UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization and Manipulation of Lipid Catabolism in Eukaryotic Microalgae

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Oceanography

by

Emily Margaret Trentacoste

Committee in Charge:

William Gerwick, Chair
Mark Hildebrand, Co-chair
Bianca Brahamsha
Michael Burkart
Susan Golden
Stephen Mayfield

2014

The Dissertation of Emily Margaret Trentacoste is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____
Co-chair

_____
Chair

University of California, San Diego

2014

DEDICATION

This dissertation is dedicated to

My parents for knowledge, wisdom and integrity.
My brother and sister for inspiration, guidance and support.
My friends for love, life and happiness.

EPIGRAPH

For all at last return to the sea

Rachel Carson

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I'd like to thank my advisers and mentors, Bill Gerwick and Mark Hildebrand, for their unending support, advice and guidance. They supported me not only in the research aspects of my dissertation, but also in my goals for my future career. Their invaluable advice will undoubtedly continue to aid me in all my endeavors. I'd also like to thank Lena Gerwick for her mentorship and guidance throughout my research. I'd like to acknowledge my dissertation committee for all of the inspiration and advice they have given me throughout my PhD. They have been instrumental in the success of my research as well as my career. Finally, I'd like to thank my co-authors for their tireless work and unending support in aiding me in completing this research.

Chapter 1, in part, is being prepared for submission as a review paper in 2014. Emily Trentacoste, Mark Hildebrand and William Gerwick.

Chapter 2.2, in part, is in prep to be submitted by Sarah Smith in 2014. The dissertation author is the primary investigator of the data reported in Chapter 2; Sarah Smith is the primary investigator of the manuscript to be submitted. Chapter 2.3 is, in full, included in The Proceedings of the National Academy of Sciences 2013, 110 (49): 19748-19753. Trentacoste, E. M., Shrestha, R. P., Smith, S. R., Glé, C., Hartmann, A. C., Hildebrand, M., & Gerwick, W. H. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is included in The Proceedings of the National Academy of Sciences 2013, 110 (49): 19748-19753. Trentacoste, E. M., Shrestha, R. P., Smith, S. R., Glé, C., Hartmann, A. C., Hildebrand, M., & Gerwick, W. H. The dissertation author was the primary investigator and author of this paper.

Chapter 4 is currently being completed and will be prepared for submission in 2014. Emily Trentacoste, Mark Hildebrand and William Gerwick. The dissertation author was the primary investigator on these studies.

Chapter 5, in full, is included in Photosynthesis Research 2014, doi: 10.1007/s11120-014-9985-8. Trentacoste, E. M., Martinez, A.M., Zenk, T. The dissertation author was the primary author of this paper.

VITA

EDUCATION

Scripps Institution of Oceanography, UC-San Diego, San Diego, CA

Ph.D. in Oceanography: GPA 3.9 **2014**

M.S. in Marine Biology: GPA 3.9 **2011**
Dissertation Research: Characterization and manipulation of lipid metabolism in the diatom *Thalassiosira pseudonana*

Dartmouth College, Hanover, NH **2009**

B.A. in Biological Sciences: GPA 3.4
Senior Thesis: Identification of regulators of biofilm formation in *Pseudomonas aeruginosa*

RESEARCH EXPERIENCE

Scripps Institution of Oceanography, San Diego, CA **2009-present**
Graduate student in Drs. Bill Gerwick & Mark Hildebrand Labs

Dartmouth College, Hanover, NH **2008-2009**
Undergraduate Researcher in Dr. George O'Toole Laboratory

Walter Reed Army Institute of Research, Silver Spring, MD **Jun-Sep 2006**
Laboratory Assistant in Dr. Malabi Venkatesan Laboratory

Walter Reed Army Institute of Research, Silver Spring, MD **2004**
Science & Engineering Apprenticeship Program Member in Dr. Malabi Venkatesan Laboratory

COMMUNICATION & TEACHING EXPERIENCE

Policy & Regulation Intern **- Sapphire Energy, Inc.** **Mar-May 2013**
Wrote major policy report, responses and briefing notes, edited white papers

Volunteer Biology Teacher **- Junipero Serra High School** **2011-present**
Developed and taught labs to bring marine science to classrooms

Concept Mapping Science Communication Workshop Participant **- Center for Ocean Sciences Education Excellence (COSEE)- California** **Oct 2011**
Taught ocean concepts to undergrads employing new teaching tool

***Volunteer Skype Outreach Communicator* - COSEE- California** **2009-2010**
Taught middle school students marine biology concepts over Skype

***Genetics Teaching Assistant* - Dartmouth College** **2008-2009**
Assisted with undergraduate genetics labs

PUBLICATIONS & PATENTS

Trentacoste, E.M., Zenk, T. & Martinez, A.M. (2014) The place of algae in agriculture: policies for algal biomass production. *Photosynth. Res.* doi: 10.1007/s11120-014-9985-8 (in press).

Trentacoste, E.M., Shrestha, R.P., Smith, S.R., Glé, C., Hartmann, A., Hildebrand, M. & Gerwick. W.H. (2013) Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. *Proc. Natl. Acad. Sci. USA*. 110 (49): 19748-19753.

Trentacoste, E.M., Shrestha, R.P, Hildebrand, M. & Gerwick. W.H. U.S. Patent Application No. SD2012-190-1, http://techtransfer.universityofcalifornia.edu/NCD/23046.html, (filed May 17, 2012).

Coates R.C., **Trentacoste E.M.**, Gerwick W.H. (2013) *Bioactive and Novel Chemicals from Microalgae. In Handbook of Microalgal Culture* Edition 2, eds Richmond A, Hu Q (Wiley-Blackwell, Oxford) pp. 504-531.

Hildebrand, M., Polle, J.E.W., Abbriano, R.A., Traller, J.C., **Trentacoste, E.M.**, Smith, S.R. & Davis, A.K. (2013) Metabolic and cellular organization in evolutionarily diverse microalgae as related to biofuels production. *Curr. Opin. Chem. Biol.* 17(3): 506-514.

PUBLIC PRESENTATIONS

Trentacoste, E.M. The two side of algae biofuels: building both molecular and political toolboxes. Food & Fuel for the 21st Century, La Jolla, California (March 2014). Oral presentation.

Trentacoste, E.M. The place of algae in agriculture: where it is, where it should be & how to get there. Algae Biomass Summit, Orlando, Florida (October 2013). Oral presentation.

Trentacoste, E.M., Shrestha, R., Smith, S.R., Glé, Hartmann, A.C., Hildebrand, M. & Gerwick. W.H. Increased lipid accumulation without compromising growth: metabolic engineering of lipid catabolism in *Thalassiosira pseudonana*. Algae Biomass Summit, Orlando, Florida (October 2013). Oral presentation.

Trentacoste, E.M., Shrestha, R., Smith, S.R., Glé, Hartmann, A.C., Hildebrand, M. & Gerwick. W.H. Engineering microalgae for production systems: metabolic manipulation without GMO classification. Society for Industrial Microbiology Annual Conference, San Diego, California (August 2013). Oral presentation.

Trentacoste, E.M., Shrestha, R., Smith, S.R., Glé, Hartmann, A.C., Hildebrand, M. & Gerwick. W.H. Increased lipid accumulation without compromising growth: metabolic engineering of lipid catabolism in *Thalassiosira pseudonana*. Molecular Life of Diatoms, Paris, France (June 2013). Oral presentation.

Trentacoste, E.M., Shrestha, R., Smith, S.R., Glé, Hartmann, A.C., Hildebrand, M. & Gerwick. W.H. Metabolic engineering of lipid catabolism in *Thalassiosira pseudonana* increases lipid accumulation without compromising growth. Algal Biomass, Biofuels & Bioproducts, Toronto, Canada (June 2013). Oral presentation.

Trentacoste, E.M., Hull, J., Shrestha, R., Smith, S.R., Glé, Hartmann, A.C., Hildebrand, M. & Gerwick. W.H. Metabolic engineering of lipid catabolism in *Thalassiosira pseudonana* increases lipid accumulation without compromising growth. Food & Fuel for the 21st Century, San Diego, CA (April 2013). Poster.

HONORS & AWARDS

***Knauss Marine Policy Fellowship* – Awarded by NOAA Sea Grant 2014-2015**
1-year postdoctoral fellowship to conduct policy work for Office of Aquaculture in NOAA Fisheries

***P.E.O. Scholar Award* – Awarded by P.E.O. International 2013**
Competitive educational award for women pursuing doctoral degrees.

***Best Student Oral Presentation* – Awarded by Society for Industrial Microbiology & Biotechnology (SIMB) Aug 2013**
Award given for best student presentation at annual SIMB conference.

***NIH Marine Biotechnology Training Fellowship* – Awarded by National Institutes of Health (NIH) 2011-present**
2-year biotechnology fellowship to support dissertation research. Included industrial internship component, completed with Sapphire Energy.

***GK-12 Scripps Fellowship* - Awarded by Scripps Classroom Connection 2010-2011**
Partnered with high school teacher to develop activities & lessons for 10th grade Biology class to incorporate the marine realm. Created and led labs, lessons & activities throughout the year. Published all original materials from lessons online.

***Scripps Innovation Prize Finalist* - Awarded by Young Presidents Organization 2009**
Created business plan & model with two local CEOs and fellow graduate student. Presented to and discussed business plan with >70 of San Diego's CEOs.

***Young Women of Distinction Award* - Awarded by Girl Scouts of the USA 2005**

Highest award in Girl Scouts. Gold Award Project was chosen as one of twenty-five in the nation for this award. Attended and presented at Girl Scouts National Council Session & Convention to hundreds of Girl Scout leaders.

***Girl Scout Gold Award* - Awarded by Girl Scouts of the Nation's Capital** **2005**

Created, planned & led Engineering Day for local middle school girls. Worked with Lockheed Martin Corporation to fund day. Wrote handbook being used globally to recreate day.

ABSTRACT OF THE DISSERTATION

Characterization and Manipulation of Lipid Catabolism in Eukaryotic Microalgae

by

Emily Margaret Trentacoste

Doctor of Philosophy in Oceanography

University of California, San Diego – 2014

William Gerwick, Chair
Mark Hildebrand, Co-chair

The search for new sources of sustainable and renewable energy is one of the most important issues of our time. Broadening our energy base not only thwarts the issues of dependence on foreign oil and national energy security, but also creates a more stable and sustainable energy sector. Biomass is one such source of renewable energy that has been recently developed over the last few decades, and microalgae were identified

half a century ago as potential producers of fuel-relevant molecules. Members of this group can produce a variety of molecules including lipids that can be converted into liquid fuel molecules, carbohydrates that can be fermented into ethanol, and biohydrogen. This dissertation work focuses in particular on the pathways associated with lipid production in microalgae.

This work shows that it is possible to increase lipid accumulation in microalgae through metabolic engineering without affecting growth. The pathway manipulated here is lipid catabolism, or the breakdown of lipids. This work used transcriptomics to guide the identification of a target lipase in the lipid catabolism pathway. Knock-down of this enzyme in the diatom *Thalassiosira pseudonana* generated strains that grew comparably to wild-type but exhibited increased TAG accumulation during exponential growth, stationary phase and nutrient starvation-induced lipid accumulation.

This dissertation also describes a novel approach for using native sequences to conduct metabolic engineering. By using the knock-down phenotypes of increased TAG accumulation and increased membrane intactness, we were able to generate and select knock-down strains using only native sequences of *T. pseudonana*, thus exempting these strains from the classification of "genetically modified organism" in the U.S.

Finally, this dissertation looks beyond the science of algal biofuels to investigate and analyze the policy landscape for cultivating algal biomass. The large-scale cultivation of algae is in essence agriculture, and it is imperative that federal agricultural programs are expanded to cover and support algae as equal commodities. Overall this dissertation expands our knowledge of lipid metabolism in microalgae, provides methods for increasing lipid yields in production systems, and explores the support that is available

and needed to transform research and development efforts into a commercially relevant industry.

1.0 Chapter 1

INTRODUCTION

1.1 Algal Biofuels

1.1.1 History of Algal Biofuels

Over the last few decades the development of renewable, sustainable, clean and domestic fuels has become increasingly important to mitigate a variety of global problems including diminishing fossil fuel supply (Hirsch 2007, Murray and King 2012) and rising CO_2 levels (Keeling et al 1995). Biomass-derived fuels have been developed as alternative energy sources for decades (Sheehan et al 1998), particularly as replacements or additives to transportation fuels. “First-generation biofuels” were focused on ethanol and biodiesel fuels, and were developed primarily from terrestrial plant crops such as corn, soy, rapeseed and palm. “Second-generation biofuels” evolved away from undesirable competition with the agricultural sector and focused on plant-derived lignocellulosic alcohols (Naik et al 2010). Combating the low biofuel productivity associated with lignocellulosic sources, “third-generation biofuels” have focused on microalgae as a fuel source (Singh et al 2011).

1.1.2 Microalgae & Their Metabolism

Microalgae are a large, diverse group of unicellular, generally aquatic, photosynthetic organisms. They span multiple clades and evolutionary histories, but are all thought to stem from a common ancestral event in which a heterotrophic eukaryote acquired a photosynthetic cyanobacterium, resulting in the plastid. From this primary endosymbiotic event evolved Glaucophyta, Rhodophyta (red algae) and Chloroplastida (green algae and

plants). Additional secondary endosymbioses, in which heterotrophic eukaryotes engulfed either a green or red alga gave rise to a number of other groups including haptophytes, cryptophytes, stramenopiles and euglenophytes. The primary endosymbiotic event and an example secondary endosymbiotic event are shown in Figure 1-1. Microalgae have evolved to be practically ubiquitous throughout the globe, and their varied distributions and evolutionary histories are reflected in extremely diverse metabolic capabilities (Andersen 2013).

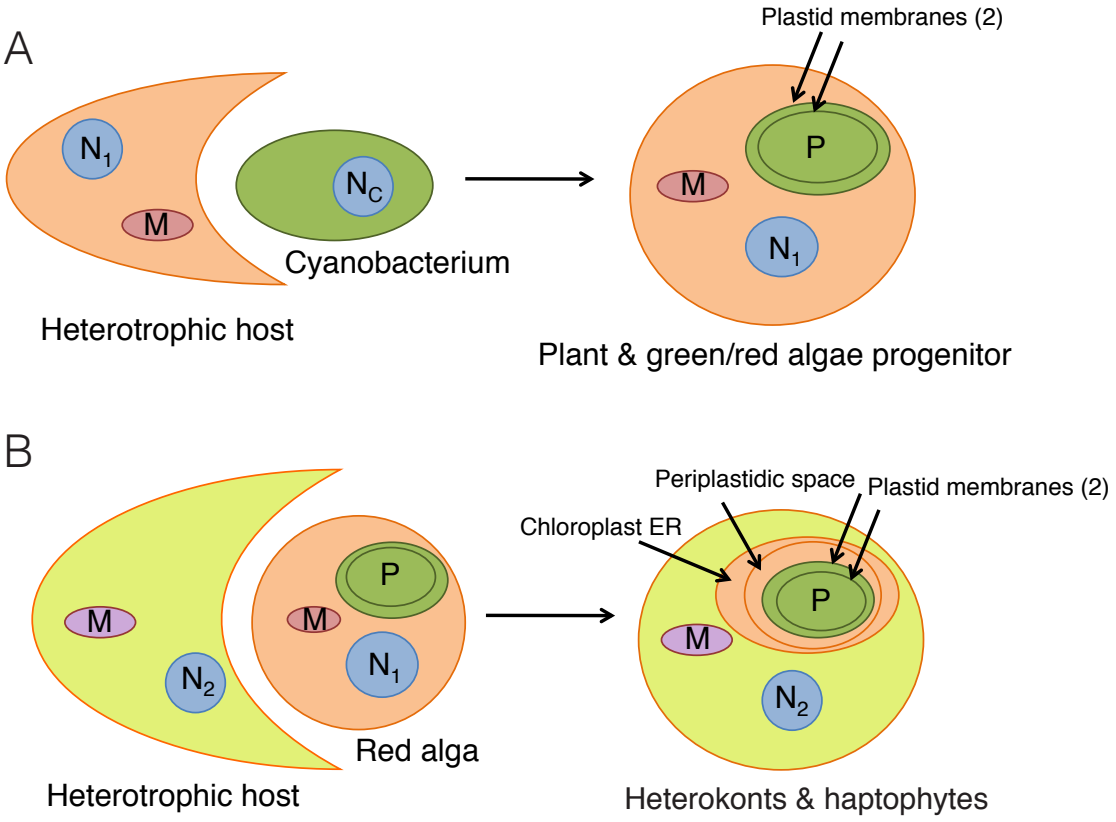


Figure 1-1.Evolutionary histories of modern plants and various classes of microalgae. *(A)* Primary endosymbiosis involving heterotrophic host and cyanobacterium endosymbiont. The endosymbiont mitochondrion and nucleus (N_c) disappeared after transfer of genes to host nucleus (N_1). The resulting organism containing a chloroplast surrounded by two membranes was the progenitor for the plant, green algae and red algae lineages. *(B)* Secondary endosymbiosis event involving new heterotrophic host and red algal endosymbiont. The endosymbiont mitochondrion and nucleus (N_1) disappeared after transfer of genes to host nucleus (N_2). Two new membranes surround the chloroplast, one from from the red algal endosymbiont's plasma membrane and one from the host's ER, generating the periplastid space and chloroplast ER. The resulting organisms was the progenitor of heterokonts (including diatoms, brown algae and chrysophytes) and haptophytes.

These diverse metabolisms produce a myriad of compounds with anthropogenic relevance including nutraceuticals and supplements, such as the carotenoids produced by *Dunaliella* and *Haematococcus* and polyunsaturated fatty acids (PUFAs) produced by various species (Borowitzka 2013) (Fig. 1-2). Some microalgae produce compounds of biotechnological interest including fluorescent compounds, such as phycoerythrin, and many produce isoprenoid molecules that can be used in food and over-the-counter products (Andersen 2013).

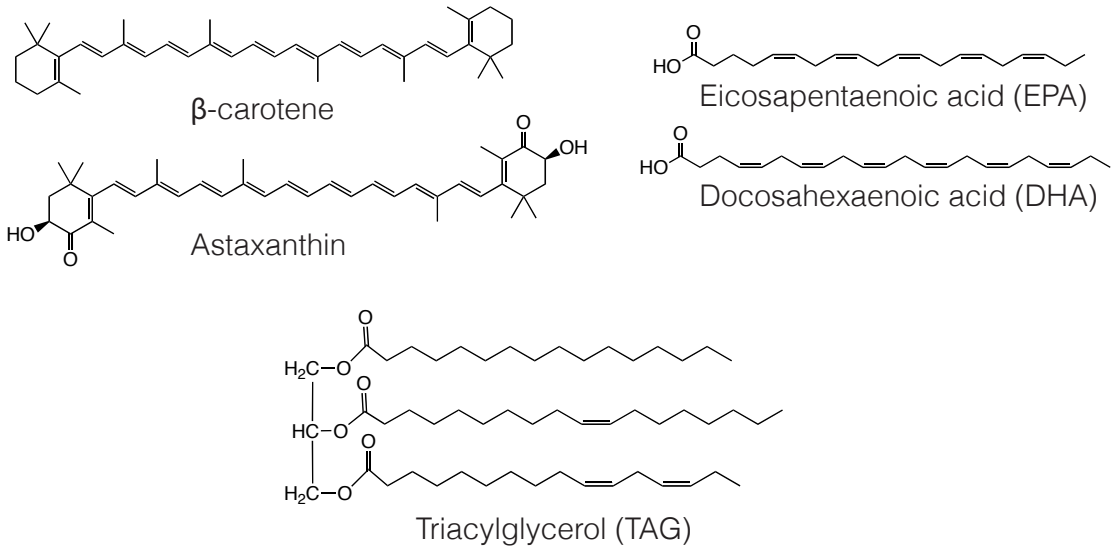


Figure 1-2. Molecules of interest from microalgae.
Microalgae produce various molecules of anthropogenic interest. β-carotene and astaxanthin are orange carotenoid pigments of the terpene class. β-carotene is used as a nutraceutical supplement and a treatment for certain disorders and is produced in large quantities by *Dunaliella* species. Astaxanthin, produced in large quantities by *Haematococcus pluvialis*, is used as a human nutraceutical and a fish feed supplement to provide natural pigmentation. EPA and DHA are omega-3 PUFAs produced by many species of microalgae. They are used as nutraceuticals and food additives, especially due to their anti-inflammatory properties. TAG is a neutral lipid consisting of three fatty acid moieties (which can differ from those pictured here) attached to a glycerol backbone. Many species of eukaryotic microalgae use TAG as a long-term carbon storage product. TAG can be extracted and converted into fuel molecules.

1.1.3 Producing Fuel from Microalgae

Recently microalgae have also been as developed as potentially relevant sources for fuel molecules. Microalgae were identified as attractive sources of biofuel because they bypass many production barriers and physiological obstacles of terrestrial plants and can produce a variety of fuel products. Various microalgal species have the ability to produce large quantities of lipid while sequestering CO_2, particularly neutral lipids in the form of triacylglycerol (TAG) (Fig. 1-2). TAG can be converted through trans-esterification to fatty acid methyl esters (FAMEs) making biodiesel (Hossain et al 2008), or refined into other fuel constituents (Pienkos and Darzins 2009). Total lipids and other biomass constituents can be converted into crude oil alternatives through thermochemical processes such as hydrothermal liquefaction (López Barreiro et al 2013). Figure 1-3 provides an overview of the process and pipeline of converting algal biomass into liquid fuels. Moreover, microalgal carbohydrates can be fermented into ethanol, and some species can produce biohydrogen (Radakovits et al 2010). In addition to their diversity of products, microalgae are attractive as fuel sources because many species have short generation times compared to terrestrial plants (thus reaching production maturity more quickly) and can be grown on brackish or saline water, thus avoiding the use of unsustainable quantities of freshwater, an increasingly limited resource (Dismukes et al 2008).

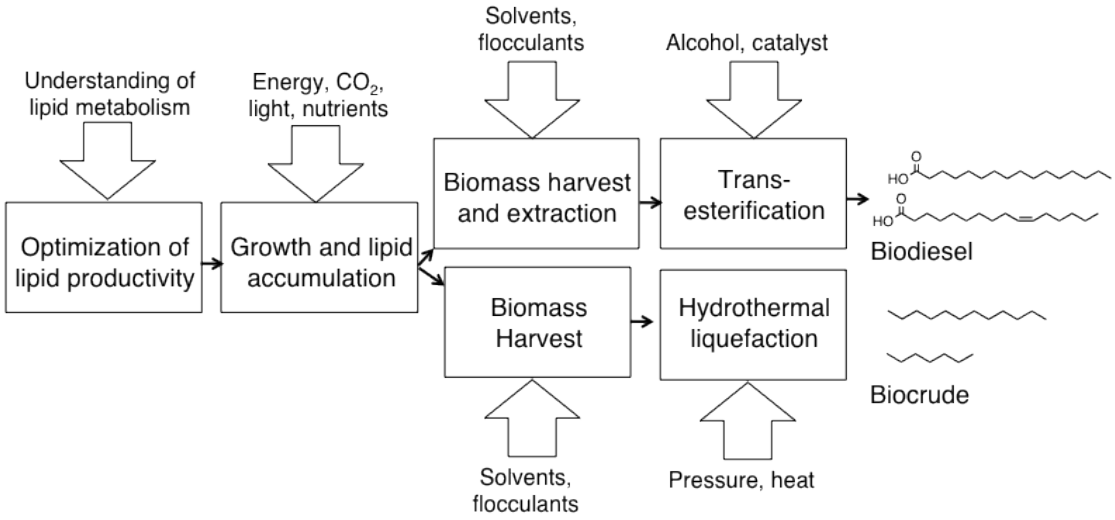


Figure 1-3. Algal biofuel pipeline for lipid-based liquid fuels.
The production of algal biofuels requires various inputs to reach a final product. Optimization of strains and lipid production at the R&D stage requires a deeper understanding of lipid metabolism. Cultivation of algal biomass requires light, energy, CO_2 and nutrients. Extraction and transesterification processes can produce biodiesel while hydrothermal liquefaction and similar processes produce biocrude, a crude oil alternative.

Microalgae can be grown either in outdoor ponds or in photobioreactors (PBRs) on non-arable land, thus evading competition with agricultural production (Fig. 1-4). Algae do, however, require other inputs for cultivation including water, nutrients, consistent temperatures and consistent year-round sunlight. Taking all of these factors into account, a recent study by the Pacific Northwest National Laboratory (PNNL) identified ~90,000 sites in the U.S. that would be suitable for cultivation of algae, comprising ~5.5% of the contiguous U.S. land mass and consisting predominantly of shrub/scrub landscape (Fig. 1-5). These sites exclude any cropland, urban land, protected lands, wetlands, wilderness, or significantly sloping landscapes (Wigmosta et al 2011).

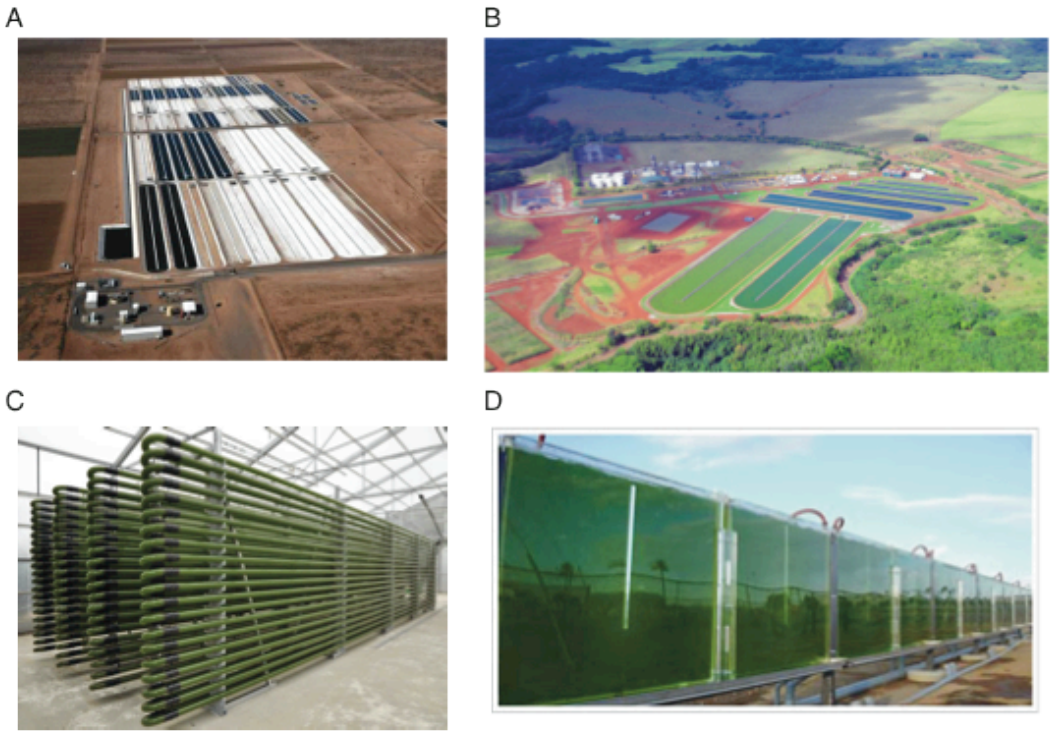


Figure 1-4. Algae cultivation platforms.
Microalgae can be cultivated in open-air ponds, typically designed as shallow raceways, such as those of Sapphire Energy, Inc. in New Mexico *(A)* and Hawaii Bioenergy in Hawaii *(B)*. Alternatively, enclosed photobioreactors can be used in a variety of designs, such as those of IGV Biotech *(C)* and Arizona State University *(D)*.

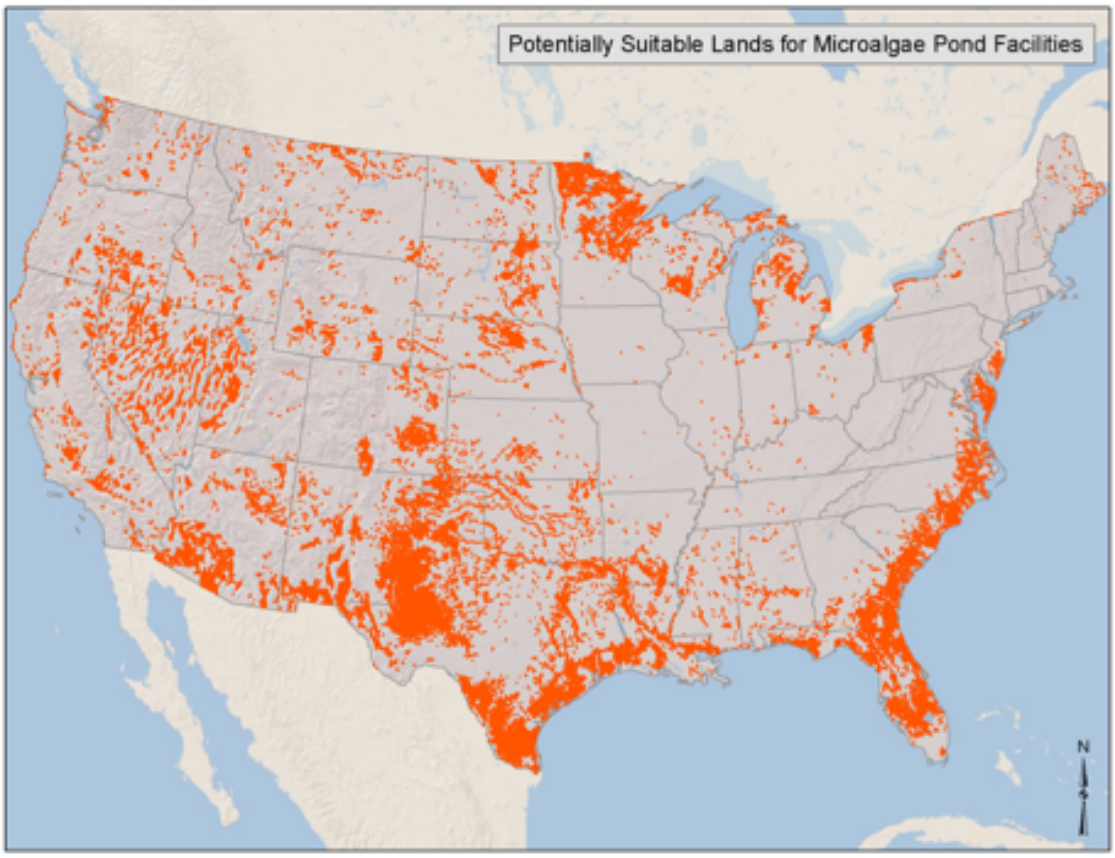


Figure 1-5. Suitable sites for microalgal pond facilities in the U.S. From: Pacific Northwest National Laboratories (Wigmosta et al 2011).

The recognition of microalgae as potential biofuel producers has led to the development over the last few decades of a billion dollar global algal biofuel industry (SBI 2012) and a surge in research and development (R&D) efforts on lipid production in microalgae. The U.S. Department of Energy's Aquatic Species Program (ASP), in effect from 1978 to 1996, provided an extensive analysis on microalgal strain selection and optimization for lipid production, but determined at the time that increased lipid yields would be necessary for the economic feasibility of algal fuels (Sheehan et al 1998). Thirty-five years later, lipid yield is still one of the most influential barriers in the economics of producing biofuels from algae (Davis et al 2011).

1.1.4 Molecular Tools for Microalgae

Since the sequencing of the first cyanobacterial genome in 1996 (Kaneko et al 1996) (that of *Synechocystis* sp. strain PCC6803) and the first eukaryotic microalgal genomes in 2004 (those of the diatom *Thalassiosira pseudonana* (Armbrust et al 2004) and the red extremophile *Cyanidioschyzon merolae* (Matsuzaki et al 2004)), significant advances in sequencing technologies have allowed for the description of many more algal genomes (Gimpel et al 2013). Beyond classically studied model organisms, such as the green alga *Chlamydomonas reinhardtii*, cheaper and faster sequencing has allowed for the elucidation of the genomes of more production-relevant strains such as *Nannochloropsis gaditana* (Radakovits et al 2012). The molecular toolbox for microalgae, including transformation, overexpression, localization and knock-downs, have also advanced in recent years, opening the door for genetic and metabolic manipulations of biochemical pathways (Liu and Benning 2013, Shrestha et al 2013, Trentacoste et al 2013).

1.2 Carbon Flux in a Microalgal Cell

Photosynthetically fixed carbon is used for almost every process in the cell and travels through a wide variety of interconnected biochemical pathways (Fig. 1-8). Beyond the core carbon metabolism pathways of glycolysis, gluconeogenesis and pyruvate metabolism, carbon is utilized for membranes, amino acids, pigments, nucleotides, small metabolites, signal molecules, etc. The flux of carbon into these different pathways is highly dependent upon the physiological state of the cell, as exemplified by the redirection of carbon into lipid biosynthesis in many microalgae

during environmental stress such as nutrient limitation and changes in pH. Carbon flux is additionally dependent upon the lineage (Hildebrand et al 2013) and even species of microalgae and accordingly the cellular organization and partitioning (Smith et al 2012). The intricate connectedness of different metabolisms within a microalgal cell implies that there are many pathways in competition with lipid biosynthesis for carbon; each merits attention as a point of regulation and thus a potential area for manipulation.

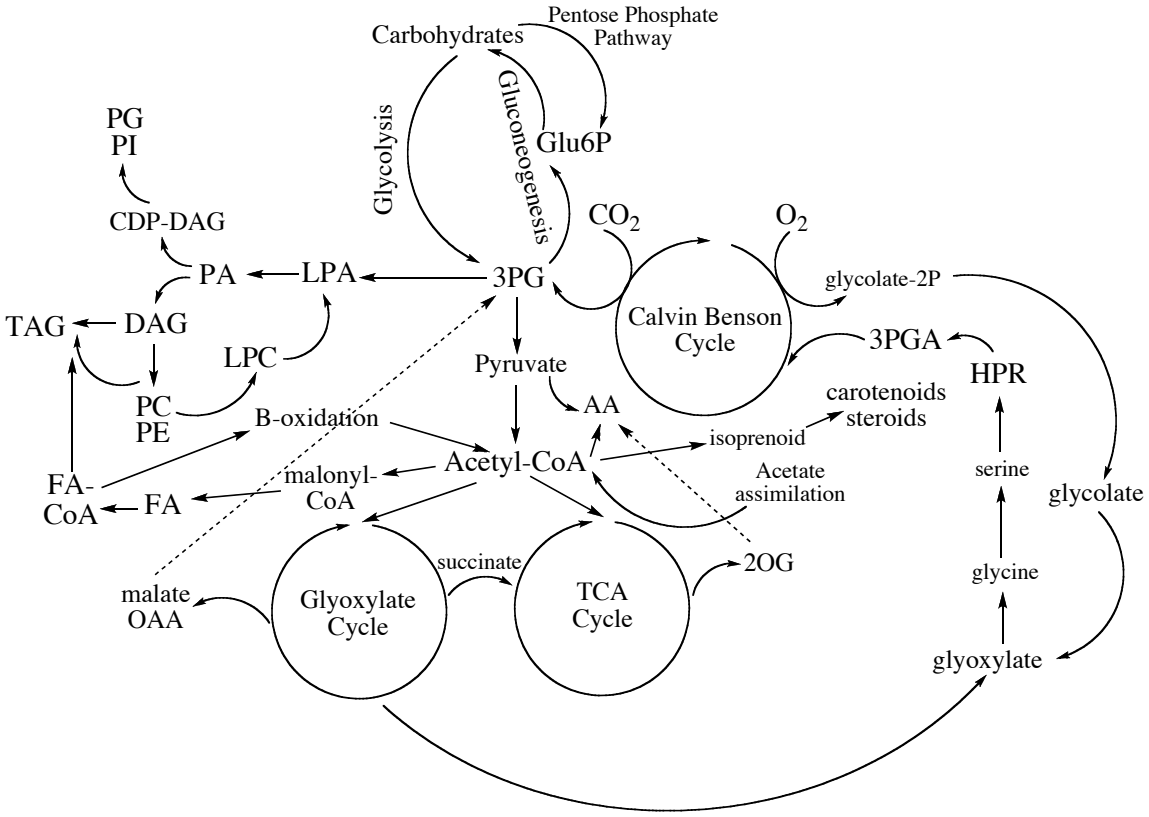


Figure 1-6. Examples of interconnected microalgal metabolisms.
Carbon can take a variety of paths to multiple fates in a microalgal cell. Some of these pathways and their connections are shown here. Subcellular locations are not shown because they often differ between microalgal lineages. 3PG: 3-phosphoglyceraldehyde; Glu6P: Glucose-6-phosphate; LPA: lysophosphatidic acid; PA: phosphatidic acid; CDP-DAG: CDP-diacylglycerol; PG: phosphatidylglycerol; PI: phosphatidylinositol; DAG: diacylglycerol; TAG: triacylglycerol; PC: phosphatidylcholine; PE: phosphatidylethanolamine; FA: fatty acid; OAA: oxaloacetate; AA: amino acids; 2OG: 2-oxoglutarate; HPR: hydroxypyruvate; 3PGA: 3-phosphoglycerate; glycolate-2P: glycolate-2-phosphate.

1.2.1 Photosynthesis

It is becoming increasingly apparent that the regulation of lipid biosynthesis is affected more by substrate availability than by presence and activities of enzymes (Collén et al 2004, Fan et al 2012, Ramanan et al 2013). Like glycolysis and gluconeogenesis (Smith et al 2012), lipid biosynthesis in microalgae may be largely driven by mass action enzymes and involve a few major regulatory points. The first pathway providing substrates for lipid biosynthesis is the sequestration of carbon from photosynthesis and the Calvin-Benson Cycle. Efficient and active photosynthesis provides fixed carbon for cellular processes, and lipid biosynthesis is partially controlled by the ability of the photosynthetic apparatus to absorb photons. In fact, at high light intensities when photosynthesis is saturated, the efficiency of lipid production decreases (Dillschneider et al 2013).

Manipulating photosynthetic efficiency is one possible way to increase substrate availability for lipid biosynthesis. The light-harvesting complexes (LHCs) in microalgae maximize light absorption under low-light conditions, and thus must frequently dissipate excess energy under saturating conditions (Fig. 1-9). Knock-down of LHCs has been proposed as a method to increase photosynthetic efficiency in microalgae (Gimpel et al 2013). The silencing of all twenty LHC isoforms in *C. reinhardtii* resulted in strains with 68% less chlorophyll, increased light penetration, and increased photosynthetic quantum yield; under high-light conditions the resulting strains also showed an increased growth rate (Mussgnug et al 2007). More recently the knock-down of three LHCs in an H2-producing *C. reinhardtiii* mutant strain similarly resulted in a light green phenotype and

increased light-to-biomass conversion efficiency (Oey et al 2013). Subsequent to the light reactions of photosynthesis, the Calvin-Benson cycle also controls carbon assimilation, thus emphasis has been placed on finding strains with high CO_2-fixing capabilities (Murakami and Ikenouchi 1997).

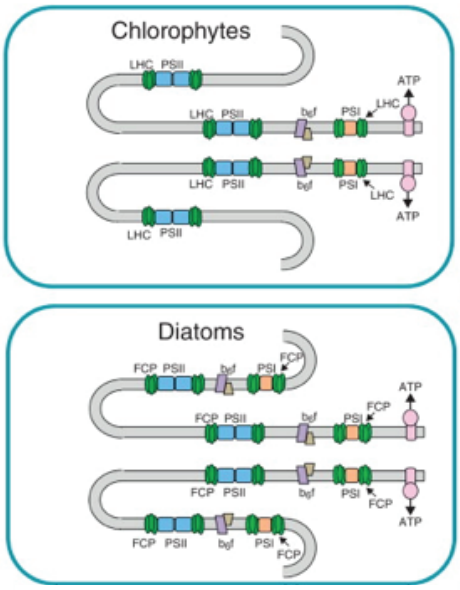


Figure 1-7. Light harvesting complexes.
Light harvesting apparatus are present in the thylakoid membranes to harvest and transfer energy into adjacent reaction systems. Chlorophytes (green algae) use light harvesting complexes (LHC), while stramenopiles, including diatoms, use fucoxanthin chlorophyll binding proteins (FCPs). From Hildebrand *et al.*, 2013.

1.2.2 Routes for Sequestered Carbon

1.2.2.1 Carbohydrates

Photosynthetically assimilated carbon can travel a variety of routes in the cell (Fig. 1-7). Under nutrient replete growth conditions, carbon is preferentially distributed to synthesize new membranes for dividing cells and into pyruvate metabolism to generate acetyl-CoA for the Citric Acid Cycle. Excess sequestered carbon is typically put into carbohydrates (such as starch or chrysolaminarin) as short-term energy storage molecules

(Fan et al 2012, Hildebrand et al 2013, Li et al 2011, Siaut et al 2011) that store reducing power. Directing glyceraldehyde-3-phosphate (G3P) into carbohydrate biosynthesis also recycles inorganic phosphate for photosynthesis (Nelson and Cox 2000). Carbohydrates are thought to be preferentially biosynthesized and utilized in the short-term because they require less ATP and NADPH to synthesize than lipids (Dillschneider et al 2013), and because it is more thermodynamically efficient for the cell to synthesize and utilize glucose polymers than lipids (Sorgüven and Özilgen 2013).

While disruption of carbohydrate biosynthesis has been a proposed method to increase lipids (Li et al 2010, Work et al 2010), this conclusion should be extrapolated with caution, as the accumulation of carbohydrates varies greatly between microalgal species. While some species show direct competition between carbohydrates and lipids for storage of carbon, others use carbohydrate breakdown as a direct source of carbon for lipid biosynthesis, and consequently may require carbohydrate production for lipid production (Fernandes et al 2013, Gardner et al 2013, Mizuno et al 2013, Roessler 1998). Additionally, some species of *Chlorella* (Brányiková et al 2011) and *Tetraselmis* (Yao et al 2012) have been shown to only accumulate starch instead of lipid.

1.2.2.2 Membrane Lipids

Under favorable growth conditions, a significant amount of carbon sequestered by microalgae is used to synthesize new membrane components for dividing cells. Stressful conditions seem to direct carbon away from membrane biosynthesis into TAG, which may be due to cessation of the cell cycle (Guckert and Cooksey 1990). Phospholipids, like TAG, are synthesized via transfer of acyl groups onto a G3P backbone, and many of

the intermediates in each pathway are the same. It has recently been shown in many microalgae that polar lipids, often from plastid membranes, are sources of acyl groups for TAG biosynthesis (Goncalves et al 2013, Li et al 2012, Siaut et al 2011, Yoon et al 2012), and an intimate connection between polar and neutral lipid pools is becoming increasingly apparent (Trentacoste et al 2013).

1.2.2.3 Pyruvate Metabolism

Pyruvate metabolism directs carbon into a variety of biochemical pathways in the cell and is therefore a key regulatory point in carbon allocation in microalgae (Guerra et al 2013). After conversion from G3P, pyruvate can directly be used for amino acid biosynthesis, or can be decarboxylated into acetyl-CoA. Acetyl-CoA is intricately linked to lipid metabolism because carboxylation of acetyl-CoA to malonyl-CoA is the first committed step of lipid biosynthesis, and also because the breakdown of fatty acids through ß-oxidation contributes to the acetyl-CoA pool. Acetyl-CoA can also be used to make isoprenoids, the building blocks of steroids, carotenoids and pigments. Some microalgae decrease pigments, which are carbon-rich molecules, during lipid accumulation (Klok et al 2013), perhaps because they compete with lipid as a carbon sink (Fernandes et al 2013). Alternatively, some *Dunaliella* strains accumulate carotenoids during stressful conditions (Lamers et al 2008) in conjunction with and interdependent on TAG accumulation (Rabbani et al 1998).

Acetyl-CoA also enters the TCA cycle to drive the production of reducing equivalents and ATP, both of which are required for *de novo* lipid biosynthesis (Dillschneider et al 2013). Thus, shuttling of acetyl-CoA into the TCA cycle cannot

necessarily be considered competitive to lipid biosynthesis. Intermediates of the TCA cycle are also used to make amino acids. Exogenous acetate assimilation may also contribute to the acetyl-CoA pool. Many microalgae have the ability to assimilate acetate from the environment (Cooksey 1974, Gibbs et al 1986, Syrett et al 1964), and to incorporate it into lipids when it provides excess carbon (Fan et al 2012, Goodson et al 2011, Ramanan et al 2013). Finally, acetyl-CoA can be incorporated into the glyoxylate cycle, which results in the net production of succinate.

Not only is carbon flux incredibly complex in an algal cell, but lipid pools are intricately connected to other parts of cellular metabolism. For example, as discussed above, for most microalgae there is probably not a single deciding branch point between carbohydrate and lipid biosynthesis, as often these two pools are connected during lipid accumulation. Moreover, major differences exist between lineages of microalgae (Hildebrand et al 2013) and even species within a lineage (Smith et al 2012), thus particular manipulations may not be applicable beyond the example strain. In order to tease apart the complexity of carbon metabolism and begin to isolate promising points for manipulation, it is necessary to explore the state of the cell during lipid accumulation, and the implications of potential manipulations on the biology of the cell.

1.3 Microalgal Lipid Metabolism

While improvements along the algal biofuel pipeline (Fig. 1-3) can bolster economic viability of the process, recent techno-economic analyses suggest that the most impactful factor is increasing lipid yields from microalgae (Davis et al 2011). As molecular techniques for transformation and genetic and metabolic manipulation have

advanced for microalgae, this aspect strain of development and optimization for biofuel production has become more feasible, thus enabling progression past the conclusions of the ASP. Strain improvement efforts have been both targeted and random and have focused on a spectrum of pathways including lipid metabolism, carbohydrate metabolism, and photosynthesis. The results of these efforts have substantially increased knowledge of the pathway of lipid metabolism and its complex interactions with other cellular pathways.

1.3.1 The Pathway of Lipid Metabolism

TAG biosynthesis involves the transferring of fatty acyl groups onto a glycerol backbone and has been shown in microalgae to occur either in the endoplasmic reticulum (ER) or the plastid (Fig. 1-6). The existence of both pathways and portion of TAG synthesis provided by each is most likely dependent on the species of algae. The transfer of acyl groups to form TAG can occur through two major routes: the Kennedy Pathway involving transfer of acyl-CoA units onto diacylglycerol (DAG), catalyzed by DAG acyltransferase (DGAT), and an acyl-CoA-independent pathway in which acyl groups are transferred from phospholipids, catalyzed by phospholipid:DAG acyltransferase (PDAT) (Yoon et al 2012).

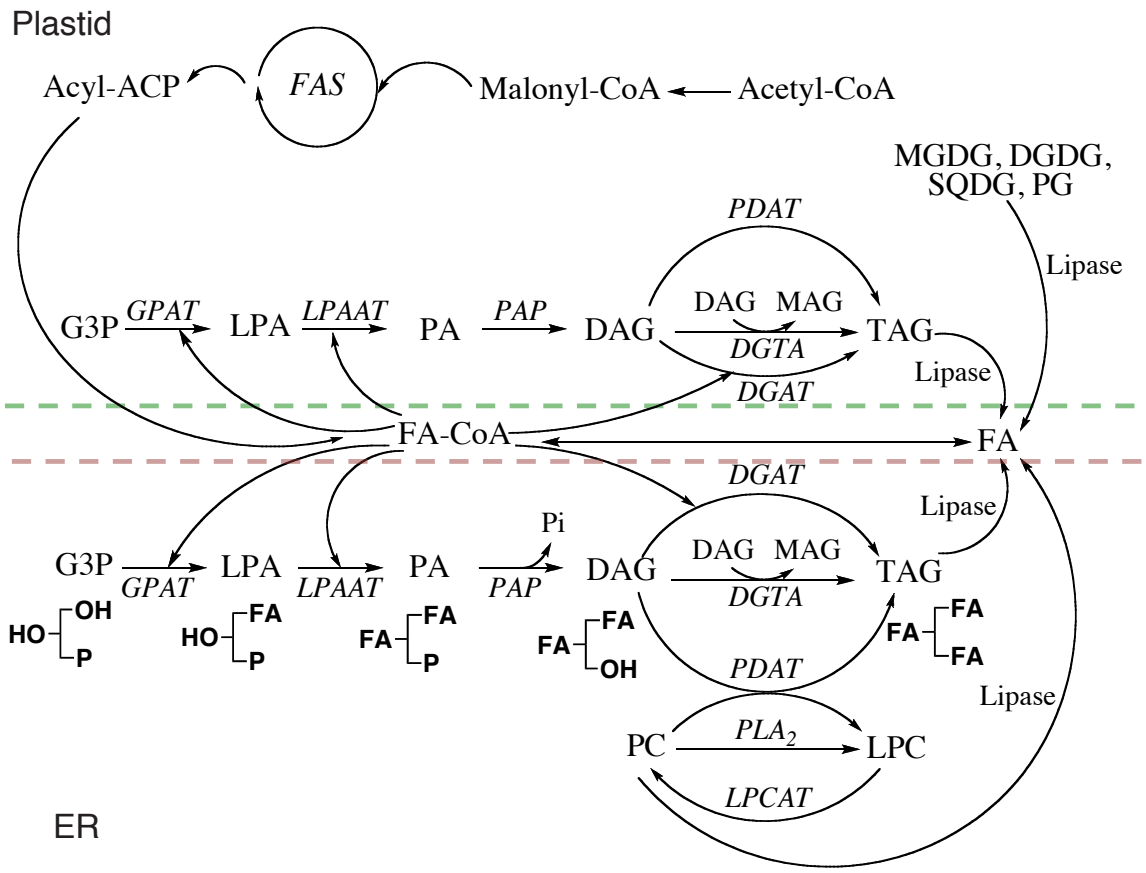


Figure 1-8. TAG biosynthetic pathway.
Fatty acid biosynthesis occurs in the plastid. TAG biosynthesis has been shown to occur in both the plastid and ER in some microalgal species. Basic structures of intermediates in TAG biosynthesis are shown in the ER pathway; structures are the same for the plastid pathway. Enzymes catalyzing reactions are shown in italics. *FAS:* fatty acid synthase; *GPAT:* glycerol-3-phosphate acyltransferase; *LPAAT*: lysophosphatidic acid acyltransferase; *PAP*: phosphatidic acid phosphatase; *DGAT*: diacylglycerol acyltransferase; *DGTA*: diacylglycerol transacylase; *PDAT*: phospholipid-diacylglycerol acyltransferase; *PLA_2:* phosphlipase A2; *LPCAT:* lysophosphatidylcholine acyltransferase. G3P: glycerol-3-phosphate; LPA: lysophosphatidic acid; PA: phosphatidic acid; DAG: diacylglycerol; TAG: triacylglycerol; MAG: monoacylglycerol; PC: phosphatidycholine; LPC: lysophosphatidylcholine; FA-CoA: fatty acyl CoA; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; SQDG: Sulfoquinovosyl diacylglycerol; PG: phosphatidylglycerol.

The Kennedy pathway involves subsequent transfers of acyl-CoA molecules onto a glycerol-3-phosphate (G3P) backbone. G3P acyltransferase (GPAT) and lysophosphatidic acid (LPA) acyltransferase (LPAAT) catalyze the first two acylations to form LPA and phosphatidic acid (PA), respectively. PA phosphatase (PAP) dephosphorylates PA to form DAG, and DGAT catalyzes the final addition of an acyl group to form TAG. Analysis of DGATs showed differences in the number and types of isoforms present, even within individual algal lineages (Chen and Smith 2012). The plastidial Kennedy pathway is considered of prokaryotic origin, and thought to have arisen from the cyanobacterial ancestor of algae, while the ER Kennedy pathway is eukaryotic.

The acyl-CoA independent pathway is mediated through PDAT. PDAT has been shown to use phospholipids, galactolipids and DAG as substrate for acyl transfer onto DAG in *C. reinhardtii* and is integral in membrane lipid turnover (Yoon et al 2012). Membrane phospholipids contribute a significant portion of fatty acids for TAG in *P. tricornutum* suggesting that PDAT or other membrane turnover enzymes are integral during neutral lipid synthesis (Burrows et al 2012). An initial analysis found different numbers of PDAT isoforms between microalgal species, implying that this pathway may be as complex as DGAT and the Kennedy pathway across algal lineages.

The fatty acyl groups used to synthesize TAG can come from a variety of sources. As mentioned above, phospholipids, galactolipids and other DAG molecules can directly contribute their acyl constituents. The acyl-CoA groups used in the Kennedy pathway come from the cell's dynamic acyl-CoA pool. *De novo* fatty acid biosynthesis, fatty acid

release from complex lipids, and membrane remodeling/editing all contribute acyl-CoA groups to this pool. In microalgae, *de novo* fatty acid biosynthesis occurs in the plastid and begins with the carboxylation of acetyl-CoA to form malonyl-CoA by acetyl-CoA carboxylase (ACCase), which after being transferred to an acyl carrier protein (ACP) undergoes elongation to form 16-carbon palmitic acid catalyzed by a series of enzymatic domains on fatty acid synthase (FAS). Palmitic acid can then be further processed, elongated, desaturated and finally activated to form different fatty acid species to contribute to the acyl-CoA pool (Blatti et al 2013).

1.3.2 Omics Analyses of Microalgal Lipid Metabolism

The prevalence of "-omics" analyses has drastically increased for microalgae in recent years with the sequencing of genomes from new species. Genomic studies provide information on the potential pathways existing in species and the evolutionary relationships between species. Transcriptomic analyses are useful in providing a snapshot of the state of an organism under environmental conditions at the mRNA level, thus providing a general overview of the genetic and metabolic response to such conditions. Proteomics can provide a more specific picture of the response of pathways on the enzymatic level, and the integration of transcriptomics and proteomics can shed light on different levels of pathway regulation. Metabolomic analyses, including lipidomics, provide information on the physiological state of the cell and changes in flux of metabolites through particular pathways.

1.3.2.1 Genomes & Genomics

As sequencing technologies have improved in recent years, the number of microalgal genomes sequenced has continued to expand. These genomes have been extremely influential in the initial elucidation of lipid metabolism in microalgae as well as the development of molecular techniques for genetic engineering. Many transformation and expression techniques were originally developed in *C. reinhardtii* (Eichler-Stahlberg et al 2009) as a model organism for green algae, but the use of molecular techniques is expanding to other species of microalgae more relevant to biofuel production as more genomes become available. For example, the genome for the oleaginous microalga *Nannochloropsis gaditana* gave insight into lipid biosynthesis in this species and allowed for the development of transformation techniques in this marine alga considered promising for production (Radakovits et al 2012). The genome of *Nannochloropsis oceanica*, another potential biofuel-producing strain, also revealed enzymes involved in lipid production (Pan et al 2011).

As more microalgal genomes become available, comparative genomics can be used to reveal conserved and unique aspects of metabolic pathways including those of lipid metabolism. One genomic study of the diatoms *T. pseudonana*, *P. tricornutum* and *Fragilariopsis cylindrus* identified major differences between species in fundamental carbon pathways (Smith et al 2012), and another study comparing *T. pseudonana* and *P. tricornutum* found differences in carbon and carbohydrate metabolism as well, including the most basic pathway of CO_2 fixation (Kroth et al 2008). Comparative genomics of *N. gaditana* with photosynthetic and non-photosynthetic stramenopiles revealed a set of genes potentially involved in conveying photosynthetic capacity (Radakovits et al 2012). These two studies show that although related, microalgae can sometimes have substantial

differences in metabolic pathways (Hildebrand et al 2013), and an understanding of these differences is imperative when trying to improve a strain for biomass or lipid yields.

1.3.2.2 Transcriptomics

Just as DNA sequencing has become cheaper, faster and easier in recent years, so has technology to analyze RNA transcripts. This has allowed for sequencing and quantification of total microalgal transcriptomes under a variety of conditions. Analyzing transcript abundances during lipid accumulation can elucidate major players in lipid biosynthesis, reveal other cellular responses during this phenomenon, and identify key enzymes to target for manipulation. A number of studies on transcript abundance have been performed in *C. reinhardtii* under nitrogen-limited conditions, which induces lipid accumulation (Boyle et al 2012, Lv et al 2013, Miller et al 2010). Under these conditions, protein biosynthetic enzymes and photosynthetic enzymes were downregulated, while enzymes involved in lipid biosynthesis (including fatty acid and glycerolipid biosynthesis), membrane turnover and remodeling were upregulated, including multiple acyltransferases and lipases. These findings indicate that some enzymes involved in lipid biosynthesis and membrane turnover may be good targets for genetic manipulation to increase TAG synthesis. The results from these studies were corroborated by another nitrogen-deprivation experiment in *C. reinhardtii* using RT-PCR (Msanne et al 2012).

RNA-seq is also becoming a useful tool for elucidating metabolic pathways in microalgae with unsequenced genomes. Sequenced transcripts can be used to query nucleotide databases for homologous sequences, providing a rudimentary annotation of

genes. This strategy was employed to elucidate the enzymes involved in carbohydrate, fatty acid and TAG metabolic pathways in *Dunaliella tertiolecta* (Rismani-Yazdi et al 2011), an unsequenced microalga with potential as a biofuel production organism. The identification of the genes and enzymes involved in these pathways will help direct genetic engineering efforts for strain improvement and allow for comparative genomics analyses.

1.3.2.3 Proteomics

Little work has been done to date to analyze microalgal proteomes despite the fact that transcriptomics data does not fully characterize regulation in a cell. Proteomics is becoming increasingly used to understand lipid pathways as it becomes evident that TAG biosynthesis is under multiple levels of regulation. Proteomic analysis of *C. vulgaris* under lipid accumulation-inducing nitrogen limitation conditions revealed increased levels of fatty acid and glycerolipid biosynthetic enzymes, especially ACCase and DGAT (Guarnieri et al 2011, Guarnieri et al 2013). A concomitant downregulation in enzymes involved oxidizing lipids was found, especially AMP-activated kinase (AMPK), which inhibits ACC, thus revealing another possible level of regulation that could explain those previous results, and thus another potential target for genetic engineering. A large increase in malic enzyme was also detected, which generates NADPH that is necessary for lipid biosynthesis. When nitrogen replete and deplete conditions were compared, a number of cell cycle progression enzymes were only found under replete conditions, which corroborate the decrease in growth exhibited by nitrogen-limited strains (Guarnieri et al 2013).

Proteomics of isolated lipid droplets in *C. reinhardtii* detected a number of enzymes involved in glycerolipid biosynthesis, including GPAT, LPAT and PAP, in association with the droplet, as well as many acyltransferases, lipases and LACSs (Moellering and Benning 2010, Nguyen et al 2011). The presence of these enzymes supports the role of oil bodies as sites of acyl-group exchange involved in TAG biosynthesis, degradation and homeostasis. The proteomics approach, however, still exhibits a number of obstacles. While genomics and transcriptomics allow analysis of almost every gene or transcript present, proteomics can currently only provide information for a subset of proteins detected. Proteomics approaches are also less sensitive than other –omics approaches and have a more limited dynamic range.

1.3.2.4 Metabolomics

Metabolomic analyses can reveal changes in flux of metabolites through pathways under different conditions in response to transcript or protein changes. Metabolome analysis of the green alga *Pseudochoricystis ellipsoidea* under nitrogen starvation conditions revealed a decrease in chloroplastic lipids, amino acids and metabolites involved in nitrogen assimilation and nitrogen transport, but an increase in neutral lipid molecules (Ito et al 2013). Lipidomics of *Chlamydomonas nivalis* revealed a similar decrease in chloroplastic lipids (Lu et al 2013). An analysis of the metabolome of *Scenedesmus obliquus* uncovered an increase in the metabolite ethanolamine during lipid accumulation; this connection was further supported when treatment of *S. obliquus* cultures with ethanolamine increased lipid levels (Cheng et al 2012). Although some changes in metabolites can be predicted under certain conditions, it's important to note

that metabolite levels are significantly different depending on the experimental condition, such as between different nutrient limitation regimes (Bölling and Fiehn 2005).

From these omics analyses in microalgae emerge a few patterns in the cellular response that results in lipid accumulation. Transcriptomic and proteomic analyses reveal an increase in biosynthetic machinery necessary for lipid production, but a simultaneous increase in enzymes involved in lipid turnover. Metabolomic analyses corroborate these results, revealing increases in neutral lipids and decreases in chloroplast membrane lipids. A decrease in photosynthetic machinery seems to be a universal response to nitrogen deprivation. However, while general patterns can be drawn across species, it is increasingly apparent that lipid accumulation is under many levels of regulation, and that the precise mechanisms differ depending on the environmental trigger, and enzymatic machinery of the species.

1.3.3 Previous Engineering Attempts

One of the original engineering strategies for improving lipid production in microalgae was the overexpression of ACCase in the diatom *Cyclotella cryptica* (Fig. 1-7). ACCase is considered to catalyze the first committed and rate-limiting step of fatty acid biosynthesis, and thus represents a key regulatory point in the pathway. ACCase activity was shown to be correlated to fatty acid biosynthesis in plants and TAG accumulation in *C. cryptica in situ*. ACCase expression levels were also shown to correlate to fatty acid biosynthesis in animals (Roessler and Ohlrogge 1993). Overexpression of ACCase in *C. cryptica* resulted in increased ACCase levels and activity, but no change in lipid content in the cell (Sheehan et al 1998). It has since been

shown in multiple microalgae that ACCase expression levels increase during lipid accumulation. However, expression of several FA biosynthesis enzymes in *C. reinhardtii* was recently proposed to be regulated by acetate availability, thus substrate availability may be the limiting factor in directing of carbon into fatty acid biosynthesis (Ramanan et al 2013).

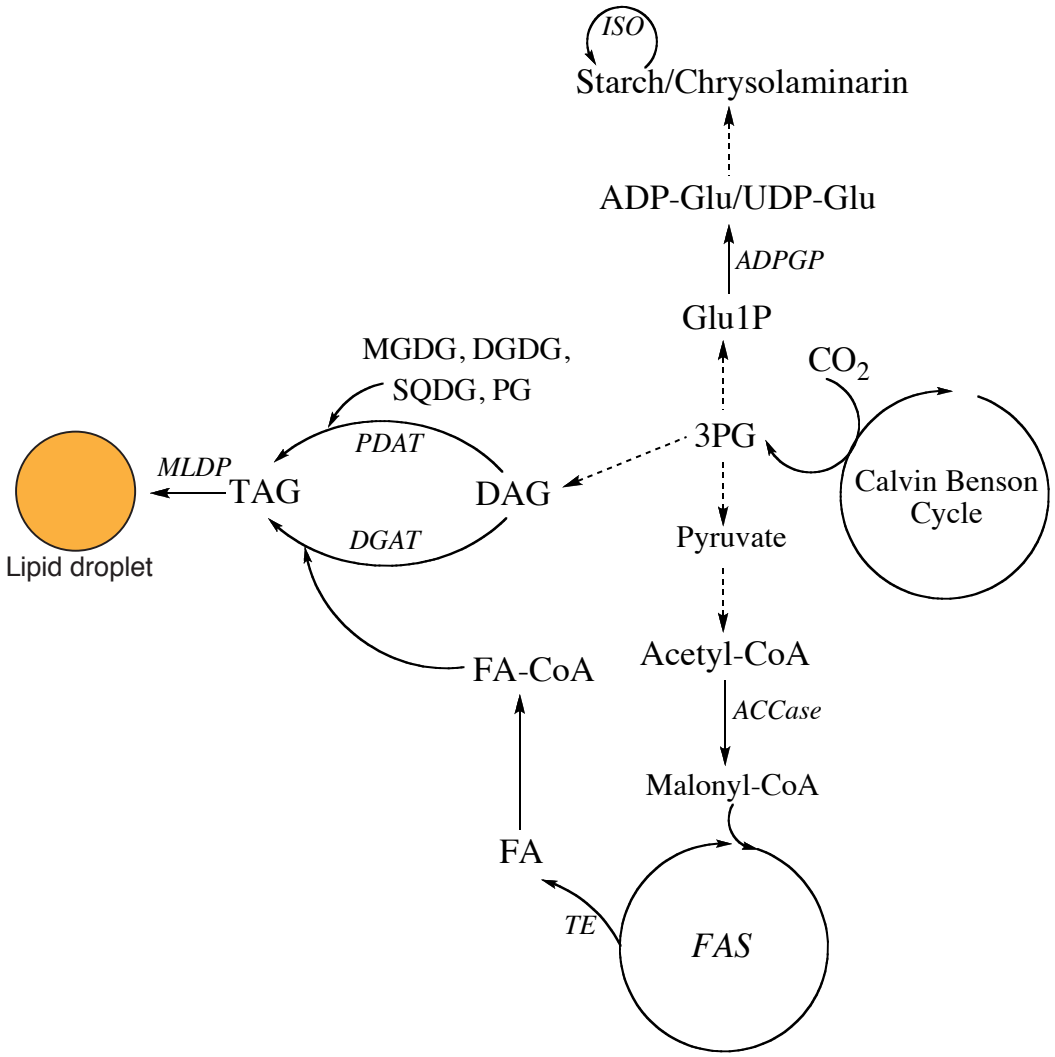


Figure 1-9. Manipulations of microalgal lipid metabolism.
Schematic of microalgal metabolism showing manipulations that have been performed on various parts of lipid metabolism and its connected pathways. Enzymes that have been manipulated are shown in italics. *FAS*: fatty acid synthase; *TE:* thioesterase; *ACCase*: acetyl-CoA carboxylase; *ADPGP*: ADP-glucose pyrophosphorylase; *ISO:* isoamylase; *PDAT*: phospholipid-diacylglycerol acyltransferase; *DGAT:* diacylglycerol acyltransferase; *MLDP:* major lipid droplet protein. FA: fatty acid; 3PG: 3-phosphoglyceraldehyde; Glu1P: glucose-1-phosphate; ADP-Glu: ADP-Glucose; UDP-Glu: UDP-Glucose; DAG: diacylglycerol; TAG: triacylglycerol; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; SQDG: Sulfoquinovosyl diacylglycerol; PG: phosphatidylglycerol.

Thioesterase (TE), which terminates fatty acid biosynthesis and releases palmitic acid from FAS, has also been a target for manipulation because it terminates fatty acid biosynthesis and thus determines the chain length of the fatty acid produced (Fig. 1-7). Recent attempts to overexpress TEs in *C. reinhardtii* and *P. tricornutum* have resulted in higher proportions of shorter chain fatty acids (Blatti et al 2012, Radakovits et al 2011). In *C. reinhardtii,* protein-protein interaction between TE and ACP was shown to be essential for transfer of the fatty acid chain to TE prior to the final cleavage step of fatty acid biosynthesis (Blatti et al 2012). The authors showed that this protein-protein interaction is extremely specific, and that while *C. reinhardtii* chloroplastic ACP and TE could interact, TEs from plants would not interact with *C. reinardtii* ACP. Additionally, *C. reinhardtii*'s mitochondrial ACP did not interact with its chloroplastic TE. Specific, targeted overexpression of *C. reinhardtii*'s chloroplastic TE, driven by chloroplast promoters and RNA elements, resulted in an increase in shorter-chain fatty acids, specifically 14-carbon myristic acid, which was not seen when plant TEs were overexpressed. This work elucidated the role of protein-protein interactions in fatty acid biosynthesis and the importance of specific targeting for metabolic engineering (Blatti et al 2012).

In *P. tricornutum*, two plant TEs catalyzing the release of lauric and myristic acids were overexpressed. The resulting strains showed variable increases in the production of these fatty acids. Additionally, overexpression strains accumulated higher total lipid than wild-type, but grew more slowly, and thus the total lipid yield was no higher. In cyanobacteria overexpression of TEs leads to fatty acid secretion, but the *P.*

tricornutum TE overexpression strains did not reveal a similar result, suggesting that secretion of fatty acids is a more complex process in eukaryotic microalgae (Radakovits et al 2011). Work is currently being done to modify fuel molecule secretion as well (Doshi et al 2013).

Looking beyond fatty acid biosynthesis, the enzymes involved in TAG biosynthesis have also been targets for engineering (Fig. 1-7). Studies in plants have shown that this is a feasible method to increase lipid production. However, the overexpression of three endogenous DGAT enzymes in *C. reinhardtii* resulted in no change in the quantity or quality of neutral or polar lipids, suggesting that TAG biosynthesis is either not regulated at this step or is controlled more by substrate availability (La Russa et al 2012). Microalgae have also been shown to contain more isoforms of DGAT than other eukaryotes (Chen and Smith 2012); only three of *C. reinhardtii*'s five DGAT isoforms were targeted for overexpression. Additionally, only one of the three overexpressed DGATs was found to be upregulated during lipid accumulation in *C. reinhardtii* (Msanne et al 2012), thus the opportunity for future engineering exists.

The acyl-CoA independent pathway of TAG biosynthesis has also been targeted for manipulation (Fig. 1-7). *C. reinhardtii* was found to have one PDAT, and knockdown of this enzyme resulted in decreased TAG biosynthesis, increased MGDG, PG and SQDG (chloroplast membrane lipids) and decreased growth. Therefore this enzyme was thought to function in using chloroplast membrane lipids as substrates for acyl transfer to DAG. Interestingly, this PDAT enzyme was found to be more important in biosynthesizing TAG during exponential growth rather than stress-induced TAG

accumulation (Yoon et al 2012), suggesting that the different routes to TAG biosynthesis play different functional roles in *C. reinhardtii*, further demonstrating the complexity of lipid metabolism.

As previously discussed, in a proteomics study in *C. reinhardtii*, proteins associated with the lipid droplet, including acyl-CoA synthetases, lipoxygenases, and vesicular trafficking enzymes were identified. The most abundant protein, denoted major lipid droplet protein (MLDP), was knocked down using RNAi techniques in this species (Fig. 1-7). The resulting strains accumulated larger lipid droplets but did not accumulate any more lipid than wild-type, suggesting that this enzyme is involved in controlling lipid droplet size or fusion (Moellering and Benning 2010).

1.3.4 Engineering Related Pathways

Rather than focus on biosynthesis of lipids, other engineering efforts have focused on disrupting pathways that compete with lipids as carbon sinks. Carbohydrates are generally thought to be short-term storage molecules that, compared to TAG, are easier for cells to synthesize and faster for cells to use. A mutant with disrupted ADP-glucose pyrophosphorylase (AGPase) was isolated in *C. reinhardtii* (Fig. 1-7) after insertional mutagenesis. AGPase catalyzes the formation of ADP-glucose and is considered the first committed step in starch biosynthesis. ADPase mutants in *C. reinardtii* accumulated higher amounts of TAG under nutrient-limited conditions compared to wild-type, however the resulting strains showed severe defects in growth (Li et al 2010). Another starchless *C. reinhardtii* mutant was isolated and found to lack isoamylase function, which is important in determining the structure of starch granules (Fig. 1-7). This mutant

accumulated more lipid, but also showed defects in growth and division (Work et al 2010). These results suggest that while carbohydrate biosynthesis competes with lipid for carbon, and that disrupting it can potentially direct more carbon into lipids, the starch pool is essential for growth and division, at least in this organism.

The manipulations performed thus far on lipid metabolism in microalgae have revealed characteristics of the regulation of this pathway. Figure 1-7 provides an overview of these manipulations. Overexpressing biosynthetic enzymes has had either no or moderate effects on lipid levels, suggesting that synthesis of lipids is controlled more by substrate availability than by enzyme levels and activity. Accordingly, decreasing carbon flux into competing sinks, such as carbohydrate biosynthesis, can increase lipid accumulation. This implies that decreasing carbon flux into other competing pathways may have a similar effect. Because lipid synthesis may be based on substrate availability, it is useful to explore carbon flux in the cell to determine potential pathways to manipulate.

1.4 Lipid Accumulation

Lipid typically accumulates in microalgae under conditions when the cell is unable to use a portion of the carbon it assimilates. These conditions, such as nutrient limitation, can cause a variety of secondary effects on the cell as well, such as the decrease in proteins often exhibited after nitrogen limitation. Because manipulations for increased lipid accumulation are often looking to mimic the lipid phenotypes of these states, it may be useful to explore these secondary effects as possible points of pathway engineering.

1.4.1 Causes of Lipid Accumulation

1.4.1.1 Nutrient Limitation

Nutrient limitation is by far the most well studied trigger for lipid accumulation in microalgae. While responses to nitrogen limitation are the most universally studied (Hu et al 2008), various species have also been found to accumulate lipid in response to phosphorus (Lu et al 2013, Reitan et al 1994), iron (Urzica et al 2013), and silicon (Traller & Hildebrand 2013). The extent and nature of the cell's physiological response often differs between the limiting nutrient regime as well as the species (Hockin et al 2012, Yu et al 2009), suggesting that while some responses are conserved, such as lipid accumulation and decreased growth, the mechanism and regulation behind them vary.

Nitrogen limitation is traumatic to a cell's physiology, as nitrogen is required for key metabolites such as proteins and nucleic acids. Accordingly, microalgae exhibit decreased growth in response to nitrogen deprivation and delayed lipid accumulation due to scavenging of internal nitrogen stores to keep basic cellular processes functioning (Hildebrand et al 2012, Simionato et al 2013). Beyond decreased protein content, a universal response to nitrogen deprivation is a decrease in photosynthetic apparatus and light-harvesting pigments (Berges et al 1996, Goncalves et al 2013, Hockin et al 2012, Klok et al 2013, Rhiel et al 1985, Simionato et al 2013, Turpin 1991).

Under most nutrient limitation regimes, the energy and carbon supply outpaces anabolism, which can lead to an overreduction of the photosynthetic machinery and the creation of harmful reactive oxygen species (ROS) (Klok et al 2013). A decrease in pigments and photosynthetic machinery is one way to avoid this photoinhibition. Specific

parts of the photosynthetic machinery can decrease in abundance, particularly photosystem II (PSII) (Berges et al 1996), but often photosynthetic membranes within the plastid (Rhiel et al 1985, Turpin 1991) or the plastid itself degrades (Goncalves et al 2013, Siaut et al 2011).

However, despite its decrease during nutrient limitation, photosynthesis is required for *de novo* fatty acid biosynthesis during lipid accumulation. In order to maintain some photosynthetic capacity the cell can also reorganize its photosynthetic apparatus to direct electrons into other pathways for protection against overreduction. For example, it was found in *Nannochloropsis gaditana* that linear electron flow (LEF) through photosystem II (PSII) decreased during nitrogen limitation while cyclic electron flow (CEF) around PSI increased. The increase in CEF protects the cell from photoinhibition and allows for photosynthesis to continue functioning (Simionato et al 2013). Unlike LEF, which produces both ATP and NADPH necessary for lipid biosynthesis, CEF only results in ATP production. Thus, it was concluded that a significant amount of the NADPH produced by the remaining LEF must go toward lipid biosynthesis. NADPH can also be produced in the cell by the pentose phosphate pathway, and this pathway was found to be upregulated in *Chlorella protothecoides* under nitrogen limitation conditions (Xiong et al 2010).

1.4.1.2 Photoprotection

The production of lipid is also a method of protection against photoinhibition as it serves as a highly reduced electron sink. Increasing photon flux while keeping nutrients constant caused TAG accumulation in *N. oleoabundans*, suggesting that TAG synthesis

can relieve excess light absorption (Klok et al 2013). When nitrogen is unavailable for the synthesis of proteins, dumping fixed carbon into lipid relieves a strong reducing intracellular environment (Guerra et al 2013).

1.4.1.3 Membrane Recycling

It is becoming increasingly apparent that in addition to *de novo* lipid biosynthesis, microalgae harvest intracellular carbon for TAG biosynthesis as well (Roessler 1988), especially from cellular membrane lipids. Membrane recycling of structural lipids (phospholipids and plastid galactolipids) has been shown to provide acyl groups for TAG biosynthesis in many microalgae (Goncalves et al 2013, Li et al 2012, Siaut et al 2011, Simionato et al 2013, Yoon et al 2012) and to be potentially responsible for up to 40% of TAG synthesis in *P. tricornutum* (Burrows et al 2012). It's been proposed that during iron limitation in *C. reinhardtii*, *de novo* fatty acids are even incorporated first into membrane lipids before being remobilized for TAG synthesis (Urzica et al 2013)

1.4.1.4 The Cell Cycle

It has also been proposed that TAG biosynthesis is not directly a result of nutrient limitation, but is rather linked to the inhibition of the cell cycle caused by nutrient limitation. TAG levels naturally cycle in dividing cells as can be seen during light:dark cycles when cells accumulate TAG during the day to use for division at night. Different stages of the cell cycle are more sensitive to specific stresses (Claquin et al 2002), thus if an environmental perturbation stops or lags the cell cycle at a place where 1) TAG

biosynthesis outpaces utilization or 2) TAG is being rapidly biosynthesized, the result would be increased TAG accumulation. This idea was tested by Guckert & Cooksey using high pH to induce stress under nutrient replete conditions. High pH caused inhibition of the cell cycle in *Chlorella* at a point in the cycle immediately preceding the typical TAG utilization period; this led to TAG accumulation even in the presence of replete nutrients (Guckert and Cooksey 1990). Treating cells with nocodazole, a microtubule-base cell cycle inhibitor caused TAG accumulation in diatoms as well, corroborating the hypothesis that TAG accumulation under stress is linked more to cell cycle inhibition than to the specifics of the perturbation (Hildebrand et al 2012).

The concept that TAG accumulation is linked to cell cycle inhibition explains why in all of the above cases, lipid production occurs only during decreased growth. However, maintaining high growth rates and high biomass accumulation is imperative for algal biofuel production on large economic scales (Borowitzka 1992), and engineering efforts that increase lipid content without decreasing growth or biomass can significantly reduce production cost and increase the economic viability of algal biofuels (Davis et al 2011). Metabolic manipulations that have increased TAG have all decreased growth of the resulting strain (Li et al 2010, Radakovits et al 2011, Work et al 2010), but if TAG accumulation occurs during nutrient starvation because the cell cycle stops at the point in which synthesis outpaces utilization, there may be manipulations that produce a similar imbalance of synthesis and degradation independent of the cell cycle, thus resulting in TAG accumulation without a decrease in growth.

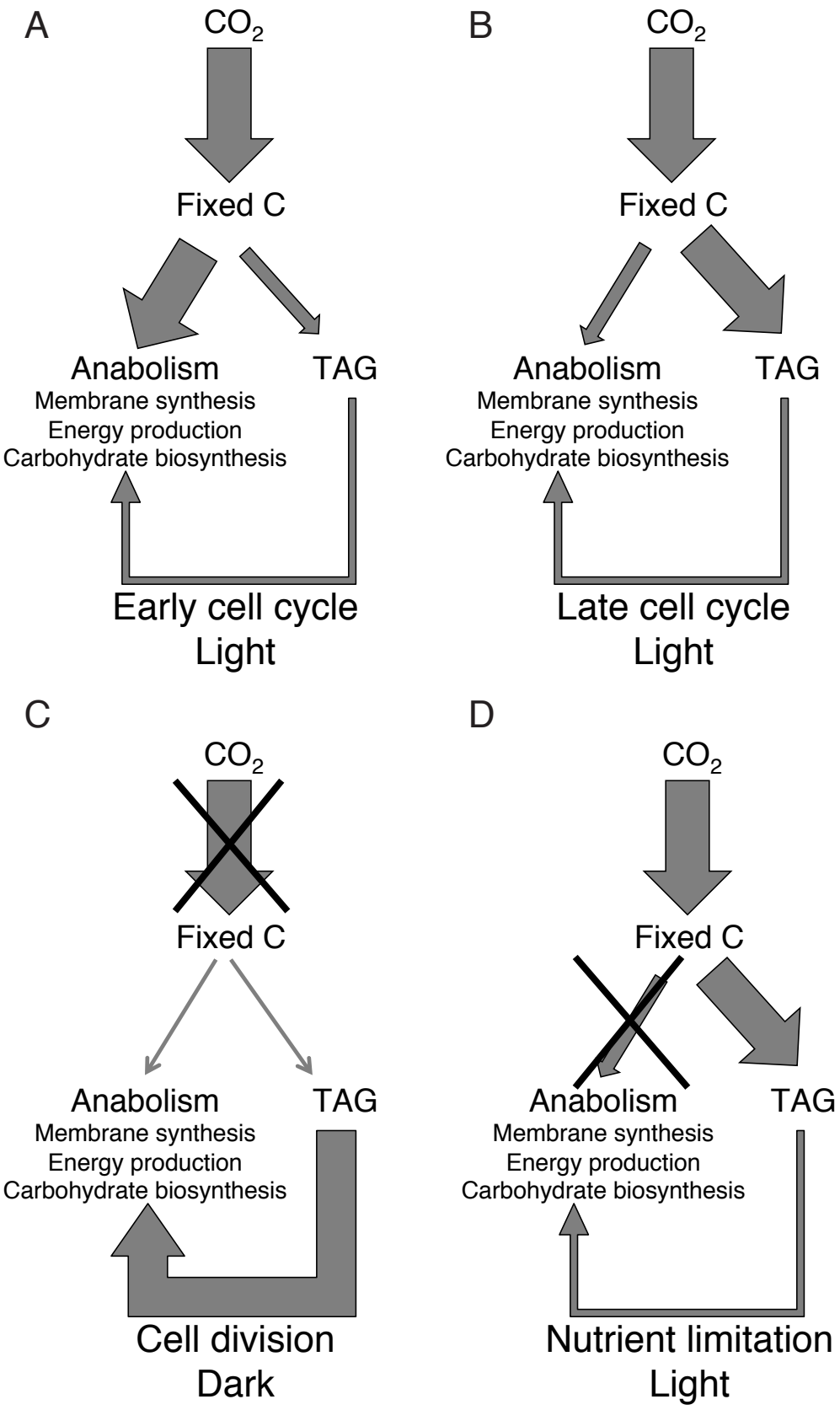
1.4.2 The Cycle of TAG

Exploring the changes in TAG levels during the cell cycle is necessary to assess the possibility of finding manipulations that decouple the two. The role of TAG as storage for reducing equivalents during photosynthetic stress has already been discussed, but TAG can also serve as a reservoir for fatty acids for membrane building. TAG accumulation allows the cell to store specific fatty acid species, such as PUFAs that are important membrane components, for use in rapid synthesis of membranes (Borowitzka 1992, Solovchenko 2012). When environmental conditions change, such as temperature, different fatty acid species are required to maintain membrane structural integrity, and often *de novo* biosynthesis of these fatty acids is not fast enough to respond to perturbations. Remobilization of TAG allows the rapid transfer of preexisting fatty acyl species to membrane components, and thus serves an important function in membrane remodeling.

Due to its multiple functions, TAG is in constant flux throughout the cell cycle (Fig. 1-10). TAG utilization also plays a role in promoting cell division, especially during dark periods and upon nutrient replenishment. When nutrient limitation ends, TAG stores are rapidly depleted to provide building blocks for membrane synthesis and other cellular processes (Bölling and Fiehn 2005, Fernandes et al 2013, Hodgson et al 1991, Khozin-Goldberg et al 2005, Siaut et al 2011). Likewise during light:dark cycling TAG is synthesized during the day (when photosynthetic carbon fixation outpaces carbon utilization) and is mobilized in the dark to promote membrane synthesis allowing for a round of cellular division in the dark (Chauton et al 2013, Fernandes et al 2013, Klein 1987, Sheehan et al 1998, Sukenik and Carmeli 1990) (Fig. 1-11). Polar membrane lipids are typically synthesized early in the cell cycle to build new cellular components (*e.g.* at

the end of a dark period) followed by a transition to TAG synthesis (*e.g.* during light periods) (Sheehan et al 1998), and TAG is utilized during mitosis directly before cell division (Chauton et al 2013, Guckert and Cooksey 1990). In some species, such as *T. pseudonana*, the cell cycle goes through two progressions during light:dark cycling (once in the day and once at night), thus polar membrane lipids are not only synthesized during a dark period.

Figure 1-10. The cycle of TAG.
TAG accumulation or utilization is dependent on the cell cycle, the light conditions, and environmental conditions. Here a few examples are shown of carbon flow under specific light and cell cycle conditions. At early points in the cell cycle, photosynthetically fixed carbon is used primarily for anabolism such as membrane building and energy generation (*A*). Towards the end of the cell cycle, preceding cell division, more carbon is directed in TAG biosynthesis (*B*). In dark conditions, there is no supply of new fixed carbon, and cellular division takes place utilizing TAG reserves synthesized previously in the light (*C*). Nutrient limitation, such as nitrogen starvation, prevents the anabolism of many metabolites, and an increased amount of fixed carbon is directed into TAG (*D*). Some nutrient limitation regimes and cell cycle inhibitors stop the cell cycle directly before division, arresting the cell in a condition similar to (*B*)


A
CO_2
Fixed C
Anabolism
Membrane synthesis
Energy production
Carbohydrate biosynthesis
TAG
Early cell cycle
Light
B
CO_2
Fixed C
Anabolism
Membrane synthesis
Energy production
Carbohydrate biosynthesis
TAG
Late cell cycle
Light
C
CO_2
Fixed C
Anabolism
Membrane synthesis
Energy production
Carbohydrate biosynthesis
TAG
Cell division
Dark
D
CO_2
Fixed C
Anabolism
Membrane synthesis
Energy production
Carbohydrate biosynthesis
TAG
Nutrient limitation
Light

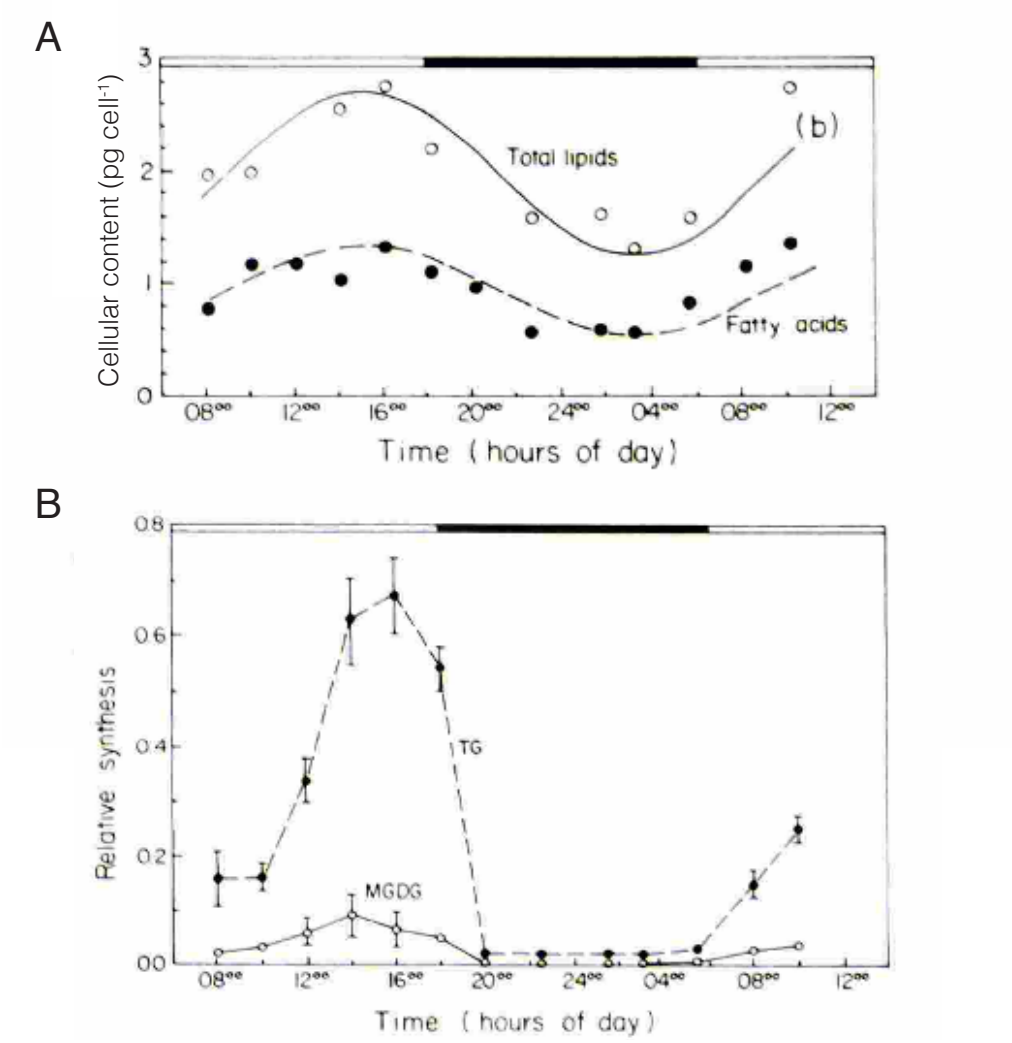


Figure 1-11. TAG cycling during light and dark periods in *Nannochloropsis* sp. During light:dark cycling, TAG is typically synthesized during light periods and utilized for cellular division during dark periods. From Sukenik & Carmeli, 1990.

From the progression of TAG levels in the algal cell emerges a pattern of constant synthesis and utilization (Fig. 1-12), in which accumulation occurs when carbon sequestration outpaces anabolism and when TAG biosynthesis outpaces utilization. Thus, it may be possible that manipulations on pathways other than TAG biosynthesis that recreate this scenario could produce accumulation as well. One such manipulation is the disruption of lipid catabolism, which could recreate the imbalance of TAG synthesis and utilization that leads to accumulation (Fig. 1-13).

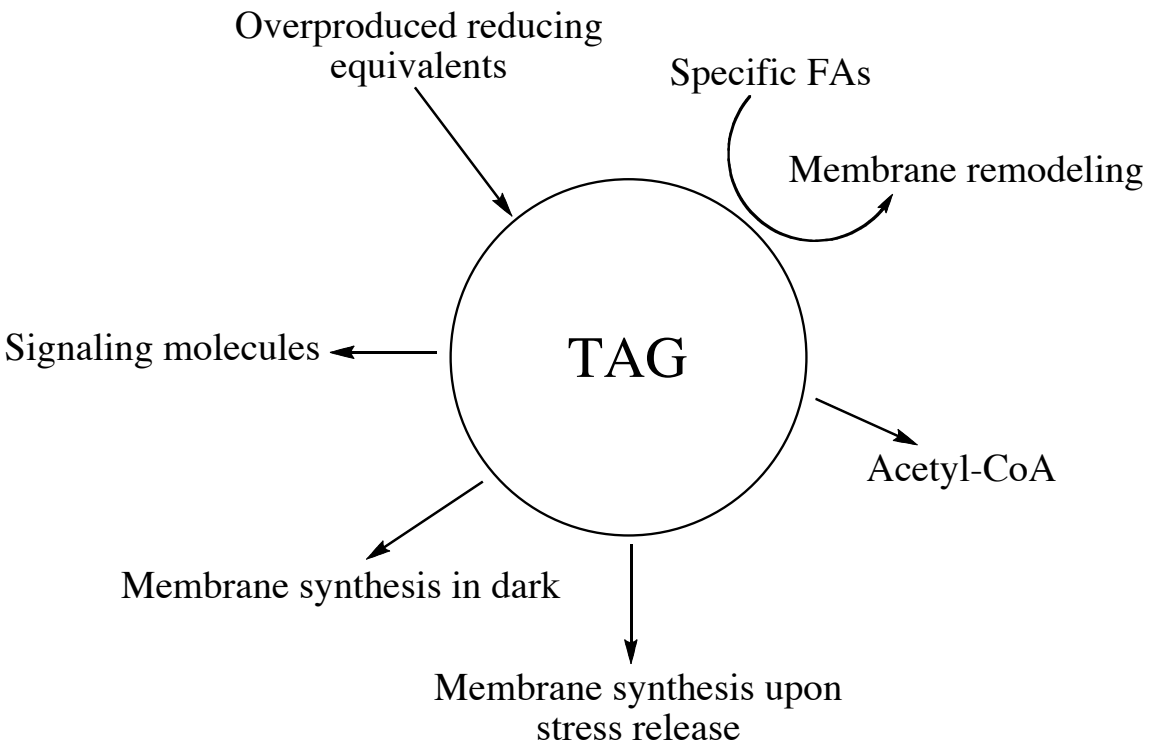


Figure 1-12. Flux of TAG.
TAG is constantly synthesized and utilized in microalgal cells to serve a variety of roles.

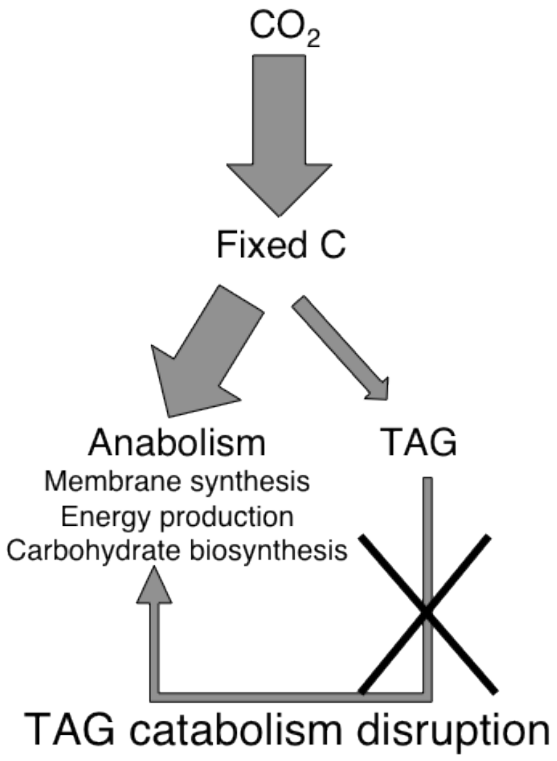
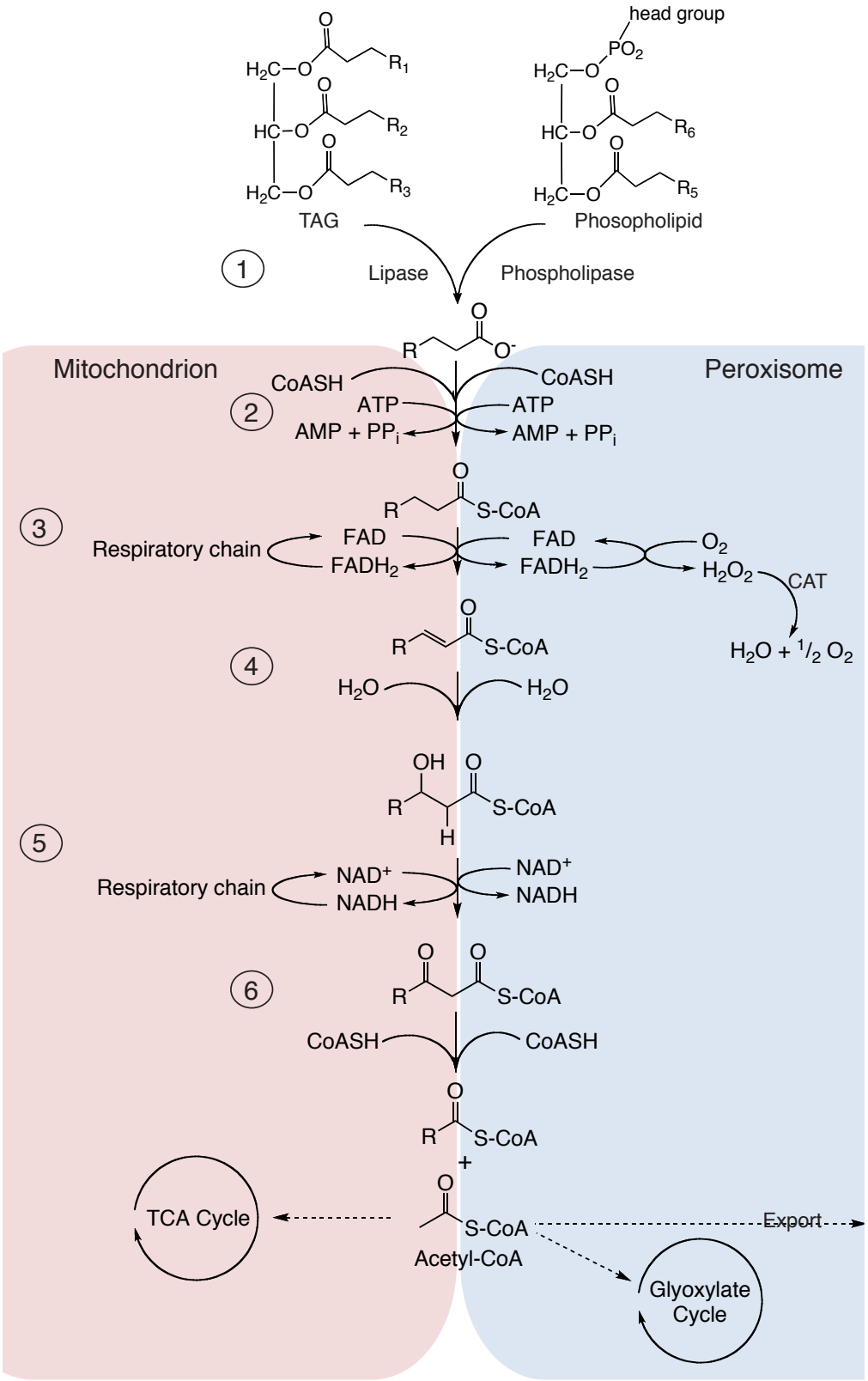


Figure 1-13. Disruption of lipid catabolism.
Disrupting the breakdown of TAG could lead to increased TAG accumulation. However, because the flux of fixed carbon in anabolic processes is not affected, simultaneous growth can occur.

1.4.3 Lipid Catabolism

The breakdown of lipids is a coordinated cellular process involving multiple subcellular locations and biochemical pathways. An overview of lipid catabolism is given in Figure 1-14. The first step in the breakdown of complex lipids is catalyzed by lipases or phospholipases, which hydrolyze the ester linkage between fatty acids and the glycerol backbone of the lipid molecule, releasing free fatty acids. Lipases and phospholipases often exhibit substrate and stereochemical specificity, hydrolyzing fatty acids of certain chain lengths at specific positions on the glycerol backbone. Free fatty acids released from hydrolysis are then subsequently processed through the iterative cycle of ß-oxidation, which releases individual acetate units as acetyl-CoA. ß-oxidation occurs in both the mitochondria and peroxisome, and the location is dependent upon the chain length of the free fatty acid.

Figure 1-14. Overview of lipid catabolism.
Complex lipids such as TAG and phospholipids are broken down to release free fatty acids, which are subsequently broken down to release acetyl-CoA subunits via β-oxidation in either the mitochondrion on peroxisome. Numbered circles denote the individual steps of catabolism. Lipases or phospholipases (1) catalyze the release of free fatty acids from complex lipids. After transport into the mitochondrion or peroxisome, free fatty acids are activated to acyl-CoA forms (2) by long-chain acyl-CoA synthetase (LACS). Subsequent dehydration (3), hydration (4), dehydration (5) and cleavage (6) are carried out by different enzymes in the mitochondrion and peroxisome. The resulting acetyl-CoA subunit can enter the TCA cycle in the mitochondrion; in the peroxisome acetyl-CoA enters the glyxoylate cycle or is exported. The resulting shortened fatty acyl-CoA chain can re-enter the β-oxidation pathway.


head group
TAG
Phosopholipid
1
Lipase
Phospholipase
Mitochondrion
Peroxisome
CoASH
CoASH
2
ATP
ATP
AMP + PPi
AMP + PPi
S-CoA
3
Respiratory chain
FAD
FADH2
FAD
FADH2
O2
H2O2
CAT
H2O + 1/2 O2
4
H2O
H2O
OH
H
5
Respiratory chain
NAD+
NADH
NAD+
NADH
6
CoASH
CoASH
+
TCA Cycle
Acetyl-CoA
Export
Glyoxylate Cycle

Short-chain fatty acids (6 carbons and smaller) can pass through the mitochondrial membrane unassisted, however medium and long-chain fatty acids (up to 22 carbons) must be transported through the membrane via the carnitine transport system. Very long-chain fatty acids (longer than 22 carbons) must first be transported into the peroxisome where they undergo a few rounds of ß-oxidation to become long-chain fatty acids before they can be transported into the mitochondria for further breakdown. Additionally, it's been suggested that polyunsaturated fatty acids are preferentially broken down in the peroxisome (Poirier et al 2006). The process of ß-oxidation involves the same reactions in both the mitochondria and peroxisome, but is catalyzed by unique and independent sets of enzymes that evolved from common genetic ancestors (Nelson and Cox 2000). Figure 2-1 highlights the differences in ß-oxidation between these two organelles.

Active transport into the mitochondria and peroxisome requires activation of fatty acids to their acyl-CoA form by AMP-dependent long-chain acyl CoA synthetases (LACSs). LACSs activate fatty acids for a number of pathways including elongation, lipid synthesis, and ß-oxidation; therefore the substrate and subcellular location of LACSs are important in determining function. The iterative pathway of ß-oxidation involves dehydrogenation, hydration followed by another dehydrogenation, and cleavage of an acetyl-CoA. In mitochondria the first dehydration is carried out by acyl-CoA dehydrogenase (ADH). The first dehydration step is carried out by acyl-CoA oxidase (ACO) in the peroxisome. These enzymes catalyze the formation of a double bond between carbons 2 and 3 of the fatty acid chain and are dependent upon the presence of flavin adenine dinucleotide (FAD) as an electron acceptor, forming $FADH_2$. In the

mitochondria, electrons are transferred to the respiratory chain for ATP generation from $FADH_2$. In the peroxisome the high-potential electrons are instead transferred onto water, forming hydrogen peroxide. To combat the potential build-up of harmful reactive oxygen species in the cell, peroxisomes contain a catalase to convert the hydrogen peroxide into water and oxygen.

In the mitochondria, a trifunctional enzyme complex containing two subunits, alpha and beta, carries out the subsequent hydration, dehydration, and cleavage steps of ß-oxidation. The enoyl-CoA hydratase function hydrates the double bond between carbon 2 and 3. The hydroxyacyl-CoA dehydrogenase function uses NAD+ to convert the hydroxyl group on carbon 3 to a ketone. Finally, the thiolase function cleaves the ß-ketoacyl-CoA, inserting a thiol group between carbons 2 and 3 and producing an acetyl-CoA molecule and the shortened remaining acyl-CoA chain. In the peroxisome, the hydration and second dehydration steps are carried out by a bifunctional enzyme complex, while a separate thiolase catalyzes the final cleavage step.

Lipid catabolism has been characterized in other organisms, but is not well understood in microalgae. Extensive knockout studies in *A. thaliana* of lipid catabolism enzymes have shown that this strategy does in fact increase lipid accumulation in the resulting mutants (Table 1-1). These studies, which primarily focused on seed oil mobilization, often resulted in impaired growth phenotypes (Graham 2008). However, because seedling establishment and growth is dependent on lipolysis of stored lipids until the photosynthetic apparatus is formed, one cannot extrapolate these results to microalgae, which go through neither seed nor germination stages.

Table 1-1. Knockout studies of lipid catabolism genes in *A. thaliana*.
LACS: long-chain acyl-CoA synthetase; AOX: acyl-CoA oxidase; MFP: multifunctional protein; MDH: malate dehydrogenase; CS: citrate synthase.

Target	Gene	Increased lipids?	Decreased growth?
Lipase	*sdp1*	Yes	Yes
LACS	*lac6 & lac7*	Yes	Yes
AOX	*acx1 & acx2*	Yes	Yes
MFP	*mfp2*	Yes	Yes
Thiolase	*kat2*	Yes	Yes
MDH	*pmdh1 & pmdh2*	Yes	Yes
CS	*csy2 & csy3*	Yes	Yes

Although TAG mobilization is used in microalgae for cellular division under certain conditions, carbohydrates are often the primary storage product (Chauton et al 2013, Li et al 2011), are mobilized alongside TAG (Klein 1987, Sukenik and Carmeli 1990), and in some case are preferentially utilized over TAG (Siaut et al 2011). Thus we hypothesize that disrupting lipid catabolism in microalgae would result in increased lipid accumulation without affecting growth and division to the same extent as previously attempted manipulations (such as the disruption of carbohydrate biosynthesis).

1.4.4 The Glyoxylate Cycle

Acetyl-CoA subunits released from fatty acid breakdown can be recycled throughout various metabolic processes including the citric acid cycle, lipogenesis, and the glyoxylate cycle. The glyoxylate cycle represents a short version of the citric acid cycle and resides solely in specialized peroxisomes called glyoxysomes. It is an important biochemical pathway that allows cells to build carbohydrate sfrom fat stores because it results in the next production of succinate by bypassing the steps in the citric

acid cycle in which carbon is lost as CO_2. Like the citric acid cycle the glyoxylate cycle involves the production of citrate from oxaloacetate and acetyl-CoA by citrate synthase and the subsequent conversion to isocitrate by aconitase. Two enzymes unique to the glyoxylate cycle, isocitrate lyase and malate synthase catalyze, respectively, the cleavage of isocitrate into glyoxyate and succinate, and the condensation of glyoxylate with acetyl-CoA to form malate. The cycle continues to produce oxaloacetate from malate via malate dehydrogenase. The succinate released from one turn of the cycle can be converted into other intermediates for use in the citric acid cycle or gluconeogenesis. Figure 2-2 shows an overview of the glyoxylate cycle.

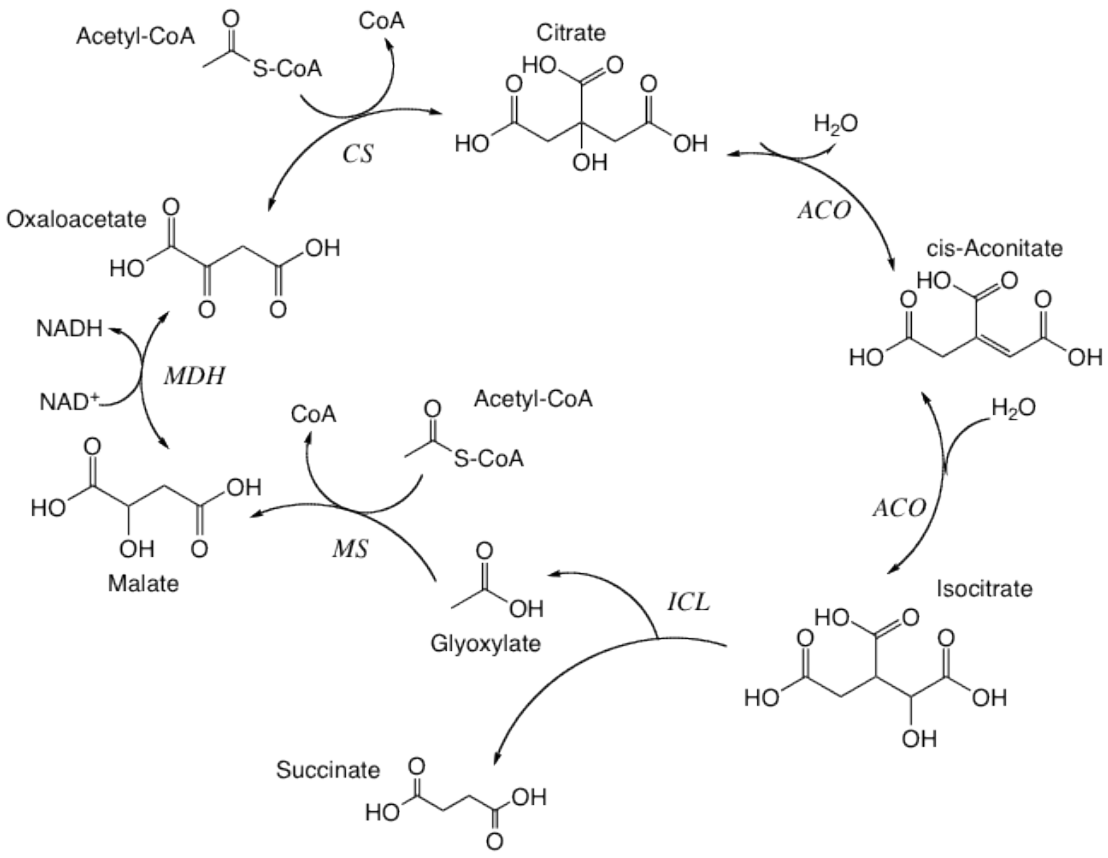


Figure 1-15. Glyoxylate cycle.
The glyoxylate cycle is a shunted version of the TCA cycle that results in the net production of succinate. Enzymes catalyzing reactions are shown in italics. *ACO*: aconitase; *ICL*: isocitrate lyase; *MS*: malate synthase; *MDH*: malate dehydrogenase; *CS*: citrate synthase.

1.5 The Diatom *Thalassiosira pseudonana*

We chose to perform this work in the centric diatom *T. pseudonana*. Diatoms are a unique class of eukaryotic microalgae characterized by cell walls made of silica. As members of the Stramenopiles they arose from a secondary endosymbiotic event in which an ancestral heterotrophic eukaryote engulfed a red alga (Armbrust 2009). In the present day oceans diatoms are one of the most prevalent phytoplankton and are extremely vital to global geophysical cycles: they account for 20% of global primary productivity (Nelson et al 1995) and up to 50% of CO_2 export from the atmosphere (Dugdale and Wilkerson 1998). Diatoms are blooming organisms, able to reproduce rapidly and to high densities given the right nutrient conditions.

Many of these characteristics of diatoms make them excellent candidates as sources for algal biofuels. Many species can grow rapidly to high densities, can grow in salt- rather than fresh-water, and some species are productive in production pond settings. Silicon is essential for growth in most diatoms, and limiting this nutrient triggers TAG accumulation. Silicon limitation, unlike nitrogen limitation, does not cause side effects of decreased photosynthetic efficiency or protein content (Hildebrand et al 2012). Engineering diatoms for lipid production is becoming more feasible as more diatom genomes become available. The centric diatom *Thalassiosira pseudonana* (Fig. 1-14) became a model organism when its genome was sequenced, representing the first genome of a marine phytoplankton (Armbrust et al 2004). Since the sequencing of its genome, *T. pseudonana* has been the focus of genomic, transcriptomic and metabolic studies, and many molecular techniques have been developed for it including transformation,

fluorescent protein expression (Poulsen et al 2007), and protein knock-down. Because of its status as a model organism, and because of the extensive molecular techniques developed for it, *T. pseudonana* was chosen as the organism in which to conduct this thesis research.

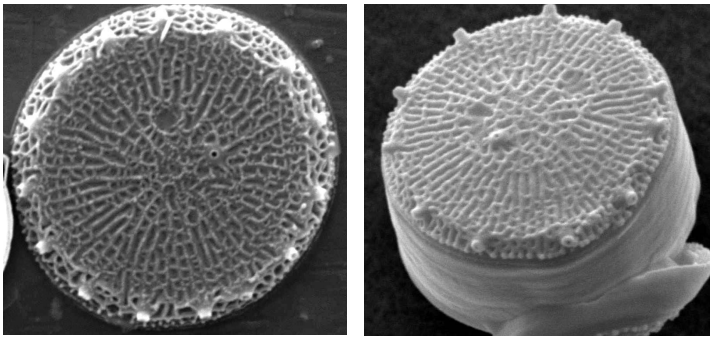


Figure 1-16. *Thalassiosira pseudonana*
From M. Hildebrand.

1.6 Metabolic Engineering Approaches

Metabolic engineering can be carried out in microalgae through either targeted or random strategies. Random mutagenesis allows for the creation and screening of multiple phenotypes, however this comes with the caveat that screening for a particular phenotype is often a long, laborious process. Mutagenesis can be used in conjunction with flow cytometry to sort out high lipid-producing mutants (Bougaran et al 2012, Doan and Obbard 2012, Manandhar-Shrestha and Hildebrand 2013), but it is difficult to elucidate where the mutations occur, thus the resulting strains provide no information on the lipid metabolism pathway. Targeted manipulations, conversely, allow for the correlation of a phenotype to a particular pathway and enzyme. Engineering of specific genes and enzymes requires advanced molecular techniques, such as transformation, overexpression

and knock-down, and target-guiding information, such as transcriptomics. These techniques have been increasingly developed for various microalgae over the last few decades (Borowitzka et al 2013).

1.7 Dissertation Contents

Chapter 2 describes the pathway of lipid catabolism and the identification of this pathway in *T. pseudonana*. Homologous enzymes in the pathways of lipid catabolism, the glyoxylate cycle and photorespiration were identified. A transcriptomics data set generated for *T. pseudonana* under lipid accumulation-inducing silicon starvation conditions was probed for gene expression changes in the genes involved in these three pathways. I hypothesized that lipid catabolism genes would be downregulated, and that in particular, significantly downregulated lipases would be could targets for metabolic engineering. I found that, contrary to my expectation, there was no general downregulation of the lipid catabolism pathway. I did, however detect changes in the glyoxylate cycle and photorespiratory pathways.

Despite observing no significant trend in lipid catabolism genes, I identified one hydrolase, Thaps3_264297, that was significantly downregulated throughout lipid accumulation. The enzyme associated with this gene was found to be homologous to human ABHD4, a lipase, and human CGI-58, an acyltransferase involved in lipid catabolism. Functional analysis of Thaps3_264297 revealed lipase, phospholipase and acyltransferase activities, suggesting that this enzyme maintained similar functions in lipid catabolism in *T. pseudonana*. The first half of Chapter 2 is in-prep for submission,

and the second have has been published in *The Proceedings of the National Academy of Sciences*.

Chapter 3 represents the major element of the dissertation and describes the knock-down of Thaps3_264297 in *T. pseudonana* and subsequent characterization of transgenic strains. Knock-down was carried out using interfering RNA and antisense techniques, but antisense was found to be the more robust method. Knock-down strains showed comparable growth to wild-type (WT) under continuous light and light:dark cycling. These strains accumulated more TAG than WT in exponential and stationary phase, as well as during silicon starvation conditions. The knock-down strains also exhibited increased membrane intactness during silicon starvation compared to WT.

The above phenotypes suggest that Thaps3_264297 is involved in lipid homeostasis rather than rapid TAG utilization, a finding that is corroborated by similar disruption of this enzyme in other organisms. The effect of the knock-down on membranes and polar lipids imply a role for Thaps3_264297 in both TAG and membrane lipids, and suggest a close connection between these metabolite pools. Chapter 3 is published in its entirety in *The Proceedings of the National Academy of Sciences*.

Chapter 4 describes work that utilizes the results of Chapter 3 to build more production-friendly strains. Genetically modified organisms (GMOs) must past a strict permitting process through the Environmental Protection Agency (EPA) in the U.S., and no transgenic microalgal strain has yet to proceed through these regulations. Strains made using only native sequences are not considered GMOs and thus are exempt from this permitting system. I built a native sequence knock-down construct using antisense DNA to Thaps3_264297 and transformed the linear construct into *T. pseudonana*. The mixture

of transgenic and WT cells was subjected to three rounds of silicon starvation, sorting for high lipid-producers, and nutrient replenishment. Final sorted cells were plated to select for single colonies, and the presence of the construct confirmed using PCR.

Chapter 5 consists of a review on the policies behind algal biomass production. Algal biomass production, no matter the end-product, involves cultivation practices similar to those of agriculture and aquaculture. However, while many federal support programs exist for agriculture and aquaculture, these programs have yet to be applied to large-scale algae cultivation. Chapter 5 reviews the programs that should be available for algal biomass production, and analyzes and assesses the reasons for which these programs have not been extended to algae. This work has been submitted for publication in *Photosynthesis Research*.

Chapter 6 is a concluding chapter providing a synthesis of the results from this dissertation and how they enhance the current picture of lipid metabolism in microalgae.

1.8 Acknowledgements

Chapter 1, in part, is being prepared for submission as a review paper in 2014. Emily Trentacoste, Mark Hildebrand and William Gerwick.

1.9 References

Andersen RA (2013). The Microalgal Cell. *Handbook of Microalgal Culture*. John Wiley & Sons, Ltd. pp 1-20.

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WWY, Lane

TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsar DS (2004). The Genome of the Diatom Thalassiosira Pseudonana: Ecology, Evolution, and Metabolism. *Science* **306:** 79-86.

Armbrust EV (2009). The life of diatoms in the world's oceans. *Nature* **459:** 185-192.

Berges JA, Charlebois DO, Mauzerall DC, Falkowski PG (1996). Differential Effects of Nitrogen Limitation on Photosynthetic Efficiency of Photosystems I and II in Microalgae. *Plant Physiology* **110:** 689-696.

Blatti JL, Beld J, Behnke CA, Mendez M, Mayfield SP, Burkart MD (2012). Manipulating Fatty Acid Biosynthesis in Microalgae for Biofuel through Protein-Protein Interactions. *PLoS ONE* **7:** e42949.

Blatti JL, Michaud J, Burkart MD (2013). Engineering fatty acid biosynthesis in microalgae for sustainable biodiesel. *Next Generation Therapeutics • Energy* **17:** 496-505.

Borowitzka M (1992). Algal biotechnology products and processes — matching science and economics. *Journal of Applied Phycology* **4:** 267-279.

Borowitzka M (2013). High-value products from microalgae—their development and commercialisation. *Journal of Applied Phycology* **25:** 743-756.

Borowitzka MA, Moheimani NR, Rasala B, Gimpel J, Tran M, Hannon MJ, Miyake-Stoner SJ, Specht EA, Mayfield SP (2013). Genetic Engineering to Improve Algal Biofuels Production. *Algae for Biofuels and Energy*. Springer Netherlands. pp 99-113.

Bougaran G, Rouxel C, Dubois N, Kaas R, Grouas S, Lukomska E, Le Coz JR, Cadoret JP (2012). Enhancement of neutral lipid productivity in the microalga Isochrysis affinis Galbana (T-Iso) by a mutation-selection procedure. *Biotechnology and Bioengineering* **109:** 2737-2745.

Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ, Gallaher SD, Johnson S, Benning C, Pellegrini M, Grossman A, Merchant SS (2012). Three Acyltransferases and Nitrogen-responsive Regulator Are Implicated in Nitrogen Starvation-induced Triacylglycerol Accumulation in Chlamydomonas. *Journal of Biological Chemistry* **287:** 15811-15825.

Brányiková I, Maršálková B, Doucha J, Brányik T, Bišová K, Zachleder V, Vítová M (2011). Microalgae—novel highly efficient starch producers. *Biotechnology and Bioengineering* **108:** 766-776.

Burrows E, Bennette N, Carrieri D, Dixon J, Brinker A, Frada M, Baldassano SN, Falkowski PG, Dismukes GC (2012). Dynamics of Lipid Biosynthesis and Redistribution in the Marine Diatom Phaeodactylum tricornutum Under Nitrate Deprivation. *BioEnergy Research* **5:** 876-885.

Bölling C, Fiehn O (2005). Metabolite Profiling of Chlamydomonas reinhardtii under Nutrient Deprivation. *Plant Physiology* **139:** 1995-2005.

Chauton MS, Winge P, Brembu T, Vadstein O, Bones AM (2013). Gene Regulation of Carbon Fixation, Storage, and Utilization in the Diatom Phaeodactylum tricornutum Acclimated to Light/Dark Cycles. *Plant Physiology* **161:** 1034-1048.

Chen JE, Smith AG (2012). A look at diacylglycerol acyltransferases (DGATs) in algae. *Photosynthetic microorganisms for bio-fuel production from sun light* **162:** 28-39.

Cheng J-S, Niu Y-H, Lu S-H, Yuan Y-J (2012). Metabolome analysis reveals ethanolamine as potential marker for improving lipid accumulation of model photosynthetic organisms. *Journal of Chemical Technology & Biotechnology* **87:** 1409-1418.

Claquin P, Martin-Jézéquel V, Kromkamp JC, Veldhuis MJW, Kraay GW (2002). Ucoupling of silicon compared with carbon and nitrogen metabolisms and the role of the cell cycle in continous cultures of Thalassiosira pseudonana (Bacillariophyceae) under light, nitrogen and phosphorus control. *Journal of Phycology* **38:** 922-930.

Collén PN, Camitz A, Hancock RD, Viola R, Pedersén M (2004). Effects of nutrient deprivation and resupply on metabolites and enzymes related to carbon allocaiton in Gracilari tenuistipitata (Rhodophyta). *Journal of Phycology* **40:** 305-314.

Cooksey KE (1974). Acetate metabolism by whole cels of Phaeodactylum tricornutum Bohlin *Journal of Phycology* **10:** 253-257.

Davis R, Aden A, Pienkos PT (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy* **88:** 3524-3531.

Dillschneider R, Steinweg C, Rosello-Sastre R, Posten C (2013). Biofuels from microalgae: Photoconversion efficiency during lipid accumulation *Bioresource Technology* **142:** 647-654.

Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Energy biotechnology / Environmental biotechnology* **19:** 235-240.

Doan TTY, Obbard JP (2012). Enhanced intracellular lipid in Nannochloropsis sp. via random mutagenesis and flow cytometric cell sorting *Algal Research* **1:** 17-21.

Doshi R, Nguyen T, Chang G (2013). Transporter-mediated biofuel secretion. *Proceedings of the National Academy of Sciences* **110:** 7642-7647.

Dugdale RC, Wilkerson FP (1998). Silicate regulation of new production in the equatorial Pacific upwelling *Nature* **391:** 270-273.

Eichler-Stahlberg A, Weisheit W, Ruecker O, Heitzer M (2009). Strategies to facilitate transgene expression in Chlamydomonas reinhardtii. *Planta* **229:** 873-883.

Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C (2012). Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in Chlamydomonas reinhardtii. *Plant and Cell Physiology* **53:** 1380-1390.

Fernandes B, Teixeira J, Dragone G, Vicente AA, Kawano S, Bišová K, Pribyl P, Zachleder V, Vitova M (2013). Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga Parachlorella kessleri *Bioresource Technology* **144:** 268-274.

Gardner RD, Lohman E, Gerlach R, Cooksey KE, Peyton BM (2013). Comparison of CO2 and bicarbonate as inorganic carbon sources for triacylglycerol and starch accumulation in Chlamydomonas reinhardtii *Biotechnology and Bioengineering* **110:** 87-96.

Gibbs M, Gfeller RP, Chen C (1986). Fermentative Metabolism of Chlamydomonas reinhardii: III. Photoassimilation of Acetate. *Plant Physiology* **82:** 160-166.

Gimpel JA, Specht EA, Georgianna DR, Mayfield SP (2013). Advances in microalgae engineering and synthetic biology applications for biofuel production. *Current Opinion in Chemical Biology* **17:** 489-495.

Goncalves E, Johnson J, Rathinasabapathi B (2013). Conversion of membrane lipid acyl groups to triacylglycerol and formation of lipid bodies upon nitrogen starvation in biofuel green algae Chlorella UTEX29. *Planta* **238:** 895-906.

Goodson C, Roth R, Wang ZT, Goodenough U (2011). Structural Correlates of Cytoplasmic and Chloroplast Lipid Body Synthesis in Chlamydomonas reinhardtii and Stimulation of Lipid Body Production with Acetate Boost. *Eukaryotic Cell* **10:** 1592-1606.

Graham IA (2008). Seed Storage Oil Mobilization. *Annual Review of Plant Biology* **59:** 115-142.

Guarnieri MT, Nag A, Smolinski SL, Darzins A, Seibert M, Pienkos PT (2011). Examination of Triacylglycerol Biosynthetic Pathways via De Novo Transcriptomic and Proteomic Analyses in an Unsequenced Microalga. *PLoS ONE* **6:** e25851.

Guarnieri MT, Nag A, Yang S, Pienkos PT (2013). Proteomic analysis of Chlorella vulgaris: Potential targets for enhanced lipid accumulation. *Journal of Proteomics* **93:** 245-253.

Guckert JB, Cooksey KE (1990). Triglyceride accumulation and fatty acid profile changes in Chlorella (Chlorophyta) during high pH-induced cell cycle inhibition. *Journal of Phycology* **26:** 72-79.

Guerra LT, Levitan O, Frada MJ, Sun JS, Falkowski PG, Dismukes GC (2013). Regulatory branch points affecting protein and lipid biosynthesis in the diatom Phaeodactylum tricornutum *Biomass and Bioenergy* **59:** 306-315.

Hildebrand M, Frigeri LG, Davis AK (2007). Synchronized growth of Thalassiosira pseudonana (Bacillariophyceae) provides novel insights in cell-wall synthesis process in relation to the cell cycle. *Journal of Phycology* **43:** 730-740.

Hildebrand M, Davis AK, Smith SR, Traller JC, Abbriano R (2012). The place of diatoms in the biofuels industry. *Biofuels* **3:** 221-240.

Hildebrand M, Abbriano RM, Polle JEW, Traller JC, Trentacoste EM, Smith SR, Davis AK (2013). Metabolic and cellular organization in evolutionarily diverse microalgae as related to biofuels production. *Current Opinion in Chemical Biology* **17:** 506-514.

Hirsch RL (2007). Peaking of World Oil Production: Recent Forecasts. DOE/NETL.

Hockin NL, Mock T, Mulholland F, Kopriva S, Malin G (2012). The Response of Diatom Central Carbon Metabolism to Nitrogen Starvation Is Different from That of Green Algae and Higher Plants. *Plant Physiology* **158:** 299-312.

Hodgson P, Henderson RJ, Sargent J, Leftley J (1991). Patterns of variation in the lipid class and fatty acid composition of Nannochloropsis oculata (Eustigmatophyceae) during batch culture. *Journal of Applied Phycology* **3:** 169-181.

Hossain AS, Salleh A, Boyce AN, Naqiuddin M (2008). Biodiesel fuel production from algae as renewable energy. *American Journal of Biochemistry and Biotechnology* **4:** 250.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal* **54:** 621-639.

Ito T, Tanaka M, Shinkawa H, Nakada T, Ano Y, Kurano N, Soga T, Tomita M (2013). Metabolic and morphological changes of an oil accumulating trebouxiophycean alga in nitrogen-deficient conditions. *Metabolomics* **9:** 178-187.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996). Sequence Analysis of the Genome of the Unicellular Cyanobacterium Synechocystis sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-coding Regions. *DNA Research* **3:** 109-136.

Keeling CD, Whorf TP, Whalen M, van der Plicht J (1995). Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. *Nature* **375:** 666-670.

Khozin-Goldberg I, Shrestha P, Cohen Z (2005). Mobilization of arachidonyl moieties from triacylglycerols into chloroplastic lipids following recovery from nitrogen starvation of the microalga Parietochloris incisa. *Biochimica et Biophysica Acta* **1738:** 63-71.

Klein U (1987). Intracellular Carbon Partitioning in Chlamydomonas reinhardtii. *Plant Physiology* **85:** 892-897.

Klok AJ, Martens DE, Wijffels RH, Lamers PP (2013). Simultaneous growth and neutral lipid accumulation in microalgae. *Bioresource Technology* **134:** 233-243.

Kroth PG, Chiovitti A, Gruber A, Martin-Jezequel V, Mock T, Parker MS, Stanley MS, Kaplan A, Caron L, Weber T., Maheswari U, Armbrust EV, Bowler C (2008). A Model for Carbohydrate Metabolism in the Diatom Phaeodactylum tricornutum Deduced from Comparative Whole Genome Analysis. *PLoS ONE* **3:** e1426.

La Russa M, Bogen C, Uhmeyer A, Doebbe A, Filippone E, Kruse O *et al* (2012). Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga Chlamydomonas reinhardtii. *Journal of Biotecnology* **162:** 13-20.

Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH (2008). Exploring and exploiting carotenoid accumulation in Dunaliella salina for cell-factory applications. *Trends in Biotechnology* **26:** 631-638.

Li X, Moellering ER, Liu B, Johnny C, Fedewa M, Sears BB, Kuo MH, Benning C (2012). A Galactoglycerolipid Lipase Is Required for Triacylglycerol Accumulation and Survival Following Nitrogen Deprivation in Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 4670-4686.

Li Y, Han D, Hu G, Dauvillee D, Sommerfeld M, Ball S, Hu Q (2010). Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. *Metabolic Engineering* **12:** 387-391.

Li Y, Han D, Sommerfeld M, Hu Q (2011). Photosynthetic carbon partitioning and lipid production in the oleaginous microalga Pseudochlorococcum sp. (Chlorophyceae) under nitrogen-limited conditions. *Bioresource Technology* **102:** 123-129.

Liu B, Benning C (2013). Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology* **24:** 300-309.

Lu N, Wei D, Chen F, Yang S-T (2013). Lipidomic profiling reveals lipid regulation in the snow alga Chlamydomonas nivalis in response to nitrate or phosphate deprivation. *Process Biochemistry* **48:** 605-613.

Lv H, Qu G, Qi X, Lu L, Tian C, Ma Y (2013). Transcriptome analysis of Chlamydomonas reinhardtii during the process of lipid accumulation. *Genomics* **101:** 229-237.

López Barreiro D, Prins W, Ronsse F, Brilman W (2013). Hydrothermal liquefaction (HTL) of microalgae for biofuel production: State of the art review and future prospects. *20th European Biomass Conference* **53:** 113-127.

Manandhar-Shrestha K, Hildebrand M (2013). Development of flow cytometric procedures for the efficient isolation of improved lipid accumulation mutants in a Chlorella sp. microalga. *Journal of Applied Phycology* **25:** 1643-1651.

Matsuzaki M, Misumi O, Shin-i T, Maruyama S, Takahara M, Miyagishima S, Mori T, Nishida K, Yagisawa F, Nishida K, Yoshida Y, Nishimura Y, Nakao S, Kobayashi T, Momoyama Y, Higashiyama T, Minoda A, Sano M, Nomoto H, Oishi K, Hayashi H, Ohta F, Nishizaka S, Haga S, Miura S, Morishita T, Kabeya Y, Terasawa K, Suzuki Y, Ishii Y, Asakawa S, Takano H, Ohta N, Kuroiwa H, Tanaka K, Shimizu N, Sugano S, Sato N, Nozaki H, Ogasawara N, Kohara Y, Kuroiwa T (2004). Genome sequence of the ultrasmall unicellular red alga Cyanidioschyzon merolae 10D *Nature* **428:** 653-657.

Miller R, Wu G, Deshpande RR, Vieler A, Gärtner K, Li X, Moellering ER, Zauner S, Cornish AJ, Liu B, Bullard B, Sears BB, Kuo MH, Hegg EL, Shachar-Hill Y, Shiu SN, Benning C (2010). Changes in Transcript Abundance in Chlamydomonas reinhardtii following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology* **154:** 1737-1752.

Mizuno Y, Sato A, Watanabe K, Hirata A, Takeshita T, Ota S, Sato N, Zachleder V, Tsuzuki M, Kawano S (2013). Sequential accumulation of starch and lipid induced by sulfur deficiency in Chlorella and Parachlorella species. *Bioresource Technology* **129:** 150-155.

Moellering ER, Benning C (2010). RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in Chlamydomonas reinhardtii. *Eukaryotic Cell* **9:** 97-106.

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Heriberto C (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae Chlamydomonas reinhardtii and Coccomyxa sp. C-169. *Phytochemistry* **75:** 50-59.

Murakami M, Ikenouchi M (1997). The biological CO2 fixation and utilization project by rite (2) — Screening and breeding of microalgae with high capability in fixing CO2 —. *Energy Conversion and Management* **38, Supplement:** S493-S497.

Murray J, King D (2012). Climate policy: Oil's tipping point has passed. *Nature* **481:** 433-435.

Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, Schenk PM, Kruse O, Hankamer B (2007). Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. *Plant Biotechnology Journal* **5:** 802-814.

Naik SN, Goud VV, Rout PK, Dalai AK (2010). Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews* **14:** 578-597.

Nelson DL, Cox MM (2000). *Lehninger Principles of Biochemistry*, 3rd edn. Worth Publishers: New York, NY.

Nelson DM, Tréguer P, Brzezinski MA, Leynaert A, Quéguiner B (1995). Production and dissolution of biogenic silica in the ocean: Revised global estimates, comparison with regional data and relationship to biogenic sedimentation. *Global Biogeochemical Cycles* **9:** 359-372.

Nguyen HM, Baudet M, Cuiné S, Adriano J-M, Barthe D, Billon E, Bruley C, Beisson F, Peltier G, Ferro M, Li-Beisson Y (2011). Proteomic profiling of oil bodies isolated from the unicellular green microalga Chlamydomonas reinhardtii: With focus on proteins involved in lipid metabolism. *Proteomics* **11:** 4266-4273.

Oey M, Ross IL, Stephens E, Steinbeck J, Wolf J, Radzun KA, Kugler J, Ringsmuth AK, Kruse O, Hankamer B (2013). RNAi Knock-Down of LHCBM1, 2 and 3 Increases Photosynthetic H₂ Production Efficiency of the Green Alga Chlamydomonas reinhardtii. *PLoS ONE* **8:** e61375.

Pan K, Qin J, Li S, Dai W, Zhu B, Jin Y, Yu W, Yang G, Li D (2011). Nuclear monoploidy and sexual propagation of Nannochloropsis oceanica (Eustigmatophyceae) as revealed by its genome sequence. *Journal of Phycology* **47:** 1425-1432.

Pienkos PT, Darzins A (2009). The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioproducts and Biorefining* **3:** 431-440.

Poulsen N, Berne C, Spain J, Kröger N (2007). Silica Immobilization of an Enzyme through Genetic Engineering of the Diatom Thalassiosira pseudonana. *Angewandte Chemie* **46:** 1843-1846.

Rabbani S, Beyer P, Lintig Jv, Hugueney P, Kleinig H (1998). Induced β-Carotene Synthesis Driven by Triacylglycerol Deposition in the Unicellular Alga Dunaliella bardawil. *Plant Physiology* **116:** 1239-1248.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010). Genetic Engineering of Algae for Enhanced Biofuel Production. *Eukaryotic Cell* **9:** 486-501.

Radakovits R, Eduafo PM, Posewitz MC (2011). Genetic engineering of fatty acid chain length in Phaeodactylum tricornutum. *Metabolic Engineering* **13:** 89-95.

Radakovits R, Jinkerson RE, Fuerstenberg SI, Tae H, Settlage RE, Boore JL, Posewitz MC (2012). Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropsis gaditana. *Nature* **3:** 686.

Ramanan R, Kim B-H, Cho D-H, Ko S-R, Oh H-M, Kim H-S (2013). Lipid droplet synthesis is limited by acetate availability in starchless mutant of Chlamydomonas reinhardtii. *FEBS Letters* **587:** 370-377.

Reitan KI, Rainuzzo JR, Olsen Y (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. *Journal of Phycology* **30:** 972-979.

Rhiel E, Mörschel E, Wehrmeyer W (1985). Correlation of pigment deprivation and ultrastructural organization of thylakoid membranes incryptomonas maculata following nutrient deficiency. *Protoplasma* **129:** 62-73.

Rismani-Yazdi H, Haznedaroglu B, Bibby K, Peccia J (2011). Transcriptome sequencing and annotation of the microalgae Dunaliella tertiolecta: Pathway description and gene discovery for production of next-generation biofuels. *BMC Genomics* **12:** 148.

Roessler PG (1988). Effects of silicon deficiency on lipid composition and metabolism in the diatom Cyclotella cryptica. *Journal of Phycology* **24:** 394-400.

Roessler PG, Ohlrogge JB (1993). Cloning and characterization of the gene that encodes acetyl-coenzyme A carboxylase in the alga Cyclotella cryptica. *Journal of Biological Chemistry* **268:** 19254-19259.

SBI (2012). Algae Biofuels Technologies – Global Market and Product Trends 2010-2015. Specialists in Business Information: New York, NY.

Sheehan J, Dunahay T, Benemann J, Roessler P (1998). A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory: Golden, Colorado. p 296.

Shrestha RP, Haerizadeh F, Hildebrand M (2013). Molecular Genetic Manipulation of Microalgae: Principles and Applications. *Handbook of Microalgal Culture*. John Wiley & Sons, Ltd. pp 146-157.

Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylides C, Li-Beisson Y, Peltier G (2011). Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnology* **11:** 7.

Simionato D, Block MA, La Rocca N, Jouhet J, Maréchal E, Finazzi G, Morosinotto T (2013). The Response of Nannochloropsis gaditana to Nitrogen Starvation Includes De Novo Biosynthesis of Triacylglycerols, a Decrease of Chloroplast Galactolipids, and Reorganization of the Photosynthetic Apparatus. *Eukaryotic Cell* **12:** 665-676.

Singh A, Nigam PS, Murphy JD (2011). Renewable fuels from algae: An answer to debatable land based fuels. *Bioresource Technology* **102:** 10-16.

Smith SR, Abbriano RM, Hildebrand M (2012). Comparative analysis of diatom genomes reveals substantial differences in the organization of carbon partitioning pathways. *Algal Research* **1:** 2-16.

Solovchenko AE (2012). Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. *Russian Journal of Plant Physiology* **59:** 167-176.

Sorgüven E, Özilgen M (2013). Thermodynamic efficiency of synthesis, storage and breakdown of the high-energy metabolites by photosynthetic microalgae. *Energy* **58:** 679-687.

Sukenik A, Carmeli Y (1990). Lipid synthesis and fatty acid composition in Nannochloropsis sp. (Eustigmatophyceae) grown in light-dark cycle. *Journal of Phycology* **26:** 463-469.

Syrett PJ, Bocks SM, Merrett MJ (1964). The Assimilation of Acetate by Chlorella vulgaris. *Journal of Experimental Botany* **15:** 35-47.

Traller JC, Hildebrand M (2013). High throughput imaging to the diatom Cyclotella cryptica demonstrates substantial cell-to-cell variability in the rate and extent of triacylglycerol accumulation. *Algal Research* **2**: 244-252.

Trentacoste EM, Shrestha RP, Smith SR, Glé C, Hartmann AC, Hildebrand M, Gerwick WH (2013). Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. *Proceedings of the National Academy of Sciences* **110:** 19748-19753.

Turpin DH (1991). Effects of inorganic N availability on algal photosynthesis and carbon metabolism. *Journal of Phycology* **27:** 14-20.

Urzica EI, Vieler A, Hong-Hermesdorf A, Page MD, Casero D, Gallaher SD, Kropat J, Pellegrini M, Benning C, Merchant SS (2013). Remodeling of Membrane Lipids in Iron-starved Chlamydomonas. *Journal of Biological Chemistry* **288:** 30246-30258.

Wigmosta MS, Coleman AM, Skaggs RJ, Huesemann MH, Lane LJ (2011). National microalgae biofuel production potential and resource demand. *Water Resources Research* **47:** W00H04.

Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, Laurens LML, Dismukes GC, Posewitz MC (2010). Increased Lipid Accumulation in the Chlamydomonas reinhardtii sta7-10 Starchless Isoamylase Mutant and Increased Carbohydrate Synthesis in Complemented Strains. *Eukaryotic Cell* **9:** 1251-1261.

Xiong W, Liu L, Wu C, Yang C, Wu Q (2010). 13C-Tracer and Gas Chromatography-Mass Spectrometry Analyses Reveal Metabolic Flux Distribution in the Oleaginous Microalga Chlorella protothecoides. *Plant Physiology* **154:** 1001-1011.

Yao C, Ai J, Cao X, Xue S, Zhang W (2012). Enhancing starch production of a marine green microalga Tetraselmis subcordiformis through nutrient limitation. *Bioresource Technology* **118:** 438-444.

Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012). Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 3708-3724.

Yu E, Zendejas F, Lane P, Gaucher S, Simmons B, Lane T (2009). Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum (Baccilariophyceae) during starvation. *Journal of Applied Phycology* **21:** 669-681.

2.0 Chapter 2

IDENTIFICATION AND CHARACTERIZATION OF LIPASE THAPS3_264297

2.0.1 Abstract

A first step in developing improved strains for algal biofuels is gaining a better understanding of the metabolic pathways involved in lipid metabolism to aid in the identification of appropriate targets for engineering. We previously hypothesized that targeting lipid catabolism for disruption could lead to increased lipid accumulation without affecting growth. To identify targets within the lipid catabolism pathway, we used a transcriptomics-guided approach to find genes within this pathway that were downregulated during lipid accumulation. Transcriptomics provides a snapshot of the cell's genetic response to different environmental conditions, such as nutrient deprivation. We identified dynamic patterns of gene expression within the lipid catabolism pathway and the connected pathways of the glyoxylate cycle and photorespiration. However, we found no general downregulation of lipid catabolism, suggesting that lipid turnover continues to occur during lipid accumulation. The transcrptomics analysis did identify a significantly dowregulated hydrolase, Thaps3_264297, which is homologous to the highly conserved human CGI-58 and ABHD4 proteins. We functionally characterized Thaps3_264297, showing that it hadlipase, phospholiapse and acyltransferase activity, and thus concluded it was an appropriate target for disruption in *Thalassiosira pseudonana*.

2.1 Introduction

The pathway of lipid catabolism is relatively under-explored in microalgae. While many of the enzymes involved in lipid breakdown are thought to be conserved between algae and higher plants, little work has been done to identify and characterize key enzymes. A number of ß-oxidation enzymes were identified and characterized in *Euglena gracilis* (Graves and Becker 1974) as well in algae from the *Mougeotia, Bumilleriopsis* and *Eremosphaera* genera (Stabenau et al 1984). These studies identified differences in subcellular localization of ß-oxidation enzymes in the various algae, suggesting diverse evolution of the pathway. Few studies have looked at lipolytic activity in microalgae, but a few microalgal lipases have been functionally characterized due to their potential use for various biotechnological applications such as manufacturing lipids and therapeutic compounds (Godet et al 2012). Since these initial investigations in algal lipid catabolism, the interest in algae for biofuel purposes has spurred numerous –omics analyses on lipid metabolism, many of which look to identify and analyze parts of the catabolic pathway.

To further elucidate the metabolic landscape of lipid metabolism in microalgae, and to identify potential targets for engineering, we identified, using homology, enzymes involved in lipid catabolism and related pathways in the diatom *T. pseudonana*. We identified all portions of the catabolic pathway including phospholipases, lipases, complete mitochondrial and peroxisomal ß-oxidation pathways, a glyoxylate pathway and a photorespiration pathway. To guide engineering target identification, we analyzed the transcript abundances of the genes for these enzymes during lipid accumulation in *T. pseudonana*. The target enzyme identified using this procedure was then characterized to determine its homology, phylogeny and function.

2.2 Transcriptomic analysis of lipid catabolism genes in *T. pseudonana* during lipid accumulation

2.2.1 Background

Most diatoms, including *T. pseudonana*, are enclosed within a siliceous cell wall called the frustule. The building of the frustule and cell division are dependent upon the availability of silicon. Under silicon-deficient or silicon-starved conditions, the cell cycle of silicon-dependent species is arrested; some have also been characterized to accumulate lipids during this time period of growth cessation (Yu et al 2009). *T. pseudonana* has been shown to arrest its cell cycle within 8 hours of silicon starvation with the majority of cells arresting in the G1 phase of growth (Hildebrand et al 2007). A number of studies have shown that *T. pseudonana* accumulates lipids, primarily in the form of TAG-containing lipid droplets, during silicon starvation, and that this effect is consistent and reproducible.

Omics approaches provide a snapshot of cellular metabolism and the state of biochemical processes under certain conditions. Transcriptomics analyses in particular assess the transcript levels of a vast number of genes, thus providing information on the regulation of various pathways as well. In order to begin to elucidate the cellular function of lipid catabolism, its connection to related pathways, to explore its regulation and to identify possible engineering targets in this pathway, we analyzed transcript abundances of genes in this pathway over 24 hours of silicon starvation-induced lipid accumulation in *T. pseudonana*. Transcript analysis was done using Affymetrix whole genome tiling arrays in collaboration with Dr. Andrew Allen at the J. Craig Venter Institute. This approach determines relative changes in transcript levels.

2.2.2 Results & Discussion

2.2.2.1 Lipases/Phospholipases

Potential enzymes in the lipid catabolism pathway and related pathways were identified in *T. pseudonana* using conserved catalytic domains and BLAST searches for homology. We sequentially identified members of the pathway beginning with lipases and phospholipases, which catalyze the first step of lipid breakdown and the release of free fatty acids. We identified 90 genes in the genome that contain esterase/lipase/thioesterase domains. Of these, 28 show a significant increase in transcript levels (31%) and 21 show a significant decrease in transcript levels (23%) (Fig. 2-1A). 29 proteins were annotated to have phospholipase activity (Fig. 2-2A). Of these proteins, 12 showed a significant increase in transcript level during lipid accumulation (41%) and 7 showed a significant decrease in transcript level (24%) (Fig. 2-1B). Out of the 90 genes with esterase/lipase/thioesterase domains, 20 are annotated to be Class 3 lipases, which is a family of lipases that specifically catalyze the hydrolysis of TAGs (Fig. 2-2B). Of these 20 predicted TAG lipases, 3 show a significant increase in transcript abundance (15%), and 1 showed a significant decrease (5%) (Fig. 2-1C).

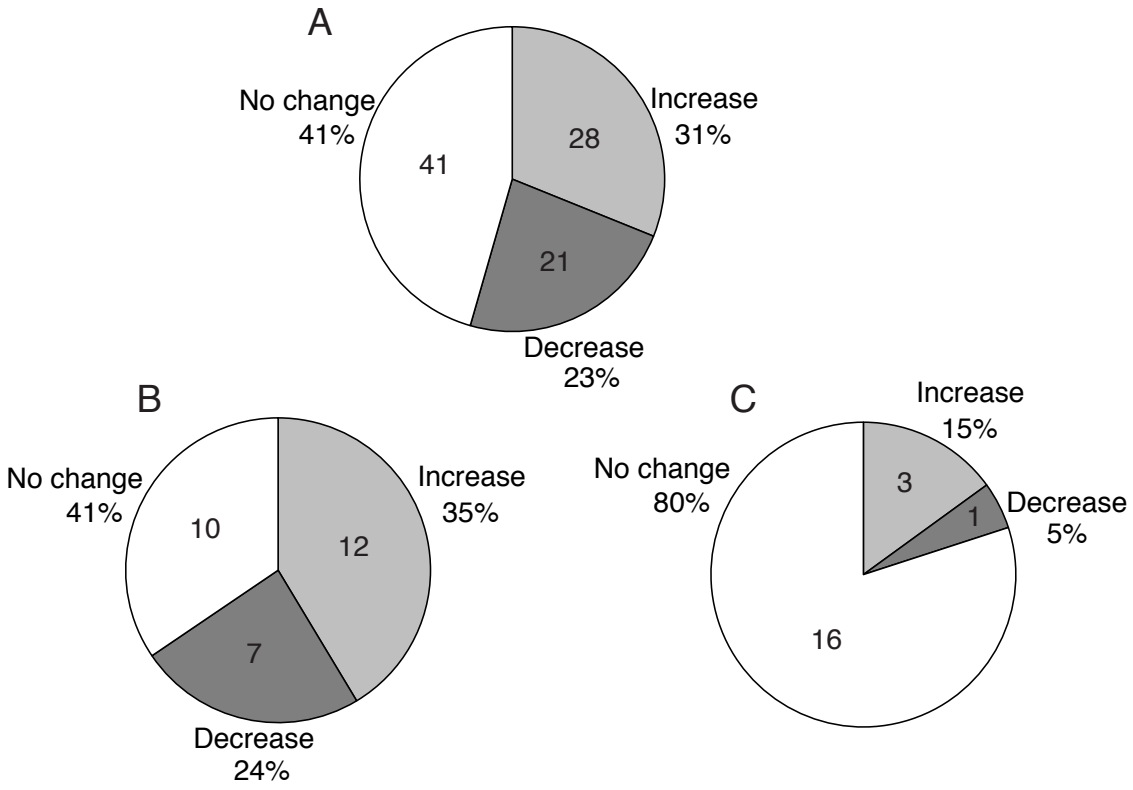
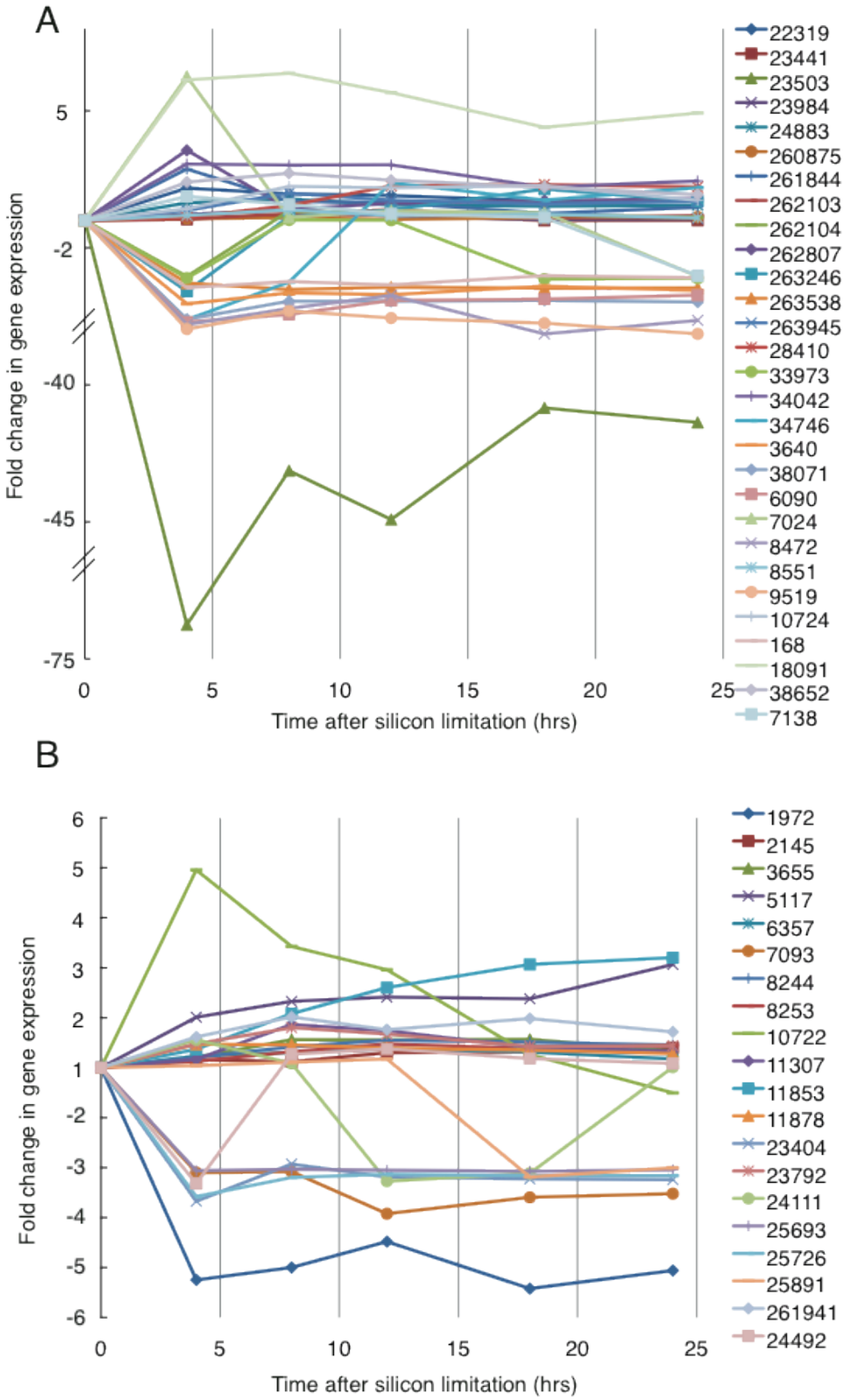


Figure 2-1. Overview of changes in lipase expression.
Proportions of lipase genes (*A*), phospholipase genes *(B)* and class 3 TAG lipase genes (*C*) showing significantly increased, significantly decreased, or no change in expression over 24 hours of silicon starvation.

Figure 2-2. Overview of phospholipase and TAG lipase expression patterns.
Fold change in transcript levels of phospholipase (*A*) and class 3 TAG lipase (*B*) genes over 24 hours of silicon starvation relative to the 0-hour time point. Protein IDs are shown in legends.


A
Fold change in gene expression
5
-2
-40
-45
-75
0
5
10
15
20
25
Time after silicon limitation (hrs)
22319
23441
23503
23984
24883
260875
261844
262103
262104
262807
263246
263538
263945
28410
33973
34042
34746
3640
38071
6090
7024
8472
8551
9519
10724
168
18091
38652
7138
B
Fold change in gene expression
6
5
4
3
2
1
-2
-3
-4
-5
-6
0
5
10
15
20
25
Time after silicon limitation (hrs)
1972
2145
3655
5117
6357
7093
8244
8253
10722
11307
11853
11878
23404
23792
24111
25693
25726
25891
261941
24492

Lipid synthesis during triacylglycerol accumulation is a combination of *de novo* synthesis as well as recycling of membrane lipids (Goncalves et al 2013, Siaut et al 2011). During this time organelle membrane remodeling and breakdown may be important sources for fatty acids. The abundance of phospholipases with increased transcript abundance (41% of annotated phospholipases) supports the recycling of membranes for triacylglycerol synthesis. An alternative route to the traditional Kennedy pathway of TAG biosynthesis involves the acylation of DAG from phospholipids by the enzyme PDAT (Yoon et al 2012). The potential PDATs we identified in *T. pseudonana*'s genome (protein IDs 261132, 8051 and 24413) show an increase in transcript abundance, suggesting that the breakdown of phospholipids is being used as a source of acyl-CoAs for TAG biosynthesis. Additionally, the localization of lipases can be informative of their function. For example, the two most upregulated lipase genes are localized to the chloroplast and are similar to other plastid phospholipases, further suggesting that phospholipids in the chloroplast are being used to harvest acyl-CoAs for TAG synthesis. The pattern of Class 3 lipases (triacylglycerol lipases) did not show a general pattern in transcript abundance (Fig. 2-2B), suggesting that either lipase activity is unchanged and lipid turnover is occurring to some extent, or regulation of these lipases does not occur at the level of transcription. This is concordant with other the findings of other transcriptomic analyses during lipid accumulation in microalgae (Miller et al 2010).

2.2.2.2 LACSs

Long-chain free fatty acids released by phospholipases and lipases must be activated by LACSs before breakdown in either the mitochondria or peroxisome,

however, LACSs are also pivotal in activating fatty acids for elongation and complex lipid synthesis. We identified 11 LACSs in the *T. pseudonana* genome, of which 4 show a significant decrease in transcript level during silicon starvation-induced lipid accumulation, and 5 show a significant increase (Fig. 2-3). Most of the LACS have no discernable localization sequence and may be cytosolic. Two LACSs (protein IDs 21299 and 11953) were targeted to the mitochondria. Protein 21299 showed a significant decrease in transcript abundance. One LACS (protein ID 29867) was targeted to the peroxisome and showed a slight increase in transcript abundance suggesting the downregulation of fatty acid activation for mitochondrial import. While localization of LACSs can be predictive of function, it has been suggested that the family to which the LACS belongs can also be informative. LACSs from families 1 and 5 are thought to function primarily for TAG biosynthesis (Coleman et al 2002). Interestingly, *T. pseudonana*'s predicted family 1 LACS (ACS1) shows an increase in transcript abundance, as does a predicted family 5 LACS (protein ID 262242), suggesting that these enzymes could function in TAG synthesis rather than oxidation. We also identified enzymes potentially involved in the carnitine transport system for the mitochondria (protein IDs 5924, 13065 and 31762), however these showed no significant change in transcript abundance.

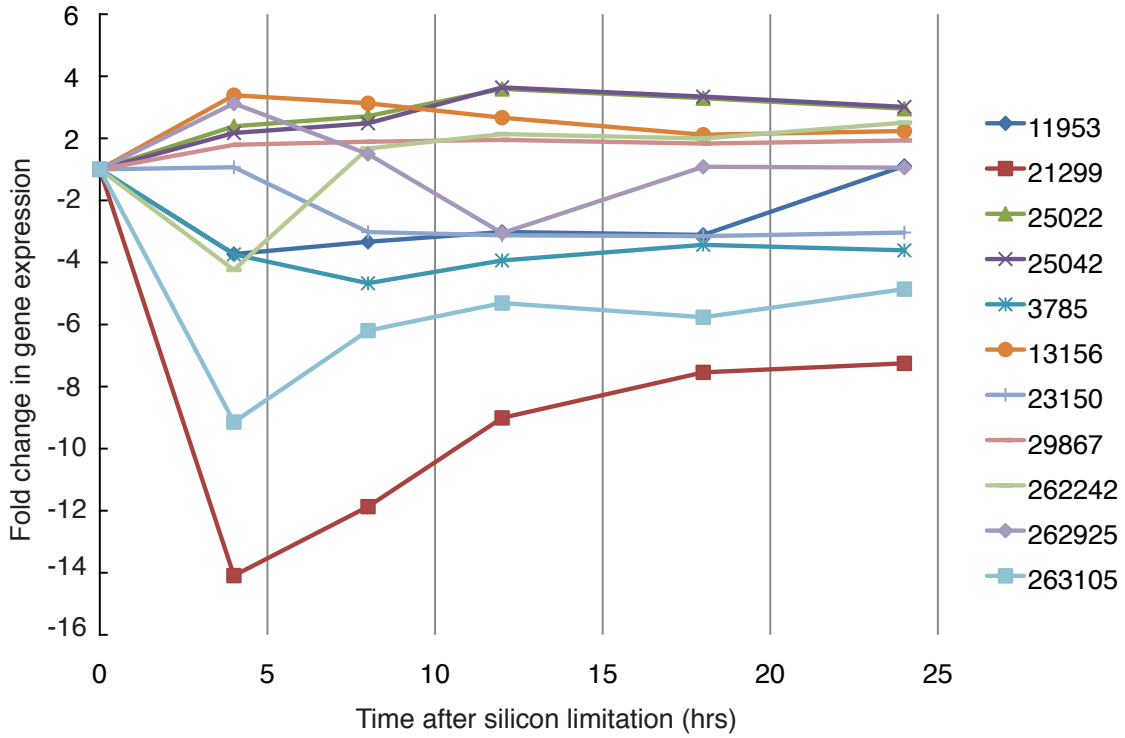


Figure 2-3. Overview of LACS expression patterns.
Fold change in gene expression of LACS genes over 24 hours of silicon starvation relative to the 0-hour time point. Protein IDs are shown in legend.

2.2.2.3 ß-oxidation

The subsequent steps in ß-oxidation can also offer predictions on the state of this metabolic pathway in the peroxisome and mitochondria (Fig. 2-4). Three ADHs were identified in *T. pseudonana* (protein IDs 35710, 269127, 269316), all localized to the mitochondria and all exhibiting a significant increase in transcript level during lipid accumulation. One peroxisomal ACO was identified (protein ID 263878) and showed a slight increase in transcript level. Both subunits of the mitochondrial trifunctional enzyme complex were identified and targeted to the mitochondria (protein IDs 26365 [alpha], 3627 [beta]), but only the alpha subunit showed a significant increase in transcript

abundance. The peroxisomal bifunctional enzyme complex was identified (protein ID 268338) and showed a significant increase in trancsript abundance during silicon starvation. The peroxisomal thiolase identified (protein ID 36742) showed no change.

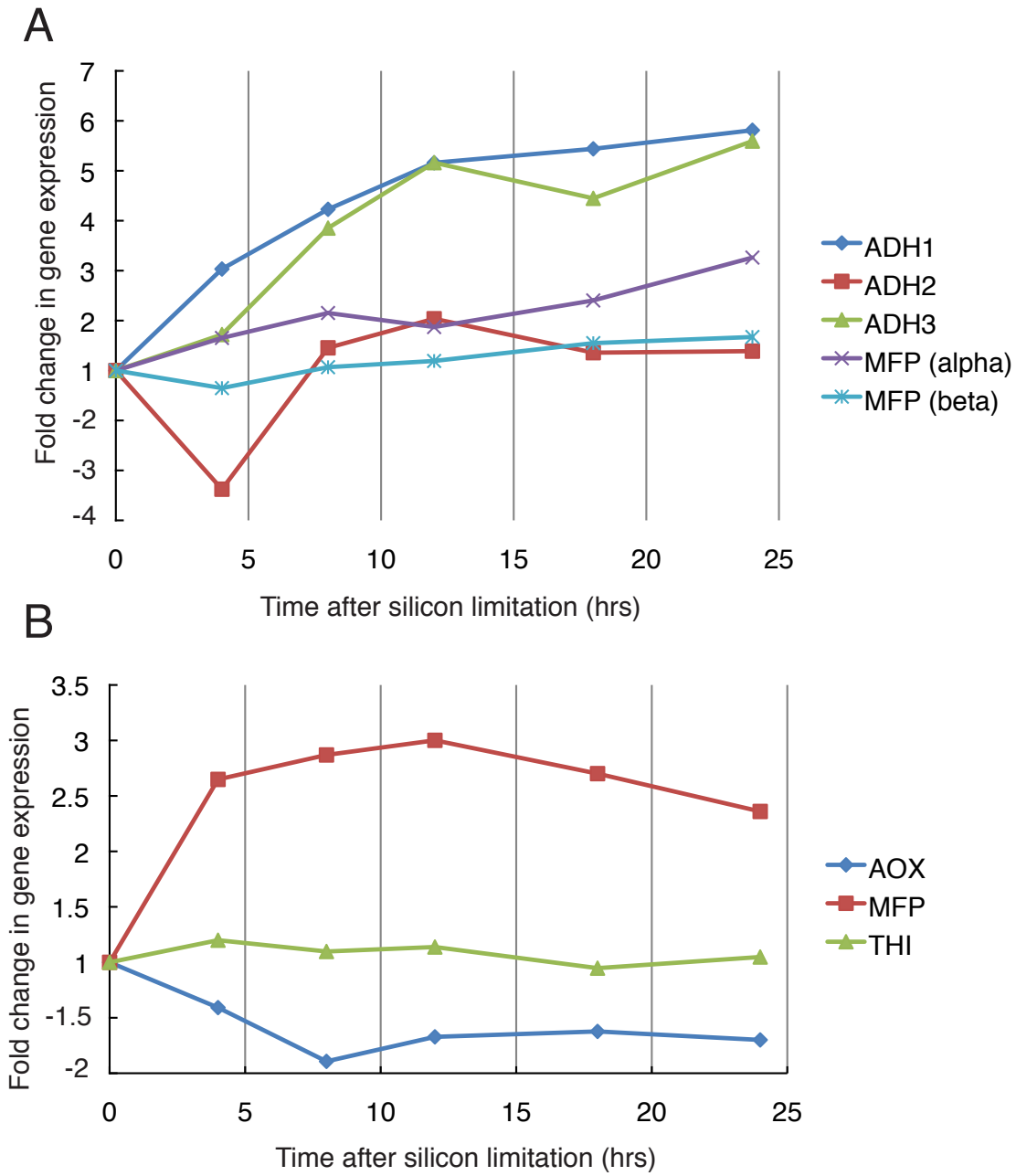


Figure 2-4. Overview of ß-oxidation expression patterns.
Fold change in gene expression of ß-oxidation in the mitochondria (*A*) and peroxisome (*B*) genes over 24 hours of silicon starvation relative to the 0-hour time point. ADH: acyl-CoA dehydrogenase; MFP: multifunctional protein; AOX: acyl-CoA oxidase; THI: thiolase.

Five proteins involved in the iterative steps of ß-oxidation were found to be targeted to the mitochondria (3 ADH, 2 trifunctional enzyme subunits). Four of these enzymes showed a significant increase in transcript abundance during silicon limitation. This predicts that fatty acids already in the mitochondria as well as short-chain fatty acids are undergoing ß-oxidation during lipid accumulation. Three enzymes involved in ß-oxidation were found to be targeted to the peroxisome (1 ACO, 1 bifunctional enzyme, 1 thiolase). The ACO showed a slight decrease in transcript level, the bifunctional enzyme showed a significant increase and the thiolase showed no change. Oxidation of lipids in the peroxisome would probably not provide a significant source of acetyl-CoA subunits, as very long-chain fatty acids are transported out to the mitochondria once they are shortened to long-chain fatty acids. Therefore, changes in transcript abundance in oxidation enzymes in the peroxisome could be in response to changes in concentration of substrates in the organelle.

2.2.2.4 Glyxoylate cycle

Acetyl-CoA units released from ß-oxidation in the peroxisome can be incorporated into the glyoxyate cycle. Because glyoxylate cycle processing results in the net production of succinate, it allows lipid breakdown products to be used for carbohydrate biosynthesis. It also used in some organisms to enable growth on acetate, as it provides a mechanism by which to incorporate the two-carbon acetate units into gluconeogenesis. Although the glyoxylate cycle has been predominantly characterized in

plants due to its instrumental function in oilseed lipid mobilization (Graham 2008), it has also been implicated in virulence (Lorenz and Fink 2002).

The genes, enzymes and activities associated with the glyoxylate cycle of been identified and characterized in a number of microalgae. We similarly identified in the *T. pseudonana* genome all the genes necessary for a complete glyxoylate cycle (Fig. 2-5). Interestingly, *T. pseudonana* only contains one potential citrate synthase enzyme (protein ID 1141), which shows targeting to mitochondria and no difference in transcript abundance throughout lipid accumulation. Although it was originally thought that all organisms had a peroxisomal citrate synthase, it has been shown in other organisms that enzymes involved in the glyoxoylate cycle are differentially localized throughout the cell (Kunze et al 2006), and the diatom *P. tricornutum* has been shown to dually localize enzymes to the peroxisome and mitochondria (Fabris et al 2012). Citrate synthase activity was also solely identified in the mitochondria in *Tetrahymena pyreformis*, even though a functional glyoxylate cycle was present (Müller et al 1968).

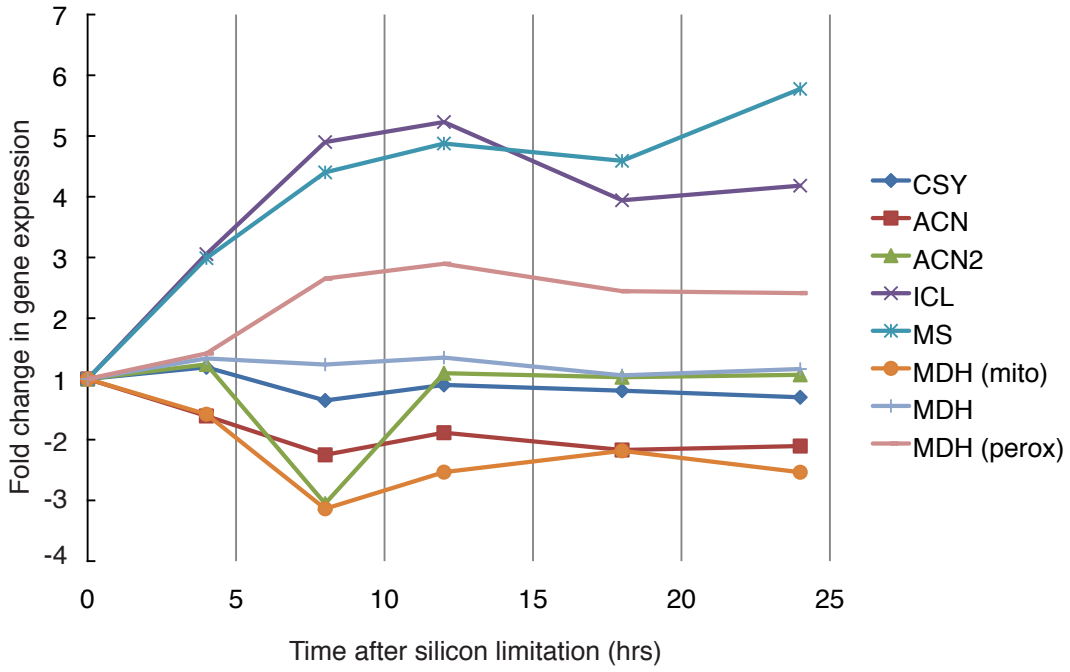


Figure 2-5. Overview of glyoxylate cycle expression patterns.
Fold change in gene expression of glyoxylate cycle genes over 24 hours of silicon starvation relative to the 0-hour time point. CSY: citrate synthase; ACN: aconitase; ICL: isocitrate lyase; MS: malate synthase; MDH: malate dehydrogenase.

We identified two potential aconitase enzymes (protein IDs 42704 and 268965); neither showed definite targeting and only 268965 was significantly downregulated. The next two enzymes in the glyxoylate pathway, isocitrate lyase (ICL) and malate synthase (MS), are only involved in this pathway and thus considered markers of the presence of the cycle. We identified both of these enzymes in *T. pseudonana*, and while ICL (Protein ID 35523) showed no definitely localization, MS (protein ID 26293) was targeted to the peroxisome. While ICL is typically thought to be peroxisomal (Müller et al 1968), it has also been found to be associated with the lipid droplet in *C. reinhardtii* (Moellering and Benning 2010). Both enzymes showed almost identical patterns of upregulation during lipid accumulation.

Finally, we identified three malate dehydrogenase (MDH) enzymes with differing targeting and expression patterns during lipid accumulation. The first MDH (protein ID 25953) had no definite targeting and showed no change in transcript abundance. The second MDH (protein ID 20726) was targeted to the mitochondria and showed significant downregulation. This is most likely the MDH involved in the TCA cycle. The third MDH (protein ID 41425) was targeted to the peroxisome and showed a significant increase in transcript abundance during lipid accumulation.

The identification of these enzyme and their patterns suggest that a functional glyxoylate cycle exists in *T. pseudonana*. The upregulated response of glyoxylate cycle-specific enzymes ICL, MS and peroxisomal MDH suggest that the cycle is responding to experimental conditions. Upregulation of these enzymes would indicate a potential increase in glyoxylate cycle activity, but determining the cause of this is more complex.

2.2.2.5 Photorespiration

Besides its role in incorporating acetyl-CoA units released from peroxisomal ß-oxidation, the glyxoylate cycle is further intricately connected to photorespiration in microalgae. Photorespiration is a necessary pathway in photosynthetic organisms due to the dual carboxylating and oxygenating nature of the enzyme RuBisCo. While the favored carboxylation of the substrate RuBp produces two molecules of 3-phosphoglycerate, the inevitable oxygenation of this substrate produces one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate. The recycling of phosphoglycolate through photorespiration intersects other metabolic pathways such as nitrogen metabolism and serine and glycine metabolism.

Photorespiration is thought to protect higher plants against photoinhibition (Kroth et al 2008). Although the process is characterized to a lesser extent in algae, it has similarly been hypothesized to alleviate light-induced stress on photosystems (Parker and Armbrust 2005). Diatoms have been shown to be capable of photorespiration (Schnitzler Parker et al 2004) and exhibit differential regulation of photorespiration under various environmental stimuli. *Thalassiosira* sp. have been shown to increase transcripts of the glycine decarboxylase T-protein, a key enzyme in the photorespiration, under high light conditions. Glycolate export into media did not substantially increase, suggesting that glycolate was recycled within the cell through photorespiration (Parker and Armbrust 2005, Schnitzler Parker et al 2004).

The *T. pseudonana* genome contains a number of proteins homologous to photorespiration enzymes (Kroth et al 2008). A potentially complete photorespiratory

pathway can be described, and the majority of the genes in this pathway show an increase in transcript abundance under the conditions described here (Fig. 2-6). The first step of photorespiration, the dephosphorylation of phosphoglycolate to glycolate, is catalyzed by phosphoglycolate phosphatase (PGP). The two potential PGPs in the *T. pseudonana* genome (25544, 18078) increase in transcript level during experiment, and both peak in transcript levels at four hours. The conversion of glycolate into glyoxylate can be carried out by glycolate oxidase in the peroxisome, glycolate dehydrogenase in the mitochondria, or glyoxylate reductase. *T. pseudonana* contains two potential glycolate oxidase enzymes (406, 3353). One is localized to the peroxisome (GOX1) and shows no change in transcript level. The other shows no definitive localization but has an increase in transcript level with a peak at four hours (GOX2). The genome also contains a potential glyoxylate reductase (35871) localized to the mitochondria that shows a similar profile of increase.

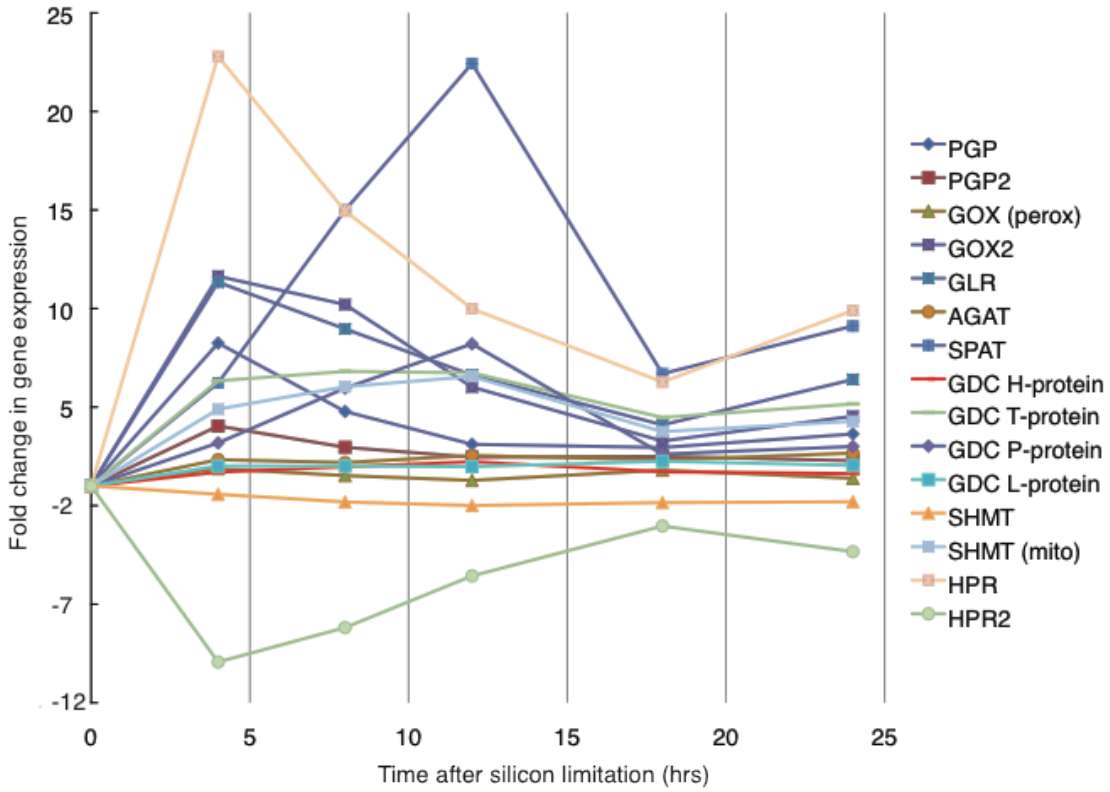


Figure 2-6. Overview of glycolate cycle expression patterns.
Fold change in gene expression of glyocolate cycle genes over 24 hours of silicon starvation relative to the 0-hour time point. PGP: phosphoglycolate phosphatase; GOX: glycolate oxidase; GLR: glyoxylate reductase; AGAT: alanine:glyoxylate aminotransferase; SPAT: serine:pyruvate aminotransferase; GDC: glycine decarboxylase; SHMT: serine hydroxymethyltransferase; HPR: hydroxypyruvate reductase.

An amino group is then transferred from alanine to glyoxylate to produce glycine via alanine:glyoxylate aminotransferase. This aminotransferase also catalyzes the transfer of an amino group from serine to produce hydroxypyruvate later in photorespiration. *T. pseudonana* contains one potential alanine:glyoxylate aminotransferase (AGAT, 2775) with unknown localization and one serine:pyruvate aminotransferase (SPT, 22208) localized to the mitochondria. Both enzymes have increased transcript levels while the SPT has a peak transcript level at 12 hours.

The next step in photorespiration involves the conversion of glycine to serine with the release of carbon dioxide and ammonia. This step is catalyzed by a complex of glycine decarboxylase and serine hydroxymethyltransferase. The glycine decarboxylase protein contains four subunits, the H-, T-, P-, and L-proteins. *T. pseudonana* contains potential genes for all four subunits (28251, 36208, 39799, 36716), and all show increased transcript levels in the dataset. The genome contains two potential serine hydroxymethyl transferase enzymes. One, localized to the peroxisome (26031), shows a decrease in transcript levels. The other, localized to the mitochondrion (269942), shows increased transcript levels with a peak at 12 hours.

After serine is converted to hydroxypyruvate via the serine:pyruvate aminotransferase described above, hydroxypyruvate reductase catalyzes the production of glycerate from hydroxypyruvate. The *T. pseudonana* genome contains two potential hydroxypyruvate reductases (2846, 261750), both localized to the mitochondrion. 2846 shows a significant increase in transcript levels with a peak at 4 hours. 261750 is also a

potential glycerate kinase, but shows a significant decrease in transcript levels in the data set.

T. pseudonana contains genes for a potentially complete photorespiratory pathway. The majority of the pathway exists in the mitochondrion, however glycolate oxidase and serine hydroxymethyltransferase isozymes are targeted to the peroxisome. The photorespiratory enzymes targeted to the mitochondrion are all upregulated. PGP, GOX2, glyoxylate reductase, and hydroxypyruvate reductase all show transcript peaks at 4 hours. Serine-pyruvate aminotransferase, the H- and P-proteins of GDC, and serine hydroxymethyltransferase all show transcript peaks at 12 hours.

The increase in transcripts of photorespiratory enzymes could correlate to the increase in irradiance when the cultures are diluted for transfer to silicon-free media. The cells, experiencing a sudden increase in light, may increase photorespiration to alleviate photosystem stress. Alternatively, photorespiration could be increased to provide intermediates of metabolic pathways such as nitrogen and amino acid metabolism. The increase in photorespiratory transcripts is unlikely to be involved in carbon assimilation because transcripts of the final enzyme involved in the recycling of phosphoglycolate, glycerate kinase, are significantly decreased. It is possible, however, that *T. pseudonana* contains a unique glycerate kinase that has yet to be identified. Despite the lack of a definite glycerate kinase, glycolate can also be recycled through the cell via the glyoxylate cycle. As discussed previously, the glyxoylate cycle enzymes of *T. pseudonana* are upregulated under these experimental conditions.

In summary, most of the enzymes in *T. pseudonana*'s photorespiration pathway show an increase in transcript level. The enzymes involved in recycling glycolate into

nitrogen and amino acid metabolism are localized to the mitochondria while the enzymes involved in assimilation of glycolate into the glyoxylate cycle show peroxisomal targeting. Increase in photorespiration could occur for many reasons, however the increased transcript levels seem to most correlate with previous studies showing a response to increased irradiance.

2.2.3 Methods

Transcriptomic data was obtained from S. Smith and M. Hildebrand and was generated by a whole genome tiling microarray of the *T. pseudonana* genome. Enzymes involved in each pathway were determined using NCBI's BLAST analysis for sequence similarity and Conserved Domain database. Incomplete protein sequences were completed manually using comparison to the Expressed Sequence Tags (EST) database and by manual extension to the immediate previous start codon or next immediate stop codon in the genome. Subcellular localization predictions were obtained using the publicly available algorithms Predotar, MITOProt, ChloroP and PTS1.

2.3 Identification and characterization of target lipase Thaps3_264297

2.3.1 Background

To identify appropriate targets for engineering lipid catabolism, we hypothesized that targeting lipases, as opposed to ß-oxidation enzymes, would have the least detrimental effects on other pathways and parts of metabolism. Disrupting lipase activity would potentially lead to a buildup of the TAG substrate – molecules of interest for fuels – as opposed to a buildup of acyl-CoA intermediates in the ß-oxidation pathway. We

hypothesized that the cell may downregulate TAG lipases during TAG accumulation to avoid futile competition between the processes, and thus considered downregulated lipases potential targets for knock-down. Although there was no overall pattern of lipase/esterase transcript abundance, 23% of these genes showed a decrease in transcript levels. Further analysis of these lipases revealed a homologue (29% identity) of the conserved enzyme comparative gene identification 58 (CGI-58). CGI-58, also known as α/ß hydrolase domain-containing protein 5 (ABHD5), is an enzyme in humans that, when mutated, causes Chanarin-Dorfman syndrome, a neutral lipid storage disease characterized by excessive accumulation of lipid droplets in cells of various tissues (Akiyama et al 2003). CGI-58 has been shown to be associated with the lipid droplet (Yamaguchi et al 2004) through protein-protein interactions and to be involved in its breakdown (Yamaguchi et al 2007). In humans it activates TAG lipase (Lass et al 2006, Yamaguchi and Osumi 2009) and exhibits lysophosphatidic acid acyltransferase activity thought to facilitate the transfer of acyl groups from storage lipids to phospholipids (Ghosh et al 2008, Montero-Moran et al 2010) and to produce signaling lipids (Lord et al 2012).

2.3.2 Results & Discussion

T. pseudonana's potential homologue of CGI-58, Thaps3_264297, maintained a greater than two-fold decrease in transcript abundance throughout the lipid accumulation phase (Fig. 2-7). It was one of the most downregulated lipases in the genome with nearly a four-fold decrease in transcript abundance at 8 hours of silicon limitation. Analysis of the annotation of Thaps3_264297 revealed the protein product was incomplete,

predicting 393 amino acids but neither a start nor stop codon. Sequencing of cDNA generated from isolated total RNA revealed a 505-amino acid protein product for Thaps3_264297:

MSPFSFLSSVHLTQRIQSLRDAEASLLDFAKSRFSPPFHPLTAHDESNHDWEIIDTP
ITPPSIFRNGSSCQVFDDQDAVLKLHGVKVVNKRMASDVKQQPAPLVLLHGYAN
GSLYFYRNLMGLSHFHFGSIYALDMLGWGLSSRPTFDLQLLGDDNGDTNDDKRS
NEHKQVASAEHFFVESLESWRKQHDLPKITLAGHSMGGYLSVAYAEKYPQHVE
RLILLSPVGVPERKEEDGVRINSLPFYMRGIVKITRYLFEKGVTPGSFLRALPLSKS
KSMVDSYILNRLPAIQCEEERKHLSEYLYQNSMLPGSGEYCLSQILTAGAFARIPL
VDRIPEIKSNDNKDGMEVHFVYGENDWMDFKGGIDVQRLCFNKRTEWEKQKN
NNESPPPKVFLHGVRNAGHLLMLDNYEEFNSALIIAAGGEDRLPSNFPRPVEFVC
NEVAASISDSVNCNVKREVLNEMGASAFFRGSRWDRRLQKKDGEGVNNDSVDD
IGCDEKKMEEQLA*

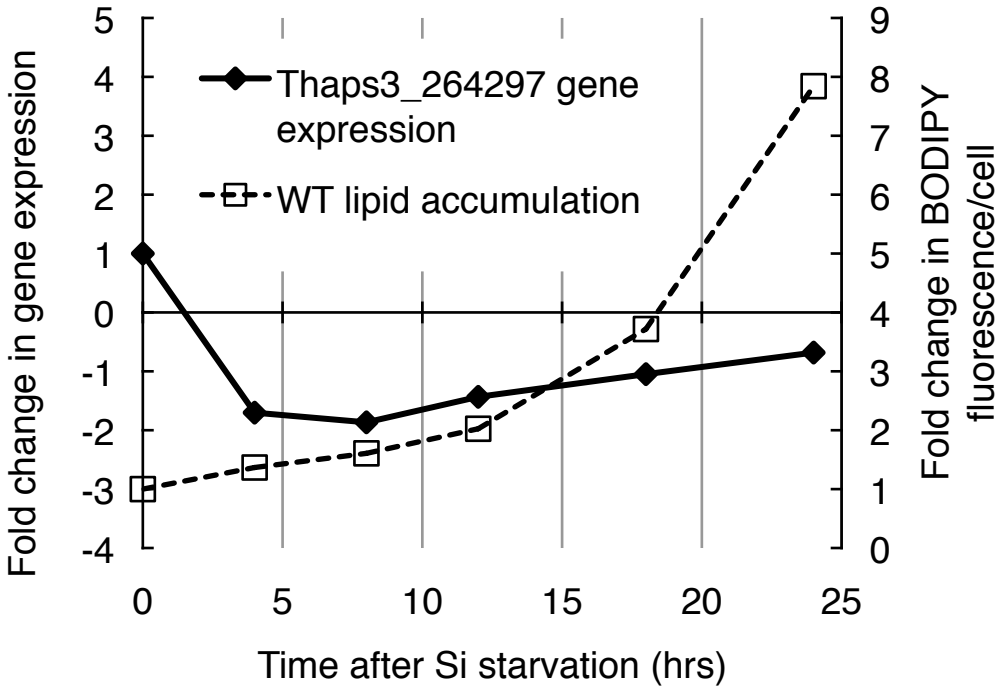


Figure 2-7. Gene expression of Thaps3_264297. Fold change in abundance of Thaps3_264297 transcript (primary axis) during silicon starvation-induced lipid accumulation in WT *T. pseudonana* (fluorescence of BODIPY, a neutral lipid dye, shown on secondary axis).

Homologs of CGI-58 were found to be ubiquitous throughout eukaryotes with at least one enzyme found in every eukaryotic genome probed. A multiple sequence alignment of the Thaps3_264297 protein with human CGI-58, as well as CGI-58 homologues from other organisms, revealed three conserved motifs that support interaction with lipids (Fig. 2-8A): the His-Gly dipeptide, a domain found in many lipases thought to define the boundaries of the lipid-binding pocket (Schrag et al 1997), the GXSXG motif, a conserved domain characteristic of hydrolytic enzymes including lipases, esterases and serine proteases (Cygler et al 1993), and the acyltransferase domain $H(X)_4D$ (Heath and Rock 1998).

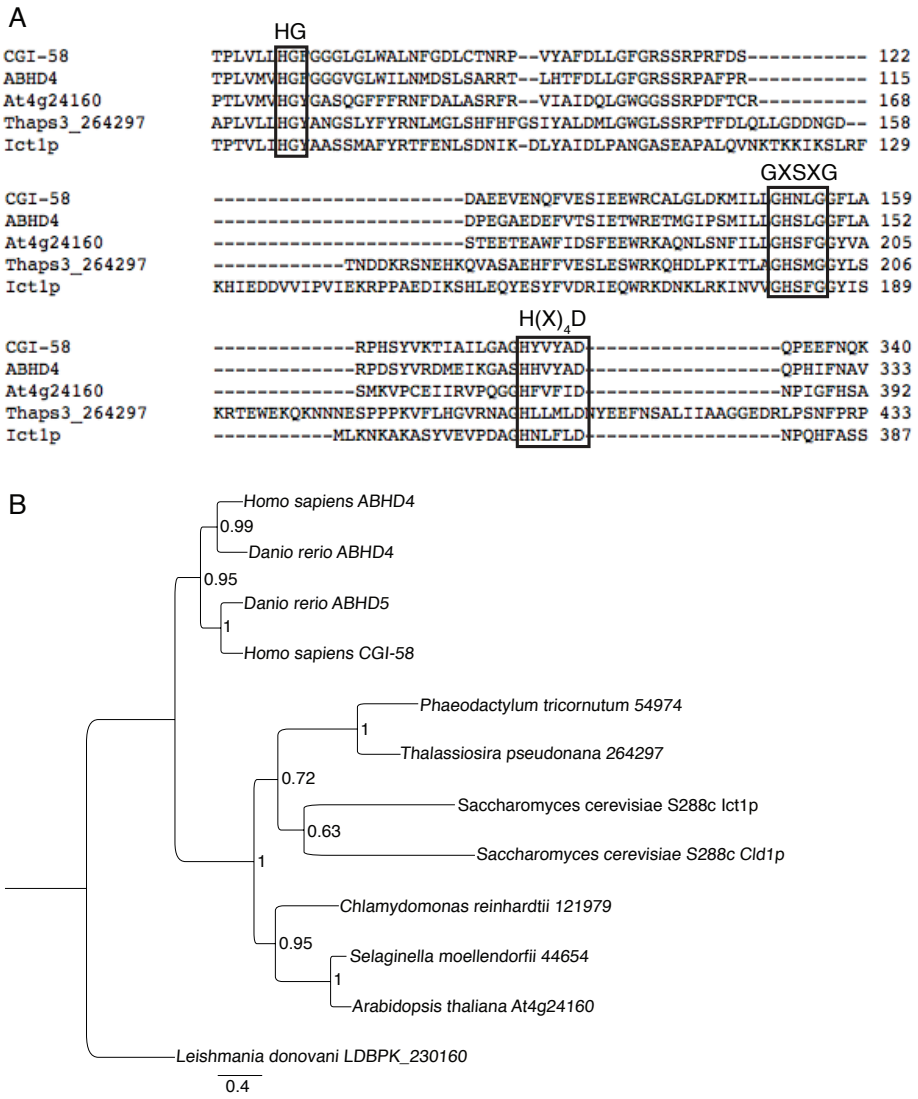


Figure 2-8. Thaps3_264297 is a homolog of CGI-58.
Partial alignment (*A*) of Thaps3_264297 amino acid sequence with CGI-58 and orthologues ABHD4 (*H. sapiens*), At4g24160 (*A. thaliana*), and Ict1p (*S. cerevisiae*). Boxes indicate conserved domains: His-Gly lipid binding domain (HG), catalytic serine lipase motif (GXSXG), acyltransferase motif ($H(X)_4D$). (*B*) Phylogenetic tree of CGI-58 homologues. Protein is indicated either by name or protein ID.

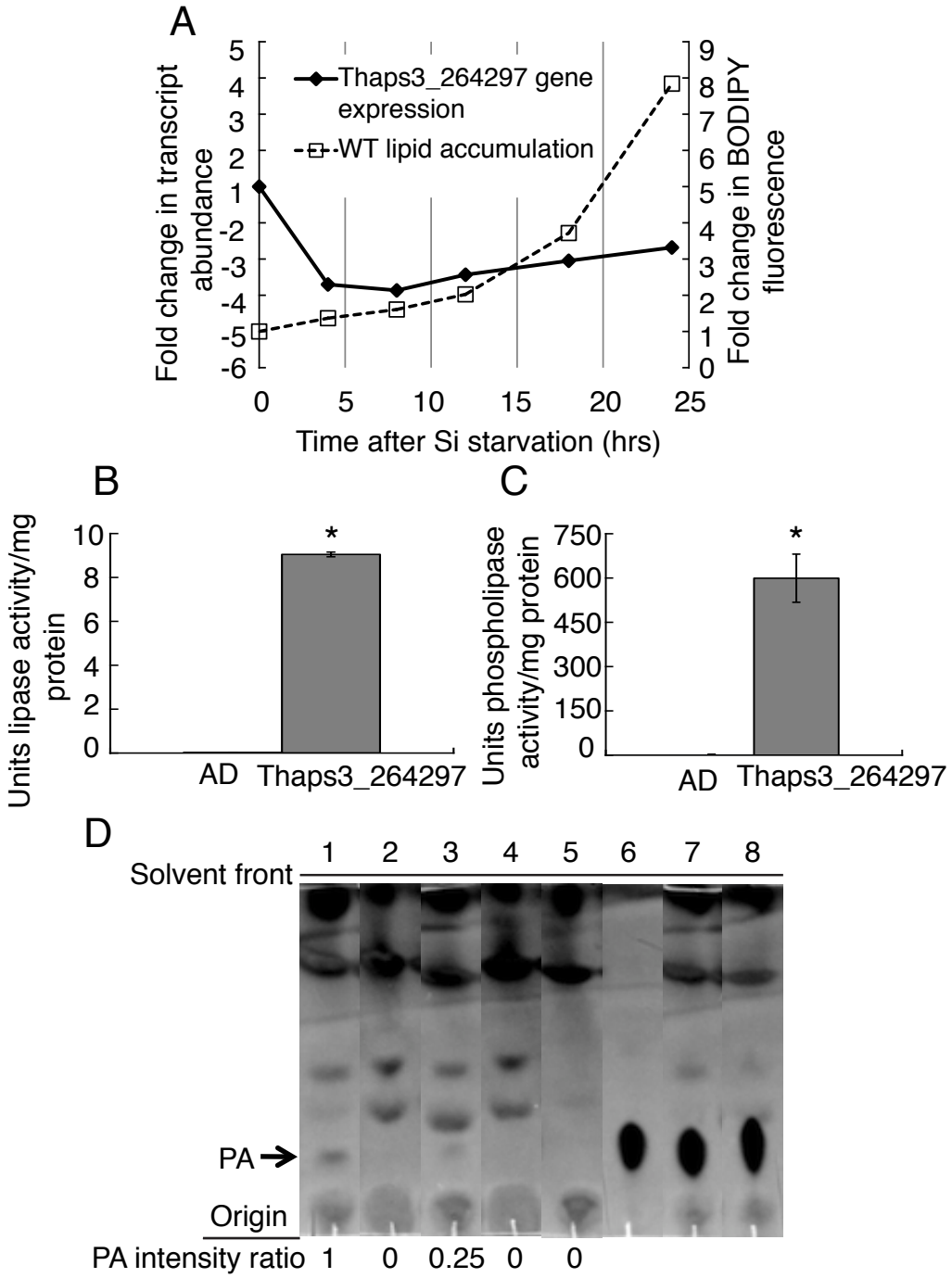
The catalytic serine of the lipase active site has changed to an asparagine in human CGI-58 (ABHD5), thus abolishing lipase activity of the enzyme. Interestingly, all vertebrates analyzed contained two paralogs of CGI-58 – ABHD5 and ABHD4. While ABHD5 does not contain a lipase active site, ABHD4 maintains the catalytic serine in these organisms. Additionally, the CGI-58 homologs in eukaryotes that only contain one homolog, such as algae and plants, maintain the catalytic serine and are more similar to ABHD4. A phylogenetic analysis of Thaps3_264297 and CGI-58 homologs revealed that the enzyme from *T. pseudonana* is more closely related to homologs from yeast, green algae and plants than to those of vertebrates (Fig. 2-8B). This evolutionary pattern of CGI-58 homologs and the ubiquitous nature of this enzyme, especially the lipase form, suggest that ABHD4 was the ancestral form and CGI-58 evolved from a duplication event somewhere in vertebrate history. While CGI-58 has evolved to have a supporting role of activating lipases, ABHD4 maintains its function as a lipase.

Homologs of CGI-58 found in both *Saccharomyces cerevisiae* and *A. thaliana* exhibit lysophosphatidic acid acyltransferase activity, phospholipase and TAG lipase activity (Ghosh et al 2008, Ghosh et al 2009). Overexpression of the CGI-58 homologue in *S. cerevisiae* resulted in a lower TAG content and higher phospholipid content than the wild-type (Ghosh et al 2008). Conversely, knocking out the CGI-58 homologue in *A. thaliana* resulted in a Chanarin-Dorfman-like excessive accumulation of lipid droplets in the leaves of the mutant plants (James et al 2010).

To assess the role of Thaps3_264297 in lipid catabolism in *T. pseudonana* we expressed His6-tagged recombinant Thaps3_264297 protein in *Escherichia coli* under the

control of the *lac* promotor. Soluble protein was expressed and purified using Ni-NTA resin. *In vitro* assays of either purified recombinant protein or Thaps3_264297-expressing cell lysates revealed TAG lipase (Fig. 2-9A), phospholipase A2 (Fig. 2-9B) and lysophosphatidic acid acyltransferase activities (Fig. 2-9C). The known lipase inhibitor tetrahydrolipstatin (Orlistat) inhibited recombinant Thaps3_264297 enzyme activity (Fig. 2-9D). These data indicated Thaps3_264297 is most likely involved in lipid catabolism.

Figure 2-9. Functional characterization of Thaps3_264297.
Lipase activity (*A*) compared to negative control alcohol dehydrogenase (AD) (n=3). (*B*) Phospholipase activity compared to negative control AD (n=3). Error bars, SEM. Statistical analyses performed using student's t-test, * $P < 0.05$, *** $P < 0.001$. (*C*) Lanes from TLC plate exhibiting lipid extracts from lysophosphatidic acid acyltransferase activity assay reactions using oleoyl-CoA and lysophosphatidic acid as substrates. TLC plate was stained with primuline and analyzed under UV. Activity is demonstrated by production of phosphatidic acid (PA). Reactions consisted of (1) substrates with lysates of *E. coli* expressing recombinant Thaps3_264297, (2) lysates with no substrate, (3) substrates with lysates of *E. coli* expressing an empty control vector, (4) control lysates with no substrates, and (5) substrates with no lysates. Standard PA is shown in 6, co-spotted in 7 with extracts from reaction 1 to show co-migration, and co-spotted in 8 with extracts from reaction 3 to show co-migration. (*D*) Increased lipase activity of lysates of *E. coli* expressing recombinant Thaps3_264297 over control lysates (n=3). Lipase activity can be inhibited using 100 µM tetrahydrolipstatin (Orlistat), a common lipase inhibitor. Error bars, SEM. Statistical analyses performed using student's t-test, *,$P < 0.05$.


A
Fold change in transcript abundance
Fold change in BODIPY fluorescence
Thaps3_264297 gene expression
WT lipid accumulation
Time after Si starvation (hrs)
B
Units lipase activity/mg protein
AD
Thaps3_264297
C
Units phospholipase activity/mg protein
AD
Thaps3_264297
D
Solvent front
1 2 3 4 5 6 7 8
PA
Origin
PA intensity ratio 1 0 0.25 0 0

When human CGI-58 was expressed in human CDS fibroblasts (with abolished CGI-58 activity), a small but reproducible percentage of fatty acids released from radiolabeled TAG were found to be incorporated into phospholipids, an observation not seen in CDS fibroblasts lacking CGI-58 (Montero-Moran et al 2010). This suggests that while CGI-58 is most likely multifunctional, at least one function is the breakdown of TAG molecules and subsequent shuttling of some acyl groups into phospholipids. This function was dependent upon acyl-CoA synthetase activity, suggesting that an activated acyl-CoA intermediate is necessary to funnel fatty acids released from TAG into phospholipids.

The main function of CGI-58 is thought to be one of maintaining lipid homeostasis. In single-celled organisms lipid homeostasis is important for regulating the flux of fatty acids through various metabolic pathways. Fatty acids are essential structural components of membranes, and the specific fatty acids incorporated affect membrane stability and fluidity. Fatty acids are also important as signaling molecules, sources of carbon for carbohydrate biosynthesis and components of rich energy stores such as neutral lipids. CGI-58 and its homologs are thought to play a role in dictating the mobilization of TAG molecules for these various pathways in the cell. The multifunctional lipase, phospholipase and acyltransferase activities we observed in Thaps3_264297 suggest that this enzyme is, in fact, a CGI-58 homolog and maintains similar functions in *T. pseudonana*.

2.3.3 Materials & Methods

2.3.3.1 Phylogeny of Thaps3_264297

Homologous sequences to Thaps3_264297 were obtained from a tBLASTn search and aligned using the ClustalW algorithm. The best-fitting amino acid substitution model optimized by maximum likelihood (ML) was selected using corrected Akaike/Bayesian Information Criterion (AIC/BIC) in Modeltest 3.0. Evolutionary histories were inferred using the Bayesian inference algorithm in Topali using MrBayes 3.1 with four Metropolis-coupled MCMC chains (one cold and three heated) ran for 1,000,000 generations. The first 25% were discarded as burn-in and the following data set was being sampled with a frequency of every 100 generations.

2.3.3.2 Construction of vectors, transformation and expression

All PCR was performed using Pfx high-fidelity DNA polymerase (Invitrogen) according to the manufacturer's instructions. All ligation reactions were performed using T4 DNA ligase (New England Biolabs). All sequencing was performed by Seqxcel (San Diego, CA). Total RNA was extracted using Trizol (Invitrogen) and cDNA synthesized using SuperScriptIII (Invitrogen). The expression vector used to heterologously express Thaps3_264297 in *E. coli* was constructed from the pet298b+ (Novagen) expression vector. Thaps3_264297 was amplified from total *T. pseudonana* cDNA using the primers 5' GGGCATATGTCGCCATTTTCCTTCTTGTCC 3' and 5' CCCGAGCTCTCAAGCCAATTGCTCCT 3'. The product was cloned into the pet28b+ expression vector using restriction sites NdeI and SacI to create pCgi58 with an N-terminal His-tag. The expression vector was transformed into GC5 chemically competent cells (Genesee Scientific, San Diego, CA) for propagation, isolated and transformed into

BL21 *E. coli* (Stratagene). Expression was induced when cultures hit mid-log phase (OD_{700} 0.5-0.7) with 0.1 mM IPTG at 30^{o} C for 2.75 hours and cells were subsequently harvested via centrifugation and frozen at -80 ^{o} C until further analysis.

2.3.3.3 Protein identification, purification and blotting

Soluble proteins were isolated and purified from *E. coli* using Ni-NTA resin as described in the Qiagen Expression Handbook. Proteins were separated using SDS-PAGE and gels visualized under UV (Nusep).Western blots using Thermo Pierce mouse 6XHis primary anti-His tag antibody and Thermo Pierce Goat anti-mouse horseradish peroxidase-conjugate secondary antibody were used to detect recombinant protein. Blots were visualized with Bio-Rad's Chemidoc XRS+ System.

2.3.3.4 Enzymatic activity assays

Lipase activity was determined using the Quantichrom Lipase Assay Kit (Bioassay Systems), using purified *Candida albicans* lipase (Invitrogen) to generate a standard curve, and purified alcohol dehydrogenase (Invitrogen) as a negative control. The lipase inhbitor tetrahydrolipstatin (Orlistat) was used at 100 μM to inhibit lipase activity. Phospholipase A2 activity was determined using the EnzCheck Phospholipase A2 Assay (Life Technologies). Lysophosphatidic acid acyltransferase activity assay reaction mixtures contained 20 μM oleoyl-CoA (VWR), 100 μM lysophosphatidic acid (Caymen Chemical), and 20 μg cell lysate in reaction buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl). Reactions were carried out at room temperature for 30 min and terminated by extracting lipids according to Bligh & Dyer[47]. Lipids were analyzed by

TLC using chloroform:methanol:water (65:25:4) and visualized under UV (302nm) after staining with primuline. Spots were quantified using the ChemiDoc XRS+ System and Image Lab software (Bio-Rad). Di-oleoyl phosphatidic acid (Sigma) was used as a standard for the reaction product.

2.4 Acknowledgements

We thank Sarah Smith for generation of the *T. pseudonana* transcriptomic data set and Dr. Andy Allen for data and statistical analysis. The transcriptomics analysis, in part, is in prep to be submitted by Sarah Smith in 2014. The dissertation author is the primary investigator of the data reported here; Sarah Smith is the primary investigator of the manuscript to be submitted. The characterization of Thaps3_264297 is, in full, included in The Proceedings of the National Academy of Sciences 2013, 110 (49): 19748-19753. Trentacoste, E. M., Shrestha, R. P., Smith, S. R., Glé, C., Hartmann, A. C., Hildebrand, M., & Gerwick, W. H. The dissertation author was the primary investigator and author of this paper.

2.5 References

Akiyama M, Sawamura D, Nomura Y, Sugawara M, Shimizu H (2003). Truncation of CGI-58 Protein Causes Malformation of Lamellar Granules Resulting in Ichthyosis in Dorfman-Chanarin Syndrome. *Journal of Investigative Dermatology* **121:** 1029-1034.

Coleman RA, Lewin TM, Van Horn CG, Gonzalez-Baró MR (2002). Do Long-Chain Acyl-CoA Synthetases Regulate Fatty Acid Entry into Synthetic Versus Degradative Pathways? *The Journal of Nutrition* **132:** 2123-2126.

Cygler M, Schrag JD, Sussman JL, Harel M, Silman I, Gentry MK, Doctor BP (1993). Relationship between sequence conservation and three-dimensional structure in a

large family of esterases, lipases, and related proteins. *Protein Science* **2:** 366-382.

Fabris M, Matthijs M, Rombauts S, Vyverman W, Goossens A, Baart GJE (2012). The metabolic blueprint of Phaeodactylum tricornutum reveals a eukaryotic Entner–Doudoroff glycolytic pathway. *The Plant Journal* **70:** 1004-1014.

Ghosh AK, Ramakrishnan G, Rajasekharan R (2008). YLR099C (ICT1) Encodes a Soluble Acyl-CoA-dependent Lysophosphatidic Acid Acyltransferase Responsible for Enhanced Phospholipid Synthesis on Organic Solvent Stress in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **283:** 9768-9775.

Ghosh AK, Chauhan N, Rajakumari S, Daum G, Rajasekharan R (2009). At4g24160, a Soluble Acyl-Coenzyme A-Dependent Lysophosphatidic Acid Acyltransferase. *Plant Physiology* **151:** 869-881.

Godet S, Hérault J, Pencreac'h G, Ergan F, Loiseau C (2012). Isolation and analysis of a gene from the marine microalga Isochrysis galbana that encodes a lipase-like protein. *Journal of Applied Phycology* **24:** 1547-1553.

Goncalves E, Johnson J, Rathinasabapathi B (2013). Conversion of membrane lipid acyl groups to triacylglycerol and formation of lipid bodies upon nitrogen starvation in biofuel green algae Chlorella UTEX29. *Planta* **238:** 895-906.

Graham IA (2008). Seed Storage Oil Mobilization. *Annual Review of Plant Biology* **59:** 115-142.

Graves LB, Becker WM (1974). Beta-Oxidation in Glyoxysomes from Euglena. *The Journal of Protozoology* **21:** 771-774.

Heath RJ, Rock CO (1998). A Conserved Histidine Is Essential for Glycerolipid Acyltransferase Catalysis. *Journal of Bacteriology* **180:** 1425-1430.

Hildebrand M, Frigeri LG, Davis AK (2007). Synchronized growth of Thalassiosira pseudonana (Bacillariophyceae) provides novel insights into cell wall synthesis processes in relation to the cell cycle. *Journal of Phycology* **43:** 730-740.

James CN, Horn PJ, Case CR, Gidda SK, Zhang D, Mullen RT, Dyer JM, Anderson RGW, Chapman KD (2010). Disruption of the Arabidopsis CGI-58 homologue produces Chanarin–Dorfman-like lipid droplet accumulation in plants. *Proceedings of the National Academy of Sciences* **107:** 17833-17838.

Kroth PG, Chiovitti A, Gruber A, Martin-Jezequel V, Mock T, Parker MS, Stanley MS, Kaplan A, Caron L, Weber T, Maheswari U, Armbrust EV, Bowler C (2008). A

Model for Carbohydrate Metabolism in the Diatom Phaeodactylum tricornutum Deduced from Comparative Whole Genome Analysis. *PLoS ONE* **3:** e1426.

Kunze M, Pracharoenwattana I, Smith SM, Hartig A (2006). A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochimica et Biophysica Acta* **1763:** 1441-1452.

Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, Kienesberger P, Strauss JG, Gorkiewicz G, Zechner R (2006). Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metabolism* **3:** 309-319.

Lord CC, Betters JL, Ivanova PT, Milne SB, Myers DS, Madenspacher J, Thomas G, Chung S, Liu M, Davis MA, Lee RG, Crooke RM, Graham MJ, Parks JS, Brasaemle DL, Fessler MB, Brown HA, Brown JM (2012). CGI-58/ABHD5-Derived Signaling Lipids Regulate Systemic Inflammation and Insulin Action. *Diabetes* **61:** 355-363.

Lorenz MC, Fink GR (2002). Life and Death in a Macrophage: Role of the Glyoxylate Cycle in Virulence. *Eukaryotic Cell* **1:** 657-662.

Miller R, Wu G, Deshpande RR, Vieler A, Gärtner K, Li X, Moellering ER, Zäuner S, Cornish AJ, Liu B, Bullard B, Sears BB, Kuo M, Hegg EL, Shachar-Hill Y, Shiu S, Benning C (2010). Changes in Transcript Abundance in Chlamydomonas reinhardtii following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology* **154:** 1737-1752.

Moellering ER, Benning C (2010). RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in Chlamydomonas reinhardtii. *Eukaryotic Cell* **9:** 97-106.

Montero-Moran G, Caviglia JM, McMahon D, Rothenberg A, Subramanian V, Xu Z, Lara-Gonzalez S, Storch J, Carman GM, Brasaemle DL (2010). CGI-58/ABHD5 is a coenzyme A-dependent lysophosphatidic acid acyltransferase. *Journal of Lipid Research* **51:** 709-719.

Müller M, Hogg JF, de Duve C (1968). Distribution of Tricarboxylic Acid Cycle Enzymes and Glyoxylate Cycle Enzymes between Mitochondria and Peroxisomes in Tetrahymena pyriformis. *Journal of Biological Chemistry* **243:** 5385-5395.

Nelson DL, Cox MM (2000). *Lehninger Principles of Biochemistry*, 3rd edn. Worth Publishers: New York, NY.

Parker MS, Armbrust E (2005). Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric

diatom Thalassiosira pseudonana (Bacillariophyceae). *Journal of Phycology* **41:** 1142-1153.

Poirier Y, Antonenkov VD, Glumoff T, Hiltunen JK (2006). Peroxisomal β-oxidation—A metabolic pathway with multiple functions. *Biochimica et Biophysica Acta* **1763:** 1413-1426.

Schnitzler Parker M, Armbrust E, Piovia-Scott J, Keil RG (2004). Induction of photorespriation by light in the centric diatom Thalassiosira weissflogii (Bacillariophyceae): molecular characterization and physiological consequences. *Journal of Phycology* **40:** 557-567.

Schrag JD, Cygler M, Byron Rubin EAD (1997). Lipases and αβ hydrolase fold. *Lipases, Part A: Biotechnology*. Academic Press. pp 85-107.

Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylides C, Li-Beisson Y, Peltier G (2011). Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnology* **11:** 7.

Stabenau H, Winkler U, Säftel W (1984). Enzymes of β-Oxidation in Different Types of Algal Microbodies. *Plant Physiology* **75:** 531-533.

Yamaguchi T, Omatsu N, Matsushita S, Osumi T (2004). CGI-58 Interacts with Perilipin and Is Localized to Lipid Droplets: Possible Involvement of CGI-58 Mislocalization in Chanarin-Dorfman Syndrome. *Journal of Biological Chemistry* **279:** 30490-30497.

Yamaguchi T, Omatsu N, Morimoto E, Nakashima H, Ueno K, Tanaka T, Satouchi K, Hirose F, Osumi T (2007). CGI-58 facilitates lipolysis on lipid droplets but is not involved in the vesiculation of lipid droplets caused by hormonal stimulation. *Journal of Lipid Research* **48:** 1078-1089.

Yamaguchi T, Osumi T (2009). Chanarin–Dorfman syndrome: Deficiency in CGI-58, a lipid droplet-bound coactivator of lipase. *Biochimica et Biophysica Acta* **1791:** 519-523.

Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012). Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 3708-3724.

Yu E, Zendejas F, Lane P, Gaucher S, Simmons B, Lane T (2009). Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum (Baccilariophyceae) during starvation. *Journal of Applied Phycology* **21:** 669-681.

3.0 Chapter 3

METABOLIC ENGINEERING OF LIPID CATABOLISM IN *THALASSIOSIRA PSEUDONANA*

3.0.1 Abstract

Biologically derived fuels are viable alternatives to traditional fossil fuels, and microalgae are a particularly promising source, but improvements are required throughout the production process to increase productivity and reduce cost. Metabolic engineering to increase yields of biofuel-relevant lipids in these organisms without compromising growth is an important aspect of advancing economic feasibility. We report that the targeted knock down of the multifunctional lipase/phospholipase/acyltransferase Thaps3_264297 increased lipid yields without affecting growth in the diatom *Thalassiosira pseudonana*. Antisense-expressing knock-down strains 1A6 and 1B1 exhibited wild-type-like growth and increased lipid content under both continuous light and alternating light/dark conditions. Strains 1A6 and 1B1 respectively contained 2.4- and 3.3-fold higher lipid content than wild-type during exponential growth, and 4.1- and 3.2-fold higher lipid content than wild-type after 40 hours of silicon starvation.

Analyses of fatty acids, lipid classes and membrane stability in the transgenic strains suggest a role for Thaps3_264297 in membrane lipid turnover and lipid homeostasis. These results demonstrate that targeted metabolic manipulations can be used to increase lipid accumulation in eukaryotic microalgae without compromising growth.

3.1 Introduction

The development of alternative fuels is becoming increasingly urgent as the world supply of fossil fuels decreases and atmospheric CO_2 levels continue to rise. Microalgae and cyanobacteria have been identified as promising biological sources of various fuel-relevant molecules including lipids, ethanol and hydrocarbons (Radakovits et al 2010). Eukaryotic microalgae have received particular recognition due to the ability of many species to accumulate triacylglycerol (TAG), especially under nutrient limitation. These neutral lipids can be converted to fatty acid methyl esters (FAMEs), the main components of biodiesel (Sharif Hossain et al 2008), through trans-esterification, or refined into other fuel constituents (Pienkos and Darzins 2009). Total lipids and other biomass constituents can be converted into crude oil alternatives through thermochemical processes such as hydrothermal liquefaction (López Barreiro et al 2013). The economic feasibility, however, of microalgae as a source of biofuels is dependent upon improvements throughout the production process (Coates et al 2013), and one of the most influential improvements would be to increase lipid yields (Davis et al 2011, Radakovits et al 2010). The traditional strain discovery and selection process is one viable approach for finding high-lipid containing strains (Sheehan et al 1998); however, the recent development of molecular techniques for microalgal strain optimization offers an alternative method to increase lipid production.

To date, engineering efforts for lipid metabolism have focused on increasing lipid biosynthesis or blocking the competing pathways of carbohydrate formation (Radakovits et al 2010). However, of the strategies that have successfully increased lipids, all have

resulted in decreased growth in the engineered strains (Li et al 2010, Radakovits et al 2011, Wang et al 2009, Work et al 2010). Maintaining high growth rates and high biomass accumulation is imperative for algal biofuel production on large economic scales (Borowitzka 1992), and engineering efforts that increase lipid content without decreasing growth or biomass can significantly reduce production cost and increase the economic viability of algal biofuels (Davis et al 2011). Lipid catabolism has largely been ignored as a relevant pathway for engineering, despite being a competing pathway to lipid biogenesis. Lipid breakdown is hypothesized to play roles in quickly providing acyl groups for membrane reorganization as environmental conditions change, contributing to polar lipid synthesis during dark cycles, and remobilizing cell membranes upon release from nutrient stress (Harwood and Guschina 2009, Kainz et al 2009, Solovchenko 2012).

We hypothesized that the targeted knock-down of lipid catabolism, and specifically lipases which catalyze the release of free fatty acids from lipids, could increase lipid accumulation. We proposed that unlike disrupting carbohydrate pools, which are the primary carbon storage product of many microalgae (Chauton et al 2013, Li et al 2011), knock-down of lipid catabolism would have less impact on the primary carbon pathways associated with growth. This work was performed in the diatom *Thalassiosira pseudonana*, a model lipid-accumulating diatom species (Yu et al 2009) with an available genome sequence (Armbrust et al 2004). Diatoms have long been attractive sources of biodiesel-type fuels (Hildebrand et al 2012, Sheehan et al 1998), and recent advancements have enabled metabolic engineering in these organisms (De Riso et al 2009, Poulsen et al 2006).

In Chapter 2 we described the identification of lipase/phospholipase/-acyltranferase Thaps3_264297 using transcriptomics as a guide. Functional characterization confirmed that this enzyme was involved in lipid catabolism and an appropriate target for knock-down. Here we report the knock-down of this enzyme in *T. pseudonana*, and the screening and characterization of resulting strains. Antisense-containing knock-down strains show no decrease in growth relative to the wild-type (WT) strain, but exhibit increased lipid accumulation during both nutrient-replete and nutrient-limited conditions. These results demonstrate that targeted metabolic manipulations can be used to increase accumulation of fuel-relevant molecules in eukaryotic microalgae with no negative effects on growth.

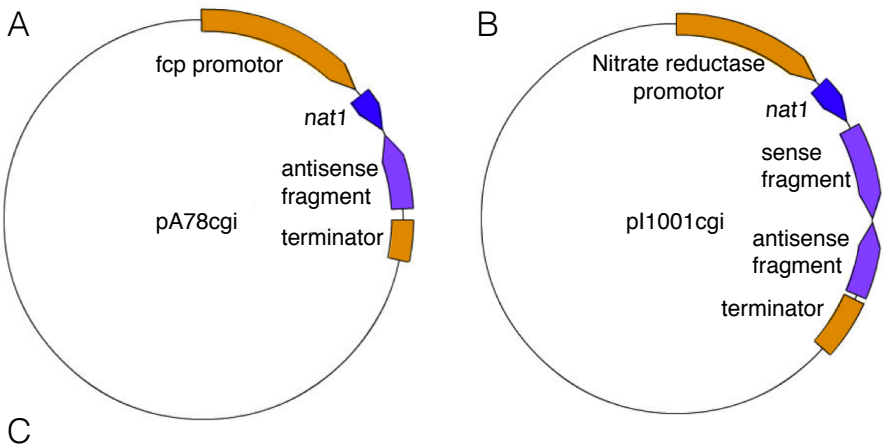
3.2 Results

3.2.1 Knock-down of Thaps3_264297 using antisense and interfering RNA (RNAi)

Knock-down of Thaps3_264297 was carried out in *T. pseudonana* using antisense and RNAi approaches. Two constructs, pA78cgi and pI1001cgi, encoding antisense and RNAi respectively, were designed to a portion of Thaps3_264297 (Fig. 3-1). Expression of pA78cgi was driven by the fucoxanthin chlorophyll a/b-binding protein (fcp) promoter, while pI1001cgi was under the control of an inducible nitrate reductase promoter (Poulsen et al 2006). The constructs were transformed into WT *T. pseudonana* and seven transformants (three antisense strains and four RNAi strains) were screened for growth, lipid accumulation under nutrient-replete conditions, and lipid accumulation under nutrient-deplete conditions. All three antisense strains responded similarly, showing comparable growth to WT and increased lipids compared to WT under nutrient-

replete and deplete conditions; RNAi strains demonstrated variable results (Fig. 3-2). Two randomly selected antisense strains (1A6 and 1B1 containing antisense-encoding pA78cgi) and the two RNAi strains (3C2 and 3D6 containing RNAi-encoding pI1001cgi) displaying the best growth were chosen for further characterization analysis. Knock-down in these four transformants was verified using both immunoblotting (Fig. 3-3A) and functional enzyme assays (Fig. 3-3B).

Figure 3-1. Knock-down constructs for Thaps3_264297.
Design of antisense (*A*) and RNAi (*B*) knock-down constructs. Constructs contained the *T. pseudonana fcp* and nitrate reductase promotors, respectively, and the nourseothricin resistance gene, *nat1*. pA78cgi contained a 500-bp antisense fragment to Thaps3_264297. PI1001cgi contained a 600-bp sense fragment of Thaps3_264297 immediately followed by a 500-bp antisense fragment. (*C*) shows the full sequence of Thaps3_264297 and the portion of the sequence contained in the sense fragment of the construct (blue arrow to orange arrow) and in the antisense fragment of the construct (green arrow to blue arrow).



C

ATGTCGCCATTTTCCTTCTTGTCCTCAGTCCACCTCACCCAACGAATCCAATCCCTACGC-

GACGCAGAGGCAAGTCTACTCGACTTTGCCAAGTCACGATTCTCTCCTCCCTTTCATCCTCTGACGGCGCATGATG

AATCTAACCACGATTGGGAGATTATTGATACACCCATCACTCCTCCATCCATCTTTCGCAATGGATCGTCATGCCAAG

TGTTTGACGACCAAGATGCAGTGCTCAAGTTGCATGGAGTCAAGGTAGTCAACAAACGCATGGCATCGGATGTTA

AACAACAACCTGCTCCATTGGTTCTGCTCCACGGATACGCCAATGGGTCACTTTACTTTTATCGCAATTTGATGGG

ACTTAGCCACTTTCACTTTGGGTCGATTTATGCATTGGATATGCTTGGATGGGGATTGAGCAGTCGGCCTACGTTCG

ACCTGCAGTTACTAGGCGATGACAACGGCGATACCAACGATGACAAACGCAGCAACGAGCACAAACAGGTGGCA

TCGGCAGAGCACTTCTTTGTGGAGTCACTAGAATCATGGAGGAAGCAGCACGATCTACCAAAGATCACCCTCGCT

GGACACAGTATGGGTGGTTATCTCAGCGTGGCTTATGCAGAAAAGTATCCGCAACACGTAGAAAGGCTCATTCTAC

TCTCTCCAGTGGGTGTTCCTGAGCGGAAGGAGGAAGATGGCGTTCGCATCAATAGTCTGCCGTTTTACATGAGGG

GAATCGTCAAGATAACTCGCTATCTGTTTGAAAAGGGCGTCACTCCTGGATCGTTCCTACGTGCTCTTCCACTCTCC

AAATCAAAATCTATGGTGGATTCTTATATTCTCAATAGGTTGCCTGCTATTCAGTGTGAAGAGGAGAGAAAGCATCT

TAGTGAATATTTGTATCAGAATAGCATGCTTCCCGGCAGTGGAGAGTATTGTTTATCGCAGATACTGACCGCGGGAG

CCTTTGCACGAATTCCATTGGTAGATCGCATTCCTGAAATAAAGTCAAATGACAACAAAGATGGGATGGAAGTGCA

CTTTGTCTACGGTGAGAATGACTGGATGGACTTCAAAGGTGGCATTGATGTTCAACGATTGTGTTTCAATAAGAGA

ACTGAGTGGGAAAAGCAAAAGAACAACAATGAATCGCCTCCACCCAAAGTATTCTTGCATGGAGTAAGAAATGCA

GGGCATTTGCTCATGTTAGACAACTATGAAGAGTTTAACTCTGCATTGATTATTGCTGCCGGTGGAGAGGATAGAT

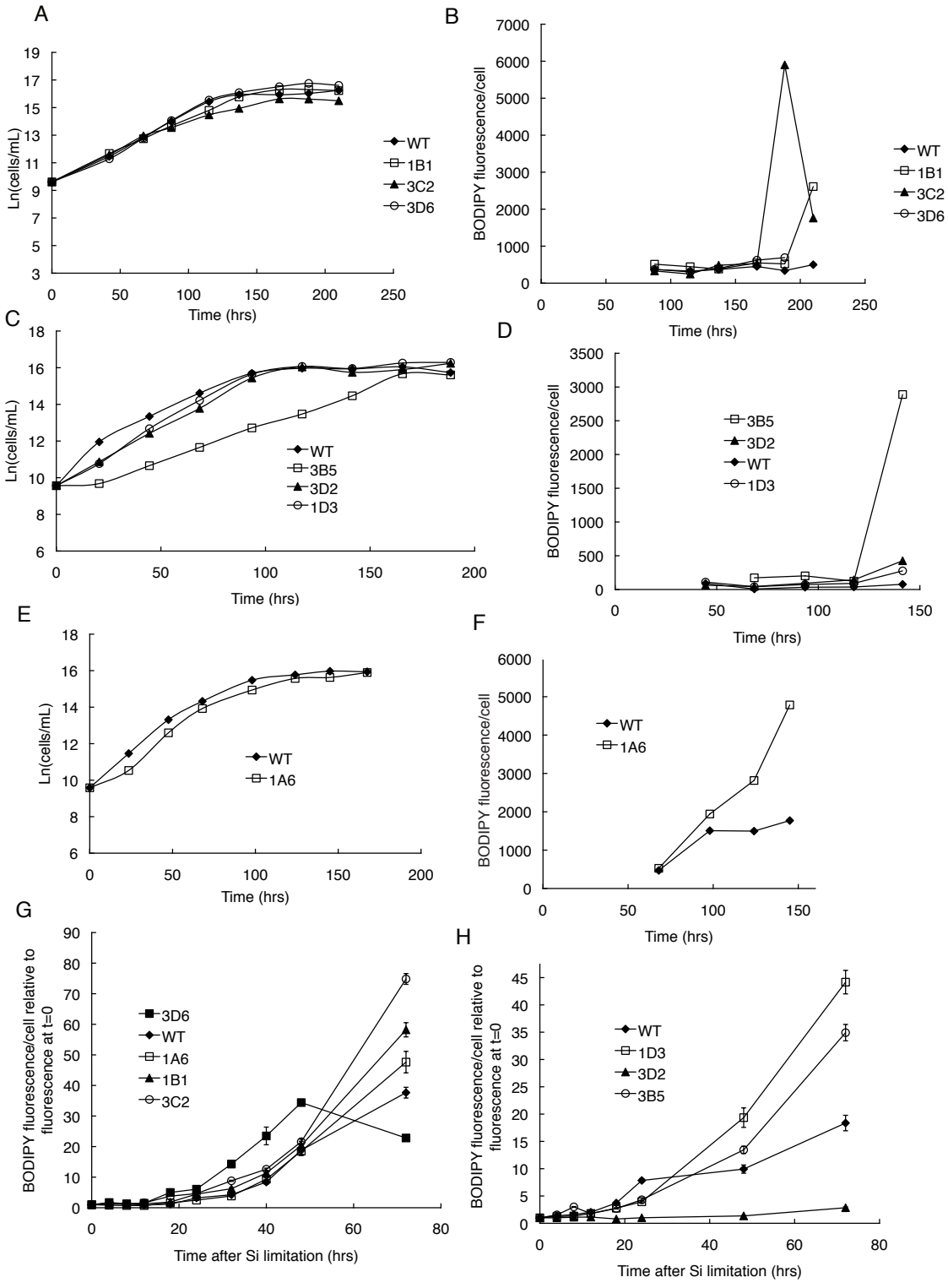
TGCCATCAAACTTTCCTCGTCCTGTAGAGTTTGTGTGTAATGAAGTTGCTGCATCCATTTCTGATTCTGTCAATTGCA

ATGTCAAGAGAGAAGTATTGAACGAGATGGGTGCATCTGCGTTCTTTAGAGGGTCTCGGTGGGATCGTCGTTTGC

AAAAGAAGGACGGAGAAGGCGTTAACAACGATTCGGTTGACGATATTGGATGCGATGAGAAGAAGATGGAGGA

GCAATTGGCTTGA

Figure 3-2. Screening of transformants for growth and lipid accumulation.
Initial screening of 3 antisense (1A6, 1B1, 1D3) and 4 RNAi (3B5, 3C2, 3D2, 3D6) knock-down strains for growth and TAG during nutrient replete (*A-F)* and nutrient deplete (*G,H*) conditions under continuous light. Growth was determined using cell density (*A, C, E)*. TAG was monitored using BODIPY fluorescence per cell as determined by imaging flow cytometry (*B, D, F, G, H)*. Error bars are SEM from imaging flow cytometry (n>100).


A
Ln(cells/mL)
Time (hrs)
WT
1B1
3C2
3D6
B
BODIPY fluorescence/cell
Time (hrs)
WT
1B1
3C2
3D6
C
Ln(cells/mL)
Time (hrs)
WT
3B5
3D2
1D3
D
BODIPY fluorescence/cell
Time (hrs)
3B5
3D2
WT
1D3
E
Ln(cells/mL)
Time (hrs)
WT
1A6
F
BODIPY fluorescence/cell
Time (hrs)
WT
1A6
G
BODIPY fluorescence/cell relative to fluorescence at t=0
Time after Si limitation (hrs)
3D6
WT
1A6
1B1
3C2
H
BODIPY fluorescence/cell relative to fluorescence at t=0
Time after Si limitation (hrs)
WT
1D3
3D2
3B5

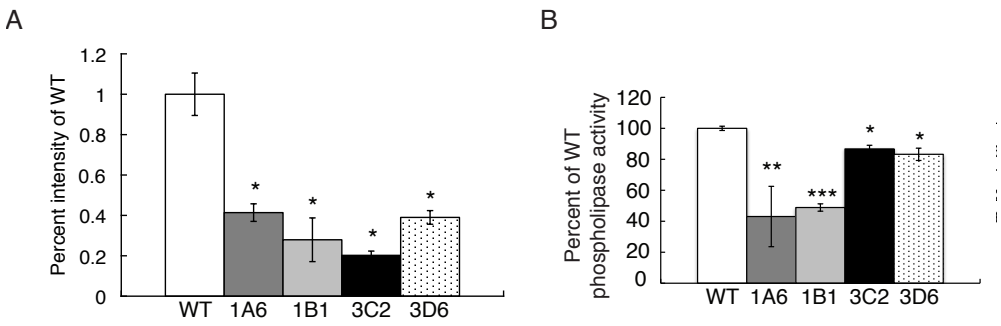


Figure 3-3. Confirmation of Thaps3_264297 knock-down.
(*A*) Relative intensities of Thaps3_264297 protein from immunoblots of transgenic strains compared to WT using anti-Thaps3_264297 antibodies. Blots were visualized with Bio-Rad's Chemidoc XRS+ System, band intensities quantified using Image Lab software, and normalized to β-tubulin (Santa Cruz Biotechnology) control (n = 3). (*B*) Functional enzyme assays of phospholipase activity in soluble cell lysates of WT and transgenic strains (n = 3). Error bars, SEM. Statistical analyses performed using student's t-test, *,$P < 0.05$.

3.2.2 Strains 1A6 and 1B1 show uncompromised growth

To assess the effects of Thaps3_264297 knock-down on growth and division, we analyzed growth rates and cell densities. Strains 1A6 and 1B1 grew comparably to WT, while strains 3C2 and 3D6 exhibited decreased growth with significantly slower doubling times and decreased cell density during stationary phase (Fig. 3-4A). We utilized the inducible promoter of strain 3C2's RNAi construct to determine that the decrease in growth in this strain is derived from expression of the construct, as the growth rate and cell density of 3C2 in repressive media are not appreciably different from WT (Fig. 3-4B). However, the growth defects of 3C2 and 3D6 do not correlate to the degree of Thaps3_264297 knock-down (Fig. 3-3A, B), indicating that secondary effects of integration, expression or processing of the RNAi construct may be occurring in these strains – a phenomenon often seen in plant transformations (Filipecki and Malepszy

2006). We also investigated the growth of 1A6 and 1B1 under a 12 hr:12 hr light:dark regime for 116 hours. Strains 1A6 and 1B1 grew comparably to WT under light:dark conditions (Fig. 3-4C), demonstrating similar growth rates to WT during both the light and dark periods (Fig. 3-4D).

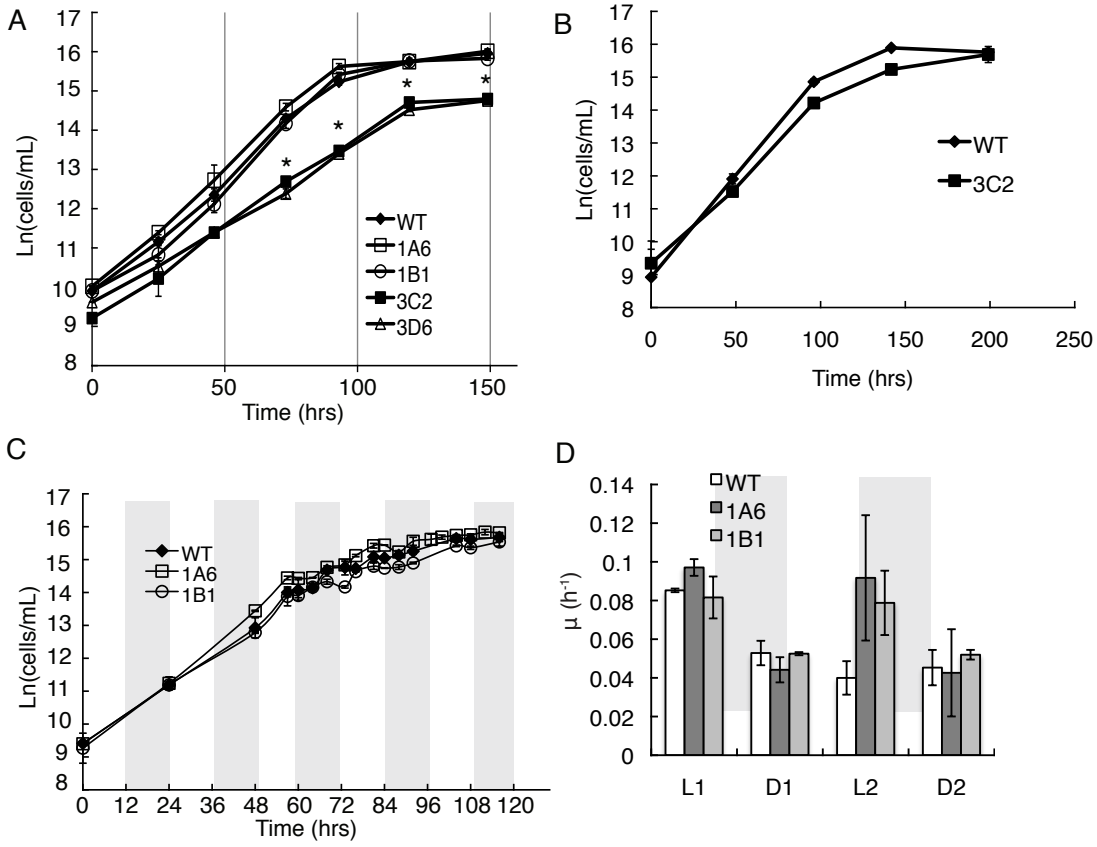
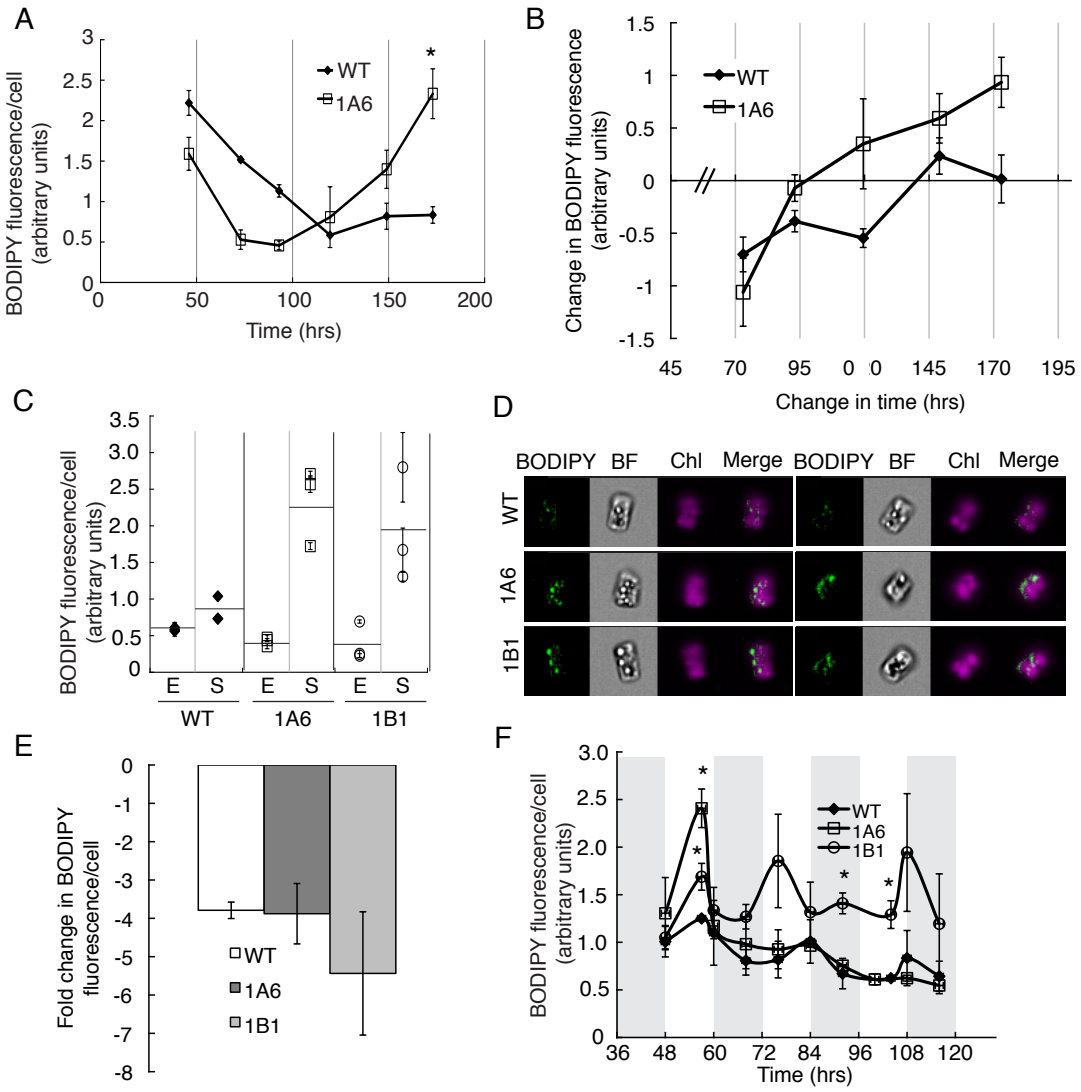


Figure 3-4. Growth analysis of knock-down strains.
(*A*) Growth of transgenic strains compared to WT as determined by cell density (n=3). (*B*) Growth of WT and strain 3C2 grown in repressive media (conditions under which knock-down construct is not expressed) as determined by cell density (n=3). Error bars, SEM. *(C)* Growth during 12 hr: 12 hr light:dark cycle as determined by cell density (n=2). Shaded regions denote dark periods. *(D)* Growth rate of transgenic strains compared to WT during light:dark cycle. Shaded regions denote dark periods. L1 = 1st light period, D1 = 1st dark period, etc.

3.2.3. 1A6 and 1B1 exhibit increased lipid accumulation

To determine if inhibition of Thaps3_264297 affected lipid accumulation, we monitored relative TAG levels in strains not showing compromised growth (1A6 and 1B1) with imaging flow cytometry using the fluorescent neutral lipid dye BODIPY as a proxy (Xu et al 2013). Under continuous light conditions, strain 1A6 began accumulating TAG one day earlier than WT, and at an accelerated rate (Fig. 3-5A, B). Strains 1A6 and 1B1 contained more TAG per cell in stationary phase than WT (Fig. 3-5C, D) despite similar depletion of TAG stores during exponential phase (Fig. 3-5E). Under a light:dark regime, both 1A6 and 1B1 were able to deplete TAG during dark periods similarly to WT, but both transgenic strains contained higher TAG than WT upon entering the first dark period (Fig. 3-5F). In the latter light periods, WT and 1A6 accumulated less TAG than they depleted, whereas 1B1 continued to accumulate and deplete TAG to consistent levels.

Figure 3-5. Analysis of lipid accumulation in knock-down strains during nutrient replete conditions.
(*A*) Increased rate, onset and extent of TAG accumulation in 1A6 over WT during nutrient-replete growth and stationary phase as determined by BODIPY fluorescence as a proxy for TAG (n=3). (*B*) Change in BODIPY fluorescence between time-points in WT and 1A6 during nutrient replete growth and stationary phase (n=3). X-axis depicts change in time since inoculation. (*C*) Increased BODIPY fluorescence and TAG accumulation of 1A6 and 1B1 over WT in stationary phase. E = exponential phase; S = stationary phase. Error bars are SEM from imaging flow cytometry (n>100). Horizontal lines depict average of 3 graphed populations. (*D*) Imaging flow cytometry images of representative cells selected because their BODIPY fluorescence per cell was the average of the population for three replicates of WT, 1A6 and 1B1 in stationary phase. BODIPY and chlorophyll fluorescence (Chl) are both shown. (*E*) Depletion of lipids in WT, 1A6 and 1B1 during exponential phase depicted as fold change in BODIPY fluorescence compared to initial time point (n=3). (*F*) BODIPY fluorescence of WT, 1A6 and 1B1 during 12:12 light:dark cycling. Error bars, SEM. Error bars, SEM. Statistical analyses performed using student's t-test, *,$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


A
BODIPY fluorescence/cell (arbitrary units)
Time (hrs)
WT
1A6
*
B
Change in BODIPY fluorescence (arbitrary units)
Change in time (hrs)
WT
1A6
C
BODIPY fluorescence/cell (arbitrary units)
E S E S E S
WT 1A6 1B1
D
BODIPY BF Chl Merge BODIPY BF Chl Merge
WT
1A6
1B1
E
Fold change in BODIPY fluorescence/cell
WT
1A6
1B1
F
BODIPY fluorescence/cell (arbitrary units)
Time (hrs)
WT
1A6
1B1
*

WT *T. pseudonana* accumulates abundant TAG under silicon-limited conditions (Yu et al 2009). To test the effect of Thaps3_264297 knock-down on nutrient starvation-induced TAG accumulation, we compared WT with the transgenic strains 1A6 and 1B1 after transfer into silicon-limited media to induce cell cycle arrest and lipid accumulation. Relative TAG levels were determined and lipid droplet formation was monitored over a time course of 72 hours using imaging flow cytometry of cells stained with BODIPY. Strains 1A6 and 1B1 showed significantly increased TAG accumulation per cell compared to WT within 40 hours of silicon starvation (Fig. 3-6A, B), and continued to accumulate lipids at a faster rate than WT through 72 hours. A high-resolution time series revealed increased lipid accumulation in 1B1 as early as 24 hours after silicon starvation (Fig. 3-6C). At 72 hours lipid droplets of 1A6 and 1B1 were larger and more pronounced (Fig. 3-6D).

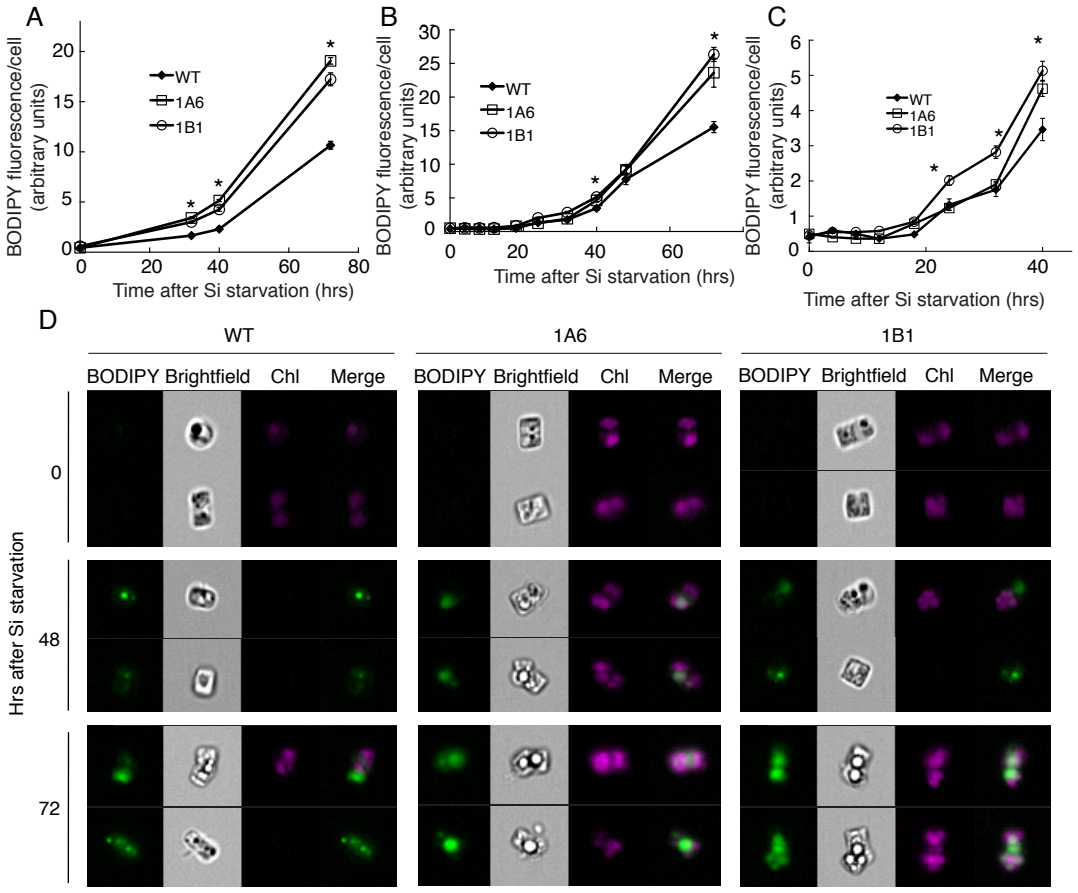


Figure 3-6. Increased TAG accumulation in 1A6 and 1B1 during silicon starvation. (*A*) Increased BODIPY fluorescence in 1A6 and 1B1 over WT during silicon starvation-induced lipid accumulation. (*B*) A high-resolution time series of TAG accumulation in WT, 1A6 and 1B1 during silicon starvation. Error bars are SEM from imaging flow cytometry (n>100). Statistical analyses performed using student t-test, *, $P < 0.05$ and signifies both 1A6 and 1B1 fluorescence intensities are significantly higher than WT. (*C*) Increased BODIPY fluorescence in 1A6 and 1B1 over WT during silicon starvation-induced lipid accumulation. Error bars are SEM from imaging flow cytometry (n>100). Statistical analyses performed using student t-test, *, $P < 0.05$. (*D*) Imaging flow cytometry images of representative cells depicting average BODIPY fluorescence of the population for WT, 1A6 and 1B1 at 0, 48 and 72 hours of silicon starvation. BODIPY and chlorophyll fluorescence (Chl) are both shown.

To compare levels of biofuel-relevant lipids between WT and transgenic strains, total lipids from 1A6, 1B1 and WT were extracted after 0 hours of silicon starvation during exponential growth, and after 40 hours of silicon starvation. During exponential growth 1A6 and 1B1 contained significantly higher TAG and total lipid content than WT (Fig. 3-7A, B). Because growth is not affected in 1A6 and 1B1, overall lipid yields increased (Table 3-1). An increase in biomass was also observed in 1A6 and 1B1 (Table 3-1). After 40 hours of silicon starvation 1A6 and 1B1 contained significantly more TAG and total lipid that WT (Fig. 3-7A, B, C). TAG content corresponded to BODIPY fluorescence (Fig. 3-7D), suggesting that many of the originally screened transgenic strains also contained increased TAG over WT after silicon starvation.

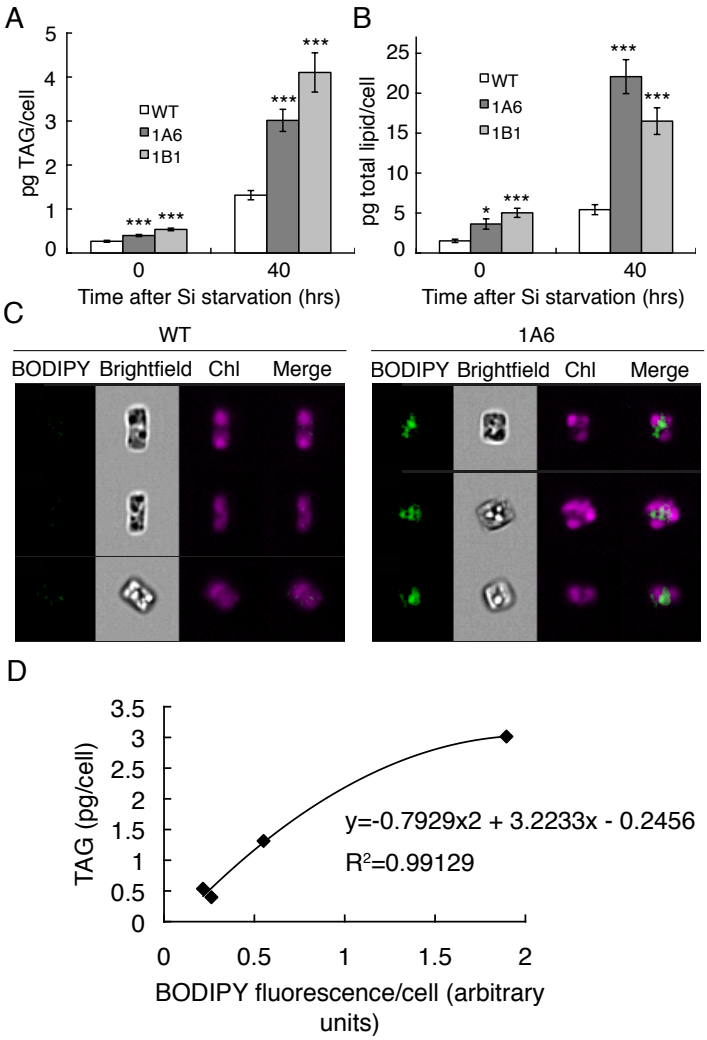


Figure 3-7. Quantification of TAG accumulation in 1A6 and 1B1 during silicon starvation.
(*A*) Quantification of pg of TAG per cell in WT, 1A6 and 1B1 at 0 and 40 hours after silicon starvation (n=8). *(B)* Quantification of pg of total lipid per cell in WT, 1A6 and 1B1 at 0 and 40 hours after silicon starvation (n=8). *(C)* Imaging flow cytometry images of representative cells depicting average BODIPY fluorescence of the population for WT and 1A6 after 40 hours of silicon starvation. BODIPY and chlorophyll fluorescence (Chl) are both shown. *(D)* BODIPY fluorescence per cell as determined by imaging flow cytometry correlated to TAG content (pg/cell) for 1A6, 1B1 and WT samples. Error bars, SEM. Statistical analyses performed using student t-test, *, $P < 0.05$, ***, $P < 0.001$.

Table 3-1. TAG accumulation during exponential phase and nutrient deprivation.

	WT	1A6	1B1
Biomass (mg/L)	9.98	20.1	26.7
Lipid yield (mg/L)	1.52	3.63	5.03

After 0 and 40 hours of silicon starvation, complex lipid species were also extracted from 1A6 and WT and converted to FAMEs for analysis. Fatty acid profiling revealed quantities of most of the FAME species detected were increased in 1A6, but their relative abundances differed slightly in that levels of 16:0 and 18:2 fatty acids were particularly enriched after 40 hours (Fig. 3-8).

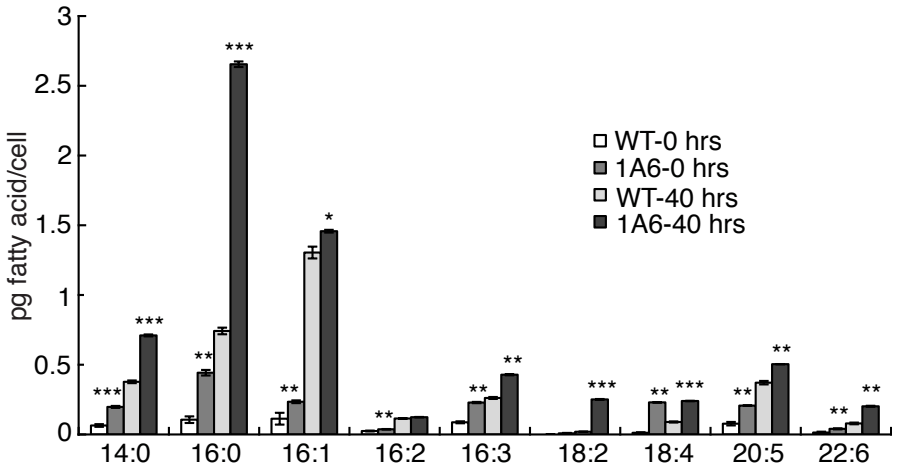


Figure 3-8. FAME-profiling of 1A6 and WT during silicon starvation.
Quantification of individual fatty acid species of 1A6 and WT after 0 and 40 hours of silicton starvation (n=2). Error bars, SEM. Statistical analyses performed using student t-test, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

3.2.4 Analysis of membrane lipids during lipid accumulation

Initial visual observations of silicon-limited cells using imaging flow cytometry revealed increased cellular debris of WT cells over 72 hours indicating their degradation – an observation not seen in 1A6 and some other transgenic strains (Fig. 3-9). Because CGI-58 exhibits phospholipase and acyltransferase activity, we investigated the role of Thaps3_264297 in membrane turnover and the effect of Thaps3_264297 knock-down on cellular membranes. To determine if the visual degradation of cells correlated to damaged or compromised membranes, the nucleic acid stain SYTOX Orange, which passes more easily through compromised membranes, was used to assess relative membrane integrity between WT, 1A6 and 1B1 (Gerken et al 2013). Under silicon-limited conditions, typically 80% of *T. pseudonana* cells are arrested in G1 phase (Hildebrand et al 2007) and contain similar DNA levels, thus differences in SYTOX fluorescence are not attributable to differences in DNA quantity. 1A6, 1B1 and WT were subjected to silicon starvation and analyzed after 8 hours (when cell cycle arrest occurs and lipid accumulation begins) and 48 hours for SYTOX Orange fluorescence using imaging flow cytometry. While both strains showed increased SYTOX Orange fluorescence over time, revealing increased membrane degradation throughout silicon starvation, the WT cells showed significantly more fluorescence per cell relative to 1A6 and 1B1 at both time points, thus indicating less membrane degradation of 1A6 and 1B1 cells (Fig. 3-10A, B). To assess the correlation between SYTOX staining and membrane lipid content, polar lipids were quantified after 40 hours of silicon starvation in WT and strains 1A6 and 1B1. Polar lipid content was 4.6-times higher in 1A6 and 3.2-times higher in 1B1 over WT

after 40 hours, suggesting that polar lipid turnover was also reduced in response to knock-down of Thaps3_264297 (Fig. 3-11).

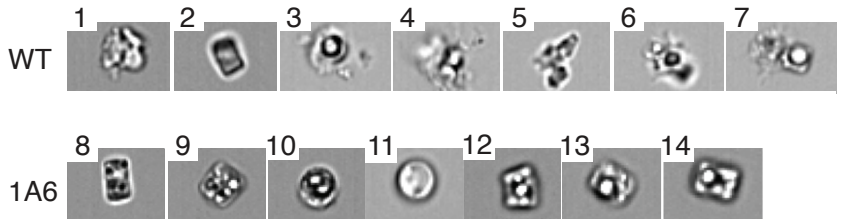


Figure 3-9. Increased intactness of transgenic strains during silicon limitation. Images represent randomly selected cells of WT and 1A6 after 48 hours (cells 1-2 and 8-10) and 72 hours (cells 4-7 and 11-14) of silicon starvation. Cells of 1A6 seem to be more intact overall, whereas the majority of WT cells are either partially (cells 3, 6, 7) or completely (cells 1, 4, 5) degraded.

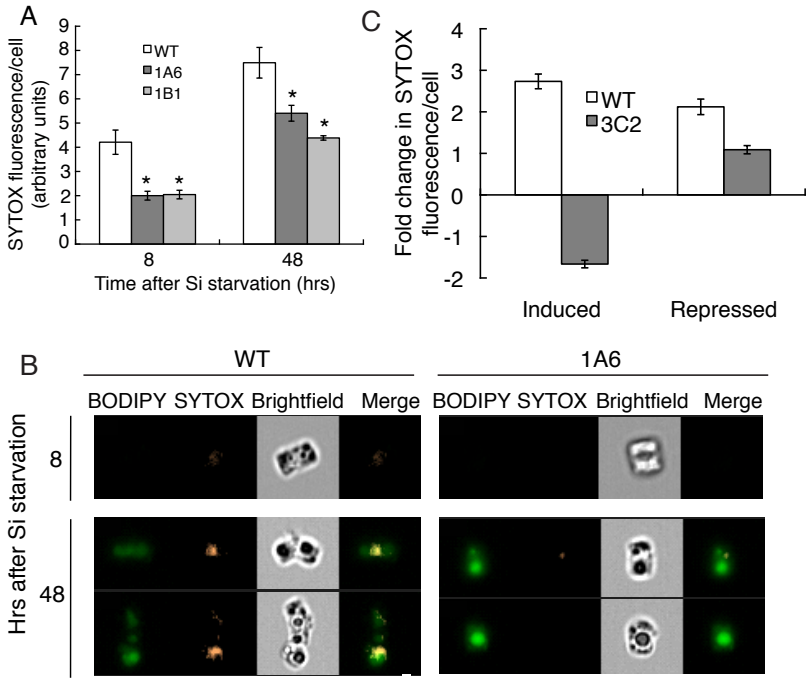


Figure 3-10. Increased membrane intactness during silicon starvation.
(*A*) SYTOX fluorescence per cell in WT, 1A6 and 1B1 at 8 and 40 hours of silicon starvation. Error bars are SEM from imaging flow cytometry (n>100). (*B*) Representative images of cells of 1A6 and WT after 8 and 48 hours of silicon starvation exhibiting the average SYTOX fluorescence of the population for each strain. (*C*) Decreased membrane permeability is correlated to expression of the knock-down construct. Fold change in SYTOX fluorescence after 48 hours of silicon starvation in WT and strain 3C2 when RNAi construct is under induced or repressed conditions.

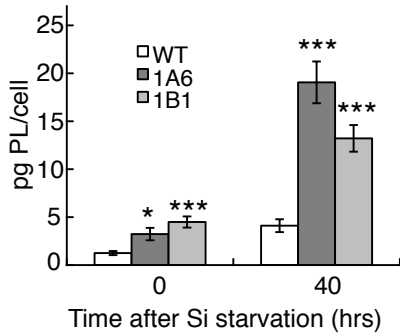


Figure 3-11. Increased polar lipid levels during silicon starvation.
Quantification of pg of polar lipids (PL) per cell in WT, 1A6 and 1B1 at 0 and 40 hours of silicon starvation (n=8). Error bars, SEM. Statistical analyses performed by student's t-test, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

We utilized the inducible promoter of the RNAi construct of 3C2 to further confirm that decreased membrane degradation was a consequence of Thaps3_264297 knock-down. When silicon starvation was carried out under conditions that enabled expression of the RNAi construct, SYTOX fluorescence decreased in 3C2 and increased in WT during the 48 hours of silicon starvation. Conversely, when silicon starvation was carried out in repressive media, 3C2 showed an increase in SYTOX fluorescence, indicative of an increase in membrane degradation. (Fig. 3-10C).

3.3 Discussion

Metabolic engineering of eukaryotic microalgae to develop improved lipid production strains for algal biofuels has become increasingly feasible with advancements in the understanding of lipid metabolism and the manipulation of microalgal metabolic pathways (Radakovits et al 2010). Only a few attempts have been made to engineer lipid

metabolism in microalgae, and to our knowledge, no targeted manipulation to date has significantly increased lipid yields without simultaneously decreasing growth. While these manipulations have had little success in increasing overall lipid yields, they have shed light on lipid pathway regulation (Moellering and Benning 2010, Radakovits et al 2011, Sheehan et al 1998). We hypothesized that disrupting lipid catabolism could increase lipid accumulation without negatively affecting growth. Lipid catabolism involves the release of free fatty acids by lipases and the subsequent breakdown of these fatty acids through β-oxidation. It has been ignored as a relevant pathway for targeted manipulation, perhaps because extensive knockout studies in *A. thaliana*, which primarily focused on seed oil mobilization, have resulted in numerous mutants of lipid catabolism with impaired growth phenotypes (Graham 2008). However, because seedling establishment and growth is dependent on lipolysis of stored lipids until the photosynthetic apparatus is formed, one cannot extrapolate these results to microalgae, which go through neither seed nor germination stages.

Although previous transcriptomic analyses in other microalgae found variable expression of annotated lipases during lipid accumulation (Miller et al 2010), we expanded our study to include all enzymes containing a lipase motif, such as some hydrolases, and identified a small number of down-regulated enzymes in *T. pseudonana*, including Thaps3_264297 (Fig. 2-9). Thaps3_264297 is homologous to human CGI-58, which has been shown to be associated with lipid droplets and their breakdown (Yamaguchi and Osumi 2009), and to be involved in the transfer of acyl groups between storage lipids and phospholipids (Montero-Moran et al 2010). We found Thaps3_264297 to exhibit lipase, phospholipase and lysophosphatidic acyltransferase activities (Fig. 2-

11), which have also been shown for At4g24160 and Ict1p, CGI-58 homologs in *A. thaliana* and *S. cerevisiae*, respectively (Ghosh et al 2008, Ghosh et al 2009). These activities correspond to the conserved catalytic domains of these enzymes (Fig. 2-10) and indicate their roles in lipid turnover.

Mutation of CGI-58 in humans causes Chanarin-Dorfman syndrome, a neutral lipid storage disease characterized by excessive accumulation of lipid droplets in cells of various tissues (Yamaguchi and Osumi 2009). Knockout of the CGI-58 homolog in *A. thaliana* resulted in a Chanarin-Dorfman-like excessive accumulation of lipid droplets in the leaves of the mutant plants; interestingly, these mutant plants did not show any defects in growth or reproduction, suggesting that the CGI-58 homolog was not involved in TAG mobilization of seeds, but rather in maintaining lipid homeostasis in vegetative cells (James et al 2010). We have similarly shown that knock-down of the CGI-58 homolog in *T. pseudonana* results in increased accumulation of TAG droplets, total lipid production, and lipid yields without negatively affecting growth, cell division or biomass under continuous light and light:dark cycling (Fig. 3-4, 3-5, 3-6, 3-7; Table 3-1). While increasing lipid yields is important for economic feasibility, maintaining high growth rates is also imperative for reducing cost of production (Borowitzka 1992). Additionally, biomass accumulation is a critical factor in current extraction regimes (López Barreiro et al 2013), and growth throughout light:dark cycles is necessary for outdoor production. The conserved role of this enzyme across taxa implicates that our methods are applicable to increase lipid yields from other biofuel production strains as well.

We found that during growth, knock-down strains depleted TAG to a similar level as WT and depleted TAG during dark periods, but contained more TAG throughout

exponential and stationary phase (Fig. 3-5, 3-7). These results suggest that Thaps3_264297 may not be involved in mobilizing TAG for rapid growth, but rather, like *A. thaliana*'s homolog, in lipid homeostasis or remodeling, therefore allowing the knock-down to grow similarly to WT. This suggests diverse and independent functions for distinct TAG lipases, which is consistent with the variable expression levels of lipases seen in transcriptomic analyses, and demonstrates the importance of such analyses in guiding targeted manipulations. We have shown that knock-down of the CGI-58 homolog in *T. pseudonana* also results in increased polar lipid levels and membrane integrity during silicon starvation (Fig. 3-9, 3-10, 3-11), indicating a role for this enzyme in membrane turnover during nutrient starvation-induced lipid accumulation and is beneficial to conversion processes such as hydrothermal liquefaction. Although these conversion processes can convert whole cell biomass to biocrude, it has been shown that high lipid content results in the highest biocrude yields (Biller and Ross 2011, Sawayama et al 1999). Knockout of the CGI-58 homolog in *A. thaliana* similarly resulted in an increase in polar lipids (James et al 2010). It has recently been shown that although 60% of the TAG accumulated under nutrient-limited conditions in the diatom *Phaeodactylum tricornutum* is derived from de novo lipid biosynthesis, substantial turnover of phospholipids also occurs (Burrows et al 2012). A secondary route to TAG biosynthesis has also been proposed involving intracellular membrane turnover by the enzyme PDAT (Boyle et al 2012, Yoon et al 2012). Our SYTOX staining results indicate that plasma membrane phospholipids may also be a source of acyl groups for TAG biosynthesis in WT, and while lipid remodeling may normally occur during accumulation, it is disrupted by knock-down of Thaps3_264297.

Lipid pools have been increasingly recognized as dynamic entities with many functions beyond long-term energy storage. Lipid droplets, for example, are known to associate with other organelles (Goodman 2008) and to distribute neutral lipids, phospholipids, lysophospholipids and acyl groups throughout the cell and between TAG and membranes (Zehmer et al 2009). Our results indicate that the multifunctional lipase, phospholipase and acyltransferase activities of Thaps3_264297 support a role in maintaining and modifying cellular lipid pools by interacting with these different lipid species. Although disruption of this process does not compromise growth, it drastically affects levels of internal lipid pools, as well as abundances of individual fatty acids (Fig. 3-8). Buildup of TAG molecules from decreased lipase activity could contribute to the overabundance of 16:0 and 18:2 fatty acids in strain 1A6 after lipid accumulation. Palmitic acid is a prevalent species in *T. pseudonana* netural lipids (Yu et al 2009). Substrate specificity of Thaps3_264297 and the increase in polar lipids in this strain could also contribute to the overabundance of 16:0 and 18:2 seen in 1A6 compared to WT. Linoleic acid is derived from and contributes to to the phospholipid pool, especially phosphatidylcholine (PC) and phosphatidylglycerol (PG) (Guschina and Harwood 2006). A skewed distribution of this particular fatty acid in the knock-down strain further suggests a role for Thaps3_264297 in maintaining neutral and polar lipid balance. Analysis of fatty acid species in individual lipid pools may give insights into the mechanisms behind the skewed fatty acid profile of 1A6.

Our results indicate that disrupting lipid catabolism can be used to produce strains with favorable characteristics for biofuel production. We have shown that knock-down strains yield more lipid than WT under multiple production schemes including

continuous growth as well as nutrient limitation conditions. Although it was not possible for us to assess the performance of the transgenic lines under authentic production conditions, our ability to mimic light:dark conditions supported the concept that at least some of the benefits we documented under controlled laboratory conditions could translate into a production system.

As the ability to engineer algal strains for their fuel molecules progresses, the elucidation of lipid metabolism and its connected pathways becomes increasingly important and relevant. A deeper understanding of carbon flux throughout the full range of cellular processes is necessary to optimize both growth and lipid accumulation in microalgal cultures. Our results demonstrate that lipid catabolism is an integral part of the metabolic processing of lipids throughout cell growth as well as during nutrient starvation, and is intimately involved in both lipid homeostasis and accumulation. We have shown that engineering this pathway is a unique and practical approach for increasing lipid yields from eukaryotic microalgae without compromising growth.

3.4 Materials & Methods

3.4.1 Strains and culture conditions

T. pseudonana was grown in batch culture with shaking or stirring and aeration under either continuous illumination or a 12 hr: 12 hr light:dark cycle at 150 μmol m^{-2} s^{-1} at 18 to 20°C in sterile artificial seawater medium (ASW) supplemented with biotin and vitamin B12 at 1 ng L^{-1} (Darley and Volcani 1969). Repressive media contained ammonia as the nitrogen source instead of nitrate (Poulsen et al 2006). To induce silicon starvation, cultures were grown to a mid-log phase density of $2x10^6$ cells/mL in ASW,

pelleted and washed twice with silicon-free ASW, then inoculated at $1x10^6$ cells/mL into silicon-free ASW in polycarbonate bottles. Silicon starvation experiments were carried out under continuous illumination.

3.4.2 Vectors, expression, and protein purification

Knock-down vectors pA78cgi and pI1001cgi were constructed using Invitrogen's Multi-Site Gateway Cloning Protocol and transformed into *T. pseudonana* using microparticle bombardment with a BioRad PDS-1000 using established procedures (Dunahay et al 1995), and the presence of integrated constructs was confimed using PCR. Details of vector construction are given in Fig. S3. Polyclonal antibodies against Thaps3_264297 were raised in rabbits using purified, soluble recombinant Thaps3_264297 (Pro-Sci, Inc., Carlsbad, CA). Soluble proteins were isolated and purified from *E. coli* using Ni-NTA resin as described in the Qiagen Expression Handbook. Soluble protein was isolated from *T. pseudonana* by boiling with Laemmli buffer and centrifuging at 4^oC at 10,000xg for 20 min. Proteins were separated using SDS-PAGE and gels visualized under UV (Nusep). Western blots using Thermo Pierce mouse 6XHis primary anti-His tag antibody and Thermo Pierce Goat anti-mouse horseradish peroxidase-conjugate secondary antibody were used to detect recombinant protein. Polyclonal antibodies were used to probe *T. pseudonana* lysates with goat anti-rabbit horseradish peroxidase-conjugate secondary antibodies. Immunoblots were detected and quantified using ChemiDoc XRS+ System and Image Lab software (Bio-Rad).

3.4.3 Enzymatic activity assays

Lipase activity was determined using the Quantichrom Lipase Assay Kit (Bioassay Systems), using purified *Candida albicans* lipase (Invitrogen) to generate a standard curve, and purified alcohol dehydrogenase (Invitrogen) as a negative control. Phospholipase activity was determined using the EnzCheck Phospholipase A2 Assay (Life Technologies). Lysophosphatidic acid acyltransferase activity assays contained 20 μM oleoyl-CoA (VWR), 100 μM lysophosphatidic acid (Caymen Chemical), and 20 μg cell lysate in reaction buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) and were carried out at room temperature for 30 min and terminated by extracting lipids according to Bligh & Dyer (Bligh and Dyer 1959). Lipids were analyzed by TLC using chloroform:methanol:water (65:25:4) and visualized under UV (302nm) after staining with primuline. Spots were quantified using the ChemiDoc XRS+ System and Image Lab software (Bio-Rad). Di-oleoyl phosphatidic acid (Sigma) was used as a standard for the reaction product.

3.4.4 Imaging flow cytometry

Imaging flow cytometry data were collected on an Amnis ImageStream^X at 60X magnification. For SYTOX staining, frozen and fresh cell pellets were tested and found to differ only slightly in response, an order of magnitude less than the relative differences between strains or timepoints, thus it was determined that frozen pellets could be used for SYTOX analysis. Frozen cell pellets were resuspended in 2.3% NaCl solution and stained with either 2.3 μg/mL BODIPY for 15 min or 250 nM SYTOX Orange for 20 min, excited with a 488 nm laser at 10mW, and brightfield and fluorescent images were

collected for between 10,000-20,000 events. Amnis IDEAS 4.0 Software was used to analyze raw image files. Cutoffs for in-focus and single cells were determined manually, and images were screened to remove those of debris.

3.4.5 Lipid analysis

For lipid analysis, cultures (1L, ~1x10^6 cells/mL) were centrifuged and rinsed with 0.4 M ammonium formate. Cells were lyophilized, placed under N_2 (g) atmosphere and stored at -80°C until analysis. Lipids were extracted following the method of Folch et al (Folch et al 1957). Fatty acids were determined by transmethylation using 14% boron trifluoride/methanol at 70°C (Christie 2003). The concentrations of FAMEs were determined with a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (GC/MS-FID). FAMEs were identified by comparison of retention times to authentic lipid standards (68A and 68D, Nu-Check Prep, USA). Quantification of C14 to C18 and C20 to C24 fatty acids was performed relative to known concentrations and FID peak areas of C13 and C19 FAME internal standards, respectively. The fatty acid methylation reaction efficiency was determined from the C15 FA internal standard added to each lyophilized algal pellet.

Lipid class composition was determined using the method of Carilli et al. (Carilli et al 2012). Briefly, crude lipid extracts were fully dried under nitrogen gas, re-suspended in 500 μL of chloroform and 1 μL immediately spotted on silica-coated quartz Chromarods (S-III, Iatron Laboratories, Inc.). After spotting, the crude lipid extracts were separated into lipid classes by first developing for 25 minutes in hexane:diethyl ether:acetic acid (99:1:0.05) (v:v:v), drying at ~100^{o}C for four minutes, then developing

in hexane:diethyl ether:acetic acid (80:20:0.1) (v:v:v) for 25 minutes and drying again. Chromatograms were generated for each rod using flame ionization detection (FID) via an Iatroscan TLC-FID MK-5 (Iatron Laboratories, Inc.) and LabView software (National Instruments). The concentration of each lipid class was calculated using retention times of known standards.

3.5 Acknowledgements

Thanks to L. Gerwick and N. Engene (UCSD) for technical advice. ET is supported by NIH Marine Biotechnology Training Grant Fellowship 5T32GM067550. We gratefully acknowledge support by the California Energy Commission's (CEC) "California Initiative for Large Molecule Sustainable Fuels", Agreement Number: 500-10-039. Additional support to MH from the Air Force Office of Scientific Research (AFOSR) grants FA9550-08-1-0178 and FA9550-08-1-0178, US DOE grants DE-EE0001222 and DE-EE0003373, and NSF grant CBET-0903712 is acknowledged. This report does not necessarily represent the views of the CEC, its employees, or the State of California.

Chapter 3, in full, is included in The Proceedings of the National Academy of Sciences 2013, 110 (49): 19748-19753. Trentacoste, E. M., Shrestha, R. P., Smith, S. R., Glé, C., Hartmann, A. C., Hildebrand, M., & Gerwick, W. H. The dissertation author was the primary investigator and author of this paper.

3.6 References

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsar DS (2004). The Genome of the Diatom Thalassiosira Pseudonana: Ecology, Evolution, and Metabolism. *Science* **306:** 79-86.

Biller P, Ross AB (2011). Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. *Bioresource Technology* **102:** 215-225.

Bligh E, Dyer WJ (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37:** 911-917.

Borowitzka M (1992). Algal biotechnology products and processes — matching science and economics. *Journal of Applied Phycology* **4:** 267-279.

Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ, Gallaher SD, Johnson S, Benning C, Pellegrini M, Grossman A, Merchant SS (2012). Three Acyltransferases and Nitrogen-responsive Regulator Are Implicated in Nitrogen Starvation-induced Triacylglycerol Accumulation in Chlamydomonas. *Journal of Biological Chemistry* **287:** 15811-15825.

Burrows E, Bennette N, Carrieri D, Dixon J, Brinker A, Frada M, Baldassano SN, Falkowski PG, Dismukes GC (2012). Dynamics of Lipid Biosynthesis and Redistribution in the Marine Diatom Phaeodactylum tricornutum Under Nitrate Deprivation. *BioEnergy Research* **5:** 876-885.

Carilli J, Donner SD, Hartmann AC (2012). Historical temperature variability affects coral response to heat stress. *PloS one* **7:** e34418.

Chauton MS, Winge P, Brembu T, Vadstein O, Bones AM (2013). Gene Regulation of Carbon Fixation, Storage, and Utilization in the Diatom Phaeodactylum tricornutum Acclimated to Light/Dark Cycles. *Plant Physiology* **161:** 1034-1048.

Christie WW (2003). *Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids*. Amer Oil Chemists Society.

Coates R, Trentacoste E, Gerwick WH (2013). Bioactive and Novel Chemicals from Microalgae. *Handbook of Microalgal Culture*. John Wiley & Sons, Ltd. pp 504-531.

Darley WM, Volcani BE (1969). Role of silicon in diatom metabolism: A silicon requirement for deoxyribonucleic acid synthesis in the diatom Cylindrotheca fusiformis Reimann and Lewin. *Experimental Cell Research* **58:** 334-342.

Davis R, Aden A, Pienkos PT (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy* **88:** 3524-3531.

De Riso V, Raniello R, Maumus F, Rogato A, Bowler C, Falciatore A (2009). Gene silencing in the marine diatom Phaeodactylum tricornutum. *Nucleic Acids Research* **37:** e96-e96.

Dunahay TG, Jarvis EE, Roessler PG (1995). Genetic transformation of the diatoms Cyclotella cryptica and Navicula saprophila. *Journal of* Phycology **31:** 1004-1012.

Filipecki M, Malepszy S (2006). Unintended consequences of plant transformation: A molecular insight. *Journal of Applied Genetics* **47:** 277-286.

Folch J, Lees M, Sloane-Stanley G (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226:** 497-509.

Gerken H, Donohoe B, Knoshaug E (2013). Enzymatic cell wall degradation of Chlorella vulgaris and other microalgae for biofuels production. *Planta* **237:** 239-253.

Ghosh AK, Ramakrishnan G, Rajasekharan R (2008). YLR099C (ICT1) Encodes a Soluble Acyl-CoA-dependent Lysophosphatidic Acid Acyltransferase Responsible for Enhanced Phospholipid Synthesis on Organic Solvent Stress in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **283:** 9768-9775.

Ghosh AK, Chauhan N, Rajakumari S, Daum G, Rajasekharan R (2009). At4g24160, a Soluble Acyl-Coenzyme A-Dependent Lysophosphatidic Acid Acyltransferase. *Plant Physiology* **151:** 869-881.

Goodman JM (2008). The Gregarious Lipid Droplet. *Journal of Biological Chemistry* **283:** 28005-28009.

Graham IA (2008). Seed Storage Oil Mobilization. *Annual Review of Plant Biology* **59:** 115-142.

Guschina IA, Harwood JL (2006). Lipids and lipid metabolism in eukaryotic algae **45:** 160-186.

Harwood JL, Guschina IA (2009). The versatility of algae and their lipid metabolism. *Biochimie* **91:** 679-684.

Hildebrand M, Frigeri LG, Davis AK (2007). Synchronized growth of Thalassiosira pseudonana (Bacillariophyceae) provides novel insights into cell wall synthesis processes in relation to the cell cycle. *Journal of Phycology* **43:** 730-740.

Hildebrand M, Davis AK, Smith SR, Traller JC, Abbriano R (2012). The place of diatoms in the biofuels industry. *Biofuels* **3:** 221-240.

James CN, Horn PJ, Case CR, Gidda SK, Zhang D, Mullen RT, Dyer JM, Anderson RGW, Chapman KD (2010). Disruption of the Arabidopsis CGI-58 homologue produces Chanarin–Dorfman-like lipid droplet accumulation in plants. *Proceedings of the National Academy of Sciences* **107:** 17833-17838.

Kainz M, Brett MT, Arts MT, Guschina I, Harwood J (2009). Algal lipids and effect of the environment on their biochemistry. *Lipids in Aquatic Ecosystems*. Springer New York. pp 1-24.

Li Y, Han D, Hu G, Sommerfeld M, Hu Q (2010). Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii. *Biotechnolgoy and Bioengineering* **107:** 258-268.

Li Y, Han D, Sommerfeld M, Hu Q (2011). Photosynthetic carbon partitioning and lipid production in the oleaginous microalga Pseudochlorococcum sp. (Chlorophyceae) under nitrogen-limited conditions. *Bioresource Technology* **102:** 123-129.

López Barreiro D, Prins W, Ronsse F, Brilman W (2013). Hydrothermal liquefaction (HTL) of microalgae for biofuel production: State of the art review and future prospects. *Biomass and Bioenergy* **53:** 113-127.

Miller R, Wu G, Deshpande RR, Vieler A, Gärtner K, Li X, Moellering ER, Zäuner S, Cornish AJ, Liu B, Bullard B, Sears BB, Kuo M, Hegg EL, Shachar-Hill Y, Shiu S, Benning C (2010). Changes in Transcript Abundance in Chlamydomonas reinhardtii following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology* **154:** 1737-1752

Moellering ER, Benning C (2010). RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in Chlamydomonas reinhardtii. *Eukaryotic Cell* **9:** 97-106.

Montero-Moran G, Caviglia JM, McMahon D, Rothenberg A, Subramanian V, Xu Z, Lara-Gonzalez S, Storch J, Carman GM, Brasaemle DL (2010). CGI-58/ABHD5 is a coenzyme A-dependent lysophosphatidic acid acyltransferase. *Journal of Lipid Research* **51:** 709-719.

Pienkos PT, Darzins A (2009). The promise and challenges of microalgal-derived biofuels. *Biofuelds, Bioproducts and Biorefining* **3:** 431-440.

Poulsen N, Chesley PM, Kröger N (2006). Molecular genetic manipulation of hteh diatom Thalassiosira pseudonana (Bacillariophyceae). *Journal of Phycology* **42:** 1059-1065.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010). Genetic Engineering of Algae for Enhanced Biofuel Production. *Eukaryotic Cell* **9:** 486-501.

Radakovits R, Eduafo PM, Posewitz MC (2011). Genetic engineering of fatty acid chain length in Phaeodactylum tricornutum. *Metabolic Engineering* **13:** 89-95.

Sawayama S, Minowa T, Yokoyama SY (1999). Possibility of renewable energy production and CO2 mitigation by thermochemical liquefaction of microalgae. *Biomass and Bioenergy* **17:** 33-39.

Sharif Hossain ABM, Salleh A, Boyce AN, Chowdhury P, Naqiuddin M (2008). Biodiesel Fuel Production from Algae as Renewable Energy. *American Journal of Biochemistry and Biotechnology* **4:** 250-254.

Sheehan J, Dunahay T, Benemann J, Roessler P (1998). A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory: Golden, Colorado. p 296.

Solovchenko AE (2012). Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. *Russian Journal of Plant Physiology* **59:** 167-176.

Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U (2009). Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless Chlamydomonas reinhardtii. *Eukaryotic Cell* **8:** 1856-1868.

Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, Laurens LML, Dismukes GC, Posewitz MC (2010). Increased Lipid Accumulation in the Chlamydomonas reinhardtii sta7-10 Starchless Isoamylase Mutant and Increased Carbohydrate Synthesis in Complemented Strains. *Eukaryotic Cell* **9:** 1251-1261.

Xu D, Gao Z, Li F, Fan X, Zhang X, Ye N, Mou S, Liang C, Li D (2013). Detection and quantitation of lipid in the microalga Tetraselmis subcordiformis (Wille) Butcher with BODIPY 505/515 staining. *Bioresource Technology* **127:** 386-390.

Yamaguchi T, Osumi T (2009). Chanarin–Dorfman syndrome: Deficiency in CGI-58, a lipid droplet-bound coactivator of lipase. *Biochimica et Biophysica Acta* **1791:** 519-523.

Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012). Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 3708-3724.

Yu E, Zendejas F, Lane P, Gaucher S, Simmons B, Lane T (2009). Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum (Baccilariophyceae) during starvation. *Journal of Applied Phycology* **21:** 669-681.

Zehmer JK, Huang Y, Peng G, Pu J, Anderson RGW, Liu P (2009). A role for lipid droplets in inter-membrane lipid traffic. *Proteomics* **9:** 914-921.

4.0 Chapter 4

GENERATION AND SELECTION OF NATIVE SEQUENCE THAPS3_264297 KNOCK-DOWN STRAINS

4.0.1 Abstract

Increasing lipid yields in microalgae is becoming possible as metabolic engineering techniques advance and understanding of lipid metabolism improves. However, genetically engineered strains that contain inserted intergeneric DNA are considered genetically modified organisms (GMOs) by the Environmental Protection Agency in the United States. Regulations imposed by the Toxic Substances Control Act require GMOs to undergo a strict permitting process to be grown outdoors. As many algal biofuel companies use outdoor ponds for biomass cultivation, a metabolic engineering method that bypasses GMO classification would be desired and impactful. We generated a knock-down construct of Thaps3_264297 using only native *Thalassiosira pseudonana* sequences and transformed the construct into the diatom. We used the phenotypes of increased TAG accumulation and increased membrane intactness seen in Thaps3_264297 knock-down strains as selectable markers for transformants. Three rounds of silicon starvation, fluorescence activated cell sorting, and nutrient replenishment were used to enrich the composite population for transgenic strains. The resulting composite population showed a more robust TAG accumulation profile under nutrient limitation than the WT culture. Single clones were isolated to identify strains with an incorporated knock-down construct.

4.1 Introduction

The evolution of algal biofuel efforts from R&D to commercial scales is dependent upon improvements in lipid yields (Davis et al 2011). As molecular techniques for manipulating microalgae (specifically for production system strains) continue to develop, metabolic engineering becomes a feasible strategy to guide yield improvements; in fact, genetic manipulation may be absolutely necessary for some algal biofuels systems to ever become economically successful. The use of engineered strains in production systems is strongly dependent upon the type of production system planned for cultivation.

While many microalgae can also be growth heterotrophicaly or mixotrophically (Brennan and Owende 2010), here we focus on photoautotrophic cultivation. One of two typical cultivation systems are generally employed for large-scale growth of microalgal biomass: outdoor open ponds and enclosed photobioreactors. Closed photobioreactors allow for greater control over many factors during microalgal growth including temperature, CO_2 delivery and preventing contamination. However, photobioreactors are substantially more costly and energy intensive than open pond alternatives (Jorquera et al 2010). Open ponds are typically closed-loop, shallow, oval raceways and include methods for mixing and circulation. Open ponds are relatively cheap, consume little energy, and are easy to maintain. However, with open ponds come problems of contamination as well as lack of control over temperature and light fluctuations (Brennan and Owende 2010).

Another major difference between open ponds and photobioreactors is the type of regulation involved in permitting cultivation of certain algal strains. In the U.S., genetically engineered microalgae are considered toxic substances by the Environmental

Protection Agency (EPA). Under the Toxic Substances Control Act (TSCA), the EPA strictly controls and regulates the release of toxic substances into the environment through a permitting process that involves the submission of information on the algal strain through TSCA Experimental Release Application (TERA) and the subsequent review and regulation by the EPA (TSCA of 1976, 1976). Because photobioreactors are typically completely enclosed systems, they are exempt from the EPA's toxic substance regulation requirements and can be used to grow genetically-modified strains (Henley et al 2013). Open ponds, however, can only be used for cultivation of genetically-modified microalgae if the strain is submitted to and passes the EPA's toxic substance permit requirements. Although a number of strains are currently in EPA's permitting pipeline, and experimental scale permits have been granted, no genetically modified strain has successfully been accepted for large-scale growth at the present time (Henley et al 2013).

The EPA permit process regulates microorganisms that are considered "new," and defines these to be any 'intergeneric' microorganisms. The EPA further describes an intergeneric organism as one that "is formed by the deliberate combination of genetic material originally isolated from organisms of different taxonomic genera." Therefore, genetically modified organisms (GMOs) whose modifications only include genetic material from within its genus are *not* considered intergeneric or new by the EPA, and are thus not subject to TSCA regulation (TSCA of 1976, 1976).

Some commercial scale microalgal growers have bypassed the TSCA reporting requirements by generating and cultivating microalgal mutants using mutagenesis techniques that don't involve genetic elements, such as UV or chemical mutagenesis. Mutants generated this way are not considered intergeneric, and are thus not subject to

TSCA screening. Direct and specific manipulations of metabolic pathways, however, typically involve the insertion of specific genetic elements as well as selectable markers such as antibiotic resistance genes, which are most often intergeneric.

In order to introduce genetic manipulation elements into microalgae, current methods of transformation rely on heterologous selectable markers, for example against antibiotics or herbicides, which has two drawbacks, 1) transformants are classified as GMOs (as previously discussed), and 2) introduction of antibiotic- or herbicide-resistant genes to the environment engenders the spread of these genes to other organisms, which has detrimental effects (e.g. antibiotic-resistant strains of bacteria) (Daniell 2002). Another method of transformation is by complementation of metabolic deficiencies (Stevens and Purton 1997), which involves a time-consuming process of generation, selection, characterization of appropriate mutations, and maintenance of mutants for each species under specially formulated growth medium.

As discussed in Chapter 3, the knock-down of Thaps3_264297 using antisense methods results in strains with a number of characteristic phenotypes including increased TAG accumulation, uncompromised growth and more intact membranes after nutrient limitation. If knock-down strains were mixed with wild-type *T. pseudonana*, these phenotypic factors could be used to select transgenic cells. While the two strains would grow comparably, higher TAG accumulation could be selected for using BODIPY staining and fluorescence-assisted cell sorting (FACS) (Manandhar-Shrestha and Hildebrand 2013). More intact membranes potentially translate to increased cell viability, and thus, if the cells were transferred to nutrient-replete medium after nutrient

deprivation, more transgenic cells should be viable compared to wild-type, thereby increasing the proportion of transgenic cells in the population.

I hypothesized that, implementing this strategy, knock-down strains of Thaps3_264297 could be generated using only native *T. pseudonana* sequences, and transformants could be selected using the discussed phenotypes. Antisense knock-down constructs were generated using only sequences amplified from *T. pseudonana*'s genome. After transformation, three rounds of phenotypic selection were used to enrich the mixed population for transgenic strains before the culture was plated to select individual clones.

4.2 Results & Discussion

4.2.1 Transformation & Selection

Linear native sequence knock-down constructs for Thaps3_264297 were generated using antisense DNA to the same portion of Thaps3_264297 as discussed in Chapter 3 (Fig. 3-1C). The construct consisted of the fucoxanthin chlorophyll a/b-binding protein (fcp) promoter, the antisense region of Thaps3_264297, and the fcp terminator sequence (Fig. 4-1). After transformation of the construct in wild type (WT) *T. pseudonana*, the resulting harvested culture contained a composite culture of WT and transgenic cells.

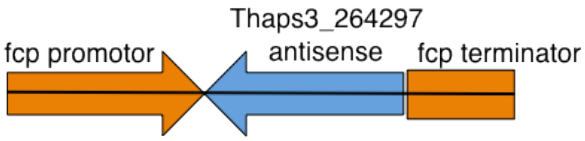


Figure 4-1. Native sequence knock-down construct.
Knock-down construct for Thaps3_264297 consisted of the full fcp promotor, an antisense portion of Thaps3_264297, and the fcp terminator. All portions of the construct were originally amplified from *T. pseudonana* DNA.

In order to sort out transgenic knock-down cells that exhibit increased TAG accumulation, the composite culture was subjected to three rounds of silicon starvation followed by nutrient replenishment. As discussed in Chapter 3, knock-down strains exhibited increased membrane intactness, which can translate to increased viability. Thus, the starvation/replenishment cycle was used as an enrichment strategy for the knock-down cells. In the first round of nutrient limitation, the composite culture was subjected to 40 hours of silicon starvation alongside a WT culture. FACS was used to sort out high lipid-producing cells that simultaneously contained high chlorophyll content as a potential marker for high viability. The same high BODIPY and high chlorophyll fluorescence parameters were used to sort both the WT culture and the composite culture, and a slightly higher percentage of the composite population fit into these desired parameters (Fig. 4-2).

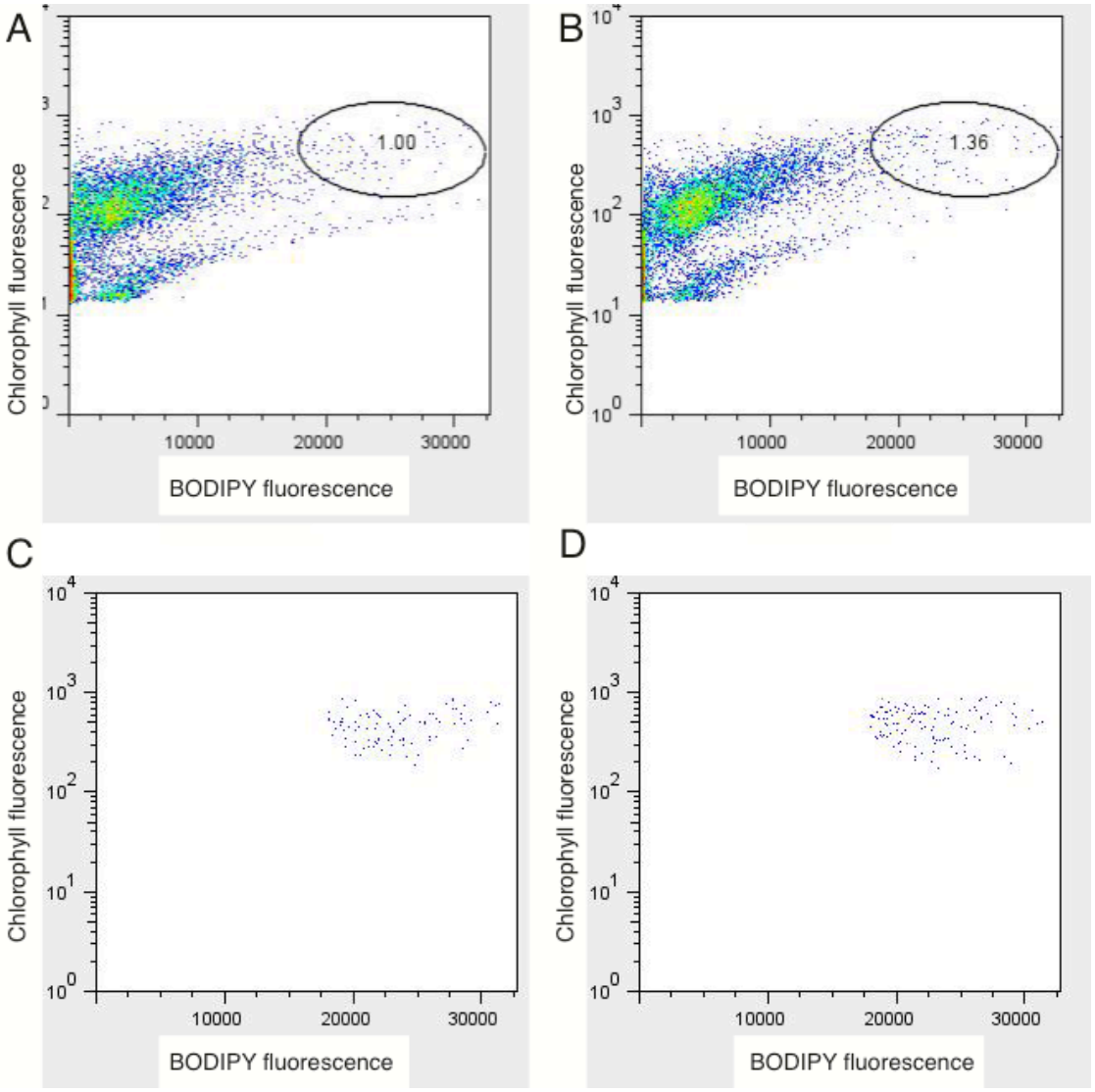


Figure 4-2. First sort of knock-down and WT mixture.
FACS was used to sort out high lipid-producing cell with high chlorophyll. Scatter plots show the BODIPY and chlorophyll fluorescence of individual cells in the WT population (*A*) and knock-down mixture (*B*). Cells falling within the circle were sorted. This sorted population is shown for WT in (*C*) and the mixture in (*D*).

The remaining unsorted composite culture was starved for a further 20 hours and then transferred to silicon-replete medium. Replenished cells were grown over multiple days to higher density before being subjected to a second round of silicon starvation. After 40 hours of silicon starvation, the composite culture was similarly sorted using FACS and compared to WT. Again an increased portion of the composite population fell within the high BODIPY/high chlorophyll parameters as compared to WT (Fig. 4-3).

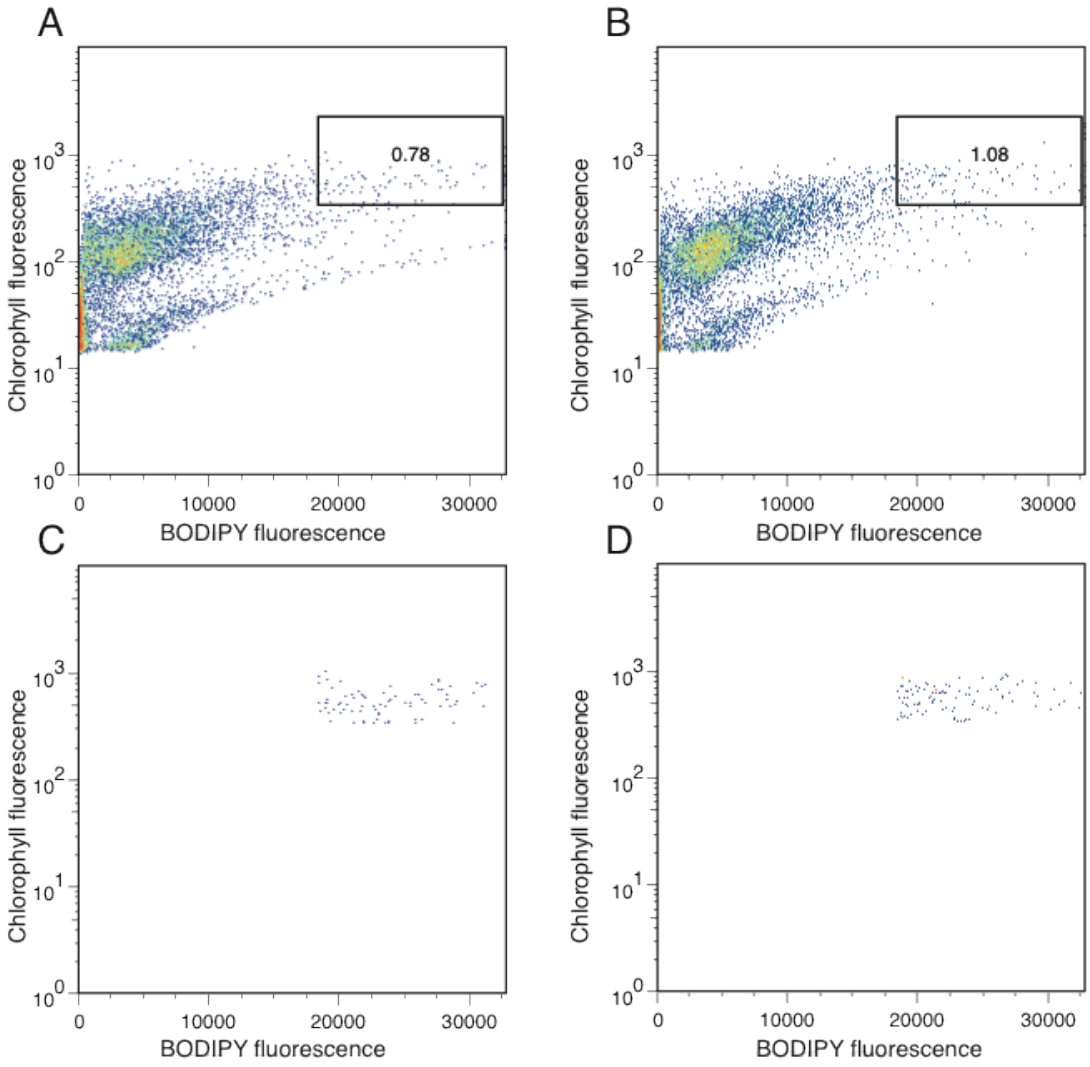


Figure 4-3. Second sort of knock-down and WT mixture.
FACS was used to sort out high lipid-producing cell with high chlorophyll. Scatter plots show the BODIPY and chlorophyll fluorescence of individual cells in the WT population (*A*) and knock-down mixture (*B*). Cells falling within the box were sorted. This sorted out population is shown for WT in (*C*) and the mixture in (*D*).

In this round, the sorted cells were allowed to recover for one week in nutrient replete media. This recovered culture was subjected to a final round of 40 hours of silicon starvation alongside WT and sorted using FACS. The composite population responded differently from WT, showing increased TAG accumulation across the population (Fig. 4-4) as reflected by an increased average BODIPY fluorescence. Parameters were set to sort out cells within the top 5% of the BODIPY fluorescence spectrum; the WT culture contained no cells that fell within these parameters. The differing response under silicon starvation of the final composite population compared to WT shows that the composite population is indeed enriched for high chlorophyll, high lipid-containing cells.

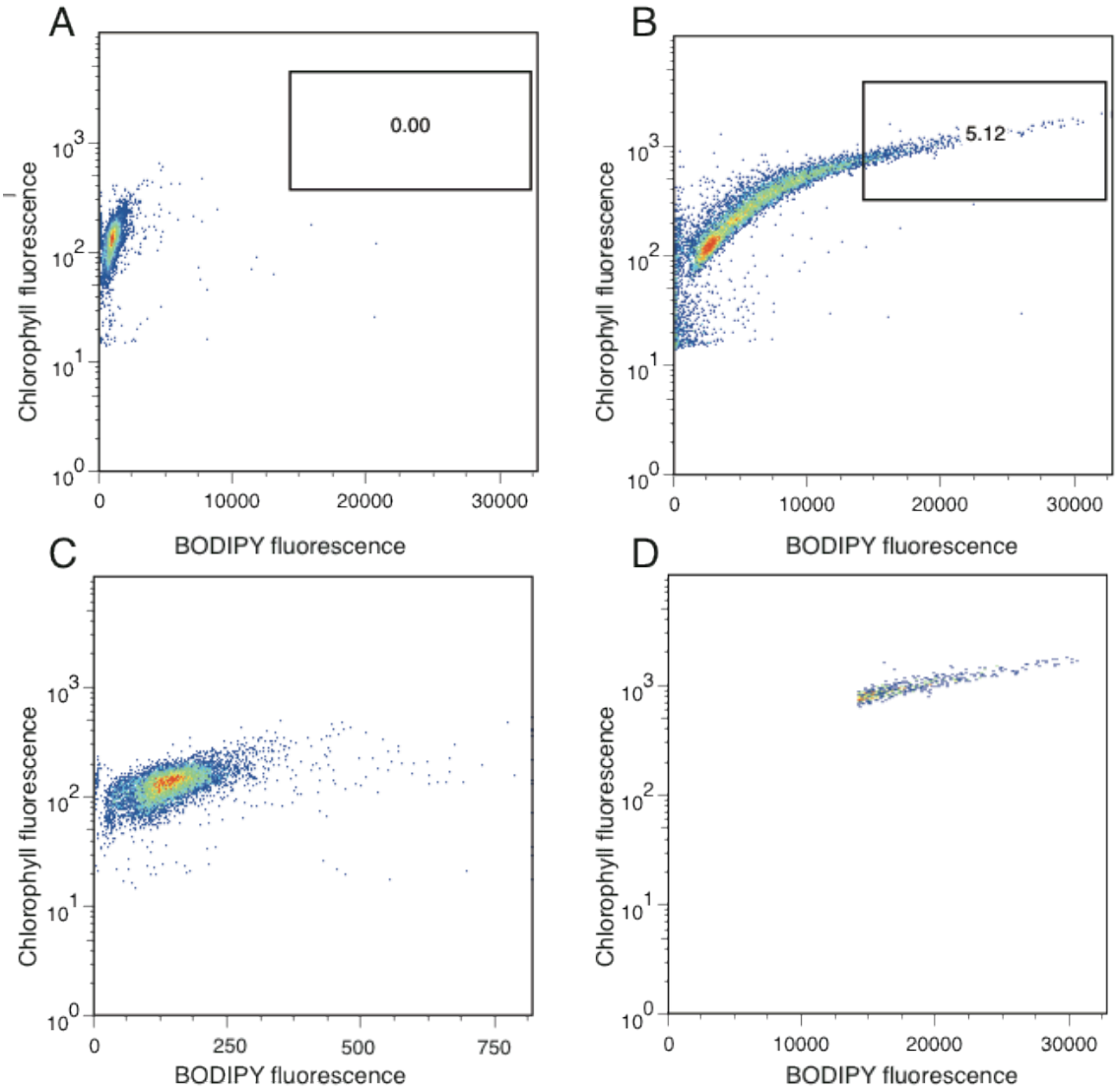


Figure 4-4. Final sort of knock-down and WT mixture.
FACS was used to sort out high lipid-producing cell with high chlorophyll. Scatter plots show the BODIPY and chlorophyll fluorescence of individual cells in the WT population (*A*) and knock-down mixture (*B*). Cells falling within the box were sorted. (*C*) shows a close-up of the WT population to show distribution. (*D*) shows the population sorted from the mixture.

4.2.2 Confirmation of knock-down construct incorporation

Cells from the 3rd sort of the composite culture were plated to select single colonies whose genomic DNA was probed for presence or absence of the knock-down construct (Fig. 4-5). Out of the colonies probed, 1 in 6 contained the transformed construct. Typically, microparticle bombardment results in transformation efficiencies of 1 tranformed cell for every million, thus the sorting scheme used here has significantly enriched the composite population for transgenic cells. The sorting process also selects for more viable, higher lipid-producing WT cells as well, which can explain why not every cell in the final population is a transformant.

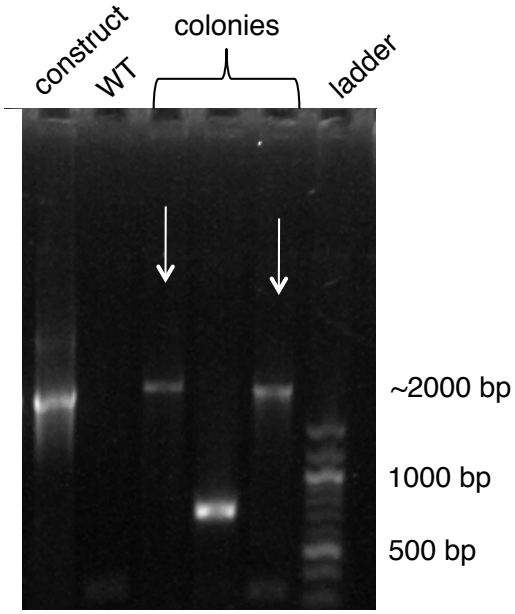


Figure 4-5. PCR of genomic DNA from isolated colonies in final composite culture. Primers specific to portions of the inserted construct were used to probe genomic DNA of isolated colonies to confirm presence of construct.

This work serves as a proof-of-concept that the phenotypes associated with knock-down of Thaps3_264297 can be used as selectable markers. Further confirmation of the validity of this approach would involve analyzing growth and lipid content of the resulting isolated transgenic strains, qPCR of the composite culture using primers specific to the construct at each stage of enrichment to demonstrate increased proportion of transformants, repeated procedure with more consistent timing and increased rounds of starvation and sorting, and generation of a construct that contains no foreign Gateway sequences. Sequencing of the construct's genomic neighborhood in isolated transformants can also determine if they arise from one predominant strain, or if multiple transformants have been selected.

The technique described in this chapter enables only native sequences to be used in order to perform targeted engineering of this particular gene. However, this construct could theoretically be used as a selectable marker in other manipulations that involve native sequences as well. Overexpression or knock-down constructs of other specific targets can be amended to contain an adjacent sequence of Thaps3_264297 antisense DNA, which would act similarly to an antibiotic resistance gene. The Thaps3_264297 antisense sequence would convey the knock-down phenotypes onto transformants, which could then be selected out using the scheme provided here.

4.3 Materials & Methods

4.3.1 Vector construction and PCR

Linear knock-down constructs were amplified from vectors constructed using Invitrogen's Multi-Site Gateway Cloning Protocol. Constructs contained the fcp promotor, Thaps3_264297 antisense fragment and fcp terminator. Linear constructs were transformed into *T. pseudonana* using microparticle bombardment with a BioRad PDS-1000 using established procedures (Dunahay et al 1995).

Genomic DNA was isolated from individual diatom colonies using three freeze/thaw cycles followed by centrifugation. Colonies were grown in liquid culture, pelleted, resuspended in sterile water, and subjected to freeze/thaw cycles using 1 minute in liquid nitrogen to freeze and 2 minutes at 65^{o} to thaw. After a final incubation for 5 minutes at 95^{o}, samples were pelleted and supernatant was collected for DNA. DNA was probed for presence or absence of the knock-down construct using forward primer 5' AGCTTGCGCTTTTTCCGAGAAC 3' and reverse primer 5' GGGAGAACTGGAGCAGCTACTAC 3'.

4.3.2 Strains and culture conditions

T. pseudonana was grown in batch culture with shaking or stirring and aeration under either continuous illumination 150 μmol m^{-2} s^{-1} at 18 to 20°C in sterile artificial seawater medium (ASW) supplemented with biotin and vitamin B12 at 1 ng L^{-1} (Darley and Volcani 1969). To induce silicon starvation, cultures were grown to a mid-log phase density of $2x10^{6}$ cells/mL in ASW, pelleted and washed twice with silicon-free ASW, then inoculated at $1x10^{6}$ cells/mL into silicon-free ASW in polycarbonate bottles. Cultures were plated on ASW plates containing 1.5% Bacto-Agar and grown for 10 days

under continuous illumination. Colonies were picked into 24-well plates and allowed to grow for a week before PCR.

4.3.3 Fluorescence-assisted cell sorting

Approximately 2-5 x 10^7 cells from silicon-starved cultures were harvested via centrifugation at 4500 rpm for 10 min. Cell pellets were resuspended in 1 mL of ASW and stained with 2.3 µg/mL BODIPY for 15 min. Stained cultures were passed through a filter to separate out debris and run on a Becton Dickinson Influx sorting flow cytometer. Cells were excited with a 488 nm laser, BODIPY emission evaluated at 530/40 nm and chlorophyll emission evaluated at 692/40 nm. Isolation parameters included approximately the top 1% in terms of BODIPY fluorescence.

4.4 Acknowledgements

Chapter 4 is currently being completed and will be prepared for submission in 2014. Emily Trentacoste, Mark Hildebrand and William Gerwick. The dissertation author was the primary investigator on these studies.

4.5 References

Brennan L, Owende P (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14:** 557-577.

Daniell H (2002). Molecular strategies for gene containment in transgenic crops. *Nature* **20:** 581-586.

Darley WM, Volcani BE (1969). Role of silicon in diatom metabolism: A silicon requirement for deoxyribonucleic acid synthesis in the diatom Cylindrotheca fusiformis Reimann and Lewin. *Experimental Cell Research* **58:** 334-342.

Davis R, Aden A, Pienkos PT (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy* **88:** 3524-3531.

Dunahay TG, Jarvis EE, Roessler PG (1995). Genetic tansformation of the diatoms Cyclotella cryptica and Navicula saprophila. *Journal of Phycology* **31:** 1004-1012.

Henley WJ, Litaker RW, Novoveská L, Duke CS, Quemada HD, Sayre RT (2013). Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. *Algal Research* **2:** 66-77.

Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML (2010). Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. *Bioresource Technology* **101:** 1406-1413.

Manandhar-Shrestha K, Hildebrand M (2013). Development of flow cytometric procedures for the efficient isolation of improved lipid accumulation mutants in a Chlorella sp. microalga. *Journal of Applied Phycology* **25:** 1643-1651.

Stevens DR, Purton S (1997). Genetic engineering of eukaryotic algae: progress and prospects. *Journal of Phycology* **33:** 713-722.

Toxic Substances Control Act (TSCA) of 1976, Pub. L. no. 94-469, 90 Stat. 2003 (1976).

5.0 Chapter 5

THE PLACE OF ALGAE IN AGRICULTURE: POLICIES FOR ALGAL BIOMASS PRODUCTION

5.0.1 Abstract

Algae have been used for food and nutraceuticals for thousands of years, and the large-scale cultivation of algae, or algaculture, has existed for over half a century. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. It is crucial during this period that institutional frameworks (i.e., policies) support and promote development and commercialization and anticipate and stimulate the evolution of the algal biomass industry as a source of renewable fuels, high value protein and carbohydrates and low-cost drugs. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture. Despite this overlap, algaculture has not yet been afforded a position within agriculture or the benefits associated with it. Various federal and state agricultural support and assistance programs are currently appropriated for crops, but their extension to algal biomass is uncertain. These programs are essential for nascent industries to encourage investment, build infrastructure, disseminate technical experience and information, and create markets. This review describes the potential agricultural policies and programs that could support algal biomass cultivation, and the barriers to the expansion of these programs to algae.

5.1 Introduction

Algae are simple, photosynthetic, generally aquatic organisms that, like plants, use energy from sunlight to sequester carbon dioxide (CO_2) from the atmosphere into biomass through photosynthesis. Plants evolved from ancient algae ancestors, and the photosynthetic machinery in both plants and algae originally came from the same source: cyanobacteria (Falcón et al 2010, Fehling et al 2007). Although algae and plants differ in many ways, the fundamental processes, such as photosynthesis, that make them so distinguished among Earth's organisms and valuable as crops, are the same.

Certain strains of algae have been used for anthropogenic purposes for thousands of years, including as supplements and nutraceuticals (Kiple & Ornelas 2000) and in the fertilization of rice paddies (Tung & Shen 1985). As early as the 1940s other strains were identified as possible fuel sources (Borowitzka et al 2013a) because of their ability to produce fuel or fuel precursor molecules. Large-scale production and cultivation systems, including photobioreactors and outdoor open ponds, were developed in the early 1950s in the U.S., Germany, Japan and the Netherlands (Borowitzka et al 2013b, Tamiya 1957). By the onset of the U.S. Department of Energy's (DOE) Aquatic Species Program in the U.S. in 1980, various species of microalgae and cyanobacteria were being produced and farmed on commercial scales around the world, and had been for over 20 years, mostly for the health food and nutritional supplement industries (Borowitzka et al 2013b).

Microalgae have evolved to be practically ubiquitous throughout the globe, and their varied distributions and evolutionary histories (Fehling et al 2007) are reflected in extremely diverse metabolic capabilities between species (Andersen 2013). These diverse metabolisms produce a myriad of compounds with anthropogenic relevance including

nutraceuticals, such as the carotenoids produced by *Dunaliella* and *Haematococcus* (Borowitzka 2013), the polyunsaturated fatty acids (PUFAs) produced by various species (Ratledge 2004), and the high-value proteins and carbohydrates available in whole-cell supplements of *Spirulina* and *Chlorella* (Görs et al 2010, Khan et al 2005). Some microalgae produce compounds of biotechnological interest including fluorescent compounds, such as phycoerythrin, and many produce isoprenoid molecules that can be used in food and over-the-counter products (Andersen 2013).

Microalgae have also been identified as attractive sources of biofuel because different species can produce a variety of fuel products. Various microalgal species have the ability to produce large quantities of lipid while sequestering CO_2, particularly neutral lipids in the form of triacylglycerol (TAG), which can be converted to fatty acid methyl esters (FAMEs), the main components of biodiesel (Hossain et al., 2008), through trans-esterification, or refined into other fuel constituents (Pienkos & Darzins 2009). Total lipids and other biomass constituents can be converted into crude oil alternatives through thermochemical processes such as hydrothermal liquefaction (Barreiro et al 2013). Microalgal carbohydrates can be fermented into ethanol, and some species can produce biohydrogen (Radakovits et al 2010). In addition to their diversity of products, microalgae are attractive as fuel sources because many species grow relatively fast compared to terrestrial plants and can be grown on brackish or saline water, thus avoiding the use of unsustainable quantities of freshwater, an increasingly limited resource (Dismukes et al 2008). Table 5-1 provides an overview of some commercial algal products and potential sources.

Table 5-1. Commercial products from algae.

Product	**Use**	**Example source**	**Reference**
β-Carotene	supplement	*Dunaliella*	(Lamers et al 2008)
Astaxanthin	supplement	*Haematococcus*	(Lorenz & Cysewski 2000)
Whole-cell nutraceuticals	supplement	*Spirulina* *Chlorella*	(Khan, et al 2005) (Görs et al 2010)
Aquaculture feed	animal feed	*Tetraselmis* *Isochrysis*	(Gladue & Maxey 1994) (Gladue & Maxey 1994)
Polyunsaturated fatty acids (PUFAs)	supplement	*Crypthecodinium* *Shizochytrium*	(Jiang et al 1999) (Spolaore et al 2006)
Phycoerythrin	biotechnology	Red algae	(Pulz & Gross 2004)
Fuel molecules	energy	*Botryococcus* *Scenedesmus* *Neochloris*	(Ashokkumar & Rengasamy 2012) (Mandal & Mallick 2009) (Gouveia et al 2009)
Anticancer drugs	pharmacueticals	*Symploca*	(Coates et al 2013)

Algaculture, or the farming of algae (Savage 2011), merges the requirements of traditional terrestrial plant agriculture such as sunlight, water, CO_2, nutrient inputs, and harvesting systems with additional aquaculture requirements such as self-contained aquatic systems, water quality and waste disposal/recycling (Fig. 5-1). Because of their capability to produce commodities that span multiple markets, including those of health food, nutraceuticals, pharmaceuticals, animal feed, chemicals and energy, algae are

uniquely versatile crops (Rosenberg et al 2008). These diverse metabolic capabilities are due, in part, to the diversity of strains found within the algal lineage. Algae strains grown for food purposes, such as *Spirulina*, have a starkly different metabolic profile from strains grown for energy, such as *Scenedesmus*. The diversity of their end-products, and their cultivation using both agriculture and aquaculture practices make algae unique among other agricultural products.

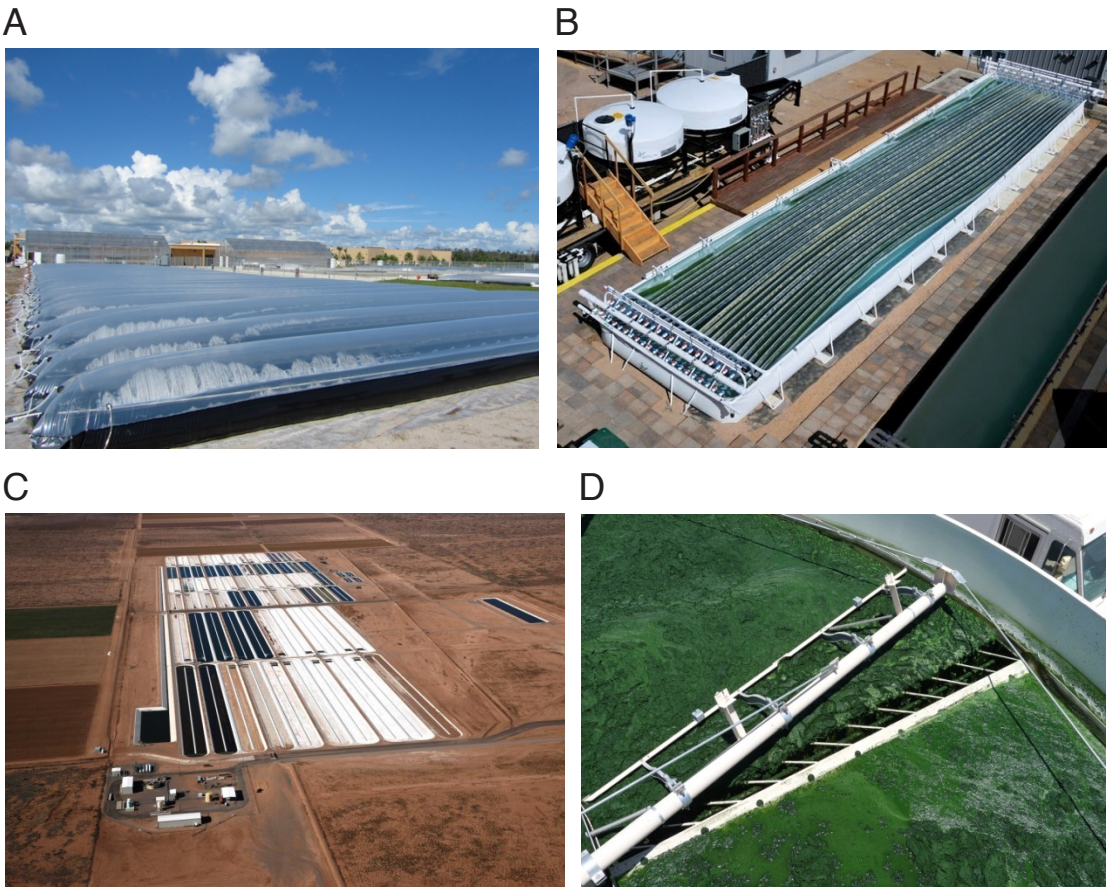


Figure 5-1. Algaculture in the U.S.
Algaculture can take place in closed photobioreactors, like those of Algenol in Florida (*A*) and Solix Biosystems in Colorado (*B*), or in open ponds like those of Sapphire Energy, Inc. in New Mexico (*C*). Like agriculture, algae cultivation requires growth as well as harvesting infrastructure, such as that of Sapphire Energy Inc.(*D*).

Despite significant overlap with both traditional agriculture and aquaculture (which Congress has defined as agriculture, including that of aquatic plants) (Food & Agriculture Act of 1977, 1977), algaculture has not yet been afforded an official position within Title 7 of the U.S. Code (USC) for Agriculture. There are currently a number of other crops that share commonalities with algae in their cultivation practices or diversity of end-use markets, but these have all been designated a place within Title 7. For example, the commercial cultivation of aquatic plants, such as seagrass, is eligible for a diverse array of agricultural programs. Similarly, the farming of terrestrial crops for renewable energy, which shares the same end market and purpose as many algal farming operations, benefits from its definition as agriculture.

Funding for research and development of algal biomass cultivation has increased over the last decade, and has led to the emergence of research programs, private projects, demonstration- and commercial-scale facilities across the U.S. (Fig. 5-2). The increase is primarily due to the growth of the algal biofuel industry in response to the demand for alternative fuel sources driven by the Renewable Fuel Standards (RFS) (Tyner 2013). While the use of algae as functional food or feed ingredients is also on the rise (Ibañez & Cifuentes 2013) there are currently few federal program resources focused in this area. The production of algae for any end-product is a two-phase process involving the farming and cultivation of algal biomass followed by processing of the harvested biomass. The ability of the algal biomass industry to access federal programs that support the agricultural phase is imperative for future growth. This report analyzes the place of algae

in the current agricultural policy and funding landscape, and the opportunities and pitfalls that exist for algae within this policy framework.

Figure 5-2. Algae projects in the U.S.
Algal biomass projects exist in almost every state in the U.S. Blue pins denote a research institution, green denote a private project or company.

5.2 Agricultural programs

Congress has legislated a number of renewable energy programs that can be applied to algae such as the Bioenergy Program for Advanced Biofuels, the Rural Energy for America Program, the Biomass Research & Development Initiative and various grants and loans established in the 2008 Farm Bill in section 9003 of the USC (Food, Conservation, & Energy Act of 2008, 2008). These programs, however, focus on research and development of algae for fuels at smaller scales. While this initial investment in research & development (R&D) is essential to build knowledge, expertise and technology around algae, the industry is now entering the formative stage of large-

scale commercialization, which requires broader coordination among federal agencies and support infrastructure to gain proper alignment at the federal and state level required for a successful industry.

5.2.1 Biomass Crop Assistance Program

The Biomass Crop Assistance Program (BCAP) was established in the 2008 farm bill (Food & Conservation Act of 2008, 2008) to financially assist farmers wishing to establish, produce and deliver biomass feedstocks. BCAP's purpose is to promote farming of bioenergy crops. The program provides either one-time establishment payments, annual payments, or matching payments to help with harvest, storage and transportation of biomass. Proposals for BCAP funding are submitted to the FSA and can come from either producers or conversion facilities (Schnepf 2011). While many traditional biofuel crops are currently eligible for BCAP funding, such as switchgrass and most non-food biomass, the 2008 farm bill specifically excluded algae from participation in the matching payment side of BCAP but qualifies algae for establishment payments through BCAP (Food & Conservation Act of 2008, 2008).

5.2.2 Support programs

Congress has appropriated numerous federal agencies, such as the USDA and DOE, funds and authorization to implement programs that aid and support development of agriculture and aquaculture resources (Table 5-2). Since the passage of the original Agricultural Adjustment Act of 1933, each subsequent farm bill has evolved to address arising relevant issues in agriculture. This frequently involves drafting new programs or

expanding existing programs to new developing technologies. The 1977 farm bill (Food & Agriculture Act of 1977, 1977) expanded the definition of agriculture to include aquaculture, thus spurring the development of industry in the U.S. The 2002 farm bill was the first to include a title (9003) on energy (Farm Security & Rural Investment Act of 2002, 2002), enabling the initial research and development of biofuels and bioenergy and set the stage for bio-based energy standards in the 2005 and 2007 energy bills.

Table 5-2. Overview of federal support programs.
Agricultural and energy support programs provided by the USDA and DOE. Solid circle denotes all crops within that category are eligible for programs; white circle denotes no crops within that category are eligible; half-solid circle denotes that support is dependent on the end-use of the crop.

Program	Description	Program Crops	Specialty Crops	Aquaculture	Algae
Farm Service Agency					
Commodity Operations	Price and market support programs to purchase, deliver, dispose of designated commodities for domestic and foreign markets	●	●	●	○
Conservation Programs	Conservation related programs	●	●	●	◑
Direct/Counter Cyclical	Income support	●	●	●	◑
Disaster Assistance	Disaster assistance insurance	●	●	●	◑
Economic and Policy	Market development assistance	●	●	●	○
Biomass Crop Assistance	Production incentive	●	●	●	◑
Farm Loan Program	Operating and capital Loans	●	●	●	◑
Energy Assistance					
Biorefinergy Assistance	Loan guarantee/grant program to assist in the development of Advanced Biofuels	○	●	●	●
Bioenergy Program for Advanced Biofuels	Producer payments to expand production of Advanced Biofuels	○	●	○	●
Rural Energy For America	Loan and Grant program for individual farmers	●	●	●	◑
Biomass Research and Development Initiative	Research and development, demonstration of biofuels and biobased products	●	●	●	◑

The current farm bill, primarily through the arm of the USDA and associated agencies, funds a large number of assistance programs for agriculture and aquaculture (Food, Conservation & Energy Act of 2008, 2008). All of the major farm price and income support programs comprising the farm safety net are available only to the "program crops" of corn, cotton, wheat, tobacco, peanuts, rice and some new oil crops such as sunflower and oilseed. The main farm safety net programs restricted to program crops include the Marketing Assistance Loan, Countercyclical Payment, Average Crop Revenue Election and Direct Payment programs. Additional programs, such as the Feedstock Flexibility Program for sugar, also instill price control while simultaneously attempting to bridge the gap with biofuel producers looking to meet RFS standards. These programs ensure that market prices for "program crops" never fall below a certain limit and provide direct income support or revenue assistance. Farmers of specialty crops, such as fruits and vegetables, aquaculture crops, horticulture crops, and livestock are eligible for a range of support programs outside of the safety net. These programs provide extension services, loans, crop insurance and incentives for improving environmental quality of farms (Mercier 2011).

5.2.2.1 Extension services

Some of the most important benefits allotted to agriculture and aquaculture in the U.S. are research, teaching and extension services. Extension services are some of the oldest programs in U.S. agriculture, dating back to the Smith-Lever Act of 1914 that established a link between universities and the USDA (Smith-Lever Act, 1914). The

purpose of the programs has always been to 1) develop applications for agricultural research and 2) provide instruction on agricultural technologies to farmers. Today the Cooperative Extension Service program of the USDA provides funding through the National Institute of Food and Agriculture to support programs that connect scientific agricultural research with local farmers. Extension services are administered through regional offices that bring expertise from land-grant universities to local levels to instruct farmers in emerging technologies that can increase productivity.

Extension services are essential for disseminating information about innovative research and technologies throughout the agricultural industry. They also play an extremely important role in providing more immediate assistance to issues faced by local farmers, and in developing plans that address regional problems. The application of USDA's extension services to aquaculture in the 1981 farm bill was instrumental in expanding the industry and coordinating research and commercialization efforts (Agriculture & Food Act of 1981, 1981).

5.2.2.2 Federal crop insurance programs

The additional support programs available for all farmers are important for the continuing success of non-program crops. These programs provide assistance for the development, commercialization and continuation of farms and provide incentives for environmentally sound farming practices. The largest of these programs, in which all farmers (including those of aquaculture and livestock) can participate, is the crop insurance program. The original crop insurance program began in 1938 and only covered major crops (Agricultural Adjustment Act of 1938, 1938), but the passing of the Federal

Crop Insurance Act of 1980 expanded the program to be universal (Federal Crop Insurance Act of 1980, 1980). Crop insurance is run by the USDA Risk Management Agency (RMA) and paid for by the separate Federal Crop Insurance Corporation (FCIC).

Over 100 crops are currently eligible for the Federal Crop Insurance (FCI) program, in which farmers pay a subsidized premium for insurance delivered by private companies. While program crops are eligible for revenue-based loss insurance, specialty crops typically only participate in physical crop-loss insurance. If a crop is ineligible for the program, it can still be insured through the Non-insured Crop Disasters Assistance program, established in the 1996 farm bill and run by the Farm Service Agency (FSA), which functions similarly to FCI (Federal Agriculture Improvement & Reform Act of 1996, 1996). Sea grass, a similar crop to algae that requires a blend of agriculture and aquaculture, is eligible for Non-Insured Crop Disasters Assistance. Additional insurance support is available for all farmers to cover losses from natural disasters under the Supplemental Revenue Assurance Program (SURE). This program provides additional assistance beyond crop insurance to farmers who experience a decrease in revenue due to natural disasters and is only available for crops that are enrolled in one of the crop insurance programs.

The expansion of crop insurance programs to specialty crops, aquaculture and livestock was important for the development and protection of these industries. Farms of these commodities are all affected by the same environmental factors as those of program crops, such as lower-than-expected production due to droughts, natural disasters, soil quality, water availability, etc. The farming of algae is equally susceptible to different but similar factors that affect biomass and crop yields.

5.2.2.3 Farm loan programs

Farm loans are essential in successful agriculture as up-front capital is needed to make purchases of inputs such as fertilizer, equipment, land, etc. Most farm loans are authorized by the Consolidated Farm and Rural Development Act (1961) and can be in the form of direct loans, guaranteed loans or emergency loans. Direct loans cover input purchases and farmland purchases, require farmers to complete financial training courses and are given preferentially to beginning farmers. Guaranteed loans are available in coordination with banks and emergency loans can help cover natural disasters.

5.2.2.4 Environment & conservation programs

Agriculture, aquaculture and livestock farms have traditionally been eligible for a number of federal programs that incentive environmentally friendly practices and resource conservation. Most notable, the Environmental Quality Incentives Program (EQIP), introduced in the 1996 farm bill, provides technical and financial assistance to farmers to increase the environmental quality of their farmland. EQIP funds are distributed by states in competitive programs that focus either on innovation of novel conservation practices or water enhancement, including enhancing water quality and conservation. EQIP also works in partnership with farms to aid in farm design that promotes environmental quality and resource conservation.

The Conservation Stewardship Program (CSP) awards funds to farmers that have adopted uncompensated practices across their entire operation for overall conservation. To be eligible for CSP funds farmers must be sustaining conservation of a certain

resource and must demonstrate improvement and maintenance of conservation practices. Farmers can receive both EQIP support and CSP rewards. The final environmental program, the Agricultural Management Assistance (AMA) Program was established in the Agricultural Risk Protection Act of 2000 to address the fact that crop insurance is heavily concentrated among "program crops" in only a few states. The AMA provides assistance for conservation practices in a select 16 states.

The algae industry, which has most recently been associated with renewable energy production with the added constraints of reducing greenhouse gas emissions and being cost-competitive with fossil fuels, has already made substantial technological advances in freshwater conservation and nutrient recycling for commercial-scale production. In order to be categorized as advanced biofuel, the overall process of algal fuel production must represent a 50% decrease in GHG emission compared to fossil fuels (Energy Independence & Security Act of 2007, 2007). A study conducted by the University of Virginia found that commercial scale production of algae-to-energy can result in a 68% reduction in overall greenhouse gas emissions when compared to traditional fossil petroleum (Liu et al 2013). Additionally, to increase economic feasibility, algae can be grown on non-potable saline or waste water and nutrients can be recycled, drastically mitigating freshwater use and fertilizer inputs. The company BioProcess Algae, for example, has successfully utilized waste outputs of water, heat and CO_2 from corn ethanol fermentation to cultivate algal biomass for various end products. Coupling algal cultivation with waste outputs from other industrial processes provides cost-effective and sustainable solutions to cultivation barriers.

5.2.2.5 Marketing Services

Agricultural products are frequently subjected to market analyses by the USDA such as economic and census reports. As the commercialization of algae progresses, market analyses will be advantageous to assess the strengths and weaknesses of the industry, the interplay between the agricultural and energy aspects of algae, and the outlook of the industry. The USDA also provides marketing assistance to farmers through financial assistance, research and promotion. To successfully break into the agricultural market, algae would benefit from the marketing services available from the USDA.

5.2.2.6 State programs

Defining the commercial cultivation of algae as agriculture provides opportunities at the state level as well. Many states offer additional loan and financing programs, especially for first-time farmers, such as "Aggie Bonds" that encourage private lenders to loan to beginning farmers. Beyond financial assistance, states can control laws associated with agricultural property and zoning. For example, the Ohio state legislatures recently defined alga as agriculture to allow use value assessments of alga land for tax purposes, thus lowering property taxes for land used for commercial algaculture (OH-H.R., 2012). The law additionally limits the authority of zoning laws to restrict algae on lands. Although decisions on specific investments in algae development are made at the regional and local levels, a federal initiative is still imperative to establish and influence direction and focus for the industry, as well as to develop guidance for new algae programs.

5.3 Application of agricultural programs to algae

Opportunities currently exist for algae cultivation to expand commercialization within agriculture if it were defined as such. The most notable is the potential to fill a large void in agriculture of the use of non-arable land to produce renewable hydrocarbons and high value protein. Unlike terrestrial crops, algae do not require fertile soil or arable land for growth, thus expanding the areas of the country in which algae can be cultivated. Algae do require other inputs such as salt or freshwater, nutrients, and consistent year-round sunlight. Taking all of these factors into account, a recent study by the Pacific Northwest National Laboratory (PNNL) identified ~90,000 sites in the U.S. that would be suitable for algaculture, comprising ~5.5% of the contiguous U.S. land mass and consisting predominantly of shrub/scrub landscape. These sites exclude any cropland, urban land, protected lands, wetlands, wilderness, or significantly sloping landscapes (Wigmosta et al 2011). To compare, agricultural land currently utilizes over 40% of the total U.S. land mass.

The USDA currently asserts jurisdiction of algae as an agricultural crop, and can potentially offer agricultural safety net programs to algal biomass companies. Despite the role of the USDA in overseeing agricultural programs for algae, barriers still exist to the application of these programs. Many of these barriers exist at the federal and state levels, and stem from lack of an overall national plan for the development of algaculture, from the overlapping jurisdictions of other federal agencies over different aspects of algae cultivation, (Fig. 5-3), and from the diverse end products generated by algae.

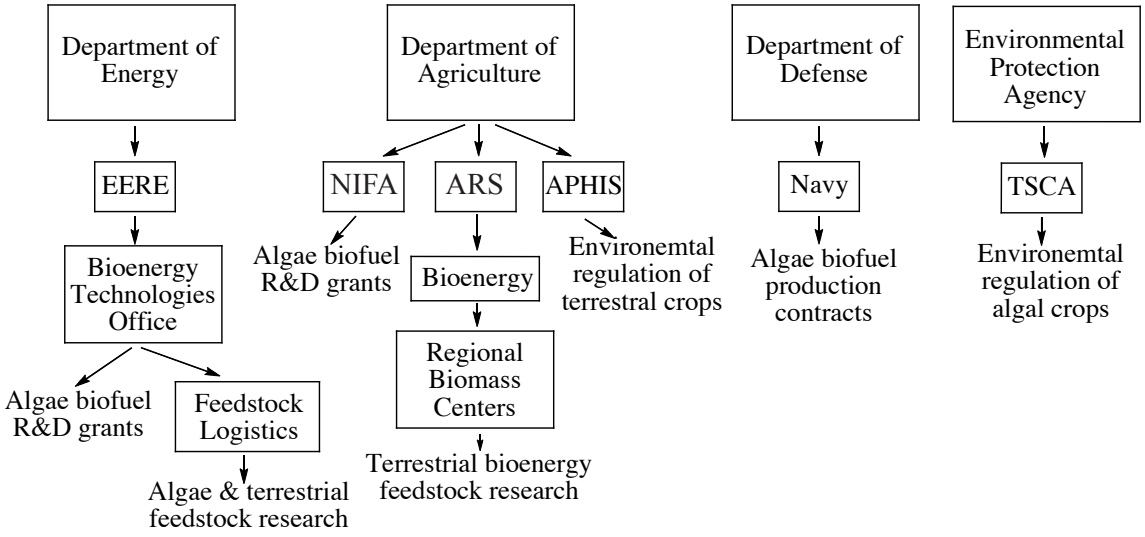


Figure 5-3. Federal agency jurisdiction over algae vs. terrestrial crops.
Four different federal departments hold jurisdiction over various aspects of algae cultivation, research and products. EERE – Energy Efficiency & Renewable Energy; NIFA – National Institute of Food & Agriculture; ARS – Agricultural Research Service; APHIS – Animal & Plant Health Inspection Service; TSCA – Toxic Substance Control Act.

Agencies that currently hold some responsibility over algae are the DOE, USDA, DOD and EPA. The DOE has been involved in algae biofuel research since the onset of the 25-year long Aquatic Species Program (ASP) in 1980 and has done extensive research on both algal biology and large-scale cultivation under its Biomass Program (Sheehan et al 1998). Findings have been reported in both the ASP close-out report and the National Algal Biofuels Technology Roadmap (U.S. DOE, 2010). The DOE also appropriates funding for grants and loans to industry and academic partners doing algae biofuel R&D. The DOD also appropriates R&D grants and participates in demonstrations for algal biofuel use. It has currently entered contracts for developing commercial-scale production. While the USDA is responsible for regulatory oversight and approval,

biotechnology and environmental regulation of genetically modified crops, the EPA has asserted jurisdiction for the permitting of genetically engineered algae varieties under its Toxic Substance Control Act, further supporting the notion of uncoordinated and overlapping federal support and regulation of the algae industry. There are also statutory limitations for the USDA's support of algae. Existing law, although not defined well and left open to individual programs for interpretation, may have the ability to support algae when used to produce a feed or food; the same standard, however, is not applied to algae if the end product is used to produce energy. None of these inconsistencies exist for the program crops (*e.g.* corn); they qualify for the vast array of USDA assistance no matter what products they support.

The USDA asserts responsibilities for agricultural policies pertaining to algae, but the end-use of algae as an energy source has created uncertainty in the applicability of these policies to algal cultivation. While a clear case can be made for expanding these programs for algal biomass used for food and nutraceutical purposes, there are still holes in the existing framework to accommodate algal biomass grown for bioenergy purposes. Because algae are such unique crops in their diversity of end product potential, no precedent exists to determine if a particular algae cultivation facility is eligible for agricultural programs or not. The USDA currently has no clear methodology for evaluating algal biomass producers within the agricultural landscape.

The uncertainty in algae's eligibility under agriculture is further exacerbated by insufficient communication about algal policies between the USDA's national leadership and its state and regional offices. The USDA's work, including decisions on application of policies to various USDA state offices, is primarily carried out in the field through

more local offices, but while the national office claims jurisdiction over algae, there is again no precedent for state offices to follow. For example, the USDA's five Regional Biomass Centers, which are designed to lead research in sustainable biomass production, currently specifically exclude algae to avoid DOE overlap. Extension services, such as those provided under the Smith-Lever Act, would be appropriate to link regional USDA centers with local institutions and algae cultivators to develop methodology for evaluating algal biomass production under the agricultural framework.

Another notable barrier is the lack of an overall algae-specific plan to move algae past R&D and into the formative stages of commercialization. The DOE has written an algae-specific roadmap, but this is primarily a summary of technologies that were available at the time and directions for R&D, without specific suggestions for moving into development and commercial stages (U.S. DOE 2010). Since then, a number of reports have been published agreeing that commercialization of algae, particularly for biofuels, is feasible given certain improvements in the production process (NRC 2012; ANL et al 2012). Furthermore, since these reports, many of these improvements have been made and technologies have been developed that successfully demonstrate the ability to sustainably cultivate and harvest algae on large scales. While continued R&D is imperative to maintain and drive such improvements in the overall production process, it is now more important than ever for federal agencies to map out the next stage of the scale-up process.

The overlapping jurisdiction of algae, lack of a national plan, and specifically the assumption of major responsibility by the DOE, has caused the focus of algal policies to primarily revolve around its downstream use for energy, and to overlook expansion of

policies that would support its most basic properties as a crop. Consistent, long-term federal policies are essential for scaling up biomass production of algae for energy, carbohydrates, protein and many other products (U.S. DOE 2012). The farming of algae requires biology, cultivation, harvest and biomass processing practices, modeled after agricultural systems, which require independent and unique support networks for commercialization from those required for the downstream conversion of biomass into fuel (such as extraction, conversion and biorefining processes).

5.4 Looking forward

While we have discussed the successes for algae in the U.S. agricultural framework and the pitfalls that still exist, we can also identify areas of progress. Individual states have taken initiative to pave the way in recognizing algal cultivation as agriculture. In 2012 two states, Arizona and Ohio, specifically amended their laws to define algaculture as part of agriculture. While these changes had different specific effects in each state, they were both carried out with the purpose of increasing investment in algaculture and attracting the industry to those states. In Ohio, the recognition of algae farming as agriculture allows land used for algae cultivation to be eligible for the same land use valuation as agriculture, thus allowing lower property taxes for algae farms. It also limits the authority of zoning laws to restrict algaculture on lands. The Ohio legislation was proposed with widespread support from many factions including the Farm Bureau, the Poultry Association and the Soybean Association (OH-H.R., 2012). In Arizona, state trust lands can now be leased for algaculture, and algae farmland is eligible for lower property taxes afforded to traditional farmland (AZ-H.R., 2012a, b). In 2013

Iowa also passed a similar bill defining land used for algal cultivation as agricultural (IA-H.R., 2013).

Arizona's bills have allowed for the development of a national testbed for algal biomass production, led by Arizona State University. This multi-regional private and public partnership, funded by the DOE, focuses on developing algal cultivation on large, economically relevant scales and involves coordination between facilities in Arizona, Ohio, California, Hawaii and Georgia. Other public-private partnerships include the California Center for Algal Biotechnology (Cal-CAB), which coordinates and promotes research, commercialization and public education projects.

5.5 Conclusions

Large-scale cultivation of algae, or algaculture, has existed for over half a century. More recently, algaculture for food and fuel purposes has begun the transition from R&D and pilot-scale operations to commercial-scale systems. It is crucial during this period that institutional frameworks, *i.e.* policies, support and promote development and commercialization. While the U.S. government has supported the R&D stage of algaculture for biofuels over the last few decades, it is imperative that policies anticipate and stimulate the evolution of the industry to the next level.

Large-scale cultivation of algae merges the fundamental aspects of traditional agriculture and aquaculture. Despite this overlap, algaculture has not yet been afforded an official position within agriculture or the benefits associated with it. Recognition of algaculture as part of agriculture under the USDA at national, regional and local levels will expand agricultural support and assistance programs to algal cultivation, thus

encouraging progression of the industry. The U.S. is currently the world leader in algal biomass technology and hosts a disproportionate number of companies devoted to the industry (Fig. 5-4). Continued federal support and initiatives will provide the spark needed to drive algaculture into the next stage of commercialization.

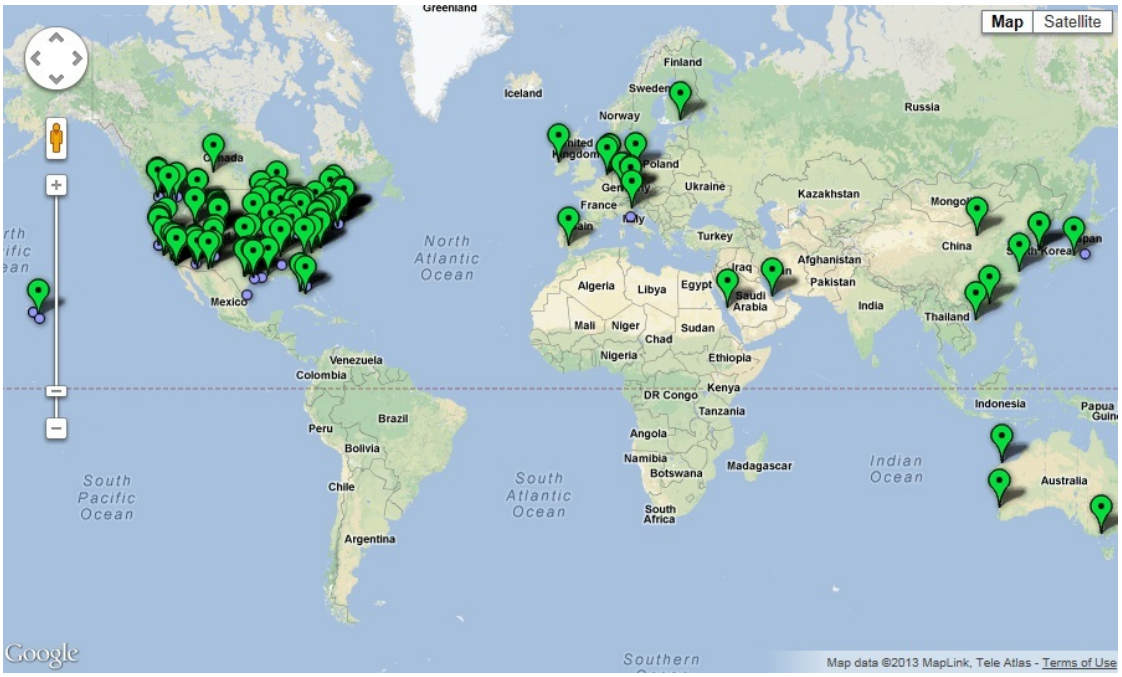


Figure 5-4. The global algal biomass industry.
Locations of algal biomass projects, production and companies around the world.

5.6 Acknowledgements

This work was completed during an industrial internship at Sapphire Energy, Inc. The internship was possible due to the NIH Marine Biotechnology Training Grant, which funded Emily Trentacoste during this work. We thank Laurie Purpuro for her help in updating sections of this report. Chapter 5, in full, has been submitted to *Photosynthesis Research* for publication in 2014. Emily M. Trentacoste, Timothy Zenk, Alice M. Martinez. The dissertation author was the primary author of this paper.

5.7 References

Agriculture & Food Act of 1981, Pub. L. no. 97-98, 95 Stat. 1213 (1981).

Agricultural Adjustment Act of 1938, Pub. L. no. 75-430, 52 Stat. 31 (1938).

Argonne National Laboratory (ANL), National Renewable Energy Laboratory (NREL), Pacific Northwest National Laboratory (PNNL). *Renewable Diesel from Algal Lipids: An Integrated Baseline for Cost, Emissions, and Resource Potential from a Harmonized Model.* ANL/ESD/12-4; NREL/TP-5100-55431; PNNL-21437. Argonne, IL: ANL; Golden, CO: NREL; Richland, WA: PNNL (2012).

AZ-H.R. 2225, 50th Legislature, 2nd Sess., (2012a).

AZ-H.R. 2226, 50th Legislature, 2nd Sess., (2012b).

Andersen RA (2013). The Microalgal Cell *Handbook of Microalgal Culture* (pp. 1-20): John Wiley & Sons, Ltd.

Ashokkumar V, Rengasamy R (2012). Mass culture of Botryococcus braunii Kutz. under open raceway pond for biofuel production. *Bioresource Technology* **104**: 394-399.

Borowitzka M (2013a). High-value products from microalgae—their development and commercialisation. *Journal of Applied Phycology* ***25***: 743-756.

Borowitzka MA, Moheimani NR, Borowitzka M (2013b). Energy from Microalgae: A Short History *Algae for Biofuels and Energy.* Springer Netherlands. Vol. 5 pp -15.

Coates R, Trentacoste E, Gerwick WH (2013). Bioactive and Novel Chemicals from Microalgae. *Handbook of Microalgal Culture*. John Wiley & Sons, Ltd. pp 504-531.

Consolidated Farm & Rural Development Act of 1961, Pub. L. no. 87-128, 75 Stat. 294 (1961).

Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Energy biotechnology / Environmental biotechnology* **19**: 235-240.

Energy Independence & Security Act of 2007, Pub. L. no. 110-140 (2007).

Falcón LI, Magallón S, Castillo A (2010). Dating the cyanobacterial ancestor of the chloroplast. *The ISME Journal* **4**: 777-783.

Fehling J, Stoecker D, Baldauf SL, Falkowski PG, Knoll AH (2007). Photosynthesis and the Eukaryote Tree of Life. *Evolutionf of primary producers in the sea.* New York: Academic Press pp 75-107.

Farm Security & Rural Investment Act of 2002*,* Pub. L. no. 107-171, 116 Stat. 134 (2002).

Federal Agriculture Improvement & Reform Act of 1996, Pub. L. 104-127, 110 Stat. 888 (1996).

Federal Crop Insurance Act of 1980, Pub. L. no. 96-365, 94 Stat. 1312 (1980).

Food and Agriculture Act of 1977*,* Pub. L. no. 95-113, 91 Stat. 913 (1977).

Food*,* Conservation, & Energy Act of 2008. Pub. L. no. 110-234, 122 Stat. 923 (2008).

Gladue R, Maxey J (1994). Microalgal feeds for aquaculture. *Journal of Applied Phycology* **6**:131-141.

Gouveia L, Marques A, da Silva T, Reis A (2009). Neochloris oleabundans UTEX #1185: a suitable renewable lipid source for biofuel production. *Journal of Industrial Microbiology & Biotechnology* **36**: 821-826.

Görs M, Schumann R, Hepperle D, Karsten U (2010). Quality analysis of commercial Chlorella products used as dietary supplement in human nutrition. *Journal of Applied Phycology* **22**: 265-276.

Ibañez E, Cifuentes A (2013). Benefits of using algae as natural sources of functional ingredients. *Journal of the Science of Food and Agriculture* **93:**703-709.

Jiang Y, Chen F, Liang SZ (1999). Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate Crypthecodinium cohnii. *Process Biochemistry* **34**: 633-637.

Khan Z, Bhadouria P, Bisen PS (2005). Nutritional and Therapeutic Potential of Spirulina. *Current Pharmaceutical Biotechnology* **6**: 373-379.

Kiple KF, Ornelas KC (2000). *The Cambridge world history of food*. Cambridge: Cambridge University Press.

Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH (2008). Exploring and exploiting carotenoid accumulation in Dunaliella salina for cell-factory applications. *Trends in Biotechnology* **26**: 631-638.

Liu X, Saydah B, Eranki P, Colosi LM, Mitchell, GB, Rhodes J, Clarens AF (2013). Pilot-scale data provide enhanced estimates of the life cycle energy and emissions

profile of algae biofuels produced via hydrothermal liquefaction. *Bioresource Technology* **148**: 163-171.

Lorenz RT, Cysewski GR (2000). Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends in Biotechnology* **18**: 160-167.

López Barreiro D, Prins W, Ronsse F, Brilman W (2013). Hydrothermal liquefaction (HTL) of microalgae for biofuel production: State of the art review and future prospects. *Biomass and Bioenergy* **53**: 113-127.

Mandal S, Mallick N (2009). Microalga Scenedesmus obliquus as a potential source for biodiesel production. *Applied Microbiology and Biotechnology* **84**: 281-291.

Mercier S (2011). *Review of U.S. Farm Programs*. Washington, D.C.

National Research Council (NRC) (2012). *Sustainable Development of Algal Biofuels in the United States*. Washington, D.C.

Pienkos PT, Darzins A (2009). The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioproducts and Biorefining* **3**: 431-440.

Pulz O, Gross W (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* **65**: 635-648.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010). Genetic Engineering of Algae for Enhanced Biofuel Production. *Eukaryotic Cell,* **9**: 486-501.

Ratledge C (2004). Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* **86**: 807-815.

Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ (2008). A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Current Opinion in Biotechnology* **19**: 430-436.

Savage N (2011). Algae: The scum solution. *Nature* **474**: S15-S16.

Schnepf R (2011). Renewable Energy Programs and the Farm Bill: Status and Issues. Congressional Research Service. 7-5700.

Sheehan J, Dunahay T, Benemann J, Roessler P (1998). *A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae*. Golden, Colorado: National Renewable Energy Laboratory.

Smith-Lever Act, ch. 79, 38 Stat. 372, 7 U.S.C. 341 et. seq. (1914).

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* **101**: 87-96.

Tamiya H (1957). Mass Culture of Algae. *Annual Review of Plant Physiology* **8**: 309-334.

Tung HF, Shen TC (1985). Studies of the Azolla pinnata—Anabaena azollae symbiosis: Concurrent growth of Azolla with rice. *Aquatic Botany* **22**: 145-152.

Tyner WE (2013). Policy Update: The US renewable fuel standard up against the wall. *Biofuels* **4**: 475-477.

U.S. DOE (2010). *National Algal Biofuels Technology Roadmap.* Washington, D.C.: Office of Energy Efficiency and Renewable Energy.

U.S. DOE (2012). *Biomass Multi-year Program Plan*. Washington, D.C.: Office of Energy Efficiency and Renewable Energy.

Wigmosta MS, Coleman AM, Skaggs RJ, Huesemann MH, Lane LJ (2011). National microalgae biofuel production potential and resource demand. *Water Resources Research* **47**: W00H04.

6.0 CHAPTER 6

CONCLUSIONS

From my characterization and manipulation of lipid catabolism emerges a complex image of the role and mechanisms involved in lipid accumulation in microalgae. Lipids are constantly being synthesized and utilized in the cell, and the view of lipid droplets as static storage entities has been robustly shattered. Understanding the physiological states that lead to lipid accumulation, the regulation of accumulation and the mechanisms behind it are all important in order to engineer strains and/or production systems capable of producing economically relevant lipid yields.

The complexity of the regulation of lipid accumulation is becoming more apparent as different parts of the lipid metabolic pathway are elucidated and manipulated. Lipid accumulation was once thought to occur as a direct response to stressful conditions, such as nutrient limitation. However, an alternate view suggests that lipid accumulation occurs in these instances because the cell cycle has stopped at a point where lipid synthesis outpaces utilization (Guckert and Cooksey 1990). The work in Chapter 3 develops this view further and shows that lipid accumulation is neither a direct response to nutrient limitation nor to cell cycle inhibition, but rather is fundamentally a result of lipid synthesis outpacing utilization.

This idea is supported by work using brefeldin A (BFA) to suppress TAG mobilization in *C. reinhardtii* (Kato et al 2013). BFA prevents vesicle formation, which is instrumental in TAG mobilization, by preventing replacement of GTP by GDP in the Arf1 complex. Arf1 is responsible for assembling vesicles and regulates TAG

mobilization. Treatment of cells with BFA leads to increased lipid body formation in a dose-dependent manner. These results further demonstrate that inhibiting TAG mobilization is sufficient to increase lipid content.

Our understanding of the mechanisms involved in lipid accumulation is also evolving. While *de novo* biosynthesis of lipids is thought to be a major contributor to lipid accumulation, the role of membrane turnover is becoming increasingly recognized as another major source of acyl groups (Goncalves et al 2013, Li et al 2012, Siaut et al 2011, Yoon et al 2012). The work in Chapter 3, and specifically the result that knock-down of the lipase/acyltransferase Thaps3_264297 leads to higher neutral and polar lipids and increased membrane intactness, further demonstrates an intimate connection between polar membrane lipid pools and neutral lipid accumulation. Supporting this, acyltransferases and phospholipases have been found in physical association with TAG lipid droplets in microalgae (Moellering and Benning 2010, Nguyen et al 2011).

Previous omics analyses, as well as the transcriptomic analysis of *T. pseudonana* described in Chapter 2, reveal the multi-level regulation of lipid accumulation and the complexity of its interconnectedness to other metabolic pathways in the cell. For example, in this study as well as previous studies, we observed differential expression of a number of lipases and phospholipases (Boyle et al 2012, Lv et al 2013, Miller et al 2010), which could be involved in membrane turnover during lipid accumulation. We also observed differential expression of long-chain acyl-CoA synthetases, suggesting that the activation of fatty acids could be a regulation point for directing lipids for modification or breakdown. Observations of continued or increased expression of ß-

oxidation genes and increased peroxisomal glyxoylate cycle gene expression suggest that lipid catabolism and lipid recycling is still active during lipid accumulation.

Chapter 2's transcriptomic analysis, as well as previous analyses, revealed that many different enzymes with similar predicted functions exist in the cell (*e.g.* lipases, DGAT), but the actual function of these enzymes may be quite different (Chen and Smith 2012, Msanne et al 2012). When performing targeted manipulation of a pathway, it is imperative to understand the distinctions between these enzymes to ensure an appropriate target is identified. Transcript or protein level analyses provide methods to distinguish the functions of homologous enzymes based on their response to specific growth conditions. For example, different DGAT isoforms show very different responses during lipid accumulation, which may provide clues as to which isoforms are involved in nutrient starvation-induced lipid synthesis (Msanne et al 2012). We used this method in Chapter 2 to predict that the lipase Thaps3_264297, which was downregulated during lipid accumulation, was involved in lipid catabolism.

It is becoming increasingly apparent that lipid biosynthesis is more dependent on substrate availability than the physiological state of the cell or the enzymes present (Collén et al 2004, Fan et al 2012, Ramanan et al 2013). This may explain why attempts to increase lipid accumulation by overexpression of biosynthetic enzymes has been thus far unsuccessful (La Russa et al 2012, Sheehan et al 1998), but increasing photon input increases TAG accumulation when nutrients are held constant (Klok et al 2013). The work in Chapter 3 shows that when TAG mobilization is disrupted photosynthesis can still provide enough substrate for continued TAG synthesis as well as other cellular functions. This contradicts the notion that carbon flux is an "either-or" scenario. If the

substrate is available, TAG will be synthesized. If synthesis outpaces utilization, TAG will accumulate.

We have demonstrated that carbon is constantly flowing through multiple pathways, including lipid biosynthesis and degradation, throughout the growth stages of the cell. This is supported by other studies, which have shown that specific TAG biosynthetic enzymes, such as PDAT in *C. reinhardtii*, are more important during exponential growth than nutrient limitation-induced lipid accumulation (Yoon et al 2012). This further demonstrates the complexity of the regulation of lipid accumulation, as different biosynthetic routes may be more active at different growth stages.

Because lipids are constantly being synthesized and utilized, the question arises of the role of TAG utilization throughout cellular growth. Some ideas concerning this issue were discussed in Chapter 1, but it is worth revisiting the function of TAG as a transient reservoir for particular fatty acyl moieties, and the use of lipid catabolism to process and alter these species. It's long been known that TAG is used to provide acyl groups for new membrane synthesis during cell division (Borowitzka 1992, Hodgson et al 1991, Klein 1987, Sukenik and Carmeli 1990), but it has recently been shown that ß-oxidation may be used to alter fatty acids in TAG as well. When yeast cells overexpressing a DGAT from *T. pseudonana* were fed exogenous DHA, a high level of DHA ß-oxidation breakdown intermediates were found in the TAG pool, suggesting that not only was ß-oxidation being used to alter the DHA before incorporation into TAG, but that this particular DGAT could use these intermediates for lipid biosynthesis (Xu et al 2013). In Chapter 2 the transcriptomic analysis showed that ß-oxidation is not downregulated in cells accumulating lipids, suggesting that it is still occurring. Fatty acid remodeling for use in

TAG synthesis may be one role of this pathway during accumulation. For example, longer chain fatty acids can be partially broken down then reincorporated into TAG. In Chapter 3 we demonstrated that certain fatty acids were disproportionately increased in knock-down strains suggesting that TAG containing these particular fatty acid species may be preferentially utilized in WT cells.

This dissertation has expanded on novel views of the regulation, role and mechanisms of lipid accumulation in microalgal cells. TAG is constantly synthesized, but accumulates for a variety of reasons including photoprotection, disrupted anabolism in response to nutrient limitation, and cell cycle arrest. TAG is also constantly utilized for a variety of functions including membrane biosynthesis for division, lipid homeostasis, and lipid and membrane remodeling in response to environmental changes such as light and temperature. An intimate connection also exists between lipid pools. Just as neutral lipids can contribute to polar membrane biosynthesis, membrane lipids can contribute to neutral lipid biosynthesis. However, the fate of carbon is not necessarily an “either-or” scenario between these different pools. As long as photosynthesis is functioning and lipid biosynthesis outpaces utilization, substrate is continually provided for the pathway and accumulation can occur without disrupting other metabolic pathways. An overview of this new picture of TAG cycling is shown in Fig. 6-1.

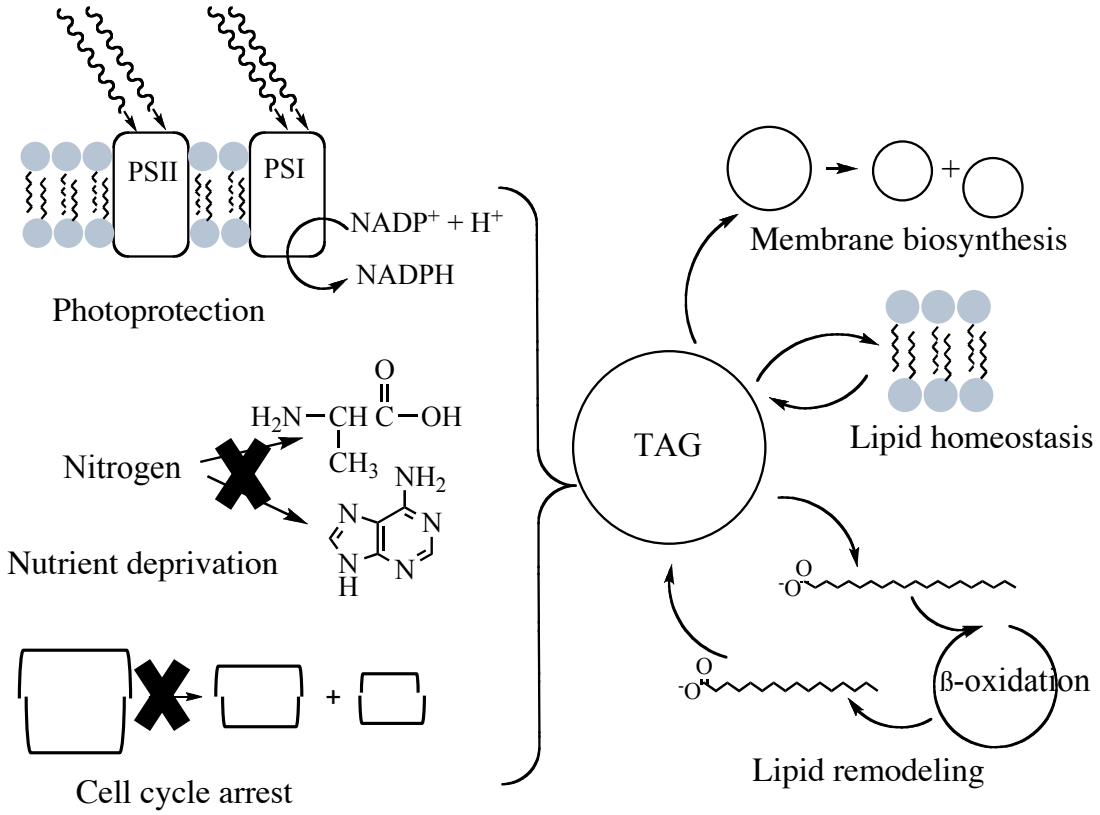


Figure 6-1. A new view of TAG cycling in microalgae.
TAG is constantly synthesized and utilized in microalgal cells. The left shows a number of situations that lead to TAG production. Excess electrons generated from photosynthesis can be used to synthesize TAG. During nutrient limitation, when anabolism of many metabolites such as amino acids and nucleotides slows, fixed carbon continues to flow into TAG. The same occurs when the cell cycle is arrested, either by nutrient limitation, pH or chemicals. The right shows uses for TAG in the cell. TAG is utilized for membrane biosynthesis and cellular division during dark periods and upon release from nutrient deprivation. TAG plays a role in lipid homeostasis by providing and reserving specific fatty acid species needed for membranes and other lipid species. Finally, fatty acids from TAG can be remodeled using ß-oxidation and either reserved or used for membrane remodeling, such as in response to rapid environmental changes like those of temperature and light. TAG accumulation occurs in a cell when the processes from the left outpace the processes on the right.

While Chapters 2 and 3 increase our understanding of lipid metabolism in microalgae, manipulations are only relevant for biofuels purposes if they can be deployed in a production system. My work in Chapter 4 demonstrated that the phenotypes imparted on knock-down strains can be used as selectable markers for native sequence transgenic strains. Knock-down constructs containing only native sequences could theoretically occur spontaneously from homologous recombination within the genome of *T. pseudonana*, thus the resulting strains are not considered GMOs in the U.S. and can bypass the lengthy permitting process for large-scale cultivation. We hypothesize that this native sequence knock-down construct can be amended to include other targets for manipulation, such as overexpression or knock-down of other endogenous genes. The Thaps3_264297 antisense sequence can be included in manipulation constructs adjacent to another overexpression or knock-down sequence, just like an antibiotic selection gene, thus conferring the lipase knock-down phenotype onto transformants. Subsequent selection of transgenics using the knock-down phenotype would allow for targeted engineering of many pathways without GMO classification.

For algal biofuels to progress to an economically relevant scale, policies supporting the industry must progress alongside scientific developments. Chapter 5 revealed that although policies exist in the U.S. that could potentially lend support to the growing algal biofuel industry, their expansion to algae is still uncertain. It will be imperative to develop clear guidelines for policies and programs supporting algal biomass cultivation.

As the algal biofuel industry grows, it becomes increasingly necessary to gain a deeper understanding of lipid metabolism in microalgae in order to manipulate strains and systems. To develop productive algal biofuel systems it is important to understand which metabolic pathways are important for lipid production and growth, how they are connected, and how they respond to various environmental and cellular cues. This work has enhanced the current knowledge of lipid metabolism by revealing the interconnectedness of pathways such as lipid catabolism, the glyoxylate cycle and photorespiration. It has shown that enzymes with similar function, such as lipases, most likely have very different roles temporally and spatially in cells. It has provided a method by which to improve strain performance by decoupling the cell cycle from lipid accumulation. Finally, it has developed new tools for the future so that looking forward we can continue to explore and modify microalgae for producing fuels. This expanded knowledge and molecular toolbox can be used to further develop production strains for the future of the algal biofuel industry.

References

Borowitzka M (1992). Algal biotechnology products and processes — matching science and economics. *Journal of Applied Phycology* **4:** 267-279.

Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J *et al* (2012). Three Acyltransferases and Nitrogen-responsive Regulator Are Implicated in Nitrogen Starvation-induced Triacylglycerol Accumulation in Chlamydomonas. *Journal of Biological Chemistry* **287:** 15811-15825.

Chen JE, Smith AG (2012). A look at diacylglycerol acyltransferases (DGATs) in algae. *Photosynthetic microorganisms for bio-fuel production from sun light* **162:** 28-39.

Collén PN, Camitz A, Hancock RD, Viola R, Pedersén M (2004). EFFECT OF NUTRIENT DEPRIVATION AND RESUPPLY ON METABOLITES AND ENZYMES RELATED TO CARBON ALLOCATION IN GRACILARIA TENUISTIPITATA (RHODOPHYTA)1 **40:** 305-314.

Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C (2012). Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in Chlamydomonas reinhardtii. *Plant and Cell Physiology* **53:** 1380-1390.

Goncalves E, Johnson J, Rathinasabapathi B (2013). Conversion of membrane lipid acyl groups to triacylglycerol and formation of lipid bodies upon nitrogen starvation in biofuel green algae Chlorella UTEX29. *Planta* **238:** 895-906.

Guckert JB, Cooksey KE (1990). TRIGLYCERIDE ACCUMULATION AND FATTY ACID PROFILE CHANGES IN CHLORELLA (CHLOROPHYTA) DURING HIGH pH-INDUCED CELL CYCLE INHIBITION1 **26:** 72-79.

Hodgson P, Henderson RJ, Sargent J, Leftley J (1991). Patterns of variation in the lipid class and fatty acid composition of Nannochloropsis oculata (Eustigmatophyceae) during batch culture. *Journal of Applied Phycology* **3:** 169-181.

Kato N, Dong T, Bailey M, Lum T, Ingram D (2013). Triacylglycerol mobilization is suppressed by brefeldin A in Chlamydomonas reinhardtii. *Plant and Cell Physiology* **54:** 1585-1599.

Klein U (1987). Intracellular Carbon Partitioning in Chlamydomonas reinhardtii. *Plant Physiology* **85:** 892-897.

Klok AJ, Martens DE, Wijffels RH, Lamers PP (2013). Simultaneous growth and neutral lipid accumulation in microalgae **134:** 233-243.

La Russa M, Bogen C, Uhmeyer A, Doebbe A, Filippone E, Kruse O *et al* (2012). Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga Chlamydomonas reinhardtii. *Photosynthetic microorganisms for bio-fuel production from sun light* **162:** 13-20.

Li X, Moellering ER, Liu B, Johnny C, Fedewa M, Sears BB *et al* (2012). A Galactoglycerolipid Lipase Is Required for Triacylglycerol Accumulation and Survival Following Nitrogen Deprivation in Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 4670-4686.

Lv H, Qu G, Qi X, Lu L, Tian C, Ma Y (2013). Transcriptome analysis of Chlamydomonas reinhardtii during the process of lipid accumulation **101:** 229-237.

Miller R, Wu G, Deshpande RR, Vieler A, Gärtner K, Li X *et al* (2010). Changes in Transcript Abundance in Chlamydomonas reinhardtii following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology* **154:** 1737-1752.

Moellering ER, Benning C (2010). RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in Chlamydomonas reinhardtii. *Eukaryotic Cell* **9:** 97-106.

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB *et al* (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae Chlamydomonas reinhardtii and Coccomyxa sp. C-169 **75:** 50-59.

Nguyen HM, Baudet M, Cuiné S, Adriano J-M, Barthe D, Billon E *et al* (2011). Proteomic profiling of oil bodies isolated from the unicellular green microalga Chlamydomonas reinhardtii: With focus on proteins involved in lipid metabolism **11:** 4266-4273.

Ramanan R, Kim B-H, Cho D-H, Ko S-R, Oh H-M, Kim H-S (2013). Lipid droplet synthesis is limited by acetate availability in starchless mutant of Chlamydomonas reinhardtii **587:** 370-377.

Sheehan J, Dunahay T, Benemann J, Roessler P (1998). A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory: Golden, Colorado. p 296.

Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P *et al* (2011). Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnology* **11:** 7.

Sukenik A, Carmeli Y (1990). LIPID SYNTHESIS AND FATTY ACID COMPOSITION IN NANNOCHLOROPSIS SP. (EUSTIGMATOPHYCEAE) GROWN IN A LIGHT-DARK CYCLE1 **26:** 463-469.

Xu J, Kazachkov M, Jia Y, Zheng Z, Zou J (2013). Expression of a type 2 diacylglycerol acyltransferase from Thalassiosira pseudonana in yeast leads to incorporation of docosahexaenoic acid β-oxidation intermediates into triacylglycerol**:** n/a-n/a.

Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012). Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga Chlamydomonas reinhardtii. *The Plant Cell Online* **24:** 3708-3724.