

Characterization of Thylakoid Immunophilins in *Arabidopsis*

By

Veder Joel García

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Plant Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Sheng Luan, Chair

Professor Lewis Feldman

Professor Randy Schekman

Spring 2013

Characterization of Thylakoid Immunophilins in *Arabidopsis*

Copyright 2013

By

Veder Joel García

Abstract

Characterization of Thylakoid Immunophilins in *Arabidopsis*

by

Veder Joel García

Doctor of Philosophy in Plant Biology

University of California, Berkeley

Professor Sheng Luan, Chair

The FK506-binding proteins (FKBPs) and cyclophilins (CYPs), collectively called immunophilins, were originally discovered as cellular receptors for immunosuppressive drugs FK506 and cyclosporine A. A common feature of immunophilins is their peptidyl-prolyl cis/trans isomerase (PPIase) activity that often functions in protein folding. These proteins exist in a wide range of organisms from bacteria to animals and plants. The abundance and diversity of immunophilins identified to date underlie the functional versatility of this protein family. In plants, immunophilins are present in various cellular compartments and, in particular, they constitute a significant portion of the chloroplast proteome. Recent studies suggest that chloroplast immunophilins play important roles in assembly and maintenance of photosynthetic complexes. However, the majority of chloroplast immunophilins await detailed functional characterization.

As a first step to investigate plant chloroplast immunophilins, an evolutionary analysis was performed on sequences derived from eight representative species across a large evolutionary time-scale; from algae to higher plants. Multiple blast searches in these genomes resulted in identification of fifty CYPs and seventy-three FKBPs. Sequence analysis and phylogenetic tree reconstruction indicated that chloroplast immunophilin proteins are largely conserved from green algae to higher plants.

Reverse genetics was employed in *Arabidopsis*, coupled with molecular and biochemical analyses, to examine the function of chloroplast immunophilins. Among them, CYP37 plays a critical role in response to high light stress. A *cyp37* mutant displayed defect in anthocyanin accumulation under high light. Consistently, *cyp37* accumulated higher levels of reactive oxygen species as compared to wild-type plants. Moreover, leaf chlorophyll fluorescence analysis indicated a higher electron transport rate in the mutant. However, thylakoid membrane protein composition was not significantly altered. Further yeast two-hybrid assays identified *cyt f* and PsbM as CYP37 interactors, suggesting that CYP37 may be involved in regulation of *cytb6f* complex or photosystem II (PSII). Taken together, these results suggest CYP37 plays a role in defense mechanisms under high light stress conditions.

Functional redundancy appears to be a major feature among chloroplast immunophilins. Data collected herein suggest that AtCYP28 might be functionally redundant with other immunophilins as plants lacking CYP28 do not exhibit any phenotypic change under a number of stress conditions. To further address the potential functional redundancy of immunophilins, we generated double mutants between four FKBP single mutants (AtFKBP16-3, 16-4, 17-1, and 20-2). Among the double mutants produced, only the *fkbp20-2/fkbp16-3* showed a significant phenotypic change under normal growth conditions. Plants lacking these two FKBP s were stunted in growth and their photosynthetic efficiency was compromised. FKBP20-2 interacted with CP43 and FKBP16-3 interacted with CP47 in a yeast two-hybrid assay. As both CYP43 and CP47 are subunits of the PSII complex, our data suggest that FKBP20-2 and FKBP16-3 may work together in the assembly/maintenance of PSII.

In conclusion, the work presented in this thesis supports the hypothesis that chloroplast immunophilins have distinct and overlapping functions. They serve as critical regulators in photosynthesis-related processes including the assembly of photosynthetic complexes and adaptation to various light intensities in the natural environment.

Acknowledgements and Dedication

As I am ready to submit my thesis, I reflect on the journey that took me to Berkeley. It all began from the moment that Dr. Todd Cooke gave me the opportunity to set foot on a lab for the first time in my life, when I was an undergraduate at the University of Maryland-College Park. It was him who encouraged me to continue my education in plant biology research and guided me throughout the process of looking for graduate schools. I am grateful to him for his support and guidance and I am convinced I would not be in Berkeley writing these acknowledgements had I not met him.

I would like to thank my thesis advisor Dr. Sheng Luan for his continuous support and guidance and for welcoming in his lab. I am most impressed with his enthusiasm for science and easygoing personality, which not only encouraged me during my Ph.D. studies but also will benefit my future career. Special thanks go to the faculty members whom have served on my thesis committee and/or qualifying exam committee: Dr. Sarah Hake, Dr. Jennifer Fletcher, Dr. Randy Shekman, Dr Mark Schlissel, and Dr. Lewis Feldman. In particular, I would like to thank Dr. Feldman for his continuous support and for always providing a dose of positivism every time I visited his office both during my qualifying exam preparation as well as during my thesis.

My life was enriched by everyday interactions with people in the laboratory and the PMB department. I would like to thank all the Luan Lab members, past and present. Special thanks go to Dr. Hong Li who introduced immunophilin proteins to me during my lab rotation; Dr. Sung-Chul Lee for his support and mentorship during the first two years of my graduate studies. Also, Dr. Renjie Tang for his continuous encouragement to complete my thesis and for sharing his outstanding scientific knowledge during the almost two years we have overlapped. His advice and criticism has influenced my future scientific career; Dr. Taegun Kwon for his continued support and encouragement that he has given me even after he left the lab. Dr. Aigen Fu for being the master mind in our lab for chloroplast research and providing me with most of the protocols that I used throughout my thesis work. In addition all the following lab members have taught me valuable lessons and made my life in Berkeley both a learning and enjoyable experience: Dr. Kai He, Dr. Xin Hou, Dr. Yufen Che, Thomas Kleist, Xiaojiang Zheng, Dr. Hye-Sun Cho, Qing Ma, Yangping Li, Weimin Jiang. I will surely miss our trips to Oakland buffet.

I also want to thank my family for their love, support, and continuous encouragement. I am especially indebted to my mother, Maria García, to whom I dedicate this thesis. She is the most hard-working person I have ever met and without her sacrifice I would not be who I am now. She has always given me an extra push to overcome my obstacles.

Thank you all!

TABLE OF CONTENTS

List of Figures	iv
List of Tables	v
List of Abbreviations	vi
CHAPTER 1	
INTRODUCTION AND BACKGROUND: LITERATURE REVIEW	1
1.1 Discovery and Classification of Immunophilins	2
1.2 Immunosuppression Mechanism in Brief	2
1.3 Peptidyl Prolyl Isomerase Activity and Protein Folding.....	3
1.4 Immunophilins: Widespread Proteins	4
1.4.1 Bacteria Immunophilins	5
1.4.2 Fungal Immunophilins	6
1.4.3 Metazoa Immunophilins	6
1.4.3.1 Human immunophilins.....	7
1.5 Discovery of Plant Immunophilins	7
1.5.1 Cyclophilins	8
1.5.2 FKBP.....	10
1.6 Biological Functions of Plant Immunophilins	10
1.6.1 Epigenetic Regulation of Transcription and Gene Silencing.....	10
1.6.2 Plant Stress Responses	12
1.6.3 Plant Hormone Signaling	12
1.7 The Immunophilins in the Chloroplast	14
1.8 Aims of Thesis	15
CHAPTER 2	
EVOLUTIONIONARY RELATIONSHIPS OF CHLOROPLAST IMMUNOPHILINS FROM ALGAE TO HIGHER PLANTS	17
2.1 Functions of Chloroplast Immunophilins	18
2.1.1 AtCYP38 and AtFKBP20-2 Function in PSII Assembly	19
2.1.2 AtFKBP13 is Associated with <i>Cytb₆f</i> Complex.....	19
2.1.3 AtCYP20-2 and AtFKBP16-2 are Linked to Photosystem I and NAD(P)H Dehydrogenase.....	20
2.1.4 Aims in this Chapter	21
2.2 Results and Discussion	21
2.2.1 Identification and Nomenclature of Chloroplast Immunophilins	21
2.2.2 Chloroplast Cyclophilin Diversity in Lower and Higher Plants.	24
2.2.3 Chloroplast FKBP Diversity in Lower and Higher plants.	26
2.2.4 Concluding Remarks.....	32
2.3 Methods.....	36
2.3.1 Retrieval of FKBP and CYP Sequences	36
2.3.2 Sequence Analysis-Phylogenetic Tree Construction	37
2.3.3 Nomenclature of Immunophilin Proteins.....	37
CHAPTER 3	
FUNCTIONAL CHARACTERIZATION OF IMMUNOPHILINS IN THE THYLAKOID LUMEN BY REVERSE GENETICS: NOVEL FUNCTIONS AND REDUNDANCY	38
3.1 Introduction.....	39
3.2 PART I: The Arabidopsis Thylakoid Lumen Cyclophilin 37 is Involved in High Light Stress Response.	39
3.2.1 Results and Discussion	39
3.2.1.1 AtCYP37 localizes to the thylakoid lumen and its protein level increases under high light ..	39
3.2.1.2 The <i>cyp37</i> mutant displays defects in anthocyanin accumulation under high light.....	40

3.2.1.3 High light stress affects electron transport rate in <i>cyp37</i> , but photosynthetic complex damage is similar to WT plants.....	41
3.2.1.4 CYP37 interacts with photosynthetic subunits Cyt <i>f</i> and PSII subunit PsbM.....	43
3.2.2 AtCYP37 Concluding Remarks	43
3.3 PART II: A number of Stress Treatments do not Affect Growth or Development of CYP28 Knock-out Arabidopsis Plants.	48
3.3.1 Results and Discussion	48
3.3.1.1 Isolation of <i>cyp28</i> allele and CYP28 subcellular localization.....	48
3.3.1.2 Lack of CYP28 does not affect plant growth or development under a number of stress conditions.....	49
3.3.1.3 CYP28 interacts with a number of photosynthetic subunits	50
3.3.2 AtCYP28 concluding Remarks	50
3.4 PARTIII: Lack of Phenotypes in AtFKBPs Single Mutants, Obstacles and Potential Solutions to Decipher Thylakoid Immunophilin Functions.....	52
3.4.1 Obstacles in Thylakoid Immunophilin Characterization.	53
3.4.2 Analysis of Double Immunophilin Mutants.....	54
3.5 PART IV-Materials and Methods	57
3.5.1 Plant Materials and Growth Conditions.....	57
3.5.2 Chloroplast Isolation and Preparation of thylakoids	58
3.5.3 Chloroplast Fractionation.....	58
3.5.4 Blue Native PAGE and 2D-SDS PAGE	58
3.5.5 Chlorophyll Fluorescence Measurement	58
3.5.6 Anthocyanin Measurements.....	59
3.5.7 Yeast two hybrid	59
3.5.8 Detection of Reactive Oxygen Species.....	59
CHAPTER 4	
GENERAL SUMMARY	60
4.1 General Summary	61
REFERENCES	63

List of Figures

Figure 1.1 Diagram of the mechanism of immunosuppression by cyclosporine A	3
Figure 1.2 Schematic diagram of a peptidyl prolyl-cis-trans bond isomerization by PPIase.....	4
Figure 1.3 Schematic diagram of photosynthesis and organization of chloroplast within the plant cell. .	14
Figure 2.1 Schematic presentation of the photosynthetic electron transport chain.	18
Figure 2.2 Multiple sequence alignment of cyclophilins in <i>Synechocystis</i> sp. PCC6803, <i>C. reinhardtii</i> , <i>P. patens</i> , <i>S. moellendorffii</i> , and <i>A. thaliana</i>	27
Figure 2.3 Phylogenetic tree with evolutionary relationships of cyclophilins in cyanobacteria, green algae, moss, lycophyte and Arabidopsis	28
Figure 2.4 Multiple sequence alignment of cyclophilins in <i>O. Sativa</i> , <i>P. trichocarpa</i> , <i>Z. mays</i> and <i>A. thaliana</i>	29
Figure 2.5 Unrooted phylogenetic tree of cyclophilins relationship in higher plants: rice, maize, poplar and Arabidopsis.	30
Figure 2.6 Multiple sequence alignment of FKBP s in <i>C. reinhardtii</i> , <i>P. patens</i> , <i>S. moellendorffii</i> , and <i>A. thaliana</i>	33
Figure 2.7 Phylogenetic tree with evolutionary relationships of FKBP s in green algae, moss, lycophyte and Arabidopsis.	34
Figure 2.8 Multiple sequence alignment of FKBP s in <i>O. Sativa</i> , <i>P. trichocarpa</i> , <i>Z. mays</i> and <i>A. thaliana</i>	35
Figure 2.9 Unrooted phylogenetic tree of FKBP s relationship in higher plants: rice, maize, poplar and Arabidopsis.	36
Figure 3.1 Characterization of the <i>cyp37</i> mutant and CYP37 thylakoid localization.	45
Figure 3.2 Light-induced anthocyanin accumulation is reduced in <i>cyp37</i> mutants.	46
Figure 3.3 Light response curves for chlorophyll fluorescence and analysis of thylakoid membrane protein complexes in <i>cyp37</i>	47
Figure 3.4 CYP37 physically interacts with PS II subunit PsbM and binds to an intermediate photosynthetic complex.	48
Figure 3.5 Characterization of the <i>cyp28</i> mutant.	51
Figure 3.6 CYP28 localizes to thylakoid lumen.	51
Figure 3.7 <i>cyp28</i> phenotype characterization and analysis of thylakoid member protein complexes.	52
Figure 3.8 CYP28 physically interacts with several thylakoid membrane proteins.	52
Figure 3.9 <i>fkbp20-2/fkbp16-3</i> exhibits stunted growth and overall photosynthetic efficiency is significantly altered.....	56
Figure 3.10 FKBP20-2 and FKBP16-3 associate with CYP43 and CP47 respectively	57

List of Tables

Table 1.1 List of all Arabidopsis single and multi-domain immunophilins and subcellular localization	9
Table 2.1 Chloroplast cyclophilin proteins present in the cyanobacterium (<i>synechocystis</i> sp. PCC6803), green alga (<i>C reinhardtii</i>), moss (<i>P patens</i>), lycophyte(<i>S moellendorffii</i>), rice (<i>O sativa</i>), poplar(<i>P trichocarpa</i>), maize (<i>Z Mays</i>) and Arabidopsis (<i>A. thaliana</i>).....	22
Table 2.2 Chloroplast FKBP proteins present in the green alga, moss, lycophyte, rice, poplar, maize and Arabidopsis	23
Table 3.1 List of FKBP single and double mutants screened for phenotypes.....	55
Table 3.2 List of Arabidopsis FKBP proteins and their respective number of cysteine residues.....	56

List of Abbreviations

CsA	Cyclosporin A
CYP	Cyclophilin
FKBP	FK506-binding protein
PPIase	Peptidyl-prolyl cis-trans isomerase
PTGS	Post-translational gene silencing
TPR	Tetratricopeptide repeat
PSII	Photosystem II
PSI	Photosystem I
Cyt <i>b₆f</i>	Cytochrome <i>b₆f</i>
NDH	NAD(P)H dehydrogenase
PC	Plastocyanin
RFes	Rieske iron-sulfur protein
ROS	Reactive oxygen species
NPQ	Non-photochemical quenching
ΦPSII	Quantum efficiency of PSII photochemistry
BN/PAGE	Blue Native gel electrophoresis
2D-SDS	Two dimensional sodium dodecyl sulfate gel
hr	hour/s
HL	High light
LL	Low light
NBT	Nitro blue tetrazolium
MS	Murashige and Skoog medium
Y2H	Yeast two-hybrid
WT	Wild type

Amino acids

L	Leucine
W	Tryptophan
A	Adenine
H	Histidine

CHAPTER 1

INTRODUCTION AND BACKGROUND: LITERATURE REVIEW

1.1 Discovery and Classification of Immunophilins

Few proteins have had more exciting beginnings than that offered by the ubiquitous immunophilin family. The story dates back more than three decades ago, when scientist discovered a fungal cyclic peptide (cyclosporine A) which subsequently was demonstrated to serve as a potent immunosuppressant clinically used for organ transplantations (Borel et al., 1977). Several years following the discovery of cyclosporine A, another immunosuppressive agent, FK506, was discovered as a macrolide from soil bacteria (Kino et al., 1987; Tanaka et al., 1987). Soon, a search for the cellular targets of these immunosuppressants began. In 1984, Handschumacher et al. purified from bovine thymocytes an abundant cytosolic cyclosporine A-binding protein, which they termed “cyclophilin.” Meanwhile, an enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) was discovered and linked to protein folding (Fischer et al., 1984). Remarkably, the story heightens when two independent research groups showed that the PPIase enzyme was identical to the cyclophilin protein and published evidence that PPIase and cyclophilin were one and the same protein (Fischer et al., 1989; Takashi et al., 1989). In parallel, Harding et al. (1989) identified a FK506-binding protein which did not cross react with antisera against cyclophilin, thus they termed it “FKBP” for FK506-binding protein. Also, they showed that this FKBP was a *cis-trans* peptidyl-prolyl isomerase (PPIase) as well. Later, other groups reported that the FKBP can also bind another immunosuppressant, rampamycin, which is structurally similar to FK506 (Dumont and Su., 1995). Hence, although cyclophilins (CYPs) and FKBP proteins share little sequence homology, they were collectively named ‘immunophilins’ based on their property of binding to these immunosuppressants and their common PPIase activity. Up to now, immunophilins have been shown to be ubiquitous proteins present in a wide variety of organisms including bacteria, fungi, animals, and plants where they are involved in a diversity of functions to be discussed in this chapter.

1.2 Immunosuppression Mechanism in Brief

Both cyclosporine A (CsA) and FK506 have a similar immunosuppression mechanism in which the first step is to bind to the PPIase pocket of CYP or FKBP, respectively, forming a complex; CsA-CYP, or FK506-FKBP (Schreiber and Crabtree, 1992; Ho et al., 1996). However, inhibition of the PPIase activity by either drug does not play a role in immunosuppression as it was shown that other drug analogues can inhibit PPIase activity but does not function in immunosuppression (Bierer et al., 1990; Sigal et al., 1991). Further studies revealed that the drug-immunophilin complex (CsA-CYP or FK506-FKBP) interacts with and inhibits the activity of calcineurin, a calcium-activated calmodulin-dependent serine/threonine phosphatase that is responsible for dephosphorylation of a nuclear factor of activated T-cells (NF-AT), which in turn is required for downstream activation of important genes in the T-cell, such as interleukin 2 (IL-2), essential for an immune response (See Figure 1.1; Schreiber and Crabtree, 1992;

Ho et al., 1996). Thus, the signaling cascade required for immune response is interrupted by the immunophilin-drug complex. However, these drug dependent functions of immunophilins have only clinical but no physiological relevance in mammalian cells.

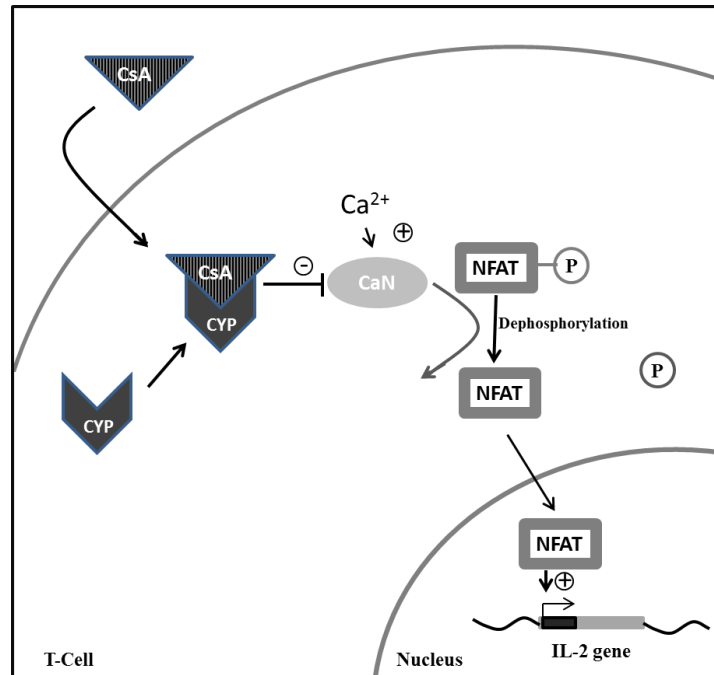


Figure 1.1 Diagram showing mechanism of immunosuppression by cyclosporine A. (See paragraph 1 in section 1.2 for details), CsA, cyclosporine A; CYP, cyclophilin; NFAT, nuclear factor of activated T cells; P, phosphate; IL-2, interleukin 2. (Adapted from Stepkowski, 2000).

1.3 Peptidyl Prolyl Isomerase Activity and Protein Folding

The dynamic nature of protein folding dictates most aspects of cellular life. Newly synthesized proteins must fold into a precise three-dimensional structure to carry out their biological functions, and in many instances chaperone proteins are needed to achieve the proper three-dimensional structure. Peptidyl-prolyl *cis-trans* isomerases (PPIase E.C. 5.1.2.8) are enzymes with the capability to accelerate folding of proteins by catalyzing the interconversion of *cis* to *trans* isomers of peptide bonds with the amino acid proline (Fig. 1.2; Brands et al., 1975; Fischer et al., 1984; Fischer and Aumüller, 2003). Generally, the planar peptide bonds in proteins are mainly in the *trans* conformation, however, proline residues are distinct because they have a relatively high intrinsic probability of being in the *cis* conformation which is unfavorable and consequently a rate-limiting step for the folding of newly synthesized proteins or unfolding of proteins (Reimer and Fischer, 2002). Thus, the isomerization of peptidyl-proline bonds can serve

as a molecular switch by controlling the structure and function of pre-existing enzymes and proteins.

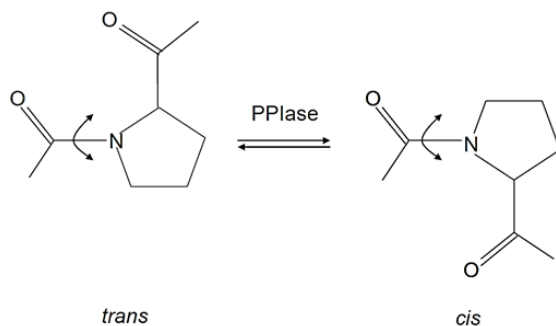


Figure 1.2 Schematic diagram of a peptidyl prolyl-*cis-trans* bond isomerization by PPIase.

Apart from CYPs and FKBP, parvulins are a third known family of PPIases, with no homologous sequence relationship to immunophilins, which is present in both eukaryotic and prokaryotic organisms (Fischer and Aumüller, 2003; not to be further discussed here). Additionally, despite the assignment of immunophilins as chaperone proteins due to their PPIase domains, studies suggest immunophilins can have chaperone activities separate from the PPIase activity (Barik, 2006).

1.4 Immunophilins: Widespread Proteins

Since the discovery of FKBP12 and cyclophilin 18a (also known as cyclophilin A), the first immunophilins in mammalian cells, as targets for immunosuppressant drugs, biochemical and sequence analyses have led to the identification of a large number of immunophilin homologs in various organisms (Galat, 2003; Lücke and Wiwad, 2011). It becomes evident that immunophilins constitute a ubiquitous and highly conserved family of proteins present in both prokaryotic and eukaryotic organisms. The residues which comprise the PPIase domain/FK506 binding site of human FKBP12, the PPIase/CsA binding site of human Cyp18a, define, respectively, an “FKBP domain” and a “cyclophilin domain” that are remarkably conserved within the family members and across species (Galat, 2003). Due to the large number of members within the FKBP and CYPs, a standardized nomenclature has been developed; the proteins are named by the initials of the organisms, for instance Hs, *Homo sapiens*, or At, *Arabidopsis thaliana*, followed by the domain name (either FKBP or CYP) and the predicted molecular weight in kilo Daltons. Interestingly, immunophilins often contain, in addition to the FKBP or

CYP domain, other accessory domains, such as tetratricopeptide repeats (TPR), WD40 domains (a 40 amino-acid conserved sequence containing a Trp-Asp dipeptide at its C terminus), RNA binding domains, Leu-rich repeats, among others, which underlie structural and functional diversity within the protein family (Galat, 2003; He et al., 2004; Barik, 2006; Lücke and Wiwad, 2011). To further illustrate immunophilin functions across species, within the next few pages, a brief up-to-date review in bacteria, yeast, animals, and a more detailed emphasis on plants will be discussed.

1.4.1 Bacteria Immunophilins

Several pathogenic bacteria possess immunophilin and immunophilin-like proteins, which have been found to play a role in virulence in a range of species, including the bacteria *Legionella pneumophila* (Cianciotto et al., 1989), *Chlamydia trachomatis* (Lundemose et al., 1993) and *Neisseria gonorrhoeae* (Leuzzi et al., 2005). For instance, the gene LpMip (*L. pneumophila* macrophage infectivity potentiator or LpFKBP25) encodes an FKBP protein required for the adhesion to collagen and its PPIase activity renders the extracellular matrix sensitive to serine protease which allows the invasion of the *L. pneumophila* through the epithelial barrier thus allowing efficient invasion of macrophages (Cianciotto et al., 1989; Köhler, 2003). Interestingly, a recent study has shown that an FKBP in *Burkholderia pseudomalle*, in contrast to the previously described virulence-associated FKBP of pathogenic bacteria, lacks characteristic PPIase activity *in vitro*, but is essential for intracellular replication and *in vivo* virulence of the pathogen (Norville et al., 2011). In *Escherichia coli*, four FKBP-like proteins, varying from 16kDa to 29kDa, and two cyclophilins, one in the periplasm and the other in the cytoplasm, with molecular masses of 18 kDa and 19 kDa, have been identified (Hacker et al., 1993; Blattner et al., 1997). One of these *E. coli* FKBP, a periplasmic protein designated as FkA, possesses chaperone activity and is essential for imported colicin M toxicity. Nevertheless, combinational mutations of the four FKBP are not required for cell viability at least under laboratory conditions (Saul et al., 2004; Justice et al., 2005; Hullmann et al., 2008). Although prokaryotic cyclophilins have been found to be active enzymes in *in vitro* studies, limited work has been carried out to identify their *in vivo* cellular functions (Liu et al., 1990; Schmidt et al., 1996). For instance, the exported *E. coli* cyclophilin, EcCYP20-2, shows high *in vitro* folding capabilities, but no cellular function can be attributed so far (Kleerebezem et al., 1995). Although a diverse range of prokaryotes genomes encode immunophilins, only a few immunophilins have been isolated and studied; they are mainly targeting the bacterial cell surface and are involved in macrophage infection. More recent studies are emerging with evidence for broader functions such as involvement in stress tolerance (Sodeberg and Cianciotto, 2008; Obit et al., 2011; Roset et al., 2013).

1.4.2 Fungal Immunophilins

The genome of the budding yeast *Saccharomyces cerevisiae* encodes 12 immunophilins including eight CYPs (CPRs) and four FKBP (FPRs) (Dolinski et al., 1997; Arevalo-Rodriguez et al., 2004). Although these immunophilins are localized to diverse cellular compartments such as ER, vacuole, nucleus, mitochondria, and cytosol; molecular and genetic studies show that either individually or in combination, these proteins are dispensable for viability of yeast cells (Dolinski et al., 1997). Nevertheless, further reports reveal that the cyclophilin Cpr1 (localized to nucleus) plays a critical role in modulating the activity of histone-deacetylase complexes and is involved in regulation of meiosis (Arevalo-Rodriguez et al., 2000; Pijnappel et al., 2001; Arevalo-Rodriguez et al., 2005). Likewise, an FKBP Fpr1 has been reported to bind to the non-histone chromatin binding protein Hmo1p and may regulate its assembly or function (Dolinski et al., 1999). Additionally, the mitochondrial cyclophilin, Cpr3, is involved in protein refolding after import into mitochondria (Matouschek et al., 1995). Cyclophilin Cpr7 has been established to work together with Hsp90 in transcriptionally regulating the heat shock response in *S. cerevisiae* (Duina et al., 1998). Hence, evidence is growing to support the notion that immunophilins play a diverse array of cellular functions in the yeast life cycle.

1.4.3 Metazoan Immunophilins

Many immunophilin proteins contain both the PPIase and protein-protein interaction motifs, and/or motifs unique to each member of the family that are associated with subcellular compartmentalization and functional specialization (Galat, 2003). Remarkably as well, a large gene expansion of immunophilins in the metazoan genomes as compared to protozoans correlates with increased complexity in the organisms within this kingdom. In the nematode *Caenorhabditis elegans*, eighteen separate CYPs and eight FBKPs are found while in the fruit fly *Drosophila melanogaster*, fourteen CYPs and eight FBKPs are present (Galat, 2003; Pemberton et al., 2005; Bell et al., 2006; Galat, 2012). The *C. elegans* immunophilins consist of members localized in the cytosol, secretory pathway organelles, and mitochondria (Bell et al., 2006). A single domain cyclophilin (CYP-3) with a divergent loop motif is exclusively expressed in excretory cells at the larval stages of *C. elegans*. This protein is suggested to be involved in cellular responses to stress caused by changes in the redox environment or by up-regulation of cellular activity. While the multi domain CYP-4, which contains a unique N-terminal and C-terminal extension, is involved in proper muscle protein folding and sexual differentiation of the hermaphrodite germ-line (Page and Winter, 1998; Belfiore et al., 2004; Bell et al., 2006). A well-studied CYP, *ninaA*, in *D. melanogaster*, is a membrane bound ER protein required for biogenesis of the light sensing rhodopsin (Rh1) protein. Studies have shown that PPIase activity of *NinaA* allows for proper Rh1 folding, and in addition, *NinaA* is involved as a chaperone, escorting Rh1 through the secretory pathway

from the endoplasmic reticulum to the photosensitive rhabdomeric membranes (Colley et al., 1991; Starnes et al., 1991, Baker et al., 1994). A cytoplasmic FKBP (*shutdown*), previously reported to be required for fertility in *Drosophila*, has recently been shown to be a component of the piRNA (pathway that preserves the integrity of the gametic genome) biogenesis machinery (Munn and Steward, 2000; Preall et al., 2012). Another example is the ER targeted FKBP14 that has multiple functions in *Drosophila* in which loss of FKBP14 gives rise to specific defects in eye, bristle, and wing development. *FKBP14* mutants genetically interact with components of the Notch signaling pathway, indicating that these phenotypes are associated, at least in part, with de-regulation of Notch signaling (van de Hoef et al., 2013).

1.4.3.1 Human immunophilins

Cyclophilin A and FKBP12 were the first immunophilin members discovered in mammalian cells as they were the purified targets bound to CsA and FK506, respectively (Haendler et al., 1987; Fretz et al., 1991). Since this breakthrough, additional members and their drug-independent cellular functions have been identified. The human genome encodes seventeen CYPs and thirteen FKBP. In general, high molecular weight immunophilins have a more complex architecture and are not related to the immunosuppression process, participating in a diversity of cellular functions (Lander et al., 2001). The smaller single domain FKBP12 and FKBP12.6 proteins are the most comprehensively studied; FKBP12 and FKBP12.6 (also known as FKBP1A and FKBP1B, respectively) are essential subunits of the ryanodine receptor (RyR) Ca²⁺ release channel in the sarcoplasmic reticulum. Aberrant RyR2–FKBP12.6 interactions have been proposed to be the underlying cause of channel dysfunction in acquired and inherited cardiac disease (Jayaraman et al., 1992; Timerman et al., 1993; Zissimopoulos et al., 2012). While another well studied pair of homologous immunophilins is the larger multi-domain FKBP51 and FKBP52. Both of these proteins possess double FKBP domains at their N-terminal half and additional TPR repeats at their C-terminal half, however, three-dimensional crystallographic structures reveal differences in the domain-domain orientations (Riggs et al., 2007; Schulke et al., 2010). FKBP51 and FKBP52 are implicated in steroid receptor-mediated signaling by binding to Hsp90 through the C-terminal TPR domains, although the two FKBP play opposite roles in this complex (Cioffi et al., 2011; Galigniana et al., 2012). The above is just a few examples of ever expanding array of diverse functions of immunophilins in mammalian systems.

1.5 Discovery of Plant Immunophilins

In the early 1990's, the discovery of plant immunophilins at the molecular level was pioneered by Gasser *et al.* (1990) by means of cDNA cloning of the first cyclophilin gene sequences from tomato (*Lycopersicon esculentum*), maize (*Zea Mays*) and oilseed

rape (*Brassica napus*). In addition to high degree of conservation of these plant proteins compared to their mammalian counterparts, it was shown that the tomato CYP, expressed and purified from in *E. coli*, had PPIase activity. Around the same time, Luan et al utilized CsA and FK506 affinity column to purify both CYPs and FKBP from fava bean and maize (Luan et al., 1994a) and subsequently cloned the genes encoding several immunophilins (Luan et al., 1994b; Luan et al., 1996). In the past decade, sequence analyses following a number of genome sequencing projects, have allowed for molecular identification of immunophilins in a number of higher plants (He et al., 2004; Vallon, 2005; Ahn et al., 2010; Yu et al., 2012, Wang et al., 2012). In recent years, a number of researchers have carried out valuable functional characterizations of individual immunophilins, but comprehensive identification and genome-wide analysis of all immunophilins in a specific higher plant species had only been carried out in *Arabidopsis thaliana* a dicot, and more recently in a monocot; rice, *Oryza sativa japonica nipponbare* (He et al., 2004; Ahn et al., 2010). The *Arabidopsis* genome encodes 23 FKBP isoforms and 29 CYP isoforms, whereas the *Oryza* genome encodes 29 FKBP isoforms and 27 CYP isoforms (He et al., 2004; Ahn et al., 2010). Classification and complete phylogenetic analyses have served as a strong base for subsequent functional characterization of plant immunophilins, especially in *Arabidopsis* due to its rich genetic resources. Interestingly, mining of the unicellular green alga *Chlamydomonas reinhardtii* genome, uncovered 23 genes encoding FKBP isoforms and 25 genes encoding CYP isoforms, which is comparable in number to vascular plants such as *Arabidopsis* and *Oryza*. In addition, it appears that the immunophilin expansion in the green lineage occurred following divergence of red and green algae (Vallon, 2005). Consequently, the genome sequencing of green organisms has shed light on the large number of protein members within the immunophilin superfamily, which are not only varied in form and function but also cellular locations.

1.5.1 Cyclophilins

The *Arabidopsis* cyclophilin family is composed of 29 members which can be sub-grouped into two major classes; single-domain and multi-domain members. Single domain cyclophilins are characterized by possessing only their conserved CYP domain (PPIase domain), whereas multiple-domain cyclophilins possess additional unique domains besides their characteristic CYP domain (Romano et al., 2004). The single domain class is further categorized by having nine members targeted to the cytoplasm, five to the secretory pathway, five to the chloroplast and two to mitochondria (Table 1.1; Romano et al., 2004). Although most of the subcellular locations of these proteins await experimental corroboration, studies have confirmed some of these predictions, especially for the chloroplast localized proteins (Lippuner et al., 1994; Peltier et al., 2002; Schubert et al., 2002; Edvardsson et al., 2003). The first confirmation was for AtCYP20-3 when it was shown by *in-vitro* import assays to be localized in the chloroplast stroma (Lippuner et al., 1994). AtCYP20-2 has been isolated from both luminal and thylakoid membranes

fractions (Peltier et al., 2002; Schubert et al., 2002; Edvardsson et al., 2003). Three other cyclophilins, including AtCYP37, AtCYP26-2, and AtCYP38, have been shown to be in the thylakoid lumen by proteomic studies (Peltier et al., 2002; Schubert et al., 2002).

Eight *Arabidopsis* genes encode multi-domain cyclophilins; one targeted to the chloroplast thylakoid lumen, three to the cytosol, and four to the nucleus (Table 1.1; Romano et al., 2004). The thylakoid luminal AtCYP38 is composed of an N-terminal leucine zipper domain, a central acidic region and the C-terminal CYP domain (PPIase), although recent studies have shown it is not an active PPIase (Vasudevan et al., 2012). The cytosolic AtCYP40 is featured with three C-terminal tetratricopeptide repeats (TPRs) and a conserved CYP domain. While the two other cytosolic (AtCYP57, AtCYP59), and two nuclear cyclophilins (AtCYP63, AtCYP95) possess motifs characteristic of RNA-interacting proteins, such as the RNA binding domain (RRM), the Glu-Lys (EK) domain, and Arg/Ser (RS)-rich domain, suggesting their potential involvement in regulation of RNA processing (Romano et al., 2004). Interestingly, another nuclear cyclophilin, AtCYP71 bears two N-terminal WD40 repeats which are motifs known to be present in all eukaryotes with basic functions such as signal transduction and transcription regulation. In *Arabidopsis* CYP71 has been shown to be involved in regulation of gene repression and organogenesis (Li et al., 2007). On the other hand, the nuclear AtCYP65 contains a U-box domain near its N-terminus. The U-box is a highly conserved domain classified as an ubiquitin ligase (E3s) and found in proteins from yeast to humans (Hatakeyama et al., 2001). Cyp-60, a nuclear localized human homolog of AtCYP65, has been shown to interact with the proteinase inhibitor eglin c and exhibits E3 activity (Wang et al., 1996).

Table 1.1 List of all *Arabidopsis* single and multi-domain immunophilins and subcellular localization.

Cytosol		Chloroplast		Nucleus		Secretory Pathway		Mitochondria
Single Domain								
AtFKBP12	AtCYP19-1	AtFKBP13	AtFKBP18	AtFKBP15-3		AtFKBP15-1	AtCYP21-1	AtCYP21-3
AtCYP18-1	AtCYP19-2	AtFKBP16-1	AtFKBP19	AtFKBP20-1		AtFKBP15-2	AtCYP21-2	AtCYP21-4
AtCYP18-2	AtCYP19-3	AtFKBP16-2	AtFKBP20-2			AtCYP19-4	AtCYP23-1	
AtCYP18-3	AtCYP22-1	AtFKBP16-3	AtCYP20-2			AtCYP20-1		
AtCYP18-4	AtCYP26-1	AtFKBP16-4	AtCYP20-3					
		AtFKBP17-1	AtCYP26-2					
		AtFKBP17-2	AtCYP28					
		AtFKBP17-3	AtCYP37					
Multiple Domains								
AtFKBP42	AtCYP40	AtCYP38		AtFKBP43	AtCYP59			
AtFKBP62	AtCYP57			AtFKBP53	AtCYP65			
	AtCYP63			AtFKBP65	AtCYP71			
				AtFKBP72	AtCYP95			

1.5.2 FKBP

Twenty-two FKBP are encoded in the Arabidopsis genome; consisting of sixteen single domain and six multi-domain members (He et al., 2004). Like the cyclophilin family, FKBP are also targeted to various cellular compartments; two to the secretory pathway, three to the cytosol, six to the nucleus, and eleven to the chloroplast (Table 1.1; He et al., 2004). All chloroplast-targeted FKBP contain typical thylakoid lumen transit peptides featured with double Arg residues followed by a hydrophobic region common to luminal proteins translocated via the Δ pH-dependent pathway. It has been corroborated that AtFKBP13 protein is synthesized and imported into the chloroplast in a precursor form and then translocated across the thylakoid membrane by the Δ pH-dependent pathway (Gupta et al., 2002). The ER-localized FKBP15-1 and 15-2, both have a 25-amino acid signal peptide at the N terminus and a typical ER retention signal at the C terminus (He et al., 2004). Moreover, four of the six multi-domain FKBP are characteristic of having a single (AtFKBP42) or triple (AtFKBP62, -65, -72) FKBP domain (s), a TPR domain, and a putative calmodulin-binding domain (He et al., 2004). Interestingly, these two multi-FKBP domain resemble the mammalian FKBP51 and FKBP52, which possess two FKBP domains and are involved in steroid mediated signaling (Schulke et al., 2010, discussed in section 1.4.3.1).

1.6 Biological Functions of Plant Immunophilins

In addition to the diversity in subcellular localization, functional characterization of plant immunophilins indicate they are involved in an array of functions as well. Although the majority of these proteins in plants remain to be characterized, current reports show involvement in regulation of gene expression, heat stress, chloroplast protein-complex biosynthesis among others (Lima et al., 2006; Fu et al., 2007; Bissoli et al., 2012; Karali et al., 2012; Trumpkin, 2012; Bannikova et al., 2013). Some of the specific functions of well-studied immunophilins are presented next.

1.6.1 Epigenetic Regulation of Transcription and Gene Silencing.

Post-translational gene silencing (PTGS) is a conserved eukaryotic gene regulatory mechanism that is important for a variety of biological processes such as development, stress response, and defense (Yu and Kumar, 2003). AtCYP40, a multi-domain cyclophilin conserved among eukaryotes, consist of N-terminal CYP domain and a C-terminal tetratricopeptide repeats (TPR) triplet separated by two putative nuclear targeting signals, has been linked to PTGS in *Arabidopsis* (Iki et al., 2012). *Atcyp40* (also known as *squint*, *sqn*) knock out mutants show reduced leaf number and alteration in leaf morphology associated to reduced microRNA activity (Berardini et al., 2001). Specifically, the mutant phenotype is largely attributed to elevated expression of

microRNA regulated genes as a result of decrease activity of ARGONAUTE1, AGO1, which is involved in miRNA-directed gene repression in *Arabidopsis* (Baumberger, 2005; Smith et al., 2009; Early et al., 2010). Like the mammalian CYP40, plant CYP40 interacts with HSP90 through the TPR domains and this interaction is indispensable for AtCYP40 function *in planta* (Earley and Poethig, 2011). More recently, using extracts of evacuated tobacco protoplasts, investigators showed that CYP40 and other TPR containing proteins associate with AGO1 in an HSP90-dependent manner (Iki et al., 2012). Explicitly, they showed that CYP40 facilitates HSP90-mediated RISC (RNA-induced silencing complex) assembly in plants by promoting or stabilizing the binding of small RNA duplexes to AGO1 (Iki et al., 2012). Both CYP and TPR domains of CYP40 were shown to be required for assembly of the RISC complex. Thus, these results suggest that CYP40 in combination with HSP90 and AGO1 plays a key role in RISC assembly in plants.

In another example, *Arabidopsis cyp71* mutant plants exhibit defective morphology and development of lateral organs, defective shoot apical meristem, altered flower morphology, and arrested root growth among other pleiotropic defects (Li et al., 2007). The nuclear-localized AtCYP71 is made up of four WD40 repeats at the N-terminal region and a CYP domain at the C-terminal region (He et al., 2004). The drastic phenotypes observed in this cyclophilin mutant are correlated with the important role of CYP71 in the regulation of epigenetic gene silencing and organogenesis. CYP71 directly associates with chromatin histone H3 and mediates methylation of H3K7 at the locus of some regulatory genes, such as *KNAT1* and *STM* that are known to regulate meristem activity and organogenesis in *Arabidopsis* (Li et al., 2007). Hence, these results indicated that CYP71 targets histone H3 through the WD40 repeat domain and may modulate histone structure by the protein foldase (CYP) domain. Chromatin, composed of histones and DNA, is the primary carrier of epigenetic information in higher eukaryotes, and further analysis of CYP71 function has been reported to be connected to chromatin assembly and histone modification in *Arabidopsis* (Li and Luan, 2011). CYP71 can genetically and physically interact with LHP1, a histone modification protein. Double mutants of these two genes results in a stronger phenotype, compared to single mutants (either *cyp71* or *lhp1*), causing sterility and lethality under normal conditions. Additionally, CYP71 was identified as partner of CAF-1 (Chromatin Assembly Factor-1); a trimeric complex functioning in nucleosome assembly pathway in plants (Li and Luan, 2011). Likewise, another immunophilin nuclear protein, AtFKBP53 has been identified as a new histone chaperone in plants that functions in chromatin remodeling and regulation of transcription as well (Li et al., 2010). Thus, immunophilin proteins are surging as key players in regulation of gene expression through epigenetic mechanisms.

1.6.2 Plant Stress Responses

The Arabidopsis multi-domain AtFKBP62 (ROF1) and AtFKBP65 (ROF2) proteins, share similar domain structure and high sequence identity. Both of these proteins are composed of triple FKBP domains, a TPR domain, and a calmodulin-binding domain (He et al., 2004). Although ROF1 and ROF2 are highly similar, studies show that both proteins are involved in long term acquired thermotolerance through different mechanisms (Meiri and Breiman, 2009; Meire et al., 2010). An initial study showed that ROF1 and ROF2 were heat-stress induced although differentially regulated (Aviezer-Hagai et al., 2007). Interestingly, Arabidopsis *rof1* mutants collapse after 24-48 hour between acclimation at 37 °C and exposure to 45 °C (Meiri and Breiman, 2009). Under normal conditions, ROF1 binds to heat shock proteins HSP90.1 via its TPR domain, and localizes to the cytoplasm. In plants exposed to heat stress, the ROF1-SHP90.1 complex transfers to the nucleus, dependent upon the presence of the heat stress transcription factor HsfA2, which interacts with HSP90.1 but not with ROF1 (Meiri and Breiman, 2009). In the *rof1* mutants the expression of small heat shock proteins (HSPs) regulated by HsfA2 was dramatically reduced suggesting ROF1 is involved in prolongation of thermotolerance by sustaining the levels of small HSPs essential for survival at high temperature (Meiri and Breiman, 2009). Similarly, ROF2 is a heat stress protein but *rof2* mutant plants are resistant to heat stress and small heat shock proteins are highly expressed contrary to what was observed in ROF1 mutants (Meiri et al., 2010). Further study showed that ROF2 transcription is controlled by HsfA2, which is also necessary for maintaining high levels of ROF2 during recovery from heat stress. ROF2 localized to the nucleus, but contrary to ROF1, translocation to the nucleus is independent of the presence of HSP90.1 or HsfA2 (Meiri et al., 2010). In conclusion, these studies indicate that these two proteins have antagonistic functions; ROF1 contributes to the transcription activity of HsfA2 but ROF2, in the presence of ROF1, abolishes this activity. Equally, the mammalian FKBP51/FKBP52 orthologues of ROF1/ROF2 have been shown to have antagonist functions in steroid receptor-mediated signaling (discussed in section 1.4.3.1). Recent studies have also implicated ROF2 in modulation of intracellular pH homeostasis in Arabidopsis whereas ROF1 has been reported to play an important role in osmotic/salt stress responses in germination of Arabidopsis seedlings through its interaction with a phosphatidylinositol-phosphate protein (Bissoli et al., 2012; Karali et al., 2012). ROF1 and ROF2 hint to the tangible multifunction carried out by immunophilins depending of different stress conditions and plant developmental stages.

1.6.3 Plant Hormone Signaling

Like other multicellular organisms, plants use long-range signaling to communicate between distant organs and to coordinate overall growth. A number of plant hormones such as auxin, ethylene and brassinosteroids (BR), among others, have been implicated in various aspects of growth regulation. Mutants defective in hormone

synthesis, perception, or response often show drastic changes in their appearance and in growth habits (Haubrick, 2006; Forestan, 2012; Rodrigues, 2012). AtFKBP42 was first identified through genetic screening and subsequent map-based cloning of Arabidopsis mutants *twisted dwarf 1 (twd1)* and *ultracurvata 2 (ucu2)*, which show a number of pleiotropic defects such as reduced height and disoriented growth of all organs, but develop fertile flowers and seeds (Geisler et al., 2003, Perez-Perez et al., 2004). Genetic and molecular analysis of the *twd1/ucu2* mutant indicated that FKBP42 is involved in the auxin and brassinosteroid (BR) signaling pathways (Perez-Perez 2004). By genetic manipulation of *TWD1* expression, it has been recently shown that TWD1 affects shootward root auxin reflux and, thus, downstream developmental traits, such as epidermal twisting and gravitropism of the root (Wang et al., 2013). Importantly, via its inactive FKBP domain, the FKBP42 protein physically interact with the multidrug resistance/P-glycoprotein (PGP) ATP-binding cassette (ABC) transporters PGP1 and PGP19 that can mediate export of the plant hormone auxin (Geisler et al., 2003). The *pgp1 pgp19* double mutant phenocopies developmental defects of *twd1*, suggesting that TWD1 plays a positive regulatory role in PGP-mediated auxin transport, which controls plant growth and development (Geisler et al., 2003; Wu et al., 2010). Indeed, further biochemical studies consolidated that FKBP42 modulates auxin efflux activities of PGP1 and PGP19 at the cellular and whole plant levels (Bouchard et al., 2006). In addition to the regulation of the transporting activity, FKBP42 appear to be also important for proper targeting of PGP1/19 transporters towards the plasma membrane (PM). Mutations in *TWD1* caused ER retention of PGP1/ABCB1, PGP19/ABCB19 as well as ABCB4. Thus, mis-localized auxin transporters probably result in abnormal spread of auxin signaling into the elongation zone of *twd1* roots that ultimately causes the twisted cell files featured in the *twd1* mutant roots (Wu et al., 2010). Recently, it has also been suggested that TWD1 co-localizes and interacts with PGP1/ABCB1 on specific lateral PM domains of epidermal cell files, which is critical for the reversal of apoplastic reflux and separation of basal and apical auxin streams in the roots (Wang et al., 2013). Interestingly, besides PM-anchored PGP transporters, vacuolar ABC transporters AtMRP1 and its close homologue AtMRP2 were also shown to physically and functionally interact with FKBP42 via the C-terminal tetratricopeptide repeat domain (Geisler et al., 2004). In summary, it seems that FKBP42 targets membrane transporters in plants to fulfill its essential function in regulation of auxin-mediated plant growth and development.

Another hormone related immunophilin is AtCYP18-3, also referred to as ROC1. ROC1 has been recently shown to link phytochrome and cryptochrome to BR sensitivity (Trupkin et al., 2012). Phytochromes (red light receptors) and cryptochromes (UV-A/Blue light receptors) are photoreceptors that regulate hypocotyl growth under specific conditions, by suppressing negative gravitropism, modulating phototropism and inhibiting elongation (Quail, 2005; Yu et al., 2010). Partial loss-of-function of the cytosolic single domain cyclophilin/ROC1 mutants exhibit long hypocotyls under blue and under far-red light with specific defects in phytochrome A and cryptochrome 1. Consistently, other seedling phenotypes such as cotyledon unfolding under red light, anthocyanin accumulation, block of greening, cotyledon expansion, were opposite in

gain- and loss-of function mutants (Trupkin et al., 2012). BR and light signaling converge to control several aspects of plant growth and development. This study further showed that mutations at BR signaling genes, such as the transcription factor BES1, and BR synthesis inhibitors eliminated the *roc1* mutant phenotype (Trupkin et al., 2012). Specifically, *roc1* mutant had altered patterns of phosphorylation of BES1, a known point of control of sensitivity to BR, correlated with altered expression levels of genes targeted by BES1. Hence, this suggests that ROC1 expression is partially enhanced by phytochromes and cryptochromes, and in turn ROC1 decreases abundance of partially phosphorylated form of BES1, therefore reducing sensitivity to BR. Furthermore, in a recent gain-of-function *roc1* mutant, the plants exhibited reduced stem elongation and increased shoot branching, with these phenotypes strongly affected by temperature and photoperiod (Ma et al., 2013). In this study, although *roc1* mutation did not interfere with gibberellic acid (GA) biosynthesis or signaling, GA antagonized *roc1* mutation on stem elongation (Ma et al., 2013). Thus, taken together these two mentioned studies, AtCYP18-3 may be involved not only in brassinosteroid signaling but also indirectly involved in other hormone related networks such as gibberellic acid.

1.7 The Immunophilins in the Chloroplast

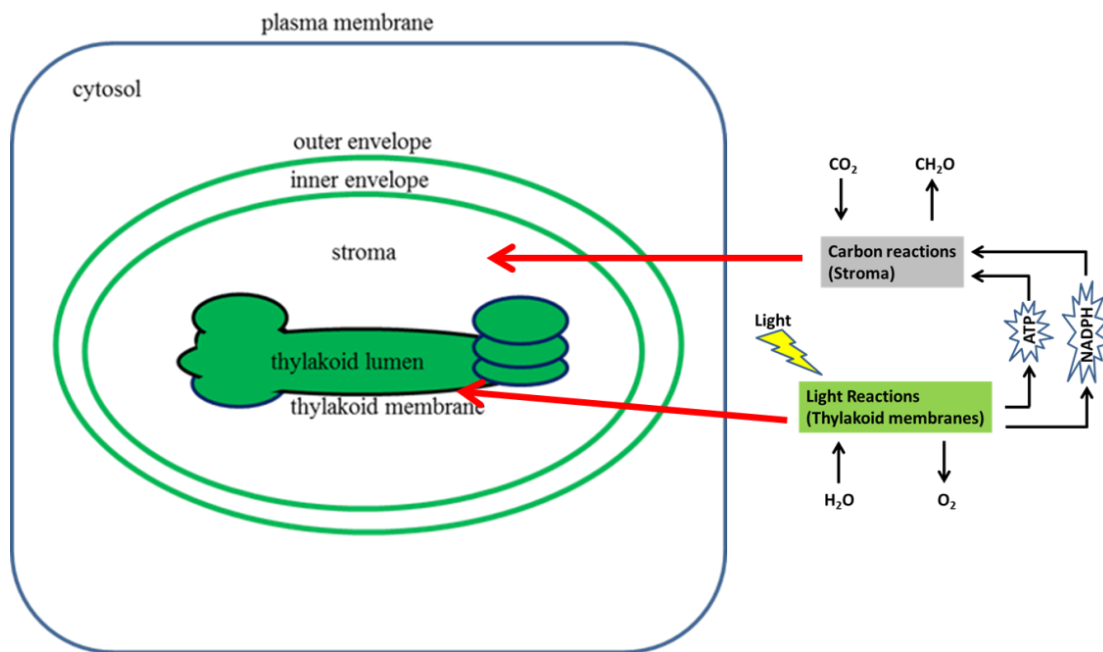


Figure 1.3 Schematic diagram of photosynthesis and organization of the chloroplast within the plant cell. This organelle is composed of an outer and inner envelope, a soluble stroma space (carbon reactions), and thylakoid membranes (light reactions) surrounding the thylakoid lumen.

The chloroplast is the characteristic organelle of plants and green algae, which allows these organisms to capture sunlight energy to generate organic compounds from inorganic compounds via photosynthesis, a key aspect of life on earth. The light-dependent reactions take place in the thylakoid membranes and light-independent reactions (carbon fixation) take place in the stroma (Fig. 1.3). Besides these two partitions, the chloroplast is surrounded by an outer envelope and an inner envelope enclosing an intermembrane space mainly involved in intracellular communication (Rochaix, 2011). About 2,500 to 3,000 proteins are believed to be housed in the chloroplasts, the vast majority of which are nucleus-encoded, synthesized in precursor form in the cytosol, and translocated into the chloroplast post-translationally (Leister, 2003; Richly and Leister 2004; Armbruster et al., 2011). In addition to photosynthesis, chloroplasts are responsible for a number of essential functions, such as lipid metabolism, starch and amino acid biosynthesis, assimilation of ammonia into amino acids, assembly of iron-sulphur complexes, among others (Balk and Lobreaux, 2005; Finkemeier and Leister, 2010; Spetea, 2012). Not long ago, it was thought that the narrow aqueous space within the thylakoid membranes, the thylakoid lumen, only function was to serve as a sink for protons (H^+) generated during the light-dependent reactions (Fig. 1.3). However, now with the era of proteomics, studies estimate that about 100 proteins localize to this compartment (Schubert et al., 2002; Peltier et al., 2002). Proteins in the thylakoid lumen, in addition to well-documented functions such as photosynthetic and linear electron transfer, are now emerging to be involved in protein folding, proteolysis, nucleotide metabolism, photoprotection, redox signaling, and Ca^{2+} signaling (Gupta et al., 2002; Komenda et al., 2008; Pesaresi et al., 2009; Yin et al., 2010; Armbruster et al., 2011; Spetea, 2012). All these functions are evidence to the complex environment in the thylakoid lumen. However, knowledge of the functional networks of luminal proteins is still in its infancy, especially when many luminal proteins remain to be functionally characterized.

Immunophilins are a significant component of the chloroplast proteome. Remarkably, in *Arabidopsis*, seventeen immunophilins (six cyclophilins and eleven FKBP) are in the chloroplast and all, with exception of one, are predicted to reside in the thylakoid lumen suggesting that such large numbers of proteins may play essential functions in this compartment (He et al., 2004; Romano et al., 2004; Edvardsson et al., 2007). Evidence is increasing for immunophilin involvement in proper folding and assembly of photosynthetic super-complexes (detailed discussion in chapter 2; Lima et al., 2006; Fu et al., 2007). Thus, although the photosynthetic multi-protein complexes composition, structure and function are known, the mixture of assembly and maintenance factors is still being determined. Understanding of the intricate biogenesis, maintenance, and controlled turnover of these photosynthetic complexes is a significant aspect of the present chloroplast research.

1.8 Aims of Thesis

The work of this thesis was based on the knowledge that the immunophilin family

is one of the predominant groups identified in the thylakoid lumen but most of these proteins were not functionally characterized. It was with this idea on structural diversity, subcellular localization, and specialized function that I aimed at further elucidating functionality of immunophilin proteins in the *Arabidopsis* chloroplast thylakoid lumen, specifically:

- Phylogenetic and Evolutionary analyses of chloroplast immunophilins across species using representative members from cyanobacteria to higher plant (Chapter II)
- Using reverse genetics and biochemical approaches to functionally characterize *Arabidopsis* chloroplast thylakoid lumen immunophilins: Cyclophilin 37, Cyclophilin 28 and FKBP20-2/FKBP16-3 (Chapter III).

CHAPTER 2

EVOLUTIONIONARY RELATIONSHIPS OF CHLOROPLAST IMMUNOPHILINS FROM ALGAE TO HIGHER PLANTS

2.1 Functions of Chloroplast Immunophilins

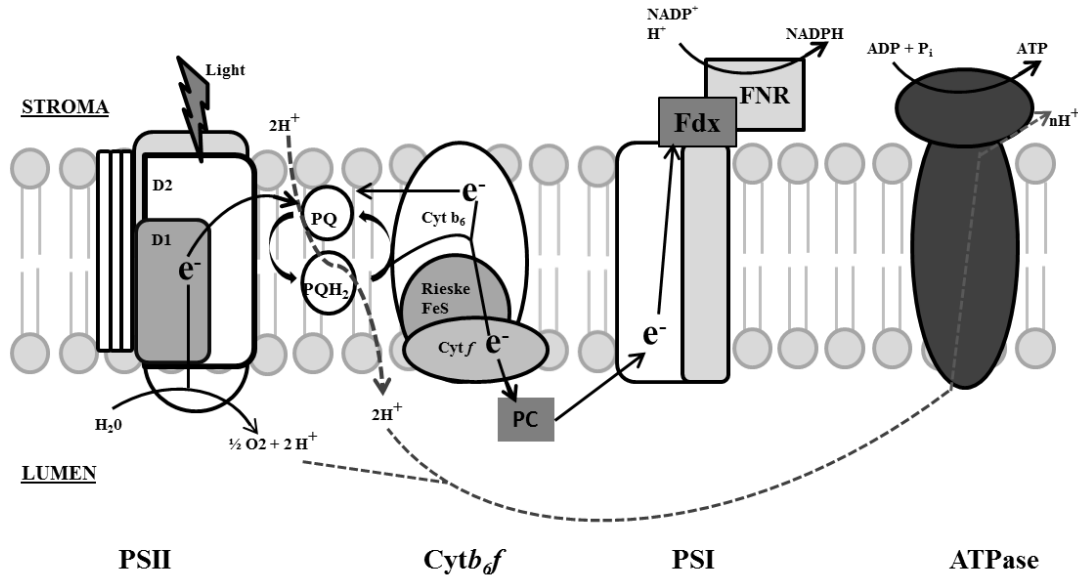


Figure 2.1 Schematic presentation of the photosynthetic electron transport chain. The four main membrane complexes involved in photosynthesis are illustrated. Photosystem II, PSII; Cytochrome *b₆f*, Cyt *b₆f*; Photosystem I, PSI; and ATP synthase, ATPase. Transfer of electrons is indicated by solid lines.

Photosystem II (PSII), photosystem I (PSI), the cytochrome *b₆f* complex (cyt *b₆f*), and the proton translocation ATP synthase (ATPase) are the four photosynthetic complexes embedded in the thylakoid membrane functioning in the primary reactions of photosynthesis (Buchanan et al., 2000). Although these photosynthetic protein complexes are well characterized in terms of their functions, their assembly and maintenance have remained relatively unresolved (Rochaix 2011; Chi et al., 2012). As discussed in Chapter I of this thesis, immunophilins are a large and diverse protein family, which are generally present in all subcellular compartments in plants. They are involved in various cellular processes such as epigenetic regulation of transcription and gene silencing, plant stress responses and plant hormone signaling (Chapter I section 1.6). Nevertheless, an intriguing feature of this large immunophilin family is the large number of isoforms that are located in the chloroplast (He et al., 2004). In Arabidopsis, 5 cyclophilins and 11 FKBP are predicted to reside in the thylakoid lumen in addition to one cyclophilin residing in the stroma. The only known stromal immunophilin, AtCYP20-3, has been associated with photosynthetic electron transport and redox signals in order to regulate the cysteine-based thiol biosynthesis pathway in response to light and oxidative stress (Dominguez-Solis et al., 2008). Of the 16 immunophilins in the lumen, only AtCYP20-2 and AtFKBP13 have been confirmed as active PPIases (Edvardsson et

al., 2003; Edvardsson et al., 2007), and functional characterization has been carried out for only three FKBP (AtFKBP13, AtFKBP16-2, and AtFKBP20-2) and two cyclophilins (AtCYP20-2 and AtCYP38) so far, as discussed hereafter.

2.1.1 AtCYP38 and AtFKBP20-2 Function in PSII Assembly

PSII catalyzes the first set of photosynthetic reactions, including the oxidation of water and production of molecular oxygen and reducing equivalents. This multi-protein complex exists mainly in the dimeric form, with each monomer containing about 30 subunits (Buchanan et al., 2000; Rochaix, 2011). The PSII complex is particularly prone to photo-oxidative damage, and as a result, it is continually impaired and needs to be repaired. It has been shown that both AtFKBP20-2 and AtCYP38 play a role in the assembly of PSII (Lima et al., 2006; Fu et al., 2007; Sirpio et al., 2008). The redox-active AtFKBP20-2 has been found to be required for the accumulation of PSII super-complexes (Lima et al., 2006). *fkbp20-2* mutant plants exhibit reduced plant growth, consistent with the reported lower rate of PSII activity. However, the mechanism of how FKBP20-2 function in maintaining PSII remains to be determined (Lima et al., 2006). Likewise, AtCYP38, a multi-domain cyclophilin which does not possess PPIase activity, plays a critical role in the assembly and maintenance of PSII as well (Fu et al., 2007; Sirpio et al., 2008; Vasudevan et al., 2012). Mutant Arabidopsis plants missing CYP38 are stunted in growth and hypersensitive to light. A further investigation into the molecular compositions of photosynthetic complexes reveals that the mutant fails to accumulate the PSII supercomplex (Fu et al., 2007; Sirpio et al., 2008). Studies suggest that CYP38 assists the proper folding and insertion of D1 and CP43 into the PSII complex and in addition plays a role in the correct assembly of the oxygen evolving complex (Vasudevan et al., 2012). Intriguingly, the AtCYP38 ortholog in spinach, TLP40, regulates the activity of a PSII-specific protein phosphatase within the thylakoid membrane (Vener et al., 1999; Rokka et al., 2000).

2.1.2 AtFKBP13 is Associated with *Cytb₆f* Complex

The *cytb₆f* complex in the thylakoid membranes functions as a plastoquinol–plastocyanin oxidoreductase that links PSII and PSI. In addition to some small subunits, the *cytb₆f* is composed of four major subunits: *cyt_f*, *cyt b₆*, Rieske-FeS (Iron Sulfur) and PetD (Buchanan et al., 2000; Rochaix, 2011). The redox regulated AtFKBP13, has been reported to interact with the Rieske protein and affect its accumulation in the cytochrome *b₆f* complex (Buchanan and Luan, 2005; Gupta et al., 2002; Gopalan et al., 2004; Edvardsson et al., 2007). Silencing of the AtFKBP13 gene in Arabidopsis altered the accumulation of the Rieske protein, suggesting that AtFKBP13 regulates import or assembly of the Rieske protein (Gupta et al. 2002). A recent independent study in wheat has also shown that FKBP13 interacts with the Rieske protein (Gollan et al., 2011).

Although, it was initially proposed that FKBP13 regulates assembly of the Rieske protein during its translocation to the thylakoid lumen by interaction of pre-cursor AtFKBP13, recent findings contradict such suggestion and the mechanism of the FKBP13-Rieske interactions remains unresolved (Gupta et al., 2004; Ingelsson et al., 2009; Gollan et al., 2011). On the other hand, AtFKBP13 is the only chloroplast FKBP protein to possess PPIase activity (Shapiguzov et al., 2006). However, a recent study found that immunophilin PPIase activity is a dispensable function, thus suggesting that the functions of immunophilins in the thylakoid are not directly related to their PPIase capacity (Ingelsson et al., 2009).

2.1.3 AtCYP20-2 and AtFKBP16-2 are Linked to Photosystem I and NAD(P)H Dehydrogenase

In addition to the previously mentioned photosynthetic complexes, higher plants also contain a low abundance protein complex known as the NAD(P)H dehydrogenase (NDH) complex in the thylakoid membrane. This NDH complex functions in PSI cyclic electron flow and chlororespiration (Rumeau et al., 2007). The linear electron transport mediated by PSII and PSI produces both ATP and NADPH, whereas PSI cyclic electron transport preferentially contributes to ATP synthesis without the accumulation of NADPH. In brief, NDH recycles electrons from ferredoxin to plastoquinone and subsequently to PSI through the cytochrome (Cyt) *b₆f* complex. Although this is a secondary process compared to the linear electron flow, evidence indicates that chloroplast NDH is required to alleviate stromal over-reduction under stress conditions (Munekage et al., 2004). Both AtCYP20-2 (PnsL5, photosynthetic NDH subunit of lumenal location) and AtFKBP16-2 (PnsL4), were shown to be associated with the lumenal subcomplex of the NDH complex, stabilizing the interaction between NDH and PSI (Penget al., 2009, Sirpio et al., 2009). AtCYP20-2 is a single domain cyclophilin that is regulated by light (Romano et al., 2004). This cyclophilin is found to be strongly reduced in *ndh* mutants that are deficient in the NDH membrane domain (Sirpio et al., 2009). AtCYP20-2 is detected in NDH–PSI supercomplex, consistent with the findings that CYP20-2 co-localizes with NDH–PSI on Blue native gels, and co-migrates with NDH subunits as well (Majeran et al., 2008; Sirpio et al., 2009; Peng et al., 2009). AtCYP20-2 protein is the only luminal cyclophilin in Arabidopsis shown to possess PPIase activity in vitro, but it remains unclear whether PPIase activity is involved in the CYP20-2 association with NDH in plants (Edvardsson et al., 2003; Shapiguzov et al., 2006). Nevertheless, plants lacking AtCYP20-2 do not lead to severe malfunction of NDH, indicating potential redundancy in the function of luminal immunophilins. Similarly, AtFKBP16-2, a single domain FKBP, has been shown that in RNAi knock-down plants, the mutant plants lacking FKBP16-2 are defective in the accumulation of the NDH complex (Peng et al., 2009). Additionally it has been shown that FKBP16-2 is absent in some NDH mutants. Thus, although the specific mechanism of these two

immunophilins and NDH association are not yet determined, it is possible they are involved in protein folding and/or in complex assembly.

2.1.4 Aims in this Chapter

Immunophilins play key roles in the chloroplast and it is of relevance to understand their evolutionary trajectory within photosynthetic organisms. Although genome-wide analyses have been carried out in some plants such as *Arabidopsis* and rice, an evolutionary investigation of chloroplast immunophilins in the green lineage is still lacking. The goal of this chapter is to focus on the evolutionary aspect of chloroplast immunophilins from algae to higher plants. Specifically, this work analyzes immunophilin proteins in cyanobacteria (*Synechocystis* sp. PCC 6803), and those in the chloroplast of green algae (*Chlamydomonas reinhardtii*), moss (*Physcomitrella patens*), lycophyte (*Selaginella moellendorffii*), monocots (*Oryza sativa* and *Zea mays*) and dicots (*Arabidopsis thaliana* and *Populus trichocarpa*).

2.2 Results and Discussion

2.2.1 Identification and Nomenclature of Chloroplast Immunophilins

An increasing number of available full-genome sequences of plant species have enabled us to further address the conservation and evolution of chloroplast immunophilins. In an attempt to gain insights into their distribution among different photosynthetic organisms, we searched the sequenced genomes of representative organisms across the evolutionary spectrum; from algae to higher plants. Also, we included the cyanobacterium, *synechocystis* sp strain PCC6803, to address the ancestral relationships within different orthologs. Although several sequenced genomes are currently available, we selected photosynthetic species that are well known or that are used as model systems throughout plant biology. The unicellular green alga, *chlamydomonas reinhardtii*, is a single-cell organism which for more than a decade has served as a model system for studying photosynthesis among other basic cellular processes (Nield et al., 2004). More recently, the moss *Phycometrella patents* has emerged as a powerful genetically tractable model plant system as well (Prigge and Bezanilla, 2010). Mosses such as *P. patens* occupy a key evolutionary position halfway between green algae and flowering plants. While the lycophyte, *Selaginella moellendorffii*, is a member of one of the oldest lineages of vascular plants (Banks et al., 2012). In addition to lower plants, we investigated the relationship of immunophilins among higher plants. The well-known monocots, maize (*Zea Mays*) and rice (*Oryza zativa*) were included, in addition to dicot plants; poplar (*Populus trichocarpa*) and *Arabidopsis* (*Arabidopsis thaliana*).

To identify members of the immunophilin family targeted to the chloroplast in different organisms, multiple blast searches against Arabidopsis chloroplast immunophilins were conducted in customized databases. To circumvent missing any possible candidates that cannot result from sequence blast, we subsequently collected all the immunophilins regardless of subcellular localization in those organisms. And based on the analysis by ChloroP software, we selected only chloroplast-targeted proteins for further evaluation. Together, fifty CYPs and seventy-three FKBP s were found among the different species studied (Table 2.1 and Table 2.2). Specifically, a total of two (0 FKBP s, 2 CYP s) immunophilins were found in the cyanobacterium, sixteen (10 FKBP s and 6 CYP s) in the green alga, nineteen (10 FKBP s and 9 CYP s) in the moss, fifteen (10 FKBP s and 5 CYP s) in the lycophyte, seventeen (10 FKBP s and 7 CYP s) in rice, nine-

Table 2.1 Chloroplast cyclophilins present in the cyanobacterium (*Synechocystis* sp. PCC6803), green alga (*C. reinhardtii*), moss (*P. patens*), lycophyte (*S. moellendorffii*), rice (*O. sativa*), poplar (*P. trichocarpa*), maize (*Z. mays*) and Arabidopsis (*A. thaliana*).

Protein name	Locus ID	aa ^a	% Identity ^b
<i>Arabidopsis thaliana</i>			
AtCYP20-2	At5g13120	259	-
AtCYP20-3	At3g62030	313	-
AtCYP26-2	At1g74070	317	-
AtCYP28	At5g35100	281	-
AtCYP37	At3g15520	466	-
AtCYP38	At3g01480	437	-
<i>Synechocystis</i> sp. PCC 6803			
slI0227 Syn 6803	NP_440031	246	*
slI0408 Syn 6803	NP_442180	403	*
<i>Chlamydomonas reinhardtii</i>			
CrCYP20-2	Cre12.g544150.t1.2	244	52.5
CrCYP20-3	Cre12.g496000.t2.1	201	63.9
CrCYP26-2	Cre03.g174750.t1.2	314	27
CrCYP28	Cre12.g561000.t1.3	338	38.1
CrCYP37	Cre06.g303300.t1.2	399	42.6
CrCYP38	Cre03.g3930.t1.0	413	47.3
<i>Physcomitrella patens</i>			
PpCYP20-2	Pp1s196_68V6.1	248	55.3
PpCYP20-3	Pp1s133_102V6.1	246	54.1
PpCYP26-2	Pp1s4_490V6.1	363	36.3
PpCYP28	Pp1s396_19V6.1	358	52
PpCYP37a	Pp1s204_121V6.1	476	52
PpCYP37b	Pp1s37_111V6.1	477	51.9
PpCYP38a	Pp1s90_50V6.1	453	59.9
PpCYP38b	Pp1s211_59V6.1	442	60.9
PpCYP38c	Pp1s149_77V6.1	451	54.5
<i>Selaginella moellendorffii</i>			
SmCYP20-2	Selmo229229	230	59.9
SmCYP26-2	Selmo79858	241	48.4
SmCYP28	Selmo57543	228	52.9
SmCYP37	Selmo73714	361	58
SmCYP38	Selmo413851	395	54
<i>Populus trichocarpa</i>			
PtCYP20-2	Potri.003G167700	265	64.7
PtCYP20-2b	Potri.001G060200	264	65.5
PtCYP20-3	Potri.002G185200	266	64
PtCYP26-2	Potri.012G058700	328	58.3
PtCYP28	Potri.006G189900	289	70
PtCYP37	Potri.001G404700	482	69.7
PtCYP38	Potri.017G072800	448	74.7
<i>Oryza sativa</i>			
OsCYP20-2	Os05g01270.1	251	67.1
OsCYP20-3	Os01g18210.1	239	62.8
OsCYP22	Os03g59700.1	205	60
OsCYP26-2	Os01g02080.1	305	52.9
OsCYP28	Os08g19610.2	328	58.4
OsCYP37	Os07g37830.1	466	63.5
OsCYP38	Os08g29370.1	428	75
<i>Zea mays</i>			
ZmCYP20-2a	GRMZM2G076544_T01	249	69.1
ZmCYP20-2b	GRMZM2G087570_T01	252	68.3
ZmCYP20-3	GRMZM2G063244_T01	243	63
ZmCYP22	GRMZM2G057329_T01	202	60
ZmCYP26-2	GRMZM2G132811_T01	304	51.1
ZmCYP28	GRMZM2G085885_T03	320	57.3
ZmCYP37	GRMZM2G117968_T01	440	61.4
ZmCYP38	GRMZM2G015967_T01	427	75.4

a, full length amino acid sequence; b, percent identity to Arabidopsis orthologs. *identity to more than one cyclophilin.

Table 2.2 Chloroplast FKBP s present in the green alga, moss, lycophyte, rice, poplar, maize and *Arabidopsis*.

Protein name	Locus ID	aa ^a	% Identity ^b
<i>Arabidopsis thaliana</i>			
AtFKBP13	At5g45680	208	-
AtFKBP16-1	At4g26555	207	-
AtFKBP16-2	At4g39710	217	-
AtFKBP16-3	At2g43560	223	-
AtFKBP16-4	At3g10060	230	-
AtFKBP17-1	At4g19830	229	-
AtFKBP17-2	At1g18170	247	-
AtFKBP17-3	At1g73655	234	-
AtFKBP18	At1g20810	232	-
AtFKBP19	At5g13410	256	-
AtFKBP20-2	At3g60370	242	-
<i>Chlamydomonas reinhardtii</i>			
CrFKBP13	Cre16.g675550.t3.1	197	44.6
CrFKBP16-1	Cre07.g315700.t1.1	247	33.5
CrFKBP16-2	Cre16.g675500.t1.2	176	36.9
CrFKBP16-3	Cre12.g530300.t1.2	209	53.9
CrFKBP16-4	Cre17.g733250.t1.2	229	36.9
CrFKBP17-2	Cre11.g1871.t1.0	268	39.9
CrFKBP17-3	Cre10.g454250.t1.2	241	24.2
CrFKBP18	Cre10.g466850.t1.1	170	30.3
CrFKBP19	Cre12.g534700.t1.2	216	56.3
CrFKBP20-2	Cre13.g577850.t1.1	269	46.4
<i>Physcomitrella patens</i>			
PpFKBP13	Pp1s347_30V6.1	225	52.7
PpFKBP16-1	Pp1s64_202V6.1	224	42.7
PpFKBP16-3	Pp1s143_175V6.1	221	59.4
PpFKBP16-4	Pp1s26_209V6.1	232	59.6
PpFKBP17-1	Pp1s226_114V6.1	243	50.9
PpFKBP17-2	Pp1s15_267V6.1	294	48.8
PpFKBP18	Pp1s215_83V6.1	296	52.4
PpFKBP19a	Pp1s297_17V6.1	271	58.3
PpFKBP19b	Pp1s15_317V6.1	269	58.1
PpFKBP20-2	Pp1s52_210V6.1	300	52.2
<i>Selaginella moellendorffii</i>			
SmFKBP13a	Selmo122401	166	56.8
SmFKBP13b	Selmo48620	126	53.8
SmFKBP16-1	Selmo68680	115	62.6
SmFKBP16-3	Selmo99440	152	70.3
SmFKBP16-4	Selmo75290	150	78.8
SmFKBP17-1	Selmo16793	148	70.9
SmFKBP17-2	Selmo15857	178	57.3
SmFKBP18	Selmo16278	153	68.9
SmFKBP19	Selmo96291	164	77.9
SmFKBP20-2	Selmo441577	219	50.3

Protein name	Locus ID	aa ^a	% Identity ^b
<i>Populus trichocarpa</i>			
PtFKBP13	Potri001G075500.1	216	66.5
PtFKBP16-1	Potri0001s47130.1	205	67.8
PtFKBP16-2	Potri005G079700.1	227	66.8
PtFKBP16-3a	Potri007G134600.1	238	71.7
PtFKBP16-3b	Potri017G017500.1	246	71.3
PtFKBP16-4	Potri016G096600.1	238	70.1
PtFKBP17-1	Potri012G119800.1	230	67.3
PtFKBP17-2	Potri012G048300.1	259	71.4
PtFKBP18	Potri005G254900.1	226	66.1
PtFKBP19	Potri001G068300.1	246	71.2
PtFKBP20-2	Potri014G045600.1	268	69.5
<i>Oryza sativa</i>			
OsFKBP13	Os06g45340.1	219	52.4
OsFKBP16-1	Os02g10590.1	212	55.8
OsFKBP16-2	Os02g51570.1	231	59.3
OsFKBP16-3	Os08g42850.1	261	66.5
OsFKBP16-4	Os07g09040.1	240	64.4
OsFKBP17-1	Os02g07220.1	213	61.3
OsFKBP17-2	Os03g50080.1	252	62.8
OsFKBP18	Os02g02550.1	242	60.4
OsFKBP19	Os07g04160.2	237	66
OsFKBP20-2	Os07g30800.1	196	55.4
<i>Zea mays</i>			
ZmFKBP13	GRMZM2G095333_T01	214	53.8
ZmFKBP16-1	GRMZM2G012340_T01	206	58.6
ZmFKBP16-2	GRMZM2G035708_T01	227	59.9
ZmFKBP16-3	GRMZM2G030494_T01	202	69.5
ZmFKBP16-4	GRMZM2G001956_T01	221	75.8
ZmFKBP17-1	GRMZM2G041060_T01	210	61.6
ZmFKBP17-2	GRMZM2G068963_T01	254	62.5
ZmFKBP17-3	GRMZM2G057374_T01	254	44.2
ZmFKBP18	GRMZM2G150337_T03	207	58.6
ZmFKBP19	GRMZM2G155753_T04	232	66.7
ZmFKBP20-2	GRMZM2G488458_T01	250	65.1

a, full length amino acid sequence; b, percent identity to *Arabidopsis* orthologs. Note: the cyanobacterium *synechocystis* is not included due to not possessing any chloroplast FKBP.

teen (11 FKBP s and 8 CYP s) in maize, eighteen (11 FKBP s and 7 CYP s) in poplar, compared to seventeen (11 FKBP s and 6 CYP s) isomers in *Arabidopsis* (Table 2.1 and Table 2.2). Overall FKBP s identified in green algae, rice, maize and poplar are consistent, except minor discrepancies (later discussed), with previous results of genome-wide analyses of immunophilins (Vallon, 2005; Ahn et al., 2010; Wang et al., 2012; Yu

et al., 2012). Similarly CYPs identified in rice and green algae are consistent with previous results (Vallon, 2005; Ahn et al., 2010).

In this study, the nomenclature was assigned according to conventional rules in previously published studies (He et al., 2004; Romano et al., 2005). The proteins are designated either FKBP or CYP with prefix letters to indicate the species of origin (for example At for *Arabidopsis thaliana*), and a suffix number to indicate molecular weight. For better comparison in this report, all proteins suffix numbers in the different species were assigned according to their ortholog isoforms in *Arabidopsis* rather than solely based on their estimated molecular weights (He et al., 2004). When multiple orthologs were identified, a letter was added to the name. For instance, moss CYP37a and moss CYP37b are both orthologous to AtCYP37 thus are referred to as PpCYP37a and PpCYP37b.

2.2.2 Chloroplast Cyclophilin Diversity in Lower and Higher Plants.

A significant question in understanding chloroplast CYPs is associated with the evolution and diversification of these proteins in species across the evolutionary spectrum. Interestingly, we find that lower plants such as moss generally have the similar level of diversity as that of vascular plants such as *Arabidopsis*. This appears to be the result of an early diversification of the gene family in the common ancestor of land plants and green algae. The flowering plant, *Arabidopsis thaliana*, encodes six chloroplast cyclophilins (AtCYP20-2, AtCYP20-3, AtCYP26-2, AtCYP28, AtCYP37, and AtCYP38). The lycophytes, a non-seed vascular plant, possesses four chloroplast cyclophilins (SmCYP20-2, SmCYP26-2, SmCYP28, SmCYP37, and SmCYP38). The *Physcomitrella patens*, a moss, contains nine cyclophilins (PpCYP20-2, PpCYP20-3, PpCYP26-2, PpCYP28, PpCYP37a, PpCYP37b, PpCYP38a, PpCYP38b, and PpCYP38c). The green alga, *Chlamydomonas reinhardtii*, includes six cyclophilins (CrCYP20-2, CrCYP20-3, CrCYP26-2, CrCYP28, CrCYP37, and CrCYP38) in the chloroplast. However, the cyanobacterium, *Synechocystis* sp PCC6803 only contains two chloroplast cyclophilins (ssll0227 and ssll0408). Although from a global perspective the same diversity of cyclophilins has been conserved across the plant lineage, a number of differences are conspicuous. For instance we did not identify an ortholog for CYP20-3 in the lycophyte. Also, it is noteworthy that across the evolutionary spectrum specific genes appear to have duplicated. In the case of the moss, multiple orthologs of AtCYP37 and AtCYP38 are found. Both, PpCYP37a and PpCYP37b are retrieved as the closest proteins to AtCYP37. PpCYP37a and PpCYP37b share 85% identity and are about the same molecular weight (Table 2.1). Alignment of these proteins with *Arabidopsis* CYP37 shows an overall well-conserved amino acid sequence with minor substitutions (Fig 2.1). Also both PpCYP37a and PpCYP37b are 52 and 51.9 percent, respectively, identical to AtCYP37, thus very likely these proteins arose from a duplication event (Table 2.1, Fig. 2.2). Interestingly, three CYP38 orthologs (PpCYP38a, PpCYP38b, and PpCYP38c) are found in moss as well. The three proteins are similar in length, ranging from 453, 442,

and 451 amino acids, respectively (Table 2.1, Fig 2.2). *P. patens* is an established organism which has experienced whole genome duplication; polyploidy (Rensing et al., 2013). It is presumed that during genome duplication, duplicated segments are lost quickly during the early phases of diploidization, unless a selective advantage is introduced which might retain certain genes (Ma and Gustafson, 2005; Innan and Kondrashov, 2010). Thus, these multiple orthologs might be due to gene duplication in moss. However, it is surprising that out of all the six cyclophilin orthologs in moss, only CYP37 and CYP38 have retained duplicates, which might implicate the importance of their function in moss development and survival. In contrast to the moss, single cyclophilin orthologs are found in the green alga, *C. reinhardtii*, suggesting that cyclophilins evolved early in a common ancestor of land plants and green algae. Further support for this hypothesis is found in the cyanobacterium *Synechocystis*, which only has two homologues of plant chloroplast cyclophilins (ssl10227 and ssl10408, Table 2.1; Vallon, 2005). A proteomic study of the thylakoid of *Synechocystis* has confirmed the presence of ssl10408 in this compartment (Srivastava et al., 2005). Chloroplasts originated more than 1 billion years ago, when a free-living cyanobacterium became an endosymbiont in a eukaryotic host cell, giving rise to a eukaryotic plant (Miyagishima, 2011). Consequently, it is not surprising to find a steep increase in number and diversity of immunophilin isoforms during the evolution from cyanobacteria to higher plants.

Furthermore, in order to analyze the evolutionary relationships among the chloroplast CYP members in these species, a phylogenetic tree was constructed based on the alignments of SynCYP (sl10227, sl10408), CrCYP, PpCYP, SmCYP, and AtCYP protein sequences (Fig 2.2). Because of the highly conserved amino acid sequences of CYP proteins, the neighbor-joining method of the MEGA software was adopted to construct the phylogenetic trees for the combined 28 chloroplast CYPs found in all these organisms. Generally, respective orthologs in green algae, moss, and lycophyte were found to share high sequence similarity to Arabidopsis CYPs, thereby indicating that CYPs between lower and higher plants are highly conserved throughout plant evolution (Fig 2.3). Interestingly, phylogenetic tree reconstruction analysis shows that one of the cyanobacterium CYPs (ssl10408) is grouped closer to CYP38 and CYP37 branches, whereas the other CYP (ssl10227) is closely grouped to the rest of the CYPs branches (Fig 2.3). Additionally, contrary to the clear evolutionary relationship observed for CYP26-2, CYP28, CYP37 and CYP28, the phylogenetic analyses show intertwined evolutionary relationship among CYP20-2 and CYP20-3. This result suggests that these two genes arose from an early gene duplication event but have potentially divergent functions. This hypothesis could be supported by the fact that the close homologues CYP20-2 and CYP20-3 in Arabidopsis localize to the thylakoid and the stroma, respectively (Dominguez et al., 2008, Sirpiö et al., 2009).

Similarly, analysis of cyclophilin proteins from the monocots (maize and rice) and dicots (poplar and Arabidopsis) further confirms an overall established chloroplast cyclophilin diversification in higher plants (Fig 2.4 and Fig 2.5). The genome analyses indicate maize contains eight cyclophilins (ZmCYP20-2a, 20-2b, 20-3, 22, 26-2, 28, 37, and 38). Poplar includes seven cyclophilins (PtCYP20-2a, 20-2b, 20-3, 26-2, 28, 37, and

38). And rice has seven isomers (OsCYP20-2, 20-3, 22, 26-2, 28, 37, and 38). Among these higher plants, a high similarity in sequence and primary protein structure is observed (Table 2.1 and Fig 2.4). However, OsCYP22 and ZmCYP22, the orthologs of AtCYP22 that is putatively cytosolic, scored high for targeting to the chloroplast based on N-terminal sequence analyses as described in methods and in agreement with a previous report in rice (Ahn et al., 2010). Analysis of AtCYP22 orthologs in the other species in this study scored poorly for chloroplast targeting (not shown). Another significant difference observed within higher plants is the presence of duplicate CYP20-2 orthologs in maize and poplar. In each case both proteins are very close in size but have a few amino acid substitutions. Interestingly, ZmCYP20-2a and ZmCYP20-2b in maize are on separate chromosome, 8 and 6, respectively (Data not shown). A constructed phylogenetic tree based on the alignments of the 28 CYP proteins present in rice, maize, poplar and Arabidopsis indicates a close evolutionary relationship (Fig 2.4 and Fig 2.5). The phylogenetic tree was generated using the neighbor-joining approach as described in methods section. The unrooted phylogenetic tree shows six well defined groups representing the corresponding cyclophilin orthologs in each species in addition to a seventh branch for CYP22 in maize and poplar. Overall, these results suggest the same level of diversity is observed from lower plant to higher plants and that a major diversification of chloroplast immunophilins arose in the evolution of photosynthetic organisms from cyanobacteria.

2.2.3 Chloroplast FKBP Diversity in Lower and Higher plants.

Proteomic analyses previously exposed a surprisingly large number of FKBP associated with the thylakoid membrane and lumen in Arabidopsis (Schubert et al., 2002; Peltier et al., 2002). Subsequently, Arabidopsis genome-wide analysis revealed that half of FKBP (11 of 22) in Arabidopsis are targeted to the chloroplast thylakoid, directed to this compartment by cleaved N-terminal transit peptides in the precursor proteins (Romano et al., 2004). Our results from database mining and analysis of evolutionary relationships indicate that FKBP from green algae (*Chlamydomonas reinhardtii*), moss (*Physcomitrella patens*), lycophyte (*Selaginella moellendorffii*), monocots (*Oryza sativa* and *Zea mays*) and dicots (*Arabidopsis thaliana* and *Populus trichocarpa*) are well conserved both in terms of number and similarity (Table 2.2). However, we did not find any chloroplast FKBP in the cyanobacterium *Synechocystis* sp. PCC 6803. The green alga encodes ten FKBP (CrFKBP13, 16-1, 16-2, 16-3, 16-4, 17-2, 17-3, 18, 19, and 20-2). Similarly, moss encode ten FKBP (PpFKBP13, 16-1, 16-3, 16-4, 17-1, 17-2, 18, 19a, 19b, and 20-2). And the lycophyte has ten FKBP (SmFKBP13a, 13b, 16-1, 16-3, 16-4, 17-1, 17-2, 18, 19, and 20-2) as well. Compared to the eleven FKBP (AtFKBP13, 16-1, 16-2, 16-3, 16-4, 17-1, 17-2, 17-3, 18, 19, and 20-2) in Arabidopsis, indicating diversity of FKBP arose before the diversification of land plants (Table 2.2). However, modifications and duplications have occurred throughout the evolution from lower to

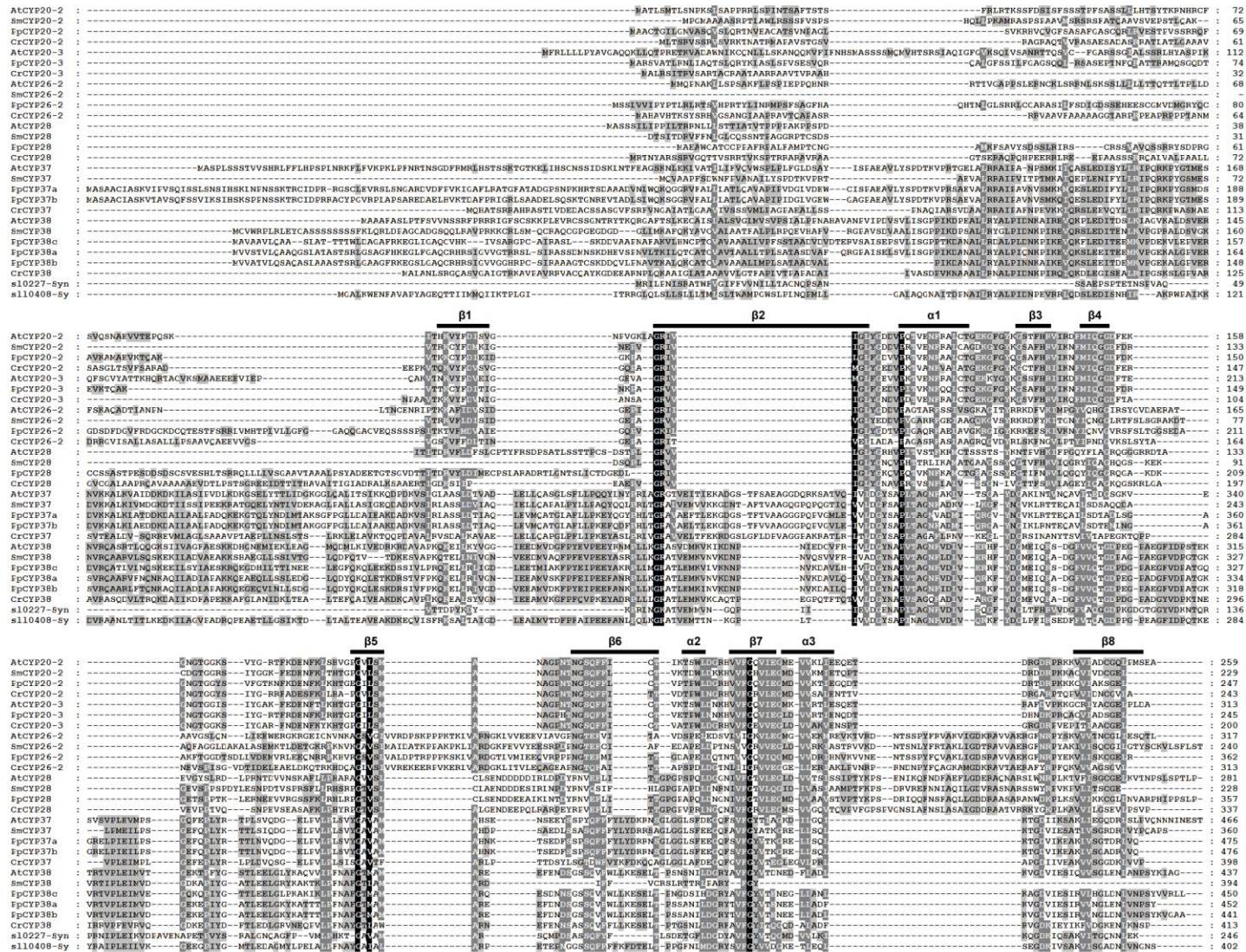


Figure 2.2 Multiple sequence alignment of CYPs in *Synechocystis* sp. PCC6803, *C. reinhardtii*, *P. patens*, *S. moellendorffii*, and *A. thaliana*. Secondary structure features (a-helix and b-sheets) derived from the human CYPa according to previous reports (Galat et al., 2003; Romano et al., 2004). Backgrounds indicate percent of amino acid similarity: black 100%, dark grey 60%, light gray 20%.

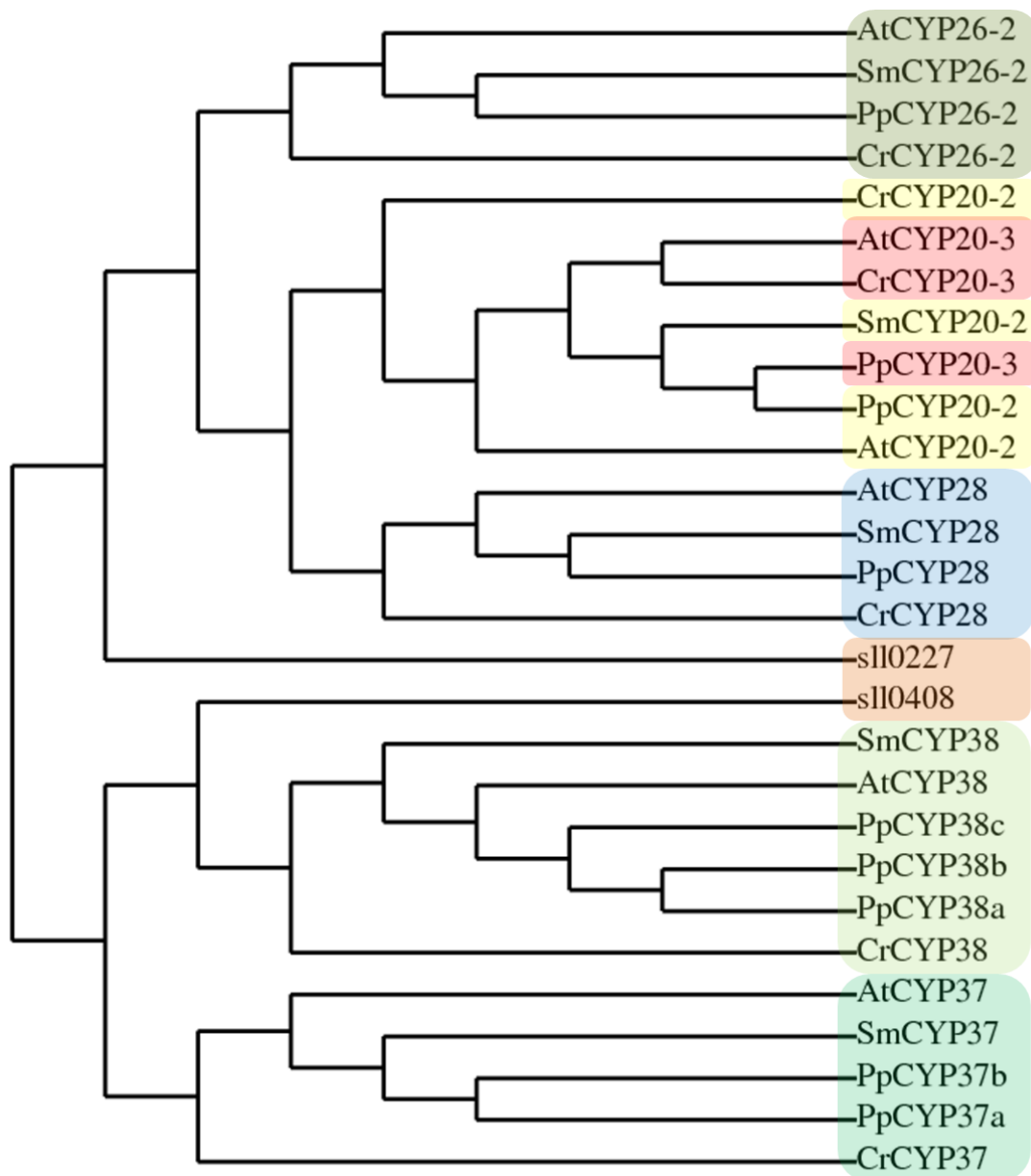


Figure 2.3 Phylogenetic tree with evolutionary relationships of Cyclophilins in *Synechocystis* sp. PCC6803 (SII), *C. reinhardtii* (*Cr*), *P. patens* (*Pt*), *S. moellendorffii* (*Sm*), and *A. thaliana* (*At*). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA software (Tamura et al., 2011). Color shades identify similar orthologs.

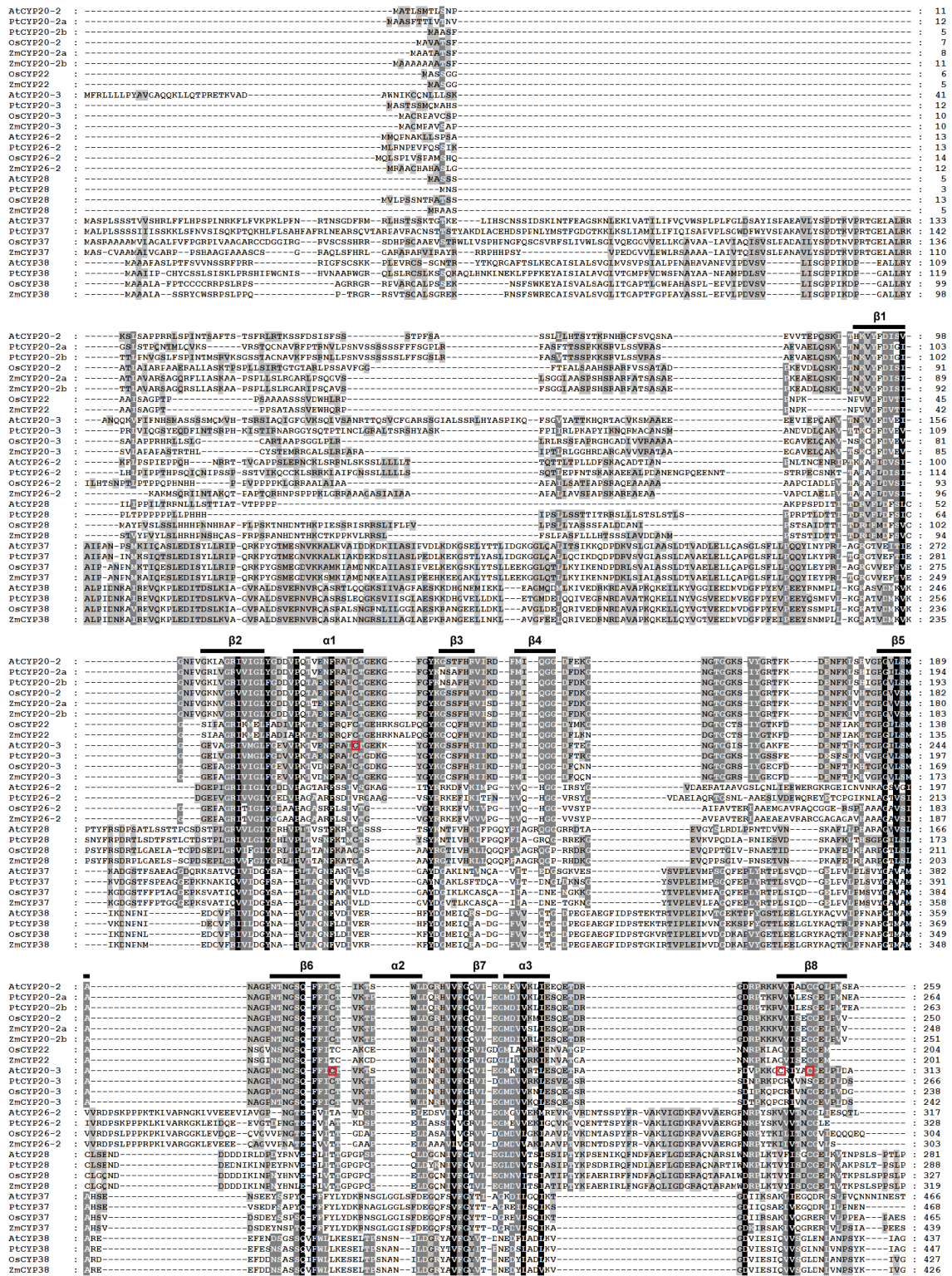


Figure 2.4 Multiple sequence alignment of cytophilins from *O. Sativa* (*Os*), *P. trichocarpa* (*Pt*), *Z. mays* (*Zm*) and *A. thaliana* (*At*). Secondary structure features (a-helix and b-sheets) derived from the human CYP4 according to previous reports (Galat et al., 2003; Romano et al., 2004). Backgrounds indicate percent of amino acid similarity: black 100%, dark grey 60%, light gray 20%. The four cysteine residues involved in redox control of AtCYP20-3 are boxed in red.

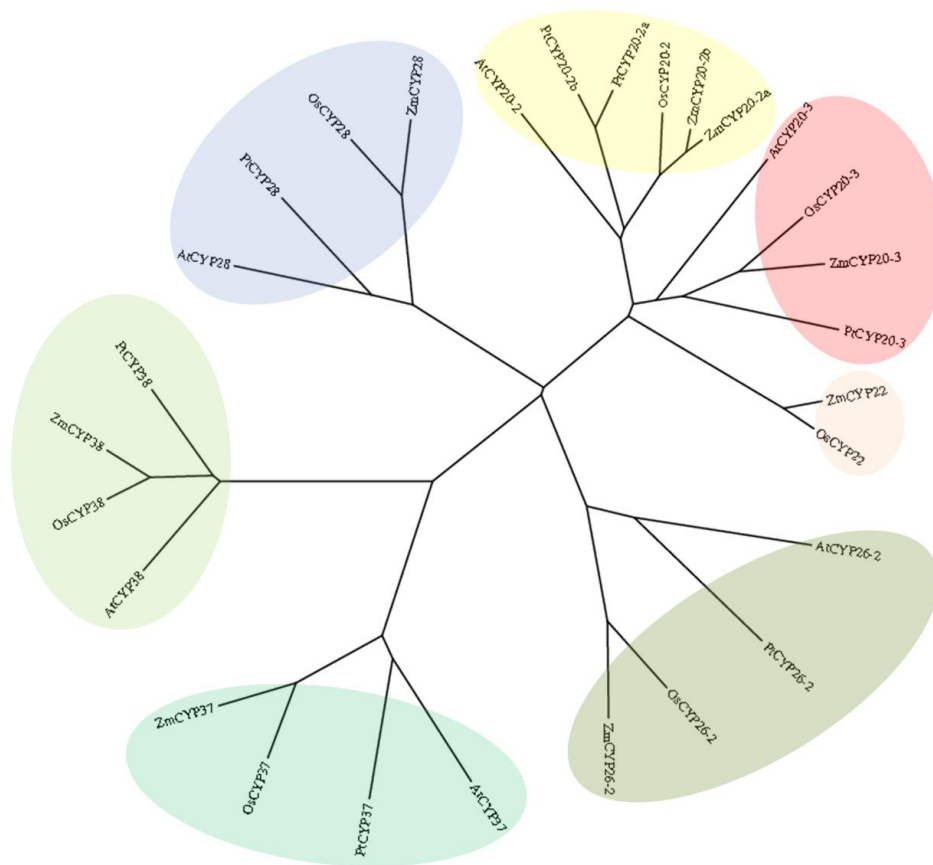


Figure. 2.5 Unrooted phylogenetic tree of cyclophilins relationship in higher plants: rice, maize, poplar and Arabidopsis. The phylogenetic analysis was based on the sequence alignments by ClustalX as described in Methods. Color shades highlight grouped orthologs proteins from all the species analyzed.

higher plants. For instance, *C. reinhardtii*, does not encode a chloroplast-targeted FKBP17-1 ortholog. Using Arabidopsis CYP17-1 as a query in the green alga genome only identifies CrFKBP19. Moving from water to land was a pivotal step in the evolution of land plants thus functional characterization of FKBP17-1 in moss and higher plants will likely provide evidence to understand the lack of this gene in green algae. Noticeably, moss encodes for duplicates orthologs of FBKP19, however, FKBP19 is not related to FKBP17-1 in species where it is present. As previously discussed in this chapter (Section 2.2.2), *P.patens* is a well-recognized species that has gone through genome duplication (Rensen et al., 2013). But similarly to what is observed in cyclophilins, not all FKBP19s have duplicates indicating the maintenance of these duplicates could be the result of specific selection pressures for these particular proteins. AtFKBP19 awaits functional characterization in higher plants thus it is unclear the significance that moss has maintained two very similar copies of FKBP19. At the same

time, no AtFKBP16-2 or AtFKBP17-3 ortholog could be identified in the moss. On the other hand, the lycophyte *S. moellendorffii*, has a duplicate of FKBP13 (SmFKBP13a and SmFKBP13b) and absence of FKBP16-2 (Table 2.2). In Arabidopsis, AtFKBP13 has been shown to be redox regulated by redox changes as a result of two disulfide-bridges, C56-C96 and C185-C190 (Fig 2.6, red boxes; Gopalan et al., 2004). The two pairs of cysteine are conserved in AtFKBP13 orthologs of green algae, moss and the lycophyte SmFKBP13b, while SmFKBP13a is missing one cysteine residue thus suggesting SmFKBP13a might have different functions than its ortholog; AtFKBP13. Accordingly, all these results indicate that across evolutionary space different selective pressures might have shape FKBP diversity. Similarly, chloroplast FKBP genes among higher plants, such as the monocots (maize and rice) and the dicots (poplar and Arabidopsis) have remained constant (Table 2.2). Maize encodes eleven FKBP similar to Arabidopsis. Poplar also contains eleven FKBP as well but does not encode for FKBP17-3 and has duplicate orthologs for FKBP16-3 (Table 2.2). While rice, only has ten FKBP isomers, lacking FKBP17-3 as in poplar. Our results show that FKBP17-3 is present in only 3 species (*A. thaliana*, *Z. mays* and *C. reinhardtii*) and absent in the other four species investigated in this study (Table 2.2). Previous reports have suggested FKBP17-3 and FKBP17-2 arose from a duplication event and thus FKBP17-3 might have been lost due to its redundancy with FKBP17-2 (Gollan et al., 2012).

Further analysis of the evolutionary relationships of FKBP from lower and higher plants by phylogenetic trees constructed based on the alignments of either lower plants compared to Arabidopsis, or higher plants compared to Arabidopsis, confirms overall ancient diversification of FKBP. The multiple full length sequence alignment of the FKBP identified in the corresponding species was performed using ClustalX and a neighbor-joining method used to construct the phylogenetic tree (Fig 2.6, Fig 2.7 and Fig. 2.8, Fig 2.9) According to the phylogenetic tree generated from lower plants and Arabidopsis, each corresponding FKBP forms independent evolutionary related group, except for FKBP16-2 with FKBP13, and FKBP17-2 with FKBP17-3 (Fig 2.7). For instance, There is no clear separation in the evolutionary relationship between FKBP16-2 and FKBP13, further adding evidence these two proteins arose from gene duplication as previously suggested (Fig 2.7; Gollan et al., 2012). Another example of ambiguous relationship, as already mentioned, is FKBP17-3 and FKBP17-2 in which there is no clear split-up among these orthologs in the tree as well. A number of studies have indicated FKBP multi-gene family in higher plants have evolved through gene duplication, but based from our results it appears that only a few FKBP such FKBP13-FKBP16-2 and FKBP17-3-FKBP17-2 pairs are clear candidates as products of duplication events, nonetheless, the major diversification of these proteins occurred in an ancient common ancestor of green algae and land plants.

2.2.4 Concluding Remarks

In this study we investigated the evolutionary relationship among chloroplast immunophilins in eight representative species over a large evolutionary time-scale, including cyanobacteria, green algae, non-angiosperms, monocots, and dicots (Table 2.1 and Table 2.2). Sequence analyses and phylogenetic tree reconstruction indicate that chloroplast immunophilin proteins (cpCYPs and cpFKBPs) are highly diverse and such diversity is largely conserved from eukaryotic lower plants to higher plants. Six cpCYPs are present in higher plants as well as in lower plants, while the prokaryotic cyanobacterium possesses only two cyclophilins. Meanwhile, about 10-11 FKBP are present in higher plants, which are comparable with the 10 FKBP in green algae and non-angiosperms. In *Arabidopsis*, functional characterization so far indicate immunophilins are mainly involved in maintaining the stability of photosynthetic protein complexes although the exact regulatory mechanisms are largely unknown. Recently, immunophilins in the chloroplast have also been reported to be redox controlled (Gopalan et al., 2004; Lima et al., 2006; Motohashi et al., 2006). For instance, AtCYP20-3 containing four cysteines (2 pairs) has been shown to be redox regulated by thioredoxin (Motohashi et al., 2006; Dominguez-solis et al., 2008). Similarly, AtFKBP13 with two pairs of cysteines has been shown to be regulated by redox changes (Gopalan et al., 2004; Shapiguzov et al., 2006). Our results show that cysteine residues are highly conserved in a number of immunophilins suggesting that formation of disulfide bridges and redox signaling potentially serves as a major regulatory mechanism in these proteins. AtCYP26-2 and AtCYP28 have three and five cysteines, respectively. By contrast, AtCYP38 with one cysteine and AtCYP37 with no cysteines are unlikely to be redox regulated. Other potential targets for redox regulation are AtFKBP16-3 and AtFKBP17-3 with two cysteines each. AtFKBP16-1, AtFKBP16-4, AtFKBP17-1, AtFKBP17-2, AtFKBP18, and AtFKBP19 contain one or no cysteines and are not able to form any intramolecular disulfide bridge.

Additionally, although immunophilins are generally referred to as PPIases, strong evidence suggests the functions of chloroplast immunophilins may be independent of PPIase capabilities (Ingelsson et al., 2009). Most of these proteins targeted to the chloroplast do not contain the required residues for PPIase activity (He et al., 2004; Gollan et al., 2012). Consistently, most of them appear to be inactive PPIases, except AtFKBP13 and AtCYP20-2 which have been shown to possess PPIase activity although this capability appears to be dispensable for *Arabidopsis* development (Shapiguzov et al., 2006; Edvardsson et al., 2007; Gollan et al., 2011). Likewise, the absence of key residues for PPIase in immunophilins from lower plants further implies that PPIase is not required for immunophilin functions in the chloroplast. Consequently, functional characterization of these proteins is pivotal to our understanding of lumenal protein functions and dynamics.

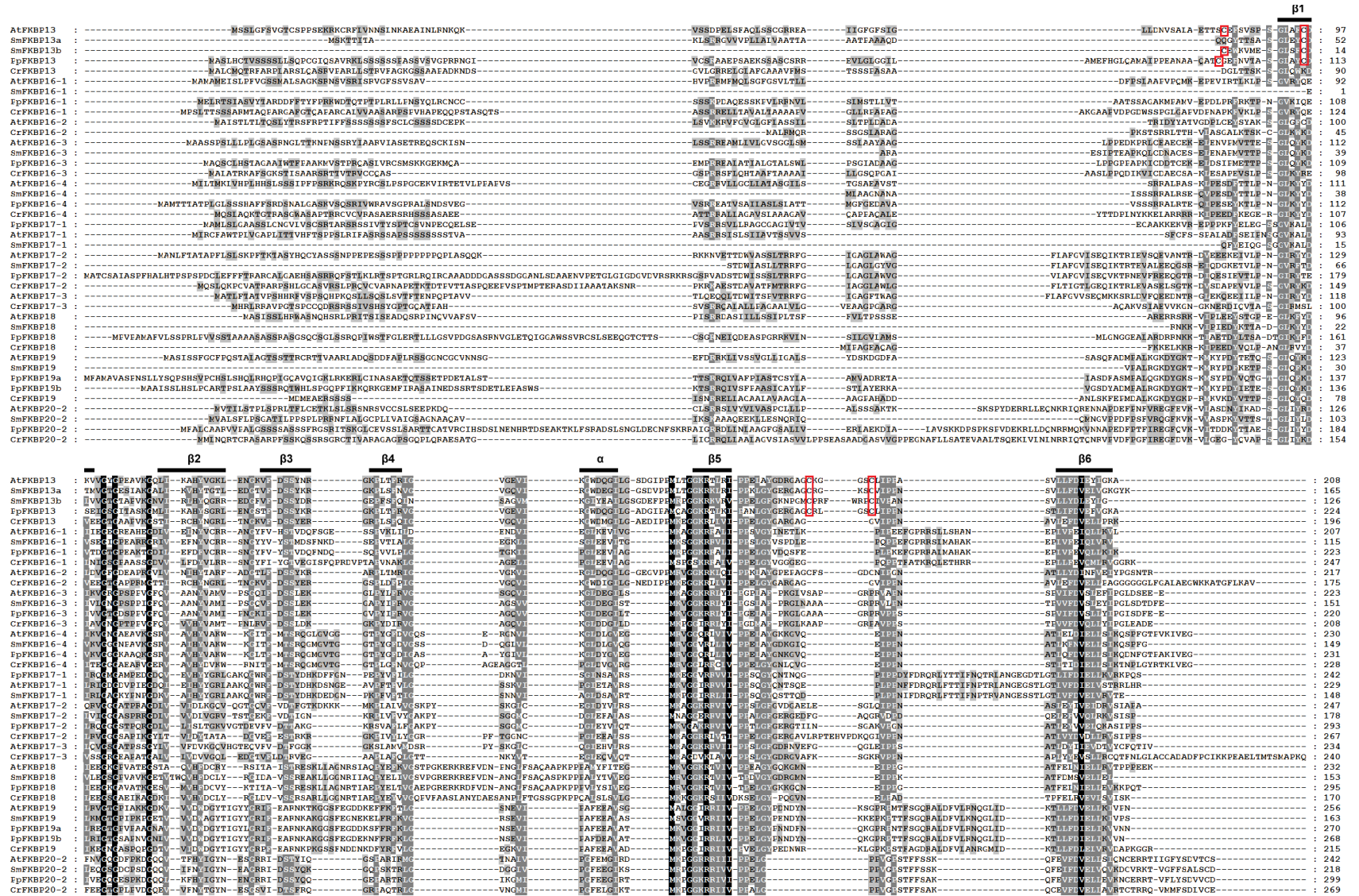


Figure 2.6 Multiple sequence alignment of FKBP in *C. reinhardtii*, *P. patens*, *S. moellendorffii*, and *A. thaliana*. Secondary structure features (α -helix and β -sheets) derived from the human FKBP12 according to He et al., 2005. Backgrounds indicate percent of amino acid similarity: black 100%, dark grey 60%, light gray 20%. The four cysteine residues involved in redox control of AtFKBP13 are boxed in red.

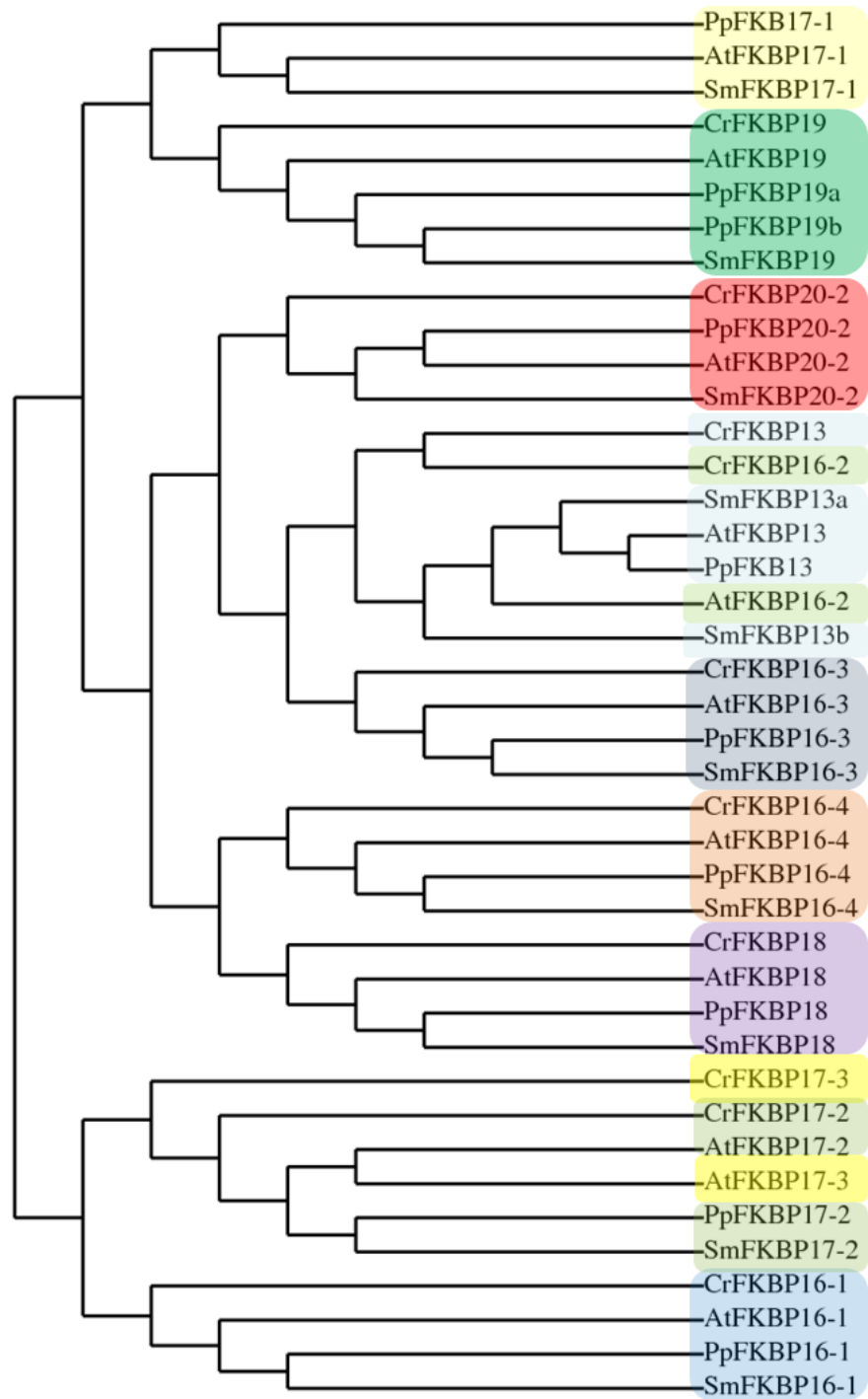


Figure 2.7 Phylogenetic tree with evolutionary relationships of FKBP proteins in green algae, moss, lycophyte and Arabidopsis. Color shades indicate orthologous proteins in the different species.

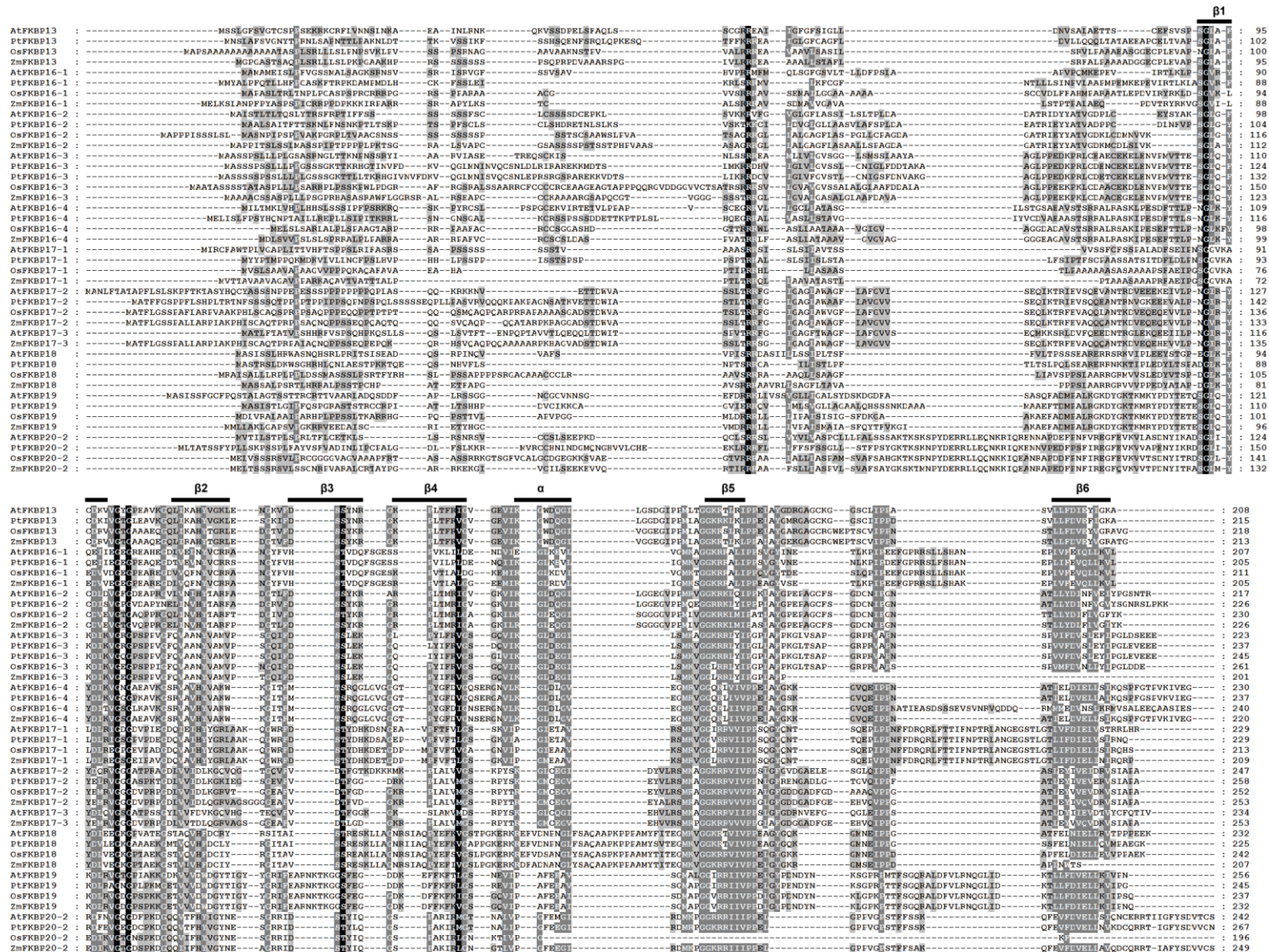


Figure 2.8 Multiple sequence alignment of FKBP from *O. Sativa*, *P. trichocarpa*, *Z. mays* and *A. thaliana*. Secondary structure features (a-helix and b-sheets) derived from the human FKBP according to He et al., 2005. Backgrounds indicate percent of amino acid similarity: black 100%, dark grey 60%, light gray 20%.

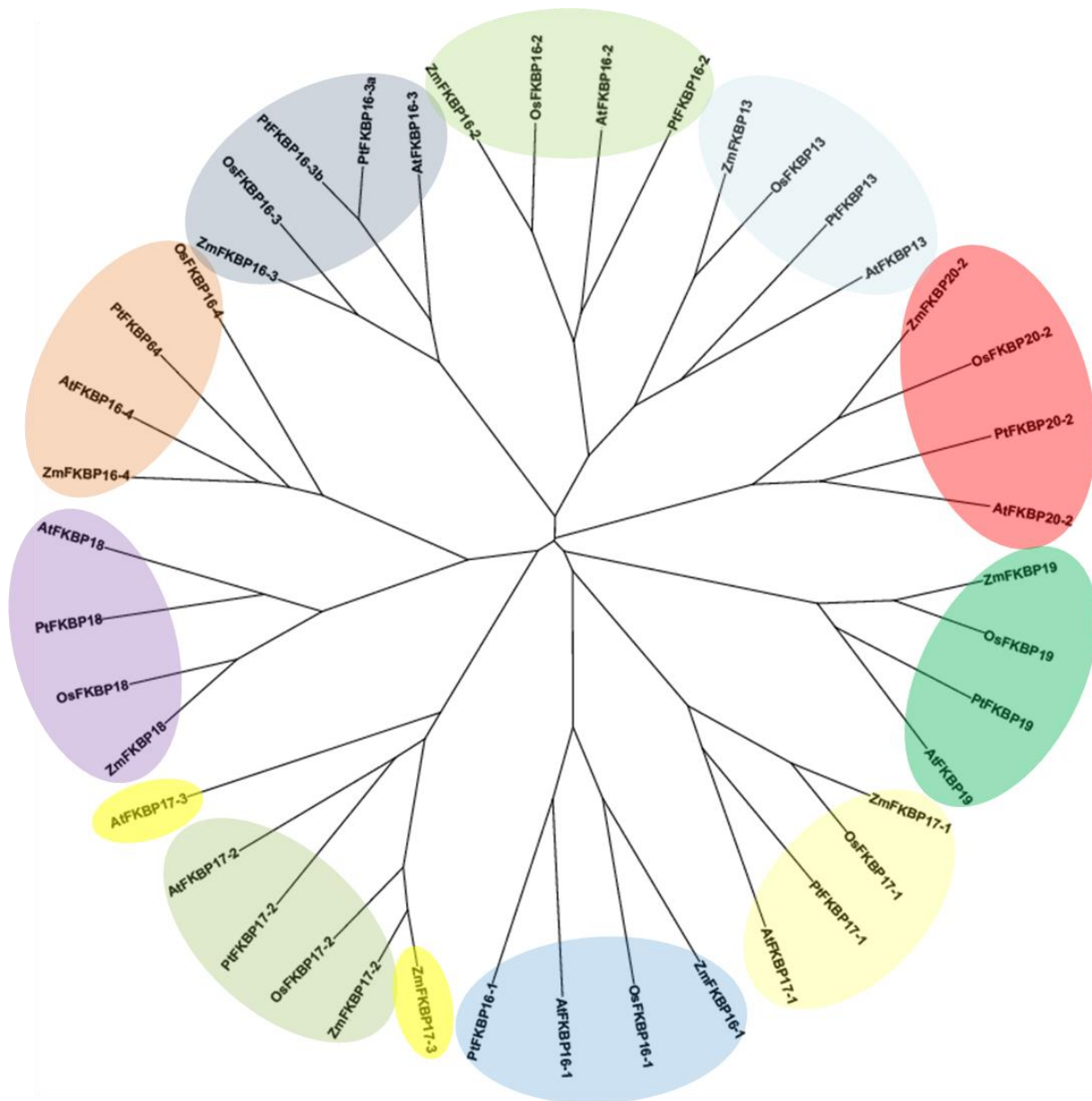


Figure 2.9 Unrooted phylogenetic tree of FKBP relationship in higher plants: rice, maize, poplar and Arabidopsis. The phylogenetic analysis was based on the sequence alignments by ClustalX as described in Methods.

2.3 Methods

2.3.1 Retrieval of FKBP and CYP Sequences

The genomes of all the organisms investigated in this work have been sequenced. Filtered proteins and CDS have also become available in a number of web servers of which all can be accessed through <http://www.plantgdb.org/>. An initial search for Arabidopsis chloroplast immunophilins protein orthologs in rice, maize, poplar,

lycophytes, moss, and green algae was performed using the Department of Energy's Joint Genome Institute (JGI) Phytozome server (<http://www.phytozome.net/>). Additionally, to confirm all chloroplast proteins were identified protein BLAST searches for the entire immunophilin protein family were performed in either the independent genomes server (for ease of access: <http://www.plantgdb.org/>) or in NCBI (<http://www.ncbi.nlm.nih.gov/>).

2.3.2 Sequence Analysis-Phylogenetic Tree Construction

All full length amino acid sequence alignments were performed using ClustalX (<http://www.clustal.org/>) program with default parameters. The ClustalX generated alignment was used to construct Phylogenetic trees with MEGA software (<http://megasoftware.net>) using Neighbor-joining parameters. Representations of the calculated trees were visualized using TreeDyn (<http://www.treedyn.org/>).

2.3.3 Nomenclature of Immunophilin Proteins

All immunophilin protein names were assigned according to previous plant nomenclature conventions such as in *Arabidopsis* (Romano et al., 2004). Where multiple orthologs to the *Arabidopsis* isoforms were identified, a letter was added to the names (e.g. PpCYP37a and PpCYP37b are very similar to *Arabidopsis* CYP37).

CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF IMMUNOPHILINS IN THE THYLAKOID LUMEN BY REVERSE GENETICS: NOVEL FUNCTIONS AND REDUNDANCY

3.1 Introduction

Photosynthesis sustains almost all living organisms directly or indirectly. The thylakoid membrane/lumen system inside the chloroplasts provides the structural platform for photosynthetic transformation of sunlight into metabolic energy forms (see Chapter I). Nevertheless, while photosynthesis is the major function of the thylakoid, this compartment also serves as a sensor that reconfigures its own components in response to environmental and metabolic needs. For instance, thylakoids host functions including protein folding, proteolysis, photo-protection, and redox signaling among others (Armbruster et al., 2011; Spetea, 2012). About 100 proteins reside in the thylakoid lumen, including a unique group of immunophilins of sixteen members representing the largest protein family known in this compartment (Romano et al., 2005). As discussed in Chapter II, a number of studies indicate thylakoid immunophilins are involved in regulating the assembly of photosynthetic membrane protein complexes through interaction with specific photosynthetic subunits. For example, single mutation in AtCYP38 or AtFKBP20-2 significantly affect photosystem II (PSII) assembly, whereas the redox regulated AtFKBP13 has been shown to associate with subunits of the Cytochrome *b₆f* complex (Fu et al., 2007, Lima et al., 2006; Gupta et al., 2004). Additionally, AtCYP20-2 and AtFKBP16-2 are involved in regulating the assembly of NADPH dehydrogenase (NDH) enzyme complex and photosystem I (PSI) (Peng et al., 2009). Since only a fraction of thylakoid immunophilins have been characterized, it is pivotal to investigate the remaining immunophilins for better understanding of functions of these proteins in the lumen, thus advancing our knowledge on photosynthetic protein assembly and other potential regulatory processes. Work presented in this chapter is an attempt to shed new light on immunophilin proteins in the thylakoid lumen. In part I, evidence for the involvement of AtCYP37 in high light stress response is described. Part II discusses the possibility that AtCYP28 is functionally redundant as plants lacking CYP28 do not exhibit any phenotype under a diverse number of stresses. Finally, work in Part III addresses the immunophilins with potential functional redundancy by presenting preliminary work on FKBP double mutants: *fkbp20-2/fkbp16-3*.

3.2 PART I: The Arabidopsis Thylakoid Lumen Cyclophilin 37 is Involved in High Light Stress Response.

3.2.1 Results and Discussion

3.2.1.1 AtCYP37 localizes to the thylakoid lumen and its protein level increases under high light: AtCYP37 with a 466 amino acid sequence is the largest cyclophilin isoform in the chloroplast. All chloroplast cyclophilins share poorly conserved primary structures which potentially underline their functional diversity.

Nevertheless, from a phylogenetic perspective, AtCYP37 appears to be more related to AtCYP38, the second largest chloroplast immunophilin. Immunophilins have been so far suggested to function in maintenance and assembly of photosynthetic machinery; for instance, AtCYP38 is required for proper PSII assembly (Fu et al., 2007). Thus, we were intrigued to determine if AtCYP37 might have similar functions. To examine the function of CYP37, we analyzed a T-DNA insertion mutant in the *CYP37* gene (At3g15520). Sequence analysis showed that the foreign T-DNA insertion site is in the ninth exon of the coding region (Fig. 3.1A). The transcript of *CYP37* was undetectable by RT-PCR in the mutant (Fig. 3.1B), indicating it was a knock out allele. To further confirm this, the mutant was subjected to western blotting using a specific polyclonal antibody against CYP37. This analysis showed there was no protein present in the mutant plants (Fig. 3.1D).

CYP37 contains a chloroplast targeting sequence and proteomic studies have reported the presence of CYP37 in the thylakoid (Schubert et al., 2002). To further document CYP37 localization in the thylakoid, isolated chloroplasts were fractionated into stromal, thylakoid membrane and thylakoid lumen extracts. Each fraction was analyzed by immunoblot with a specific polyclonal antibody against CYP37 or specific polyclonal antibodies against ferredoxin-thioredoxin reductase (FTR) localized to stroma, the rieske iron-sulfur (RFeS) protein in the thylakoid membrane, and plastocyanin (PC) in thylakoid lumen, respectively. The results confirm that CYP37 is a resident in the thylakoid lumen (Fig 3.1C).

Additionally, gene expression analysis by public microarray data and other reports revealed that CYP37 is expressed in photosynthetic tissue (Romano et al., 2004). In order to test whether expression of CYP37 is affected by light, protein levels were tested in plants grown under low light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) and at different time points (8, 16, and 24 hours) after plants were transferred to continuous high light ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$). Interestingly, CYP37 protein was greatly increased during the first 8 hours and subsequently decreased to a stable level after the 16-hour exposure to high light (Fig.3.1E). This data suggest CYP37 maybe important for rapid acclimation response at the onset of plant exposure to high light.

3.2.1.2 The *cyp37* mutant displays defects in anthocyanin accumulation under high light: Although *cyp37* is a knock-out line, the mutant plants grown under normal light ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$), either short-day (10 hr light/14 hr dark) or long-day conditions (16 hr light/8 hr dark), did not exhibit any obvious phenotypic changes as compared to the wild type (Fig 3.2A). As described in this work, CYP37 levels are highly induced upon exposure to high light, further screen to look for defects in the mutant was pursued by exposing the mutants to high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) under a 10 hr light/14 hr dark cycle (Fig 3.2C). Remarkably, contrary to our expectation, *cyp37* plants were less compromised in their resistance to high light treatments than WT plants. Under these light conditions mutant plants remained green for a longer period compared to WT which showed purple color just two to three days after exposure to high light stress (Note: plants

in figure 3.2C are five days after high light exposure). Also, transition to flowering was delayed in mutant plants. Under normal conditions, both the WT and mutant plants did not accumulate significant amounts of anthocyanin; and both flower at similar times. After two days of high light/dark cycle, WT plants accumulated 350 μ g of anthocyanin per gram of fresh weight whereas the mutant accumulated 204 μ g, a 40% less in comparison with WT (Fig. 3.2B). Thus, it appears that although the mutant plants can sense high light stress, they displayed a delayed response in activating photoprotection mechanisms such as anthocyanin biosynthesis.

Generally, plants have evolved a number of defense mechanism typically involving protective and adaptive mechanisms to cope with stresses including high light (Dickison and Chang, 2011; Kreslavski et al., 2012). Exposure to high light disturbs the balance between absorbed light energy and capacity of its photochemical utilization resulting in photoinhibition as reactive oxygen species (ROS) are generated which can be very detrimental to plant survival. This disturbance activates photo-protective defense mechanisms such as dissipation of light and removal of ROS. For instance, high light induces synthesis of anthocyanins that protect plants from light-induced photodamage (Krieger-Liszky et al., 2008; Havaux et al., 2009). Generally, anthocyanins function as sunscreens to attenuate the visible and ultraviolet light penetrating the leaves and thus reducing excitation pressure. Furthermore these molecules serve as antioxidants that aid in protection against oxygen radicals (Zhang et al., 2012). It is intriguing that *cyp37* plants are delayed in accumulation of anthocyanin implying that this immunophilin might be linked to sensing of light stress and activation of defense mechanisms. Relevant to this finding, it is interesting to investigate whether ROS levels are different in the mutant and WT plants. An initial analysis of ROS accumulation in *cyp37* mutant plants grown for 2 weeks in low light (20 μ mol m⁻² s⁻¹) and then transferred to high light conditions for five hours showed the mutants accumulate a high concentration of superoxide, O₂⁻ (Fig 3.2D). It has been previously reported that high accumulation of ROS can inhibit anthocyanin production thus the question remains to be investigated whether *cyp37* reduction in anthocyanin biosynthesis is due to a direct link or as a consequence of higher ROS levels (Gadjev et al., 2006). Superoxide is one of three major reactive oxygen species produced under high light conditions, in addition to hydrogen peroxide, H₂O₂, and hydroxyl radicals, OH[•] (Asada, 2006). Thus, to have a better understanding of ROS behavior in the *cyp37* mutant, both hydrogen peroxide and hydroxyl radicals will still need to be investigated.

3.2.1.3 High light stress affects electron transport rate in *cyp37*, but photosynthetic complex damage is similar to WT plants: Typically, when plants are exposed to high light the photosynthetic activity declines rapidly, as a result of photo-inactivation, also known as “photoinhibition” (Campbell and Tyystjärvi, 2012). This phenomenon occurs as the rate of photodamage to PSII exceeds the rate of repair of damaged PSII. *cyp37* mutant plants have decreased accumulation of anthocyanin under high light, thus we wanted to further investigate if photosynthetic efficiency is different

in the mutant relative to WT plants. To pursue this possibility, we took advantage of chlorophyll fluorescence measurement which can assess overall photosynthetic efficiency with a number of parameters in the leaves of whole plants (Maxwell & Johnson, 2000). In brief, light energy absorbed by chlorophyll molecules in a leaf can undergo three different routes: photochemical quenching, being dissipated as heat (non-photochemical quenching or NPQ) or being re-emitted as light-chlorophyll fluorescence. These three processes occur in competition, thus by measuring chlorophyll fluorescence, information about photochemistry and heat dissipation can be gained. Using a pulse amplitude modulation (PAM) fluorometer, we measured chlorophyll fluorescence in plants grown under normal and high light conditions. Mutant plants grown under normal conditions showed no difference in photosynthetic efficiency compared to WT (Data not shown). However, mutant plants after a 3-day high light-exposure have a higher electron transport rate than the WT (Fig 3.3A). The overall photosynthetic efficiency, represented by Φ_{PSII} , the quantum efficiency of PSII photochemistry at different photon flux, is not significantly altered (Fig. 3.3A). It is surprising that the mutant has a higher electron transport rate, suggesting that the mutant is more efficient in utilizing the light energy, opposite to our initial hypothesis that CYP37 might be involved in maintenance or assembly of photosynthetic machinery.

In addition to further dissecting CYP37 function; we were interested in investigating whether the different protein complexes (such as PSII, PSI, *Cyt_{b6/f}*) are affected in this mutant under high light conditions which could potentially explain the phenotype. To investigate this, thylakoid membranes, from WT and mutant, were isolated and analyzed by Blue native (BN) gel electrophoresis followed by two dimensional separation (2D) and silver staining. The advantage of this method is that it allows us to visualize individual complexes in their native states using the non-denatured procedure and further 2D analysis provides an overview of the major individual subunits of the respective complexes. Thylakoid membranes of WT and mutant plants show normal photosynthetic complex distribution as expected since the mutant does not show any phenotype under normal conditions (Fig 3.3B) Further 2D analyses reveals the major subunits of the different complexes and intermediate complexes are similar in both WT and mutant (Fig 3.3B). For instance, the abundance of individual PSII super complex bands (PSII SC) are not altered in the blue native gel, and silver staining of the subsequent 2D gels show all the major subunits of this complex are equally present in both WT and mutant. Similarly other complexes such as photosystem I (PSI), cytochrome *b_{6/f}* complex (*Cyt b_{6/f}*), the light harvesting complex trimer (LHCII-T) are not affected either. To our Surprise, overall protein complexes appear to behave similarly in both WT and mutant plants exposed to high light for three days (Fig. 3.3C). Although protein levels decreased under high light, both WT and mutant appear to be affected to the same extent (Fig. 3.3C). However, it must be noted that with this method it is difficult to compare the specific abundance of each individual protein between WT and mutant, especially low molecular weight subunits, because the gels were processed separately. Instead, western blotting analysis needs to be carried out on individual subunits by

loading samples in the same gel. This will further provide evidence if the mutant is affected in accumulation of any of photosynthetic subunits.

3.2.1.4 CYP37 interacts with photosynthetic subunits Cyt *f* and PSII subunit PsbM: Immunophilins, regardless of their subcellular location, appear to fulfill their functions by interacting with their target proteins (discussed in Chapter I and Chapter II). To test this possibility with CYP37, we used the yeast two-hybrid (Y2H) assays to identify interacting proteins from the thylakoid lumen and proteins protruding from the thylakoid membrane into the thylakoid lumen as well. We cloned CYP37 cDNA encoding the mature sequence into the expression vector pGBT9.BS (BD, bait). Also, more than 100 proteins from the thylakoids, either soluble (e.g. experimentally confirmed or predicted) and thylakoid lumen exposed membrane proteins (e.g. luminal exposed domains of PSII and PSI subunits) were cloned into pGADGH (AD, prey). Yeast cells carrying CYP37-BD and individual thylakoid proteins in AD plasmids were grown on selection media (synthetic medium lacking Leu (L), Trp (W), and His (H), SC-LWH). Contrary to other immunophilins such as CYP28 (discussed in part II of this chapter) which have numerous interacting partners, CYP37 only interacted with two proteins, cytochrome *f* and PsbM (Fig 3.4A). Cyt *f* is part of the cytochrome *b₆f* complex involved in transferring electrons from PSII to PSI, while PsbM is a relatively uncharacterized low molecular weight subunit of PSII complex (Kurisu et al., 2003; Kawakami et al., 2011). It seems that CYP37 may presumably not interact with both of these two proteins *in vivo*; otherwise, a more detrimental effect might have been observed in the absence of CYP37 in *planta*. Furthermore, Y2H can also produce false positive results. Further *in-vivo* interaction procedures will be carried out to confirm CYP37 interacting partners. An initial 2D BN/PAGE analysis suggested that CYP37 is associated with an unidentified intermediate complex larger than the Cyt *b₆f* complex (Fig. 3.4B). This result may indicate that interaction with Cyt *f* in the yeast two-hybrid may be a false positive. As PsbM antibody was not available, we did not confirm if CYP37 migrates together with this protein in the same complex which could provide further evidence to their potential interaction. Analysis of CYP37 accumulation in a *psbM* mutant background may also provide insights in the functional relevance of CYP37-PsbM interaction.

3.2.2 AtCYP37 Concluding Remarks

In this study, evidence for the characterization of the thylakoid lumen CYP37 in Arabidopsis is presented. Plants lacking CYP37 do not exhibit any observable phenotype under normal conditions, but respond differently to high light exposure. Under high light, mutant plants accumulate less anthocyanin than WT and their electron transport rate is increased. An increase electron transport rate could indicate the mutant is able to utilize light more efficiently than WT thus reducing the pigment accumulation. However, initial ROS accumulation analysis show the mutant accumulates more superoxide, thus hinting to another hypothesis in which anthocyanin biosynthesis is inhibited as potentially result of “too much” reactive oxygen species rather than more efficient photosynthetic

capabilities. Additionally, analyses of photosynthetic protein complex formation and damage by BN/2D PAGE of WT and mutant isolated thylakoid membranes indicate both are equally affected. Interestingly, CYP37 interacts with *cyt f* and PsbM subunits of the *cytb₆f* complex and PSII complex respectively in the Y2H assay. Although, *cyt f* might be a false positive interaction as BN/2D PAGE shows CYP37 binds to an unidentified photosynthetic intermediate protein complex larger than *cytb₆f*. Taken all these data together, it appears CYP37 might not be directly involved in assembly or maintenance of super complexes but potentially working in a pathway involved in light stress response. More than 3,000 nuclear genes in plants encode plastid proteins, while the chloroplast genome contains about 100 genes (Martin et al., 2002). It has been suggested that a tight relationship between plastid development/regulation and nuclear gene expression must exist. Specifically, chloroplast retrograde signaling, in which the plastid emits signals that regulate nuclear gene expression to match the status of the chloroplast has been recently highlighted to play an important role in the acclimation of plant function to environmental stress (Woodson et al., 2013; Chi et al., 2013). For instance, molecular components of retrograde signaling during drought, high light and wounding stress responses are just beginning to be addressed (Li et al., 2013). Anthocyanin genes are encoded in the nucleus thus we hypothesize AtCYP37 might be potentially linked to retrograde signaling as the mutant has a delayed response to high light relative to normal plants. A number of experiments would need to be carried out to decipher the phenotype. Firstly, complete analyses of ROS molecules accumulating in the mutant as well as expression of markers gene involved in anthocyanin biosynthesis would provide insights about anthocyanin deficiency in the mutant. Specifically, these experiments would allow us to determine if the mutant is “blind” (or partially blind) to high light stress or if anthocyanin biosynthesis inhibition is a downstream effect unlinked to gene expression (retrograde signaling). Secondly, western blot analyses of individual protein abundance from the different photosynthetic protein complexes in the mutant would help in determining any subtle changes in protein accumulation not detected by BN/2D PAGE analysis. Thirdly, a careful analysis of the rate of photodamage and recovery will potentially dissect defects in the photosynthetic response in the mutant. For instance, this can be accomplished by inhibiting protein synthesis with lincomycin as previously reported and monitoring photosynthetic efficiency changes at different time points (Fu et al., 2007). Fourthly, complementation of the *cyp37* will provide key confirmation the phenotype observed is caused by absence of CYP37. Therefore, characterization of this immunophilin will very likely provide interesting evidence to strengthen the functional diversity of immunophilins.

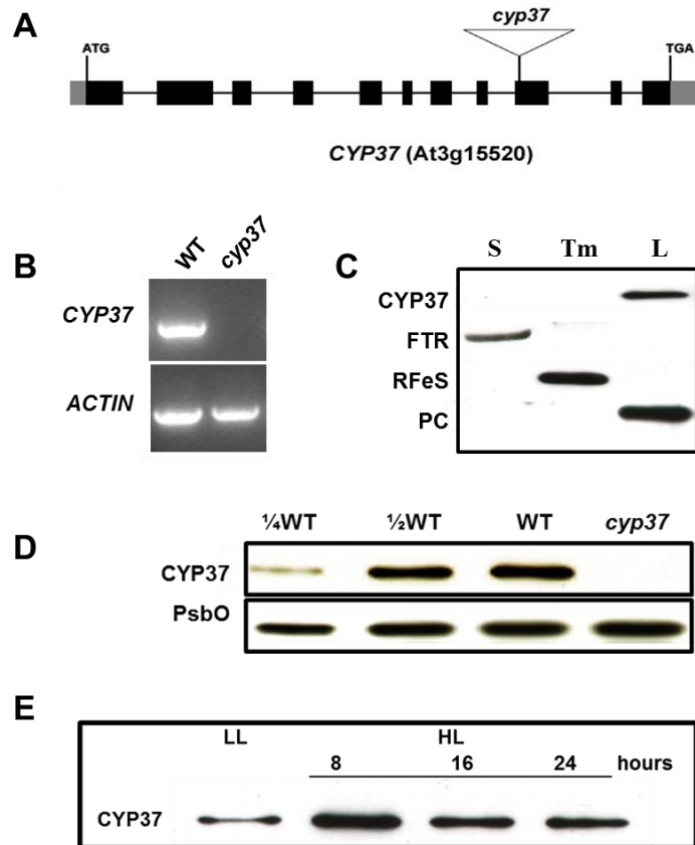


Figure 3.1 Characterization of the *cyp37* mutant and CYP37 thylakoid localization. (A) Localization of T-DNA insertion site in *CYP37* genomic DNA. (B) RT-PCR analysis of *CYP37* mRNA in the WT and *cyp37* mutant. (C) Sub-organelle localization of CYP37 in chloroplast compartments: stroma (S), thylakoid lumen (L), and thylakoid membrane (T). Purified chloroplasts were used to fractionate protein and each fraction was loaded to SDS-PAGE and analyzed by specific polyclonal antibodies against CYP37; PC, Plastocyanin (lumenal marker); FTR, ferredoxin-thioredoxin reductase (stromal marker); and RFeS, Rieske iron-sulfur protein (thylakoid membrane marker). (D) Western blot indicating CYP37 protein levels in WT and *cyp37* (PsbO used as an internal control). (E) CYP37 protein levels in 4 week old plants under low light (LL, $20 \mu\text{mol m}^{-2} \text{s}^{-1}$) and protein levels under high light (HL, $300 \mu\text{mol m}^{-2} \text{s}^{-1}$) at different time points (8, 16 and 24 hours) after transfer from low light to continuous high light. Chlorophyll concentration used as standard.

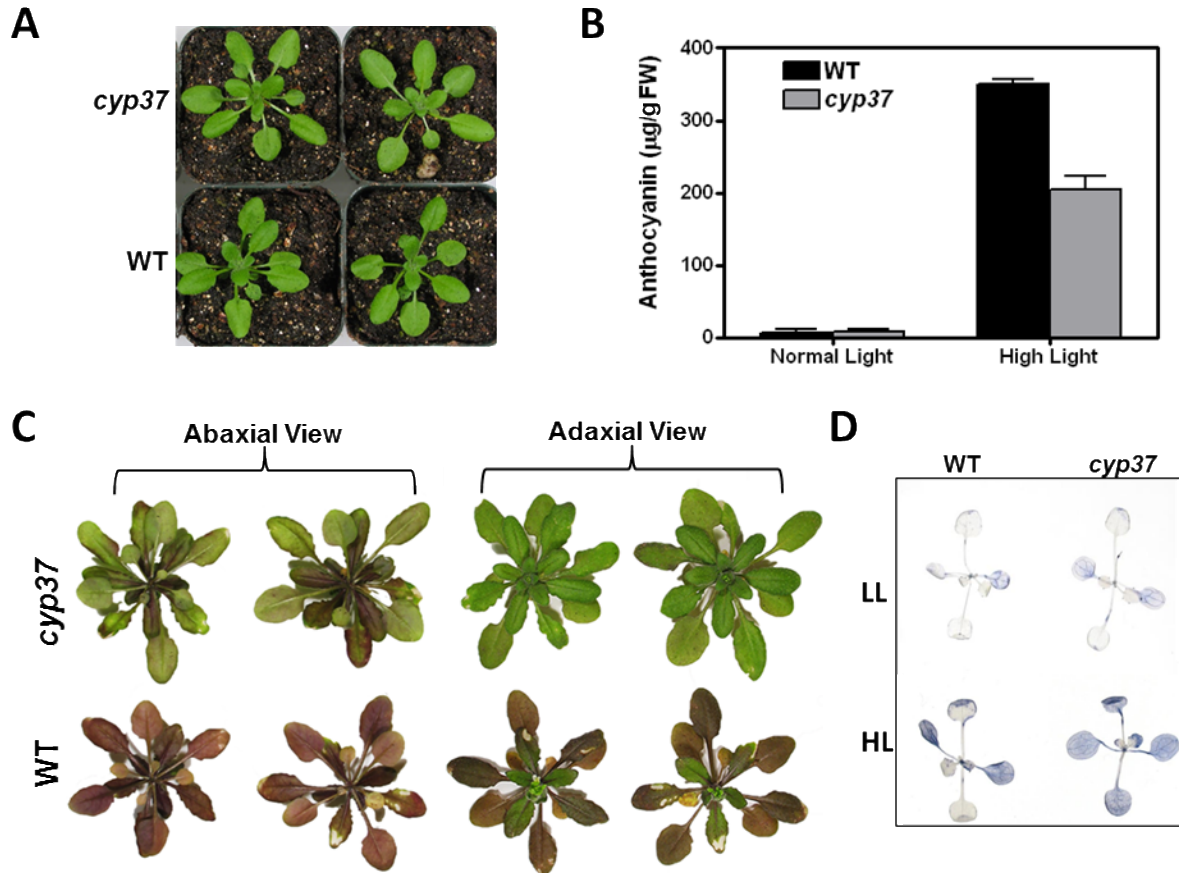


Figure 3.2 Light-induced anthocyanin accumulation is reduced in *cyp37* mutants. (A) 4-week old WT and mutant plants grown under normal long day conditions ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16 hr light/8 hr dark,). (B) Anthocyanin accumulation ($\mu\text{g/g}$ fresh weight) in 4-week old plants after 2-day exposure to high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$, 10 hr light/14 hr dark). (C) 4-week old plant phenotype after exposure to high light treatment for 5 days. (D) Nitro blue tetrazolium (NBT) staining for superoxide accumulation in 2-week old plants after 5 hours exposure to high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) conditions, low light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) used as control (LL-low light, HL-high light).

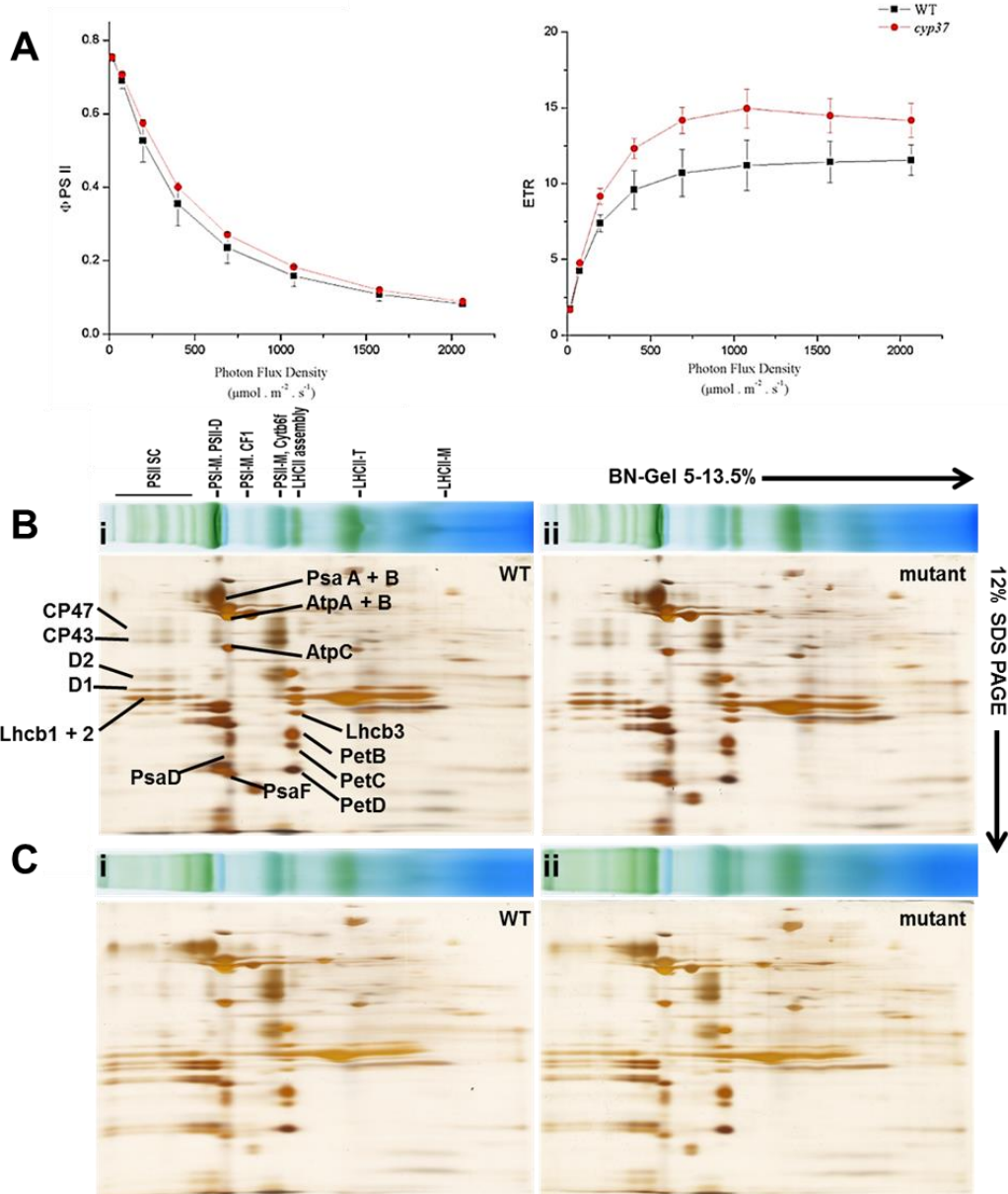


Figure 3.3 Light response curves for chlorophyll fluorescence and analysis of thylakoid membrane protein complexes in *cyp37*. (A) Chlorophyll fluorescence analyses of wild-type (WT) and *cyp37* plants exposed to high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) for two days (10 hr light/14 hr dark). Data represent means \pm SE ($n = 3$). Efficiency of PSII photochemistry (ΦPSII) and electron transport rates (ETR). (B) i-WT, ii-mutant; Thylakoid protein complexes (PSI-M, PSI monomer; PSII-D, PSII dimer; LHCII, PSII light-harvesting complex; LHCII-T, PSII light-harvesting complex trimer; LHCII monomer, PSII light-harvesting complex monomer) from WT and mutant plants grown under normal short day (10 hr light/14 hr dark) conditions were separated by 5–13.5% Blue Native Gel (BN). Native gel slices were further analyzed by 2D SDS/PAGE and silver staining (representative proteins labeled). (C) i-WT, ii-mutant; Thylakoid protein complexes from plants exposed to high light for 3-days (10 hr light/14 hr dark) and analyzed as described for panel A. Protein identification in 2D gels was according to previous report (Fu et al., 2007).

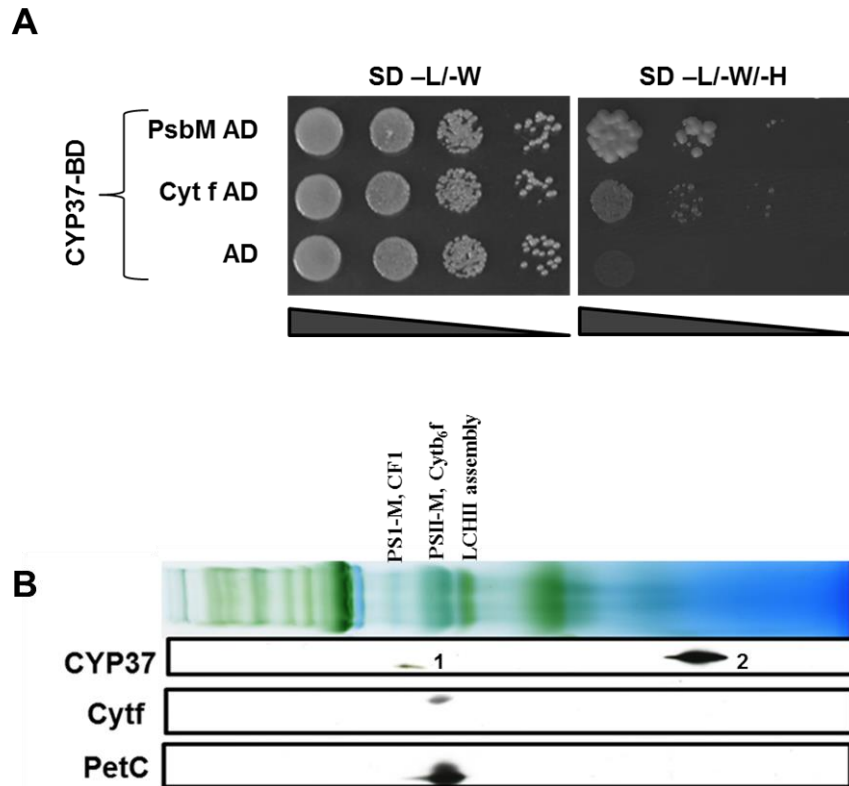


Figure 3.4 CYP37 physically interacts with PS II subunit PsbM and binds to an intermediate photosynthetic complex. (A) DNA encoding mature CYP37 sequence was cloned into the BD containing vector and used to screen a thylakoid membrane/lumenal mini-protein library in the Yeast-two-hybrid system (as described in methods). The growth of yeast, when transformed with these constructs, on selection media indicates protein–protein interaction of the corresponding proteins. Shaded Triangle represents dilution series, SD-L/-W is control media and SD-L/-W/-H/-A is selection media. AD is empty vector for control. (B) BN/2D PAGE analysis of CYP37 binding to photosynthetic complexes using CYP37 specific polyclonal antibody, for reference *cyt f* and Pet C proteins were immunoblotted as well with their respective antibodies. 1-bound CYP37 to unidentified complex, 2-CYP37 soluble form.

3.3 PART II: A number of Stress Treatments do not Affect Growth or Development of CYP28 Knock-out Arabidopsis Plants.

3.3.1 Results and Discussion

3.3.1.1 Isolation of *cyp28* allele and CYP28 subcellular localization: AtCYP28 is a divergent 290 amino acid single-domain cyclophilin isoform predicted to be localized in the thylakoid lumen. To examine the function of CYP28, we analyzed a T-DNA insertional mutant in the *CYP28* gene (At5g35100). Sequence analysis showed that the T-DNA insertion site is in the first exon of the coding region, 370bp downstream of ATG

(Fig. 3.5A). The mRNA of *CYP28* was undetectable by RT-PCR in the mutant (Fig. 3.5B), indicating that the T-DNA insertion (*cyp28*) disrupted the expression of the *CYP28* gene. Further immunoblot analysis showed there was no protein present in the mutant when subjected to a specific polyclonal antibody against CYP28 (Fig. 3.5C).

CYP28 possess N-terminal twin arginine motifs followed by a number of basic residues, which are characteristics in transit peptide sequences of proteins targeted to the chloroplast thylakoid lumen compartment. To examine the subcellular localization of CYP28, we constructed a plasmid with GFP fused to CYP28 and transferred it into tobacco (*Nicotiana benthamiana*) leaves. The CYP28 GFP fusion protein was specifically located in the chloroplast (Fig. 3.6A). Furthermore, isolated Arabidopsis chloroplasts were fractionated to determine the precise sub-organelle location of CYP28 in the chloroplast. The chloroplast fractionation followed by immunoblot analysis with the anti-CYP28 specific polyclonal antibody indicated CYP28 resides in the thylakoid lumen (Fig. 3.6B).

3.3.1.2 Lack of CYP28 does not affect plant growth or development under a number of stress conditions: To evaluate the consequences of inactivation of the gene, we examined the *cyp28* mutant and wild-type plants under normal growth conditions (16 hr light/8 hr dark) throughout their life cycle and observed no phenotypic differences (Fig 3.7A). Microarray data and published studies indicate immunophilins are generally induced by light (Romano et al., 2004). Light is a key requisite for photosynthesis, but at the same time it is well-documented that high light may also cause damage to plants by producing reactive oxygen species (ROS) as the chloroplast is a major source of ROS under stress conditions (Foyer et al., 2011). To investigate whether *cyp28* mutant is affected by light conditions, both mutant and WT were grown or exposed to various light conditions in search of potential defects. However, similar to normal conditions, no visible changes were observed under these stressful light conditions. As discussed in Chapter I, the plant chloroplast has diverse functions, thus a number of different stress conditions such as osmotic, salt, and different ROS inducing treatments were carried out at germination and post germination with unsuccessful outcomes (Data not shown). Although mutant plants were developmentally similar to WT, it was still possible the mutant differed from wild-type at the molecular level. For instance we investigated physiological processes, such photosynthetic efficiency, that might indicate subtle deficiencies/changes in the mutant. Thus, we analyzed photosynthetic efficiency using chlorophyll fluorescence from intact leaves. The *Fv/Fm* ratio, which represents the maximum efficiency of PSII photochemistry, was similar in both mutant and WT plants indicating that overall photosynthetic capabilities are not affected in this mutant (Fig. 3.7B). Further dissection of the different photosynthetic complexes through BN gel electrophoresis analysis confirmed no difference in accumulation of protein complexes in this mutant (Fig 3.7C). Thus, these results imply that CYP28 might be required for a specific function under elusive conditions not tested in this study. Alternatively, it may have a redundant function with other CYP family proteins.

3.3.1.3 CYP28 interacts with a number of photosynthetic subunits: In the context of their function as chaperones or related regulatory capacities, immunophilins typically interact with their target proteins (Described in Chapter I and Chapter II). To hunt for CYP28 interactors, we used a yeast two-hybrid system to screen for potential thylakoid luminal protein targets. In addition to soluble luminal proteins (e.g. experimentally confirmed or predicted), the side loops or segments of thylakoid membrane proteins exposed to the lumen (e.g. PSII and PSII subunit domains) were also tested as well. Interestingly, CYP28 physically interacted with PSII subunits (CP43Z and CP47E), PS I subunits (PsaF), and *Cyt_b_f* subunits (Cyt *f* and RFes) (Fig. 3.8). It is intriguing that CYP28 has multiple interactors whereas some of other immunophilins tested under this system yields no interactors or one or two (e.g. AtCYP37 only has two interactors). Further *in-vivo* and *in-planta* protein-protein approaches will be required to assess the true CYP28 interactors of physiological significance. Additionally, analyses of CYP28 in different photosynthetic mutant backgrounds may expose the elusive role of CYP28 in the thylakoid lumen.

3.3.2 CYP28 Concluding Remarks

The AtCYP28 is a single domain cyclophilin containing no other known domains, and with well-conserved orthologous proteins in all photosynthetic organisms investigated in this study (see Chapter II). AtCYP28 is localized in the thylakoid lumen. Interestingly, the knock-out mutant does not exhibit any phenotype under normal or several different stress conditions such as high light and other inducing stress inducing ROS. The thylakoid lumen plays a complex role in key processes during plant development and in the event of stress conditions (For review: Spetea, 2012). Thus, CYP28 might potentially participate in a specific stress network response which is unrelated to the different conditions investigated in this work. A clue to the potential function of CYP28 might be found in its protein sequence; it contains five cysteine residues, raising the question whether CYP28 might be linked to redox as it has been shown for FKBP13 (See Chapter II; Buchanan and Luan, 2005). The thiol/disulfide redox network is an intricate system that involves redox input elements, redox transmitters, redox targets, and redox sensors (Dietz et al., 2008; for further review: König et al., 2012). Recent studies indicate that redox regulation in the lumen is critical for plant development (Dietz, 2010). It is worth noting that CYP28 is the only luminal immunophilin with five cysteine residues, one more than the redox regulated FKBP13 which only possesses four cysteine residues (Gopalan et al., 2004). Thus, a redox-related quest for CYP28 is intriguing and worth pursuing. Due to limited time I am not able to pursue this quest.

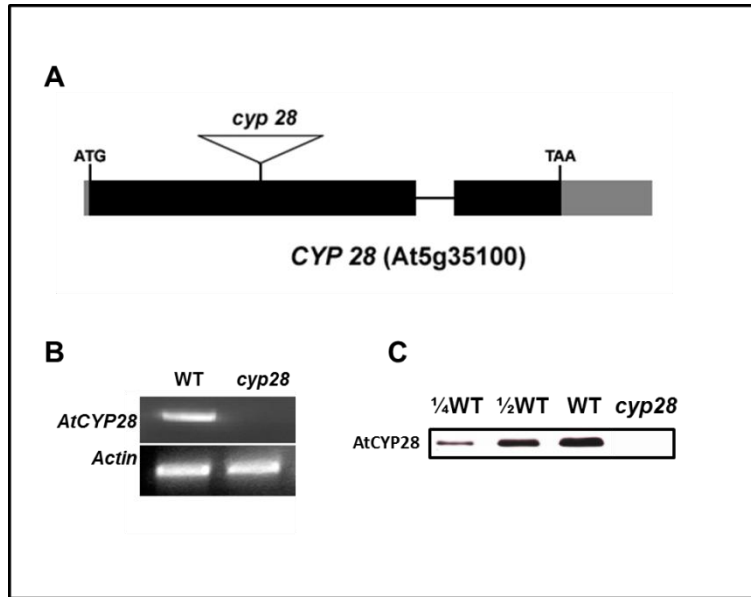


Figure 3.5 Characterization of the *cyp28* mutant. (A) Localization of T-DNA insertion site in *CYP28* genomic DNA. (B) RT-PCR analysis of *CYP28* mRNA in the WT and *cyp28* mutant. (C) Western blot showing CYP28 protein levels in WT and *cyp28*. Chlorophyll concentration used as standard.

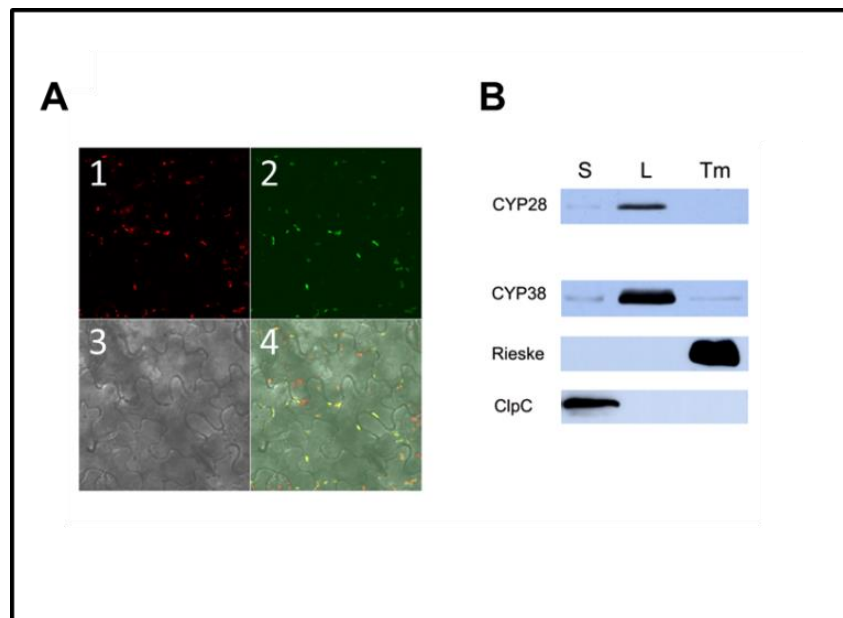


Figure 3.6 CYP28 localizes to thylakoid lumen. (A) CYP28-GFP tag chloroplast localization in *N. Benthamiana*; (1) chlorophyll autofluorescence, (2) CYP28-GFP fluorescence, (3) Bright field, and (4) overlay of panels 1, 2 and 3. (B) Localization of CYP28 in chloroplast compartments: stroma (S), thylakoid lumen (L), and thylakoid membrane (T). Purified chloroplasts were used to fractionate protein and each fraction was loaded to SDS-PAGE gels and analyzed by antibodies against CYP28, PC (luminal marker), ClpC (stromal marker), and Rieske (thylakoid membrane marker).

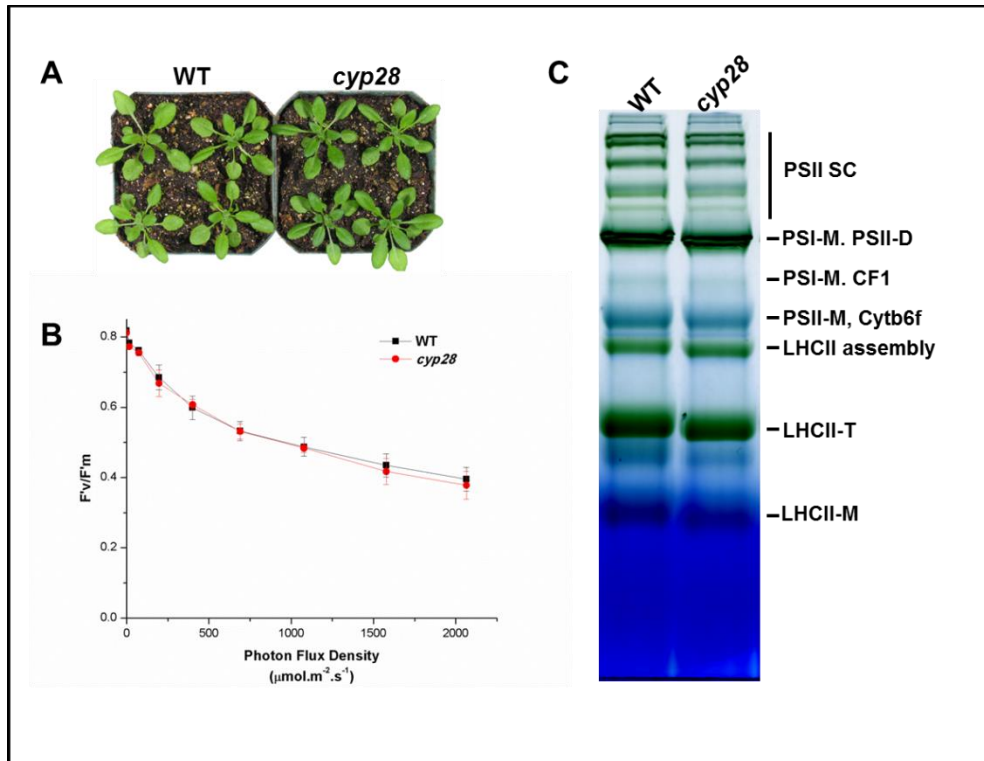


Figure 3.7 *cyp28* phenotype characterization and analysis of thylakoid member protein complexes. (A) 4-week old plant grown in long day conditions (16 hr light/8 hr dark). (B) Chlorophyll fluorescence analyses of WT and *cyp28* plants grown in greenhouse for four weeks. (C) Blue native gel analysis of thylakoid proteins of WT and *cyp28* (15 μg chlorophyll as loading standard).

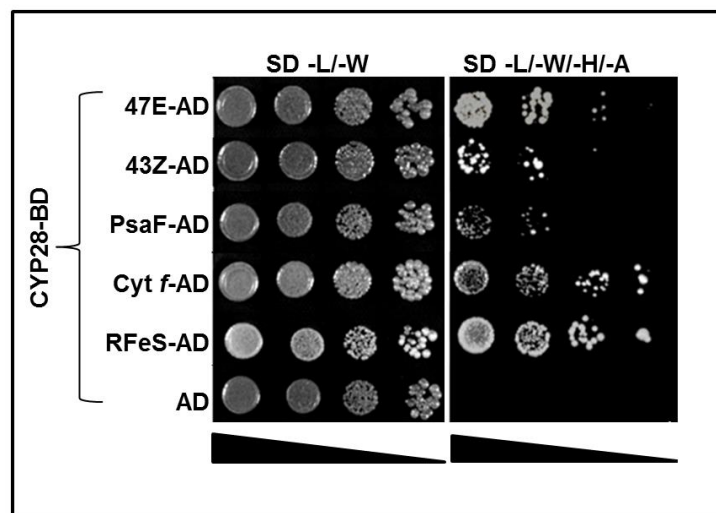


Figure 3.8 CYP28 physically interacts with several thylakoid membrane proteins. DNA encoding mature CYP28 sequence was cloned into the BD containing vector and used to screen a thylakoid membrane/luminal mini-protein library in the yeast two-hybrid system. The growth of yeast, when transformed with these constructs, on selection media indicates protein–protein interaction of the corresponding proteins.

3.4 PARTIII: Lack of Phenotypes in AtFKBPs Single Mutants, Obstacles, and Potential Solutions to Decipher Thylakoid Immunophilin Functions.

The aim of this work is to highlight the major constraints in approaching immunophilin functions in the thylakoid and also to briefly introduce the initial work progress in characterizing double and triple immunophilin mutants.

3.4.1 Obstacles in Thylakoid Immunophilin Characterization.

In reverse genetics, the disruption of a target gene serves as a centrally important tool for exploring the consequences of the loss of gene function. However, it is common for researchers to meet the challenge that single mutants do not display any obvious phenotype. This is not surprising, as organisms such as plants have intricate gene networks that function intertwined with several processes in which simultaneous players may overlap (Kuepfer et al., 2005; khana et al., 2006; Pasek et al., 2006). Although the publically available T-DNA insertional mutant pool is substantial, quite a few *Arabidopsis* genes still lack knock-out T-DNA insertional alleles. Thus at times, functional characterization of proteins is delayed by the unavailability of knock out lines, driving researchers to look for alternative approaches such as RNA interference to knock down the gene of interest.

Due to the unavailability of T-DNA insertional mutants during our characterization of thylakoid immunophilins, I started my thesis work by using RNA interference (RNAi) to knockdown the expression of some immunophilin genes. A glucocorticoid-mediated transcriptional induction system was employed (For review see Aoyama and Chua, 1997). In brief, this system is composed of a constitutively expressed chimeric transcription factor (GVG) which consists of a DNA-binding domain, a trans-activating domain and a glucocorticoid receptor domain. In the absence of the synthetic glucocorticoid dexamethasone (DEX), GVG remains inactive in the cytosol, but in the presence of DEX it is activated and translocated to the nucleus where it activates the expression of a transgene targeting the gene of interest. Although I was able to reduce mRNA level in some of the genes tested, further characterization of these RNAi-lines was discontinued due to a number of artifacts resulting from this RNAi system. For instance, control transgenic plants harboring the empty vector of this system would sometimes display anomalies similar to the observable “phenotype” in the mutant plants (Data not shown). However, it is important to note that more recently some more robust RNAi systems have been developed with little side effects as reported, which could still potentially serve as a tool to explore the uncharacterized immunophilins (Gilchrist and Haughn, 2010; Ahn et al., 2011; Schmidt et al., 2012).

Now, as immunophilin knock-out T-DNA lines become available, a new challenge arose: no observable phenotype. Although most of the different thylakoid immunophilins do not appear to have originated from gene duplication (See Chapter II), some may be functionally redundant. Consequently, it is feasible that construction of multiple mutants

among similar immunophilins would help to reveal their function. In the next part, I will describe an initial approach investigating potential immunophilin redundancy in the thylakoid lumen.

3.4.2 Analysis of Double Immunophilin Mutants

There are 16 immunophilins present in the thylakoid lumen and only a few of the T-DNA available mutants show pronounced phenotypes as described in chapter II. In particular four single domain FKBP (FKBP16-3, -16-4, 17-1, and -20-2) with T-DNA insertion have been previously screened by our lab. Among them, only AtFKBP20-2 mildly showed stunted growth (Lima et al., 2006; TABLE 3.1). These four single domain FKBP are very similar in size and protein structure as they lack any additional domains, and thus a collection of double mutants were generated by crossing single mutants to study the combined effect of these proteins (Table 3.1). Three (*fkbp26-3/fkbp16-3*, *fkbp20-2/fkbp16-4*, *fkbp17-1/fkbp16-4*) out of these six double mutants still did not show any visible phenotype. Two other double mutant combinations (*fkbp20-2/fkbp17-1*, *fkbp17-1/fkbp16-3*) had subtle changes such as plants slightly turning yellowish only under continuous high light conditions ($> 300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), however in this initial observation the overall growth was not affected. Of special interest is the only double mutant *fkbp202/fkbp16-3* which exhibits remarkable growth defects under normal conditions (Fig. 3.9A). In line with the stunted growth phenotype in the *fkbp20-2* mutant, the double mutant is even smaller in size (Fig. 3.9A). Also, in a preliminary analysis the overall photosynthetic capabilities in this double mutant were further compromised compared to either of the single mutants (Fig. 3.9B). Thus, it seems that these two FKBP might somehow have related or partially overlapping functions, in which FKBP20-2 plays a more critical step thus destabilizing the system in its absence. While FKBP16-3 is presumably playing a minor role, which can only be manifested in absence of FKBP16-3 with other proteins such in this case with FKBP20-2. More hints to their potentially related functions come from the yeast two-hybrid screens (Fig 3.10). A screen for partner proteins yielded physical interaction of FKBP16-3 with CYP47 and FKBP20-2 with CP43 (Fig 3.10). Both CP47 and CP43 are subunits of the core complex of PSII. Assembly of PSII is highly coordinated and occurs through a number of distinct assembly intermediates. The assembly of this multi-protein complex initiates with formation of the reaction center complex (composed of D1 and D2), followed by association of CP43/CP47 subunits, and subsequent integration of smaller subunits and the assembly of the water-splitting complex (For detailed review see Rochaix 2011; Nixon et al., 2010). Lima et al reported *fkbp20-2* affects PSII assembly although the mechanism remains unresolved. The assembly of PSII multi-protein complex is a coordinated process involving a number of proteins in which most remain to be discovered. Thus, the generation of the *fkbp16-3/fkbp20-2* double mutant may add an important piece of information how these proteins coordinate to maintenance/assemble PSII.

As discussed in Chapter II, several chloroplast immunophilins might be under redox regulation. For instances, it has been shown that AtFKBP13 and AtCYP20-3 are redox regulated (Gopalan et al., 2004; Dominguez-Solis et al., 2008). Both FKBP20-2 and FKBP16-3 have at least one pair of cysteine residues, which affect the CP43 and CP47 interaction in Y2H when mutated, respectively (Fig. 3.10, Table 3.2). It is intriguing that FKBP16-3 no longer interacts with CYP47 when one of the cysteine residues is mutated. By contrast, a similar mutation in FKBP20-2 did not abolish its interaction with CYP43 but further stabilized the interaction with CYP47. Complementation studies in the double mutant using mutated version would clarify the significance of this redox regulation at the physiological level.

Here, our work gives a good example to support the hypothesis that chloroplast immunophilins tend to have overlapping functions in a certain physiological process. This functional redundancy could also partially explain why there are so many immunophilins in a relatively small proteome of the plant chloroplast. Future efforts will be directed to elucidate the detailed function of all the immunophilins and their target proteins through a genetic analysis of multiple mutants, couple with biochemical and molecular studies.

Table 3.1 List FKBP single and double mutants screened for phenotypes.

T-DNA single mutants	Phenotype
<i>fkbp16-3</i> (SALK_026390C)	No
<i>fkbp16-4</i> (SALK_099864)	No
<i>fkbp17-1</i> (SALK_080914C)	No
<i>fkbp20-2</i> (SALK_134696)	smaller plants/PSII assembly. ^a
T-DNA double mutants	Phenotype
<i>fkbp16-3/fkbp16-4</i>	No
<i>fkbp16-3/fkbp20-2</i>	Light yellow/smaller
<i>fkbp20-2/fkbp16-4</i>	No
<i>fkbp20-2/fkbp17-1</i>	Slightly pale in high light ^b
<i>fkbp17-1/fkbp16-4</i>	No
<i>fkbp17-1/fkbp16-3</i>	Slightly pale in high light ^b

*T-DNA lines identification number are in parenthesis and were obtained from the Arabidopsis resource center (Columbus, Ohio).^a Lima et al., 2006. ^b Phenotype only observed under continuous high light conditions (> 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)

Table 3.2 List of Arabidopsis FKBP s and their respective number of cysteine residues.

Gene ID	Cysteine	Cysteine pair
AtFKBP13	4	2(1NT/1CT)
AtFKBP16-1	1	0
AtFKBP16-2	4	2(1NT/1CT)
AtFKBP16-3	2	1(NT)
AtFKBP16-4	0	0
AtFKBP17-1	0	0
AtFKBP17-2	1	0
AtFKBP17-3	2	1
AtFKBP18	1	0
AtFKBP19	0	0
AtFKBP20-2	3	1(CT)

* Cysteine pairs either form in the N-terminal (NT) or C-terminal (CT) regions of the protein.

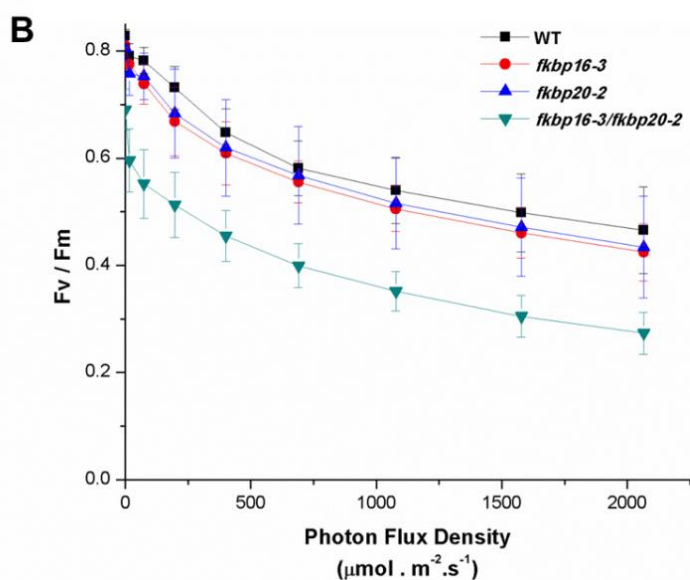
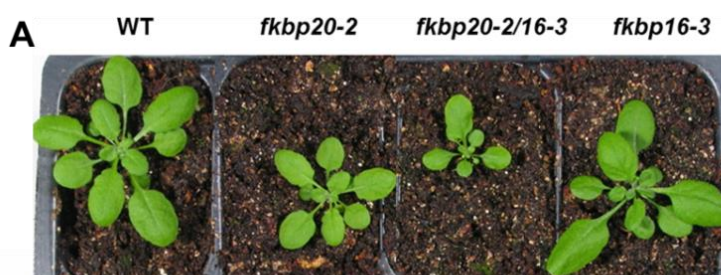


Figure 3.9 *fkbp20-2/fkbp16-3* exhibits stunted growth and overall photosynthetic efficiency is significantly altered. (A) 4-week old plant grown in long day conditions (16 hr light/8 hr dark). **(B)** Chlorophyll fluorescence analyses of WT and double mutant plants grown in greenhouse for four weeks.

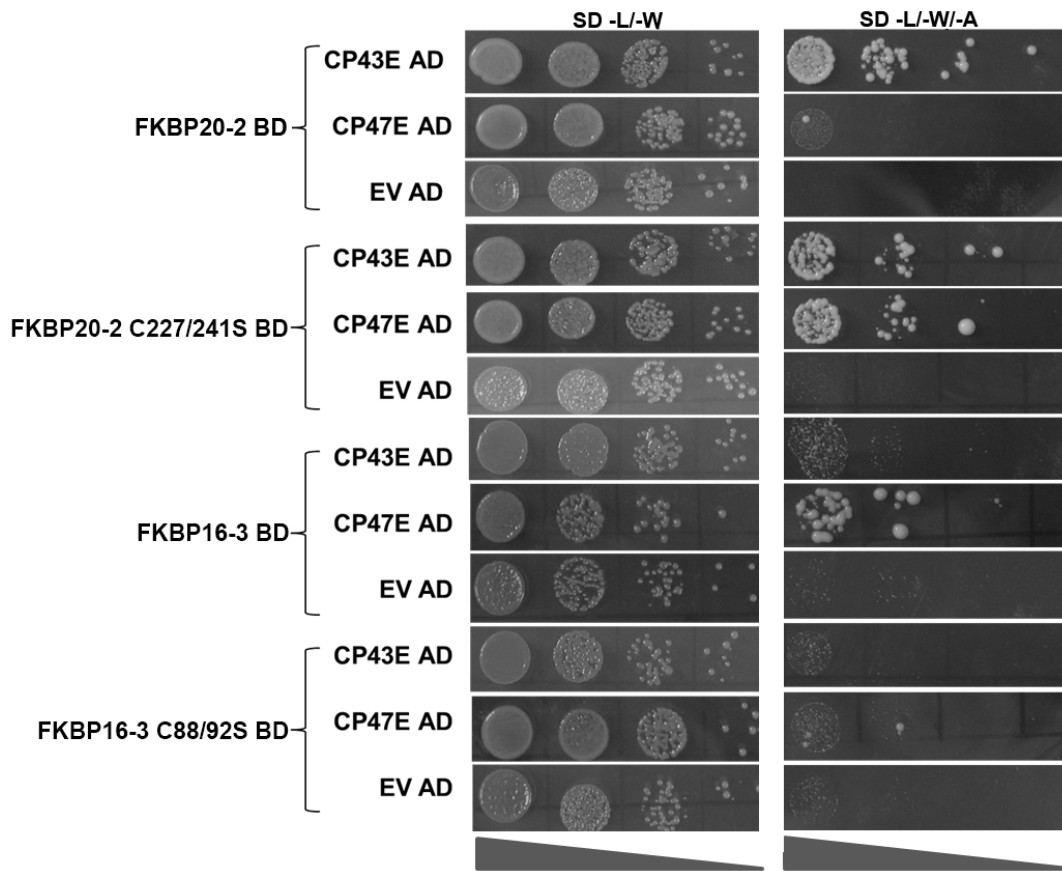


Figure 3.10 FKBP20-2 and FKBP16-3 associate with CYP43 and CP47 respectively: DNA encoding mature FKBP20-2 or FKBP16-3 sequence was cloned into the BD containing vector. Mutated versions (cysteine to serine residue) of FKBP20-2 and FKBP16-3 were cloned as well. Each construct were used to screen a thylakoid membrane/luminal mini-protein library in the Yeast-two-hybrid system. The growth of yeast, when transformed with these constructs, on selection media indicates protein–protein interaction of the corresponding proteins.

3.5 PART IV-Materials and Methods

3.5.1 Plant Materials and Growth Conditions

Arabidopsis thaliana, ecotype Columbia-0, was used in these studies. The T-DNA insertion mutants for the respective genes were obtained from the Arabidopsis Resource Center (Columbus, OH): AtCYP37, SK5097 (stock CS100179); AtCYP28, SALK_071190C; AtFKBP20-2, SALK_134696; AtFKBP16-3, SALK_026390C. Homozygous lines were isolated by confirming the T-DNA insert by PCR. RT-PCR was used to verify expression levels in homozygous plants. RNA isolation, cDNA synthesis, and RT-PCR were performed according to standard procedures. Null mutant was further confirmed with immunoblot analysis using an antibody against CYP37 and CYP28, respectively. For soil-grown plants, sown seeds were cold-treated for at least 3 days, and then

transferred to the indicated growth conditions. For plate-grown plants, seeds were surface-sterilized with isopropanol for 3 minutes and 50% bleach for 3 minutes and then washed 5 times with sterilized distilled water. The washed seeds were plated on 0.8% agar plates, containing half-strength MS medium with .5% sucrose, cold treated for 3 days and then grown under same growth conditions as soil grown plants.

3.5.2 Chloroplast Isolation and Preparation of thylakoids

Thylakoid membranes were isolated as previously described (Fu et al., 2007). For Blue native gel, Arabidopsis leaf tissues were homogenized in ice-cold homogenization buffer (0.33M sorbitol/ 10mM EDTA/ 50mM Hepes, pH 8.0). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA). The filtrate was centrifuged (at 8,000 x g for 5 min at 4°C). The pellet was re-suspended in homogenization buffer, overlaid on 40% Percoll solution (40%, wt/vol, Percoll / 330mM sorbitol/ 30mM Mops-KOH, pH 7.8/2 mM EDTA/2 mM ascorbate) and centrifuged (at 8000 x g for 15 min at 4°C). Isolated thylakoids were resuspended in the same buffer to a final Chlorophyll concentration of 1 mg/ml.

3.5.3 Chloroplast Fractionation

10-20 grams of Arabidopsis leaves were harvested and chloroplast isolated as previously described (Lima et al., 2006). Further fractionation included washing and re-suspension of intact chloroplast in 0.33M sorbitol/50mM Hepes, pH 8.0 buffer. Chloroplast were lysed with the following lysing buffer; 62.5 mM Tris-Cl, pH 7.5 2 mM MgCl, and incubated for 5 min on ice. Stromal fraction was obtained by centrifugation for 15 min at 4°C (14000 x g). In brief, thylakoid membrane fraction and lumenal fraction were obtained by pre-treatment with 0.1% DM and lysing buffer and 30 min centrifugation (Fu et al., 2007).

3.5.4 Blue Native PAGE and 2D-SDS PAGE

Blue native gel electrophoresis was performed as previously described (Lima et al., 2006; Fu et al., 2007). For 2D-SDS PAGE, lanes of blue native gel were excised with a razor blade and incubated in 2-X SDS sample buffer containing 5% β -mercaptoethanol for 15 minutes at 25 °C and then for 15 minutes at 70 °C. Each lane with denatured proteins was placed on top of a 12% SDS PAGE gel and subjected to second dimension separation. After electrophoresis, gels were stained with silver (Schwenkert et al., 2006).

3.5.5 Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence was measured with a FMS2 fluorometer (Hansatech, Norfolk, UK) attached to intact leaves that had been dark adapted overnight or for 15 minutes for plants that had been exposed to high light treatment. Plants were subjected to a saturating light pulse and then illuminated for a programmed series of 5-min periods of increasing light intensities. Between steps in actinic photon flux density, the minimum fluorescence in the light-adapted state (F_o') was determined during a 1-s period of far-red illumination. The maximum efficiency of PSII photochemistry (F_v/F_m) was defined as

$(F_m - F_o)/F_m$. Efficiency of PSII electron transport, Φ_{PSII} , was defined as $(F'_m - F_s)/F'_m$. F'_m is the maximum fluorescence in any light-adapted states, F_s is the steady-state value of fluorescence immediately before the light flash, and F_m is the maximum fluorescence in the dark-adapted state.

3.5.6 Anthocyanin Measurements

Anthocyanins were extracted in water from leaves (0.1 grams) by grinding in liquid nitrogen. After centrifugation at 16,000g for 10 minutes, the supernatant was diluted with 0.4 M sodium acetate (pH 4.5) and 25 mM potassium chloride (pH 1.0). Total monomeric anthocyanin was measured by recording the A_{520} as described by Giusti and Wrolstad, 2001.

3.5.7 Yeast two-hybrid

DNA encoding mature protein sequence of CYP37, CYP28, FKBP20-2, FKBP16-3, respectively, was cloned into a binding domain (BD) vector (pGBT9BS) and an activation domain (AD) vector (pGADGH). The resulting BD and AD constructs were selectively introduced into the yeast strain AH109 by a lithium acetate-mediated transformation method (Ito et al., 1983). Yeast transformants carrying both BD and AD vectors were initially selected on synthetic complete agar (SC)-Leu-Trp medium. To screen protein-protein interaction, exponentially grown yeast cells were harvested and adjusted to OD₆₀₀ = 0.5 with sterilized double-distilled water and diluted 1/10, 1/100, and 1/1000. Five-microliter yeast cells were spotted onto the interaction selection media (synthetic complete agar-Leu-Trp-His and/or SC-Leu-Trp-His-Ade) to score growth as an indicator of protein-protein interaction (Li et al., 2006). To identify potential interaction partners for CYP37 in thylakoids, we constructed a chloroplast luminal protein/domain yeast two-hybrid library. DNA fragments encoding experimentally identified luminal proteins, predicted luminal proteins (Peltier et al., 2002; Schubert et al., 2002), luminal domains of PSII major proteins, low molecular mass subunits (Shi and Schröder, 2004; Loll et al., 2005), luminal domains of photosystem I subunits (Amunts et al., 2007), and Cytb₆f complex (Stroebel et al., 2003) were cloned into the AD vector pGADGH to form the mini-library. This library was screened against BD vectors harboring DNA sequences encoding mature proteins of the different immunophilins studied in this thesis.

3.5.8 Detection of Reactive Oxygen Species

For superoxide detection, 2-week-old plants grown on ½ MS media with .5% sucrose were vacuum-infiltrated with 0.1 mg/mL nitroblue tetrazolium in 25 mM Hepes buffer, pH 7.6. Samples were incubated at room temperature in the dark for 2 h. To remove chlorophylls, the stained samples were transferred to 80% ethanol and incubated at 70°C for 10 min.

CHAPTER 4
GENERAL SUMMARY

4.1 General Summary

The overall aim of this thesis was to study the functions of immunophilins in the thylakoid lumen of chloroplasts using *Arabidopsis* as a model plant. Immunophilins constitute a ubiquitous and highly conserved family of proteins which are classified into two sub-families, namely FK506-binding proteins (FKBPs) and cyclosporine-binding proteins (cyclophilins, CYPs). Genetic and biochemical studies have identified immunophilin homologs from a wide variety of organisms including plants. Immunophilins are present in various subcellular compartments. Specifically, immunophilins are a significant component of the chloroplast proteome. For instance, of 52 immunophilins present in *A. thaliana*, 16 are in the chloroplast thylakoid lumen suggesting that these proteins play essential roles in this relatively uncharacterized subcellular compartment. Indeed, current studies indicate chloroplast immunophilins serve as critical players in the maintenance/stability of photosynthetic complexes. However, more than one third of these proteins await characterization. Thus, we began this thesis work with the goal of contributing to our understanding of these proteins in the thylakoid lumen.

Firstly, through phylogenetic analysis we investigated the evolutionary relationships of chloroplast immunophilins across plant genomes ranging from cyanobacteria and green algae to higher plants. Altogether, we collected 50 cyclophilins and 73 FKBPs in various plant species. Phylogenetic tree construction revealed a well conserved evolutionary relationship among the respective groups of orthologs. Minor discrepancies were observed such as homolog duplications in the moss or missing homologs in rice and poplar. A hypothesis for this “random” duplication might be the result of key functions carried out by these specific proteins. For instance, CYP38 is required for PSII assembly in *Arabidopsis*, and moss contains three orthologs of this protein. Also, we only identified two CYPs, but no FKBPs in cyanobacteria thylakoids, suggesting a significant diversification of these proteins during the evolution from cyanobacteria to land plants. These results indicate chloroplast immunophilins are structurally conserved from green algae to higher plants, which could imply conserved functions as well.

Secondly, we took a reverse genetic approach to elucidate the functions of *Arabidopsis* immunophilins. In particular, we focused on the functional analysis of the thylakoid lumen-localized CYP37, which was up-regulated by high light. Plants lacking CYP37 were “insensitive” to high light stress and showed delayed accumulation of anthocyanins. Molecular evidence indicates that CYP37 interacted with PsbM and/or Cyt *f*; however, photosynthetic complex assembly was not significantly affected in the mutant. Our results suggest that CYP37 might be functioning in light stress response.

Additionally, in this thesis we characterize several other chloroplast immunophilins such as AtCPY28. Although we confirmed its localization in the thylakoid lumen, loss of function mutant did not show any detectable phenotype under a number of stress treatments such as high light and ROS. Proteins in the thylakoid lumen are involved in a wide variety of functions and conditions. Thus, CYP28 may be required

only under specific conditions not tested in this study or it is functionally redundant with other immunophilin proteins.

Lastly, to provide some evidence of potential redundancy among the chloroplast immunophilins, we generated a collection of AtFKBP double mutants from the following single mutants: *Atfkbp16-3*, *-16-4*, *17-1*, and *-20-2*. Surprisingly, only one double mutant (*fkbp20-2/fkbp16-3*) displayed an obvious phenotype. Growth and photosynthetic efficiency in this double mutant were greatly impaired. Furthermore, molecular evidence using Y2H indicate that FKBP20-2 and FKBP16-3 target PSII subunits CP43 and CYP47, respectively. Taken together, our results indicate these two FKBP might have partial functional redundancy as both might be implicated in the initial step of PSII assembly by their interaction with CYP43/CP47.

In conclusion, the work presented in this thesis supports the hypothesis that chloroplast immunophilins have strong functional redundancy in regulation of photosynthesis-related processes including the assembly of photosynthetic complexes and adaptation to various light intensities in the natural environment.

REFERENCES

- Ahn, J., Kim, D. W., You, Y., Seok, M., Park, J., Hwang, H. & Cho, H. (2010). Classification of rice (*Oryza sativa* L. Japonica nipponbare) immunophilins (FKBPs, CYPs) and expression patterns under water stress. *BMC plant biology*, 10 (1), 253.
- Ahn, C. S., Han, J. A., Lee, H. S., Lee, S., & Pai, H. S. (2011). The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. *The Plant Cell Online*, 23 (1), 185-209.
- Amunts, A., Drory, O., and Nelson, N. (2007). The structure of a plant photosystem I supercomplex at 3.4 Å resolution. *Nature* 447: 58–63.
- Aoyama, T., & Chua, N. H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. *The Plant Journal*, 11(3), 605-612.
- Arévalo-Rodríguez, M., Cardenas, M. E., Wu, X., Hanes, S. D., & Heitman, J. (2000). Cyclophilin A and Ess1 interact with and regulate silencing by the Sin3–Rpd3 histone deacetylase. *The EMBO journal*, 19(14), 3739-3749.
- Arevalo-Rodriguez, M., Wu, X., Hanes, S. D., & Heitman, J. (2004). Prolyl isomerases in yeast. *Front. Biosci*, 9(1-3), 2420-2446.
- Arévalo-Rodríguez, M., & Heitman, J. (2005). Cyclophilin A is localized to the nucleus and controls meiosis in *Saccharomyces cerevisiae*. *Eukaryotic cell*, 4(1), 17-29.
- Armbruster, U., Pesaresi, P., Pribil, M., Hertle, A., & Leister, D. (2011). Update on chloroplast research: new tools, new topics, and new trends. *Molecular plant*, 4(1), 1-16.
- Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology*, 141(2), 391-396.
- Aviezer-Hagai, K., Skovorodnikova, J., Galigniana, M., Farchi-Pisanty, et al., (2007). Arabidopsis immunophilins ROF1 (AtFKBP62) and ROF2 (AtFKBP65) exhibit tissue specificity, are heat-stress induced, and bind HSP90. *Plant molecular biology*, 63(2), 237-255.
- Baker, E. K., Colley, N. J., & Zuker, C. S. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *The EMBO journal*, 13(20), 4886.
- Balk, J., & Lobréaux, S. (2005). Biogenesis of iron–sulfur proteins in plants. *Trends in plant science*, 10(7), 324-331.
- Banks, J. A., Nishiyama, T., Hasebe, M., Bowman, J. L., Gribskov, M., dePamphilis, C., & Manning, G. (2011). The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. *Science*, 332(6032), 960-963.

- Bannikova, O., Zywicki, M., Marquez, Y., Skrahina, T., Kalyna, M., & Barta, A. (2013). Identification of RNA targets for the nuclear multidomain cyclophilin atCyp59 and their effect on PPIase activity. *Nucleic acids research*, *41*(3), 1783-1796.
- Barik, S. (2006). Immunophilins: for the love of proteins. *Cellular and Molecular Life Sciences CMLS*, *63*(24), 2889-2900.
- Baumberger, N., & Baulcombe, D. C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(33), 11928-11933.
- Belfiore, M., Pugnale, P., Saudan, Z., Puoti, A., 2004. Roles of the *C. elegans* cyclophilin-like protein MOG-6 in MEP-1 binding and germline fates. *Development* *131*, 2935–2945.
- Bell, A., Monaghan, P., & Page, A. P. (2006). Peptidyl-prolyl cis–trans isomerases (immunophilins) and their roles in parasite biochemistry, host–parasite interaction and antiparasitic drug action. *International journal for parasitology*, *36*(3), 261-276.
- Berardini, T. Z., Bollman, K., Sun, H., & Scott Poethig, R. (2001). Regulation of vegetative phase change in Arabidopsis thaliana by cyclophilin 40. *Science Signaling*, *291*(5512), 2405.
- Bierer, B. E., Somers, P. K., Wandless, T. J., Burakoff, S. J., & Schreiber, S. L. (1990). Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science*, *250*(4980), 556-559.
- Bissoli, G., Niñoles, R., Fresquet, S., Palombieri, S., Bueso, E., Rubio, L., et al (2012). Peptidyl-prolyl cis-trans isomerase ROF2 modulates intracellular pH homeostasis in Arabidopsis. *The Plant Journal*, *70*(4), 704-716.
- Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., et al. (1997). The complete genome sequence of Escherichia coli K-12. *science*, *277*(5331), 1453-1462.
- Borel, J. F., Feurer, C., Magnee, C., & Stähelin, H. (1977). Effects of the new anti-lymphocytic peptide cyclosporin A in animals. *Immunology*, *32*(6), 1017.
- Bouchard, R., Bailly, A., Blakeslee, J. J., Oehring, S. C., Vincenzetti, V., Lee, O. R., et al (2006). Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. *Journal of Biological Chemistry*, *281*(41), 30603-30612.
- Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975). Consideration of the possibility that the slow step in protein denaturation reactions is due to cis-trans isomerism of proline residues. *Biochemistry*, *14*(22), 4953-4963.
- Breusegem, F. (2006). Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiology*, *141*(2), 436-445.
- Buchanan, B. B., & Luan, S. (2005). Redox regulation in the chloroplast thylakoid lumen: a new frontier in photosynthesis research. *Journal of experimental botany*, *56*(416), 1439-1447.

- Buchanan, B. B., Gruissem, W., & Jones, R. L. (2000). *Biochemistry & molecular biology of plants* (pp. 1177-1189). Rockville: American Society of Plant Physiologists.
- Campbell, D. A., & Tyystjärvi, E. (2012). Parameterization of photosystem II photoinactivation and repair. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1817(1), 258-265.
- Chi, W., Ma, J., & Zhang, L. (2012). Regulatory factors for the assembly of thylakoid membrane protein complexes. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367(1608), 3420-3429.
- Chi, W., Sun, X., & Zhang, L. (2013). Intracellular Signaling from Plastid to Nucleus. *Annual review of plant biology*, Vol. 64
- Cianciotto, N. P., Eisenstein, B. I., Mody, C. H., Toews, G. B., & Engleberg, N. C. (1989). A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infection and Immunity*, 57(4), 1255-1262.
- Cioffi, D. L., Hubler, T. R., & Scammell, J. G. (2011). Organization and function of the FKBP52 and FKBP51 genes. *Current opinion in pharmacology*, 11(4), 308-313.
- Colley, N. J., Baker, E. K., Stamnes, M. A., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is required in the secretory pathway. *Cell*, 67(2), 255-263.
- Dickinson, B. C., & Chang, C. J. (2011). Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature chemical biology*, 7(8), 504-511.
- Dietz, K. J. (2008). Redox signal integration: from stimulus to networks and genes. *Physiologia Plantarum*, 133(3), 459-468.
- Dietz, K. J., & Pfannschmidt, T. (2011). Novel regulators in photosynthetic redox control of plant metabolism and gene expression. *Plant physiology*, 155(4), 1477-1485.
- Dolinski, K. J., & Heitman, J. (1999). Hmo1p, a high mobility group 1/2 homolog, genetically and physically interacts with the yeast FKBP12 prolyl isomerase. *Genetics*, 151(3), 935-944.
- Dolinski, K., Muir, S., Cardenas, M., & Heitman, J. (1997). All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, 94(24), 13093-13098.
- Dominguez-Solis, J. R., He, Z., Lima, A., Ting, J., Buchanan, B. B., & Luan, S. (2008). A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. *Proceedings of the National Academy of Sciences*, 105(42), 16386-16391.
- Duina, A. A., Kalton, H. M., & Gaber, R. F. (1998). Requirement for Hsp90 and a Cyp-40-type cyclophilin in negative regulation of the heat shock response. *Journal of Biological Chemistry*, 273(30), 18974-18978.
- Dumont, F. J., & Su, Q. (1995). Mechanism of action of the immunosuppressant rapamycin. *Life sciences*, 58(5), 373-395.

- Earley, K. W., & Poethig, R. S. (2011). Binding of SQUINT to Hsp90 is required for the function of this Cyclophilin 40-related protein in Arabidopsis. *Journal of Biological Chemistry*, 18; 31(2): 267–278
- Earley, K., Smith, M., Weber, R., Gregory, B., and Poethig, R. (2010) An endogenous F-box protein regulates ARGONAUTE1 in *Arabidopsis thaliana*. *Silence* 1:15
- Edvardsson, A., Eshaghi, S., Vener, A. V., & Andersson, B. (2003). The major peptidyl-prolyl isomerase activity in thylakoid lumen of plant chloroplasts belongs to a novel cyclophilin TLP20. *FEBS letters*, 542(1), 137-141.
- Edvardsson, A., Shapiguzov, A., Petersson, U. A., Schröder, W. P., & Vener, A. V. (2007). Immunophilin AtFKBP13 sustains all peptidyl-prolyl isomerase activity in the thylakoid lumen from *Arabidopsis thaliana* deficient in AtCYP20-2. *Biochemistry*, 46(33), 9432-9442.
- Finkemeier, I., and Leister, D. (2010). Plant chloroplasts and other plastids. In *Encyclopedia of Life Sciences (ELS)* (Chichester:John Wiley & Sons, Ltd).
- Fischer G, Bang H, Mech C. (1984) Nachweis einer enzymkatalyse die cis-trans-isomerisierung der peptidbindung in prolinhaltigen petiden. *Biomedica Biochimica Acta* 43: 1101–1111.
- Fischer, G., & Aumüller, T. (2003). Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes. In *Reviews of physiology, biochemistry and pharmacology* (pp. 105-150). Springer Berlin Heidelberg.
- Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989). Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins.
- Forestan, C., & Varotto, S. (2012). The role of PIN auxin efflux carriers in polar auxin transport and accumulation and their effect on shaping maize development. *Molecular plant*, 5(4), 787-798.
- Fretz, H., Albers, M. W., Galat, A., Standaert, R. F., Lane, W. S., Burakoff, S. J., et al & Schreiber, S. L. (1991). Rapamycin and FK506 binding proteins (immunophilins). *Journal of the American Chemical Society*, 113(4), 1409-1411.
- Fu, A., He, Z., Cho, H. S., Lima, A., Buchanan, B. B., & Luan, S. (2007). A chloroplast cyclophilin functions in the assembly and maintenance of photosystem II in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 104(40), 15947-15952.
- Gadjev, I., Vanderauwera, S., Gechev, T. S., Laloi, C., Minkov, I. N., Shulaev, V., et al & Van Breusegem, F. (2006). Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiology*, 141(2), 436-445.
- Galat, A. (2003). Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity-targets-functions. *Current topics in medicinal chemistry*, 3(12), 1315-1347.

- Galat, A. (2012). Functional diversity and pharmacological profiles of the FKBP and their complexes with small natural ligands. *Cellular and Molecular Life Sciences*, 1-33.
- Galigniana, N. M., Ballmer, L. T., Toneatto, J., Erlejman, A. G., Lagadari, M., & Galigniana, M.D. (2012). Regulation of the glucocorticoid response to stress-related disorders by the Hsp90 binding immunophilin FKBP51. *Journal of neurochemistry*, 122(1), 4-18.
- Gasser, C. S., Gunning, D. A., Budelier, K. A., & Brown, S. M. (1990). Structure and expression of cytosolic cyclophilin/peptidyl-prolyl cis-trans isomerase of higher plants and production of active tomato cyclophilin in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, 87(24), 9519-9523.
- Geisler, M., Girin, M., Brandt, S., Vincenzetti, V., Plaza, S., Paris, N., ... & Martinoia, E. (2004). Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. *Molecular biology of the cell*, 15(7), 3393-3405.
- Geisler, M., Kolukisaoglu, H. Ü., Bouchard, R., Billion, K., Berger, J., Saal, B., et al & Schulz, B. (2003). TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. *Molecular biology of the cell*, 14(10), 4238-4249.
- Gilchrist, E., & Haughn, G. (2010). Reverse genetics techniques: engineering loss and gain of gene function in plants. *Briefings in functional genomics*, 9(2), 103-110.
- Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current protocols in food analytical chemistry*.
- Gollan, P. J., Bhave, M., & Aro, E. M. (2012). The FKBP families of higher plants; exploring the structures and functions of protein interaction specialists. *FEBS letters*. 586:20
- Gollan, P. J., Ziemann, M., & Bhave, M. (2011). PPIase activities and interaction partners of FK506-binding proteins in the wheat thylakoid. *Physiologia Plantarum*, 143(4), 385-395.
- Gopalan, G., He, Z., Balmer, Y., Romano, P., Gupta, R., Héroux, A., et al. (2004). Structural analysis uncovers a role for redox in regulating FKBP13, an immunophilin of the chloroplast thylakoid lumen. *Proceedings of the National Academy of Sciences of the United States of America*, 101(38), 13945-13950.
- Gupta, R., Mould, R. M., He, Z., & Luan, S. (2002). A chloroplast FKBP interacts with and affects the accumulation of Rieske subunit of cytochrome b_f complex. *Proceedings of the National Academy of Sciences*, 99(24), 15806-15811.
- Hacker, J., & Fischer, G. (1993). Immunophilins: structure—function relationship and possible role in microbial pathogenicity. *Molecular microbiology*, 10(3), 445-456.
- Haendler, B., Hofer-Warbinek, R., & Hofer, E. (1987). Complementary DNA for human T-cell cyclophilin. *The EMBO journal*, 6(4), 947.

- Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984). Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science*, 226(4674), 544-547.
- Harding, M. W., Galat, A., Uehling, D. E., & Schreiber, S. L. (1989). A receptor for the immuno-suppressant FK 506 is a cis-trans peptidyl-prolyl isomerase. *Nature*, 341(6244), 758-760.
- Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., & Nakayama, K. I. (2001). U box proteins as a new family of ubiquitin-protein ligases. *Journal of Biological Chemistry*, 276(35), 33111-33120.
- Haubrick, L. L., & Assmann, S. M. (2006). Brassinosteroids and plant function: some clues, more puzzles. *Plant, cell & environment*, 29(3), 446-457.
- Havaux, M., Ksas, B., Szewczyk, A., Rumeau, D., Franck, F., Caffarri, S., & Triantaphylidès, C. (2009). Vitamin B6 deficient plants display increased sensitivity to high light and photo-oxidative stress. *BMC plant biology*, 9(1), 130.
- He, Z., Li, L., & Luan, S. (2004). Immunophilins and parvulins. Superfamily of peptidyl prolyl isomerases in Arabidopsis. *Plant physiology*, 134(4), 1248-1267.
- Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, et al. (1996). The mechanism of action of cyclosporin A and FK506. *Clinical immunology and immunopathology*, 80(3), S40-S45.
- Hullmann, J., Patzer, S. I., Römer, C., Hantke, K., & Braun, V. (2008). Periplasmic chaperone FkpA is essential for imported colicin M toxicity. *Molecular microbiology*, 69(4), 926-937.
- Iki, T., Yoshikawa, M., Meshi, T., & Ishikawa, M. (2011). Cyclophilin 40 facilitates HSP90-mediated RISC assembly in plants. *The EMBO journal*, 31(2), 267-278.
- Ingelsson, B., Shapiguzov, A., Kieselbach, T., & Vener, A. V. (2009). Peptidyl-Prolyl Isomerase Activity in Chloroplast Thylakoid Lumen is a Dispensable Function of Immunophilins in Arabidopsis thaliana. *Plant and cell physiology*, 50(10), 1801-1814.
- Innan, H., & Kondrashov, F. (2010). The evolution of gene duplications: classifying and distinguishing between models. *Nature Reviews Genetics*, 11(2), 97-108.
- Jayaraman, T., Brillantes, A. M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., & Marks, A. R. (1992). FK506 binding protein associated with the calcium release channel (ryanodine receptor). *Journal of Biological Chemistry*, 267(14), 9474-9477.
- Justice, S. S., Hunstad, D. A., Harper, J. R., Duguay, A. R., Pinkner, J. S., Bann, J., et al. (2005). Periplasmic peptidyl prolyl cis-trans isomerases are not essential for viability, but SurA is required for pilus biogenesis in Escherichia coli. *Journal of bacteriology*, 187(22), 7680-7686.
- Karali, D., Oxley, D., Runions, J., Ktistakis, N., & Farmaki, T. (2012). The Arabidopsis thaliana Immunophilin ROF1 Directly Interacts with PI (3) P and PI (3, 5) P2 and Affects Germination under Osmotic Stress. *PloS one*, 7(11), e48241.
- Kawakami, K., Umena, Y., Iwai, M., Kawabata, Y., Ikeuchi, M., Kamiya, N., & Shen, J. R. (2011). Roles of PsbI and PsbM in photosystem II dimer formation and stability

- studied by deletion mutagenesis and X-ray crystallography. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1807(3), 319-325.
- Khanna, R., Shen, Y., Toledo-Ortiz, G., Kikis, E. A., Johannesson, H., Hwang, Y. S., & Quail, P. H. (2006). Functional profiling reveals that only a small number of phytochrome-regulated early-response genes in Arabidopsis are necessary for optimal deetiolation. *The Plant Cell Online*, 18(9), 2157-2171.
- Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka M, Aoki Hatsuo & Imanaka, H. (1987). FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. *The Journal of antibiotics*, 40 (9), 1249.
- Kleerebezem, M., Heutink, M., & Tommassen, J. (1995). Characterization of an Escherichia coli rotA mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase. *Molecular microbiology*, 18(02), 313-320.
- Köhler, R., Fanghänel, J., König, B., Lüneberg, E., Frosch, M., Rahfeld, J. U., et al. (2003). Biochemical and functional analyses of the Mip protein: influence of the N-terminal half and of peptidylprolyl isomerase activity on the virulence of Legionella pneumophila. *Infection and immunity*, 71(8), 4389-4397.
- Komenda J, Nickelsen J, Tichy´ M, Pra´sil O, Eichacker LA, Nixon PJ (2008) The cyanobacterial homologue of HCF136/YCF48 is a component of an early photosystem II assembly complex and is important for both the efficient assembly and repair of photosystem II in Synechocystis sp. PCC 6803. *J Biol Chem* 283:22390–22399
- König, J., Muthuramalingam, M., & Dietz, K. J. (2012). Mechanisms and dynamics in the thiol/disulfide redox regulatory network: transmitters, sensors and targets. *Current opinion in plant biology*, 15(3), 261-268.
- Kreslavski VD, Fomina IR, Los´ DA, Allakhverdiev SI (2012) Heat induced impairments and recovery of photosynthetic machinery. In: Josipovic S, Ludvig E (eds) Heat stress: causes, treatment and prevention, chapter 4. Nova Science Publishers, Inc, New York
- Krieger-Liszkay, A., Fufezan, C., & Trebst, A. (2008). Singlet oxygen production in photosystem II and related protection mechanism. *Photosynthesis Research*, 98(1-3), 551-564.
- Kuepfer, L., Sauer, U., & Blank, L. M. (2005). Metabolic functions of duplicate genes in *Saccharomyces cerevisiae*. *Genome research*, 15(10), 1421-1430.
- Kurusu, G., Zhang, H., Smith, J. L., & Cramer, W. A. (2003). Structure of the cytochrome b6f complex of oxygenic photosynthesis: tuning the cavity. *Science*, 302(5647), 1009-1014.
- Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., et al. (2001). Initial sequencing and analysis of the human genome. *Nature*, 409(6822), 860-921.
- Leister, D. (2003). Chloroplast research in the genomic age. *Trends Genet.* 19, 47–56.
- Leuzzi, R., Serino, L., Scarselli, M., Savino, S., Fontana, M. R., Monaci, E., et al. (2005). Ng-MIP, a surface-exposed lipoprotein of Neisseria gonorrhoeae, has a peptidyl-

- prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. *Molecular microbiology*, 58(3), 669-681.
- Li, B., Kronzucker, H. J., & Shi, W. (2013). Molecular components of stress-responsive plastid retrograde signaling networks and their involvement in ammonium stress. *Plant signaling & behavior*, 8(2), 0-1.
- Li, H., & Luan, S. (2010). AtFKBP53 is a histone chaperone required for repression of ribosomal RNA gene expression in Arabidopsis. *Cell research*, 20(3), 357-366.
- Li, H., & Luan, S. (2011). The cyclophilin AtCYP71 interacts with CAF-1 and LHP1 and functions in multiple chromatin remodeling processes. *Molecular plant*, 4(4), 748-758.
- Li, H., He, Z., Lu, G., Lee, S. C., Alonso, J., Ecker, J. R., & Luan, S. (2007). A WD40 domain cyclophilin interacts with histone H3 and functions in gene repression and organogenesis in Arabidopsis. *The Plant Cell Online*, 19(8), 2403-2416.
- Lima, A., Lima, S., Wong, J. H., Phillips, R. S., Buchanan, B. B., & Luan, S. (2006). A redox-active FKBP-type immunophilin functions in accumulation of the photosystem II supercomplex in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, 103(33), 12631-12636.
- Lippuner, V., Chou, I. T., Scott, S. V., Ettinger, W. F., Theg, S. M., & Gasser, C. S. (1994). Cloning and characterization of chloroplast and cytosolic forms of cyclophilin from Arabidopsis thaliana. *Journal of Biological Chemistry*, 269(11), 7863-7868.
- Liu, J., & Walsh, C. T. (1990). Peptidyl-prolyl cis-trans-isomerase from Escherichia coli: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proceedings of the National Academy of Sciences*, 87(11), 4028-4032.
- Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005). Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438: 1040–1044.
- Luan S, Albers MW, Schreiber SL. (1994a). Light-regulated, tissue-specific immunophilins in a higher plant. *Proceedings of the National Academy of Sciences, USA* 91: 984–988.
- Luan S, Kudla J, Grussem W, Schreiber SL. (1996). Molecular characterization of a FKBP-type immunophilin from higher plants. *Proceedings of the National Academy of Sciences, USA* 93: 6964–6969.
- Luan S, Schreiber SL. (1994b). pCyP B: a chloroplast-localized, heat shock-responsive cyclophilin from fava bean. *Plant Cell* 6: 885–892.
- Lucke, C., & Weiwad, M. (2011). Insights into immunophilin structure and function. *Current medicinal chemistry*, 18(35), 5333-5354.
- Lundemose, A. G., Kay, J. E., & Pearce, J. H. (1993). Chlamydia trachomatis Mip-like protein has peptidylprolyl cis/trans isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of chlamydial infection. *Molecular microbiology*, 7(5), 777-783.

- Ma, X. F., & Gustafson, J. P. (2005). Genome evolution of allopolyploids: a process of cytological and genetic diploidization. *Cytogenetic and genome research*, 109(1-3), 236-249.
- Ma, X., Song, L., Yang, Y., & Liu, D. (2013). A gain-of-function mutation in the ROC1 gene alters plant architecture in Arabidopsis. *New Phytologist*, 197(3), 751-762.
- Majeran, W., Zybaïlov, B., Ytterberg, A. J., Dunsmore, J., Sun, Q., & van Wijk, K. J. (2008). Consequences of C4 differentiation for chloroplast membrane proteomes in maize mesophyll and bundle sheath cells. *Molecular & Cellular Proteomics*, 7(9), 1609-1638.
- Martin W, et al. (2002) Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc Natl Acad Sci USA* 99(19):12246–12251.
- Matouschek, A., Rospert, S., Schmid, K., Glick, B. S., & Schatz, G. (1995). Cyclophilin catalyzes protein folding in yeast mitochondria. *Proceedings of the National Academy of Sciences*, 92(14), 6319-6323.
- Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of experimental botany*, 51(345), 659-668.
- Meiri, D., & Breiman, A. (2009). Arabidopsis ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90. 1 and affecting the accumulation of HsfA2-regulated sHSPs. *The Plant Journal*, 59(3), 387-399.
- Meiri, D., Tazat, K., Cohen-Peer, R., Farchi-Pisanty, O., Aviezer-Hagai, K., Avni, A., & Breiman, A. (2010). Involvement of Arabidopsis ROF2 (FKBP65) in thermotolerance. *Plant molecular biology*, 72(1-2), 191-203.
- Miyagishima, S. Y. (2011). Mechanism of plastid division: from a bacterium to an organelle. *Plant physiology*, 155(4), 1533-1544.
- Motohashi, K., & Hisabori, T. (2006). HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. *Journal of Biological Chemistry*, 281(46), 35039-35047.
- Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K. I., Endo, T., Tasaka, M., & Shikanai, T. (2004). Cyclic electron flow around photosystem I is essential for photosynthesis. *Nature*, 429(6991), 579-582.
- Munn, K., & Steward, R. (2000). The shut-down gene of Drosophila melanogaster encodes a novel FK506-binding protein essential for the formation of germline cysts during oogenesis. *Genetics*, 156(1), 245-256.
- Nield, J., Redding, K., & Hippler, M. (2004). Remodeling of light-harvesting protein complexes in Chlamydomonas in response to environmental changes. *Eukaryotic cell*, 3(6), 1370-1380.
- Nixon, P. J., Michoux, F., Yu, J., Boehm, M., & Komenda, J. (2010). Recent advances in understanding the assembly and repair of photosystem II. *Annals of botany*, 106(1), 1-16.
- Norville, I. H., Breitbach, K., Eske-Pogodda, K., Harmer, N. J., Sarkar-Tyson, M., Titball, R. W., & Steinmetz, I. (2011). A novel FK-506-binding-like protein that

- lacks peptidyl-prolyl isomerase activity is involved in intracellular infection and in vivo virulence of *Burkholderia pseudomallei*. *Microbiology*, 157(9), 2629-2638.
- Obi, I., Nordfelth, R., & Francis, M. (2011). Varying dependency of periplasmic peptidylprolyl cis-trans isomerases in promoting *Yersinia pseudotuberculosis* stress tolerance and pathogenicity. *Biochemical Journal*, 439(2), 321-332.
- Page, A.P., Winter, A.D., (1998) A divergent multi-domain cyclophilin is highly conserve-ed between parasitic and free-living nematode species and is important in larval muscle development. *Mol. Biochem. Parasitol.* 95, 215–227.
- Pasek, S., Risler, J. L., & Brézellec, P. (2006). The role of domain redundancy in genetic robustness against null mutations. *Journal of molecular biology*, 362(2), 184-191.
- Peltier, J. B., Emanuelsson, O., Kalume, D. E., Ytterberg, J., Friso, G., Rudella, A., et al. (2002). Central functions of the lumenal and peripheral thylakoid proteome of *Arabidopsis* determined by experimentation and genome-wide prediction. *The Plant Cell Online*, 14(1), 211-236.
- Pemberton, T. J., & Kay, J. E. (2005). Identification and comparative analysis of the peptidyl-prolyl cis/trans isomerase repertoires of *H. sapiens*, *D. melanogaster*, *C. elegans*, *S. cerevisiae* and *Sz. pombe*. *Comparative and functional genomics*, 6(5-6), 277-300.
- Peng, L., Fukao, Y., Fujiwara, M., Takami, T., & Shikanai, T. (2009). Efficient operation of NAD (P) H dehydrogenase requires supercomplex formation with photosystem I via minor LHCI in *Arabidopsis*. *The Plant Cell Online*, 21(11), 3623-3640.
- Pérez-Pérez, J. M., Ponce, M. R., & Micol, J. L. (2004). The ULTRACURVATA2 gene of *Arabidopsis* encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. *Plant Physiology*, 134(1), 101-117.
- Pesaresi P, Scharfenberg M, Weigel M, Granlund I, et al. (2009) Mutants, overexpressors, and interactors of *Arabidopsis* plastocyanin isoforms: revised roles of plastocyanin in photosynthetic electron flow and thylakoid redox state. *Mol Plant* 2:236–248.
- Pijnappel, W. P., Schaft, D., Roguev, A., Shevchenko, A., Tekotte, H., Wilm, M., et al & Stewart, A. F. (2001). The *S. cerevisiae* SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program. *Genes & development*, 15(22), 2991-3004.
- Preall, J. B., Czech, B., Guzzardo, P. M., Muerdter, F., & Hannon, G. J. (2012). shutdown is a component of the *Drosophila* piRNA biogenesis machinery. *RNA*, 18(8), 1446-1457.
- Prigge, M. J., & Bezanilla, M. (2010). Evolutionary crossroads in developmental biology: *Physcomitrella patens*. *Development*, 137(21), 3535-3543.
- Quail, P. H. (2005) Phytochrome Overview. IN WADA, M., SHIMAZAKI, K. & IINO, M. (Eds.) *Light Sensing in Plants*. Tokyo, Springer-Verlag. pp 21-35.

- Reimer, U., & Fischer, G. (2002). Local structural changes caused by peptidyl-prolyl cis/trans isomerization in the native state of proteins. *Biophysical chemistry*, 96(2-3), 203.
- Rensing, S. A., Beike, A. K., & Lang, D. (2013). Evolutionary importance of generative polyploidy for genome evolution of haploid-dominant land plants. In *Plant Genome Diversity Volume 2* (pp. 295-305). Springer Vienna.
- Richly, E., and Leister, D. (2004). An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of Arabidopsis and rice. *Gene*. 329, 11–16.
- Riggs, D. L., Cox, M. B., Tardif, H. L., Hessling, M., Buchner, J., & Smith, D. F. (2007). Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. *Molecular and cellular biology*, 27(24), 8658-8669.
- Rochaix, J. D. (2011). Assembly of the photosynthetic apparatus. *Plant Physiology*, 155(4), 1493-1500.
- Rodrigues, C., Vandenberghe, L. P. D. S., de Oliveira, J., & Soccol, C. R. (2012). New perspectives of gibberellic acid production: a review. *Critical reviews in biotechnology*, 32(3), 263-273.
- Rokka, A., Aro, E. M., Herrmann, R. G., Andersson, B., & Vener, A. V. (2000). Dephosphorylation of photosystem II reaction center proteins in plant photosynthetic membranes as an immediate response to abrupt elevation of temperature. *Plant Physiology*, 123(4), 1525-1536.
- Romano P, Gray J, Horton P, Luan S (2005) Plant immunophilins: functional versatility beyond protein maturation. *New Phytologist* 166: 753-769
- Romano, P. G., Edvardsson, A., Ruban, A. V., Andersson, B., Vener, A. V., Gray, J. E., & Horton, P. (2004). Arabidopsis AtCYP20-2 is a light-regulated cyclophilin-type peptidyl-prolyl cis-trans isomerase associated with the photosynthetic membranes. *Plant physiology*, 134(4), 1244-1247.
- Romano, P. G., Horton, P., & Gray, J. E. (2004). The Arabidopsis cyclophilin gene family. *Plant Physiology*, 134(4), 1268-1282.
- Roset, M. S., Fernández, L. G., DelVecchio, V. G., & Briones, G. (2013). Intracellularly Induced Cyclophilins Play an Important Role in Stress Adaptation and Virulence of *Brucella abortus*. *Infection and immunity*, 81(2), 521-530.
- Rumeau, D., Peltier, G., & Cournac, L. (2007). Chlororespiration and cyclic electron flow around PSI during photosynthesis and plant stress response. *Plant, cell & environment*, 30(9), 1041-1051.
- Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4), 406-425.
- Saul, F. A., Arie, J. P., Vulliez-le Normand, B., Kahn, R., Betton, J. M., & Bentley, G. A. (2004). Structural and functional studies of FkpA from *Escherichia coli*, a cis/trans peptidyl-prolyl isomerase with chaperone activity. *Journal of molecular biology*, 335(2), 595-608.

- Schmidt, B., Tradler, T., Rahfeld, J. U., Ludwig, B., Jain, B., Mann, K., et al. (1996). A cyclophilin-like peptidyl-prolyl cis/trans isomerase from *Legionella pneumophila*—characterization, molecular cloning and overexpression. *Molecular microbiology*, *21*(6), 1147-1160.
- Schmidt, N., Merker, M., & Becker, D. (2012). Novel high-throughput RNAi vectors for plant biotechnology. *Plant Breeding*, *131*(3), 453-456.
- Schreiber, S. L., & Crabtree, G. R. (1992). The mechanism of action of cyclosporin A and FK506. *Immunology today*, *13*(4), 136-142.
- Schubert, M., Petersson, U. A., Haas, B. J., Funk, C., Schröder, W. P., & Kieselbach, T. (2002). Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. *Journal of Biological Chemistry*, *277*(10), 8354-8365.
- Schülke, J. P., Wochnik, G. M., Lang-Rollin, I., Gassen, N. C., Knapp, R. T., Berning, B., et al. (2010). Differential impact of tetratricopeptide repeat proteins on the steroid hormone receptors. *PloS one*, *5*(7), e11717.
- Schwenkert, S., Umate, P., Dal Bosco, C., Volz, S., Mlčochová, L., et al. & Meurer, J. (2006). PsbI affects the stability, function, and phosphorylation patterns of photosystem II assemblies in tobacco. *Journal of Biological Chemistry*, *281*(45), 34227-34238.
- Shapiguzov, A., Edvardsson, A., & Vener, A. V. (2006). Profound redox sensitivity of peptidyl-prolyl isomerase activity in *Arabidopsis* thylakoid lumen. *FEBS letters*, *580*(15), 3671-3676.
- Shi, L. X., & Schröder, W. P. (2004). The low molecular mass subunits of the photosynthetic supracomplex, photosystem II. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1608*(2), 75-96.
- Shirane, M., & Nakayama, K. I. (2002). Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nature cell biology*, *5*(1), 28-37.
- Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., et al. (1991). Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A?. *The Journal of experimental medicine*, *173*(3), 619-628.
- Sirpiö, S., Holmström, M., Battchikova, N., & Aro, E. M. (2009). AtCYP20-2 is an auxiliary protein of the chloroplast NAD (P) H dehydrogenase complex. *FEBS letters*, *583*(14), 2355-2358.
- Sirpiö, S., Khrouchtchova, A., Allahverdiyeva, Y., Hansson, M., Fristedt, R., Vener, A. V., et al. (2008). AtCYP38 ensures early biogenesis, correct assembly and sustenance of photo-system II. *The Plant Journal*, *55*(4), 639-651.
- Smith, M. R., Willmann, M. R., Wu, G., Berardini, T. Z., Möller, B., Weijers, D., & P oethig, R. S. (2009). Cyclophilin 40 is required for microRNA activity in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *106*(13), 5424-5429.
- Söderberg, M. A., & Cianciotto, N. P. (2008). A *Legionella pneumophila* peptidyl-prolyl cis-trans isomerase present in culture supernatants is necessary for optimal growth at low temperatures. *Applied and environmental microbiology*, *74*(5), 1634-1638.

- Spetea, C. (2012). Role of chloroplast thylakoid lumen in photosynthetic regulation and plant cell signaling. In *Progress in Botany 73* (pp. 207-230). Springer Berlin Heidelberg.
- Srivastava, R., Pisareva, T., & Norling, B. (2005). Proteomic studies of the thylakoid membrane of *Synechocystis* sp. PCC 6803. *Proteomics*, 5(18), 4905-4916.
- Stamnes, M. A., Shieh, B. H., Chuman, L., Harris, G. L., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila* rhodopsins. *Cell*, 65(2), 219-227.
- Stepkowski, S. M. (2000). Molecular targets for existing and novel immunosuppressive drugs. *Expert reviews in molecular medicine*, 2(1), 1-23.
- Stroebel, D., Choquet, Y., Popot, J.L., & Picot, D. (2003). An atypical haem in the cytochrome b(6)f complex. *Nature* 426: 413–418.
- Takahashi, N., Hayano, T., & Suzuki, M. (1989). Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin. *Nature*, 337(6206), 473-475.
- Tanaka, H., Kuroda, A., Marusawa, H., Hatanaka, H., Kino, T., Goto, T., et al. (1987). Structure of FK506, a novel immunosuppressant isolated from *Streptomyces*. *Journal of the American Chemical Society*, 109(16), 5031-5033.
- Timerman, A., Ogunbumni, E., Freund, E., Wiederrecht, G., Marks, A. & Fleischer, S. (1993). The calcium release channel of sarcoplasmic reticulum is modulated by FK506-binding protein. *J. Biol. Chem.* 268, 22992-22999.
- Trupkin, S. A., Mora-García, S., & Casal, J. J. (2012). The cyclophilin ROC1 links phytochrome and cryptochrome to brassinosteroid sensitivity. *The Plant Journal*, 71(5), 712-723.
- Vallon, O. (2005). Chlamydomonas immunophilins and parvulins: survey and critical assessment of gene models. *Eukaryotic Cell*, 4(2), 230-241.
- van de Hoef, D. L., Bonner, J. M., & Boulianne, G. L. (2013). FKBP14 is an essential gene that regulates Presenilin protein levels and Notch signaling in *Drosophila*. *Development*, 140(4), 810-819.
- Vasudevan, D., Fu, A., Luan, S., & Swaminathan, K. (2012). Crystal structure of *Arabidopsis* cyclophilin38 reveals a previously uncharacterized immunophilin fold and a possible autoinhibitory mechanism. *The Plant Cell Online*, 24(6), 2666-2674.
- Vener, A. V., Rokka, A., Fulgosi, H., Andersson, B., & Herrmann, R. G. (1999). A cyclophilin-regulated PP2A-like protein phosphatase in thylakoid membranes of plant chloroplasts. *Biochemistry*, 38(45), 14955-14965.
- Wang, B. B., Hayenga, K. J., Payan, D. G., & Fisher, J. M. (1996). Identification of a nuclear-specific cyclophilin which interacts with the proteinase inhibitor eglin c. *Biochemical Journal*, 314(Pt 1), 313.
- Wang, B., Bailly, A., Zwiewka, M., Henrichs, S., Azzarello, E., Mancuso, S., et al. (2013). *Arabidopsis* TWISTED DWARF1 Functionally Interacts with Auxin Exporter ABCB1 on the Root Plasma Membrane. *The Plant Cell Online*, 25(1), 202-214.

- Wang, L., Shao, Y., Zhao, Y., He, H., Cheng, B., & Xiang, Y. (2012). Genome-Wide Analysis of FK506-Binding Protein Genes in *Populus trichocarpa*. *Plant Molecular Biology Reporter*, 30(4), 915-928.
- Woodson, J. D., Perez-Ruiz, J. M., Schmitz, R. J., Ecker, J. R., & Chory, J. (2013). Sigma factor-mediated plastid retrograde signals control nuclear gene expression. *The Plant Journal*, 73(1), 1-13.
- Wu, G., Otegui, M. S., & Spalding, E. P. (2010). The ER-localized TWD1 immunophilin is necessary for localization of multidrug resistance-like proteins required for polar auxin transport in *Arabidopsis* roots. *The Plant Cell Online*, 22(10), 3295-3304.
- Yin L, Lundin B, Bertrand M, Nurmi M, et al. (2010) Role of thylakoid ATP/ADP carrier in photoinhibition and photoprotection of photosystem II in *Arabidopsis*. *Plant Physiol* 153:666–677
- Yu, H., & Kumar, P. P. (2003). Post-transcriptional gene silencing in plants by RNA. *Plant cell reports*, 22(3), 167-174.
- Yu, X., Liu, H., Klejnot, J., & Lin, C. (2010). The cryptochrome blue light receptors. *The Arabidopsis book/American Society of Plant Biologists*, 8.
- Yu, Y., Zhang, H., Li, W., Mu, C., Zhang, F., Wang, L., & Meng, Z. (2012). Genome-wide analysis and environmental response profiling of the FK506-binding protein gene family in maize, *Zea mays*. *Gene*, 498(2), 212-222.
- Zhang, Q., Su, L. J., Chen, J. W., Zeng, X. Q., Sun, B. Y., & Peng, C. L. (2012). The antioxidative role of anthocyanins in *Arabidopsis* under high-irradiance. *Biologia Plantarum*, 56(1), 97-104.
- Zissimopoulos, S., Seifan, S., Maxwell, C., Williams, A. J., & Lai, F. A. (2012). Disparities in the association of the ryanodine receptor and the FK506-binding proteins in mammalian heart. *Journal of cell science*, 125(7), 1759-1769.