby the Car in question would be Fx. As in higher plants, a concerted action of several mechanisms cannot be excluded so far (see especially Walla et al., Chap. 9). However, except for the gear-shift model, all of these mechanisms require a conformational change, as that measured using FT-IR on whole cells of *C. meneghiniana* upon NPQ induction (M. T. A. Alexandre, A. Mezzetti, C. Ilioaia, K. Gundermann, A. A. Pascal, R. van Grondelle, C. Büchel, B. Robert, unpublished). Since FCPa in centric diatoms was shown to respond to all factors influencing NPQ in vivo, whereas FCPb only reacts to aggregation, whatever phenomenon is responsible for NPQ has to be present in FCPa, but not necessarily in FCPb.

Using Stark fluorescence spectroscopy on isolated FCPa and FCPb complexes in the soluble and aggregated states, a new red emission band with a low fluorescence yield was detected upon induction of thermal energy dissipation in FCPa, but not in FCPb (Wahadoszamen et al. 2014). This band featured remarkable spectral similarities to a red emission band recently detected in LHCII under dissipative conditions (Wahadoszamen et al. 2012). In principle, this band could arise from Chl-Chl or Chl-Car mixed excitonic charge transfer states, induced by small conformational changes upon aggregation (see also Walla et al., Chap. 9). However, the absence of excitonically coupled Chl pairs in FCPs (Papagiannakis et al. 2005; Premvardhan et al. 2008, 2009, 2010; Gildenhoff et al. 2010a, b) argues against a Chl-Chl charge transfer state responsible for the red emission. This red shifted emission band may thus originate from a Chl-Car charge transfer state in FCPa, that is not present in FCPb under the same conditions. It has recently been shown that Fx molecules in FCP are active in Chl-Car triplet-triplet energy transfer (Di Valentin et al. 2012), pointing to a protective role of Fx. Thus it is possible that the vicinity and the orientation of one or more Chls with respect to Fx is such that a small conformational change may induce the required Chl-Car interaction responsible for the new red shifted emission band with a low fluorescence yield. Such a feature could then serve as the thermal energy dissipation channel in the quenched FCPa. No data about the molecular action of Dt are available so far.

VII Proposed Mechanisms for the Involvement of FCPs in NPQ

Since clear differences exist between centrics and pennates in the general features of NPQ and with respect to FCPs, models should be able to distinguish between those two cases. We will first consider the pennates here, and then describe the centrics in more detail, since more data about molecular details are available in the latter group.

As described above, the precise localization of Lhcx proteins is still unclear in pennate diatoms. However, involvement of Lhcx1 in NPQ was established by Bailleul et al. (2010) using knock-down strains, in which no differentiation was made between qI and qE. In addition, a linear correlation between Dt content and NPQ exists. The slope is species dependent and not all Dt synthesized under HL actively participates in NPQ. This led to the hypothesis that Dt has to be ‘activated’ by lumen acidification. In pennates, more of the newly synthesized Dt is active in NPQ as compared to centrics (Lavaud and Lepetit 2013, see Chap. 20 for details). These observations led to a model, in which the portion of Dt active in NPQ is bound to Lhc complexes close to PS II reaction centers, and resembling the Q2 site as proposed by Miloslavina et al. (2009) (Fig. 11.3a–c). Whether this site consists of Lhcx1, whether this protein is present near the reaction center as a monomer or as part of (possibly transient) oligomers, and whether other Lhcx proteins participate, remains to be elucidated. Most likely, the Lhcx proteins also bind the Dt pool functioning in NPQ, but as long as no biochemical evidence is available, this remains speculative. The proteins responsible for NPQ should then undergo a conformational change upon binding of Dt and/or lowering of luminal pH, resulting in increased heat dissipation (Goss et al. 2006). This hypothesis was extended by Lepetit et al. (2012) in stating that it is probably Lhcx1 that acts on FCP complexes by inducing a conformational change, and that Lhcx1 is not necessarily the protein binding Dt (Bailleul et al. 2010). This allosteric model was adopted from higher plants, but still lacks direct evidence at the molecular level. In addition, *P. tricornutum* was shown to exhibit variable 710 nm fluorescence at room temperature, especially when cells were cultivated under red light (Fujita and Ohki 2004). This room-temperature emission was attributed to FCPs associated with PS II. 77 K emission at 710 nm in *P. tricornutum* was later shown to correlate with the capacity for NPQ (Lavaud and Lepetit 2013). Therefore, an additional quenching center was postulated, which amplifies NPQ when dissociated from PS II, and fluoresces at 710 nm. The amount of this amplification depends on the growth conditions of the cells,

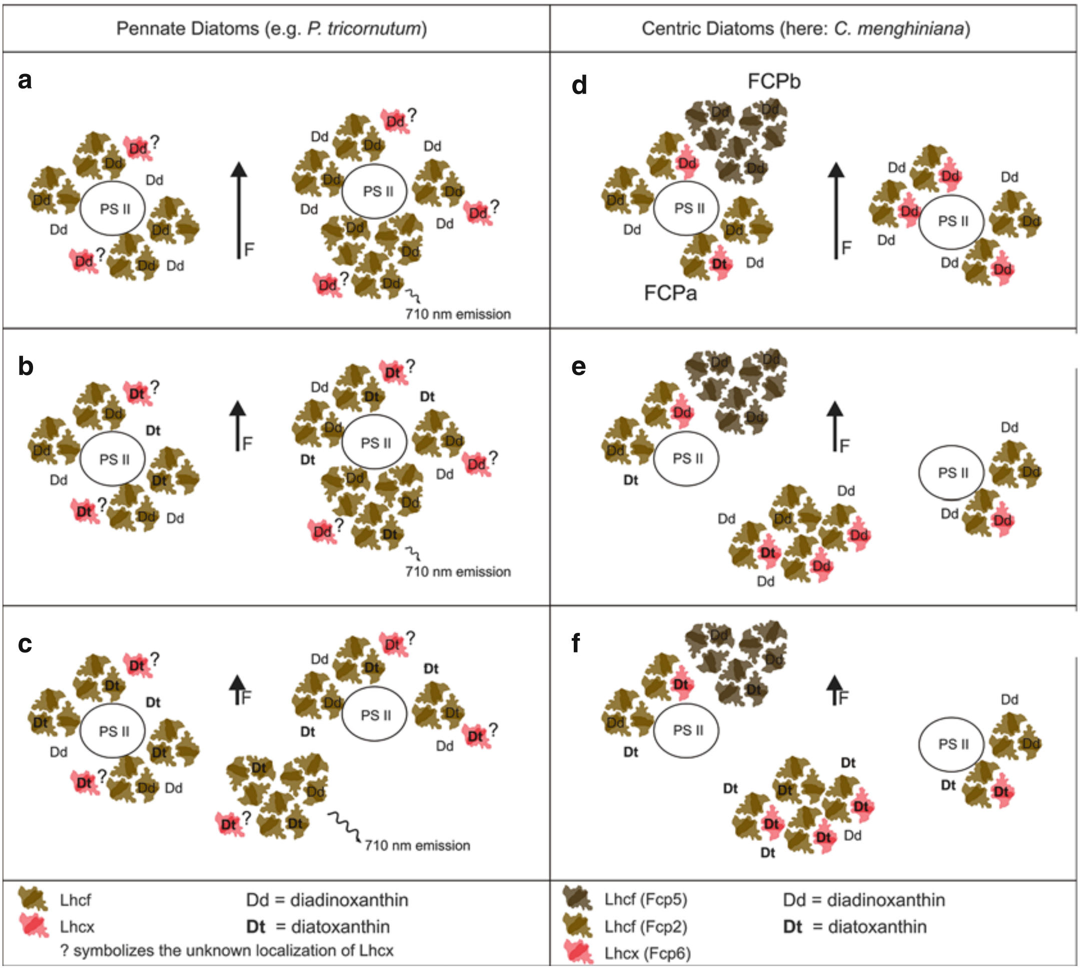

Fig. 11.3. Proposed model for the molecular architecture underlying the quenching of fluorescence in diatoms. Note that in the model the arrangement around PS II (*circles*) is completely arbitrary, since no data exist about the precise localization of FCP complexes. The different fluorescence yield (*F*) of the FCPs after prolonged darkness (**a, d**), at the beginning (**b, e**) and during steady state of HL illumination (**c, f**) is symbolized by the length of the *arrows*. In (**a**–**c**) the model for pennates is depicted. No data about the localization of Lhcx proteins (*red*) is available so far, except that they are missing in isolated trimers, which are built of Lhcf peptides (*brown*) only. In darkness, mainly Dd is found (**a**) Upon illumination and the build-up of a pH gradient, Dt is synthesized and either found in the lipid phase or bound to the FCP proteins (**b**) inducing a decrease in fluorescence yield. Oligomeric FCP complexes, which are characterized by a 710 nm emission, are hypothesized to amplify NPQ by detaching from PS II (Lavaud and Lepetit 2013) (**c**) Note that no biochemical evidence for these oligomers exists so far. In (**d**–**f**) the model for centrics, as developed for *C. meneghiniana*, is shown. Trimeric FCPa complexes contain Fcp2 (Lhcf, *brown*) and some of them Fcp6 (Lhcx, *red*) subunits, whereas the oligomeric FCPb is built of Fcp5 (Lhcf, *dark brown*) (**d**). Upon illumination and built-up of a pH gradient, FCPa complexes aggregate aided by the Dd found in the membrane and the fluorescence yield is thereby reduced (**e**) Under continued illumination the Dt additionally synthesized further enhances the quenching (**f**) which is also the case for the Dt found at the beginning of an illumination period after shorter dark adaptation. See text for further explanations (Adopted from Gundermann and Büchel (2012)).

and is related to their maximal NPQ capacity. The spectroscopic fingerprint of this center, i.e., the variable 710 nm emission, does not exist in centrics, and these differences are underlined by the different decay-associated spectra provided by Miloslavina et al. (2009). The center might resemble Q1, but with another molecular basis as compared to centrics (see Fig. 11.3 and below). The 77 K emission at 710 nm is already present to some extent in the dark-adapted state, especially in *P. tricornutum* strains exhibiting a high NPQ capacity, which was interpreted as already aggregated, i.e., oligomeric FCP complexes (Lavaud and Lepetit 2013), but no FCP displaying this spectral feature has been isolated thus far. No evidence exists for participation of Lhcx in either center, and the molecular role of Lhcx thus remains elusive.

In centrics, no data using knock-down strains are available for the involvement of any of the Lhcx proteins in NPQ. Park et al. (2010) reported a correlation between Lhcx mRNA levels and both NPQ and Dt content for *Chaetoceros neogracilis*. Zhu and Green (2010) observed a relationship between Lhcx6 accumulation and NPQ in *T. pseudonana*, but correlation was better for only the qI component than for total NPQ. Since Lhcx1 is already expressed in a substantial amount under LL, and no NPQ is measurable under those light conditions, the latter authors hypothesized that Lhcx1 might instead have a structural role in thylakoid membrane organization. Thus, no Lhcx protein being specifically involved in the qE part of NPQ was identified, and the question which Lhcx plays a role in NPQ remains open. At the level of isolated FCP complexes, most work was done using *C. meneghiniana*. As described above, FCPa, the trimeric complex containing Fcp6 as the protein almost identical to Lhcx1/2 of *T. pseudonana*, was shown to change its fluorescence yield depending on aggregation, pH, and Dd and Dt content. FCPb, the higher oligomer consisting solely of Lhcf polypeptides, was not studied intensively. Aggregation also reduced FCPb fluorescence yield, but neither pH nor Dt content had an influence.

Still, this work enables a first rough model for the involvement of FCPs in NPQ of centrics: in vivo, Dd is found mainly after prolonged darkness and FCPa is not aggregated (Fig. 11.3d–f), leading to a high fluorescence yield. Note that Fcp6 content in the FCPa pool differs depending on the acclimation of cells (HL or LL) (Beer et al. 2006), and that some Dt might still be found in the dark depending on the duration of the preceding light adaptation. During illumination, a pH gradient is built-up in vivo, and will lead to rapidly induced NPQ (Grouneva et al. 2008), mirrored on molecular level solely by aggregation, and thus a decreased fluorescence yield of FCPa at lower pH. This aggregation is presumably aided by Dd present in the membrane. The slower part of NPQ in vivo is correlated with increasing Dt levels (Grouneva et al. 2008), which also leads to decreased fluorescence yield of FCPa in vitro. An additional conformational change upon binding of Dt might be included as well, since a conformational change of FCPs in whole cells upon formation of NPQ and Dt accumulation was detected via FT-IR and Resonance Raman spectroscopy (M. T. A. Alexandre, A. Mezzetti, C. Ilioaia, K. Gundermann, A. A. Pascal, R. van Grondelle, C. Büchel, B. Robert, unpublished). In the latter model, the lowering of fluorescence yield by Dt and aggregation might represent the sum of two independent mechanisms, while a synergistic effect cannot be ruled out. The aggregated state of FCPa is characterized by an additional red fluorescence emission band with strong charge-transfer character but very low fluorescence yield, which is in contrast to the variable 710 nm emission seen in steady state spectroscopy in pennates upon NPQ induction. The model excludes FCPb, since FCPa is responsible for Q2 (Dt-dependent quenching) and is presumably also involved in Q1 (aggregation-dependent quenching). Thus, all features seen by Miloslavina et al. (2009) can be explained solely by FCPa, if one assumes that Q1 and Q2 are different mechanisms taking place in a single protein complex. On the other hand, it cannot be ruled out that Q1 (the aggregation site) contains FCPb as well.

VIII Conclusions and Outlook

In summary, Dt-, pH-, and aggregation-dependent changes of the fluorescence yield of isolated FCPs were demonstrated in vitro, while all these factors were also demonstrated to exert an influence on NPQ both in pennates and centrics in vivo. However, the molecular mechanism of NPQ in diatoms remains elusive, since still too little is known about its molecular basis. No spectroscopic evidence is available for the action of Dt. Only PS II complexes without FCPs (Nagao et al. 2010), but no super-complexes of PS II consisting of PS II cores and functionally attached FCPs have been isolated so far, and it remains to be explored which of the several Lhcx proteins is located in an appropriate place to directly or indirectly influence NPQ. A lot of knowledge concerning correlations of NPQ with e.g., absorbance changes at 552 nm, Dt content, 710 nm fluorescence and the influence of pH has been accumulated in vivo and for isolated FCPs, but little is known about the cause(s) for NPQ. In addition, many publications point to differences between the various groups of diatoms, e.g., centrics and pennates. Diatoms are ecologically extremely important since they account for about 25 % of yearly global biomass production (Falciatore and Bowler 2002). One of the features enabling this success is the diatoms' ability for fast adaptation to different light intensities. Thus, research interest is increasing, and fascinating aspects of the mechanisms underlying the sometimes extremely large NPQ seen in diatoms will certainly emerge in the future.

Acknowledgments

This work was funded by the Deutsche Forschungsgemeinschaft (Bu812/4-1, Bu812/4-2) and the European Union (Marie Curie ITN 238017 'Harvest').

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Chapter 12

Involvement of a Second Xanthophyll Cycle in Non-Photochemical Quenching of Chlorophyll Fluorescence: The Lutein Epoxide Story

Raquel Esteban
Department of Plant Biology and Ecology,
University of the Basque Country (UPV/EHU), Bilbao 48080, Spain
Agrobiotechnology Institute,
IdAB-CSIC-UPNA-Government of Navarre, Pamplona E-31192, Spain

and

José I. García-Plazaola
Department of Plant Biology and Ecology,
University of the Basque Country (UPV/EHU), Bilbao 48080, Spain

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*Author for Correspondence, e-mail: raquel.esteban@ehu.es

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_12, © Springer Science+Business Media Dordrecht 2014

Summary

The lutein epoxide (Lx) cycle (Lx-L cycle), initially described in tomato fruits, remained unexplored during the following 25 years until it was re-discovered in the stems of the parasitic plant *Cuscuta reflexa*. Since then, 15 years of continuous research have revealed that the Lx-L cycle is present in a wide diversity of species, allowing us to make significant progress in our understanding of this carotenoid cycle. However, due to its absence in some traditional model plant species, much of its functional significance remains to be clarified. We have therefore provided an overview of the current knowledge of this cycle as well as an ecological perspective of the possible benefits derived from the possession of two xanthophyll cycles (all plants possess a xanthophyll cycle involving violaxanthin, antheraxanthin, and zeaxanthin, i.e., the VAZ cycle, whereas only some species possess the Lx-L cycle). In the Lx-L cycle, Lx is de-epoxidized upon illumination, by the action of violaxanthin de-epoxidase (VDE), and transformed into lutein (L). Correlative evidence supports a role for this newly formed L (ΔL) in the regulation of thermal energy dissipation as assessed from the non-photochemical quenching of chlorophyll fluorescence (NPQ). The reversibility of this reaction in darkness differentiates two modes of operation, one "completed" in which the initial Lx pool is restored and one "truncated" in which ΔL remains for a longer period. The first, described in sun and shade leaves of a few unrelated species, apparently provides fine-tuning for the adjustment of photoprotective energy dissipation complementary to that exerted by the VAZ cycle. On the other hand, the "truncated" cycle is widespread amongst woody plants, but is restricted to shaded environments where it may act as a rapid switch for the photosynthetic apparatus from a photosynthetically highly efficient state to a photoprotected one. The latter may be necessary for chloroplast acclimation after transition to high light, such as that which occurs following gap formation in forests or budbreak and subsequent leaf greening in trees.

I Introduction

"*A second epoxide cycle of lesser amplitude was observed between lutein and lutein monoepoxide*". This sentence came at the end of the summary of a report published in *Planta* by Haim Rabinowitch and his former supervisors, Pierre Budowski and Nachum Kedar, 38 years ago (Rabinowitch et al. 1975). Using thin layer chromatography with astonishing precision, they were able to identify, for the first time, the simultaneous operation of two xanthophyll cycles in green tomato fruits, for which they even

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Abbreviations: A – Antheraxanthin; *A*-Lx – Accumulative Lx pool; Chl – Chlorophyll; *C*-Lx – Constitutive Lx pool; *D*-Lx – Dynamic Lx pool; DPS – De-epoxidation state of the xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin and zeaxanthin; F_m – Maximal chlorophyll fluorescence emission in the dark-adapted state; F_v – variable chlorophyll fluorescence emission in the dark-adapted state; F_v/F_m – Intrinsic efficiency (or quantum yield) of photosystem II in the dark-adapted state; L1 – Intra-protein site L1; L2 – Intra-protein site L2; LHC – Light-harvesting complex; Lhcb5 – Chlorophyll a-b binding protein; LHCII – Major light-harvesting complex of photosystem II; Lx – Lutein epoxide; Lx-L cycle – Lutein epoxide cycle involving the carotenoids lutein epoxide and lutein; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PFD_i – Incident photon flux density; PS II – Photosystem II; qE – ΔpH-dependent component of NPQ; V – Violaxanthin; V1 – Peripheral V1 site; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin and zeaxanthin; VDE – Violaxanthin de-epoxidase; Z – Zeaxanthin; ZEP – Zeaxanthin epoxidase; α-C – α-carotene; β-C – β-carotene; ΔL – Newly formed L from Lx de-epoxidation

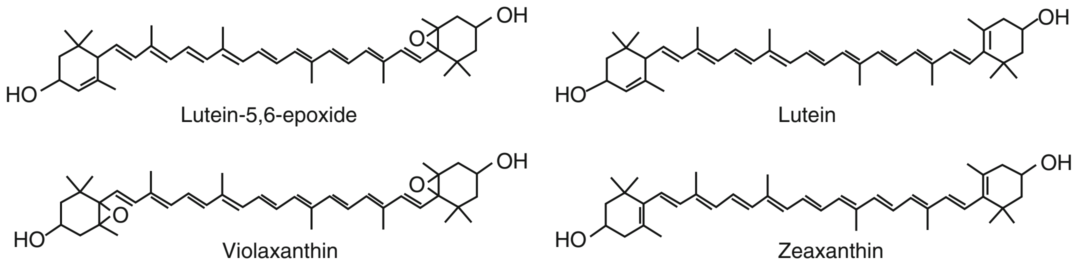

Fig. 12.1. The structure of the main molecules involved in the Lx-L and the VAZ cycles: Lx, L, V and Z.

hypothesized a photoprotective function. Unfortunately, as many other beautiful pieces of science published before the 1980s, this finding was overlooked for more than 25 years.

However, since the xanthophyll cycle involving lutein-5,6-epoxide (Lx) and lutein (L; Fig. 12.1) was re-discovered by Bungard and co-workers (Bungard et al. 1999), interest in this cycle has led a number of research groups to elucidate and explore the operation and significance of this Lx-L cycle. As a result, it is now known that the Lx-L cycle is present in many taxonomic groups; yet, it is still overlooked and underappreciated. For instance, Lx is absent from figures in some of the recent reviews of plant carotenoid biosynthetic pathways (Hirschberg 2001; DellaPenna and Pogson 2006; Cazzonelli 2011), despite the fact that Lx is produced by epoxidation of the 3-hydroxy-β-ring of L (García-Plazaola et al. 2007). Furthermore, many models of non-photochemical quenching of chlorophyll fluorescence (NPQ) are based exclusively on the xanthophyll cycle involving the carotenoids violaxanthin (Fig. 12.1), zeaxanthin (Fig. 12.1), and the intermediate antheraxanthin (VAZ cycle; Ahn et al. 2008; Johnson et al. 2009), while the potential role of the Lx-L cycle has been neglected, despite its apparent involvement in energy dissipation (García-Plazaola et al. 2007). This chapter reviews recent insights into photoprotection in plants possessing the two xanthophyll cycles, with a specific focus on advances in understanding of the Lx-L cycle and its ecophysiological significance.

II Discovery and Presence of Lutein Epoxide in Plant Tissues and Plastids

Chloroplast pigment composition is remarkably constant in vascular plants, mosses, and green algae (subkingdom Viridiplantae). Virtually all species contain two chlorophylls (*a* and *b*) and six major carotenoids (Young et al. 1997): β-carotene (β-C), neoxanthin, L, V, A, and Z, the last three of which can be interconverted and constitute the VAZ cycle. In addition to this basic composition, some plants also contain significant amounts of other non-ubiquitous pigments, the concentrations of which are regulated by environmental conditions. Lactucaxanthin (Demmig-Adams et al. 1995; Demmig-Adams and Adams 1996a), partially replacing L, also has a constitutive character (Phillip and Young 1995). Rhodoxanthin (Han et al. 2003) and anhydroeschscholtzxanthin (Hormaetxe et al. 2004) are found in leaves exposed to long-lasting environmental stress combined with high light, while the other two, α-carotene (α-C) (Thayer and Björkman 1990) and Lx (García-Plazaola et al. 2007), accumulate in certain taxa mainly in low irradiance environments. Both of the latter compounds are synthesised through the alpha branch of the carotenoid biosynthetic pathway. This branch starts with the cyclization of lycopene forming α-C that contains a β-ionone and an ε-ionone ring (contrasting with the β-branch in which two β-ionone rings are formed). The α-branch shows marked differences between seasons (Adams and Demmig-Adams 1994) and is also predominant in shade environments

(Demmig-Adams and Adams 1992; Logan et al. 1996; Matsubara et al. 2009). These observations agree with the upregulation of ε-cyclases induced by low light (Hirschberg 2001). In addition, the regulation of this branch is different depending on species, since not all shade plants accumulate α-C, as is the case for *Vinca minor* that can grow in extremely deep shade without accumulating α-C (Demmig-Adams 1998). However, when the latter carotenoid is present, its concentration is invariably higher in shade versus sun leaves. Based on these data, an important role for α-branch carotenoids in shade acclimation has been proposed (Caliandro et al. 2013).

Hydroxylation of the ε- and β-rings of α-C generates L. Subsequently, Lx can be generated by the epoxidation of L, presumably by the action of a zeaxanthin epoxidase (ZEP) (García-Plazaola et al. 2007). As shown for isolated thylakoids, α-C replaces β-C in the core complexes, while Lx replaces VAZ cycle pigments or L in the pigment-protein complexes of both photosystems (Matsubara et al. 2007).

Furthermore, Lx has been detected in plant organs other than leaves, such as petals (Meléndez-Martínez et al. 2006; Tai and Chen 2000), floral receptacles (Miller et al. 2009), fruits (Rabinowitch et al. 1975; Razungles et al. 1996; Gandul-Rojas et al. 1999; Esteban et al. 2010b), stems (Bungard et al. 1999; Esteban et al. 2010b), seeds (Edelenbos et al. 2001), and cotyledons (Esteban et al. 2009a). In some of these structures, Lx is present in chromoplasts where it plays a role as a coloring agent (typically exemplified in petals and pericarps) important to reproductive success, whereas in others Lx is located in photosynthetically active thylakoids. The present chapter deals only with Lx present in chloroplasts, and for simplicity we will designate “Lx species” as those in which Lx is present in photosynthetic organs.

When present in chloroplasts, Lx is rapidly transformed to L upon illumination, and the presence of Lx can thus typically be taken as an indicator of an active Lx-L cycle. The initial finding of this cycle in the parasitic plants *Cuscuta reflexa* (Bungard et al. 1999) and *Amyema miquelii* (Matsubara et al. 2001) led to the suggestion that the Lx-L cycle might be restricted to parasitic plants (Matsubara et al. 2003). However, in the following year, the Lx-L cycle was described in the tree *Quercus ilex* (Llorens et al. 2002) and in other species of the same genus (García-Plazaola et al. 2002). During the following years, the distribution of Lx and, presumably, of the Lx-L cycle was explored in a wide range of taxa (Matsubara et al. 2003; García-Plazaola et al. 2004) and some of the first model species for the study of this cycle (e.g., *Inga* sp. trees) were established (Matsubara et al. 2005).

III Lutein Epoxide Is Present in a Diversity of Species

A Taxonomic Distribution and Evolutionary Aspects

The finding of Lx in some parasitic species in the late 1990s (Bungard et al. 1999; Matsubara et al. 2003) grew in relevance when the list of species containing Lx increased to 59 % of the species analyzed (García-Plazaola et al. 2004). This percentage was subsequently further addressed by surveys of plants from tropical, subtropical, and temperate biomes by Matsubara et al. (2009) and Esteban et al. (2010a), with those possessing the Lx-L cycle accounting for 22 and 45 %, respectively, of species analyzed. The different level of Lx accumulation among closely related species (Esteban et al. 2007), together with its presence in a broad range of rather unrelated plant taxa (Matsubara et al. 2009), led researchers to delve into the evolutionary aspects of the Lx-L cycle compared to the VAZ cycle. The ubiquitous distribution of the latter cycle, present in all photosynthetic organisms belonging to the green lineage of

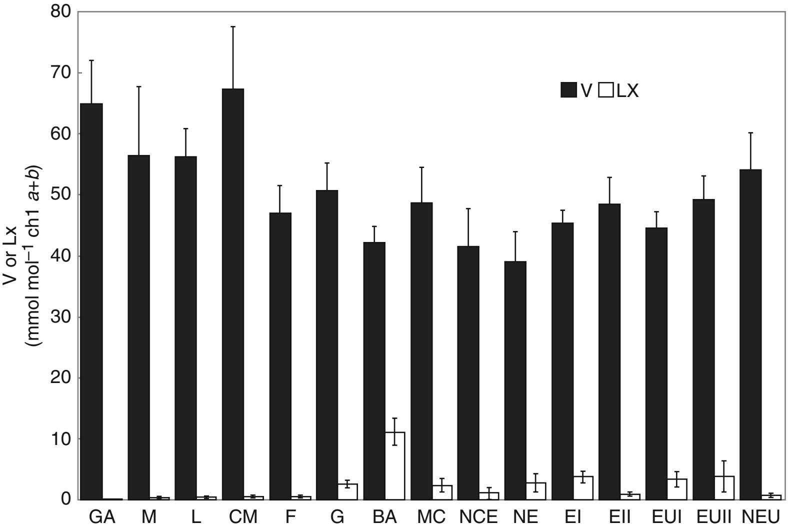

Fig. 12.2. Average content and distribution of the pigments V (*black columns*) and Lx (*white columns*) in each of the taxonomic groups analyzed in the study of 266 species among taxonomic groups (Esteban et al. 2009b). Data represent means ± S.E. Acronyms for each group are as follow: *GA* green algae, *M* musci, *L* Liverworts, *CM* club mosses, *F* ferns, *G* Gymnosperms, *BA* basal angiosperms, *MC* monocotyledons, *NCE* non-core eudicots, *NE* non-eurosids, *EI* eurosid I, *EII* eurosid II, *EUI* euasterid I, *EUII* euasterid II, *NEU* non-euasterids.

the plant kingdom, differs markedly from the non-ubiquitous Lx-L cycle that is restricted to certain taxonomic groups (Fig. 12.2; García-Plazaola et al. 2004). With respect to the taxa surveyed thus far, Lx is absent in green algae, rare among bryophytes, and quite common in several unrelated groups of gymnosperms (Pinaceae and Gingkoaceae; García-Plazaola et al. 2004). Among angiosperms, Lx is frequent in basal angiosperms, with high Lx contents in species of the families Myristicaceae and Lauraceae (García-Plazaola et al. 2004; Matsubara et al. 2008; Esteban et al. 2009b), but also in advanced groups like Rosids (Fagaceae, Fabaceae) and Asterids (Ericaceae, Cornaceae, Verbenaceae, Caprifoliaceae) (García-Plazaola et al. 2004; Esteban et al. 2009b; Matsubara et al. 2008, 2009) (Fig. 12.2).

Based on its absence in more primitive groups, it seems that Lx is not an ancient characteristic in plants. While Lx is generally highly conserved at the family level (García-Plazaola et al. 2004; Esteban et al. 2007), Lx concentration, nevertheless, varies greatly among species belonging to the same family or even the same genus. This is exemplified by congeneric species, such as *Cinnamomum japonicum* and *Persea americana* with a high Lx content versus *Cinnamomum camphora* and *Persea indica* with a low Lx content (Esteban et al. 2007). This result contrasts with those from other families, such as the parasitic Loranthaceae and Viscaceae, in which a high fidelity in Lx content at the genus level has been documented (Matsubara et al. 2003).

Therefore, one may ask why Lx is not ubiquitous in plants. Two limitations at the biosynthetic level may explain this, e.g., substrate availability for Lx formation (namely L) and/or enzyme (epoxidase) activity. Based on the absence of Lx in the *Arabidopsis thaliana* mutant *lutOE*, that accumulates increased amounts of L at the expense of V (Pogson and Rissler 2000), it

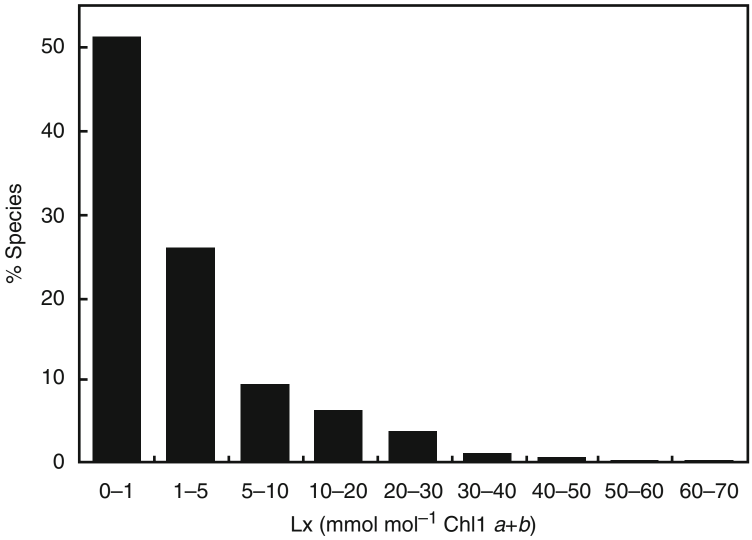

Fig. 12.3. Lutein epoxide content frequency distribution in plant species (%). Recompilation of data from the main surveys of species carried out to search for Lx species (Matsubara et al. 2003, 2008, 2009; García-Plazaola et al. 2000; Esteban et al. 2009b). Note that only 13 % of the species analyzed in these surveys possess an Lx content higher than 10 mmol mol^{-1} Chl1 *a*+*b*.

appears unlikely that L availability is restrictive for Lx synthesis in non-Lx-accumulating species. On the other hand, occurrence of Lx in some, but not all, groups of higher plants has been associated with differential regulation and/or substrate specificity of the enzyme ZEP, with a higher affinity of ZEP for L in Lx-forming species (Matsubara et al. 2003; García–Plazaola et al. 2007). It was therefore suggested that there may have been multiple and independent appearances of the Lx-L cycle during the course of evolution and that the ability to form Lx may be a consequence of a recurrent mutation in the ZEP enzyme increasing its affinity for L (Matsubara et al. 2003; García-Plazaola et al. 2007). However, no biochemical evidence is thus far available to support this suggestion. When a phylogenetic tree based on ZEP sequences of 30 ZEP genes belonging to 22 angiosperm species was constructed (Esteban et al. 2009a), presence or absence of Lx was not found to be restricted to any evolutionary branch, but was instead randomly distributed among different clades (Esteban et al. 2009b).

B Lutein Epoxide and Primary Species in Which It Has Been Investigated

The differences in Lx content among taxa can be considerable: concentrations lower than 10 mmol Lx mol^{-1} Chl *a* + *b* are commonly seen among angiosperms, whilst concentrations exceeding the latter value, are relatively rare. For example, among the 56 species examined in the tropical forest of Panama, only 14 % exhibited more than 10 mmol Lx mol^{-1} Chl *a*+*b* (Matsubara et al. 2009). This is also illustrated by Fig. 12.3, which includes data from the main surveys carried out to identify species that accumulate Lx (García-Plazaola et al. 2000; Matsubara et al. 2003, 2008, 2009; Esteban et al. 2009b). Concerning active participation in a Lx-L cycle, only those species with Lx concentrations equal to or higher than 5–10 mmol mol^{-1} Chl *a*+*b* appear to carry out de-epoxidation (Esteban et al. 2010b), and these species are referred to as Lx species. The key Lx species investigated thus far, categorized by their level of woodiness, are described below.

Non-woody Lx species: All herbaceous Lx species identified thus far are parasitic, with the exception of *Cucumis sativus* (Cucurbitaceae family) with an Lx content of around 20 mmol mol^{-1} Chl *a*+*b* (Esteban et al. 2009a; Matsubara et al. 2009). The Lx content of parasitic species, which in the case of holoparasites possess very little or no chlorophyll, are high when expressed on a chlorophyll basis, e.g., ~90 mmol mol^{-1} Chl *a*+*b* in *Cuscuta reflexa* (family Convolvulaceae; Bungard et al. 1999; Snyder et al. 2005), ~50 mmol mol^{-1} Chl *a*+*b* in *Cassytha glabella* (family Lauraceae; Close et al. 2006), and ~40 mmol mol^{-1} Chl *a*+*b* in *Amyema miquelii* (family Loranthaceae; Matsubara et al. 2003).

Woody Lx species: The buds of *Lonicera demissa* Rehd, also containing very little chlorophyll, possessed the highest Lx level thus far reported (on a chlorophyll basis) of 166 mmol Lx mol^{-1} Chl *a*+*b* (García-Plazaola et al. 2004). On the other hand, foliar Lx levels in woody species typically range between 10 and 80 mmol Lx mol^{-1} Chl *a*+*b*. These latter species include *Inga marginata* (80–20 mmol Lx mol^{-1} Chl *a*+*b*; Matsubara et al. 2005, 2007, 2008), *Inga sapindoides* (~30 mmol Lx mol^{-1} Chl *a*+*b*; Matsubara et al. 2008), *Albizia guachapele* (~30 mmol mol^{-1} Chl *a*+*b*; Matsubara et al. 2009), and members of the family Lauraceae, such as *Umbellularia californica* (~50 mmol Lx mol^{-1} Chl *a*+*b*; Esteban et al. 2007), *Laurus nobilis* and *Cinnamomum japonicum* (~30 mmol Lx mol^{-1} Chl *a*+*b*; Esteban et al. 2007), *Persea americana* (50–10 mmol Lx mol^{-1} Chl *a*+*b*; Förster et al. 2009; 2011; Matsubara et al. 2013), and *Ocotea foetens* (20–60 mmol Lx mol^{-1} Chl *a*+*b*; Esteban et al. 2010a). The latter two species, as well as *Inga* species and three *Virola* species of the family Myristicaceae (*V. elongata*, *V. sebifera*, and *V. surinamensis* with ~60 mmol Lx mol^{-1} Chl *a*+*b*; Matsubara et al. 2009), have been used to intensively study the light-driven conversion of Lx to ΔL (L enhancement due to de-epoxidation of Lx).

IV Lutein Epoxide Cycle Operation

A Lutein Epoxide Distribution in Thylakoids

The kinetics of Lx and V de-epoxidation are similar, with V conversion to Z being somewhat faster than the Lx conversion to ΔL (García-Plazaola et al. 2007). This fact, together with a similar partitioning of V and Lx across pigment-protein complexes, as shown in thylakoids fractionated from *I. sapindoides* leaves (Matsubara et al. 2005, 2007), suggests that both carotenoids, and indeed both Lx-L and VAZ cycles, compete for the same binding sites in chloroplast pigment-protein complexes. In monomeric LHCs, Lx is bound to the internal L2 site, while both the peripheral V1 and the internal binding site L2 of trimers can be occupied by Lx (Matsubara et al. 2007). When illuminated, ΔL formed from Lx de-epoxidation replaces Lx in V1 and L2 in trimers and in L2 in monomeric antenna proteins, displacing VAZ pigments as well. While de-epoxidation of Lx to ΔL has been also observed in photosystem I (Matsubara et al. 2007), the precise location of these pigments in the PS I-LHCI supercomplex has not been established.

B Xanthophyll Interconversions: Lutein Epoxide Participates in Both “Completed” and “Truncated” Cycles

In all Lx species tested so far, Lx undergoes light-driven de-epoxidation to L (ΔL) that occurs simultaneously with de-epoxidation of V in the VAZ cycle when leaves are illuminated. This de-epoxidation further augments the already large pool of L in plants (L+ΔL) (Nichol et al. 2012). Lutein epoxide de-epoxidation in the light does not convert all Lx to ΔL, and a non-photoconvertible fraction remains (apparently less available to the de-epoxidase). The proportion of photoconvertible Lx is similar (from 10 to 50 %, depending of the species) to that of V (for a detailed description see García-Plazaola et al. 2007). Several in vitro (Goss 2003) and in vivo

(García-Plazaola et al. 2003) studies indicate that de-epoxidation of Lx is catalyzed by VDE (as is V expoxidation), with both de-epoxidation reactions being inhibited by dithiothreitol (García-Plazaola et al. 2003). However, Lx de-epoxidation rates are always slower than those of V (García-Plazaola et al. 2007; Esteban et al. 2010a). This difference suggests that Lx may be more strongly bound to LHCs and less available for de-epoxidation or, alternatively, that VDE has a lower affinity for Lx. In fact, the presence of two epoxide groups in V can be expected to increase the probability of being de-epoxidized by VDE. Interestingly, in experiments performed with shade leaves of *P. americana* exposed to high light, an initial decrease in L preceded de-epoxidation of Lx (Förster et al. 2009; Matsubara et al. 2013). This effect has been interpreted as a consequence of the capacity of L to scavenge reactive oxygen species and be subject to a certain level of photodestruction in this process (Peng et al. 2006).

Under low light, ΔL may eventually be epoxidized back to Lx by the enzyme ZEP, leading to a “completed” Lx-L cycle. In the majority of Lx species, however, ΔL remains for days or even weeks. Two types of Lx-L cycles have therefore been discerned, i.e., a “truncated” Lx-L cycle with no overnight recovery of the initial Lx pool and a “completed” Lx-L cycle with nearly full recovery of the Lx pool in one dark period (Fig. 12.4). Examples of the truncated Lx-L cycle are seen in leaves in the deeply shaded inner canopy of *Quercus rubra* (García-Plazaola et al. 2003), *Inga sapindoides* (Matsubara et al. 2005), *Persea americana* (Esteban et al. 2007, 2008; Förster et al. 2009, 2011; Jia et al. 2013), and herbaceous *Cucumis sativus* (Esteban et al. 2009a). The truncated Lx-L cycle also appears to operate in the enclosed buds of some woody plants, where an irreversible conversion of Lx to L following bud-burst takes place (García-Plazaola et al. 2004). On the other hand, the parasitic *C. reflexa* (Bungard et al. 1999), *Amyema miquelii* (Matsubara et al. 2001), *Virola elongata* (Matsubara et al. 2009), and *Ocotea foetens* (Esteban et al. 2010a) exhibit a “completed” Lx-L cycle.

Even in species operating the Lx-L cycle on a daily basis, Lx restoration in the dark is typically delayed compared with restoration of the V pool (Matsubara et al. 2009; Esteban et al. 2010a). In the case of *C. reflexa*, recovery of Lx pools in the short-term under low light was not accompanied by a concomitant decrease in L (Snyder et al. 2005), suggesting that cycling was due to de-novo synthesis of Lx, and does not constitute a proper cycle. However, in *O. foetens*, experiments with norflurazon (an inhibitor of carotenoid biosynthesis) have shown that an increase in Lx content under low light was due to reversible epoxidation of ΔL to Lx (Esteban et al. 2010a); the latter species thus possess a true Lx-L cycle. In this scenario, ΔL re-epoxidation to Lx is apparently the limiting step for the complete operation of the Lx-L cycle. Different binding strengths of L and Lx to LHCs and different affinities of the enzymes (Matsubara et al. 2007) may be responsible for different operations of the Lx-L cycle in species with a “truncated” versus “completed” Lx-L cycle.

V Lutein Epoxide Function

A Lutein Epoxide and Lutein in Relation to Quenching Mechanisms

Due to its similarities with the VAZ cycle, a photoprotective role for the Lx-L cycle was proposed since the very first studies (Bungard et al. 1999; Matsubara et al. 2001; García-Plazaola et al. 2002; Llorens et al. 2002). Correlative evidence for a putative modulation of photoprotective thermal energy dissipation (as assessed through changes in NPQ) by the Lx-L cycle activity was later obtained by taking advantage of the slower (or null) rate of ΔL conversion to Lx in darkness. This way, a differential induction of VAZ and Lx-L cycles that would otherwise operate at the same time was achieved. Experiments with DTT treated leaves of *Q. rubra* (Garcia-Plazaola et al. 2003), *I. marginata* (Matsubara et al. 2008), *O. foetens* (Esteban et al. 2010a), and *P. americana* (Förster et al. 2011) demonstrated

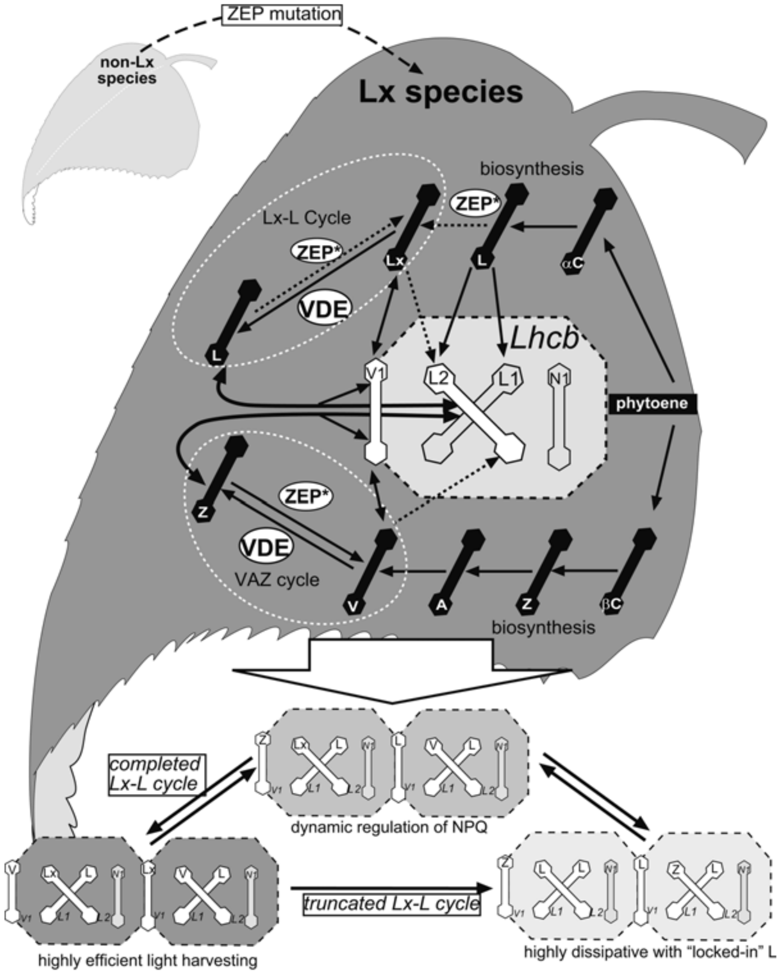

Fig. 12.4. Possible binding sites of xanthophylls involved in the Lx-L and VAZ cycles. A mutation in ZEP increases its affinity for L (ZEP*), generating Lx. Both Lx and V can be found in sites V1 and L2. After illumination Lx is converted to ΔL and V to Z, competing for the same binding sites. When the Lx-L cycle is "completed", the initial epoxidized xanthophylls are formed again and the initial situation is restored. When the Lx-L cycle is "truncated", ΔL is maintained, probably in L2, locking in a slowly reversible mechanism facilitating thermal energy dissipation that underlies NPQ.

faster NPQ induction and/or a higher level of maximal NPQ during light exposure of leaves (all starting with a highly epoxidized VAZ cycle and no Z), retaining ΔL versus leaves no retaining ΔL (Fig. 12.5). These observations are consistent with a role of L in the induction of the ΔpH-dependent component of NPQ (qE), as has been demonstrated in *Arabidopsis* mutants with a low level of β-pathway carotenoids and an enhanced content of α-carotene and L (Li et al. 2009). Furthermore, analysis of carotenoid radical cation formation strongly suggested that L can substitute for Z in qE in Z-deficient mutants (Li et al. 2009). However, it needs to be kept in mind that a role of L in NPQ of Z-deficient mutants does not allow unequivocal conclusions about the role of L in wild type. Direct biochemical evidence of the mechanistic relationship between the operation of the Lx-L cycle and NPQ modulation is still lacking because of the absence of adequate molecular tools and/or model species and the impossibility of separating Lx and V de-epoxidation.

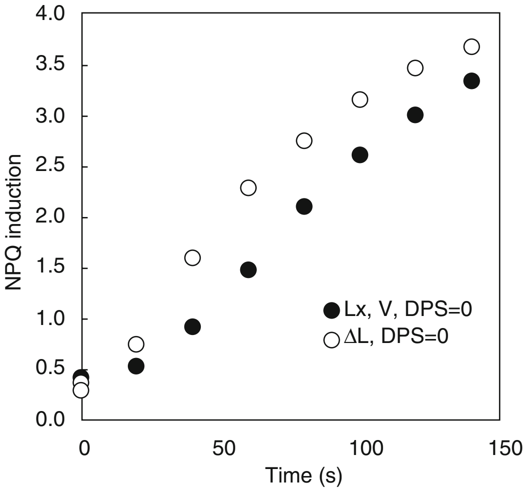

Fig. 12.5. Non-photochemical quenching kinetics in seedlings of *O. foetens* measured after 25 h of recovery in low light from 3-min (*black circles*) or 10-min (*white circles*) with strong illumination. The first treatment (3 min; *black circles*) led to a complete recovery of the initial Lx and V pools and, consequently, with a de-epoxidation state of the VAZ cycle (DPS) of zero. While in the second treatment (10 min; *white circles*) most of ΔL remained, with only the V pool completely restored (DPS = 0).

Despite the lack of characterization of the biochemical mechanism of NPQ regulation by Lx-L cycle activity, some general patterns can be drawn from the above-mentioned experiments. Basically, four functional traits characterize Lx-L cycle-associated modulation of NPQ. First, ΔL formed in the light and retained in the dark enhances the capacity for NPQ formation independently of the effect of A+Z (Llorens et al. 2002; Matsubara et al. 2008; Jia et al. 2013). Second, engagement of NPQ is faster in leaves with ΔL not containing A+Z prior to light exposure (Matsubara et al. 2003; Garcia-Plazaola et al. 2003). Third, dark relaxation of ΔL-associated NPQ is also faster than that of ΔL-AZ-associated NPQ and similar to that of the ΔpH component of NPQ (Förster et al. 2011). Fourth, ΔL does not influence the photoinhibitory component of NPQ, as (i) suggested by the fact that photosystem II (PS II) efficiency (as estimated from the ratio of variable over maximal chlorophyll fluorescence emission, F_v/F_m) is independent of ΔL (Matsubara et al. 2008) and (ii) as demonstrated by the analysis of fluorescence lifetime-resolved imaging (Matsubara et al. 2011), showing that, unlike Z, retention of ΔL does not stabilize fluorescence quenching in the absence of ΔpH. However any thermal dissipation of singlet-excited Chl is expected to lower F_v/F_m (Kitajima and Butler 1975), and the absence of F_v/F_m lowering would thus suggest that L might instead quench triplet excited Chl or singlet oxygen. Taking all these trends together, it can be concluded that Lx-L cycle activity provides a complementary mechanism of photoprotection, which at the same time may not compromise carbon assimilation in light-limited environments.

B Lutein Epoxide and Light Harvesting

When maintained under low irradiance for a prolonged period of time, Lx continues to accumulate for several weeks in Lx plants (Matsubara et al. 2008; Förster et al. 2009; Esteban et al. 2010a) before reaching saturation. This continuous accumulation of Lx suggests a role for Lx other than acting as a simple reservoir of photoconvertible L. In vitro reconstitution experiments of LHC proteins (Lhcb5) with Lx instead of L resulted in a higher Chl fluorescence yield (Matsubara et al. 2007), suggesting a higher efficiency of energy transfer from Lhcb5 to reaction centers, a lesser dissipative loss, and improved photosynthetic efficiency when Lx is bound to LHCs in shade leaves (Matsubara et al. 2007). This observation was later supported by in vivo studies of the fluorescence induction kinetics in I. marginata leaves (Matsubara et al. 2008), although gas exchange measurements were not fully consistent with this hypothesis.

VI Why Two Xanthophyll Cycles?

A Comparison Between VAZ and Lx-L Cycles

Parallelism between the Lx-L cycle and the widespread VAZ cycle has been an intuitive idea since its discovery. As discussed

Table 12.1 Comparison of the two cycles present in higher plants: Lx-L cycle and VAZ cycle in terms of presence, evolution, co-location in the LHC, pigments pool, response to light, cycle characteristics, regulation and functions

	VAZ cycle	Lx-L cycle
Presence	In all higher plants. Ubiquitous pigment	Non-ubiquitous pigment
Evolution	Early in evolution	Lx is not an ancient character. Fidelity at family level
Co-location in the LHC	V occupies V1 and L2	Lx occupies V1 and L2
Pigment pool	V pool is similar among species for comparable conditions	Lx content varied among species
Response to light	V pool scales positively with irradiance	Three types of responses: scales positively, negatively (more frequent) or no response
Enzymes	VDE	VDE
	ZE	ZE?
Operation	"Completed" cycle	"Completed" cycle/truncated cycle
	In all photosynthetic tissues studied so far	Leaves, stems and leaves
Cycle kinetics	Fast de-epoxidation	De-epoxidation slower than V
	Slow epoxidation	If exists, much slower than V
Regulation	In the short- and long-term	In the short- and long-term
Function	Photoprotection and energy dissipation	Light harvesting
		Photoprotection and energy dissipation
NPQ development	Participates in qE, qI	Only participates in qE
		Faster relaxation

previously, both cycles share some resemblances in the fraction of photoconvertible xanthophylls (Matsubara et al. 2002; García-Plazaola et al. 2003), in their correlative link with thermal energy dissipation (Matsubara et al. 2001; Llorens et al. 2002; García-Plazaola et al. 2003; Förster et al. 2009, 2011), and in the co-location of V and Lx within pigment protein complexes of photosynthetic membranes (Matsubara et al. 2003, 2005). On the other hand, there are also several dissimilarities between the two cycles. These differences, summarized in Table 12.1, mainly include inter-specific differences in the presence, operation, putative function, evolution, and behavior of the two cycles under light exposure. As will be discussed later, VAZ pool size increases with increasing irradiance and environmental stress (Demmig-Adams and Adams 1992; Niinemets et al. 1998, 1999, 2003; Adams and Demmig-Adams, Chap. 2), while Lx is commonly seen in unstressed, shaded versus sun leaves, such as inner canopy and long-lived leaves (Matsubara et al. 2003, 2005; García-Plazaola et al. 2007) or light protected tissues such as buds (García-Plazaola et al. 2004).

B A Tentative Unified Model

The variable Lx levels and the existence of several types of Lx-L cycles complicate exploring the physiological function of these carotenoids. Towards a tentative unifying model of Lx-L cycle operation, we suggest the existence of three different Lx pools: (i) a small constitutive pool (*C*-Lx) with concentrations of around 5 mmol Lx mol^{-1} Chl *a+b* present under prolonged sun exposure or at noon in all Lx species. This pool is non-convertible, analogous to the non-convertible pool of V in the VAZ cycle in annuals (Verhoeven et al. 1999; Jahns et al. 2001). This constitutive pool of Lx was bound to L1, the internal binding sites of trimeric LHCII, in *Inga* (Matsubara et al. 2007). The second pool corresponds to the dynamic pool (*D*-Lx), of less than 20 mmol Lx mol^{-1} Chl *a*+*b* and subject to de-epoxidation and epoxidation on a daily basis. This pool is specific to species with a "completed" Lx cycle, such as *O. foetens* and *V. elongata* (Matsubara et al. 2009) that recover the Lx pool overnight. The third pool is the accumulative pool (*A*-Lx), continuously accumulated (at least for several weeks) during shade acclimation of, e.g., *I. sapindoides* (Matsubara et al. 2007), *P. americana* (Förster et al. 2011), and *O. foetens* (Esteban et al. 2010a). Lutein epoxide slowly accumulates in LHCs in low light and is correlated with efficient light harvesting by PS II (Matsubara et al. 2007).

Species with a "truncated" Lx cycle may thus possess *C*-Lx and *A*-Lx. Conversely, species with a "completed" Lx cycle can exhibit *C*-Lx, *A*-Lx and *D*-Lx. These differences may reflect distinct physiological or ecological roles of the carotenoids participating in the Lx-L cycle. The existence of these different Lx pools may explain some of the apparent lack of consistency between results obtained with different model species. Our model is based on kinetic studies of the performance of the Lx-L cycle in different species, but still needs to be corroborated with biochemical studies.

VII Ecological Significance of Two Xanthophyll Cycles

A Lutein Epoxide Cycle Operation in the Field

The majority of research on the Lx-L cycle has been conducted using long-term shade-acclimated leaves that contain substantial amounts of Lx. In most experiments, these leaves are suddenly exposed to high light intensities and the responses to these treatments are analyzed. It might be argued that this experimental approach is somewhat artificial, and might exacerbate Lx photoconversion to unphysiological levels. However, a number of field experiments demonstrated that the Lx-L cycle is not just a biochemical curiosity observable only under experimental conditions, but is indeed a physiological phenomenon in natural plant canopies. So far, these observations have been performed in different environments and under diverse ecological conditions around the world, covering biomes from tropical to temperate forests.

Some of the best field measurements were performed in the tropical dry forest in Panama. In the tree *Virola elongata*, Matsubara et al. (2009) documented the complete operation of the Lx-L cycle. In sun leaves of this species, 60 % of the initial Lx pool was converted into L during the morning (simultaneously 70 % of the V pool was de-epoxidated). During the subsequent night, a complete restoration of the Lx pool took place, although Lx recovery was delayed by several hours in comparison with that of V. In contrast, when the daily operation of the Lx-L cycle in tree canopies was monitored in species with a "truncated" cycle, such as *I. marginata*, Lx was not converted to ΔL, while the VAZ cycle was fully operative (Matsubara et al. 2008).

Similar results as those obtained in Panama were also found in the subtropical laurel forest of the Canary Islands. Esteban et al. (2007, 2010a) described the "completed" operation of the Lx-L cycle in the late successional tree *O. foetens*. In the innermost canopy levels, seedlings of this species activated the Lx-L cycle in response to very brief sunflecks. Indeed, both Lx and V pools showed a dynamic behaviour and similar rates of conversion and reconversions (Esteban et al. 2010a). On the other hand, the Lx pool was stable in the related trees species *Laurus azorica*, *Persea indica* and *Apollonias barbujana*.

Mediterranean ecosystems, characterized by a two-season pattern of environmental stress (summer drought and cold winter temperatures), provided additional ecological information. The Lx-L cycle was described in several evergreen oaks, including one of the dominant tree species in the Mediterranean basin, *Q. ilex* (Llorens et al. 2002). The study of seasonal changes in pigment composition revealed a decrease in basal Lx content during periods of sustained environmental stress, which occurred at the same time as a higher overnight retention of the de-epoxidized forms of the VAZ cycle (García-Plazaola et al. 1999). The daily dynamics of Lx changes were also investigated in *Laurus nobilis* growing in the understory of an oak forest and, as reported for other species with "truncated" cycles, no changes were observed during daily exposure to light in low light environments (Esteban et al. 2007). Long-term seasonal adjustment and overnight retention of de-epoxidized xanthophylls (ΔL and Z) were observed in this species (Esteban et al. 2008).

In temperate forests, the study of seasonal changes in Lx pool size of parasitic plants *A. miquelii* (Matsubara et al. 2002), *Cassytha glabella* and *Cassytha pubescens* (Close et al. 2006) and in *Acacia melanoxylon* (Watson et al. 2004) revealed a close correlation with changes in V pool size, and an inverse relationship with sustained depressions of photochemical efficiency. Irreversible formation of L from Lx has been also described in spring during the process of bud break, when leaf primordia are transformed to leaves (Garcia-Plazaola et al. 2004).

Some of these patterns were confirmed in intact plants growing in artificial environments, such as Biosphere 2 (Matsubara et al. 2005; Nichol et al. 2012), Humid Tropics Biome at the Eden Project (Matsubara et al. 2005; Matsubara et al. 2007), or large greenhouses (Esteban et al. 2010a). Botanic gardens are also a source of ecophysiological information, and some processes involving Lx to L conversions were described in intact plants, such as the transition from chromoplast to chloroplast in floral receptacles of *Nelumbo nucifera* (Miller et al. 2009), which is analogous to the irreversible photoconversion of Lx to L after budbreak (García-Plazaola et al. 2004) or the accumulation of Lx during leaflet development in *Ceratozamia robusta* (Cardini et al. 2006).

Overall, these studies under natural conditions highlight the fact that dynamic inter-conversions between Lx and ΔL are relevant on a daily basis only for species that exhibit a "completed" Lx-L cycle. On the other hand, in the majority of species with a "truncated" Lx-L cycle, Lx de-epoxidation occurs in response to a sudden change in environmental conditions (severe environmental stress) or developmental stage (budbreak, floral development) that demands a higher level of photoprotection (Matsubara et al. 2011).

B Ecophysiological Significance

Recent surveys of woody plants (Esteban et al. 2007; Matsubara et al. 2008, 2009) have revealed an accumulation of high Lx levels in shade leaves and inner-canopy leaves of many species. For example, field measurements in avocado (Förster et al. 2011) and in *Inga* spp. (Matsubara et al. 2008) showed a different Lx composition between inner- and outer-canopy leaves. This was also confirmed by studies in the tropical dry forest in Nicaragua, where Lx distribution scaled negatively with irradiance in the species *Pithecellobium* sp. (Esteban R and García-Plazaola JI, unpublished data 2013; Fig. 12.6). Evidently, Lx accumulates in deeply shaded inner canopy leaves infrequently exposed to light strong enough to activate the VDE enzyme (Matsubara et al. 2005, 2007). Although the presence of Lx is a rather common phenomenon in shade environments for those species capable of accumulating Lx, sun exposure does not appear to limit Lx accumulation in sun leaves of some species. Elevated levels of Lx has been observed in sun leaves of species such as *O. foetens* and *L. azorica* (Esteban et al. 2007), *Virola elongata* (Matsubara et al. 2009), and in the parasitic plants *C. reflexa* (Snyder et al. 2005) and *Cassytha* sp. (Close et al. 2006), as well as *U. californica* with a very high Lx content in both sun and shade leaves (Esteban et al. 2007). Therefore, although a negative response of Lx content to increased growth irradiance is the more common behavior, a

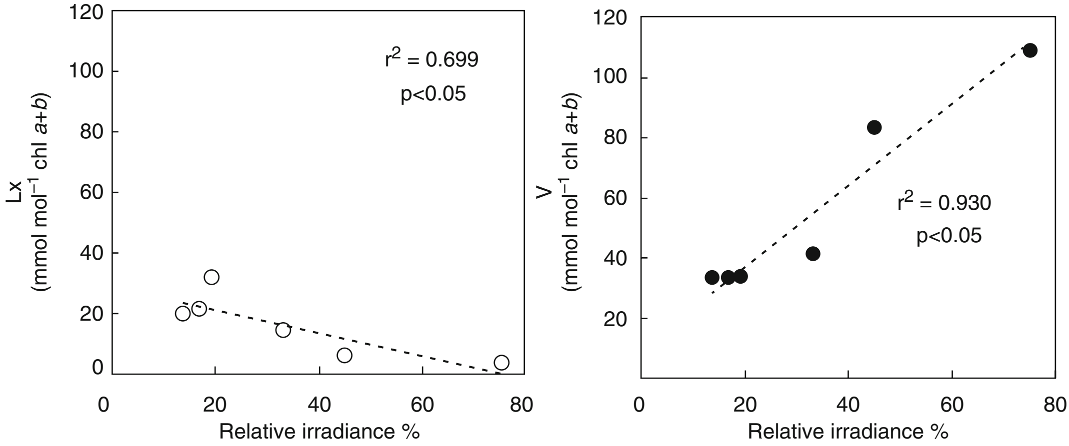

Fig. 12.6. Variation in foliar Lx and V contents in relation to the irradiance transmitted (% relative irradiance) by a natural canopy of *Pithecelobium* sp. in Juan Venado Nature Reserve (Nicaragua).

positive Lx response to increased growth irradiance is not rare among Lx-rich species.

The co-existence of both VAZ and Lx-L cycles in some plant species led us to advocate for a physiological or ecophysiological role for the Lx-L cycle that may be different or complementary to that of the VAZ cycle. This function will presumably vary depending on which version of the Lx-L cycle ("truncated" or "completed") a species has as well as with the plant organ (e.g., fruit, stem, leaf) under consideration. Therefore, we will contemplate a hypothetical forest, where different Lx species co-habit (Fig. 12.7) and where the Lx-L cycle may fulfill various ecophysiological roles. We propose seven different possible scenarios:

<u>Leaves with a "truncated" Lx-L cycle</u>: Seedlings or inner shade-canopy leaves of Lx species are usually enriched in Lx due to slow ZEP activity and the absence of VDE activity in these shaded environments (Matsubara et al. 2005, 2007). Sudden changes in irradiance, such as a gap forming in the middle of the forest, contribute to quick and irreversible de-epoxidation of Lx to ΔL, with the latter presumably contributing to enhanced photoprotective thermal energy dissipation reflected in the rapid development of NPQ (Matsubara et al. 2008; Jia et al. 2013). A "truncated" cycle may represent, under some extreme circumstances, an emergency mechanism of special relevance for long-term downregulation of photosynthetic efficiency, supplementing retention of Z+A and their sustained engagement in energy dissipation in response to prolonged environmental stress, such as that represented in cases of sudden exposure to high photon flux density experienced during the generation of forest gaps or heavy pruning in orchards.

<u>Leaves with a "completed" Lx-L cycle</u>: These leaves differ from the previous ones in that ΔL is restored to Lx (as Z is reconverted to V in the VAZ cycle) in the absence of other stresses. This reversibility facilitates alternation between photoprotection in response to excess light versus high light-harvesting efficiency when light levels become non-excessive. This restoration subsequent to high-light exposure depends on the intensity and duration of the illumination, as well as the length of the subsequent dark or low-light period (Esteban et al. 2010a). Short sunflecks in the forest environment would favor operation of a reversible Lx-L cycle while longer sunflecks might continuously stimulate thermal dissipation. Modulation of NPQ via de-epoxidation and epoxidation of the VAZ cycle (Demmig-Adams and Adams 1996b), rapid engagement

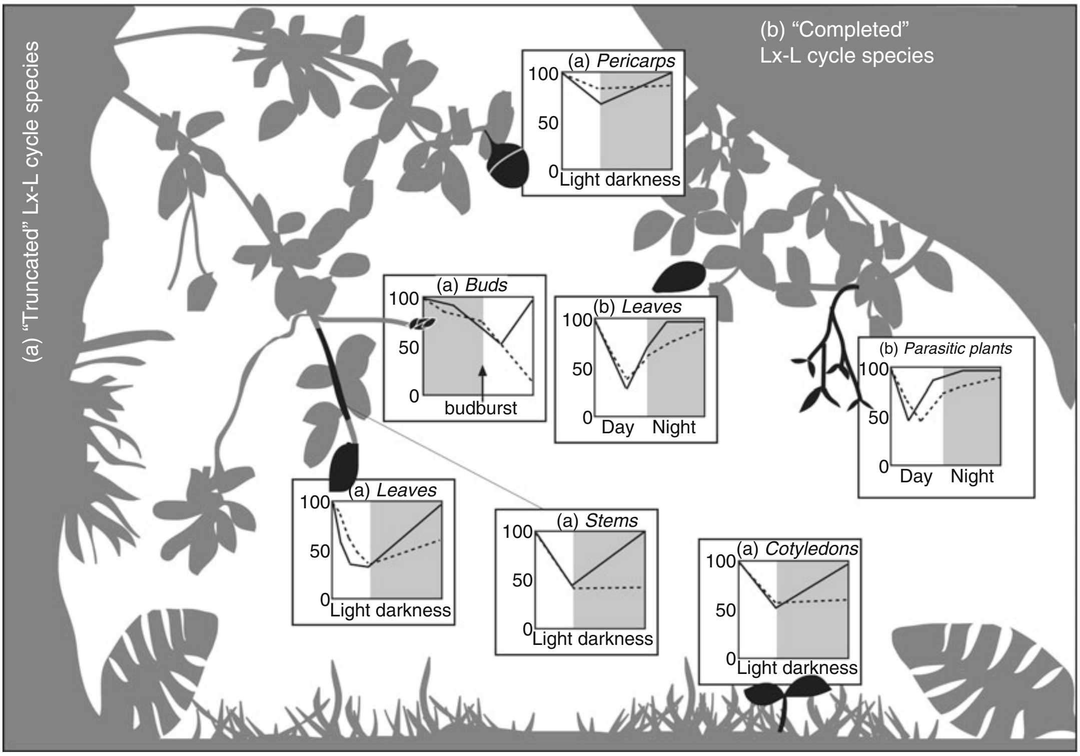

Fig. 12.7. Idealized representation of the diverse modes of Lx-L cycle operation in natural canopies: truncated Lx-L cycle (**a**) and completed Lx-L cycle (**b**). In the insets the dynamics of Lx (*dashed line*) and V (*solid line*) are in relation to high light episodes (% of total pool size). In species with a completed cycle, daily changes are shown; in buds, responses to budbreak; and in species with "truncated" cycle, as no changes are observed on a daily basis, responses to high light treatments are represented Data were obtained from Matsubara et al. (2001), García-Plazaola et al. (2004), and Esteban et al. (2008, 2009a, 2010a, b).

and disengagement of Z+A (Adams et al. 1999), together with the different epoxidation kinetics of the Lx-L cycle, may allow a finer modulation of light harvesting and photoprotection.

Parasitic plants: Holoparasitic plants have minimal photosynthetic activity and acquire the majority of reduced carbon from the host (Jeschke et al. 1994). It has been demonstrated in *Cuscuta* (Snyder et al. 2005), as well as in hemiparasitic plants (Matsubara et al. 2003), that once Lx is de-epoxidated to ΔL, recovery in the cycle is slow. The Lx-L cycle has been demonstrated to fulfill a primarily photopretective role in hemiparasitic plants (Matsubara et al. 2003), which rely on photosynthesis for carbon assimilation.

Buds: When these structures break in spring, Lx is rapidly and irreversibly converted to ΔL (García-Plazaola et al. 2004), typically in conjunction with the activation of diverse photoprotection mechanisms (Hughes et al. 2007). The same effect is observed when shade leaves are suddenly exposed to high light intensity. The irreversible conversion of Lx to L after bud-burst may reflect a role of L in the stabilization of new light-harvesting complexes.

Stems: Lx may also be present in stems, as has been demonstrated in *P. americana* (Esteban et al. 2010b). The light driven de-epoxidation of Lx to ΔL (Esteban et al. 2010b) was supported by experiments with stems of avocado, so here we might assume that the dynamic regulation of photosynthetic activity is also dependent upon the Lx-L cycle.

Pericarps: The Lx cycle may fulfil different functions in leaves and fruits. High contents of Lx (~40 mmol Lx mol^{-1} Chl $a+b$) have been found in some fruits such as beans (Esteban et al. 2010b). This may be due to low ZEP activity and the absence of VDE activity, as occurs in deeply shaded inner canopy leaves (Matsubara et al. 2005, 2007). However, these tissues are not exposed to light, and the function of such Lx accumulation may be similar to other carotenoids in fruits that serve to attract pollinators to flowers and other animals in order to disperse seeds (Hirschberg 2001). Future research is needed in this direction.

Cotyledons: The ecophysiological function of the Lx-L cycle in these structures growing in the forest understory may resemble what occurs in cotyledons of cucumber (Esteban et al. 2009a). Photosynthetic cotyledons need to grow fast, and maximization of light harvesting efficiency is thus important. However, when faced with excessive light, the conversion of the Lx pool to ΔL and ΔL engagement in photoprotection may be crucial to survival.

In a nutshell, the Lx-L cycle, as an additional cycle with different kinetics to that of the VAZ cycle, features an array of responses, thus presumably increasing the diversity of physiological solutions to excess light in nature. We postulate that the existence of two xanthophyll cycles may represent an advantage for plants in allowing a more precise adjustment and modulation to fluctuating environmental conditions. Such a response could be of great adaptive value in forest environments, where plants are exposed to sudden irradiance and environmental changes, and their success depends on the ability to maximize carbon gain while simultaneously preventing damage (Adams et al. 1999).

VIII Perspectives

The publication of more than 25 papers has resulted in a detailed view of the Lx-L cycle and the significance of the photoprotective process that may arise from it. Important pieces in the Lx puzzle have now been elucidated, such as the distribution of Lx in the Plantae kingdom, the two modes of Lx-L cycle operation (the “truncated” and “completed” Lx-L cycles), the possible distribution in thylakoids, and the apparent ecophysiological significance of the Lx-L cycle. However, there is still much to determine. A number of active areas of study, e.g., the ecophysiological significance of Lx in fruits, differences in the biochemical properties of ZEP between Lx and non-Lx species, and the comparison of pigment location between a “truncated” Lx-L cycle and a “completed” Lx-L cycle may fill these gaps. The main problem for this last objective is that most Lx species are woody plants, which complicates the task of performing molecular studies. Cucumber, the only non-parasitic herbaceous Lx species found to date, may provide excellent material for further investigation of the Lx-L cycle story.

Acknowledgments

The authors acknowledge the support of research grants BFU 2010-15021, UPV/EHU-GV IT-299-07, and a Fellowship from the Basque Government, the doctor specialization grant from the UPV/EHU, and the JAE-Doc fellow from the Spanish National Research Council (CSIC) received by RE. We also thank Brian Webster for language editing. We are greatly indebted to all the researchers involved in the Lx-L cycle for advancing knowledge in this field, and especially to our friends and colleagues Barry Osmond and Shizue Matsubara for their helpful comments and continuous advice in the endeavor to understand what is behind the Lx-L cycle. We also thank Marisol Jimenez, Domingo Morales, Patricia Brito, Eduardo Jimenez and Beatriz Fernandez-Marín for their constant help and support during field studies in Tenerife. Finally, we thank the useful comments of the reviewers and the editor.

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Chapter 13

PsbS-Dependent Non-Photochemical Quenching

Matthew D. Brooks
Department of Plant and Microbial Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA 94720-3102, USA
Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Stefan Jansson
Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, Umeå, Sweden

and

Krishna K. Niyogi*
Department of Plant and Microbial Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA 94720-3102, USA
Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

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*Author for Correspondence, e-mail: niyogi@berkeley.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_13,

Summary

Much of the research on the thermal dissipation of excess absorbed light, measured as non-photochemical quenching (NPQ) of chlorophyll fluorescence, has been focused on a major, rapidly induced and rapidly reversible component of fluorescence quenching termed energy-dependent quenching, feedback de-excitation, or qE. A breakthrough in this field came with the discovery of the involvement of the photosystem II subunit S (PsbS) protein, a thylakoid membrane protein required for qE induction and relaxation. In this chapter, we discuss the history of how PsbS was first identified as a photosystem II subunit, the genetic characterization that defined its important role in qE, and the biochemical work describing how PsbS might regulate photosynthetic light harvesting. We emphasize how mutants affecting PsbS (i.e., *npq4)* have been invaluable tools in defining structural and spectroscopic changes associated with qE, and how these mutants have contributed to our current understanding of the physiological role of the thermal dissipation process accompanied by non-photochemical fluorescence quenching.

I Introduction

Energy-dependent fluorescence quenching or feedback de-excitation (qE) is the fastest component of the non-photochemical quenching (NPQ) of chlorophyll fluorescence, inducing and relaxing within seconds to minutes, and used as a measure of thermal dissipation of singlet-excited chlorophyll *a*, the primary mechanism by which plants cope with excess energy in fluctuating light conditions (Murchie and Niyogi 2011; Jahns and Holzwarth 2012; Ruban et al. 2012). Thermal dissipation of excess absorbed light is thought to limit the production of reactive oxygen species by decreasing the lifetime of singlet-excited chlorophyll (Chl) *a* and reducing the excitation pressure on photosystem (PS) II (Niyogi 1999). The signal that activates thermal dissipation (measured as qE) is a low lumen pH, which occurs when the trans-thylakoid proton gradient is formed faster than it is dissipated by ATP synthase (Kanazawa and Kramer 2002; Takizawa et al. 2008). In this way, thermal dissipation (measured as qE) acts as a feedback mechanism, switching the PS II antenna to a quenching state in response to the (excess) light perceived.

There are at least three requirements for the activation of thermal dissipation (qE) in vascular plants. The first is, as stated above, the trans-thylakoid pH gradient formed in the light (Wraight and Crofts 1970). By preventing formation of, or removing, the pH gradient, using uncouplers, the qE component of NPQ can be abolished (Briantais et al. 1979). Conversely, using chemicals (Takizawa et al. 2008; Johnson and Ruban 2011), or decreas-

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Abbreviations: BBY – Berthold-Babcock-Yocum; Co-IP – Co-immunoprecipitation; CP24 – Chlorophyll protein of 24 kDa molecular mass; CP26 – Chlorophyll protein of 24 kDa molecular mass CP29 – Chlorophyll protein of 29 kDa molecular mass; DAD – Diaminodurene; DCCD – Dicyclohexylcarbodiimide; LHC – Light-harvesting complex; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PS – Photosystem; PsbO – Photosystem II subunit O; PsbP – Photosystem II subunit P; PsbS – Photosystem II subunit S; qE – Energy-dependent, rapidly reversible component of NPQ, also termed energy-dependent quenching or feedback de-excitation; QTL – Quantitative trait locus; V – Violaxanthin; VDE – Violaxanthin de-epoxidase; Z – Zeaxanthin ing CO_2 fixation (Schreiber et al. 1986; Fichtner et al. 1993), in order to increase the pH gradient can increase qE. There is also evidence that qE can be modulated in vivo by fine-tuning the pH gradient via cyclic electron transport (Heber and Walker 1992; Miyake et al. 2005) or changes to the conductivity of ATP synthase (Kanazawa and Kramer 2002; Avenson et al. 2004). The chemical dicyclohexylcarbodiimide (DCCD) that binds to protonatable residues in hydrophobic environments was also shown to inhibit qE (Ruban et al. 1992b). It was later shown that the light-harvesting complex (LHC) proteins to which DCCD binds are CP26 and CP29, suggesting that the low pH can be sensed through lumen-exposed residues, and implicating the latter two proteins in qE (Walters et al. 1994; Pesaresi et al. 1997).

The second requirement for qE in vivo is the xanthophyll (VAZ) cycle, in particular the conversion of the carotenoid violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) (Demmig-Adams 1990; Niyogi 1999). V is converted to Z through the action of V de-epoxidase (VDE), a lumenal enzyme of the lipocalin family of proteins (Yamamoto et al. 1999). The first evidence for the role of the VAZ cycle in qE came from the correlation between Z levels and the amount of quenching upon exposure of leaves to high light (Demmig et al. 1987). This relationship was confirmed by the identification of mutants affecting VDE (*npq1*) and Z epoxidase (*npq2*) that also affected NPQ (Niyogi et al. 1998). Despite years of study, the exact role of Z in qE remains controversial, owing largely to the lack of consensus on the molecular mechanism of qE (Holt et al. 2004; Jahns and Holzwarth 2012; Ruban et al. 2012). A direct role in qE has been proposed for Z through either an energy transfer (Owens 1994; Frank et al. 2000; Ma et al. 2003) or charge-transfer (Holt et al. 2005; Ahn et al. 2008; Avenson et al. 2008, 2009) mechanism from Chl to Z (see also Walla and Fleming, Chap. 9). Recently, it has also been shown that this function is not specific to Z, as accumulation of lutein can partially compensate for the qE defect in *npq1* (Li et al. 2009). Alternatively, an indirect role for Z in qE has been put forward, in which Z acts as an allosteric regulator, controlling the sensitivity of qE to the pH gradient and/or conformational changes within the LHCs where qE occurs (Crouchman et al. 2006; Johnson et al. 2008, 2012; Ruban et al. 2012). These roles for Z are not necessarily mutually exclusive, and models have been put forth to explain Z involvement in NPQ by different roles depending on site and type of quenching (Jahns and Holzwarth 2012).

The third requirement for qE in plants, and the focus of this chapter, is the thylakoid membrane protein PS II subunit S (PsbS). While this protein has been the topic of a great deal of research over the last 15 years, a surprisingly large amount of uncertainty remains regarding its location within the thylakoid membrane, what other protein(s) it interacts with, and the biochemical mechanism by which it is involved in NPQ. In this chapter, we will describe experiments and proposed models leading to our current understanding of PsbS-dependent qE and discuss what questions remain.

II Discovery of PsbS and Involvement in qE

A Initial Discovery as a Photosystem II Subunit

The PsbS protein was initially identified as a component of PS II-enriched Berthold-Babcock-Yocum (BBY) preparations (Ghanotakis and Yocum 1986) and by co-immunoprecipitation (Co-IP) with the oxygen-evolving complex subunits PsbO and PsbP (Ljungberg et al. 1984, 1986). While these results initially suggested that PsbS might be a component of the oxygen-evolving complex, subsequent experiments showed that isolated PS II core complexes depleted in PsbS did not lose any oxygen-evolving ability (Ghanotakis et al. 1987). Sequencing of the *psbS* cDNA revealed that it encoded a protein with similarity to LHC proteins (Kim et al. 1992; Wedel et al. 1992). PsbS

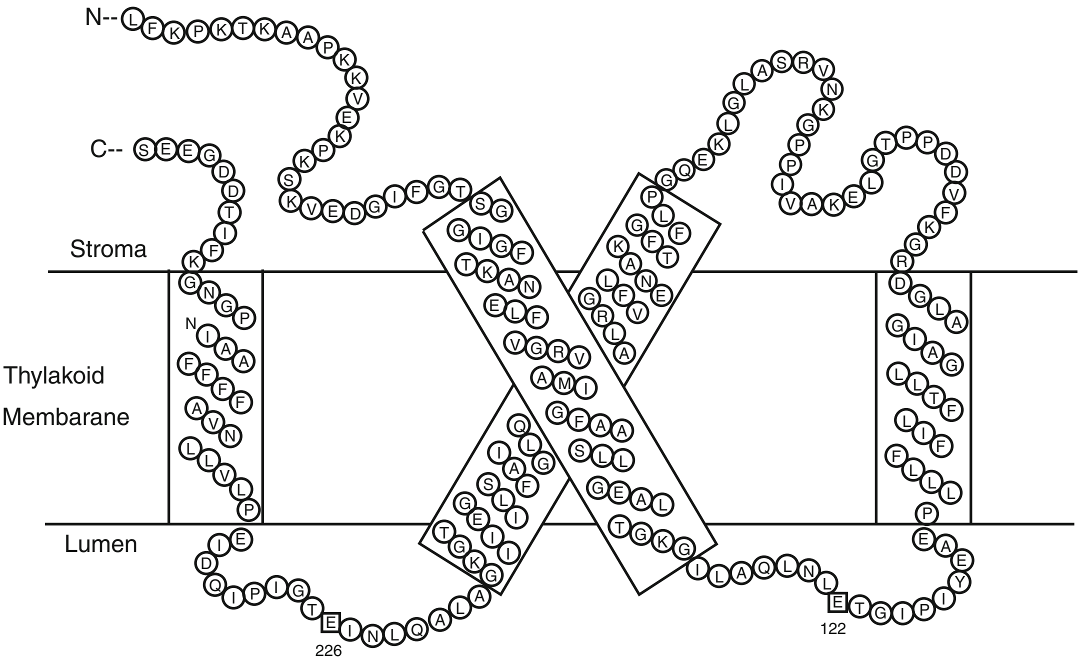

Fig. 13.1 Proposed topology of PsbS in the thylakoid membrane. Two glutamate residues shown to be involved in sensing of lumen pH are marked by *squares*.

is unique, however, as it contains four rather than the typical three transmembrane helices (Fig. 13.1). The similarity between the first/third and second/fourth helices suggested that PsbS likely arose through duplication of a two-helix LHC protein (Kim et al. 1992). Analysis of extant LHC protein sequences indicates that PsbS and typical light-harvesting proteins arose via independent duplication events (Engelken et al. 2010).

B Involvement in qE Revealed Through a Genetic Screen

More than 15 years after the initial discovery of the PsbS protein, a functional role for PsbS in NPQ (and thus presumably thermal dissipation) was first established through a genetic approach. Using video imaging of chlorophyll fluorescence to screen for qE-deficient mutants, the *npq4* mutant was isolated (Li et al. 2000). Unlike the previously characterized *npq1* and *npq2* mutants, *npq4* plants contained a normal xanthophyll (VAZ) cycle, yet lacked qE (Fig. 13.2) and ΔA_{535} (Li et al. 2000; Peterson and Havir 2000), a spectroscopic signature correlated to qE (Ruban et al. 1993). The *npq4* mutation was mapped to the gene encoding PsbS, and this protein was demonstrated to be necessary for qE in plants (Li et al. 2000). It was also shown that, despite a complete lack of PsbS protein in the *npq4-1* allele, all other PS II subunits accumulated to normal levels, and other photosynthetic properties were unaffected. Later, rice PsbS knockout mutants (Zulfugarov et al. 2007; Ishida et al. 2011; Kasajima et al. 2011) and *Populus* PsbS RNAi lines (Fig. 13.3) were shown to have a similar phenotype as *npq4*, confirming that the function of the protein is conserved.

Since the discovery that PsbS is essential for qE (and presumably thermal dissipation of excess energy) in plants, the *npq4* mutant has been a crucial tool for exploring possible mechanisms involved in qE. While *npq1* mutants have also been used as qE-deficient controls, the role of Z as an antioxidant (Havaux et al. 2007), and the trace amounts of Z remaining in the *npq1* mutant complicates the interpretation of results. Likewise,

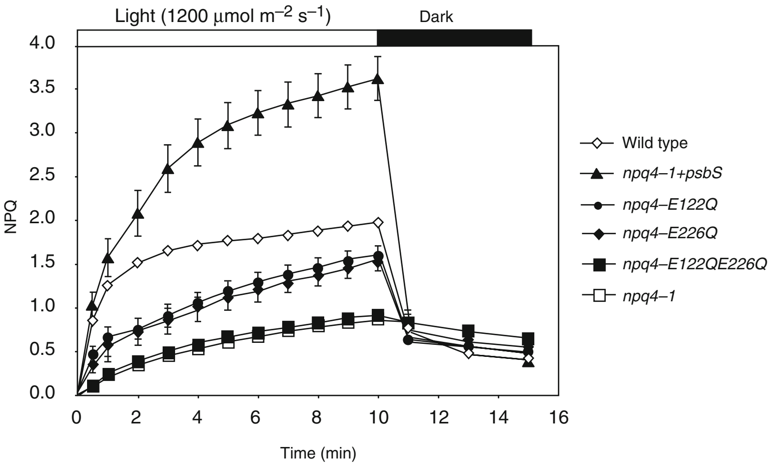

Fig. 13.2 Induction and relaxation of NPQ in mutants affecting PsbS. NPQ was measured during 10 min of illumination with high light (1,200 μmol photons m^{-2} s^{-1}), followed by relaxation in the dark for 5 min. Data are means ± SE (n = 8), from Li et al. (2004).

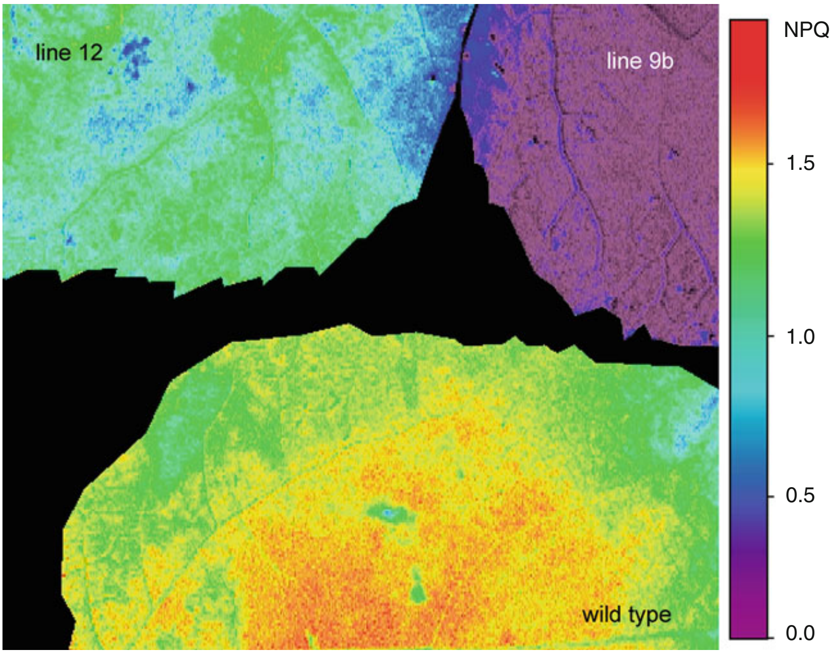

Fig. 13.3 Chlorophyll fluorescence imaging of NPQ in leaves of wild type and two independent PsbS RNAi lines (9b and 12) of *Populus*.

the use of pH uncouplers to prevent qE may also have other effects on the cell. Because PsbS has thus far been implicated solely in NPQ, and specifically qE, with no other effect on light harvesting or photosynthesis, the *npq4* mutant is an ideal control for studies examining the spectroscopic and structural changes associated with qE.

III Biochemical Function of PsbS

A Does PsbS Bind Pigments?

The similarity between PsbS and other members of the LHC superfamily suggested a role of PsbS as a pigment-binding protein. On the other hand, many of the amino acids that coordinate chlorophylls, and that are conserved in most LHC proteins, are absent in PsbS (Dominici et al. 2002). Early studies, in which PsbS was isolated from solubilized BBY membranes, indicated that the protein bound five chlorophyll molecules (Funk et al. 1994, 1995b). Unlike other LHCs, however, PsbS accumulated in etiolated tissues (Funk et al. 1995a) and chlorophyll-deficient mutants of barley (Dominici et al. 2002), indicating that the protein is stable in the absence of chlorophyll. Subsequently, two research groups purified PsbS from thylakoids by the same method as above, as well as using other techniques, and were unable to observe any chlorophyll binding for PsbS (Aspinall-O'Dea et al. 2002; Dominici et al. 2002). In addition, while all other recombinant LHCs require pigments in order to fold in vitro, recombinant PsbS protein does not exhibit such a requirement (Dominici et al. 2002; Wilk et al. 2013). Binding of Z to purified PsbS in vitro was suggested to cause a red shift in the pigments's absorbance, similar to the ΔA_{535} signature observed in vivo (Aspinall-O'Dea et al. 2002). This result has been questioned, however, as the signal attributed to PsbS-xanthophyll interaction was shown to be the result of Z aggregation (Bonente et al. 2008), and PsbS reconstituted into liposomes along with or without Z did not show any differences (Wilk et al. 2013). Interestingly, mutagenesis of the glutamate-arginine ion pairs, modeled as Z ligands in PsbS (Haripal et al. 2006), resulted in a complete loss of PsbS function without affecting protein stability and accumulation (Schultes and Peterson 2007). There is thus no firm evidence that PsbS is a "typical" pigment-binding protein, although it is possible that PsbS somehow interacts with at least carotenoid molecules to fulfill its function.

B Insights into How PsbS Functions in qE

The amount of PsbS protein present modulates a plant's maximal qE level. This effect was hypothesized based on the semi-dominant nature of *npq4* heterozygous plants obtained from the backcross to wild type; the heterozygotes had an intermediate level of qE relative to the parental strains (Li et al. 2000). In subsequent work the PsbS mRNA and protein levels were shown to correlate to the amount of qE in *npq4* heterozygotes, wild type and homozygous *npq4* plants (Li et al. 2002a). The dosage effect (the amount of PsbS protein determining the amount of qE) was further confirmed by transforming wild-type plants with an additional copy of the *psbS* gene and showing that these plants accumulated more protein and were able to reach NPQ values approximately twice as high as wild type (Li et al. 2002b; Fig. 13.2). These results suggested that differences in qE capacity observed among plant species and in response to high light (Demmig-Adams and Adams 1992; see also Demmig-Adams et al., Chap. 24) might result from changes in PsbS expression. While this has been shown to be the case in certain situations (Demmig-Adams et al. 2006), it is not always the case as discussed below.

1 PsbS as a Lumen pH Sensor

The presence of several conserved acidic residues on each of the two lumen-exposed loops of PsbS (Li et al. 2000) suggested that the protein may be capable of sensing lumenal pH. It has been demonstrated that, just as CP26 and CP29, PsbS is also able to bind DCCD (Dominici et al. 2002), consistent with the hypothesis that protonatable amino acid residues occur in PsbS. Two glutamate residues, one on each lumen-exposed loop of PsbS, were identified by site-directed mutagenesis as being critical for qE function in vivo (Li et al. 2002c, 2004). Mutation of either one of these residues (E122Q or E226Q) reduced qE capacity to approximately one-third of the control level, while a double point mutant expressed wild-type levels of protein but lacked all qE (Fig. 13.2). Finally, DCCD-binding by PsbS was completely abolished in the E122Q/E226Q double mutant, suggesting that the latter two residues are solely responsible for the pH-sensing capability of PsbS (Li et al. 2004).

2 Interactions with Other Photosynthetic Complexes

Interactions between PsbS and other thylakoid proteins have not been resolved completely. As mentioned in the previous section, PsbS was originally found to be associated with the oxygen-evolving complex (Ljungberg et al. 1984, 1986). Co-IP experiments in various crop species, using antibodies raised against PsbS, resulted in the appearance of a band at approximately 42 kDa (Bergantino et al. 2003). This band was determined to be a PsbS dimer based on migration in the gel and the lack of signal when blotted with other PS II subunits of similar sizes. Since formation of this band was pH dependent, it was concluded that PsbS is present as a homodimer in the dark, and that low pH induces monomerization in the light (Bergantino et al. 2003). It would thus be interesting to explore whether or not mutation of the pH-sensing glutamates affects monomer-to-dimer transition. Co-IP was also used to show interaction between PsbS and several photosynthetic complexes, including not only LHCII components and CP29 but also, surprisingly, several PS I, cytochrome b_6f, and ATPase subunits (Teardo et al. 2007). More recently, a direct interaction between PsbS and LHCII was demonstrated when both proteins were reconstituted into proteo-liposomes (Wilk et al. 2013). Cross-linking experiments performed in several laboratories have failed to provide informative results.

3 Bypassing the Need for PsbS in qE

Treatment of thylakoids with chemicals has been a traditional way of investigating NPQ, and revisiting several of those experiments with mutants has proven informative. A particularly interesting example is the use of diaminodurene (DAD), shown in the 1970s to cause a higher than normal ΔpH by facilitating artificial electron transport around PS I (Wraight and Crofts 1970; Hauska and Prince 1974). Recently, the effects of DAD on isolated chloroplasts from wild type and *npq* mutants were compared (Johnson and Ruban 2011; Johnson et al. 2012). Surprisingly, while control thylakoids behaved as expected, DAD was able to induce qE to an equal and significantly higher level in both wild type and *npq4* (Johnson and Ruban 2011). Similar results were demonstrated for *lut2 npq1* mutants lacking the xanthophylls involved in qE (Johnson et al. 2012). The authors used these results to suggest that the role of PsbS and xanthophylls in qE is to modulate the pKa of lumen-exposed residues involved in qE and allow qE at a physiologically relevant lumen pH in vivo (Johnson and Ruban 2011; Johnson et al. 2012; Ruban et al. 2012).

IV Does PsbS Affect the Organization of Photosynthetic Complexes?

More recently, it has been proposed that the conformational change occurring as a result of PsbS protonation may affect the organization of complexes within the grana membrane. It was shown that the amount of PsbS protein affected Mg^{2+}-induced thylakoid restacking, possibly by altering interactions between PS II and LHCII complexes (Kiss et al. 2008). This effect on thylakoid restacking did not depend on the capability of PsbS to function in qE, but rather solely on PsbS protein level as demonstrated using mutants in the pH-sensing glutamate residues. Evidence for a role in membrane organization also came from sucrose-gradient fractionation of *Arabidopsis* thylakoids and the observation that a band (called band 4), containing CP29, CP24 and LHCII trimers, was present in dark-acclimated samples but disassociated upon high light treatment in wild type (Betterle et al. 2009). In the *npq4* mutant, band 4 remained even after high light treatment and, even more strikingly, dissociation of this complex was reduced in the single glutamate mutants by approximately 50 %. The latter authors concluded that, while dissociation of band 4 was not sufficient for qE, it is possible that this complex stabilizes an unquenched light-harvesting state. They also noted that PsbS is likely to have other roles besides allowing the dissociation of band 4, as knockouts of CP24 constitutively lacked band 4, yet contained disconnected unquenched LHCs and were able to activate NPQ upon exposure to high light (Kovacs et al. 2006; Betterle et al. 2009).

Electron microscopy has also been used to investigate the location of PsbS and how it affects grana organization. Using single particle analysis of cryo-images, Nield et al. (2000) determined that the electron density of PS II-LHCII super-complexes does not allow for the presence of a protein containing a fourth transmembrane helix, such as PsbS, and that PsbS is more likely associated with LHCII-rich regions of the membrane. NPQ was also correlated to structural changes in thylakoid properties and grana organization using freeze fracture and thin-section electron microscopy, and from these results it was suggested that NPQ requires the dissociation and aggregation of LHCII (Johnson et al. 2011a, b). Subsequent work used the *npq4* mutant and PsbS-overexpressing lines to show that PsbS is able to increase fluidity of the membrane and reduce ordering of PS II complexes (Kereïche et al. 2010; Goral et al. 2012). That PsbS affected the fluidity of the membrane was supported by fluorescence recovery after photobleaching (FRAP) experiments, a technique that uses a strong laser pulse to bleach a region of the thylakoid membrane, and then follows the kinetics of recovery of chlorophyll fluorescence (Goral et al. 2012). Using FRAP it was shown that in the *npq4* mutant, PS II complexes had a reduced mobility within the thylakoid membrane, whereas mobility increased in PsbS-overexpressing lines.

A drawback to most of the techniques used to analyze grana organization is that they are rarely performed on intact leaves, but rather on isolated chloroplasts or grana membranes. The time it takes to prepare the samples is also important given how rapidly qE relaxes. Care must be taken when interpreting results particularly from experiments using detergent-solubilized membranes, since it is unclear what effect various detergents have on interactions between complexes and proteins or on extracting specific proteins from the membrane. For instance, in sucrose gradients PsbS has been found either solely as a monomer (Nield et al. 2000), or in an aggregated form (Caffari et al. 2009), depending on which isomer of dodecyl maltoside was used to solubilize BBY membranes.

V Using Spectroscopic Measurements to Understand the Mechanism of qE

A Multiple Models of NPQ

Spectroscopy is another frequently used tool used to investigate qE. As mentioned previously, qE has been correlated with specific spectroscopic changes, such as the ΔA_{535} absorbance change (Ruban et al. 1993). A dosage-dependent effect of PsbS after illumination with high light has been demonstrated by measuring chlorophyll fluorescence lifetimes of wild type, *npq4* and PsbS overexpressors (Li et al. 2002a, b). While most current research indicates that quenching occurs in the LHCs associated with PS II (LHCII), there is some evidence that PS II reaction centers can also form quenching complexes during photoinhibitory stress (Huner et al. 2006). Higher plants have six LHCII proteins associated with PS II (Lhcb1-6), with Lhcb1-3 forming the major trimeric antenna while Lhcb4-6 exist as monomers (Peter and Thornber 1991; Jansson 1999). The observation that isolated LHCII trimers form aggregates in vitro with spectroscopic features that have been correlated with qE in vivo (Ruban et al. 1992a; Phillip et al. 1996) has led to the proposal of the LHCII aggregation model (Horton et al. 2008; Ruban et al. 2012). In this four-state model, aggregation of LHCII trimers is controlled cooperatively by PsbS protonation and Z formation, which presumably allow for more rapid formation of the quenching state.

Another model put forth, and in many ways complementary to the aggregation model, proposes the presence of two distinct quenching sites, Q1 and Q2 (Holzwarth et al. 2009; Jahns and Holzwarth 2012). This model is based on spectroscopic measurements and global target analysis on mutants with varying amounts of PsbS as well as the *npq1* mutant (Holzwarth et al. 2009). Based on this analysis the authors suggested that detached LHCs can aggregate and lead to the formation of Q1, and that this quenching site may in fact be formed upon dissociation of band 4, as described above. The formation of a far-red spectral component assigned to Q1 has been seen during qE formation in both aggregated LHCII (Ruban and Horton 1992; Miloslavina et al. 2008) and intact leaves (Lambrev et al. 2010). Z was shown to enhance this effect, but to not be required, again suggesting an allosteric role (Johnson and Ruban 2009). The Q2 site in the model by Holzwarth et al. (2009) remains associated with PS II and is Z-dependent, but presumably does not require PsbS. More kinetic information will be required to determine whether or not the quenching at this site is, in fact, qE-like, or if it might fit better with one of the slower components of NPQ.

B The Molecular Mechanism

The molecular mechanism responsible for qE (and presumably thermal dissipation of excess absorbed light) is more contentious. One proposed mechanism involves an aggregation-dependent conformational change affecting a lutein located in the L1 site of trimeric LHCII, which positions this lutein molecule as a quencher of Chl through an energy transfer mechanism (Ruban et al. 2007; however, see Demmig-Adams et al., Chap. 24, for relationships between NPQ in nature and foliar zeaxanthin versus lutein levels). Alternatively, it has been suggested that the same conformational change results in quenching through a Chl-Chl charge transfer mechanism (Miloslavina et al. 2008; Müller et al. 2010). In contrast to these scenarios, where Z plays a strictly allosteric role, mechanisms for qE based on direct involvement of Z either through energy or electron transfer from Chl to Z have been proposed (Demmig-Adams 1990; Frank et al. 1994; Dreuw et al. 2003; Holt et al. 2004). Evidence supporting a carotenoid charge-transfer mechanism came from observations of the formation of Z radical cation formed under qE conditions in isolated thylakoids but absent in thylakoids of *npq4* (Holt et al. 2005). While this signal was observed in Lhcb4-6 in vitro (Ahn et al. 2008; Avenson et al. 2008, 2009), no evidence of any radical cation could be found in isolated LHCII trimers (Avenson et al. 2009).

Excitonic coupling between the S1 state of carotenoids and Chl has been observed to be correlated with the amount of quenching and the formation of the red-shifted band in isolated LHCs (Bode et al. 2009; Liao et al. 2010a, b) and in vivo (Bode et al. 2009). This has led to the proposal that, when electronic coupling between the two molecules is sufficient, energy can be trapped in this short-lived state, and further increases in coupling can lead to the charge transfer state (Bode et al. 2009; Liao et al. 2012; see also Walla and Fleming, Chap. 9). Recently, Wilk et al. (2013) reconstituted LHCII along with PsbS and Z in liposomes and showed that the amount of chlorophyll fluorescence quenching observed in these samples was higher relative to liposomes containing LHCII in the absence of PsbS or Z, and the authors were able to correlate this quenching with the electronic coupling measured by two-photon excitation. Importantly, these experiments were performed at LHCII concentrations low enough to avoid aggregation quenching. It remains to be seen whether this phenomenon is specific to LHCII or if the minor complexes are also able to interact with PsbS and Z to form this quenching state, and whether the Z cation radical can be observed in these liposomes.

VI Physiological Function of qE and PsbS

A qE Is Important Under Fluctuating Light Conditions

Despite some remaining uncertainty regarding how PsbS functions at a biochemical level, the *npq4* mutant has been invaluable in studies aimed at defining the physiological function of qE in plants. One important question in the field is what effect qE has on plant growth and fitness. Using a variety of lines containing various amounts of PsbS, and therefore qE, this question can be addressed directly. There is at least one report suggesting that under continuous high light, *npq4* plants were inhibited in growth relative to wild type (Graßes et al. 2002), while others have reported that growth was similar to wild type (Havaux and Niyogi 1999; Külheim et al. 2002). Experimental variation, such as a difference in the age of plants transferred to high light, may account for this discrepancy. For instance, it has been shown that young but not mature leaves of *npq1* mutants are able to acclimate to high light via other mechanisms, such as increasing the amount of tocopherols (Havaux et al. 2007).

When plants are grown in fluctuating light environments, a more obvious PsbS-dependent growth and fitness phenotype was observed (Külheim et al. 2002; Frenkel et al. 2009; Krah and Logan 2010). One explanation for the more dramatic difference seen under fluctuating versus constant light is that qE might be important to reduce the excitation pressure on PS II and prevent photoinhibition during rapid changes in light intensity. This explanation is supported by experiments showing that exposure of low light-grown plants to higher light intensities resulted in greater excitation pressure in *npq4* mutants than in wild type (Li et al. 2002b; Johnson and Ruban 2010), but lower excitation pressure in PsbS-overexpressing lines than in wild type (Li et al. 2002b).

B PsbS Prevents Photoinhibition

Using the *npq4* mutant, qE was shown to prevent high light-induced photoinhibition measured as a persistent decrease in variable fluorescence (Graßes et al. 2002; Li et al. 2002b; Takahashi et al. 2009; for a discussion on photoinhibition, see Adams et al., Chap. 23). While such a role for qE had previously been demonstrated in *npq1* plants (Havaux and Niyogi 1999; Havaux et al. 2000), the additional function of Z in the prevention of lipid peroxidation makes it difficult to isolate the qE-dependent effects (Havaux et al. 2007). How qE protects PS II from photoinhibition remains unresolved, due to conflicting results from leaves treated with a chloroplast protein translation inhibitor and exposed to high light. Graßes et al. (2002) reported that the increased photoinhibition observed in *npq4* was not attributable to increased degradation of D1 protein, but rather to a downregulation of PS II activity by another mechanism(s). In another study, Takahashi et al. (2009) compared wild type to *npq4* and found that the amount of photoinhibition was the same in the absence and presence of the inhibitor, leading to the conclusion that the additional photoinhibition in qE-deficient plants is caused by a lower rate of de novo chloroplast protein synthesis. Most recently, Roach and Krieger-Liszkay (2012) repeated the latter experiment and found that the difference may depend on the light intensity used for high light treatment. It should also be noted that the three groups all used a different inhibitor in their experiments. A more extensive investigation of the effect of a lack of qE (and presumably thermal dissipation) on photoinhibition using a combination of light intensities and chemical inhibitors will be important in future work.

Several groups have previously observed that the amount of photoinhibition cannot be entirely explained by damaged PS II and have proposed that other slowly relaxing NPQ pathways could be responsible (Walters and Horton 1993; Adams et al. 2006; see also Adams et al., Chap. 23, and Demmig-Adams et al. Chap. 24). The results, mentioned above, might suggest that, in some situations, the rapidly reversible form of thermal dissipation indicated by qE may protect plants as well as prevent an activation of slowly relaxing NPQ pathways that might decrease photosynthesis in fluctuating light. An increase in another form of quenching with slower kinetics could explain the decreased photochemical efficiency and the small, yet significantly higher, amount of NPQ that is observed in *npq4* compared to wild type after high light treatment. Alternatively, the remaining NPQ in high-light-exposed *npq4* plants may have the same mechanism as qE, but form more slowly in the absence of PsbS (Johnson and Ruban 2010; see also Demmig-Adams et al., Chap. 24).

The observation that overexpression of PsbS can lead to increased levels of qE in *Arabidopsis* and prevent photoinhibition raises several questions. What is the optimal amount of qE (and NPQ)? Why do some plants not maximize their qE capacity and does this imply that there are drawbacks of having too much qE (see also Demmig-Adams et al. 2013 for a discussion of trade-offs)? And finally, can altered levels of PsbS be used to optimize crop productivity?

1 Natural Systems Control qE Through PsbS

It has been observed that different species are capable of different levels of qE depending largely on whether they were grown in full sun or in the shade (Demmig-Adams and Adams 1994; see also Demmig-Adams et al., Chap. 24). Variations in qE capacity that depend on growth light conditions have also been documented within a species, and it has been shown that shade or low-light-grown plants had less rapidly reversible NPQ and higher levels of photoinhibition when compared to sun or high-light-grown plants (Demmig-Adams et al. 1998; Mishra et al. 2012; see also Demmig-Adams et al., Chap. 24). With the discovery that PsbS is critical for rapidly reversible thermal dissipation (qE), this analysis was extended to show that the amount of PsbS could be altered depending on growth conditions and affect the capacity of qE (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006). Quantitative trait locus (QTL) mapping identified PsbS expression level as a determinant of qE capacity in rice cultivars (Kasajima et al. 2011).

However, it should be emphasized that PsbS may not be the only factor contributing to the qE and that plants may adapt in other ways. For example, comparison of *Arabidopsis* ecotypes that exhibited different levels of qE revealed equal levels of PsbS (Jung and Niyogi 2009), despite the earlier observations of a PsbS dosage effect on qE (Li et al. 2002a, b). QTL mapping with two of the ecotypes suggested that the genes responsible had not been identified previously by genetic approaches using induced mutants (Jung and Niyogi 2009). Moreover, when qE levels were compared in a set of *Arabidopsis* ecotypes, grown under lab and field conditions, there was no correlation between PsbS and qE levels, and in the field most lines had qE levels similar to a PsbS-overexpressing line (Mishra et al. 2012). It cannot be excluded that possible differences in electron transport capacity among ecotypes contributed to different qE levels. Nevertheless, it appears that qE level is determined not only by a plant's genetic makeup but can also be adjusted dynamically based on environmental conditions, and that factors other than PsbS level may affect qE level

2 Too Much of a Good Thing

If qE protects leaves from photoinhibition, why do plants often not increase their expression levels of PsbS to the point that saturates qE? PsbS-overexpressing lines indeed had larger rosettes than wild-type plants when grown under a light regime with a single daily sunfleck, suggesting that increasing qE capacity is beneficial under such conditions (Logan et al. 2008). However, the latter conditions are unlikely to be representative of the more common conditions – with frequent, rapid fluctuations in light intensity – plants experience in nature. Field experiments with PsbS-overexpressing lines grown alongside wild-type plants revealed no significant difference in seed production between the two groups (Frenkel et al. 2009). It has thus been proposed that NPQ capacity is optimized in nature and reflects a balance between the gains from an increased protection from photoinhibition versus potential losses to photosynthesis that might occur during the induction of NPQ. This possibility was examined by measuring photosynthetic assimilation in transgenic rice with varying levels of PsbS, and it was shown that PsbS-overexpressing lines had lower assimilation rates when measured during fluctuating illumination (Hubbart et al. 2012). The possible interplay between NPQ and other stress responses has also led to the suggestion that, when a plant contains more PsbS than necessary, other stress response pathways could become compromised (Frenkel et al. 2009). In particular, both herbivores (Johansson Jänkänpää et al. 2013) and pathogens (Göhre et al. 2012) appear to be able to discriminate between plants with different PsbS levels, perhaps due to changes in gene expression and chloroplast metabolism as a consequence of increased ROS production in plants lacking PsbS (Roach and Krieger-Liszkay 2012; see also Demmig-Adams et al. 2013). Finally, it has also been reported that overexpression of PsbS can inhibit cyclic electron flow and lead to photoinhibition of PS I (Roach and Krieger-Liszkay 2012). This last result is particularly interesting in view of potential interactions between PsbS and PS I subunits (Teardo et al. 2007) (see above). Further research will be necessary to clarify both positive and negative potential effects of PsbS overexpression.

VII Evolutionary Aspects of PsbS

From an evolutionary perspective, it appears as though PsbS-mediated capacity to rapidly modulate NPQ arose before, or at the latest around, the time when plants started to inhabit the terrestrial ecosystem. This latter ability is likely to have been a crucial innovation, as it appears to be widely conserved in land plants. Genes encoding PsbS have been found in the genomes of sequenced green algae, strongly suggesting that PsbS evolved in the common ancestor of green algae and plants (Koziol et al. 2007; see also Morosinotto and Bassi, other Chap. 14). However, there is presently no experimental evidence for PsbS-dependent NPQ in any green alga (Bonente et al. 2008; Niyogi and Truong 2013). Instead, green algae, such as *Chlamydomonas reinhardtii*, use a different LHC-related protein, called LHCSR, for qE (Peers et al. 2009; see also Morosinotto and Bassi, Chap. 14). Both LHCSR- and PsbS-dependent qE have been shown conclusively to co-exist in the moss *Physcomitrella patens* (Alboresi et al. 2010; Gerotto et al. 2012), but LHCSR was presumably subsequently lost during the early evolution of vascular plants, leaving only PsbS-dependent qE (Niyogi and Truong 2013). In the moss, the induction kinetics of PsbS-dependent qE appear to be faster than the LHCSR-dependent qE, which might explain why LHCSR was lost while PsbS was retained (Gerotto et al. 2012).

Although PsbS is not the only mechanism plants possess to modify NPQ capacity, the rapid kinetics of PsbS-dependent qE make the latter an attractive component of plant defense against photo-oxidative stress, and natural selection has presumably maintained this trait in all vascular plant lineages investigated so far. Since the capacity of the PsbS-mediated system is not constant, PsbS level, and hence qE capacity, may be under selection by the plant's natural environment. Enhanced PsbS levels clearly enhance qE capacity in *Arabidopsis* that, however, possesses only the relatively low maximal qE capacity typically seen in rapidly growing annual species compared with other species (especially evergreens) adapted to more extreme environments (see also Demmig-Adams et al., Chap. 24). The level of PsbS in a plant is, of course, selected by evolution to maximize fitness, and less-than-maximal PsbS might be explained by compromised defense against biotic attack, losses to photosynthetic efficiency during the induction or relaxation of NPQ, or something else.

VIII Conclusions

Several important questions remain to be answered concerning the role of PsbS in qE. Plants with varying amounts of PsbS, in several species beyond *Arabidopsis* and rice, will be needed to elucidate all aspects of the effect of PsbS-dependent NPQ on plant fitness and productivity. Furthermore, sequencing the genomes and characterizing more species between *Chlamydomonas reinhardtii* and vascular plants may help understand what drove the switch from LHCSR-dependent to PsbS-dependent qE, and what function PsbS has where it does not appear to be involved in NPQ.

Conflicting reports on pigment binding, localization, and interaction with other components have led to many different proposals over the years as to how PsbS might be involved in the mechanism of qE. Despite these different views, consensus is forming around the idea that, (1) a conformational change within PsbS occurs upon protonation, which (2) promotes dissociation of a subset of LHCs from the PS II core, and (3) leads to the formation of the qE quenching site. The connection between each of these steps, however, remains poorly defined. The location of PsbS within the grana membrane and/or PS II super-complex is an important piece of information that remains elusive despite several attempts by many labs. In order to help define the molecular trigger for qE, it would also be useful to have a structure of PsbS and, if possible, determine what changes occur in the protein upon protonation. Advances in X-ray crystallography and solid-state nuclear magnetic resonance techniques could lead to exciting results in this area.

Acknowledgments

This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy, FWP number 449B. K.K.N. is an investigator of the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation (through Grant GBMF3070).

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Chapter 14

Molecular Mechanisms for Activation of Non-Photochemical Fluorescence Quenching: From Unicellular Algae to Mosses and Higher Plants

Tomas Morosinotto
Dipartimento di Biologia, Università di Padova,
Via Ugo Bassi 58B, 35121 Padova, Italy

and

Roberto Bassi*
Dipartimento di Biotecnologie, Università di Verona,
Strada le Grazie 15, 37134 Verona, Italy

Summary

All oxygenic photosynthetic organisms have mechanisms for regulation of light-harvesting efficiency in response to variable light intensity. Non-photochemical quenching of chlorophyll fluorescence (NPQ) is a marker for the rapid dissipation of excess excitation energy as heat, activated in order to prevent formation of reactive oxygen species. Although widespread among oxygenic photosynthetic organisms, NPQ is activated through distinct

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*Author for Correspondence, e-mail: roberto.bassi@univr.it

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_14, © Springer Science+Business Media Dordrecht 2014

molecular effectors depending on taxa. In unicellular green algae as well as other algal groups (such as diatoms), NPQ activity depends on a light-harvesting complex (LHC)-like protein, called light-harvesting-complex stress-related (LHCSR). In land plants, such as *Arabidopsis thaliana*, NPQ instead relies on a different LHC-like protein: PsbS. This protein responds to low lumenal pH via protonation of two glutamate residues essential for activity. PsbS induces a reorganization of thylakoid membrane complexes upon lumen acidification, which is indispensable for the generation of the dissipative state. Upon reorganization, two distinct quenching components are generated: one is tightly connected to the photosystem II (PS II) reaction center and the other is located in a domain of the PS II antenna system, disconnected from the reaction center. Low lumenal pH also induces activation of the violaxanthin (V) de-epoxidase enzyme, leading to the synthesis of zeaxanthin (Z), which, in turn, up-regulates heat dissipation/NPQ upon binding to antenna proteins. PsbS-dependent NPQ is present in land plants and in the moss *Physcomitrella patens*. Although the *PsbS* gene sequence is conserved in all green algae whose genome has been sequenced thus far, the corresponding protein was not found to be accumulated in algal cells under any of many growth conditions explored, suggesting it may be a pseudogene. PsbS and LHCSR play a similar role in activating protective heat dissipation of excess light by responding to low lumenal pH. In contrast to PsbS, LHCSR exhibits a clear capacity for binding xanthophylls and chlorophylls. Thus LHCSR appears to combine the pH-responsive activity typical of PsbS with the capacity for binding pigments, chlorophylls and carotenoids, as a basis for engagement of energy dissipation reflected in NPQ. LHCSR appears to directly participate in energy dissipation possibly avoiding the need for reorganization of thylakoid complexes.

I Introduction: All Oxygenic Photosynthetic Organisms Exhibit NPQ Activity

Photosynthetic reaction centers (RC) harness solar energy for driving electrons from water to NADP$^+$. Electron flow is coupled to H$^+$ transfer from chloroplast stroma to thylakoid lumen, building a proton gradient for ATP synthesis (Nelson and Ben Shem 2004). ATP and NADPH are then exploited for carbon fixation. Photosynthetic organisms are exposed to strong and rapid changes in light intensity, temperature and water availability in their natural environment. In response to some of these conditions, limitation in photochemical quenching leads to increased Chl excited state (^{1}Chl*) lifetime and increased probability of Chl *a* triplet formation (^{3}Chl*) by intersystem crossing. Chl triplets react with molecular oxygen ($^3O_2$) to yield potentially harmful reactive oxygen species (ROS), responsible for photoinhibition and oxidative stress (Barber and Andersson 1992). These events are counteracted by photo-protection mechanisms

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Abbreviations: C2S2 – Photosystem II supercomplex composed of 2 core complexes and 2 LHCII trimers; Car – Carotenoid; C-B – Calvin-Benson; Chl – Chlorophyll; F_m – (F_m') maximal fluorescence in dark- (or light - respectively) adapted cells; KO – Knock-out; Lhca (Lhcb) – Light harvesting complex of photosystem I (or II respectively); LhcbM – Polypeptide of the major light-harvesting complex of photosystem II; LHCI – Light-harvesting complex protein of photosystem I; LHCII – Major light-harvesting complex of photosystem II; LHCSR – Stress-related light-harvesting complex; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OCP – Orange carotenoid-binding protein; Pi – Inorganic phosphate; PS I (II) – photosystem I (II); PsbS – Photosystem II subunit S; qE, qZ, qI – energy-dependent quenching, zeaxanthin-related quenching, “photoinhibitory” quenching; RC – Reaction Center; ROS – Reactive oxygen species; TP – Triose phosphate; V – Violaxanthin; WT – Wild type; Z – Zeaxanthin; ΔpH – Proton gradient

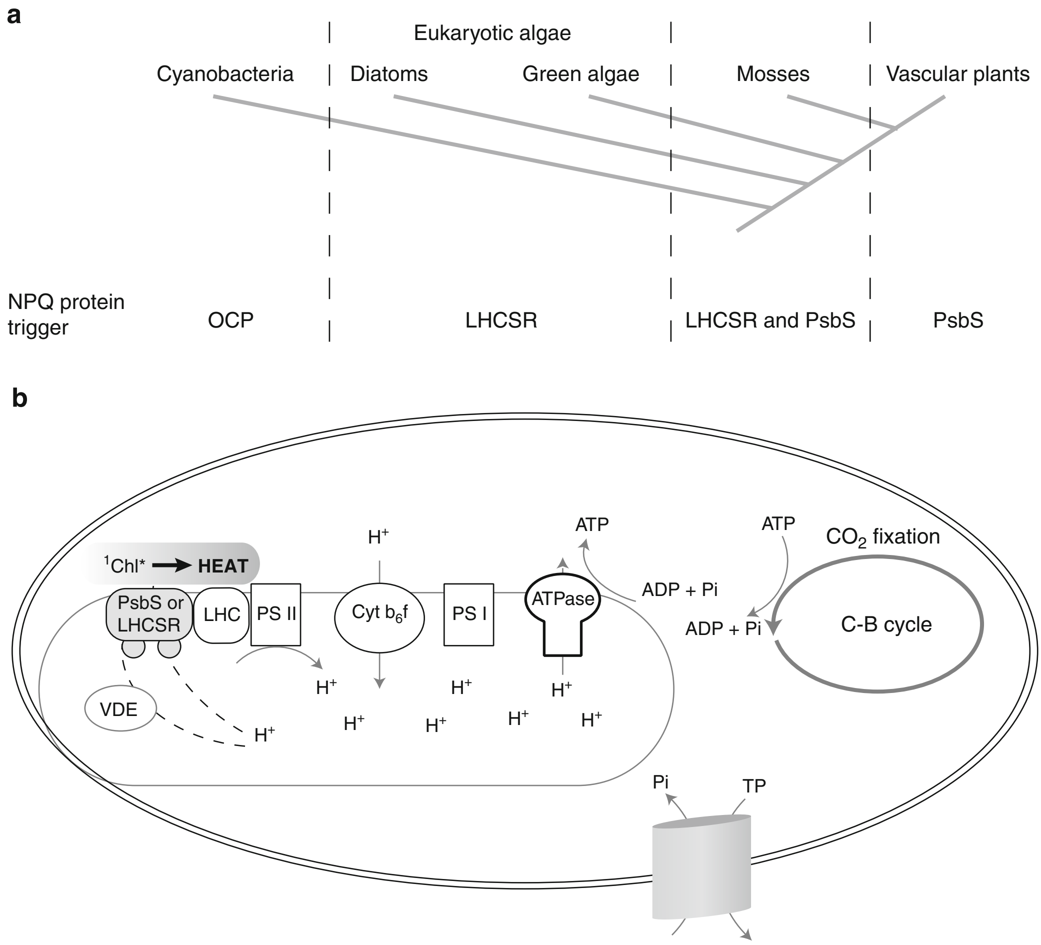

Fig. 14.1. NPQ response in different photosynthetic organisms. (**a**) Scheme of the evolution of oxygenic photosynthetic organisms. Different groups with distinct proteins involved in NPQ activation are reported. References are cited in the text. (**b**) Simplified scheme for feedback system involved in NPQ activation in photosynthetic eukaryotes. *TP* triose phosphate.

consisting either of scavenging of ROS (Asada 1999) or prevention of ROS production through down-regulation of triplet formation (Dall'Osto et al. 2012), quenching of ^{3}Chl* by xanthophylls (Havaux and Niyogi 1999), or dissipation of excess ^{1}Chl* (Niyogi 2000; Külheim et al. 2002). Among regulatory mechanisms, the thermal dissipation of excited Chl singlet states (^{1}Chl*), measured as non-photochemical quenching of Chl fluorescence (NPQ; see Papageorgiou and Govindjee, Chap. 1, and Logan et al., Chap. 7), is of major general importance. The ability to activate an NPQ response has been observed in all organisms performing oxygenic photosynthesis including cyanobacteria, algae and plants (Fig. 14.1a). Despite its widespread occurrence, NPQ has distinct features in different taxa, possibly shaped by adaptation to different environmental conditions. In cyanobacteria, NPQ depends on the presence of a soluble carotenoid-binding protein, OCP (Wilson et al. 2007; see Kirilovsky et al., Chap. 22). In eukaryotic algae and plants, NPQ depends on the presence of members of the extended LHC protein family. In particular, light harvesting-complex stress-related (LHCSR) has been shown to be essential in green algae and diatoms where mutants depleted in this protein have a reduced level of NPQ (Peers et al. 2009; Bailleul et al. 2010). In vascular plants, NPQ instead depends on the presence of a photosystem II subunit called PsbS (Li et al. 2000; Kasajima et al. 2011). PsbS is an LHC-like protein, sharing evolutionary origin with antenna proteins, and yet possesses special features, such as the presence of four trans-membrane helices rather than three found in all eukaryotic antenna proteins (Li et al. 2000).

Three components contributing to NPQ in eukaryotic organisms have been distinguished in the most recent studies: Energy-dependent quenching (qE), zeaxanthin-dependent quenching (qZ) and photoinhibitory quenching (qI) (Nilkens et al. 2010; see Holzwarth and Jahns, Chap. 5, and Logan et al., Chap. 7). The first type (qE) depends on lumen acidification and develops within seconds upon an increase in light intensity (Niyogi 2000). qE also relaxes on a 1–2 min timescale in the dark (Horton et al. 1996; Demmig-Adams et al. 1996). In dark-adapted leaves, zeaxanthin (Z) is accumulated within a few minutes of exposure to excess light and causes enhancement of NPQ (Horton et al. 1996; Nilkens et al. 2010; Förster et al. 2011; Jahns and Holzwarth 2012; Nichol et al. 2012). If Z is already present before illumination (e.g., mutants constitutively accumulating Z or leaves in natural environments), it induces a faster qE activation upon exposure to light (see Adams and Demmig-Adams, Chap. 2, and Demmig-Adams et al., Chap. 24). Zeaxanthin accumulation is also responsible for a slower component going in parallel with back conversion of Z into violaxanthin (V), which takes up to 1 h in lab experiments (Dall'Osto et al. 2005; Reinhold et al. 2008). The slowest quenching component, qI, is associated with PS II photoinhibition and it occurs under a variety of conditions of plant sink limitation (Adams et al. 2013, Chap. 23). Its relaxation requires hours to days to recover and is sensitive to chloroplast translation inhibitors (Walters and Horton 1991).

A Eukaryotic Systems Have a Feedback NPQ Response

In eukaryotic organisms under conditions of rapid growth, the largest fraction of NPQ consists of the qE component, whose activation depends on generation of a pH gradient across the thylakoid membrane, as shown in early studies on NPQ inhibition by nigericin (Walters and Horton 1991; Gilmore et al. 1998; see Strand and Kramer, Chap. 18). The NPQ dependence on lumenal pH generates a feedback loop between the production and consumption of the products of the light reactions. As depicted schematically in Fig. 14.1b, photosynthetic complexes in the thylakoid membranes convert light energy into NADPH and concomitantly build up a proton gradient (ΔpH) for ATP synthesis. ATP is then used by the Calvin-Benson (C-B) cycle for CO_2 fixation, yielding inorganic phosphate (Pi) and ADP, which maintains ATPase activity. Additional sources of Pi in the chloroplast are coupled with the export of triose phosphates as the products of the C-B cycle. Any excess in light absorption rate with respect to the cell's ability to hydrolyze ATP causes a depletion of Pi in the chloroplast, limiting ATPase activity and consequently proton export from the thylakoid lumen (see Strand and Kramer, Chap. 18). The imbalance between light absorption rate and use of ATP thus translates into an increase in proton concentration in the lumen, as the major signal activating photoprotection responses (Fig. 14.1b). This scheme shows how NPQ is the result of a feedback mechanism to adjust light-harvesting efficiency to the effective cell capacity for energy use through photochemical reactions. On a longer timescale, the consumption of reduced carbon by the plant's sinks also influences NPQ capacity (see Adams et al., Chap. 23). It is worth mentioning, however, that this picture is only valid for eukaryotic organisms, since NPQ in cyanobacteria is an on-off mechanism depending on photo-conversion of OCP only, and is independent of ΔpH (see Kirilovsky et al., Chap. 22).

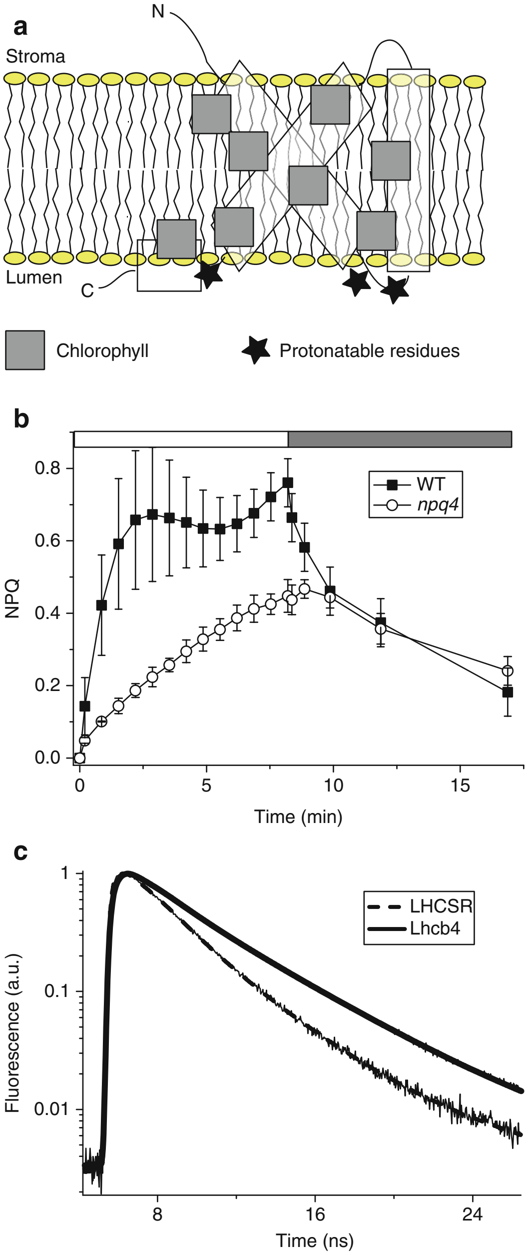

Fig. 14.2. LHCSR is active in algal NPQ. (**a**) Scheme of LHCSR structure with indication of the presence of DCCD-binding lumenal residues that can be protonated (*black stars*). (**b**) NPQ (calculated as $F_m/F_m'-1$) measured in *Chlamydomonas* cells of WT and *npq4* mutants depleted in LHCSR2, but still retaining the LHCSR1 isoform. *White and gray bars* above the middle panel indicate that light is switched on and off, respectively. Growth of cells and NPQ measurements were performed as in Bonente et al. (2012). (**c**) Comparison of fluorescence lifetime decay curves of LHCSR (*dashed line*) and an antenna complex (Lhcb4, *solid line*). *a. u.* arbitrary units.

II LHCSR Is Responsible for NPQ Activity in Mosses and Many Algal Taxa

It has been shown that NPQ in the green alga *Chlamydomonas reinhardtii* (Chlorophyta) and in two species of diatoms depends on the LHC-like protein LHCSR/LHCX (also known as Li818 in an older nomenclature (Gagne and Guertin 1992; Peers et al. 2009; Zhu and Green 2010; Bailleul et al. 2010; Fig. 14.2a, b). LHCSR-like sequences have been identified in all eukaryotic algal groups, suggesting that algae rely on LHCSR-like proteins for NPQ. While no LHCSR genes are present in any known vascular plant, LHCSR has been identified, e.g., in the moss *Physcomitrella patens* (Alboresi et al. 2008). Generation of Knock Out (KO) lines specifically depleted in LHCSR demonstrated that this protein plays a major role in moss NPQ activity (Alboresi et al. 2010; Gerotto et al. 2012).

In most organisms analyzed so far, a strong dependence of LHCSR levels on light intensity has been observed. While LHCSR is constitutively present even in low-light-acclimated cells in some algal species (Zhu and Green 2010; Gerotto et al. 2011), in other species, such as *Chlamydomonas reinhardtii*, acclimation to light environment has a large influence on NPQ activity, with low light-grown cells capable of only a low level of NPQ (Peers et al. 2009). These cells, however, strongly up-regulate both LHCSR protein level and NPQ activity upon acclimation to high light (Peers et al. 2009). In all species, including the moss *Physcomitrella patens*, modulation of LHCSR accumulation by growth light environment seems to be a common strategy to optimize NPQ amplitude based on environmental conditions experienced.

A Properties of the LHCSR Protein

Similar to many other eukaryotic antenna proteins, LHCSR has three trans-membrane helices (Fig. 14.2b) and binds pigments, both Chl and carotenoids (Bonente et al. 2011). LHCSR is active in organisms with different pigment composition, like diatoms, where the major carotenoid is fucoxanthin, or green algae, where lutein is the most abundant xanthophyll (see Büchel, Chap. 11, and Finazzi and Minagawa, Chap. 21). LHCSR is thus likely to be flexible in binding various carotenoids, although it does not bind neoxanthin (Bonente et al. 2011). Since LHCSR is present in organisms with different accessory Chls (Chl c and b), it is likely to also bind different Chl types, although the *Chlamydomonas* LHCSR3 showed a strong affinity for Chl a (Bonente et al. 2011). One major difference between antenna proteins and LHCSR is their ability to dissipate energy as heat. In fact, even as an isolated protein in detergent solution, LHCSR shows a short fluorescence lifetime, implying it efficiently dissipates energy as heat (Bonente et al. 2011). This observation suggests that LHCSR operates in thermal energy dissipation by receiving energy from nearby antenna complexes and catalyzing dissipation of excited states into heat. Since this ability to dissipate energy is present in the isolated protein in vitro at neutral pH, LHCSR appears to thermally dissipate a fraction of its excitation energy constitutively (Fig. 14.2c). Upon illumination with high light, the decrease in lumenal pH further increases this quenching activity (Bonente et al. 2011).

B Additional Factors Involved in LHCSR-Dependent NPQ Activity: Carotenoids and Lhcb Subunits

While LHCSR is apparently indispensable for NPQ activity in eukaryotic algae, other factors are also involved. It has been shown that, in *Chlamydomonas reinhardtii*, depletion of one major component of the PS II antenna complex, LhcbM1, causes a major decrease in NPQ activity (Elrad et al. 2002; see Finazzi and Minagawa, Chap. 21), while depletion of other, closely related major components of the PS II antenna, LHCBM2 and 7, has no effect on NPQ activity (Ferrante et al. 2012). Although direct experimental evidence is still missing, this suggests that LHCSR may specifically interact with LhcbM1 upon lumen acidification.

Carotenoids also play a major role in NPQ in both plants and algae (Niyogi et al. 1997, 2001). Carotenoid composition of photosynthetic organisms is not constant but depends on environmental conditions. In particular, xanthophylls are interconverted by xanthophyll cycles that consist of conversion of V to Z in green algae (and plants) and, in addition, of diadinoxanthin to diatoxanthin in diatoms (see Finazzi and Minagawa, Chap. 21). These reactions are typically reversed when organisms return to low light. Xanthophyll cycles are operated by violaxanthin de-epoxidase and activated by low lumenal pH, as is NPQ induction (Arnoux et al. 2009). The de-epoxidized xanthophylls apparently affect NPQ in several organisms, with their level being correlated with NPQ amplitude (see Adams and Demmig-Adams, Chap. 2). In diatoms, NPQ activity is closely associated with high diatoxanthin level, suggesting that this pigment affects LHCSR quenching activity (Ruban et al. 2004; Bailleul et al. 2010). The same holds for the moss *Physcomitrella patens*, where LHCSR–dependent NPQ is clearly up-regulated by Z (Pinnola et al. 2013). In contrast, in other species the influence of the xanthophyll cycle may be small. For instance, the *npq1* mutant of *Chlamydomonas reinhardtii*, unable to synthesize Z, exhibits NPQ levels similar to those observed in wild type (WT), suggesting LHCSR quenching ability in this species does not respond to Z (Bonente et al. 2011). These data suggest that specific properties of LHCSR protein may be different depending on species and modulate features of NPQ response in vivo.

III PsbS Is Responsible for NPQ Activity in Plants

Genomes of vascular plants do not contain *lhcsr* genes (Alboresi et al. 2008). Instead, they include a different LHC-like protein, PsbS, that is responsible for NPQ activation in plants (Li et al. 2000; see Brooks et al., Chap. 13). Mutants depleted in this protein are, in fact, unable to activate a NPQ response (Fig. 14.3b). Clear evidence for PsbS-dependent qE activity has been found in several plant species, including *Arabidopsis*, rice and the moss *Physcomitrella patens*, based on the phenotype of PsbS-less mutants. PsbS-encoding genes are absent in diatoms or red algae but have been identified in the genomes of several green algae, suggesting their conservation in the common ancestor of green algae and plants *Viridiplantae* (Gerotto and Morosinotto 2013). However, even in algae with PsbS-encoding genes, the corresponding polypeptides have never been detected (Koziol et al. 2007; Bonente et al. 2008b; Engelken et al. 2010; Gerotto and Morosinotto 2013). The PsbS-like gene, at least in *Chlamydomonas*, thus appear to be a pseudogene since it does not allow for accumulation of the corresponding protein. Even when the PsbS gene was over-expressed, no differential NPQ activation was observed with respect to WT (Bonente et al. 2008b). During *Viridiplantae* evolution, the first organism for which NPQ activity can be associated with PsbS is the moss *Physcomitrella patens* that contains a *PsbS* gene and accumulates the corresponding proteins in thylakoid membranes (Alboresi et al. 2010). This finding suggests that algae might have recruited PsbS on their way to land colonization to increase non-photochemical quenching efficiency relative to that allowed by LHCSR. The PsbS-dependent mechanisms then became the only ones responsible for qE in vascular plants. Consistent with this hypothesis, recent evidence suggests that a PsbS–dependent NPQ activity might also be present in *Charales* and *Coleochetales*, as algal taxa later derived from plant ancestors (Gerotto and Morosinotto 2013). At present, the mechanistic differences between LHCSR- and PsbS-induced non-photochemical quenching processes that might have led to substitution of LHCSR with PsbS still need to be clarified.

A Properties of the PsbS Protein

PsbS is evolutionarily related to LHC proteins and yet has unique features, such as the presence of four trans-membrane helices (Fig. 14.3a) rather than three as in most members of the family (Engelken et al. 2010; see Brooks et al., Chap. 13). PsbS is thought to be the sensor for NPQ activation upon lumen acidification (Li et al. 2002). An explanation for this pH dependence at the molecular level was provided by the identification of two glutamate residues, the protonation of which is fundamental for NPQ activation; mutants (e.g., *npq4*) carrying substitutions with non-protonatable glutamine residues showed no qE despite accumulation of the PsbS protein to WT levels or higher (Li et al. 2000; Li et al. 2004; see Brooks et al., Chap. 13).

PsbS lacks most of the residues conserved in LHC proteins as Chl ligands (Dominici et al. 2002). Only two arginine residues, also known to stabilize protein structure by generating salt bridges between adjacent trans-membrane helices (Remelli et al. 1999), are conserved (Fig. 14.3a), suggesting structural stabilization rather than pigment binding might be the reason for their conservation in PsbS. The pigment-binding capacity of PsbS has been the subject of debate since the identification of its key role in NPQ; early reports suggested that PsbS binds both Chl and xanthophylls, implying it could be the site of quenching of Chl excited states, similar to LHCSR (Funk et al. 1995). Yet, alternative views have been proposed (Aspinall-O'Dea et al. 2002), suggesting that only Z and no Chl is bound to PsbS. Finally, the binding of any pigment has been disputed, suggesting that PsbS may not be a pigment-binding protein at all (Dominici et al. 2002; Bonente et al. 2008a).

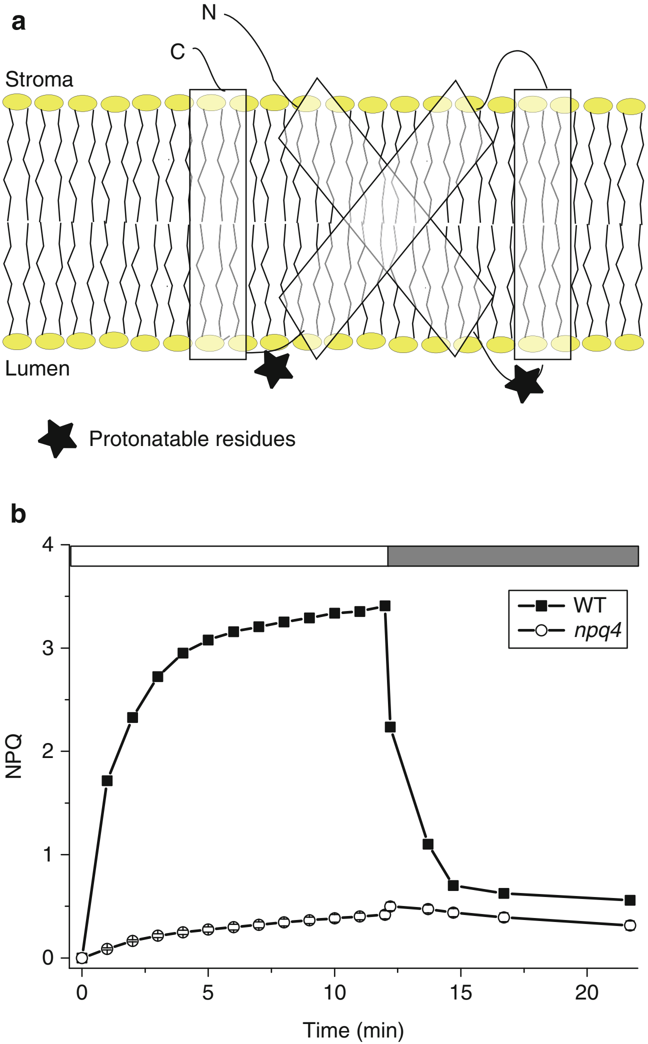

Fig. 14.3. PsbS is active in plants' NPQ. (**a**) Scheme of PsbS structure with protonatable residues (*black stars*). (**b**) NPQ (calculated as $F_m/F_m'-1$) in *Arabidopsis* WT (*black squares*) and PsbS KO (*npq4*, *empty circles*). *White and gray bars* above the panel indicate that light is switched on and off, respectively. Growth of plants and NPQ measurements were performed as in De Bianchi et al. (2008).

B Additional Factors Involved in NPQ Activation: Carotenoids and Lhcb Subunits

While fundamental for the activation of qE, PsbS is not sufficient for activation to full amplitude, and the xanthophylls Z and lutein are also required (Niyogi et al. 2001). Lack of Z, as in the *npq1* mutant, decreases qE to 30 % of WT in *Arabidopsis*, while constitutive Z accumulation, as in the *npq2* mutant, makes the onset of qE faster (Fig. 14.4a). Since Z formation from V is typically faster than its epoxidation back to V by Z epoxidase, Z remains present for about 1 h after returning to low light in the model plant *Arabidopsis thaliana*. The presence of this slowly epoxidated Z pool is responsible for a fraction of NPQ named qZ (Nilkens et al. 2010). qZ is likely due to Z binding to antenna complexes, with Lhcb5 being particularly important for this slow component (Dall'Osto et al. 2005). Besides Z, lutein is also a player in NPQ; lutein-deficient mutants exhibit slower initial

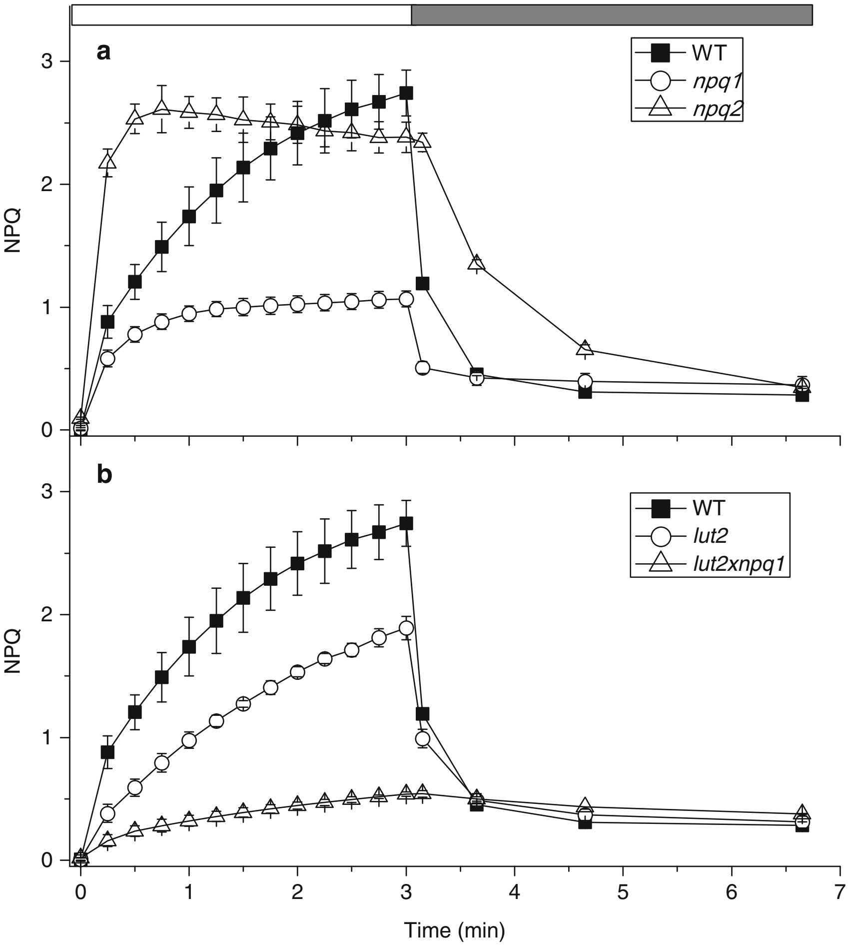

Fig. 14.4. NPQ dependence on additional factors. (**a**) NPQ in *Arabidopsis* WT, *npq1* and *npq2* mutants (black squares, white circles and empty triangles, respectively). *npq1* mutants are unable to synthesize zeaxanthin while *npq2* accumulate this xanthophyll constitutively. (**b**) NPQ (calculated as $F_m/F_m'-1$) in *Arabidopsis* WT, *lut2*, *lut2npq1* and *npq4* mutants (*black squares, white circles, empty triangles* and *black diamonds*, respectively). *lut2* mutants are unable to synthesize lutein while *npq4* are depleted of PsbS. *White and gray bars* above the panel indicate that light is switched on and off, respectively. Growth of plants and NPQ measurements were performed as in De Bianchi et al. (2008).

NPQ kinetics and a reduced NPQ amplitude over longer periods of time (Fig. 14.4b). Over-accumulation of lutein, as in the *szl-1npq1* mutant (Li et al. 2009) compensates in part for the lack of Z in this double mutant. Consistently, lack of both lutein and Z, as in the *npq1lut2* double mutant, yields an NPQ phenotype of qE absence similar to the *PsbS*-less mutant *npq4* (Fig. 14.4b). Since PsbS most likely binds little or no pigments, including xanthophylls, the strong effect of Z and lutein cannot be explained without the involvement of other xanthophyll-binding proteins. The xanthophyll–dependent effect on NPQ likely occurs through their binding to LHC proteins that are well known to constitutively bind lutein as a major component and undergo exchange of V with Z upon exposure to excess light conditions (Morosinotto et al. 2002). Consistent with a major role of antenna proteins in NPQ activation, extensive depletion of Lhcb proteins strongly reduces NPQ activity (Havaux et al. 2007), while selective deletion of individual Lhcb gene products yields weaker phenotypes (Andersson et al. 2003; De Bianchi et al. 2008, 2011) suggesting a functional redundancy within this protein sub-family (see Horton, Chap. 3).

C Molecular Mechanism of PsbS Activity: Modulation of Thylakoid Structural Organization

Since the first isolation of PsbS-deficient *Arabidopsis* mutants (Li et al. 2000), many research groups have investigated the molecular mechanism for its function, which, however, proved to be elusive. Due to its lack of pigment cofactors, PsbS is unlikely to be the site of the quenching reaction(s) dissipating Chl excited states. Yet, in the absence of PsbS, other *npq* mutations show no influence on NPQ, including *npq2* and *szl1*, overaccumulating Z and lutein, respectively, indicating an essential role of PsbS (Li et al. 2009). Recent proposals suggest that PsbS is active as a "facilitator" of the quenching reaction that occurs in Lhcb proteins rather than in PsbS itself (Betterle et al. 2009; Johnson et al. 2011; see Holzwarth and Jahns, Chap. 5, Brooks et al., Chap. 13, and Ruban and Mullineaux, Chap. 17). Antenna complexes associated with PS II have been shown to undergo conformational transitions between states with distinct lifetimes, making them able to shift between a light-harvesting and a dissipative state (Moya et al. 2001; Kruger et al. 2011; see Krüger et al., Chap. 6, and Robert et al., Chap. 10). In their conformation with long lifetimes (highly efficient in light harvesting), Lhcb complexes transfer energy to the PS II reaction center, while in their short-lifetime conformation a significant fraction of absorbed energy is dissipated as heat before being trapped by the PS II reaction center, thus reducing the probability of light-induced damage under excess light conditions.

Although lifetime transitions have been experimentally assessed in isolated LHC proteins (Moya et al. 2001; Krüger et al. 2011, 2012; see Krüger et al., Chap. 6), the state of individual LHC molecules within the thylakoid membranes and factors affecting it are not easily accessible (van Oort et al. 2010). Early work with Lhcb proteins reconstituted in liposomes has shown that the probability of protein-protein interactions is a powerful modulator of Chl fluorescence lifetime (Moya et al. 2001). The recent finding that PsbS is essential for re-organization of PS II-LHCII super-complexes within grana partition membranes provides a model for PsbS-mediated NPQ activation; in low light, V-binding Lhcb proteins are organized in super-complexes locked in their long-lifetime (light-harvesting) conformation (see Ruban and Mullineaux, Chap. 17). Lumen acidification, acting through activation of PsbS, induces dissociation of the external layer of the PS II-LHCII antenna system, causing re-organization of pigment-protein complexes into distinct membrane domains containing either C2S2 particles (PS II dimer + Lhcb4 + Lhcb5 + LHCII; Boekema et al. 1999; see Garab, Chap. 16, and Ruban and Mullineaux, Chap. 17) or most of the LHCII antenna plus Lhcb6, respectively (Betterle et al. 2009; Johnson et al. 2011). Chl fluorescence lifetime analysis in vivo has identified two different quenching sites each associated to one of the two compartments, suggesting altered pigment-protein interactions resulting from reorganization of the thylakoid membrane leads to a transition into the quenching state (Miloslavina et al. 2011; see Holzwarth and Jahns, Chap. 5). This model can also explain the enhancing effect of Z on NPQ, since Z synthesis, also activated by low lumenal pH, enhances the rate of supercomplex dissociation (Betterle et al. 2009) and binds to Lhcb proteins, thereby enhancing their quenching activity (Moya et al. 2001; Lambrev et al. 2010).

IV PsbS- vs. LHCSR-Dependent NPQ: Differences and Similarities

PsbS and LHCSR have common features (Figs. 14.2a and 14.3a), i.e. they are both LHC-like trans-membrane thylakoid proteins essential for qE and indispensable for NPQ activity. In addition, both proteins have protonatable residues (Li et al. 2004; Bonente et al. 2011) that are likely to detect the low lumenal pH essential for triggering qE, and whose mutation to non-protonable residues abolish PsbS activity in qE (Li et al. 2002). Both PsbS- and LHCSR- dependent mechanisms depend on ΔpH and their activities are both modulated by specific xanthophylls. Differences include presence of Chl and xanthophyll ligands in LHCSR but not in PsbS, implying that the former, but not the latter, might be the actual site of the quenching activity. PsbS is permanently located in grana thylakoids, in close contact with PS II super-complexes (Harrer et al. 1998), while the precise location of LHCSR remains to be determined. Isolation of a PS II-LHCII super-complex including LHCSR (Tokutsu et al. 2012; see Finazzi and Minagawa Chap. 21) suggests its close contact with PS II-rich domains, even though the easy extractability with mild detergents might suggest a peripheral location of LHCSR with respect to grana partitions (Richard et al. 2000).

A Proposed Molecular Mechanisms of Excess Energy Dissipation

Despite many studies of various *npq* mutants and of isolated LHC proteins, fundamental questions about Chl fluorescence quenching remain to be answered, e.g., concerning the identity of the (one or more) quenching species and the mechanism by which quenching is catalyzed. There may possibly be more than one qE quenching mechanism/site.

At least two models have been proposed for the physical mechanism of quenching:

Model 1: Aggregation-dependent LHCII quenching. This model was proposed based on the early evidence that aggregation of isolated LHC proteins, induced by low detergent concentration and/or low pH, causes a decrease in Chl fluorescence lifetime (Horton et al. 1991; see Horton, Chap. 3). Aggregation was later shown to be instrumental in catalyzing conformational change(s) within the LHCII protein, and the spectral signatures associated with this event were interpreted to indicate formation of a tight interaction between lutein and a Chl *a* molecule (Ruban et al. 2007). Extrapolation to NPQ in vivo relies on similarities between spectral changes in leaves upon NPQ induction and those upon aggregation of the isolated protein (Pascal et al. 2005; Ruban et al. 2007). Yet, LHCII aggregation quenching is insensitive to the presence or absence of Z in the preparation (Ballottari et al. 2010), while most of NPQ in vivo is dependent on Z synthesis as shown by *npq1* mutants (Niyogi et al. 1998).

Model 2: Charge-transfer (CT) quenching mechanism. In this model, qE activation involves a charge separation between a Chl-Z heterodimer that produces a transient Z radical cation ($Z^{\cdot+}$) that rapidly (50–200 ps) relaxes to the uncharged state. The spectroscopic signal of $Z^{\cdot+}$ was detected in isolated monomeric Lhcbs (Avenson et al. 2008) and mutation analysis of Chl-binding sites in Lhcb4 enabled to propose that a specific Chl pair (Chl A5 and Chl B5) is involved in this charge-transfer event (Ahn et al. 2008; see Walla and Fleming, Chap. 9). The charge transfer model is also based on the observation that, upon xanthophyll cycle activation, V in the monomeric Lhcb antenna complexes can be replaced by Z (Morosinotto et al. 2002). Both Lhcb4-6 subunits involved in the PsbS-dependent NPQ and LHCSR for the algal-type mechanism were observed to undergo transient formation of radical cations (Holt et al. 2005; Ahn et al. 2008; Bonente et al. 2011) while the major trimeric LHCII complex did not.

Recent analysis of ultrafast Chl fluorescence kinetics in intact leaves of *Arabidopsis* (Miloslavina et al. 2008; Miloslavina et al. 2011; see Holzwarth and Jahns, Chap. 5) provided evidence that at least two different quenching sites contribute to qE in vivo: a PsbS-dependent site located in LHCII that becomes detached from PS II and aggregates upon illumination, and a PsbS-independent site located within the minor antennae attached to PS II. Furthermore, Car S_1–Chl excited state coupling was recently measured in isolated LHCII and correlated with qE amplitude in vivo in mutants such as *npq1*, *npq2* and *lut2* (Bode et al. 2009; see Polivka and Frank, Chap. 8). On this basis, it was proposed that a short-lived, low energy excitonic Car–Chl state, formed upon lumen acidification, may also function in vivo as an excess energy dissipation valve (see Walla et al., Chap. 9).

V Concluding Remarks: Why NPQ Evolved from LHCSR to PsbS

Plants started colonizing land about 450 Ma ago and evolved new morpho-physiological traits to adapt to the conditions of the terrestrial environment, such as flooding/desiccation cycles, low and high temperatures, increased exposure to UV radiation and excess visible light (Waters 2003; Becker and Marin 2009). Oxygen is more abundant and diffuses faster in the atmosphere than in water (Scott and Glasspool 2006), and acts as a competitor for the fixation of CO_2 in photosynthesis because of the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Maurino and Peterhansel 2010) and as a generator of reactive species (Niyogi 1999). Furthermore, the availability of carbon dioxide, the final acceptor of electrons extracted from water by the oxygen-evolving complex, depends on the stomatal opening in plants, which may become restricted when water availability is diminished under climatically stressful conditions (Waters 2003). These conditions increase the probability of harvesting light in excess of what can be utilized by the photochemical reactions, thus requiring optimization of photoprotective mechanisms in plants.

It may be asked why plants only retained the PsbS-dependent mechanism. The answer probably does not lie in the amplitude of the NPQ response, since algae and plants have been shown to be active in inducing NPQ of different amplitudes depending on the species and growth conditions. An alternative explanation can be inferred from the observation that LHCSR shows a level of constitutive quenching, while PsbS does not (Bonente et al. 2011). PsbS, when inactive, has essentially no influence on the photon-harvesting efficiency of the antenna proteins, thus allowing plants to use light as efficiently as possible under light-limited conditions. In contrast, LHCSR efficiently dissipates energy even when isolated in detergent solution (Bonente et al. 2011), a condition that induces longer fluorescence lifetimes than those measured from LHC proteins in vivo or in lipid membranes (Moya et al. 2001; Miloslavina et al. 2008; van Oort et al. 2010), suggesting that LHCSR may dissipate a fraction of the harvested energy even in low light conditions. One way to overcome this problem is to accumulate LHCSR only when needed, upon exposure to strong light, as observed in *Chlamydomonas* (Peers et al. 2009).

However, this strategy leaves cells vulnerable to radiation damage by any abrupt changes in light intensity, conditions where the physiological role of PsbS was shown to be particularly relevant (Külheim et al. 2002; Allorent et al. 2013). Therefore, PsbS' ability to finely regulate thermal energy dissipation may provide a selective advantage in the terrestrial environment where fast and pronounced fluctuation of light irradiance is common (Külheim et al. 2002). In this context, it should also be considered that other mechanisms provide protection from constitutive strong illumination in plants, such as excess light-evading chloroplast movements (Brugnoli and Björkman 1992), leaving a more important role for NPQ in short-term regulation. Figure 14.5 shows the dependence of NPQ activity on light intensity for *Arabidopsis thaliana*, with its PsbS-dependent NPQ system, versus *Chlamydomonas reinhardtii*, performing NPQ through LHCSR. Both organisms were grown under high light conditions to compensate for the different inducibility of the key proteins upon acclimation (Ballottari et al. 2007; Bonente et al. 2012). Here it can be observed that the NPQ response in *Chlamydomonas* is already activated in low

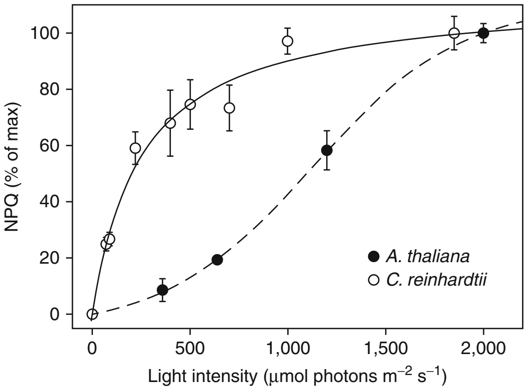

Fig. 14.5. Dependence of NPQ on actinic light intensity in *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*. Both organisms were acclimated to high light intensity (400 and 1,600 μmol photons m^{-2} s^{-1}, respectively) for 5 days before measurement. Data are redrawn from Ballottari et al. (2007) and Bonente et al. (2012).

light and saturates at relatively low light intensity. In contrast, *Arabidopsis* plants respond proportionally to light intensity, extending their photoprotection effect over a much wider light intensity range. Thermal dissipation levels are very low in limiting light intensity where photo-damage is unlikely. Although this relation needs to be extended to a wider range of species before being considered a general property of PsbS- versus LHCSR-dependent systems, these differences nonetheless suggest that the different response to light intensity might be a reason for the transition from algal to plant-like light regulation systems.

Acknowledgments

RB thanks the EEC projects “Sunbiopaths”, “Harvest” and “Accliphot” for supporting research on regulation of photosynthesis in plants and algae. Luca Dall’Osto, Matteo Ballottari, Alberta Pinnola and Alessandro Alboresi are acknowledged for discussions and help. TM acknowledges financial support from “Cassa di Risparmio di Padova e Rovigo” (CaRiPaRo) Foundation and the University of Padova (grants CPDA089403 and CPDR104834).

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Chapter 15

Are Chlorophyll-Carotenoid Interactions Responsible for Rapidly Reversible Non-Photochemical Fluorescence Quenching?

Herbert van Amerongen*
Laboratory of Biophysics, Wageningen University,
8128, Wageningen 6700 ET, The Netherlands

Summary

Photoprotective thermal energy dissipation (as assessed via non-photochemical quenching of singlet-excited chlorophyll a, NPQ) in plants is driven by various mechanisms occurring over different time scales. The rapid and reversible part of NPQ, also called qE (for energy-dependent quenching), was demonstrated to correlate with the twisting of a neoxanthin molecule in the light-harvesting antenna as observed by resonance Raman spectroscopy (Nature 450: 575–578, 2007). Interestingly, the extent of fluorescence quenching correlates with the change in Raman signal in different situations: during NPQ in vivo, during fluorescence quenching upon aggregation of LHCII (the major light-harvesting complex in plants), and in crystals of LHCII. In the same study, it was proposed that the quenching is caused by excitation energy transfer from chlorophyll *a* to lutein in LHCII after a structural change that correlates with the twisting of the neoxanthin. However, this view has been challenged by others for different reasons. Here we discuss the arguments in favor and against this mechanism. A short overview is given of the spectroscopic data on chlorophyll-carotenoid interactions in plant light-harvesting systems, the changes in interactions upon aggregation or crystallization, and the possible relationship to the mechanism of NPQ.

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*Author for Correspondence, e-mail: herbert.vanamerongen@wur.nl

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_15,

I Introduction

Many plants occur in (strongly) varying light conditions. In low light, plants make optimal use of light-harvesting complexes to increase the absorption cross-section of the photosystems, but in high light the probability of triplet formation increases substantially, leading to the formation of potentially deleterious ROS (reactive oxygen species). Plants have developed various photoprotective mechanisms; in high light, non-photochemical quenching of chlorophyll fluorescence (NPQ) is induced, most of which represents a collection of processes leading to the quenching (via heat formation) of the singlet excited state of chlorophylls (Chls) and thus of Chl fluorescence from the photosynthetic apparatus (for an excellent recent review, see Jahns and Holzwarth 2012; see Logan et al., Chap. 7).

The induction of NPQ proceeds on different time scales resulting in various stages of NPQ. The fastest phase occurring on a time scale from seconds to minutes is called qE, and is triggered by the proton gradient across the thylakoid membrane (see Strand and Kramer, Chap. 18). More specifically, a low pH in the lumen leads to the protonation of a membrane protein called PsbS that is crucial for qE, although its exact role is still unknown (see also Brookes et al., Chap. 13). Another prerequisite for NPQ is the xanthophyll cycle in which violaxanthin (V) is de-epoxidized to zeaxanthin (Z) via the intermediate antheraxanthin. Although Z formation correlates with induction of NPQ (Demmig et al. 1987; Demmig-Adams 1990), qE also occurs in the absence of Z (Holzwarth et al. 2009) albeit the magnitude of qE is lower (however, see Demmig-Adams et al., Chap. 24, for the conclusion that plants in nature invariably exhibit NPQ in the presence of Z). For systems containing both Z and PsbS, Holzwarth and coworkers identified a red-shifted fluorescence component of 400 ps in the presence of qE. However, in the absence of Z this fluorescence component was still present, but its lifetime was somewhat longer (600 ps), indicating that quenching still occurs but is somewhat less efficient (Holzwarth et al. 2009).

Protonation of PsbS is thought to induce detachment from photosystem II (PS II) of some antenna complexes that subsequently aggregate and become quenched, a process termed Q1 quenching by Holzwarth and coworkers (see e.g., Jahns and Holzwarth 2012 and Holzwarth and Jahns, Chap. 5). In 2009, Betterle et al. (2009) found that, in high light, PsbS leads to disassembly of a complex termed B4C (band 4 complex). B4C consists of two minor antenna complexes, CP29 and CP24, as well as the M-trimer of LHCII (moderately bound light-harvesting complex II), and B4C can typically be isolated separately from other complexes on a sucrose gradient. The B4C sub-complex is normally part of the C2S2M2 PS II supercomplex composed of a core dimer (C2), two each of the minor antenna complexes CP29, CP26 and CP24, two strongly bound (S) LHCIIs, and two moderately bound (M) LHCII trimers (Fig. 15.1; see Dekker and Boekema 2005 and Caffarri et al. 2009 for further explanation). In the latter supercomplex, all light energy captured by the antenna is efficiently transferred to a reaction center (RC) (Caffarri et al. 2011). In high light and in the presence of PsbS, B4C is not present, and it was

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Abbreviations: B4C – Band 4 complex; C2 – Dimeric core of PS II; C2S2M2 – Supercomplex of PS II consisting of a dimeric core surrounded by the outer light-harvesting complexes (LHCs): 4 major (LHCII) and 6 minor ones (two each of CP24, CP26 and CP29); Car – Carotenoid; Chl – Chlorophyll; Chl 610 – Chl A1; Chl 612 – Chl A2; CP24, CP26, CP29 – Minor light-harvesting complexes of photosystem II; CT – Charge-transfer; DES – De-epoxidation state; EET – Excitation energy transfer; LHC – Light-harvesting complex; LHCII – Light-harvesting complex II; LHCII-M – Moderately coupled LHCII trimer; LHCII-S – Strongly coupled LHCII trimer; Lut1 – Lutein 1; N – Neoxanthin; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PS I – Photosystem I; PS II – Photosystem II; qE – Energy-dependent quenching; RC – Reaction center; ROS – Reactive oxygen species; V – Violaxanthin; Z – Zeaxanthin

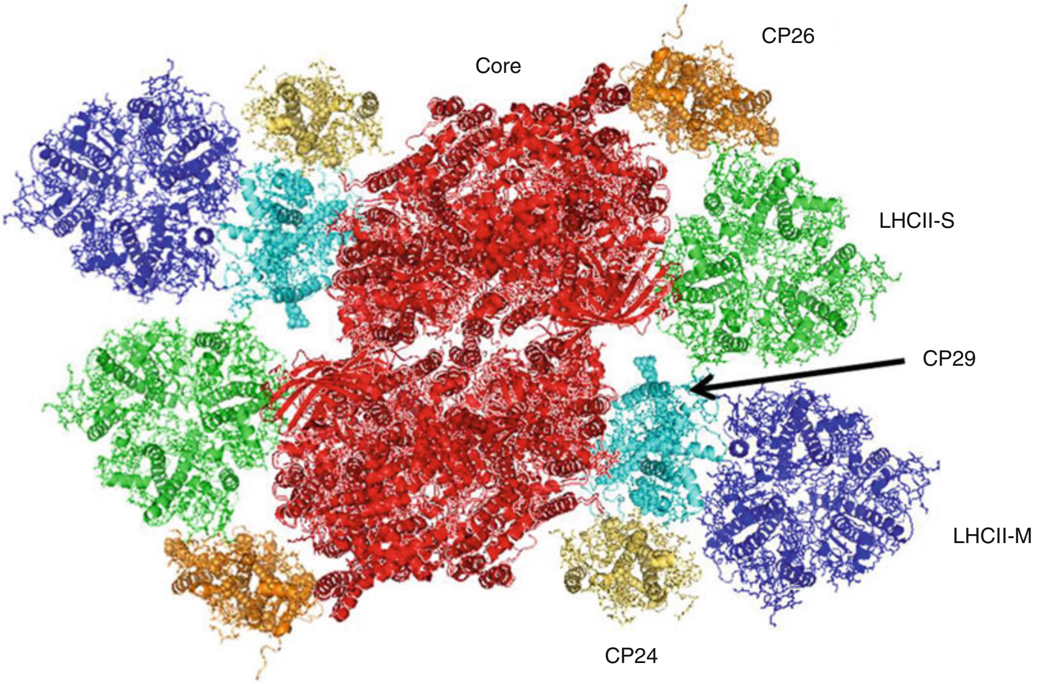

Fig. 15.1 Organization of photosystem II in a supercomplex. The supercomplex shows C_2 symmetry and consists of two cores in the middle, surrounded by the outer light-harvesting complexes *(LHCs)*: four major (LHCII) and six minor ones (two each of CP24, CP26 and CP29). The outer LHCs transfer excitation energy to the cores. Each core consists of two inner antenna complexes named CP43 and CP47 and the reaction center *(RC)* where charge separation takes place. LHCII-M, moderately coupled LHCII trimer of the PS II supercomplex; LHCII-S, strongly coupled LHCII trimer of the PS II supercomplex.

therefore concluded that trimer M and CP24 are detached from the supercomplex under the latter conditions, and thus cannot transfer energy to the reaction center, while CP29 remains attached (Betterle et al. 2009). While such a situation may disturb the balance between PS II and photosystem I (PS I), it was recently demonstrated that some LHCII remains attached to PS I under nearly all light conditions (Wientjes et al. 2013), but detaches during light stress (Tikkanen et al. 2011; Wientjes et al. 2013). It is thus possible that the complexes detached from PS I and PS II aggregate and become quenched.

Subsequently, during the slower phase of NPQ formation occurring on a time scale of around half an hour (also called qZ), more Z accumulates and leads to quenching of the remainder of PS II (this process is termed Q2 quenching by Holzwarth et al. 2009). It might well be that, for this quenching, the required molecules are those Z that replace V in the light-harvesting complexes. It has been found that the minor antenna complexes CP24, CP26 and CP29 can form Z^+ Chl^- radical pairs that lead to fluorescence quenching, whereas this was not observed for the major antenna complex LHCII (Ahn et al. 2008; Avenson et al. 2008, 2009; see also Walla et al., Chap. 9).

On an even longer time scale, the so-called qI quenching takes place, a process that has been speculated to be caused by photoinhibition (but see also Demmig-Adams et al., Chap. 24). In the present chapter, however, I will focus on the fast phase of NPQ, i.e., on qE. The generally accepted view is that low lumenal pH induces a conformational change, or a change in protein-protein interaction, controlled by PsbS (see also Brooks et al., Chap. 13) and modulated by Z (Jahns

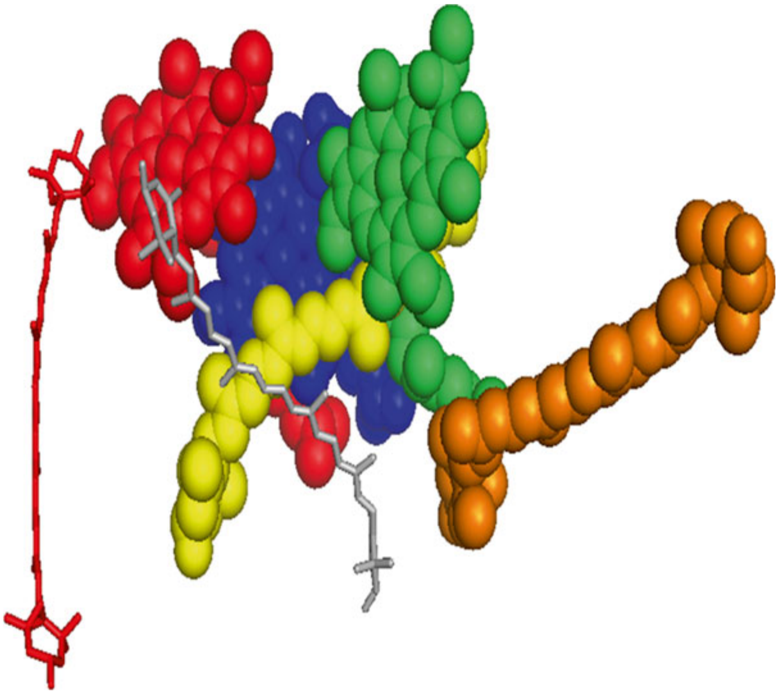

Fig. 15.2 Part of the structure of LHCII (see Liu et al. 2004; Standfuss et al. 2005) showing only a few relevant pigments. Lutein 1 is shown in *yellow* and is in Van der Waals contact with Chl 610 (A1) given in green and Chl 612 (A2) given in *blue*. The *red* Chl is Chl 611 (formerly called B2). The *orange* carotenoid is neoxanthin (*N*). The *grey* carotenoid is lutein 2 and the xanthophyll (violaxanthin-antheraxanthin-zeaxanthin) cycle carotenoid is given in *red*.

and Holzwarth 2012). Zeaxanthin is either an allosteric modulator of controlling the efficiency, kinetics or apparent pK of qE (Jahns and Holzwarth 2012) or it quenches directly via excitation energy transfer (EET) from Chl to Z (Owens 1994; Frank et al. 2000) or via a charge-transfer step (Holt et al. 2005; Ahn et al. 2008; Avenson et al. 2008, 2009; see also Walla et al., Chap. 9). Interestingly, Gruszecki et al. (2010) found that Z stimulates oligomerization of LHCII, and it is well known that oligomerization/aggregation of LHCII can lead to strong fluorescence quenching (see e.g., Horton et al. 2005; Van Oort et al. 2007a).

II Molecular Mechanism of qE

Different models have been proposed in recent years to explain the underlying molecular mechanism of qE. Holt et al. (2005) concluded that formation of a radical pair Z^+ Chl^- is largely responsible for the quenching during NPQ, and this was further corroborated in subsequent studies (Ahn et al. 2008; Avenson et al. 2008, 2009). However, as mentioned above, Z is not required for qE under certain circumstances, and it is more likely that the former radical pair may be involved in qZ.

Ruban et al. (2007) found that the amount of qE in vivo correlates with the increase of a band in the Raman spectrum that reflects twisting of a neoxanthin (N) molecule present in the light-harvesting antenna, which can thus be used as a reporter of qE. Earlier, it had been found that crystals of LHCII are also quenched, and show the same characteristic change in the Raman spectrum of N (Pascal et al. 2005). More recently, it was demonstrated that, in densely packed PS II membranes, N is twisted and quenching also occurs, whereas a slight dilution of the membrane with lipids leads to untwisting of the N molecule and to improved light harvesting, i.e., less quenching (Haferkamp et al. 2010). A corresponding change in the Raman signal also correlates with the amount of quenching in LHCII aggregates. Since femtosecond transient absorption spectroscopy showed that the excitations in these aggregates are quenched by lutein 1 (Lut1), also called pigment number 620 in (Liu et al. 2004), it was proposed that EET from Chl *a* to Lut1 may also be responsible for qE (Ruban et al. 2007). In particular, Chls 610–612 were suggested to be important for the latter transfer, as they form the lowest-energy pigments in LHCII, and Chls 610 (also termed Chl A1) and 612 (Chl A2) are in Van der Waals contact with Lut1. In Fig. 15.2, part of the structure of LHCII is shown, emphasizing the pigments that may be relevant. Apparently, aggregation leads to a conformational change in LHCII that induces a change in interaction between Chl and Lut1. A change in interaction between Chl *a* and carotenoids (Cars) in LHCII upon aggregation had, in fact, already been observed in earlier studies using triplet-minus-singlet spectroscopy (Barzda et al. 1998; Lampoura et al. 2002).

However, there is no consensus that a conformational change leads to the quenching of Chl excitations by lutein. It was, for instance, stated by Barros et al. (2009) that controlled thermal energy dissipation in plant photosynthesis is not brought about by a conformational switch within the antenna (LHCII), based, e.g., on their conclusion that LHCII crystals are not quenched. However, this conclusion was based on calculations of the average fluorescence lifetime of their crystals that turned out to be erroneous (see also Van Oort et al. 2011). The original results showing quenching of LHCII crystals (Pascal et al. 2005) were subsequently confirmed by Van Oort et al. (2011) and the extent of quenching turned out to depend on the crystal packing, hinting again at a correlation between LHCII structure and fluorescence lifetime.

To address the magnitude of the conformational change of LHCII required for substantial quenching, Van Oort et al. (2007b) performed time-resolved fluorescence measurements on trimeric LHCII at hydrostatic pressures up to 400 MPa. Upon raising the pressure, quenching was induced, accompanied by an increase of the amplitude of a short lifetime (~500 ps) component (that was already present at atmospheric pressure with very small amplitude), at the expense of the amplitude of the unquenched ~4 ns component. By determining the pressure dependence of the corresponding amplitudes, it was deduced that the conformational change leading to the quenching was accompanied by a change in protein volume of only 0.006 %. In addition to the increase of the contribution of the 500 ps component, it was also observed that an even faster component was created, with a lifetime of around 25 ps, as well as an even shorter one. It was concluded that a very small conformational change may lead to a substantial change in excited-state lifetime.

The results of Ruban et al. (2007) were questioned by Holzwarth and coworkers (Muller et al. 2010), who performed similar femtosecond transient absorption measurements on aggregated LHCII as were done by Ruban et al. (2007), but did not observe a signal attributable to lutein or any other carotenoid, and therefore discarded the model that quenching occurs via lutein 1. Instead, Holzwarth and coworkers discovered another spectroscopic marker for qE, namely a fluorescence band above 700 nm with a short lifetime that, like the N Raman signal, was observed both in vivo and with aggregated (oligomeric) LHCII (Miloslavina et al. 2008; Holzwarth et al. 2009; Lambrev et al. 2010). The latter band was attributed to a charge-transfer (CT) state created by two Chl molecules on different trimers that come together upon aggregation. This state is thought to be in equilibrium with an exciton state created upon aggregation and with a similar excited-state energy as the CT state, albeit slightly lower in energy than the other LHCII exciton states. The CT state is short-lived and was proposed to be responsible for the quenching, either by quenching excitations directly or acting as a quencher intermediate. In order to explain the observed spectra, it had to be assumed that the CT is nearly isoenergetic with the intertrimer LHCII exciton state and the forward and backward rates between them were concluded to be nearly identical.

However, it is not clear how this model would account for the fact that there are many more intra-trimer states that apparently do not participate in excitation distribution, despite the fact that they are nearly isoenergetic. Including these states would require a faster backward rate from the CT state to the exciton state(s), and this would not fit the model. Possibly, such a fast backward rate can be compensated in the model of Holzwarth and coworkers by a faster quenching rate for the CT state. Such a CT state, in general, corresponds to strong electron-phonon coupling, and this might also explain why Pieper et al. (1999) were not able to burn holes above 683 nm in their hole-burning experiments on oligomers of LHCII. It should be pointed out that, subsequent to the measurements of Pieper et al. (1999), absorption Stark measurements were performed on oligomeric LHCII to test whether CT could indeed be observed above 683 nm, but no CT was observed (at least not in absorption; Palacios et al. 2003).

As mentioned above, the generally accepted view is that the proton gradient ΔpH induces a conformational change in one of the light-harvesting complexes or a change in protein-protein interaction. At first glance, it seems difficult to discriminate between aggregation and conformational change because the putative conformational change typically occurs upon aggregation as a result of lowering detergent concentration (for further discussion of the influence of detergents on excitonic interactions in LHCII, see Garab, Chap. 16). However, Ruban and coworkers (Ilioaia et al. 2008, 2011) circumvented this problem by embedding LHCII trimers in a gel, thereby preventing aggregation, and subsequently lowering detergent concentration. Despite the fact that aggregation was not allowed to take place, LHCII was quenched, the characteristic N twisting occurred, a change in conformation of one of the luteins was observed, and an increased carotenoid-chlorophyll interaction appeared to be present (see also below) (Ilioaia et al. 2011). The latter findings argue against quenching by a CT state due to interacting Chls on different trimers. Nevertheless, it is still possible that a conformational change within a trimer might cause changes in Chl-Chl interactions and lead to a red-shifted CT state (Kruger et al. 2011; Wientjes et al. 2012). Very recently, Wahadoszamen et al. (2012) performed fluorescence Stark measurements on trimeric and oligomeric LHCII, and also observed the Chl-Chl CT band and deduced the presence of yet another one, which was attributed to an intra-monomeric carotenoid Chl pair. These measurements are not conclusive regarding the role of the different CT states in quenching.

If a change in Chl-Car interaction is responsible for the quenching, how does this occur? One possibility might be that EET takes place from a Chl *a* Q_y state to the lutein S_1 state that is very short lived (several picoseconds), leading to quenching of Chl fluorescence. Such an effect would require lowering of the energy of the S_1 state during qE as compared to the unquenched case, which might happen via a conformational change. Quantum-chemical calculations indicate that EET might indeed be possible from Chl 612 to Lut1 (Duffy et al. 2013), but on the other hand it was found that the S_1 state is rather insensitive to structural deformation (Macernis et al. 2012), whereas a lowering of the energy level seems to be needed.

Another possible qE mechanism is based on the occurrence of strong excitonic interaction between one or more of the Chl *a* Q_y states and the S_1 state of Lut1. It was pointed out by van Amerongen and van Grondelle (2001) that there already is excitonic interaction between these states in trimeric LHCII (see below), which leads to some delocalization of the excitation over both states and, therefore, to some shortening of the Chl excited-state lifetime. Such a shortening was indeed reported by

Naqvi et al. (1999). It was proposed by van Amerongen and van Grondelle (2001) that an increase in delocalization, due to, for instance, a change in interaction between Chl and Car (e.g., in case of a conformational change), might lead to a shortening of the excited-state lifetime and thus induce NPQ.

The presence of (excitonic) interactions between Chls *a* and carotenoids in trimeric LHCII has been demonstrated, both implicitly and explicitly, by many spectroscopic methods, such as (sub)picosecond EET from Car to Chl (Peterman et al. 1997; Gradinaru et al. 2000; Croce et al. 2001), triplet-minus-singlet spectroscopy (Van der Vos et al. 1991; Peterman et al. 1995; Naqvi et al. 1997; Barzda et al. 1998; Lampoura et al. 2002), femtosecond experiments, in which Chl was excited and an instantaneous carotenoid bleach was observed (Gradinaru et al. 2003), and steady-state spectroscopic measurements on LHCII and minor antenna complexes and mutants thereof (Mozzo et al. 2008). All of these latter studies show that substantial interactions exist, and imply that an increase of these interactions can influence the Chl excited-state lifetime, while not, however, proving that strong Chl-Car interactions are responsible for qE. More recently, a beautiful series of experiments was performed by Walla and coworkers (Bode et al. 2009; Liao et al. 2010a, b), comparing the fluorescence of photosynthetic systems upon alternating one- and two-photon femtosecond excitation pulses that selectively excite Chls and Cars, respectively. The latter authors demonstrated a direct correlation between the electronic Car S_1 – Chl Q_y interaction and quenching in both isolated LHCII and intact leaves. The same authors later performed a systematic study with isolated (aggregated) LHCII of varying pigment composition and demonstrated correlations among the following four parameters: (1) The rate of EET from Car to Chl, (2) rate of EET from Chl to Car, (3) exciton coupling, and (4) the amount of quenching, exactly as predicted from assuming that exciton coupling between Chl and Car is responsible for excited-state quenching.

Since it was also found that aggregated LHCII lacking Chl A2 (Chl 612) was still quenched, it is not simply such that coupling between A2 and Lut1 is fully responsible for quenching. The same method of comparing the fluorescence of photosynthetic systems upon alternating one- and two-photon femtosecond excitation pulses was used on LHCII quenched in gels upon lowering the detergent concentration (Ilioaia et al. 2011), and indeed showed an increase in Car-Chl interaction. Furthermore, the characteristic N twisting was observed in the latter study together with a change in lutein conformation. These results are consistent with the conclusion of Ruban et al. (2007) that Lut1 of LHCII is the quencher of excitations/fluorescence during qE as well as, possibly, Lut1 in the minor antenna complexes, since both pigments show similar Chl-Car coupling (Mozzo et al. 2008). In addition, it was demonstrated that the mechanism of quenching is not EET from a Chl Q_y state to the Lut1 S_1 state, but is instead a result of excitonic coupling between these states.

III Conclusions

Based on the results and arguments presented above, we arrive at the following model. At the initial stage of NPQ, the qE stage, the proton gradient across the thylakoid membrane, or more precisely the low pH in the lumen, leads to protonation of the PsbS protein, which then causes detachment of part of the antenna from the PS II core. The detached antenna becomes quenched due to a conformational change of LHCII, and possibly also of some minor antenna complexes, which leads to strong excitonic interaction between the S_1 state of Lut1 and one or more Chl *a* Q_y states, thereby shortening the Chl excited-state lifetime. The quenching effect may be increased by additional aggregation of antenna complexes caused by Z originating from de-epoxidation of V, as induced by the same proton gradient, and by quenching via a Chl-Chl charge-transfer state.

The conclusions presented in the present chapter further illustrate that lutein is a quencher in NPQ. More information about the role of lutein in NPQ can be found in Chap. 12 by Esteban and Garcia-Plazaola that focuses on the lutein epoxide-lutein cycle. However, as is also discussed in Chaps. 2, 3, 7, and 24, there is certainly also an important role for zeaxanthin.

Acknowledgments

I would like to thank Dr. Roberta Croce for helpful discussions and for providing the figures. I am also obliged to Drs. B. Demmig-Adams, G. Garab, A.R. Holzwarth, and T. Polivka for helpful comments and suggestions. I would like to acknowledge support from the research programme of BioSolar Cells, cofinanced by the Dutch Ministry of Economic Affairs.

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Chapter 16

Structural Changes and Non-Photochemical Quenching of Chlorophyll *a* Fluorescence in Oxygenic Photosynthetic Organisms

GyőzőGarab*
Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

Summary

This chapter focuses on the nature, physical mechanism and physiological significance of structural changes at different levels of structural complexity of the photosynthetic apparatus of oxygenic photosynthetic organisms – with special emphasis on non-photochemical quenching (NPQ) of the singlet-excited state of chlorophyll *a*. The dual role of the antenna system, light harvesting under low light and thermal dissipation under high light, requires substantial structural flexibility. Indeed, reversible structural changes induced by excess-light excitation have been observed in different organisms via several techniques, including

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*Author for Correspondence, e-mail: garab.gyozo@brc.mta.hu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_16, © Springer Science+Business Media Dordrecht 2014

electron microscopy, light scattering measurements, circular dichroism spectroscopy, and small-angle neutron scattering. These investigations have revealed reorganizations at the level of (i) the ultrastructure of thylakoid membranes, affecting repeat distances, (ii) macro-organization of light-harvesting antenna complexes within the membrane, perturbing the ordered arrays of the complexes, as well as (iii) isolated light-harvesting antenna systems. In some cases, correlations between the observed reorganizations and NPQ have been well established. In many cases, however, the relationship between changes in the macro-organization of the light-harvesting antenna or thylakoid membranes and NPQ remains to be explored. Nevertheless, the currently available data strongly suggest that, while an overall reorganization is a necessary condition for adjusting the functional activities to different growth light intensities and for photoprotection, in particular, reorganization per se is not sufficient for NPQ (that also requires the presence of effector molecules, such as, e.g., PsbS or zeaxanthin). This chapter also deals with the effect of dissipation of excess excitation energy on the photosynthetic apparatus: light-induced reversible structural changes in different antenna systems in vivo and in vitro, with rates linearly proportional to the intensity of excess light (that is not used for photosynthesis). These structural changes have been proposed to be driven by what is termed a thermo-optic mechanism: elementary structural changes elicited by ultrafast local heat transients due to the dissipation of photon energy, a photophysical feedback mechanism that appears to modulate NPQ and regulate enzymatic functions in light-harvesting antenna complexes.

I Introduction

The photophysical processes in photosynthesis (absorption of light, funneling of excitation energy into photochemical reaction centers, and dissipation of excess excitation energy in the pigment system) are largely determined by the architecture of the photosynthetic apparatus (for basic introduction and reviews, see, e.g., Sybesma 1989; Cramer and Knaff 1990; Van Amerongen et al. 2000; Renger and Renger 2008; Blankenship 2014, Ostroumov et al., Chap. 4). The highly organized thylakoid membranes and the molecular organization of the pigment-protein complexes assure efficient utilization of absorbed light energy in photosynthesis. At the same time, dynamic features at different levels of structural complexity allow functional plasticity of photosynthesis under different environmental conditions. In this chapter, an overview of the main static and dynamic features of the (macro-) organization of thylakoid membranes that potentially play roles in non-photochemical quenching (NPQ) of singlet-excited chlorophyll (Chl) *a* is provided. We focus here on different elements of the structural flexibility of the photosynthetic machinery, including changes in the multilamellar membrane systems, reorganization of protein arrays in the membrane, as well as on the ability of some antenna complexes to undergo light-induced reversible conformational changes. Furthermore, we discuss the underlying physical mechanisms of structural alterations, including the mechanism associated

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Abbreviations: $\Delta\mu_{H^+}$ – Electrochemical potential gradient of protons across the thylakoid membrane; CD – Circular dichroism; Chl – Chlorophyll; EPR – Electron paramagnetic resonance; FCP – Fucoxanthin–chlorophyll *a*/*c* protein; LHCII – Light-harvesting complex II; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OCP – Orange carotenoid protein; PG – Phosphatidylglycerol; PMS – Phenazine methosulphate; PS I – Photosystem I; PS II – Photosystem II; psi – Polymer or salt-induced; Q – Scattering vector; qE – Energy-dependent quenching: qI – Largely irreversible quenching originally associated with photoinhibition of PS II; qT – Quenching arising from state transitions; RD – Repeat distance; SANS – Small-angle neutron scattering with the heat-dissipation of excess excitation energy in light-harvesting antenna complexes, which represents a photophysical feedback mechanism.

II The Macro-Organization of Thylakoid Membranes

A Multilamellar Membrane Systems

In oxygenic organisms virtually all light reactions take place in the thylakoid membranes. These membranes contain the two photochemical systems, with their associated membrane-intrinsic or membrane-bound light-harvesting protein complexes, as well as the cytochrome b6f complex and the ATP synthase (see Fig. 16.1 in Papageorgiou and Govindjee, Chap. 1). The thylakoid membranes separate the inner aqueous phase, the lumen, from the outer aqueous phase, called the stroma (or cytoplasmic side, in cyanobacteria). This separation of the two aqueous phases and the impermeability of membranes to protons and ions are of vital importance for membrane energization, i.e., for maintaining the electrochemical potential gradient for protons across the thylakoid membrane, $\Delta\mu_H^+$, which is generated upon primary charge separation and consecutive redox reactions of the photosynthetic electron transport system. This gradient includes a transmembrane electrical field as well as a pH gradient; the two together provide energy for ATP synthesis (see a detailed summary of various aspects of photosynthesis in Eaton-Rye et al. 2012).

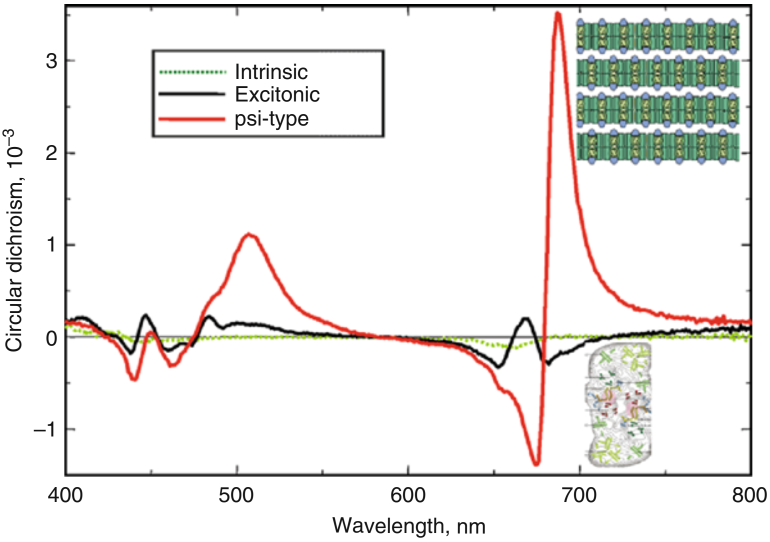

Fig. 16.1. Circular dichroism spectra exhibited by thylakoid pigments at identical pigment concentrations (20 μg Chl [a + b] ml^{-1}) in samples at different levels of structural complexity: *intrinsic CD* due to (weak) molecular asymmetries of the isolated pigments (here measured in 80 % acetone; for easier comparison, the spectrum is multiplied by a factor of 5); short-range *excitonic* interactions between pigment molecules within pigment-protein complexes and possibly on adjacent complexes in supercomplexes (in the figure represented by the PS II core (Dekker and Boekema 2005), with the spectrum dominated by the CD bands of LHCII and measured on isolated pea thylakoid membranes suspended in low-salt, hypotonic medium); the same membranes suspended in isotonic medium in the presence of Mg^{2+}-ions give rise to intense *psi-type CD* bands, indicating that the pigment-protein complexes are organized in large domains with long-range chiral order of their chromophores (and protein complexes), as schematically illustrated. Note that spectra from higher-organization-level structures contain the spectra from lower-organization-level structures: for instance, the spectrum of the intact thylakoid membranes is the superposition of intrinsic, excitonic and psi-type CD bands. Spectra are reproduced from Garab and van Amerongen (2009).

The thylakoid membranes in most organisms are multilamellar, a feature that has evidently evolved to increase the efficiency of light capture. The thylakoid-membrane system is formed from a continuous membrane, enclosing a single, contiguous inner aqueous phase with interconnected lumenal spaces (for a discussion of structure and function of plastids, see various chapters in Wise and Hoober 2006). In higher-plant chloroplasts, this was established mainly by "conventional" electron microscopy and by electron tomography (reviewed by Mustárdy and Garab 2003; see also Shimoni et al. 2005; Mustárdy et al. 2008; Daum et al. 2010; Austin and Staehelin 2011). At the other, lower end of the phylogenetic tree of oxygenic photosynthesis are cyanobacteria, in which evidence for the formation of a continuous, highly connected network has been found by Nevo et al. (2007), using electron tomography. In terms of evolution, network formation of the thylakoid membranes can probably be explained by the advantage it provides, since a continuous membrane more readily allows for additional growth of the entire thylakoid membrane system without independent centers of synthesis for each thylakoid. The continuity of the membrane is also important during lateral trafficking of some complexes between the stacked and unstacked thylakoid membrane regions.

Multilamellar membrane networks have additional advantages for energy conversion. Rapid equilibration prevents occurrence of large differences in $\Delta\mu_H^+$ between different layers of thylakoid membranes. Fast equilibration currents have been observed experimentally (Fowler and Kok 1974) in both lateral and transmembrane directions (Osváth et al. 1994). In this context, an interesting unsolved problem is the optical behavior of multilamellar systems, given that their sizes are commensurate with the wavelength of the visible light, which can thus lead to internal interference patterns (Paillotin et al. 1993) and inhomogeneities in $\Delta\mu_H^+$. Hence, *both the optical properties and membrane energization appear to be features with strong characteristics arising from the entire multilamellar assembly rather than from individual membrane layers*.

B Ordered Arrays of Protein Complexes

The multilamellar organization of thylakoid membranes is closely correlated with their internal organization. This is clearly illustrated in granal chloroplasts, where the two photosystems are laterally separated. Photosystem II (PS II) core complexes, along with their associated light-harvesting complexes (LHCII), reside preferentially in the stacked (grana) region. In contrast, the unstacked (stroma) lamellae, which interconnect neighboring grana as well as different layers of the granum, are enriched in photosystem I (PS I), its light-harvesting complex (LHCI), and the ATP synthase (Andersson and Anderson 1980). This organization assures the highest overall packing density of the membranes because LHCII and the PS II supercomplex display very low levels of protrusion into the stroma, while PS I and the ATP synthase have large, stroma-exposed structures (see Dekker and Boekema 2005 and references therein).

The lateral segregation (*sorting*) of the two photosystems evidently requires molecular recognition and a lateral driving force. These two features result from the high aggregation propensity of LHCII and LHCII-PS II super-complexes, which form densely packed large domains (*packing*) with flat surfaces on the stromal side (Mustárdy and Garab 2003; Mustárdy et al. 2008). After (and only after) sorting and packing, these membrane regions become capable of tight *stacking*, which stabilizes this molecular macroassembly and the membrane system. These processes depend mainly on the membranes' LHCII content and are largely governed by electrostatic forces (Arntzen 1978; Barber 1982; Garab 1996; Chow et al. 2005; Garab 2014). The LHCII-enriched macrodomains possess a strong spectroscopic signature: due to the long-range chiral order of the complexes (and thus of their chromophores), they exhibit a giant circular dichroism (CD) signal, characterized by psi-type CD bands (Garab et al. 1991; Barzda et al. 1994; Garab and van Amerongen 2009). These bands are present in intact thylakoid membranes, but absent in disorganized, laterally homogenous thylakoids, suspended in hypotonic low salt medium (Fig. 16.1).

We note that psi-type aggregates are three-dimensional macroaggregates, which contain a high density of interacting chromophores, have the appropriate size commensurate with the wavelength of the measuring light, and have a long-range chiral order of their chromophores (Keller and Bustamante 1986; Garab 1996; Tinoco et al. 1987; Garab and van Amerongen 2009). Concerning terminology, the term psi-type, polymer or salt-induced, had been originally introduced for condensed DNA aggregates, such as supercoiled isolated DNA, which had been shown to exhibit giant CD, and which could not be put together from the CD of their constituents; the term chiral is simply a chemical term, which means that a molecule is not superimposable on its mirror image. Nuclei, chromosomes viruses and some protein aggregates belong to the category of hierarchically organized structures of high level structural complexity which exhibit psi-type features (Tinoco et al. 1987).

In the highly organized lamellar aggregates of LHCII and granal thylakoid membranes, intense psi-type CD signals with non-conservative and anomalously shaped bands and long differential scattering tails outside the absorbance bands are superimposed onto the excitonic and intrinsic CD signals originating from short-range pigment-pigment interactions and molecular asymmetries inside the individual (super-) complexes (Garab 1996; Garab and van Amerongen 2009).

The existence of extended, ordered arrays in the grana is also clearly indicated by the presence of LHCII- and PS II-enriched semi-crystalline arrays (Miller and Staehelin 1976; Dekker and Boekema 2005; Kouril et al. 2012). The frequency of these ordered arrays depends on the organization of PS II super-complexes, as indicated by the effects of the presence or the absence of minor PS II antenna complexes and the PsbS protein (Kovács et al. 2006; Kiss et al. 2008). The relative abundance of such ordered arrays is, furthermore, greater in low-light grown leaves compared to leaves grown in higher light, suggesting a functional significance in efficient light-energy utilization (Kirchhoff et al. 2007; Kouril et al. 2013). However, the available data do not allow a firm conclusion about the role of these semi-crystalline domains, mainly because the frequency of their occurrence is rather low (usually lower than 10 %). It is also unclear if detergent treatment, applied at low concentrations during the preparation of membranes for electron microscopy, enhances or diminishes the semi-crystalline order. CD experiments, showing that the psi-type bands are dramatically decreased by very low concentration of detergents (O. Zsiros, L. Kovács and G. Garab, unpublished), suggest that detergents diminish rather than enhance semi-crystalline order. On the other hand, it is known that the magnitude of psi-type CD in granal thylakoids is considerably weaker than in microcrystalline LHCII (Fig. 16.2; Simidjiev et al. 1997; Miloslavina et al. 2012). Taken together, the available electron microcopy and CD data strongly suggest that *the stacked regions contain large ordered arrays of (super-)complexes*, *but the overall long-range order is probably weaker than in the semi-crystalline domains*. Nevertheless, the highly organized macrodomains evidently provide a structural basis for excitation-energy migration over long distances and thus provide conditions for efficient light-energy utilization.

C Functional Domain Sizes and the Effective Radii of Quenchers

With regard to energy migration and NPQ within grana, or in the multilamellar system of thylakoids in general, it is important to know the functional domain sizes in which excitation energy diffuses freely. This question is closely related to the “classical” problem of connectivity between PS II units and in general to the lake versus puddle model (Stirbet 2013; Blankenship 2014). Clearly, the efficiency of the antenna to supply excitation energy to the reaction centers and the

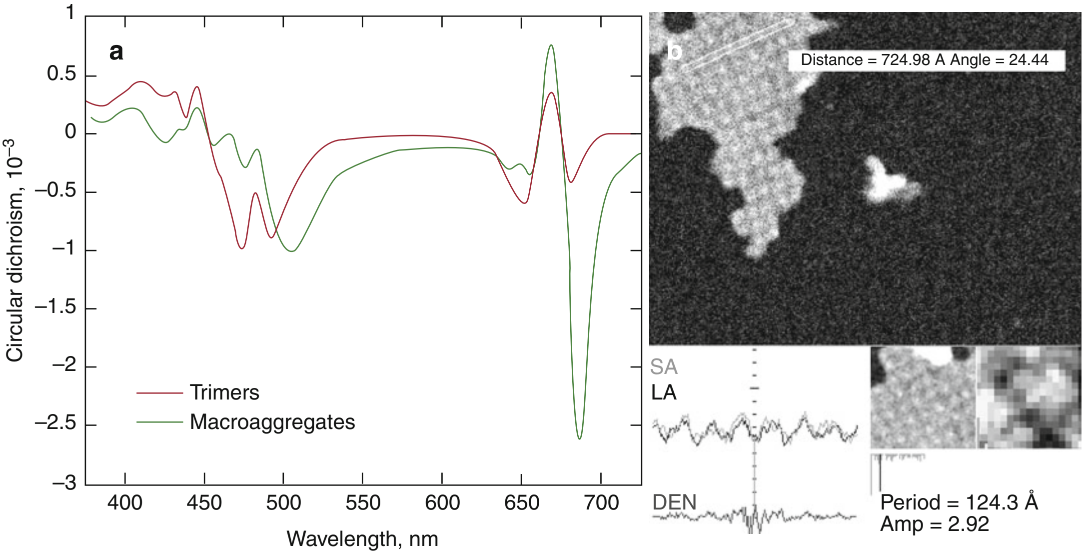

Fig. 16.2. Comparison of circular dichroism spectra of tightly stacked microcrystalline lamellar aggregates of LHCII and loosely stacked lipid:LHCII lamellae (**a**) as well as a scanning-transmission-electron microscopy (*STEM*) image of unstained microcrystalls also showing the periodicity of 128 ± 3 Å of the microcrystals containing particles with a mass estimated at 118 ± 7 kDa corresponding to LHCII trimers (**b**). Modified after Miloslavina et al. (2012); analysis (courtesy of Dr. Geoffrey Hind). Notice the giant size of the psi-type band at (−)680 nm in the microcrystalline aggregate, which is much more intense than the corresponding band in the loosely stacked lamellar aggregates of LHCII or the psi-type bands in isolated thylakoid membranes (Fig. 16.1), indicating a significantly stronger long-range order of trimers in the microcrystals than in the lipid matrix in vitro or in vivo – the excitonic bands, e.g., the (-)650 nm band, can be used as a basis of comparison.

connectivity of photosynthetic units depend on excitation diffusion length. At the same time, the latter also determines the effective radius of a quencher.

For LHCII aggregates, van Oort et al. (2007) have shown that *quenchers induced in trimers can also trap excitations arising in complexes that do not contain quenchers themselves*. Stern-Volmer plots of fluorescence quenching (determined from fluorescence lifetimes) induced by exogenous compounds have provided data on the functional domain size of LHCII ensembles in vitro and in vivo (Lambrev et al. 2011). Both in LHCII aggregates and in plant thylakoid membranes, conservative estimates for the functional domain sizes gave values of or corresponding to 15–30 LHCII trimers. These data thus show that, because of excitation-energy diffusion in a large domain, *a single quencher molecule can be efficient for several photosynthetic units*. This suggests that a minor compound or complex, e.g., the PsbS protein (Niyogi et al. 2005), even if present in sub-stoichiometric amounts, might efficiently act as a quencher. Assuming that excitation-energy transfer within a complex is much faster than the intercomplex energy transfer and that quenching ability is due to a specific pigment-protein complex, Valkunas et al. (2011) used a model, termed the coarse-grained model, and concluded that one NPQ trap is capable of quenching up to 100 monomeric complexes in LHCII aggregates. Further, Caffarri et al. (2011) showed that quenchers at low concentration are more efficient in the PS II supercomplex than in external LHCII trimers.

Lambrev et al. (2011) showed that unstacking does not significantly alter the domain size, which is in good agreement with the data of Kirchhoff et al. (2004). On the other hand, variations in the macro-organization of complexes, which affects the frequency of semi-crystalline arrays (Boekema et al. 2000; Kouril et al. 2012, 2013) and/or the magnitude of the psi-type CD, might lead to changes in functional domain size. However, this will only affect the NPQ capability of membranes if the domain size limits NPQ.

In most models, it is implicitly assumed that the domain size does not change upon transition from unquenched (low-light) state to quenched (high-light) state. In general, however, this assumption does not seem to be valid. As proposed by Holzwarth et al. (2009), *the PsbS-dependent quenching in leaves originates from detached LHCII* (see Holzwarth and Jahns, Chap. 5; for the role of PsbS, see Brooks et al., Chap. 13). This means that it is not antenna quenching but rather detachment of part of the antenna complexes that reduces the excitation-energy pressure on the reaction center. Under these conditions, quenching provides protection against photooxidation in the detached complexes (without a reaction center as the sink for excitation energy) via lowering the lifetime of fluorescence, and Chl *a* triplet formation. Photoprotection of the reaction center presumably depends more directly on another quenching mechanism – regulated at the zeaxanthin-dependent quenching site in the minor antenna complexes (Jahns and Holzwarth 2012). In line with these different functions of the two proposed quenching sites, a detailed analysis (Lambrev et al. 2012) has revealed that *NPQ and actual photoprotection are not necessarily linearly related*. Further, by using artificial quenchers of Chl *a* singlet excited state, Santabarbara et al. (2001) concluded that quenching of PS II fluorescence does not protect the D1 protein against light-induced degradation in thylakoids.

A large fraction of LHCII trimers are only loosely associated with the PS II supercomplex or are found in LHCII-only domains (Boekema et al. 2000), and these readily detach from PS II as suggested by Holm et al. (2005). Light-induced detachment of the phycobilisome antenna has also been observed in cyanobacteria under conditions that trigger NPQ (Stoitchkova et al. 2007). In agreement with the mechanism proposed by Stoitchkova et al. (2007), i.e., with the thermo-optic mechanism (see below), Tamary et al. (2012) have suggested that the heat generated by non-radiative energy dissipation in the phycobilisome induces alterations in thermo-labile elements, most likely in certain rod and core linker polypeptides, which then leads to disruption of excitation-energy migration and promotes dissociation of the phycobilisome from the thylakoid membranes (for further details, see section "Light-Induced Reversible Reorganization in Isolated LHCII").

III Structural Flexibility of Thylakoid Membranes

The fact that sun and shade plants differ in the number of grana layers (Anderson et al. 2012) shows that the overall membrane organization of chloroplasts is adjusted to the available energy supply. Membrane reorganization is achieved via changes in the composition of the photosynthetic machinery, which, in turn, brings about changes in ultrastructure. As pointed out by Anderson et al. (2012), a similar remodeling of membrane ultrastructure as seen during long-term acclimation of plants can also be induced by rapid transitions from one irradiance to another.

A Involvement of Structural Changes in Light-Adaptation Mechanisms

The kinase- and phosphatase-dependent state transitions are essentially based on LHCII mobility between the two photosystems and the stacked and unstacked regions of the membranes. Structural reorganization is thus proposed to play a key role in this regulatory mechanism (Allen and Forsberg 2001; Chuartzman et al. 2008; Minagawa 2011; Tikkanen and Aro 2012; Wientjes et al. 2013), which manifests itself as the qT component of NPQ, i.e., as state-transition quenching (Horton et al. 1996; see also Finazzi and Minagawa, Chap. 21). Photoinhibition, the continuously maintained qI component of NPQ (see also

Adams et al. 2013, Adams et al., Chap. 23; Demmig-Adams et al., Chap. 24) also involves substantial structural changes. This is most evident during the processes related to the repair mechanism of damaged PS II reaction-center complexes, which move from the stacked to the unstacked region (Yamamoto 2001; Vass 2012 and references therein). Photoinhibitory treatment of thylakoids has been shown to eliminate long-range order of chromophores (Gussakovsky et al. 1997), which strongly suggests that *the dissipative regime is associated with a certain degree of disorder in the array of complexes*, *rather than with the long-range order* (*and semi-crystalline arrays*) *of the complexes*. Experiments by Janik et al. (2013) using x-ray diffraction, infrared imaging microscopy, confocal laser scanning microscopy, and transmission electron microscopy on lipid-protein membranes composed of LHCII isolated from spinach and thylakoid lipids, monogalcatosyldiacylglycerol and digalactosyldiacylglycerol, revealed substantial differences between LHCIIs isolated from dark-adapted and high-light (HL) treated plants. Lipid-LHCII membranes, when isolated from dark-adapted leaves assemble into planar multibilayers, in contrast with the lipid-LHCII-HL membranes, which form less ordered structures. These data are in broad agreement with the above conclusion. For the role of protein disorder in NPQ at the level of individual complexes, see Krüger et al., Chap. 6.

With regard to the energy-dependent NPQ component, qE, the role of structural changes is far less evident; the question of whether or not there are reorganizations at different levels of structural complexity is not yet settled. An involvement of major structural changes is indicated, e.g., by the slow kinetics of qE relative to the build-up and decay of the transmembrane ΔpH (Horton et al. 1994), and a similar lag phase observed in ΔCD compared to ΔpH (Barzda et al. 1996). Experiments showing that the quenched state, after its formation, can be trapped by rapidly lowering the temperature is consistent with the involvement of a major reorganization associated with NPQ (Lambrev et al. 2007a). A major lateral reorganization of thylakoid membrane complexes, associated with ΔpH and dependent on PsbS, has also been observed by electron microscopy (Betterle et al. 2009; Johnson et al. 2011, also see Ruban and Mullineaux, Chap. 17).

PsbS, a key player in energy-dependent NPQ, may modulate the proportion of supercomplexes organized into semi-crystalline arrays; PsbS also determines the shape of the psi-type CD signal (Kiss et al. 2008). Decreased formation of ordered semi-crystalline arrays in the presence of PsbS was accompanied, and perhaps caused, by increased mobility of Chl proteins within the thylakoid membrane (Damkjaer et al. 2009). An increased flexibility at the macro-organization level in the presence of PsbS is correlated with the magnitude of NPQ, in perfect agreement with the idea that NPQ involves extensive reorganization in the membrane (Horton et al. 1996; Istokovics et al. 1997; Betterle et al. 2009; Johnson et al. 2011; Goral et al. 2012) and with the hypothesis of allosteric regulation of qE (Horton et al. 2005). It is interesting to note that phosphorylation of LHCII also leads to a substantial increase in the flexibility of thylakoid membranes, as reflected by enhancement of heat- or light-induced reorganizations in the membranes (Várkonyi et al. 2009). This might be important for the qT component of NPQ.

B NPQ Is Associated with Reversible Dismantling of Ordered Arrays of Protein Complexes

Kouril et al. (2013) found that the ability of PS II to form semi-crystalline arrays in the grana is higher in low-light grown than in high-light grown *Arabidopsis*, suggesting that the dense packing and *long-range order of complexes in the semi-crystalline arrays is important for efficient energy transfer between PS II rather than for NPQ*. This notion is supported by the following experimental data. In the case of the diatom

Table 16.1 Relative amplitude (ΔCD/CD) and initial rate (ΔCD/Δt) of the light-induced reversible CD changes and the magnitude of non-photochemical quenching (NPQ) in high-light grown (HL, 180–200 μmol photons m^{-2} s^{-1}) and low-light grown (LL, 15–20 μmol photons m^{-2} s^{-1}) *P. tricornutum* cells

	HL	LL
ΔCD/CD	0.33	0.05
Rate (ΔCD/Δt, s^{-1})	3.9×10^{-6}	1.3×10^{-6}
NPQ	3.38	1.17

The CD changes were induced by blue light of a photon flux density of 800 μmol photons m^{-2} s^{-1}; relative amplitudes and initial rates were determined at 698 nm (cf. Szabó et al. 2008). NPQ was calculated after 10 min of actinic-light illumination (white light, 828 μmol photons m^{-2} s^{-1}) using the Stern-Volmer approach according to Bilger and Björkman (1990) and Demmig-Adams (1990). Experimental data (n = 3, SD ≤20 %) of M. Szabó and G. Garab, unpublished

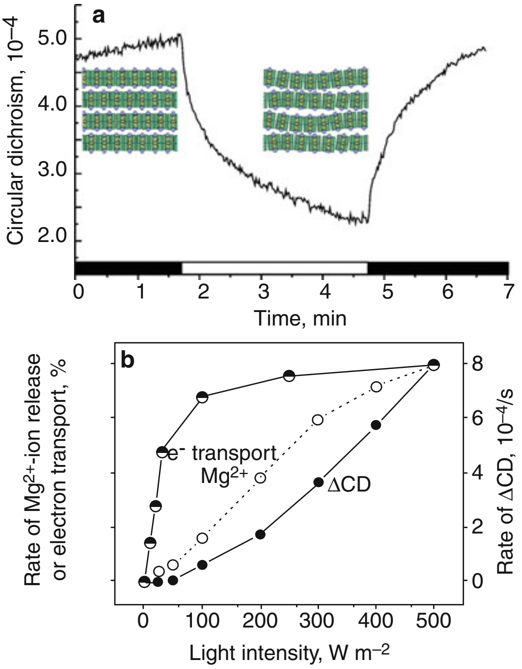

Fig. 16.3. Light-induced (red light, ~1,000 μmol photons m^{-2} s^{-1}, i.e., about 200 W m^{-2}) dark-reversible structural changes in the macro-organization of isolated pea thylakoid membranes monitored by amplitude variations of the psi-type circular dichroism band at 510 nm (**a**) as well as the dependence on exciting light intensity of the electron transport rate, the initial rate of CD changes, and the light-induced reversible release of Mg^{2+}-ions (**b**). *Black* and *white horizontal bars* indicate dark and light periods, respectively; the schematic illustration shows that upon illumination of the sample with excess light, the long-range order of the complexes is perturbed (panel **a**). Very similar kinetics are displayed by all main psi-type CD bands, indicating an overall reorganization of the chiral macrodomains in the grana (Garab et al. 1988; Holm et al. 2005). Panel **b** is reproduced from Garab (2014).

Phaeodactilum tricornutum, low light-grown cells exhibited considerably stronger psi-type CD than high light-grown cells, indicating that more complexes are assembled in ordered arrays in low light-grown than in high-light grown cells (Szabó et al. 2008, 2010). Moreover, high-light grown cells exhibited a considerably higher structural flexibility and larger NPQ as reflected in light-induced reversible changes in the psi-type CD (Table 16.1). Although NPQ in diatoms depends mainly on the amount of deepoxidized xanthophylls (Goss and Jakob 2010), the correlation between the level of structural flexibility and NPQ is remarkable. Furthermore, granal (plant) chloroplasts in the dark exhibit intense psi-type CD bands that are found to reversibly diminish in excess light (Fig. 16.3a), and, remarkably, the rate of decrease is proportional to the intensity of excess light (Fig. 16.3b; Garab et al. 1988; Barzda et al. 1996). It thus seems that *NPQ in plants is associated with reversible dismantling of long-range order arrays of the (super-)complexes*. It is also important to realize that structural changes are confined to the psi-type CD band, without noticeably affecting the excitonic bands, i.e., *without affecting the molecular architecture of bulk pigment-protein complexes*. This also holds true for irreversible, photoinhibitory treatments (Gussakovsky et al. 1997; Cseh et al. 2005). We note, however, that the sensitivity of excitonic CD, especially if the changes affect only a small fraction of the complexes, might not be sufficient to detect minor alterations. More sensitive techniques, e.g., single molecule spectroscopy, have revealed dynamic behavior of solubilized LHCII trimers (Krüger et al., Chap. 6). In addition, resonance Raman spectroscopy is a highly sensitive technique to detect subtle conformational changes in LHCII, even in whole leaves (Pascal et al., Chap. 10). We also note that alterations in the structure that affect the fluorescence yield do not necessarily involve changes in the excitonic CD; instead, conformational changes in the outer loop segments of LHCII, e.g., near the phosphorylation site can also result in alterations in the fluorescence yield (Zer et al. 1999).

At first sight, disassembly of ordered arrays of complexes under NPQ conditions seems to be in conflict with the role of LHCII aggregation in NPQ (Horton et al. 1991), which had been supported by numerous data in the literature (cf. Horton et al. 2005 and references therein; see also Horton, Chap. 3). In order to resolve the apparent contradiction, we *propose that* (*i*) *extended ordered arrays of LHCII cannot be responsible for quenching and*, *in contrast*, (*ii*) *small*, *irregular aggregates of the complexes*, *formed during disassembly of arrays with long-range order* (i.e., *during the disassembly of ordered macro-aggregates*), *possibly containing* "*irregular*" *contacts between adjacent LHCII trimers*, *are likely to be involved in qE and qI*. This kind of irregular contact can lead to the formation of charge-transfer complexes that can act as quenchers in detached LHCIIs (Miloslavina et al. 2008).

Data obtained at low pH are consistent with the above proposal. As revealed by CD spectroscopy, the magnitude of psi-type CD decreases progressively and reversibly upon gradual lowering of the medium's pH from 7.5 to 4.5, while the same treatment induces no significant changes in excitonic CD, showing that low pH leads to disassembly of chiral macrodomains but apparently does not significantly affect the molecular architecture of the constituent complexes (Jajoo et al. 2012). This finding is in good agreement with the results obtained with reconstituted mutant LHCIIs: the lumenal loop segment between helix B and C has proven to be important in maintaining the structural stability of complexes under acidic conditions. Mutagenesis experiments have demonstrated that negatively charged residues in the latter loop regulate pigment conformation, energy transfer efficiency, and structural stability under different pH environments (Yang et al. 2008). Exchanging glutamic acid at position 94 with glycine (E94G) destabilized the 3_{10} helix in the lumenal loop, resulting in an acquired pH sensitivity of the complex, and affected the excitonic bands assigned to the neoxanthin-binding domain. While Chl fluorescence yield of reconstituted solubilized wild-type trimeric complexes exhibited virtually no sensitivity to pH variation (under conditions preventing aggregation at low pH), the serine-to-glycine S123G mutant exhibited a low-pH induced reversible quenching (Liu et al. 2008) – showing that in wild type the lumenal loop of LHCII is largely insensitive to variations in pH. Belgio et al. (2013) have also shown that, in the absence of aggregation, quenching in LHCII at low pH is affected by single-amino-acid mutagenesis in the lumenal loop. Also, Petrou et al. (2014) have shown that isolated wild type LHCII trimers, solubilized in 200 μM n-dodecyl β-d-maltoside preventing low-pH-induced aggregation, display virtually no sensitivity to pH changes – in contrast to samples at lower detergent concentrations, at 6 and 20 μM (i.e., below the critical micelle concentrations of 120 μM). These data strongly suggest that *low lumenal pH affects the macro-organization of the complexes rather than the molecular organization of LHCII trimers*.

C Light-Induced Reversible Changes in Membrane Repeat Distances

Small-angle neutron scattering (SANS) method has been applied to obtain information on structure and structural plasticity of thylakoid membranes in different organisms (Nagy et al. 2011, 2013). SANS provides structural information, averaged over the entire sample volume in the neutron beam, under physiologically relevant conditions. Other than the use of D_2O for contrast variations, the sample does not require any treatment, such as fixation or staining, and even whole organisms, algal cells and leaves can be used. The signal-to-noise ratio for single runs allows time resolution from 1–2 min down to about 5–10 s, depending on the sample and the intensity of the neutron beam (Nagy et al. 2013; Ünnep et al. 2014). In a series of SANS experiments on different organisms, Nagy et al. (2011, 2012, 2013) determined spatially averaged repeat distance (RD) values of thylakoid membranes in cyanobacterial and algal cells and isolated plant thylakoid membranes. In addition, these SANS experiments provided clear evidence for the occurrence of small, but easily discernible light-induced membrane reorganization, the magnitude and rate of which depended on the actinic light intensity (Nagy et al. 2011, 2012, 2013; for wild type and phycobilisome-mutant cyanobacterial cells, see also Liberton et al. 2013).

By using SANS on isolated plant thylakoid membranes, rapidly reversible reorganizations have been shown to occur under conditions of cyclic PS I electron transport in the presence of phenazine methosulphate (PMS). In reasonable agreement with early light-scattering kinetic experiments and electron microscopy data of Murakami and Packer (1970), light-induced shrinkage of the thylakoid RDs was observed that was fully reversible. This reduction in thylakoid RD displayed complex kinetics with time constants of several tens of seconds to minutes and was sensitive to removal of the pH gradient across the thylakoid membrane (Nagy et al. 2011). However, a direct correlation between changes in RD and qE could not be made, partly because PMS quenches Chl fluorescence (Wientjes and Croce 2012), and also because isolated thylakoids exhibit poor NPQ. Nevertheless, these investigations demonstrated that *thylakoid membranes inherently possess a very high structural flexibility*, which had been observed earlier under essentially the same conditions by using CD spectroscopy (Garab et al. 1988; Barzda et al. 1996; see also Garab 2014). In order to correlate NPQ and RD changes in higher plants, we suggest that these experiments be carried out on leaves, and in parallel with fluorescence measurements. This is feasible but technically less straightforward than with isolated thylakoids or algal cells. Experimental data suggest that the nature of changes might be different in intact leaves than in isolated thylakoid membranes and upon operation of the PS I cycle (Ünnep et al. 2014).

In the diatom *Phaeodactylum tricornutum*, in which membranes are organized into stacked groups of three (Pyszniak and Gibbs 1992), RD in the dark is about 170 Å (Fig. 16.4a), which evidently requires a tight packing of membranes, given the homogenous distribution of the two photosystems in the membrane and the relatively large protrusion of PS I (see above and Fig. 16.4b). In fact, topographically this can only be solved by placing the fucoxanthin-Chl protein complex (FCP) opposite to PS I, and the ATP synthase on the end membranes (Fig. 16.4b). Lepetit et al. (2012) have proposed that the inner membranes contain domains enriched in PS II as well as oligomerized peripheral FCP complexes, whereas the PS I complexes with their specific FCP antenna are found in the outer membranes. Thus, this partial segregation of PS II and PS I might offer a better explanation for the small RD values obtained in this diatom. Nevertheless, in order to utilize $\Delta\mu_H{}^+$ generated in the middle thylakoid membranes, its lumenal phase must be interconnected with the “upper” layer to form a network; elucidation of this question requires an electron tomography study.

Upon illumination, the RD of *P. tricornutum* is increased by about 10 Å (Fig. 16.4a) and the rate of this change is significantly accelerated at higher light intensities (Nagy et al. 2012). The same holds true for light-induced CD changes observed earlier (Szabó et al. 2008). Both *ΔSANS and ΔCD occur on the time scale of minutes with very similar kinetics to NPQ*. The changes in SANS and psi-type CD could be mimicked by osmotic treatments: upon adding high concentrations (100–600 mM) of sorbitol, the SANS peaks were shifted to higher Q values and the amplitude of the psi-type CD decreased, with these changes being the opposite to the changes induced by light. (Q is the scattering vector, given in Å^{-1} units, and for the first order Bragg peak, Q*, $\text{RD} = 2\pi/\text{Q}^*$.)

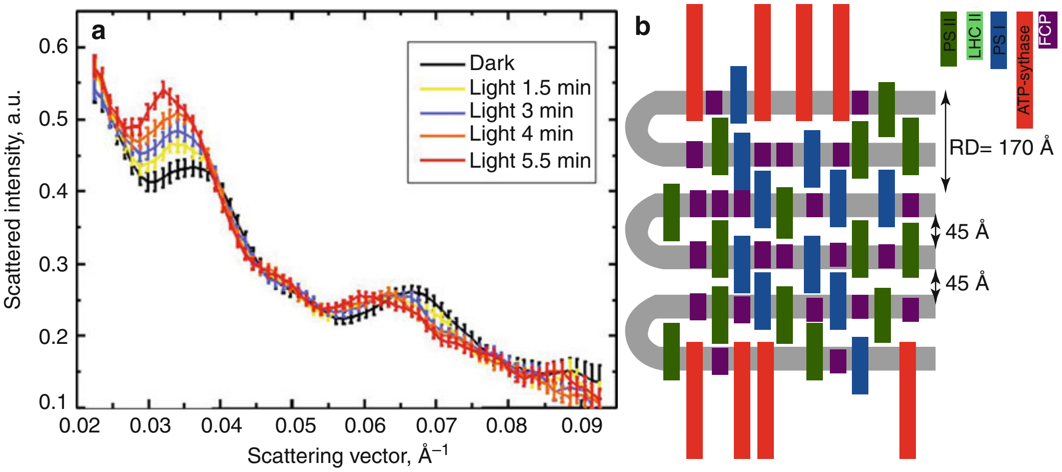

Fig. 16.4. Small-angle neutron scattering (*SANS*) profiles of the diatom *Phaeodactylum tricornutum* in the dark and upon the illumination of cells with *white* light of 150 μmol photons m^{-2} s^{-1} light intensity for different periods of time as indicated (**a**) as well as a schematic structural model showing the possible arrangement of the main protein complexes in thylakoid membranes in the dark (LHCII is shown only for comparison) (**b**). From the variations of the SANS profile it can be seen that light induces a small (about 1 nm) expansion of the membrane repeat distances, which appears to originate mainly from expansion of the inter-thylakoidal space, as reflected by the shift in the higher Q (scattering vector) peak, parallel to the shift of the peak at around 0.03 Å^{-1}. These changes occur much faster in strong light and are fully reversible in the dark (cf. Nagy 2012, from where the figures are reproduced).

Hence, the light-induced CD and SANS changes could unequivocally be assigned to the expansion of RDs. This appears to affect both the overall RD and the interthylakoidal space, as indicated by the involvement of two SANS peaks: the first, at lower Q value, is defined by the overall RD, whereas the second is tentatively assigned to originate from adjacent membrane pairs (Nagy et al. 2011, 2013). Kinetic correlations have been found between rapid light-induced and slow dark-reversible structural changes (ΔSANS and ΔCD) and NPQ. More importantly, progressive reversible shrinkage, induced by gradually increasing the sorbitol concentration, led to a continuous and reversible decrease of the maximal attainable NPQ. It can thus be inferred that *NPQ depends on the mobility of complexes*, and that the *light-induced increase in the interthylakoidal space facilitates this mobility*. Further, the effects of NPQ inhibitors on ΔSANS and ΔCD also suggest a close correlation between the overall membrane reorganization and NPQ (Nagy et al. 2012). These correlations strongly suggest a direct *link between thylakoid ultrastructural changes and NPQ* – indicating a key role for ultrastructural reorganization in the regulation of excitation-energy utilization in thylakoid membranes in vivo.

D Mechanistic Link Between the Structural Changes in Thylakoid Membranes and NPQ

We ask what the mechanistic link between ΔSANS or ΔCD and NPQ is. It is not easy to identify the link between the two phenomena since structural features reflected by SANS or the psi-type CD are "global" properties belonging to the entire membrane system, while fluorescence arises from chlorophylls of individual PS II units. There are several possibilities: (i) global reorganization is brought about by local, elementary structural changes that are also responsible for NPQ in individual PS II units – these elementary structural changes might have a cumulative aspect, yielding global changes; (ii) the global structural changes affect the fluorescence properties of individual complexes in the membrane, e.g., via modulation of surface charges, and thus affecting the overall fluorescence yield of PS II units; and (iii) the two phenomena are largely independent of each other, although they might act in the same direction and might be a part of the same regulatory mechanism.

Data available in the literature do not allow firm conclusion with regard to the above possibilities, and the answer might be different for different types of organisms and/or for different regulatory mechanisms. The PAL mutant of *Synechocystis* PCC6803, lacking phycobilisomes and possessing only CP47 and CP43, the core antenna complexes of PS II, shows no NPQ (Wilson et al. 2007), but exhibits RD changes quite similar to those in diatoms (Nagy et al. 2011). Similar light-induced changes were observed in wild-type and other phycobilisome mutants of *Synechocystis* cells (Liberton et al. 2013), demonstrating the robustness of these structural changes. Hence, at least in the PAL mutant of *Synechocystis*, RD changes do occur independently of NPQ, and thus the first possibility (see above) can be ruled out for *Synechocystis*, a cyanobacterium, suggesting that *changes in the multilamellar organization of thylakoid membranes appear to be a necessary, but not sufficient condition for NPQ to occur*. NPQ evidently requires involvement of additional components, such as the orange carotenoid protein (OCP) in cyanobacteria (see Kirilovsky et al., Chap. 22).

It would be interesting to determine if specific changes in RD are associated with OCP-related NPQ (Rakhimberdieva et al. 2004; Wilson et al. 2006). Further experiments are needed on different organisms, especially using NPQ and state-transition mutants, not only to clarify the role of structural changes affecting membrane ultrastructure during light adaptation and photoprotection of photosynthetic organisms, but also to understand the exact nature and mechanism of structural changes under different environmental conditions.

The mechanism of changes in the multilamellar organization in cyanobacteria and diatoms is not well understood. Liberton et al. (2013) have shown that changes in SANS profile were diminished upon treating cells with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) for 30 min in light (DCMU is an inhibitor of electron flow between the primary plastoquinone electron acceptor of PS II, Q_A, and the plastoquinone pool). In *P. tricornutum*, RD changes are largely insensitive to the uncoupler NH_4Cl (Nagy et al. 2011), a molecule that shuttles protons across membranes thereby removing the pH gradient, which thus does not appear to be the dominant driving force of the structural reorganization, while its role cannot be ruled out entirely. Preliminary data indicate that both ΔCD and ΔSANS exhibit a much weaker sensitivity to uncouplers and electron transport inhibitors than anticipated, but are quite sensitive to dithiothreitol, an inhibitor of the xanthophyll cycle (Nagy et al. 2011; M. Szabó, G. Nagy, R. Ünnep O. Zsiros, P. Lambrev and G. Garab, unpublished). Membrane reorganization might be driven by changes in membrane surface charge; distribution of ions in the aqueous phase as well as on the membrane-water interface are determined by charge movement, local and transmembrane electric field, and the proton gradient (Zimányi and Garab 1989). Variations in surface charges can also be induced directly by light, as shown by the light-induced reversible Mg^{2+}-release by isolated LHCII, a dissipation-assisted, thermo-optically driven reaction, i.e., induced by ultrafast local thermal transients due to photon energy dissipation in the vicinity of the cation-binding site (Garab et al. 2002; Garab 2014; see below and in IV.A).

As far as the role of reversible RD changes is concerned, we can put forward a hypothesis, which suggests that *extension of repeat distances, and of the inter-thylakoidal space in particular, makes the membrane system more flexible, permitting additional reorganization to occur, structural changes related to adjustments of the organism to light.* In tightly stacked membranes, the protein components are largely immobilized since LHCIIs on adjacent granal membranes are also electrostatically stacked on each other (Standfuss et al. 2005). Indeed, in unstacked thylakoid membranes more than 50 % of protein complexes are mobile, whereas this number drops to about 20 % in stacked grana regions (Kirchhoff et al. 2013). Conversely, in order to allow dynamic reorganization in the lamellae, membranes might need to be unstacked.

IV Structural and Functional Plasticity of Light-Harvesting Antennas

The astounding variability of light-harvesting antennas in different species along the evolutionary tree, as well as changes in antenna composition occurring over both fast and slow time scales in response to environmental conditions (Green et al. 2003; Larkum et al. 2003), point to significant regulatory roles played by the light-harvesting antenna in oxygenic photosynthetic organisms. Since antenna complexes account for the largest fraction of proteins embedded in the membrane and/or associated with it, and thus also function as structural proteins, they constitute the most important link between the molecular architecture of individual antenna complexes and the macro-organization of thylakoid membranes. This link and the regulatory function of light-harvesting antenna complexes explain the substantial interest in the spectral, structural and dynamic properties of antenna complexes in vitro and in vivo. In particular, questions related to NPQ, modulation of light-harvesting efficiency, and fluorescence lifetime in relation to the molecular architecture of different light-harvesting antenna complexes are discussed by several authors in this book (Papageorgiou and Govindjee, Chap. 1; Ostroumov et al., Chap. 4; Holzwarth and Jahns, Chap. 5; Polivka and Frank, Chap. 8; van Amerongen, Chap. 15; Bilger, Chap. 19).

In the following sections, I would like to direct attention to two special questions, the molecular architecture of light-harvesting antenna complexes in their native state(s) versus their detergent-solubilized or crystalline states, and the dynamic properties of isolated complexes. The discussion will be confined to LHCII.

A The Molecular Architecture of Detergent-Solubilized and Crystalline LHCII Versus Its Native State

The structure of LHCII is known at near atomic resolution (Liu et al. 2004; Standfuss et al. 2005). These structures serve as the basis of interpretation for its structural flexibility, inferred from different sets of experiments (see below), although according to some authors for LHCII no flexibility is allowed by the structure (Barros and Kühlbrandt 2009). The structures also serve as the basis for the understanding of all steady-state and ultrafast spectroscopic features of LHCII.

It is widely assumed that information from LHCII crystal structure can be applied to both the native state of LHCII in the thylakoid membrane and solubilized trimers. While this is true for the most important features, deviations have been revealed with respect to some of the details. Also, as long as spectral features of isolated complexes cannot be fully modeled (see, e.g., Georgakopoulou et al. 2007; Müh and Renger 2012), differences between the crystal structure and solubilized trimers may be responsible for the discrepancies.

When comparing crystals with the native conformation of LHCII, a striking difference is that neighboring trimers are in a non-natural, up-down orientation in stacked two-dimensional crystals (Standfuss et al. 2005). In the icosahedral proteoliposome assembly of LHCII (Liu et al. 2004), it is the non-natural packing of trimers that differs from the in vivo situation. As concluded by Dockter et al. (2012), who used double electron-electron resonance spectroscopy allowing the measurement of distances between two spin-labeled residues of LHCII, the non-natural packing of complexes might distort protein conformation in the crystal, especially in the hydrophilic domains.

A systematic comparison of CD spectra of solubilized LHCII trimers and aggregated

LHCII, both with each other and with corresponding spectral features in the thylakoid membranes, has revealed significant differences in the organization of pigment molecules (Lambrev et al. 2007b). It was found that, while CD spectra of lipid-enriched small lamellar aggregates of LHCII closely resemble those of unstacked thylakoid membranes, solubilized trimers display significantly different bands in the Soret region (Lambrev et al. 2007b) (Fig. 16.5). Upon solubilization of thylakoid membranes or LHCII aggregates, the CD spectra become very similar to the corresponding spectra of isolated detergent-solubilized trimers, showing that the main differences cannot be accounted for by their different composition, but are rather due to the effect of detergents. Solubilization also results in a dramatic increase in Chl fluorescence lifetime (Lambrev et al. 2007b), in agreement with earlier reports (Ruban and Horton 1992). Based on the above data we can conclude that, while *lamellar aggregates of LHCII retain the native organization of complexes in the thylakoid membrane*, *detergent-solubilized trimers do not*, *and instead undergo perturbations*. Detergent solubilization affects the orientation of carotenoids with respect to the membrane plane and perturbs a few excitonic CD bands probably arising from carotenoid: Chl interactions (Lambrev et al. 2007b); it also leads to a diminution in the efficiency of triplet–triplet energy transfer from Chl *a* to lutein and a rise in both the triplet formation yield and the fluorescence yield of Chl *a* (Naqvi et al. 1999). Upon the removal of detergent from gel-trapped isolated trimers, Ilioaia et al. (2008) observed similar alterations, but with the opposite sign – both in CD spectra and in fluorescence lifetimes. Taken together, these observations show that spectral and fluorescence lifetime differences cannot be accounted for by changes in pigment composition, but must rather originate from differences in LHCII molecular architecture of LHCII.

While discussing the effects of detergent and its removal, Rutkauskas et al. (2012) have argued that, "since the detergent micelle is thought to mimic the native membrane environment, its removal has a profound effect on the condition of LHCII resulting in its quenching." However, the observations described above suggest that detergent micelles do not, in fact, mimic the native environment. Instead, we note the following: (i) the spectral features in the native membranes differ very significantly from samples in the presence of detergents; and (ii) different detergents cause different distortions in CD, showing that they all perturb the native structure, albeit to different extent and probably in slightly different domains (Fig. 16.5).

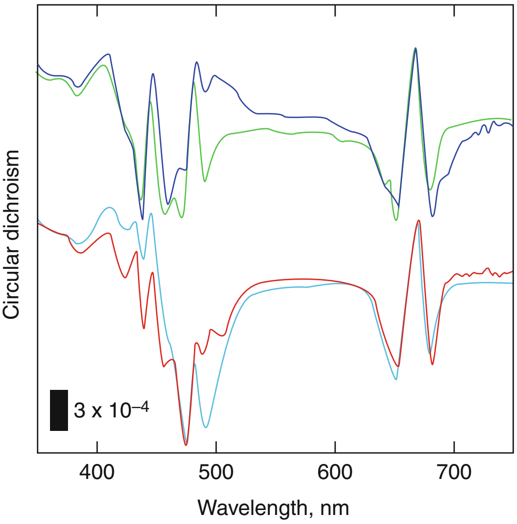

Fig. 16.5. (*Upper* pair of spectra) Circular dichroism spectra of isolated thylakoid membranes suspended in low salt, hypotonic medium (*dark blue trace*) and small aggregates of LHCII (*green trace*) and (*lower* pair of spectra) spectra of isolated LHCII trimers solubilized in α-DM (n-dodecyl-α-D-maltoside, *red trace*) and in β-DM (n-dodecyl β-d-maltoside, *light blue trace*). Note that the spectrum of small aggregates of LHCII (containing detergent only at low concentration) closely resembles the spectrum of washed thylakoid membranes, with most differences accounted for by the presence of PS I in thylakoid membranes, but differing significantly from detergent-solubilized trimers. In these latter samples, some of the excitonic interactions are evidently perturbed, and this perturbation depends on the chemical nature of the detergent (with two displayed here) (P. Lambrev, K. Pawlak, P. Akhtar and G. Garab, unpublished; see also Lambrev et al. 2007b).

As shown by the above data, the molecular architecture of both the crystal structure and the detergent-solubilized isolated trimers deviates to some extent from the native state. *The crystal structure does not account for the structural flexibility of some of the polypeptide segments in the aqueous phases*, *whereas detergents perturb the orientation of some carotenoids and a few pigment-pigment interactions*. Future studies on LHCII embedded in lipid membranes, using electron paramagnetic resonance (EPR) spectroscopy (Dockter et al. 2012) with improved sensitivity, and CD measurements on reconstituted LHCII-lipid vesicles and/or on small stacked lamellar crystals at low temperature, are needed for a better understanding of the problem. Also, it is not yet known if LHCII crystals resemble detergent-solubilized trimers or lamellar aggregates. Part of the difficulty is that the microcrystalline lamellar aggregates of LHCII, which are probably similar to stacked two-dimensional crystals (Standfuss et al. 2005), exhibit very strong psi-type CD bands that mask the much weaker excitonic bands (Simidjiev et al. 1997; see Fig. 16.2). Answers to questions concerning the in vivo relevance of fine details of crystal structure on the one hand and of data obtained on solubilized trimers on the other, are important for NPQ studies, since both detergent-solubilized LHCII trimers and crystals are frequently used as reference materials for the in vivo case. Further, it would be equally important with regard to theoretical modeling of the spectral features of LHCII, during which the spectra of either the native or the detergent-solubilized trimers are to be interpreted using the crystal structure. Last but not least, an answer to the above questions is important for understanding the nature and mechanism of the structural flexibility of LHCII in vitro and in vivo.

B Light-Induced Reversible Reorganizations in Isolated LHCII

The first experimental evidence demonstrating structural and functional flexibility of isolated LHCII was provided by Jennings et al. (1991), who showed that LHCII is capable of undergoing light-induced reversible fluorescence quenching with a rate proportional to the intensity of excitation. Thermodynamic analysis of these fluorescence transients has shown that these transients are associated with conformational changes in protein complexes that are more pronounced in lamellar aggregates than in trimers (Barzda et al. 1999). Direct experimental evidence for the capability of lipid-enriched lamellar aggregates of LHCII to undergo light-induced reversible changes has been obtained via CD spectroscopy (Barzda et al. 1996). Figure 16.6 shows this type of CD changes and their peculiar, non-Arrhenius temperature dependence (Cseh et al. 2005), the significance of which will be discussed in a later section. The loosely stacked lamellar aggregates, isolated by the method of Krupa et al. (1987) and Simidjiev et al. (1997), exhibit psi-type features, although much weaker than displayed by microscrystalline LHCII (Simidjiev et al. 1997; see Fig. 16.2). These lipid:LHCII macroassemblies, but not the much more rigid microcrystalline lamellar aggregates (that are largely depleted of bulk lipids; Simidjiev et al. 2011), exhibit light-induced reversible changes in their main, psi-type CD bands. These changes in the macro-organization of complexes are further enhanced by externally added thylakoid lipids (Simidjiev et al. 1998). The reorganizations in LHCII lamellar aggregates closely resemble the light-induced, reversible changes observed in isolated thylakoid membranes (Garab et al. 1988); further, the excitonic CD bands are not affected, showing that *the light-induced reversible reorganizations in isolated LHCII or thylakoid membranes do not noticeably affect the bulk pigment-pigment interactions in trimeric pigment-protein complexes*.

Studies by Garab et al. (2002) and Dobrikova et al. (2003) have revealed that prolonged exposure of isolated LHCII or thylakoid membranes to high light leads to monomerization of LHCII trimers, which can also be identified by excitonic CD fingerprinting. Physiologically, *monomerization of LHCII in high light*, a process

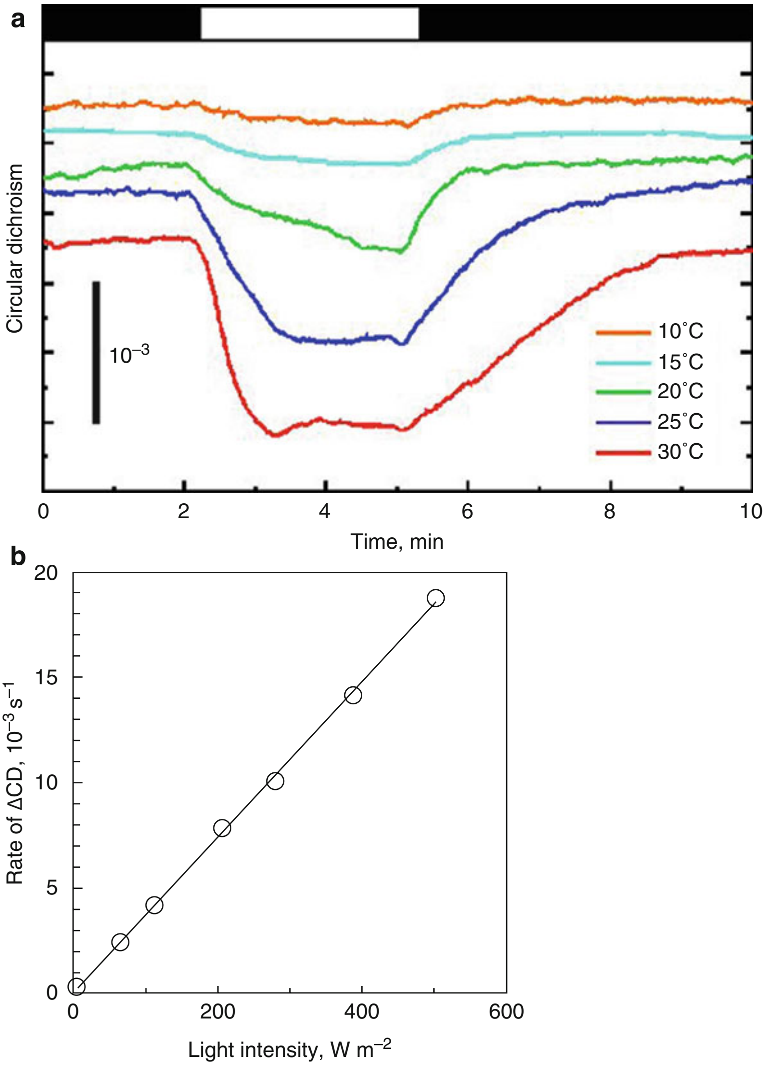

Fig. 16.6. Light-induced dark-reversible changes in the amplitude of the (−)495 nm psi-type CD band of loosely stacked lamellar aggregates of LHCII elicited by ~1,000 µmol red photons m^{-2} s^{-1} at different temperatures (**a**) and dependence of the CD changes on light intensity (**b**) (Cseh et al. 2005). *Black* and *white horizontal bars* in panel **a** indicate dark and light periods, respectively (kinetic traces recorded by S. Rajagopal). These data, the peculiar temperature and light-intensity dependences, can be explained satisfactorily using the model of thermo-optic mechanism, i.e., elementary structural changes induced by ultrafast thermal transients in the close vicinity of the dissipation site of the photon energy (Cseh et al. 2000, 2005). Panel **b** reproduced from Cseh et al. (2005).

enhanced by phosphorylation (Várkonyi et al. 2009), *is important in the proteolytic removal of LHCII* – since only the monomers are accessible to the appropriate protease (Yang et al. 2000).

Reorganization of thylakoid membranes – similar to that observed for LHCII, although facilitated by ΔpH – is also largely independent of the photochemical activity of thylakoids (Garab et al. 1988; Barzda et al. 1996; Istokovics et al. 1997). Light-induced CD changes occur at rates linearly proportional to the light intensity above photosynthetic saturation in isolated thylakoid membranes (see Fig. 16.3; Barzda et al. 1996; Garab et al. 2002; Cseh et al. 2005). Remarkably, release of Mg^{2+}-ions occurs with a similar light-intensity dependence as ΔCD (Garab et al. 1998; Garab 2014). The membranes' response with a structural change proportional to excess light intensity is thus similar to the response of LHCII lamellar aggregates (cf. Figs. 16.3 and 16.6). In other words, *thylakoid membranes and lamellar aggregates of LHCII appear to be capable of responding specifically to the fraction of light that is not used for photosynthesis, with structural changes being proportional to the intensity of excess light*. Clearly, this unique feature per se makes these structural changes excellent candidates for involvement in light adaptation and photoprotection of plants.

Although there are observations on the variability of these structural changes among different plants and different growth conditions, systematic investigations have yet to reveal the specifics of this structural flexibility, e.g., in lipid composition or polyamine content of membranes and the location of dissipation sites in the complexes. With regard to NPQ, and as pointed out above, *direct and very tight correlation between global reorganization and locally determined fluorescence yield changes are not expected*. In addition to structural reorganization, *NPQ also requires the presence of effector molecules*, such as the PsbS protein and zeaxanthin. Nevertheless, structural changes in LHCII and NPQ are evidently not independent of each other, and it seems likely that *NPQ depends on the structural flexibility of the antenna system*.

What is the physical mechanism underlying the structural changes? Based on a series of systematic studies (reviewed by Garab 2014), it appears that *light-induced reversible structural changes in isolated LHCII and in the thylakoids induced by excess light can be accounted for by a novel, thermo-optic mechanism*: ultrafast thermal transients (heat packages), arising from dissipated excitation energy can lead to elementary structural transitions in the close vicinity of the dissipation site due to the presence of "built-in" thermal structure-instabilities (Cseh et al. 2000, 2005). Such an elementary, dissipation-assisted structural change might occur near the site of the terminal emitter, in the outer loop segment of LHCII, in the highly flexible N-terminus section (cf. Dockter et al. 2012). This polypeptide section, in the vicinity of the lowest energy terminal emitter Chl-*a* molecules responsible for fluorescence emission (Novoderezhkin et al. 2005), contains a phosphorylation site and has been shown to be capable of undergoing light-induced reversible conformational changes (Zer et al. 1999); it might also contain cation-binding sites (cf. Garab 2014). Dissipation of excitation energy causes a thermal transient with a sizeable temperature jump in the close vicinity (~1 nm) of the dissipation site (Cseh et al. 2000) and with a decay time of about 20–200 ps (Gulbinas et al. 2006). The relaxation of the protein might not be complete, and might thus, with some (usually very low) probability induce an elementary structural change, especially if the heat (temperature) jump induces a cation release (as has been observed both for LHCII and for isolated thylakoid membranes; Garab et al. 1998; Garab et al. 2002, see Garab 2014). The probability for a sizeable structural change to occur is evidently higher with the involvement of cation release. Cations are involved in protein-protein interactions that are responsible for the formation of LHCII:PS II macrodomains and the stacking of thylakoid membranes (Garab et al. 1991; Mustárdy and Garab 2003; Garab 2014). Cations that are released via thermo-optic mechanism, i.e., due to appearance of a local heat transient near the cation-binding site, will not be rebound immediately after the heat jump, but may instead bind on a much slower time scale. In fact, the cations (Mg^{2+}) reversibly released from lamellar aggregates of LHCII upon illumination with continuous light can be detected on the time scale of seconds to minutes (Garab et al. 1998). Of course, a light-induced, dissipation-assisted release of cations (i.e., a release assisted by the local heat transient) would have to be much faster; this release appears to manifest itself in a fast (<200 ns, instrument limited) electric signal, most likely from charge displacement, upon excitation of oriented LHCII lamellae with a 20 ns laser pulse (Garab et al. 2002). Cation release, in turn, may affect the array of LHCII complexes held together via electrostatic forces (Garab et al. 1991). Hence, over an illumination period of a few minutes, such structural changes might lead to macroscopically observable structural changes detected as changes in the psi-type CD signal of LHCII macroassemblies in vitro or in vivo (Barzda et al. 1996; Cseh et al. 2000; Dobrikova et al. 2003; Cseh et al. 2005).

Even though effects of ultrafast local heat transients arising from the dissipation of excess excitation energy receive less attention than other photophysical and photochemical events, such effects are not unique to photosynthetic antennae. *Hot molecules*, formed by internal conversion from an excited singlet state to a highly vibrationally-excited ground electronic state having very high equivalent temperatures, can facilitate various reactions and play a role as an intermediate step of, e.g., multiphoton dissociations (Nakashima and Yoshihara 1989). Also, both visible- and UV-photoinduced reduction has been shown to occur mainly in the ground state of hot cytochrome c, i.e., following an ultrafast internal conversion of oxidized cytochrome c upon UV or visible excitation; modest heating of the sample has also been shown to lead to its efficient thermal reduction (Lowenich et al. 2008). These two additional examples point to the significance of dissipation of excess excitation energy in different molecular assemblies. It appears that, in light-harvesting antenna systems, dissipation causes only relatively mild effects. Rather than facilitating photochemistry, it appears that *the heat packages resulting from dissipation primarily affect the stacking interactions and the macro-organization of complexes*. In the hierarchically organized structures of thylakoid membranes, these are the most thermally unstable assemblies, held together mainly by weak, electrostatic interactions (Garab et al. 1991; Dobrikova et al. 2003). Disassembly of thermally more stable units, such as trimers, requires higher temperatures and higher light intensities (more dissipation; Garab et al. 2002).

According to the mechanism proposed by Cseh et al. (2000) and Garab et al. (2002), cation-binding sites play an important role in structural changes. This is consistent with the finding that amino acids at the N-terminus of LHCII possess high structural flexibility. EPR measurements showed that the nine amino acids at the N terminus, that have not been resolved in crystal structures, exhibit very broad and possibly bimodal distance distributions, suggesting existence of two families of preferred conformation (Dockter et al. 2012).

In the light of the results discussed above and the thermo-optic mechanism the following scheme is proposed to illustrate the link between dissipation-assisted structural changes and NPQ in thylakoid membranes (Fig. 16.7).

The N-terminus of LHCII acts as a ligand for one phosphatidylglycerol (PG) molecule, which is critical for maintaining the trimeric structure. We note that monomerization of LHCII trimers, which adhere via PG molecules, can also be achieved thermo-optically: monomerization can be induced either by elevating the temperature to about 55–60 °C or by intense light at 25 °C (see Garab et al. 2002). According to the model using the thermo-optic effect, the mechanism of light-induced monomerizetion is identical to the thermally induced trimer-to-monomer transition. With light exposure, in particular upon dissipation of unused excitation energy, the local temperature may transiently reach the temperature where monomerization of LHCII trimers occur. The “addition” of local transient temperature to the ambient temperature also explains the peculiar temperature-dependence of the light-induced reversible changes in the macro-array of LHCII trimers shown in Fig. 16.6.

The threonine and serine residues at the N-terminus are also sites for phosphorylation, where light-induced conformational changes have been documented (Zer et al. 1999). It has been shown that accessibility of these residues to tryptic cleavage depends on pre-illumination of isolated LHCII. As an important consequence, *phosphorylation of LHCII is regulated by light at the substrate level* (Zer et al. 1999, 2003; Vink et al.

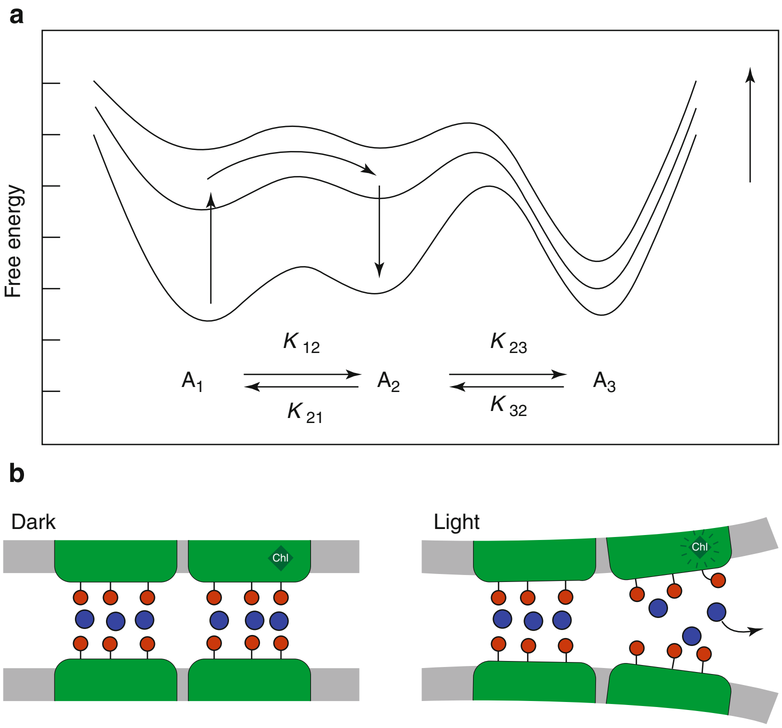

Fig. 16.7. Potential profile of a thermo-optically induced reorganization (**a**) and schematic illustration of the unstacking of two adjacent layers of LHCII-containing lamellae (**b**). Panel **a** shows that transitions from state A_1 to states A_2 and A_3 can occur via elevating the temperature; in this case, the entire molecular assembly will undergo the same transition. In contrast, upon dissipating an excitation (photon energy) near the site prone to an elementary structural change (A_1 to A_2), the transition occurs only locally, due to the ultrafast rise of the local temperature, which is followed by rapid "cooling". (Evidently, this local, elementary structural transition can only be achieved if the "combined" local and ambient temperatures allow the transition, thus explaining the strong non-Arrhenius temperature dependences (Fig. 16.6, see Cseh et al. 2005). Panel **b** illustrates that, upon photon-energy dissipation in the pigment bed (*green areas*) by a "heat-radiating" Chl molecule on the stromal side (e.g., in the domain of terminal-emitter Chl *a* molecules), near a cation-binding site (*red balls*), a Mg^{2+}-ion (*dark-blue ball*) might be released, which, in turn, leads to local unstacking. Panel **a**, reproduced from Garab et al. 2002; panel **b** is from Hind et al. (2014).

2004). These results are consistent with the proposed thermo-optic mechanism and the involvement of cation-binding sites. Also, the high similarity of the light-induced structural changes in LHCII-containing chiral macrodomains in vitro and in vivo, as well as the light-induced regulation of LHCII phosphorylation observed both in isolated LHCII and in vivo, are consistent with the suggestion that *the ability of LHCII to undergo reorganization* in vitro *is transferred to the thylakoid membranes. Furthermore, this inherent structural flexibility of LHCII plays a significant role in at least two regulatory processes*, *phosphorylation and*, as discussed above, *the proteolytic removal of LHCII. LHCII's inherent flexibility might also play a role in NPQ* (see below).

Using lamellar aggregates isolated according to Krupa et al. (1987), Gruszecki and coworkers (Gruszecki et al. 2009, 2010a, b; Zubik et al. 2011, 2012) focused their attention on the problem of molecular configuration of LHCII-bound xanthophylls and the effect of light-induced isomerization changes on the quenching of Chl fluorescence. In particular, Gruszecki et al. (2009, 2010a) observed, by means of resonance Raman scattering technique, that, upon strong illumination, violaxanthin changed from a more twisted to a more relaxed molecular configuration. For a comprehensive treatment of NPQ-related structural changes in LHCII as revealed by Raman spectroscopy, see Pascal et al., Chap. 10. Since the more twisted molecular configuration of violaxanthin is characteristic of the pigment located within the binding pocket in LHCII, Gruszecki et al. (2009) concluded that over-excitation of antenna protein results in the liberation of violaxanthin from the protein environment, accompanied by its transfer into the lipid phase of the thylakoid membrane.

In fact, such a process, as discussed above, is necessary for the operation of the xanthophyll cycle because violaxanthin de-epoxidation is an enzymatic reaction that takes place in the lipid environment of the membrane (Schaller et al. 2011). The fact that violaxanthin is a xanthophyll localized in the trimeric structures of LHCII at the border of the trimer-forming monomeric subunits suggests that a light-driven change of the molecular configuration of this xanthophyll might be associated with, and accompany, the thermo-optically induced LHCII trimer-to-monomer transition reported by Garab et al. (2002). Monomeric LHCII has been demonstrated by a single-molecule approach to have a higher excitation-quenching capacity as compared to trimeric complexes and, therefore, such a mechanism (i.e., monomerization of LHCII by high light) can be considered to be involved in the photoprotection of plants (Gruszecki et al. 2010a). The observation that light induces molecular configuration changes of LHCII-bound neoxanthin from 9′-cis to 9′,13- and 9′,13′-dicis forms, and that this is a spontaneously reversible reaction, implies that this change may have physiological significance (Zubik et al. 2011). On the basis of their experiments, Zubik et al. (2011, 2012) suggested that a light-driven change of the molecular configuration of LHCII-bound neoxanthin removes steric hindrance and enables close contact of the two neighboring LHCII trimers, which results in the formation of low-energy Chl spectral forms that can act as energy traps.

Given the fact that gross reorganization in lamellar aggregates of LHCII and in intact thylakoid membranes closely resemble each other, and taking into account reports on the involvement of substantial reorganization related to qE, it seems safe to conclude that *LHCII plays a key role in the reorganization associated with qE*. At the same time, we stress that *these structural changes on their own*, *in the absence of effector molecules*, *do not explain the NPQ process*. Nevertheless, the propensity of LHCII to undergo reorganization might be directly responsible for the structural changes associated with qE.

C Conformational Switches in LHCII

In a series of experiments using single-molecule fluorescence microscopy, it has been shown that *detergent-solubilized LHCII trimers are capable of undergoing rapid conformational changes*, switching between the light-energy-utilizing (fluorescent) state and the dissipative (non-fluorescent) state (see Krüger et al., Chap. 6). This type of molecular switch, which also exists in bacterial (Rutkauskas et al. 2004) and PS I light-harvesting complexes (Krüger et al. 2011), has been suggested to play a role in NPQ in PS II (Krüger et al. 2012). These conformational transitions might explain the ability of LHCII to participate in substantial light-induced reversible reorganizations, such as those outlined above. Theoretical models have been published that provide interpretations of the manifestations of the dynamic disorder in the complexes. It is not yet clear whether and how these reorganizations and the associated fluorescence changes are related to light-induced reversible fluorescence quenching observed in macroscopic LHCII samples (Jennings et al. 1991; Barzda et al. 1999) and to the reorganizations in macroscopic LHCII samples reported by others (Barzda et al. 1996; Zer et al. 1999; Gruszecki et al. 2010b). Krüger et al. (2010) have shown that, just as in thermo-optically induced changes (Cseh et al. 2000), where the light intensity is orders of magnitude lower, steady-state heating of the sample does not occur in the sample and thus cannot explain the above observations. Nevertheless, as discussed by Krüger et al. (Chap. 6), ultrafast thermal transients in the vicinity (1 nm) of dissipation centers might represent a perturbation in the molecular structure and influence the operation of the conformational switch in the light-harvesting antenna complex.

V Conclusions and Outlook

In this chapter, an overview of static and dynamic properties of the photosynthetic apparatus at different levels of structural complexity, relevant to NPQ, has been provided. Stern-Volmer analyses show that, in intact thylakoid membranes, generation of quenchers of the excited state of Chl *a* can, in principle, lead to substantial reduction in the lifetime of the singlet excited state of Chl *a* even if their concentration is very low. The currently available experimental data strongly suggest that organization of the pigment-protein complexes and super-complexes into long-range order arrays in the membrane provides the structural basis for diffusion of excitation energy over long distances and might thus favor both photosynthetic utilization of the absorbed light energy and NPQ of excited Chl *a*. However, it appears that in plants the dissipative regimes are associated with dismantling of the long-range order arrays. In general, NPQ appears to depend heavily on the structural flexibility of the multilamellar membrane system as the main structural unit and on the macro-organization of complexes in the thylakoid membranes; this flexibility allows gross reorganizations in the pigment system without noticeable changes in bulk pigment-pigment, excitonic interactions. It seems clear, however, that while reorganization appears to be necessary for NPQ to occur, such reorganization on its own is only partially or indirectly responsible for the quenching that also requires the presence of special "effector" molecules, such as zeaxanthin or PsbS in plants (Adams and Demmig-Adams, Chap. 2; Brooks et al., Chap. 13; Demmig-Adams et al., Chap. 24), diatoxanthin in diatoms and brown algae (Büchel, Chap. 11; Lavaud and Goss, Chap. 20), light-harvesting complex stress-related proteins (LHCSR) in unicellular algae (Morosinotto and Bassi, Chap. 14), and OCP in cyanobacteria (Kirilovsky et al., Chap. 22).

A comparison of data for in vivo and in vitro systems reveals that, while some of the properties of detergent-solubilized trimers and of crystals deviate significantly from the native states, the properties of lipid:LHCII lamellar aggregates closely resemble the in vivo features. Most importantly, lipid:LHCII lamellar aggregates possess the ability to undergo light-induced, thermo-optically driven (i.e., dissipation-assisted) reversible reorganizations (Barzda et al. 1996), mimicking those observed in isolated thylakoid membranes (Garab et al. 1988) (see also Garab 2014). Native thylakoid membranes, similar to lipid:LHCII macroassemblies, are capable of responding to excess light with reorganization that is proportional to the unused excitation above the saturation of photosynthesis. A photophysical feedback mechanism, that is most probably the thermo-optic mechanism, plays key roles in important enzymatic regulatory processes, such as phosphorylation of LHCII and its proteolytic removal. The structural changes induced by excess light (i.e., by unused excitation dissipated to heat) and giving rise to ultrafast local heat transients might also induce a transition to a state of controlled dissipation in the system.

The nature of the structural changes, and the exact physical mechanisms underlying different structural reorganizations, are yet to be elucidated. In particular, the dissipation-assisted reorganizations are of high potential relevance for light adaptation, photoprotection and NPQ in various organisms. Our understanding is also quite rudimentary with regard to the structural requirements and mechanism of safe dissipation of unused excitation energy in light-harvesting complexes, which is probably as important for evolution and physiology of oxygenic photosynthetic organisms as the energy-utilization process itself.

Acknowledgments

I am indebted to Dr. Petar Lambrev for fruitful discussions and for his CD data, Dr. Geoffrey Hind for the analysis of scanning transmission electron microscopy images of LHCII microcrystals and helpful advice, Dr. Krzysztof Pawlak for his help in preparing the illustrations, and Drs. Gergely Nagy, Subramanyam Rajagopal and Milán Szabó for providing some of their unpublished figures. I am grateful to Profs. Govindjee, Barbara Demmig-Adams and Herbert van Amerongen, and Dr. Tjaart Krüger for critical reading of the manuscript and helpful comments. This work was supported by grants from NKTH-OTKA (CNK 80342), NIH-A*STAR (TÉT 10-1-2011-0279) and TÁMOP-4.2.2.A-11/1/KONV-2012-0060.

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Chapter 17

Non-Photochemical Fluorescence Quenching and the Dynamics of Photosystem II Structure

AlexanderV. Ruban and Conrad W. Mullineaux*
School of Biological and Chemical Sciences, Queen Mary University of London,
Mile End Road, London E1 4NS, UK

Summary

High-energy state quenching (qE) is the rapidly reversible component of non-photochemical quenching in chloroplasts. Recent experimental approaches indicate that establishment of the photoprotective qE state in green plant chloroplasts requires a large-scale re-organization of photosystem II and its light-harvesting antenna complexes (LHCII) within the grana membrane, with formation of spatially segregated domains of aggregated LHCII complexes. Consequently, qE formation requires mobility of chlorophyll-protein complexes within thylakoid membranes, and factors that control such mobility are major determinants of rapid qE formation. These factors include the complement of some minor light-harvesting proteins as well as the PsbS subunit of photosystem II. We review the evidence for this scenario, including studies of the organization of chlorophyll-protein complexes by freeze-fracture electron microscopy and studies of the mobility of chlorophyll-protein complexes by fluorescence recovery after photobleaching. Finally, we present an integrated working model for the structural events involved in qE formation, and the factors that trigger the transition. This model incorporates available evidence on the roles of proton binding to LHC complexes and to PsbS, and of xanthophyll cycle carotenoids.

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*Author for Correspondence, e-mail: c.mullineaux@qmul.ac.uk

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_17,

I Introduction

It is now generally accepted that the rapidly reversible (qE) component of non-photochemical quenching (NPQ) of chlorophyll fluorescence, as an indicator of photoprotective thermal dissipation of excess energy absorbed by plants, depends on functional changes in the light-harvesting antenna of photosystem II (PS II) involving the formation of quenching centers within the antenna that convert excitation energy to heat (Ruban et al. 2012). The nature of these quenching centers is still under debate, but there is general agreement that their formation is stimulated by low pH within the thylakoid lumen, presence of the xanthophyll cycle carotenoid zeaxanthin, and presence of the PsbS subunit of PS II (for more detail on the PsbS protein, see Brooks et al., Chap. 13) within the PS II antenna system (Ruban et al. 2012).

In principle, formation of quenching centers could occur without any major rearrangement of the PS II light-harvesting antenna. Quenching centers could be formed by subtle conformational changes within individual light-harvesting antenna proteins and/or changes in the complement of carotenoids bound to antenna proteins (Ilioaia et al. 2008). However, there is increasing evidence that the formation of quenching centers is, in fact, tied to a larger-scale rearrangement of complexes within the thylakoid membrane system. This evidence comes both from biochemical and spectroscopic approaches that probe the interactions of antenna proteins within super-complexes, and from structural approaches that examine the layout, or “landscape”, of proteins within the intact thylakoid membrane.

Here, we review the evidence that large-scale rearrangement of proteins within the thylakoid membrane is associated with the formation of quenching centers and the induction of NPQ. We focus particularly on the structural evidence. A large-scale rearrangement of proteins requires significant lateral movement of protein complexes within the thylakoid membranes. This could be problematic, as thylakoids are densely packed with protein, and movement of integral thylakoid membrane proteins is very restricted due to macromolecular crowding, particularly within the grana stacks, where the vast majority of PS II reaction centers and light-harvesting complexes are located (Kirchhoff et al. 2002, 2008). We therefore review approaches to probing the mobility of complexes in grana membranes, and discuss evidence suggesting that the kinetics of quenching-state formation strongly depend on the mobility of PS II components within the membrane. Finally, we present and

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Abbreviations: AFM – Atomic force microscopy; B4C – Supercomplex of CP24, CP29 and trimeric LHCII-M; C2S2M2 – Supercomplex containing dimeric PS II core 2 LHCII-S trimers and 2 LHCII-M trimers; CP24 – Monomeric light-harvesting antenna complex encoded by the *lhcb6* gene; CP26 – Monomeric light-harvesting antenna complex encoded by the *lhcb5* gene; CP29 – Monomeric light-harvesting antenna complex encoded by the *lhcb4* gene; EFs – Exoplasmic (lumenal) fracture face from stacked thylakoid membrane region; EFu – Exoplasmic (lumenal) fracture face from unstacked thylakoid membrane region; FFEM – Freeze-fracture electron microscopy; FRAP – Fluorescence recovery after photobleaching; LHC – Light-harvesting complex; Lhcb1 – Major LHCII protein encoded by the *lhcb1* gene; Lhcb2 – Major LHCII protein encoded by the *lhcb2* gene; LHCII – Light-harvesting chlorophyll *a*/*b*-binding protein of photosystem II; LHCII-M – Moderately-bound LHCII trimer; LHCII-S – Strongly-bound LHCII trimer; LHCSR – Stress-related member of the LHC protein superfamily product of the *LI818* transcript in *Chlamydomonas reinhardtii;* NPQ – Non-photochemical quenching of chlorophyll fluorescence; PFs – Protoplasmic (stromal) fracture face from stacked thylakoid membrane region; PFu – Protoplasmic (stromal) fracture face from unstacked thylakoid membrane region; PS II – Photosystem II; PsbS – S subunit of photosystem II; qE – High-energy state quenching as the rapidly reversible component of NPQ; Stn7 – Chloroplast-located protein kinase encoded by the *stn7* gene of *Arabidopsis thaliana;* Stn8 – Chloroplast-located protein kinase encoded by the *stn8* gene of *Arabidopsis thaliana* discuss an integrated working model that links the triggering events of NPQ to structural rearrangements and the formation of quenching centers.

II Reorganization of the PS II Antenna During NPQ Formation: Biochemical and Spectroscopic Evidence

Initial suggestions that formation of the NPQ photoprotective state might involve a general reorganization of the PS II light-harvesting antenna came from some suggestive similarities between spectroscopic signatures observed upon formation of NPQ in vivo and upon aggregation of isolated LHCII complexes in vitro (Ruban and Horton 1992; Ruban et al. 1992). This prompted the idea that formation of the NPQ-photoprotective state might involve formation of LHCII aggregates in vivo with similarities to the artificial aggregated states produced in vitro (Horton et al. 1991, 1996). A wide range of spectroscopic techniques, including 77 K fluorescence spectroscopy (Ruban and Horton 1992), circular and linear dichroism (Ruban et al. 1997a), resonance Raman (Ruban et al. 2007) and two-photon excitation spectroscopy (Bode et al. 2009) all demonstrate comparable features of NPQ in vivo and LHCII aggregation in vitro. Furthermore, packing of LHCII into the three-dimensional crystals used for high-resolution X-ray crystallography also induces a quenched state with some resemblance to the NPQ state in vivo (Pascal et al. 2005).

Although the latter studies are suggestive, they do not provide any direct information on the organization of LHCII in the quenched state in vivo. Any aggregates of LHCII formed in the intact thylakoid membrane in vivo are unlikely to be directly comparable to aggregates of detergent-solubilized LHCII formed in vitro in the absence of a membrane. However, spectroscopic studies in intact thylakoid membranes provide strong suggestions for the presence of chiral macrodomains composed largely of LHCII, their reorganization under a variety of conditions including NPQ induction and their involvement in the regulation of light-harvesting (Goss and Garab 2001; Holm et al. 2005; see also Garab, Chap. 16). Recent studies using time-resolved fluorescence spectroscopy on leaves (Miloslavina et al. 2008) and intact chloroplasts (Johnson and Ruban 2009) provided further evidence of LHCII aggregation in vivo by revealing the red-shifted chlorophyll fluorescence band – a fingerprint of aggregation – in the qE state. Furthermore, Holzwarth et al. (2009) argued that their decay-associated fluorescence spectroscopy provided evidence for a functional detachment of part of the LHCII pool from PS II during the formation of qE (see also Holzwarth and Jahns, Chap. 5). This finding is suggestive of a large-scale re-organization, by which LHCII complexes rearrange to associate more closely with other LHCII complexes rather than with PS II.

Biochemical techniques provide a more direct approach for probing the organization of PS II reaction centers and light-harvesting antennae, and changes accompanying the formation of the NPQ-photoprotective state. In brief, thylakoid membranes adapted to states with and without non-photochemical quenching can be gently solubilized and the photosynthetic super-complexes separated (for example by sucrose-density-gradient centrifugation). The composition of the isolated super-complexes can be probed (for example by Western blotting) to characterize differences in super-complex organization between the low light- and the NPQ-photoprotective state. Use of mutants with altered NPQ characteristics provides a powerful way to link the ability to perform NPQ in vivo with the ability to change the composition of super-complexes.

An early use of this approach came from Lokstein et al. (2002), who showed that xanthophyll biosynthesis mutants of *Arabidopsis* have a perturbed organization of PS II-LHCII super-complexes. The latter authors concluded that the effects on NPQ in the xanthophyll biosynthesis mutants were a consequence of their altered PS II-LHCII macro-organization. Kiss et al. (2008) used *Arabidopsis* mutants to show that PS II-LHCII super-complexes could be more readily extracted from thylakoid membranes by detergent solubilization when higher levels of the PsbS protein were present. This finding suggests a role for PsbS in controlling interactions within the PS II light-harvesting antenna. Since PsbS plays a key role in NPQ, this, in turn, suggests that PS II-LHCII macro-organization is important for NPQ induction. Betterle et al. (2009) probed the composition of LHCII super-complexes in detail, comparing the composition of super-complexes isolated from thylakoids prepared from dark-adapted and light-adapted *Arabidopsis* leaves. The latter authors found that NPQ induction was associated, in particular, with dissociation of the "B4C" complex composed of a trimer of LHCII-M (moderately-bound LHCII) plus monomers of the minor light-harvesting proteins CP24 and CP29. Furthermore, dissociation of the B4C complex requires the presence of the PsbS protein (Betterle et al. 2009).

Although the models provided by the authors of the latter studies differ in their details, taken together, these studies provide strong evidence for a link between NPQ induction and changes in the macro-organization of the PS II light-harvesting antenna.

III Reorganization of the PS II Antenna During NPQ Formation: Structural Evidence

The biochemical approaches described in section II are unable to provide a complete picture of the macro-organizational changes involved in formation of the NPQ-photoprotective state, since the super-complexes are examined following membrane solubilization rather than in the intact membrane. All information on the natural larger-scale organization of the PS II light-harvesting antenna is lost when membranes are solubilized.

However, there are a number of techniques with the potential to determine the layout of protein complexes within the intact thylakoid membrane, including negative-staining electron microscopy, freeze-fracture electron microscopy (FFEM), atomic force microscopy (AFM), and cryo-electron tomography. AFM has revealed the layout of complexes in grana membranes, but suffers from the disadvantage that, to allow high resolution, only individual, isolated layers of membrane fixed to a solid support can be examined (Kirchhoff et al. 2002). The non-physiological condition of the membrane limits the usefulness of AFM for high-resolution probing of the effects of a rapid physiological switch, such as NPQ. Cryo-electron tomography has perhaps the greatest potential for probing the structural changes involved in NPQ, since the latter technique is potentially able to visualize the layout of complexes within an entire intact chloroplast rapidly frozen in a particular physiological state. However, with current technology, sufficient resolution can only be obtained using isolated grana stacks rather than intact chloroplasts (Kouřil et al. 2011). Therefore, the main techniques currently in use are negative-staining and freeze-fracture electron microscopy.

Negative-staining electron microscopy provides higher resolution, but has the disadvantage that the membranes must be isolated and fixed during preparation, which limits the ability to probe rapid physiological changes. Nevertheless, Betterle et al. (2009) were able to use negative-staining electron microscopy to show changes in PS II macro-organization associated with NPQ induction in *Arabidopsis* grana membranes. Quantification of the images revealed a lower mean separation between PS II core complexes (i.e., closer packing of these complexes) following NPQ induction. This change did not occur in a mutant lacking the PsbS protein (Betterle et al. 2009), which is suggestive of a dissociation of the outer part of the LHCII antenna from PS II, allowing the smaller, modified PS II super-complexes to pack together more closely. However, since LHCII could not be directly resolved, the images do not give any direct information on the arrangement of LHCII complexes in the NPQ state.

FFEM generally provides lower resolution than negative-staining electron microscopy, but offers considerable other advantages. Images can be obtained via FFEM from intact chloroplasts rapidly frozen in different physiological states, and both PS II and LHCII can be visualized, albeit in different membrane fracture faces. Johnson et al. (2011) used FFEM to probe the organization of both PS II and LHCII in intact spinach chloroplasts either dark-adapted or following NPQ induction (Fig. 17.1). Quantification of the images revealed a substantial change in macro-organization following NPQ induction. Consistent with the results of Betterle et al. (2009), the mean nearest-neighbor distance among PS II complexes decreased following NPQ induction (indicating closer packing), and mean PS II particle size decreased. At the same time, mean nearest-neighbor distances between LHCII complexes also decreased, indicating formation of clustered domains containing only LHCII (Fig. 17.2). This re-organization was promoted both by exposure to increased light sufficient to induce qE (350 µmol photons m^{-2} s^{-1} for 5 min) and by the presence of zeaxanthin, which strengthens the argument that changes in macro-organization are directly associated with NPQ. The results of Johnson et al. (2011) provide the first direct evidence that NPQ induction is accompanied by formation of segregated domains of clustered LHCII proteins. These domains are very likely to be the site of thermal de-excitation (of singlet-excited chlorophyll a) in the PS II antenna, similar to the model proposed by Holzwarth and co-workers (Miloslavina et al. 2008; Fig. 17.3).

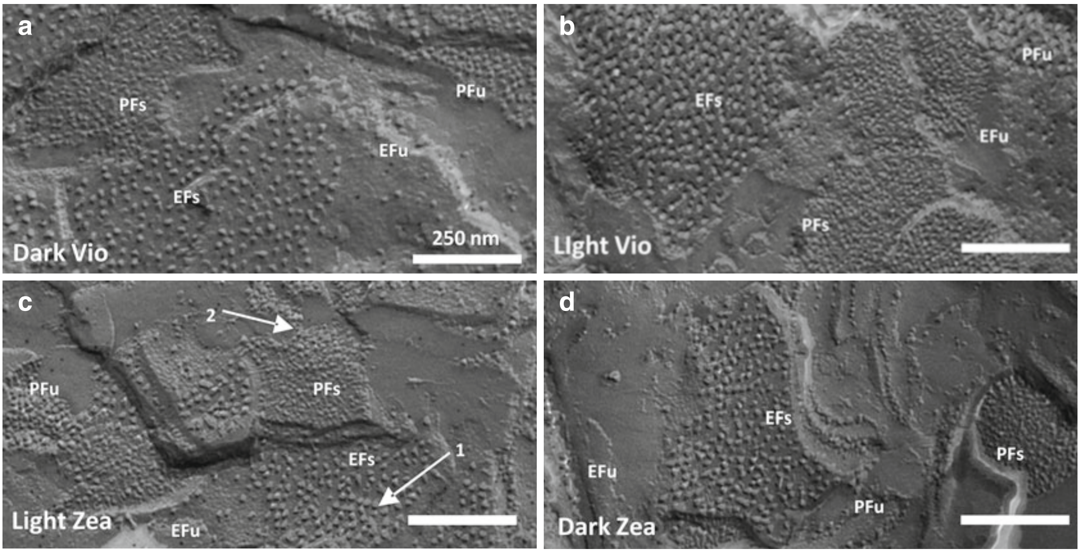

Fig. 17.1. Freeze-fracture electron micrographs showing thylakoid membrane structures from intact spinach chloroplasts adapted to different conditions: (**a**) Dark + violaxanthin (Vio), (**b**) Light + violaxanthin, (**c**) Light + zeaxanthin (Zea), (**d**) Dark + zeaxanthin. Inset *arrows* in (**c**) highlight areas with enhanced clustering of PS II (*1*) and LHCII (*2*). Labels: *EFs and PFs* exoplasmic (lumenal) and protoplasmic (stromal) fracture faces of the stacked grana regions of the thylakoid membrane, *EFu and PFu* exoplasmic and protoplasmic fracture faces of unstacked (stromal lamellae) regions of the thylakoid membrane (Reproduced from Johnson et al. (2011). Copyright American Society of Plant Biologists).

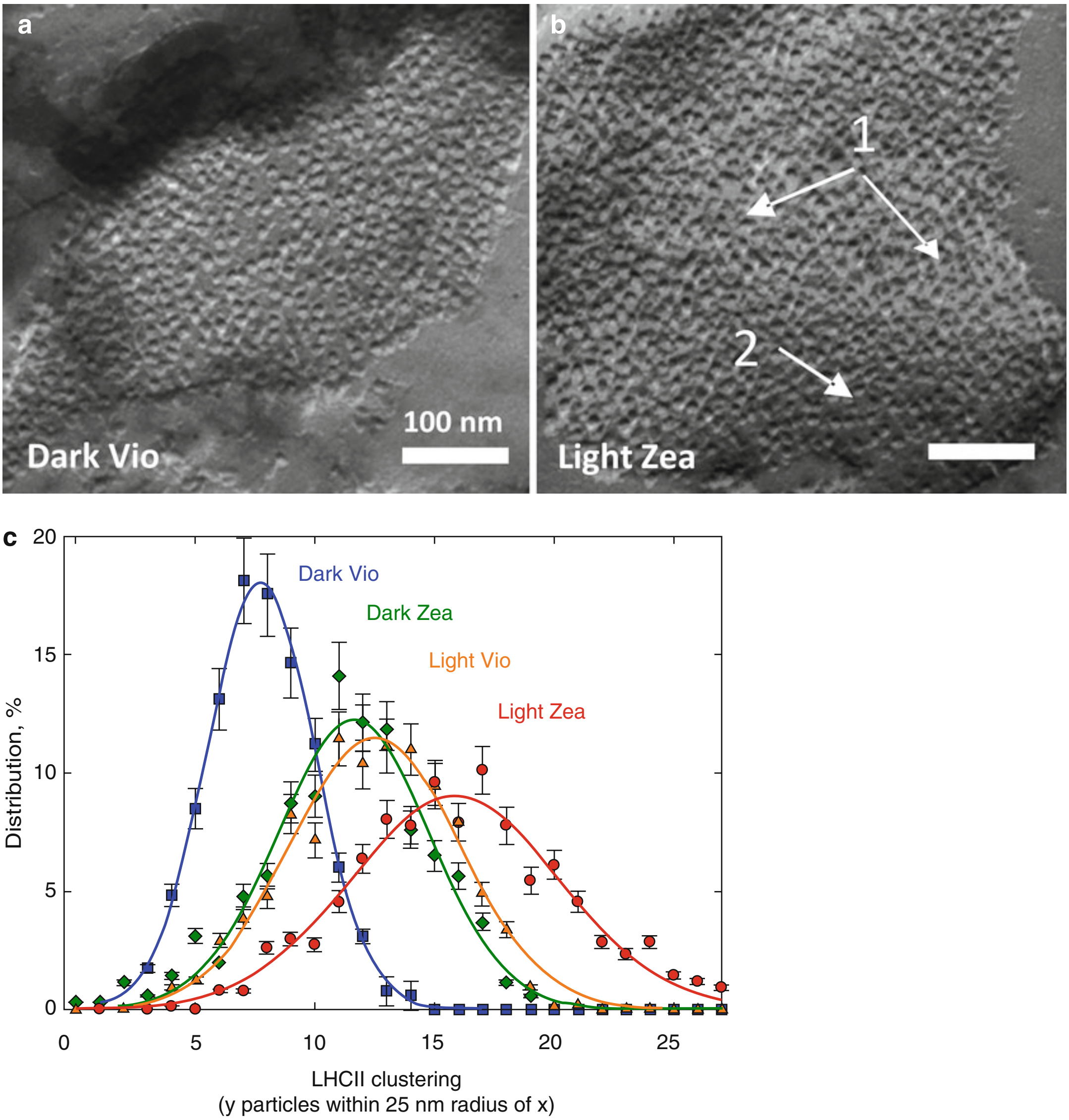

Fig. 17.2. Higher magnification view of LHCII PFs in freeze-fracture electron micrographs of intact spinach chloroplasts. (**a**) Dark + Violaxanthin (Vio) and (**b**) Light + Zeaxanthin (Zea). Inset *arrows* highlight areas with enhanced (*1*) and reduced (*2*) clustering of LHCII. Analysis of LHCII PFs from freeze-fracture electron micrographs of intact spinach chloroplasts. Graphs showing the percentage distribution of (**c**) LHCII clustering (number of neighboring LHCII particles within a 25 nm radius of a given LHCII particle).

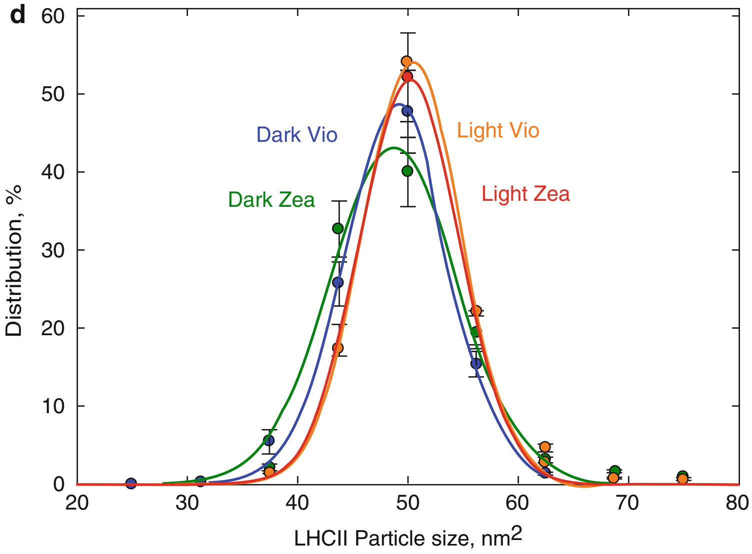

Fig. 17.2. (continued) and (**d**) LHCII particle size (area) in Dark + Violaxanthin (*blue squares*), Light + Violaxanthin (*orange triangles*), Light + Zeaxanthin (*red circles*), and Dark + Zeaxanthin (*green diamonds*) intact spinach chloroplasts. Data are averaged from three independent experiments, ± standard error of the mean. Reproduced from Johnson et al. (2011). Copyright American Society of Plant Biologists.

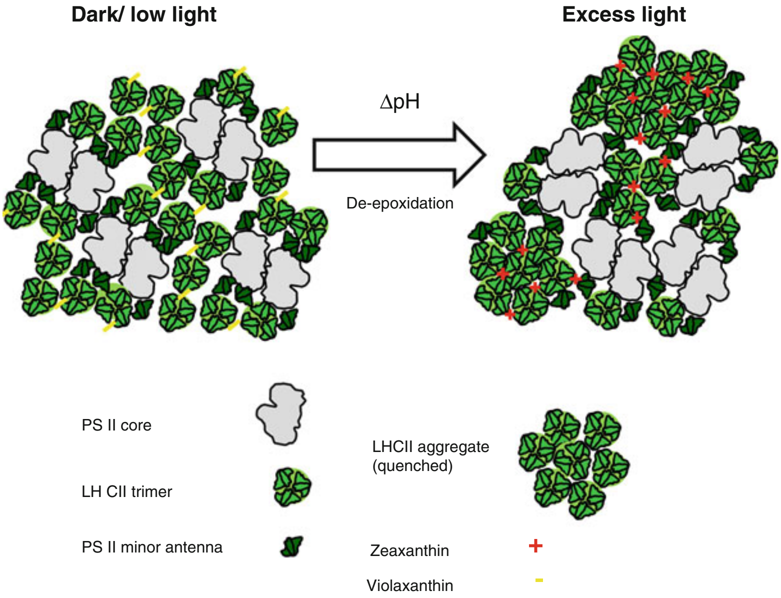

Fig. 17.3. Structural model of NPQ related re-organization of thylakoid grana membranes. In the dark and low light LHCII is distributed fairly evenly in the grana forming large C2S2M2 supercomplexes with PS II and minor antenna proteins. In excess light, ΔpH triggers a conformational change within LHC complexes that causes the partial dissociation of the PS II-LHCII supercomplex and leads to LHCII aggregation. De-epoxidation of violaxanthin to zeaxanthin promotes LHCII aggregation and thus NPQ. Reproduced from Johnson et al. (2011). Copyright American Society of Plant Biologists.

IV Mobility of Chlorophyll-Protein Complexes Within Thylakoid Membranes

Given the evidence that NPQ induction involves large-scale changes in the organization of PS II and its light-harvesting antenna (outlined in sections II and III), a complete model of NPQ must take into account the mobility of chlorophyll-protein complexes in thylakoids. Mobility of protein complexes within grana membranes is especially relevant, as grana are the site of the macro-organizational changes associated with NPQ induction (Goss and Garab 2001; Holm et al. 2005; Lambrev et al. 2007; Betterle et al. 2009; Johnson et al. 2011).

Grana membranes are particularly densely packed with membrane-integral proteins that occupy around 70–80 % of membrane area (Kirchhoff et al. 2002). At such high densities, macromolecular crowding is likely to provide a major impediment to protein diffusion (Kirchhoff et al. 2004). The dense packing of protein complexes in grana membranes is necessary for efficient light harvesting (Haferkamp et al. 2010), but comes at the expense of restricted mobility of complexes for turnover and regulation (Mullineaux 2008). Mobility of at least some of the chlorophyll-binding proteins in the grana was inferred from biochemical studies that demonstrated redistribution of complexes between grana and stroma lamellae as a result of adjustments such as state transitions (Drepper et al. 1993). More recently, fluorescence recovery after photobleaching (FRAP) with a laser-scanning confocal microscope was employed to directly observe diffusion of chlorophyll-proteins in plant thylakoid membranes. Initial studies used isolated spinach grana membranes fused into larger membrane patches on an artificial lipid bilayer support (Kirchhoff et al. 2008). These studies showed that the majority (~75 %) of chlorophyll fluorescence is completely immobile on the timescale of the measurement (indicating that it arises from chlorophyll-protein complexes that do not detectably relocate in the membrane). However, a small population of chlorophyll-proteins (accounting for about 25 % of chlorophyll fluorescence) was able to diffuse at an appreciable rate (lateral diffusion coefficient of ~0.005 μm^2 s^{-1}). This finding suggests that the majority of chlorophyll-proteins in the grana are locked in place by a combination of supra-molecular interactions and macromolecular crowding. Nevertheless, there is sufficient diffusion space for a subpopulation of complexes (probably including some of the LHCII) to move between the other complexes. At the measured diffusion coefficient, this mobile population would be able to migrate from the center to the periphery of a granum within a few seconds (Kirchhoff et al. 2008), which indicates that substantial rearrangement of complexes may be possible on the same timescale as NPQ induction.

Subsequent studies used FRAP to probe chlorophyll-protein mobility within the thylakoid membranes of intact spinach and *Arabidopsis* chloroplasts (Goral et al. 2010, 2012; Johnson et al. 2011). The topography of intact thylakoid membranes is too complex and heterogeneous for simple measurement of diffusion coefficients by confocal FRAP (Mullineaux and Sarcina 2002). Nevertheless, some aspects of protein mobility can be detected in this system by measuring the "mobile fraction" of chlorophyll-protein complexes (giving rise to chlorophyll fluorescence) that migrate between separate grana on a timescale of a few minutes. The size of this mobile fraction (typically about 15 % of chlorophyll-protein complexes contributing to chlorophyll fluorescence) proved to be a robust indicator of chlorophyll-protein mobility within the thylakoid membrane system, although it gives no specific information on the diffusion coefficient (Goral et al. 2010). Protein mobility is significantly modulated by physiological adaptation. For example, mobility increases following photoinhibition (for more on photoinhibition, see Adams and Demmig-Adams, Chap. 2, Adams et al., Chap. 23, and Demmig-Adams et al., Chap. 24), and this effect is dependent on the presence of the Stn7 and Stn8 protein kinases (Goral et al. 2010; Herbstová et al. 2012). Formation of the rapidly reversible NPQ-photoprotective state, on the other hand, significantly decreases protein mobility, presumably as a consequence of the segregation and clustering of PS II and LHCII discussed in section III (Johnson et al. 2011). Protein mobility is, furthermore, significantly altered in *Arabidopsis* mutants with alterations in the complement of minor LHC proteins (Goral et al. 2012).

These findings also suggest that granal protein mobility may be significantly different in different plant species, and in differently acclimated plants (for a summary of differences in NPQ induction and extent as dependent on plant species and growth conditions, see Demmig-Adams et al., Chap. 24). The fact that the NPQ-photoprotective state appears to require significant rearrangement of protein complexes in the grana raises the possibility that the mobility of chlorophyll-proteins in the grana may be a major factor controlling the kinetics of NPQ formation.

V Induction of NPQ Correlates with Mobility of Protein Complexes

Evidence in support of chlorophyll-protein mobility as one of the factors controlling the kinetics and amplitude of NPQ induction comes from a study by Goral et al. (2012), who used a range of *Arabidopsis* mutants deficient in specific PS II light-harvesting proteins (Lhcb1 and Lhcb2, CP26, CP24, CP29), as well as with alterations in the expression of the PsbS protein. Some of these light-harvesting antenna mutants had previously been shown to have strong perturbations in NPQ formation (Li et al. 2000; Anderson et al. 2003; Kovacs et al. 2006). The latter mutants also exhibited significant variations in chlorophyll-protein mobility as judged from FRAP, and in the extent of formation of two-dimensional, semi-crystalline arrays of PS II complexes, as judged from FFEM (Goral et al. 2012). There is a positive correlation between protein mobility in the dark-adapted state and NPQ formation upon exposure to light. There is, furthermore, a negative correlation between the extent of PS II array formation (arrays defined as semi-crystalline patches of ordered PS II complexes, with a minimum of four aligned PS II centers at their longest axis) and NPQ formation (Fig. 17.4; Goral et al. 2012). The PsbS mutants follow the same trend, i.e., the PsbS-deficient *npq4* mutant (Li et al. 2000) shows decreased protein mobility in the dark-adapted state, increased formation of PS II arrays and reduced ability to develop NPQ (Fig. 17.4). By contrast, the L17 mutant that over-expresses PsbS shows the opposite effects, i.e., increased protein mobility in the dark-adapted state, no detectable formation of PS II arrays, and enhanced NPQ formation (Fig. 17.4; Goral et al. 2012).

The results reviewed above suggest a simple model for the previously established crucial role of the PsbS protein in NPQ formation (Li et al. 2000). In the dark-adapted state, PsbS inhibits the formation of PS II arrays and tight packing of PS II super-complexes. It may be such that the presence of PsbS at sub-stoichiometric levels (i.e., fewer than 1 PsbS per PS II on average) introduces heterogeneity to PS II super-complexes, thus inhibiting their tight packing. A looser packing of PS II complexes in the presence of PsbS could facilitate protein mobility in the grana membranes, and this could, in turn, allow a much more rapid rearrangement of complexes following light exposure and the formation of a pH gradient across the thylakoid membrane. In this model, PsbS acts primarily as a “lubricant” of the membrane, promoting flexibility and rapid adaptation by

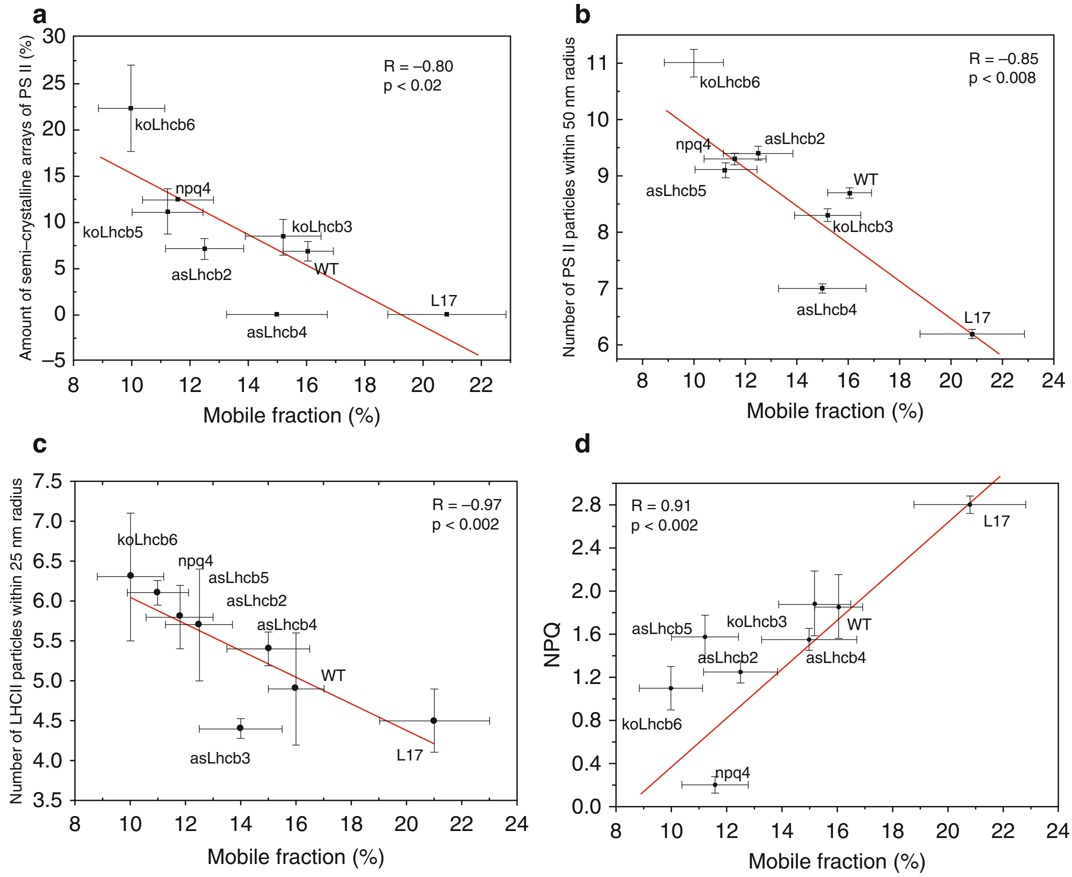

Fig. 17.4. Relationships between the mobility of chlorophyll-proteins in *Arabidopsis thaliana* light-harvesting antenna and PsbS mutants with different parameters obtained from analysis of freeze-fracture electron micrographs and NPQ values. Negative correlations between the mobility and (**a**) the abundance of semi-crystalline arrays present in the grana membranes, (**b**) PS II clustering and (**c**) LHCII clustering. (**d**) A positive correlation between the mobility of chlorophyll-proteins in the dark and the amount of NPQ formed after light treatment. Correlation excluding the *npq4* and L17 PsbS mutants is still significant with R = 0.82 and $p = 0.045$. Error bars = standard error. R = Pearson correlation coefficient, p = statistical significance of the correlation. Reproduced from Goral et al. (2012).

preventing PS II super-complexes from locking into a rigid macro-organization.

It should be noted that PsbS is probably not the only physiologically important factor with such an effect, since Kouřil et al. (2013) recently showed that high-light-adapted *Arabidopsis* plants exhibit a much lower content of PS II semi-crystalline arrays, despite the fact that PsbS expression was not increased (and in fact somewhat decreased) in their high-light growth conditions (in contrast to other authors who found increased PsbS expression in high light, e.g., Demmig-Adams et al. 2006; Zarter et al. 2006; Ballotari et al. 2007). Furthermore, PsbS is absent in many algae, where another light-harvesting protein (LHCSR: a member of the LHC superfamily induced under stress conditions) appears to play a similar role in facilitating NPQ (Peers et al. 2009; see also Morosinotto and Bassi, Chap. 14; Finazzi and Minagawa, Chap. 15). It will be interesting to see whether LHCSR may also affect mobility and/or crystalline packing of PS II super-complexes.

VI An Integrated Model for NPQ Formation in Plants

Figure 17.5 depicts a simple working model that links changes in macro-organization discussed in sections II, III, IV, and V with the roles of trans-thylakoid ΔpH, PsbS and the xanthophyll cycle carotenoids. It must be noted that this remains a working model that requires further experimental testing. Formation of the quenching state is assumed to be driven co-operatively by ΔpH and de-epoxidation of violaxanthin to zeaxanthin (for associations between zeaxanthin and NPQ in plants under natural conditions, see Adams and Demmig-Adams, Chap. 2, Adams et al., Chap. 23, and Demmig-Adams et al., Chap. 24). Since ΔpH formation triggers the concomitant efflux of Mg^{2+} from the thylakoid lumen (Barber et al. 1974), exposure to light increases the H^+ concentration on the lumenal side of the membrane and the Mg^{2+} concentration on the stromal side. Mg^{2+} electrostatically screens acidic amino acids on the lumenal and stromal surfaces of LHCII and is crucial not only to the stacking of thylakoid membranes but also to the stability of the PS II-LHCII super-complexes themselves (Barber 1982). For further discussion of the effects of electrostatic interactions on grana stacking, see Standfuss et al. (2005).

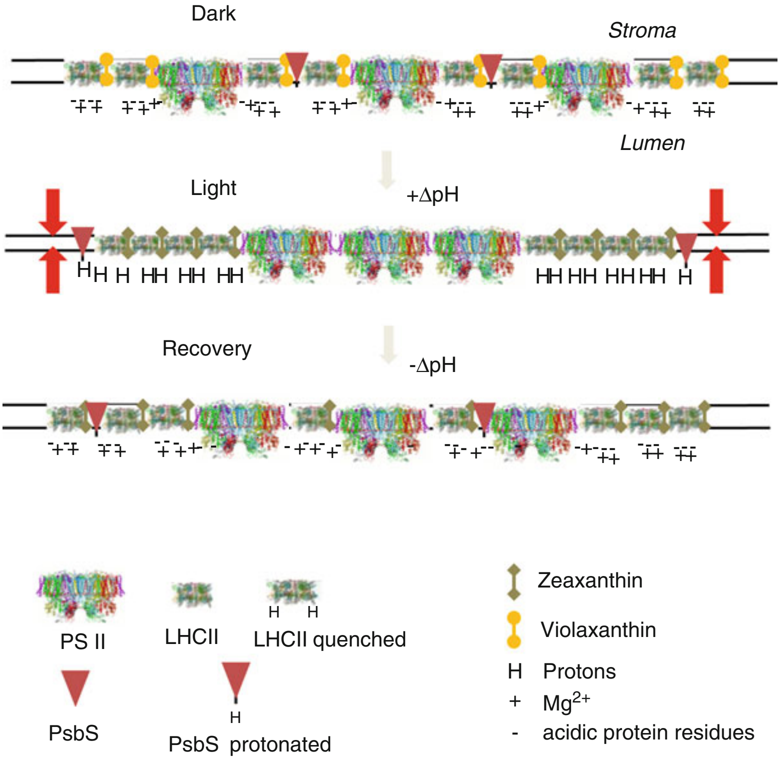

Fig. 17.5. Schematic model depicting macro-structural events underlying NPQ formation in the photosynthetic membrane. In the dark and low light, short range coulombic repulsion forces between LHCII proteins and the anti-aggregation effect of violaxanthin maintain the unquenched state, while Mg^{2+} electrostatically screens negative charges stabilizing PS II-LHCII association. PsbS disrupts the order of the PS II-LHCII macrostructure maintaining protein mobility. In excess light, an influx of protons into the lumen neutralizes acidic protein residues triggering synthesis of zeaxanthin and abolishing the repulsive forces between LHCII; attractive van der Waals interactions, strengthened by hydrophobic zeaxanthin, lead to segregation of LHCII from PS II and its aggregation; the accompanying decrease in membrane thickness (*red arrows*) leads to the enhancement of dissipative pigment-pigment interactions within LHCII. The exact location of PsbS in each state remains unknown.

We thus suggest that it is ultimately the redistribution of ions that triggers the excess light-induced formation of the quenching state, following previous suggestions by Lambrev et al. (2007) and Garab et al. (1998; see also Garab, Chap. 16). In our model, protonation of acidic protein residues on the lumenal thylakoid membrane side neutralizes these residues completely, thereby weakening short-range coulombic repulsion forces between LHCII proteins within grana and allowing hydrophobic and van der Waals interactions to foster self-association among LHCII proteins along with their partial segregation from PS II (Figs. 17.3 and 17.5). It will be interesting to see whether the macro-organizational changes associated with NPQ formation (Figs. 17.1 and 17.2) can be induced artificially by a combination of changes in pH and Mg^{2+} concentration.

Conversion of the more hydrophilic violaxanthin into the more hydrophobic zeaxanthin was found to enhance the NPQ-related structural change, consistent with the ability of zeaxanthin to promote LHCII aggregation in vitro (Ruban et al. 1997b). Condensed states of LHCII in vitro, such as aggregates and crystals, facilitate dissipative pigment interactions within individual complexes, thereby efficiently shortening the chlorophyll excited-state lifetime (Ruban and Horton 1992; Pascal et al. 2005; Johnson and Ruban 2009). The non-linear relationship between structural changes and NPQ may be explained by a non-linear dependence of both energy transfer and excitonic interactions (for excitonic interactions, see also Walla et al., Chap. 9 and Ostroumov et al., Chap. 4) on inter-pigment distance, possibly causing NPQ amplitude to rise as LHCII becomes increasingly condensed.

PsbS presumably controls the rate at which the NPQ-associated change in PS II-LHCII macro-organization can occur, most likely by enhancing protein mobility within the thylakoid membrane. It is also possible that the looser and more mobile membrane organization promoted by PsbS facilitates the effect of zeaxanthin by promoting percolation of xanthophyll molecules into the LHCII antenna. PsbS is therefore an elegant response of evolution to the need to maintain a membrane packed with large light-harvesting complexes in a sufficiently fluid state to allow rapid regulatory re-organization of the protein landscape.

Acknowledgments

We thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) and Engineering and Physical Sciences Research Council (EPSRC), The Royal Society, The Leverhulme Trust and EU FP7 (Marie Curie ITN “HARVEST”) for financial support for our research.

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Chapter 18

Control of Non-Photochemical Exciton Quenching by the Proton Circuit of Photosynthesis

Deserah D. Strand
*Department of Plant Biology, and MSU-DOE Plant Research Laboratory,
Michigan State University, 612 Wilson Rd 106, East Lansing, MI 48824, USA*
and
David M. Kramer*
*Department of Plant Biology, Department of Biochemistry and Molecular Biology,
and MSU-DOE Plant Research Laboratory, Michigan State University,
612 Wilson Rd 106, East Lansing, MI 48824, USA*

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*Author for Correspondence, e-mail: kramerd8@msu.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_18, © Springer Science+Business Media Dordrecht 2014

Summary

This chapter discusses our current understanding of the chloroplast proton circuit, i.e., those reactions that involve the storage and utilization of light energy in the transfer of protons, and its importance for regulating photosynthetic light-capture/electron-transfer reactions. The photosynthetic machinery of plants is finely tuned to balance the needs for efficient light capture with an avoidance of photodamage by regulating the capture of light energy, via thermal dissipation of excess excitation energy (assessed from non-photochemical quenching, NPQ, of chlorophyll fluorescence) by regulating light-driven electron transfer processes. In addition to driving ATP synthesis at the chloroplast ATP synthase, the thylakoid electrochemical gradient of protons or proton-motive force (*pmf*) plays a central role in regulating NPQ. The trans-thylakoid proton concentration gradient (ΔpH) component of *pmf* triggers the "energy-dependent", or qE component of NPQ, which protects photosystem II from photodamage and regulates electron transfer through the cytochrome b_6f complex, thereby preventing damage to photosystem I. The extent and mode of storage in ΔpH and ΔΨ of *pmf* are regulated by several processes that respond to the metabolic, or physiological, state of the organism. The extent of *pmf* is determined by proton influx (via linear and alternative electron flows) into the thylakoid lumen, and proton efflux through the chloroplast ATP synthase. Both processes are modulated by, or responsive to, environmental conditions and resulting metabolic fluctuations. Proton influx is controlled by linear electron flow and a series of alternative electron flow pathways, possibly including cyclic electron flow around photosystem I, the Mehler peroxidase reaction (or water-water cycle), and oxidation of plastoquinol by the plastid terminal oxidase. The fraction of *pmf* stored as ΔpH is also regulated by plastidic ionic strength or luminal buffering capacity, altering the sensitivity of pH-dependent processes to *pmf*. The integrated regulation of these processes is an open, active area of research.

I Introduction

Sunlight, harnessed in photosynthesis, is the energy source for most life on our planet. Energy from absorbed sunlight presents a challenge for photosynthetic organisms because within the light reactions of photosynthesis, highly reactive redox intermediates are formed that can generate harmful reactive oxygen species (ROS). Thus, plants must regulate photosynthetic light harvesting and electron transfer to avoid photodamage, especially when light input exceeds the capacity of photosynthesis to safely process it (Allahverdiyeva and Aro 2012). To meet this need, essentially every process in photosynthesis is regulated to balance minimization of deleterious side reactions with the need for efficient capture of solar energy (Kramer and Crofts 1996; Kramer and Evans 2011). This regulation is critical for efficient energy conversion, since most of the energy captured by an ecosystem is lost within the light reactions of photosynthesis, much of it dissipated in photoprotective processes, the level of which can be estimated from the non-photochemical quenching (NPQ) of chlorophyll fluorescence (Long et al. 2006; Zhu et al. 2007; Hambourger et al. 2009).

Abbreviations: APX – Ascorbate peroxidase; AsA – Ascorbate (ascorbic acid); CEF – Cyclic electron flow around photosystem I; Fd – Ferredoxin; FNR – Ferredoxin NADP$^+$ reductase; FQR – Ferredoxin:quinone reductase; LEF – Linear electron flow; MDA – Monodehydroascorbate; MDH – NADP malic enzyme; MPR – Mehler peroxidase reaction; NDA2 – Type II NADH:plastoquinone oxidoreductase; NDH – NADPH:plastoquinone oxidoreductase; NPQ – Non-photochemical quenching (of chlorophyll fluorescence); PGR5 – Proton gradient regulation 5; PGRL1 – PGR5-Like protein; *pmf* – Proton motive force; PQR – Plastoquinone reductase; PS I – Photosystem I; PS II – Photosystem II; PTOX – Plastid terminal oxidase; qE – 'Energy dependent' quenching; ROS – Reactive oxygen species; SOD – Superoxide dismutase

This is a critical point: even though the rate-limiting steps in photosynthesis occur in biochemical processes that follow the initial storage of energy by the light reactions, these limitations inevitably result in the loss of energy by one of two mechanisms: backup of electrons and protons resulting in long-lived excitation states (and potential ROS formation), or in activation of processes that "dump" or dissipate energy in excess of that needed to sustain the downstream processes. As discussed below, the former mechanism can result in the buildup of deleterious intermediates while the second prevents their generation, but, if not tightly regulated, could lead to excessive energy loss.

Over developmental time scales, this regulation can occur by altering such processes as gene expression, protein accumulation, and the synthesis of pigments and antioxidants (Pfannschmidt 2003; Eberhard et al. 2008). However, plants must also respond to rapid fluctuations in environmental conditions such as light and temperature, requiring equally rapid regulation at the level of the photosynthetic machinery itself (Adams et al. 1999; Müller et al. 2001; Cruz et al. 2005; Demmig-Adams et al., Chap. 24).

One of the major mechanisms for this regulation (and the main topic of this book) is NPQ of antenna excitation energy, a key component of which, qE ("energy dependent" quenching), is triggered by acidification of the thylakoid lumen caused by the transthylakoid proton concentration gradient, or ΔpH component of thylakoid proton-motive force (*pmf*) (see Brooks et al., Chap. 13). The *pmf* is generated by the light-induced electron transfer reactions and, in turn, drives photophosphorylation of ADP to ATP. In this chapter, we review our understanding of how the process of activating qE is intimately integrated into the entire energy storage system to balance energy capture and storage.

A The Electron and Proton Circuits of Photosynthesis

The light reactions of oxygenic photosynthesis consist of two highly integrated energy-storing circuits. The "electron circuit" stores energy through a series of light-driven electron transfer reactions, via the linear electron flow (LEF) pathway (Fig. 18.1; reviewed by Papageorgiou and Govindjee, Chap. 1; Ort and Yocum 1996; Eberhard et al. 2008;

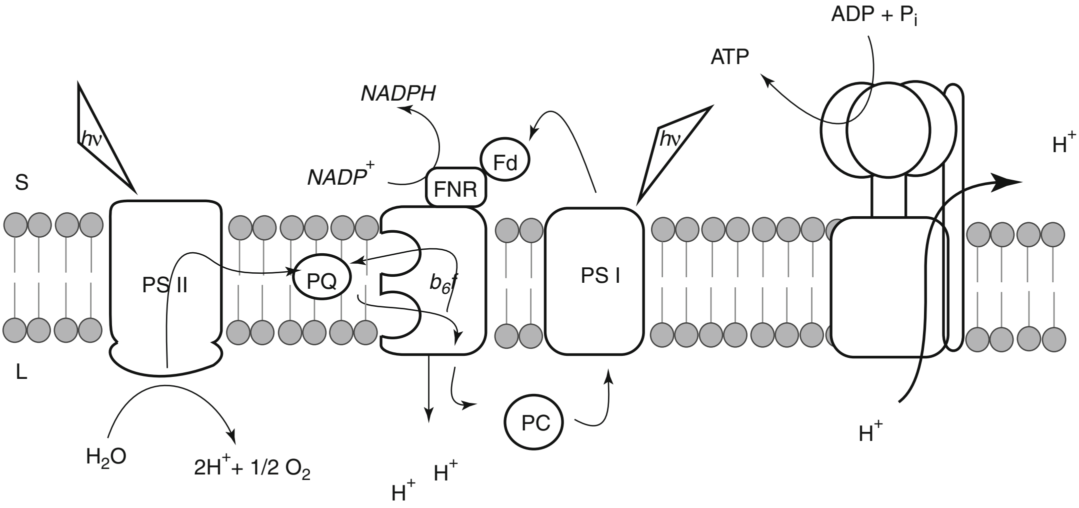

Fig. 18.1. The light-dependent electron and proton transfer reactions of photosynthesis. Electrons are extracted from water and passed through an electron transport chain. Protons are translocated into the lumen generating a *pmf* to drive ATP synthesis. Stroma (*S*), Lumen (*L*), Photosystem I (*PS I*), Photosystem II (*PS II*), Plastoquinone (*PQ*), Ferredoxin-NADP$^+$ reductase (*FNR*), Ferredoxin (*Fd*), Plastocyanin (*PC*), cytochrome b_6f (b_6f).

Cournac and Baker 2012). Light energy is captured by pigments in light-harvesting complexes and shunted to a special subset of chlorophylls in photosystem I (PS I) and photosystem II (PS II) (reviewed, e.g., by Ostroumov et al., Chap. 4; Melis 1996). The photochemically excited chlorophylls in PS II extract electrons from water and transfer them through an electron transfer chain, reducing plastoquinone (PQ) to plastoquinol (PQH_2), then to the cytochrome b_6f complex, plastocyanin, and finally to PS I. An additional photon of light energy is introduced at PS I, driving electron transfer to ferredoxin (Fd) and $NADP^+$, which are then used to drive reductive processes in the cell.

The "proton circuit" is energetically and mechanistically coupled to the electron circuit to store energy in an electrochemical gradient of protons between the stroma and thylakoid lumen across the thylakoid membrane, or *pmf*, which drives the synthesis of ATP through the ATP synthase that spans the thylakoid membrane. Protons are released into the thylakoid lumen during water oxidation at PS II. Additional protons are translocated via the Q-cycle, an enzymatic process catalyzed by the cytochrome b_6f and related complexes involving the oxidation and reduction of plastoquinones and subsequent electron and proton transfer reactions that results in proton translocation across the thylakoid membrane (Cape et al. 2006; Cooley et al. 2008; Cramer et al. 2011).

B The Proton Circuit Is a Central Regulator of Photosynthesis

When light absorption exceeds the capacity of the downstream reactions of photosynthesis, reactive intermediates may accumulate potentially leading to photoinhibition and photodamage, although the relationship between reactive intermediates and photoinhibition is still controversial (reviewed in Osmond and Förster 2008; see also Adams et al., Chap. 23; Demmig-Adams et al., Chap. 28). Plants have multiple mechanisms of avoidance and dissipation of excess light, including activation of processes underlying NPQ, changes in leaf orientation, chloroplast movement, rearrangement of the photosynthetic machinery, and changes in photosynthetic gene expression (Pfannschmidt 2003; Baginsky and Link 2008; Demmig-Adams et al. 2006; Allahverdiyeva and Aro 2012; Logan et al., Chap. 7).

This chapter focuses on how the activation of a process reflected in NPQ and other processes that regulate light capture and electron flow interact with the proton circuit of photosynthesis. We concentrate mainly on these processes in higher plants and, where appropriate, in algae. The case of cyanobacteria is not covered here because, in these organisms, NPQ is controlled by factors not directly related to *pmf* (see Kirilovsky et al., Chap. 22).

Much of the dynamic regulation of photosynthesis directly involves the thylakoid proton circuit, which modulates light capture, photoprotection, and electron transfer allowing the light reactions to respond to changes in the physiology of the organism. Chloroplasts are thought to protect themselves from photodamage by dissipating excess light energy, as reflected in NPQ (Müller et al. 2001; Adams and Demmig-Adams, Chap. 2; Demmig-Adams et al., Chap. 24). The major, rapidly reversible component of NPQ in plants is termed qE (for energy-dependent quenching), and is triggered by the ΔpH component of *pmf*, which acidifies the lumen, thereby activating violaxanthin deepoxidase, catalyzing the conversion of violaxanthin to antheraxanthin and zeaxanthin (see Eskling et al. 2001), and protonating the antenna-associated protein PsbS (Li et al. 2004a; Brooks et al., Chap. 13).

Photosynthetic electron flow is regulated at the cytochrome b_6f complex by acidification of the lumen. As a result of the buildup of the ΔpH component of *pmf*, plastoquinol oxidation is slowed at the Q_o site (quinol oxidation) (Kramer et al. 2004; Takizawa et al. 2007). This prevents accumulation of electrons on highly reducing components of the acceptor side of PS I, which can lead to generation of reactive oxygen species (ROS) and/or affect redox regulation of metabolism.

In extreme cases, lumen acidification can sensitize PS II to light-induced destabilization (Krieger and Weis 1993; Kramer et al. 1999). There is evidence that this can occur in vivo under low CO_2 or environmental stress, such as drought (Takizawa et al. 2007). Because it produces inactive PS II, which quenches excitation energy and decreases electron transfer, photoinhibition may represent a regulatory "tactical retreat" to prevent overexcitation and damage to DNA or other cellular components (for a similar suggestion, see Demmig-Adams et al., Chap. 28; Tyystjärvi and Aro 1996; Nishiyama et al. 2006; Sarvikas et al. 2006).

The proton circuit is, in turn, modulated by secondary regulatory mechanisms, particularly at the ATP synthase, allowing the light reactions to respond to changes in the physiology of the organism. The activity of the ATP synthase is downregulated to retard proton efflux from the thylakoid lumen, increasing *pmf* and leading to down-regulation of light capture by qE and regulation of electron transfer reactions at the cytochrome b_6f complex (Kanazawa and Kramer 2002; Avenson et al. 2004, 2005a, b; Kramer et al. 2004; Cruz et al. 2005). The fraction of *pmf* (stored as ΔpH and electric field $\Delta\Psi$) affects the relationship between *pmf* and lumen acidification, further adjusting the pH-dependent responses underlying NPQ (Kramer et al. 2003; Avenson et al. 2004). In addition, the responses of qE to lumen pH can be modulated by altering the expression of qE-related proteins (Li et al. 2004a; Zhang et al. 2009; Murchie and Niyogi 2011; see also Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14).

These "flexibility mechanisms", which may adjust *pmf*-dependent NPQ responses (with *pmf* modulation of these processes being the focus of this chapter) to physiological and metabolic changes, fall into two categories (Kramer et al. 2004). "Type I" mechanisms adjust the "qE response" (defined as the activation state of qE as a function of LEF) by altering light-driven proton-transfer reactions via activation of alternative cyclic electron transfer processes and, because proton efflux from the lumen is generally coupled to ATP synthesis, affect the ratio of ATP/NADPH arising from photosynthetic electron transport. "Type II" flexibility mechanisms, on the other hand, adjust the qE response without altering the ATP/NADPH balance. The distinction between Type I and Type II mechanisms is critical because, despite both having an effect on qE, they have very different consequences for downstream metabolism, i.e., the requirement for the ATP/NADPH ratio to precisely match that required by downstream reactions may pose substantial constraints on the activation of Type 1 mechanisms.

II Type I Flexibility Mechanisms: Non-Photochemical Quenching (NPQ) of Chlorophyll Fluorescence and Balancing of the Chloroplast Energy Budget

Proton and electron transfer reactions in photosynthetic complexes are tightly coupled and consumption of photosynthetic products must thus match production to prevent buildup of reactive intermediates (Allen 2002; Eberhard et al. 2008; Kramer and Evans 2011). The ratio of ATP/NADPH from LEF is thought to be fixed at 1.3, approximately 13 % less than the 1.5 required for the Calvin-Benson cycle and other metabolic processes in the chloroplast (reviewed in Kramer and Evans 2011). The ATP and NADPH consumption within the chloroplast is also dynamic and dependent on the activation of multiple processes and thus requires rapidly adjustable plasticity in the relative output of ATP and NADPH. At least five processes have been proposed to remedy a deficit of ATP in the chloroplast (Backhausen et al. 1994; Scheibe 2004; Eberhard et al. 2008; Kramer and Evans 2011), as discussed in the following sections.

A Malate Valve

The "malate valve" (Fig. 18.2, reviewed in Scheibe 2004) involves shuttling of reducing equivalents from the chloroplast to other cellular compartments. NADP-malic enzyme

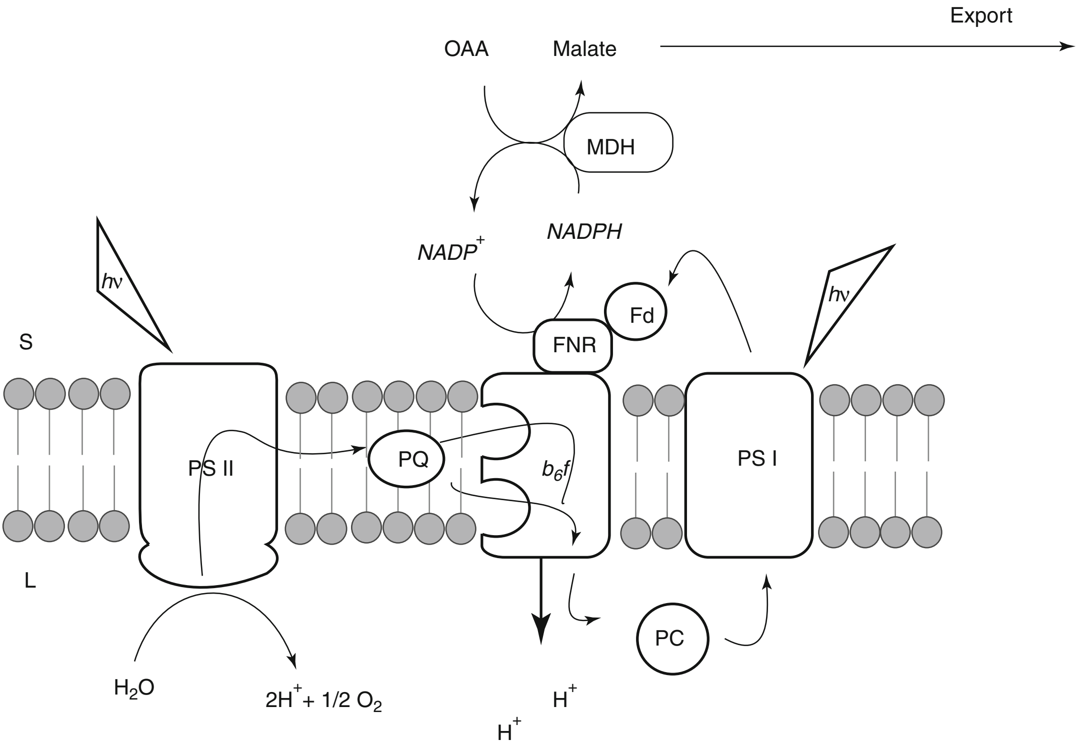

Fig. 18.2. The malate valve. NADPH is consumed in the production of malate from oxaloacetate (*OAA*) via NADP-Malate dehydrogenase (*MDH*). The malate is then exported from the chloroplast.

(MDH) uses NADPH to reduce oxaloacetate to malate. The malate can then be exported from the chloroplast to the mitochondria to generate ATP from respiration. In this process, ATP production is maintained while NADPH is only transiently reduced, allowing it to help balance the chloroplast ATP/NADPH ratio. MDH is inhibited by $NADP^+$, suggesting a control mechanism involving NADPH levels, which would be consistent with an involvement in balancing production of ATP and NADPH in the chloroplast. On the other hand, it has been argued that the activity of the malate valve is too low to account for substantial ATP/NADPH rebalancing under permissive conditions, i.e., conditions like those in the laboratory, where loss of productivity to environmental stress is minimal (Fridlyand et al. 1998; Scheibe et al. 2005), although there is also evidence that malate valve activity is increased under prolonged stress where the ratio of ATP/NADPH consumption may be higher (Scheibe 2004). Supporting this evidence, Hebbelmann et al. (2012) found upregulation of antioxidant pathways in mutants lacking MDH when exposed to high light, suggesting that the malate valve may play expanded roles under non-laboratory conditions.

B Mehler Peroxidase Reaction (MPR)

In the MPR, also referred to as the water-water cycle (Fig. 18.3), electrons from LEF are shunted away from NADPH reduction and instead reduce O_2 to superoxide at PS I (Robinson 1988; Asada 1999, 2006). Superoxide is then dismutated to H_2O_2 and O_2 via superoxide dismutase and then H_2O_2 is reduced to H_2O via ascorbate peroxidase. This leads to production of ATP via LEF-coupled accumulation of protons in the thylakoid lumen, without net reduction of NADPH (Asada 1999). The MPR appears to

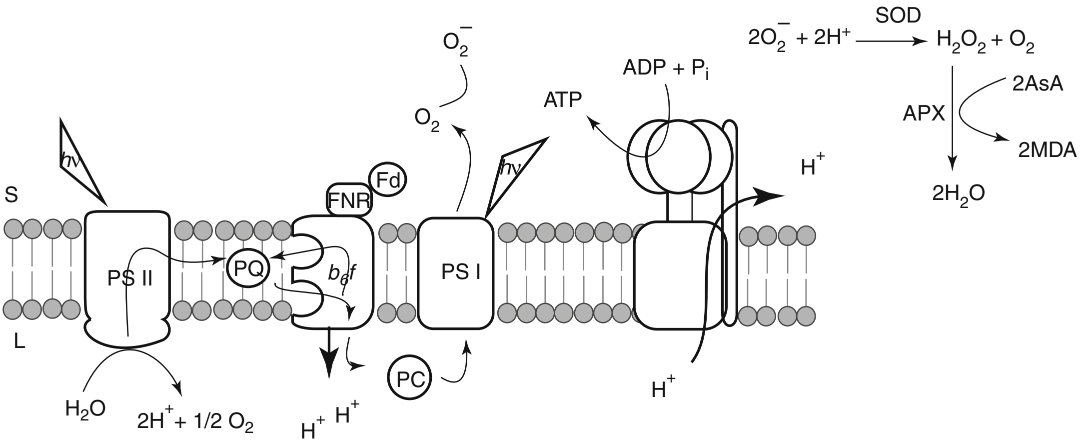

Fig. 18.3. The Mehler peroxidase reaction. Electrons from PS I reduce O_2 to superoxide (O_2^-) that is dismutated to H_2O_2 via superoxide dismutase (*SOD*), and then reduced to H_2O and O_2 via ascorbate peroxidase (*APX*), oxidizing ascorbate, or ascorbic acid (*AsA*), to monodehydroascorbate (*MDA*).

contribute significantly to ATP synthesis and photoprotection in cyanobacteria and bryophytes, but appears to have limited in vivo capacity in vascular plants and algae under permissive steady state conditions (Fridlyand et al. 1998; Ruuska et al. 2000; Clarke and Johnson 2001; Heber 2002; Driever and Baker 2011; Proctor and Smirnoff 2011; and reviewed in Badger et al. 2000). It has been reported that MPR may have an important role during activation of photosynthesis to prevent over-reduction of the electron transport chain under excess light (Asada 2000; Makino et al. 2002; Ort and Baker 2002).

C Plastid Terminal Oxidase (PTOX)

Chloroplasts also contain an alternate oxidase (PTOX) that oxidizes PQH_2 and reduces O_2 to H_2O (Fig. 18.4, reviewed in Joët et al. 2002a). Electron flow from PS II through PTOX could generate ATP from proton accumulation at PS II; however, its capacity would be limited by the fact that only one proton is expected to be deposited into the lumen per electron transferred from PS II through PTOX. In vascular plants, PTOX is important in developing plastids (Aluru et al. 2006) and some alpine plants (Rumeau et al. 2007; Laureau et al. 2013), although its contribution to ATP/NADPH balance in steady-state photosynthesis in most species is probably small (reviewed in Eberhard et al. 2008; Kramer and Evans 2011). It has recently been proposed that PTOX works in conjunction with the chloroplast NADPH: plastoquinone oxidoreductase (NDH, see below) to tune the redox state of the photosynthetic electron transport chain, and possibly acts as an electron sink under high-light conditions in tomato (Trouillard et al. 2012; Laureau et al. 2013).

D Exchange of ATP and ADP + P_i with Stroma

The ATP budget could also be balanced by direct exchange of ATP and ADP + inorganic phosphate (P_i) among chloroplast, cytosol, and mitochondria. Early suggestions that chloroplasts directly exchange ATP and ADP + P_i with the cytosol were questioned by observations of slow ATP and ADP exchange in isolated chloroplasts (Lilley et al. 1975; Stitt et al. 1982), leading to the generally accepted view that the chloroplast ATP budget is "self-contained". However, this has not been rigorously tested under physiologically relevant conditions, opening up the possibility that ATP and ADP + P_i exchange may, in fact, be sufficiently rapid to allow highly flexible energy balancing.

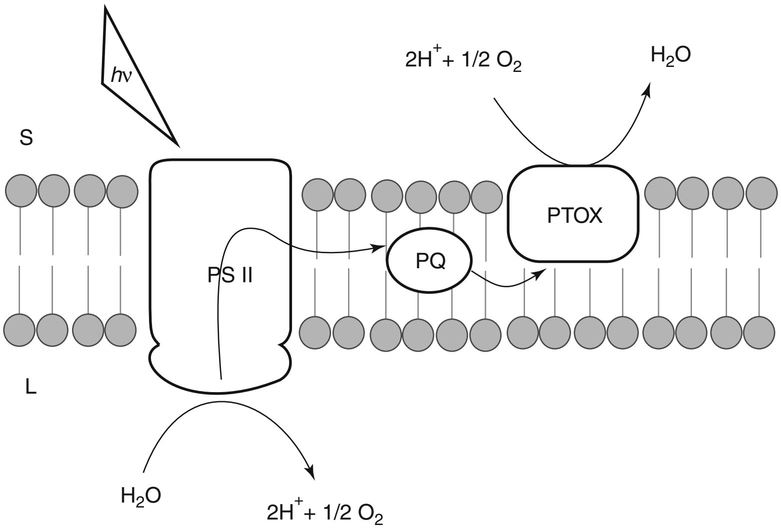

Fig. 18.4. The plastid terminal oxidase (*PTOX*). Electrons from PS II reduce plastoquinone (*PQ)* that is, in turn, oxidized via PTOX, bypassing the Q-cycle. Proton translocation into the lumen is only possible via the plastoquinone reductases (PS II in this figure).

E Cyclic Electron Flow (CEF) Around Photoststem I (PS I)

CEF is strongly implicated in the ATP/NADPH balance (Joliot and Joliot 2006; Joliot et al. 2006; Eberhard et al. 2008; Johnson 2010; Joliot and Johnson 2011; Kramer and Evans 2011). CEF involves photochemistry in PS I, but not PS II, resulting in oxidation of plastocyanin and reduction of Fd. Electrons from PS I are transferred back into the PQ pool in a ferredoxin-dependent process involving a plastoquinone reductase (PQR). Following its formation by PQR, PQH_2 is oxidized by the cytochrome b_6f complex via the Q-cycle (Cape et al. 2006; Cooley et al. 2008; Cramer et al. 2011), resulting in proton translocation into the thylakoid lumen that generates *pmf*. This *pmf* is then used to drive ATP synthesis. The electrons are passed back to PS I via plastocyanin. This process results in ATP formation, with no net NADPH production, altering the ATP/NADPH stoichiometry formed by the light reactions.

CEF appears to be minimally activated under non-stressed conditions in C3 plants, when LEF nearly meets the ATP required for chloroplast metabolism (Harbinson et al. 1989; Avenson et al. 2005a; Cruz et al. 2005; Fan et al. 2007; Laisk et al. 2007; Livingston et al. 2010a, b). However, CEF is upregulated when high ATP demand is greater, e.g., under environmental stress (Clarke and Johnson 2001; Rumeau et al. 2007; Jia et al. 2008; Kohzuma et al. 2009), when carbon-concentrating mechanisms or C4 photosynthesis are engaged (Kubicki et al. 1996; Finazzi et al. 2002), or during induction of photosynthesis when dark-adapted plants are suddenly exposed to light (Joët et al. 2002b; Joliot et al. 2004). CEF may also play a regulatory role via acidification of the thylakoid lumen (Golding et al. 2004; Kramer et al. 2004; Joliot and Johnson 2011). These ATP/NADPH balancing and regulatory roles for CEF are not independent, since ATP/NADPH balance is critical for maintaining *pmf*, and thereby regulating qE or electron flux through the cytochrome b_6f complex. However, CEF is clearly not essential for qE since qE can be observed in the absence of CEF (Avenson et al. 2005a) when other processes, including the regulation of the ATP synthase and partitioning of *pmf* into ΔpH and $\Delta\Psi$ components, can account for regulation of qE responses (Kanazawa and Kramer

2002; Avenson et al. 2004; Kramer et al. 2004; Cruz et al. 2005).

Several labs have isolated mutants, in *Arabidopsis* and tobacco, exhibiting high rates of CEF (*hcef*). Characterization of these mutants has established that CEF can run at high rates even in C3 plants, but must also be highly regulated in the wild type to prevent over-acidification of the thylakoid lumen. The high rates of CEF in *hcef* mutants has allowed more facile probing of key CEF processes to explore its regulation and biochemical pathways. The genetic loci of some of these *hcef* mutants have been identified (Gotoh et al. 2010; Livingston et al. 2010a), yielding hints about the mechanism and regulation of this process.

One high CEF mutation (*hcef1*) was found in the Calvin-Benson cycle enzyme fructose-1,6-bisphosphatase (Livingston et al. 2010a). Despite similar effects on overall photosynthesis, suppressing the expression of glyceraldehyde phosphate dehydrogenase (Livingston et al. 2010b) and fructose bisphosphate aldolase (Gotoh et al. 2010) led to increased CEF, while suppression of other enzymes, including Rubisco small subunit (Livingston et al. 2010b), ATP synthase and the Rieske protein of b_6f (Maiwald et al. 2003), or the triose-phosphate/phosphate translocator (Walters et al. 2004), did not lead to an apparent increase in CEF. These contrasting effects suggest that CEF, as discussed in more detail below, may be regulated by specific intermediates or byproducts, rather than simply by slowing overall photosynthesis.

1 CEF Pathways

The pathway(s) and regulatory mechanisms of CEF are currently the subject of debate. The identity of the PQR is as yet unresolved and several protein complexes have been proposed, but have yet to be fully characterized. Several putative PQRs, and many additional associated proteins have been proposed (Fig. 18.5), such as Proton Gradient Regulation 5 (PGR5) and the PGR5-Like

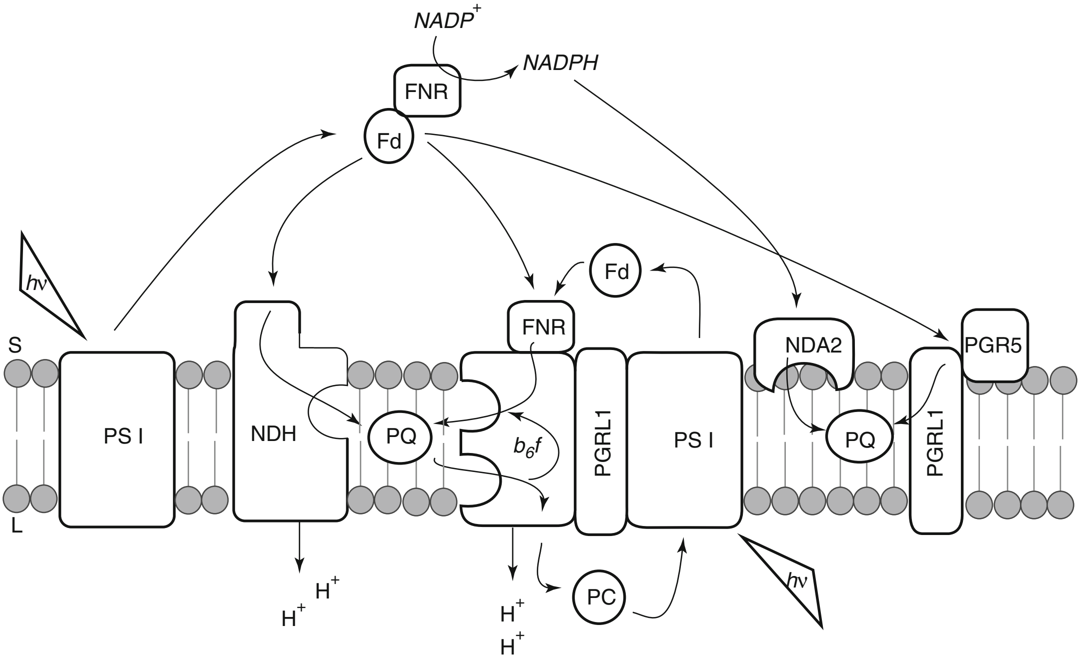

Fig. 18.5. Proposed routes of cyclic electron flow around photosystem I. (1) The thylakoid NAD(P)H dehydrogenase complex (*NDH*). (2) A PS I-b_6f supercomplex. (3) A Type II (non-proton pumping) NAD(P)H dehydrogenase complex (*NDA2*). (4) A ferredoxin plastoquinone reductase thought to be comprised of a complex containing PGRL1 and PGR5.

protein (PGRL1) (Munekage et al. 2002; DalCorso et al. 2008). Some of these are plant-specific, such as the NADPH dehydrogenase complex (NDH, Endo et al. 1998) or algae-specific, such as type II (non-proton pumping) NADH:plastoquinone oxidoreductase, NDA2 (Desplats et al. 2009). We note that these pathways are not necessarily mutually exclusive, and may either operate in parallel (Hosler and Yocum 1987; Scheller 1996; Munekage et al. 2004; Ducruet et al. 2005) or differentially under specific conditions (Takabayashi et al. 2005; Gotoh et al. 2010; Iwai et al. 2010).

(a) Chloroplast NDH Pathway

One proposed route of CEF is through the thylakoid NAD(P)H dehydrogenase complex (NDH, Fig. 18.5; Endo et al. 1998). The NDH complex is highly conserved in cyanobacteria and green plants across all phyla (Martin and Sabater 2010). The ~550-KDa (Burrows et al. 1998) plant complex consists of >24 subunits, 11 of which are chloroplast encoded (Suorsa et al. 2009) and are (with a few notable exceptions) highly homologous to mitochondrial and bacterial respiratory Complex I (Sazanov et al. 1998). The identification of novel plant-specific NDH-associated proteins suggests that the plastid NDH has diverged in structure and regulation from Complex I and cyanobacterial NDH-1 (Suorsa et al. 2009; Peng et al. 2009). Complex I contains an integral membrane hydrophobic arm with at least three proton channels (Sazanov et al. 1998); the genes coding for these channels are conserved in NDH (Casano et al. 2004; Suorsa et al. 2009).

In Complex I, proton pumping is attributed to conformational changes in conserved lysine and glutamate residues that are also conserved in chloroplast NDH (Casano et al. 2004; Efremov et al. 2010; Efremov and Sazanov 2011). The specific and generally high level of conservation of the NDH complex in vascular plants suggests that the same mechanism operates in plant chloroplasts. The occurrence of proton pumping in NDH would make CEF a very efficient ATP-generating process and imply an important role for the NDH complex in maintaining the thylakoid ATP budget.

In support of involvement of NDH in CEF, the *hcef1* mutant shows constitutively high CEF that requires the NDH complex (but not PGR5 that is involved in an alternative pathway; see below). These results suggest that NDH can catalyze high rates of CEF, at least under certain conditions (Livingston et al. 2010a). Direct participation of NDH in photosynthesis is further supported by a report that NDH is activated by formation of a super-complex with PS I, implying a direct participation in photosynthesis rather than a function in non-photosynthetic plastids (Peng et al. 2009).

Because chloroplast NDH levels are relatively low (NDH:PS I ~1:20; Peng et al. 2009), it is not clear whether this enzyme is able to catalyze high rates of CEF under all conditions. However, NDH subunits accumulate at much higher levels under environmental stresses (Horváth et al. 2000; Casano et al. 2001; Li et al. 2004b) and in *hcef1* (Livingston et al. 2010a). Increased expression is also seen in C4 bundle-sheath chloroplasts, where the required ratio of ATP/NADPH is higher (Takabayashi et al. 2005). It has also been observed that CEF and NDH activity increase in vitro upon exposure to H_2O_2, as expected in response to environmental stresses (Casano et al. 2001; Lascano et al. 2003; Gambarova 2008; Livingston 2010; D.D. Strand, A.K. Livingston, R. Vink, V. Maurino and D. Kramer, unpublished). Thus, NDH may be activated both by increased expression and at the enzyme level to catalyze CEF that is highly efficient in ATP synthesis under environmental stresses. On the other hand, it is almost certain that other CEF pathways operate in other systems and under different environmental conditions, as discussed in the following sections.

(b) Super-Complex Pathways

While *Chlamydomonas reinhardtii* lacks genes for chloroplast NDH (Maul et al. 2002), a CEF competent super-complex comprised of PS I, LHCI, LHCII, cytochrome b_6f, ferredoxin NADP$^+$ reductase (FNR), and PGRL1 has been isolated (Iwai et al. 2010). In *C. reinhardtii*, it has been observed that CEF activation correlates with an antenna state transition to PS I. State transitions modulate partitioning of photosynthetic antenna complexes between PS I and PS II (Allen 2002). State 1 is induced when PS I is preferentially excited, resulting in the continued association of LHCII with PS II, and under these conditions LEF is operating (Depège et al. 2003). When light conditions favor PS II, the redox state of the PQ pool activates phosphorylation of LHCII by the STT7/STN7 kinase and LHCII associates with PS I in state 2 (Bellafiore et al. 2005). It is under these state 2 conditions that the super-complex was isolated (Iwai et al. 2010). Similar super-complex formation has been suggested to facilitate CEF in higher plants (Joliot and Joliot 2002; Eberhard et al. 2008), but has not been fully characterized. In addition, NDH is proposed to require formation of a super-complex with PS I (Peng et al. 2009).

Overall, it appears that rearrangement of thylakoid protein complexes into super-complexes associated with processes like state transitions may regulate CEF and other photosynthetic functions. It may therefore be important that *C. reinhardtii* appears to have a much larger capacity for state transitions than higher plants: up to 80 % vs ~15 % (Delosme et al. 1996; Allen 2002; Bellafiore et al. 2005), respectively, consistent with the reported higher capacity for CEF in *C. reinhardtii*.

The specific electron-transfer pathway for super-complex CEF is not known, although it has been proposed that heme c_i of the cytochrome b_6f complex may act as a conduit for electrons from the stroma to the plastoquinone reductase (Q_i) site (Hasan et al. 2013). It is also intriguing that CEF super-complex shows relatively high rates of electron transfer from the cytochrome b_6f complex to P700 despite the fact that the experiments were performed in isolated complexes with plastocyanin concentrations much lower than those expected in thylakoids (Iwai et al. 2010). The channeling of electron transfer by close association in super-complexes could be important for controlling the fraction of electrons sent into LEF or CEF, as discussed in Breyton et al. (2006), Peng et al. (2009), and Johnson (2010).

(c) FQR Pathways

CEF has also been proposed to be facilitated by an antimycin A-sensitive PQR termed ferredoxin:quinone reductase (FQR; Bendall and Manasse 1995). While its identity has yet to be fully elucidated, this complex has been proposed to be NDH, or a complex comprised of PGRL1 and PGR5 (Endo et al. 1998; Munekage et al. 2002; DalCorso et al. 2008; Hertle et al. 2012).

Shikanai and coworkers (Munekage et al. 2002, 2004) identified a mutant (*pgr5*) lacking the ability to maintain *pmf-dependent* NPQ and deficient in the small membrane-associated protein PGR5. This mutant was determined to be lacking the ability to perform FQR-mediated PQ reduction, based on in vitro chlorophyll reduction assay kinetics similar to antimycin A inhibition. Avenson et al. (2005a) calculated that the PGR5 pathways could contribute up to 13 % of proton flux in vivo under steady-state conditions in *Arabidopsis thaliana*. However, they also showed that many of the phenotypes associated with *pgr5* can be attributed not to CEF, but to effects on ATP-synthase activity, calling into question the physiological importance of PGR5-catalyzed CEF. Similarly, Aro and coworkers (Suorsa et al. 2012) have presented evidence that the main function of

PGR5 is in the regulation of PS I redox state, perhaps via effects on ATP synthase, and PGR5 is not involved in CEF.

Work on the *hcef1* mutant likewise implied that PGR5 is dispensable for CEF (Livingston et al. 2010a). The *hcef1 pgr5* double mutant retained the same elevated rates of CEF as *hcef1*, implying that, at least in this high CEF mutant, PGR5 is not needed. In contrast, elevated CEF was completely lost in the *hcef1 crr2-2* double mutant that lacks NDH (Livingston et al. 2010a), implying that NDH is required for elevated CEF, either directly as a PQ reductase or indirectly via regulatory or developmental effects. Elevated CEF in tobacco plants with antisense-suppressed glyceraldehyde phosphate dehydrogenase (*gapR*) showed no sensitivity to antimycin A, an inhibitor of the FQR pathway (Livingston et al. 2010b). This is in agreement with the proposed role of PGR5 in regulation of LEF but not CEF (Suorsa et al. 2012). Similar observations were made with another high CEF mutant, *fba3-1* deficient in fructose-1,6-bisphosphate aldolase (Gotoh et al. 2010). In this mutant, however, it has been proposed that the PGR5-dependent pathway may compensate for the absence of NDH. Overall, these results do not support PGR5 as the primary route of CEF, but rather suggest multiple roles for PGR5 in maintaining photosynthetic processes.

In addition to its proposed role in the *C. reinhardtii* super-complex, PGRL1 has recently been suggested to act as a PQR (Hertle et al. 2012). PGRL1 is an integral thylakoid-membrane protein (DalCorso et al. 2008) that associates with PGR5 and PS I in higher plants, and associates with the CEF super-complex in *C. reinhardtii* (Iwai et al. 2010). Loss of PGRL1 leads to a decrease in Fd-dependent dark PQ reduction as assayed by chlorophyll fluorescence, similar to the loss seen in PGR5 (Munekage et al. 2002). Identification of conserved cysteine residues, binding of a metal cofactor, and the ability of recombinant PGRL1 to reduce dimethyl-*p*-benzoquinone (DMBQ) (dependent on the presence of PGR5) suggests that PGRL1 is a quinone reductase (Hertle et al. 2012). However, some caution with regard to this interpretation is warranted. Inhibition of the observed FQR activity, performed with artificial quinones, required antimycin A concentrations 3 orders of magnitude higher than are effective in thylakoids (Cleland and Bendall 1992; Bendall and Manasse 1995; Munekage et al. 2002).

(d) NDA2 Pathway

For *C. reinhardtii*, it has been proposed that CEF is catalyzed by a type II NADH:plastoquinone oxidoreductase, termed NDA2, related to those found in bacteria, fungi, plants, and protists (Desplats et al. 2009). NADH, which is reduced during metabolism but not directly by PS I, appears to be the preferred substrate for NDA2, suggesting a primary role in metabolism-related PQ reduction (i.e., not directly related to the light-driven CEF; Desplats et al. 2009). Consistent with this role, NDA2 appears to be important for redox poising of the plastoquinone pool (Jans et al. 2008) and hydrogen production under sulfur deficiency (Mignolet et al. 2012), i.e., functionalities not directly associated with CEF. However, NDA2 also shows partial (10 %) activity with NADPH (Desplats et al. 2009), perhaps allowing it to operate in CEF, although a contribution to photosynthetic ATP production is yet to be demonstrated.

2 *Regulation of CEF*

Several signals for CEF regulation have been proposed, including Calvin-Benson cycle intermediates, state transitions, ATP/ADP ratios, NADPH or Fd redox states, and/or ROS (Finazzi et al. 2002; Joliot and Joliot 2002; Lascano et al. 2003; Breyton et al. 2006; Fan et al. 2007; Gambarova 2008; Livingston et al. 2010b). Given the numerous pathways that may be involved, it is likely that multiple regulatory processes are employed, and each pathway may have different signals for activation. This is thus an active area of research, albeit with little resolution to date. A few important advances are discussed in the following.

Livingston et al. (2010b) assayed major photosynthetic metabolites from mutants affected in photosynthesis with elevated (*hcef1* and *gapR*) and unchanged (anti-sense Rubisco small subunit tobacco mutant; *ssuR*) CEF, respectively. None of the metabolites measured (fructose-6-phosphate, dihydroxyacetone phosphate, fructose bisphosphate, phosphoglyercerate, ribulose-5-phosphate, glucose-5-phosphate, phosphoenolpyruvate) correlated with the activation of CEF, indicating that these metabolites are unlikely to be simple regulators of CEF.

It has been proposed that CEF in *C. reinhardtii* is regulated by state transitions (discussed above). The transition to state 2 has been shown to correlate with activation of CEF in algal systems under specific conditions (Finazzi et al. 2002; Iwai et al. 2010). It has been shown, however, that state 2 is not absolutely required for CEF in *C. reinhardtii* (Cardol et al. 2009) and more recent work has shown that CEF can proceed in the absence of state transitions, leading to suggestions that redox status, and not phosphorylation state, controls CEF (Takahashi et al. 2013; Lucker and Kramer 2013). It has recently also been shown that STN7 is not required for activation of CEF in *Arabidopsis thaliana* (Shikanai 2010).

Consistent with a role in balancing the chloroplast's energy budget, it has been suggested that ATP/ADP or NADPH/NADP$^+$ ratios can regulate CEF (Joliot and Joliot 2002). CEF has been proposed to be activated during photosynthetic induction, thus increasing ATP supply when this supply is limiting the Calvin-Benson cycle (Joliot and Joliot 2002, 2006). However, this conclusion is not consistent with previous spectroscopic findings (Klughammer and Schreiber 1994). Furthermore, metabolic profiling of *hcef* mutants showed no significant differences in ATP/ADP or NADPH/NADP$^+$ ratios in wild type versus mutant lines with elevated CEF (Livingston et al. 2010b), suggesting that regulation of CEF may instead involve ROS.

ROS have been implicated in a number of signaling processes, such as those regulating growth and environmental stress response (Bolwell et al. 2002; Kar 2011; Huang et al. 2012). The *hcef1* mutant accumulates significantly higher levels of H_2O_2 compared to wild-type *A. thaliana* (Livingston 2010). With the discovery of the role of H_2O_2 in the activation of the NDH complex by phosphorylation (Lascano et al. 2003), CEF was established as a target of ROS. Indeed, infiltration with H_2O_2 also leads to increased CEF, the level of which correlates with the concentration of H_2O_2 (Livingston 2010). However, H_2O_2 has also been shown to inhibit several steps in the Calvin-Benson cycle, knockdown/knockout mutants of which show an *hcef* phenotype (Kaiser 1979; reviewed in Livingston et al. 2010b). CEF in the *hcef1* and *gapR* mutants is independent of the antimycin A-sensitive pathway (Livingston et al. 2010a, b). In *hcef1*, and possibly also in *gapR*, ROS accumulation, or another signal downstream of Calvin-Benson cycle inactivation, may lead to phosphorylation and activation of NDH. Therefore, it is possible that FQR or other paths of CEF, are also regulated by additional factors independent of ROS.

III Type II Flexibility Mechanisms: Regulation of (*pmf*) Partitioning and ATP Synthase Activity and the Consequences for NPQ

While Type I flexibility mechanisms alter the ATP/NADPH ratio available for downstream metabolism, Type II mechanisms alter *pmf* without changing this ratio. Included in the latter category is regulation of the ATP synthase and changes in *pmf* partitioning between ΔpH and $\Delta\Psi$. Downregulation of the ATP synthase increases *pmf*, and subsequently the qE response, while alterations in *pmf* partitioning may alter qE response to total *pmf*.

A The Chloroplast ATP Synthase Is a Central Regulator of Photosynthesis

The enzymatic activity of the chloroplast ATP synthase is rapidly modulated in response to metabolic or physiological conditions, allowing it to regulate both the light reactions, via effects on the proton circuit, and the assimilatory reactions, via effects on chloroplast ATP and P_i levels (Kramer et al. 2004; Avenson et al. 2005b). Under low CO_2, environmental stresses, and feedback-limiting conditions at high CO_2, ATP synthase activity is rapidly and reversibly downregulated, slowing proton efflux from the lumen (Kanazawa and Kramer 2002; Avenson et al. 2005b). The resulting buildup of *pmf* activates downregulation of the antennae via processes underlying qE, and slows electron transfer at the cytochrome *b_6f* complex, thus downregulating the light reactions (Kramer et al. 2004).

Because many of the assimilatory enzymes of photosynthesis are controlled by stromal ATP, ADP, and P_i levels (Sharkey 1990), changes in the activity of the ATP synthase should act to co-regulate or coordinate the light and assimilatory reactions (Takizawa et al. 2008). Decreasing ATP synthase activity should result in decreased ATP and increased ADP and P_i, regulating assimilatory and metabolic reactions.

Several possible mechanisms can be proposed for metabolism-related ATP synthase regulation. It has been well documented that chloroplast ATP synthase is regulated by redox modulation of γ-subunit thiols (reviewed in Ort and Oxborough 1992). Recently, Kohzuma et al. (2013) further tested this hypothesis by modifying the conserved acidic residues on the γ-subunit. This manipulation modified the redox potential of the thiols, but the mutant complex still maintained wild-type responses to metabolic limitations. Thus, redox switching and metabolic regulation of ATP synthases have different mechanisms and physiological roles. Redox regulation appears to act as a sensitive on-off switch, activating ATP synthase even in very low light (Kramer and Crofts 1989; Kramer et al. 1990), possibly to prevent "wasteful" ATP hydrolysis in the dark (Wu et al. 2007).

In one possible model for metabolic regulation of ATP synthase, the availability of stromal P_i can be drawn down to below its K_M at the ATP synthase, e.g., under limiting internal CO_2 (Kanazawa and Kramer 2002) or under feedback limitation resulting from accumulation of photosynthetic products under high CO_2 levels (Sharkey 1990; Paul and Foyer 2001). In agreement with these models, Takizawa et al. (2008) found that lowering P_i levels decreases the activity of ATP synthase, leading to downregulation of photosynthesis. It has also been observed that downregulation of the ATP synthase occurs at high CO_2 levels, when P_i availability is thought to limit photosynthesis (Kiirats et al. 2009). These results are consistent with, but do not establish, a direct causal relationship between P_i levels and ATP synthase activity.

It has also been proposed that ATP synthase can be regulated by phosphorylation and subsequent binding of a 14-3-3 protein (de Boer 2002; del Riego et al. 2006). Although the phosphorylation state of the ATP synthase appears to change in response to light exposure of dark-adapted plants (Reiland et al. 2009), no measurements of phosphorylation in response to metabolic status have been reported. The 14-3-3-protein family is known to interact with enzymes involved in chloroplast metabolism (Huber 2007), but a direct role in phosphorylation of the ATP synthase has yet to be shown, and 14-3-3 proteins may be nonspecific.

B Regulation of pmf Partitioning

Chemiosmotic coupling can be driven by *pmf* via either of its two components, proton diffusion potential, ΔpH, and transthylakoid electric field, ΔΨ. Early work on isolated thylakoids suggested that the contribution of ΔΨ to thylakoid *pmf* was low, but more recent work showed that ΔΨ can make significant contributions to *pmf* (Cruz et al. 2001). Although the two components of *pmf* are thermodynamically equivalent, they have very different consequences for the regulation of photosynthesis (Kramer et al. 2004). Buildup of the ΔpH component decreases the pH of the thylakoid lumen, activating qE and slowing electron transfer at the cytochrome b_6f complex (Takizawa et al. 2007). Modulation of the fraction of ΔpH and ΔΨ to total *pmf* can change the sensitivity of qE to the *pmf*, and thus act as an additional level of photoprotective regulation at limiting CO$_2$, elevated temperatures, or other environmental stresses (Avenson et al. 2004; Finazzi, et al. 2006; Takizawa et al. 2007; Zhang et al. 2009). While the mechanism of this modulation partitioning is currently not understood, it is clear that several factors determine the storage of ΔΨ, i.e., membrane capacitance, ionic strength, and buffering capacity of the lumen (Cruz et al. 2001). It is unlikely that thylakoid membrane capacitance would change sufficiently rapidly to account for the observed changes in ΔΨ/ΔpH. On the other hand, ion channels in the plasma membrane, chloroplast inner envelope and thylakoid membranes can control ion movements across the thylakoid. In the absence of counter-ion movement across the membrane, we would expect the buffering capacity of the lumen to maintain a higher fraction of ΔpH than ΔΨ (Fig. 18.6a). Active ion channels for Mg^{2+}, Ca^{2+}, Cl^-, and K^+ have been confirmed to occur in thylakoids (Schönknecht et al. 1998; Enz et al. 1993; Pottosin and Schönknecht 1996), and Cruz et al. (2001) proposed the regulation of chloroplast ion balance as the major mechanism of adjusting ΔpH/ΔΨ (Fig. 18.6b). In accord with this proposal, a gated K^+ channel was found to be required for maintaining ΔpH in cyanobacteria (Zanetti et al. 2010; Checchetto et al. 2012). Consistent with the view that *pmf* partitioning is controlled by ion movements, it was recently found that disruption of a thylakoid K^+ channel in plants resulted in higher partitioning of *pmf* into ΔΨ, with consequences for the regulation of photosynthesis by the proton circuit (read Carraretto et al. 2013).

An additional regulatory mechanism was proposed by Ioannidis et al. (2012) who

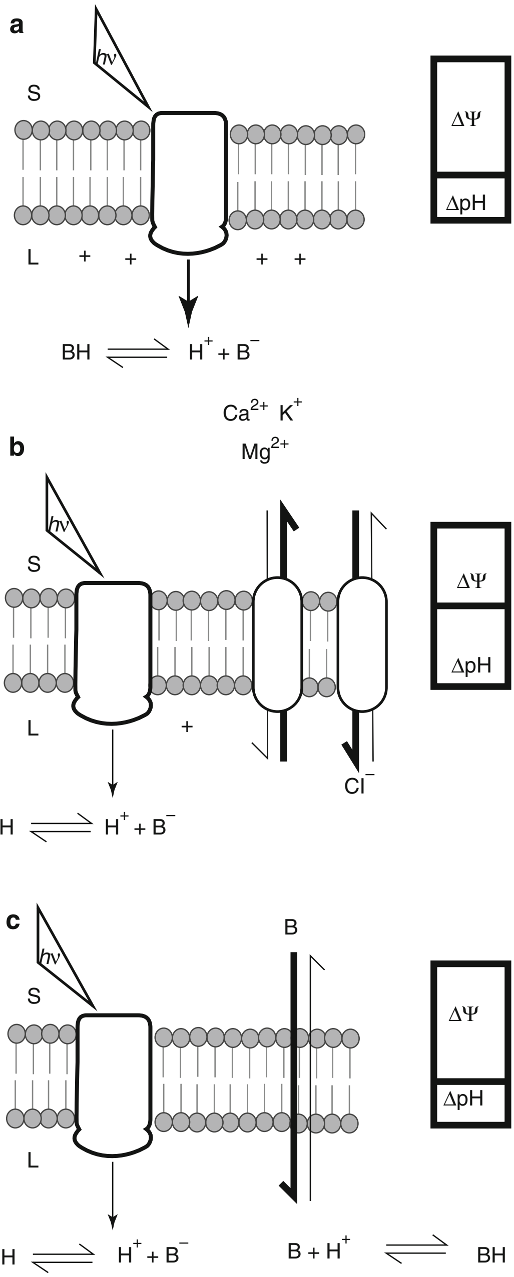

Fig. 18.6. Proposed mechanisms of changes in *pmf* partitioning. (**a**) In the absence of counter ion movement, ΔΨ should predominate. (**b**) Counter ion movement (i.e., lumenal cation efflux/anion influx) would allow increased ΔpH. (**c**) A membrane permeable weak base would increase the buffering capacity of the lumen, collapsing the ΔpH. This diagram was by Nicholls and Ferguson (2002).

showed that putrescine, a polyamine acting as a weak base and involved in plant stress responses, can regulate $\Delta\Psi/\Delta pH$ by modulating the effective proton buffering capacity of the lumen. This work supports a "biological weak base" model (Fig. 18.6c), in which molecules like polyamines may allow for rapid modulation of *pmf* partitioning.

IV Concluding Remarks

The proton circuit plays a central role in governing the responses of processes underlying NPQ, predominantly by activating the qE response, but also by governing the rate of electron transfer through the cytochrome b_6f complex and thus modulating the PQ-pool redox state. The proton circuit also provides a very flexible means by, which metabolic or physiological states of the chloroplast influence NPQ responses, e.g., via regulating CEF or ATP synthase. It is obvious that, although we are beginning to delineate the interacting processes, a large number of important questions remain open.

One way to assess whether our understanding of the proton circuit and its influence on NPQ is complete, and to identify additional important open questions, is to compare physiological responses with numerical simulations. There have been several attempts to model *pmf* and the qE response to *pmf* (Cruz et al. 2001; Takizawa et al. 2007; Nedbal et al. 2009; Rubin and Riznichenko 2009; Yin et al. 2009; Zhu and Long 2009; Zaks et al. 2012). These models are based on broad simplifications, but show qualitatively reasonable *pmf* and responses that reflect the general trends seen in vivo. The recent model of Zaks et al. (2012) is noteworthy in that it incorporates several possible regulatory mechanisms for qE, and provides a solid framework for future inclusions of more complex processes. However, these models also highlight the fact that we know little about several regulatory mechanisms, especially CEF and *pmf* partitioning that are clearly important for balancing the proton circuit (Harbinson 2012).

Acknowledgments

We thank Dr. Jeffrey Cruz and Dr. Nick Fisher for helpful discussions. The authors are funded by Grant DE-FG02-11ER16220 from the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US Department of Energy.

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Chapter 19

Desiccation-Induced Quenching of Chlorophyll Fluorescence in Cryptogams

Wolfgang Bilger
Botanisches Institut der Christian-Albrechts-Universität zu Kiel,
Am Botanischen Garten 3-9, Kiel 24118, Germany

Summary

Desiccation-resistant cryptogams in the dry state display a strong reduction of basal chlorophyll fluorescence emission, F_o. In this state, variable fluorescence, F_v ($=F_m - F_o$), is also lost. The occurrence of a combination of these two phenomena is restricted to cryptogams, i.e., mosses, cyanobacteria, algae, and, hence, lichens containing the latter two groups as photosynthetic symbionts, also termed photobionts. Fluorescence lifetime has been shown to be considerably shortened in the desiccated state, indicating that a large portion of the fluorescence decrease (or quenching) is due to enhanced thermal dissipation of energy from the singlet-excited state of chlorophyll a, leading to non-photochemical quenching of chlorophyll fluorescence. Desiccation-induced quenching has been extensively characterized and has similar properties in cyanobacterial and green algal lichen photobionts and mosses. This quenching is not inhibited by the electron transport inhibitor DCMU, the uncoupler nigericin, or the inhibitor of zeaxanthin formation, dithiothreitol. Since desiccation-induced quenching is, however, inhibited by glutaraldehyde and released by heating above 40–50 °C, this quenching may be caused by a conformational change of a PS II protein. Based on fluorescence decay-associated spectra determined via chlorophyll fluorescence lifetime measurements, it was proposed that a long-wavelength chlorophyll form is involved in desiccation-induced quenching. There is also evidence that desiccation-induced quenching protects cryptogams against high-light damage in the desiccated state.

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*Author for Correspondence, e-mail: wbilger@bot.uni-kiel.de

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_19,

I Introduction

Water shortage is a general problem for land plants. One extreme adaptation to this stress factor is tolerance of cellular desiccation down to a relative water content of a few percent (Lüttge et al. 2011). Whereas very few vascular plants are able to tolerate such low water contents in their vegetative organs, many cryptogams, i.e., almost all lichens, a large number of mosses, and many algae and cyanobacteria, survive complete desiccation (Alpert 2000; Kranner et al. 2008; Green et al. 2011). While, among algae and cyanobacteria, land-dwelling (aeroterrestrial) organisms are especially desiccation-tolerant, some marine algae also tolerate desiccation. It has been proposed that desiccation tolerance is an ancient trait of land plants that was lost during the evolution of vascular plants (Oliver et al. 2000; Proctor et al. 2007).

II The Phenomenon of Desiccation-Induced Fluorescence Quenching

Chlorophyll fluorescence has often been used to study the function of the photosynthetic apparatus during desiccation of desiccation-tolerant vascular plants (Eickmeier et al. 1993; Augusti et al. 2001; Georgieva et al. 2005) and cryptogams (Wiltens et al. 1978; Sigfridsson and Öquist 1980; Jensen and Feige 1987; Lange et al. 1989; Calatayud et al. 1997; Deltoro et al. 1998a, b; Csintalan et al. 1999; Heber et al. 2000; Satoh et al 2002; Harel et al. 2004; Lüttge et al. 2008). Early studies of the effects of desiccation on chlorophyll fluorescence focused more on the changes in energy distribution between the photosystems, but also noted a decrease of fluorescence emitted by photosystem II (PS II) in various algae and lichens (Sigfridsson 1980; Sigfridsson and Öquist 1980; Öquist and Fork 1982a, b). With the advent of easy access to the measurement of modulated fluorescence, the desiccation-induced quenching of basal or “dark” fluorescence yield, F_o, and maximal fluorescence yield, F_m, could be readily followed at room temperature (Lange et al. 1989; Jensen and Feige 1991).

With the loss of 95 % of water content, all metabolic activity in plant cells ceases, i.e., a state of cryptobiosis is induced. In this state, a loss of all variable fluorescence ($F_v = F_m - F_o$) is commonly observed in cryptogams (e.g., Lange et al. 1989; Harel et al. 2004; Nabe et al. 2007). This loss is accompanied by a strong decline of F_o. This phenomenon is illustrated in Fig. 19.1 by comparing fluorescence traces of an aeroterrestrial green alga, *Trebouxia asymmetrica*, that was isolated from a lichen (Friedl and Gärtner 1988), with those of a desiccation-sensitive green microalga, *Chlorella vulgaris*. Before drying, both algae responded to application of a saturating light pulse with a strong increase of fluorescence (trace a). In the dry state, *T. asymmetrica* displayed a very low fluorescence yield (F_o), and no further reaction to the light pulse was observed (trace b). In contrast, after desiccation in darkness PS II function was only partially inhibited in *C. vulgaris*, as the remaining fluorescence increase upon a saturating light pulse shows (trace b). The substantial increase of F_o may be, at least partly, attributed to continuing functionality of PS II itself combined with a loss of activity in electron transport downstream of PS II, which would lead to enhanced fluorescence due to lasting reduction of the primary electron acceptor in PS II, Q_A. One hour after rehydration (trace c), the desiccation-induced decline of F_o was reversed and variable fluorescence restored in *T. asymmetrica*. On the other hand, *C. vulgaris* no longer showed evidence for a functional PS II following rehydration (trace c), illustrating a lack of desiccation resistance

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Abbreviations: Chl – Chlorophyll; Chl_{740} – Long-wavelength emitting chlorophyll species putatively acting as a quencher; DTT – Dithiothreitol; F_m – Maximal chlorophyll fluorescence in the dark-adapted state; F_o – Minimal chlorophyll fluorescence in the dark-adapted state basal fluorescence; F_v/F_m – Intrinsic efficiency (or quantum yield) of photosystem II in the dark-adapted state; PS I – Photosystem I; PS II – Photosystem II

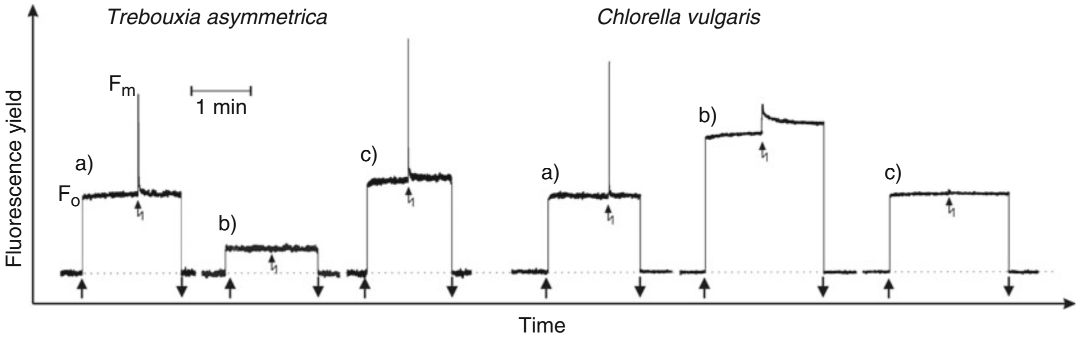

Fig. 19.1 Fluorescence traces of Trebouxia asymmetrica and Chlorella vulgaris before desiccation (a), in the desiccated state (b), and 1 h after rewetting (*c*). Algae were grown in liquid culture as in Wieners et al. (2012) at 50 μmol photons m^{-2} s^{-1} and before measurement placed on a glass-fiber filter disc (type 13,400, Sartorius, Göttingen, Germany) at a high chlorophyll concentration. Filter discs were slowly dried in darkness in air of approximately 50 % relative humidity at 22 °C. Filter discs were rewetted from below in order to minimally disturb the orientation of the algal layer. Fluorescence measurements were conducted using a PAM 2,000 fluorometer (Walz GmbH, Effeltrich, Germany). Where indicated, saturating flashes of 0.6 s duration and an irradiance of 4,000 μmol photons m^{-2} s^{-1} were applied to induce F_m. *Up* and *downward arrows* at the bottom of the traces indicate when the measuring light was turned on or off, respectively. The timescale is shown by a horizontal bar in the figure. Measurements by P. C. Wieners (unpublished, 2013).

(Chen and Hsu 1995). The response of *T. asymmetrica* is typical for desiccation-resistant cryptogams, whereas the response of *C. vulgaris* can be observed not only in desiccation-sensitive cryptogams but also in vascular plants, even if they are desiccation tolerant (see below). The particular way in which water is lost or removed from the organism apparently does not play a role, since similar observations can be made when cryptogams are osmotically dehydrated (Chakir and Jensen 1999; Jensen et al. 1999; Hirai et al. 2004; Hajek et al. 2006; Kosugi et al. 2009).

In principle, a decline of F_o need not be caused by true (non-photochemical) fluorescence quenching in the form of enhanced thermal dissipation. Desiccation per se can severely affect optical properties of the organisms leading to decreased fluorescence emission as a mere physical phenomenon (see discussion below). However, it has been shown in several lichens and a moss that desiccation was accompanied by a strong decrease in fluorescence lifetime (Veerman et al. 2007; Komura et al. 2010; Miyake et al. 2011; Yamakawa et al. 2012). This decrease shows that, at least a large part of, the observed decline of F_o is indeed caused by non-photochemical fluorescence quenching. Accordingly, decreases of F_o caused by desiccation will be denoted as desiccation-induced quenching in the following.

The comparison of the two microalgae (Fig. 19.1) shows that strong desiccation-induced quenching of F_o in *T. asymmetrica* is apparently not caused by a simple physical process such as a change in the optical properties of the sample, since it is not observed in *C. vulgaris*. In spite of the presumable occurrence of optical changes in the filter paper upon which the algae were suspended, opposite changes in fluorescence yield were induced in the two algae. Furthermore, a qualitative correlation between the decline of F_o and the loss of photochemical function in PS II can be observed in Fig. 19.1, suggesting a potential mechanistic relationship between the two phenomena.

A correlation between desiccation-induced quenching and the loss of F_v was also observed when fluorescence was measured during the time course of desiccation (Heber et al. 2007; 2011). Figure 19.2 shows the relationship between the decline of F_v and F_o during slow desiccation in near darkness of the lichen *Parmelia sulcata*. A similar close correspondence between losses of

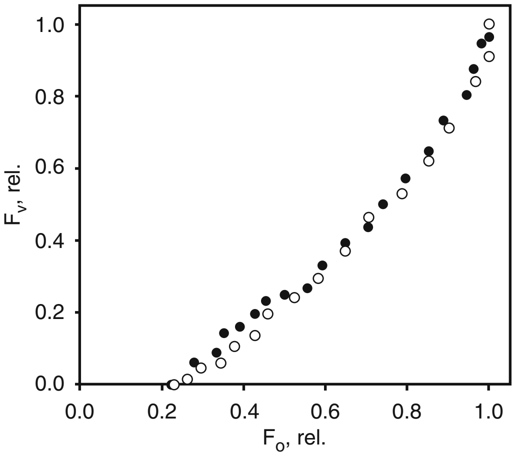

Fig. 19.2 Relationship between the decline of ground fluorescence F_o and F_v ($=F_m - F_o$) during slow desiccation in darkness of the lichen Parmelia sulcata containing a green alga of the genus Trebouxia as photobiont (The data were replotted from Heber et al. (2007; *open circles*) and Heber et al. (2011; *closed circles*)).

F_v and of F_o was reported by Bukhov et al. (2004) for the lichen *Hypogymnia physodes* during rehydration.

III Occurrence Within the Plant Kingdom

As a rule, organisms showing desiccation-induced quenching are desiccation-tolerant, but not all desiccation-tolerant organisms display this kind of quenching. It rather appears to be restricted to cryptogams, from cyanobacteria to mosses. Based on its peculiar properties, one may hypothesize that the mechanism of desiccation-induced quenching in these different groups is the same (see below). Desiccation-induced quenching has been shown to be absent from the desiccation-tolerant fern *Polypodium vulgare* (Kopecky et al. 2005) and the desiccation-tolerant flowering plant *Haberlea rhodopensis* (Georgieva et al. 2005), and there are no reports of such quenching from other desiccation-tolerant ferns or angiosperms. It has been pointed out that the small early land plants were probably poikilohydric with desiccation tolerance being a prerequisite for life on land (Oliver et al. 2000; Proctor et al. 2007). This early trait was lost in the evolution of vascular plants and presumably evolved secondarily in the comparatively few extant desiccation-tolerant ferns and angiosperms. It may be of significance that the presence of desiccation-induced quenching correlates with the ancient trait of desiccation tolerance but not with its "reinvention" through evolution.

Among cyanobacteria, the most investigated genus with regard to desiccation tolerance and desiccation-induced quenching is *Nostoc* (Lange et al. 1989; Hirai et al. 2004; Fukuda et al. 2008) that is also an important symbiont in lichens. Also, desiccation-induced quenching observed in soil crusts presumably mainly originated from cyanobacteria of the genus *Microcoleus* as the dominant member of these communities (Harel et al. 2004; Ohad et al. 2005).

Desiccation-induced quenching and complete loss of F_v/F_m has been reported for aeroterrestrial green algae from tree bark (Lange et al. 1989; Lüttge and Büdel 2010) and algae isolated from lichens (Wieners et al. 2012). Interestingly, Kosugi et al. (2009) found reduced quenching and an incomplete loss of F_v/F_m in the green microalga *Trebouxia* sp. when it was cultured or freshly isolated from the lichen *Ramalina yasudae*, while this response was complete in the intact lichen. These authors concluded that some factor present in the lichen could be responsible for full quenching as the addition of an aqueous extract from the lichen induced fluorescence quenching in wet algae. In fact, Kosugi et al. (2013) showed that the lichen *R. yasudae* contained high concentrations of the sugar alcohol arabitol, and that addition of this compound to isolated *Trebouxia* sp. cells during desiccation enhanced quenching of PS II fluorescence in the dry state as determined by fluorescence decay kinetics. Furthermore, Wieners et al. (2012) showed that desiccation-induced quenching was stimulated by the simultaneous presence of low concentrations of Zn^{2+} and I^- ions in the culture medium of *T. asymmetrica*.

Lichens often serve as handy experimental objects, providing easy access to green algal and cyanobacterial photobionts (e.g., Lange et al. 1989; Kosugi et al. 2009; Heber et al. 2011). As a symbiosis between a fungus and a photobiont, lichens are, however, optically complex. Photobionts are usually located in a deeper layer of the lichen. Both the light that excites chlorophyll fluorescence and the emitted fluorescence have to pass a layer of fungal hyphae that screens the photobionts against direct irradiation. Changes in the optical properties of the lichen structure during drying will strongly affect fluorescence yield. It has been reported that transmittance of the cortical layer of lichens decreases by 30–70 % upon drying (Ertl 1951; Gauslaa and Solhaug 2001). Accordingly, a significant part of the decrease in chlorophyll fluorescence in a drying lichen may be due to optical changes. Fluorescence decreases due to changes in screening versus true thermal dissipation of absorbed light can be distinguished by measuring fluorescence lifetime. Veerman et al. (2007) showed that in the lichen *Parmelia sulcata*, an often-used experimental organism (see Fig. 19.2), structural changes in the lichen thallus contributed less than 25 % to overall fluorescence decline that was caused primarily by enhanced thermal dissipation. It should also be pointed out that desiccation-induced quenching of a similar magnitude has been observed with isolated or free-living algae or cyanobacteria (Lange et al. 1989; Wieners et al. 2012).

Desiccation-induced quenching can be observed in desiccation-tolerant (Nabe et al. 2007; Lüttge et al. 2008; Li et al. 2010; Yamakawa et al. 2012) but not in desiccation-sensitive mosses (Nabe et al. 2007). In two shade-adapted moss species, *Hylocomium splendens* and *Rhytidium squarrosum*, expression of desiccation-induced quenching was lost during the humid autumn and winter seasons, but appeared in late spring and summer when the weather was dry (Heber et al. 2006a). Acclimation of the extent of quenching to growth conditions was also seen in the green-algal lichen *Lobaria pulmonaria*. Sun-grown thalli exhibited larger desiccation-induced quenching than shade grown ones (Heber et al. 2010). Furthermore, for isolated lichen algae of the genus *Trebouxia*, this type of quenching was enhanced by increased growth irradiance (Wieners PC, unpublished). Such phenotypic variation in the extent of desiccation-induced quenching not only further demonstrates that its appearance is not a simple physical consequence of desiccation, but also that some species are able to regulate its presence by hitherto unknown mechanisms.

Despite the broad taxonomic origin of organisms for which desiccation-induced quenching has been reported, its basic features are, remarkably, identical: F_o is decreased to less than 50 % and variable fluorescence is virtually completely lost (e.g., Satoh et al. 2002; Kopecky et al. 2005; Nabe et al. 2007).

IV Photosystem Activity in the Dry State

What is the state and function of PS II when fluorescence emission is strongly quenched in organisms experiencing desiccation? Loss of F_v can be understood as a loss of charge separation in PS II. In the desiccated lichen *Cladonia convoluta*, all peaks of thermoluminescence curves disappeared (Sass et al. 1996). This result could be interpreted as indicating either a total absence of charge separation or an inhibition of light emission in the dry state, but the two possibilities could not be distinguished (Sass et al. 1996).

Some photoreactions in PS II are apparent when extremely high irradiance is applied to organisms in the desiccated state (Heber 2008; Heber et al. 2010, 2011; Yamakawa et al. 2012). The reactions are highly variable and appear to depend on the particular state of PS II as a function of the actual degree of desiccation. Increases as well as decreases of fluorescence can be observed in different organisms (Heber et al. 2011). While more systematic analyses are necessary to further resolve the character of these reactions, it is clear that these activities have very low quantum yield and do not appear to play an important role under natural light conditions.

Interestingly, in desiccation-tolerant mosses or green-algal lichens PS I is not, or only partially, inhibited by desiccation as its function could still be detected by the measurement of absorbance changes at 820 nm (ΔA820) (Heber et al. 2000; Nabe et al. 2007; Kosugi et al. 2009). Whereas P700 was oxidized by illumination with white or far-red light in all desiccated organisms, data for P700 reduction in a subsequent dark phase are controversial. Reversal of ΔA820 upon darkening was inhibited for two desiccation-tolerant mosses and the lichen *Ramalina yasudae* as well as its photobiont (Nabe et al 2007; Kosugi et al. 2009), indicating the absence of linear and cyclic electron transport. Similar observations were made by Bukhov et al. (2004) with the desiccated green-algal lichen *Hypogymnia physodes*. In the latter lichen, P700 oxidation induced by single turnover flashes was largely reversible, but to only 40 % after a 50-ms flash, and reversibility was completely missing after longer flashes. This result demonstrates that in this lichen only a very limited number of electron donors for $P700^+$ were available in the desiccated state, presumably due to an inhibition of electron transport. On the other hand, even after longer flashes ΔA820 quickly reversed in a dry moss and in two dry lichen species investigated by Heber et al. (2000). Notably, rapid reversal of 80 % of the light induced P700 absorbance change was still possible in the desiccation-sensitive moss *Marchantia polymorpha*, which also maintained an F_v/F_m level of 0.2 in the desiccated state (Nabe et al. 2007). In the cyanobacterium *Nostoc commune*, P700 absorbance changes were inhibited in the dry state (Satoh et al. 2002). The continuing function of PS I in green algae and mosses proves that desiccation per se is not leading to inhibition of electron transfer in a photosystem, suggesting that inhibition of PS II in the dry state requires a regulatory mechanism.

V The Mechanism of Desiccation-Induced Quenching

The molecular events that lead to desiccation-induced quenching are not resolved yet. However, a large variety of experimental manipulations of the latter quenching and inhibitor studies made it possible to narrow down its mechanism. Much experimental evidence has accumulated suggesting that desiccation-induced quenching is caused by a structural change in a protein, presumably a pigment-protein complex. The strongest evidence for the latter hypothesis is inhibition of desiccation-induced quenching by glutaraldehyde, a compound that crosslinks proteins and inhibits further structural changes in these proteins (Coughlan and Schreiber 1984). Such an inhibition has been demonstrated for green algal lichens (Heber 2008; Heber et al. 2007, 2010, 2011), cyanobacterial lichens (Heber et al. 2011), and mosses (Heber et al. 2007; Yamakawa et al. 2012). In the presence of 0.2–0.25 % glutaraldehyde, the desiccation-induced decline of fluorescence almost completely disappeared. The applied glutaraldehyde concentrations, that had to be increased to 2 % for cyanobacterial lichens (Heber et al. 2011), also inhibited electron transport. Notably, in the desiccated state a fluorescence increase in response to a saturating light pulse was still visible in the presence of glutaraldehyde.

The hypothesized structural change leading to desiccation-induced quenching may also require time to develop. It has been observed that the extent of this quenching was enhanced when samples were slowly desiccated over several hours as compared to rapid desiccation over a period of only 1 h (Heber et al. 2007; Gasulla et al. 2009). Several hours seem short to reach complete desiccation considering the rate of water loss of higher plant leaves, but lichens lacking a cuticle lose water more quickly. More rapid desiccation led not only to incomplete quenching, but F_v was not completely suppressed, again illustrating the close relationship between quenching of F_o and loss of variable fluorescence. It is important that similar observations were made in a moss as well as in cyanobacterial and green-algal lichens (Heber et al. 2007). Given the importance of the rate of desiccation, one may conclude that desiccation conditions, especially the water vapor pressure gradient between organism and air, should be carefully controlled for comparison with other studies. This has not always been the case in previously published reports.

Heating of dry lichen thalli or a moss led to increased levels of F_o (Heber and Shuvalov 2005; Heber et al. 2007). Concomitant with the rise of F_o, the fluorescence increase induced by a saturation pulse (increased levels of F_m) also appeared. These changes were reversible as long as a temperature of about 60 °C was not exceeded. This phenomenon has been interpreted as being due to a change in the conformation of proteins, presumably a reversal of the conformational change that induces F_o quenching (Heber et al. 2007).

Ongoing electron transport is not necessary for the formation of desiccation-induced quenching. The latter quenching is typically monitored during desiccation in complete darkness. This condition ensures that reaction centers stay open during desiccation, which is a prerequisite for the determination of F_o. Even the presence of low light in the dry state will cause the acceptor side to be reduced due to being stabilized by blocked electron transport in the desiccated thylakoid membrane. On the other hand, fluorescence yield of open or closed reaction centers is practically identical in a desiccated sample, due to loss of variable fluorescence. Accordingly, application of DCMU to the moss *Rhytidiadelphus squarrosus* did not inhibit desiccation-induced quenching (Heber et al. 2006a, b).

A high-energy state (characterized by, e.g., a trans-thylakoid pH gradient) of the thylakoid membrane is also not required for desiccation-induced quenching since its formation is not inhibited by addition of the uncoupler nigericin to desiccating green-algal lichen thalli (Heber 2008; Heber et al. 2010). The same insensitivity to uncouplers was seen in desiccating mosses (Heber et al. 2006a, b; Nabe et al. 2007; Yamakawa et al. 2012). Together with the induction of desiccation-induced quenching in darkness, these findings show convincingly that proton translocation coupled to electron transport is not necessary for the formation of the quenching state. This is in strong contrast to the light-induced non-photochemical quenching seen in higher plants during exposure to excessive light (Demmig-Adams et al., Chap. 24).

At the same time, light, while not being required, can nevertheless influence the extent of desiccation-induced quenching in desiccation-resistant cryptogams. When lichens and mosses were desiccated in the presence of weak to modest actinic light, quenching of F_o was enhanced (Kopecky et al. 2005; Heber 2008; Heber et al. 2010; Yamakawa et al. 2012). Light-induced enhancement of desiccation-induced quenching was also implied when lichens collected in the field in the dry state were subjected to hydration followed by renewed desiccation in darkness (Heber et al. 2010). In this case, the new F_o reached after desiccation in darkness was higher than that observed directly after collection from the field in the air-dry state. This indicates that in the field, where lichens typically dry out while being irradiated, F_o is more strongly quenched. One may assume that this situation is very common since a high water-saturation deficit of air leading to desiccation usually goes hand in hand with sunshine, while at night air humidity is higher. Desiccation of lichens is further enhanced by sunshine due to additional heating of thalli in the sun. In a field study on the cyanobacterial lichen *Peltigera rufescens*, loss of PS II activity due to desiccation was much more common during the day than at night (Leisner et al. 1996). In darkness, PS II quantum yield was always inhibited when the thalli froze. Freezing also causes cellular desiccation (Levitt 1980).

Heating of green-algal lichen thalli dried in the light caused a considerably higher fluorescence increase than when the thalli were dried in darkness (Heber 2008). This suggests that light-induced enhancement of desiccation-induced quenching was reversed by heating, which would also indicate that light-induced enhanced quenching is caused by a similar molecular mechanism as the quenching induced by desiccation in darkness.

Zeaxanthin is formed during desiccation in the light (Heber et al. 2010). Strong water loss directly inhibits metabolic reactions such as CO_2 fixation (Kaiser 1987). Under such circumstances, even comparatively low irradiance will be excessive and may therefore enhance zeaxanthin formation. A zeaxanthin-related non-photochemical quenching may be further enhanced by desiccation-induced quenching. On the other hand, concurrent formation of zeaxanthin does not seem to be a prerequisite for the occurrence of desiccation-induced quenching, as experiments using an uncoupler show (Heber 2008; Heber et al. 2010). This is further corroborated by experiments in which lichens or mosses were incubated in a solution of dithiothreitol, which inhibits formation of zeaxanthin from violaxanthin. This treatment did not affect desiccation-induced quenching (Kopecky et al. 2005; Nabe et al. 2007; Heber 2008; Yamakawa et al. 2012).

Further insight into the nature of the quenching state of PS II has been provided by measurements of fluorescence lifetime and fluorescence decay-associated spectra (DAS) of three different green algal lichen species (Veerman et al. 2007; Komura et al. 2010; Miyake et al. 2011) and the moss *Rhytidium rugosum* (Yamakawa et al. 2012). All results are consistent in that fluorescence decay at room temperature was at least one order of magnitude faster in the dry versus the wet state. This proves that the desiccation-induced fluorescence decline in the investigated lichens and the moss was indeed largely due to enhanced thermal dissipation. Decay-associated spectra showed a PS II-associated component at 740 nm indicating a long-wavelength emitting species (Chl_{740}) coupled to PS II that is absent in higher plants and cyanobacteria investigated thus far (Veerman et al. 2007; Komura et al. 2010). Chl_{740} had a very short lifetime in the desiccated state at room temperature and at 77 K. This component has to be distinguished from PS I-associated long wavelength emission whose decay was not or only slightly accelerated in the dry state (Veerman et al. 2007; Miyake et al. 2011). A general conclusion from these investigations was that, in the dry state, thermal dissipation in PS II is efficiently competing with charge separation because of dissipation's higher rate constant. Through measurements conducted at 5 K, Miyake et al. (2011) were able to resolve an additional decay component not associated with Chl_{740} that was slower and therefore less effective than the former. Miyake et al. (2011) suggested that quenching due to Chl_{740} was occurring at the antenna complexes of PS II, whereas the second and somewhat less efficient component may either be located in the antenna or the reaction center.

Dissipation in the Chl b-containing light-harvesting complexes was also deduced from excitation spectra of desiccated green-algal lichens or free-living green algae (Bilger et al. 1989; Kopecky et al. 2005). These authors observed preferential loss of fluorescence excitation around 480 nm where Chl b absorbs strongly. However, Heber et al. (2007) could not reproduce this observation.

The concept of strong competition between thermal dissipation and charge separation in PS II does not require that photoreactions per se be inhibited in the desiccated state. This view is consistent with the observation of small fluorescence and long-wavelength absorbance changes upon illumination with very strong light of desiccated lichens and mosses (e.g., Heber 2008; Heber et al. 2010; Yamakawa et al. 2012).

VI Photoprotective Function of Thermal Dissipation Associated with Desiccation-Induced Quenching

Photoinhibition of photosynthesis caused by exposure to high irradiance is strongly enhanced when consumption of absorbed light is reduced due to stress-induced inhibition of photosynthetic CO_2 fixation (Takahashi and Murata 2008). The imbalance between absorption of light and photon usage is extreme in a desiccated organism whose metabolism is completely arrested. Destruction of the photosynthetic apparatus in dry leaves of vascular plants is evidenced by the bleaching of herbarium plant specimens over time. In contrast, lichens have been shown to be much better protected against high light stress in the desiccated versus the wet state (Demmig-Adams et al. 1990). This is likely due to the photoprotective function of thermal dissipation associated with desiccation-induced quenching. Increased thermal dissipation of excited states in the dry condition has been assumed to compete successfully with energy conservation by charge separation (Veerman et al. 2007; Heber 2008). Such a mechanism would causally link disappearance of F_v and desiccation-induced quenching and explain the co-occurrence of the two phenomena during drying. Inhibition of desiccation-induced quenching by glutaraldehyde as well as by slow heating of dry thalli also results in the appearance of fluorescence increases by saturating light pulses indicating charge separation. One may therefore postulate that the drastic increase in thermal dissipation effectively prevents PS II from being able to transfer electrons. This would avoid formation of reduced acceptors that could recombine to form Chl triplet states as the main cause for formation of singlet oxygen (Krieger-Liszkay 2005).

Evidence for a photoprotective function was provided by experiments in which desiccation-induced quenching was manipulated. Heber (2008) varied the rate of desiccation, which resulted in a higher quenching after slow desiccation. For a green-algal and a cyanobacterial lichen, he showed that slowly versus rapidly desiccated thalli experienced less decrease of F_v/F_m after strong illumination in the dry state. Sun-grown thalli of *L. pulmonaria* displaying higher desiccation-induced quenching were also more resistant against high light than shade-grown thalli (Heber et al. 2010). Wieners et al. (2012) manipulated desiccation-induced quenching by supplying the green lichen alga *T. asymmetrica* with low concentrations of Zn^{2+} and I^- ions. Enhanced quenching was coupled with less decline of F_v/F_m after photoinhibitory treatment.

Although strong evidence indicates that desiccation-induced thermal dissipation is protecting against high-light-induced PS II inactivation (photoinhibition), prevention of damage under natural conditions remains to be linked to the latter quenching. The lichen *L. pulmonaria* exhibits strong desiccation-induced quenching (Heber et al. 2010), but is nevertheless sensitive to extended solar exposure in the dry state (Gauslaa and Solhaug 1999). Survival of exposure to high solar irradiance depends on concomitant heat resistance, which is low in the latter lichen. Screening of the photobionts by the cortex (the uppermost layer) of the thallus may contribute to the extraordinary resistance of desert lichens. The cortex is often pigmented or strongly reflectant. For mosses and free-living algae and cyanobacteria, even less data are available, making further investigations under field conditions necessary.

VII Conclusion

The available data as a whole show that only cryptogams are able to induce thermal dissipation in PS II during desiccation; this dissipation is so strong that it is able to almost fully outcompete charge separation. This is stronger than typical light-induced non-photochemical quenching in many vascular plants that corresponds to roughly a 50 %

reduction of F_v'/F_m'. (While the latter value is typical for annual plants, evergreen plants can persist throughout entire growth-precluding seasons in a photosynthetically inactive state, where virtually all light absorbed by their green needles or leaves is in excess, with near-zero PS II efficiencies; see Demmig-Adams et al., Chap. 24.) The desiccation-induced quenching process has specific characteristics that, in contrast to light-induced NPQ, are similar across all groups of cryptogams. However, no clear molecular mechanism for the desiccation-induced quenching has been established thus far. There are indications that the latter quenching can be modulated during acclimation of the organisms. However, it remains unclear which specific environmental factors induce this acclimation and via what mechanisms. In any case, it is highly probable that desiccation-induced quenching is contributing to the survival of desiccated cyanobacteria, lichens, and mosses in full sunlight in a state when all absorbed light must be considered excessive.

Acknowledgments

Mareike Jezek and Paul Christian Wieners are thanked for help with preparation of the manuscript.

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Chapter 20

The Peculiar Features of Non-Photochemical Fluorescence Quenching in Diatoms and Brown Algae

Johann Lavaud*
UMR 7266 'LIENSs, CNRS/University of La Rochelle, Institute for Coastal Research and Environment (ILE),
2 rue Olympe de Gouges, La Rochelle cedex 17000, France

and

Reimund Goss
Department of Plant Physiology, Institute of Biology, University of Leipzig,
Johannisallee 21-23, Leipzig 04103, Germany

Summary

Diatoms and brown algae are major contributors to marine primary production. They are biologically diverse, with thousands of different species, and are extremely successful, occupying almost every marine ecosystem ranging from the coastal-estuarine to deep-sea regions. Their ecological success is based in part on their ability to rapidly regulate photosynthesis in response to pronounced fluctuations in their natural light environment. Regulation of light

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*Author for correspondence, e-mail: johann.lavaud@univ-lr.fr

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_20, © Springer Science+Business Media Dordrecht 2014

harvesting, and the use of excitation energy, is largely based on effective dissipation of excessive energy as heat. Thermal dissipation of excitation energy is assessed as non-photochemical quenching of chlorophyll *a* fluorescence (NPQ). NPQ depends strongly on the conversion of xanthophylls: diadinoxanthin (Dd) to diatoxanthin (Dt) in the Dd-Dt cycle of diatoms and violaxanthin (V) to zeaxanthin (Z), via the intermediate antheraxanthin (A), in the VAZ cycle present in brown algae. Xanthophyll cycle (XC)-dependent thermal energy dissipation underlying NPQ represents one of the most important photoprotection mechanisms of diatoms and brown algae. In the present chapter, we review the biochemistry of XC enzymes with a special focus on co-substrate requirements and regulation of enzyme activity. In addition, we present a new model for the structural basis of XC-dependent NPQ in diatoms based on the latest experimental findings. In the last section, we highlight the importance of XC-dependent photoprotection for the ecological success of diatoms and brown algae in their natural environments.

I Introduction

In marine ecosystems, the main in situ abiotic driving forces for autotrophic growth are temperature, nutrients and light, the latter showing the highest variations in amplitude and frequency (MacIntyre et al. 2000; Kirk 2011). Consequently, the response of algae to their natural light environment is influenced by at least one irradiance-dependent process (Li et al. 2009). Because rapid light fluctuations are unpredictable, this process must be fast and flexible. The xanthophyll (XC)-dependent non-photochemical quenching of chlorophyll (Chl) *a* fluorescence (NPQ) turns out to provide such features. Photoprotective thermal energy dissipation that is reflected in NPQ is one of the main physiological processes used by algae to respond to the fluctuations of their natural light environment (Lavaud 2007; Brunet and Lavaud 2010; Goss and Jakob 2010; Depauw et al. 2012; Niyogi and Truong 2013). Diatoms and brown macroalgae can exert a strong NPQ (Lavaud et al. 2002a; Ruban et al. 2004; Lavaud 2007; Garcia-Mendoza and Colombo-Pallotta 2007).

Diatoms, as well as macrophytic brown algae (Phaeophyceae), belong to the stramenopiles that are classified as Chromista

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Abbreviations: A – Antheraxanthin; Asc – Ascorbate; Chl *a* – Chlorophyll *a;* Chl $a_{710-712}$ – Chlorophyll *a* fluorescence emission band between 710 and 712 nm; Dd – Diadinoxanthin; DDE – Diadinoxanthin de-epoxidase; DEP – Diatoxanthin epoxidase; DES – Diadinoxanthin de-epoxidation state; Dt – Diadinoxanthin; DTT – Dithiothreitol; FCP – Fucoxanthin chlorophyll protein; FCPa, FCPb, FCPo, Fcp6 – Fucoxanthin chlorophyll protein complex 'a', fucoxanthin chlorophyll protein complex 'b', fucoxanthin chlorophyll protein complex 'o', fucoxanthin chlorophyll protein '6' (=Lhcx1); H^+ – Protons; H_{II} – MGDG inverted hexagonal phase; LHC – Light-harvesting complex; LHC7, LHC8 – Light-harvesting complex protein '7', light-harvesting complex protein '8'; Lhcf, Lhcx – Light-harvesting complex protein binding fucoxanthin, light-harvesting complex protein 'x'; LHCSR – Stress-related light-harvesting complex protein (=LI818); LI818 – Light-induced light-harvesting complex protein '818'; MGDG – Monogalactosyldiacylgycerol; MPB – Microphytobenthic; Ndh – NAD(P)H dehydrogenase; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OEC – Oxygen evolving complex; PQ – Plastoquinone; PS II – Photosystem II; PS II RC – Photosystem II reaction center; Q1 and Q2 – Quenching sites 1 and 2; qE – 'Energy- or pH-dependent' quenching; qI – 'Photoinhibitory' quenching; qT – 'State-transition' quenching; SQDG – Sulfoquinovosyldiacylglycerol; V – Violaxanthin; VDE – Violaxanthin de-epoxidase; XC – Xanthophyll cycle; Z – Zeaxanthin; ZEP – Zeaxanthin epoxidase; Δ522 nm – Absorption change at 522 nm; ΔpH – Transthylakoid proton gradient;

(Kooistra et al. 2007). The chloroplasts of the Chromista are most likely the result of different endosymbiotic events, including a significant gene transfer from red algal and from prasinophyte cells (Frommolt et al. 2008; Moustafa et al. 2009). Among the genes that were retained are the ones encoding for the XC enzymes violaxanthin (V) de-epoxidase (VDE) and zeaxanthin (Z) epoxidase (ZEP). It was suggested that the latter genes were retained because, through the control of thermal energy dissipation underlying NPQ, the XC provides an efficient photoprotection mechanism, thereby improving photosynthetic performance (Frommolt et al. 2008). The plastids of the stramenopiles are further characterized by the existence of four envelope membranes and by a different pigment assemblage (Wilhelm et al. 2006; Lavaud 2007; Lepetit et al. 2012). The thylakoid membranes are arranged in regular stacks of three with no grana systems as in vascular plants (Pyszniak and Gibbs 1992).

Diatoms show a fascinating biodiversity with up to 100,000 different species divided into three clades and two morphological groups: the pennates and the centrics (longitudinal versus radial symmetry, respectively) (Kooistra et al. 2007). Planktonic diatoms evolved from benthic forms (Kooistra et al. 2007). Diatoms are almost equally distributed in marine and limnic habitats (Falkowski et al. 2004) and they are major contributors to marine primary production (Armbrust 2009). The light-harvesting system of diatoms consists of the fucoxanthin-chlorophyll protein (FCP) complexes that form oligomers whose organization is groups-/species-dependent (see Büchel, Chap. 11). In addition to the peripheral, main light-harvesting complex (LHC), which serves as antenna for both photosystem I (PS I) and II (PS II), diatoms contain a specific PS I FCP complex (Veith et al. 2009; Lepetit et al. 2010). FCP complexes are apoproteins of up to 18–19 kDa (Wilhelm et al. 2006; Lavaud 2007; Lepetit et al. 2012). The pigments of the XC cycle involving diadinoxanthin (=Dd) and diatoxanthin (=Dt) are bound to FCPs or located in a monogalactosyldiacylglycerol (MGDG) shield around the peripheral FCP complexes (Lepetit et al. 2010). These complexes are the site, where the majority of energy dissipation that results in NPQ takes place (see Büchel, Chap. 11).

Macrophytic brown algae are important coastal primary producers (Enriquez and Borowitzka 2011). There are about 2,000 known species (de Reviers and Rousseau 1999) whose phylogeny is still under debate (Philips et al. 2008). Their morphology is extremely diverse, with forms ranging from microscopic filaments to the well-known macrophytes like the Fucales and the Laminariales. Most of them are sessile organisms with few pelagic (open water) species. The LHC system of brown algae is also composed of FCP complexes (Dittami et al. 2010). The latter are isolated as trimeric units of 20-kDa apoproteins (De Martino et al. 1997; Fujii et al. 2012) that can possibly form higher dodecameric structures (Katoh et al. 1989; Passaquet et al. 1991). These complexes exhibit pigmentation similar to that of the corresponding complexes in diatoms, with the exception of the XC pigments that are the same as in green algae and vascular plants, i.e., V, antheraxanthin (A), and Z (Passaquet et al. 1991).

The variability of the XC-NPQ responses among lineages/genera/species has been a matter of growing debate. It was suggested that XC-NPQ diversity, and to a larger extent the diversity of photoadaptative strategy and capacity, would contribute to promoting competitive exclusion and spatial co-existence and/or temporal succession of algae in both pelagic and benthic (region along the bottom) systems (see Brunet and Lavaud 2010; Goss and Jakob 2010). The high efficiency of photoprotective XC-dependent energy dissipation underlying NPQ could also explain the amazing capacity of diatoms and brown algae to exploit differences in underwater light climate and to occupy a wide range of ecological niches.

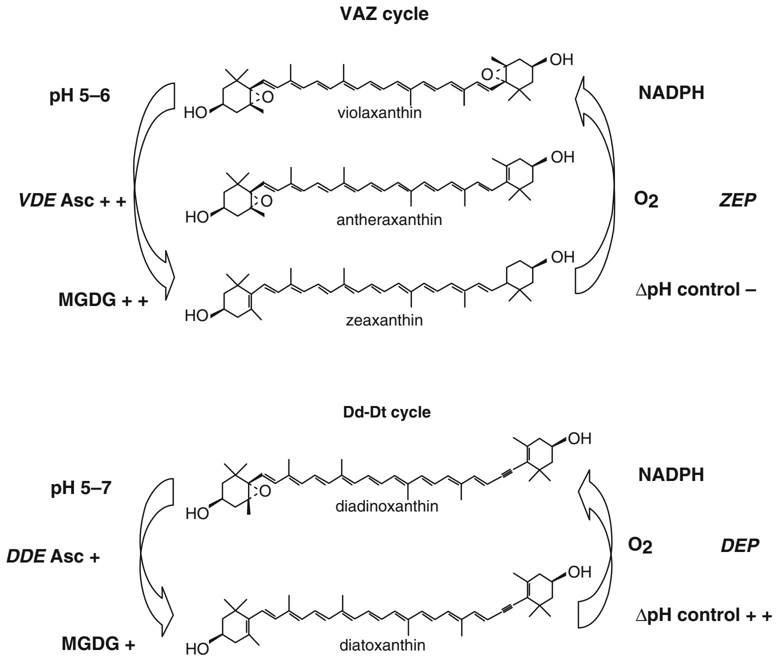

Fig. 20.1. Reaction sequence and enzymes of the VAZ and the Dd-Dt cycles. The VAZ cycle is the xanthophyll cycle (XC) of vascular plants as well as green and brown algae, the Dd-Dt cycle is the main XC of the algal classes Bacillariophyceae (diatoms), Xanthophyceae, Haptophyceae and Dinophyceae. The figure depicts the cofactor requirements of the enzymes VDE and DDE as well as ZEP and DEP. Symbols after the cofactors indicate that *high* (++) or only *low* (+) concentrations are needed for high enzyme activity. The figure shows that the transthylakoid proton gradient inhibits diatoxanthin (*Dt*) epoxidation (high ΔpH control, ++) whereas zeaxanthin (Z) epoxidation is unaffected by ΔpH (−). The figure also depicts the pH range of the thylakoid lumen, where VDE and DDE are active (for more details, see the text). *VDE/DDE* violaxanthin and diadinoxanthin de-epoxidase, respectively, *ZEP/DEP* Z and Dt epoxidase, respectively, *Asc* ascorbate, *MGDG* monogalactosyl-diacylglycerol, *V* violaxanthin, *Dd* diadinoxanthin.

II Xanthophyll Cycle-Dependent NPQ

A Xanthophyll Cycles of Diatoms and Brown Algae

1 Diatoms

Diatoms possess both the VAZ and the Dd-Dt cycle (Lohr 2011). While the Dd-Dt cycle is the main XC and is essential for NPQ, the VAZ-cycle pigment V serves as precursor pigment in the biosynthesis of Dd and the main light-harvesting xanthophyll fucoxanthin (Lohr 2011; Dambek et al. 2012). In line with their function as xanthophyll synthesis intermediates, the VAZ-cycle pigments are only present in significant amounts after exposure to prolonged excess light, which triggers profound de-novo carotenoid synthesis (Lohr and Wilhelm 1999).

The Dd-Dt cycle (Fig. 20.1) consists of a de-epoxidation step from Dd, with one epoxy group, to the epoxy-free Dt (Lohr 2011). The back epoxidation reaction re-introduces the epoxy group into the Dt molecule. De-epoxidation from Dd to Dt takes place under excess light intensities but may also be triggered during long periods of darkness (Jakob et al. 1999, 2001; Cruz et al. 2011) (see section II.C). Epoxidation is usually observed during periods of low light or darkness that follow excess light exposure (Goss et al. 2006a).

De-epoxidation is catalyzed by the enzyme Dd de-epoxidase (DDE) while Dt epoxidase (DEP) triggers the epoxidation.

Important differences exist between the latter two enzymes and the respective enzymes of the VAZ cycle of vascular plants, VDE and ZEP. DDE exhibits a different pH-dependence (Jakob et al. 2001); while VDE activity occurs at pH values of 6.5 or lower, DDE activity can already be observed at neutral pH values. The pH optimum of DDE is also shifted to higher pH values (5.5) in isolated thylakoids of diatoms as compared to VDE (Jakob et al. 2001; Grouneva et al. 2006). These differences imply that, in diatoms, Dd de-epoxidation can be triggered by a lower light-driven proton gradient across the thylakoid membrane (ΔpH) (see section II.C). It is not clear if DDE activation at less acidic pH values results in a conformational change of the enzyme as has been proposed for VDE of vascular plants. Inactive VDE is monomeric, and forms a dimer after exposure to low/acid pH values (Arnoux et al. 2009; Saga et al. 2010). It has been proposed that the dimeric form of VDE is able to simultaneously de-epoxidize both epoxy groups of V (Arnoux et al. 2009). Such a direct de-epoxidation of V to Z is, however, in contrast to the various studies that describe V de-epoxidation under natural conditions in intact plant leaves. These studies have shown that a significant accumulation of A can occur during the morning with increasing light intensities and during the afternoon when the irradiance decreases (Adams and Demmig-Adams 1992).

Since Dd de-epoxidation only comprises one de-epoxidation step, the greatest advantage of dimeric VDE, i.e., simultaneous de-epoxidation of both epoxy-groups of V, does not apply to the Dd-Dt cycle. Additionally, DDE exhibits a significantly lower K_M value (than VDE) for the co-substrate of de-epoxidation, ascorbate (Grouneva et al. 2006). The K_M value for isolated DDE of *Cyclotella meneghiniana* is 0.7 mM versus 2.3 mM for VDE of spinach. DDE is also able to maintain efficient de-epoxidation in the presence of very low concentrations of ascorbate, although this leads to a shift of the pH-optimum towards lower pH values (Grouneva et al. 2006). The optimized co-substrate usage of DDE indicates that the diatom cell is not providing a high concentration of ascorbate for Dd de-epoxidation.

A further, albeit more indirect, optimization is seen in the lipid requirement of the de-epoxidation. Efficient Dd de-epoxidation depends on presence of the main thylakoid lipid MGDG (Goss et al. 2005, 2007). MGDG serves to solubilize the hydrophobic pigment Dd, making it accessible for DDE. For the complete solubilization of a certain amount of Dd, a lower concentration of MGDG is sufficient than for the same number of V molecules (Goss et al. 2005, 2007). This increased solubilization efficiency of Dd is in line with the often large XC pigment pool of diatoms with values of up to 800 mM Dd + Dt per M Chl *a* (Lavaud et al. 2003; Lepetit et al. 2010). A higher number of Dd molecules can presumably be incorporated into the MGDG phase, resulting in increased de-epoxidation (Lavaud et al. 2003). A high concentration of Dt in the thylakoid lipid phase is in line with increased photoprotection by Dt (Lepetit et al. 2010). Additionally, MGDG forms what are termed inverted hexagonal (H_{II}) phases essential for efficient de-epoxidation (Latowski et al. 2002; Goss et al. 2005, 2007). H_{II} phases are likely associated with the FCP complexes and represent the docking sites for DDE once the enzyme has been activated by ΔpH (Lepetit et al. 2010, 2012), i.e., the MGDG shield around the LHC targets DDE to the site where the majority of Dd is located.

The DEP also shows significant differences compared to ZEP, especially with regard to the epoxidation kinetics that can be almost 20-fold higher (Goss et al. 2006a). Consequently, while Z epoxidation in vascular plants and green algae is slow compared to V de-epoxidation (Goss et al. 2006b; Schaller et al. 2012), Dt epoxidation is fast and can almost equal the Dd de-epoxidation kinetics. The extremely fast Dt epoxidation must be viewed in conjunction with the differences in the mechanism of Dt- and Z-dependent NPQ (Goss et al. 2006a; Goss and Jakob 2010) (see section II.B.1). While

Z normally quenches Chl *a* fluorescence non-photochemically in the presence of ΔpH, Dt maintains a stable NPQ after ΔpH relaxation dissipation. This stable quenching is comparable to a certain type of stable, ΔpH-independent Z quenching seen in evergreen plants during the winter or subjected to water stress, or low light-grown plants subjected to excess light (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006; see Adams et al., Chap. 23, and Demmig-Adams et al., Chap. 24). Due to the stable Dt quenching, a complete disengagement of Dt from thermal energy dissipation and relaxation of NPQ is presumably possible only by efficient removal of Dt from its FCP-binding sites. However, the possibility of an extremely fast Dt epoxidation poses a problem for the Dd-Dt cycle when, during excess light illumination, a fast net accumulation of Dt is needed to trigger thermal energy dissipation resulting in NPQ (Lavaud et al. 2002b; Goss et al. 2006a; Lepetit et al. 2013) (see section II.B.1). Without an efficient control of Dt epoxidation, excess light could not result in a fast and strong increase of Dt concentration. Therefore, it is of great importance that DEP be under the strict control of light-driven ΔpH, i.e., ΔpH almost completely inhibits epoxidation of Dt (Mewes and Richter 2002; Goss et al. 2006a). The mechanism of this inactivation is, however, still unknown. Nevertheless, upon exposure to gradually increasing light intensities, a complete inactivation of the DEP can be circumvented (Dimier et al. 2009).

With respect to the localization of the XC enzymes, the pH-dependent activation of DDE and the NADPH requirement of DEP indicate that the enzymes are situated in the thylakoid lumen and on the stromal side of the thylakoid membrane, respectively, as it has been described for VDE and ZEP of vascular plants (Jahns et al. 2009; Latowski et al. 2011). The MGDG-requirement of Dd de-epoxidation indicates that DDE, like VDE (Schaller et al. 2010), binds to MGDG-enriched regions of the thylakoid membrane that are located in the vicinity of the FCP complexes (Lepetit et al. 2010). The binding of DDE to MGDG-enriched regions occurs after the DDE pH-activation. Whether DEP is a peripheral, loosely-bound membrane protein, as it has been recently suggested for ZEP (Schaller et al. 2012), remains to be assessed.

Genetic studies of the Dd-Dt cycle demonstrated that the genes encoding for DDE and DEP are closely related to the respective genes of the Prasinophytes (Frommolt et al. 2008). The comparable structure of the gene arrangement in diatoms and Prasinophytes, i.e., a cluster of the DDE-VDE gene with one of the multiple copies of the DEP-ZEP gene, further strengthens the notion that the Chromista have acquired the XC genes through an early endosymbiosis involving a primitive green alga, in addition to the later endosymbiosis events with red algae. A recent study reported the genetic manipulation of the DDE gene in *Phaeodactylum tricornutum* (Lavaud et al. 2012). By suppressing the transcript level of the DDE in vivo, it was possible to inhibit up to 50 % of Dt synthesis and to strongly reduce the extent of NPQ, as well as to disturb the ΔpH-Dt-NPQ relationship. Such approaches will help to decipher the mechanism underlying NPQ in diatoms in the future (see section II.B.1).

2 Brown Algae

Unlike diatoms, brown algae only possess the VAZ cycle (Rodrigues et al. 2002; Gévaert et al. 2003; Garcia-Mendoza and Colombo-Pallotta 2007). The VAZ cycle (Fig. 20.1) consists of a two-step de-epoxidation from the di-epoxy xanthophyll V to Z via the intermediate A (Yamamoto et al. 1962; Hager 1967a, b). This reaction sequence, taking place under excess light (as is the case for the VAZ cycle in plants and the Dd-Dt cycle in diatoms), is reversed in low light or in darkness. The enzymes catalyzing the forward and backward reaction are VDE and ZEP, respectively. Almost nothing is known about the biochemistry of these enzymes in brown algae. Since the overall characteristics of the XC, i.e., kinetic, rates of the first and second de-epoxidation and epoxidation steps, and ratio of the first to the second de-epoxidation and epoxidation step, are generally comparable to vascular plants (Rodrigues et al. 2002), the properties of the VAZ-cycle enzymes in brown algae are most likely also comparable. However, V de-epoxidation may be slightly slower and Z epoxidation slightly faster in brown algae (Garcia-Mendoza et al. 2011). Brown algae thus exhibit slow Z epoxidation kinetics similar to those in vascular plants (Garcia-Mendoza and Colombo-Pallotta 2007). This means that, during a low-light or dark period following excess light illumination, a significant amount of Z remains bound to the FCP complexes. As described above for Dt in diatoms (see also section II.B.1), Z in brown algae also seems to be able to contribute to a sustained NPQ even after ΔpH relaxation (Garcia-Mendoza and Colombo-Pallotta 2007). Hence the structure/organization of the FCP complexes appears to be responsible for this stable Dt- or Z-dependent NPQ. In the LHC II complexes of vascular plants such a pronounced stable Z-dependent NPQ is normally not seen after ΔpH relaxation (Goss et al. 2006a), but can be found in plants exposed to excess light for extended time periods with or without additional stresses (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006; see also Adams et al., Chap. 23, and Demmig-Adams et al., Chap. 24). It remains an open question as to why brown algae are unable to epoxidize Z to V with comparably fast kinetics as observed for the conversion of Dt to Dd in diatoms. Based on the observation that the conversion rate of A to V in darkness decreases in *M. pyrifera* exposed to longer illumination periods (Ocampo-Alvarez et al. 2013), it has been proposed that the slow epoxidation reaction represents an adaptive strategy of brown algae to cope with light stress conditions. Maintaining A in the antenna system may allow for a faster response to saturating light by accelerating Z accumulation and NPQ induction (Garcia-Mendoza et al. 2011).

B Mechanism of Xanthophyll-Dependent NPQ

1 Diatoms

In diatoms, NPQ is mainly composed of qE, i.e., pH- or energy-dependent quenching (see Papageorgiou and Govindjee, Chap. 1, and Logan et al., Chap. 7 for a definition of the different quenching components). Indeed, qT, the NPQ component based on state-transitions, i.e., the reversible, wavelength-dependent translocation of part of the PS II antenna to PS I, seems to be absent in diatoms (Owens 1986). It is, however, still unclear if a light intensity-dependent structural reorganization of PS II and PS I, which might lead to an increased spillover of excitation energy, takes place. The origin and mechanism of qI, the slowly reversible so-called “photoinhibitory” quenching, remain unclear, although it is likely that XC pigments are involved (Demmig-Adams and Adams 2006; see also Adams et al., Chap. 23, and Demmig-Adams et al., Chap. 24). Mechanistic models of xanthophyll-dependent NPQ in diatoms have been proposed before (Lavaud 2007; Goss and Jakob 2010; Lepetit et al. 2012) and significant recent advances have been made since (Miloslavina et al. 2009; Gundermann and Büchel 2012; Lavaud and Lepetit 2013). The following tentative NPQ model for pennate diatoms (Fig. 20.2, see Büchel, Chap. 11 for a model for centric diatoms) is based on the recent observation of two quenching sites (Q1 and Q2) in diatoms and vascular plants (Miloslavina et al. 2009; Jahns and Holzwarth 2012). The model includes differences observed between diatom cells exhibiting a low versus high NPQ amplitude (see also section III.A).

The quenching site Q2 is proposed to be located in a part of the LHC antenna that remains attached to the PS II core; its quenching properties presumably rely on an aggregation state induced by lowering of the luminal pH and further depends on the thylakoid membrane lipid environment as shown in vitro (Gundermann and Büchel 2012) and

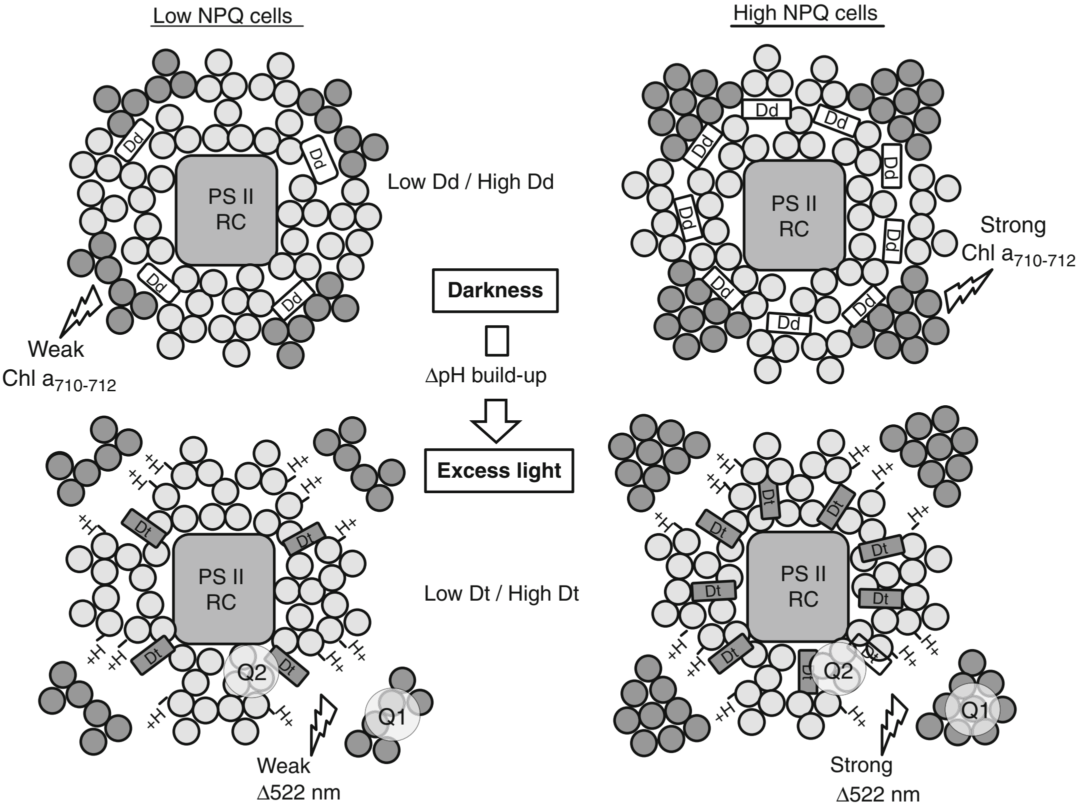

Fig. 20.2 A working hypothesis for xanthophyll-dependent energy dissipation (resulting in NPQ) in pennate diatoms depicting the organization of the PS II LHC antenna system in "low NPQ" and "high NPQ" cells dark-acclimated and exposed to excess light conditions, respectively (see also Büchel, Chap. 11). It should be noted that this model is based mainly on observations in P. tricornutum; differences observed in other species are discussed in the text (see section III.A). Light and dark-grey circles are Fcp polypeptides forming trimers and higher FCP oligomeric complexes, respectively; the latter are loosely bound to the PS II LHC antenna system and can disconnect under excess light exposure. Chl $a_{710-712}$ chlorophyll a fluorescence emission band between 710 and 712 nm, *Dd* diadinoxanthin, *Dt* diatoxanthin, H^+ protons, *PS II RC* photosystem II reaction center, *Q1/Q2* quenching sites 1 and 2, *ΔpH* trans-thylakoid proton gradient, *Δ522 nm* absorption change at 522 nm. Δ522 nm is a spectroscopic fingerprint that provides information on the engagement of Dt in energy dissipation (Ruban et al. 2004; Lavaud and Lepetit 2013), i.e., its effective involvement in NPQ. It is similar to the Δ525–Δ535 nm in land plants (Jahns and Holzwarth 2012; Ruban et al. 2012). The model depicted here aims to illustrate the effective involvement of Dt as an allosteric regulator of a conformational change in the LHC antenna. The conformational change is believed to be an aggregation of part of the LHC antenna generated by the pronotation of FCP-binding sites (Goss et al. 2006a; Lavaud and Kroth 2006) (see also Büchel, Chap. 11). Chl $a_{710-712}$ is a spectroscopic fingerprint illustrating the ability to form oligomeric FCP complexes that disconnect from the PS II core under excess light conditions (Miloslavina et al. 2009; Lepetit et al. 2012) in order to amplify Dt-dependent energy dissipation and thus NPQ (Lavaud and Lepetit 2013). See the text for further details.

on the binding of Dd-Dt (Miloslavina et al. 2009). The quenching Q1 is proposed to be located in a part of the LHC antenna that disconnects from PS II upon illumination and forms oligomeric FCP complexes (potentially including both Lhcf and Lhcx proteins, see below) (Miloslavina et al. 2009). The quenching property of Q1 appears to be relatively independent of the presence of Dd-Dt in vitro (Gundermann and Büchel 2012). Chl $a_{710-712}$ oligomeric FCP complexes are already present in dark- and low light-adapted cells (Miloslavina et al. 2009; Chukhutsina et al. 2013; Lavaud and Lepetit

2013). However, until the latter disconnect from PS II, their dissipative property remains negligible. Presence of large and stable Chl $a_{710-712}$ FCP oligomers in high NPQ, dark-acclimated cells, might be induced by the decrease of the thylakoid lipid ratio MGDG/SQDG (Lepetit et al. 2012). We propose that disconnection of Chl $a_{710-712}$ FCP oligomers upon illumination is generated by both protonation of the Lhcf/Lhcx proteins involved in NPQ (Goss et al. 2006a; Lavaud and Kroth 2006) and conversion of Dd to Dt (see below and Enriquez et al. 2010). Once disconnected, the Chl $a_{710-712}$ FCP oligomers show a spatial arrangement of pigment-protein complexes that establishes new Chl *a*-Chl *a* interactions reducing the lifetime of excited Chl *a* (Miloslavina et al. 2009), thereby dissipating excessive excitation energy as heat at the Q1 site in addition to the Q2 site. Recent data obtained in *P. tricornutum* support such a mechanism; they revealed that FCP macrodomains undergo a fast and reversible light-induced reorganization that correlates with the kinetics of NPQ (Szabo et al. 2008).

The exact regulatory role of Dt remains under debate; two alternatives have been proposed that are not mutually exclusive (Fig. 20.2; Enriquez et al. 2010; see also Holzwarth and Jahns Chap. 5): (1) direct quenching of excited Chl *a* at the Q2 site by Dt via a transfer of energy from the respective S_1 states of Chl *a* and Dt as measured in vitro, or (2), together with proton binding, de-epoxidation of Dd to Dt could support a conformational change of the Dd-Dt-binding Lhcf/Lhcx proteins, thereby promoting disconnection of the FCP oligomers and formation of the Q1 site. The latter possibility (2) is supported by (i) the sensitivity of the Δ522-nm fingerprint to the ΔpH-uncoupler NH_4Cl and to the Dd de-epoxidation-inhibitor dithiothreitol (DTT) and (ii) numerous studies that succeeded in uncoupling ΔpH, Dt and NPQ in several pennate and centric species (Lavaud et al. 2002b, 2012; Goss et al. 2006a; Lavaud and Kroth 2006; Grouneva et al. 2009; Cruz et al. 2011; Gundermann and Büchel 2012). The latter studies showed three essential features of NPQ in diatoms: (1) a certain magnitude of the ΔpH is needed for complete induction of NPQ, (2) NPQ can be modulated to a certain extent by Dt in the absence of a ΔpH, once Dt had been synthesized in the presence of a ΔpH, and (3) Dt is mandatory for NPQ. The possibility of a coupling between both a direct and an indirect effect of Dt in NPQ is consistent with the synergistic effect of ΔpH and Z in the NPQ/qE allosteric model of vascular plants (Jahns and Holzwarth 2012; Ruban et al. 2012). Nevertheless, a significant part of NPQ can be maintained in the dark without any ΔpH as long as Dt is present (Goss et al. 2006a; Lepetit et al. 2013), see also section II.A.1).

The persistence of Dt in the dark was proposed to be responsible for keeping the FCP oligomers in an aggregated state (Goss et al. 2006a). The latter feature would be in line with the persistence of part of the quenching at the Q2 (and/or Q1) site(s) in the dark, i.e., as long as the majority of the Dt molecules are not converted back to Dd, the FCP oligomers cannot reconnect to PS II (Fig. 20.2). Retention of Dt and its engagement in thermal energy dissipation resulting in sustained NPQ in the dark were reported for several species, including pennate and centric representatives (Goss et al. 2006a; Lavaud and Lepetit 2013). Because its dark relaxation is slow, this sustained component of NPQ, also called "dark NPQ" (see section III.B), probably contributes in part to qI, together with photo-damage/-inactivation of PS II (Wu et al. 2012). Sustained "dark NPQ" is often observed after a prolonged exposure to excess light (Lavaud and Lepetit 2013), which exhausts the pool of NADPH+H^+ and leads to a slower epoxidation of Dt back to Dd in the dark (Goss et al. 2006a). Hence, at least part of the "dark NPQ" could be interpreted as a slow recovery from the light-dependent reversible reorganization of the LHC system. "Dark NPQ" is also observed after a prolonged exposure to darkness (several hours), where it is induced by Dt molecules generated by a chlororespiratory ΔpH (see section II.C).

The identity of the proteins involved in formation of the Q1 and Q2 sites remains unclear. These proteins are obviously different among diatom species (see Büchel, Chap. 11 for further details). In the centric diatom *C. meneghiniana*, the basic organizational units of FCPs are trimers (FCPa) and hexamers (FCPb) composed of different Lhcf/Lhcx polypeptides. Their respective features (binding of Dd and Dt, ability to aggregate, dependence of the quenching property on pH and Dt) support the possible identification of FCPa as Q2 and FCPb as Q1 (Miloslavina et al. 2009; Gundermann and Büchel 2012; Lepetit et al. 2012), although such a proposal remains to be further evaluated. In the pennate diatom *P. tricornutum*, a clear differentiation between FCP fractions that form trimers or hexa- to nonameric complexes (termed "FCPo") does not exist (Lepetit et al. 2007, 2010; Joshi-deo et al. 2010). FCPa contains the Fcp6 polypeptide, a member of the Lhcx family (Gundermann and Büchel 2012), the functional ortholog of LHCSR in green algae (Niyogi and Truong 2013 and see also Morosinotto and Bassi, Chap. 14; Finazzi and Minagawa, Chap. 21), termed Lhcx1 in *P. tricornutum*. It was proposed that Fcp6 may interact with Fcp2, a member of the Lhcf family, to form a quenching center (Gundermann and Büchel 2012) that we hypothesize to be Q2. While Fcp2 might be responsible for the pH sensitivity of FCPa (Gundermann and Büchel 2012), and could therefore drive the above-mentioned aggregation of FCPs at the Q2 site, the role of Fcp6/Lhcx1 remains unclear. While the latter complexes' ability to bind protons has not been completely confirmed, they appear to bind Dt (Lepetit et al. 2010; Zhu and Green 2010; Gundermann and Büchel 2012).

It has recently been demonstrated that the transcription of some Lhcx genes is co-regulated with the Dd+Dt content via the redox state of the PQ pool (Lepetit et al. 2013). In *P. tricornutum*, an increase, via genetic engineering, of the in-vivo Lhcx1 level generates a higher NPQ (Bailleul et al. 2010; Depauw et al. 2012). Zhu and Green (2010) proposed a structural role for Lhcx1 that fits well with its presence in significant amounts in low light-acclimated cells (Bailleul et al. 2010; Schellenberger Costa et al. 2013; Lepetit et al. 2013). Nevertheless, this proposal is not in contradiction with Lhcx1 providing Dd- and Dt-binding sites or the amount of Dd and -Dt bound to the LHC system being modulated by the amount of Lhcx1 as a function of strains/species and of the organism's acclimation to light (Lavaud and Lepetit 2013). There is currently no information on the Lhcf/Lhcx composition of the FCP oligomers responsible for Chl $a_{710-712}$.

In addition to Dt-dependent NPQ, part of the thermal dissipation of the excessive excitation energy can be independent of Dt synthesis. The latter NPQ is a PS II reaction center-type quenching, may include recombination of Q_A^- and the S2/S3 state of the oxygen-evolving complex (OEC) (Eisenstadt et al. 2008), and is most likely caused by an excess light-induced structural change of the PS II core complex itself. Earlier measurements (Lavaud et al. 2002c; Feikema et al. 2006) revealed the existence of cyclic electron flow within PS II that includes an electron transfer pathway from the acceptor to the donor side (Lavaud 2007). This process has been interpreted as a protection mechanism against acceptor side photoinhibition in the OEC and donor side photoinhibition in PS II where OEC is temporarily disabled.

2 Brown Algae

Little is known about the mechanism of xanthophyll-dependent NPQ in brown algae (Enriquez and Borowitzka 2011). Although the latter algae display a different XC (see section II.A.2), it seems that the NPQ of brown algae shows similar features to that of diatoms. As observed for diatoms, NPQ is mainly composed of the qE component, as a wavelength-dependent qT seems to be absent (Fork et al. 1991). However, the possibility of an excess light-dependent qT in brown algae has yet to be studied systematically. Also similar to the situation in diatoms, ΔpH alone cannot generate NPQ (Lavaud et al. 2002b; Lavaud and Kroth 2006); NPQ requires the concomitant binding of A and Z to the LHC system (Garcia-Mendoza and Colombo-Pallotta 2007; Garcia-Mendoza et al. 2011). The relationship between NPQ and Z (and A) is similar to that of diatoms, demonstrating an obligatory role of Z and A (Garcia-Mendoza and Colombo-Pallotta 2007; Ocampo-Alvarez et al. 2013). Strikingly, this relationship depends on light acclimation and the size of the VAZ pool (Ocampo-Alvarez et al. 2013), as is also the case in diatoms (Schumann et al. 2007). Because NPQ in brown algae reacts as in diatoms to DTT, ΔpH uncouplers (NH_4Cl, nigericin), and shows the same differences in pre-illuminated versus dark-adapted samples (Garcia-Mendoza and Colombo-Pallotta 2007; Garcia-Mendoza et al. 2011), it is likely to display similar mechanistic features as well (see section II.B.1).

Alternatively, NPQ in brown algae was proposed to depend only on the synthesis of Z and A, and thus would be controlled by the activity of the XC enzymes and by the number of quenching sites in the light-harvesting complex (Ocampo-Alvarez et al. 2013). Such a ΔpH-independent but Z- and A-dependent NPQ would be comparable to the slowly reversible, pH-independent form of NPQ in evergreen vascular plants persisting throughout harsh seasons (Demmig-Adams et al. 2006; see also Demmig-Adams et al., Chap. 24). In general, it can be concluded that the considerable NPQ observed in some diatoms (Lavaud et al. 2002a; Ruban et al. 2004) and brown algae (Garcia-Mendoza and Colombo-Pallotta 2007) is independent of the specific nature of the XC pigments, i.e., Dt or Z and A. Instead, NPQ most likely depends on the ability to synthesize a large amount of either Dt or Z and A (Lavaud et al. 2003; Garcia-Mendoza and Colombo-Pallotta 2007) and on the spatial organization and the protein composition of the FCP complexes (Lavaud 2007; see section II.A.1).

The Lhcf/Lhcx proteins involved in thermal energy dissipation and thus NPQ in brown algae have not been identified yet. However, the recent sequencing of the *Ectocarpus silicosus* genome highlighted their large diversity with about 40 *Lhcf* genes and 13 *Lhcx* genes (Dittami et al. 2010). Interestingly, and comparable to the situation in diatoms, an FCP complex with a high content of XC pigments exists (De Martino et al. 1997, 2000); it most likely is one of the NPQ sites (Q2). In *M. pyrifera*, a large VAZ pool is found in blades growing at the surface and exposed to high and changing light conditions (Ocampo-Alvarez et al. 2013). This is concomitant with a higher expression of two LI818 isoforms (“LHC7” and “LHC8”) (Konotchick et al. 2013), as the functional orthologs of the diatom Lhcx and the green algal LHCSR (Niyogi and Truong 2013 and see also Morosinotto and Bassi, Chap. 14; Finazzi and Minagawa, Chap. 21). It is thus very likely that LI818 proteins are involved in the NPQ process in brown algae, although a thorough biochemical investigation is needed to decipher their exact role.

C Regulation of Xanthophyll Cycle-Dependent NPQ

Because Dt and Z are mandatory for NPQ in diatoms and brown algae, respectively, the most important factors for regulation of the majority of energy dissipation underlying NPQ are the kinetics of the build-up and relaxation of the ΔpH and the availability of the co-substrates of the de-epoxidation and epoxidation reactions (summarized in Fig. 20.1). In general, Dd de-epoxidation is faster than the respective conversion of V to A and Z in vascular plants and brown algae (Lavaud et al. 2002b; Goss et al. 2006a). The faster kinetics are caused by the almost immediate activation of DDE due to the shift of its pH-optimum to higher pH-values (Jakob et al. 2001). This shift is accompanied by complete inhibition of Dt epoxidation by establishment of the ΔpH (Mewes and Richter 2002; Goss et al. 2006a; see also section II.A.1). Rapid Dd de-epoxidation correlates with a very fast induction of NPQ

(Lavaud et al. 2002a, c; Lavaud and Kroth 2006). The almost immediate generation of NPQ is further aided by the existence of an NPQ component that shows extremely fast kinetics after the onset of illumination (Grouneva et al. 2008). This component relies on establishment of the ΔpH and depends on the concentration of Dt already present at the onset of illumination (Lavaud and Kroth 2006; Grouneva et al. 2008). A comparably fast NPQ component that depends on the presence of Z at the onset of actinic illumination has also been observed in vascular plants (Demmig-Adams et al. 1989; Kalituho et al. 2007; see also Demmig-Adams et al., Chap. 24).

Due to the prominent differences in the pH-dependent activation of DDE of diatoms and VDE of vascular plants, low pH-gradients are already sufficient to activate DDE of diatoms (see section II.A.1). This means that, in addition to light-dependent DDE activation, Dd de-epoxidation can also be triggered by a chlororespiratory ΔpH in the dark (Jakob et al. 1999, 2001; Cruz et al. 2011). Although establishment of a ΔpH by chlororespiration is rather slow, significant synthesis of Dt occurs during prolonged periods of darkness. In *P. tricornutum*, accumulation of Dt is further supported by inhibition of DEP by the chlororespiratory ΔpH (Cruz et al. 2011). Dt epoxidation is also limited when the co-substrate of DEP, NADPH, is not available in sufficient concentration. Such a partial inhibition of Dt epoxidation occurs during dark periods that follow excess light illumination (Goss et al. 2006a). It was proposed that both the ongoing Calvin-Benson Cycle and the chlororespiratory electron flow act as sinks for NADPH, thereby depriving DEP of its cosubstrate (Grouneva et al. 2009). In contrast, the conversion of Dt to Dd is very fast and efficient after a transition from high to low light (Goss et al. 2006a; Grouneva et al. 2009), probably because electron transport is sufficient to generate the NADPH needed for Dt epoxidation. Both the fast deprivation of NADPH during dark periods following excess light illumination and the maintenance of NADPH synthesis during low light illumination is in line with the assumption that diatoms contain a significantly smaller pool of $NADP^+$/NADPH compared to vascular plants (Goss et al. 2006a, b; Grouneva et al. 2009).

The synthesis of Dt in darkness results in the establishment of NPQ (Jakob et al. 1999, 2001; Cruz et al. 2011). However, the quenching efficiency of Dt generated by a chlororespiratory ΔpH may be lower than that of Dt formed by a light-driven ΔpH (Cruz et al. 2011). In *Thalasiosira pseudonana*, a high concentration of Dt can be induced in the dark by anaerobic conditions without development of a correspondingly high NPQ (Cruz et al. 2011). The return to aerobic conditions strongly increases the quenching efficiency of Dt, most likely by a significant increase of lumen acidification via a combination of electron transport from the plastoquinone (PQ) pool to oxygen by PQ oxidase and proton translocation by a type-1 NAD(P)H dehydrogenase (Ndh) (Cruz et al. 2011).

Chlororespiration also contributes to Dt synthesis and NPQ during excess light illumination (Eisenstadt et al. 2008; Grouneva et al. 2009). This, however, depends on the diatom species (Grouneva et al. 2009). The complete inhibition of light-driven linear electron transport by DCMU in *P. tricornutum* does not lead to a breakdown of ΔpH. In contrast, electron flow from the stroma to the PQ pool and the PQ oxidase, in combination with a type-1 Ndh, maintains a chlororespiratory ΔpH that leads to further Dd de-epoxidation and NPQ (see above). Since this latter electron flow is not coupled to $NADP^+$ reduction, the substrate of DEP is not synthesized, thus restricting a competing epoxidation of Dt to Dd. In the diatom *C. meneghiniana*, the first steps of the alternative electron flow are comparable and also result in donation of electrons from stromal sources to the PQ pool. Further electron transfer reactions are, however, different and include electron transport from the PQ pool to PS I, which results in the production of NADPH. Chlororespiratory electron transport in *C. meneghiniana* does not lead to the generation of a ΔpH, most likely due to the absence of electron donation to PQ oxidase and due to involvement of a type-2 Ndh that does not translocate protons. Consequently, chlororespiratory-dependent Dd de-epoxidation and NPQ is not observed as long as the generation of NADPH simultaneously enables epoxidation of preformed Dt to Dd.

III Importance of the Xanthophyll Cycle and NPQ in the Field

A Diatoms of the Phytoplankton

Planktonic diatoms live in a three-dimensional environment characterized by strong physical driving forces coupled with a depth-dependent attenuation of the irradiance. They usually undergo passive movements determined by their sinking rate and the water turbulence. Many studies have explored the in-situ functioning of the XC and NPQ in planktonic diatoms in different ecosystems and in relation to changes of the diurnal, seasonal, and latitudinal underwater light climate (see Brunet and Lavaud 2010). On a yearly scale, the pool size of Dd+Dt, the extent of Dd de-epoxidation, and NPQ correlate well with day length and seasonal changes in irradiance. On a daily scale, Dt and NPQ track the course of the sun and the water depth, showing an increase at midday and in the upper layer of the water column. In that framework, the dynamics of the Dd+Dt pool size and Dt synthesis were often used to characterize the coupling of phytoplankton photosynthesis with light and hydrodynamics. While changes in the pool size of Dd+Dt provide information on the average irradiance to which the diatom cells have been exposed during the past hours/days, the dynamics of Dt synthesis can be used as a tracer for cell movements within the euphotic zone (Brunet and Lavaud 2010; Hashihama et al. 2010). The dynamics of the XC and NPQ, together with other physiological processes (MacIntyre et al. 2000), thus appear relevant for the regulation of the photosynthetic productivity of planktonic diatoms in the mixed upper layer of the water column, especially in the context of a regular exposure to excessive light intensities (Alderkamp et al. 2010; Brunet and Lavaud 2010) coupled with other environmental stresses (Petrou et al. 2011) and during bloom conditions (Fujiki et al. 2003).

Planktonic diatoms, and to a larger extent planktonic microalgae, show a large inter-group/species XC and NPQ diversity (Lavaud et al. 2007; Brunet and Lavaud 2010; Goss and Jakob 2010). It was proposed that the XC-NPQ activity and efficiency may be influenced by ecological niche adaptation, i.e., the light environment of the habitat from which the strains/ecotypes originate (Lavaud 2007; Brunet and Lavaud 2010). In other words, the XC and NPQ efficiency would be a functionally adaptive trait that would participate in the cost/benefit balance of photoadaptative strategies (McKew et al. 2013) and that could, in part, explain the geographical/spatial species distribution and their temporal succession (Lavaud 2007; Brunet and Lavaud 2010). Dimier et al. (2009) even proposed a classification of ecological groups of phytoplankton based on their XC dynamics.

The hypothesis of XC and NPQ as functional traits was investigated by a comparison of representatives of the main phytoplankton groups (Casper-Lindley and Bjorkman 1998; Meyer et al. 2000; van Leeuwe et al. 2005; Wagner et al. 2006; Kropuenske et al. 2009; van de Poll et al. 2011). In general, diatoms seem to cope well with a fluctuating light climate, i.e., they can exert strong NPQ (Lavaud et al. 2002a; Ruban et al. 2004; Lavaud 2007). This feature is an adaptive advantage in deeply-mixed aquatic habitats: it drives, in part, the competition for light among phytoplankton groups that ultimately participate in species succession independent of other environmental cues (Huisman et al. 2004; Edwards et al. 2013). Interestingly, it has been debated how modifications of upper-ocean turbulence may have shaped the functional and evolutionary ecology of planktonic diatoms and may currently influence their geographical distribution (Falkowski et al. 2004; Tozzi et al. 2004). The ecophysiological connection to XC-NPQ diversity of planktonic diatoms was investigated by comparing strains/ecotypes from different pelagic habitats (estuary, coast, semi-enclosed bay, open ocean) (Lavaud et al. 2004, 2007; Strzepek and Harrison 2004; van de Poll et al. 2006; Petrou et al. 2011), including different seasonal distributions (Dimier et al. 2007) and pico-phytoplankton representatives (Giovagnetti et al. 2012). A special focus was recently given to diatom representatives of polar ecosystems (van Leeuwe et al. 2005; van de Poll et al. 2006, 2011; Kropuenske et al. 2009; Mills et al. 2010; Petrou et al. 2011). Species/ecotypes adapted to a more stable light environment (semi-enclosed bays/open ocean versus estuaries/coasts) seem to rely more on regulatory processes other than NPQ and the PS II electron cycle (see section II.B.1), which are likely better suited to cope with more gradual light changes: (1) a fast turnover of the PS II D1 protein (Wu et al. 2012; Lavaud and Campbell unpublished results 2013), and (2) employment of Dt in the prevention of lipid peroxidation (Lepetit et al. 2010; Lavaud and Lepetit 2013). Finally, the adjustment of light harvesting pigment content (Brunet et al. 2011) and of the metabolic capacity, and especially the balance of energy between lipids and carbohydrates, is of importance for the response to dynamic light regimes of diatoms with different NPQ capacity (Su et al. 2012).

A recent report suggested a molecular basis for the diversity in NPQ capacity observed among planktonic diatoms based on the mechanistic model described in “Diatoms” (Lavaud and Lepetit 2013). A high capacity for NPQ seems to depend on a large amount of Dt present at the Q2 quenching site (see Fig. 20.2) and the capacity to form many/large Q1 quenching sites (Lavaud and Lepetit 2013). In addition, the number of Lhcx proteins present in the light-harvesting system seems to be crucial (Bailleul et al. 2010; Zhu and Green 2010; Lepetit et al. 2013). NPQ capacity is based more on the plastidic distribution of Dt and its effective involvement in NPQ than on the total amount of Dd+Dt and the de-epoxidation state of the XC (DES), i.e., species/ecotypes can show the same Dd+Dt content and DES with a drastically different NPQ amplitude and kinetics (Lavaud and Lepetit 2013). For that reason, the slope of the relationship between Dt and NPQ appears to be a good indicator of the adaptation of diatom species/ecotypes to a specific light regime and, possibly, an aquatic niche (Lavaud et al. 2004, 2007; Lavaud and Lepetit 2013).

B *Diatoms of the Microphytobenthos*

The in-situ light response of diatoms of the microphytobenthic (MPB) community has received less attention. This is probably due to the technical challenge of measuring the photosynthetic activity of the MPB community in its major natural habitat, i.e., estuarine mudflats (though MPB organisms can also be found in surf beaches, coral reef lagoons, and shallow coastal waters) (Underwood and Kromkamp 1999; Perkins et al. 2011). Because they inhabit sediments, MPB diatoms experience a different light climate than planktonic diatoms. The fluctuations of irradiance at the surface of mudflats are similar to those on a land surface, with the succession of emersion/immersion periods adding a regular, sharp light/dark photoperiod. Additionally, other environmental cues (especially temperature and salinity) can vary rapidly between extremes (Underwood and Kromkamp 1999) and generate stressful conditions.

The in-situ pool size of Dd+Dt and XC operation in the MPB diatoms depends strongly on light and temperature as a function of the tidal cycle, season, and latitude (van Leeuwe et al. 2009; Chevalier et al. 2010; Jordan et al. 2010). The DES correlates well with higher daily and latitudinal irradiances (van Leeuwe et al. 2009; Chevalier et al. 2010; Jordan et al. 2010) and with light distribution in the sediments, i.e., a higher DES is observed at the surface (Jesus et al. 2009; Cartaxana et al. 2011). The NPQ of the MPB diatoms in situ can reach high values above 5–6 (Serôdio et al. 2005, 2008, 2012). In general, NPQ correlates well with DES with an especially clear increase at midday emersion (exposure to high irradiance) in summer (Serôdio et al. 2005; Chevalier et al. 2010). The seasonal pattern of NPQ is strongly influenced by both irradiance and temperature in a complex manner (Serôdio et al. 2005, 2012; Jesus et al. 2006). These general trends of light and temperature control were also reported for isolated benthic diatoms (Perkins et al. 2006; Salleh and McMinn 2011; Serôdio and Lavaud 2011).

Of considerable interest are differences in the ability of species/groups to cope with excessive light/UV exposure (Waring et al. 2006; Jesus et al. 2009; Cartaxana et al. 2011), which is similar to the situation in planktonic diatoms (see section III.A). Differences in adaptation are believed to potentially impact spatial distribution (muddy versus sandy habitats) as well as species succession within the MPB biofilm during emersion and different seasons (Tuji 2000; Serôdio et al. 2005; Underwood et al. 2005). Epipsammic species (firmly attached to sediment particles) use physiological processes such as the XC and energy dissipation underlying NPQ for their photoprotection (Jesus et al. 2009; Cartaxana et al. 2011). Epipelic (freely living in sediment particles) motile species rely on both physiology and “behavioral photoprotection”, i.e., the cells use their vertical mobility to escape from excess light (Consalvey et al. 2004; Perkins et al. 2010).

The respective importance of physiological and behavioral photoprotection has long been a topic of interest and has shown major recent advances (Jesus et al. 2006; Mouget et al. 2008; Perkins et al. 2010; Cartaxana et al. 2011; Serôdio et al. 2012). In general, both XC-dependent photoprotection and behavioral photoprotection provide the MPB diatoms with a strong capacity to resist periods of prolonged excessive light (Blanchard et al. 2004; Serôdio et al. 2008, 2012; Perkins et al. 2010), including strong resistance to UV radiation (Waring et al. 2007; Mouget et al. 2008). Additionally, MPB diatoms are usually characterized by a strong “dark NPQ” (Perkins et al. 2011) (see section II.B.1), which presumably allows the cells to maintain their antenna system in a basal dissipative state and fully activate their NPQ machinery more rapidly upon sudden light exposure. Such a system would be especially relevant for the MPB diatoms that can spend more than 18 h per day in the dark (immersion and night emersion) before being exposed to sunlight again.

C Brown Algae

Because most of the main macrophytic brown algae are sessile organisms, their light environment strongly depends on the water depth where they are located. The light environment of immerged brown algae is similar to that of planktonic diatoms at a given depth, while the light environment of emerged thalli bears greater semblance to that of the benthic diatoms. Brown algae are able to perform XC-dependent energy dissipation reflected in NPQ, with high NPQ reported for some species adapted to harsh environments, i.e., species living at the air-water interface and in the upper zone of the intertidal rocky shore (Harker et al. 1999; Colombo-Pallotta et al. 2006). Increases in XC carotenoid levels, conversion of those carotenoids to the forms responsible for energy dissipation, and their engagement in energy dissipation underlying NPQ are involved in the response to natural irradiance changes, including excessive light exposure and UV radiation (Uhrmacher et al. 1995; Hanelt et al. 1997; Schofield et al. 1998; Apprill and Lesser 2003), sometimes coupled with other environmental stresses, especially desiccation (Harker et al. 1999) and high temperature (Poulson et al. 2011). For the same individual, the level of XC-dependent NPQ can be diverse among different parts of the thallus, dependent on the respective age of the thallus parts (Colombo-Pallotta et al. 2006; Poulson et al. 2011) as well as life-cycle stage (sporophytes versus gametophytes) (Hanelt et al. 1997). For instance, in *Laminaria saccharina*, gametophytes are more resistant to excessive light stress than sporophytes, partially due to a stronger XC, which may influence their respective distribution on the sea shore (Hanelt et al. 1997).

The zonation of brown macroalgal species strongly depends on their depth distribution in and out of the water column (depending on the tide) as a function of irradiance and the light climate, with the exception of pelagic species like *Sargassum* spp. (Schofield et al. 1998). Inter-species XC-NPQ variability correlates with depth distribution, showing a higher NPQ capacity for the species located on the upper-shore (Harker et al. 1999; Rodrigues et al. 2002; Nitschke et al. 2012). In this case, the higher NPQ capacity (usually twice as high) correlates with an increase of the pool size of VAZ and a greater synthesis of Z (Harker et al. 1999; Rodrigues et al. 2002). The depth distribution and the related XC-dependent NPQ acclimation to irradiance also holds true for the same species in a given ecosystem (Apprill and Lesser 2003). All intertidal species increase the level of NPQ during emersion; for instance, the acclimative response of *L. digitata* to midday emersion involves Z synthesis and increased NPQ resulting from increased thermal energy dissipation (Gévaert et al. 2002, 2003; Apprill and Lesser 2003). *Laminaria digitata* also exhibits a XC-NPQ seasonal pattern involving an increase of the VAZ pool size in spring compared to winter (Gévaert et al. 2002). The canopy-forming *Macrocystis pyrifera* provides a striking example (Colombo-Pallotta et al. 2006; Konotchick et al. 2013; Ocampo-Alvarez et al. 2013). Due to its height (about 18–20 m), this alga occupies the entire water column, such that the blades close to the surface are exposed to an underwater light environment with higher irradiance and stronger fluctuations. Within a single organism, the XC-dependent capacity for NPQ varies dramatically with depth (fivefold), with NPQ values being linearly related to water depth. As is also the case for intertidal species, NPQ differences are correlated with VAZ pool size and the capacity to synthesize Z and A, and with the differential expression of LI818 isoforms (Colombo-Pallotta et al. 2006; Konotchick et al. 2013; Ocampo-Alvarez et al. 2013). Finally, in pelagic species that live at the air-surface interface, the XC conversions follow the daily course of the sun and NPQ strictly correlates with the synthesis and the accumulation of Z (Schofield et al. 1998).

Interestingly, the strongest competitors with brown algae in the intertidal zone are the red algae (Wiencke and Bischof 2012). Based on the current state of knowledge, it is not clear if red algae contain an operable XC (Carnicas et al. 1999; Ursi et al. 2003). Despite this ongoing debate (Goss and Jakob 2010), red algae are characterized by a high, stable concentration of Z, thus demonstrating the importance of de-epoxidized xanthophylls for efficient photoprotection.

IV Conclusion

Investigations of the XC and photoprotective thermal energy dissipation (as reflected in NPQ) in diatoms and brown algae are of high importance for two obvious reasons: (1) the algae are essential primary producers in marine ecosystems, and, (2) their productivity is based mainly on light availability that can fluctuate drastically in aquatic habitats. XC-dependent energy dissipation (NPQ) appears to be the central mechanism for the rapid adjustment of light harvesting and photochemistry of the cell, thereby enabling diatoms and brown algae to maintain an optimal productivity in a permanently changing light environment. The most recent advances provide new insights into the regulation of the XC and the underlying mechanism of NPQ (Goss and Jakob 2010; Depauw et al. 2012; Lepetit et al. 2012, 2013) and the species/strain diversity of the XC and NPQ, including the latter features' potential eco-physiological importance (Brunet and Lavaud 2010; Petrou et al. 2011; Lavaud and Lepetit 2013). There are numerous areas that deserve further attention in the near future, starting with the investigation of the protein partners involved in the regulation of NPQ (especially the Lhcx proteins and the Chl $a_{710-712}$ FCP complexes) and experiments to confirm the importance of the XC-dependent process(es) underlying NPQ as a functional trait essential to the ecology of diatoms and brown algae. To further investigate the latter, more thorough in-situ studies are needed that take advantage of recent improvements in fluorescence methodology and analysis of NPQ (Perkins et al. 2011; Enriquez and Borowitzka 2011; Lefebvre et al. 2011; Serôdio and Lavaud 2011; Nitschke et al. 2012). To gain further knowledge about the mechanistic basis of NPQ in diatoms and brown algae, genomic/genetic tools in conjunction with sophisticated spectroscopic and physiological measurements will have to be performed. While such approaches have already shown their utility in diatoms (Materna et al. 2009; Bailleul et al. 2010; Depauw et al. 2012; Lavaud et al. 2012; Lepetit et al. 2013), this field has just opened up for brown algae (Dittami et al. 2010; Konotchick et al. 2013).

Acknowledgments

JL thanks the Centre National de la Recherche Scientifique (CNRS), the Université de La Rochelle (ULR), the Contrat Plan Etat Région (CPER) 'Littoral', the Région Poitou-Charentes (CG17), the Deutsche Forschungsgemeinschaft (DFG, grant LA2368/2-1), the Deutsche Akademische Austauschdienst (DAAD), and Egide/Campus France (grants 27377TB and 28992UA) for their financial support. RG thanks the DFG (grants Go818/6-1, Go818/7-1) for its financial support. The two authors thank B. Lepetit for his help building and drawing the model of Fig. 20.2. JL dedicates this work to his mentors Pr. J.-C. Duval and Dr. A.-L. Etienne.

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Chapter 21

High Light Acclimation in Green Microalgae

Giovanni Finazzi
Unité Mixte Recherche 5168, Laboratoire Physiologie Cellulaire et Végétale,
Centre National Recherche Scientifique, Grenoble 38054, France

Commissariat à l'Energie Atomique et Energies Alternatives,
l'Institut de Recherches en Technologies et Sciences pour le Vivant,
Grenoble 38054, France

Université Grenoble 1,
Grenoble 38041, France

Institut National Recherche Agronomique,
Grenoble 38054, France

and

Jun Minagawa*
Division of Environmental Photobiology, National Institute for Basic Biology,
38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan

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*Author for Correspondence, e-mail: minagawa@nibb.ac.jp

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_21,

Summary

Absorption of light in excess of the capacity for photosynthetic electron transport can be detrimental for photosynthetic organisms. Mechanisms exist to protect chloroplasts from damage, which are in general associated with photosystem II and collectively assessed via non-photochemical quenching (NPQ) of chlorophyll fluorescence. Non-photochemical quenching comprises several components, including (1) qE (high-energy state quenching, as the rapid component of NPQ) as a measure of thermal dissipation linked to the development of a low pH in the thylakoid lumen and possibly (2) qT (state transition-dependent quenching, as the slower component of NPQ) as a measure of antenna size reduction involving phosphorylation and migration of antenna proteins from PS II to PS I. The relative amplitude and efficiency of these processes is extremely variable in different photosynthetic organisms (the relative amplitude of the latter is especially more prominent in microalgae than vascular plants), likely reflecting the different molecular machineries and/or regulation of the effectors for the processes underlying qE and state transitions in these organisms. The present review focuses on NPQ in green microalgae and summarizes changes in the latter two NPQ components in photosynthetic microalgae (in particular green algae including *Ostreococcus*, *Chlamydomonas*, *Dunaliella*, and *Chlorella*). We also relate these changes to possible differences between their molecular machineries, which reflect specific responses of each organism to the constraints existing in its environmental niche. Moreover, alternative photoprotective responses based on changes in the electron flow modes/efficiencies are also presented along with an interpretation as to how these mechanisms can provide a benefit for specific photosynthetic organisms.

Abbreviations: A – Antheraxanthin; bf – The cytochrome b_6f complex; CEF – Cyclic electron flow; Chl – Chlorophyll; CP26 – 26 kD minor monomeric light-harvesting complex II protein; CP29 – 29 kD minor monomeric light-harvesting complex II protein; CP43 – 43 kD chlorophyll-binding inner antenna protein; CP47 – 47 kD chlorophyll-binding inner antenna protein; Cys – The amino acid cysteine; D1 – D1 subunit of photosystem II; D2 – D2 subunit of photosystem II; DBMIB – 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DCCD – Dicyclohexylcarbodiimide; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fd – Ferredoxin; F_m – Maximum fluorescence; FNR – Ferredoxin-NADP$^+$ oxidoreductase; His – The amino acid histidine; HQNO – 2-heptyl-4-hydroxyquinoline-N-oxide; LEF – Linear electron flow; LHC – Light-harvesting complex; Lhcb1 – Light-harvesting complex II protein type I; Lhcb2 – Light-harvesting complex II protein type II; LHCBM – Light-harvesting complex II major protein; LHCSR – Light harvesting complex stress related protein; NPQ – Non-photochemical quenching of chlorophyll fluorescence; *p* – PS II connectivity parameter; P700 – Reaction center chlorophyll of photosystem I; Pc – Plastocyanin; pK – Negative logarithm of an equilibrium constant; PP2C – Protein phosphatase type 2C; PPH1 – Phosphatase 1; PQ – Plastoquinone; PQH_2 – Plastoquinol; PS – Photosystem; PsaA – PsaA subunit of photosystem I; PsaH – PsaH subunit of photosystem I; PsaI – PsaI subunit of photosystem I; PsaK – PsaK subunit of photosystem I; PsaL – PsaL subunit of photosystem I; PsaO – PsaO subunit of photosystem I; PsbS – PsbS subunit of photosystem II; PTOX – Plastoquinone terminal oxidase; Q_A – First quinone acceptor in the PS II reaction center; qE – ΔpH-dependent component of non-photochemical quenching; qI – Photoinhibition-dependent component of non-photochemical quenching; qT – State transition-dependent component of non-photochemical quenching; RNAi – Ribonucleic acid interference; Ser – The amino acid serine; TAP38 – Thylakoid-associated phosphatase 38; Thr – The amino acid threonine; V – Violaxanthin; VHL_R – Very high light resistant nuclear mutants of *Chlamydomonas reinhardtii;* Z – Zeaxanthin

I Introduction

The absorption of light in excess of the capacity for photosynthetic electron transport is potentially damaging to photosynthetic organisms (Horton et al. 1996; Niyogi 1999). That capacity is, however, variable, depending mainly on the sink strength of CO_2 assimilation. Under conditions where electron flow rate and/or the capacity to assimilate the reducing power generated by photosynthesis is lower than the light energy input, protective responses are seen. This can also happen in relatively dim light (e.g., Finazzi and Forti 2004), but as a general rule photoprotective mechanisms are amplified under excess illumination to avoid oxidative stress generated by uncontrolled formation of reactive oxygen species. Several protective responses have been characterized in the past. They mostly belong to two groups (see also, Logan et al., Chap. 7), i.e., (i) processes that reduce photodamage by downregulating the efficiency of light harvesting of the photosynthetic apparatus and (ii) mechanisms that adjust electron transport capacity to the light absorption by diverting photogenerated reducing equivalents to alternative sinks when CO_2 assimilation is not commensurate to the absorbed light energy input (Eberhard et al. 2008). Processes belonging to the first group are all associated with photosystem (PS) II downregulation; such processes can therefore be easily visualized following changes in the PS II fluorescence yield at room temperature. Because their onset is associated with a decrease of maximum fluorescence (F_m, all PS II centers having a reduced Q_A quinone acceptor), these processes all contribute to what is collectively referred to as the non-photochemical quenching (NPQ) of chlorophyll (Chl) fluorescence emission (reviewed by Horton et al. 1996; see also Papageorgiou and Govindjee, Chap. 1; Logan et al. Chap. 7). The different components of NPQ have commonly been defined based on the dark relaxation of quenching when the light is switched off. The fastest relaxing component in plants is (i) qE, also termed "high energy state quenching". It represents an increased thermal dissipation capacity that develops within the PS II antenna due to the acidification of the lumen pH (e.g., Briantais et al. 1979; see also Strand and Kramer, Chap. 18). A second one is (ii) qT, the intermediate phase, which reflects state transitions, i.e., a change in the relative antenna sizes of PS II and PS I due to the reversible phosphorylation and migration of antenna-proteins (light-harvesting complex proteins of PS II, or LHCII) (Allen 1992). The last one is (iii) qI, a slowly-reversible component that was previously attributed to photoinhibition, i.e., damage to PS II reaction centers (Aro et al. 1993; but see Adams et al., Chap. 23 for an alternative view of photoinhibition). While this attribution of the NPQ components based on their rates of dark relaxation has been challenged by several authors (e.g., Adams et al. 1995; Dall'Osto et al. 2005; Joliot and Finazzi 2010; see also Demmig-Adams et al., Chap. 24), these terms are still in use within the community.

While the physical mechanisms leading to these NPQ components are, in principle, conserved among photosynthetic organisms, these organisms' capacity to develop NPQ varies extensively (Fig. 21.1). For example, large differences in NPQ capacity are seen among different microalgae (Bonente et al. 2008). In the diatom model *Phaeodactylum tricornutum*, NPQ values can vary by a factor of 6 depending on growth conditions (Lavaud et al. 2002a; see, also Lavaud and Goss, Chap. 20). In the picoeukaryote *Ostreococcus*, big differences in the level of NPQ are seen among different ecotypes (Cardol et al. 2008), leading to different light sensitivity (Six et al. 2009). However, these differences disappear if the same ecotypes are exposed to specific light growth regimes for several months (Six et al. 2008), suggesting either a very long acclimation capacity, or the onset of an adaptation response. Differences are also seen within the same species depending on the experimental conditions. In the chlorophyte *Chlamydomonas reinhardtii*, NPQ capacity can vary from ~0 to ~2, based on the growth conditions

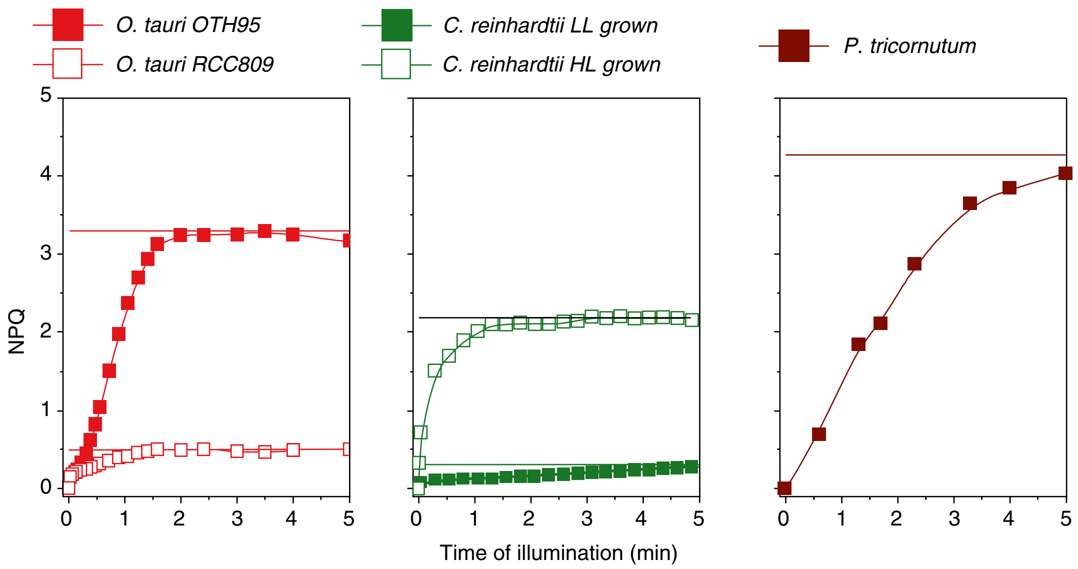

Fig. 21.1 NPQ capacity of two green microalgae (a prasinophyte Ostreococcus tauri and a chlorophyte Chlamydomonas reinhardtii) compared with NPQ in a diatom Phaeodactylum tricornutum. NPQ was estimated as $(F_m / F_m')-1$ *according to Bilger and Björkman (1990). Two O. tauri ecotypes (OTH95, a surface/high light ecotype; filled red squares) and RCC 809 (a low light open ocean ecotype that chronically experiences Fe starvation leading to reduced LEF capacity; open red squares; Cardol et* al. 2008) are shown. Two extreme conditions are presented for *C. reinhardtii*: growth in low light (40 μmol photons m^{-2} s^{-1}) in mixotrophic condition (acetate) to prevent the accumulation of LHCSR3 in the thylakoids (low light = LL; *green squares*) and high light (400 μmol photons m^{-2} s^{-1} = HL; *open green squares*) in phototrophic condition (without acetate) to induce a full LHCSR3 accumulation. *Phaeodactylum tricornutum* was grown in a natural seawater-derived F/2 medium under moderate light as described in Bailleul et al. (2010).

(Finazzi et al. 2006; Peers et al. 2009). This suggests that specific factors exist that can modulate the extent of NPQ depending on the specific genetic/environmental conditions that characterizes every organism by acting on a common physical platform.

In the following part of this review, we will summarize data concerning the physiological responses of green microalgae to changes in light environment, with an emphasis on qE and qT at the level of both antenna downregulation and (in the few cases studied so far) of electron flow acclimation.

II ΔpH-Dependent Energy Quenching (qE) in Green Microalgae

Microalgae represent an ideal system to study the mechanisms and regulation of photosynthesis because they grow faster than vascular plants and can be easily isolated and manipulated. While photoprotective mechanisms are not necessarily the same in plants and algae, studies with microalgae have provided a relevant contribution to elucidating the mechanisms of plant photoprotection. State transitions were first described in *Chlorella pyrenoidosa* (Bonaventura and Myers 1969), while studies with *Chlamydomonas reinhardtii* have revealed essential regulatory processes governing qE (Niyogi 1999). Discovered in the 1970s in vascular plants, qE corresponds to a decreased fluorescence quantum yield associated with lumen acidification in the light (Wraight and Crofts 1971). In both vascular plants and green algae, qE is linked to the deepoxidation of the xanthophyll violaxanthin (V) to zeaxanthin (Z) (Yamamoto et al. 1962; Demmig-Adams and Adams 1990; see also Demmig-Adams et al., Chaps. 2 and 24). Induction of qE, as an estimate of the level of thermal energy dissipation activity, is catalyzed by a thylakoid-bound deepoxidase activated by a low luminal pH. V deepoxidation is counterbalanced by Z epoxidation, which is catalyzed by an epoxidase activity. Thermal energy dissipation takes place in specific PS II antennae and requires protein cofactors acting as pH sensors and/or as quenching amplifiers (see also Horton, Chap. 3). These cofactors are different in vascular plants and green microalgae, as revealed by the comparative analysis of NPQ mutants in *Arabidopsis thaliana* and *C. reinhardtii* (Peers et al. 2009; see also, e.g., Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14).

A Chlamydomonas

Chlamydomonas reinhardtii is a motile (due to two flagella), unicellular alga (~10 μm in diameter) living in freshwater environments. It is a model organism used in the study of photosynthesis, flagella, and green microalgal physiology in general (Eberhard et al. 2008; Minagawa 2011). Thanks to the genetic evidence first provided by *C. reinhardtii* and then by the characterization of *Arabidopsis* mutants with a modified NPQ response, huge progress has been made in elucidating the site and the mechanism of qE. Since many of the mutants were isolated based on PS II fluorescence and turned out to be deficient in the VAZ cycle, there is now a consensus that the major form of quenching occurs within the PS II antenna, although reports exist suggesting that in some microalgae (e.g., diatoms and *Chlamydomonas*) quenching could also occur in the PS II core (Lavaud et al. 2002b; Pocock et al. 2007).

Several common elements exist that are implicated in the qE type of quenching in vascular plants and microalgae: xanthophyll deepoxidation, linked to the establishment of a proton gradient across the thylakoid membranes is the committed step for qE onset in both kingdoms. However, differences exist at the level of the protein machinery that acts as a quenching effector. In vascular plants, lumen acidification is probed by two glutamic acids present on PsbS, the putative S subunit of PS II (Li et al. 2000, 2004; see also Brooks et al., Chap. 13). This protein acts as a quencher amplifier in a dose related manner (Li et al. 2000). However, the gene for this protein, which is present in the nuclear genome of *C. reinhardtii*, is not expressed in this alga even under stress conditions (Finazzi et al. 2006; Bonente et al. 2008; see also Morosinotto and Bassi, Chap. 14) with the only exception of nitrogen starvation (Miller et al. 2010). In *C. reinhardtii*, the role of the quenching amplifier is mostly played by the light harvesting complex stress related protein LHCSR3 (Peers et al. 2009; see also Brooks et al., Chap. 13; Morosintoo and Bassi, Chap. 14). This protein is a member of an ancient family of LHC found in the green lineage, including *Ostreococcus* and *Chlamydomonas*, up to the moss *Physcomitrella patents* (Alboresi et al. 2010; Gerotto and Morosinotto 2013). LHCSR proteins are also active in diatoms as evidenced by genomic analysis in both *Phaeodactylum* and *Thalassiosira* (Nymark et al. 2009; Zhu and Green 2010; see also Büchel, Chap. 11; Lavaud and Goss, Chap. 20) and functional studies in *Phaeodactylum* (Bailleul et al. 2010). Although the genetic studies mentioned above demonstrate that PsbS and LHCSR3 are required for qE to develop in vascular plants and algae, respectively, their exact role is still not defined. While PsbS cannot bind pigments, LHCSR3 is capable of binding Chl *a* and *b*, as well as xanthophylls (Bonente et al. 2011). Therefore, the latter protein could be directly involved in the light-harvesting process as a “specialized” antenna protein (see also paragraph below), i.e., a function that seems to be very unlikely in the case of PsbS.

Recent experiments by some of us (Tokutsu and Minagawa 2013) have shed light on the role of LHCSR3 in quenching. By comparing the PS II super-complex with LHCII from the wild type *C. reinhardtii* cells and *npq4*, the mutant lacking both the *LHCSR3.1* and *LHCSR3.2* genes (Peers et al. 2009), the former authors found that the LHCSR3 protein was only present in the PS

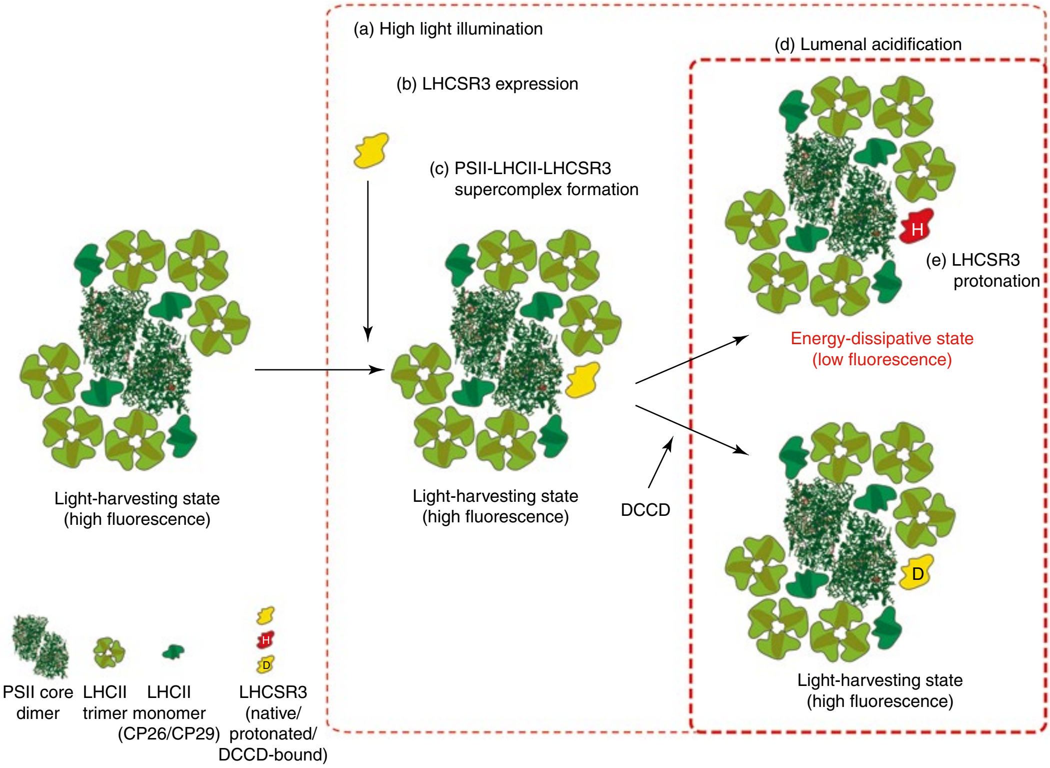

Fig. 21.2 A model for the induction of qE in C. reinhardtii. When C. reinhardtii cells are exposed to high light for several hours (a), the expression of LHCSR3 is induced (b). LHCSR3 is then associated with the PS II-LHCII supercomplex to form the PS II-LHCII-LHCSR3 supercomplex (c). Though the PS II-LHCII-LHCSR3 supercomplex is still in a light-harvesting state under dark or low light conditions, it becomes energy-dissipative upon protonation of LHCSR3 (e) in the acidified thylakoid lumen under high light conditions (d). DCCD-binding to the PS II-LHCII-LHCSR3 supercomplex inhibits the conversion of PS II-LHCII-LHCSR3 supercomplex from the light-harvesting state to the energy-dissipative state. The crystal coordinates were obtained from the Protein Data Bank: PS II core, 3ARC

II super-complex from the high-light-grown wild type cultures, but not those from the low-light-grown wild type or high-light-grown *npq4*. The purified PS II super-complex containing LHCSR3 was in a high fluorescent state at a neutral pH (7.5), as evaluated by single-photon counting, but in an energy-dissipative state at pH 5.5, similar to the effect of lumen acidification following illumination of thylakoid membranes. The switching from a light-harvesting state to an energy-dissipative state observed in the LHCSR3-containing PS II super – complex was sensitive to dicyclohexylcarbodiimide (DCCD), a protein-modifying agent specific to protonatable amino acid residues. It is therefore likely that the PS II-LHCII-LHCSR3 super-complex formed in high-light-grown *C. reinhardtii* cells is capable of thermal energy dissipation upon protonation of LHCSR3 (Fig. 21.2).

Owing to the involvement of different qE effectors, physiological differences can be observed between the NPQ responses of green microalgae and vascular plants. While PsbS is present even in low-light-grown plants (Demmig-Adams et al. 2006), and therefore provides constitutive photoprotection, LHCSR3 is only induced upon exposure to high light for several hours in algae. Once accumulated, LHCSR3 apparently provides a quenching capacity that is also evident at moderate light (Bonente et al. 2012) or under short periods of illumination

(Peers G, personal communication). The observation that LHCSR3 must be induced to promote the onset of qE obviously raises the question of the signal(s) leading to the expression of the *LHCSR3* gene. *LHCSR3* transcripts accumulate under both high light and nutrient stress conditions in *C. reinhardtii* (Zhang et al. 2004; Naumann et al. 2007). This would be expected from a physiological point of view, because this protein is needed to counterbalance the light stress related to saturation of the electron flow machinery, which can be induced either by excess light or by a reduced photosynthetic performance due to nutrient limitation.

However, recent data have shown an unexpected regulation of LHCSR3 accumulation in *C. reinhardtii*. Petroutsos et al. (2011) showed that knockdown mutants (*cas-kd*) with reduced levels of the chloroplast putative a calcium sensor are unable to properly induce the expression of LHCSR3 upon transfer to high light (HL). Therefore, these cells show a compromised NPQ phenotype and a photosensitivity comparable to that seen in *npq4* mutants (Peers et al. 2009). However, as opposed to the situation in *npq4*, induction of LHCSR3 could be rescued by addition of external calcium in the medium. This finding suggests that a calcium sensor and Ca^{2+} are critically involved in the regulation of the HL response and particularly in the control of LHCSR3 expression. At present, it is not known if this control stems from a direct regulation of the *LHCSR3* gene or from a more indirect regulatory mechanism related to the well-known effect of calcium on stress responses in plant cells (Reddy et al. 2011).

Consistent with the possible existence of "specialized" antenna proteins involved in quenching modulation, genetic analysis in *C. reinhardtii* has led to the identification of light-harvesting complexes directly responsible for quenching onset. Depletion of the light-harvesting complex major protein 1 (LHCBM1) in the *npq5* mutant (Elrad et al. 2002) decreases the capacity for thermal energy dissipation, but not the ability to perform state transitions. On the other hand, an opposite behavior is observed in mutants affected in LHCBM2/7 (Ferrante et al. 2012), implying that the different LHCII polypeptides have specific roles in NPQ. Besides LHCSR3 and LHCBM1, other gene products may be involved in high light acclimation in *C. reinhardtii*. In 2001, Förster et al. (2001) isolated very high-light resistant nuclear mutants (VHL_R) of *C. reinhardtii*, i.e., strains that grew at light intensities (1,500–2,000 μmol photons m^{-2} s^{-1}) lethal to wild type. Phenotypic analysis of these (four) strains indicated that in some of them the VHL_R phenotype was due to slower PS II electron transfer, reduced connectivity between PS II centers, and decreased PS II efficiency, indicating that optimal PS II function was not critical for survival in very high light. While no clear link could be established between the VHL_R phenotype and NPQ, these mutants appear to be affected in the proper assembly of antenna components and/or of the photosystems. Additional work is clearly needed to further decipher the molecular basis of this peculiar phenotype.

B *Dunaliella*

Dunaliella sp. are motile, unicellular, green algae (size ~10 μm) found in both freshwater and marine environments. The most common species is *D. salina*, a halophilic alga found in sea salt fields. To survive in this environment, *D. salina* has high concentrations of β-carotene to protect against intense light (Oren 2005) and high concentrations of glycerol to provide protection against low osmotic pressure (Oren 2005). Other strategies have been developed in this microalga to withstand other forms of environmental stress. Most of them involve a regulation of light harvesting and electron flow capacity. Recent studies (Einali et al. 2013) have shown, however, that cyclic electron flow around PS I (CEF-PSI) is substantially induced in high light. This induction allows for the development of high levels of qE in PS II by augmenting the acidification of lumen pH (Einali et al. 2013). This finding is in line with the conclusion that, in vascular plants, protons derived from CEF-PSI are extremely active in regulating qE (Joliot and Alric 2013).

Comparative studies using the *zea1* mutant, that constitutively accumulates Z, have shown that NPQ kinetics were largely Z-independent in this alga and may have been related to an enhanced capacity to create proton motive force (Thaipratum et al. 2009). Two possible explanations can be proposed to interpret this effect. (i) A saturating concentration of Z may already be produced in wild type cells following high light exposure, thus rendering the capacity to over-accumulate Z ineffective. Alternatively, it is (ii) possible that a high capacity of building ΔpH (via CEF-PSI) may make qE generation less dependent on the accumulation of de-epoxidized xanthophylls. Following previous suggestions by Ruban and coworkers (Horton et al. 2000; see also Horton, Chap. 3), conversion of V to Z may shift the apparent pK of protonatable residues associated with qE toward less acidic values (from 4.7 to 5.7). Although such a role of Z is apparently essential for qE development under natural conditions, fluorescence quenching can also occur at low amounts of Z, provided that the lumen proton concentration is high enough. Thus, due to high CEF activity, qE may depend less strongly on xanthophyll deepoxidation in *Dunaliella*.

Some unique quenching mechanisms have been identified in *Dunaliella*. Results in *D. tertiolecta* show significant changes in the PS II connectivity parameter *p* (Joliot and Joliot 1964) during onset of qE in this alga upon transition from dark to subsaturating light (Ihnken et al. 2011). This finding suggests that the energetic connectivity among PS II antenna proteins may change during illumination, and contribute in a still-undefined manner to PS II downregulation (see also Ruban and Mullineaux, Chap. 17). However, this effect appears to be confined to low light conditions in *Dunaliella* as no such effect was seen in high light, indicating that the main qE response still relies on the “classic” qE mechanism depending on ΔpH.

C *Chlorella*

While *C. reinhardtii* (and *Dunaliella* to a minor extent) have been exploited to assess the molecular bases of qE using genetic approaches, *Chlorella* species have been widely employed in the past to assess the biophysical and physiological features of qE in microalgae. *Chlorella* is a genus of unicellular microalgae without flagella that is cosmopolitan in freshwater. Circular dichroism studies performed on *Chlorella fusca* have shown that, besides promoting sustained accumulation of Z, lumenal acidification leads to the formation of large chiral aggregates (Goss and Garab 2001). These aggregates, which are fully reversible in the dark, could provide a physical network for long-distance migration of excitation energy, thereby facilitating thermal energy dissipation by de-epoxidized xanthophylls within several photosynthetic units. Nonlinear laser fluorimetry has been applied to assess the consequences of xanthophyll de-epoxidation in *C. pyrenoidosa*. Fadeev et al. (2012) found that Z accumulation significantly increases the rate constant of singlet-singlet annihilation of the Chl *a* excited state, in agreement with the proposed occurrence of a conformational change in the LHCII following qE onset (Ruban et al. 2007). *Chlorella vulgaris* cells have also been used to investigate the relationship between xanthophylls and the kinetics of qE onset and relaxation in comparison with other photosynthetic protists. In diatoms (*Phaeodactylum tricornutum*, *Thalassiosira pseudonana*), the kinetic performance of diatoxanthin epoxidase (i.e., the enzyme involved in the reverse step of the diadinoxanthin-diatoxanthin conversion in these algae) is extremely low, and apparently sets the rate of qE relaxation, which is at least ten times slower than in *Chlorella vulgaris* (Goss et al. 2006). Conversely, no such relationship is seen in *Chlorella*, where qE relaxation is much faster than the epoxidation kinetics because it follows the kinetics of ΔpH relaxation (Joliot and Finazzi 2010). Additional interesting features of NPQ have also been elucidated by studying *Chlorella*.

Seaton et al. (1996) reported that an algal virus infecting *Chlorella* (strain Pbi) induces NPQ even at very low light. This quenching state, which is not related to conversion of V to antheraxanthin (A) and Z, is reminiscent of the enhanced NPQ observed in vascular plants upon pathogen infection. It is therefore tempting to suggest that algae too possess the capacity to undergo an "acquired systemic response to excess excitation energy and to pathogens", which results in an enhanced energy quenching response following application of external stimuli of a different nature (e.g., light stress or wounding), as proposed for vascular plants (Karpinski et al. 2003).

D *Ostreococcus*

Ostreococcus is a prasinophytic alga, which is a prominent member of the picoplankton (i.e., the fraction of plankton composed of cells between 0.2 and 2 μm), phylogenetically located at the early branch of the green lineage. In *Ostreococcus*, qE development exhibits the same mechanism illustrated in *Chlamydomonas*, where both the VAZ cycle (Cardol et al. 2008) and LHCSR3 (Peers et al. 2009) are involved. This finding indicates that these qE effectors are strongly conserved phylogenetically, as this alga (and the *Prasinophyceae* in general) is at the base of the evolutionary tree of plants, and its antenna complexes are rather different from those of plants (Six et al. 2005) in terms of their possible structure (Goss et al. 2000) and pigment content (Six et al. 2005). Moreover, a unique physiological character of qE has been reported in *Ostreococcus*. Functional studies comparing ecotypes of *Ostreococcus* species collected from different environments have suggested that the major determinant for NPQ development in this alga is the extent of proton motive force (in line with previous findings in other microalgae). In an *Ostreococcus* ecotype isolated close to the limit of the photic zone (RCC 809), the magnitude of the pmf turned out to be controlled by the cell capacity to reroute a substantial fraction of electron flow to establish a water-to-water cycle involving PS II and a potential PQ terminal oxidase (PTOX) (Cardol et al. 2008). This pathway bypasses electron transfer through the cytochrome b_6f complex and allows the pumping of additional protons into the thylakoid lumen. By promoting the generation of a large ΔpH even under restricted electron flow conditions, this process facilitates photoprotection of PS II in strains where PS I accumulation is restricted by iron limitation (Cardol et al. 2008).

III State Transition-Dependent Quenching (qT) in Green Microalgae

The two photosystems (PS I and PS II) in the thylakoid membranes possess distinct pigment systems with distinct absorption characteristics (Fig. 21.3). While PS I has a broad absorption peak in the far-red region as well as peaks in the blue and red regions, PS II has a distinct action spectrum lacking far-red absorption. Thus, an imbalance of energy distribution between the two photosystems may occur in natural environments, where light quality and quantity fluctuate over time (Allen 1992; Bellafiore et al. 2005). Since the two photosystems are connected in series under normal conditions, vascular plants, mosses, and green algae constantly balance their excitation levels to ensure optimal efficiency of electron flow. It is via state transitions occurring under such conditions that the harnessed energy is redistributed to minimize unequal distribution. The phenomenon known today as "state transition" was discovered independently by Murata (Murata 1969) and Bonaventura and Myers (1969). Norio Murata, who had been studying fluorescence properties of a red microalga (*Porphyridium cruentum*), observed that Chl fluorescence in the far-red region, emanating primarily from the PS I antenna, was increased upon selective excitation of phycoerythrin, which constitutes the PS II peripheral antenna in red algae. On the other hand, Celia Bonaventura and Jack Meyers had been studying a green microalga (*Chlorella*

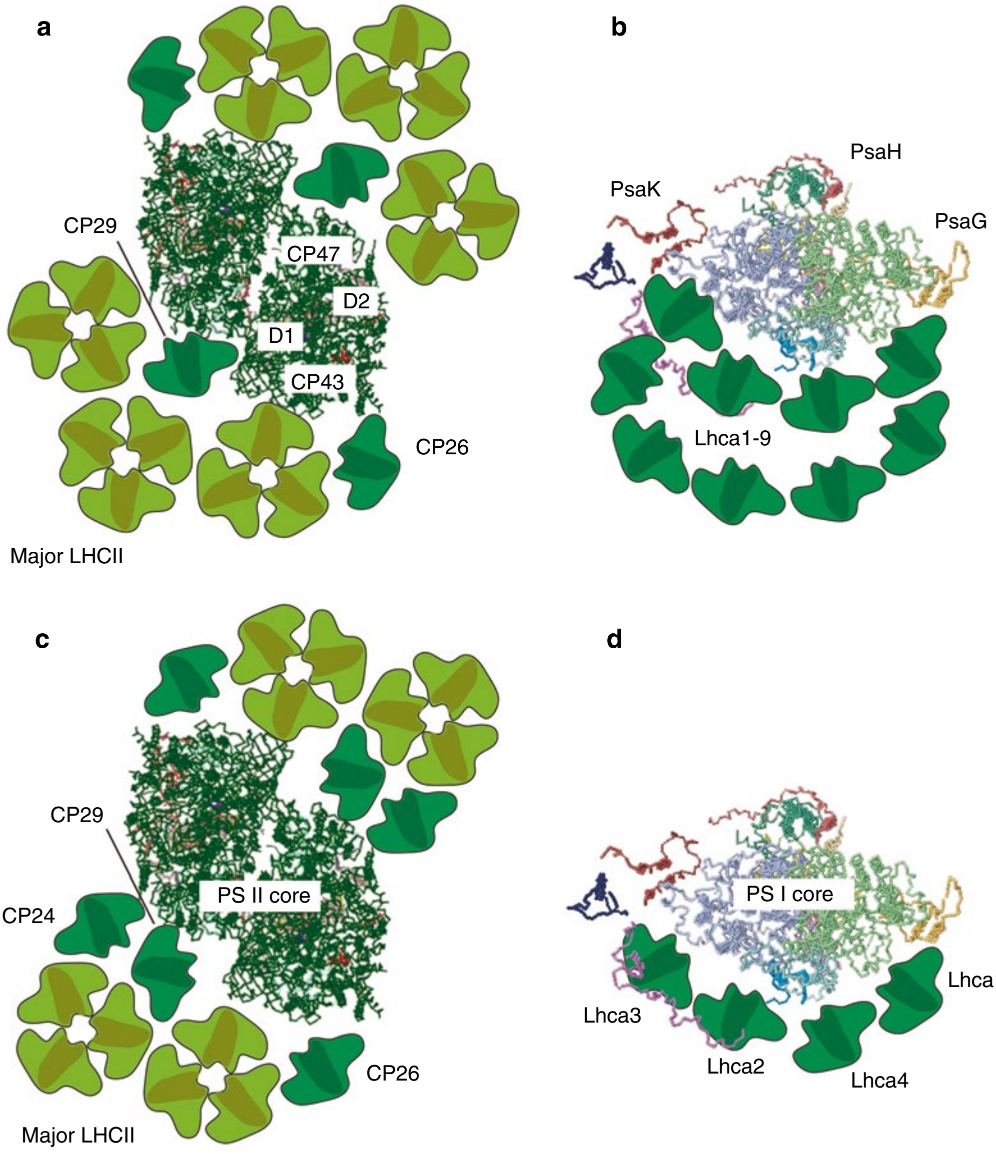

*Fig. 21.3 Supramolecular organization of PS II-LHCII and PS I-LHCI supercomplexes in green microalgae and vascular plants. Top views of the PS II-LHCII supercomplex (***a***) and the PS I-LHCI supercomplex (***b***) from C. reinhardtii* based on the single-particle image analysis by Tokutsu et al. (2012) and Drop et al. (2011), respectively. Top views of the PS II-LHCII supercomplex from spinach (**c**) and the PS I-LHCI supercomplex from pea (**d**) based on the single-particle image analysis by Dekker and Boekema (2005) and the crystallography of the PS I-LHCI supercomplex (Amunts et al. 2010). All top view images are from the luminal sides. The PS II and PS I core structures were taken from the coordinates determined by crystallography 3ARC.pdb and 2WSC.pdb, respectively.

pyrenoidosa) and observed that the yield of Chl fluorescence in the red region emanating primarily from the PS II antenna was decreased, and that oxygen evolution rate was increased, due to the increased oxidation of PS II after energy redistribution from PS II to PS I. The two groups reached the same conclusion that light-harvesting ability of the two photosystems was regulated to balance distribution of excitation between the two photosystems. State 1 is characterized by a higher Chl fluorescence yield from PS II at room temperature, as a higher ratio of fluorescence intensity in the red region as compared with the far-red region at 77 K, a higher quantum yield of PS II reactions, and a lower quantum yield of PS I reactions. Conversely, State 2 is characterized by a lower Chl fluorescence yield from PS II at room temperature, a lower ratio of fluorescence intensity in the red region as compared with the far-red region at 77 K, a lower quantum yield of PS II, and a higher quantum yield of PS I reactions. Thus, cells in State 2 show high NPQ. Although the situation when State 2 arises is quite different from that when qE arises, the emerging evidence (Iwai et al. 2010; Nagy et al. 2014) tells us the two may share some of the underlying mechanisms.

Although the green microalga *Chlorella pyrenoidosa* is one of the first organisms in which state transitions were reported (Bonaventura and Myers 1969), most of the molecular research on this phenomenon was performed on the major model green microalga *Chlamydomonas reinhardtii*. The following sections will therefore focus mostly on the recent findings obtained using the latter organism. Examples of such findings obtained with *C. reinhardtii* include the involvement of the cytochrome b_6f complex in control of LHCII phosphorylation, identification of LHCII kinase, mapping phosphorylated residues in LHCII, the molecular reorganization of photosystem supercomplexes, and the interplay between other physiological responses, such as CEF and qE.

A LHCII Phosphorylation

Chloroplasts contain the highest concentration of plant phosphoproteins (Bhalla and Bennett 1987). Among the latter, LHCII phosphorylation was first observed in illuminated pea chloroplasts supplied with [^{32}P]orthophosphate, in which Thr residues were reversibly phosphorylated upon illumination (Bennett 1977). Inhibition of LHCII phosphorylation by the PS II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) indicated that this light-dependent phosphorylation was activated through electron flow rather than a process mediated by a kinase directly activated by light (Allen and Bennett 1981). Phosphorylation of LHCII coincided with a decrease in the yield of Chl fluorescence from PS II (Bennett et al. 1980; Chow et al. 1981; Horton and Black 1981). Low-temperature fluorescence spectra showed that LHCII phosphorylation resulted in a decreased yield of the PS II fluorescence band in the red region relative to that of the PS I band in the far-red region (Bennett et al. 1980; Horton and Black 1981), suggesting that LHCII phosphorylation increased the proportion of absorbed excitation energy transferred to PS I. This was further confirmed by the increase of the Chl/P700 ratio and quantum efficiency of PS I (Telfer et al. 1984; Samson and Bruce 1995; Delosme et al. 1996).

Information about the phosphorylated residues was fragmented until genome sequences became available, allowing proteomic studies using mass spectrometry to be applied. Vener and colleagues mapped the phosphorylated amino acid residues in thylakoids of *C. reinhardtii* during state transitions (Turkina et al. 2006a, b). Two phospho-LHCII proteins were found in State 2 cells, including LHCBM1 (major LHCII type IV) and CP29. CP29 was heavily phosphorylated; Thr-7 and -33 were phosphorylated irrespective of the state, and Thr-17 and Ser-103 were phosphorylated only in State 2 (Turkina et al. 2004, 2006b). Thr-27 in the major LHCII and Thr-7 in CP29 were commonly phosphorylated in *C. reinhardtii* and vascular plants under State 2 promoting conditions (Michel et al. 1991; Hansson and Vener 2003). The fact that these residues phosphorylated in a redox-dependent manner and other residues phosphorylated in a light-dependent manner in D1, D2, CP43, CP26, and LHCBM4, −6, −9, and −11 (major LHCII type I) cluster at the interface between the PS II core and the peripheral LHCII proteins suggests that these residues play a role in undocking peripheral LHCII proteins during state transitions as well as in thermal energy dissipation under high-light conditions (Turkina et al. 2006b).

B Redox Control

Imbalances in excitation of the two photosystems are sensed by the redox state of the intersystem electron transfer carrier in the thylakoid membranes, which regulates LHCII phosphorylation (Fig. 21.4). Several initial studies suggested that plastoquinone (PQ) acts as a redox regulator of LHCII phosphorylation. Reduced ferredoxin (Fd) (Bennett 1979), dithionite (Allen et al. 1981), and duroquinol (Allen and Horton 1981), all of which are known to reduce PQ in isolated thylakoids, activated LHCII phosphorylation in the dark, whereas DCMU, which blocks PS II, inhibited light-dependent LHCII phosphorylation (Horton and Black 1981), indicating that LHCIIs are phosphorylated when the PQ pool is reduced. Further analysis revealed that it is not the reduced PQ molecule (PQH_2) per se that is critical for activation of the protein kinase but the binding of PQH_2 to the cytochrome b_6f complex. Wollman and Lemaire (1988) found that *C. reinhardtii* mutants lacking the cytochrome b_6f complex were unable to phosphorylate LHCII while other thylakoid phospho-proteins were unaffected. Bennett et al. (1988) reported that, while an inhibitor of the stromal side of the cytochrome b_6f complex (Qi-site), 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), did not affect activation of LHCII phosphorylation by PQH_2, a luminal-side inhibitor of the cytochrome b_6f complex, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), did inhibit LHCII phosphorylation, suggesting that the binding of PQH_2 to the Qo-site of the cytochrome b_6f complex activates LHCII kinase(s) (Fig. 21.4). Results consistent with this interpretation were provided by later studies using spectroscopy (Vener et al. 1997) and site-directed mutants around the Qo-site (Zito et al. 1999). There are also reports claiming that LHCII kinase(s) is/are not only activated by a reduced PQ pool, but also deactivated by the reduced thioredoxin pool that is downstream of PS I in the stroma of chloroplasts in pumpkin (Rintamäki et al. 2000) and pea (Hou et al. 2002), and in *C. reinhardtii* (Vink et al. 2004).

Although the activity of LHCII kinase was identified in 1977 (Bennett 1977), biochemical attempts to isolate specific LHCII kinases have been unsuccessful (Lin et al. 1982; Coughlan and Hind 1986, 1987; Sokolenko et al. 1995). The identities of the kinase and phosphatase of LHCII had thus remained elusive until recently. The most likely candidate for the LHCII kinase was identified in a genetic screen using *C. reinhardtii*. Using the characteristic fluorescence changes related to a State 1-to-2 transition, Rochaix and his co-workers isolated several mutants that lack state transitions (Fleischmann et al. 1999). One such mutant, *stt7*, was demonstrated to be deficient in a Ser/Thr kinase in chloroplast thylakoid membranes (Depège et al. 2003), where LHCIIs were barely phosphorylated. Subsequently, a mutant lacking an ortholog of STT7, STN7, also showed impairment in state transitions in *A. thaliana* (Bellafiore et al. 2005). The STT7/STN7 kinase contains a single membrane-spanning domain and is localized in thylakoid membranes. Immunoprecipitation assays indicated that STT7 could interact with LHCII, the cytochrome b_6f complex, and PS I subunits (Lemeille et al. 2009). Two Cys-residues (conserved in all STT/STN7 orthologs in various species) near the lumen-exposed N-terminus of STT7 are critical for the kinase's activity and might be potential targets for thioredoxin-mediated inhibition (Lemeille et al. 2009).

Reversible phosphorylation of LHCII in thylakoids during state transitions implies that phosphatases participate in the process and that at least a portion of those are membrane associated. The activities of such thylakoid protein phosphatases have been reported to be redox independent and constitutively high (Silverstein et al. 1993). A biochemical approach demonstrated that protein phosphatases of different families were

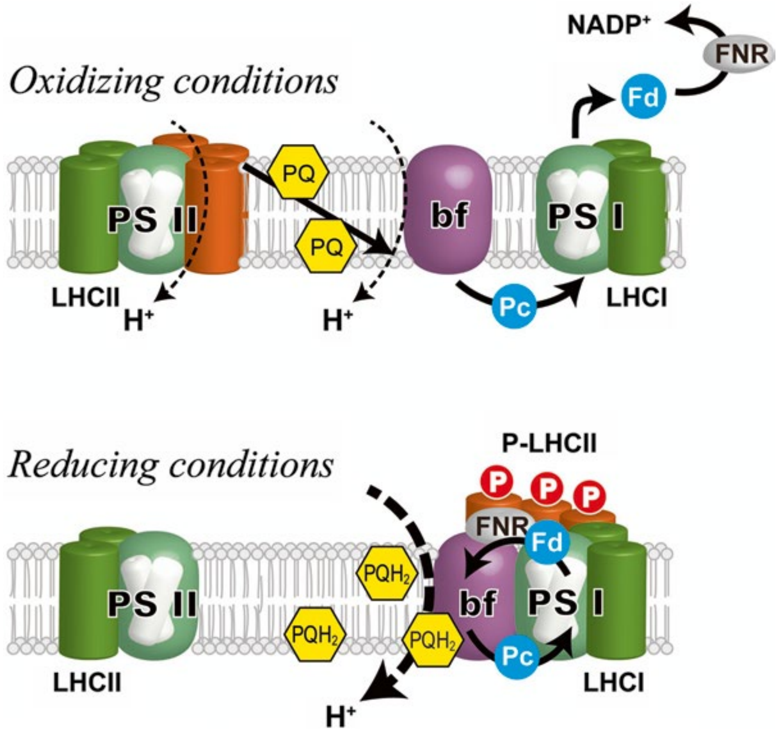

Fig. 21.4 Schematic representation of the regulation of state transitions and electron flow. Oxidizing conditions, when PS I is preferentially excited and the PQ pool is oxidized. Under these conditions, LHCIIs are bound to PS II (State 1). The photosynthetic electron flow proceeds in LEF mode generating a proton gradient across the thylakoid membrane that is used for ATP production, as well as NADPH. Reducing conditions, migration of the mobile LHCIIs (orange) occurs on the PsaH side of PS I that is on the opposite side of the LHCI belt to establish State 2. The cytochrome b_6f complex and ferredoxin NADP$^+$ reductase (FNR) are also bound to PS I to form a super-supercomplex (CEF supercomplex), which is required to establish CEF between PS I and the PQ pool generating mostly ATP. bf the cytochrome b_6f complex, Fd ferredoxin, Pc plastocyanin.

involved in the reversible phosphorylation of thylakoid phosphoproteins (Sun et al. 1989; Hast and Follmann 1996; Hammer et al. 1997). The LHCII phosphatase activity was shown to depend on the presence of divalent cations, and was not inhibited by microcystin and okadaic acid (Sun et al. 1989; Hammer et al. 1997). These findings strongly suggested involvement of a protein phosphatase type 2C (PP2C) in phospho-LHCII dephosphorylation. Recently, two groups reported a putative thylakoid-bound phosphatase in *A. thaliana*, termed TAP38/PPH1, which is specifically required for dephosphorylation of LHCII, but not of PS II core proteins (Pribil et al. 2010; Shapiguzov et al. 2010). The latter phosphatase, which belongs to a family of monomeric PP2C, is mainly associated with stroma lamellae of the thylakoid membranes. Loss of the TAP38/PPH1 phosphatase leads to an increase in the antenna size of PS I and impairs State 2-to-1 transition (Shapiguzov et al. 2010), while overexpression of the gene decreases LHCII phosphorylation and inhibits State 1-to-2 transition (Pribil et al. 2010; Shapiguzov et al. 2010). The nature of the LHCII phosphatase(s) in *C. reinhardtii*, which has no TAP38/PPH1 counterpart (Pribil et al. 2010), still needs to be clarified.

C Molecular Remodeling of Photosystems

How can LHCII phosphorylation decrease energy distribution to PS II and increase energy distribution to PS I? Lateral heterogeneity in the distribution of the photosystems between appressed and non-appressed membranes has been observed: the non-appressed region (stroma lamella) is highly enriched in PS I, and the appressed region (grana lamella) is highly enriched in PS II and LHCII (Andersson and Anderson 1980). LHCII in the non-appressed region is more phosphorylated than in the appressed region (Andersson et al. 1982; Bassi et al. 1988), suggesting that phosphorylation of LHCII in the appressed region causes migration of LHCII from PS II-enriched appressed region to PS I-enriched non-appressed region (Kyle et al. 1983; Larsson et al. 1983; Black et al. 1986). Initially, the association of LHCII proteins with PS I was shown indirectly as changes in the quantum yield of PS I and PS II during state transitions (Samson and Bruce 1995).

Scheller and his colleagues studied the association of the mobile LHCII with PS I via cross-linking and antisense approaches. *Arabidopsis thaliana* plants without PsaH and PsaL (PsaH subunit and PsaL subunit of photosystem I) (Lunde et al. 2000), as well as those without PsaO (PsaO subunit of photosystem I) (Jensen et al. 2004), were highly deficient in state transitions. Since these small PS I subunits are located on the side of the PS I core devoid of LHCI (Ben-Shem et al. 2003; Fig. 21.3), these PS I subunits were hypothesized to constitute a specific binding site for mobile LHCII. Later, binding of an LHCII trimer, and possibly of several LHCII monomers, was observed near this site in an electron microscopic study of thylakoids from *A. thaliana* partially solubilized by digitonin (Kouřil et al. 2005). In the absence of the docking-site-protein PsaH, LHCII still underwent phosphorylation in State 2, and a significant portion of phospho-LHCII remained attached to PS II. Similarly, phospho-LHCIIs were also observed to be a part of the PS II antenna in PS I-deficient *C. reinhardtii* mutants (Delosme et al. 1996).

Despite an accumulation of physiological observations, the structural basis for state transitions has remained obscure because of a lack of information about the identity of the mobile LHCII polypeptide(s) (Allen and Forsberg 2001). Only recently have several lines of direct biochemical evidence shown the binding of LHCII proteins to PS I (Pesaresi et al. 2002; Zhang and Scheller 2004; Kouřil et al. 2005; Takahashi et al. 2006). Such an association was first observed in the *A. thaliana psae1-1* mutant; a fraction of LHCII was associated with PS I when the mutant plants were exposed to low-light (State 2-favoring) conditions, giving rise to a high-molecular-mass protein-pigment complex (Pesaresi et al. 2002). This large complex, however, seemed to be an aggregated product, because the mutant did not show state transitions, probably owing to a low level of PsaH. The next approach was to crosslink the mobile LHCII proteins with the PS I-LHCI super-complex in *A. thaliana* (Zhang and Scheller 2004). In State 2, more major LHCII proteins, including Lhcb1 and −2, were crosslinked to the PsaH, PsaI, and PsaL subunits than in State 1. A further report was provided by a study of *C. reinhardtii*, where the PS I −LHCI/II super-complex isolated from State 2 cells contained two minor monomeric LHCII (CP26 and CP29) and one major trimeric LHCII (LHCBM5), suggesting a pivotal role for minor monomeric LHCIIs in state transitions (Takahashi et al. 2006). Mobile LHCII proteins were also observed in single-particle images of PS I-LHCI/II super-complexes. As described above, Boekema and colleagues (Kouřil et al. 2005) reported a large density along the side of PsaH/L/A/K in electron micrographs of *A. thaliana* PS I, which they assigned to the LHCII trimer. Barber and colleagues located a smaller density near PsaH in *C. reinhardtii*, which they assigned to CP29 (Kargul et al. 2005). The significance of the minor LHCIIs in state transitions in *C. reinhardtii* was further supported by an RNA interference (RNAi) study, where one of the two minor LHCII proteins, either CP29 or CP26, was knocked down (Tokutsu et al. 2009). Both the CP29 and CP26 RNAi mutants exhibited reductions in PS II antenna size during a State 1-to-2 transition, as reflected by increased qT, low-temperature fluorescence spectra, and functional absorption cross section. However, the undocked LHCIIs from PS II did not re-associate with PS I in the CP29-RNAi mutant because antenna size of PS I was not complementarily increased. On the other hand, mobile LHCIIs in the CP26-RNAi mutant re-associated with PS I, whose PS I-LHCI/II super-complex was visualized on a sucrose density gradient, thus clarifying that CP29, not CP26, is crucial when mobile LHCIIs re-associate with PS I under State 2 conditions in *C. reinhardtii*.

Studies on the migration of LHCII proteins have focused primarily on their re-association with PS I. However, polypeptide composition, supramolecular organization, and phosphorylation of PS II complexes under both State 1 and State 2 conditions were recently studied in *C. reinhardtii* (Iwai et al. 2008). Three PS II fractions – the PS II core complex, the PS II-LHCII super-complex, and the multimer of PS II-LHCII super-complex (PS II megacomplex) – were affinity-purified from a mutant carrying a His-tagged CP47. Gel filtration and electron microscopy showed that the megacomplex was predominant in State 1, whereas the core complex was predominant in State 2, indicating that LHCIIs are dissociated from PS II upon a State 1-to-2 transition. Moreover, in State 2, strongly phosphorylated LHCII type I (LHCBM3, −4, −6, and −9) was found in the super-complex, but not in the megacomplex. Phosphorylated minor LHCIIs (CP26 and CP29) were only found in their unbound form. The PS II subunits, including the CP43 and D2 proteins, were most highly phosphorylated in the core complex. It has been hypothesized that phosphorylated major LHCIIs do not necessarily dissociate themselves from PS II in *A. thaliana* (Lunde et al. 2000). In *C. reinhardtii*, LHCBM3, −4, −6, and −9 (LHCII type I) remain associated with PS II upon phosphorylation (Iwai et al. 2008). Phosphorylation of LHCII type I is induced earlier in the course of a transition from State 1 to State 2, and the latter protein complex is mostly localized in the super-complex, not in the megacomplex. Phosphorylation of LHCII type I thus probably causes division of the megacomplex into single super-complexes. In *C. reinhardtii*, the minor monomeric LHCIIs are shuttled to PS I, thus acting as linkers between PS I and major trimeric LHCII during a transition to State 2 (Kargul et al. 2005; Takahashi et al. 2006; Tokutsu et al. 2009). Although neither CP26 nor CP29 was phosphorylated while being associated with the PS II-LHCII megacomplex or PS II-LHCII supercomplex, both CPs were phosphorylated when dissociated from PS II, suggesting that this dissociation was caused by their phosphorylation. The minor monomeric LHCIIs border the major LHCII trimers and the PS II core (Harrer et al. 1998; Yakushevska et al. 2003), as shown in Fig. 21.3, and the hyper-phosphorylated residues in CP29 were indeed mapped at the interface of the PS II core and the peripheral antenna proteins (Turkina et al. 2006b). Therefore, phosphorylation of CP26 and CP29 likely triggers the undocking of the entire peripheral antenna during the transition to State 2 in *C. reinhardtii*. Detailed information about the specific phosphorylated sites in LHCII and the minor antennas as well as in other PS II subunits, and their comparison between *Chlamydomonas* and vascular plants, can be found in Vener (2007).

D Another Role of State Transitions in Chlamydomonas

Although the "power balancing (Haldrup et al. 2001)" role of state transitions to address an imbalance of energy distribution between the two photosystems has been generally accepted, recent studies may suggest another balancing role of state transitions in *C. reinhardtii*, which is in contrast to the situation in vascular plants, where (the small) state transitions may be important for photoprotection of PS I as recently proposed (Grieco et al. 2012). When most of the LHCII antenna is associated with PS I in *C. reinhardtii*, the quantum yield of the resulting complex increases greatly under conditions leading to State 2 (e.g., anaerobiosis). Thus, ATP can be produced without sustained generation of reducing equivalents. Consistently, a systematic transition to State 2 has been seen in *Chlamydomonas* under nutrient deprivation (reviewed in Davies and Grossman 1998). Moreover, in the case of another abiotic stress, high light, a decrease in the ability to perform linear electron flow (LEF) was observed (Finazzi et al. 2001), likely because of photoinhibition, while no impact was found on the ability to perform CEF. Under more physiological conditions, an enhanced PS I activity, i.e., intermediate conditions between State 1 and State 2, may contribute to balancing the ATP/NADPH ratio for assimilation of CO_2. Such "extra" ATP cannot be provided solely by LEF (Berry and Rumberg 1999; see also Wollman 2001; Finazzi and Forti 2004; Rochaix 2007 for further discussion). It should be noted that, although a correlation exists between transition to State 2 and activation of CEF around PS I in *Chlamydomonas* (e.g., Finazzi 2005), recent data (Terashima et al. 2012; Takahashi et al. 2013) indicate that the relationship between CEF and state transitions is not a cause-and-effect one, but is mostly phenomenological.

IV The Dual Strategy to Cope with High Light in Green Microalgae

In vascular plants, the state transition capacity is always much lower than that of qE (see below). While state transitions lead to quenching of around 20 % of PS II fluorescence, thermal energy dissipation reflected in qE can diminish the maximum fluorescence yield by about 2/3 (corresponding to an NPQ value of ~2 for an annual plant species according to Bilger and Björkman 1990 and even higher values of 4 and above for evergreen plant species; see Demmig-Adams et al., Chap. 24). Moreover, state transitions are active only under limiting light absorption, while qE mostly develops when photon flux exceeds the capacity of the photosynthetic machinery. No overlap would be possible between the two processes because studies in vascular plants and microalgae have demonstrated that qT is inhibited in high light (Aro and Ohad 2003). This inhibition could be due to either a down-regulation of STT7/STN7 kinase (due to negative feedback mediated by a thioredoxin; Rintamäki et al. 2000) or to a light-induced structural change within the LHCII moiety, which would make its phosphorylation site inaccessible to the kinase (Vink et al. 2004). Independently of the exact mechanism involved in this phenomenon, an inhibition of state transitions in high light is consistent with the notion that this phenomenon only allows balancing of absorption cross section of the two photosystems in limiting light, while qE induces massive downregulation of PS II efficiency (due to enhanced thermal dissipation of the excitation energy arising from absorbed photons) under oversaturating light intensity. However, it was shown that the inhibition of LHCII phosphorylation in high light seen in (fast-growing) annual plants did not take place in a (slow-growing) evergreen plant species (Cleland et al. 1990) that exhibits a much higher NPQ capacity than annual species (Demmig-Adams et al., Chap. 24), and a possible role in photoprotection under excess light of protein re-arrangements resembling state transitions in evergreen plants should receive future attention.

Recent experiments conducted in our laboratories have also challenged the current understanding of state transitions for the case of *C. reinhardtii*. Unlike low light-treated cells, cells exposed to high light for several hours to induce accumulation of LHCSR3 (Peers et al. 2009) not only display a high capacity to develop qE, but also show a State 2 phenotype that is not reversed by strong aeration to promote reoxididation of the PQ pool via the combined activity of the mitochondrial respiratory and of the chloroplast chlororespiratory chains (Bulté et al. 1990; Allorent et al. 2013). These results suggest that both phenomena (qE and qT) are induced by conditions suitable to induce

NPQ, and that they are therefore intimately linked in this alga. Such a link can be viewed in the context of the regulatory mechanisms of state transitions. In high light, a net reduction of the PQ pool is expected, because the rate of PQH_2 generation by PS II becomes faster than the rate of PQH_2 oxidation by the cytochrome b_6f complex, which is the slowest step of electron flow (e.g., Joliot and Johnson 2011). This situation should lead to a net accumulation of reducing equivalents (PQH_2) upstream of the cytochrome b_6f complex. In the dark, PQH_2 may bind to the cytochrome b_6f complex (Finazzi 2005), thereby activating STT7 kinase because the inhibitory mechanisms preventing LHCII phosphorylation described so far are not active in the dark.

The relationship between state transitions and qE in high light-treated *C. reinhardtii* cells is observed not only in dark-adapted cells but upon retransfer of the cells to the light. Comparison of fluorescence profiles of high light-treated wild type, *stt7* (locked in State 1), *npq4* (devoid of qE), and *stt7*/*npq4* cells shows that several NPQ phases exist in *C. reinhardtii*. Some of them are related to the transition from State 2 to State 1 in the light (and the recovery of State 2 in the dark). Moreover, two quenching phases can be observed that correspond to qE. These distinct phases reflect the reversible migration of LHCSR3 from PS I to PS II (and vice versa) during state transitions (Allorent et al. 2013). Consistent with this notion, LHCSR3 can be phosphorylated by STT7 (Bonente et al. 2011), i.e., the same kinase involved in LHCII phosphorylation. Thereby the role of LHCSR3 as a specialized antenna complex of PS II seems to be extremely likely.

The functional interaction between state transitions (protein migration between the photosystems) and qE (net thermal dissipation of energy) likely provides cells with an additional layer of photoprotection. Both state transitions and qE seem to be required for optimum photoprotection in high light exposed *C. reinhardtii* cells, as shown by the enhanced photosensitivity of the *stt7 npq4* double mutant (Allorent et al. 2013). This behavior has not been reported in plants (Breitholtz et al. 2005). Comparative analysis of the different mutant strains (*stt7*, *npq4*, *npq4*/*stt7*) reveals that state transitions could play a photoprotective role during the early phase of high light acclimation of the cells while, in steady state, qE would perform most of the photoprotective functions, as already reported in vascular plants. This observation likely provides an explanation for early observations that state transitions are far larger in this alga than in vascular plants (Delosme et al. 1996). Indeed, the induction of qE during a dark to high light transition is a much slower process in this alga than in vascular plants, owing to the long time required to accumulate LHCSR3 during high light exposure (e.g., Peers et al. 2009). Occurrence of a sustained transition to State 2 during this period may alleviate the high light pressure on PS II, and protect it from photodamage. Up to now, the observed synergy between state transitions and qE seems to be confined mainly to *Chlamydomonas*, probably because this alga is the only one studied so far where induction of qE capacity is a slow process. Diatoms, where LHCSR proteins also modulate qE (Nymark et al. 2009; Zhu and Green 2010), do not undergo state transitions (Ting and Owens 1993), but LHCX1 (the LHCSR isoform responsible for most of qE) is constitutively expressed in the pennate diatom *Phaeodactylum tricornutum*. Similarly, in the moss *Physcomitrella patens*, where both PsbS and LHCSR3 contribute to qE (Alboresi et al. 2010), the LHCSR isoform that modulates quenching is constitutively accumulated in the thylakoids. It is therefore tempting to speculate that the large state transition capacity observed in *Chlamydomonas* may have evolved to provide this alga with a high degree of flexibility to cope with abiotic stresses. Indeed, the possibility to modulate PS II absorption to a large extent (by lowering its antenna size via state transitions), to regulate the ATP/NADPH synthesis capacity (via the changes in LEF vs CEF ratio that also follow state transitions; Iwai et al. 2010;

Cardol et al. 2011), as well as the capacity to vary qE extent by moving LHCSR3 to PS I when needed could provide a benefit during both light and nutrient stress.

V Additional Photoprotective Mechanisms Based on Electron Flow

While thermal energy dissipation, as reflected in qE (and state transitions to some extent), plays a major role in providing photoprotection in green algae, a few examples exist in these organisms where changes in electron flow capacity are directly involved in preventing the occurrence of photodamage. Recent studies in *C. reinhardtii* (Houille-Vernes et al. 2011) have shown that the product of one of the two genes encoding for PQ terminal oxidase (PTOX2) is involved in photoprotection. PTOX is a di-iron enzyme bound to the thylakoid membranes (Carol et al. 1999) of vascular plants. This protein is encoded by a nuclear gene present in a single copy in vascular plants and responsible for non-photochemical oxidation of the PQ pool (reviewed in Carol and Kuntz 2001). In *C. reinhardtii*, the *ptox2* mutant exhibits a lower fitness than wild type when grown under photoautotrophic conditions. Moreover, double mutants lacking both the cytochrome b_6f complex and PTOX2 were more sensitive to light stress than single mutants lacking either the cytochrome b_6f complex or PTOX2 (Houille-Vernes et al. 2011). This observation suggests that PTOX2 provides an efficient electron sink under extreme conditions where the PQ pool is over-reduced, thereby lowering the electron pressure on PS II and limiting photodamage. In *C. reinhardtii*, this enzyme operates at a rate of around 5 electrons per second (Houille-Vernes et al. 2011), which is approximately ten times higher than that measured in vascular plants (Trouillard et al. 2012). This finding suggests that even at such a relatively low rate (the PQ oxidation by the cytochrome b_6f complex has a maximum rate of ~100 s^{-1}), PTOX can act as a safety valve under stress conditions. The notion that PTOX can actively prevent photodamage is not limited to the case of *C. reinhardtii*.

Previous studies conducted in both prokaryotes and eukaryotes (reviewed in Zehr and Kudela 2009) have suggested a similar role in Fe–starved photosynthetic organisms occurring in the open ocean (where Fe is not available in sufficient amounts). Fe limitation results in a massive downregulation of the Fe-rich PS I complex (to release iron for essential functions; Moseley et al. 2002). Under these circumstances, the PS I concentration becomes limiting for photosynthetic electron flow, which leads to over-reduction of PS II acceptors and possible photoinhibition (e.g., Barber and Andersson 1992). Some cyanobacteria (Bailey et al. 2008; Mackey et al. 2008) and some ecotypes of *Ostreococcus* (Cardol et al. 2008) circumvent this limitation by rerouting excess electrons generated by PS II into a water-to-water cycle catalyzed by PTOX. Such an alternate electron flow, of up to 50 % of PS II-generated electrons, has some advantages when compared to photoprotective strategies based on downregulating light harvesting. Indeed, development of qE requires a ΔpH across the thylakoid membrane, which cannot be maintained by LEF- or CEF-driven electron flow when both are inhibited because of low PS I concentration. In this case, operation of the water-water cycle, coupled to generation of a proton gradient (Cardol et al. 2008), may fulfill the double role of relieving the redox pressure on PS II and allowing building of a proton motive force for both qE generation and ATP synthesis (Cardol et al. 2011) in Fe-limited cells. A comparison with previous observations in diatoms and cyanobacteria (Strzepek and Harrison 2004; Bailey et al. 2008) suggests that electron diversion downstream of PS II may be widespread among prokaryotic and eukaryotic components of phytoplankton. In oceanic diatoms (*Thalassiosira oceanica*), however, increased expression of LHCSR-like orthologs may also contribute to successful adaptation to a constitutively Fe-poor environment (Lommer et al. 2012).

VI Conclusion

Previous work on green microalgae has been instrumental to derive information on the molecular mechanisms of light acclimation (state transitions, high energy quenching). This progress was facilitated by the identification via molecular tools of mutants lacking such acclimation and of the corresponding genes. Moreover, the large diversity of environmental niches occupied by these organisms has allowed derivation of important information on the physiology and ecology of the light response of these organisms. Several questions remain to be solved in this field, i.e., the molecular mechanism of thermal energy dissipation during high-energy quenching, the fate of phosphorylated LHCII during state transitions in limiting light and upon exposure to excess light, and changes in chloroplast ultrastructure that follow "qE" and state transitions. Thanks to their extreme flexibility in modifying the photosynthetic machinery in response to changes in environmental conditions, their rapid growth, and the fact they can be easily manipulated, microalgae as targets of future research have the potential to make further contributions to the understanding of the processes associated with NPQ.

Acknowledgments

GF wishes to thank the financial support from the Agence Nationale de la Recherche, grant "phytadapt" n°NT09_567009, the Labex GRAL (Grenoble Alliance for Integrated Structural Cell Biology) and the Région Rhone Alpes. JM wishes to thank the financial support from the NEXT Program initiated by the Council for Science and Technology Policy Grant (GS026). Support from the Japanese Society of Technology – Conseil National de la Recherche Scientifique cooperative program on Marine Genomics and Marine Biology to GF and JM is also acknowledged.

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Chapter 22

Mechanisms Modulating Energy Arriving at Reaction Centers in Cyanobacteria

Diana Kirilovsky*
Commissariat à l'Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay (iBiTec-S), Gif sur Yvette, France

Centre National de la Recherche Scientifique (CNRS), UMR 8221, 91191 Gif sur Yvette, France

and

Radek Kaňa and Ondřej Prášil*
Laboratory of Photosynthesis, Institute of Microbiology, Center Algatech, Czech Academy of Sciences, Opatovicky mlyn, CZ 37981 Třeboň, Czech Republic

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*Author for correspondence, e-mail: diana.kirilovsky@cea.fr; prasil@alga.cz

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_22, © Springer Science+Business Media Dordrecht 2014

Summary

Cyanobacteria possess several photoprotective mechanisms involving relatively rapid (<few minutes) changes in the photosynthetic apparatus to dynamically adjust the amount of irradiance arriving at photosystem II (PS II). Photoprotection in cyanobacteria differs from that in plants and algae, with both of the latter possessing transmembrane chlorophyll- and carotenoid-binding light-harvesting complexes. Most cyanobacteria harvest light via a large extra-membrane complex, the phycobilisome (PBS) that contains blue and red phycobiliproteins. Thus far, three prominent and rapid processes have been identified that control effective PS II antenna size in cyanobacteria: state transitions, blue-green-light-induced thermal dissipation of excess energy absorbed by PBS, and PBS decoupling. While the latter two have a mostly photoprotective function, the process of state transitions also optimizes energy distribution between the two photosystems.

For blue-green-light-induced energy thermal dissipation, a water soluble photoactive Orange Carotenoid Protein (OCP) is essential. The OCP acts as a light-intensity sensor and energy-dissipation-inducer and is the only photoactive protein known thus far with a carotenoid as its sensor. Strong blue-green light induces structural changes in the OCP that lead to the formation of its "red-active form". The red OCP, by interacting with the PBS core, increases thermal energy dissipation at the level of antenna and decreases the energy arriving at reaction centers. To recover full antenna (light-harvesting) capacity under low light conditions, a second protein is required, the "Fluorescence Recovery Protein" (FRP) that plays a key role in dislodging the red OCP protein from the PBS and accelerates OCP conversion to the inactive orange form. In this chapter, we review the current understanding of the mechanism of OCP-mediated fluorescence and energy dissipation in cyanobacteria.

Despite decades of research on state transitions in cyanobacteria, the underlying mechanism of this phenomenon remains unresolved. Two mechanisms of state transitions occurring at low light intensities have been suggested for cyanobacteria. In the first, a physical movement of PBS or PS I monomers leads to redistribution of energy absorbed by PBS between PS II and PS I. In the second suggested mechanism, excitation-energy spillover regulates redistribution of absorbed light between PS II and PS I. Controversy exists as to whether state transitions involve long-range displacement of proteins (PBS, PS I) and how changes in plastoquinone redox state trigger energy redistribution between the photosystems.

Several new reports now indicate that cyanobacteria can modulate energy transfer within PBS or from PBS to PS II reaction centers. Such a "decoupling" could represent an alternative safety valve for excess energy dissipation, especially in those groups of cyanobacteria that do not possess OCP-related NPQ.

Abbreviations: F_m, F_m' – Maximal fluorescence yield (in the dark or in the presence of actinic light, resp.); F_o, F_o' – Minimal, intrinsic fluorescence yields (in the dark or in the presence of actinic light, resp.); F_s – Steady-state fluorescence yield in the light-acclimated state; F_v, F_v' – Variable fluorescence (in the dark or in the presence of actinic light); APC – Allophycocyanin; Chl – Chlorophyll; CP43 – Chlorophyll protein antenna 43 kD; CP47 – Chlorophyll protein antenna 47 kD; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea (also called Diuron); ECN – Echinenone; Elip – Early light induced protein; FRAP – Fluorescence recovery after photobleaching; FRP – Fluorescence recovery protein; hECN – 3-hydroxy-echinenone; Hlip; High-light induced protein; ICT – Intramolecular charge transfer; IsiA – Iron-induced light harvesting protein in cyanobacteria; L_{CM} – Phycobilisome core-membrane linker polypeptide (Anchor polypeptide LCM) ApcE; NPQ – Non-photochemical quenching of chlorophyll excited state; OCP – Orange carotenoid protein; PBS – Phycobilisome(s); PC – Phycocyanin; PE – Phycoerythrin; PEC – Phycoerythrocyanin; PQ – Plastoquinone; PQH_2 – Plastoquinol; PS I – Photosystem I; PS II – Photosystem II; PsbU – Subunit of the oxygen evolving complex of PS II; Q_A – The first (primary) plastoquinone electron acceptor of PS II; Q_B – The second (secondary) plastoquinone electron acceptor of PS II; qP – Photochemical quenching; RC – Reaction center; ROS – Reactive oxygen species; RpaC – Regulator of phycobilisome association C protein

I Introduction

Light is essential for photosynthetic organisms that convert solar energy into chemical energy and produce reducing power, which is used for the synthesis of organic carbon molecules. However, light becomes dangerous when the energy arriving at the reaction centers of the photosynthetic apparatus exceeds the energy consumption by various cellular processes. In this case, the photosynthetic electron transport chain becomes reduced and reactive oxygen species (ROS) accumulate, especially at the level of both photosystems, photosystem I (PS I) and photosystem II (PS II; see Pospíšil 2012). This has the potential to inactivate the photosynthetic apparatus, a result also referred to as photoinhibition of photosynthesis (for recent reviews, see Adams et al. 2013; Ohad et al. 2011; Tyystjärvi 2008; Vass 2012 and Adams et al. Chap. 23). The translational and transcriptional machineries can also be inactivated by ROS, leading to cell death (Nishiyama et al. 2001). A disequilibrium between energy absorbed and energy consumed can also occur at lower irradiance, e.g., under nutrient starvation or low CO_2 conditions. Cyanobacteria have developed acclimation responses, involving protein synthesis and degradation to maintain the balance between energy absorbed and energy used, in order to decrease ROS accumulation and/or remove ROS. Under light- and nutrient-stress conditions, cyanobacteria, via repression or up-regulation of specific genes, decrease the levels of photosystems and phycobilisomes (PBS) and of the cyanobacterial antennae, and increase CO_2 fixation capacity and overall cellular metabolism. Genes encoding ROS-scavenging enzymes are also up-regulated. Mechanisms specific to particular stresses, and involving newly synthesized proteins, are induced or increased. We recommend a review by Muramatsu and Hihara (2012) that deals with genes and proteins involved in the high light acclimation of cyanobacteria.

Cyanobacteria have also evolved photoprotective mechanisms that involve rapid changes in the photosynthetic apparatus to cope with abrupt and fluctuating changes in the quality and intensity of light: state transitions, transient PBS disconnection and Orange Carotenoid Protein (OCP) related photoprotection. These mechanisms, all either decreasing the energy arriving at the reaction centers or changing the relative amount of energy arriving at PS II, diminish ROS accumulation and thus potential cell damage. The common feature of these three mechanisms is the involvement of the PBS and their association with changes in the yield of PS II fluorescence. Thus, the triggering and unfolding of these photoprotective mechanisms can be easily followed using fluorescence kinetic measurements.

This review will describe in detail the photoprotective mechanisms involving rapid changes in the PBS and/or in the relationship between PBS and the photosystems. A brief description of the PBS and the fluorescence methods used to follow these processes will be included in the first part of the chapter. In addition, other cyanobacterial photoprotective mechanisms in which the PBS are not involved will be briefly described.

II Phycobilisomes

Phycobilisomes, that are attached to the outer surface of the thylakoid membranes (Gantt and Conti 1966), are composed of several types of chromophorylated (chromophore-binding) phycobiliproteins and linker peptides needed for the structural organization and functioning of PBS (for reviews, see (Glazer 1984; Grossman et al. 1993; MacColl 1998; Tandeau de Marsac 2003; Adir 2005). PBS have a core from which rods radiate (Fig. 22.1). The major core protein is allophycocyanin (APC), and while the rods in most freshwater cyanobacteria contain only phycocyanin (PC), phycoerythrin (PE) or phycoerythrocyanin (PEC) are found in the distal end of the rods in many marine cyanobacteria.

In most of cyanobacterial strains, the core of PBS consists of three cylinders, each formed by four APC trimers. The upper cylinder is formed by αAPC-βAPC trimers that

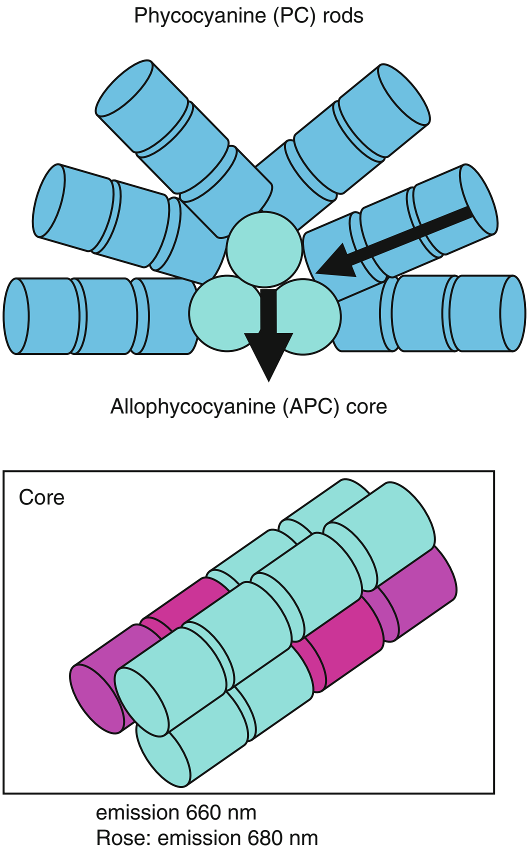


Fig. 22.1. Schematic representation of phycobilisomes (*PBS*) of *Synechocystis* PCC 6803. The PBS contain six rods formed by three hexamers of phycocyanin (*PC*) and a core formed by three cylinders of allophycocyanine (*APC*). Each cylinder contains four allophycocyanin trimers. In the core, APC trimers emit at 660 nm and four APC trimers emit at 680 nm. Two of the 680 nm emitting trimers contain the ApcD subunit (*rose*) and the other two contain the ApcF and ApcE subunits (*dark rose*).

exhibit maximal fluorescence emission at 660 nm (APC_{660}). Each basal cylinder contains two APC_{660} and two APC_{680} trimers. In the latter trimers, the αAPC or the βAPC is replaced by another APC-like subunit, ApcD, ApcF or ApcE. All these subunits carry a phycocyanobilin chromophore. In one APC_{680} trimer, one αAPC is replaced by an ApcD, and in the other, one βAPC is replaced by ApcF and one αAPC by the N-terminal domain of ApcE. The energy is transferred from the PC in the rods to the APC in the core. The APC_{680} trimers transfer the energy absorbed by the PBS to the chlorophyll (Chl) antenna and reaction centers of PS II and PS I (Mullineaux 1992; Rakhimberdieva et al. 2001). The subunit ApcE was previously termed L_{CM} because, in addition to transferring energy to the photosystems, it is also involved in the interaction between the PBS and thylakoids (Glazer and Bryant 1975; Redlinger and Gantt 1982; Capuano et al. 1991, 1993). Due to its more complex composition, ApcE is much larger than the other subunits that range in mass from 75 to 125 kDa. It has been proposed that ApcE provides a flexible surface allowing interaction with multiple membrane components such as PS II and PS I (Mullineaux 2008b), possibly through the Arm 2 domain (Ajlani and Vernotte 1998). ApcE is primarily composed of alkaline amino acids, which suggests that electrostatic interactions occur between PBS and PS II surfaces (Arteni et al. 2009). PS II is negatively charged by phospholipids and sulpholipids (Loll et al. 2007), which is in contrast to the PS I trimer that contains fewer negatively charged lipids buried between monomers or under peripheral subunits (Fromme et al. 2001).

III Fluorescence Measurements

A Measurements of Fluorescence Quenching in Cyanobacteria

The kinetics of fluorescence yields can be monitored using modulated fluorometers utilizing the pulse saturation method and only recording the fluorescence excited by non-actinic pulses (Schreiber et al. 1995). Since fluorescence excited by other sources of light is not recorded by the latter detection system, measurements can be done using different intensities and qualities of actinic light or changing the actinic light during the measurement. Minimal fluorescence level (F_o) is determined using low-intensity (non-actinic) modulated light to illuminate dark-adapted cells. Under these conditions, all reaction centers of PS II are open and no measurable charge separation is induced. Maximal fluorescence level (F_m and F_m', i.e., maximal fluorescence in the fully relaxed state following a sufficiently long absence of light and maximal fluorescence during exposure to actinic light) is measured by applying pulses of intense light that transiently close all PS II centers, thereby removing photochemical quenching (qP). Under continuous illumination, the steady-state fluorescence level (F_s), that depends on the redox state of the one-electron acceptor plastoquinone, $Q_{A,}$ is measured. Depending on the wavelength of the modulated-low-intensity measuring light, one can excite either Chl or PBS, or both. For example, the fluorescence yields (F_o, F_s and F_m') detected by fluorometers using excitation light of 650 nm can be emitted from both Chl and from PBS (Campbell et al. 1998). Thus, a decrease in the fluorescence yield could be the result of a diminution of either PBS emission or Chl-antenna emission, or be caused by a decrease of excitation energy transfer from the PBS to PS II. Figure 22.2 shows typical traces of PAM measurements.

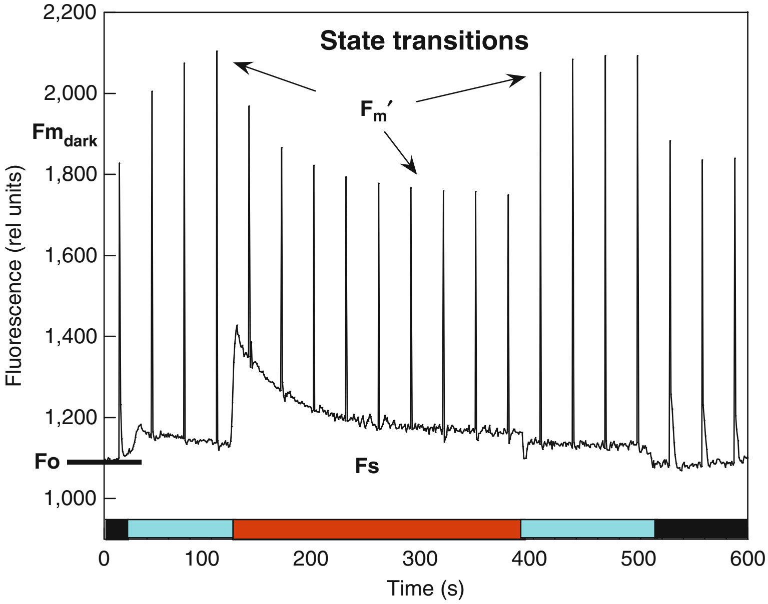
State transitions, that regulate redistribution of energy between PS II and PS I, are induced by changes in the redox state of the PQ pool. Exposure of photosynthetic organisms to light absorbed preferentially by PS I (blue or far-red light for cyanobacteria) induces a relative increase of PS II Chl fluorescence yield (state 1). Conversely, illumination with light absorbed predominantly by PS II (orange or green light for cyanobacteria) causes a relative decrease of PS II Chl fluorescence yield (state 2). Dark-adapted cyanobacterial cells are in state 2 due to reduction of the PQ pool by respiratory substrates (Dominy and Williams 1987; Mullineaux and Allen 1990), which is responsible for the low maximal fluorescence level in darkness (F_m). Upon illumination by low-intensity blue light, the maximal level of F_m' is reached (Fig. 22.2a). The OCP-mediated photoprotection is associated with decreases of all fluorescence levels (F_o, F_s and F_m') induced by strong blue-green light (Fig. 22.2b). The decrease of fluorescence is related to the quenching of phycobilisome emission (Wilson et al. 2006). The minimal intensity of blue-green light required for inducing fluorescence quenching depends on the concentration of cells, the relationship between PBS and PS II under different growth or stress conditions, and on the cyanobacterial strain.

B Cyanobacterial Emission Spectra

To distinguish various possible sources (quenching of PBS emission versus Chl quenching; fluorescence quenching versus state transition) of differences in the yield of PS II fluorescence observed with the kinetic fluorometers, measurements of emission spectra either at room or low (77 K) temperature are useful.

At room temperature, emission at 660 nm is related to PBS (APC emission), while emission at 680 nm arises mostly from PS II-associated Chl. At 77 K, the fluorescence emission at 715–730 nm originates from PS I, whereas the peaks at 685 and 695 nm originate from PS II. The peak at 685 nm corresponds to the emission of the PBS terminal emitters as well as to the emission of Chls of CP43 (inner antenna of PS II). The peak at 695 nm originates in Chls of CP47 (second inner antenna of PS II) and reaction center (RC) II, while peaks at 650 and 660 nm are associated with emission of PC and APC, respectively (Fig. 22.3). For early reviews on this topic, see Fork and Mohanty (1986) and Mimuro (2004).

When cells are excited at 430 nm, fluorescence emitted primarily by Chl *a* is observed. In this case, the level of fluorescence depends on excitation-energy transfer within PS II and PS I Chl antennae. When cells are excited at 600 nm (light principally absorbed



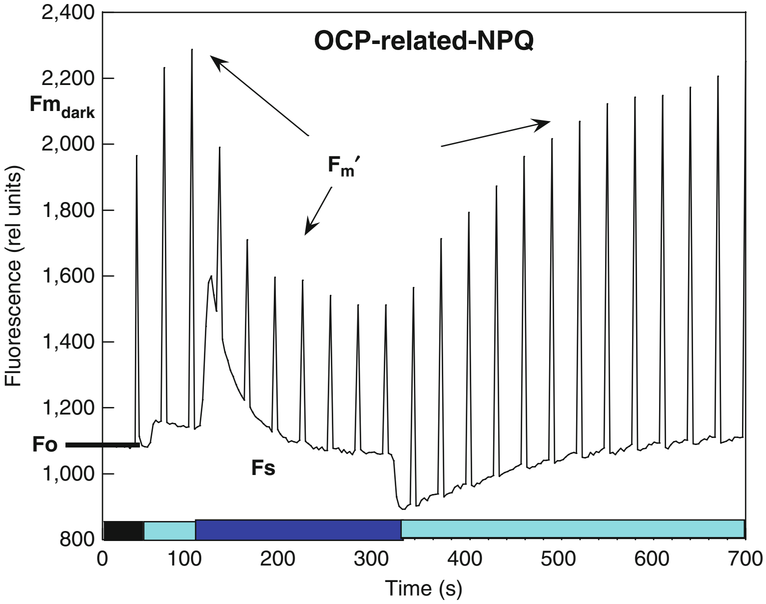


Fig. 22.2 Examples of changes in fluorescence levels by *orange light* and different intensities of *blue light* measured with kinetic fluorometer (PAM, Walz, Germany). Dark adapted cells (in state 2) were illuminated with low intensities of *blue-green light* (400–550 nm, 80 μmol photons m^{-2} s^{-1}) inducing the transition to state 1. Then the cells were illuminated with low intensities of *orange light* (600 ± 20 nm, 50 μmol photons m^{-2} s^{-1}) or strong *blue-green light* (400–550 nm, 1,800 μmol photons m^{-2} s^{-1}). In both cases, a decrease of F_m' is observed, but the mechanisms underlying each are different. Orange light induces transition to state 2 and strong blue-green light induced the OCP-mediated-NPQ mechanism. Saturating pulses (0.4 s duration, 4,000 μmol photons m^{-2} s^{-1}) were applied to measure F_m and F_m' levels in darkness and in light, respectively. F_m = maximal fluorescence in darkness, F_m' = maximal fluorescence during illumination. F_s steady-state fluorescence, F_o minimal fluorescence in darkness.

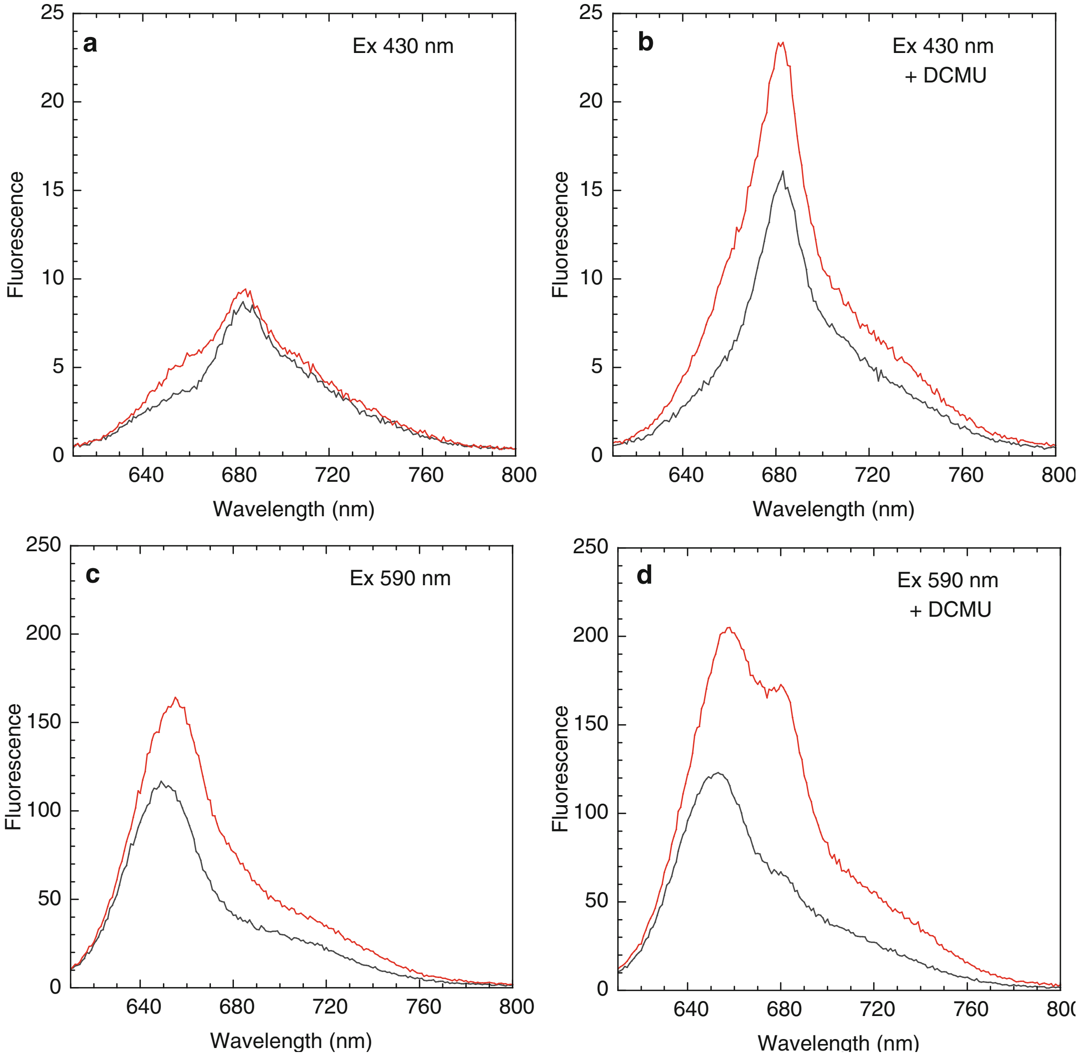


Fig. 22.3. Room temperature fluorescence spectra of unquenched (*red*) and quenched (*black*) Synechocystis PCC 6803 cells. The spectra were obtained by excitation at 430 nm (**a**, **b**) or at 590 nm (**c**, **d**) in the absence (**a**, **c**) or presence of DCMU (**b**, **d**). The *Synechocystis* cells were illuminated for 5 min with strong blue-green light (400–550 nm, 2,400 μmol photons m^{-2} s^{-1}) to induce fluorescence quenching.

by PC), fluorescence emission from both phycobiliprotein and Chl are detected. In the latter case, fluorescence level depends on processes occurring in the PBS and/or during the excitation energy transfer from the PBS to the PS II (Chl-antennae CP43 and CP47 and RC II).

In the case of the OCP-related photoprotective mechanism, fluorescence spectra generated by excitation at 430 nm are similar in control and quenched cells at room temperature and at 77 K (Wilson et al. 2006). In contrast, in the spectra obtained by excitation at 600 nm, all peaks are smaller in quenched cells, indicating a quenching of the PBS fluorescence emission and a concomitant decrease in the energy transfer from the PBS to the photosystems (Fig. 22.3) (Wilson et al. 2006, 2007). When spectra are recorded in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; an inhibitor of photosynthetic electron transport), a rather large decrease of 680-nm emission related to Chl is observed when the spectra are excited at 430 or 600 nm. DCMU inhibits electron transport between the primary (Q_A) and secondary (Q_B) quinones in PS II. In the presence of DCMU, PS II centers are closed and photosynthetic electron flow is not possible. Under these conditions, the energy absorbed by Chl migrates to PBS where it can be thermally dissipated. Thus, in the presence of DCMU, Chl fluorescence quenching is also observed.

During state transitions, the relative intensities of PS II and PS I fluorescence bands change, suggesting that state transitions modulate energy transfer between photosystems and between PBS and the photosystems. For discussions on state transitions, see Allen and Mullineaux (2004) and Papageorgiou and Govindjee (2011).

IV Brief Description of Cyanobacterial Photoprotective Mechanisms Not Involving Phycobilisomes

In this review, we focus on three photoprotective mechanisms involving PBS. In addition, cyanobacteria have evolved several other mechanisms to protect themselves from high irradiance. We mention here, as examples, three types of "photoprotective" proteins involved in these mechanisms: (1) Hlips, one-helix high-light-induced proteins, are part of the Elip (early light induced protein) family, light-harvesting-like proteins that contain a Chl-binding site but are not involved in light harvesting. Hlips accumulate under high light and play an important role in cell survival under these conditions (He et al. 2001; Bhaya et al. 2002; Havaux et al. 2003). These proteins appear to be important for Chl synthesis and storage as well as PS II repair (Vavilin and Vermaas 2002; Havaux et al. 2003; Xu et al. 2004; Vavilin et al. 2007; Hernandez-Prieto et al. 2011). (2) IsiA, another protein of the light-harvesting-protein family, is induced by iron starvation (Laudenbach and Straus 1988; Burnap et al. 1993) but also by other stress conditions (Jeanjean et al. 2003; Yousef et al. 2003; Havaux et al. 2005). Under iron starvation, 18 IsiA molecules encircle trimeric PS I complexes and function as a PS I antenna (Bibby et al. 2001; Boekema et al. 2001). IsiA also accumulates as large empty rings (without PS I) that are in a strongly quenched state, suggesting that they may be responsible for the thermal dissipation of absorbed energy (Yeremenko et al. 2004). (3) Under low CO_2 conditions, the four genes encoding flavodiiron (Flv) proteins in *Synechocystis* cells are up-regulated (Vicente et al. 2002; Zhang et al. 2009; Allahverdiyeva et al. 2011). Flv1 and Flv3 function in a Mehler-like (involving molecular oxygen as an electron acceptor and producing water) reaction (Vicente et al. 2002; Allahverdiyeva et al. 2011). The latter two proteins transfer electrons from PS I to oxygen, producing water instead of ROS. Under low CO_2, 60 % of electrons from water splitting in PS II go to molecular oxygen via Flv1 and Flv3 (Allahverdiyeva et al. 2011), decreasing the reduced state of the photosynthetic electron transport chain generated by low CO_2. Flv2 and Flv4 accept electrons from PS II or from the plastoquinone (PQ) pool, and are involved in PS II photoprotection (Zhang et al. 2009, 2012).

V The OCP-Related Photoprotective Mechanism

It was initially generally believed that NPQ of Chl fluorescence in cyanobacteria was only related to state transition or to photoinhibition (i.e., inactivation of PS II) (Campbell et al. 1998). However, some observations in blue-green algal lichens suggested the possible existence of a zeaxanthin related photoprotective mechanism involving energy and fluorescence quenching (Demmig-Adams et al. 1990; Adams et al. 1993). Then, El Bissati et al. (2000) showed that strong blue-green light induced strong fluorescence quenching that was not related to PS II inactivation or to reduction of the PQ pool known to trigger state 2-transition, but this fluorescence quenching was related to energy dissipation at the level of the PBS. It was suggested that the latter blue-light induced fluorescence quenching that further increases under iron-starvation conditions was related to the IsiA protein (Bailey et al. 2005; Cadoret et al. 2005; Joshua et al. 2005). However, several years later, three different research groups working with IsiA, PS II and PBS *Synechocystis* PCC 6803 (in the following referred to simply as *Synechocystis*) mutants clearly demonstrated that blue-light-induced fluorescence quenching was related to PBS fluorescence quenching and not to IsiA (Rakhimberdieva et al. 2004, 2007a; Scott et al. 2006; Wilson et al. 2006, 2007). Fluorescence quenching is correlated with a decrease of effective antenna size and of the energy arriving at the reaction centers (Wilson et al. 2006; Rakhimberdieva et al. 2010; Gorbunov et al. 2011). The latter mechanism protects cells from photoinactivation under high-light (Wilson et al. 2006) and fluctuating-light conditions (Boulay et al. 2008). Although this mechanism is widespread in PBS-containing cyanobacteria strains, around 20 % of species lack it (Kirilovsky and Kerfeld 2012). For example, *Synechoccocus elongatus* 7942 and *Thermosynechococcus elongatus* (and *T. vulcanus*), often used in studies of cyanobacterial photosynthesis, lack this photoprotective mechanism and are more sensitive to fluctuating high-light conditions (Boulay et al. 2008; Kirilovsky and Kerfeld 2012; see also below).

A The Orange Carotenoid Protein

Wilson et al. (2006) demonstrated that a soluble carotenoid protein binding a ketocarotenoid, the Orange Carotenoid Protein (OCP), is essential for triggering blue-green-light-induced fluorescence quenching. In the absence of OCP, no photoprotective thermal dissipation occurs at the level of PBS (Wilson et al. 2006). Moreover, the amplitude of fluorescence quenching (and thermal dissipation) depends on OCP concentration (Wilson et al. 2007, 2008; Gorbunov et al. 2011; Kuzminov et al. 2012). Both *Synechocystis* mutant cells overexpressing OCP and *Synechocystis* WT cells when exposed to stress conditions such as high light or iron starvation, exhibit an elevated OCP-to-PBS ratio. In these cells with a high OCP to PBS ratio, blue-green light induces 60–70 % of maximal fluorescence (Wilson et al. 2007, 2008; Boulay et al. 2008; Gorbunov et al. 2011), correlated with a decrease of the functional cross-section of PS II by 53 % (Gorbunov et al. 2011). Less pronounced fluorescence quenching (30–40 %) was observed in *Synechocystis* cells grown under low- or medium-light conditions and containing only one OCP per 2–3 PBS (Wilson et al. 2007, 2008). These results suggest that OCP-related photoprotection becomes more important in the presence of combined stress, especially under stress conditions inducing an imbalance between the number of PBS and reaction centers.

Holt and Krogmann (1981) first described the presence of a soluble OCP protein, binding a ketocarotenoid molecule, in three cyanobacterial strains, i.e., *Arthrospira maxima*, *Microcystis aeroginosa* and *Aphanizomenon flos-aqua*. Wu and Krogmann (1997) then isolated OCPs from *Arthrospira maxima* and *Synechocystis*; they also deduced that OCP is encoded by the *Synechocystis slr1963* gene. Homolog genes to *slr1963* are present in a large majority of PBS-containing cyanobacteria (Kirilovsky and Kerfeld 2012). Kerfeld's research group has structurally characterized the OCP from *Arthrospira maxima* (PDB ID: 1M98; Kerfeld et al. 2003) and from *Synechocystis* (PDB ID: 3MG1; Wilson et al. 2010) and found these two proteins to be essentially identical

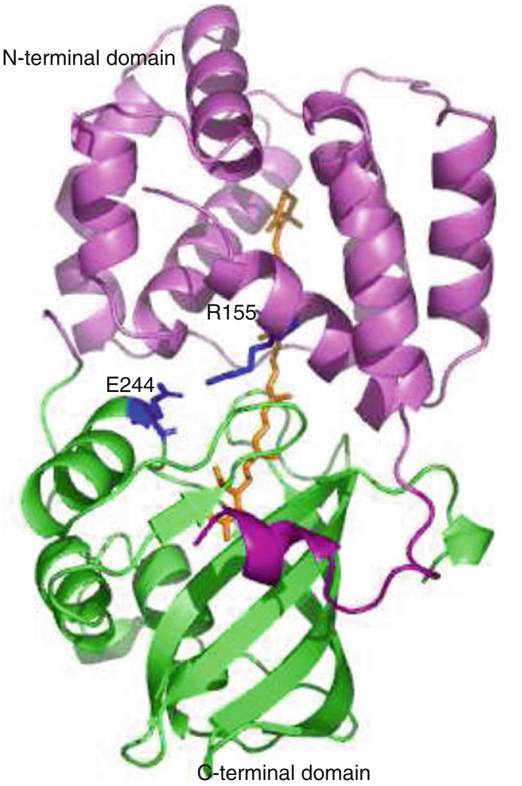


Fig. 22.4. The structure of the OCP. The N-terminal domain is in violet (*uppermost*) and the C-terminal domain is in *green*. The carotenoid is shown in *orange*. The residues Arg155 and Glu244, which form a hydrogen bond that stabilize the orange inactive form of the OCP, are shown in *blue. The figure was made with pymol.*

(Fig. 22.4). The first crystal structure of OCP was reported before OCP's function was known. Since 2008, functional in vivo and in vitro studies of OCP based on the reported structures have elucidated the role of several conserved amino acids in OCP activity (Wilson et al. 2008, 2010, 2011, 2012).

OCP is composed of two domains, i.e., an α-helical N-terminal domain (amino acid residues 15–165) unique for cyanobacteria and an α/β C-terminal domain (residues 190–317) that is a member of the nuclear transport factor 2 (NTF2) superfamily of folding proteins (Fig. 22.4). The two domains, connected by a long flexible linker (about 25 residues), interact via two regions: (1) the first 19 amino acids of the OCP extend from the N-terminal domain and form part of the C-terminal domain (Wilson et al. 2010) and (2) the second interface, across which the carotenoid spans the protein, contains a salt bridge between the Arg 155 (in the N-terminal domain) and the Glu 244 in the C-terminal domain (Fig. 22.4). This salt bridge stabilizes interaction between the N-terminal and C-terminal domains giving a "closed structure" to the protein. The carotenoid 3-hydroxy-echinenone (hECN) spans both domains (Kerfeld et al. 2003; Wilson et al. 2010) and is in all-trans configuration (Kerfeld et al. 2003; Polivka et al. 2005; Wilson et al. 2008). Its keto (carbonyl) group forms hydrogen bonds with amino acid residues Tyr 201 and Trp 288 in the C-terminal domain (Kerfeld et al. 2003; Wilson et al. 2010, 2011). The binding of the hydroxyl ring in the N-terminal domain is stabilized by the ring's interaction with aromatic amino acids (Tyr 44 and Trp 110; Kerfeld et al. 2003; Wilson et al. 2010, 2011).

B OCP Photoactivity

The OCP is a photoactive protein (Wilson et al. 2008); absorption of blue-green light by the ketocarotenoid chromophore induces conformational changes in the carotenoid and the protein (Wilson et al. 2008). In darkness, OCP is orange (OCP^o), and its absorbance spectrum has two maxima at 476 and 496 nm, with a shoulder at 440 nm. The photoactivated protein (OCP^r) is red and its absorption spectrum with a maximum at 510 nm loses the resolution of the vibrational bands. The rate of OCP^r accumulation largely depends on light intensity (Wilson et al. 2008) and, in a minor manner, on temperature (Wilson et al. 2012). In darkness in vitro, OCP^r spontaneously converts to OCP^o in a reaction largely dependent on temperature (Wilson et al. 2008). The presence of the carbonyl group in the carotenoid (Punginelli et al. 2009) and of the amino acid residues Tyr201 and Trp288 in the protein (Wilson et al. 2011) are essential for photoactivity. In addition to binding the ketocarotenoids hECN or ECN (the latter lacking the hydroxyl group), OCP is able to bind carotenoids lacking a keto group, especially zeaxanthin (Punginelli et al. 2009; Wilson et al. 2011). However, the yellow zeaxanthin-OCP is not photoactive and remains yellow even under very high intensities of blue-green light (Punginelli et al. 2009).

OCP^r is the active form of the protein that mediates fluorescence quenching and thermal dissipation at the level of PBS. The concentration of OCP^r is the only known parameter that predicts the amplitude of photoprotection (Wilson et al. 2008, 2010; Punginelli et al. 2009; Gwizdala et al. 2011;). This indicates that OCP-related photoprotection in cyanobacteria is independent of the existence of a reduced photosynthetic electron transport chain and/or a high ΔpH across the thylakoid membrane as indicators of stress conditions leading to ROS accumulation. In all other organisms performing oxygenic photosynthesis (including red algae that contain PBS), a photoprotective antenna mechanism decreasing energy arriving at PS II is triggered by acidification of the lumen; this latter mechanism is equivalent to the OCP-related mechanism. In these other organisms, the amplitude of photoprotection is directly linked to the stress situation leading to ROS accumulation. Unlike in plants or algae, low levels of CO_2 or nutrient starvation that lead to the reduction of the electron transport chain will not induce a rapid increase of photoprotection in cyanobacteria. The apparent lower efficiency of the OCP-related mechanism may be the reason for the evolutionary disappearance of OCP as the trigger of a photoprotective mechanism in red algae and other organisms. It is noteworthy that expression of the *ocp* genes in cyanobacteria is upregulated under stress conditions (Hihara et al. 2001; Fulda et al. 2006; Wilson et al. 2007; Blot et al. 2011), and that the redox state of the photosynthetic electron transport chain seems to be involved in this regulation (D Kirilovsky 2013, unpublished data).

Absorption of blue-green light induces changes in the position and conformation of the keto-carotenoid in the OCP, i.e., apparent conjugation length increases by about one conjugated bond and the carotenoid assumes a less distorted, more planar structure (Wilson et al. 2008), which leads to modification of the excited states of the carotenoid (Berera et al. 2012; Polívka et al. 2013). This reaction has a very low yield and as a consequence, OCP^r significantly accumulates only under high irradiance (Wilson et al, 2008). Hydrogen bonds between the carotenoid's carbonyl group and the protein in OCP apparently play a crucial role in modulating and stabilizing an intramolecular charge-transfer (ICT) state (Polívka et al. 2005, 2013; Chábera et al. 2010; Berera et al. 2012). Carotenoid ICT states can be involved in thermal energy dissipation (Berera et al. 2006). For the excited state of OCP^r, the contribution of the ICT state is enhanced compared with OCP^o (Berera et al. 2012), thus increasing thermal energy dissipation.

C OCP-Phycobilisome Interaction and the Quenching Site

Light-minus-dark Fourier Transform Infrared (FTIR) spectra revealed that light absorption by OCP also induces large conformational changes in the protein that are essential for OCP interaction with the PBS (Gwizdala et al. 2011; Wilson et al. 2012). OCP^r exhibits a less rigid helical structure (loosening of α-helices) and a compaction (strengthening) of the β-sheet domain with some loop changes (Wilson et al. 2008). These changes provoke the breakage of the R155-E244 salt bridge, leading to an open conformation of the protein (Wilson et al. 2012). Only the open OCP^r is able to bind to PBS (Gwizdala et al. 2011; Wilson et al. 2012). As already said, in the orange form, the presence of the R155-E244 salt bridge stabilizes a "closed" conformation precluding OCP^o binding to the PBS. The light-induced domain motion exposes the surface of the N-terminal domain containing the amino acid residue Arg155. Wilson et al. (2012) demonstrated that the positive charge of this residue is essential for OCP^r binding to PBS. The opening of the protein, and the possible interaction between Arg155 and negative charges around one of the bilin chromophores of the core of the PBS, permit a closer interaction between the carotenoid and the bilin, allowing for efficient excitation-energy transfer from the excited bilin to the carotenoid. The lifetime of the ICT and S1 states of hECN in the OCP (0.6 and 3.2 ps), which is about three orders of magnitude shorter than that of the excited bilin, makes hECN a very efficient quencher (Berera et al. 2012; Polívka et al. 2013). Thus, energy absorbed by the PBS can be harmlessly dissipated as heat (Berera et al. 2012). Alternatively, the small distance between the carotenoid and the bilin may allow charge transfer between the bilin and the carotenoid, causing the very fast (240 ± 4 femtoseconds)$^{-1}$ and efficient fluorescence quenching (80 %) observed in several studies (Tian et al. 2011, 2012). Ultrafast transient absorption measurements of OCP-phycobilisome complexes will be needed to distinguish between charge transfer or energy transfer.

The binding of only one molecule of OCP^r is able to induce quenching of almost all PBS fluorescence. The binding of OCP^r is light independent and can occur in darkness in vitro (Gwizdala et al. 2011) and in vivo (Gorbunov et al. 2011; Rakhimberdieva et al. 2011). OCP^r is able to quench the fluorescence of a PBS mutant, labeled as CK. lacking all rods (CK PBS, containing only the core) in vivo and in vitro (Wilson et al. 2006; Gwizdala et al. 2011), but is unable to quench the fluorescence of rods when the core is absent (Wilson et al. 2006; Gwizdala et al. 2011). Thus, OCP^r evidently binds to the core of PBS. However, this binding is less stable (at least in vitro) when the rods of the PBS are absent (Gwizdala et al. 2011). The presence of only one PC hexamer is sufficient to stabilize the binding (Gwizdala et al. 2011).

As described above, the core of *Synechocystis* PBS consists of three cylinders, each one formed by four APC trimers (Fig. 22.1). The trimers containing only αAPC-βAPC exhibit maximal fluorescence emission at 660 nm (APC$_{660}$). The trimers containing ApcD or ApcF and ApcE have an emission maximum at 680 nm (APC$_{680}$). Three reports from different research groups clearly demonstrated that ApcD and ApcF are not required for OCP-related fluorescence quenching in *Synechocystis* (Jallet et al. 2012; Stadnichuk et al. 2012) or *Anabaena* PCC7120 (Dong and Zhao 2008). The role of ApcE is less clear. Results from three studies strongly suggest that OCP^r interacts exclusively with one of the APC$_{660}$ trimers (Tian et al. 2011, 2012; Jallet et al. 2012). Since OCP^r is able to quench fluorescence of a phycobilisome lacking the bilin attached to ApcE, ApcE is apparently not involved in the OCP-related NPQ mechanism and OCP likely interacts with one of the αβAPC trimers emitting at 660 nm (Jallet et al. 2012). This was demonstrated using spectrally resolved picosecond fluorescence measurements with a streak-camera (Tian et al. 2011, 2012). Tian and coworkers studied *Synechocystis* cells (WT, ΔOCP and overexpressing OCP) and isolated phycobilisomes (WT, CK, only the core) and the mutant named CB (the core plus rods containing only one PC hexamer) in both the quenched and unquenched states. A compartmental model was constructed to fit the data, and the fit of the quenched state was calculated by adding an additional decay rate, kq, to various compartments and keeping all other rates as for unquenched samples. Only quenching of the APC$_{660}$ compartment led to a satisfactory fit to the data from cells and isolated PBS (Tian et al. 2011, 2012). Kuzminov et al. (2012), using non-linear laser fluorimetry to elucidate the site of quenching in the PBS, proposed that both APC$_{660}$ and APC$_{680}$ trimers can be quenched. A possible interaction between OCP^r and ApcE was also suggested by the observation of a low level of fluorescence quenching when isolated ApcE and OCP were illuminated together (Stadnichuk et al. 2012). However, since this experiment was carried out in the presence of 2 M urea and formic acid (pH 2–3) to maintain solubility of ApcE, ApcE and OCP were partially denatured under these conditions and this denaturation may have led to a spurious interaction between the OCP and the ApcE chromophore. While the possibility that OCP binds an APC_{680} trimer in some PBS, or that OCP binding allows the simultaneous quenching of 660 and 680 nm emission, has not been ruled out, this possibility seems unlikely. Gwizdala et al. (2011) obtained results suggesting that only one very specific OCP binding site exists per phycobilisome. It is improbable that the OCP carotenoid is able to interact with bilins present in different trimers.

D The Fluorescence Recovery Protein

Another protein, the Fluorescence Recovery Protein (FRP), has an essential role in OCP-mediated photoprotection. When "quenched" cyanobacteria cells are exposed to low light conditions or are incubated in darkness, recovery of PBS fluorescence is observed (Wilson et al. 2006, 2007; Rakhimberdieva et al. 2007a; Boulay et al. 2010). Fluorescence recovery is strongly temperature dependent (Rakhimberdieva et al. 2007a; Wilson et al. 2008; Gorbunov et al. 2011), and absence of the protein encoded by the *slr1964* gene in *Synechocystis* strongly inhibits fluorescence recovery (Boulay et al. 2010). Thus, FRP, a non-chromophore-binding, soluble protein with a theoretical mass of about 13 kDa, turns off OCP-mediated photoprotection (Boulay et al. 2010). FRP contains between 106 to 111 amino acids in most of the cyanobacterial strains sequenced thus far (Boulay et al. 2010). Only the FRPs of *Microcystis* NIES 843 and *Synechocystis* seem to be longer based on cyanobase.org data. These longer FRPs have an N-terminal extension of about 24 amino acids (Boulay et al. 2010). However, it has now been demonstrated that in *Synechocystis* WT cells, FRP begins at Met26 and contains only 111 amino acids; this "short" FRP has a weaker interaction with the thylakoids and a higher activity (see below) than the "long" FRP (Boulay et al. 2010; Gwizdala et al. 2013). Crystals of the FRP that diffract to 3 Å have been reported (Liu et al. 2011), and a 2.5 Å structure has recently been determined (Sutter et al. 2013). The active form of the FRP is a homodimer; FRP is all helical, consisting of an extended helical stalk (residues 1–65) and a compact C-terminal head domain (residues 66–109). All contacts for dimerization are located in the stalk (Sutter et al. 2013). A patch of highly conserved residues are involved in dimerization and activity. Trp50, Asp54, His53, and especially Arg60, are very important for FRP activity (Sutter et al. 2013).

FRP interacts only with the C-terminus domain of OCP^r (Boulay et al. 2010; Sutter et al. 2013) and greatly accelerates OCP^r to OCP^o reversion (Boulay et al. 2010), apparently by lowering the activation energy of the OCP^r to OCP^o conversion (Wilson et al. 2012). When added to OCP^r-PBS complexes, FRP induces or accelerates fluorescence recovery, indicating that FRP promotes detachment of the OCP from the phycobilisomes (Gwizdala et al. 2011). Synthesis of OCP and FRP must be strictly regulated to obtain a high OCP to FRP ratio under conditions in which photoprotection is needed. Indeed, high concentrations of FRP inhibit photoprotection by destabilization of OCP^r (Gwizdala et al. 2011, 2013). Thus, the amplitude of the OCP-related photoprotective mechanism may be regulated not only by the concentration of OCP but also by that of FRP. A high OCP-to-FRP ratio is necessary to induce photoprotection (Gwizdala et al. 2011, 2013). While Boulay et al. (2010) proposed that the *ocp* and *frp* genes may be co-translated in some specific conditions, more recent results do not support this hypothesis. A strong transcriptional terminator was detected between *ocp* and *frp* genes that renders co-transcription of these two genes improbable (Gwizdala et al. 2013).

E A Model for OCP-Related Photoprotection

Three components are essential and sufficient for blue-green light to induce photoprotection in cyanobacteria, i.e., OCP, PBS and FRP (see Fig. 22.5). Blue-green light triggers OCP activation by inducing conformational changes in the carotenoid and the

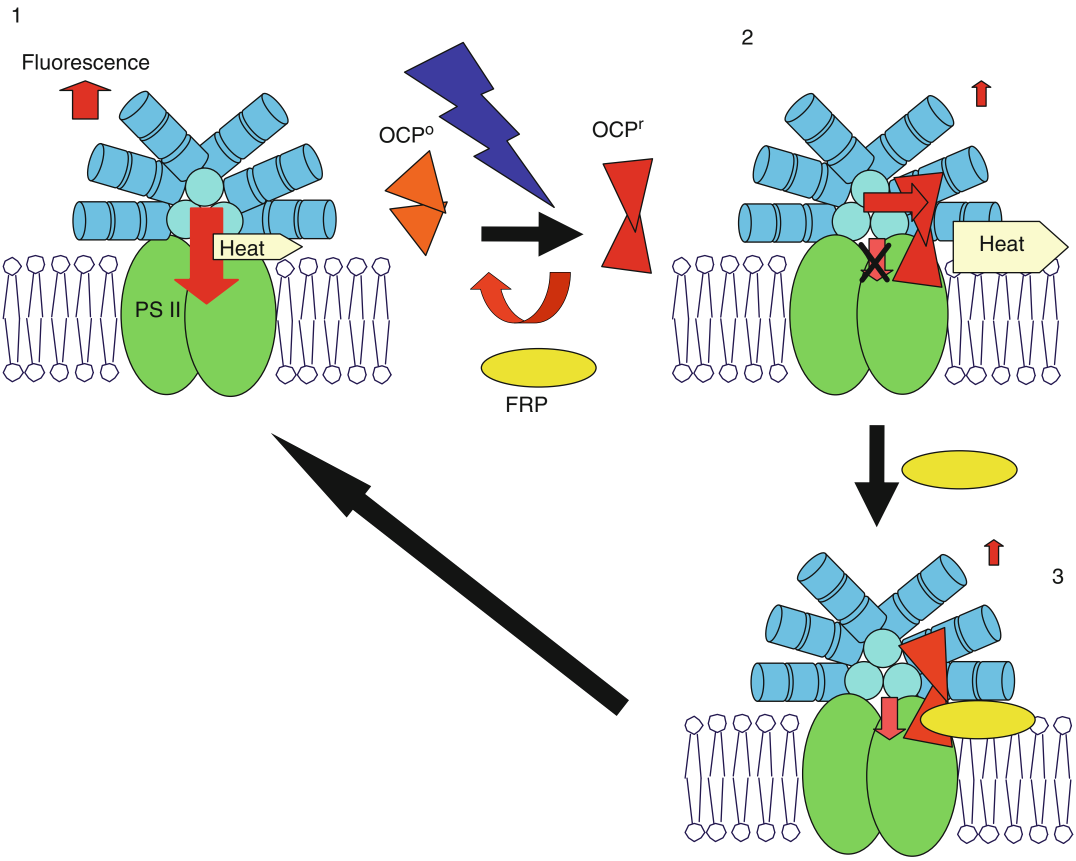


Fig. 22.5. Schematic of the OCP-related photoprotective mechanism. Under low and moderate light conditions, most of the absorbed energy by the PBS is delivered to the photosystems. High irradiance induces conformational changes in the OCP converting the orange inactive form (*OCP^o*) to the red active form (*OCP^r*). Only *OCP^r* can interact with the PBS or the FRP. These interactions are light independent. The FRP concentration is low in order to avoid a rapid reconversion of OCP^r to OCP^o. Thus, most of OCP^r interact with PBS inducing energy and fluorescence quenching by energy transfer from the bilin to the OCP or by charge transfer between both chromophores. OCP is the inducer of photoprotection and the energy quencher. FRP, under light and dark conditions, interacts with the OCP^r bound to the PBS and induces its conversion to the orange form and its detachment from the PBS. In darkness, in the absence of OCP^r, in several minutes all the PBS become free of OCP and most of energy is again transmitted to photosystems.

protein that are essential for OCP binding to the PBS and to fluorescence quenching. Only the activated red protein is able to bind to the PBS. In cells, the amplitude of fluorescence quenching depends on the concentration of OCP^r and on the affinity of OCP^r for the PBS. In turn, the concentration of OCP^r depends on OCP concentration, light intensity, temperature and FRP concentration. Indeed, OCP^r can interact with FRP and rapidly converts to OCP^o. The stability of the unbound OCP^r can also influence rate and amplitude of fluorescence quenching. Attachment of OCP^r to the PBS stabilizes the red-activated form. Attached OCP^r will quench (dissipate) excess energy and PBS fluorescence via charge transfer between a bilin of an APC$_{660}$ trimer and the carotenoid of OCP^r, or via energy transfer from excited-state bilin to the ICT and S1 states of hECN in OCP^r. FRP, which is active in darkness and in light, by interacting with attached

OCP^{r}, induces conversion to OCP^{o} and almost simultaneous detachment of the latter from the PBS. Under strong illumination, a new OCP^{r} will rapidly attach to the PBS that will "remain" quenched. In darkness or low light, OCP^{r} concentration is zero or very low, and fluorescence recovery will be observed, accompanied by recovery of the full size of the antenna.

VI State Transitions

The effective size of the PS II antenna in cyanobacteria can also be controlled by state transitions that redistribute excitation energy between the two photosystems. State transitions help to optimize the efficiency of photosynthesis under a changing light environment (Melis et al. 1989) on a time scale of seconds to diurnal cycles (Sherman et al. 1998). State transitions were first described for PBS-containing red algae (Murata 1969), green algae (Bonaventura and Myers 1969), and later for cyanobacteria and higher plants (see, reviews, Mullineaux and Emlyn-Jones 2005; Ruban and Johnson 2009; Minagawa 2011; also see Papageorgiou and Govindjee 2011). Two light-induced states are proposed: '*state 1*' – induced by light preferentially absorbed by PS I (blue or far-red light in cyanobacteria) characterized by an increase of the effective cross-section of PS II and '*state 2*' – induced by light preferentially absorbed by PS II (orange or green light in cyanobacteria) and characterized by an increase of the effective cross-section of PS I (Figs. 22.2 and 22.5). State transitions are manifested by changes in PS II and PS I Chl fluorescence, with higher PS II fluorescence in state 1 and higher PS I fluorescence in state 2, respectively. This phenomenon can be clearly observed in 77 K fluorescence spectra of cyanobacterial cells. When state transition kinetics are followed by kinetic fluorometry at room temperature, only the PS II fluorescence changes are detected (Fig. 22.2). State transitions do not affect PBS emission, which is a typical effect observed during PBS decoupling from photosystems (see Sect. VII for details) or during OCP-related photoprotection (see Sect. V).

The mechanism of state transitions in higher plants and in green algae is relatively well understood (however, see Tikkanen et al. 2012), but there is no general consensus in cyanobacteria (Bald et al. 1996; Sherman et al. 1998; Mullineaux 1999; Allen and Mullineaux 2004; Mullineaux and Emlyn-Jones 2005). Like in higher plants, state transitions in cyanobacteria are triggered by redox shifts of the PQ-pool (Mullineaux and Allen 1986, 1990) and the occupancy of the Qo site of the Cyt b_6f complex (Mao et al. 2002; Huang et al. 2003), and involve changes in the size of PS I and PS II antennae (Mullineaux et al. 1986). In contrast to plants and green algae, phosphorylation of the antenna seems not to be involved in cyanobacteria (Biggins and Bruce 1989; Mullineaux 1999). We still do not know how energy redistribution between the two photosystems proceeds. It is not clear if a long-range displacement of proteins (PBS, PS I complex) is necessary in vivo (Joshua and Mullineaux 2004) or if minor protein re-arrangement may explain the observed results (McConnell et al. 2002). Two general mechanisms have been proposed for state transitions in cyanobacteria: (1) *The mobile antennae model* in which changes occur in the physical association of PBS with PS II and PS I, requiring mobility of either PBS (Mullineaux et al. 1997; Mullineaux and Emlyn-Jones 2005; Xu et al. 2012) or PS I (Schluchter et al. 1996); (2) *The energy spill-over model* that includes redistribution of energy from PS II Chls to Chls of PS I with a minimal requirement for PBS or mobility of the photosystems (Biggins and Bruce 1989; Bruce et al. 1989; McConnell et al. 2002). Some models combine the two mechanisms – energy spillover and mobile antennae (Koblizek et al. 1998; McConnell et al. 2002; Zhang et al. 2007; see Fig. 22.6, below).

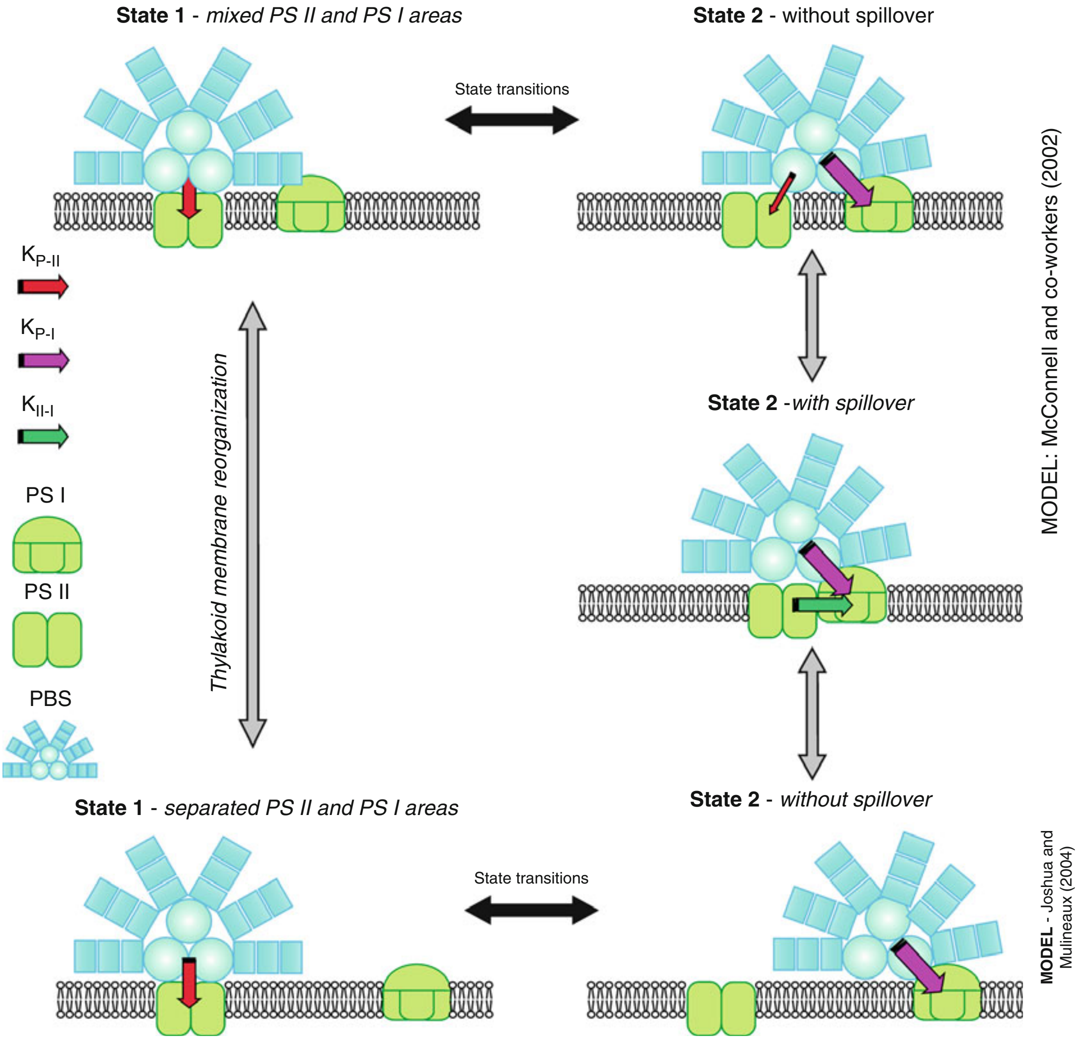


Fig. 22.6. Proposed mechanism of state transitions in cyanobacteria. The mechanism of state transition represents a process of light redistribution between PS II and PS I. It can proceed either through only a slight coupling or decoupling of proteins (Photosystem I – PS I, Photosystem II – PS II, phycobilisomes – PBS) without the necessity of their long-distance movement (McConnell et al. 2002). State transitions are then accompanied by changes in the rate of excitation flow between PS II and PS I (K_{II-I} – energy), between PBS and PS I (K_{P-I}) or PBS and PS II (K_{P-II}). The model proposed no special areas with only PS I or with only PS II (mixed PS II and PS I areas). An alternative model of state transition has been suggested (Joshua and Mullineaux 2004) based on measurements of PBS mobility by the Fluorescence Recovery After Photobleaching method (see Kaňa 2013 for description). The latter model has proposed separate PS II and PS I areas and PBS movement between them. State transitions then represent changes in the ratio of energy transfer from PBS to PS II and to PS I; it requires PBS mobility and partial PBS decoupling from photosystems as sterical hindrance of PS I subunits does not allow free PBS diffusion on the thylakoid membrane surface.

A *Trigger of State Transitions*

State transitions can rectify a temporal imbalance in PS I versus PS II excitation under changing light conditions. Imbalance in the activity of PS I and PS II is reflected in the redox state of electron carriers between the two photosystems (PQ pool, cytochrome b_6f complex and plastocyanin). The most probable signal triggering state transitions is thus the redox state of the PQ pool (Mullineaux and Allen 1990). Preferential excitation of PS II (PS I) in state 2 (state 1) induces reduction (oxidation) of the PQ pool that triggers state 2-to-state 1 (state 1-to-state 2) transition. Mao et al. (2002) and Huang et al. (2003) clearly demonstrated that the redox state of the PQ pool regulates state transitions via Cyt b_6f similar to that in plants and green algae, with binding of PQH_2 to the Qo site inducing state-2 transition. Binding of dibromothymoquinone to the Qo site also triggers state-2 transition, even in the presence of DCMU or 2,6-dimethyl-benzoquinone. Under these conditions, the PQ pool is more oxidized. In higher plants and green algae, PQ pool reduction, via the Qo site of Cyt b_6f, triggers activation of the stn7 kinase that phosphorylates the external light-harvesting antenna of PS II, leading to the latter's release and migration toward PS I (Lemeille and Rochaix 2010). While some initial reports indicated reversible phosphorylation of PBS-associated components in cyanobacteria during state transitions (Allen et al. 1985), there is, however, no conclusive evidence that protein phosphorylation is involved in the regulation of cyanobacterial light-harvesting (Biggins and Bruce 1989; Mullineaux 1999). The signal transduction pathway that links the PQ redox to state transitions in cyanobacteria thus remains unknown.

In cyanobacteria, changes in PQ pool reduction are caused not only by light-dependent activity of PS II (linear electron transport) or PS I (cyclic electron transport around PS I; Satoh and Fork 1983), but also influenced by respiratory pathways (Mullineaux and Allen 1986; Dominy and Williams 1987). Respiration and photosynthesis share the same PQ-pool and Cyt b_6f complex (Scherer 1990). The respiratory reduction of the PQ pool is the reason why cyanobacteria remain in the low-fluorescent state 2 in darkness (Mullineaux and Allen 1986; Aoki and Katoh 1982). In contrast, green algae or higher plants, due to oxidation of the PQ pool under dark conditions, are in state 1 in darkness (Bennoun 1982). Compared to respiratory pathways, PS I cyclic electron transfer in cyanobacteria appears to make a relatively small contribution to triggering state transitions to state 1 (Huang et al. 2003). In contrast, cyclic electron flow activity around PS I is increased while respiratory activity is low in state 2 (Ma et al. 2007).

B Redistribution of Light Absorbed by Phycobilisomes

PBS have been known for a long time to serve primarily as the light-harvesting antennae of PS II in cyanobacteria. However, PBS can interact and transfer absorbed energy to both photosystems (Mullineaux and Holzwarth 1991; Mullineaux 1992, 1994). As PBS are mobile along the thylakoid membrane in vivo (Mullineaux et al. 1997; Sarcina et al. 2001), their mobility between photosystems has been suggested as one possible mechanism of state transitions in cyanobacteria (Joshua and Mullineaux 2004). Joshua and Mullineaux (2004) proposed that PBS interact only transiently with reaction centers, and that state transitions represent a change in the dynamic equilibrium of PBS interaction with PS II and PS I. However, little is known about the mechanism involved in PBS disconnection from PS II (or from PS I) and its subsequent connection with the other photosystem (see Sect. VII).

Due to the structural differences between PS II and PS I, a different interaction of PBS with the photosystem's stromal surfaces must be assumed. PS II is a supercomplex with an almost flat surface with no extra-membrane proteins on its stromal side. In contrast, PS I has several subunits on its stromal side, extending by more than 3 nm into the cytoplasm, including the F subunit as the probable binding site for core-containing PBS (Fromme et al. 2001). It is unlikely that PBS binding to both PS I and PS II occurs via the same mechanism, since PBS binding to PS I requires an additional tilting of the PBS core due to the ridged surface of PS I. The latter hypothesis is supported by changes in the orientation of the PBS core relative to the membrane during transition to state 2 (Bruce and Biggins 1985) and by the flexibility of the PBS rods (Arteni et al. 2009). In contrast, no such flexibility is necessary for PBS binding to PS II that can accommodate PBS directly on its top (Mullineaux 2008b).

Shifts in energy transfer from PBS to respective photosystems during state transitions occur predominantly via the terminal emitters of the PBS core (Mullineaux 1994). PBS core is essential for state transitions. While cyanobacterial mutants containing PBS without rods can still perform state 2-to-state 1 transition with similar kinetics to that of wild-type cells (Zhao et al. 2001), cyanobacterial mutants containing only rods are incapable of state transitions. Moreover, the recently discovered type of PBS that is devoid of the central core CpcG2-PBS (Kondo et al. 2005), that preferentially transfers energy to PS I, is not involved in state transitions (Dong et al. 2009; Kondo et al. 2009). State transitions have recently been described for the evolutionarily ancient cyanobacterium *Gloeobacter violaceus* that possesses a core, but does not have a typical hemidiscoidal structure (Bernat et al. 2012). Typical features of state transitions in cyanobacteria with hemidiscoidal phycobilisomes, such as a pronounced fluorescence rise during state 2-to-state 1 transition, are missing in *Acaryochloris marina* (Kaňa et al. 2012a), a Chl d-containing cyanobacterium with rod-like phycobiliproteins without a core (Theiss et al. 2011). This finding shows that unresolved mechanisms, different from state transitions, exist in strains containing rod-PBS without a PBS core.

As already mentioned, most cyanobacteria contain a hemidiscoidal type of PBS that interacts with photosystems through the PBS core composed of trimeric $(\alpha\beta)_3$ discs of allophycocyanin (APC) stacked into cylinders (Fig. 22.1; Glazer 1989). In the core, APC680 trimers containing minor APC subunits (ApcD, ApcF and ApcE) are involved in energy transfer to photosystems. Since deletion of ApcE prevents PBS assembly, the role of ApcE in state transitions could not be studied. ApcD and ApcF subunits are essential in state transitions and energy redistribution from PBS (Mullineaux 2008b). Cyanobacteria mutants lacking ApcD and/or ApcF are unable to perform state transitions (Ashby and Mullineaux 1999; Dong et al. 2009; Dong and Zhao 2008). In *Synechococcus sp.* PCC 7002, ApcD appears to be essential for PBS-to-PS I energy transfer (McConnell et al. 2002; Dong et al. 2009) and in the absence of ApcD, cyanobacteria cells seem to be trapped in state 1 with PBS bound to PS II (Dong et al. 2009). ApcF appears to transfer energy to PS II in *Synechococcus* sp. PCC 7002 (Zhao et al. 1992; Dong et al. 2009). The role of ApcD and ApcF in *Synechocystis* cells is less clear. While lack of ApcF strongly decreases energy transfer to both photosystems, lack of ApcD only slightly affects energy transfer to both photosystems (Ashby and Mullineaux 1999).

The 9 kDa RpaC (Regulator of phycobilisome association C) protein has been recognized as an important protein controlling PBS interaction with PS II (Emlyn-Jones et al. 1999; Joshua and Mullineaux 2005). While *Synechocystis* mutants without RpaC are incapable of PBS-dependent state transitions, they still exhibit the energy-spillover mechanism of direct energy flow between photosystems (McConnell et al. 2002). The latter mechanism also still occurs in the mutant without ApcD (McConnell et al. 2002). RpaC was initially proposed as a signal transducer (Emlyn-Jones et al. 1999), but was later suggested to be the factor regulating PS II-PBS interaction (Joshua and Mullineaux 2005) – without RpaC, PBS seems to be permanently attached to PS II in state 1 (Emlyn-Jones et al. 1999). Transcription of the *rpaC* gene is downregulated under high light (Hihara et al. 2001), suggesting the importance of RpaC for cell adaptation to low light (Mullineaux and Emlyn-Jones 2005).

C Protein Mobility and Membrane Reorganization During State Transitions

Since PS II is almost immobile (Mullineaux et al. 1997; Kirchhoff et al. 2008; Kaňa et al. 2009b) and PBS is mobile, a temporal linkage between PBS and PS II has been proposed (Mullineaux et al. 1997). However, this model should be further tested as the strength of phycobilisome coupling to the photosystems is unknown (Kaňa et al. 2013). Since treatments that inhibit PBS diffusion (e.g., high phosphate concentration) also inhibit state transitions, it has been proposed that movement of PBS is essential for state transitions (Mullineaux et al. 1997; Joshua and Mullineaux 2004). Indeed, diffusion of PBS and other photosynthetic pigment-proteins has been tested on a single cell level using Fluorescence Recovery After Photobleaching (FRAP; Mullineaux and Sarcina 2002; Kaňa 2013). This experimental approach shows that PBS diffuse (Mullineaux et al. 1997) with a diffusion coefficient of about 0.03 μm^2 s^{-1} for *Synechococcus sp.* PCC 7942 (Sarcina et al. 2001). The ability of PBS to move along the thylakoid membrane surface has been unequivocally demonstrated in cyanobacterial cells by synchronous monitoring of FRAP and Fluorescence Loss In Photobleaching, proving that PBS mobility is not an artifact of high intensity laser power at the site of bleaching (see data in Yang et al. 2007 and the recent review by Kaňa 2013). Even though PBS mobility alone cannot explain the state transition mechanism completely (it does not account for energy spill-over between photosystems; McConnell et al. 2002), it represents a plausible explanation for the redistribution of light-energy absorbed by PBS between the two photosystems (see Mullineaux and Emlyn-Jones 2005 for a review). A similar connection between state transitions and mobility of photosynthetic light-harvesting antennae has been revealed in higher plants, where macromolecular crowding of proteins causes PS II immobility, but a small fraction of LHCII antennae can be exchanged relatively fast (within a few seconds) between the grana (with abundant PS II) and stroma lamellae (Kirchhoff et al. 2008).

In cyanobacteria, long-term redistribution of PBS during state transition between separate PS II and PS I domains seems to be questionable. In cyanobacteria, that do not form large heterogeneities like grana/stroma of diameters of hundreds nanometers, there is no large-scale physical or functional separation of the photosystems. The model of cyanobacterial thylakoid-membrane organization (Folea et al. 2008) has revealed that dimeric PS II particles are organized into multiple rows surrounded by PS I trimers or monomers. Therefore, even minor PBS movement between these domains is able to explain the redistribution of excitation energy from PBS either to PS II or to PS I.

Even though the role of PBS mobility during state transitions seems to be confirmed (Mullineaux et al. 1997; Joshua and Mullineaux 2004; Ma et al. 2007; Yang et al. 2007), we do not know how this process is triggered or controlled (for recent reviews, see Mullineaux 2008a and Kaňa 2013). Due to steric hindrance of PS I-extrinsic subunits, a long-distance and unlimited diffusion would require PBS decoupling from the membrane surface. The mechanism and role of this process remains unexplored (see Sect. VII).

We cannot exclude the possibility that PS I mobility may also play an important role in state transitions (Schluchter et al. 1996). State transitions are inhibited at relative high temperatures (18 °C) in a lipid mutant in which thylakoids are in a solid crystalline state at 25 °C (El Bissati et al. 2000). Re-arrangement of the PS II and PS I domains (as described in Folea et al. 2008) has been suggested as a plausible process that may accompany changes in excitation energy flow observed during state transitions (see, e.g., Bald et al. 1996 for a review). Although the PS II supercomplex has been found to be immobile in all studied organisms (Kirchhoff et al. 2008; Kaňa et al. 2009b), including cyanobacteria (Mullineaux et al. 1997; Joshua and Mullineaux 2004), no data are available regarding the mobility of PS I (Mullineaux 2008a; Kaňa 2013). This is complicated by the fact that PS I in cyanobacteria in vivo can be in two forms, PS I trimer and PS I monomer (Kruip et al. 1994, 1999; El-Mohsnawy et al. 2010). PS I monomerization/trimerization is known to affect PBS diffusion; PBS diffuse several times faster between PS I monomers than in the mix of monomers and trimers (Aspinwall et al. 2004). Therefore, the earlier models of state transitions had also proposed changes in the oligomerization state of both PS I and PS II (Bald et al. 1996), in line with EM pictures of cyanobacterial thylakoid membranes in different states – in state 1 PS II, particles are aligned in rows compared to state 2 with more randomly distributed PS II particles (Olive et al. 1986, 1997). In the model of Bald et al. (1996), state 1 has a dimeric PS II organized in rows with monomeric PS I, where PBS is primarily attached to PS II. In state 2, PS I is a trimer with attached PBS and with PS II disorganized into monomers. However, the physiological importance of such a reversible and fast PS I monomerization/trimerization (e.g., due to electrostatic interactions; El-Mohsnawy et al. 2010) in state transitions remains to be confirmed. In fact, the presence of PS I trimers usually results in a higher photostability due to the higher content of red-shifted Chls that assists in the dissipation of excess absorbed energy (Karapetyan et al. 1999). On the other hand, iron deficiency in cyanobacteria causes monomerization of PS I and reduces the capacity for state transitions in vivo (Ivanov et al. 2006).

D Excitation Spillover Between Photosystems

Excitation spillover from Chls of PS II to PS I has been proposed as another mechanism for state transitions (Biggins and Bruce 1989; Bruce et al. 1989). A pure spillover model of state transitions can explain changes in the distribution of both PBS and Chl a-absorbed energy during state transitions solely by variation of the rate constant of excitation energy transfer between Chls in PS II and PS I. Such a model therefore requires strong coupling between PBS and PS II and variable coupling between Chls in PS II and PS I during state transitions, with lower PS II-PS I coupling in state 1, and higher coupling in state 2. The spillover mechanism of state transition therefore primarily regulates excitation energy flow among Chls and, in fact, it does not require PBS (Bruce et al. 1989).

McConnell et al. (2002) proposed a combined mechanism (combining spillover and state transitions) involving energy transfer from PBS to both photosystems with excitation energy spillover between PS II Chl *a* and PS I Chl *a* (Fig. 22.5). State transitions would be controlled by variation in excitation-energy transfer rates to PS I from PBS and rate constants of energy spillover from PS II to PS I. Rate constants would increase in state 2 and decrease in state 1. State 2 would be accompanied by excitation-energy spillover from PS II to PS I by stimulation of physical coupling of CP47 of PS II with Chls of PS I and by increase in energy transfer from PBS to PS I Chls (McConnell et al. 2002). The energy lost in spillover from PS II to PS I would be limited during organization of PS II into rows (Folea et al. 2008). This mechanism does not require long-scale mobility of PBS; only a slight shift (protein coupling) is expected. It has also been suggested that, to initialize movement of PBS, some PBS uncoupling from the photosystems also occurs (Kaňa et al. 2012a). This would affect the rate of energy transfer from PBS to PS II and allow long-distance movement of PBS that would otherwise be restricted by steric hindrance of PS I subunits.

E Model and Physiological Role of State Transitions

A complete model of state transitions in cyanobacteria should take into account the following processes (see Fig. 22.6): (1) Extent and efficiency of energy spillover from PS II to PS I (characterized by its high rate constant in state 2 and a low rate constant in state 1 in which spillover is restricted by organization of PS II into rows; see model in McConnell et al. 2002); (2) Extent and efficiency of energy transfer from PBS to PS II (characterized by its high rate constant in state 1 and low rate constant in state 2), which is affected by PBS decoupling from PS II, is controlled by the RpaC protein and by PBS mobility; (3) Extent and efficiency of energy transfer from PBS to PS I is affected by PS I trimerization and characterized by high rate constant in state 2 and low rate constant in state 1.

State transitions in cyanobacteria have two main physiological roles: (1) Regulation of light-energy distribution between photosystems at non-saturating irradiances (Mullineaux and Emlyn-Jones 2005); and (2) Protection from the effects of excess, saturating light energy (Dong et al. 2009; Kaňa et al. 2012a).

VII Phycobilisome Decoupling from Photosystems

In cyanobacteria, dynamic and reversible decoupling of whole phycobilisomes from PS II and/or PS I, or detachment of individual phycobiliproteins from PBS, is another mechanism that can modulate the energy arriving at the reaction centers under stress conditions. Physical modifications in the structure or attachment of PBS induce excitonic decoupling that contributes to excess energy dissipation within PBS. Experimentally, the latter phenomenon can be observed as a relative increase in phycobilin fluorescence compared to Chl fluorescence. Under the general term of decoupling, we here consider both detachment of whole PBS and partial disassembly of PBS into individual phycobiliproteins (Fig. 22.7). Since such a mechanism results in a decrease of the effective cross-section of PS II (and/or PS I), it can be considered to be photoprotective against photo-oxidative damage.

While the literature on OCP quenching and state transitions is rather extensive, only a few studies have dealt with the stress-induced dynamics of PBS in sufficient detail. Initial observations, based on 77 K emission spectroscopy, indicated that dynamic, light-dependent alteration of associations between PBS and photosystems occurs in several cyanobacterial strains (Harnischfeger and Codd 1977). Wyman et al. (1985) observed that a substantial fraction of phycoerythrin in nitrogen-replete cultures of marine *Synechoccocus* is not coupled to reaction centers. Although this phenomenon was not dynamic in the sense that it could respond on a time scale of minutes, such permanently disconnected antennae were suggested to serve as a selective light shield that restricts the absorption of green light. Dissociation of whole PBS and degradation of their components has been reported in response to UV-B irradiance in filamentous freshwater cyanobacteria *Anabaena* or *Nostoc* (Sinha et al. 1995; Lao and Glazer 1996) and single-cell *Synechococcus sp.* PCC 7942 (Sah et al. 1998) or *Synechocystis sp.* PCC 6803 (Rinalducci et al. 2006). The reported effects of UV irradiance were often lethal and it is not clear whether the observed dissociation of PBS components has any photoprotective role. Exposure of the marine cyanobacterium *Synechococcus sp.* WH 8102 to non-lethal UV irradiance resulted in disconnection

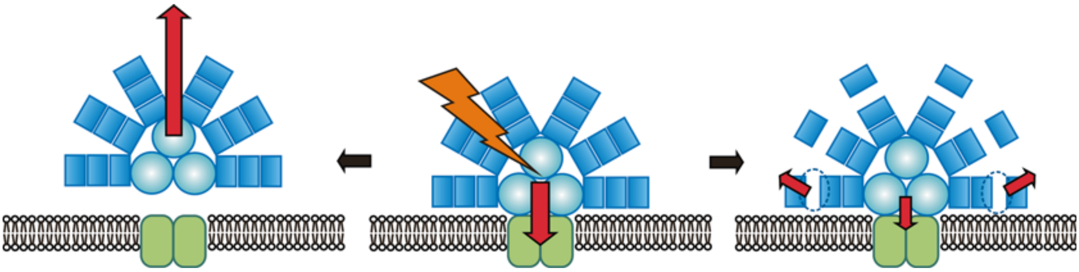


Fig. 22.7. Possible mechanisms of phycobilisome decoupling. Under normal conditions (*center*), PBS (marked in *blue*) is optimally attached to photosystems (marked in *green*) and transfers most of the absorbed light energy to reaction centers (*red arrow*). Under stress conditions, PBS can be either detached from reaction centers (*left*) or can remain attached, but partially disassemble the rods (*right*). In both cases, an increased fraction of absorbed energy is released as fluorescence and dissipated as heat within the PBS and less energy is delivered to reaction centers.

of PBS from the thylakoid membrane and partial dismantling of PBS rods (Six et al. 2007). In this case, UV irradiance disrupts the N-terminal domain of the anchor linker polypeptide ApcE (formerly L_{CM}), which leads to disconnection of the PBS complex from the thylakoid membrane. Dismantling occurs near the phycoerythrin I- phycoerythrin II junction.

The mechanism of light-induced PBS decoupling and its physiological role is now being studied. When cells of *Synechocystis sp.* PCC 6803 are exposed to stress conditions of excess irradiance or high temperature, PBS are partly excitonically decoupled (Stoitchkova et al. 2007; Tamary et al. 2012). Kaňa et al. (2012a) proposed that some mechanisms of state transitions (associated with long-distance PBS mobility) require PBS uncoupling from the photosystems (for a recent review, see Kaňa 2013). The uncoupling is followed either by PBS redistribution between photosystems, or can also be followed by disassembly of longer-term detached PBS. Because the uncoupling can be induced either by excessive irradiance or by short heat stress, it has been suggested that it is based on what is termed the thermo-optic effect (Stoitchkova et al. 2007). According to this theory (Cseh et al. 2000), non-radiative (thermal) dissipation of excess excitation energy induces fast local thermal fluctuations and instabilities that result in reorganization and structural changes in specific thermo-labile elements, such as certain rod or core linker polypeptides (Stoitchkova et al. 2007; Tamary et al. 2012). Such heat-induced alterations are assumed to modify and destabilize the molecular architecture of PBS and disrupt the transfer of excitation energy within and from PBS to reaction centers. However, PBS decoupling is also stimulated at low temperature (Manodori and Melis 1985); therefore, understanding of the role of temperature in the mechanism of PBS decoupling requires additional research. Since this effect can be induced by strong light of wavelengths absorbed not only by phycobilins, but also by Chls, it is possible that, in the latter case, PS II Chl excitation energy equilibrates with that of APC, resulting in backward energy transfer from reaction centers to the PBS core (Rakhimberdieva et al. 2007b; Tamary et al. 2012). Significant photo-induced increase of reversibly variable C-phycocyanin emission at 650 nm was observed in *Synechococcus sp.* PCC 7942 cells excited with blue light absorbed by Chls (Kaňa et al. 2009a). The 650 nm C-phycocyanin emission is variable (changes with time of irradiance) and reversible when actinic light is switched off. It has been suggested that the PsbU subunit of PS II might be the subunit controling PBS-PS II interaction in response to the state of the PS II donor side (Veerman et al. 2005). The energetic decoupling of PBS is photon dose-dependent and should also occur under lower light intensities (above some critical threshold), provided sufficient exposure time (Tamary et al. 2012). Some PBS decoupling from photosystems seems to be present at all actinic light intensities, even after short irradiation of only a few seconds (Kaňa et al. 2009a, 2012a, b). Light-dependent effects can also be induced or accentuated by other environmental stresses, such as low or high temperatures. Uncoupling is not restricted to cyanobacteria, since, similar uncoupling of B-phycoerythrin subunits within PBS rods has been observed in isolated PBS of the red alga *Porphyridium cruentum* (Liu et al. 2008). The decoupled rods dissipate a significant fraction of energy in the form of PE fluorescence.

Energetic coupling of PBS to PS II depends on formation of an active donor side of PS II (Hwang et al. 2008), suggesting that structural alterations on the lumenal side of the membrane can be allosterically transmitted to the other side of the membrane and affect the light-harvesting function of PBS. Such an effect diminishes the likelihood of photodamage to reaction centers that have either lost an intact Mn cluster or are in the process of assembling an active donor side.

Reversible and variable uncoupling of PBS plays a key role in the regulation of photosynthesis during periods of intensive nitrogen fixation in the ecologically important marine diazotrophic cyanobacterium *Trichodesmium* (Berman-Frank et al. 2001; Kupper et al. 2004, 2009). *Trichodesmium* fixes nitrogen in vegetative cells called diazocytes exclusively in the light period during the day, when overall photosynthesis is fully active. Since diazocytes possess, in addition to the strictly anaerobic enzyme nitrogenase, a fully developed photosynthetic apparatus, oxygenic photosynthesis in these cells must be temporally down-regulated during the midday peak in nitrogenase activity. The key mechanism contributing to down-regulation of PS II appear to be state transitions inducing a Mehler-reaction-dependent consumption of oxygen required for nitrogenase protection. These diazocytes are called "bright I" cells. They show almost doubled intrinsic fluorescence yield in the dark (F_o), while variable fluorescence (F_m–F_o = F_v) remains similar to that in the neighboring vegetative cells not engaged in nitrogen fixation. Under prolonged stress, many cells show quick uncoupling and rearrangement of the PBS associated with PS II. These are "bright II" cells. The enhanced quantum yield of fluorescence of phycoerythrins indicates less efficient transfer of excitation energy to the inner part of PBS. Uncoupling and reassembly of functional PBS in *Trichodesmium* is reversible and can occur within a few minutes. The fraction of bright cells and the contribution of different parts of the PBS antenna to energy dissipation changes over the course of the day. Decoupling is increased under high oxygen levels (Kupper et al. 2004) when both oxidative stress and the need for down-regulation of PS II activity during the period of nitrogen fixation are increased. In addition to exhibiting states with increased fluorescence yield, some *Trichodesmium* cells were observed in a quenched, low fluorescence state interpreted to involve aggregates of uncoupled phycobiliproteins (Kupper et al. 2004, 2009). The molecular mechanism and signaling pathway behind the dynamic changes in PBS architecture in *Trichodesmium* are currently unknown. It remains to be shown whether the phenomena observed in *Trichodesmium* "bright II" cells can be explained by the "thermo-optic" mechanism proposed for *Synechocystis*.

VIII Interaction Among Photoprotective Mechanisms in Cyanobacteria

Interaction among NPQ mechanisms in cyanobacteria can be discerned using the spectrally resolved fluorescence induction method (Kaňa et al. 2009a, 2012a) that allows for simultaneous monitoring of changes in fluorescence yield of different pigment systems. In *Synechococcus sp.* PCC 7942 lacking OCP-dependent NPQ (Boulay et al. 2008), overexcitation of PBS (by orange light) results in increased Chl *a* emission (at 685 nm) and a faster and dominant fluorescence rise of PBS fluorescence (at 650 nm; Kaňa et al. 2009a). This phenomenon has been interpreted as sign of fast PBS decoupling. An alternative explanation would be reverse energy flow from Chls of closed PS II centers to PBS. Increases in Chl *a* and PBS fluorescence can also be induced by excitation of Chls with blue light (Kaňa et al. 2009a), as is the case in many *Synechococcus* strains (Kaňa et al. 2012a) that lack an OCP analogue (fresh water *Synechococcus sp. PCC7942*) or have an OCP showing a lower similarity to the *Synechocystis* OCP (e.g., marine *Synechococcus sp.* WH 5701; Boulay et al. 2008; Kaňa et al. 2012a). In contrast, only a minor increase of Chl *a* and PBS fluorescence emission at 660 nm is induced by strong orange light in the presence of the electron transport inhibitor DCMU in *Synechocystis sp.* PCC 6803 (Kaňa et al. 2012a). The increase in fluorescence induced by strong blue-light in *Synechocystis sp.* PCC 6803 is masked by the blue-light induced OCP-dependent fluorescence quenching (Kaňa et al. 2012a).

The pronounced fluorescence increase at 682 nm (termed the SM rise; see, e.g., Papageorgiou and Govindjee 2011) and reflecting predominantly Chl *a* emission, is absent in mutants lacking state transitions (Kaňa et al. 2012a). It has therefore been suggested that the pronounced increase of fluorescence is related to state 2 – state 1 transition (Kaňa et al. 2012a). In state 1 (induced by PS I specific blue light), the increase of fluorescence is related to an increase of the effective size of the PS II antenna (more PBS connected to PS II and/or less spillover to PS I). The question is why strong orange light that already overexcites PS II induces further increase in PS II antenna cross-section that might lead to overexcitation and photoinhibition of PS II. The answer may be that PS I photoinhibition is more dangerous to the cell than PS II photoinhibition (Sonoike 2011). Since PBS also transfers energy to PS I, strong orange light (like strong blue light) also overexcites PS I, and a mechanism that decreases PS I antenna protects PS I from photodamage. Tikkanen et al. (2012) have demonstrated that, under fluctuating light, it is more important to protect PS I than PS II. We can also hypothesize that the Chl *a* fluorescence increase is related to other photoprotective mechanisms increasing radiative energy dissipation (as fluorescence) at the level of the Chl antenna (CP47, CP43) and thereby decreasing the energy arriving at the reaction centers. The magnitude of energy dissipation by this fluorescence rise is comparable to fluorescence quenching attained by OCP mechanism (Kaňa et al. 2012a).

IX Conclusions

The literature on photoprotection in cyanobacteria indicates that OCP-dependent thermal energy dissipation (leading to NPQ), state transitions, and PBS-decoupling represent three complementary photoprotective mechanisms that divert excess absorbed excitation energy from the photosystems; their individual importance differs among species and conditions. In some strains, like *Synechocystis*, the OCP-related NPQ mechanism appears to be the predominant one; in other strains of cyanobacteria, either lacking OCP (such as *Synechococcus elongatus*) or exhibiting low OCP concentrations, state transitions and/or a proposed photoprotective mechanism increasing radiative energy dissipation apparently become principal photoprotective mechanism. PBS decoupling appears to occur in all cyanobacteria. In *Synechocystis,* energy is dissipated from disconnected PBS by OCP; in OCP-lacking strains, synthesis of phycobiliproteins is rapidly decreased to lower the concentration of disconnected PBS (Boulay et al 2008).

Acknowledgments

OP and RK have been supported by projects Algatech administered by the Ministry of Education of the Czech republic, and GACR P501/12/G055 and P501/12/0304 financed by the Czech Science Foundation. DK has been supported by grants from Agence Nationale de la Recherche (ANR, Cyanoprotect Project), Centre Nationale de Recherche Scientifique (CNRS), Commisariat à l'énergie atomique et aux energies alternatives (CEA) and HARVEST EU FP7 Marie Curie Research Training Network. The authors thank Conrad W. Mullineaux for his valuable comments during revision of this chapter. We also acknowledge the comments of the editors (Govindjee, William Adams and Barbara Demmig-Adams) during the preparation of the final version.

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Chapter 23

Photosystem II Efficiency and Non-Photochemical Fluorescence Quenching in the Context of Source-Sink Balance

William W. Adams III*
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

Onno Muller
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

Institute of Bio- and Geosciences, IBG-2: Plant Sciences,
Forschungszentrum Jülich GmbH, Jülich 52425, Germany

Christopher M. Cohu
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

Dow AgroSciences, Portland, OR, USA

and

Barbara Demmig-Adams
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

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*Author for Correspondence, e-mail: william.adams@colorado.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_23, © Springer Science+Business Media Dordrecht 2014

Summary

Products of photosynthesis (reduced carbon, typically in the form of sugars), synthesized by a plant's mature source leaves, are exported to the plant's sinks for growth, storage, and/or maintenance respiration. When the rate of sugar production in source leaves exceeds the rate of export (often as a result of insufficient sink activity), sugars and starch accumulate in source leaves. In a feedback response, levels of messenger RNAs, coding for various photosynthetic proteins, of source leaves are repressed along with the levels of these proteins. Furthermore, the overall capacity for photosynthetic CO_2 fixation is also decreased. Consequently, these source leaves with decreased rates of photosynthetic electron transport utilize a lessened fraction of the light they absorb for photochemistry, and should thus increase the level of thermal dissipation of (excess) absorbed light. Thermal dissipation can be assessed as increased non-photochemical quenching (NPQ) of chlorophyll fluorescence, as well as decreased intrinsic photosystem II (PS II) efficiency, the ratio of variable to maximal chlorophyll fluorescence F_v/F_m (in darkness) or F_v'/F_m' (in the light) (F_v being $F_m - F_o$; where v stands for variable, m for maximum, and o for minimum). Greater source activity relative to sink activity within the whole plant, resulting in starch and/or sugar accumulation, indeed leads to increases in thermal dissipation and in the concentration of zeaxanthin, a xanthophyll involved in thermal dissipation. Under severe conditions, high zeaxanthin levels, high thermal energy dissipation, and low PS II efficiency in source leaves become locked-in as part of a state of chronic photoinhibition associated with high foliar levels of sugar and starch. A correlation between photoinhibition and foliar non-structural carbohydrate accumulation is a common occurrence. In this chapter, we discuss various potential underlying causes for this correlation, including the possibility that reduced growth of plants under stress conditions leads to sink limitation, foliar starch and sugar accumulation, and photoinhibition as a manifestation of photosynthetic repression under excess light.

I Introduction

Leaf photosynthesis is regulated in response to (i) the availability of light as the plant's energy source and (ii) the demand for photosynthate from the rest of the plant (sink activity). These adjustments occur over minutes, via modulation of photosynthetic proteins' enzymatic activity and actual photosynthesis rate, and over days, via regulation of photosynthetic genes resulting in profound adjustments in protein levels and in intrinsic photosynthetic capacity (Paul and Foyer 2001). While increasing growth light intensity typically leads to increased photosynthetic capacity (Anderson and Thomson 1989), extended exposure to high light intensities can result in a depression in photosynthetic capacity referred to as photoinhibition (Baker and Bowyer 1994; Kyle et al. 1987). Likewise, removal of a plant's sinks or reduction in the activity of those sinks under high growth light intensity can lead to a depression in photosynthetic capacity. In this chapter, we review the phenomenon of photoinhibition in a whole-plant context with a focus on the involvement of (i) thermal dissipation and (ii) foliar nonstructural carbohydrate levels, carbon export, and whole plant-source-sink balance. We conducted extensive searches of

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Abbreviations: A – The carotenoid antheraxanthin; CBc – Calvin-Benson cycle; F – Fluorescence; F_m, F_m' – Maximal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_o – Minimal chlorophyll fluorescence in the dark-adapted state; F_v, F_v' – Variable chlorophyll fluorescence in the dark-adapted (F_m–F_o) and light-adapted (F_m–F_o') state, respectively; F_v/F_m, F_v'/F_m' – Intrinsic efficiency (or quantum yield) of photosystem II in the dark- and light-adapted state, respectively; NPQ – Non-photochemical quenching of chlorophyll fluorescence; PFD – Photon flux density; PS II – Photosystem II; ROS – Reactive oxygen species; V – The carotenoid violaxanthin; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin, and zeaxanthin; Z – The carotenoid zeaxanthin the literature to identify any and all studies where both photoinhibition and foliar carbohydrates were characterized. We did not identify a single case in which photoinhibition was not accompanied by foliar carbohydrate accumulation. We conclude that a remarkable universal correlation exists between photoinhibition of photosynthesis and foliar starch and/or sugar accumulation under a range of different conditions and manipulations at the whole plant level for every case where both photoinhibition and foliar carbohydrates have been characterized.

II Non-Photochemical Quenching in Leaves Over Different Time Scales

Exposure of leaves to more light than can be utilized in photosynthesis typically leads to a compensatory increase in photoprotective thermal energy dissipation (for additional features involved in the acclimation to high light, see Logan et al., Chap. 7), accompanied by increases in the levels of xanthophylls associated with thermal dissipation, i.e., zeaxanthin (Z) and antheraxanthin (A) (Demmig-Adams and Adams 1996a, 2006; see also Adams and Demmig-Adams, Chap. 2 and Demmig-Adams et al., Chap. 24) and, in some species, lutein (Esteban et al. 2008, 2010; Matsubara et al. 2008; Förster et al. 2011; see also Esteban and García-Plazaola, Chap. 12). Increases in foliar zeaxanthin and antheraxanthin level occur through de-epoxidation of violaxanthin (V; Yamamoto 1979, 2006; Hager 1980; Yamamoto et al. 1999) as well as through de novo synthesis from β-carotene (Adams et al. 1999; Adams and Demmig-Adams 1992; Demmig-Adams et al. 1998). Non-photochemical quenching (NPQ) of chlorophyll fluorescence in leaves can be used to assess zeaxanthin (and antheraxanthin) facilitated thermal dissipation of excitation energy absorbed by chlorophyll molecules and not utilized photochemically in photosynthesis (Adams and Demmig-Adams 2004, 1996b; Demmig-Adams et al. 1996; Müller et al. 2001).

Under conditions of prolonged exposure to very high light levels, additional abiotic stress, and/or lasting sink limitation, assessment of thermal dissipation from non-photochemical quenching of chlorophyll fluorescence becomes problematic. Non-photochemical quenching of maximal fluorescence (see Logan et al., Chap. 7) in the light (F_m') relative to a control level of F_m in darkness is straightforward when dark-levels of F_m are high and no dark-sustained decreases in F_m are present, i.e., when the quenching of the F_m level of chlorophyll fluorescence relaxes (increases) fully (thermal energy dissipation becomes completely disengaged) during the dark period (or night) such that the true maximal level of F_m can be ascertained. Leaves of many non-herbaceous plants subjected to stress conditions engage in photoprotective thermal energy dissipation that becomes locked in a sustained form that persists even after an entire night of darkness (Adams et al. 1995b, 2002, 2004, 2006; Adams and Barker 1998; Adams and Demmig-Adams 1994, 1995, 2004; Demmig et al. 1988; Demmig-Adams and Adams 1996a, 2006; Demmig-Adams et al. 1998, 1999, 2006a, b, 2012; Ebbert et al. 2005; Verhoeven et al. 1996, 1998, 1999; Zarter et al. 2006a, b, c), thus making a straightforward calculation of NPQ (in this chapter as $F_m/F_m'-1$) impossible (Adams et al. 1995a, b, 2006; Adams and Demmig-Adams 1995, 2004; Logan et al. 2007). Under such conditions, calculations of non-photochemical quenching based on a locked-in, low value of dark F_m underestimate the level of thermal dissipation in leaves. This is a feature that can become especially prominent in slowly growing evergreen species, although it can also occur in herbaceous species (Adams et al. 1995a; Verhoeven et al. 1999). Herbaceous species, with higher rates of photosynthesis (utilization of the absorbed light) and growth, tend to (1) reduce the absorption of excess light under conditions of stress through decreases in chlorophyll content (e.g., Verhoeven et al. 1997; Logan et al. 1999; see Morales et al., Chap. 27), or (2) when conditions permit, disassemble the components of older leaves and mobilize the essential elements for the construction of new leaves that are better acclimated to the prevailing environmental conditions, and/or (3) in response to low temperature, upregulate photosynthesis (see below).

Under conditions when NPQ may be locked-in, photosystem II (PS II) efficiency estimated from the ratio of variable to maximal fluorescence (F_v/F_m in darkness or F_v'/F_m' in the light) can assist in assessing the state of PS II (Adams et al. 1995b, 2006; Adams and Demmig-Adams 2004). Intrinsic PS II efficiency declines as a consequence of either (i) increased thermal dissipation of excitation energy (even in its locked-in form) and/or (ii) inactivation of photochemistry (Kitajima and Butler 1975). For further information on the basic relationship between PS II and chlorophyll fluorescence, see Govindjee (1995, 2004). There is a strong linear relationship between the conversion state of the VAZ cycle [(Z + A)/(V + A + Z)] and the level of NPQ and between the level of Z + A per chlorophyll a and PS II efficiency F_v'/F_m' (Demmig-Adams and Adams 1996b). On the other hand, the relationship between the VAZ cycle conversion state (Z + A)/(V + A + Z) and PS II efficiency is curvilinear, and is identical whether assessed as F_v'/F_m' at midday from leaves of plants that are not experiencing additional stress or determined as F_v/F_m prior to sunrise from plants experiencing different degrees of stress (with nocturnal retention of Z + A and sustained depressions in F_v/F_m throughout an entire night of darkness) (Adams et al. 1995b, 2006). Such correlative evidence is consistent with zeaxanthin (and antheraxanthin) playing a role in the facilitation of photoprotective energy dissipation from leaves of plants on a regular, daily basis as well as on a seasonal basis under prolonged stress. Sustained depression in PS II efficiency is one of several responses of plants to excess light that is also commonly referred to as photoinhibition.

Responses of leaves to excess light that include decreased intrinsic photosynthetic capacity, decreased photon efficiency of photosynthesis (CO_2 uptake or O_2 evolution per absorbed photon in the light-limited region), and decreased intrinsic PS II efficiency (as estimated from F_v/F_m or F_v'/F_m') are a suite of phenomena collectively recognized as photoinhibition of photosynthesis (Adir et al. 2003; Baker and Bowyer 1994; Kyle et al. 1987). Discussions of photoinhibition are typically focused on long-term phenomena. However, as characterized by Kok (1956) in algae, and defined more broadly by Osmond (1994) and Osmond and Grace (1995), the above manifestations of photoinhibition can occur as features that are either rapidly reversible (dynamic photoinhibition, e.g., over the midday hours of a sunny day) or only slowly reversible upon removal of the excess light conditions (chronic photoinhibition). The involvement of zeaxanthin and thermal energy dissipation (assessed as NPQ) in dynamic photoinhibition, first documented in 1989 (Demmig-Adams et al. 1989a, b, c, 1990; see also Demmig-Adams and Adams 1990), is widely recognized (Müller et al. 2001; Adams et al. 2006; Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006a, 2012). Association of zeaxanthin with sustained, locked-in NPQ in chronic photoinhibition was demonstrated in shade plants that were transferred to high light (Demmig et al. 1987; see also Demmig-Adams et al. 1998, 2006b), and in water-stressed sun-exposed plants (Demmig et al. 1988) even prior to its suggested involvement in transient, rapidly reversible modulation of PS II efficiency. Underlying possible mechanisms responsible for photoinhibition can thus include any combination of locked-in, sustained high thermal energy dissipation and inactivation of PS II photochemistry (Adams et al. 2004, 2006). We identified retention of high levels of zeaxanthin and antheraxanthin and apparent high levels of locked-in energy dissipation together with degradation of PS II core proteins and of the oxygen-evolving complex not only in shade-grown evergreens photoinhibited by sudden prolonged high-light exposure, but also in photoinhibited overwintering evergreens growing under natural conditions in the field (Zarter et al. 2006a, c; Adams et al. 2013b; see Demmig-Adams et al., Chap. 24). The associated F_v/F_m values (as well as the associated light- and CO_2-saturated rates of photosynthetic oxygen evolution) were negligible, and while the net levels of F_o were lower in photoinhibited versus non-photoinhibited leaves/needles (Adams et al. 1995a, b; Adams and Demmig-Adams 1994; Demmig-Adams et al. 1998; Oh et al. 2013; Verhoeven et al. 1996), it is likely that this represented a mix of sustained energy dissipation-associated F_o decreases and F_o increases associated with inactivation of PS II photochemistry (Kitajima and Butler 1975).

III Changes in Source-Sink Balance

Removal of sinks (e.g., young, developing leaves and/or fruits) as consumers of photosynthate often results in decreased rates of photosynthesis in a plant's source leaves (Cheng et al. 2009; Duan et al. 2008; Mondal et al. 1978; Myers et al. 1999; Wünsche et al. 2005), except for cases with apparent intermediate carbohydrate pools that can buffer responses (Murchie et al. 2002; Nakano et al. 1995). For example, severe and highly significant depressions in photosynthesis rates were observed in leaves of peach tree branches from which all the fruits were removed (filled squares) compared to branches with a single peach (open circles; Fig. 23.1a). This downregulation of photosynthesis was accompanied, and presumably compensated for, by significantly greater employment of photoprotective thermal energy dissipation as assessed from higher NPQ levels (Fig. 23.1b) mirrored by significantly greater decreases in intrinsic PS II efficiency (Fig. 23.1c) and much higher levels of zeaxanthin + antheraxanthin (Fig. 23.1d). Both NPQ and PS II efficiency were linearly (positively for NPQ and negatively for F_v'/F_m') related to the level of Z + A (Duan et al. 2008), indicating involvement of (Z + A)-associated thermal dissipation in the depression of PS II efficiency F_v'/F_m'. In a subsequent study, these decreases in photosynthesis and increases in thermal energy dissipation were found to be correlated with an accumulation of sorbitol, sucrose, and starch in source leaves of peach after fruit removal (Cheng et al. 2009), as might be expected when the plant's sinks fail to utilize the products of photosynthesis at a rate to match that of the source leaf's rate of sugar production.

Modulation of both photosynthetic activity and thermal energy dissipation can occur in response to any changes in the balance between the respective rates of (i) carbohydrate synthesis in the leaves and (ii) carbohydrate export from leaves (and utilization in the sinks). In line with the *accumulation* of carbohydrates, decreases in photosynthesis, and increases in photoprotective thermal energy dissipation observed when *sinks* are removed from a plant, removal of most of the *source* leaves from cherry trees resulted in significant *decreases* in foliar starch levels, increases in photosynthesis rates, and higher intrinsic PS II efficiencies in the few remaining source leaves (Layne and Flore 1993; also see section "Exposure to Excess Light", this chapter).

Another approach illustrating the response of source leaves to increased levels of carbohydrates is through the feeding of sugars to source leaves. Simple feeding of sugars (glucose, sucrose) through the transpiration stream has been shown to result in significant increases in non-structural carbohydrate levels in leaves, and a downregulation of photosynthesis via decreased levels of Calvin-Benson cycle enzymes and thylakoid membrane components, including e.g., chlorophyll (a + b)-binding proteins and the D1 protein (Jones et al. 1996; Kilb et al. 1996; Krapp et al. 1991, 1993; Rolland et al. 2002; Smeekens 2000; Van Oosten and Besford 1996). Changes in chlorophyll fluorescence suggest that such sugar-feeding-induced downregulation of photosynthesis results in increased levels of NPQ indicative of greater employment of photoprotective thermal energy dissipation (Kilb et al. 1996; Krapp et al. 1993).

Another approach to overloading source leaves with carbohydrates is through the growth of plants under elevated CO_2. Such growth leads to substantial increases in leaf carbohydrate concentrations (compared to plants grown at ambient levels of CO_2) that can result in downregulation of photosynthesis (Makino and Mae 1999; Van Oosten and Besford 1996) and increased employment of compensatory thermal energy dissipation. For instance, scrub-oak trees growing in Florida under elevated (twice ambient) atmospheric CO_2 compared to plants under ambient CO_2 experienced a greater decline in the carboxylation capacity of ribulose bisphosphate carboxylase (Rubisco), and greater decreases in PS II efficiency (presumably involving

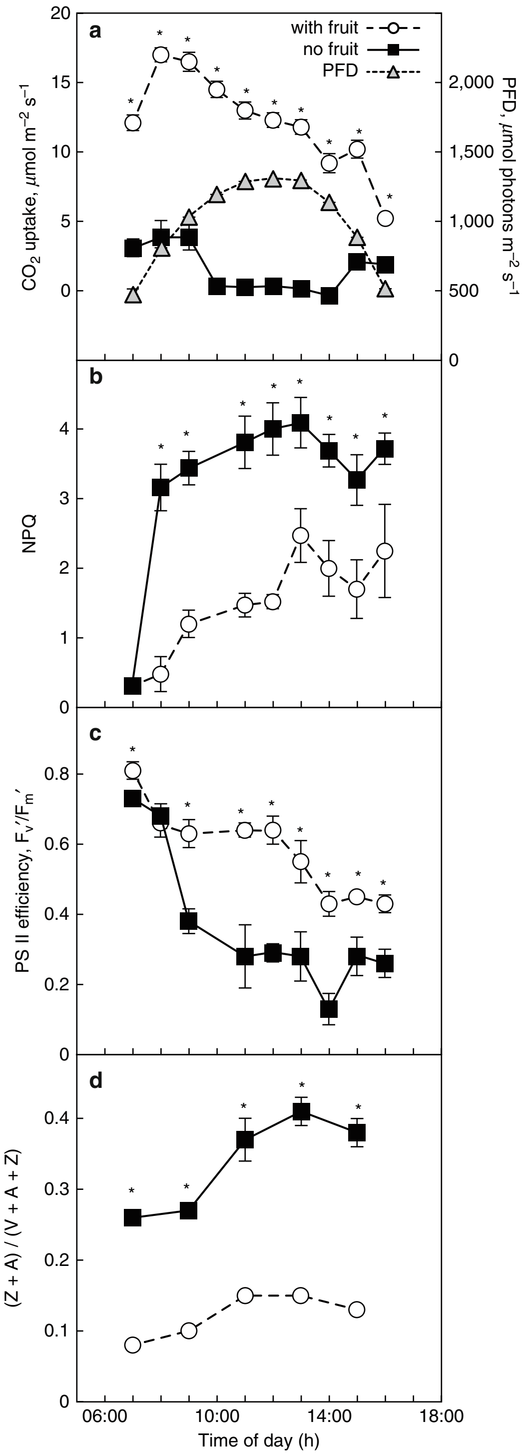

Fig. 23.1 Diurnal changes in (**a**) photon flux density (*PFD*) and net CO_2 exchange, (**b**) nonphotochemical quenching of chlorophyll fluorescence (*NPQ*) calculated as $(F_m/F_m')-1$, (**c**) the calculated efficiency of photosystem II in the light (F_v'/F_m'), and (**d**) the conversion state of the *VAZ* (Violaxanthin-Antheraxanthin-Zeaxanthin) cycle (zeaxanthin + antheraxanthin as a fraction of the total pool of violaxanthin + antheraxanthin + zeaxanthin) from source leaves of peach trees on 1 year old branches that had all fruits removed (*solid squares* and *lines*) or had one fruit that was maintained on the branch (*open circles* and *dashed lines*). Trees were growing outside (characterized on a sunny day during the summer month of June) under full sunlight. Each point represents the mean ± standard error of five replicates, except for the pigment values that are the mean ± standard error of four replicates. *Asterisks* indicate significant differences between the means at $p<0.05$ or less (*t*-tests) Data from Duan et al. (2008).

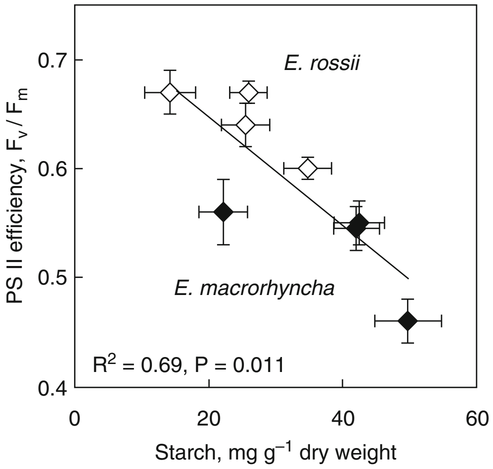

Fig. 23.2 Relationship between the amount of starch and photosystem II (*PS II*) efficiency (F_v/F_m) in leaves of two *Eucalyptus* species subjected to high temperature stress (3 h midday exposures to 45 °C and 1,250 μmol photons m^{-2} s^{-1}) with and without adequate water and at two different CO_2 levels (350 and 700 μL l^{-1}). Prior to these experimental treatments, plants were grown in a greenhouse at 20 °C and received a maximum of 1,100 μmol photons m^{-2} s^{-1} (midday), and were then placed in a growth chamber for 4 days where they experienced day/night temperatures of 35/20 °C under a 10 h photoperiod of 750 μmol photons m^{-2} s^{-1}, the latter of which provided the background conditions for the experimental treatments. Mean values ($n=7$) ± standard error are shown. Data from Roden and Ball (1996).

increased thermal energy dissipation), upon cessation of sink activity (growth arrest) during the excessively hot summer (Hymus et al. 2001). Similar results were obtained for loblolly pine growing in North Carolina during the coldest month of winter (February), when intrinsic PS II efficiency was lower in the needles of trees growing under elevated CO_2 compared to ambient CO_2 levels (Hymus et al. 1999). Furthermore, Roden et al. (1999) found that growth of *Eucalyptus* seedlings under elevated CO_2 (twice ambient) resulted in greater foliar levels of starch and total non-structural carbohydrates coupled with greater depressions in intrinsic PS II efficiency compared to ambient CO_2 levels. The correlation between increased levels of foliar starch (resulting from elevated CO_2) and greater declines in PS II efficiency (presumably involving increased photoprotective thermal energy dissipation) is well illustrated by long-term exposure of *Eucalyptus* plants to different combinations of ambient and elevated CO_2 levels with ample or limiting water supply (Fig. 23.2).

Decreased plant sink activity can also result from insufficient supply of essential nutrients for plant growth. Decreased growth of spinach plants resulting from limiting supply of nitrogen (Paul and Driscoll 1997; Verhoeven et al. 1997; Logan et al. 1999; see also Castro et al. 1995) was associated with accumulation of sugars and starch (Fig. 23.3a), downregulation of pho-

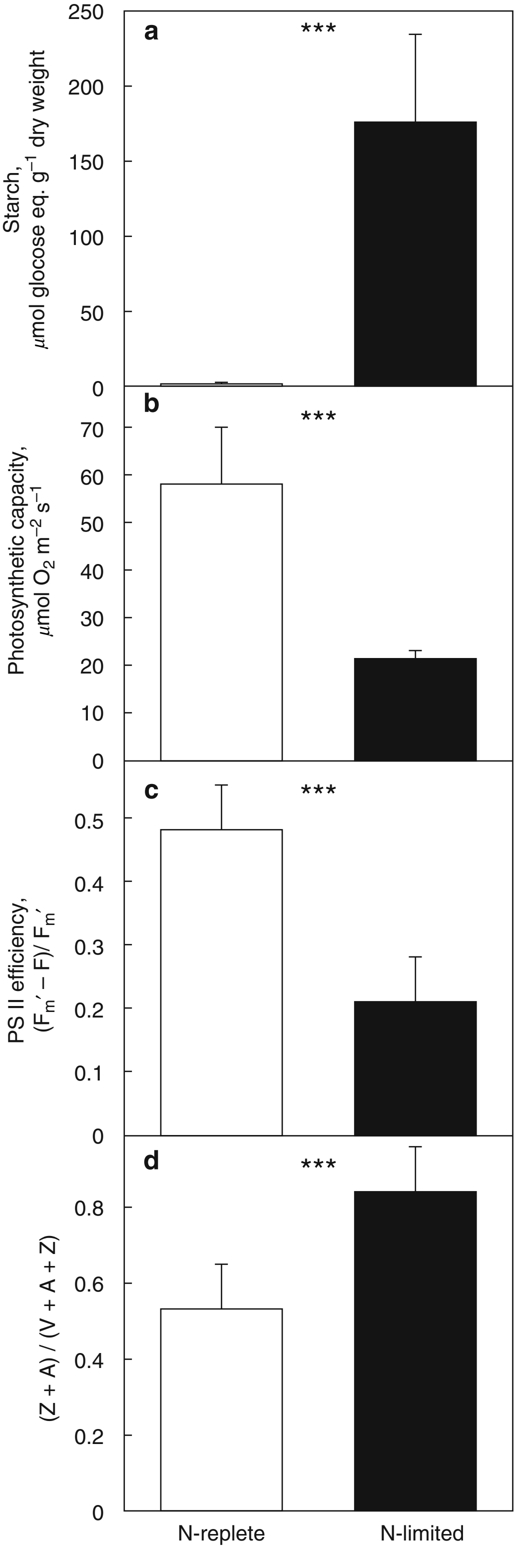

Fig. 23.3 (**a**) Foliar levels of nocturnally retained starch (sampled predawn), (**b**) light- and CO_2-saturated rates of photosynthetic oxygen evolution, (**c**) midday PS II efficiency (see text), and (**d**) midday conversion state of the VAZ cycle from mature source leaves of spinach watered daily with sufficient (14 mM nitrate) or insufficient (0.25 mM nitrate) nitrogen and growing in a fully sunlit glasshouse (midday photon flux densities of approximately 1,300 μmol photons m^{-2} s^{-1}). Means (n=4, n=3 for starch) ± standard deviations depicted, and all parameters were significantly different at $p<0.005$ (t-tests). Corresponding predawn foliar levels of sucrose were 14 ± 1 and 29 ± 8 μmol glucose equivalents g^{-1} dry weight and glucose were 7 ± 2 and 15 ± 4 μmol g^{-1} dry weight from the nitrogen replete and nitrogen limited plants. Data from Logan et al. (1999).

tosynthesis (depressed intrinsic photosynthetic capacity; Fig. 23.3b), and decreased actual PS II efficiency (Fig. 23.3c) and increased levels of zeaxanthin + antheraxanthin (Fig. 23.3d) in source leaves. Very similar responses were reported for plants grown with an insufficient supply of magnesium and sulfur (starch accumulation and decreased F_v/F_m; Godde and Hefer 1994), iron (increased NPQ, decreased PS II efficiency, and increased levels of zeaxanthin + antheraxanthin; Morales et al. 2000), or boron (sugar and starch accumulation, reduced photosynthetic enzymes and photosynthesis, and decrease in F_v/F_m; Han et al. 2009).

Overwintering evergreen plants are another case where foliar levels of soluble carbohydrates increase, and this could be viewed in a context of increased freezing tolerance as well as in a context of source-sink imbalances. For example, overwintering leaves of the evergreen groundcover *Vinca minor* possessed much higher levels of foliar sugars (Fig. 23.4a), significantly reduced intrinsic photosynthetic capacity (Fig. 23.4b), strong nocturnally-sustained depression in intrinsic PS II efficiency (Fig. 23.4c; presumably involving locked-in thermal energy dissipation absent in the summer), and nocturnal retention of high levels of zeaxanthin + antheraxanthin that was almost the entire pool of VAZ cycle carotenoids (Fig. 23.4d). While foliar sugar accumulation in overwintering plants likely serves in freezing tolerance, the possibility

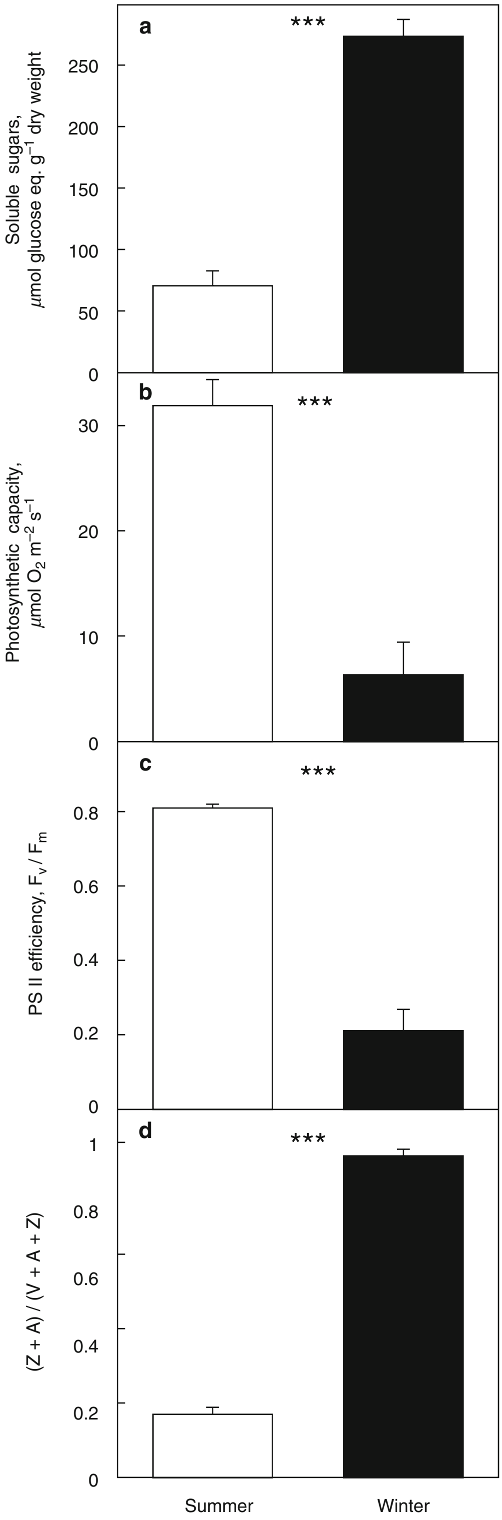

Fig. 23.4 Summer and winter levels of (**a**) total soluble sugars, (**b**) light- and CO_2-saturated rates of oxygen evolution (photosynthetic capacity), (**c**) predawn PS II efficiency (F_v/F_m), and (**d**) the conversion state of the VAZ cycle in leaves of *Vinca minor* growing in full sunlight. Means (n = 3) ± standard deviations depicted, and all parameters were statistically different at $p < 0.0001$ (*t*-tests). Data from Adams et al. (2001a, b, 2002).

of concomitant downregulation of photosynthesis (and upregulation of thermal dissipation) via feedback inhibition must not be ignored. Under conditions of (i) cold-suppressed carbohydrate export from source leaves to the rest of the plant on individual cold days in otherwise less extreme habitats or times of the year or (ii) full growth arrest under winter conditions, evergreens remain photoprotected through thermal dissipation of the excess excitation energy absorbed by the chlorophyll molecules utilizing high levels of zeaxanthin and antheraxanthin that are retained 24 h a day (Adams and Demmig-Adams 1994, 1995; Adams et al. 1995b, 2001a, 2002, 2006; Verhoeven et al. 1996, 1998; Adams and Barker 1998; Ebbert et al. 2005; Zarter et al. 2006a, b, c). Metabolic arrest due to low temperature can be distinguished from actual downregulation of photosynthetic capacity by collecting leaves or needles in the field, warming them, and determining the light- and CO_2-saturated rate of photosynthetic oxygen evolution at 25 °C (photosynthetic capacity). Photosynthetic capacity of both needles from conifers and leaves from broad-leafed evergreens can experience downregulation of photosynthesis depending on the severity of the winter conditions (Adams et al. 2001a, 2002, 2004, 2006; Verhoeven et al. 1999; Zarter et al. 2006a, b, c; Koh et al. 2009), and respond to the availability of liquid water with an upregulation of photosynthetic capacity during the spring (Zarter et al. 2006b).

Overwintering herbaceous species can exhibit a different response to low temperature. Mesophytic biennials and winter annuals have been shown to upregulate photosynthesis when grown at low temperature (Adams et al. 2013a; Holaday et al. 1992; Martindale and Leegood 1997; Strand et al. 1997, 1999) and in response to winter conditions (Adams et al. 1995a, 2001a, b, 2002, 2004; Verhoeven et al. 1999), despite an increase in foliar sugars (Strand et al. 1997, 1999; Adams et al. 2001b). Such upregulation is facilitated by increased layers of chloroplast-packed palisade mesophyll cells that arise in leaves that develop at cool compared to warm temperature (Boese and Huner 1990; Gorsuch et al. 2010; Dumlao et al. 2012; Cohu et al. 2014) that allows for the maintenance of elevated photosynthetic rates despite lower temperatures. This upregulation of photosynthetic tissue is coupled with a significantly higher number and consequently greater cross-sectional area of phloem cells (both companion cells and sieve elements) per minor loading vein in some apoplastic loaders (Adams et al. 2013a; Cohu et al. 2013a, b; 2014) or larger sugar-loading companion cells (intermediary cells) and greater vein density in some symplastic loaders (Adams et al. 2013a; Dumlao et al. 2012). These altered foliar vascular features permit maintenance of the active loading of sugars into the phloem and continued sugar export from the leaf despite the impact of lower temperature on (1) enzymatic and transporter activity and (2) the increased viscosity of the phloem sap (Adams et al. 2013a; Cohu et al. 2013a, b).

Experimental short-term transfer to low temperatures can result in responses that are similar to those of evergreen leaves/needles in the field. Renaut et al. (2005) grew poplar plants at 23 °C and found that transfer of such plants to 4 °C-growth conditions resulted in a tripling of foliar soluble carbohydrates and a highly significant decrease in PS II efficiency (presumably involving increased thermal energy dissipation).

IV Manipulation of Carbohydrate Export from Source Leaves

An impact of decreased carbohydrate export from source leaves on their photosynthetic and thermal energy dissipation rates has also been demonstrated via manipulations acting closer to the source leaves than the activity or removal of distant sinks. Accumulation of

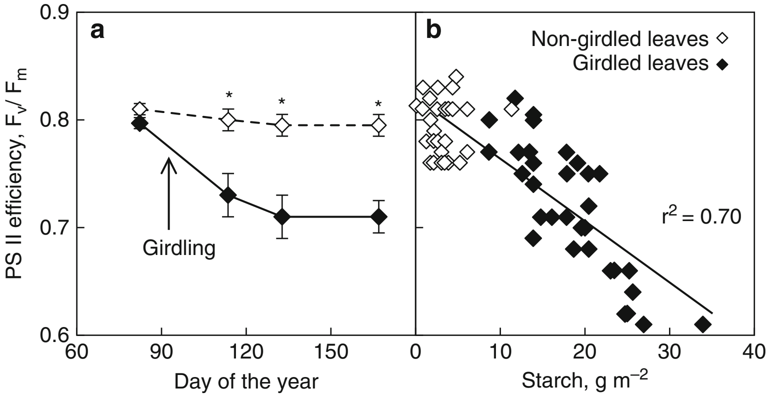

Fig. 23.5 (**a**) Impact of branch girdling on PS II efficiency (F_v/F_m) in mango leaves over the course of 70 days and (**b**) the relationship between the amount of starch and PS II efficiency (F_v/F_m) in leaves of non-girdled and girdled branches of mango trees. Trees were growing outside under full sunlight. Each point in (**a**) represents the mean ± standard error of 11 (girdled) or 7 (non-girdled) replicates, and *asterisks* indicate significant differences between the means at $p < 0.05$ (ANOVA and Multiple Comparisons of Means). Data from Urban and Alphonsout (2007).

an engineered invertase (that cleaves sucrose into glucose and fructose) in the walls of leaf mesophyll cells inhibited sucrose export from the latter cells, and resulted in foliar accumulation of nonstructural carbohydrates, a decrease in mRNA for Rubisco, a decrease in Rubisco protein, a decrease in photosynthesis rate, and a greater level of NPQ in transgenic versus non-engineered tobacco plants (Krapp et al. 1993; Stitt et al. 1990). Similar results (except for NPQ that was not determined) were observed when the stems (petioles) of wild-type tobacco, potato, or spinach were cold-girdled (fitted with a coldwater jacket to slow sugar export from the leaf through the phloem) compared to control leaves (Krapp et al. 1993; Krapp and Stitt 1995).

Actual girdling (physical removal of the phloem) of branches can result in strong downregulation of photosynthesis and (presumably) compensatory engagement of high levels of photoprotective energy dissipation. Such a presumption is based on the many studies that have shown a strong correlation between decreases in PS II efficiency and increased levels of zeaxanthin + antheraxanthin, as well as increases in NPQ (when conditions permitted the calculation of NPQ or the rate constant for energy dissipation in studies published prior to 1990) (see Adams and Demmig-Adams 1994, 1995, 2004; Demmig-Adams and Adams 1994a, 1996a, b, 2006; Adams et al. 1995a, b, 1999, 2002, 2006; Demmig-Adams et al. 1995, 1996, 1998, 1999, 2006a, b, 2012; Verhoeven et al. 1996, 1998; Demmig-Adams 1998; Zarter et al. 2006a,b,c; see also Demmig-Adams et al., Chap. 24). Urban and Alphonsout (2007) girdled branches of mango trees and observed the impact of this inhibition of carbohydrate export from leaves of girdled versus non-girdled branches over an entire season. Leaves of girdled branches accumulated significantly higher levels of soluble sugars and especially starch, experienced severe downregulation of photosynthesis, and significantly lower predawn levels of intrinsic PS II efficiency (Fig. 23.5a). Consequently, there was a strong linear relationship between the accumulation of foliar starch and nocturnally sustained decreases in intrinsic PS II efficiency (i.e., photoinhibition; Fig. 23.5b). Similar decreases in intrinsic PS II efficiency occurred in response to girdling of branches or tree trunks of several other species growing in full sunlight (Myers et al. 1999; Rivas et al. 2007; Werner and Correia 1996).

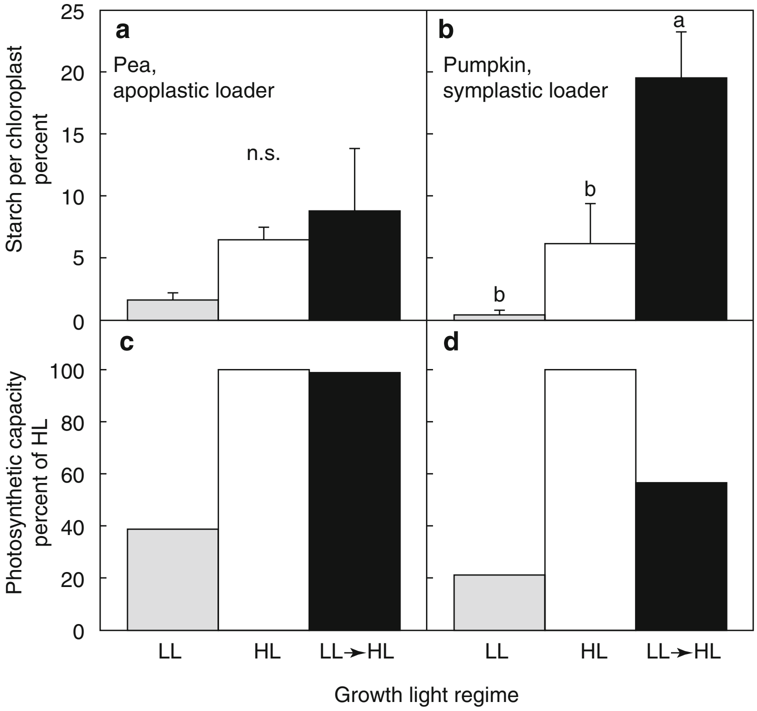

Fig. 23.6 Fractional area of the chloroplasts filled with starch grains in pea (**a**) and pumpkin (**b**) quantified from cross-sections of palisade cells (examined with electron microscopy) from fully expanded leaves and photosynthetic capacity (light- and CO_2-saturated rates of oxygen evolution determined in a leaf disc oxygen electrode) relative to that determined from fully expanded leaves of pea (**c**) and pumpkin (**d**) grown under high light (*HL*). Plants were grown under a 9 h photoperiod of 100 μmol photons m^{-2} s^{-1} (low light *LL*; fluorescent and incandescent bulbs), or under a 14 h photoperiod of 1,000 μmol photons m^{-2} s^{-1} (high light *HL*; metal halide bulbs), or developed under LL and subsequently transferred for 1 week to HL (LL → HL). Only leaves that had expanded fully under LL were characterized 1 week after transfer to HL. Means (n = 3) ± standard deviation depicted in (**a**, **b**). Different *lower case letters* indicate significant differences at $p < 0.05$ (ANOVA and Tukey-Kramer test for honestly significant differences); *n.s.* not significantly different. The relative photosynthetic capacities (**c**, **d**) were calculated from the data published by Amiard et al. (2005). Photosynthetic capacities of leaves from HL plants and LL transferred to HL plants were both significantly higher than leaves of LL pea plants (**c**), and photosynthetic capacities of pumpkin leaves were significantly different from each other for all three growth conditions (**d**).

Furthermore, an apparent physical limitation to carbohydrate export (resulting in starch accumulation) and an inability to fully upregulate photosynthesis in response to increased light availability was observed in leaves of plants that load sugars into the phloem via a symplastic route (through plasmodesmatal opening in the cell walls), but not in plants that load sugars via an apoplastic route. Pea (an apoplastic loader relying on sucrose-H^+ cotransporters to actively transport sucrose into the phloem) showed no significant increase in starch (Fig. 23.6a) and full upregulation of photosynthetic capacity (Fig. 23.6c) in leaves acclimated to low light and then transferred to high light for 1 week. Pea strongly increased cell surface area of transport cells (via companion cell wall invagination) following the transfer from low to high light (Amiard et al. 2005), and was thus presumably able to upregulate the level of sucrose transport proteins and sucrose export from leaves to avoid feedback inhibition of photosynthesis that would prevent full upregulation of photosynthetic capacity. Leaves of pumpkin (a symplastic loader relying on sucrose flow through physical plasmodesmatal pores) exhibited a

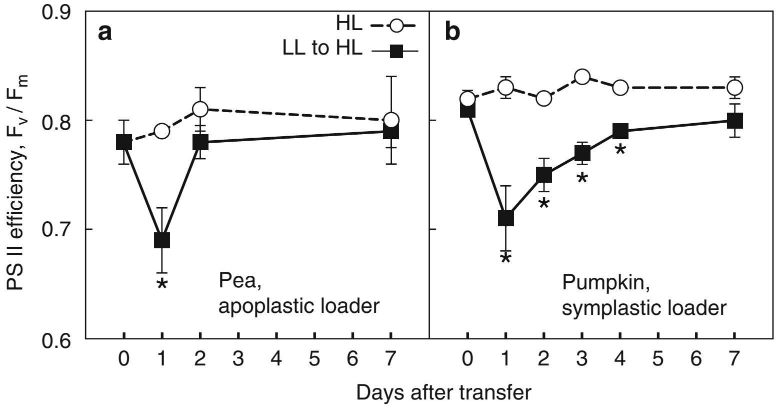

Fig. 23.7 Changes in pre-dawn PS II efficiency (F_v/F_m) in fully expanded leaves of pea (**a**) and pumpkin (**b**) either growing under a 14 h photoperiod of 1,000 μmol photons m^{-2} s^{-1} (HL; metal halide bulbs) over the course of 7 days (*open circles* and *dashed lines*) or from fully expanded leaves on plants that developed under a 9 h photoperiod of 100 μmol photons m^{-2} s^{-1} (LL; fluorescent and incandescent bulbs) and then characterized during exposure to a week of HL conditions (*solid squares* and *lines*). Each point represents the mean ± standard deviation of three replicates, and *asterisks* indicate significant differences between the means at $p < 0.05$ (*t*-tests). V. Amiard, B. Demmig-Adams, and W. W. Adams, unpublished data.

very different response to low-to-high-light transfer.

Leaves of symplastic loaders, in contrast to those of apoplastic loaders like pea and spinach, develop a significantly higher vein density (and thus a higher total number of plasmodesmatal connections leading into the phloem per leaf area) when growing in high light compared to low light (Amiard et al. 2005). This physical infrastructure for exporting sugars from symplastically loading leaves is inflexible, and does not change upon transfer of mature leaves from low to high light (Amiard et al. 2005). As a consequence, such low-to-high-light transfer results in significantly higher starch levels (Fig. 23.6b) and an inability to fully upregulate photosynthetic capacity to the level seen in leaves developed in high light (Fig. 23.6d). The above difference in the ability to upregulate photosynthesis between the two species also results in small, but significant differences in the level of nocturnally sustained depressions in intrinsic PS II efficiency over the course of a week following the transfer from low to high light (Fig. 23.7). F_v/F_m was significantly decreased only on the first day following transfer in the apoplastic loader pea (Fig. 23.7a), but was significantly decreased over multiple days following transfer of fully expanded (symplastically loading) pumpkin leaves from low to high light (Fig. 23.7b). It is likely that the nocturnally-sustained depression in PS II efficiency is a reflection of a compensatory photoprotective response to slow and incomplete upregulation of photosynthetic capacity as non-structural carbohydrate accumulated in these leaves (Fig. 23.6b) following transfer of pumpkin from low to high light.

V Exposure to Excess Light

Layne and Flore (1993), in addition to observing release from feedback downregulation of photosynthesis and PS II efficiency in a few leaves upon removing the majority of the source leaves from cherry trees (Fig. 23.8), also exposed a set of non-defoliated plants to continuous light. Continuous light exposure resulted in accumulation of greater levels of foliar starch (Fig. 23.8a), a downregulation of photosynthesis (Fig. 23.8b), and a lower intrinsic PS II efficiency (Fig. 23.8c). Intrinsic PS II

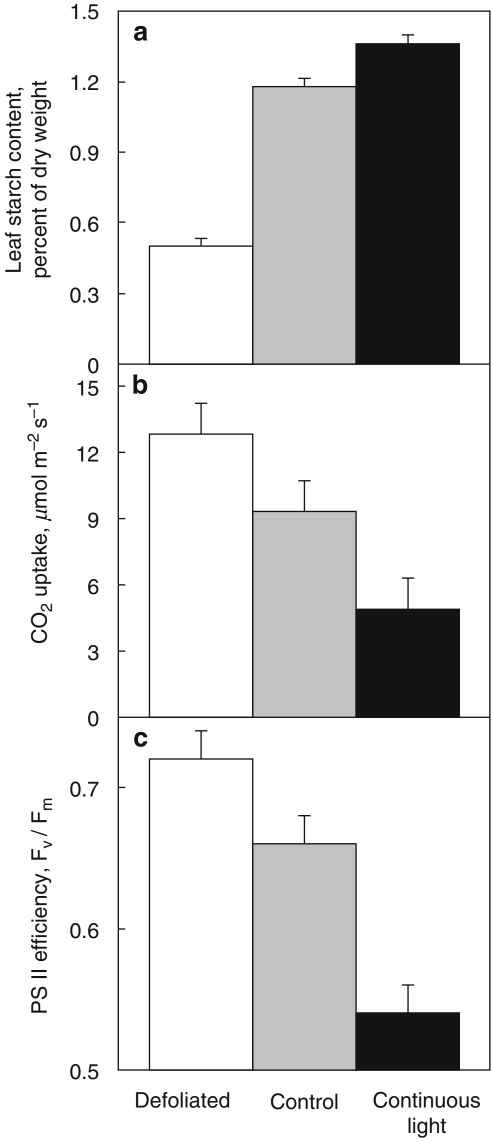

Fig. 23.8 Impact of the removal of 70 % of the leaf area (Defoliated) or exposure to light for 24 h per day (Continuous light) on (**a**) foliar starch accumulation, (**b**) CO_2 uptake, and (**c**) PS II efficiency in young cherry trees 7 days after the start of the treatments. Plants were exposed to 800 μmol photons m^{-2} s^{-1} for 14 h per day (Defoliated and Control) or 24 h per day (Continuous light) and characterized on day 7 following the beginning of the respective treatments. CO_2 uptake and PS II efficiency were determined 3 and 8 h, respectively, after the beginning of the 14 h photoperiod (for Defoliated and Control plants). PS II efficiency was ascertained following 15 min of dark adaptation. Mean values ($n \geq 8$) ± standard error are shown. Data from Layne and Flore (1993).

efficiency in the latter case was determined during the (continuous) light period, but following 15 min of dark adaptation, and thus likely involves slowly relaxing (sustained/locked-in) thermal energy dissipation and/or photoinactivation.

When shade-grown plants, particularly low-light-acclimated shade-adapted plants (e.g., rain-forest sclerophytes with inherently low growth rates) are transferred from low to high growth light environments, strong, persistent (predawn) depression in intrinsic PS II efficiency (Fig. 23.9a), high levels of nocturnally-sustained (predawn) NPQ (Fig. 23.9b) indicative of locked-in thermal dissipation, and high levels of nocturnally retained zeaxanthin + antheraxanthin (Fig. 23.9c) were observed (see also Demmig-Adams et al. 1998). Sustained engagement of thermal energy dissipation was accompanied by nocturnally retained non-structural carbohydrates (sugars and starch) that increased with each day of exposure to high light (Fig. 23.10). Thus, despite the fact that photosynthetic efficiency was strongly depressed, daily exposure to high light resulted in continuous accumulation of the products of photosynthesis considerably above the level present under low light growth conditions. Sudden exposure of shade-grown plants to high light levels likely leads to the production of high levels of reactive oxygen species that can induce photoinactivation of photosynthesis (Chen et al. 2012; Ohad et al. 2011), although some argue that reactive oxygen species only maintain the photosynthetic apparatus in an inactivated state (Murata et al. 2012; Nishiyama et al. 2011). In addition, shade-grown plants also likely feature very low sink activity as well as only slow, minimal increases in sink activity upon transition from low to high light. Shade-grown leaves of such plants furthermore possess lower vein densities than sun-grown leaves (Adams et al. 2006, 2007) and therefore presumably lower capacities for carbohydrate export from the leaves.

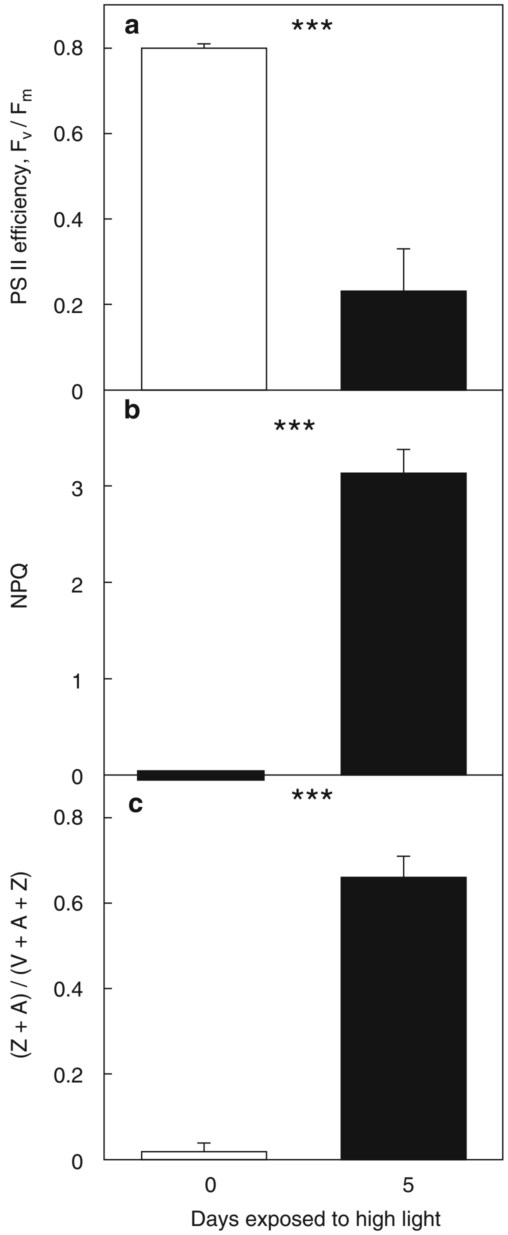

Fig. 23.9 Effect of 5 days of exposure to high light (10 h photoperiod of 700 μmol photons m^{-2} s^{-1}; metal halide bulbs) on predawn (**a**) PS II efficiency (F_v/F_m), (**b**) NPQ [$(F_m/F_m')-1$] (using the F_m value determined prior to the first day of exposure to high light), and (**c**) the conversion state of the VAZ cycle in *Monstera deliciosa* leaves grown under low light (10 μmol photons m^{-2} s^{-1}; fluorescent lamps). Leaves were characterized prior to exposure to high light (Day 0) and after 5 days of exposure to the high light conditions. Means ± standard deviation shown were significantly different at $p<0.001$ (*t*-tests). n = 5 for (**a**), n = 4 for (**b**), and n = 4 for Day 0 and 3 for Day 5 in (**c**). Data from Demmig-Adams et al. (2006b).

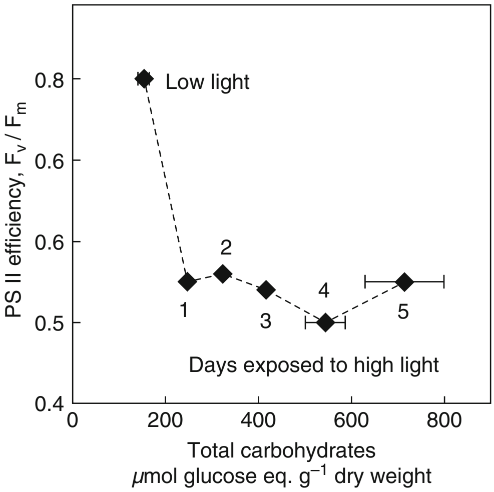

Fig. 23.10 Relationship between total non-structural carbohydrates (starch + total soluble sugars) and predawn PS II efficiency (F_v/F_m) in leaves of *Monstera deliciosa* grown in low light (10 μmol photons m^{-2} s^{-1}; fluorescent and incandescent bulbs) and transferred to high light (10 h photoperiod of 700 μmol photons m^{-2} s^{-1}; metal halide bulbs) for 5 days. Means (n = 3) ± standard deviations are shown. Data from Adams et al. (2006).

VI Sustained NPQ, Photoinhibition, and Plant Productivity

A case where it is widely accepted that the phenomenon of photoinhibition is photoprotective and involves sustained zeaxanthin-associated NPQ is the winter downregulation of evergreen plants (Adams et al. 1995b, 2001a, 2002, 2004, 2006; Demmig-Adams et al. 1999, 2006a, 2012; Gilmore and Ball 2000; Öquist and Huner 2003; Ensminger et al. 2004; Ebbert et al. 2005; Demmig-Adams and Adams 2006; Zarter et al. 2006a, b, c). On the other hand, the majority of studies on other systems tend to equate photoinhibition with damage (Adir et al. 2003; Campbell and Tyystjärvi 2012; Goh et al. 2012; Keren and Krieger-Liszkay 2011; Murata et al. 2007, 2012; Oguchi et al. 2011; Ohad et al. 2011; Takahashi and Badger 2011; Tyystjärvi 2008, 2013; Vass 2012) and assume that photoinhibition has negative consequences for plant productivity (Farage and Long 1991; Baker et al. 1994; Long et al. 1994; Ögren 1994; Raven 1994, 2011; Werner et al. 2001; Alves et al. 2002;

Losciale et al. 2010; Takahashi and Badger 2011; Goh et al. 2012; Reynolds et al. 2012; see also Zhu et al. 2004, 2010; Murchie and Niyogi 2011). The demonstration of non-structural carbohydrate accumulation in photoinhibited leaves summarized here suggests that the consequences of photoinhibition on plant productivity should be examined further.

Mathematical models, based on correlations between decreases in PS II efficiency and CO_2 uptake during the afternoon in the most sun-exposed leaves of plants, have been used to extrapolate decreases in plant productivity resulting from photoinhibition (Ögren and Sjöström 1990; Werner et al. 2001). If non-structural carbohydrate accumulation during midday exposure to full sunlight leads to temporary feedback downregulation of photosynthesis and compensatory employment of photoprotective thermal energy dissipation resulting in decreased PS II efficiency (e.g., Cheng et al. 2009; Duan et al. 2008), these assumptions should be revisited. Furthermore, assumptions that photoinhibition is responsible for decreased plant growth and productivity in response to winter stress have been made based, e.g., on correlations between photosynthetic efficiency and productivity in overwintering *Brassica napus*, as documented by Farage and Long (1991); however, cause-and-effect have not been determined. Winter conditions may have inhibited plant growth (resulting in decreased sink activity and foliar sugar accumulation) and/or induced the accumulation of sugars as cryoprotectants, leading to feedback inhibition and photoprotective downregulation of photosynthesis in source leaves that had been exposed to full sunlight. In the review presented above, all leaves that experienced photoinhibition contained higher levels of starch and/or sugars compared to leaves that experienced less or no photoinhibition. Whether photoinhibition is interpreted as damage or photoprotection, there appears to be an abundance of the products of photosynthesis in photoinhibited leaves (under both field conditions and in plants subjected to experimental conditions), calling into question whether photoinhibition limits plant productivity.

The approach taken thus far to assess the relationship between photoinhibition and plant productivity has been to determine productivity in plants experiencing photoinhibition (typically under high light conditions) versus plants not experiencing photoinhibition (typically under shaded conditions). An inherent limitation of this experimental design is its inability to compare growth in photoinhibited versus non-photoinhibited plants under the same light availability for growth, and final conclusions are therefore not available. However, no evidence was found that plants experiencing photoinhibition grew less than plants not experiencing photoinhibition. Ball (1994) and Alves et al. (2002), among others, had initially suggested that photoinhibition might impair establishment of seedlings and forest restoration efforts, but photoinhibited plants (in high light) were found to grow as much as, or more than, plants that did not experience photoinhibition (in lower light). Krause et al. (2006) found overall growth (plant biomass) of tropical tree seedlings experiencing photoinhibition (decreases in PS II efficiency associated with high zeaxanthin levels) in full sunlight to be the same as that of seedlings shaded whenever incident light intensity reached 50–80 % of full sunlight. Einhorn et al. (2004) similarly found that full sunlight-exposed beech seedlings accumulated slightly more (although not significantly) biomass than ash seedlings, despite the fact that the beech plants exhibited greater depressions in PS II efficiency. Furthermore, Blennow et al. (1998) reported that seedlings of *Eucalyptus pauciflora* experiencing the greatest photoinhibition in the field (under a combination of lower temperatures and higher light) actually grew (biomass and root growth) more than those that experienced less or no photoinhibition. Moreover, when the same species growing in full sunlight was exposed to high CO_2 levels, plants accumulated more starch and total soluble carbohydrates, experienced more photoinhibition during winter, and subsequently grew more than plants exposed to ambient levels of CO_2

(Roden et al. 1999). Close and Beadle (2003) found strong photoinhibition in full-sun-exposed (but not in shaded) seedlings of *Eucalyptus nitens* accompanied by greater overall growth (relative growth rate based on biomass) in the plants that were not shaded.

These compiled results on overall plant growth, as well as those on sugar and starch accumulation in photoinhibited leaves, suggest that downregulation of photosynthesis due to excess light may be more than compensated for by the level of energy available to continue fixing CO_2. Both environmental and/or inherent genetic limitations to growth (many perennial and evergreen species grow much more slowly than herbaceous annuals or biennials) can limit plant sink activity, and may prevent full utilization in the plant's sinks of reduced carbon supplied by source leaves under high light levels. The correlation between photoinhibition and decreased CO_2 uptake – often used to suggest that photoinhibition limits plant productivity – would be equally consistent with the assumption that limiting plant growth and sink activity may cause foliar sugar and starch accumulation leading to feedback inhibition/downregulation of photosynthesis and photoinhibition. The commonly observed accumulation of starch and/or sugars in photoinhibited leaves, as well as the greater growth under subsequent favorable environmental conditions of sun-exposed plants that experienced greater levels of photoinhibition under prior stress conditions, suggests that photoinhibition may not limit plant productivity, unless photoinhibition, or a condition associated with it, were also responsible for inhibiting foliar export of carbohydrates.

We don't wish to suggest that there are no circumstances under which the response of an experimental system to excess light might accurately be construed as photodamage. Experiments employing isolated photosystem II complexes, thylakoids, or even chloroplasts, outside of the context of the regulatory network of the cell and organism, are unlikely to respond in the same way as the photosynthetic apparatus of an intact organism. The use of electron transport inhibitors, artificial electron acceptors and/or donors, uncouplers, inhibitors of protein synthesis, etc., are furthermore likely to induce responses that would otherwise not occur. Likewise, the responses of mutants and genetically engineered lines, while useful in dissecting the role of certain proteins and components of the chloroplast, can also lead to responses and additional effects that do not occur in the wild-type line (see discussion in Adams and Demmig-Adams, Chap. 2). Furthermore, the use of experimental conditions (light intensity, temperature) that far exceed what an organism might naturally experience may also induce responses that would never be observed under physiologically relevant conditions. For instance, the first study to identify the D1 protein as the purported sight of photodamage involved the exposure of low-light grown algae to light equivalent to almost twice full sunlight (Kyle et al. 1984). Interestingly, feeding of glucose to spinach leaves exposed to very low light resulted in greater decreases in the level of the D1 protein compared to leaves that did not receive glucose (Kilb et al. 1996).

Algae have been reported to be particularly susceptible to photoinhibition (Falkowski et al. 1994; Neale 1987). Without sinks to which the products of photosynthesis can be exported, free-living algae might readily respond to an accumulation of carbohydrates through sustained downregulation of photosynthesis. Interestingly, Falkowski and Dubinsky (1981) reported that the algal symbiont of the coral *Stylophora pistillata* exhibited no photoinhibition (no decreases in photosynthetic efficiency) when exposed to light intensities 50 % greater than full sunlight. In such a symbiotic relationship, the alga presumably secretes products of photosynthesis to feed its coral host in exchange for shelter, nutrients, and elevated levels of CO_2. We have likewise found that culture of the green alga *Chlamydomonas* under conditions that stimulate the release of reduced carbon compounds into the medium stimulates the upregulation of photosynthesis in response to growth under high light levels (T.A. Burch, W.W. Adams, B.L.S. Degrenne, C.H. Englert, B.R. Mines, P.C. Nash, E.C. Boone, B. Demmig-Adams, unpublished data).

High temperatures, especially those experienced by organisms that are not adapted or acclimated to those temperatures, may lead to increased membrane fluidity that exceeds the capacity to maintain the proper arrangement of the components of photosynthetic electron transport. Such effects in the presence of light and oxygen would likely lead to the transfer of excess excitation energy captured by chlorophyll to oxygen and a cascade of damage (we have observed a rapid transition from green to brown when photosynthetic tissues are allowed to overheat). However, when leaf temperature is properly controlled, all changes in PS II efficiency can be accounted for by increases in zeaxanthin (and antheraxanthin) associated energy dissipation from even extremely shade-acclimated leaves (Demmig-Adams et al. 1998, 2006b). Furthermore, plants adapted and acclimated to habitats where tissue temperatures reach 50 °C thrive and actively employ high levels of photoprotective energy dissipation (Adams et al. 1987; Barker et al. 2002).

Some have suggested that plants growing in shaded habitats (or "low carbohydrate" conditions) may be negatively impacted by decreased PS II efficiency that occurs during exposure to sunflecks (Murchie and Niyogi 2011; Raven 1994). However, the study upon which such conclusions were based assessed changes in chlorophyll fluorescence (decrease in F_m and no effect on F_o) from *Oxalis* leaves that were prevented from folding down during a sunfleck, leading Powles and Björkman (1981) to conclude that there was "photoinhibitory injury". This assessment of fluorescence, however, was following 5 min dark adaptation before freezing in liquid nitrogen – had F_o' been assessed without dark adaptation, it likely would have been found to have decreased during exposure to high light as well (see below). There was nothing in the Powles and Björkman (1981) study to suggest, as Murchie and Niyogi (2011) stated, based on the hypothetical analysis of Raven (1994), "that the metabolic costs of high light avoidance movements by leaves during sunflecks were low compared to the repair costs of photodamage if the leaf did not move". In the Powles and Björkman (1981) study, the cost of leaf movement was not assessed, the cost of photodamage (if there was any) was not determined, foliar carbohydrate levels were not measured, and the employment of the VAZ cycle in energy dissipation was not ascertained. We found a strong, linear correlation between increases in foliar zeaxanthin content and decreases in F_m fluorescence (i.e., increases in NPQ reflective of photoprotective energy dissipation) in low-light (never exceeding 50 μmol photons m^{-2} s^{-1}) acclimated leaves of *Oxalis* in response to exposure to high light (Demmig-Adams and Adams 1994b). We would, nonetheless, concur with the other conclusion from the Powles and Björkman (1981) study, that "the leaf-folding response has the additional advantage of improving the water and heat balance of the leaves during exposure to high radiation" for such a mesophytic, herbaceous species. This species is faced with a long period of reduced water availability during the hottest months of the summer on the floor of forests growing along the west coast of the United States, even in the temperate rainforest of Washington (WW Adams and B Demmig-Adams, personal observation).

Furthermore, our characterization of plants growing on the deeply shaded floor of a subtropical rainforest that received few sunflecks (Logan et al. 1997) and the more open floor of a *Eucalyptus* forest punctuated with frequent, high-intensity sunflecks (Adams et al. 1999) showed strong employment of zeaxanthin (and antheraxanthin) associated energy dissipation (including decreases in F_o' during the sunflecks) that was rapidly disengaged for resumption of efficient light collection once the sunfleck had passed. Moreover, Pearcy and colleagues (Pearcy and Calkin 1983; Pearcy 1987; Valladares et al. 1997; Way and Pearcy 2012) have convincingly demonstrated that (1) there is a considerable enhancement of photosynthetic CO_2 fixation during sunflecks and (2) such sunfleck-enhanced photosynthesis can be responsible for up to a third of a leaf's total daily carbon gain (for plants growing in the understory, or for leaves in the inner canopy of a plant). And, as we have shown above (Figs. 23.9, and 23.10), a leaf acclimated to only 0.5 % of full sunlight subsequently exposed to high light accumulates more and more non-structural carbohydrates while maintaining the photosynthetic apparatus in a low efficiency state likely reflective of strong engagement of sustained energy dissipation. Thus, from a photosynthesis and productivity perspective, sunflecks are likely to be beneficial for plants growing in the forest understory, unless the exposure to increased radiation exacerbates another stress factor (e.g., insufficient water supply, excessively high temperatures) that is detrimental to survival.

VII Concluding Remarks

Figure 23.11 provides a schematic overview of many (but not all) of the experimental treatments reviewed in this chapter leading to the accumulation of foliar carbohydrates that can, in turn, provide feedback regulation to bring the activity of photosynthesis back into balance with the utilization of the products of photosynthesis by the rest of the plant. Such feedback can arise from the interaction of inherent genetically determined constraints (e.g., the architecture of the xylem supplying water and nutrients to the plant and of the phloem receiving sugars from source tissues and distributing those sugars to the sink tissues, plant phenology, and rate of growth, all of which are, however, subject to modulation by the environment) and environmental factors and/or experimental manipulations that can lead to imbalances in the source/sink activity of the whole plant. Experimental manipulations resulting in elevated levels of foliar carbohydrates have included removal of active sinks, feeding of sugars to the leaves, inhibition of foliar sugar export through girdling (not depicted in Fig. 23.11), exposure of leaves to elevated atmospheric carbon dioxide or continuous or high levels of light, exposure of the whole plant to low temperature (not depicted in Fig. 23.11), and subjecting plants to limited water or essential nutrients.

Plants can experience such imbalances through naturally occurring biotic and/or abiotic stresses. Biotic stresses can include, but are likely not limited to, herbivory of phloem and/or sink tissues, infection of the phloem and/or sink tissues by pathogens (fungi, bacteria, viruses, e.g., Rodriguez et al. 2010), infection of the xylem by fungi (reducing the supply of water to growing tissues), modification of the plant canopy by nest builders (insects, birds, mammals), modification of the plant architecture by parasitic plants, and trampling/dislodging of plants by large animals or herds of animals.

Abiotic stresses that can result in the loss of actively growing sinks include land shifts and rock slides, avalanches, swiftly flowing floodwaters, and severe weather events that involve strong winds, sand, hail, heavy snowfall, and/or driving rain. Growth in sites with less than completely favorable soil conditions can also limit growth of the roots and the entire plant. Such sites include those with limited soil volume (e.g., rock outcrops, or epiphytic sites on trees or shrubs) as well as sites with compacted soil that inhibit root growth, sites with an insufficient supply of water (see Hartmann et al. 2013 for maintenance of stored carbohydrate during drought) or essential nutrients (see Morales et al., Chap. 27), sites with excess nutrients or non-essential elements that reach levels that result in an inhibition of growth (see Morales et al., Chap. 27), and sites that become flooded with excess water (for species that are not acclimated to grow in an aquatic habitat, e.g., Rengifo et al. 2005) resulting in an inhibition of root functioning through a lack of oxygen to support aerobic respiration. Furthermore, as plants grow, they can eventually reach a size that exceeds their ability to supply water to the upper portions of a plant once a height is reached that is beyond the capacity of that species' vessels and/or tracheids to maintain the hydraulic conductivity required between the soil and the atmosphere, leading to a diminution of sink activity. Several other envi-

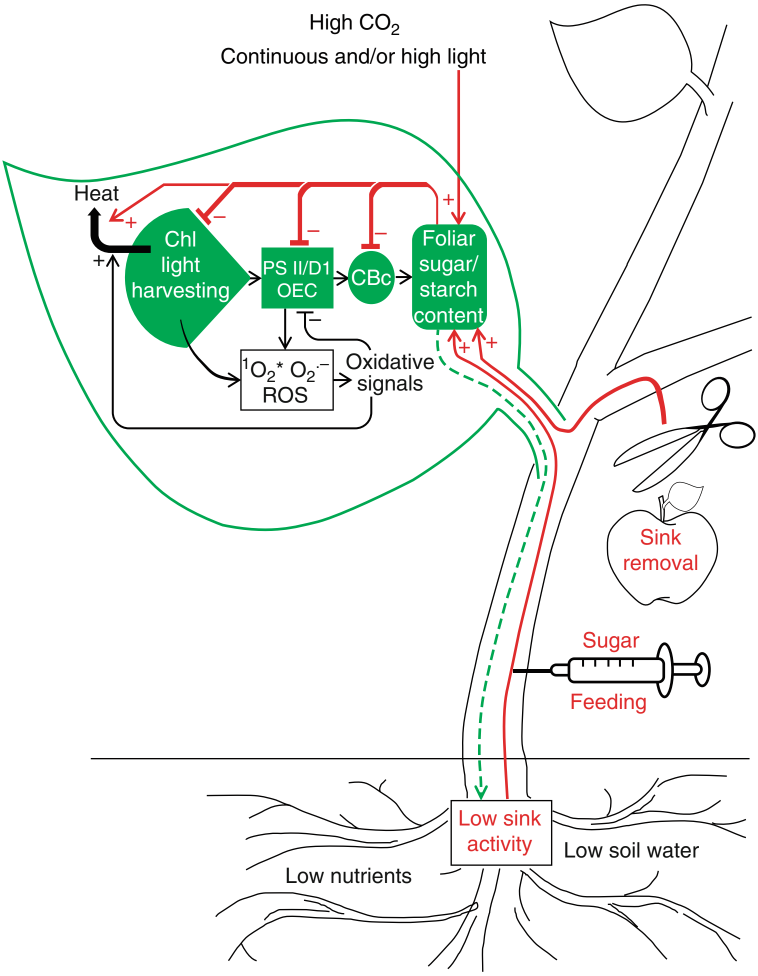

Fig. 23.11 Scheme depicting the impact of foliar carbohydrate accumulation on the components of photosynthesis (downregulation of enzymes of the Calvin-Benson cycle [*CBc*], key proteins of the electron transport chain [D1 and the oxygen evolving complex = *OEC*], and the light-harvesting chlorophyll-protein complexes [limited to some species], and increase in thermal energy dissipation [Heat, assessed through changes in non-photochemical quenching of chlorophyll fluorescence or decreases in PS II efficiency as the ratio of variable to maximal chlorophyll fluorescence] facilitated by zeaxanthin) brought about through experimental manipulations and/or environmental factors. If feedback regulation of photosynthesis as a result of the accumulation of the reduced carbon products of photosynthesis is insufficient, greater levels of the reactive oxygen species (*ROS*) singlet excited oxygen ($^1O_2^*$) and superoxide ($O_2^{\cdot-}$) will be formed leading to the generation of more oxidative signaling molecules that will upregulate thermal energy dissipation as well as lead to decreased levels of electron transport components such as D1. The *green dashed line* represents the export and transport of sugars from the leaves to sinks, the *red lines* represent positive (+) or negative (−) feedback on the accumulation of foliar carbohydrates or on components of the photosynthetic process and its photoprotection, and *black lines* represent the generation of ROS and the impact of the resulting oxidative signals on thermal energy dissipation and the D1 protein. For additional details regarding experimental manipulations and environmental conditions that can result in increased levels of carbohydrates in source leaves, see the text.

ronmental conditions can also lead to source activity that exceeds sink activity, including exposure of plants in the shade of a forest floor to high light through a sudden opening in the canopy when a tree falls, exposure to low temperature that arrests growth (and also limits sugar transport through the phloem as viscosity of the phloem sap increases), and freezing temperatures that further exacerbate the impact of non-freezing low temperature as well as eliminating the supply of (liquid) water from the soil.

The imbalance between the absorption of more light energy than can be utilized through photosynthesis, and the utilization of the products of photosynthesis by the rest of the plant, leads to a compensatory increase in photoprotective zeaxanthin-associated thermal energy dissipation that is reflected in increased levels of NPQ and decreased levels of PS II efficiency as long as the absorbed light is excessive (dynamic photoinhibition). When these imbalances become prolonged, this photoprotective thermal energy dissipation can become locked into a sustained form that remains engaged in a state primed for dissipation even in darkness (reflected in dark-sustained depressions in PS II efficiency). Furthermore, the key proteins involved in generating electrons for electron transport (the oxygen evolving complex) and in facilitating the acceptance of those electrons into the electron transport chain (D1) are often concomitantly removed. This sustained condition is commonly referred to as chronic photoinhibition (or simply photoinhibition). As we noted above, such a state of photoinhibition is more likely to be entered into by the leaves (or photosynthetic stems) of evergreen species that remain green in the face of environmental conditions that restrict growth whereas mesophytic species typically lower their chlorophyll content (and thus light-harvesting capacity), allow leaves to senesce, or finish their life-cycle completely and thereby avoid the need for locking-in photoprotective energy dissipation.

What do we actually know about the cost of photoinhibition? If – as shown here – photoinhibition is typically correlated with foliar starch and/or sugar accumulation in whole plants (at least in evergreen species, as well as some mesophytic species as shown above) under physiologically relevant conditions, the following possibilities should be considered: (1) Photoinhibition may be a consequence, rather than a cause, of an imbalance between the production and consumption of photosynthate, and (2) photoinhibition, through reduced light-harvesting efficiency and inactivation of photosystem II and of the oxygen-evolving complex, decreases the level of reactive oxygen species formation and or oxidative signal/messenger production (Adams et al. 2004, 2006, 2013b; Demmig-Adams et al. 2013). Future research should address (i) how common the association between foliar carbohydrate accumulation and either locked-in NPQ (in evergreens) or decreased chlorophyll content (e.g., in annuals) is, (ii) whether photoinhibition is induced by the same carbohydrate-related signals that trigger repression of photosynthetic genes (such as those encoding the chlorophyll binding proteins and the D1 protein; Krapp and Stitt 1995; Kilb et al. 1996), and (iii) to what extent lipid-peroxidation-derived oxylipin signals produced under photoinhibitory conditions contribute to a further modulation of plant development and reproduction (Demmig-Adams et al. 2013; see also Demmig-Adams et al., Chap. 28). One clear conclusion from the findings reported here is that additional study of photoinhibition in the whole plant context should be undertaken.

Acknowledgments

The research of BD-A and WWA was supported by the National Science Foundation (Award Numbers IOS-0841546 and DEB-1022236) and the University of Colorado at Boulder. We also thank Erik Murchie for his suggested improvements to the chapter.

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Chapter 24

Non-Photochemical Fluorescence Quenching in Contrasting Plant Species and Environments

Barbara Demmig-Adams*
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA

Seok-Chan Koh
Department of Biology, Jeju National University, Jeju 690-756, South Korea

Christopher M. Cohu
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA
Dow AgroSciences, Portland, OR, USA

Onno Muller
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA
Institute of Bio- and Geosciences, IBG-2: Plant Sciences, Forschungszentrum Jülich GmbH, Jülich 52425, Germany

and

Jared J. Stewart and William W. Adams III
Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA

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*Author for Correspondence, e-mail: barbara.demmig-adams@colorado.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_24, © Springer Science+Business Media Dordrecht 2014

Summary

This chapter summarizes the striking variation in the modulation of plant light-harvesting efficiency in nature. Extent and kinetics of changes in light-harvesting efficiency vary strongly in response to plant genetic background and plant growth environment. Concerning genetic differences, rapidly growing annuals possess higher maximal capacities for photosynthesis than slowly growing evergreens. Conversely, slowly growing evergreens possess much higher capacities for thermal dissipation of excess excitation energy (assessed via non-photochemical quenching of chlorophyll fluorescence, NPQ, in conjunction with decreases in intrinsic photosystem II efficiency). With regard to effects of growth environment, the ability to rapidly modulate light-harvesting efficiency depends on regular exposure to excess light during plant development. Leaves grown in natural sunlight, as well as leaves grown in natural shade (typically including periods of exposure to excess light during sunflecks), are capable of modulating of light-harvesting efficiency within seconds to minutes. In contrast, leaves grown under controlled conditions without regular exposure to excess light take many hours to strongly lower their light-harvesting efficiency when exposed to high light, and at least as many hours to return to a high light-harvesting efficiency. Some of the most long-lasting depressions in light-harvesting efficiency in nature – enduring throughout an entire season – are seen in overwintering evergreens. We furthermore illustrate that overwintering evergreens show similar features as photoinhibited leaves of evergreens grown under low, non-fluctuating light and subjected to prolonged experimental high light exposure. For all conditions described here for intact leaves, low intrinsic photosystem II efficiency is closely correlated with high levels of NPQ and high foliar zeaxanthin levels – irrespective of the widely different kinetics of onset and relaxation.

Abbreviations: A – Antheraxanthin; Chl – Chlorophyll; CP26, CP29 – Chlorophyll-binding proteins of molecular weights of 26 kDa and 29 kDa, respectively, as minor light-harvesting proteins associated with photosystem II; ELIP – Early light-inducible protein (light stress-responsive member of the family of light-harvesting proteins); F – Steady-state chlorophyll fluorescence under actinic illumination; F_o, F_o' – Minimal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_m, F_m' – Maximal chlorophyll fluorescence in the dark- and light-adapted state, respectively; F_v, F_v' – Variable chlorophyll fluorescence in the dark- ($F_m - F_o$) and light-adapted ($F_m'–F_o'$) state, respectively; F_v/F_m, F_v'/F_m' – Interpreted to be intrinsic efficiency (or quantum yield) of photosystem II in the dark- and light-adapted state, respectively; HLIP – High light-inducible protein (light stress-responsive member of the family of light-harvesting proteins); LHC – Light-harvesting complex; LHCII – Major light-harvesting complex of photosystem II; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OEC – Oxygen-evolving complex; PFD_i – Incident photon flux density; PS II – Photosystem II; qE, qI, qN – Quenching coefficients for energy-dependent (E), photoinhibitory (I), and non-photochemical (N) quenching of chlorophyll fluorescence, respectively; V – Violaxanthin; Z – Zeaxanthin

I Introduction

The molecular mechanisms of plant photoprotection have received much attention in studies of the modulation of light-harvesting efficiency via harmless thermal dissipation of excess excitation energy (de Bianchi et al. 2010; Ballottari et al. 2012; Horton 2012; Jahns and Holzwarth 2012; Ruban et al. 2012; Papageorgiou and Govindjee, Chap. 1; Horton, Chap. 3; Polivka and Frank, Chap. 8; Walla et al., Chap. 9; Robert et al., Chap. 10; Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14; van Amerongen, Chap. 15; Ruban and Mullineaux, Chap. 17). By necessity, many mechanistic studies are conducted using isolated components from plants grown under controlled conditions. To judge the involvement of various possible molecular mechanisms in the thermal dissipation of excess excitation energy in nature, knowledge of the responses of intact plants under natural conditions is needed. Numerous studies have been conducted on the responses of individual plant species in specific natural environments (see e.g., Morales et al., Chap. 27). The authors of the present chapter paint a comprehensive picture of plant responses in nature from an ecological and evolutionary vantage point (see also Demmig-Adams et al. 2012; García-Plazaola et al. 2012; Murchie and Harbinson, Chap. 25). We explore the astounding variation in thermal dissipation of excess excitation energy in nature and ask what the selective advantages of each different manifestation may be. We examine differences in how strongly and how fast light-harvesting efficiency is modulated (via apparent increases and decreases in thermal energy dissipation) between plants with different inherent rates of growth and photosynthesis and between plants grown in different light environments. We compare the extent of, and kinetics of changes in, (i) non-photochemical quenching of chlorophyll fluorescence (NPQ) as a measure of thermal dissipation of excess excitation energy, (ii) intrinsic photosystem II efficiency (from the ratio of variable to maximal chlorophyll fluorescence) as a measure of light-harvesting efficiency, and (iii) foliar accumulation of the xanthophyll pigment zeaxanthin.

II Principal Differences in the Allocation of Absorbed Light to Photosynthesis Versus Thermal Dissipation Between Annuals and Evergreens

Growth form and life span have a profound impact on how much absorbed light plants allocate to either photosynthesis (photochemistry and electron transport) or dissipation as heat, chiefly via thermal de-excitation of singlet excited chlorophyll (Chl) *a*. Annual plants grow rapidly throughout most of their short lifespan, and respond to increased growth light availability (from intermediate to high intensity) by further increasing their intrinsic photosynthetic capacity (Fig. 24.1a), thus presumably avoiding a need to increase their capacity for alternative, thermal dissipation (Fig. 24.1b). In contrast, long-lived evergreens have lower maximal growth rates than annuals, and respond differently from annuals to increases in growth light intensity. The evergreen characterized in Fig. 24.1, unlike the annual, does not increase its intrinsic photosynthetic capacity upon increased light availability (from intermediate to high growth light intensities; Fig. 24.1c); instead, it exhibits exceptionally strong increases in its intrinsic capacity for dissipation of excess absorbed light as heat (assessed by non-photochemical quenching of Chl fluorescence; Fig. 24.1d). The evergreen thus exhibits its maximal photosynthetic capacity already at the intermediate growth light intensity of 300 μmol photons m^{-2} s^{-1}, and is apparently incapable of further increases in intrinsic photosynthetic capacity under an even higher growth light intensity of 1,500 μmol photons m^{-2} s^{-1} (Demmig-Adams et al. 2006).

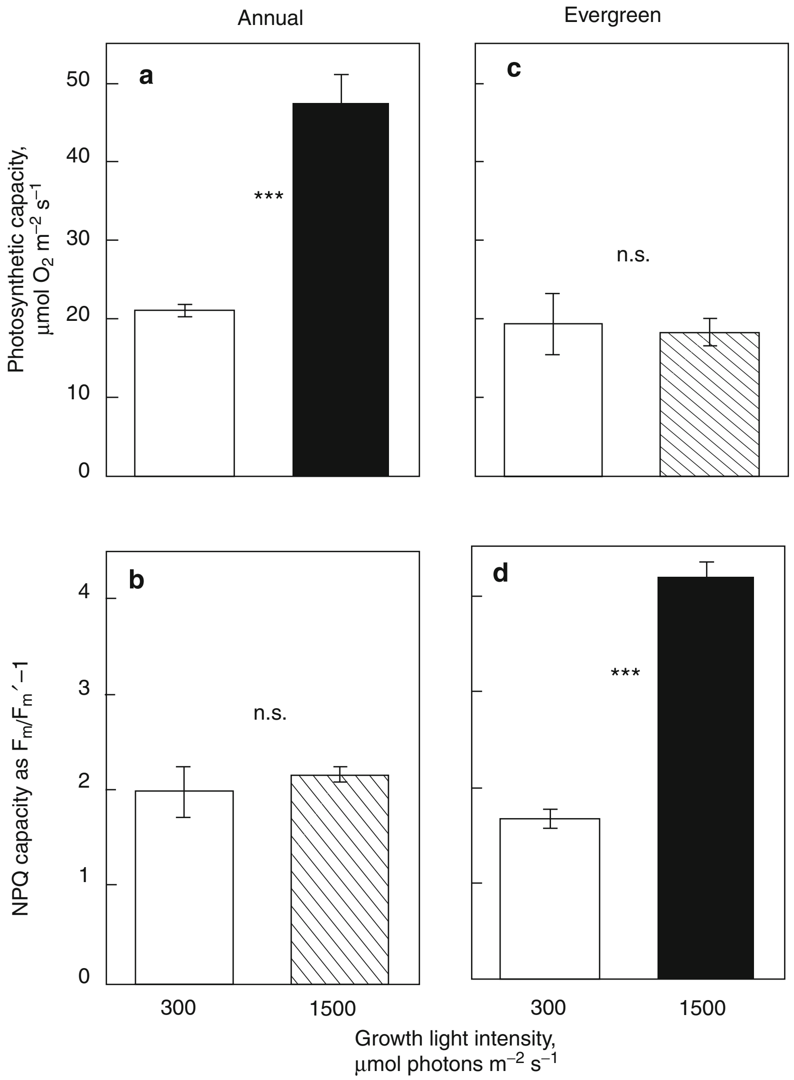

Fig. 24.1. (**a**, **c**) Maximal photosynthetic capacity (light- and CO_2-saturated rate of oxygen evolution) and (**b**, **d**) capacity for non-photochemical quenching (*NPQ*) of chlorophyll fluorescence (calculated as $F_m/F_m{}' - 1$; ascertained from excised leaf disks in a temperature-controlled chamber under 2 % O_2, no CO_2 to minimize photosynthetic electron transport and photochemical quenching, and to be able to assess the maximal level of NPQ) in spinach leaves (annual mesophyte; **a**, **b**) and *Monstera deliciosa* (evergreen; **c**, **d**) grown in a greenhouse under moderate light (peak of 300 μmol photons m^{-2} s^{-1} in an area fitted with a layer of shade cloth) or high light (peak of 1,500 μmol photons m^{-2} s^{-1} in a fully sunlit-area). Mean ± standard deviation shown (*n.s.* non-significant, *** significant at $p < 0.001$; *t*-test). Data from Demmig-Adams et al. (2006).

The above findings suggest that both annuals and evergreens possess potent protection against adverse effects of high growth light intensities – the annual mainly by virtue of utilizing the additional light for photosynthesis and growth, and the evergreen mainly by virtue of employing an unusually high capacity of thermal dissipation far exceeding the maximal dissipation capacity of annuals. To determine the maximal capacity of thermal dissipation, non-photochemical fluorescence quenching was assessed in the absence of CO_2 at low levels of oxygen, which minimizes electron transport supported by CO_2 fixation or photorespiration. This approach permits assessment of the maximal capacity

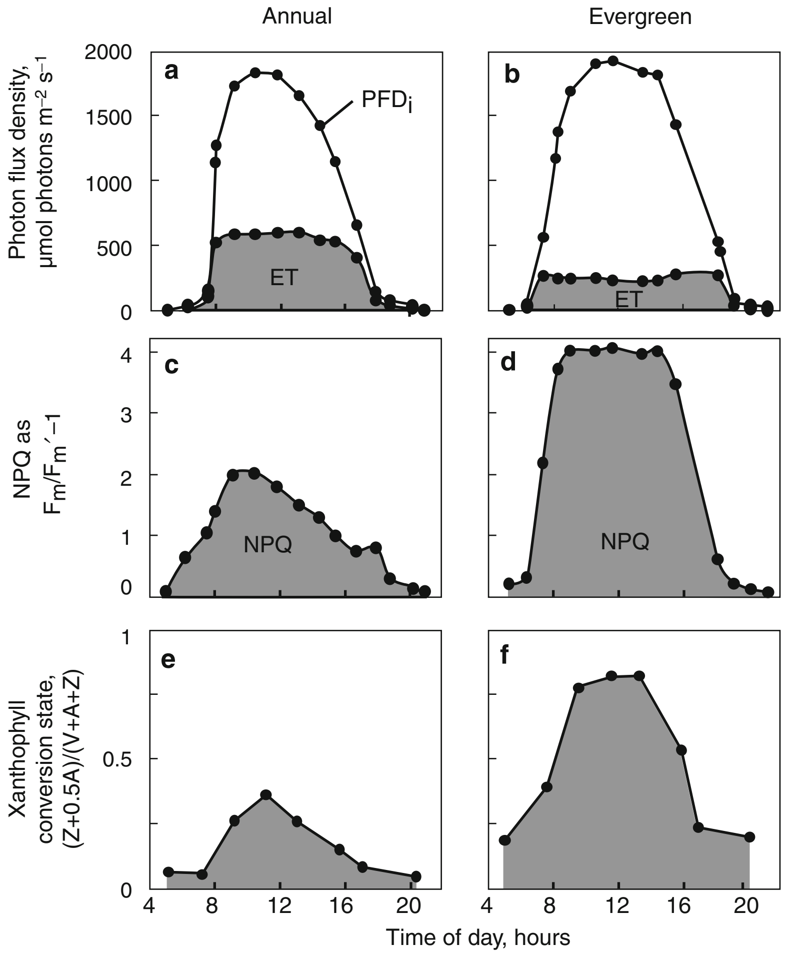

Fig. 24.2. (**a**, **b**) Incident photon flux density (PFD$_i$) on the leaf surface, and estimated utilization of absorbed light through photosystem II (PS II) photochemistry (*ET* electron transport; as estimated rate of PS II photochemistry, from $(F_m'-F)/F_m' \times PFD_i$), (**c**, **d**) level of in-situ NPQ (as $F_m/F_m'-1$), and (**e**, **f**) xanthophyll conversion state (zeaxanthin + antheraxanthin) / (violaxanthin + antheraxanthin + zeaxanthin) measured over the course of an entire day in an annual (sunflower; **a**, **c**, **e**) and in the evergreen groundcover *Vinca major* (**b**, **d**, **f**), both from south-facing leaves growing in locations exposed to full natural sunlight. Data from Demmig-Adams et al. (1996).

of thermal dissipation when electron transport rates and photochemical quenching are minimal (only enough to build up the trans-thylakoid pH gradient required for NPQ formation; Krause and Jahns 2004; Li et al. 2004) to allow a side-by-side comparison of NPQ capacity in leaves with widely different electron transport capacities.

Similar differences in photosynthesis and thermal dissipation between annuals and evergreens as illustrated for potted plants grown in a naturally-lit greenhouse (Fig. 24.1) shown above are also seen over the course of a sunny day in situ in plants growing outdoors under natural field conditions (Fig. 24.2). When growing side-by-side in full sunlight, annuals again utilize a greater fraction of absorbed light for photosynthetic electron transport (and photochemical quenching) than evergreens (Fig. 24.2a, b). Correspondingly, higher maximal levels of non-photochemical quenching (as a measure of thermal dissipation of excess absorbed light) are seen in the evergreen, with its lower photosynthesis rates, than in the annual, with its higher photosynthesis rates (Fig. 24.2c, d). Formation of zeaxanthin and antheraxanthin via xanthophyll conversions mirrors increases and decreases in NPQ over the course of the day – and greater maximal levels of zeaxanthin and antheraxanthin in the evergreen match the evergreen's greater maximal levels of NPQ (Fig. 24.2e, f; Demmig-Adams et al. 1996). Moreover, only in the evergreen does the entire pool of the xanthophylls (violaxanthin [V] + antheraxanthin [A] + zeaxanthin [Z]) become converted to Z or Z+A under peak irradiance at midday (Fig. 24.2f), while the annual converts a much lesser fraction even at peak irradiance (Fig. 24.2e). The latter observations are consistent with the notion that zeaxanthin and antheraxanthin are involved in thermal energy dissipation (see, e.g., Demmig-Adams and Adams 2006; de Bianchi et al. 2010; Ballottari et al. 2012; Demmig-Adams et al. 2012; Horton 2012; Jahns and Holzwarth 2012; Ruban et al. 2012; Horton, Chap. 3; Morosinotto and Bassi, Chap. 14; Ruban and Mullineaux, Chap. 17). Overall, these findings show that rapidly growing and photosynthesizing annuals utilize a greater fraction of the light they absorb for photosynthesis – compared to slowly growing evergreens that dissipate, via thermal dissipation, a greater fraction of the light they absorb.

Notably, neither annuals nor evergreens possess photosynthesis rates high enough to utilize all light absorbed during peak irradiance in sunny habitats even under optimal water and nutrient supply (cf. Fig. 24.2a, b). It thus appears that plants adjust their foliar chlorophyll content to maximize light absorption when irradiance is low (e.g., during morning and afternoon portions of the day), thus making light absorption during peak irradiance (over midday) highly excessive. Likewise, photosynthetic capacity is not acclimatized for full utilization of the light available during the limited time of peak irradiance, and may be adjusted to average daily light availability. Consequently, daily exposure to highly excessive light at peak irradiance, and employment of substantial levels of thermal dissipation (serving to modulate light-harvesting efficiency via safe dissipation of excess absorbed light), is a ubiquitous feature of plants growing naturally in sun-exposed habitats. In contrast, plants grown in growth chambers under low, non-fluctuating light, and without periods of excess light absorption, exhibit low maximal capacities for thermal dissipation and are unable to rapidly modulate light-harvesting efficiency, as will be discussed in the following section.

III The Ability for Strong, Rapid Modulation of Light-harvesting Efficiency Is Entrained by the Light Environment During Plant Development

A Growth Light Environment Can Affect the Capacity for Thermal Dissipation

The response of a shade plant (*Oxalis*) grown indoors under a very low, continuously non-excessive light level to exposure to a range of light intensities (photon flux density, PFD) was compared with that of a sun plant (*Malva*) grown outdoors in full sunlight. Rates of photosystem II (PS II) electron transport (estimated from photochemical quenching of Chl fluorescence) increased to high maximal levels in sun-grown *Malva*, but reached only a very low plateau in shade-grown *Oxalis* (Fig. 24.3a). Sun-grown *Malva* also exhibited a much greater maximal capacity for NPQ (and thus presumably a greater thermal dissipation capacity) than shade-grown *Oxalis* (Fig. 24.3b).

Moreover, similar differences exist between plants of the same species (e.g., the rainforest evergreen *Monstera deliciosa*) grown under either very low light (10 μmol photons m^{-2} s^{-1}) indoors or under high light levels in a naturally lit

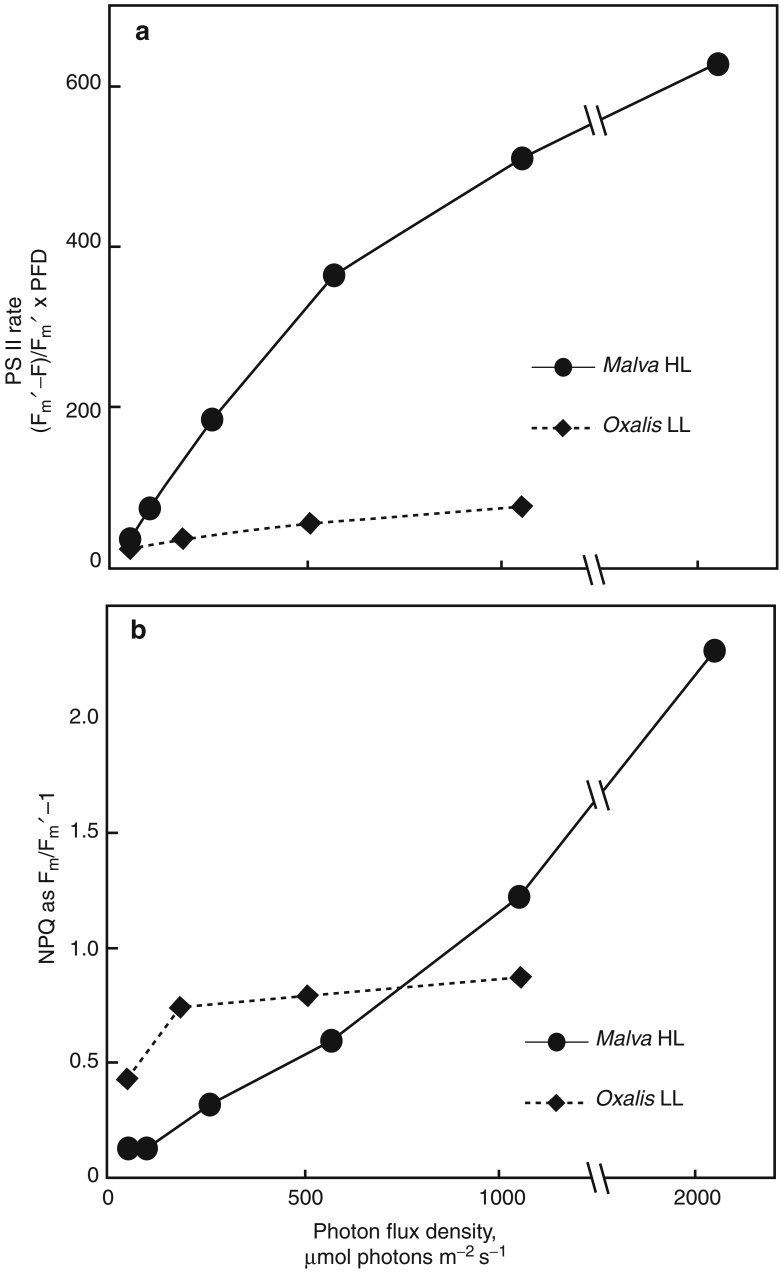

Fig. 24.3. (**a**) Estimated rate of PS II photochemistry, as $(F_m{}' - F)/F_m{}' \times$ incident photon flux density (*PFD*), and (**b**) capacity for NPQ $(F_m/F_m{}' - 1)$, both measured at different photon flux densities, for *Malva neglecta* grown outdoors in full sunlight (*HL*) and *Oxalis* cf. *trilliifolia* Hook. grown indoors under low light (*LL*). *Oxalis* data from Demmig-Adams and Adams 1994; *Malva* data unpublished from B. Demmig-Adams and W.W. Adams.

glasshouse (with the typical strong variation in light intensity over the course of the day and midday peaks of 1,500 μmol photons m^{-2} s^{-1}). Sun-grown *Monstera* shows a considerably higher capacity of NPQ (indicative of a higher thermal dissipation capacity) than low-light-grown *Monstera* (Fig. 24.4). Together, these findings indicate that growth in natural sunlight (with ample water and nutrient supply)

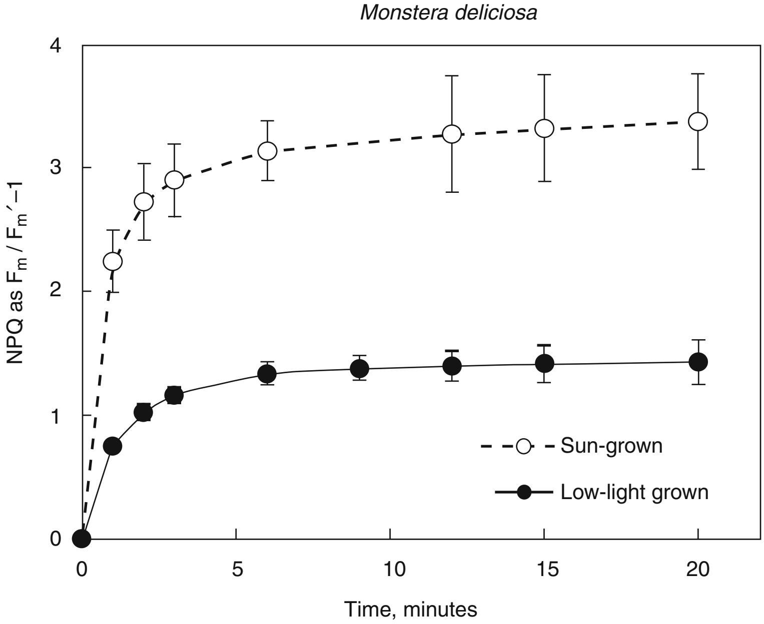

Fig. 24.4. Non-photochemical quenching (*NPQ*) of chlorophyll fluorescence (calculated as $F_m/F_m' - 1$) for *Monstera deliciosa* leaves grown in full sunlight in a naturally lit greenhouse (sun-grown; *open circles*) and grown indoors under low light (low-light grown; *black circles*). Mean ± standard deviation shown for each time point over a 20-min measurement period in a temperature-controlled chamber under 2,425 μmol photons m^{-2} s^{-1}, 2 % O_2, no CO_2 (causing the final NPQ level to represent the maximal capacity for NPQ of these respective leaves).

induces maximal capacities for both photosynthesis and thermal dissipation – while growth under low, non-fluctuating light levels indoors leads to minimal capacities for photosynthesis and thermal dissipation (see also Demmig-Adams 1998; Demmig-Adams et al. 1998, 2006). Nevertheless, maximal NPQ in *Monstera* grown in low, non-fluctuating light (Fig. 24.4) is similar to the maximal level of NPQ seen in the sun-grown annual (cf. Fig. 24.2c, shown above, for maximal NPQ levels in an annual growing in full sunlight), and the sun-grown evergreen thus displays much higher NPQ levels than either evergreens grown indoors under low, non-fluctuating light or sun-grown annuals.

The above conclusions apply to comparison of leaves of sun-grown plants with leaves of plants grown without any exposure to excess light, as is the case in leaves of plants grown indoors under low, non-fluctuating light levels. However, this does not apply to comparison with leaves of plants growing outdoors in shaded locations, where regular intermittent exposure to excessive light is common (see section III.C below).

B Plants Grown Under Low, Non-Fluctuating Light Are Incapable of Pronounced, Rapid Modulation of Light-harvesting Efficiency

Unlike leaves grown in natural, fluctuating light, leaves of evergreens grown indoors under low, non-fluctuating light levels are incapable of quickly lowering light-harvesting efficiency (via thermal dissipation) upon high light exposure. Furthermore, these latter leaves fail to quickly return to high light-harvesting efficiency when returned to low light after prolonged high light exposure (Fig. 24.5a). In contrast, leaves of the same species grown in a sun-lit glasshouse, with daily exposure to excess light, are capable of rapidly and strongly lowering light-harvesting efficiency via exceptionally high levels of thermal dissipation upon high light exposure, as well as rapidly recovering a high light-harvesting efficiency upon return to low light (Fig. 24.5a). Similar differences between sun-grown plants and plants grown under low, non-fluctuating light exist in the kinetics of their increases in NPQ upon exposure to high light and subsequent decreases in NPQ upon return to low light (Fig. 24.5b). Moreover, the conversion of violaxanthin to zeaxanthin and antheraxanthin (Z+A; Fig. 24.5c), with Z+A increasing in high light and decreasing again in low light, also takes place with very different kinetics in the two sets of plants from contrasting growth light environments. Overall, F_v'/F_m', NPQ, and Z+A changed in concert with each other throughout the entire experiment in either set of plants. The principal adjustments to high light and subsequent return to low light are similar in both sun and shade leaves. However, only the excess-light-acclimated sun leaf is capable of undergoing very pronounced adjustments almost instantly, while the leaf of the plant grown in low, non-fluctuating light (never before exposed to excess light and thus not acclimated to excess light) requires a long time for its full adjustment to high light as well as for reversal of these adjustments upon return to low light. From the delayed return to low NPQ and low Z+A levels (Fig. 24.5b, c), it appears as though zeaxanthin-associated thermal dissipation, once slowly established, becomes "locked-in" within leaves not acclimated to excess light and exposed to high light (see also Demmig-Adams et al. 1998). It is important to note that careful control of leaf temperature against overheating is required to produce the responses seen here (without any loss of chlorophyll or leaf discoloration). If leaf temperature upon sudden transfer of a low-light-grown evergreen to high light is allowed to rise (as can occur readily in high light growth chambers), the leaf can suffer heat damage and turn brown.

The syndrome seen in the leaf grown under low, non-fluctuating light indoors thus includes a version of NPQ that develops and relaxes slowly (Fig. 24.5b), Z+A formation that develops and relaxes slowly (Fig. 24.5c), and depressions of intrinsic PS II efficiency that develop and relax slowly (Fig. 24.5a). Over the 24-h exposure to high light, this leaf (grown under low, non-fluctuating light) converts its V+A+Z pool from around 60 % Z+A during the early phase of exposure (which is similar to the maximal conversion state seen in sun-grown annuals) to the much higher levels seen in sun-exposed leaves of sun-grown evergreens (Fig. 24.5c), while simultaneously dramatically increasing its NPQ level (Fig. 24.5b). We speculate that prolonged high light exposure of a leaf grown in low, non-fluctuating light (and thus not acclimated to excess light) may induce structural rearrangements necessary for high NPQ. Judging from the very slow secondary increases in Z+A levels (Fig. 24.5c), such structural rearrangements might include allowing violaxanthin de-epoxidase to access a fraction of the V pool that may not be accessible in leaves of evergreens grown indoors under low, non-fluctuating light or in annuals grown at any light level.

Throughout much of the history of research on chlorophyll fluorescence, different sub-categories of non-photochemical quenching were defined on the basis of their widely different recovery kinetics (see, e.g., Müller et al. 2001; Jahns and Holzwarth 2012). For example, rapidly reversible NPQ dependent on a trans-thylakoid pH gradient is often termed "qE" or "qN" and is generally assumed to reflect photoprotective thermal dissipation, while slowly reversible NPQ is sometimes termed "qI" for "photoinhibitory quenching" and is assumed to involve photoinactivation of photosynthesis (Krause and Jahns 2004; Dall'Osto et al. 2010). The features shown in Fig. 24.5a–c indicate that zeaxanthin-associated NPQ and modulation of intrinsic PS II efficiency can have widely different kinetics of onset and recovery depending on the acclimation of leaves to excess light. Discussion of the molecular mechanisms of rapidly reversible versus

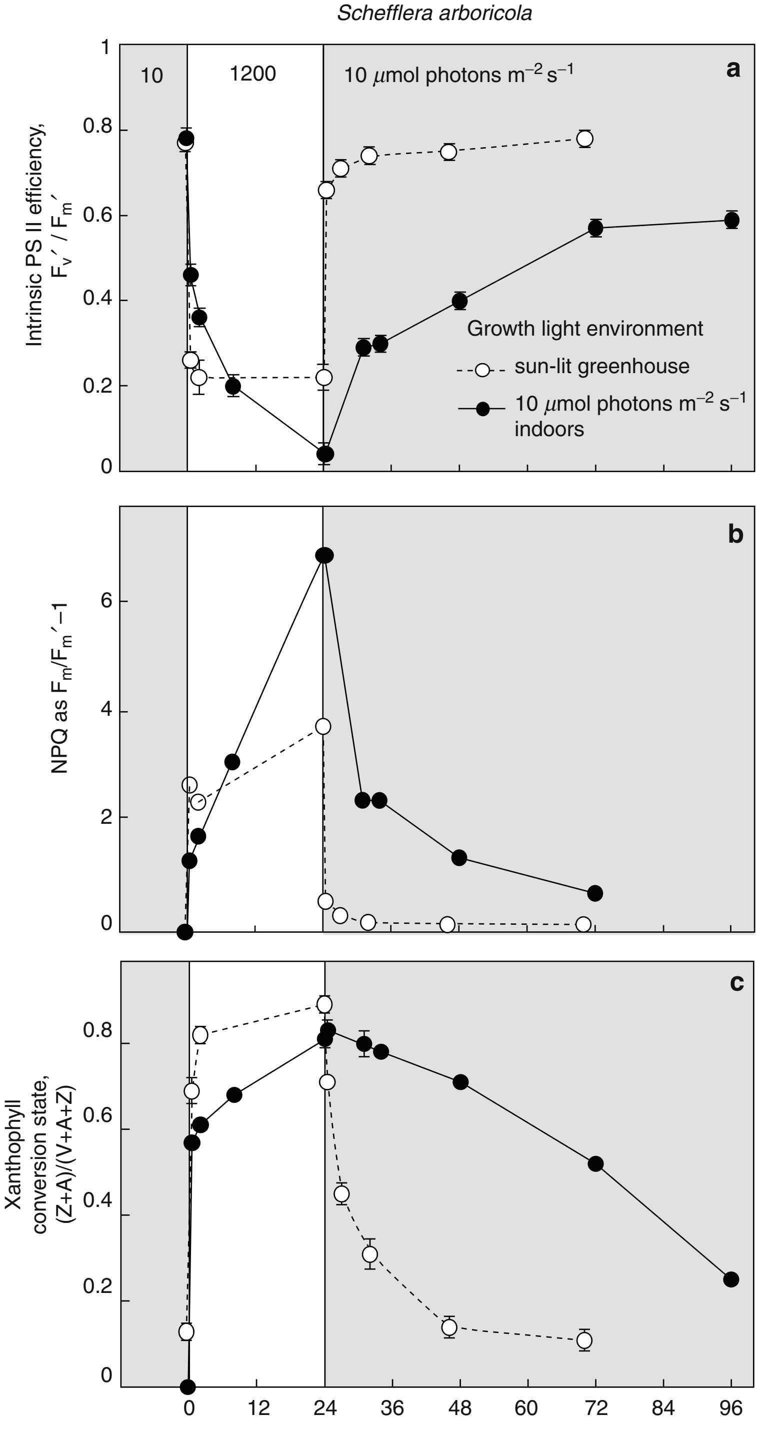

Fig. 24.5. (**a**) In situ intrinsic PS II efficiency (F_v'/F_m'), (**b**) in situ NPQ (as $F_m/F_m' - 1$), and (**c**) xanthophyll conversion state (zeaxanthin + antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin) of *Schefflera arboricola* grown indoors under a low light intensity of 10 μmol photons m^{-2} s^{-1} (*black circles*) or grown in a naturally-lit greenhouse under full sunlight with peak light intensities of about 1,500 μmol photons m^{-2} s^{-1} (*white circles*) and exposed to 1,200 μmol photons m^{-2} s^{-1} (*white region*) for 24 h in a temperature- and humidity-controlled growth chamber, followed by transfer to 10 μmol photons m^{-2} s^{-1} (*grey regions* of graph) for an additional 72 h. Data from Demmig-Adams et al. (1998).

slowly reversible adjustments to high light needs to acknowledge (i) the highly flexible kinetics of xanthophyll conversions as well as (ii) additional features associated with NPQ. Rapidly reversible (Z+A)-associated increases in NPQ (and decreases in PS II efficiency) in sun-grown leaves have been shown to be associated with changes in the trans-thylakoid pH gradient (Krause and Jahns 2004; Li et al. 2004). In contrast, slowly reversible (Z+A)-associated NPQ increases (and decreases in PS II efficiency) in leaves grown under low, non-fluctuating light levels do not depend on a trans-thylakoid pH gradient and are instead associated, e.g., with PS II protein modification (including sustained phosphorylation and, subsequently, degradation of the D1 protein of PS II, and accumulation of light-stress-related LHC proteins; Demmig-Adams et al. 2006) reminiscent of the changes in thylakoid protein composition seen in overwintering evergreens (that include sustained phosphorylation, and/or degradation of the D1 protein of PS II, degradation of the oxygen-evolving complex, and accumulation of members of light-stress-related LHC proteins, such as several early-light-inducible proteins, ELIPs, and high-light-inducible proteins, HLIPs; Ebbert et al. 2005; Zarter et al. 2006a, b, c; Wang et al. 2009). The lasting depression of intrinsic PS II efficiency seen in the latter leaves grown under low, non-fluctuating light meet the definition of "photoinhibition" of photosynthesis; the involvement of lasting maintenance of high levels of NPQ and of Z+A in photoinhibition will be discussed in section IV.

C Natural Shade Environments Promote Extremely Rapid Modulation of Light-harvesting Efficiency

In contrast to the above-described slow and persisting effects induced by high light exposure of leaves grown under low, non-fluctuating light, leaves growing outdoors in the shade of tree canopies exhibit extremely rapid adjustments to high light. Leaves under a tree canopy typically experience one or multiple sunflecks (sudden shafts of bright light piercing the canopy) over the course of each day. The appearance of such sunflecks, while seemingly random, is nevertheless repeated in a regular pattern each and every day. Figure 24.6 shows extremely rapid increases and decreases in incident PFD (Fig. 24.6a) striking understory leaves, as well as the response of leaves via almost instant increases and decreases in NPQ (Fig. 24.6b). Measurements taken on the same leaves the following day (and including brief, rapid darkening of the leaves to determine minimal chlorophyll fluorescence F_o' in the light-adapted state) reveal that both maximal (F_m') and minimal (F_o') fluorescence in the light-adapted state are quenched (Fig. 24.6c) in the manner typical for increased thermal dissipation (Adams et al. 1999). Remarkably, these excess-light-entrained shade leaves are thus capable of performing very pronounced increases and decreases in NPQ (and presumably thermal dissipation) almost instantly, closely tracking their extremely variable light environment under a tree canopy.

Xanthophyll conversions do not occur on the time scale of seconds, but instead require at least minutes to occur (Yamamoto 1979; Hager 1980). One may, therefore, wonder whether the ultra-rapid adjustments in NPQ during sunflecks experienced by understory plants occur in the absence of zeaxanthin. However, this is not the case. These understory plants exposed to a series of rapid sunflecks on a predictable daily basis in their natural environment retain, overnight, a substantial level of zeaxanthin (and antheraxanthin), and they further increase their Z+A concentrations (to conversion states higher than those seen in sun-grown annuals) in response to the first sunflecks of the day (Fig. 24.6d). The extremely rapid engagement and disengagement of NPQ in zeaxanthin-preloaded leaves receiving rapid sunflecks under a tree canopy may be facilitated by almost instantaneous increases and decreases in the trans-thylakoid pH gradient that has been linked to NPQ modulation

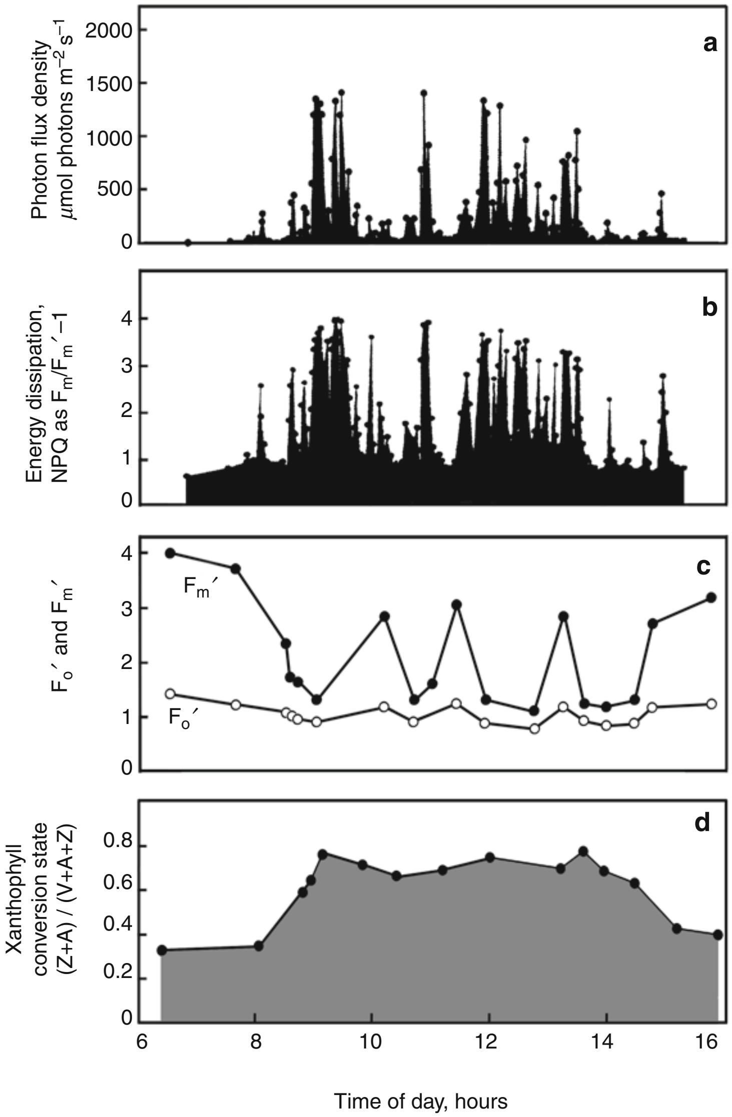

Fig. 24.6. (**a**) Incident photon flux density on the leaf surface, (**b**) in-situ NPQ (as $F_m/F_m' - 1$), (**c**) in-situ F_m' and F_o' (F_o' fluorescence obtained upon brief experimental darkening of the leaf in the field) at several time points over the course of the subsequent day in the same leaves, and (**d**) xanthophyll conversion state (zeaxanthin + antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin) over the course of an entire day in the field for a *Stephania japonica* vine growing under a canopy repeatedly pierced by shafts of intense sunlight (*sunflecks*). Data from Adams et al. (1999).

(Li et al. 2004; Kiss et al. 2008; Jahns and Holzwarth 2012). We have never observed NPQ under natural conditions in leaves free of zeaxanthin; we are also not aware of any positive evidence for the occurrence of zeaxanthin-independent NPQ in higher plants in nature.

IV Lasting Maintenance of Thermal Dissipation and Arrested Xanthophyll Conversions in Nature

A similar phenomenon to the slowly relaxing changes in leaves grown under low, non-fluctuating light and treated with prolonged high light (cf. Fig. 24.5 above) occurs in sun-exposed plants during times when there is no net carbon uptake by the leaves. Such conditions occur, for example, in temperate climates either during entire seasons, or during intermittent periods, with subfreezing temperatures (Adams and Demmig-Adams 1995). We describe below in detail that overwintering plants exhibit evidence for a continuous maintenance of high levels of NPQ and Z+A day-and-night under either intermittent or season-long subfreezing conditions. However, the accompanying mechanisms differ for intermittent versus season-long subfreezing conditions. Winter annuals and evergreens growing in environments with intermittent subfreezing periods, interspersed with milder conditions, employ a pH-dependent form of continuously maintained, (Z+A)-associated NPQ that relaxes instantly upon warming (Demmig-Adams and Adams 2006). In contrast, evergreens growing in even harsher environments with season-long subfreezing conditions arrest their growth, show no net carbon uptake for an entire season (see, e.g., Monson et al. 2002), and employ a pH-independent form of continuously maintained (Z+A)-associated NPQ that does not relax upon warming and is associated with changes in thylakoid protein composition that include sustained phosphorylation and/or degradation of the D1 protein of PS II, degradation of the oxygen-evolving complex (Ebbert et al. 2005; Zarter et al. 2006a, b, c), and accumulation of light-stress-related (ELIP and HLIP) LHC proteins (for a review, see Demmig-Adams and Adams 2006).

We will first compare a warm summer day with a subfreezing winter day in an environment where subfreezing days alternate with milder days in the winter. Figure 24.7 shows increases and subsequent decreases in NPQ (Fig. 24.7a) and in foliar Z+A concentration (Fig. 24.7b), accompanied by decreases and subsequent increases in intrinsic PS II efficiency (Fig. 24.7c), over the course of a warm, sunny summer day in the evergreen shrub *Euonymus kiautschovicus*. In contrast, on a cold winter day, high levels of Z+A (with almost the entire V+A+Z pool present as Z+A day and night) and low intrinsic PS II efficiency are maintained 24-h-a-day; the high Z+A levels and low intrinsic PS II efficiency typically seen only at midday on a summer day appear to become frozen, or "locked-in", all day and night long (Fig. 24.7e, f). The level of NPQ is likely also "locked-in" day-and-night, although NPQ cannot technically be calculated for such a case due to the low level of F_m throughout the entire day-night period (Fig. 24.7d). A portion (but not all) of the locked-in NPQ, and locked-in F_v/F_m depressions, on such a cold winter day were rapidly reversed by warming of the leaves or by treatment of leaves maintained at cold temperature with the uncoupler nigericin (Verhoeven et al. 1998). Therefore, maintenance of trans-thylakoid pH on sub-freezing winter days apparently contributes to the arrest of xanthophyll conversion, to the retention of high Z+A concentrations, and to the locking in of high NPQ and F_v/F_m depressions (see also Gilmore and Björkman 1995).

Slowly reversible changes in NPQ, Z+A, and intrinsic PS II efficiency that do ***not*** involve pH maintenance are seen under more extreme conditions (Fig. 24.8), such as those experienced by overwintering conifers growing where sub-freezing temperatures persist continuously for several winter months (Fig. 24.8a). Depression of intrinsic PS II efficiency F_v/F_m is sustained throughout the sub-freezing winter months (Fig. 24.8b); this is accompanied by low levels of F_m and F_o continuously maintained (or locked-in) 24-h-a-day for several months (Fig. 24.8c, d), and not rapidly reversible by warming of leaves (Adams et al. 1995; Verhoeven et al.1996; Zarter et al. 2006b). While, once again, NPQ cannot be technically calculated for these overwintering needles, due to the continuous low levels of dark F_m and F_o, it appears that high levels of thermal energy dissipation are also locked-in throughout sub-freezing winter months (Adams and Demmig-Adams 1994, 1995; Adams et al. 1995, 2002, 2006; Verhoeven et al. 1996, 1998; Demmig-Adams and Adams 2006; Zarter et al. 2006a, b, c). The depressed

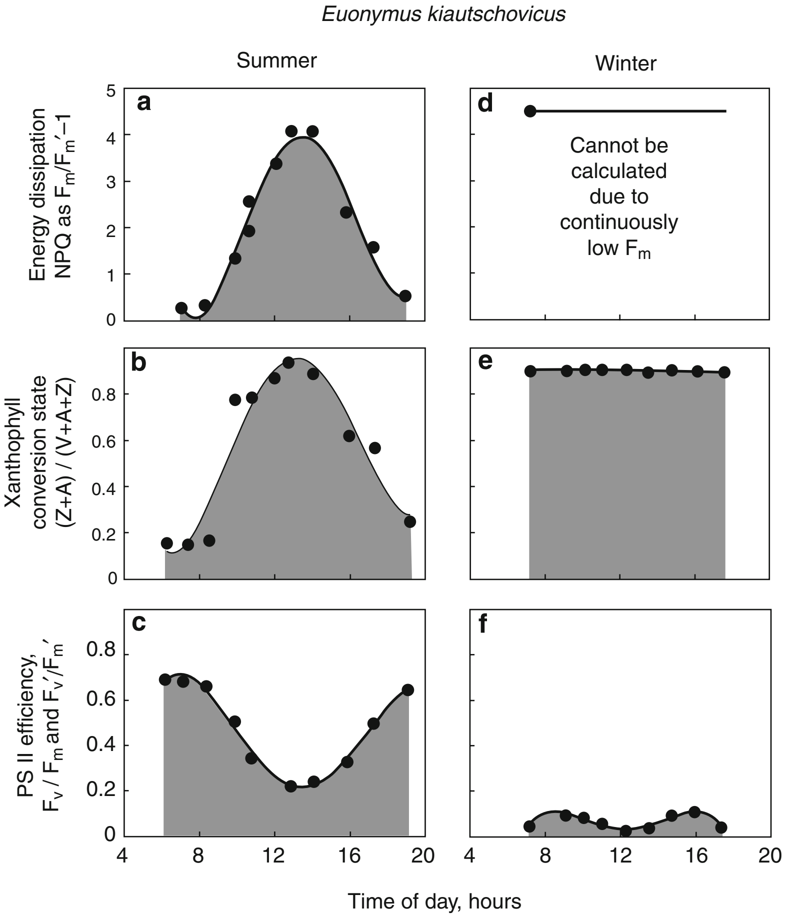

Fig. 24.7. (**a**, **d**) In-situ NPQ (as $F_m/F_m{}' - 1$), (**b**, **e**) xanthophyll conversion state (zeaxanthin + antheraxanthin) / (violaxanthin +antheraxanthin + zeaxanthin), and (**c**, **f**) in-situ intrinsic PS II efficiency (dark F_v/F_m at predawn and $F_v{}'/F_m{}'$ under natural light exposure) in leaves of the evergreen shrub *Euonymus kiautschovicus* over the course of a summer (**a**–**c**) and winter (**d**–**f**) day. Data from Verhoeven et al. (1998).

intrinsic PS II efficiency in overwintering evergreens had initially been assumed to be due to photodamage. However, the observation that sustained low PS II efficiency is accompanied by retention of Z+A led to a revision of this view, allowing for the possibility that the entire syndrome may represent a state of semi-permanent, powerful photoprotection in evergreen conifers during growth-precluding seasons (Adams and Demmig-Adams 1994; Adams et al. 1995, 2002, 2004, 2006; Verhoeven et al. 1996; Öquist and Huner 2003; Ensminger et al. 2004; Ebbert et al. 2005; Demmig-Adams and Adams 2006; Zarter et al. 2006a, b).

True to their designation as evergreens (rather than "ever-yellows"), these conifers maintain high chlorophyll contents (Fig. 24.9a) throughout the extended freezing period in the winter without growth or CO_2 uptake. While chlorophyll content is somewhat higher in the summer than in the fall and the winter, there is no difference in chlorophyll content between winter – when F_v/F_m depressions are locked-in – and spring when F_v/F_m returns to high control values and NPQ relaxes (cf. Fig. 24.8b, c). Summer and spring conditions produce little retention of Z+A overnight in darkness, whereas winter conditions lead to a pronounced arrest of

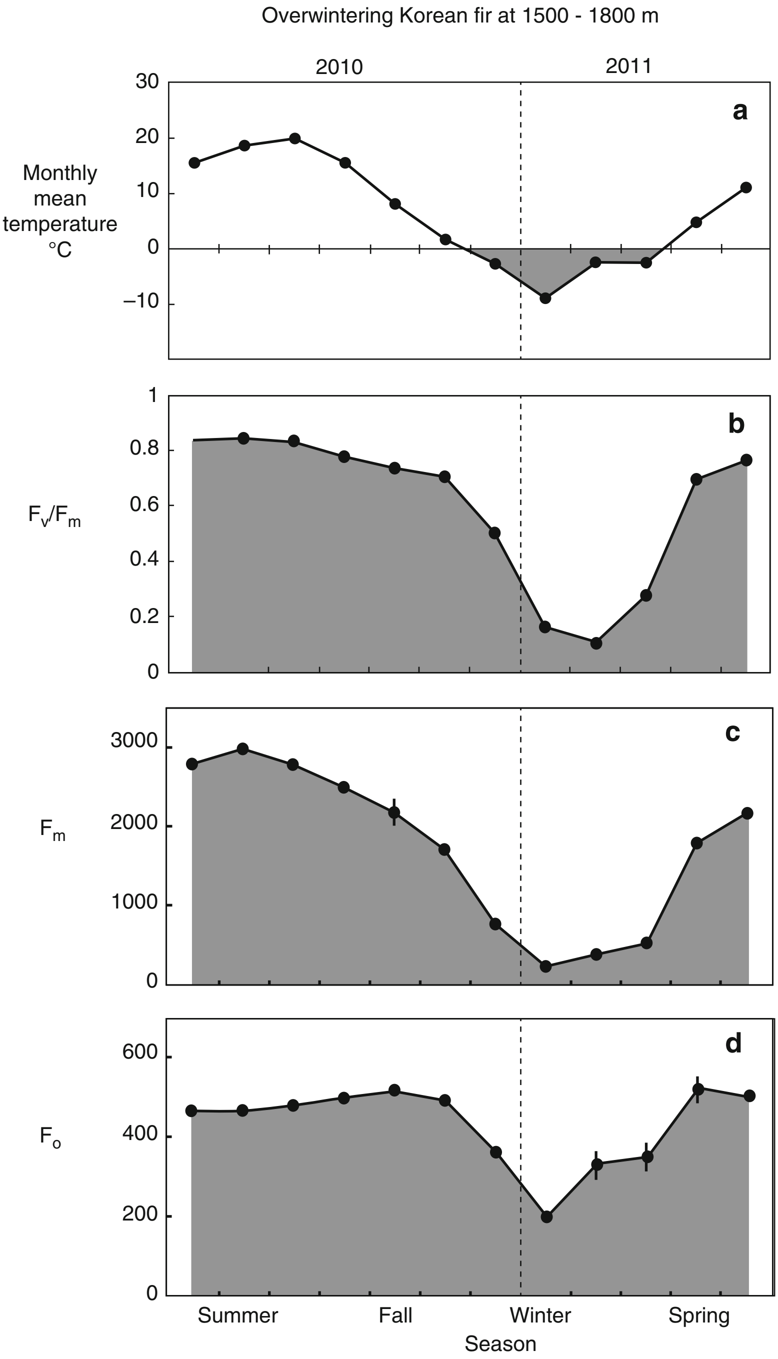

Fig. 24.8. (**a**) Monthly mean air temperature and (**b–d**) chlorophyll fluorescence (dark levels of intrinsic PS II efficiency, F_v/F_m, and dark level of maximal and minimal fluorescence yields F_m and F_o, respectively) measured monthly from early summer 2010 to late spring 2011 for needles of Korean fir trees growing at 1,500–1,800 m of elevation. Vertical dashed line represents the transition from the year 2010 to 2011. Mean ± standard deviation shown (n = 15). Data from Oh et al. (2013).

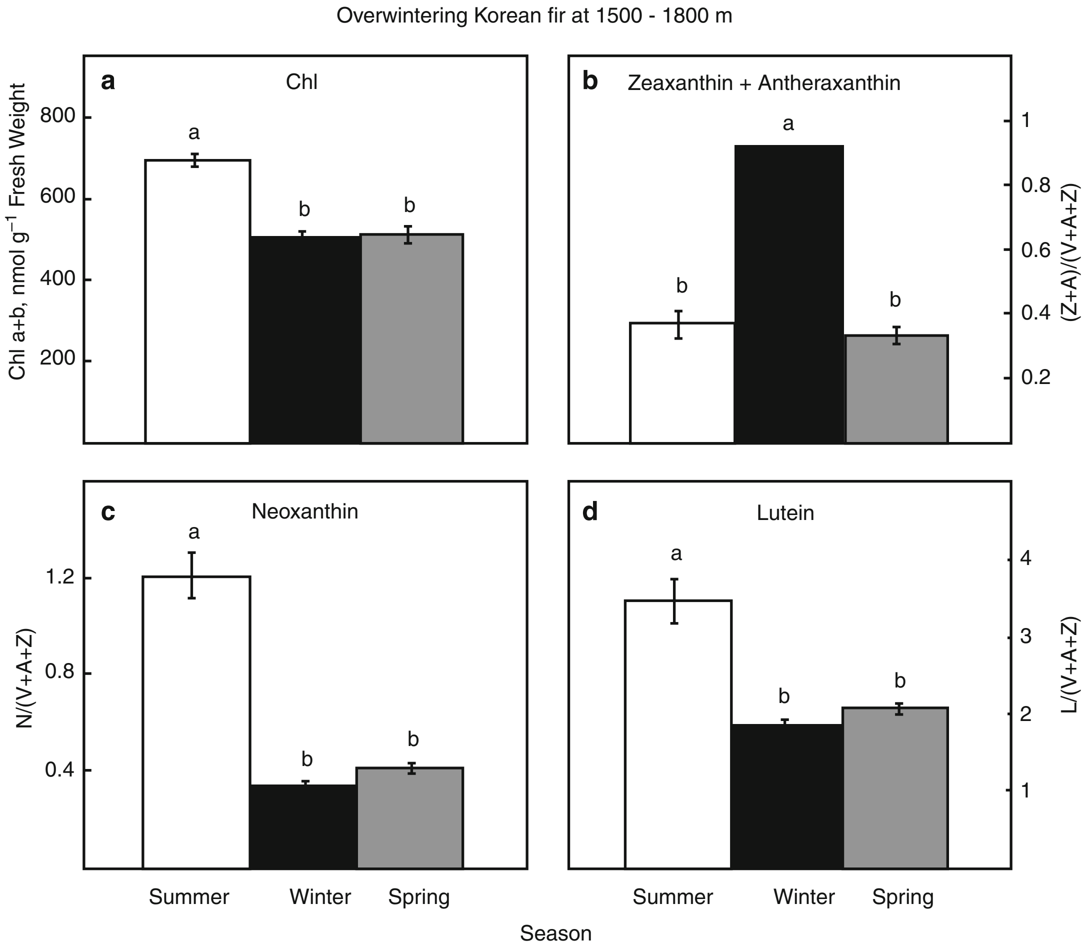

Fig. 24.9. Seasonal chlorophyll and carotenoid contents for needles of overwintering Korean fir trees growing at 1,500–1,800 m of elevation. (**a**) Chlorophyll a + chlorophyll b as nmol g^{-1} fresh weight, (**b**) zeaxanthin + antheraxanthin levels relative to the total pool of violaxanthin + antheraxanthin + zeaxanthin, and (**c**) neoxanthin and (**d**) lutein per V+A+Z pool. Mean ± standard deviation shown (n=9). Lower-case letters indicate significance at $p<0.05$. Data from Oh et al. (2013).

xanthophyll conversions and to retention of high levels of Z+A (Fig. 24.9b, again with almost the entire V+A+Z pool continuously present as Z+A) accompanied by locked-in F_v/F_m depressions (cf. Fig. 24.8b above) and apparent lock-in of high thermal energy dissipation (cf. Fig. 24.8c, d above). A similar "winter state", where high levels of zeaxanthin-associated thermal dissipation are apparently locked-in and PS II efficiency remains depressed, has also been described for other (broad-leafed) overwintering evergreens (Adams and Demmig-Adams 1995; Adams et al. 1995, 2001, 2002; Verhoeven et al. 1998; Gilmore and Ball 2000; Zarter et al. 2006c).

In addition to zeaxanthin and antheraxanthin, the xanthophyll lutein can play a role in thermal dissipation (Niyogi et al. 2001; Li et al. 2009; Matsubara et al. 2008, 2011; Esteban et al. 2008, 2010; Förster et al. 2011; Ilioaia et al. 2011; García-Plazaola et al. 2012; Jahns and Holzwarth 2012; Ruban et al. 2012; Wahadoszamen et al. 2012). Yet another xanthophyll, neoxanthin, has been used as an indicator for a conformational change of LHC proteins during the onset of NPQ (Ilioaia et al. 2011; Zubik et al. 2011; Ruban et al. 2012), but Dall'Osto et al. (2007) showed that neoxanthin is not involved in thermal dissipation (NPQ). Fig. 24.9 shows

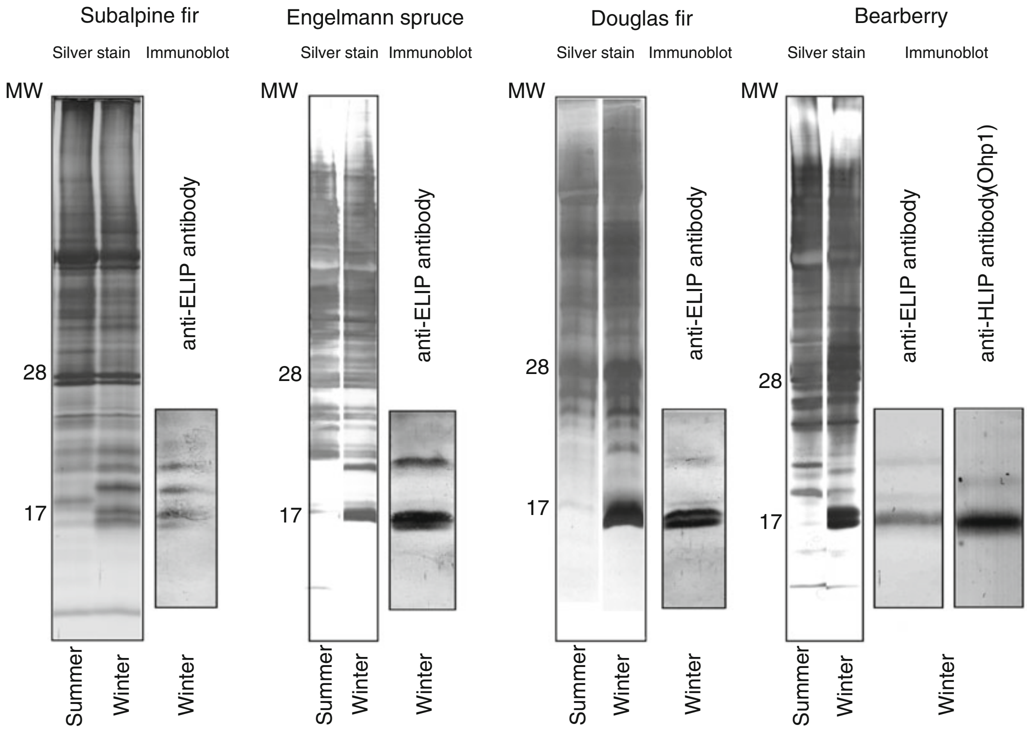

Fig. 24.10. Changes in protein composition of thylakoids isolated from three conifers (subalpine fir, Engelmann spruce, and Douglas fir) and an evergreen groundcover (bearberry) between summer and winter. For each species, a set of two silver-stained protein gels is shown (for summer and winter) as well as immunoblots (winter only) showing anti-early-light-inducible protein (*ELIP*)-reactive bands and (for bearberry only) anti-high-light-inducible protein (*HLIP*)-reactive bands (Ohp1 = one helix protein). MW = molecular weight. For methods, other details, and accompanying data on PS II efficiency and zeaxanthin levels, see Zarter et al. (2006a, b).

the levels of neoxanthin (Fig. 24.9c) and lutein (Fig. 24.9d), expressed in the same format as Z + A concentration (i.e., relative to the pool of V + A + Z), for summer, winter, and spring seasons in overwintering conifers. Neoxanthin and lutein are preferentially accumulated (relative to the V + A + Z pool) when chlorophyll content is highest in the summer, and do not show preferential accumulation in the winter when high levels of thermal dissipation are apparently locked-in. Relative to chlorophyll, neoxanthin levels decreased whenever chlorophyll b levels decreased (not shown; see Oh et al. 2013), consistent with location of an important fraction of neoxanthin in the major light-harvesting complex (LHCII) of PS II (while neoxanthin is also present in minor light-harvesting complexes CP26 and CP29; Bassi et al. 1993; Caffarri et al. 2007; Pan et al. 2011). Neither lutein nor V + A + Z decreased in concert with chlorophyll b (not shown; see Oh et al. 2013), which is consistent with their location in LHCII as well as other chlorophyll-binding complexes. Overall, the V + A + Z pool increased strongly as chlorophyll b content decreased in winter, while lutein concentrations stayed similar, and neoxanthin concentrations decreased (Oh et al. 2013). Together, these correlative data are consistent with no involvement of neoxanthin in locked-in winter photoprotection and a strong indication for an involvement of Z + A, while not allowing a potential involvement of lutein to be excluded.

The locked-in state of apparent high thermal dissipation and low PS II efficiency for the duration of sub-freezing conditions in the winter is accompanied, as already stated above, by degradation of both the oxygen-evolving complex (OEC) and PS II core proteins, as well as by severely decreased capacities of oxygen evolution to negligible levels (Demmig-Adams and Adams 2006; Zarter et al. 2006a, b, c) – and is independent of a trans-thylakoid pH gradient (cf. Verhoeven et al. 1998). Figure 24.10 shows that the latter changes in PS II protein composition were accompanied by accumulation of light-stress-related LHC proteins, such as several early-light-inducible proteins (ELIPs) and high-light-inducible proteins (HLIPs). Future studies are needed to demonstrate involvement of these proteins in photoprotection of evergreen plant species.

In contrast to overwintering evergreens, overwintering annuals do ***not*** exhibit downregulation, but instead upregulation of light- and CO_2-saturated photosynthetic capacity; furthermore, annuals do ***not*** exhibit slowly reversible depressions (sustained in the dark at warm temperatures) in intrinsic PS II efficiency, measured as F_v/F_m, or in NPQ. Annuals exhibit a larger VAZ pool size ***without*** warm-sustained retention of Z + A during the winter (see, e.g., Adams et al. 2002) or when grown at cool temperatures. The overall composition of the PS II antenna proteins of annuals is, furthermore, remarkably stable (Ballottari et al. 2007) when compared with overwintering evergreens.

The combination of OEC/PS II core protein removal, and continuous high thermal dissipation in the chlorophyll-binding complexes, has the potential to eliminate all further reactive oxygen species formation, and to thereby contribute to permitting maintenance of evergreen leaves over many months in the presence of high light absorption without any carbon uptake (Adams et al. 2004, 2006). The ecological benefit for conifers of maintaining green needles in a photosynthetically inactive and highly dissipative state – rather than dropping their needles during the winter – may lie in the ability of overwintering conifer forests to resume photosynthetic carbon uptake promptly upon snowmelt, rather than requiring growth of new leaves as is the case for winter-deciduous species. Subalpine conifer forests in Colorado show most of their annual carbon gain in the months right after snowmelt, when needles quickly resume photosynthesis after showing no net carbon uptake over the entire winter season (Monson et al. 2002). It appears that the ability of evergreens to enter a state of suspended metabolic/photosynthetic activity – with a high level of locked-in photoprotection in their evergreen leaves – may be a prerequisite for their long lifespan, over which they persist with green leaves throughout multiple harsh, growth-precluding seasons.

V Thermal Dissipation and Photoinhibition

The features of leaves grown under low, non-fluctuating light and then suddenly exposed to high light (cf. section IIa) resemble the features of overwintering evergreens (cf. section III). Both cases involve slowly developing and persisting depression in intrinsic PS II efficiency; both involve slowly developing and persisting (locked-in) increases in Z + A concentration; and, further, both involve slowly developing and persisting (locked-in) NPQ that does not involve a trans-thylakoid pH gradient. The similarities do not end here. Like conifers overwintering under extreme conditions (see section IV), leaves of evergreens grown under low, non-fluctuating light and suddenly subjected to high light treatment (section IIIb) exhibit an accumulation of foliar carbohydrates (for further details, see Adams et al., Chap. 23, and references therein), degradation of PS II core proteins (Zarter et al. 2006a, c; B. Demmig-Adams, V. Ebbert, W.W. Adams, unpublished), and accumulation of light-stress-inducible members of the family of light-harvesting proteins (HLIPs in high-light-treated *Monstera* and multiple ELIPs and an HLIP in overwintering evergreens; Demmig-Adams et al. 2006; Zarter et al. 2006a, c). While (Z + A)-associated NPQ in evergreens (with NPQ persisting at warm temperature in darkness), overwintering under the harshest conditions with months of subfreezing temperatures, do not involve a trans-thylakoid pH gradient, such a gradient is involved in (Z+A)-associated NPQ in the summer as well as during individual cold days in environments with intermittent above-freezing temperatures in the winter (with all or a portion of NPQ rapidly reversible at warm temperature in both of the latter scenarios). Therefore, the nomenclature of NPQ components by their recovery kinetics (as done, e.g., with the terms qE versus qI) should be expanded to specify that these categories may offer information about an involvement of trans-thylakoid pH gradient on the one hand, and PS II core degradation on the other hand, but should not be taken to imply differences with respect to involvement of (Z+A)-associated NPQ increases and PS II efficiency decreases. We, therefore, propose to replace the term qE with "**rapidly reversible**, **pH-dependent NPQ**" (cf. Figs. 24.2, 24.6, and 24.7a), and to differentiate the term qI into "**sustained**, **pH-dependent NPQ**" (that is long-term sustained in darkness at cold temperatures but rapidly reversible upon transfer to warm temperature; Verhoeven et al. 1998; Demmig-Adams et al. 2006, 2012) and "**sustained**, **pH-independent NPQ**" (that is long-term sustained in darkness even at warm temperatures; cf. Fig. 24.8).

As stated in section IV, for the case of overwintering conifers, there is mounting agreement that locked-in (Z+A)-associated thermal dissipation and inactivation of PS II photochemistry represent a highly photoprotected state requisite for the evergreen lifeform (Adams et al. 1995, 2001, 2002, 2004, 2006; Gilmore and Ball 2000; Öquist and Huner 2003; Ensminger et al. 2004; Demmig-Adams and Adams 2006). Consideration should also be given to the possibility that the evergreen lifeform may give rise to a similar scenario in response to prolonged experimental high light treatment of leaves grown in low, non-fluctuating light. The suggestion, given in section IIIb above, that leaves grown in low, non-fluctuating light, and thus not acclimated to excess light, exhibit similar (albeit considerably more slowly induced and reversed) responses to high light as sun-grown leaves, is reminiscent of the discussion of chronic versus dynamic photoinhibition (Osmond 1994; Osmond and Grace 1995). In the present context, cooccurrence of PS II core protein inactivation/removal, locked-in (Z+A)-associated NPQ, and persistent depression of intrinsic PS II efficiency is, in fact, consistent with the early, non-mechanistic definitions of photoinhibition. There, photoinhibition was defined as a lasting depression of photochemical efficiency and maximal photosynthesis rate (see, e.g., Powles et al. 1979), consistent with decreased PS II efficiency F_v/F_m arising from increased thermal dissipation and/or PS II core inactivation (see Demmig and Björkman 1987 after the model by Kitajima and Butler 1975).

VI Concluding Remarks

This chapter shows that the highest levels of NPQ are seen in evergreens – plants that grow slowly, have low maximal capacities of photosynthesis, and often experience high levels of excess absorbed light. Leaves' ability to rapidly modulate light-harvesting efficiency requires an exposure to fluctuating light levels during plant development. The close correlation between foliar zeaxanthin levels and high NPQ and/or low intrinsic PS II efficiency seen in intact plants – irrespective of widely different kinetics of onset and relaxation – needs to be taken into consideration in the evaluation of various molecular mechanisms of NPQ.

Acknowledgments

Work presented in this chapter was supported by the National Science Foundation (Award Numbers IOS-0841546 and DEB-1022236 to BD-A and WWA) and the

University of Colorado at Boulder. We thank Dr. Volker Ebbert for providing the protein gel images shown in Fig. 24.10. We are, furthermore, indebted to Roberto Bassi and Govindjee for their most valuable comments on this chapter.

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Chapter 25

Non-Photochemical Fluorescence Quenching Across Scales: From Chloroplasts to Plants to Communities

Erik H. Murchie
Division of Plant and Crop Sciences, School of Biosciences,
University of Nottingham, Nottingham, LE12 5RD, UK

and

Jeremy Harbinson*
Horticultural Supply Chains Group, Department of Plant Sciences,
Wageningen University, Wageningen, The Netherlands

Summary

Non-photochemical quenching (NPQ) of chlorophyll fluorescence, as a measure of photoprotective thermal dissipation of excess excitation energy (from singlet state chlorophyll a), is usually studied at the molecular, organelle, and leaf scale over

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*Author for correspondence, e-mail: jeremy.harbinson@wur.nl

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_25,

relatively short time periods, where it is most readily and easily quantified. The focus has been more on the mechanism(s) of the rapid component (energy-dependent quenching or qE) of NPQ and its basic modulation, rather than on the diversity in capacity and dynamics of NPQ (although see, e.g., Adams and Demmig-Adams, Chap. 2, Adams et al. Chap. 23, and Demmig-Adams et al. Chap. 24 for discussion of continuously sustained NPQ). In the past, it has also been difficult to measure levels of NPQ continually and at scales that reach whole plant or even community levels. This has resulted in a lack of understanding of how NPQ affects plant productivity and what may drive the adaptation and acclimation of NPQ kinetics and capacity. Thus, the role of thermal dissipation (assessed via its indicator NPQ) at the whole plant and even community scale is an understudied, albeit important topic. Thermal dissipation is by definition a protective process, and yet subject to tight metabolic control. How does regulation of thermal dissipation impact the operational state of photosynthesis? How efficient are the regulatory processes preventing photoinhibition and photooxidative stress? How does the large variation in NPQ dynamics and capacity relate to plant fitness and productivity? Does thermal dissipation impose a cost on photosynthetic rate in fluctuating environments? In this chapter, we address these questions by (1) describing the processes that give rise to thermal dissipation and the measurement of NPQ, (2) the role of thermal energy dissipation at different scales within the plant system, (3) the modulation of thermal energy dissipation capacity according to growth environment, and (4) the development of approaches for understanding how the dynamics of thermal dissipation determine plant fitness and productivity.

Abbreviations: ^{1}chl* – Singlet excited state of chlorophyll a; DCMU – 3-(3,4-dichlorophenyl)-1,1-diemthylurea: an herbicide that binds to the Q_B (plastoquinone binding) site of PS II; F' – The apparent chlorophyll fluorescence yield under steady state irradiance; F_m – The apparent chlorophyll fluorescence yield in the dark-adapted state (i.e., NPQ has been allowed to relax for a specified time) and with all Q_A reduced; F_m' – The apparent chlorophyll fluorescence yield in the light-adapted state (i.e., usually in the presence of NPQ) and with all Q_A reduced; F_o – The apparent chlorophyll fluorescence yield in the dark-adapted state with all Q_A oxidized; F_o' – The apparent chlorophyll fluorescence yield in the light-adapted state with all Q_A oxidized; F_q' – The difference between F_m' and F; F_v – The difference between F_m and $F_{o;}$ F_v' – The difference between F_m' and F_o'; k_d – The apparent first-order rate constant for the dissipation of ^{1}chl* in PS II by non-fluorescent and non-photochemical dissipation in PS II in the dark-adapted state (i.e., npd_b); k_D – The apparent first-order rate constant for the dissipation of ^{1}chl* in PS II by those non-photochemical dissipation processes that give rise to NPQ (i.e., npd_i); k_f – The apparent first-order rate constant for the dissipation of ^{1}chl* in PS II by fluorescence; k_p – The apparent first-order rate constant for the dissipation of ^{1}chl* in PS II by photochemistry; npd_b – The basal, or constitutive, non-photochemical dissipation of ^{1}chl* in PS II; npd_i – The inducible non-photochemical dissipation of ^{1}chl* in PS II; PRI – Photochemical Reflectance Index: usually calculated by [R531 − R570]/[R531 + R570], where R is the reflectance from a leaf or canopy and 531 and 570 are the wavelengths in nanometers; PS II – Photosystem II; Q_A – The primary stable electron acceptor of PS II, a quinone; qE – The component of NPQ attributed to the high-energy state quenching mechanism; qI – The component of NPQ attributed to photodamage to PS II or slowly reversible down-regulation of PS II; qP – Photochemical quenching of chlorophyll fluorescence in PS II; qT – The component of NPQ attributed to state-transitions; QTL – Quantitative trait locus; RUE – Radiation use efficiency; SVQ – Quenching of chlorophyll fluorescence by a quencher according to the Stern-Volmer model of quenching; VAZ – The violaxanthin-antheraxanthin-zeaxanthin cycle; ΔpH – The transthylakoid membrane pH difference; $\Phi_{PS\,II}$ – Operating quantum efficiency of electron transport by PS II

I Introduction

A A Problem of Scales

This chapter will focus on photosynthetic light-use efficiency by plants and the role played by those processes that give rise to the non-photochemical quenching of chlorophyll fluorescence (NPQ) when light-use efficiency decreases. This requires us to consider the scaling of processes: how do we evaluate and understand the role of a process over several orders of time and physiological complexity. At one extreme NPQ can be analyzed at the nanoscale, with the focus on mechanism and effects on the formation and lifetime of excited states of chlorophyll *a* (^{1}chl*) principally associated with photosystem II (PS II). The non-photochemical processes that dissipate ^{1}chl* (strictly speaking not all these processes are dissipative [e.g., state transitions] but for the sake of simplicity we will use this umbrella term) in PS II, and which give rise to NPQ, act at the level of the thylakoid. To fully understand the contribution these processes make to NPQ under any particular conditions it is much easier if they are understood in terms of mechanism and regulation at the local, thylakoid level (many chapters in this book focus on that level). Understanding the basic mechanisms and regulation of non-photochemical dissipation is, however, not enough to allow us to evaluate and understand it in all circumstances. ^{1}Chl* are the basic driving force behind photochemistry in PS II and the efficiency with which they are used, or quenched, by photochemistry is fundamental to photosynthetic light-use efficiency. Since NPQ arises as a result of processes that modulate formation and quenching by non-photochemical means of ^{1}chl* in PS II, the extent of NPQ reflects the activity of underlying processes that are linked to changes in photosynthetic light-use efficiency as a whole.

This last point shows that NPQ needs to be understood beyond the thylakoid level in order to be applied to plant productivity and fitness. While photosynthesis is the engine of plant productivity, it is also sensitive to limitation by non-photosynthetic constraints on plant growth, and we thus need to view photosynthesis (and NPQ) as part of the entire, functioning plant. Photosynthesis poses its own scaling problems as we move from the chloroplast to the whole plant or canopy via the cell and the leaf, and we need to consider responses that extend from the ultrashort (in the order of picoseconds) to the very long-term as plants grow and adapt, sometimes over millennia.

A particularly important feature of the scaling of photosynthesis and NPQ is time: measurements of primary productivity of vegetation occur over a timescale of days, weeks and months whereas the most commonly obtained measurements from leaves, such as assimilation rate, the light-use efficiency of PS II electron transport ($\Phi_{\text{PS II}}$), or NPQ, are of short duration. The impact of this discrepancy can seen within the integrated framework for understanding the radiation use efficiency (RUE) of plants and plant communities developed by Monteith (1977; Sinclair and Muchow 1999; Murchie and Reynolds 2012). While developed for monospecific stands of crops, this framework has also been applied to monospecific stands of wild plants (Piedade et al. 1991), showing that, at least for fast growing, short-lived crop plants, dry matter accumulation is a linear function of absorbed irradiance with the conversion efficiency or coefficient setting the slope of the relationship (e.g., Beale and Long 1995; Piedade et al. 1991). Within the Monteith framework, which is successful in describing canopy-level processes, events and physiological processes and responses are integrated over wide spatial and temporal scales. The details of the photosynthetic properties of individual leaves (including regulation of the physiology of NPQ) are submerged within the conversion coefficient of the canopy as a whole. However it is clear that photosynthetic light-use efficiency is highly variable and canopy level models of photosynthesis predict that improvements in the photosynthetic properties of leaves will have an impact on canopy carbon gain over short and long time periods (Zhu et al.

2004; Long et al. 2006; Sheehy and Mitchell 2013). Such models need ultimately to take account of whole plant level processes (such as source and sink limitations) and link effectively with crop level models. We therefore need to better understand the relationship between cumulative, short–term changes in photosynthesis (for example, as related to NPQ, photoinhibition, mid-day depression, abiotic stress or canopy development) and RUE, and to achieve this we must quantify these processes on an hourly and daily basis. Incorporating leaf level, and especially dynamic, photosynthetic properties into the Monteithian, or similar, vegetation level framework remains something of an interdisciplinary challenge for plant scientists and ecologists. How we measure and describe NPQ at all levels will be critical if we wish to understand its impact on plant productivity and this is addressed in this chapter.

B *The Importance of Flexibility of NPQ: Excess Light and Acclimation*

Photosynthesis uses the energy of absorbed photons to drive the endothermic reduction of CO_2. A hypothetical, ideally efficient photosynthetic system would be able to use the energy of absorbed light to fix carbon dioxide with a constant light-use efficiency across all natural light intensities. Such a system would display a linear relationship between light intensity and the rate of CO_2 fixation (Fig. 25.1, curve A). If naturally occurring photosynthesis behaved like this there would be only a limited need to protect the photosynthetic apparatus from some unavoidable, but minor, side reactions that occur during the photosynthetic process. Very few leaves, however, even come close to this ideal (Pearcy and Ehleringer 1984), with most having an irradiance response for CO_2 fixation that is conspicuously non-linear (Fig. 25.1, curves B–E) and the degree of non-linearity being dependent on the species and its growth environment (Björkman 1981). The curvilinear relationship between photosynthesis and irradiance implies that the light-use efficiency of photosynthesis is maximal at low irradiances where photosynthesis is wholly light-limited and then decreases as irradiance increases beyond the light-limited region. This loss of light-use efficiency results in a loss of photosynthetic productivity, which would be expected to be disadvantageous so it is reasonable to consider why it should occur. Some plants (e.g., *Camissonia claviformis* and *Amaranthus palmeri* Pearcy and Ehleringer 1984) show only a moderate loss of light-use efficiency with increasing irradiance, so this loss is not due to an intrinsic limitation in photosynthesis. The reasons for the limited photosynthetic light-use capacity seem to lie more with the costs and benefits – the economics – of photosynthesis. Simplifying a complex subject, high rates of CO_2 fixation require leaves that are expensive in terms of the

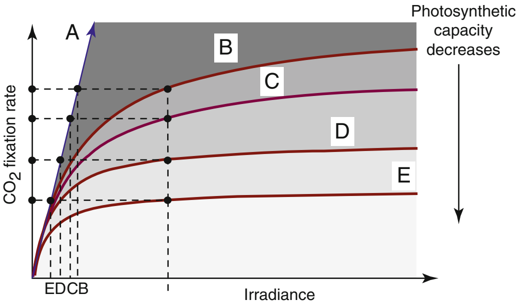


Fig. 25.1. The extent of excess irradiance associated with different CO_2 fixation irradiance curves. Curve *A* is an idealized response of a leaf without any loss of photosynthetic light-use efficiency with increasing irradiance and in which the irradiance response has a constant quantum yield that is determined by the underlying light-harvesting, energy transduction and biochemistry of photosynthesis. Curves *B* through *E* show responses from leaves with increasingly limited photosynthetic capacities and in which, by implication, photosynthetic light-use efficiency decreases with increasing irradiance once it is above the region of light-limitation (i.e., low irradiances). Assuming that the chemistry of photosynthesis does not change with increasing irradiance, the irradiance needed to produce any CO_2 fixation rate is given by the intercept with curve *A* of a horizontal line drawn from any light-response curve (shown for irradiance I). The component of excess irradiance in the imposed irradiance I is given by the difference between I and irradiances *B*, *C*, *D*, and *E* (for curves *B*, *C*, *D*, and *E* respectively): the excess irradiance therefore increases from curve *B* to curve *E*.

allocation of resources (Reich et al. 1997) and, owing to the scarcity of these resources (especially mineral nitrogen) or the difficulty of acquiring them (e.g., iron (Guerinot and Yi 1994; Kim and Guerinot 2007)), producing a leaf with high rates of CO_2 fixation is usually inefficient in terms of the cost of extra resources required to achieve the extra photosynthetic capacity compared to the marginal carbon gain that results, even in high-light environments. This also implies that when considering the scaling of leaf photosynthetic properties (such as NPQ) we need to consider the selective pressures that have driven the evolution of these properties over evolutionary time.

The decrease of the light-use efficiency of carbon dioxide fixation with increasing irradiance that is observed to a greater or lesser extent in all leaves implies that with increasing irradiance more light is being absorbed by the leaf than can be used for CO_2 fixation, i.e., there is excess light (Fig. 25.1). Light absorbed by PS II and PS I forms ^{1}chl* that are the driving force for photochemistry and thus photosynthetic electron transport. The decreasing light-use efficiency of CO_2 fixation implies that an increasing fraction of these ^{1}chl* are not being used (i.e., quenched photochemically) by electron transport activity coupled to photosynthetic metabolism. The lower the light-saturated rate of photosynthesis the more readily will increasing irradiances result in excess light and the greater will be the over production of ^{1}chl* relative to the demands of assimilation. The light-saturated rate of CO_2 fixation (photosynthetic capacity) differs among species, so the extent to which light will be in excess is species dependent. Within a species, changes in photosynthetic capacity can be produced by acclimation (Björkman 1981; Walters 2005) or stress responses, so the extent to which there is excess light is variable even in the short-term.

C The Function of NPQ Across Scales

Excess light, and more directly the production of excess ^{1}chl*, is a potential problem because of the chemistry associated with light-harvesting, photochemistry and electron and proton transport. Potentially hazardous intermediates (including ^{1}chl* itself) and by-products of side-reactions are formed during these processes. Oxidants powerful enough to oxidize water are formed in PS II, and thylakoid lumen pH below 6.0 can inactivate the oxygen-evolving complex (Krieger and Weis 1993), and in a review of the literature about lumen pH, Kramer et al. (1999) suggest that when photosynthetic metabolism is active and non-limiting lumen pH will be in the range 5.8–6.5, falling below 5 only under extreme conditions, but it is the formation of reactive oxygen species that is seen to be the greatest threat emerging from the operation of photosynthesis. On the acceptor side of PS I reductants are formed that can reduce dioxygen (O_2) to superoxide (O_2^-), which can then form hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (Foyer and Harbinson 1994; Asada 1999). The reaction centers and pigment bed of PS II can form singlet oxygen from triplet state chlorophylls (Foyer and Harbinson 1994; Niyogi 1999; Krieger-Liszkay 2005; Triantaphylidès et al. 2008). Even under the most benign of conditions, the potentially damaging chemistry of photosynthesis occurs, (e.g., photodamage to PS II Aro et al. 1993; Tyystjärvi and Aro 1996), and in the absence of any ameliorating factors (such as activation of non-photochemical dissipation of ^{1}chl* in PS II) will increase under conditions where there is excess light (i.e., when the rate of quantum absorption by photosystems I and II exceeds the demands of metabolism).

The operation and regulation of potentially protective mechanisms that might mitigate the damaging side reactions of photosynthesis is therefore critical to the long-term operation of photosynthesis. To reduce the risk of damage posed by reactive oxygen species, thylakoids and stroma are equipped with numerous protective mechanisms, such as antioxidants (Fryer 1992; Foyer and Harbinson 1994; Asada 1999), the carotenoid valve (Wolff and Witt 1969; Witt 1971; Kramer and Mathis 1980), the regulation of photosynthetic electron (and proton) transport (Genty and Harbinson 1996; Foyer et al. 2012) and processes that result in the phenomenon of non-photochemical dissipation (measured as NPQ). Several processes give rise to NPQ, but all result in a reduction in the steady-state concentration of excited, singlet states of chlorophyll *a* (^{1}chl*) in PS II when photochemical use of these states is restricted. It is the effect on ^{1}chl* density that results in a quenching, or reduction, of chlorophyll fluorescence yield – the tool by which the activity of these mechanisms is usually measured. It is important to be aware that while in terms of measurement, quantification and analysis the focus might be on the phenomenon of NPQ (i.e., the impact on fluorescence yield), the physiological significance is based on the underlying mechanisms that produce the change in the yield, not in the change in yield itself. What we observe via NPQ is the activity of processes acting to moderate the level of ^{1}chl* in PS II. These mechanisms are under physiological control because ^{1}chl* are the driving force for photochemistry and electron transport in PS II, and efficient use of ^{1}chl* by PS II (and PS I) is essential for high-light use efficiencies of photosynthesis under light-limited conditions. Consequently, the engagement of mechanisms that result in a reduction in the density of ^{1}chl* in PS II, and therefore result in NPQ, need to be controlled.

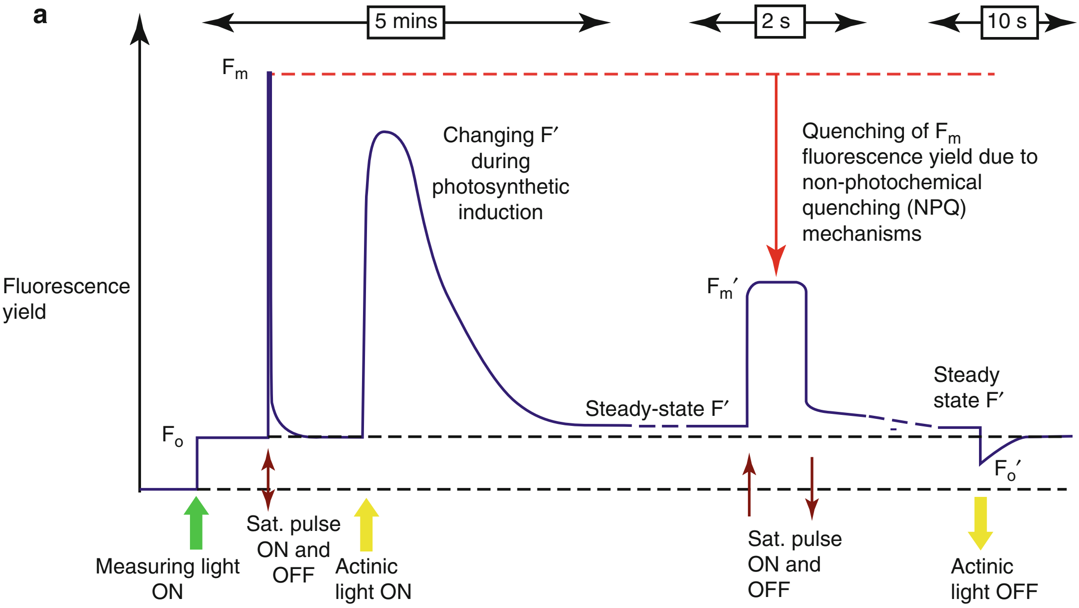
In our discussion so far it is tacitly accepted that the non-photochemical dissipation mechanisms that give rise to NPQ act in a protective manner to prevent or reduce photooxidative stress and reduce the likelihood of photoinhibition occurring. However, in fluctuating light the non-photochemical dissipation processes underlying NPQ may aid in preventing over-reduction of PS II during photosynthetic induction (i.e., the activation of photosynthesis) processes that occur following increases in irradiance. This kind of "buffering" effect on electron transport could be viewed as homeostatic, i.e., not only acting in a photoprotective manner but perhaps preventing fluctuations in ΔpH that may influence ATP: NADPH ratios. This effect would also need to be viewed alongside other mechanisms that provide a similar role for the electron transport chain, such as the water-water cycle and cyclic electron transport (Asada 1999; Johnson 2011). Support for such a role for the processes underlying NPQ comes from recent work where engineering a higher capacity for energy dissipation by transformation in rice resulted in reduced PS II electron transport and even CO_2 assimilation during photosynthetic induction (Hubbart et al. 2012). If such a dual role for thermal energy dissipation exists, one may ask whether the activity and regulation of processes underlying NPQ are optimized for photosynthetic productivity in "real" environments and whether these processes acclimate in response to changes in the environment (e.g., changes in irradiance) or physiological state of the leaf or plant?

Acclimation is highly relevant to the physiology of NPQ because it allows adjustment of both the photochemical quenching of ^{1}chl* (that by PS II is especially relevant in relation to NPQ) and non-photochemical quenching capacity (i.e., the processes giving rise to NPQ) for reducing ^{1}chl* density in PS II. Acclimation is a change in the composition of the photosynthetic apparatus in response to medium- to long-term changes in light intensity, and typically takes place over a period of days. In many cases acclimation improves the rate of photosynthesis at the new light level. For example, since under high light the supply of light is not limiting, the amount of light-harvesting pigment-protein complex is reduced while the levels of, e.g., the cytochrome b_6/f complex, thylakoid ATPase and Calvin cycle enzymes increase (Björkman 1981; Anderson et al. 1995; Yano and Terashima 2001; Walters 2005). Acclimation to light is a common process in plants that enables efficient exploitation of light and also maintains a high economy of form and function. However, there is species diversity in the pattern of acclimation of different photosynthetic components, and this is associated with ecological habitat (Murchie and Horton 1997, 1998). For example, some species that are associated with forest environments do not increase photosynthetic capacity upon transfer to high light and instead show evidence of sustained thermal dissipation (Jurik et al. 1979; Demmig-Adams et al. 2006), whilst other forest species increase photosynthetic capacity but do not alter light-harvesting antenna size (Adamson et al. 1991; Chow et al. 1991). Such species differences are probably related to the availability of nutrients and water that determine whether higher photosynthetic capacities are cost-effective (see section I.B). *Arabidopsis thaliana* has a typical "complete" acclimation that shows some accession dependency (Bailey et al. 2001; Walters 2005), but contrasts with rice leaves that show an effect of leaf development on key components, where Rubisco protein levels do not increase if light is increased after full leaf extension (Murchie et al. 2005).

The diversity of responses observed in acclimation enhances adaptive capabilities and the range of environments plants can exploit. An important consequence of acclimation is that it has the potential to limit the potential for oxidative damage; increasing photochemical capacity under high light via levels of electron transport and Calvin cycle components has the effect of lowering the amount of absorbed light energy that is in excess of photosynthetic requirements (Fig. 25.1; Demmig-Adams and Adams 2006; Murchie and Niyogi 2011). This needs to be seen in the context of acclimation of NPQ itself (see later in this chapter).

II The Basics of Chlorophyll Fluorescence and Excited States in Leaves

The tool most commonly used to measure the activity of dissipation processes (both photochemical and non-photochemical) of ^{1}chl* in leaves is chlorophyll fluorescence. Most chlorophyll fluorescence at room temperature comes from PS II (Pfündel 1998; see review by Baker 2008) so discussions about chlorophyll fluorescence focus on how changes in PS II drive changes in fluorescence. Absorption of light by chlorophyll *a* results in the formation of ^{1}chl* this same excited state is formed if the excited state is formed initially on chlorophyll *b* or a carotenoid and then transferred to chlorophyll *a* (e.g., Pålsson et al. 1994; Gradinaru et al. 1998; Croce et al. 2001; de Weerd et al. 2003a, b). All detectable fluorescence from normally organized thylakoids is produced from ^{1}chl* and none comes from excited states of accessory pigments. The intensity of fluorescence from a leaf depends on the intensity of light absorbed and the yield of fluorescence from ^{1}chl*. Many of the procedures that use chlorophyll fluorescence to describe operation and regulation of PS II use changes in light-intensity to produce controlled changes in the state of PS II (principally completely oxidizing or reducing the Q_A pool; Fig. 25.2), and measuring the irradiance response of photosynthesis in any case requires change in irradiance. These changes in light intensity produce changes in chlorophyll fluorescence intensity due to changes in the rate of ^{1}chl* formation and changes in the yield of fluorescence from ^{1}chl* arising from changes in the state of PS II. Since it is the yield of fluorescence that provides direct physiological information about photosynthesis, it is necessary to separate changes in fluorescence intensity due to changes in irradiance from those due to changes in yield. Many modern chlorophyll fluorometers measure the amount of fluorescence produced by a weak modulated measuring beam of a fixed (or highly controlled) intensity (Quick and Horton 1984; Ogren and Baker 1985; Schreiber et al. 1986), and these allow changes in fluorescence yield (strictly, a relative fluorescence yield) to be conveniently measured. In PS I the yield of fluorescence is scarcely influenced by its physiological state (Byrdin et al. 2000) and the amount of PS I fluorescence is also



b

Fluorescence parameter	Actinic irradiance on or off?	Saturation pulse on or off?	Q_A reduction state	Constitutive (or basal) non-photochemical quenching state	Inducible non-photochemical quenching state
F_o	Off	Off	Fully oxidized	Present	Absent
F_m	Off	On	Fully reduced	Present	Absent
F'	On	Off	Usually partly reduced	Present	Usually present
F_m'	Off	On	Fully reduced	Present	Usually present
F_o'	Off	Off	Fully oxidized	Present	Usually present

Fig. 25.2. (**a**) The basic measurable fluorescence parameters F_o, F_o', F_m, F_m', and F' shown in relation to the fluorescence induction curve of a dark-adapted leaf. Note the different time scales for different parts of the fluorescence response. Application of the measuring light to the leaf results in the F_o fluorescence yield, which is increased to F_m by the application of a saturating (=sat.) light pulse. Application of actinic irradiance (i.e., normally up to 2,000 μmol photons m^{-2} s^{-1}) produces further changes in fluorescence yield (F') until a stable steady-state yield is reached, normally after several minutes. Application of a saturating irradiance to the leaf in this light-adapted state will produce an increase in fluorescence yield to F_m', which is lower than F_m owing to induction of npd$_i$ (the inducible component of NPQ): the activity of npd$_i$ is $F_m/F_m' - 1$ (based on a Stern-Volmer model of quenching). Removing the actinic irradiance results in a fall in fluorescence yield to F_o' (the scale of this fall is, for most leaves, exaggerated in this diagram for the sake of clarity). (**b**) The physiological properties and conditions of measurement for each of the measurable fluorescence parameters shown in part A. Note that it is the induction, or formation, of the inducible component of NPQ (npd$_i$) that gives rise to the fall in fluorescence yield from F_m to F_m' (This figure was adapted with permission from Harbinson (2013)).

low compared to that from PS II (Pfündel 1998), so chlorophyll fluorescence is nearly always only used to measure the status of PS II. Light absorption by the leaf can change due to chloroplast movement (Brugnoli and Björkman 1992), and the amount of light partitioned to PS II rather than PS I (most fluorescence comes from PS II) can change, and these changes are not corrected for (see Logan et al., Chap. 7). Therefore changes in fluorescence yield are only "apparent" as they may also include effects due to changes in absorption of the measuring beam as a whole or the partitioning of the absorbed measuring beam between PS I and PS II. In practice a decrease in, e.g., the apparent fluorescence yield from a leaf may be due to either a decrease in the yield of formation of ^{1}chl* in PS II or an increase in the rate at which ^{1}chl* is quenched in PS II.

In vivo fluorescence is generally described by means of parameters corresponding to the (apparent) chlorophyll fluorescence yields measured when PS II is in certain specific states, defined in terms of the degree of reduction of Q_A or the presence or absence of non-photochemical quenching (F_m, F_o, F_m', F_o' and F'; Fig. 25.2). These measures are often termed "steady-state" or "PAM" (for pulse amplitude modulation) measurements to distinguish them from time-resolved measurements of fluorescence described elsewhere in this book. Here we make extensive use of parameters that are used to describe operation and regulation of PS II and that are derived from these basic chlorophyll fluorescence parameters. Non-photochemical quenching often needs to be evaluated in relation to the overall regulation and operation of PS II, so it is important that all relevant processes influencing operation and regulation of PS II can be described in a way that allows their impact or significance to be compared. This can be done by means of parameters derived from basic, measurable fluorescence metrics (F_m, F_o, F_m', F_o' and F'), and all fluorescence-derived parameters, such as F_v/F_m and F_q'/F_m' (using the terminology of Baker 2008, F_q'/F_m' is also known as $\Delta F/F_m'$ Genty et al. 1989 or $\Phi_{PS\ II}$), are based on some or all of these basic parameters.

Changes in fluorescence yield are due to changes in the lifetime of ^{1}chl* in PS II (Genty et al. 1992), i.e., the shorter the lifetime the greater the extent of quenching. In the F_o state, where photochemical quenching is maximal, the mean (in thylakoids, fluorescence lifetimes are multiphasic owing to the complexity of the quenching process, e.g., Holzwarth et al. 2009) lifetime is short – about 200–300 ps (e.g., Holzwarth et al. 2009; Wientjes et al. 2013). At F_m, when photochemistry is inactive and no processes contributing to the NPQ of chlorophyll fluorescence are engaged, average lifetime is 2 ns (e.g., Genty et al. 1992; Belgio et al. 2012). This is the maximum mean lifetime found in thylakoids and it is shorter than the lifetime found in solution. The singlet excited states of any molecule or atom are, however, fundamentally short-lived and their accumulation is ultimately limited by fluorescence decay. In the case of chlorophyll *a*, the theoretical fluorescence decay limited lifetime is about 20 ns (Connolly et al. 1982). In solution, the measured fluorescence (or ^{1}chl*) lifetime is about 5.5–6 ns (Kaplanová and Čermák 1981; Connolly et al. 1982) due to triplet state formation competing with fluorescence decay (see discussion in Foyer and Harbinson 1994). That the F_m lifetime in thylakoids is less than that in solution implies that even in the absence of photochemistry, thylakoids have additional quenching mechanisms beyond those acting on isolated chlorophyll molecules. It is not yet, however, clear where this additional quenching (or dissipation) activity resides; the 2 ns lifetime could be due to weak quenching centers in LHCII (Belgio et al. 2012) or may be due to quenching in the reaction center of PS II (Schatz et al. 1988; Lavergne and Trissl 1995).

Some important conclusions can be drawn from this. First, the finite lifetime of ^{1}chl* in the absence of photochemistry implies that even when there is no photochemistry, there are basal non-photochemical dissipation processes at work. The shorter lifetime in the F_m state compared to chlorophyll in solution implies that there are additional constitutive or basal non-photochemical dissipation processes active in PS II compared to chlorophyll in solution, and that these processes act in addition to the non-photochemical dissipation processes that give rise to NPQ. The constitutive processes are not under physiological control, while the processes that give rise to NPQ are under physiological control and can thus be seen as inducible. This dual nature of non-photochemical dissipation – with constitutive and inducible components – needs to be accounted for in any analysis of the efficiency and regulation of PS II and its analysis by means of fluorescence. So while the rise of fluorescence from F_o to F_m (Fig. 25.2) is largely due to loss of photochemical quenching of ^{1}chl* as a result of reduction of Q_A and the corresponding increase in the lifetime of ^{1}chl* in PS II, the use of chlorophyll fluorescence to measure the efficiency requires an equation that incorporates the basal non-photochemical dissipation present at F_o and F_m.

In the dark-adapted state in unstressed leaves, ^{1}chl* quenching is via fluorescence, triplet formation, photochemistry and other constitutive, or basal, dissipation mechanisms. In the light-adapted state, the non-photochemical dissipation processes contributing to NPQ engage and contribute to the quenching processes that operate in the dark-adapted state. These inducible non-photochemical dissipation mechanisms are physiologically diverse and can be divided into those diminishing the rate of formation of ^{1}chl* in PS II in the first place (state transitions and chloroplast movement – note that these processes do not act by increasing non-photochemical dissipation but as they contribute to NPQ they are conveniently lumped with other processes that do) and mechanisms that non-photochemically quench the ^{1}chl* formed in the pigment bed of PS II (energy-dependent quenching, photodamage, slowly reversible down-regulation of PS II). Note that non-photochemical quenching in vitro largely focuses on only a subset of the processes encountered in leaves: chloroplast movement can contribute to non-photochemical quenching in leaves (Brugnoli and Björkman 1992), but not in a chloroplast suspension. Slowly activated or inactivated processes, such as slowly reversible down-regulation of PS II (Demmig-Adams and Adams 2006; see also Demmig-Adams et al., Chap. 24), are likewise not actively studied in vitro. As we will need to distinguish between inducible and basal forms of non-photochemical dissipation we will use npd_i to refer to inducible dissipation processes and npd_b to refer to basal or constitutive dissipation processes.

The rate of formation of ^{1}chl* from chlorophyll *a* (the ground state) is proportional to irradiance (Fig. 25.3). Disappearance of ^{1}chl* in PS II (PS I can be treated similarly) to reform, directly or indirectly, chlorophyll *a,* is determined by kinetically first-order processes that quench ^{1}chl* (shown in a simplified way in Fig. 25.3). Increasing the magnitude of the rate constants for any of these processes results in a proportional increase in flux through the corresponding pathway; first-order rate constants can thus be seen as analogous to the diffusive conductances (resistances) used to analyze transpiration and assimilation. All basic fluorescence parameters shown in Fig. 25.2 can be expressed in terms of apparent first-order rate constants for quenching of chlorophyll *a* excited states in PS II using the approach of Butler and co-workers (e.g., Butler and Kitajima 1975; Butler 1978; Fig. 25.4a). This kinetic modeling approach has proven to be useful in deriving physiologically useful parameters from the basic set of measurable fluorescence parameters. The processes (and rate constants) included in the approach of Butler that are relevant here are fluorescence (k_f), photochemistry

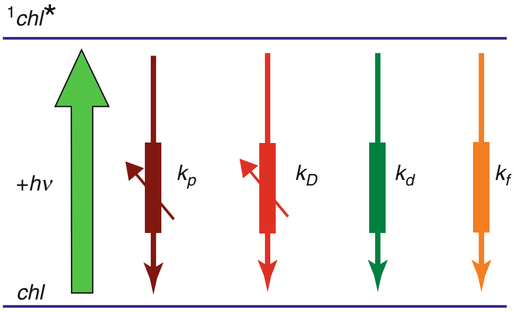


Fig. 25.3. Excitation (photon absorption: +hν) and de-excitation (k_p, k_D, k_d, and k_f) pathways for the singlet excited state of chlorophyll a (^{1}chl*) in PS II. The routes of formation via excited states of chlorophyll a and carotenoids are not included in this diagram. Each decay route can be seen as either a first-order reaction where the rate is given by k[^{1}chl*] or as a resistance through which there is a flux proportional to a driving force ([^{1}chl*]) and the reciprocal of resistance, which is proportional to the first-order rate constant. Note that the values (strictly speaking these are apparent values) for k_p and k_D are variable owing to differences in the variable efficiency of photochemistry ($\Phi_{PS\,II}$) and activity of npd_i. While this diagram is for PS II it could also be used for PS I, in which case k_D would be, in most cases, due to the presence of P_{700}^+.

a

$$F_o = k_f / (k_f + k_d + k_p) \qquad F_m = k_f / (k_f + k_d)$$

$$F_o' = k_f / (k_f + k_d + k_p + k_D) \qquad F_m' = k_f / (k_f + k_d + k_D)$$

$$F' = k_f / (k_f + k_d + k_p + k_D)$$

b

1. $$NPQ = \left(\frac{F_m}{F_m'}\right) - 1$$

2. $$NPQ = \left(\frac{F_m - F_m'}{F_m'}\right)$$

3. $$NPQ = \frac{\dfrac{k_f}{k_f + k_d} - \dfrac{k_f}{k_f + k_d + k_D}}{\dfrac{k_f}{k_f + k_d + k_D}}$$

4. $$NPQ = \frac{\dfrac{1}{k_f + k_d} - \dfrac{1}{k_f + k_d + k_D}}{\dfrac{1}{k_f + k_d + k_D}}$$

4. $$NPQ = \frac{(k_f + k_d + k_D) - (k_f + k_d)}{(k_f + k_d + k_D) \cdot (k_f + k_d)} \times \frac{(k_f + k_d + k_D)}{1}$$

6. $$NPQ = \frac{k_D}{(k_f + k_d)}$$

Fig. 25.4. (**a**) The definition of F_o, F_m, F_o', F_m', and F' in terms of the apparent first-order rate constants for fluorescence decay (k_f), basal non-fluorescent and non-photochemical losses (k_d), photochemistry (k_p), and inducible NPQ processes (k_D). (**b**) An example showing the conversion (1–6) of the equation for NPQ using the Stern-Volmer quenching model and measurable fluorescence parameters into a ratio of rate constants.

(k_p), non-radiative decay (k_d) (basal non-photochemical dissipation, npd_b, is encompassed by the k_f and k_d terms), and k_D (which accounts for inducible non-photochemical dissipation, $npd_{i,}$). Of these, the rate constants for photochemistry and non-photochemical quenching respond to the physiological condition of the leaf and are subject to short-term changes (Fig. 25.3). The yield of any de-excitation (or quenching) process is the ratio of the rate constant for that process divided by the sum of the constants for all other relevant de-excitation processes. Which rate constants for ^{1}chl* decay are relevant depends on the state of PS II, e.g., F_o fluorescence yield occurs when fluorescence, non-radiative decay (i.e., npd_b), and photochemistry are all active, but inducible non-photochemical dissipation (i.e., npd_i) processes that give rise to NPQ are absent, so $F_o = k_f/(k_f + k_d + k_p)$ (Fig. 25.4a). This approach, developed by Butler and colleagues, is simple and widely used to derive parameters to describe the operation of PS II (e.g., Krause and Weis 1991; Rohacek 2002; Hendrickson et al. 2004; Kramer et al. 2004). While the Butler model is simple and convenient, for a more detailed and rigorous analysis of PS II, more complete models of PS II chlorophyll fluorescence should be used (e.g., Lavergne and Trissl 1995).

A Quantifying the Extent of NPQ in Leaves

1 Theoretical Background

Non-photochemical quenching is most usefully quantified in terms of two of the of basic fluorescence parameters, F_m and F_m', combined in the form of the Stern-Volmer equation (Fig. 25.4b, Eqs. 1 and 2; Bilger and Björkman 1990; Atkins and De Paula 2010) that relates the concentration of a fluorescence quencher to the decrease in fluorescence yield. In vitro PS II fluorescence is susceptible to quenching by exogenous quenchers following the Stern-Volmer model (Bukhov et al. 2003) and the energy-dependent form of NPQ can be correlated with other measures of thylakoid energization using the Stern-Volmer model (Noctor et al. 1993). As NPQ is substantially (but not completely) due to the effect of putative quenching centers that dissipate ^{1}chl*, Stern-Volmer is a logical choice of model with which to describe NPQ. However not all decreases in F_m' are due to quenching or dissipation of the kind described by the Stern-Volmer model; those due to processes decreasing the formation of ^{1}chl* in PS II (state transitions and chloroplast movement) differ mechanistically from Stern-Volmer quenching. So while the Stern-Volmer quenching model is a useful tool with which to understand NPQ, the expectation that a wholly Stern-Volmer based analysis of NPQ is appropriate for all NPQ is incorrect (Lavergne and Trissl 1995; see also Logan et al., Chap. 7). The use of this Stern-Volmer based equation to describe non-photochemical quenching has largely supplanted an earlier equation introduced by Bilger and Schreiber (1986) except in certain specific cases (e.g., Adams and Demmig-Adams 2004; Adams et al. 2006; Rohacek 2010).

The Stern-Volmer quenching (SVQ) parameter for NPQ (Eqs. 1 and 2; Fig. 25.4b) is based on F_m and F_m', but its meaning in relation to other fluorescence-derived parameters can be better understood in terms of k_f, k_p, k_d and k_D (Fig. 25.4a). If F_m and F_m' are expressed in terms of kinetic parameters (Fig. 25.4a), these can be substituted into the Stern-Volmer equation (Fig. 25.4b, Eq. 3). After rearrangement (Fig. 25.4b, Eqs. 4 and 5), this simplifies to $k_D/(k_d + k_f)$: the SVQ parameter is therefore the ratio of npd_i (due to processes encompassed by k_D) and npd_b (encompassed by k_f and k_d). Notably, the SVQ parameter does not include a k_p term, which is consistent with it being derived from measurements of F_m and F_m', neither of which depend on k_p. As the aim is to use fluorescence measurements to quantify the activity of npd_i, which is variable, it is necessary to eliminate the impact of photochemical quenching (which is also variable) on fluorescence. The F_m and F_m' states are characterized by the absence of photochemistry due to the complete reduction of Q_A, so their use in a parameter describing npd_i should not be surprising.

2 Problems of Quantifying NPQ Across Different Scales

It is important to note that measurement of NPQ in plant tissue usually requires dark adaptation of leaf tissue followed by a period of illumination. Whilst possible in many circumstances, this procedure can be impractical in the field with large and frequent sample numbers. This is why the use of other parameters, such as $\Phi_{PS\,II}$ and qP, are often more prominent in eco-physiological studies using fluorescence. There have been alternative approaches, such as using F_v'/F_m' as a proxy for NPQ (Fig. 25.5), but as described above, the relationship between F_v'/F_m' and NPQ may not be linear and will depend on the prevalence of other components. Additionally there can be errors introduced through the lack of complete dark adaptation.

To accurately monitor NPQ as it tracks changes in ambient light in real time it is necessary to dark-adapt and usually to have an instrument that remains attached to the leaf for long time periods. If long-term, accumulated measurements are required then these measurements may be necessary for months without any interruption (in order to link to integrated measurements of radiation interception for example). Although pre-dawn measurements are commonly and easily made by hand, acquisition of the required number of instruments, or the sustained manpower for such long-term experiments is often not feasible, especially when measurements take place in remote areas. A commercial modular set up has recently become available that allows leaf-contact periodic measurements of F, F′, F_o and F_m' to be made simultaneously on several leaves and over long time periods (Porcar-Castell et al. 2008; Porcar-Castell 2011). The advantage of this approach is that accurate night-time values of F_o and F_m are taken, allowing more or less continuous measurements of NPQ on the same leaf. Whilst limited by the small leaf area over which measurements can be made and by the number of leaves measured, the

$$1.\quad NPQ = \left(\frac{F_m}{F_m{}'}\right) - 1 = \frac{k_D}{(k_f + k_d)}$$

$$2.\quad \frac{F_v{}'}{F_m{}'} = \frac{(F_m{}' - F_o{}')}{F_m{}'} = \frac{k_p}{(k_f + k_d + k_p + k_D)}$$

$$3.\quad NPQ = \left(\frac{F_m}{F_m{}'}\right) - 1 = \frac{(F_m - F_m{}')}{(F_m{}' - F_o{}')} \times \frac{F_v{}'}{F_m{}'}$$

$$4.\quad \Phi_{NP}{}^{c} = \Phi_{f,D} = \Phi_{NO} = \frac{F'}{F_m} = \frac{(k_f + k_d)}{(k_f + k_d + k_p + k_D)}$$

$$5.\quad \Phi_{NP}{}^{r} = \Phi_{NPQ} = \frac{F'}{F_m{}'} - \frac{F'}{F_m} = \frac{k_D}{(k_f + k_d + k_p + k_D)}$$

$$6.\quad NPQ = \left(\frac{F_m}{F_m{}'}\right) - 1 = \frac{k_D}{(k_f + k_d)} = \frac{\Phi_{NPQ}}{\Phi_{NO}}$$

Fig. 25.5. Equations used to measure the magnitude of NPQ and its impact on the operation of PS II. These are shown in terms of measurable fluorescence parameters and, where appropriate, on the apparent first order rate constants for processes that quench (or dissipate) ^{1}chl* in PS II. The measurable fluorescence parameters (F_o, $F_o{}'$, F_m, $F_m{}'$ and F′) and apparent first order rate constants for the quenching processes that determine fluorescence yield (k_f, k_d, k_p and k_D) are defined in Figs. 25.2 and 25.3 and in the text. *Equation 1*: the equation based on the Stern-Volmer relationship for fluorescence quenching that is commonly used to quantify NPQ. *Equation 2*: the relative quantum efficiency for electron transport by open (i.e., Q_A is oxidized) traps in PS II (Genty et al. 1989): qualitatively, as NPQ increases $F_v{}'/F_m{}'$ decreases. *Equation 3*: the relationship between the NPQ Eq. 1 and $F_v{}'/F_m{}'$ Eq. 2 parameters. *Equation 4*: the quantum yield of dissipation of ^{1}chl* in PS II by basal or constitutive non-photochemical quenching processes (i.e., those that were still present following dark-adaptation). The three abbreviations encountered in the literature for this yield are shown. *Equation 5*: the quantum yield of dissipation of ^{1}chl* in PS II by inducible non-photochemical quenching processes (i.e., those that were not present following dark-adaptation but were activated in the light). The two abbreviations encountered in the literature for this yield are shown. *Equation 6*: the relationship between NPQ Eq. 1 and Φ_{NO} Eq. 4 and Φ_{NPQ} Eq. 5.

latter approach opens up possibilities for experiments where high frequency of sustained logging (weeks to several months) are required without human intervention, even in isolated regions. In this way Porcar-Castell (2011) was able to determine the dominant processes operating over an entire year in boreal *Pinus sylvestris*. Sustained and rapidly reversible NPQ could be separated and were observed to operate in an "additive" manner and they concluded that low temperature was the dominant factor defining NPQ induction. This technique has yet to see its full potential realized; routine measurements are commonly made manually (e.g., Zarter et al. 2006a), but, as argued above, this type of high frequency sustained logging enables an empirical link with plant productivity indicators such as RUE.

Techniques for remote sensing of chlorophyll fluorescence through the "Fraunhofer lines" in the daylight spectrum have been used (Meroni et al. 2009; Zarco-Tejada et al. 2012). The term Fraunhofer line is, strictly speaking, misleading in this context, although it is the term that is widely used. Fraunhofer lines are absorption lines resulting from the presence of specific gases in the Sun's atmosphere, but the lines in the Solar spectrum used for fluorescence detection (688 and 770 nm) are due to absorption by molecular oxygen in the Earth's atmosphere (two of the "Telluric lines"). This type of remote fluorescence measurement could be made from aircraft and even satellites, but as it uses ambient light to excite fluorescence it is limited in its capacity to detect NPQ as there is no capacity to produce the controlled changes in the state of PS II necessary to allow the impact of npd_i to be separated from the impact of photochemical quenching of $^1chl^*$ (see section III.B). Clearly, the best approach would be a remote platform capable of continuous measurement; laser-induced fluorescence transient (LIFT) uses a laser to project a reasonably broad excitation beam onto a target area of canopy. Fluorescence emission is collected using a telescope, filtered, and detected using a large photodiode allowing remote measurement. This technique compares well with contact fluorometry and thus far has been used successfully from distances of 5–50 m or more to measure fluorescence-derived parameters, including NPQ, for ecophysiological studies (Ananyev et al. 2005; Malenovsky et al. 2009; Pieruschka et al. 2012). It can furthermore provide information on the spatial distribution of photosynthetic efficiency (e.g., Ananyev et al. 2005). This technique currently requires customized construction as there is no commercially available instrument.

The spectra of light reflected from vegetation (spectral reflectance) of plant canopies can be used to remotely assess properties such as plant nitrogen content and drought (Babar et al. 2006; Malenovsky et al. 2009). The Photochemical Reflectance Index (PRI) is one such index that can be derived from wavelengths corresponding to de-epoxidation of the VAZ cycle and chlorophyll: carotenoid ratios (Gamon et al. 1990; Peñuelas et al. 1995; Evain et al. 2004). The PRI is usually calculated as [R531 − R570]/[R531 + R570]. Here, R indicates reflectance at each wavelength. PRI has been shown to correlate with a variety of parameters including $\Phi_{PS\ II}$, NPQ, de-epoxidation state of the VAZ cycle, chlorophyll/carotenoid ratio, CO_2 uptake and even RUE. One of the most interesting conclusions from PRI work thus far is the correlation with RUE (Filella et al. 2009; Garbulsky et al. 2011). Such a link with light-use efficiency would seem intuitive; as photosynthesis reaches and exceeds saturation, a higher proportion of absorbed energy will be dissipated via the de-epoxidized components of the VAZ cycle. Additionally a higher chlorophyll: carotenoid ratio may indicate a higher photosynthetic capacity. However a mechanistic link with NPQ alone is currently confounded by the action of several other physiological factors on the PRI signal (Garbulsky et al. 2011).

III What Underlies the Diversity of NPQ?

While the phenomenon of non-photochemical quenching in leaves is defined in simple terms - a decrease of F_m' below F_m is due to npd_i - this simple definition conceals a complexity of causes. Initial analyses of the components contributing to chlorophyll fluorescence quenching were based on addition of DCMU to intact chloroplasts and algae to rapidly stop electron transport and allow relaxation of fluorescence quenching (Krause et al. 1982). This essentially destructive procedure was replaced by non-destructive techniques based on the application of saturating light pulses to measure F_m', allowing the relaxation of NPQ to be measured. A disadvantage is that the saturating pulses can disturb dark relaxation of NPQ. Nonetheless, using the saturating-pulse technique a separation between the contributions made by different quenching components based on their different relaxation times in the dark can be made (Horton and Hague 1988; Quick and Stitt 1989; Walters and Horton 1991; Rohacek 2010; see Dau and Hansen (1989) for a technique to measure time constants in illuminated leaves). The first analyses of NPQ divided it (and by implication npd_i) into three components, i.e., qE, due to “energy-dependent” quenching; qT, due to state-transitions; and qI, due to slowly reversible quenching, which was assumed to result only from photodamage to PS II (Horton and Hague 1988; Quick and Stitt 1989; Walters and Horton 1991; Rohacek 2010; for a different interpretation of qI, see Adams and Demmig-Adams, Chap. 2, Adams et al., Chap. 23, Demmig-Adams et al., Chap. 24). The halftime for qE relaxation is about 30–60 s, for qT it is about 5–10 min, and for qI it is over 30 min. The total amount of NPQ (and therefore npd_i) depends strongly on the history and physiological state of the leaf, but qT is restricted to, and dominates in, low irradiances (in light-limiting conditions or nearly so; Walters and Horton 1991; Rintamäki et al. 1997), while qE develops and increases as irradiance increases above light-limiting values. qI is characteristic of leaves that have been exposed to irradiances above saturation for prolonged periods (see Demmig-Adams et al., Chap. 24).

The association between qE and energy-dependent quenching is unambiguous even though the nature of energy-dependent quenching is still unclear (Horton et al. 2005; Holzwarth et al. 2009; Johnson and Ruban 2010; Jahns and Holzwarth 2012; see, e.g., Krüger et al., Chap. 6; Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14; van Amerongen, Chap. 15). In the case of qT and qI, however, a strict association with state-transitions and photoinhibition, respectively, is less certain.

State-transitions in plants are reported as a mechanism acting under light-limiting conditions and serving to bring into balance excitation of photosystems I and II (Allen 1992; Vener et al. 1998; Krause and Jahns 2004). It has been shown that a greater overexcitation of PS II provokes a greater state transition (Hogewoning et al. 2012), a decrease in PS II cross-section (Deng and Melis 1986), an increase in PS I size due to attachment of LHCII (Kouril et al. 2005), with complementary changes in PS II and PS I cross-section (Samson and Bruce 1995). All of the latter results are consistent with state-transitions altering the cross-sections of PS II and PS I to adjust the electron-transport balance of PS I and PS II. In addition, PsaH and PsaL subunits of PS I have been identified as essential for state-transitions (Lunde et al. 2000). However, there are still unresolved issues surrounding state-transitions that pose problems for the basic state-transition model. In some cases decreases in PS II cross-section are not accompanied by an increase in PS I cross-section (Haworth and Melis 1983), and in a study of the effect of state-transitions on the quantum yield for carbon dioxide fixation, Andrews et al. (1993) found no improvement in light-use efficiency. These apparent disagreements with the basic state-transition model have yet to be resolved. In addition to state transitions, chloroplast movements, which do contribute to the photoprotection of PS II (Park et al. 1996; Kasahara et al. 2002), could also contribute to changes in F_m' (Brugnoli and Björkman 1992), and these movements relax in a time range of minutes and could thus be confused with a state-transition. In contrast to state-transitions, however, chloroplast movement to minimize light absorption is a high-light response (Park et al. 1996; Gorton et al. 1999; Kasahara et al. 2002). The extent to which these movements can be (and have been) confused with other forms of npd_i has not been well studied.

The slowly reversible component of npd_i, qI, cannot simply be attributed to recovery from photodamage as was once believed. In addition to photodamage, slowly reversible forms of quenching can occur that share similarities with qE type quenching (Demmig-Adams and Adams 2006). As these are discussed at length elsewhere in this book (Adams and Demmig-Adams, Chap. 2, Adams et al. Chap. 23, Demmig-Adams et al. Chap. 24), they will not be described further here.

A Genes, Environment and Mechanisms

Variation in NPQ capacity (here we largely refer to qE) across species and genotypes is commonly observed and can arise from factors that can be broadly divided into those resulting from genetics and those caused by phenotypic “plasticity”, i.e., acclimation to the environment.

Acclimation of NPQ has been examined in contrasting plant types, and it is possible to draw generalizations based on growth habit and ecology. There is support for the concept that fast-growing annual mesophytes, such as crop plants, have relatively lower capacities for ΔpH-dependent, fast-relaxing NPQ, associated with higher photosynthetic capacities, whilst evergreen species with slower growth rates and lower photosynthetic capacities such as *Monstera deliciosa* have relatively higher capacity for the same form of NPQ (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006). *Monstera deliciosa* did not increase photosynthetic capacity between growth light intensities of 300 and 1,500 μmol m^{-2} s^{-1}, but exhibited a strong increase in the capacity of pH-dependent inducible NPQ (qE) and in the level of the PsbS protein. Conversely, low-light grown *Monstera* suddenly transferred to high light exhibited a sustained ΔpH-independent form of NPQ (qI) associated with continuous zeaxanthin retention and synthesis of one or two high-light inducible proteins (one-helix proteins related to PsbS). This was also associated with a rearrangement, or degradation, of PS II components to limit the proportion of absorbed light energy available for photochemistry. Annual mesophytes (including spinach and *Arabidopsis*) show an enhanced ability to acclimate (upregulate) photosynthetic capacity over the same light range, but do not show induction of qI or an increase in PsbS level whilst zeaxanthin or VAZ pool size did not increase (Demmig-Adams et al. 2006; Zia et al. 2011). Bailey et al. (2004) also did not observe a correlation with VAZ cycle pool sizes or in de-epoxidation state upon acclimation to high light in *Arabidopsis thaliana*.

This supports the general concept that the level of fast-relaxing ΔpH-dependent NPQ is in part determined by the capacity of the plant to utilize absorbed energy in photosynthesis that is in turn limited by environmental conditions, ability to acclimate intrinsic photosynthetic capacity, and interactions between both. Fast-growing species with high leaf photochemical capacity may (a) have less need for non-photochemical mechanisms of protection and (b) have a need to avoid the risk of a lowered quantum yield in a highly variable light environment that may arise from enhanced PsbS (Li et al. 2002; Hubbart et al. 2012). Hence fast-growing species predominantly make use of rapidly induced and rapid relaxing qE, but possibly at a limited maximal capacity for qE. Conversely, there may be a need to reduce metabolic cost of oxidative stress and recovery from photoinhibition (Powles and Björkman 1981; Raven 1994; Ögren 1994) in species, where the margins for carbon gain are smaller, or qI may alternatively be interpreted as representing a continuous form of photoprotection (see Adams et al. 2013, Chap. 23; Demmig-Adams et al. Chap. 24).

Diversity in mechanisms underlying NPQ was discussed by Garcia-Plazaola et al. (2012) who interpreted variation in longevity of NPQ as a "molecular memory" of previous light events, a concept previously suggested by Horton and Ruban (2004) and Murchie et al. (2009). Plants with a greater need for protective NPQ because of low photosynthetic capacity and/or extreme environmental conditions (overwintering, low water availability) would need to integrate "memory" over longer time periods.

However, this discussion does not cover the diversity in photoprotective mechanisms that can influence NPQ levels; other environmental conditions, plant processes, and components would need to be considered in a more extensive analysis. For example, Lhc-related proteins such as Elip, Sep, and Ohp are upregulated in some species during the transition to a ΔpH-independent quenching state under cold and light stress (Jansson 1999; Adamska 2001; Zarter et al. 2006a, b; Adams and Demmig-Adams, Chap. 2) and under light stress alone (Demmig-Adams et al. 2006). The existence of alternative electron sinks, such as the Mehler reaction, can provide photochemical quenching under high light without a change in photosynthetic capacity (Murchie et al. 1999; Weng et al. 2008). Other factors that limit photosynthetic capacity, and hence influence NPQ expression, are nutrient levels (Verhoeven et al. 1997; Chen et al. 2003; Morales et al. Chap. 27) and temperature (Adams and Demmig-Adams 1995; Adams et al. 1995; Savitch et al. 2002; Adams and Demmig-Adams, Chap. 2).

In general, the range of NPQ capacity resulting from acclimation to high and low light is large, typically from two to four-fold, depending on species (Johnson et al. 1993; Demmig-Adams 1998; Bailey et al. 2004; Demmig-Adams and Adams 2006; Zia et al. 2011). This implies that the role of npd_i in protection against oxidative stress and PS II damage is significant. Evidence for processes underlying NPQ in helping to prevent photoinhibition is clearly provided by work that enhanced or reduced PsbS levels by genetic modification (Li et al. 2002; Niyogi et al. 2005). However, when trying to assess the role of such processes in influencing plant productivity in natural, variable environments using models such as those described above, we run into the problem that we do not have an empirical understanding of the relationship between processes underlying NPQ and D1-degradation, in other words "photoprotective effectiveness". The relationship with NPQ is complicated by the fact that a number of metabolic factors are involved in the onset of photoinhibition (some of them well defined) and deconvoluting the effect of NPQ is thus difficult. For example, processes underlying NPQ are not the only photoprotective mechanisms in plants, and the onset of multiples stresses, such as heat and high irradiance, cause concurrent processes to reduce the likelihood of photoinhibition (Cheeseman et al. 1991; Murchie et al. 1999). Further, the VAZ cycle is a highly regulated mechanism that underlies much of NPQ (Horton et al. 1996; Demmig-Adams et al. 2012; Ruban et al. 2012), whereas the quenching by zeaxanthin of singlet oxygen or even triplet chlorophyll is comparatively unregulated (Foyer and Harbinson 1999; Baroli et al. 2003) and yet may contribute to NPQ. In addition, accurate measurement of photoinhibition in the field is straightforward from a simple measurement of F_v/F_m, usually after 20–30 min in the dark (Maxwell and Johnson 2000). This gives an indication of any sustained quenching mechanism present in the leaves, but it does not allow any separation of quenching caused by damage or zeaxanthin retention. However the extraction of underlying components of NPQ is more difficult; e.g., the separation into qE, qT and qI and the time for dark adaptation means that it is practically difficult to make a routine measurement without disrupting photosynthesis. The latter parameters allow us to determine energy partitioned into thermal dissipation (Kramer et al. 2004, and see section III.B.3), but leave the question of how effective this dissipation is in the prevention of photoinhibition.

Recently a method was proposed that used a calculated value of F_o' (Oxborough and Baker 1997) to measure qP in the dark (dark-qP) following a photoinhibitory treatment (Ruban and Murchie 2012). Using a series of PsbS and xanthophyll mutants, this method showed that dark-qP values always deviated from 1.0 at the same point at which $\Phi_{PS\ II}$ deviated from values predicted by the value of NPQ. Therefore a measurement of photoinhibition can be made "continuously" in the light (it just requires darkening briefly to identify dark-qP level) and, in principle, provides a more accurate measurement of "photoprotective" NPQ than qE. This method still requires field testing.

Despite the strong evidence that NPQ is protective it must be put into proper ecological context: the proposition that photoinhibition is a continuously maintained version of thermal dissipation (accompanied by inactivation of PS II photochemistry) under long-term sink limitation (Adams et al. 2013, Chap. 23) would affect the protective role of NPQ under these conditions. For fast growing plants, including many crop species we must consider the evidence that yield can be limited by source activity (e.g., Murchie et al. 2009) so the importance of sink limitation remains to be established.

We have shown that NPQ shows a high level of variation between species and genotypes, and that this has been rationalized by the interaction of genetic adaptation with environmental variability. What are the genetic components that determine the capacity for processes underlying NPQ? Jung and Niyogi (2009) conducted a Quantitative Trait Locus (QTL) analysis of *Arabidopsis* accessions of diverse origins that displayed high, low, and intermediate values of NPQ (qE). Two high-NPQ QTLs were identified on chromosomes 1 and 2. However, neither QTL mapped to a region with known genes for functions in qE, including PsbS or the VAZ cycle. Although it is correct that a more diverse set of growth and measuring conditions should be used to expose "true" NPQ capacity, e.g., by restriction of photochemical capacity during measurement (Demmig Adams et al. 2006), this finding is nonetheless important because it suggests that NPQ may not have a robust in vivo relationship with PsbS or zeaxanthin. NPQ may be determined by an interaction between a large number of genetically controlled metabolic and photochemical components, which is consistent with some aspects of its "kinetic diversity". For example, Johnson and Ruban (2010) demonstrated that PsbS was not necessary for the expression of high qE over long time periods, and showed that a macrostructural reorganization of PS II involving intrinsic aggregation of light-harvesting complexes may be involved, with PsbS as the accelerator for this process (Ruban et al. 2012). Moreover, PsbS was found to be effective in enhancing NPQ in the total absence of zeaxanthin, showing that interaction may not be required (Crouchman et al. 2006).

B Dynamics of Photosynthesis and Quenching in a Fluctuating Environment

1 Measurement in Response to Changes in Light Intensity

An essential property of npd$_i$ is that it competes with photochemistry as a de-excitation pathway for ^{1}chl* in PS II (see discussion in Genty and Harbinson 1996) and therefore, in principle, reduces the efficiency of photochemistry. If there is photosynthesis, there must be photochemistry, but the absence of k$_p$ in the SVQ parameter implies that it cannot provide information on the impact of npd$_i$ on the operation of PS II when there is photochemistry. If the significance of the activity npd$_i$ on operation and regulation of PS II is to be fully understood, the impact of npd$_i$ needs to be assessed in parallel with photochemistry.

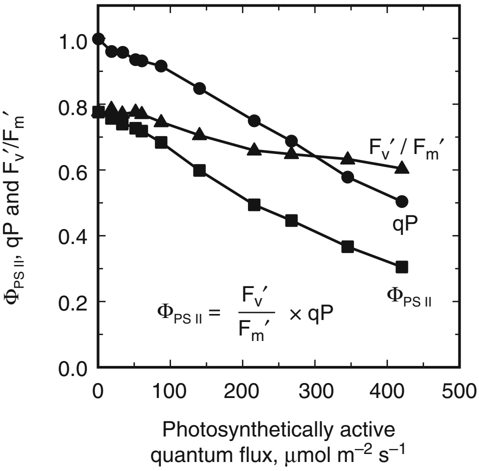


Fig. 25.6. An example of the responses of F_v'/F_m', qP and $\Phi_{PS\,II}$ to a series of increasing photosynthetically active quantum fluxes (a measure of irradiance). Measurements were made on a leaf of *Juanulloa aurantiaca.*

2 The Genty Equation and F_v'/F_m'

A method for assessing the consequences of npd$_i$ on PS II in the presence of photochemistry was developed by Genty et al. (1989) as part of their strategy for estimating the quantum yield for PS II photochemistry ($\Phi_{PS\,II} = k_p/(k_p + k_d + k_D + k_f)$) from measurements of chlorophyll fluorescence. They showed that $\Phi_{PS\,II}$ was equivalent to the product of F_v'/F_m' and qP (Fig. 25.6), where F_v'/F_m' is the efficiency of PS II when all reaction centers are open and qP is a measure of the probability that a ^{1}chl* in PS II will produce photochemistry (i.e., encounter an open trap). Note that the use of chlorophyll fluorescence to measure F_v/F_m, F_v'/F_m' or $\Phi_{PS\,II}$ produces an underestimate of the actual value of these parameters (Lavergne and Trissl 1995; Baker et al. 2007; see discussion about the Genty equation in Wientjes et al. 2013). As the qP parameter is a measure of trap openness, it is affected by the redox state of Q_A, although this dependency is non-linear (Joliot and Joliot 1964; Lavorel and Joliot 1972; and see also more recent discussions by Lavergne and Trissl 1995; Kramer et al. 2004). In a dark-adapted, unstressed leaf F_v/F_m is around 0.82 for C3 leaves (Björkman and Demmig 1987) and qP is assumed to be one, so dark-adapted $\Phi_{PS\ II}$ (based on fluorescence measurements) is around 0.82 (1.0 × 0.82).

When leaves are exposed to actinic irradiances there will usually be a decrease in qP, F_v'/F_m', or both, and this results in a proportionate decrease in $\Phi_{PS\ II}$ (Fig. 25.6). Decreases in F_v'/F_m' below the dark-adapted F_v/F_m are the result of increases in qE and qI (because state-transitions result in energy transfer to PS I, changes in qT result in no changes in F_v'/F_m'; Hogewoning et al. 2012), while decreases in qP below 1.0 are due to reduction of the Q_A pool. In the absence of any changes in qE and qI, all changes in $\Phi_{PS\ II}$ will be due to changes in qP (e.g., Hogewoning et al. 2012; i.e., excitation energy in PS II excess to the needs of photochemistry is dissipated by the npd_b mechanism), an increase in fluorescence yield, and an increase in $^1chl^*$ lifetime. On the other hand, if npd_b, and the npd_i mechanisms that give rise to qE and qI, were to dissipate all excess excitation in PS II, then qP would remain at 1.0 and all decreases in $\Phi_{PS\ II}$ below the dark-adapted value would be due to decreases in F_v'/F_m'. If a decrease in qP is taken as evidence of excess excitation of PS II (i.e., the pool of $^1chl^*$ exceeds the capacity for photochemical dissipation of this pool), then perfect regulation of $^1chl^*$ lifetime by the adjustment of npd_i to prevent excess excitation would result in qP remaining at 1.0 and all changes in $\Phi_{PS\ II}$ being due to changes in F_v'/F_m'. The effectiveness of npd_i (resulting in qE and possibly qI) as means of dissipating excess PS II excitation can be shown by comparing the changes in qP and F_v'/F_m' that accompany changes in $\Phi_{PS\ II}$ (Fig. 25.7). Unfortunately, the factor for converting F_v'/F_m' to NPQ, while simple (Fig. 25.5), has no straightforward physiological meaning. Further, while the relationship between F_v'/F_m' and NPQ is approximately linear (Fig. 25.8), it will show a stepped response if there is a state-transition (the qT component of npd_i).

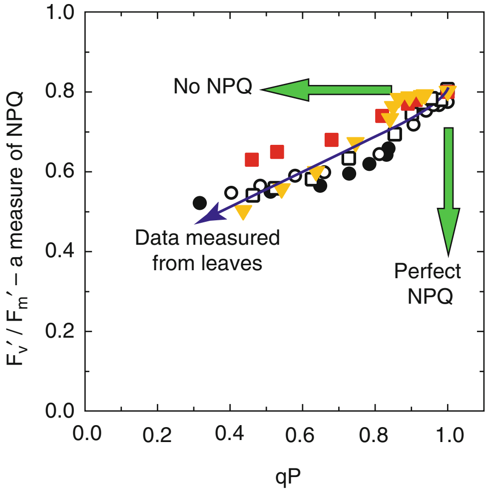


Fig. 25.7. The relationship between qP and F_v'/F_m' for leaves of *Juanulloa aurantiaca* (*black* symbols) subjected to increasing photosynthetically active quantum flux (a measure of irradiance) (0–420 μmol m^{-1} s^{-2}; *open* symbols) or decreasing CO_2 concentrations (350–35 ppm) in 2 % O_2 (*solid* symbols), or *Begonia luzonensis* (*red* symbols) or *Pisum sativum* (*yellow* symbols) subject to increasing photosynthetically active quantum flux (0–250 μmol m^{-1} s^{-2} for *B. luzonensis* and 0–1 500 μmol m^{-1} s^{-2} for *P. sativum*). Also shown are the trajectories that would be expected for the situation were all of the loss of $\Phi_{PS\ II}$ due to the activity of npd_i and the situation where there was no npd_i.

3 *Calculating the Yields of npd_b and npd_i*

The decrease of $\Phi_{PS\ II}$ from the maximal dark-adapted value is, in fact, accompanied by an increase in photochemical inefficiency. In the absence of npd_i (in the dark-adapted state, for example), even when $\Phi_{PS\ II}$ is zero (e.g., at F_m), PS II $^1chl^*$ are still being dissipated non-photochemically via npd_b mechanisms represented by k_f and k_d. This implies that in the dark-adapted state, when npd_i is absent, there are still npd_b mechanisms at play. Once

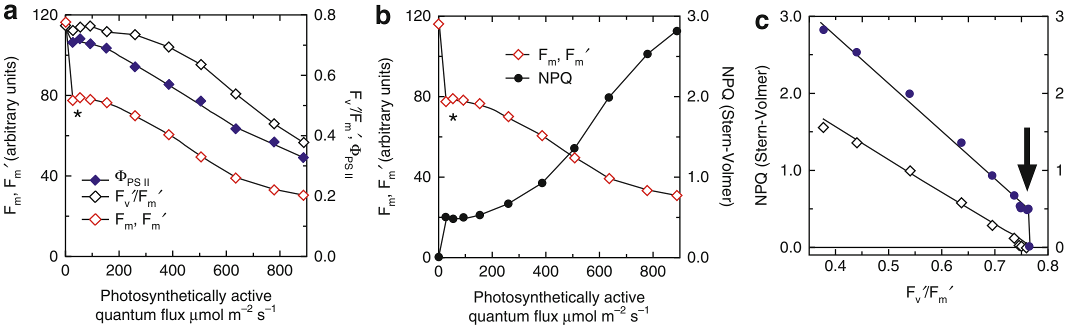


Fig. 25.8. A comparison of the responses of F_m and F_m', F_v'/F_m', and $\Phi_{PS\,II}$ (**a**) and F_m and F_m' and NPQ (Stern-Volmer) to the photosynthetically active quantum flux (a measure of irradiance) (**b**), measured from a leaf of *Prunus laurocerasus* collected from deep shade at the edge of the dysphotic zone (i.e., the zone within the canopy that is without leaves owing to low irradiance (e.g., Fig. 1.29 in Larcher 1995). In the case of this leaf there is an unusually large decrease in fluorescence yield between F_m and the first measurement of F_m' (marked with an '*'). The scale of the decrease in this case allows the consequences of low light changes in F_m and F_m' to be more clearly shown, and other than for the scale of this phenomenon is quite normal. This decrease in F_m' is not associated with any effect on F_v'/F_m' (**a**), suggesting that it was not due to qE type quenching (which is associated with a decrease in F_v'/F_m' – see Fig. 25.6) and that it may have been due to a state-transition (though this was not otherwise demonstrated). Though the fall in fluorescence yield from F_m to F_m' has no effect on F_v'/F_m' it does produce a step increase in NPQ that precedes an overall increase in NPQ with increasing irradiances **b** This change in NPQ at low photosynthetically active quantum flux without a corresponding change in F_v'/F_m' has a strong effect on the relationship between NPQ and F_v'/F_m' (**c**) The upper curve in (**c**) shows that while the relationship between NPQ and F_v'/F_m' is broadly linear (actually slightly curvilinear), the increase in NPQ corresponding to the fall in F_m at the first measurement of F_m' produces a step increase in the relationship between NPQ and F_v'/F_m' (shown by an *arrow*). If NPQ is calculated using the first measure of F_m' (marked by an '*' in **a** and **b**), which corresponds to the F_m without qE and with the maximum effect of qT, then the relationship between NPQ and F_v'/F_m' (shown by the *lower* curve in (**c**) now lacks the step increase (shown by the *arrow*) and is still broadly linear, but the slope differs from that shown in the upper curve.

npd_i has been activated in the form of qE or qI, this also dissipates $^1chl^*$. Importantly, as implied by the effect of npd_i on the efficiency of open PS II (measured by F_v'/F_m'), and by the fact that dark-adapted F_v/F_m is only around 0.82, the npd_b and npd_i mechanisms both compete significantly with photochemistry. In the absence of any npd_i, all PS II inefficiency is due to npd_b and the quantum yield for this process (termed $\Phi_{NP}{}^c$ by Cailly et al. 1996, $\Phi_{f,\,D}$ by Hendrickson et al. 2004, or Φ_{NO} by Kramer et al. 2004) is given by F'/F_m (or F_o/F_m if in the dark-adapted condition; Fig. 25.5). Once npd_i is present, it offers another dissipation pathway, and the yield of this mechanism (termed $\Phi_{NP}{}^r$ by Cailly et al. 1996 or Φ_{NPQ} by Hendrickson et al. 2004; see also Kramer et al. 2004) is given by $F'/F_m' - F'/F_m$ (Fig. 25.5). It is therefore possible to calculate the quantum yield for photochemistry, the quantum yield for dissipation by npd_b (k_f and k_d), and the quantum yield for dissipation by npd_i (k_D). Further, as NPQ is $k_D/k_f + k_d$ (Fig. 25.4b, Eq. 6) then NPQ is also Φ_{NPQ}/Φ_{NO} (Fig. 25.5, Eq. 6) – the ratio of the yields for npd_b and npd_i is identical to the Stern-Volmer quenching parameter (NPQ) used to quantify npd_i. NPQ can therefore be used to calculate Φ_{NPQ} and Φ_{NO} as $\Phi_{PS\,II} + \Phi_{NPQ} + \Phi_{NO} = 1$. For a related discussion, see also Logan et al. (Chap. 7).

4 Constraints to Productivity Arising from Responses of Quenching to Dynamic Environments

We have described how NPQ, and the underlying non-photochemical dissipation processes that give rise to it, can be quantified in leaves, and introduced the biophysical processes that give rise to NPQ. This discussion provides the necessary background to the coverage of NPQ variation across different scales (chloroplast to plant to canopy system) and how this can be rationalized from physiological and genetic viewpoints. It should be clear by now that the interpretation of NPQ values requires careful consideration of how they were measured (see also Logan et al., Chap. 7). For example, practical problems with dark adaptation and existence of different components of NPQ with different half-times for relaxation can make complex measurements and interpretation out of the lab quite difficult (Horton and Hague 1988; Quick and Stitt 1989; Walters and Horton 1991), and we argue here that the latter problems play a role in our current incomplete understanding of the contribution of NPQ to vegetation productivity. Our assessment and rationalization of diversity in the "expression" of NPQ in nature should be seen as complementing other chapters in this book (e.g., Chaps. 22, 23, 24, and 25).

The processes contributing to NPQ should be considered as integrated into the regulation of photosynthesis and as such it would seem natural to incorporate these into mathematical models that describe photosynthesis and plant productivity. However this is difficult, possibly due to lack of empirical understanding of its effectiveness in reducing photoinhibition (here defined as inactivation of PS II requiring removal and replacement of core components) or reducing quantum yield. This is especially true when we consider that many other environmental and intrinsic factors combine to influence NPQ such as temperature and nutrient content. Biochemically based models for NPQ mechanisms have been proposed, the most recent of which is pH dependent and simulates the kinetics of qE at different light intensities incorporating key components such as PsbS protonation and xanthophyll de-epoxidation (Kanazawa and Kramer 2002; Ebenhöh et al. 2011; Zaks et al. 2012). We will eventually need an extension of mechanistic models such as this to biochemical and physiological models of photosynthesis (von Caemmerer and Farquhar 1981; Zhu et al. 2007) and eventually canopy photosynthesis (Song et al. 2013).

Zhu et al. (2004) modeled the decline in quantum yield of CO_2 assimilation that occurs during sustained NPQ. During such quenching, the quantum yield and the convexity of the light response curve is altered to an extent that reduces CO_2 assimilation. If the quenching is due to zeaxanthin (or photoinhibition), then the recovery in low light recovery after a high light event may be slow. A plant canopy experiences a shifting pattern of light in time and space and therefore can be analyzed as a population of leaf segments that have differing states of recovery. By describing light distribution using ray-tracing algorithms, Zhu et al. (2004) concluded that the lag in recovery substantially reduced canopy carbon assimilation by up to 32 %.

Photoinhibition can be defined as a sustained reduction in photosynthesis caused by high light and is variously associated with a reduction in quantum yield, convexity, and light saturated rate of photosynthesis (Long et al. 1994; Murchie and Niyogi 2011). Mathematical descriptions of how photoinhibition limits canopy photosynthesis have been carried out and describe an impact on productivity (e.g., Ögren and Sjöström 1990; Long et al. 1994; Werner et al. 2001). Some processes responsible for NPQ contribute to the prevention of photoinhibition (Li et al. 2002). An argument can be made that photoinhibition itself acts in a photoprotective manner and this is set out in Chap. 23 of this book. By inference, the protection provided by those processes is a significant factor that influences productivity. Earlier in the chapter we discussed how the "protective effectiveness" of NPQ can be quantified.

So far we have largely considered the capacity for NPQ measured under steady-state light conditions. However what does this value represent? It is common to measure the capacity for thermal energy dissipation as NPQ under conditions of high light intensity when these processes are maximally activated. Alternatively the capacity for NPQ (and by implication the maximum activity of npd_i) has been measured using the less physiologically relevant conditions of 2 % O_2 and 98 % N_2 to minimize photochemistry and provoke the maximum possible level of NPQ (Demmig-Adams and Adams 2006). Another complicating factor is that a longer exposure to high light, over a period of minutes to hours, may increase NPQ level and this can even occur at low levels of PsbS protein (Johnson and Ruban 2010). Therefore great care should be taken when measurements of NPQ capacity are made and interpreted.

Light commonly fluctuates and the kinetics of NPQ induction may thus be relevant for its function in vivo. qE is induced in seconds following a sudden increase in light levels to form the ΔpH. This is consistent with its role in preventing photoinhibition during the onset of sudden illumination such as a sunfleck (Pearcy 1990; Hubbart et al. 2012; Porcar-Castell and Palmroth 2012). The induction of photosynthesis from a state of darkness or low light can place PS II into a highly reduced state, increasing the likelihood of triplet chlorophyll formation and possibly photoinhibitory damage to PS II. In addition to processes underlying NPQ, this is alleviated by a number of processes including the gradual activation of Calvin cycle enzymes, the accumulation of metabolite pool sizes, and processes such as cyclic electron transport (Johnson 2011; Carmo-Silva and Salvucci 2013). It is therefore intriguing to discuss whether an important function of processes underlying NPQ is not at steady state in which we commonly measure it but in fact to protect and regulate PS II in dynamic environments. It could be said that NPQ may act in a general way to "buffer" the electron transport chain against sudden fluctuations in irradiance. Indirect evidence for this comes from growth of the *A. thaliana* mutant npq4 lacking PsbS in natural environments or controlled fluctuating conditions (Külheim et al. 2002; Külheim and Jansson 2005). It was clear that lowered levels of NPQ were associated with a reduced level of fitness (based on seed production) in fluctuating environments only and was attributed to a decline in the quantum yield of PS II and increase in the level of photoinhibition. However, this does not distinguish between prevention of photoinhibition at steady state or that occurring during induction/light transients. Other work has suggested that reduced fitness in these experiments could be attributed to other metabolic changes induced by levels of jasmonates (Frenkel et al. 2009; Adams et al. 2013).

Is there any evidence for the mechanism of control of the rate of induction of NPQ? *A. thaliana* plants were engineered to possess increased pool sizes of VAZ cycle carotenoids by overexpressing β-carotene hydroxylase (Davison et al. 2002). These plants did not show enhanced capacity for NPQ but rather a notable tolerance to oxidative stress conditions in comparison to wild type, which was attributed to the antioxidant properties of zeaxanthin (Davison et al. 2002; Johnson et al. 2007). Additionally, the larger pool sizes affected the kinetics of induction of NPQ, which was notably slower in the overexpressors (Johnson et al. 2008). This is because the rate of NPQ formation is a result of the de-epoxidation state of the VAZ cycle pool, the half time for which is determined by the pool size itself. A similar effect was observed in rice plants that had been transformed to over express the same gene (E. H. Murchie, unpublished data). PsbS can also be used to alter the dynamics of NPQ formation: the rate of induction of NPQ is observed to be slower in plants with reduced PsbS such as npq4 and rice (Li et al. 2002; Niyogi et al. 2005; Hubbart et al. 2012). Overexpressing PsbS in L17 *A. thaliana* plants also enhanced the rate of formation and the rate of NPQ relaxation (Zia et al. 2011).

We would expect the rates of formation and relaxation to determine the overall rate of induction of NPQ in leaves. An obvious question is why plants do not constitutively over express PsbS and accumulate high VAZ cycle pool sizes. It has been suggested that this may lead to an over reduction of PS II quantum yield (Li et al. 2002). Direct evidence for this was provided by Hubbart et al.

(2012) who showed that over accumulation of the PsbS protein in rice caused a lowering of electron transport rate and CO_2 assimilation during photosynthetic induction in rice leaves in conditions that fluctuated between high light and darkness over periods of minutes. It was hypothesized that the large photosynthetic capacity of rice was responsible for the extremely rapid and high NPQ that was immediately induced upon illumination in rice. During such conditions, cyclic electron transport and the Mehler reaction are also considered important (Makino et al. 2002).

This leads to the intriguing hypothesis whereby the kinetics of induction of NPQ can be determined by the differential accumulation of PsbS and the VAZ cycle pool size. A high PsbS level that results in rapid induction and higher NPQ capacity could act antagonistically to a high xanthophyll pool size that slows induction, slows the relaxation, and has only a small impact on NPQ capacity (Murchie et al. 2009; Horton 2012). Is this consistent with comparative species observations? Indeed the patterns of acclimation exhibited by evergreens and annual mesophytes show that the former accumulate more PsbS and xanthophylls under high light indicating a need for both rapid induction and retention of thermal dissipation. By comparison, the annuals showed a limited induction of PsbS protein and xanthophyll accumulation under high light, presumably due to their high photochemical capacity and the need to reduce transient reduction of PS II quantum yield (Demmig-Adams et al. 2006). More work will need to be done on a range of genotypes to test the full range of components determining NPQ during induction, including cyclic electron flow (Makino et al. 2002; Johnson 2011; Hubbart et al. 2012).

Mechanisms for regulation of photosynthesis, and especially electron transport in fluctuating light, have attracted a lot of recent attention (Krah and Logan 2010; Grieco et al. 2012; Hubbart et al. 2012; Suorsa et al. 2012, Tikkanen et al. 2012; Carmo-Silva and Salvucci 2013). Early work showed that acclimation of photosynthesis to fluctuating light occurred but could not be fully explained by either maximum irradiance or average irradiance (Yin and Johnson 2000). If processes underlying NPQ have a critical role to play, specifically in induction during fluctuating light conditions, then is it possible that acclimation of NPQ can occur specifically in response to a fluctuating regime? To test this we need to grow plants in a range of controlled static and dynamic environments, continually monitoring over long periods the induction and decay of NPQ, using monitoring or imaging instruments. Work in natural systems suggests a key role for the VAZ cycle in acclimation to sunflecks, e.g., Adams et al (1999); plants growing in natural fluctuating-light environments exhibited extremely fast increases and decreases in inducible NPQ from second-to-second, and showed overnight retention of a pool of zeaxanthin (see also, e.g., Demmig-Adams et al., Chap. 24).

An intriguing and fundamental question is whether induction of photosynthesis is sufficient to induce acclimation of the dynamics of NPQ.

IV Conclusion

We have described the factors that contribute to the formation and the capacity of NPQ, the issues with measurement across different scales, and the rationale for NPQ variation with respect to variation in light intensity. We have focused on the rapidly formed ΔpH-dependent form of NPQ that is highly regulated by multiple mechanisms and operates to protect against light-induced stress whilst there is also some evidence that this is traded off against the risk of a reduced quantum yield and photosynthesis under some conditions. The increased technical capability to measure and accurately deconvolute NPQ in the field, coupled with increased sophistication of models that scale metabolic components to leaf, whole plant, and canopy level mean that our quantitative understanding of how NPQ operates to influence vegetative productivity will improve markedly.

Acknowledgments

JH would like to acknowledge financial support from the EU Marie Curie ITN 'Harvest' (grant 238017) and the Dutch national programme 'Biosolar Cells'. EHM acknowledges funding from the BBSRC (UK).

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Chapter 26

Beyond Non-Photochemical Fluorescence Quenching: The Overlapping Antioxidant Functions of Zeaxanthin and Tocopherols

Michel Havaux*
Department of IBEB, CEA, DSV, Laboratoire d'Ecophysiologie Moléculaire des Plantes, Saint-Paul-lez-Durance F-13108, France
CNRS, UMR 7265 Biologie Végétale et Microbiologie Environnementales, Saint-Paul-lez-Durance F-13108, France
Aix-Marseille Université, Saint-Paul-lez-Durance F-13108, France

and

José Ignacio García-Plazaola
Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Bilbao 48080, Spain

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*Author for Correspondence, e-mail: michel.havaux@cea.fr

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_26,

Summary

Tocopherols, present in plastids from all lineages of photosynthetic eukaryotes, have long been recognized as key constituents of photoprotective defenses. Membrane-bound tocopherols play an essential antioxidant role by quenching singlet oxygen and preventing the propagation of lipid peroxidation through their radical scavenging activity. However, experiments with tocopherol-deficient plants have shown a surprisingly low impact of high light, and this apparently is the result of a functional overlap of tocopherol with zeaxanthin (Z). Apart from the role of zeaxanthin in the modulation of thermal dissipation (assessed via non-photochemical quenching of chlorophyll fluorescence, NPQ), zeaxanthin molecules dissolved as free pigments in the membrane lipid phase or present at the lipid-protein interfaces may act as direct antioxidants and membrane stabilizers, having a synergistic effect with tocopherol. The existence of pools of unbound zeaxanthin is supported by numerous stress experiments that showed a much higher enhancement of the total pool of violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle pigments than that of their potential binding sites in antenna proteins. Tocopherol content is also subject to strong environmental modulation by stress factors, and the dynamics of the tocopherol and Z pools are frequently highly correlated. However, a significant proportion of the leaf tocopherol pool accumulates in plastoglobules, instead of thylakoids, where its protective role is not so clearly established. From an evolutionary perspective, there seems to be a trend from a xanthophyll cycle-based photoprotection in algae to a more diversified strategy in terrestrial plants.

I Reactive Oxygen Species and Tocopherols

A Photosynthesis, Reactive Oxygen Species and Lipid Peroxidation

Reactive oxygen species (ROS) are inevitable by-products of photosynthesis. Indeed, the photosynthetic processes involve electron-transfer reactions as well as generation of various excited molecules that can interact with O_2, leading to the formation of singlet-excited oxygen ($^1O_2$*) and of reduced forms of O_2, such as superoxide (Apel and Hirt 2004; Li et al. 2009). Superoxide can spontaneously or enzymatically disproportionate to hydrogen peroxide, ultimately leading to the hydroxyl radical (OH**.**) in the presence of metals. Moreover, due to photosynthetic O_2 evolution, the photosynthetic processes occur in an O_2-enriched environment, hence favoring O_2 activation processes. Both $^1O_2$* and OH**.** are highly reactive compounds that can readily oxidize biomolecules near their site of production. This phenomenon is amplified when light energy is absorbed in excess to what can be used by the photosynthetic processes (e.g., under stress conditions). The resulting over-reduction of the photosynthetic electron transport chain and increased lifetime of singlet-excited chlorophylls favor leakage of electron and/or excitation energy to O_2. As a consequence, massive ROS production can overwhelm the protective mechanisms, potentially resulting in oxidative damage to

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Abbreviations: Dd – Diadinoxanthin; Dd-Dt cycle – The xanthophyll cycle involving the carotenoids diadinoxanthin and diatoxanthin; Dt – Diatoxanthin; ELIPs – Early light-induced proteins; H_{II} Non-lamellar hexagonal lipid phase II; HLIPs – High-light-induced proteins; HOTE – Hydroxy-octadecatrienoic acid; L – Lutein; LCHII – Light-harvesting antenna of PS II; LHCSR – Evolutionarily ancient Light Harvesting Complex Stress-Related Protein; Lx – Lutein epoxide; Lx-L cycle – The xanthophyll cycle involving the carotenoids lutein epoxide and lutein; MGDG – Monogalactosyldiacylglycerol; MYA – Million years ago; NPQ – Non-photochemical quenching; PG – Plastoglobule; PS – Photosystem; PsbS – PS II protein PsbS; ROS – Reactive oxygen species; T – Tocopherol or tocotrienol; Toc – α-tocopherol; V – Violaxanthin; VAZ – Violaxanthin + antheraxanthin + zeaxanthin; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin, and zeaxanthin; VDE – Violaxanthin de-epoxidase; Z – Zeaxanthin the photosynthetic apparatus and reduced photosynthetic efficiency (with impacts on plant biomass production remaining to be elucidated; see Adams et al., Chap. 23, for sugar accumulation in photoinhibited leaves under all conditions thus far examined; see also Demmig-Adams et al. Chap. 28).

Because lipids in plant leaves are highly unsaturated (Douce and Joyard 1980), plant lipids are very sensitive to oxidation, and lipid peroxidation is usually a primary event associated with photo-oxidative stress in plants. High levels of unsaturated fatty acids in thylakoid membranes have, in fact, been suggested to be a sink for ROS, which could constitute a protection mechanism against propagation of oxidative stress (Mène-Saffrané et al. 2009). The major fatty acid in plant leaves is linolenic acid (C18:3; Douce and Joyard 1980). Oxidation of this molecule can be measured as an accumulation of a hydroxy fatty acid (hydroxy-octadecatrienoic acid, HOTE; Montillet et al. 2004). Depending on which double bond in C18:3 is oxidized by ROS, different HOTE isomers can be formed. Two of them, 10-HOTE and 15-HOTE, are formed specifically by attack with $^1O_2$*, while the other isomers (9-,12-,13-,16-HOTE) can be formed both by free radicals and $^1O_2$* (Mueller et al. 2006). Thus, the HOTE profile during lipid peroxidation is indicative of the type of ROS involved in the oxidation (Stratton and Liebler 1997). Since photo-oxidative damage/modifications to plant leaves has invariably been found to be associated with a lipid peroxidation signature typical of $^1O_2$* attack on lipids, it has been concluded that $^1O_2$* is the major ROS involved in photo-oxidative damage to plant leaves (Triantaphylidès et al. 2008). In agreement with the latter finding, $^1O_2$* was found to be the predominant ROS produced in *Arabidopsis* cell suspensions exposed to high light (González-Pérez et al. 2011). $^1O_2$*-specific lipid and carotenoid oxidation products were found in *Arabidopsis* leaves even in the absence of any light stress, indicating a certain level of constitutive production of $^1O_2$* in chloroplasts (Triantaphylidès et al. 2008; Ramel et al. 2012).

B Tocopherols, Major Lipid-Soluble Antioxidants of Chloroplast Membranes

Tolerance of plants to photo-oxidative stress requires mechanisms that protect plastid unsaturated lipids from oxidation by $^1O_2$, and tocopherols are believed to be a major component of this protection (Munné-Bosch and Alegre 2002; DellaPenna and Pogson 2006). Together with tocotrienols, tocopherols belong to the vitamin E family. The latter compounds are lipid-soluble and amphipathic, meaning that each molecule contains a polar moiety derived from tyrosine and a hydrophobic polyprenyl side chain originating from the isoprenoid pathway. Different tocopherols and tocotrienols (α-, β-, γ- and δ-tocopherol or -tocotrienol) vary in the number of methyl groups in the aromatic head group (Fig. 26.1a). Furthermore, the isoprenoid side chain is saturated in tocopherols and unsaturated in tocotrienols. Tocopherols occur more widely than tocotrienols in plants and are the principal vitamin E components in leaves of most dicotyledon species (Horvath et al. 2006), while tocotrienols are almost exclusively present in seeds and fruits of various unrelated taxa (Falk and Munné-Bosch 2010). In photosynthetic tissues, tocopherols are found in all chloroplastic membranes (envelopes, thylakoids), and also in plastoglobules (PGs) (Lichtenthaler 2007) that contain a large fraction of tocopherols in leaves of *Arabidopsis* and tobacco (Vidi et al. 2006; Matringe et al. 2008). Interestingly, PGs are not only involved in storage, but also in the metabolism of tocopherols (via presence of the tocopherol cyclase VTE1 protein; Bréhélin et al. 2007; Lundquist et al. 2012). These organelles are also involved in other stress responses, such as the biosynthesis of abscisic acid and jasmonic acid (Bréhélin et al. 2007; Eugeni Piller et al. 2012).

Tocopherols and tocotrienols are potent lipid-soluble antioxidants. The antioxidant activity of tocopherols results from direct chemical reaction with reactive compounds, such as lipid-based free radicals, ROS and reactive nitrogen species (Munné-Bosch and Alegre 2002; DellaPenna and Pogson 2006). A well-known mechanism involves transfer

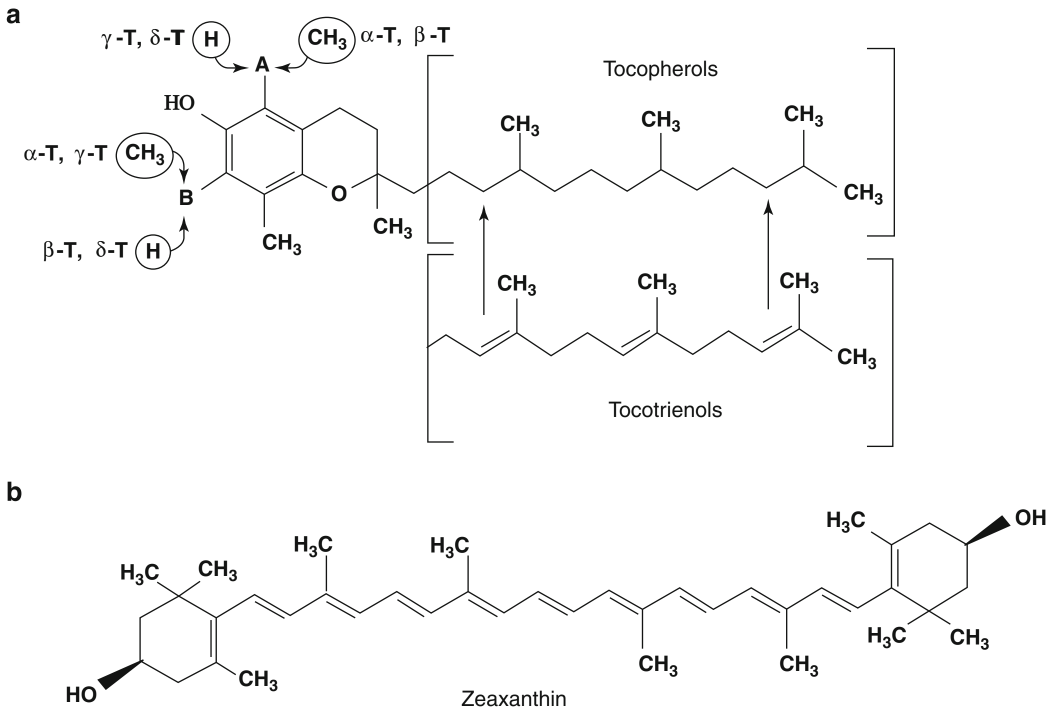

Fig. 26.1 (**a**) Chemical structures of tocopherols and tocotrienols (*T*). Combination of methyl groups in the chromanol head generates the different *α*, *β*, *δ* and *γ* forms. The saturation of the isoprenoid chain differentiates tocopherols from tocotrienols. (**b**) Structure of zeaxanthin.

of a phenolic hydrogen atom from the chromanol moiety of the tocopherol to a lipid peroxy radical, resulting in the production of a lipid hydroperoxide and a tocopheroxyl radical (Liebler et al. 1996). Lipid peroxidation propagation is thus stopped and the latter radical can be reconverted back to the corresponding tocopherol by reaction with another reductant (e.g., ascorbate) (Liebler et al. 1989). The reaction between the tocopherol radical and a second radical results in production of the corresponding tocopherolquinone and other oxidation products. In fact, α-tocopherolquinone was the only tocopherol-oxidation product detected in *Arabidopsis* leaves (Kobayashi and DellaPenna 2008). Physical quenching of $^1O_2$* constitutes yet another important role of tocopherol (Kaiser et al. 1990; Triantaphylidès and Havaux 2009). It has been estimated that, before being degraded, one molecule of α-tocopherol can deactivate up to 120 $^1O_2$* molecules by resonance energy transfer (Fahrenholz et al. 1974). Despite its relatively high efficiency, the rate constant for $^1O_2$* quenching of tocopherols is substantially lower than that of carotenoids (Di Mascio et al. 1990).

The antioxidant functions of tocopherol stated above were observed in vitro (Burton and Ingold 1981). In addition, tocopherols have been shown to be essential for seed longevity and for preventing lipid peroxidation during germination (Sattler et al. 2004). However, mature plants of the tocopherol-free *vte1* and *vte2* mutants of *Arabidopsis thaliana* (Fig. 26.2) surprisingly had no phenotype under optimal conditions and displayed only a slightly increased sensitivity to photo-oxidative stress (Porfirova et al. 2002; Havaux et al. 2005). Similarly, tocopherol-deficient *Synechocystis* mutants did not differ much from wild type (Collakova and DellaPenna 2001). While lack of obvious phenotypes may, at first glance, seem to be in contradiction with the presumed antioxidant

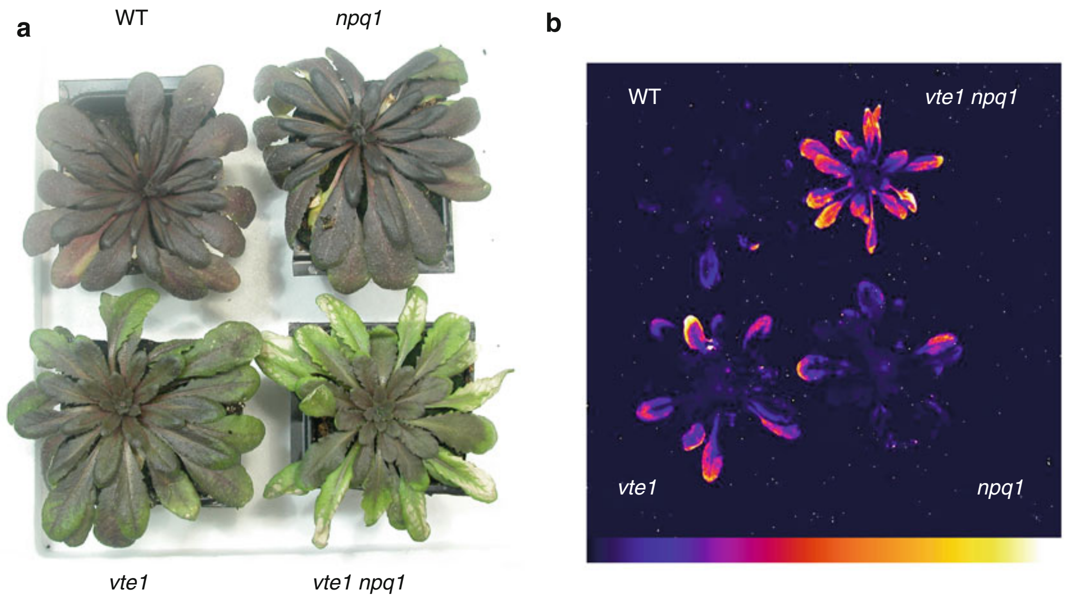

Fig. 26.2 Functional overlap between Z (and NPQ associated with Z) and tocopherol in *Arabidopsis*. WT *Arabidopsis* plants and mutants deficient in zeaxanthin (*npq1* mutation) and/or tocopherol (*vte1* mutation) were exposed for 7 days to high light at low temperature (~1,200 μmol photons m^{-2} s^{-1} at 8 °C). The high photosensitivity of the double mutant (*vte1 npq1*) compared to the single mutants and WT is illustrated by (**a**) bleaching of the leaves and (**b**) accumulation of oxidized lipids as measured by autoluminescence imaging. Color scale indicates signal intensity from 0 (*blue*) to saturation (*white*).

functions of tocopherol described above, such features can be understood when considering that tocopherols are part of a network of antioxidants in the chloroplasts, and that alternative lipid-soluble antioxidants may fulfill similar functions in the protection of lipid membranes. The carotenoid zeaxanthin (Z; Fig. 26.1b) is one such antioxidant compound, whose physiological roles overlap with those of tocopherols (see section II below).

C Other Functions of Tocopherols

Based initially on a vast amount of correlative evidence obtained in stress experiments (reviewed in Munné-Bosch 2005; Falk and Munné-Bosch 2010), and later verified through the use of *Arabidopsis* mutants lacking specific steps of tocopherol biosynthesis (see section I), the in-vivo antioxidant function of α-tocopherol is well established (Falk and Munné-Bosch 2010; Mène-Saffrané and DellaPenna 2010). Apart from the antioxidant activity described in section I, $^1O_2$* quenching by tocopherols appears to play a role in the protection of PS II reaction centers against photoinhibition. When tocopherol synthesis was chemically blocked in algae exposed to high light, the tocopherol pool was rapidly exhausted, and this was associated with PS II inactivation (Krieger-Liszkay and Trebst 2006). Moreover, when energy delivery to PS II centers was enhanced by inhibition of non-photochemical quenching (NPQ) of chlorophyll fluorescence, in *npq4 Arabidopsis* leaves, PS II photoinhibition was drastically stimulated by the absence of tocopherol (*npq4 vte1* vs *npq4*) (Havaux et al. 2005), confirming the protective role of tocopherols against PS II inactivation. This protection may involve protection of the PS II repair cycle in thylakoid membranes (Inoue et al. 2011).

The use of mutants of model plants has not only provided conclusive evidence for the antioxidant function of tocopherols, but has also revealed additional, unexpected roles of tocopherols in stress responses. For instance, at low temperature, tocopherol deficiency was reported to reduce the rate of photoassimilate export, to induce the accumulation of soluble sugars and starch, and to induce callose deposition in phloem-parenchyma transfer-cell walls (Maeda et al. 2006; reviewed in Demmig-Adams et al., Chap. 28). All these observations suggest a role for tocopherol in low-temperature adaptation and phloem loading. Other examples of tocochromanol roles related to stress tolerance but independent of photoprotection are provided by the higher tolerance to osmotic stress of mutants accumulating γ- instead of α-tocopherol (Abbasi et al. 2007), the regulation of nitric oxide levels by γ-tocopherol (Desel et al. 2007), and the protection of photosynthetic membranes at chilling temperatures by tocotrienols (Matringe et al. 2008).

II Interactions of VAZ-Cycle Pigments with Tocopherols

A Functional Overlap Between Zeaxanthin and Tocopherols

Studies of *npq* mutants of *Arabidopsis thaliana* deficient in the energy-dependent component (q_E) of non-photochemical quenching confirmed the involvement of the VAZ cycle in this energy-dissipation process (Niyogi et al. 1998). However, comparison of the photosensitivity of the PsbS-deficient mutant *npq4* (lacking q_E but possessing a normal VAZ cycle) and the *npq4 npq1* double mutant (lacking both PsbS and Z) revealed that the protective function of Z goes beyond NPQ (Havaux and Niyogi 1999). The *npq4* mutant was found to be more tolerant to photo-oxidative stress and lipid peroxidation than the *npq4 npq1* double mutant, indicating an antioxidant role for Z distinct from its role in thermal dissipation (the latter measured as NPQ). When the pool of VAZ-cycle pigments was selectively enhanced in *Arabidopsis* by over-expressing β-carotene hydroxylase, tolerance of the plants to photo-oxidative stress was increased, and this phenomenon was, again, not correlated with a corresponding increase in NPQ (Johnson et al. 2007). In the chlorophyll *b*-less *ch1* mutant lacking all light-harvesting antenna complexes (LHCII) of PS II, xanthophylls are partly free in the thylakoid membranes (Havaux et al. 2007). Mutational suppression of xanthophylls in *ch1* leaves revealed that the only xanthophyll whose absence leads to a strong increase in photosensitivity was Z, while removal of other xanthophylls or PsbS had no such effect (Havaux et al. 2007). Additional suppression of Z or tocopherol formation by crossing *ch1* with *vte1* or *npq1*, respectively, resulted in similarly photosensitive phenotypes that were further enhanced in the triple mutant *ch1 npq1 vte1*, indicating that Z and tocopherols have cumulative effects. Functional interaction between carotenoids and tocopherols was also reported in the cyanobacterium *Synechocystis* (Maeda et al. 2005).

Interestingly, when continuously grown in high light, Z-deficient *npq1* mutant leaves contained more tocopherols than the wild type (Havaux et al. 2000). Similarly, the tocopherol-deficient *vte1* mutant grown in high light accumulated more Z than the wild type (Havaux et al. 2005), and a selective accumulation of VAZ-cycle pigments was also observed during photo-acclimation of *chlP* transgenic tobacco plants partially deficient in tocopherols (Havaux et al. 2003b). These findings suggest that the absence of one protective compound was compensated by a corresponding increase in the concentration of the other. Conversely, an increased size of the VAZ-cycle pool in *chy*B transgenic *Arabidopsis* plants was accompanied by a decrease in tocopherol concentration (Johnson et al. 2007). The concept of a functional interaction between tocopherol and Z was confirmed in the double mutant *vte1 npq1* lacking both compounds and exhibiting a very high sensitivity to photo-oxidative stress conditions compared to the single mutants (Fig. 26.2). In the green alga *Chlamydomonas*, overexpression of VTE2 in the double mutant *npq1 lor1*, lacking both Z and lutein, resulted in increased tocopherol levels and a relatively greater resistance to photo-oxidation (Li et al. 2012). Taken together, the latter observations strongly support a functional overlap between tocopherols and Z in plants.

B Zeaxanthin as a Lipid Protector

In-vitro studies on artificial lipid membranes have shown that xanthophylls act as antioxidants stabilizing membrane lipids and protecting membranes from peroxidative damage (Lim et al. 1992; Sujak et al. 1999; Woodall et al. 1997; McNulty et al. 2007). Since these latter effects correspond to the functions traditionally attributed to tocopherols in both plant and animal membranes, either a functional redundancy or a synergistic interaction between Z and tocopherols is likely. Zeaxanthin-overaccumulating mutants of vascular plants and microalgae exhibit a high resistance to lipid peroxidation (Baroli et al. 2003; Havaux et al. 2004; Johnson et al. 2007), as do VTE1-overexpressing plants that contain high tocopherol levels (Liu et al. 2008). In-vitro studies have also shown that carotenoids and tocopherols exert a synergistic protection against lipid peroxidation (Wrona et al. 2004). Prevention of carotenoid consumption by effective scavenging of free radicals by tocopherols is a possible explanation for this phenomenon (Bohm et al. 1997).

Operation of the VAZ cycle is assumed to involve binding and release of violaxanthin (V) from photosynthetic pigment-binding complexes, diffusion of the xanthophyll molecule in the lipid phase and conversion of V to Z by the violaxanthin de-epoxidase (VDE) enzyme in the monogalactosyldiacylglycerol (MGDG) phase (Havaux 1998; Macko et al. 2002; Jahns et al. 2009). The presence of lipids such as MGDG, forming reversed hexagonal structures (H_{II}), is necessary for VDE activity (Latowski et al. 2004; Goss et al. 2005). Due to their small headgroup area and a critical packing parameter value higher than one, the so-called non-bilayer lipids (such as MGDG) form hexagonal structures (H_{II} phases) in an aqueous medium (Israelachvili and Mitchell 1975). These non-lamellar lipids presumably provide the three-dimensional structure needed for binding of VDE and accessibility of V to the enzyme (Latowski et al. 2012). The amount of V measured in purified LHCII was correlated with the amount of MGDG associated with the purified complex, indicating that the xanthophyll is partially present in the MGDG phase (Schaller et al. 2010). Based on current knowledge, Jahns et al. (2009) proposed a model for the operation of the VAZ cycle emphasizing the role of lipids, where lumen acidification in high light induces LHCII aggregation and formation of H_{II} phases to which VDE binds. A fraction of V is presumably released from LHCII complexes and solubilized in H_{II} lipid structures, increasing accessibility of the xanthophyll to the de-epoxidase enzyme. The ability of xanthophyll to diffuse across the thylakoid membrane was first inferred from the observation that exogenous VDE is able to efficiently convert V to Z from the stromal side of the thylakoids (Macko et al. 2002). In fact, the trans-membrane organization of the VAZ cycle, with de-epoxidation taking place on the lumen side and epoxidation on the stromal side of the membrane, in and by itself assumes that the xanthophyll pigments are mobile in the thylakoid membrane (Yamamoto et al. 1999). Therefore, it is likely that Z molecules formed via the VAZ cycle are initially present in the thylakoid lipid phase (particularly MGDG) or at the interface between lipids and light-harvesting complexes, where Z may supplement the anti-oxidative action of tocopherols.

Diatoms and some other algae possess a xanthophyll cycle different from the VAZ cycle of vascular plants, the diadinoxanthin-diatoxanthin (Dd-Dt) cycle, involving a one-step deepoxidation from the monoexpoxy xanthophyll Dd to the expoxy-free Dt (Goss and Jakob 2010). Like V de-epoxidation in vascular plants, the reversible photoconversion of Dd to Dt depends on the presence of MGDG (Goss et al. 2005). Drastic increases in the total amount of Dd-Dt cycle pigments occur in high light-grown diatom cultures, and are not necessarily correlated to more pronounced NPQ (Schumann et al. 2007). A large fraction of Dd-Dt cycle pigments formed in high light appeared to be localized within a MGDG shield surrounding the fucoxanthin-chlorophyll protein complexes (Lepetit et al. 2010), where the Dd-Dt cycle pigments play an antioxidant role distinct from NPQ. Diatoxanthin was reported to be a better antioxidant than diadinoxanthin (Lepetit et al. 2010), similar to Z versus V.

Interestingly, protection against photooxidation in some animal and human tissues, such as the eye, is also achieved by a combination of xanthophylls and tocopherol (e.g., Beatty et al. 2000). The human retina is susceptible to photo-oxidative stress because of a number of features also found in chloroplast membranes, i.e., presence of photosensitizers, high fluxes of incident light, high O_2 tension and polyunsaturated fatty acids. Beside tocopherols and Z, the eye contains substantial amounts of lutein (Beatty et al. 2000; Krinsky et al. 2003) that is also the major carotenoid found in the light-harvesting antennae of the photosystems. It should be noted, however, that lutein is most concentrated in the low light-exposed areas of the retina, while Z is more highly concentrated in the high light-exposed areas of the retina (for a review, see Demmig-Adams and Adams 2013). Thus, light-harvesting systems of plants and animals apparently share the same complement of lipid-soluble antioxidants for their photoprotection.

It has been shown that Z in the human retina is bound in a specific manner by xanthophyll-binding proteins (Li et al. 2010), and that this binding enhances the antioxidant action of Z in protecting against lipid peroxidation in vitro (Bhosale and Bernstein 2005). It is possible that, similar to xanthophylls in the retina, the function of Z as an antioxidant preventing thylakoid lipid damage is enhanced by its binding to LHCII. The idea that Z's protective effect is enhanced by interaction with antenna proteins is supported by analysis of the *npq1* mutation in the WT and *ch1* genetic backgrounds, which indicated a greater impact of a lack of Z on lipid peroxidation in LHCII-containing plants (WT vs *npq1*) versus LHCII-deficient plants (*ch1* vs *ch1 npq1*) (Dall'Osto et al. 2010). Binding of Z to LHCII may have a role in the protection of specific lipids associated with the latter protein complexes (Nussberger et al. 1993). Examination of the structural model of the major LHCII reveals that the VAZ-cycle carotenoid present in the V1-binding site encircles the bound phospholipid, and is also in close proximity to the bound molecule of digalactosyl diacylglycerol (Liu et al. 2004; Johnson et al. 2007). Thus, Z may confer antioxidant protection to bound lipids from oxidative damage caused by ROS produced in the PS II antennae. Protection of bound lipids is physiologically relevant because this protection would not only preserve antenna stability during photooxidative stress but also prevent initiation of lipid peroxidation chain reactions by eliminating the potential initiator. Based on biochemical and Raman spectroscopic data, it was hypothesized that an enhancement of the antioxidant activity of Z versus V may arise from the differential strength of binding of V and Z to the LHCII complex and by a distortion of the bound Z molecule (Johnson et al. 2007).

Stabilization of the thylakoid membrane by Z has been suggested to include control of membrane fluidity, with Z accumulation causing the membrane to become more rigid (Gruszecki and Strzalka 1991; Tardy and Havaux 1997; Szilagyi et al. 2008). Such an effect could explain the increased tolerance of Z-containing plants to heat stress (Havaux et al. 1996). It is worth mentioning that extremely thermophilic bacteria use Z derivatives, such as thermo-zeaxanthin in *Thermus thermophilus*, to stabilize their membranes at very high temperatures (Yokoyama et al. 1995). The effect of Z in increasing membrane rigidity may also act as a feedback mechanism, decreasing the rate of lateral diffusion of xanthophyll molecules and, thereby, the rate of V de-epoxidation (Latowski et al. 2002). Consistent with the latter view, the rate of xanthophyll inter-conversions was correlated with thylakoid membrane fluidity in barley cultivars (Tardy et al. 1998). Z-induced changes in membrane fluidity were suggested to arise from free Z molecules dissolved in the lipid phase (Havaux 1998). It is known that xanthophylls incorporated into liposomes exert a strong rigidifying effect on lipid bilayers (Gabrielska and Gruszecki 1996). It should be noted that a similar effect was reported for tocopherol in artificial lipid membranes (Stillwell et al. 1992). However, we cannot exclude an effect arising from the binding of Z to LHCII, either through interaction with boundary lipids or a Z-induced increase in the strength of interaction among LHCII trimers. Irrespective of the exact mechanism underlying the NPQ-independent antioxidant function of the VAZ cycle, findings from *Arabidopsis* mutants showed that the Z-tocopherol combination plays a central role in the protection of thylakoid membranes against photooxidation.

III Environmental Regulation

A What Determines Tocopherol Content in Plastids?

1 External Factors

The responsiveness of the pools of VAZ pigments and tocopherols to most stress factors is a well-established phenomenon (for a review, see Munné-Bosch 2005). This is consistent with the stress-induced activation of the expression of genes involved in tocopherol biosynthesis (Collakova and Della Penna 2003; Kanwischer et al. 2005), and the hormonal control of this activation, which includes jasmonic acid, ethylene (Munné-Bosch et al. 2004), salicylic acid (Abreu and Munné-Bosch 2009) and abscisic acid (El Kayal et al. 2006). Thus, an increase in the content of α-tocopherol generally occurs in response to a wide range of stresses, such as drought (Bartoli et al. 1999; Munné-Bosch and Alegre 2000; Peñuelas et al. 2004), low temperature (Leipner et al. 2000), high light (Hansen et al. 2003; García-Plazaola et al. 2004), ozone (Grulke et al. 2003) or ultraviolet radiation (Malanga et al. 1997). Some environmental conditions, such as presence of heavy metals (Artetxe et al. 2002; Collin et al. 2008) or severe weather conditions (García-Plazaola et al. 2008), lead to particularly rapid and dramatic increases in α-tocopherol (more than five-fold higher than basal levels). Leaf content of γ-tocopherol is also responsive to stress factors such as drought or salinity (Cela et al. 2011; Asensi-Fabado et al. 2012). Although an increase in tocopherols in response to stress represents the general trend, some studies have shown an absence of response, or even a decrease in the pools of tocopherol after exposure to severe conditions, such as photo-oxidative stress (Munné-Bosch et al. 2001b), drought stress (Munné-Bosch et al. 2001a), desiccation (Kranner et al. 2003), iron deficiency (Iturbe-Ormaetxe et al. 1995), high light (Grace and Logan 1996) or pollutants (Wingsle and Hällgren 1993). Such decreases may be attributable to consumption of tocopherol molecules associated with their antioxidant activity.

Based on the latter observations, Munné-Bosch (2005) revised the stress response model, proposing an initial response phase in which acclimation mechanisms activate tocopherol biosynthesis to counteract the stress, followed by a second phase when the stress is too severe to be fully compensated for and the tocopherol pool might become depleted. Following traditional stress theory (for a recent review, see Kranner et al. 2010), these two phases can be integrated by the concept of eustress and distress. While eustress represents stress levels enhancing the organism's function and resistance, distress refers to stress levels that cannot be compensated for by the organism, leading to antioxidant consumption and chronic alterations or death (Table 26.1). Thus, the intensity of the stress may determine positive (eustress) or negative (distress) effects. The resulting "U" or "A"-shaped response curves complicate the utility of antioxidant levels as reliable indicators of environmental stresses (Fig. 26.3).

Table 26.1 Some examples of the relative changes in the content of VAZ-cycle pigments and α-tocopherol (Toc) in response to different factors inducing eustress and distress situations

Stress factor	Eustress			Distress		
	Plant	VAZ	Toc	Plant	VAZ	Toc
Drought	*Phyllirea angustifolia*	+71	+126	*Salvia officinalis*	−45	−62
Light	*Cucurbita pepo*	+185	+79	*Vinca major*	+163	−82
Temperature	*Dicksonia antarctica*	+28	+143	*Zea mays*	−26	−38
Desiccation	*Pleopeltis mexicana*	+19	+182	*Myrothamnus flabellifolia*	+67	−92

Data, expressed as percentage of change with respect to controls, were calculated from Grace and Logan (1996), Leipner et al. (1997), Logan et al. (1998), Munné-Bosch et al. (2001a), Tausz et al. (2001), Kranner et al. (2002), Peñuelas et al. (2004), Volkova et al. (2009)

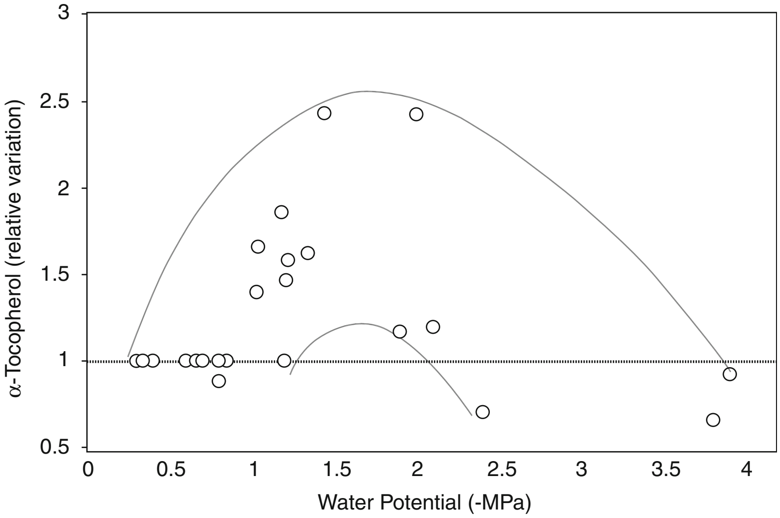

Fig. 26.3 An example of the "A-shaped" pattern of photo-protective responses to environmental stress, illustrating the relationship between the relative changes in the pool of α-tocopherol (relative to unstressed controls) and the water potential of drought-stressed plants. Data were obtained from Moran et al. (1994), Bartoli et al. (1999), Karminska-Rozek and Pukacki (2004), Sjrceli et al. (2005), Gallé et al. (2007), and Hojati et al. (2011).

2 Internal Factors

Together with environmental stresses, internal factors regulate leaf tocopherol content. It was described more than 20 years ago that foliar tocopherol content increases with leaf age in the leaves of some species (Molina-Torres and Martínez 1991; Tramontano et al. 1992). This trend was confirmed in a literature survey (Hormaetxe et al. 2005) including 65 % of species for which age-dependent tocopherol changes were available. Older leaves may contain up to 20-fold more tocopherol than young leaves of the same plant (Hormaetxe et al. 2005). This age-dependent control of the tocopherol pool is additive to a stress-dependent trend, and both components contribute to determining the actual tocopherol content of a leaf.

The physiological reason for such a progressive accumulation of tocopherol during ageing is not clear. A possible explanation was proposed more than 20 years ago on the basis of an inverse relationship between chlorophyll degradation and accumulation of

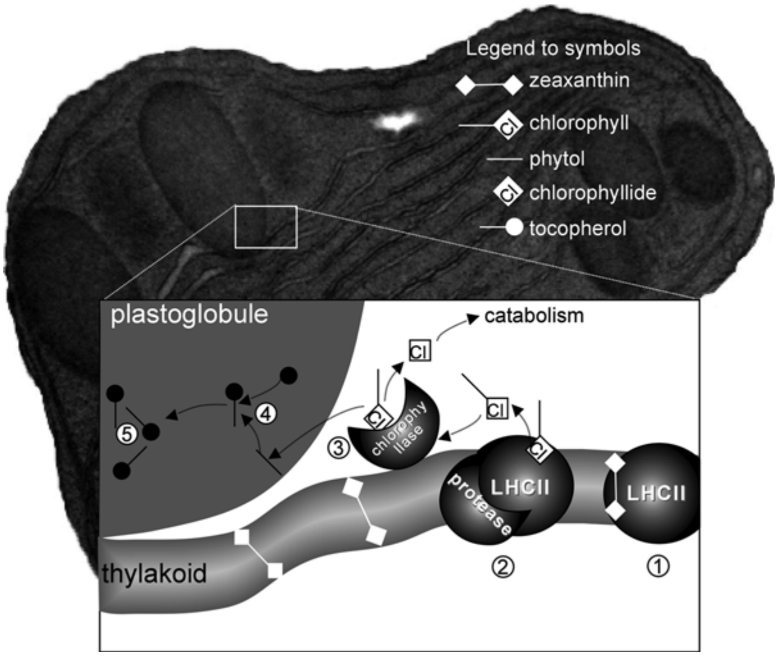

Fig. 26.4 Proposed model of the interaction among zeaxanthin, tocopherol and chlorophyll degradation. Zeaxanthin enhances instability of the PS II antennae, favoring their degradation by proteases (*1*). Chlorophyll released is immediately degraded (*2*), releasing free phytol (*3*) that is detoxified forming tocopherol (*4*) that is finally stored in PGs (*5*).

α-tocopherol in senescing leaves (Rise et al. 1989). The latter authors proposed that phytol, released from chlorophyll molecules by chlorophyllase and transformed to phytyl pyrophosphate, may be recycled and used for tocopherol synthesis. Confirmation of this hypothesis has remained elusive until recently, when the use of tocopherol-deficient mutants of *Arabidopsis* shed a great deal of light on the latter mechanism (Mène-Saffrané and DellaPenna 2010 and references therein). In essence, the presence of free phytol has harmful effects (Dörmann 2007), and phytol transformation to tocopherol and subsequent sequestration in plastoglobules (PG) might serve as a detoxification mechanism. The turnover of chlorophyll molecules may thus generate a continuous source of phytol, which would be enhanced during any reorganization of PS II antenna size (Fig. 26.4). Such an effect would explain the continuous age-dependent increase in tocopherol (Hormaetxe et al. 2005), and also some part of the dramatic stress-induced changes (see section III).

B Simultaneous Changes in VAZ-Cycle Pigments and Tocopherol

In the eustress phase, increases in the pools of tocopherol and VAZ molecules are typically elastic in nature, meaning that these pools return to their basal level after stress cessation. Simultaneous changes of VAZ-cycle xanthophylls and tocopherols in response to environmental stresses can generate a highly synchronized response, with the two pools of photo-protective molecules displaying strongly correlated changes (García-Plazaola et al. 2004). These responses can be easily interpreted by assuming a common and synchronous up-regulation of the biosynthesis of both groups of protective molecules that act cooperatively and also share a common pathway. Alternatively, the parallel, and sometimes well correlated, changes in the pools of both molecules may be caused by the unavoidable detoxification of phytol, released after chlorophyll degradation induced by Z-modulated antenna size reorganization. The latter suggestion is based on the observation that Z formed under stress conditions apparently increases instability of the PS II antenna (Havaux et al. 2004) and its susceptibility to proteases, thereby contributing to antenna-size regulation via PS II antenna degradation by proteases (Morosinotto and Bassi 2012). Irrespective of the origin of this additional tocopherol, and considering that (i) the main reservoir for tocopherols is located in PGs (see section I), (ii) number and size of these organelles typically increase in sunny exposure, and with leaf age and environmental stresses (Lichtenthaler 2007; Matringe et al. 2008; Eugeni Piller et al. 2012), and (iii) PGs play a role in activation of the biosynthesis of carotenoids and tocopherols (Bréhélin et al. 2007), it is likely that most of these newly synthesized molecules are accumulated in PGs. The latter observation adds uncertainty as to whether or not the entire tocopherol pool participates in photo-protection, especially when one considers the lack of experimental data. Alternatively, tocopherol stored in PGs could be released to the thylakoid membranes through structural connections (between PGs and thylakoids) whenever oxidative conditions demand protection (Bréhélin et al. 2007).

The pool of VAZ-cycle pigments in field-grown plants can be much higher (VAZ/Chl up to 200 mmolmol^{-1}) than those of model plants grown in controlled chambers (VAZ/Chl lower than 100 mmolmol^{-1}) (for a recent review on field data, see Demmig-Adams et al. 2012; see also Demmig-Adams et al., Chap. 24). As the number of available binding sites for VAZ-cycle pigments is limited, such a high VAZ/Chl ratio suggests that a variable proportion of these additional VAZ molecules is not bound to proteins in the thylakoids, and that the latter portion is instead acting as free protective molecules (see section I). This view is also supported by the rapid induction of carotenoid biosynthesis by high light, leading to rapid synthesis of VAZ-cycle pigments (together with lutein), which is not paralleled by increases in other structural carotenoids such as neoxanthin (Förster et al. 2009; Beisel et al. 2010). Moreover, in an experiment with supplemental illumination over field-grown leaves of deciduous trees, Niinemets et al. (2003) did not detect any sign of saturation in the continuous increase in VAZ-cycle pigments. However, one cannot exclude that additional binding sites are generated under high-light conditions. Proteins exhibiting a high homology with LHCII, such as the High-Light Induced Proteins (HLIPs) and the Early Light-Induced Proteins (ELIPs), are known to be induced by high light stress (Heddad and Adamska 2002). Interestingly, correlations between Z accumulation and strong accumulation of HLIPs and/or ELIPs were observed under stressful conditions in the field (Demmig-Adams and Adams 2006). These LHCII relatives have photoprotective properties that could involve interaction with carotenoids (Havaux et al. 2003a; Hutin et al. 2003).

C Other Connections Between Tocopherol and NPQ

No direct relationship between tocopherol and the thermal dissipation of excess excitation energy (NPQ) has been described. However, a number of indirect connections can be deduced. One such connection relates to the activation of compensatory mechanisms (see section II and Tocopherols), when some of the components of photo-protective defenses (in this case, tocopherol) are altered. For example, in the *vte1* mutant of *Arabidopsis*, the absence of tocopherol is partially compensated by increased levels of Z and NPQ (Kanwischer et al. 2005; Abbasi et al. 2009). An additional interaction between NPQ and tocopherol may arise from the role of tocopherol in photo-assimilate export at low temperature (see section I; Maeda et al. 2006). Under these conditions, sugar accumulation might inhibit carbon assimilation, thereby activating thermal dissipation of the increased levels of excess excitation energy and, possibly, photoinhibition (see also Adams et al., Chap. 23).

IV Evolutionary Considerations

A Presence of Tocopherols and VAZ-Cycle Pigments in Different Evolutionary Groups

All photosynthetic eukaryotes originated 1,500 millions years ago (MYA) from a primary endosymbiotic event that involved engulfment of a free-living cyanobacterium by a non-photosynthetic eukaryote (Yoon et al. 2004). From this initial symbiosis, two main clades diverged, i.e., the green and the red lineages (Fig. 26.5a). The former lineage evolved to plants and green algae (forming the subkingdom Viridiplantae – latin for "green plants") and the latter gave rise to the red algae (Rhodophyta) and, through a secondary endosymbiosis that occurred 1,300 MYA, to all other major groups of algae (Cryptophyta, Haptophyta and Chromista). Other secondary and tertiary endosymbiotic events generated the other groups of plastid-containing organisms (Dinophyta, Choraracniophyta, Euglenophyta, Apicomplexa) that are scattered throughout the tree of life. As all eukaryotic phototrophs (photosynthetic organisms) originated from the same cyanobacterial ancestor (Archibald 2009), their plastids may share the same biochemical machinery. This applies to photo-protective isoprenoids that include tocopherols and carotenoids (Peñuelas and Munné-Bosch 2005). Thus, tocopherol synthesis has traditionally been considered to be restricted to photosynthetic eukaryotic organisms and their cyanobacterial relatives (Lichtenthaler 2007; Falk and Munné-Bosch 2010; Mène-Saffrané and DellaPenna 2010). However,

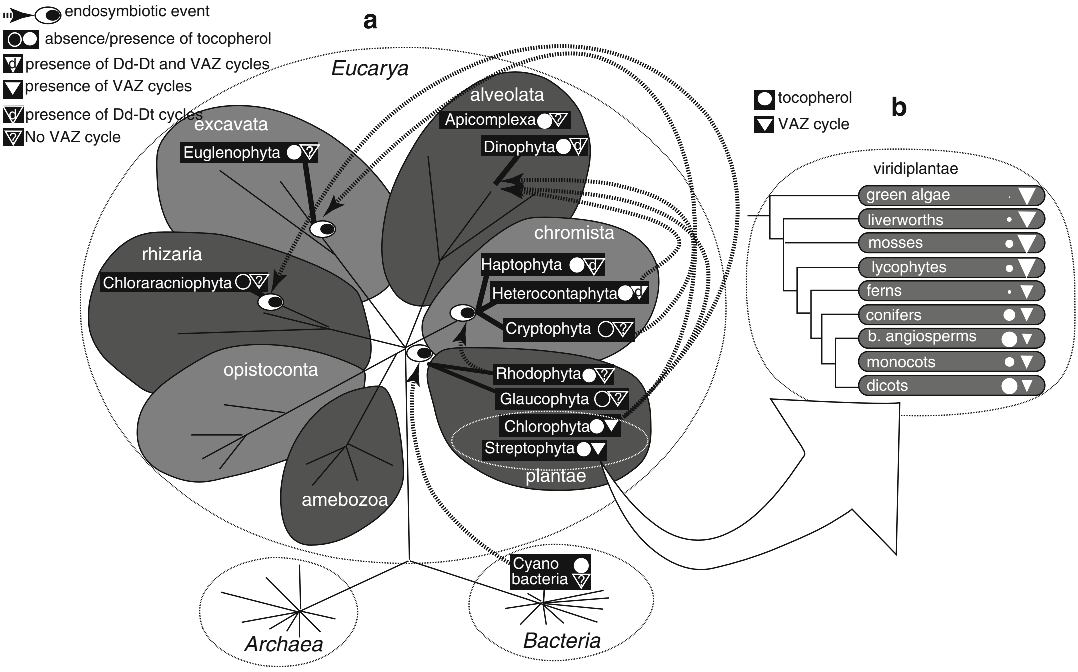

Fig. 26.5 (**a**) Presence of xanthophyll cycles (Dd-Dt and VAZ) and tocopherols in photosynthetic eukaryotes. (**b**) Average content of VAZ-cycle pigments and α-tocopherol in the main lineages of the subkingdom Viridiplantae. Relative size of symbols is proportional to the content. Data calculated from supplementary material in Esteban et al. (2009).

recent reports indicate that this is not entirely correct since tocopherol biosynthesis has been confirmed in the non-photosynthetic malaria parasite *Plasmodium falciparum* (Sussmann et al. 2011), where this pathway is being explored as a potential therapeutic target (Duke 2010). The latter organism, that is included in the apicomplexa group derived from red algae, contains a special plastid (apicoplastid), where synthesis of prenylquinones takes place (Sussmann et al. 2011). In addition, some reports have indicated the presence of tocopherols in fungi at very low concentrations (reviewed in Ferreira et al. 2009), although this presence remains to be further confirmed since no gene or pathway specifically related to tocopherol biosynthesis has been described in fungi thus far (see supplementary material in Falk and Munné-Bosch 2010). Although not yet tested in all algal groups, the presence of tocopherol has been reported for most major groups of photosynthetic organisms, including Rhodophyta (Matanjun et al. 2009), Chlorophyta (Galvez-Valdivielso et al. 2011) and Chromista, such as Pheophyta (Collén and Davison 1999), diatoms and Haptophyta (Durmaz et al. 2009), as well as others belonging to the Excavata, such as the protozoan *Euglena* (Ruggeri et al. 1985).

While carotenoids, in particular β-carotene, are also ubiquitous, xanthophylls are more taxonomically restricted (Jeffrey et al. 2011). Although Z is present in most groups, its dynamic regulation through the VAZ cycle has been only confirmed in Viridiplantae and Chromista (brown algae). The separate evolutionary history of the latter two groups suggests that the VAZ cycle evolved independently at least twice, or that it has been lost in the intermediate groups (red algae) (Coesel et al. 2008). In addition to the VAZ cycle, at least five additional xanthophyll cycles evolved in photosynthetic organisms (García-Plazaola et al. 2007). In diatoms, dinophyta and cryptophyta, the VAZ cycle is replaced by the Dd-Dt cycle, and in some species both cycles operate simultaneously (Goss and Jakob 2010). In several taxa of terrestrial plants, the VAZ cycle and the lutein-epoxide cycle (Lx-L cycle) operate concurrently (see Esteban and García-Plazaola, Chap. 12). All of these cycles generate de-epoxidized xanthophylls (Z, Dt, or L) under conditions of excess light. This fact is consistent with an involvement of these latter xanthophylls in regulation of thermal dissipation and/or a strong evolutionary pressure to develop mechanisms for the rapid regulation of light-harvesting efficiency. Since the presence of Z precedes appearance of V on an evolutionary (and biosynthetic) scale, it is attractive to assume that evolution of the VAZ cycle occurred primarily to *remove* – rather than *form* – Z, and to allow rapidly growing land plants to quickly return to a high light-harvesting efficiency in a rapidly fluctuating light environment.

The green eukaryotic photosynthetic organisms (subkingdom Viridiplantae), studied in great detail with respect to photoprotective isoprenoids, invariably exhibit the presence of α-tocopherol and several carotenoids (lutein, neoxanthin, VAZ-cycle pigments, and β-carotene) in all lineages, indicating that the associated photo-protective mechanisms are strongly conserved despite the enormous functional diversity in this group (considering, e.g., the difference between a unicellular *Chlamydomonas* and a giant *Sequoia*) (Peñuelas and Munné-Bosch 2005). Furthermore, a survey of 266 species belonging to Viridiplantae revealed two opposing evolutionary trends (Fig. 26.5b; see also Esteban et al. 2009). On one hand, tocopherols tend to accumulate in larger amounts in angiosperms than in ancient photosynthetic organisms, with tocopherol levels being below the detection threshold in 40 % of the green algae studied. On the other hand, the content of VAZ-cycle pigments is higher in green algae and bryophytes than in terrestrial vascular plants (Esteban et al. 2009). A plausible explanation for this shift in the specifics of photo-protection may be the transition from a more stable aquatic environment to highly fluctuating terrestrial ecosystems that require a more diversified strategy of photoprotection, as has also been argued for the replacement of the (presumably less flexible) LHSCR-based NPQ in algae by (highly flexible) PsbS-based NPQ in land plants (Gerotto et al. 2012; see also Morosinotto and Bassi, Chap. 14). Furthermore, despite existing functional redundancies between tocopherol and Z, the former may confer a clear advantage in the thermally unstable terrestrial environments, because its constitutive presence, unlike Z formation by VDE, is not temperature-dependent. Likewise, tocopherols are immediately ready for protection of thylakoids while Z formation requires a certain amount of time. This may apply especially to annual plant species, while evergreen plant species readily exhibit VAZ-cycle arrest in the form of Z, with long-term retention of high Z levels (see Demmig-Adams et al., Chap. 24).

V Concluding Remarks

In biological systems, shifts in the function of a trait during evolution have resulted in involvement of the same molecules, organs, or mechanisms in completely unrelated processes. This is the case for tocochromanols (in particular α-tocopherol) that display a plethora of functions, far beyond their (presumably initial) antioxidant role. Some of these functions are involved in the protection of photosynthesis, while others seem to be independent of this latter function. The use of *Arabidopsis* mutants lacking certain components of the photo-protective defense network has allowed a dissection of the roles and interactions of each compound in photoprotection. This dissection has also revealed that the role of VAZ-cycle pigments, as is the case for tocopherols, goes beyond a direct regulation of photosynthesis, with intriguing functional redundancies and/or synergisms between the two groups of compounds. The remaining challenge is to scale up from the analysis of model organisms to the study in nature of the wider diversity represented in the Plant Kingdom.

Acknowledgments

JIGP thanks the research grants BFU 2010-15021 and UPV/EHU-GV IT-624-13 for support of research related to this chapter. MH would like to thank the Marie Curie International Training Network HARVEST for financial support of the research performed on zeaxanthin and NPQ in his laboratory. JIGP also thanks Unai Artetxe and the technical and human support provided by SGIker (UPV/EHU, MICINN, GV/EJ, ESF) for the micrograph in Fig. 26.4 and Sergio Seoane for helpful comments on the manuscript.

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Chapter 27

Thermal Energy Dissipation in Plants Under Unfavorable Soil Conditions

Fermín Morales*, Javier Abadía, and Anunciación Abadía
Department of Plant Nutrition, Aula Dei Experimental Station-CSIC,
13034, 50080 Zaragoza, Spain

Summary

Unfavorable soil conditions in crops and natural habitats include limited availability of water and nutrients, presence of salts, as well as an excess of essential nutrients and heavy metals. When plants are exposed to such stresses, rates of photosynthetic carbon fixation decrease

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*Author for Correspondence, e-mail: fmorales@eead.csic.es

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_27, © Springer Science+Business Media Dordrecht 2014

for a variety of reasons, while plants continue gathering sunlight. As a consequence of the resulting imbalance between light absorption and energy utilization, plants experience what the research community has termed photoinhibition, which is considered to be a reflection of either photoprotection mechanisms or photodamage. Data reported to date suggest that under unfavorable soil conditions, photoprotection mechanisms are far more important than photodamage. Plants under stress generally dissipate thermally, i.e., as heat, a large part of the light absorbed by photosystem II in a process mediated by ΔpH, xanthophyll pigments (particularly zeaxanthin and antheraxanthin), and the photosystem II subunit S (PsbS) protein. Changes in thermal energy dissipation under unfavorable soil conditions are summarized here. Very high levels of thermal energy dissipation are frequently, but not always, accompanied by decreases in leaf chlorophyll concentration, such as those found under N and Fe deficiency, excess Al, or water stress in some species. The mechanisms of photoprotection remain largely unexplored for some of the stress situations reported here. In this chapter, we review changes in thermal energy dissipation in response to water stress, salinity, macronutrient (N, P, and K) deficiencies, micronutrient (Fe, Mn, Cu, and Zn) deficiencies and toxicities, and other metal (Cd, Pb, Al, and Hg) toxicities.

I Introduction

Frequent abiotic stresses in plants include a limited availability of nutrients and water, salinity, and an excess of nutrients and non-essential metals. These environmental stresses occur both in natural habitats and crops. Stressed plants often experience a decrease in photosynthetic carbon fixation, whereas they continue harvesting sunlight. Unfavorable soil conditions often decrease the efficiency with which solar energy is harvested and used by plants in photosynthetic reactions. This is one aspect of what the scientific community has named photoinhibition (see Adams et al., Chap. 23). Some researchers have tacitly assumed that photoinhibition results from photodamage, whereas others believe it is rather the integration of regulatory and protective adjustments.

A decreased Chl concentration can, under some circumstances, be considered a first level of photoprotection because, when significant, the lower Chl content decreases the amount of light that is absorbed (Morales et al. 1991). Changes in Chl and carotenoid levels under unfavorable soil conditions are summarized in Table 27.1. Absorbed light can either be used in photochemistry (in the absence of any stress, typically 80–85 % of absorbed light is used in photochemistry), thermally dissipated, or diverted to other processes that could be potentially deleterious. Demmig-Adams et al. (1996) proposed using modulated Chl fluorescence to assess allocation of the energy absorbed by PS II to three different fractions: P, D and X, as the fractions used in photochemistry, dissipated thermally by the antenna, and neither used nor dissipated, respectively (see also Logan et al., Chap. 7).

Abbreviations: A –Antheraxanthin; Chl – Chlorophyll; D – Fraction of light absorbed by PS II that is dissipated thermally in the antenna, e.g., as $1-\Phi_{exc.}=1-(F_v'/F_m')$; ETR – Electron transport rate; NPQ – Non-photochemical quenching of chlorophyll fluorescence; P – Fraction of light absorbed by PS II that is used in photochemistry; Pc – Fraction of P that Rubisco uses for RuBP carboxylation; Po – Fraction of P that Rubisco uses for RuBP oxygenation; PPFD – Photosynthetic photon flux density; PS I – Photosystem I; PS II – Photosystem II; Rubisco – Ribulose-1,5-*bis*phosphate carboxylase oxygenase; RuBP – Ribulose *bis*phosphate; V – Violaxanthin; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin, and zeaxanthin; X – Fraction of light absorbed by PS II that is neither used nor dissipated; Z – Zeaxanthin

Table 27.1 Summary of the changes in total Chl and carotenoid concentrations under unfavorable soil conditions

Unfavorable soil condition		
Deficiencies	Total Chl	Total carotenoids
Water stress	= or ↓	↓ or ↑
-N	↓	↓
-P	= or ↓ or ↑	=
-K	↓	nd
-Fe	↓	↓
-Mn	↓	= or ↓
-Cu	= or ↓	↓
-Zn	↓	= or ↓
Toxicities		
Salinity	= or ↓	= or ↓ or ↑
Excess Fe	=	=
Excess Mn	= or ↓	=
Excess Cu	= or ↓ or ↑	↓
Excess Zn	↓ or ↑	↓
Heavy metals		
Cd	= or ↓ or ↑	= or ↓
Pb	= or ↓	= or ↓
Al	= or ↓ or ↑	= or ↓
Hg	↓	nd

= no effect, ↑ increase, ↓ decrease, *nd* not described in the literature

The fraction P is equal to the actual PS II efficiency ($\Phi_{PS\,II}$) or yield (Genty et al. 1989), and would include electrons used in RuBP carboxylation (P_C) and oxygenation (P_O), Mehler reaction, chloro-respiration and any other electron-consuming process. For water-stressed plants, some studies have provided concurrent data sets of Chl fluorescence and gas exchange, permitting a discrimination among the major components of P (photosynthesis and photorespiration), assuming that the proportion of P allocated to other minor components is negligible (Valentini et al. 1995; Medrano et al. 2002). Our chapter includes some papers reported in the early seventies before discovery of the oxygenation activity of Rubisco (Ogren and Bowes 1971), and claims for changes in photorespiration should therefore be viewed with caution today.

Some photoprotective mechanisms act through the removal of excess excitation energy as thermal energy. The above-mentioned D fraction $(1-(F_v'/F_m'))$ and the widely used Chl fluorescence-derived NPQ parameter, e.g., as $(F_m/F_m')-1$, reflecting quenching of the first singlet Chl excited state, can be used as indicators of such energy removal via thermal dissipation (see also Logan et al., Chap. 7). The extent of D and NPQ depends on light intensity (Fig. 27.1). For the mechanistic bases of D and NPQ and how they may differ in assessing photoprotection, readers are referred to recent literature (Holzwarth et al. 2009; Ruban et al. 2012; Peguero-Pina et al. 2013). In the present chapter, we report that D and NPQ under unfavorable soil conditions are generally positively and linearly correlated (Fig. 27.2). However, when unfavorable soil conditions are considered individually, some differences between D (Fig. 27.3) and NPQ (Fig. 27.4) do arise.

The aim of the present chapter is to summarize the current knowledge on photoprotective mechanisms occurring under the most common water- and nutritient-related abiotic stresses in plants. This includes deficiencies of macro- and micronutrients, drought, salinity, excess of micronutrients, and presence of heavy metals. Flooding stress is not included because there is not enough data in the literature. Very few studies have examined responses of plants to naturally flooded (Rengifo et al. 2005) and waterlogged (Roy Chowdhury et al. 2009) areas or artificially waterlogged seedlings (Wu et al. 2013). We have considered as photoprotection-related observations those reporting either decreases in Chl (leading to lower light absorption) and/or readily reversible interconversions of the violaxanthin-antheraxanthin-zeaxanthin cycle (hereafter referred to as VAZ cycle) that produces zeaxanthin (Z) upon exposure to excess light and is associated with thermal dissipation (resulting in increases in D and/or NPQ). Permanent maintenance, or “lock-in”, of the VAZ cycle in the de-epoxidized state (Adams et al., Chap. 23) and the possible role of zeaxanthin (Z) as an antioxidant (Havaux and Niyogi 1999; Müller-Moule et al. 2003) under some particularly unfavorable soil conditions are also discussed.

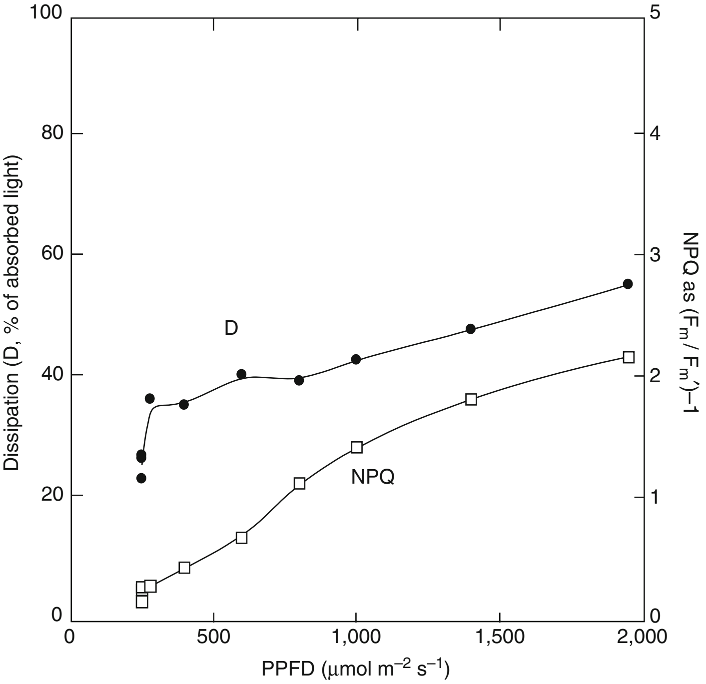

Fig. 27.1 Changes in dissipation (D, as % of absorbed light; solid circles) and NPQ (*open squares*) in response to increases in light intensity (*PPFD* photosynthetic photon flux density). Data were taken from sugar beet leaves; Morales et al. (1998).

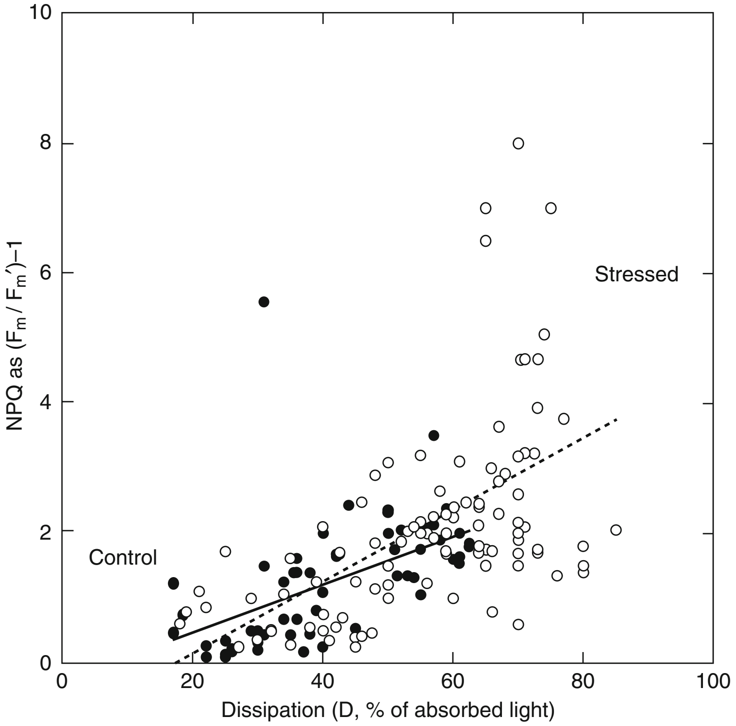

Fig. 27.2 Relationship between dissipation (D, as % of absorbed light) and NPQ in different species grown under favorable (control, *solid circles*; fit with *continuous line*) and unfavorable (stressed, *open circles*; fit with *dotted line*) soil conditions (Data were taken or recalculated from the references cited in the text).

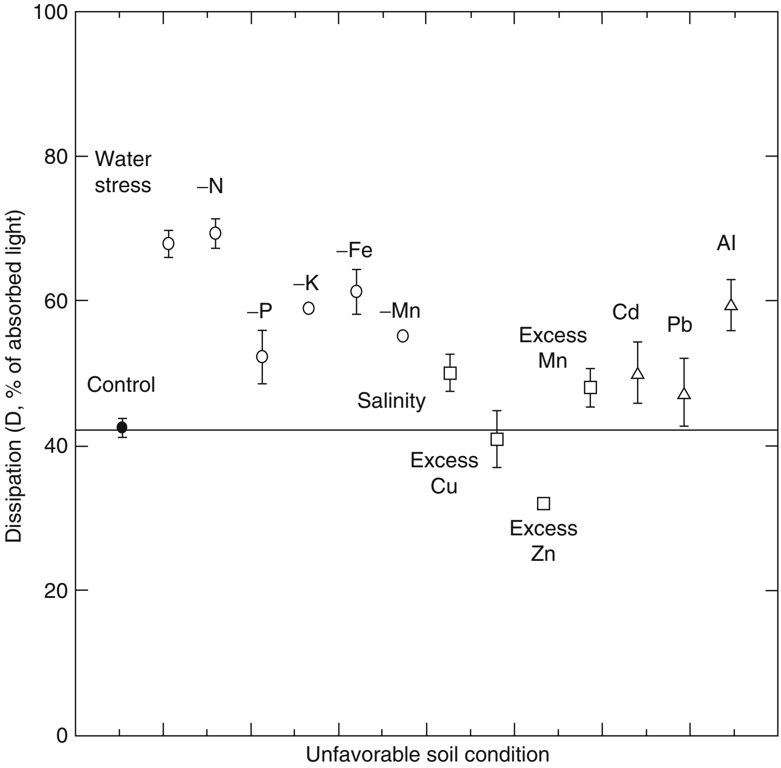

Fig. 27.3 Differences in dissipation (D, as % of absorbed light) from leaves of control plants (*closed circle* and *solid line*) compared to leaves of plants experiencing various unfavorable soil conditions including deficiencies (water stress, -N, -P, -K, –Fe and -Mn; *open circles*), toxicities (salinity, excess Cu, Zn and Mn; *open squares*), and heavy metals (Cd, Pb and Al; *open triangles*) (Data were taken or recalculated from the references cited in the text). Data are mean ± 1 SE (n ranged between 1 and 105; note that only one value was found for some stresses).

II Drought

In response to water stress, some plant species, such as barley, coffee and grapevine, maintain high Chl concentrations (Da Matta et al. 1997; Flexas et al. 2002; Flexas and Medrano 2002; Salazar-Parra et al. 2012a, b) and unaltered light-harvesting capacity. Conversely, other species, such as rice, cotton and wheat, prevent excess light absorption when water is scarce by decreasing Chl concentrations (Boo and Jung 1999; Kyparissis et al. 2000; Deeba et al. 2012; Huseynova 2012). Furthermore, droughted plants capable of moving their leaves had more rapid rates of leaf movement than well-watered plants and, at midday, the former avoided some of the excess light due to a longer period of paraheliotropism (Kao and Forseth 1991, 1992), decreasing the amount of absorbed light.

Approximately 54–72 % of the absorbed light is dissipated thermally (D) under well-watered conditions and saturating light in species such as sugar beet, cotton, bean, wheat, madrone, grapevine, tomato and watermelon (Fig. 27.1, and Flexas and Medrano 2002), while photosynthesis and photorespiration utilize as little as 12–35 and 6–9 %, respectively, of the PPFD absorbed by PS II (Flexas and Medrano 2002). While, under mild drought, D typically does not increase in the above-mentioned species, the contribution of photorespiration (and respiration; Beis and Patakas 2012) do increase somewhat at the expense of photosynthesis. This increase in photorespiration under mild water stress is probably the most common situation in agricultural and natural environments in Mediterranean habitats. In summer at midday, high vapor-pressure deficits lead to a temporary, mild leaf water deficit,

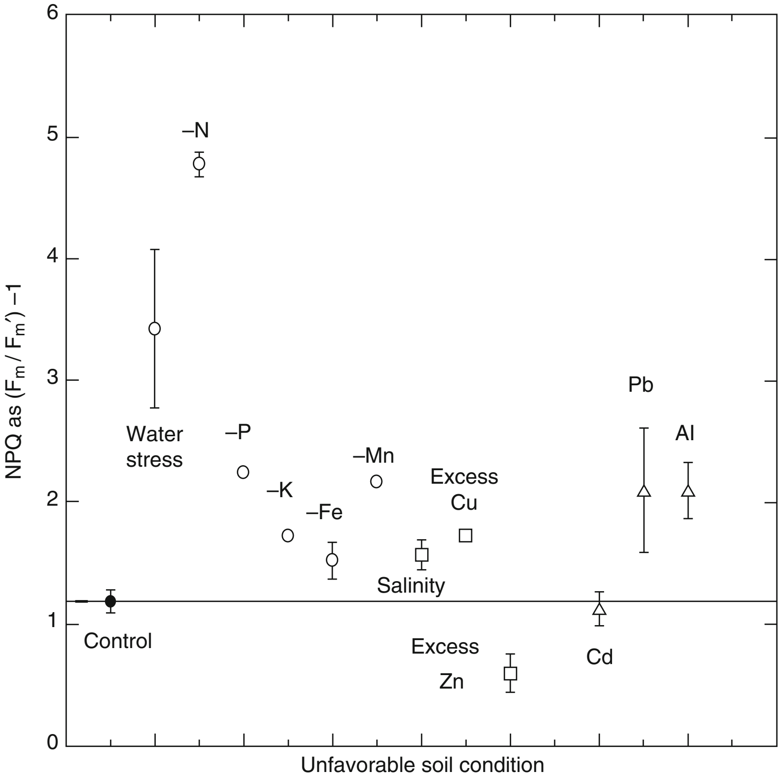

Fig. 27.4 Differences in NPQ from leaves of control plants (*closed circle* and *solid line*) compared to leaves of plants experiencing various unfavorable soil conditions including deficiencies (water stress, -N, -P, -K, –Fe and -Mn; *open circles*), toxicities (salinity, excess Cu and Zn; *open squares*), and heavy metals (Cd, Pb and Al; *open triangles*) (Data were taken or recalculated from the references cited in the text). Data are mean ± 1 SE (n ranged between 1 and 91; note that only one value was found for some stresses).

irrespective of the degree of soil water availability (Flexas and Medrano 2002).

In response to moderate to severe drought, D can increase to values as high as 70–92 % of the absorbed PPFD, with NPQ increasing several-fold (Figs. 27.3 and 27.4; Flexas and Medrano 2002; Beis and Patakas 2012; Silva et al. 2012), whereas the contribution of photosynthesis and photorespiration to the utilization of absorbed light decreases (Flexas and Medrano 2002). Most of this thermal dissipation is associated with the VAZ cycle (de-epoxidation of V to A + Z) and apparent engagement of the latter two carotenoids in thermal energy dissipation, whereas dissipation through photoinactivated photosystems is only minor (Björkman and Demmig-Adams 1994; Niyogi 1999; Flexas and Medrano 2002). Some species may retain some A + Z (15–20 % of the total V + A + Z carotenoids) overnight under summer conditions (Björkman and Demmig-Adams 1994; Faria et al. 1998; Chaves et al. 2002; Medrano et al. 2002), and the extent of this retention increased in response to water stress in some cases (Medrano et al. 2002), whereas it was unaltered by water stress in others (Chaves et al. 2002). Other species, however, retained large amounts of A + Z overnight in summer under drought (Demmig et al. 1988; Barker et al. 2002), generally accompanied by large increases in sustained NPQ (Demmig et al. 1988). In addition, the concentration of V + A + Z pigments relative to Chl exceeded 200 mmol mol^{-1} Chl in *Fraxinus ornus* under water stress, leading to speculation that Z might also serve as a chloroplast antioxidant (Fini et al. 2012). However, there are also notable exceptions to these trends. In cacao grown during either rainy or drought seasons, NPQ was unaffected (Araque et al. 2012) and D only increased from 61–65 to 62–69 % (recalculated from Araque et al. 2012).

The contribution of the Mehler reaction to energy dissipation is very low under either irrigation or drought (Flexas and Medrano 2002); values as high as 9 % of total absorbed PPFD found previously in severely stressed wheat (Biehler and Fock 1996) may have been overestimated due to methodological reasons (Flexas and Medrano 2002). The contribution of other pathways proposed to dissipate excess light under drought, such as PS II and PS I cyclic electron transport (Canaani and Havaux 1990; Katona et al. 1992; Fork and Herbert 1993), are considered to be minor. Although it is known that re-assimilation of CO_2 evolved from photorespiration may increase as drought progresses (Haupt-Herting et al. 2001), estimated rates of dissipation are similar whether or not re-assimilation is considered (Flexas and Medrano 2002).

There is no evidence for sustained photodamage in water-stressed plants, as judged by the lack of effects on maximal intrinsic PS II efficiency, estimated from the dark-adapted F_v/F_m Chl fluorescence ratio (Epron and Dreyer 1993; Faria et al. 1998; Flexas and Medrano 2002; Salazar-Parra et al. 2012b; Silva et al. 2012). However, photoinhibition may occur when drought is very severe and stomata are almost completely closed. The decrease in predawn F_v/F_m ratios in this case is still quite limited, since non-irrigated plants exhibited ratios of 0.72–0.78 (Flexas et al. 1998; Beis and Patakas 2012). Values as low as 0.60 have recently been reported in drought-stressed cacao and cotton (Araque et al. 2012; Deeba et al. 2012), but the mechanistic basis of these low values has not been investigated.

III Salinity

The response of leaf Chl and carotenoid levels to salt stress is species- and cultivar-dependent (Table 27.1). In olive, salt-stress-mediated Chl decreases ranged from nil in Arbequina (Melgar et al. 2008) to 50 % in Zard or Roghani cultivars (Mousavi et al. 2008). In barley (Morales et al. 1992; Abadía et al. 1999a) and lettuce (Mahmoudi et al. 2012), leaf Chl was not decreased by increased soil salinity, and therefore light absorption by leaves was not affected. In other species, including *Arabidopsis thaliana* (Martínez-Peñalver et al. 2012), *Atriplex portulacoides* (Benzarti et al. 2012), *Hibiscus hamabo* (Li et al. 2012b), *Salvia officinalis* (Tounekti et al. 2012), *Cassia obtusifolia* (Zhang et al. 2012a), cucumber (Shu et al. 2012) and grapevine (Fozouni et al. 2012), decreases in Chl concentration at high soil salinities have been found.

Some species increase thermal dissipation in response to soil salinity. Increased D (Fig. 27.3; Kchaou et al. 2013) and NPQ (Fig. 27.4; Benzarti et al. 2012; Kchaou et al. 2013) values have been reported, but changes are typically moderate. Increased thermal dissipation under high soil salinity has recently been related to VAZ cycle activity (Tounekti et al. 2012; Zhang et al. 2012b; Kchaou et al. 2013). Both NPQ and the de-epoxidation state of the VAZ cycle were increased in *Cassia obtusifolia* (Zhang et al. 2012a) and tomato (Zhang et al. 2012b), and a close positive correlation between the latter two parameters was found in cucumber (Liu et al. 2012) and olive (Kchaou et al. 2013). In salt-stressed *Caragana korshinskii*, thermal dissipation was slightly increased, with D (recalculated) increasing from 17–19 to 18–22 % and NPQ increasing from 0.45–0.75 to 0.61–1.11 (Yan et al. 2012). In cotton grown under increased soil salinity, however, D accounted for 37–41 % of the PPFD absorbed by PS II, values similar to those of control plants (recalculated from Brugnoli and Björkman 1992). The observation of unaffected D values is consistent with the lack of an effect of salinity on the de-epoxidation state of the VAZ cycle in the same plants (Brugnoli and Björkman 1992). Zhang et al. (2012b) also suggested Z (that accumulated up to 163–171 mmol Z mol^{-1} Chl under salinity) may serve as an antioxidant in transgenic plants with antisense-mediated suppression of Z epoxidase.

Increases of the photosynthetic electron transport rate (ETR)/net photosynthesis ratios in response to increasing soil salinity have been reported in different plant species, supporting the existence of electron-accepting reactions other than CO_2 fixation. The ETR/net photosynthesis ratio in barley increased from control values of 6.0 to12.0 in response to increased soil salinity (recalculated from Belkhodja et al. 1999). Salt stress also increased this ratio in spinach from control values of 7–8 to 11 (recalculated from Delfine et al. 1999). Dark respiration, as a process that decreases net photosynthesis, does not seem to have an effect on the latter values, and was itself not affected by salt stress in several olive cultivars (Centritto et al. 2003; Loreto et al. 2003). At this stage, it is not possible to differentiate between photorespiration, Mehler reaction, PS II cyclic electron transport, and any other electron-consuming process.

Salinity does not result in decreased F_v/F_m ratios in various plants species grown under high PPFDs in the field, indicating that salinity does not induce sustained photodamage. This was observed in halophytes such as *Artemisia anethifolia* (Lu et al. 2003) and *Atriplex portulacoides* (Benzarti et al. 2012), and also in barley (Belkhodja et al. 1999). Similar conclusions have been obtained at lower PPFDs in the annual plant species cowpea, wheat, barley, spinach, cotton, bean, tomato and cucumber (Larcher et al. 1990; Brugnoli and Lauteri 1991; Mishra et al. 1991; Brugnoli and Björkman 1992; Morales et al. 1992; Belkhodja et al. 1994; Delfine et al. 1999; Li et al. 2012a; Liu et al. 2012; Shu et al. 2012; Zhang et al. 2012b). However, perennial species may be more susceptible to salinity than annuals. For instance, salt-stressed olive leaves had F_v/F_m ratios as low as 0.5 (Loreto et al. 2003; Petridis et al. 2012). The underlying causes of these low F_v/F_m values were not fully explored.

IV Macronutrient Deficiencies: N, P and K

A Nitrogen Deficiency

In N-deficient spinach, a first level of photoprotection may be a decrease in light absorption, due to a decreased leaf content of Chl (Bottrill et al. 1970; Bungard et al. 1997; Verhoeven et al. 1997; Cheng 2003; Demmig-Adams and Adams 2003) and carotenoids (Cheng 2003). Plants grown under low N showed more rapid rates of leaf movement compared to plants receiving high levels of N and a longer period of paraheliotropism (light avoiding) at midday (Kao and Forseth 1991, 1992), decreasing the amount of absorbed light. Since light absorptance (Al-Abbas et al. 1974) is apparently less affected than leaf Chl content, the average number of photons absorbed by each Chl molecule presumably increases, resulting in an excess of absorbed PPFD relative to the amount of absorbed light used in photosynthesis.

Both D (Fig. 27.3) and NPQ (Fig. 27.4) increase under a deficiency in N availability in the soil. Rapidly reversible, VAZ cycle-associated thermal dissipation occurs in N-deficient spinach, rice and apple leaves (Verhoeven et al. 1997; Chen et al. 2003; Cheng 2003; Demmig-Adams and Adams 2003). In N-deficient spinach leaves, D accounted for 64–73 % of the light absorbed by the PS II antenna versus 36–59 % in the controls (Verhoeven et al. 1997). In leaves of apple trees grown with low N in the soil, D accounted for 35 and 60 %, respectively, of absorbed light at limiting and saturating light, whereas in trees grown with high N availability in the soil, D accounted for only 25 and 40 % under these two respective light conditions (recalculated from Chen et al. 2001). In a subsequent report on apple leaves, Cheng (2003) reported N-deficiency-mediated increases in D from 36–43 to 71–74 % and in NPQ from 1.4–1.7 to 4.7–5.1. Similar results were found in low light-grown maize (Lu and Zhang 2000).

N-deficient leaves have not been reported to exhibit evidence of alternative electron-consuming processes; the partitioning of electron flow between CO_2 assimilation and photorespiration was not affected by N deficiency in apple tree leaves (Chen et al. 2001). Respiration, however, was found to decrease in N-deficient spinach leaves (Bottrill et al. 1970).

No sustained photoinhibition appears to occur under N deficiency in spinach, as reflected by high predawn F_v/F_m ratios (Verhoeven et al. 1997). Similarly, *Clematis vitalba*, maize, and apple leaves exhibited similar dark-adapted F_v/F_m ratios, ranging from 0.86–0.80 and 0.77–0.74 for control versus N-deficient leaves, respectively (Bungard et al. 1997; Lu and Zhang 2000; Cheng 2003), with the lowest values seen in plants either growing at high PPFD or being severely N-deficient. These slightly decreased F_v/F_m ratios at predawn are likely associated with an overnight retention ("lock-in") of Z + A and sustained NPQ that may occur in N-deficient leaves (Verhoeven et al. 1997).

B Phosphorus Deficiency

Under P deficiency, leaf Chl concentration sometimes increases (Bottrill et al. 1970; Abadía et al. 1987; Marschner 1995; Arthur 2012) but not always (Al-Abbas et al. 1974; Brooks 1986; Lauer et al. 1989; Xu et al. 2007; He et al. 2011), and carotenoid concentrations remain unaffected (He et al. 2011), whereas photosynthesis saturates at lower PPFD than in the P-sufficient controls (Walker and Sivak 1985; Fredeen et al. 1989; Lauer et al. 1989; Rao and Terry 1989). The combination of these responses should result in an excess of energy excitation when P-deficient leaves are exposed to high PPFDs, leading, in turn, to potential photoinhibition.

There are few data available to speculate on how P-deficient plants protect themselves against possible excess energy. Phosphorus-deficient soybean leaves exhibit paraheliotropic (leaflet laminae parallel to incident radiation) orientation on bright, sunny days at midday rather than the normal diaheliotropic (solar tracking) orientation of control leaves and of P-deficient leaves early and late in the day (Lauer et al. 1989). Decreases in absorptance, other than those due to leaf movements, in P-deficient plants are rather small, and are accompanied by increases in reflectance and transmittance (Al-Abbas et al. 1974).

Increases in D (Fig. 27.3) and NPQ (Fig. 27.4) have been reported for P-deficient plants, suggesting VAZ cycle-associated increases in thermal dissipation of excess energy. For instance, D may reach 57–60 % in P-deficient rice versus 52–56 % in P-replete control plants (Xu et al. 2007). Takizawa et al. (2008) reported that a depletion of stromal P_i content decreases proton conductivity at the ATP synthase and leads to an acidification of the thylakoid lumen (see also Walker and Sivak 1985), with a resulting down-regulation of photosynthetic light capture accompanied by increased thermal dissipation. While Rao et al. (1986) concluded that P-deficient sugar beet plants have an altered ability to dissipate the intrathylakoid proton gradient, other studies reported unchanged D values, of about 44–47 %, under P deficiency (recalculated from He et al. 2011). No studies have thus far examined the operation of the VAZ cycle under P deficiency.

An increase in the CO_2 compensation point in low-P plants may suggest elevated respiration and/or photorespiration (Lauer et al. 1989). Dark respiration (and photorespiration, see below) is unlikely to contribute to PS II photoprotection under P deficiency, because P_i is itself a substrate for mitochondrial respiration (not including alternative oxidase that is not regulated by adenylate level). Both dark respiration and photorespiration (Bottrill et al. 1970; Terry and Ulrich 1973b) were reported to decrease under P deficiency compared to the controls (at that time, however, it was not known that Rubisco can catalyze both carboxylation and oxygenation). On the other hand, the ETR/net photosynthesis ratio decreases under P deficiency, from ca. 7–9 to 6 (recalculated from Xu et al. 2007), which suggests an absence of alternative electron sinks in P-deficient plants. Further research is required to elucidate any roles of other electron-consuming processes in photoprotection under P deficiency.

The scarce literature available suggests that no sustained photoinhibition occurs under P deficiency, as judged by the unaffected quantum efficiency measuring CO_2 fixation at limiting light in soybean (Fredeen et al. 1989), and the high F_v/F_m ratios in rice (Xu et al. 2007). On the other hand, quantum efficiency, estimated from the initial response of gas exchange (O_2 or CO_2) to light, decreased in P-deficient spinach (Brooks 1986) and soybean (Lauer et al. 1989). In the study by Brooks (1986), the F_v/F_m ratio (recalculated from Brooks' data), however, only decreased from 0.79 to 0.76 under P starvation in spinach.

C Potassium Deficiency

In some species, K deficiency is associated with low Chl concentrations (Marschner 1995; Weng et al. 2007). Furthermore, Al-Abbas et al. (1974) reported a decreased absorptance, accompanied by increases in reflectance and transmittance, in K-deficient plants, which suggests that light-havesting capacity may be decreased.

There is little information on how K-deficient plants protect the photosynthetic apparatus against possible excess energy. Increases in D (Fig. 27.3) and NPQ (Fig. 27.4) under K deficiency in rice suggest VAZ cycle-associated increases in thermal dissipation as one possible mechanism (Weng et al. 2007), although operation of the VAZ cycle under K deficiency has not yet been addressed.

The possible existence of alternative electron-consuming processes protecting PS II under K deficiency is an open question. Similarly to what happens with P deficiency (see above), dark respiration and photorespiration rates have been shown to decrease with respect to the controls in sugar beet (Terry and Ulrich 1973a). However, there are other reports showing marked increases in dark respiration (Bottrill et al. 1970) and possibly photorespiration (Weng et al. 2007) in K-deficient spinach and rice, respectively. Further research is required to elucidate the role of electron-consuming processes in photoprotection under K deficiency.

V Micronutrient Deficiencies: Fe, Mn, Cu and Zn

A Iron Deficiency

In response to Fe deficiency, leaf Chl levels have been reported to decrease. Iron-deficient leaves are thus apparently protected to some extent against excess PPFD by decreases in light absorptance, associated with decreases in the concentrations of Chls and carotenoids (Terry 1980; Morales et al. 1991; Masoni et al. 1996; Abadía et al. 1999b; Smith and Cheng 2005). Iron deficiency, furthermore, decreases absorptance from control values of 80 % (of incident PPFD) to ca. 20, 40, and 60 % in Fe-deficient leaves of sugar beet, pear, and peach, respectively (Morales et al. 1991; Abadía et al. 1999b). Therefore, Fe-deficient leaves simply do not absorb 40–80 % of the incident PPFD.

In addition, leaves affected by Fe deficiency thermally dissipate a large part of the energy absorbed by the PS II antenna. Changes in D (Fig. 27.3) appeared to be somewhat more pronounced than those in NPQ (Fig. 27.4). With severe Fe deficiency, D may reach up to 55 % (Smith and Cheng 2005) or 75–80 % (Morales et al. 1998; Larbi et al. 2006). Similar D values can also be seen early in the morning, if concomitant mild water stress is present (Morales et al. 2000b). In Fe-sufficient controls, D only accounts for ca. 25 and 54–57 % under low- and high-PPFD, respectively (Fig. 27.1). Corresponding NPQ values increased only from 1.3–2.5 to 1.3–3.2 (Morales et al. 1998; Smith and Cheng 2005; Larbi et al. 2006) with Fe deficiency. Therefore, Fe-deficiency seems to enhance more D than NPQ, but the origin of such differences has not yet been explored. In addition, it should be remarked the large range of NPQ values found in Fe-deficient leaves. This is because NPQ capacity of Fe-deficient leaves depends on the severity of the deficiency, and severely Fe-deficient leaves may have values as low as 1.3 (Morales et al. 1998). The Fe deficiency-mediated increases in D and NPQ have been related to increases in the molar ratio of Z + A to Chl and to the extent of V de-epoxidation to A + Z, which occur in daily cycles in Fe-deficient plants (Morales et al. 1990, 1994, 1998, 2000a; Smith and Cheng 2005). The VAZ cycle-related thermal dissipation in leaves of Fe-deficient sugar beet (low PPFD) is not "locked-in" overnight (Morales et al. 1990, 1998), suggesting that it constitutes a photoprotective mechanism. While in pear grown in the field (under high PPFD), VAZ cycle-related thermal dissipation is not "locked-in" in moderately Fe-deficient leaves, some de-epoxidized VAZ pigments are maintained overnight in severely Fe-deficient leaves (Morales et al. 1994, 2000a). In these latter severely deficient leaves, predawn F_v/F_m was decreased. These low predawn F_v/F_m values might suggest that severely Fe-deficient plants may experience processes considered as photoinhibitory.

Severely Fe-deficient leaves showed apparent sustained decreases in F_v/F_m ratios, associated with a component of fluorescence emission accounting for approximately 15 % of total fluorescence emission of an unknown origin (Morales et al. 2001; see also Cessna et al. 2010). It is not likely that sustained decreases in F_v/F_m ratios come from PS I fluorescence. Readers are referred to Cessna et al. (2010) and Logan et al. (Chap. 7) for details on how PS I contamination of PS II fluorescence is eliminated by excitation in the blue combined with a filter that cuts off PS I fluorescence in the far-red. This approach was used with Fe-deficient leaves in the early 1990s by Morales and co-workers (Morales et al. 1990, 1991). Using this approach, severely Fe-deficient leaves had low F_v/F_m ratios after either 30 min or 8 h of dark adaptation. Moreover, the unknown emission of Fe-deficient leaves had a lifetime of approximately 3.3 ns, very different to the 40–50 ps emission from PS I (Morales et al. 2001). Furthermore, the contribution of PS I emission at F_o decreased from 34.5 % (controls) to 20 % (Fe-deficient) of total fluorescence (Morales et al. 2001). When F_v/F_m values were corrected to eliminate such emission, the corrected F_v/F_m values of 0.66 were similar to those of the controls in the range 0.73–0.83. Without any further evidence, we cannot distinguish between an inactivation of PS II photochemistry, PS II antenna disconnection or other causes as the origin of these low F_v/F_m ratios.

Moderately Fe-deficient leaves exhibit no signs of significant photoinhibitory damage, as judged by both Chl fluorescence and gas exchange parameters. Evidence for the maintenance of a high maximal PS II energy conversion efficiency comes from measurements of the F_v/F_m ratio after dark adaptation, both in controlled environments (Morales et al. 2001) and predawn in the field (Morales et al. 2000a). Furthermore, high quantum yields of CO_2 fixation (Terry 1980) and O_2 evolution (Morales et al. 1991) are seen in moderately Fe-deficient leaves. Moreover, no sustained decreases in F_v/F_m were found in fruit trees even when Fe deficiency occurred concomitantly with other stresses, such as mild water stress, high temperatures, low air relative humidity and high PPFD (Morales et al. 2000b). Some data in the literature had initially suggested that Fe deficiency may cause decreases in maximal intrinsic PS II efficiency. For instance, low dark-adapted F_v/F_m ratios were reported for sugar beet (Morales et al. 1991). These values, however, were due to artifactually high F_o values, caused by the reduction of the plastoquinone pool occurring in Fe-deficient organisms during dark adaptation, which can be prevented by far-red pre-illumination (Belkhodja et al. 1998).

Taken together, all these data suggest that Fe-deficient leaves are well protected against oxidative damage, and explain why low-Chl leaves of Fe-deficient trees can remain stable in the field for months. Most of these leaves appear otherwise healthy, and only extremely Fe-deficient leaves show necrotic spots.

B Manganese Deficiency

Manganese (Mn) deficiency results in decreased levels of leaf Chl and carotenoids (Bottrill et al. 1970; Ohki et al. 1981; Abadía et al. 1986; Kriedemann and Anderson 1988; Pérez et al. 1993; Masoni et al. 1996; Henriques 2003; Feng et al. 2012). As a consequence, Mn deficiency reduces leaf absorptance by 2–29 %, depending on species (Masoni et al. 1996).

The VAZ cycle-associated increases in thermal dissipation under Mn deficiency are likely small. In Mn-deficient soybean, the (A + Z)/(V + A+ Z) ratio increased from 0.06–0.09 (control) to 0.08–0.19 (recalculated from Pérez et al. 1993). An interesting case is the work reported by Jiang et al. (2002) working with Mn-starved maize. As recalculated from the authors' data, NPQ decreases from 5.6 to 2.2 and the (A + Z)/(V + A + Z) ratios decrease from 0.41 to 0.12, while D increases from 31 to 55 % for Mn-starved maize compared to controls. To our knowledge, this is the only report showing opposite changes of NPQ and D with no clear explanation for what may be going on.

Photorespiration and respiration, potentially acting as alternative sinks for electrons or changing net photosynthesis, are not likely to contribute to photoprotection under Mn deficiency, since both processes decreased (Bottrill et al. 1970; Terry and Ulrich 1974) or remained unchanged (Terry and Ulrich 1974; Ohki et al. 1981) under Mn deficiency.

On the other hand, dark-adapted F_v/F_m ratios were unaffected in pecan leaves grown under Mn deprivation (Henriques 2003). Husted et al. (2009) reported a decline in dark-adapted F_v/F_m ratios for both Mn-efficient and -inefficient barley genotypes under Mn deficiency, although the decline in F_v/F_m occurred more readily in the Mn-inefficient genotype.

C Copper Deficiency

Effects of Cu deficiency on leaf Chl content are species-dependent and vary from no effect in spinach (Bottrill et al. 1970), moderate Chl decreases in cereals (Kriedemann and Anderson 1988) and *Amomum villosum* (Feng et al. 2012), and very pronounced Chl decreases in sugar beet (Henriques 1989).

Few data are available about photoprotection and alternative electron sinks in Cu-deficient plants. Changes in light scattering, as evidence for the development of NPQ, were markedly decreased in Cu-deficient leaves (Abadía et al. 1988). Bottrill et al. (1970) concluded that dark respiration is not affected. Copper-deficient leaves showed minor changes in F_o, a lack of the typical biphasic rise to F_m, and decreases in F_v to various extents (Kriedemann and Anderson 1988), although maximal intrinsic PS II efficiency was either only marginally affected (Kriedemann and Anderson 1988) or was not affected (Feng et al. 2012). This finding indicates the absence of irreversible PS II damage under Cu deficiency.

D Zinc Deficiency

Decreases in the concentrations of Chl and carotenoids, as well as chlorosis, i.e., the yellowing of leaf tissue due to larger decreases of Chl than carotenoids, and necrosis are reported in Zn-deficient plants (Bottrill et al. 1970; Marschner and Cakmak 1989; Schuerger et al. 2003; Wang and Jin 2005; Chen et al. 2008; Feng et al. 2012). All these changes should affect the capacity for light harvesting. However, the only change reported in the optical leaf properties is an increased visible reflectance (cited by Masoni et al. 1996).

Under Zn deficiency, the ETR/net photosynthesis ratio increased from 5 to 11 in the C4 plant *Paspalum notatum* (recalculated from Schuerger et al. 2003). Small increases of this ratio under water stress were interpreted as an indication of increased photorespiration in C3 plants (Flexas et al. 1999, 2002), but may reflect increased Mehler reaction in C4 plants (Fryer et al. 1998). Plant respiration, however, has been reported to decrease under Zn deficiency (Bottrill et al. 1970).

Maximal intrinsic PS II efficiency, F_v/F_m, was only slightly diminished in Zn-deficient plants (Schuerger et al. 2003; Wang and Jin 2005; Chen et al. 2008; Feng et al. 2012), which is indicative of the absence of permanent damage to the photosynthetic apparatus.

VI Micronutrient Toxicities: Fe, Mn, Cu and Zn

A Iron Toxicity

Leaf Chl and carotenoid levels were almost unaffected under Fe toxicity resulting from excessively high Fe levels in the soil (Kampfenkel et al. 1995; Kumar et al. 2008), and, therefore, the capacity to harvest light should not be reduced. Once absorbed, an increased fraction of the energy is thermally dissipated under excess Fe and respiration rates also increase (Kampfenkel et al. 1995).

Excess Fe has been suggested to lead to photoinactivation and oxidative stress (Kim and Jung 1993; Kampfenkel et al. 1995). Thylakoids from Fe-overloaded plants were more susceptible to a loss of D1 and other thylakoid proteins (Suh et al. 2002). However, maximal intrinsic PS II efficiency decreased only slightly (F_v/F_m values decreased from 0.80 to 0.76, recalculated from Kampfenkel et al. 1995; or from 0.81 to 0.79, Suh et al. 2002), which calls into question the extent and physiological significance of any PS II photoinactivation.

B Manganese Toxicity

Effects of light intensity on Mn toxicity were first shown by McCool (1935), who reported that low light-grown plants displayed fewer symptoms under excess Mn than those grown under high light. In common bean exposed to excess Mn, Chl concentration was unchanged under low light, but decreased by 48 % under high light (González et al. 1998). Excess Mn resulted in decreased photosynthetic pigment concentrations in chestnut, rose, and grapevine (Yao et al. 2012), but not in *Citrus grandis* (Li et al. 2010) and *Phytolacca americana* (Zhao et al. 2012). Kitao et al. (1997) reported that excess Mn led to decreased leaf absorptance. These findings suggest that excess Mn may decrease light harvesting capacity, the occurrence and extent of which depend on species and light intensity.

Under Mn toxicity, D has been found to increase slightly (Fig. 27.3). In white birch, D increased from 33–38 to 41–54 % (recalculated from Kitao et al. 1997). On the other hand, F_v/F_m ratios were either unaffected (Kitao et al. 1997) or only slightly affected (Li et al. 2010), which points to absence of PS II photodamage under excess Mn.

C Copper Toxicity

Excess Cu generally leads to decreased leaf Chl (Ouzounidou et al. 1994, 1997; Ouzounidou 1996; Zhao et al. 2012) and carotenoid contents (Kumar et al. 2008). However, there is one report of increases in pigment content in pea exposed to excess Cu (Wodala et al. 2012). Ouzounidou (1996) suggested that light-harvesting capacity is decreased, and leaf reflectance increases have also been found, under excess Cu (Horler et al. 1980; Schwaller et al. 1983).

Thermal dissipation increases under excess Cu (Ouzounidou 1996; Moustakas et al. 1997; Ouzounidou et al. 1997). For instance, D increased from 33–40 % in control plants to 45–52 % under excess Cu in *Thlaspi ochroleucum* and maize grown in hydroponics (recalculated from Ouzounidou 1996; Ouzounidou et al. 1997) and from 17–36 % in control plants to 25–43 % under excess Cu in runner bean plants grown hydroponically (recalculated from Maksymiec and Baszynski 1999). At the same time, NPQ increased in response to excess Cu from 1.1–1.6 to 1.6–1.9 (recalculated from Ouzounidou et al. 1997; Maksymiec and Baszynski 1999) in maize and runner bean, but remained as low as in controls (0.21–0.22) in pea (Wodala et al. 2012). These increases can be associated with changes in the VAZ cycle; the (A + Z)/(V + A + Z) ratio increased from 0.36 to 0.63 in *Secale cereale* under excess Cu (recalculated from Janik et al. 2010).

Maximal intrinsic PS II efficiency (F_v/F_m) was unaffected, or only slightly affected, by excess Cu (recalculated from Ouzounidou 1996; Ouzounidou et al. 1997; Wodala et al. 2012), which suggests absence of permanent PS II damage.

D Zinc Toxicity

In several species, excess Zn leads to decreased foliar Chl and carotenoid concentrations (Monnet et al. 2001; Schuerger et al. 2003; Vaillant et al. 2005; Sagardoy et al. 2009; Zhao et al. 2012). In soybean, however, Zn-treated plants were dark green, suggesting that inhibition of leaf expansion predominated over any effects on Chl biosynthesis or breakdown (Horler et al. 1980). The capacity for light harvesting may thus either be compromised or not, depending on the species exposed to excess Zn.

Increases of thermal dissipation under excess Zn are rather small. NPQ increased from 0.27 to 0.49, while D increased from 22 to 32 %, in sugar beet plants treated with excess Zn (Sagardoy et al. 2009). Cambrollé et al. (2013) reported that NPQ increased from 0.7 to 1.2 under excess Zn. These small changes, and the limited interconversion of V into Z within the VAZ cycle under excess Zn (Sagardoy et al. 2009), suggest that excess Zn may impair ΔpH formation.

Monnet et al. (2001) reported that excess Zn strongly decreased Rubisco carboxylase activity, possibly due to a decreased affinity of Rubisco for CO_2, whereas its oxygenase activity was unaffected or even increased. The latter authors concluded that the oxygenase activity of Rubisco appears to be an essential mechanism for the protection of the photosynthetic apparatus under Zn excess. The ETR/net photosynthesis ratios increased under Zn excess, from ca. 9 to 31–77 (recalculated from Monnet et al. 2001), which may be indicative of increased photorespiratory activity.

Excess Zn does not cause major decreases in maximal intrinsic PS II efficiency, which can be interpreted as absence of permanent photodamage. Likewise, the quantum yield of PS II-related electron transport at low PPFD was unaffected (Van Assche and Clijsters 1986). This conclusion is consistent with those of Sagardoy et al. (2009, 2010) who showed either no changes, or only small decreases, in F_v/F_m (from 0.82 to 0.76) with exposure to excess Zn. The lowest F_v/F_m values reported with Zn excess were 0.60–0.70 (Schuerger et al. 2003; Cambrollé et al. 2013).

VII Other Metal Toxicities: Cd, Pb, Al and Hg

A Cadmium Toxicity

Cadmium toxicity generally induces leaf chlorosis, in the form of major decreases in foliar Chl and carotenoid contents (Krupa et al. 1993; Larsson et al. 1998, 2001; Di Cagno et al. 2001; Larbi et al. 2002; Linger et al. 2005; He et al. 2008; Li et al. 2012c, 2012d; Mohamed et al. 2012), and this is likely due to Cd-induced Fe deficiency (Larbi et al. 2002). Cadmium has been reported to reduce Fe concentrations in soybean, sugar beet seedlings, lettuce and maize, and induce typical physiological responses of plants to limited Fe supply (Larbi et al. 2002, and references therein). Cadmium directly inhibited Chl biosynthesis in barley (Stobart et al. 1985) in short-term experiments with leaf segments. When Cd toxicity is very severe, however, plant growth is restricted and foliar Chl concentration increases (Wodala et al. 2012). In *Cannabis sativa*, effects of excess Cd on foliar Chl and carotenoid content were cultivar-dependent; leaf Chl and carotenoid content was lower following exposure to Cd in 7 out of 18 cultivars tested (Shi et al. 2012). Leaf reflectance has also been reported to increase in Cd-treated leaves (Horler et al. 1980).

Increases in de-epoxidation of the VAZ cycle under Cd toxicity (Larbi et al. 2002; Mohamed et al. 2012) were similar to those reported for Fe-deficient plants with comparable Chl levels (see section "Micronutrient deficiencies: Fe, Mn, Cu and Zn"). A higher amount of de-epoxidated xanthophylls may be attributed to a Cd-induced diminished Z-epoxidase activity. Latowski et al. (2005) reported that Cd inhibits Z epoxidation in *Lemna trisulca* and tomato but not in apricot leaves, which can be explained considering the absence of the cysteine residue in motif I of apricot epoxidase as a target site of Cd inhibitory action on this enzyme. These changes were accompanied by increases in D from 22–36 to 33–65 % (recalculated from Krupa et al. 1993; Di Cagno et al. 2001; Larbi et al. 2002). In Cd-treated *Cannabis sativa*, D increased from control values of 32 to 62 % but returned to control values with longer exposures to Cd (recalculated from Linger et al. 2005). NPQ also generally increased under excess Cd (Krupa et al. 1993; Larsson et al. 1998; Di Cagno et al. 2001; Larbi et al. 2002), although increases were small in some cases (Wodala et al. 2012). In *Arabidopsis thaliana*, however, plants grown under excess Cd exhibited lower NPQ than controls, with 0.86 and 1.26, respectively (Larsson et al. 2001).

Electron transport rates were lower in sugar beet exposed to Cd (recalculated from Larbi et al. 2002, using absorptance values reported by Morales et al. 1991 for comparable Chl contents). In turn, the ETR/net photosynthesis ratio was greater, e.g., ca. 11–15 versus 11–21, for excess Cd-treated sugar beet and rice compared with controls, respectively (recalculated from Larbi et al. 2002; He et al. 2008), which suggests that alternative electron sinks do not play a role. Using isolated chloroplasts, Weigel (1985) postulated a shift of Rubisco towards its oxygenase function under Cd toxicity. However, photorespiration rate was not affected in Cd-affected pea plants (McCarthy et al. 2001). Dark respiration was increased in protoplasts isolated from Cd-treated sugar beet plants (Greger and Ögren 1991).

Although Greger and Ögren (1991) reported a decreased maximal quantum yield of CO_2 assimilation in sugar beet, F_v/F_m ratios generally remained unaltered, or were only slightly affected, in many plant species exposed to excess Cd (Greger and Ögren 1991; Krupa et al. 1993; Larsson et al. 1998, 2001; Di Cagno et al. 2001; Larbi et al. 2002; Li et al. 2012c; Shi et al. 2012; Wodala et al. 2012). Changes in F_v/F_m ratios that did occur were only transient; they decreased from control values of 0.80 to 0.66 after 48 days of Cd treatment but recovered to values of 0.77 at the end of the experimental period (30 days later) (Linger et al. 2005). In contrast, in experiments with excised leaves immersed in solutions containing Cd, decreases in F_v/F_m were much larger and were accompanied by apparent damage to the oxygen-evolving complex and changes in D1 turnover (Geiken et al. 1998; but see Adams et al. 2013 and Chap. 23 for an alternative, photoprotective interpretation of decreased levels of D1 and oxygen-evolving complex proteins).

B Lead Toxicity

Lead has been reported to alter Chl biosynthesis (Prasad and Prasad 1987). However, in sugar beet grown in Pb-containing medium, Chl and carotenoid concentrations were only slightly decreased (Larbi et al. 2002). In other studies, effects on the Chl and carotenoid concentrations depended on plant species (Ribeiro de Souza et al. 2012), ecotypes (Liu et al. 2008), or Pb dose and exposure time (Kumar et al. 2012). Furthermore, increases in leaf reflectance were reported for plants grown in the presence of Pb (Horler et al. 1980).

Few studies have focused on the photoprotection mechanisms of Pb-treated plants. Both the VAZ cycle (Larbi et al. 2002) and D (recalculated from Larbi et al. 2002) were unaffected by Pb in whole plants. In Pb-infiltrated leaf discs, D was reported to be 50 % in controls compared to 46–61 % in Pb-treated discs, and NPQ 2.3 in controls compared to 2.5–3.1 in the Pb-treated ones (recalculated from Liu et al. 2010).

Lastly, in sugar beet and tobacco grown in the presence of Pb, the F_v/F_m ratio remained unchanged (Larbi et al. 2002; Alkhatib et al. 2011), which suggests the absence of irreversible PS II damage under excess Pb.

C Aluminum Toxicity

Under Al toxicity, leaf Chl content decreased in some species (Ohki 1986; Chen et al. 2005a, b; Jiang et al. 2008; He et al. 2011; Yang et al. 2012), was unaffected in cover crops like cowpea, black oat and lablab (Vieira et al. 2008), and increased in maize (Lidon et al. 1997). Leaf carotenoid content decreased in Al-treated maize (Lidon et al. 1997) and *Citrus grandis* (Yang et al. 2012), but increased in *Camellia oleifera* (He et al. 2011). Therefore, some species, but not others, have a compromised ability to gather light under Al stress.

Maximal intrinsic PS II efficiency, F_v/F_m, showed either no significant changes, or was slightly decreased, under Al toxicity in wheat, maize, *Festuca arundinacea*, highbush blueberry, and *Citrus reshni* (Moustakas and Ouzounidou 1994; Moustakas et al. 1995; Lidon et al. 1999; Chen et al. 2005b; Inostroza-Blancheteau et al. 2011; Jin et al. 2011); significant decreases in F_v/F_m were found in the annual species barley and tobacco (Dawood et al. 2012; Li et al. 2012e). In Al-treated *Citrus grandis* growing in a greenhouse in pots containing sand and fertirrigated with nutrient solution, F_v/F_m values as low as 0.35 have been reported after 3 h in darkness (Yang et al. 2012). These data indicate that photoinhibitory damage to PS II is very limited in some plants, and either damage or sustained photoprotection are important in other species.

Under Al stress, both D (Fig. 27.3) and NPQ (Fig. 27.4) generally increase. For instance, D increased under Al stress from 24 to 43 % (recalculated from Moustakas and Ouzounidou 1994), from 44–47 to 42–64 % (recalculated from He et al. 2011), from 58–63 to 64–85 % (recalculated from Jin et al. 2011), or from 34–36 to 38–48 % (recalculated from Moustakas et al. 1996; Chen et al. 2005a, b). Increases in D were associated with increases in the (A + Z)/(V + A + Z) ratio (Chen et al. 2005a). Regarding NPQ, either increases (from 1.5–2.4 to 2.1–3.6) (Jin et al. 2011), no changes (Lidon et al. 1997), or decreases (from 0.68 to 0.55–0.56) (Chen et al. 2005a, b) have been reported for Al-treated plants. In highbush blueberry, presence of Al in the soil induced NPQ increases (from 1.9 to 2.3–2-4) in Al-resistant cultivars and NPQ decreases (from 2.2 to 1.1–1.2) in Al-sensitive cultivars (Inostroza-Blancheteau et al. 2011). We cannot be sure if these low NPQ values were caused by incorrect calculations due to sustained decreases in F_m (and F_v/F_m) prior to exposure to light since F_v/F_m ratios ranged from 0.82 to 0.75 (Inostroza-Blancheteau et al. 2011). A low NPQ in a leaf with a dark-adapted F_v/F_m of 0.82 is indeed unlikely to be caused by pre-existing NPQ in darkness, but a leaf with a dark-adapted F_v/F_m of 0.75 could have a much-decreased dark F_m level. Unfortunately, Inostroza-Blancheteau et al. (2011) did not report F_m data.

D Mercury Toxicity

Mercury is very toxic even at low concentrations, causing decreased foliar Chl concentrations (Godbold and Hüttermann 1988; Marschner 1995; Cho and Park 2000), therefore potentially decreasing light-harvesting capacity. Very little is known with regard to possible photoprotection responses in leaves exposed to Hg. Leaf reflectance at 550 nm is increased in plants grown with Hg (Dunagan et al. 2007), and respiration remained unaffected, or was decreased, in response to Hg (Godbold and Hüttermann 1988).

VIII Conclusions and Future Research

Under unfavorable soil conditions, the rates of photosynthetic carbon fixation of plants decrease for a variety of reasons, while leaves often continue to gather sunlight. In response to some stresses, the amount of foliar Chl decreases, in turn decreasing leaf absorptance. In response to other stresses, however, Chl concentration does not change. At high PPFDs, as a consequence of an imbalance between light harvesting and the capacity for utilization of the absorbed light, plants experience what has been called "photoinhibitory" processes. The data currently suggest that, although signs of photodamage are sometimes found, photoprotection mechanisms are far more important. The relative importance of the latter two processes also depends on stress type and plant species.

Plants grown under environmental stress generally dissipate a large part of the light absorbed by PS II as heat, in a process mediated by ΔpH, the VAZ cycle, and the PsbS protein within the PS II antenna pigment bed. For contrasting models (independent or cooperative action of ΔpH, VAZ cycle, and PsbS) and debate, see recent literature (Holzwarth et al. 2009; Ruban et al. 2012; Peguero-Pina et al. 2013). Cases where high levels of thermal dissipation occur frequently, but not always, involve decreases in Chl, as has been confirmed for water stress, N and Fe deficiencies, and Al stress. Thermal dissipation usually becomes increasingly important with increasing severity of the stress. To broadly generalize the importance of thermal dissipation under unfavorable soil conditions, however, more information is needed on the extent of thermal dissipation under additional, thus-far poorly explored nutrient deficiencies and toxicities.

In most cases reviewed here, there is no evidence for major sustained photodamage under unfavorable soil conditions. Oxidative stress-related processes, traditionally regarded as damage, could alternatively be interpreted as part of redox-regulated signal transduction pathways (see Demmig-Adams and Adams 2002, 2003; see also Demmig-Adams et al. 2013). Future research should be focused to investigate these signaling pathways in plants under unfavorable soil conditions and should also examine the involvement of sink limitation and foliar carbohydrate accumulation in leaves with decreased Chl content and/or any signs of photoinhibition (Adams et al. 2013; see also Adams et al., Chap. 23).

An often-overlooked fact in studies on the effects of environmental stress on photosynthetic parameters is the existence of major gradients within the leaf, since top cell layers are exposed to higher effective PPFD than inner ones (Sun et al. 1996; Nishio 2000; Peguero-Pina et al. 2009). This heterogeneity within the leaf poses some difficulties. Some techniques widely used to study stress effects, such as gas exchange or pigment composition, integrate over all cell layers within the leaf. Chlorophyll fluorescence studies, on the other hand, focus on the effects of stress on the top cell layers only. Therefore, combining data obtained using these different techniques can lead to confusion and further work is needed in this area.

The combined use of Chl fluorescence and gas exchange techniques (Valentini et al. 1995; Medrano et al. 2002) has been successful for the separation of electron transport rate into several fractions, including that supported by photosynthesis and photorespiration, in drought-affected plants (Flexas and Medrano 2002). However, using this approach with Fe- or N-deficient plants has thus far not been possible because the apparent ratio of ETR and the sum of photosynthetic rates (net or gross) and photorespiration decreases with Fe and N deficiency (our unpublished observations and data recalculated from the literature) below the theoretical minimum value of 4.5–5 (von Caemmerer and Farquhar 1981). Likewise, computed values of 6 in P-deficient rice (recalculated from Xu et al. 2007) are unlikely to be possible. A possible explanation for this conflict between theory and apparent ratios computed for stressed plants is that ETR estimated from Chl fluorescence and photosynthetic rate measured via gas exchange may originate from different cell populations. The uppermost cell layers, contributing strongly to ETR estimation, may be more affected than deeper cell layers that, in turn, contribute more strongly to the estimation of photosynthesis. Another problem arising from leaf heterogeneity is related to thylakoid isolation. Some evidence for photodamage comes from studies carried out with isolated thylakoids, which may or may not be representative of the leaves used for isolation (Quílez et al. 1992). Methodologies should be established to assess photodamage from intact leaves.

Absoption of excessive light is avoided in some species by a canopy structure that decreases exposure of leaf surfaces to sunlight (He et al. 1996; Valladares and Pugnaire 1999) or by the presence of epidermal trichomes that intercept incoming light (Morales et al. 2002). In some species, leaves grown under low P, water stress or low N actively avoid direct radiation during the period of maximum solar intensities by placing leaves parallel to sunlight (Lauer et al. 1989; Kao and Forseth 1991, 1992). This aspect is poorly investigated for other nutrient stresses. In most cases, researchers fix leaves perpendicular to sunlight for measurements, thus not taking into account any possible paraheliotropic leaf response. For instance, it has been reported that in *Heliconia* a change in leaf orientation from 10 to 80° from the horizontal plane decreases the PPFD incident on the leaf surface by 41 % and results in increases in dark-adapted F_v/F_m from 0.63 to 0.72 (He et al. 1996). Some species may change leaf orientation, and thus exposure to light, over the course of a day. For instance, wild soybean, grown under low water availability (Kao and Tsai 1998), and *Arbutus unedo* (Gratani and Ghia 2002) exhibit more vertical leaf angles at midday. The importance of leaf movements and folding (Huang et al. 2012) should be addressed by future research.

Discrepancies among laboratories in the results for the same plant species may be related, e.g., to leaf age. For instance, older *Juncus roemerianus* plants are less tolerant to salinity than younger plants, with only the former, but not the latter, showing F_v/F_m decreases under salt stress (Touchette et al. 2012). Furthermore, in *Salvia officinalis* older leaves suffer from greater photo-oxidative salinity-related stress than young leaves, despite being located lower in the canopy and therefore presumably having a lesser need for photoprotection (Tounekti et al. 2012). Chlorosis (decreased Chl content) in response to deficiencies of Mn or Fe typically occurs in the oldest or youngest leaves (Marschner 1995). In the model species *Arabidopsis thaliana* under water stress, younger leaves dissipated more energy thermally than mature leaves (Sperdouli and Moustakas 2012). In *Pistacia lentiscus* grown under field-conditions, mature leaves suffered from water and N deficiency during late spring and summer, whereas young (emerging) and old (senescing) leaves were more sensitive to photo-oxidative stress during late fall and winter (Juvany et al. 2012). Moreover, comparative analyses of field-grown plants and plants grown under controlled conditions are needed to reveal differences in leaf morphology and other features in response to growth environment (Mishra et al. 2012; see also Demmig-Adams et al., Chap. 24).

Acknowledgments

This work was supported by Spanish grants AGL2009-09018 to AA and AGL2010-16515 to JA, and Aragón Government (A03 research group).

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Chapter 28

Chloroplast Photoprotection and the Trade-Off Between Abiotic and Biotic Defense

Barbara Demmig-Adams*, Jared J. Stewart, and William W. Adams III
Department of Ecology and Evolutionary Biology,
University of Colorado, Boulder, CO 80309-0334, USA

Summary

This chapter places two key photoprotective processes in the chloroplast (thermal dissipation of excess excitation energy and removal of reactive oxygen species, ROS, by antioxidants) into the context of whole-leaf and whole-plant function in the environment. The emerging evidence for possible trade-offs between effects of altered thermal dissipation and/or anti-oxidation capacity on plant resistance to abiotic stresses (unfavorable physical conditions) versus biotic stresses (pests and pathogens) is summarized. We conclude that more research on this topic is urgently needed, especially for specific crop species and agriculturally relevant environments, including various combinations of multiple abiotic and biotic stresses. As an example of a key redox-signaling pathway impacted by thermal dissipation and/or anti-oxidation capacity, the formation of lipid-peroxidation-derived hormonal messengers of the oxylipin family, with critical roles in plant growth, development, and defenses, is discussed. The available evidence for specific effects of the capacities of thermal dissipation and/or anti-oxidation on sugar loading into foliar phloem conduits, sugar export from leaves, whole-plant growth rate, and plant biotic defenses is reviewed. Lastly, leaf responses to moderate versus massive ROS formation via multiple feedback loops are compared and contrasted.

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*Author for Correspondence, e-mail: barbara.demmig-adams@colorado.edu

B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_28,

I Introduction

The present volume is dedicated to a key aspect of the suite of photoprotective mechanisms (see Logan et al., Chap. 7) employed by photosynthetic organisms to avoid potential damage encountered during the collection and processing of solar energy. This key photoprotective process is the thermal dissipation of excess excitation energy from the singlet-excited state of chlorophyll *a*, assessed as the non-photochemical quenching of chlorophyll fluorescence, operating in conjunction with cellular anti-oxidant networks (see Havaux and García-Plazaola, Chap. 26). Assessing the level of employment of these different photoprotective processes in different species under various environmental conditions provides information about how those species cope with stress and excess light, furthering our understanding of each species' role in the habitats where they thrive. Moreover, a better understanding of the mechanisms of thermal dissipation (and anti-oxidation) promises to provide the tools for identification, breeding, and molecular engineering of crop plants enhanced in various aspect of this component of photoprotection. It is often tacitly assumed that plants with a *greater* capacity for photoprotective thermal dissipation and/or anti-oxidation would be better protected against losses in productivity caused by unfavorable environmental conditions, thus resulting in plants with *higher yields of food and fuels* under not-always-perfect growing conditions. Do we, however, know this to be the case? Will crops with augmented photoprotection be higher-yielding crops?

The currently available evidence indeed suggests that losses in plant productivity caused by *abiotic* stresses, such as drought or unfavorable (excessively low or high) temperatures or soil mineral content, may be counteracted by augmented photoprotection (for abiotic stress tolerance of, e.g., zeaxanthin-deficient and zeaxanthin-overexpressing mutant lines, see Davison et al. 2002; Du et al. 2010; Gao et al. 2010; Wang et al. 2010; Chen et al. 2011). However, net crop productivity is affected not only by plant biomass yield, but is crucially dependent on limiting the staggering losses (over 50 %) to pests and pathogens as *biotic stresses* (Fletcher et al. 2006). In this chapter, we summarize the emerging evidence that plant resistance to pests and pathogens is affected by factors serving in thermal dissipation and/or anti-oxidation. In fact, it appears from the evidence available to date (as detailed below) that plant biotic stress resistance can be *either lowered or increased* by augmented anti-oxidation, and can be *increased* by a *decrease* in thermal dissipation capacity. Future work in specific crop species and specific environments (pointed out by, e.g., Horton, Chap. 3; Brooks et al., Chap. 13). This future work should also consider differences between defenses against pests versus herbivores as well as defenses against specific biotic agents within each of the two categories.

The call for more research into the potential costs of anti-oxidant overexpression echoes a parallel development in the medical field. For example, Jackson (2008) stated "From the initial view that ROS [reactive oxygen species] were potentially damaging and that prevention of their actions would inevitably be beneficial, we are reaching an understanding that these substances play fundamental roles in metabolism", and Poljsak and Milisav

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Abbreviations: ^{1}Chl* – Singlet-excited chlorophyll; $^1O_2$* – Singlet-excited oxygen; ^{3}Chl* – Triplet-excited chlorophyll; Chl – Chlorophyll; LHC – Light-harvesting complex; NPQ – Non-photochemical quenching of chlorophyll fluorescence; *npq1* – Non-photochemical quenching mutant #1 of *Arabidopsis* deficient in violaxanthin de-epoxidase catalyzing conversion of violaxanthin to zeaxanthin; *npq4* – Non-photochemical quenching mutant #4 of *Arabidopsis* deficient in the photosystem II protein PsbS; $O_2^{\bullet-}$ – Oxygen radical anion (superoxide); OEC – Oxygen-evolving complex; PS II – Photosystem II; PsbS – Photosystem II protein S (member of the stress-associated sub-family of light-harvesting proteins); RC – Reaction center; ROS – Reactive oxygen species; *vte* – Vitamin E-deficient *Arabidopsis* mutant lacking alpha-, beta-, gamma-, and delta-tocopherol as a result of deficiency in the enzyme tocopherol cyclase (*vte1*) or homogentisate phytyltransferase (*vte2*)

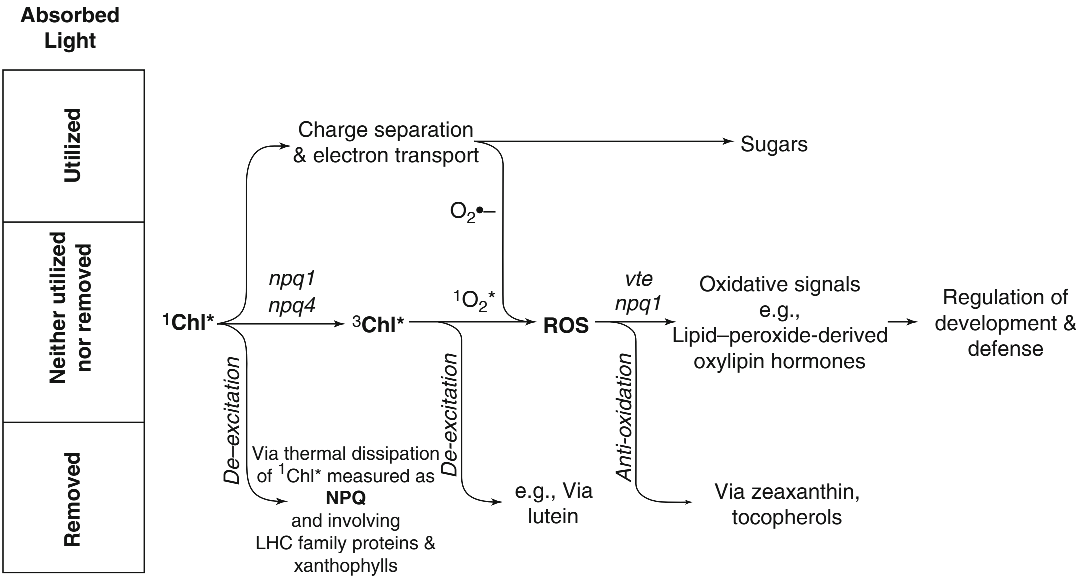

Fig. 28.1 Schematic depiction, placing excitation energy conversion during the primary light reactions of photosynthesis in the context of ROS formation and ROS-triggered signaling with an emphasis on lipid-peroxide-derived oxylipin hormones. *^{1}Chl**: singlet-excited chlorophyll, *^{3}Chl**: triplet-excited chlorophyll, *$^1O_2$**: singlet-excited oxygen, $O_2^{\bullet-}$: oxygen radical anion (superoxide), *LHC*: light-harvesting complex, *NPQ*: non-photochemical quenching of chlorophyll fluorescence from singlet-excited chlorophyll *a*, *npq1 Arabidopsis*: mutant deficient in violaxanthin de-epoxidase catalyzing conversion of violaxanthin to zeaxanthin, *npq4 Arabidopsis*: mutant deficient in the photosystem II protein PsbS, *ROS*: reactive oxygen species, *vte Arabidopsis*: mutants lacking alpha-, beta-, gamma-, and delta-tocopherol as a result of deficiency in either the enzyme tocopherol cyclase (*vte1*) or homogentisate phytyltransferase (*vte2*).

(2012) elaborated that “Oxidative stress is not necessarily an un-wanted situation, since its consequences are beneficial for many physiological reactions in cells. On the other hand, there are potentially harmful effects of ‘anti-oxidative stress’”. Furthermore, Villanueva and Kross (2012) stated that, “the normal balance between antioxidants and free radicals in the body is offset when either of these forces prevails… In summary, a hypothesis is presented that ‘antioxidant-induced stress’ results when antioxidants overwhelm the body’s free radicals.”

II Integration of Photoprotection into Whole-Plant Functioning

Strong evidence has accumulated that (i) foliar levels of reactive oxygen species (ROS), and multiple signaling networks responding to cellular redox state (as the balance between oxidants and anti-oxidants), contribute chiefly to the regulation of plant growth and development as well as the orchestration of abiotic and biotic plant defenses (for a review, see, e.g., Munné-Bosch et al. 2013), and that (ii) chloroplast ROS level and redox state is modulated by thermal energy dissipation and anti-oxidant level (Ledford and Niyogi 2005).

Figure 28.1 places thermal dissipation and anti-oxidation into the context of oxidative signaling networks regulating plant growth and development as well as plant defenses against pathogens and herbivores. When exposed to light levels that are limiting to photosynthesis, plants use as much of the light they absorb as possible in the photochemical pathway leading (via charge separation and photosynthetic electron transport) to sugar production. Plants grown in environments with fluctuating light levels harmlessly dissipate excess excitation energy (light absorbed in excess of what can be utilized photochemically) via photoprotective thermal dissipation of excessive excitation energy, which represents a non-photochemical route leading to non-photochemical quenching (NPQ) of chlorophyll fluorescence (Fig. 28.1). Excess singlet-excited chlorophyll *a* (^{1}Chl*) is de-excited via thermal dissipation (involving, as summarized in the present volume, various xanthophyll pigments and proteins of the family of light-harvesting proteins; Fig. 28.1; see also, e.g., Büchel, Chap. 11; Brooks et al., Chap. 13; Morosinotto and Bassi, Chap. 14). Remaining ^{1}Chl* (not dissipated via the combination of photochemical and non-photochemical pathways) can convert to triplet-excited chlorophyll (^{3}Chl*) that readily passes its excitation energy to ground-state oxygen (Fig. 28.1). The NPQ-deficient *Arabidopsis* mutants *npq1* (lacking violaxanthin de-epoxidase that catalyzes the conversion of violaxanthin to zeaxanthin; see, e.g., Demmig-Adams et al., Chap. 24) and *npq4* (lacking the PsbS protein; see Brooks et al., Chap. 13), both deficient in NPQ (and thermal dissipation), are likely to form elevated levels of ^{3}Chl* under excess light. While light-harvesting proteins possess potent quenchers of ^{3}Chl* (see, e.g., Dall'Osto et al. 2006), any remaining ^{3}Chl* will transfer its energy on to oxygen, forming highly reactive singlet oxygen $^1O_2$* (Fig. 28.1). Tocopherols (vitamin E) as well as zeaxanthin are potent anti-oxidant quenchers of ROS like $^1O_2$* (see Havaux and García-Plazaola, Chap. 26; concerning the terminology of vitamin E versus tocopherols, vitamins are specifically defined as compounds not synthesized in the organism in which they have essential functions, which does not apply to photosynthetic organisms proficient in the synthesis of compounds serving as vitamins in non-photosynthetic organisms. We will, therefore, refer mainly to tocopherols with occasional reference to the fact that these compounds also serve as vitamin E).

Any remaining ROS (not removed by anti-oxidants) can give rise to oxidative signals – for example, lipid-peroxidation-derived oxylipin hormones like jasmonic acid (Fig. 28.2; see also section III below) that regulate plant growth and development as well as plant biotic defense (see also Brooks et al., Chap. 13). It should be noted that ROS-dependent oxylipin production is but one of a multitude of signaling pathways controlling plant function via redox-based signals (Munné-Bosch et al. 2013). For reviews of other redox signaling networks, see Baginsky and Link (2006), Dietz et al. (2006), Foyer et al. (2006), and Mullineaux et al. (2006).

III Lipid-Peroxidation-Derived Hormones as an Example for Redox Modulation of Plant Form and Function

Tocopherol-deficient plants exhibit defects in growth and development (for a review, see Falk and Munné-Bosch 2010), in particular impaired phloem loading, decreased carbohydrate export from leaves, enhanced foliar starch accumulation (Russin et al. 1996; Botha et al. 2000; Provencher et al. 2001; Sattler et al. 2003, 2004, 2006; Hofius et al. 2004; Maeda et al. 2006, 2008), and premature senescence (Abbasi et al. 2009). Falk and Munné-Bosch (2010) pointed out that these *impairments* in the growth of tocopherol-deficient plants are most obvious when the corresponding wild type plants exhibit a *high sink demand* (i.e., high carbohydrate consumption by the plant's growing or carbohydrate-storing sinks); they stated that "the sucrose export phenotype is more clearly observed in tocopherol-deficient plants in species with a higher sink demand, such as potato or maize, and also in other species when grown at low temperature, such as *Arabidosis thaliana*." Our own work has shown that *Arabidopsis* wild type increases the number of phloem cells in its leaf veins when grown under cool versus warm temperature, which likely contributes to a maintenance of carbon export flux and elevated levels of photosynthesis despite increased phloem sap viscosity at lower temperature (Cohu et al. 2013a,b).

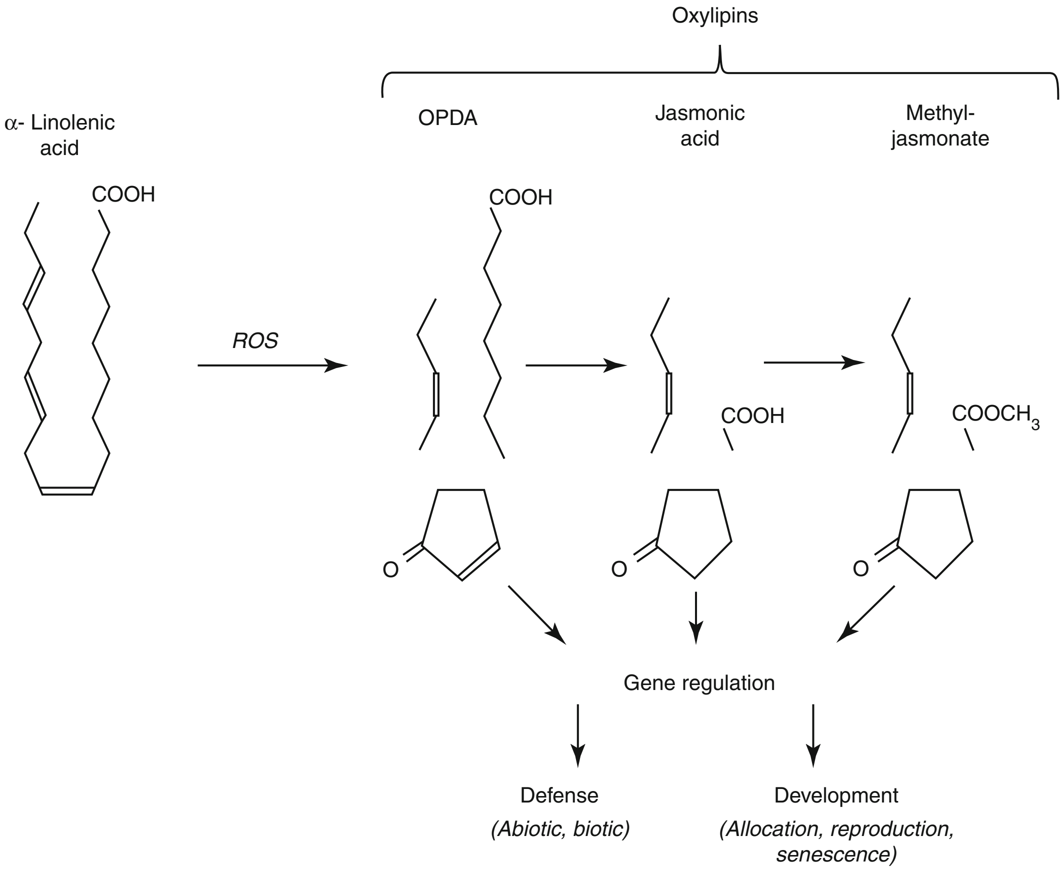

Fig. 28.2 Features of the biosynthesis of selected oxylipins with gene regulatory functions in *Arabidopsis*. *ROS* reactive oxygen species, *OPDA* 12-oxo-phytodienoic acid. Modified after Devoto and Turner (2005) and Demmig-Adams et al. (2013).

The mechanism of growth inhibition in tocopherol-deficient plants apparently involves increased levels of ROS, lipid-peroxidation-derived hormonal messengers (such as jasmonic acid and related oxylipins), and deposition of callose in cells involved in phloem loading (in the leaf's sugar-loading veins; Amiard et al. 2007). Callose formation has been shown to be associated with lipid peroxidation (Yamamoto et al. 2001), and a closing of phloem-loading routes via callose deposition occurs in plants infected by fungi (Koh et al. 2012), presumably to prevent the spread of the pathogen (Zavaliev et al. 2011). It has been shown that fungi and other pathogens utilize the plant's phloem conduits to spread themselves throughout the plant (Gilbertson and Lucas 1996; Waigman et al. 2004; Scholthof 2005). While the *benefit* of an ROS-triggered shutdown of phloem conduits may lie in a greater pathogen resistance, the *cost* of this protection would be the plant's diminished ability to export sugars from the photosynthesizing leaves for plant growth. Conversely, upregulation of foliar anti-oxidant levels, and a corresponding suppression of lipid-peroxidation-derived (and other oxidative) signals, has the potential *benefit* of preventing obstruction of phloem conduits (allowing unimpaired carbon export from source leaves to sinks), while potentially simultaneously rendering plants *more vulnerable* to succumbing to pathogen attack.

Characterized feature	Wild Type	Tocopherol ("Vitamin E")-deficient (*vte*)	PsbS-deficient (*npq4*)	Zeaxanthin-deficient (*npq1* or *npq1 lut2*)
ROS level	+ *(1)*	++ *(2)*	++ *(1)*	*(unknown)*
Oxylipin level (e.g., jasmonic acid)	+ *(3)*	++ *(5)*	++ *(3)*	++ *(4)*
Defense against herbivores	+ *(3)*	*(unknown/ complex)*	++ *(3)*	*(unknown)*
Callose deposition in phloem / putative pathogen defense	+ *(4)*	++ *(6)*	*(unknown)*	++ *(4)*

Fig. 28.3 Summary of the available evidence for a role of the anti-oxidant tocopherol, the photosystem II protein PsbS, and the xanthophyll zeaxanthin in modulating ROS and oxylipin levels as well as in plant biotic defenses. (*1*) Jänkänpää et al. 2013, (*2*) Havaux et al. 2005; Sattler et al. 2006; Semchuk et al. 2009, (*3*) Frenkel et al. 2009, (*4*) Amiard et al. 2007; Demmig-Adams et al. 2013, (*5*) Munné-Bosch et al. 2007, (*6*) Botha et al. 2000; Maeda et al. 2006. Both *npq1* and *npq1 lut2* exhibited a trend (albeit not significant for *npq1*) for increased cell wall ingrowths/reinforcements. The double mutant *npq1 lut2* exhibited even more pronounced cell wall ingrowths than *npq1* and yielded significant differences versus wild type. Both *npq1* and *npq1 lut2* were transferred from low to moderate (double mutant) or high light (*npq1*) to trigger cell wall ingrowths. The PsbS-deficient *npq4* mutant exhibited significant differences in oxylipin levels under herbivore attack in the field but not in the absence of herbivores. *npq1*, *Arabidopsis* mutant deficient in violaxanthin de-epoxidase catalyzing conversion of violaxanthin to zeaxanthin (this mutant was used to demonstrate elevated oxylipin levels in plants transferred from low growth light to high light; Demmig-Adams et al. 2013); *npq1 lut2*; *Arabidopsis* mutant deficient in both zeaxanthin (deficient in violaxanthin de-epoxidase catalyzing conversion of violaxanthin to zeaxanthin) and lutein (this mutant was used to demonstrate elevated cell wall ingrowths in phloem cells in plants transferred from low growth light to moderate light; Demmig-Adams et al. 2013); *npq4*, *Arabidopsis* mutant deficient in the photosystem II protein PsbS; *ROS* reactive oxygen species, *vte*, *Arabidopsis* mutant deficient in tocopherol synthesis and lacking alpha-, beta-, gamma-, and delta-tocopherol as a result of deficiency in either the tocopherol cyclase (*vte1*) or homogentisate phytyltransferase (*vte2*).

We note that a multitude of oxylipins is formed by either non-enzymatic or enzymatic lipid peroxidation, with both processes being modulated by ROS and anti-oxidants. Enzymatic lipid peroxidation occurs via lipoxygenases (LOX) with a catalytic iron center; ROS oxidize LOX from the inactive form LOX-Fe^{2+} to the LOX-Fe^{3+} form active in lipid peroxidation (Maccarrone et al. 1996), while anti-oxidants, such as tocopherols reduce LOX from the LOX-Fe^{3+} active form to the inactive form LOX-Fe^{2+} (Maccarrone et al. 1999). Products of both enzymatic and non-enzymatic lipid peroxidation serve as gene regulators in many organisms (see, e.g., Demmig-Adams and Adams 2010; Demmig-Adams et al. 2013).

Figure 28.3 summarizes the available evidence on altered redox state, altered oxidative messenger production, and altered biotic defenses in plants deficient in tocopherols (as key anti-oxidants: see, e.g., Havaux and García-Plazaola, Chap. 26), the PsbS protein (as a PS II protein and member of the light-stress-associated sub-family of light-harvesting proteins: see, e.g., Brooks et al., Chap. 13; Morosinottto and Bassi, Chap. 14), or the xanthophyll zeaxanthin (as a close correlate of thermal dissipation in

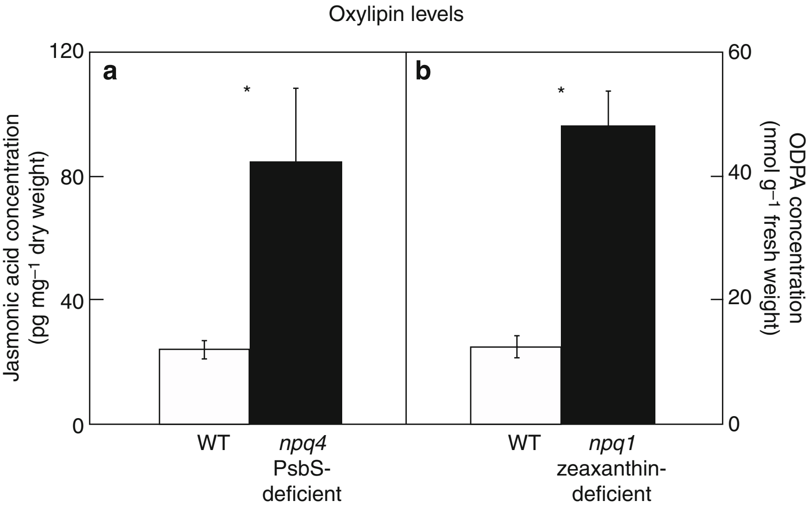

Fig. 28.4 Augmented foliar oxylipin concentrations in *Arabidopsis* mutants deficient in either of two components of the photoprotective system. (**a**) Difference in jasmonic acid concentration between wildtype *Arabidopsis* (*WT*) and the PsbS-deficient *npq4* mutant under outdoors/field conditions in the presence of herbivores (Modified after Demmig-Adams et al. 2013; based on original data from Frenkel et al. 2009). (**b**) Difference in the concentration of the jasmonic acid precursor OPDA (12-oxo-phytodienoic acid) between WT *Arabidopsis* and the zeaxanthin-deficient *npq1* mutant upon transfer from low growth light to high light (modified after Demmig-Adams et al. 2013). *, significantly different at $p<0.05$.

plants grown in nature; see, e.g., Demmig-Adams et al., Chap. 24). Here we summarize the evidence showing *increased ROS* levels, *increased oxylipin* levels, and *increased defenses* against certain pests or pathogens, respectively, in plants deficient in tocopherols (Fig. 28.3; Vitamin E-deficient, *vte*), PsbS (Figs. 28.3, 28.4a, and 28.5a; PsbS-deficient, *npq4*), or zeaxanthin (Figs. 28.3, 28.4b, and 28.5b; Zeaxanthin-deficient, *npq1* or *npq1 lut2*). Increased callose deposition in sugar-loading phloem complexes in tocopherol-deficient plants and in plants deficient in zeaxanthin and lutein has been observed (Fig. 28.5b). Furthermore, PsbS-deficient plants growing under highly variable light conditions in the field exhibit *decreased* sensitivity to a specific herbivore (Fig. 28.5a; see also Brooks et al., Chap. 13). To date, no information is available on the susceptibility of tocopherol-deficient plants to herbivore attack; future research is needed to address the possibility that the latter plants may have augmented herbivore defense, as seen in PsbS-deficient plants. However, it is clear that the defenses against multiple pests and pathogens involve multiple defense pathways that are regulated by different signaling pathways, and can have *opposite* effects (Derksen et al. 2013). For example, resistance of a range of pepper varieties against the major pepper insect pest flower thrips was *positively* correlated with foliar tocopherol content, with insect development apparently being inhibited by foliar tocopherol (Maharijaya et al. 2012). Future research is thus urgently needed to address the role of components of the photoprotection network in plant defense to *multiple* different biotic challenges (and, e.g., pests versus pathogens) as well as to *combinations* of biotic and abiotic stresses under actual field conditions (see also, e.g., Horton, Chap. 3; Brooks et al., Chap. 13). The possibility that trade-offs may exist between defenses against herbivores versus pathogens, or against any two specific biotic agents, also needs to be considered.

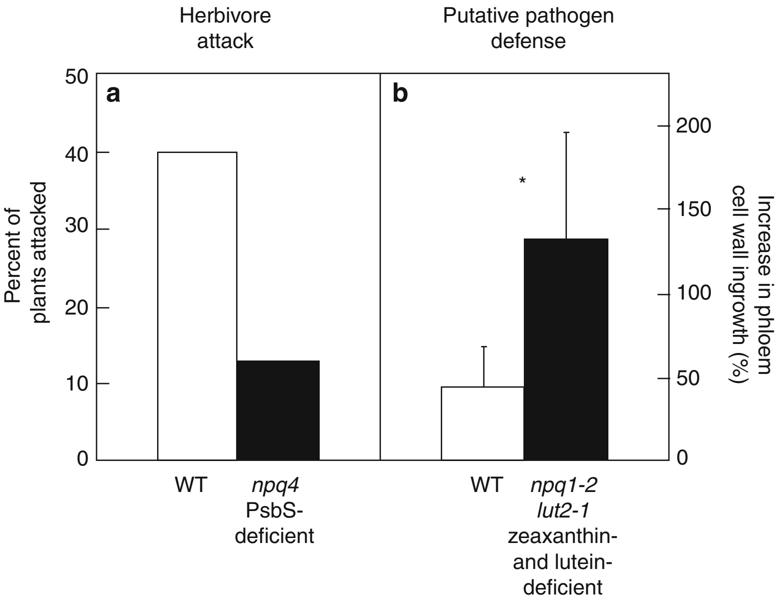

Fig. 28.5 (**a**) Augmented defense against herbivores in the PsbS-deficient *npq4* mutant and (**b**) augmented cell-wall deposition in phloem-loading complexes (as a putative defense against pathogens) in the zeaxanthin- and lutein-deficient double mutant *npq1-2 lut2*. The percent of plants attacked by herbivores was compared in wild type *Arabidopsis* (*WT*) and the PsbS-deficient *npq4* mutant under outdoors/field conditions (Modified after Demmig-Adams et al. 2013; based on original data from Frenkel et al. 2009); WT and the zeaxanthin- and lutein-deficient double mutant *npq1-2 lut2-1* were compared upon transfer from low growth light to high light (modified after Demmig-Adams et al. 2013). *, significantly different at $p < 0.05$.

IV Feedback Loops Between Photoprotection and Whole-Plant Function Under Moderately Versus Highly Excessive Light

Redox signaling networks include multiple feedback loops between primary photosynthetic reactions on the one hand, and plant growth, development and defenses on the other hand (Figs. 28.6 and 28.7). The overall outcome of the action of these multiple feedback loops apparently varies depending on how much excessive light is absorbed by a given leaf. Two contrasting scenarios are envisioned in Fig. 28.6 (*moderate* ROS formation) and Fig. 28.7 (*massive* ROS formation). Figure 28.6 depicts the putative situation in a leaf consuming/dissipating *most* of the absorbed light via the combination of photochemistry and thermal dissipation (NPQ), but also forming ROS levels sufficient to trigger production of redox signals that, in turn, fully induce synthesis of factors (i) involved in thermal dissipation (NPQ) and anti-oxidation as well as (ii) biotic defenses. It has, for example, been reported that jasmonic acid triggers de-novo synthesis of the anti-oxidant tocopherols (Sandorf and Holländer-Czytko 2002) and ascorbic acid (Wolucka et al. 2005) as well as of carotenoids (Pérez et al. 1993).

Figure 28.7 depicts a putative situation for a leaf experiencing *massive* ROS formation. Continuing massive ROS formation and, e.g., leaf death (for an overview of senescence and death, see Biswal et al. 2013) can theoretically be prevented by a *shutdown* of the sources of ROS production. Two major forms of ROS produced in the primary light reactions are electronically excited singlet oxygen, formed in light-harvesting complexes (LHCs), and singly reduced superoxide, formed with electrons ultimately stemming from charge separation

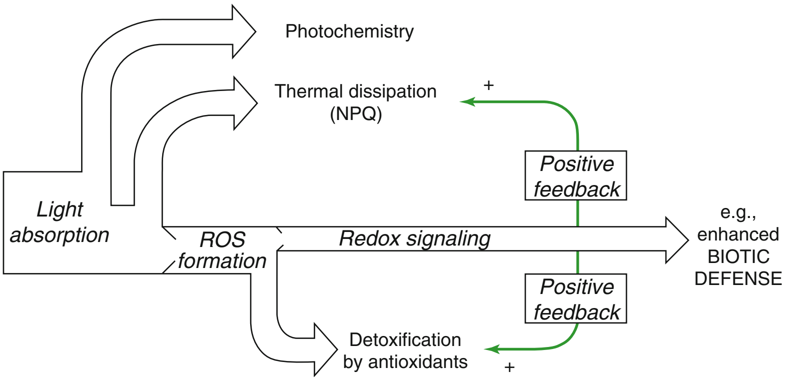

Fig. 28.6 Schematic depiction of the link between light absorption, formation of reactive oxygen species (*ROS*), redox signaling, and plant responses like biotic defense under moderate ROS formation as well as (positive) feedbacks (*green arrows*) between redox signals and (i) anti-oxidation or (ii) thermal energy dissipation in the chloroplast (leading to non-photochemical quenching of chlorophyll fluorescence, NPQ).

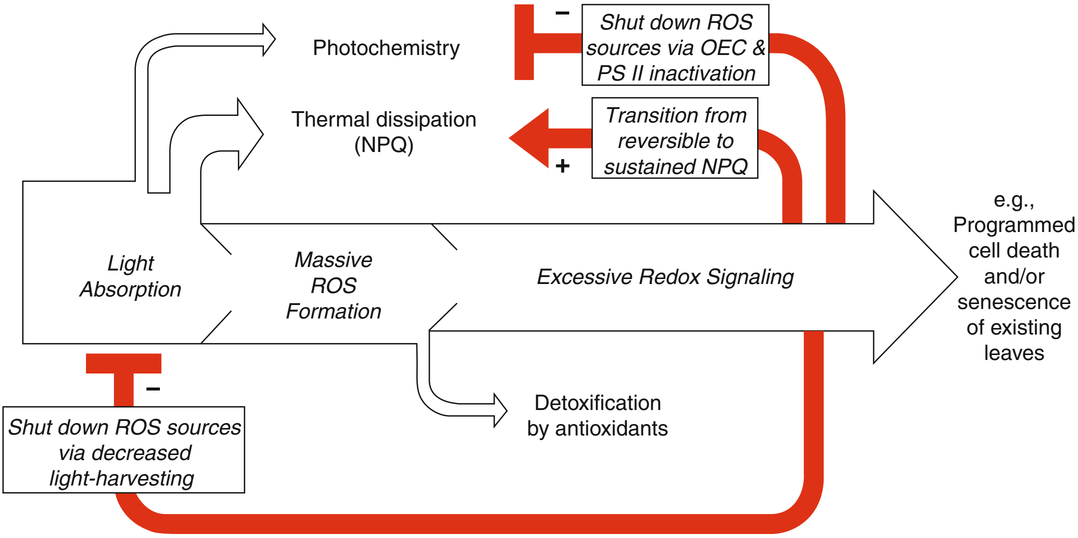

Fig. 28.7 Schematic depiction of the links among light absorption, formation of reactive oxygen species (*ROS*), redox signaling, and plant responses under massive ROS formation as well as feedbacks (*red arrow* and *red* feedback loops) between redox signals and shut-down of ROS sources via (i) inactivation of the oxygen-evolving complex and photosystem II (*PS II)* photochemistry and/or (ii) decreased light harvesting as well as (iii) transition from reversible to sustained non-photochemical quenching of chlorophyll fluorescence (*NPQ*).

in the oxygen-evolving complex (OEC) and photosystem II reaction center (PS II-RC). Consequently, degradation of LHCs and inactivation and/or degradation of OEC and PS II-RC would theoretically serve to *shut-down* ROS formation (Fig. 28.7). The inactivation and/or net degradation of PS II-RC under highly excessive light indeed involves an inhibition of de-novo synthesis of the D1 protein of the PS II-RC by ROS (e.g., Murata et al. 2007). Future research is needed to address the possibility that inactivation/degradation of PS II-RC and/or OEC does serve to shut down massive ROS formation as proposed in Fig. 28.7 – while simultaneously shutting down photosynthesis (see also, e.g., Murchie and Harbinson, Chap. 25; Morales et al., Chap. 27). Degradation of PS II-RCs and OEC, and photosynthetic shutdown ("photoinhibition"), in overwintering evergreens is associated with maintenance of high Chl (and LHC) levels, and a transition from rapidly reversible NPQ to a sustained form of NPQ locked-in for an entire season during which plant growth is arrested (Adams et al. 1995, 2002, 2004, 2006; Zarter et al. 2006a,b,c).

Adams et al. 2013 (see also Adams et al., Chap. 23) have reviewed the evidence indicating that photoinhibition (as a long-term inactivation of primary photochemistry and photosynthesis) in evergreens, as well as in deciduous perennials, is associated with foliar carbohydrate accumulation, and presumably with either sink limitation or limiting sugar export from leaves. Conversely, species other than evergreens, exposed to mineral stress, typically exhibit pronounced degradation of LHCs and elimination of excess light-harvesting capacity, which is expected to lower ROS formation without requiring sustained NPQ or OEC/PS II-RC shutdown/degradation (see Morales et al., Chap. 27).

Placement of PS II/OEC inactivation into a whole-organism context also begs for re-assessment of the assignment of such inactivation as "damage", similar to the re-evaluation efforts currently occurring in the medical field,

> Reactive oxygen species (ROS) and chronic changes in membrane lipids are not the result of accidental damage. Instead, these changes are the result of a highly evolved, stereotyped, and protein-catalyzed 'oxidative shielding' response that all eukaryotes adopt when placed in a chemically or microbially hostile environment… Research efforts need to be redirected. Oxidative shielding is protective and is a misguided target for therapy…An alternative title for this review might be, 'Oxidative stress or oxidative shielding: can 50 years of research be wrong?' (Naviaux 2012).

It is important to elucidate whether ROS-triggered responses represent actual damage or a form of prevention, favored by natural selection, of actual damage. Furthermore, accelerated death of pre-existing leaves may actually hasten redeployment of resources towards growth of new leaves better adjusted to altered environmental conditions. Lastly, what is favorable for plant fitness in natural settings may not necessarily be favorable in agricultural settings, and differences among crop species or varieties in the degree of photosynthetic shutdown in response to environmental stress might be exploited in such settings.

V Conclusions

As illustrated by many examples in plant ecology, *trade-offs* between the benefits and costs of plant adaptations make many specific strategies beneficial only in certain environments but not in others. This principle needs to be further applied to the case of plant photoprotection via thermal dissipation of excess excitation energy and/or anti-oxidation. While plant resistance against losses in productivity due to abiotic stresses may possibly be improved by augmented thermal dissipation/anti-oxidation in certain cases, plant resistance to pests and pathogens can apparently be either *lowered* or *increased* by augmented thermal dissipation/anti-oxidation. Future work is urgently needed to address these questions in specific crop species and specific agriculturally relevant environments, including various combinations of abiotic and biotic stresses as well as specific pests and herbivores.

While the benefit of a shutdown of (sugar-exporting) foliar phloem conduits triggered by reactive oxygen species may lie in a greater resistance to, e.g., specific pathogens, the cost to the plant may be a diminished ability to export sugars from photosynthesizing leaves to the plant's sinks for plant growth (and/or sugar and starch accumulation in fruits, roots, or tubers). We need to address the question of whether upregulation of foliar anti-oxidant levels and of the capacity for thermal dissipation, as well as putative corresponding suppression of oxidative signals, has the potential benefit of preventing any obstruction of phloem conduits, while possibly simultaneously coming with the cost of rendering plants more vulnerable to pathogen attack.

We suggest that research be done to further elucidate specific feedback loops between redox signals and gene regulation of the biosynthesis of factors involved in thermal dissipation and/or anti-oxidation. The possibility also needs to be addressed that inactivation/degradation of PS II reaction centers and the oxygen-evolving complex serves to shut down massive ROS formation under highly excessive light conditions. It should, furthermore, be considered that naturally occurring differences among plant species and varieties/ecotypes in maximal NPQ capacity, antioxidant levels, and lipid peroxidation levels have evolved as a result of trade-offs between the needs for specific abiotic and biotic defenses in specific environments.

Acknowledgments

We wish to thank Drs. Barry Logan and Stefan Jansson for their helpful comments on our manuscript. The work in our laboratory is currently supported by the University of Colorado at Boulder and the National Science Foundation (award number DEB-1022236).

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B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1, © Springer Science+Business Media Dordrecht 2014