BIOCHEMICAL CHARACTERIZATION OF TRIACYLGLYCEROL METABOLISM IN MICROALGAE

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ABSTRACT

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Triacylglycerol (TAG) is a storage lipid with high energy density that is widely present in animals, plants, and algae. The fact that microalgae are able to accumulate TAGs has attracted scientific and public interest due to the potential to provide biofuel feed stocks. In addition, algal TAG is able to serve as a source of polyunsaturated fatty acids that show benefits for human health. Moreover, experimental manipulation of algal TAG metabolism is relatively easy, especially in the single-cellular alga *Chlamydomonas reinhardtii*, making this organism a good model for studying TAG related events in multi-cellular organisms.

Chlamydomonas reinhardtii is the most investigated green alga, with a full set of molecular tools available. Another microalga, *Nannochloropsis oceanica*, has received research interests as it has been specifically considered for biofuel production because of its naturally high TAG content. For both *C. reinhardtii* and *N. oceanica*, the process of TAG accumulation is activated by stress induction. Transcriptomic analysis of algal cells under stress conditions has been performed to understand the expression profile of TAG metabolism related genes. Moreover, forward genetics approaches have been used to identify and characterize novel genes involved in TAG accumulation. However, the mechanisms of TAG accumulation and degradation are not yet fully understood. Here, an investigation of TAG biosynthesis in stressed

microalgae by analytical and biochemical approaches is presented, providing insights into algal TAG metabolism.

A liquid chromatography-mass spectrometry (LC-MS) based TAG profiling approach was developed. This approach is able to identify all TAG species in microalgae – more than one hundred TAG species in both *C. reinhardtii* and *N. oceanica*. In addition, accurate and robust TAG quantification was achieved, in comparison with measurements by gas chromatography (GC). Notably, internal standards were identified for LC-MS and GC, respectively, during this work. Both internal standards have the potential to be applied in the analysis of a large number of lipid samples, enabling high throughput phenotypic screening strategies.

During a series of collaborative projects, lipid analysis was performed for *C. reinhardtii* under N deprivation and hypoxia stress. Based on the comparison of lipid profiles, especially TAG profiles, between N deprived and hypoxia stressed *C. reinhardtii*, a new project was initiated, during which algal TAG accumulation under hypoxia stress was biochemically characterized. Radioactive labeling experiments demonstrated that membrane lipid remodeling is the major contributor of algal TAG biosynthesis under dark hypoxia conditions. Characterization of *C. reinhardtii* mutant strains with deficiencies in *Phospholipid: Diacylglycerol Acyltransferase 1* (*PDAT1*) showed that both TAG accumulation and degradation of membrane lipids, including diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine (DGTS) were affected in mutant strains, suggesting the *PDAT1* gene is involved in algal TAG biosynthesis during hypoxia.

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Michigan State University, my first stop in this country, brought me from thousands of miles away and hosted me for more than five years. I have now become a big fan of college sports and pride as a Spartan. The Department of Biochemistry and Molecular Biology recruited me and provided me classes, activities and financial support, all of which are essential to my PhD education.

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CHAPTER 1

Triacylglycerol synthesis and related lipid metabolism in microalgae: a literature review[1]

[1]This chapter was prepared and revised based on the publication: Liu, B., Benning, C. (2013) Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology* 24 (2): 300-309. I wrote the draft and prepared both figures of this review.

1.1. Abstract

Microalgae are attracting renewed interest from both the scientific and public communities owing to their potential applications as sustainable feed stocks for the production of biofuels and high value compounds, and environmental remediation. Recent advances in molecular and biochemical analyses of microalgae point toward interesting differences in lipid metabolism between algal species and in comparison to plants. These differences range from distinct acyl groups present in algal lipids to a possible more direct role of plastids in the assembly of TAGs with consequences for the overall subcellular organization of glycerolipid metabolism. Thus, studying lipid metabolism in microalgae points to new possible avenues of genetic engineering of lipid metabolism in this organism group, and may also inform studies of lipid metabolism in plants.

1.2. Introduction

Research in the area of cell and molecular biology of microalgae has accelerated in recent years facilitated primarily by their potential as sustainable feedstock for biofuel production (Hu et al., 2008). Triacylglycerol (TAG) is a major form of stored energy in microalgae with energy-rich acyl chains approaching the energy density of hydrocarbons (Durrett et al., 2008). Therefore, TAG biosynthesis, turnover and deposition in lipid droplets have become a focus of algal research. TAG biosynthesis is part of primary cell metabolism, integrated with membrane lipid assembly and turnover, and, therefore, needs to be considered in the full context of cellular metabolism. As photosynthetic organisms, microalgae fix carbon dioxide during the day and convert it into various products, including TAGs depending on the species of alga or specific conditions such as nutrient deprivation, a process that may be reversed by respiration during the night. The details and dynamics of microalgal TAG and membrane lipid metabolism have remained largely unexplored, but this trend has been reversed in recent years as documented by the rapid increase in publications in the area (Figure 1.1).

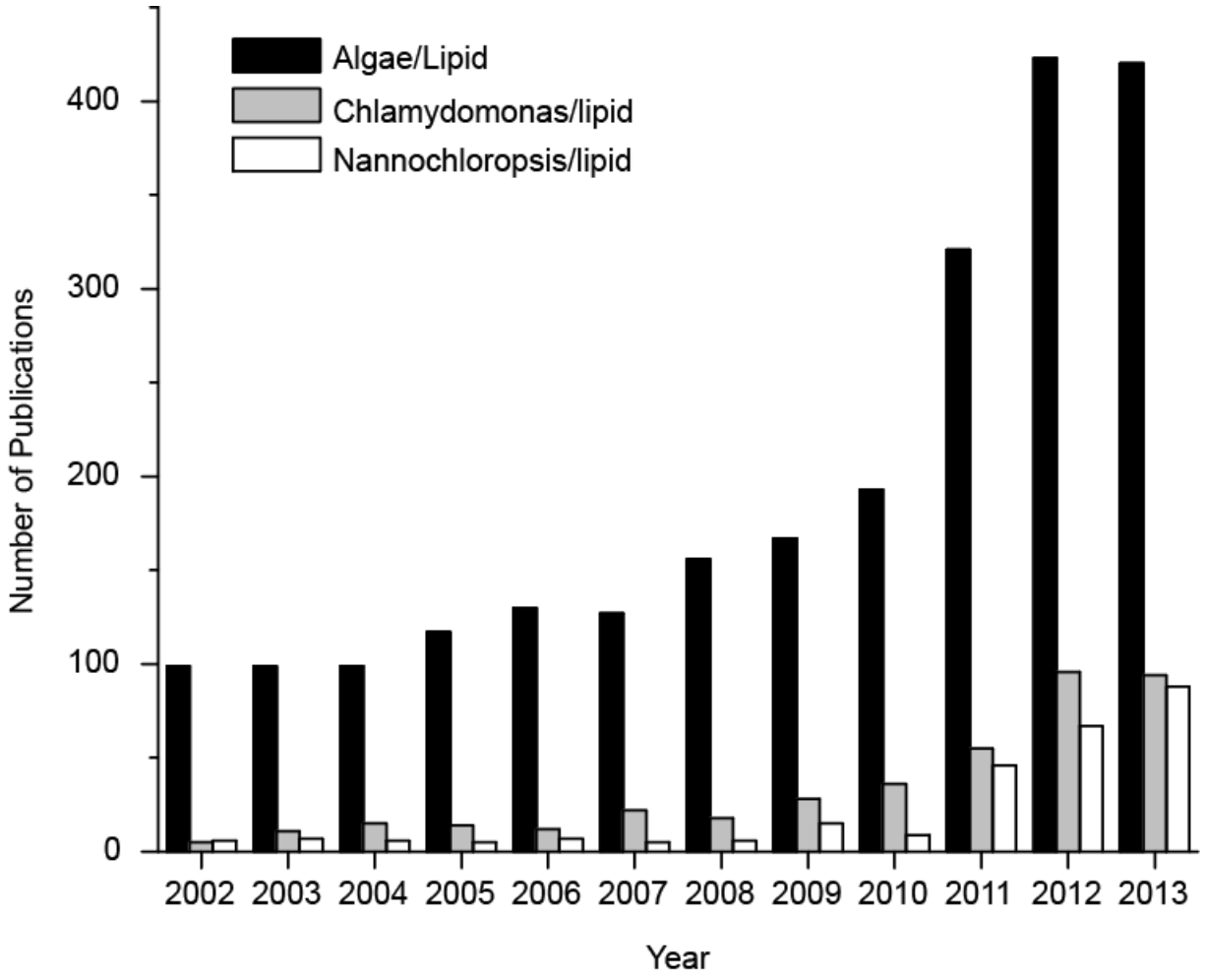

Figure 1.1. Numbers of scientific publications in the field of algal lipid research. The publication search results from Web of Science® from 2002 to 2013 are listed here. The keywords of topic were set as 'Algae' and 'Lipid', 'Chlamydomonas' and 'Lipid' and 'Nannochloropsis' and 'Lipid'. The search was performed on January 1st, 2014.

To date, more than 10 microalgal genomes have been sequenced (Blanc et al., 2010; Derelle et al., 2006; Lopez et al., 2011; Merchant et al., 2007; Palenik et al., 2007; Radakovits et al., 2012) and annotated to different extents. Among the algae species with sequenced genomes, *Chlamydomonas reinhardtii* is the most extensively studied. Although not considered as a candidate for biofuel production, Chlamydomonas currently provides the best model for algal lipid research. However, it should be noted that algae are evolutionarily highly diverse (Archibald and Keeling, 2002) and lipid metabolism is not expected to be uniform across the algal realm. Species representing different clades will likely have to be explored and compared to gain a more complete understanding of lipid metabolism across algal species under consideration for industrial exploitation. For example, algal species from genera such as Nannochloropsis, which have a higher potential for industrialized biofuel production than Chlamydomonas, are still in their infancy with regard to lipid biosynthesis research, but clearly differ from Chlamydomonas in their lipid metabolism. For example, Chlamydomonas lacks phosphatidylcholine (PtdCho) (Giroud et al., 1988) compared to other algae that have been studied, and it accumulates TAG only under stress such as nutrient depletion, while other microalgae such as Nannochloropsis already synthesize TAG under nutrient replete conditions likely instead of starch (Vieler et al., 2012). As novel hypotheses emerge for well-established model organisms such as the proposed formation of lipid droplets associated with the plastid in Chlamydomonas (Fan et al., 2011; Goodson et al., 2011) and genome sequences are being released for more species, a clearer picture of lipid metabolism in microalgae is emerging. For example, a comprehensive summary of gene identification in algal lipid metabolism was recently published (Khozin-Goldberg and Cohen, 2011).

Years of effort spent on plant models such as Arabidopsis have led to many important mechanistic insights into plant lipid metabolism. Nevertheless, plant lipid metabolism is not yet fully understood, perhaps due in part to the overwhelming complexity of lipid molecular species present in a plant cell. As will be pointed out below, many aspects of lipid metabolism in algae deviate from past findings in model plants such as Arabidopsis, and one may perhaps anticipate that discoveries in less complex, unicellular algae one day may inform our understanding of lipid metabolism in seed plants.

1.3. Molecular tool-box for algal lipid research

The fact that Chlamydomonas can be grown heterotrophically has made this microalga a popular model organism for basic photosynthesis research during the past decades (Merchant et al., 2007). Thus, the microalgal research community has develop a set of tools for Chlamydomonas over the years for the analysis of gene function that is not yet available for other microalgae (Fuhrmann, 2002). First of all, stable nuclear transformation (Kindle, 1990) in combination with a series of selectable markers (Harris, 2009) provide the basis for insertional mutagenesis approaches in Chlamydomonas, which are now being applied by many labs to the identification of genes involved in lipid metabolism. Moreover, a fully sequenced genome (Merchant et al., 2007), with improved annotation based on increasingly available RNA-seq data (Castruita et al., 2011; Fang et al., 2012; Gonzalez-Ballester et al., 2010; Lopez et al., 2011), as well as a bacterial artificial chromosome library (Nguyen et al., 2005) has facilitated mutant gene identification and phenotype complementation of Chlamydomonas mutant strains.

Targeted altering of gene expression levels by genetic engineering has been achieved in Chlamydomonas, applying RNA interference (Rohr et al., 2004) and artificial microRNA (Molnar et al., 2009; Zhao et al., 2009) techniques to decrease the RNA levels of specific genes. In addition, ectopic expression of specific nuclear transgenes in Chlamydomonas has been developed (Neupert et al., 2009). Based on these tools, reverse genetics approaches can be applied to Chlamydomonas lipid research. In addition, successful expression of codon optimized green fluorescent protein (GFP) in Chlamydomonas has been used with some success (Fuhrmann et al., 1999), suggesting an effective way to overcome the GC-rich genome and inefficiency in foreign gene expression. Moreover, GFP expression has been used to study the localization of lipid related proteins, such as a recently characterized fatty acid desaturase (Zäuner et al., 2012). However, it should be noted that none of the described tools and approaches work in all cases tested. Nuclear expression of foreign genes in Chlamydomonas remains a challenge and the generation of specific null-mutants is currently based on random insertional mutagenesis followed by large scale multiplexed PCR-based screening to identify lines with the targeted insertion (Gonzalez-Ballester et al., 2011).

As Chlamydomonas is not likely to become a species used in the production of biofuels, other microalgal species with greater production potential have recently been targeted for development of molecular tools needed to facilitate gene functional analysis and genetic engineering of metabolic pathways. For instance, for Nannochloropsis, nuclear transformation protocols have been recently published (Kilian et al., 2011; Radakovits et al., 2012). In addition, Nannochloropsis has been shown to be amenable to homologous gene replacement (Kilian et al., 2011), which should rapidly facilitate the functional analysis of genes and genetic engineering of this oleaginous algae. While algae species with biofuel potential have been receiving increasing

attention, for the time being Chlamydomonas still has the most resources available of all microalgal system and is still an excellent reference organisms to test basic principles of lipid metabolism in microalgae, in particular of the "green algal" lineage. Thus, the focus here will be on studies done with Chlamydomonas addressing mechanistic questions of lipid metabolism.

1.4. General aspects of lipid metabolism in algae

Much of our current knowledge about genes encoding enzymes involved in lipid metabolism in photosynthetic organisms is based on work with Arabidopsis (recently reviewed in (Li-Beisson et al., 2013). Adapting this plant-based knowledge to algae provided an initial framework for the tentative identification of genes presumably involved in algal lipid metabolism. As such, the network of glycerolipid metabolism was initially reconstructed in Chlamydomonas based on genomic and EST data available at that time (Riekhof et al., 2005) and was updated in 2009 (Moellering et al., 2009; Riekhof and Benning, 2009). In many cases, functional annotation suggested that Chlamydomonas has a simpler lipid metabolism network than plants (Riekhof and Benning, 2009; Riekhof et al., 2005). For instance, most predicted enzymes involved in fatty acid synthesis are encoded by single copy genes. Assuming mitochondria as well as chloroplasts require fatty acid synthesis, these enzymes might be targeted to both organelles, while in plant, specific enzymes involved in mitochondrial fatty acid biosynthesis have been identified (Li-Beisson et al., 2013). In addition, plants often have multiple genes encoding proteins with redundant enzymatic activities. For instance, Chlamydomonas has only the single copy genes *MGD1* and *DGD1* involved in the biosynthesis of the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), respectively, while the Arabidopsis genome expresses redundant activities from the

genes *MGD1*, *MGD2* and *MGD3* (MGDG biosynthesis) and *DGD1* and *DGD2* (DGDG biosynthesis). However, there are exceptions in Chlamydomonas. At least six genes predicted to encode acyl-CoA: diacylglycerol acyltransferases (DGAT) were identified in the Chlamydomonas genome, compared to two genes known to encode this activity in Arabidopsis, suggesting perhaps a prominent role for TAG biosynthesis in algal cells with a complexity that might exceed that of TAG biosynthesis in plant cells. Bulk synthesis of TAGs in microalgae generally occurs within the photosynthetically active cell, while TAG biosynthesis in plants is more prominent in specialized tissues, e.g. developing embryos or fruits that are often not photosynthetically active. On the other hand, algal TAG accumulation is usually triggered by exposure to stresses. In this regard it is similar to TAG biosynthesis in photosynthetic tissues of plants following stress exposure such as freezing (Moellering et al., 2010), when the need for membrane lipid remodeling is linked to TAG formation (Moellering and Benning, 2011). Little is known about how TAG is synthesized in plants under these stress conditions, and perhaps clues can be gathered from studying TAG metabolism in algae.

One pronounced difference between algae and plants in terms of lipid biosynthesis is that algae usually contain betaine lipids, which are thought to be able to substitute for PtdCho due to their structural similarity (Klug and Benning, 2001). Instead of a phosphate bond, the head group of betaine lipids is linked to the diacylglyceryl moiety by an ether bond. The ability to replace PtdCho with betaine lipids may allow algae to adapt to a fluctuating aquatic environment in which phosphorus is limited (Van Mooy et al., 2009). While some algal species synthesize both betaine lipids and PtdCho, Chlamydomonas produces only the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethyl-homoserine (DGTS) (Eichenberger et al., 1977).

In Arabidopsis, PtdCho is not only a structural membrane lipid, but also an important substrate for TAG biosynthesis or the modification of acyl groups that end up in the diacylglycerol backbone of other glycerolipids (Li-Beisson et al., 2013). Because of their similarities in structure and biophysical properties, DGTS might take the role of PtdCho as central intermediate in algal lipid metabolism. However, the fact that the ether bond linking the head group to the diacylglyceryl moiety in DGTS is stronger than the corresponding phosphoryl ester bond in PtdCho suggests that it might be energetically and biochemically unfavorable to convert DGTS to diacylglyceryl moieties as a precursor for other lipids such as TAGs. In addition, an enzyme directly involved in breaking the ether bond in DGTS has not yet been identified in Chlamydomonas, although, one assumes that the cell must be able to metabolize DGTS as metabolic needs change. Thus, whether DGTS has additional functions besides structural roles remains unclear. One clue was recently provided by proteomics studies (Moellering and Benning, 2010; Nguyen et al., 2011) which indicated that BTA1, the enzyme catalyzing the synthesis of DGTS (Riekhof et al., 2005), is associated in abundance with lipid droplets of Chlamydomonas. This observation suggests that DGTS contributes to the mono-layer membrane on the surface of lipid droplets in Chlamydomonas, analogous to the role phospholipids play in plants (Li-Beisson et al., 2013). However, this proposed function of DGTS will still need to be confirmed by detailed biochemical analysis of lipid droplets. If DGTS is particularly abundant in the lipid droplet peripheral membrane, the question arises how DGTS synthesis and degradation are regulated during formation and turnover of lipid droplets.

Algae are the original source for several species of polyunsaturated fatty acids prized and exploited for their health benefits (Khozin-Goldberg et al., 2011). Some aspects of fatty acid desaturation and elongation are different in algae compared to plants. For instance,

Nannochloropsis contains a considerable amount of eicosapentaenoic acid (EPA, 20:5, number of carbons : number double bonds in the acyl chain). In Chlamydomonas, several desaturases have been characterized by forward genetics approaches (Moellering et al., 2009; Riekhof and Benning, 2009) including one gene encoding a *Δ*4-desaturase associated with plastid envelopes (Zäuner et al., 2012) that is involved in the synthesis of hexadeca-4,7,10,13-tetraenoic acid (16:4). This 16:4 acyl group is nearly exclusively present in MGDG, the most abundant membrane lipid in Chlamydomonas chloroplasts. Interestingly, knock-down and over-expression of this *Δ*4-desaturase gene changed the total amount of MGDG in Chlamydomonas, altering the relative amount of 16:4 and 18:3 acyl groups in total lipid extracts in parallel. Apparently, the availability of 16:4 affects the total amount of the prevalent MGDG molecular species containing 18:3 in the *sn-1* and 16:4 in the *sn-2* position of the glycerol backbone. The molecular mechanism regulating the abundance of this particular molecular species of MGDG is unknown and there are no answers yet to the questions why 16:4 is primarily present in MGDG, or whether the respective molecular species of MGDG has a specific role in the photosynthetic membrane. Therefore, studying a loss-of-function mutant entirely lacking 16:4 or a series of mutants with decreasing amounts of this fatty acid could be rereaing.

1.5. Storage lipids in algae

In Arabidopsis, DGAT and phospholipid: diacylglycerol acyltransferase (PDAT) are two major enzymes involved in the final step of TAG synthesis (Li-Beisson et al., 2013; Zhang et al., 2009), and the same is true in Chlamydomonas (Merchant et al., 2011). There are two DGAT families identified in Chlamydomonas, type one (DGAT) and type two (DGTT), which do not

share sequence similarity (Deng et al., 2012; Merchant et al., 2011). In the Chlamydomonas genome, aside from the one *DGAT1* gene, 5 *DGTT* genes were annotated as *DGTT1* through *DGTT5* (Miller et al., 2010). Two independent studies showed the expression level of *DGAT1* and *DGTT1* increased considerably following nitrogen (N) deprivation (Boyle et al., 2012; Miller et al., 2010), a condition typically applied to algal cultures to induce TAG accumulation. Moreover, the mRNA levels of *DGAT1* and *DGTT1* increased under other nutrient stress conditions such as deprivation of sulfur, phosphorus, zinc, and iron, which like N-depletion also led to TAG accumulation (Boyle et al., 2012). Heterologous expression of *DGTT1* and *PDAT*, respectively, in an acyltransferase-deficient yeast strain could rescue TAG synthesis, providing indirect biochemical evidence for a possible role of these two enzymes in TAG biosynthesis (Boyle et al., 2012). Knock-down and over-expression of three *DGTT* genes individually in Chlamydomonas (corresponding to *DGTT1*, *DGTT3* and *DGTT4*, respectively (Miller et al., 2010)), showed a corresponding change in TAG levels (Deng et al., 2012). However, when in a separate study three *DGTT* genes (corresponding to *DGTT1*, *DGTT2* and *DGTT3* (Miller et al., 2010)) were over-expressed individually, neither total lipid nor neutral lipid levels increased in the transgenic lines (La Russa et al., 2012). These different results could be due to different levels of transgene expression in the two different studies and differences in the methods used to quantify TAGs. Taken together, at this time it still remains unclear which DGATs are primarily responsible for the accumulation of TAGs following nutrient deprivation, or whether individual isoforms have specific roles. Loss-of-function mutations in the individual genes and combinations thereof, subcellular localization of the proteins and biochemical analysis of purified individual DGAT proteins for their substrate preference may be necessary to answer these questions.

In many plants, the biosynthesis of thylakoid lipids originates from both the plastid and endoplasmic reticulum (ER) pathways (Mongrand et al., 1998). However, based on labeling studies and molecular species analysis of lipids in Chlamydomonas, it appears that the plastid pathway contributes exclusively to the *de novo* biosynthesis of thylakoid lipids (Giroud et al., 1988). One reason may be that PtdCho, a proposed intermediate in lipid trafficking between the ER and the plastid in plant cells (Li-Beisson et al., 2013), is absent from Chlamydomonas. The key distinguishing feature between lipids assembled by the ER pathway versus the plastid pathway in plants is the presence of a 16-carbon acyl group in the *sn-2* position of the glycerol backbone in lipids derived from the plastid pathway and an 18-carbon acyl group at the same position of lipids assembled at the ER (Heinz and Roughan, 1983). This is thought to be due to the difference in substrate specificity of acyltransferases at the ER versus the plastid envelopes. However, it should be noted that this class of enzymes has not been studied in algae in detail at this time, and it is possible that the plant paradigm of two pathways being involved in lipid synthesis and assembly may not occur universally in microalgae.

TAG assembly also appears to differ in plants and algae. In plants, the assembly of large amounts of storage TAGs occurs at the ER (Li-Beisson et al., 2013), but in a recent study in Chlamydomonas it was hypothesized that a large fraction of TAGs is assembled *de novo* by the chloroplast pathway following N deprivation (Fan et al., 2011). In particular, position-specific lipase treatment of TAGs determined that the *sn*-2 position of TAGs was mostly occupied by 16-carbon fatty acids, suggesting that the DAG moieties of these TAGs have been assembled through the chloroplast pathway, assuming that the above mentioned criterion for distinguishing plastid and ER-derived molecular species holds true for algae. In this context it is also intriguing to note that electron micrographs of starch-less Chlamydomonas cells showed large lipid droplets

inside chloroplasts (Fan et al., 2011; Goodson et al., 2011). Thus it seems likely that TAG metabolism in Chlamydomonas involves the plastid in ways not observed in plants, perhaps with the exception of TAG synthesis following freezing in plant tissues (Moellering et al., 2010), and that TAG molecules are assembled in the plastid envelopes exclusively or in parallel to assembly at the ER as summarized in the model in Figure 1.2. As fatty acids are synthesized in the plastid, assembling acyl groups into TAGs in the plastid envelopes would make sense. In fact, the absence of PtdCho in Chlamydomonas may impact the export of fatty acids from the plastid. In plants, the plastid PtdCho pool is rapidly labeled with nascent fatty acids prior to the appearance of acyl groups in the cytosolic acyl-CoA pool, implicating a PtdCho-based acyl exchange mechanism during acyl group export (Bates et al., 2007). Due to the absence of PtdCho in Chlamydomonas, the question of which lipid substitutes for this function of PtdCho arises when acyl groups have to be exported for extraplastidic lipid assembly. Assembling at least a fraction of the TAGs at the envelopes closer to the source of fatty acid biosynthesis inside plastids might compensate for differences in the export efficiency of acyl groups in plants and Chlamydomonas. If both chloroplast-derived and ER-derived lipid droplets are present in Chlamydomonas, one must ask can they be distinguished physically or can one identify TAG assembly enzymes involved in the two pathways? Because all known Chlamydomonas DGATs lack plastid targeting sequences, at least based on prediction using programs trained on plant chloroplast sequences, one would have to assume that the encoded enzymes have access to the outer envelope. Alternatively, it is also possible that entirely novel enzymes are involved in the assembly of TAG inside Chlamydomonas plastids.

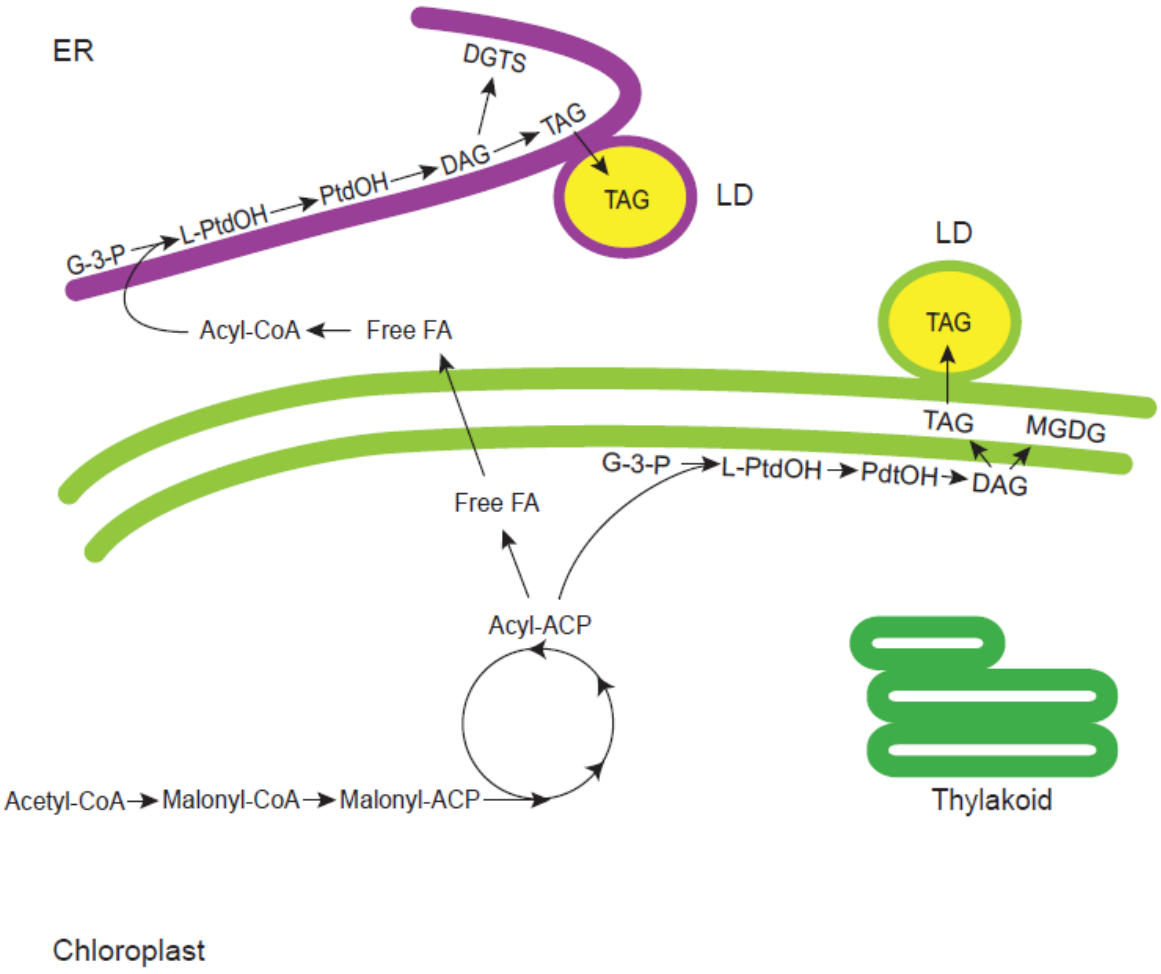

Figure 1.2. Hypothesis for TAG biosynthesis in green alga *Chlamydomonas reinhardtii*. Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FA, fatty acid; G-3-P, glycerol-3-phosphate; LD, lipid droplet; L-PtdOH, Lyso-phosphatidic acid; MGDG, monogalactosyldiacylglycerol; PtdGro, phosphatidylglycerol; PtdOH, phosphatidic acid; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

1.6. Lipid droplets in algae

To date, lipid droplets remain among the least characterized organelles, as their biological importance had not been recognized until recently (Farese and Walther, 2009). While the understanding of lipid droplet dynamics in algae is still lacking compared to mammalian lipid droplets, algal lipid droplets have garnered increasing attention as storage organelles for biofuel molecules (Merchant et al., 2011). The formation of lipid droplets in microalgae is triggered by cellular stresses (Hu et al., 2008), such as nutrient deprivation, temperature fluctuation and high light exposure. Among those stresses, N deprivation is easily accomplished through a change in growth medium and is a commonly used approach to experimentally induce lipid droplet formation. It should be noted that this process is easily reversed by replenishing N in the growth medium. Thus, the ease of manipulating the lipid droplet status of a cell by simply changing the growth medium makes microalgae excellent models to study fundamental mechanisms of lipid droplet biogenesis or mobilization, perhaps with relevance for processes involving lipid droplets in multicellular organisms. Detailed structural information and correlative physiological data throughout the formation of lipid droplets are already available for Chlamydomonas (Goodson et al., 2011; Wang et al., 2009). In addition, several independent lipid droplet-focused proteomic studies (James et al., 2011; Moellering and Benning, 2010; Nguyen et al., 2011) indicated the presence of a highly abundant, major lipid droplet protein (MLDP) with presumed perilipin (Kimmel et al., 2010) functions, the betaine lipid synthase BTA1, and other lipid metabolism related proteins. The presence of MLDP orthologs appears to be limited to the green algal clade (Peled et al., 2011). The abundance, hydrophobic properties of MLDP, and its relatively small size (28 kDa) suggest that MLDP might function as a structural component on the surface of the lipid droplet. Preliminary support for this hypothesis was derived by RNA interference silencing

of the *MLDP* gene of Chlamydomonas, which led to an enlargement of lipid droplets, although the amount of TAG per cell remained unchanged. Similarly, when the expression of the lipid droplet associated protein of plant embryos, oleosin, was suppressed, lipid droplets in Arabidopsis seedlings became larger (Siloto et al., 2006). Recently, a major lipid droplet surface protein (LDSP) of the oleogenic heterokont *Nannochloropsis oceanica* was identified and characterized (Vieler et al., 2012). This protein has a similar size, hydrophobicity and predicted secondary structure as plant oleosins, even though both proteins are unrelated at the sequence level. Heterologous expression of Nannochloropsis *LDSP* in the Arabidopsis oleosin-deficient mutant *oleo1* restored the lipid droplet size but did not complement other physiological phenotypes, such as a delay of TAG degradation in *oleo1* seedlings. This result demonstrated that LDSP and oleosin share structural roles, but have additional species-specific functions. If both are involved in the recruitment or regulation of different enzymes involved in lipid synthesis or degradation, the required protein-protein interactions and binding partners might be species-specific, explaining the observed incomplete heterologous complementation result. In fact, this observation could be the basis for separately studying structural and biochemical functions of these two proteins in ways previously not possible.

1.7. Lipid synthesis as part of primary metabolism in algae

Maximizing oil yield in microalgae by engineering will require a fundamental understanding of the integration of lipid metabolism into the overall metabolic network of the microalgal cell. Toward this end, efforts have been initiated to reconstruct the primary metabolism of Chlamydomonas and to apply metabolic modeling to probe the energy

requirements for photoautotrophic growth (Kliphuis et al., 2012). Moreover, system-wide perturbations in Chlamydomonas metabolic networks in response to the availability of ammonium have been explored at the metabolite and protein levels (Lee et al., 2012). In the future, combining these global approaches with genetic perturbation of specific aspects of the metabolic network should enable engineers to develop strategies for the optimization of microalgal oil production. Already, more focused efforts have succeeded in altering carbon partitioning. It is well known that starch and TAG accumulate following N deprivation in algae (Hicks et al., 2001; Hu et al., 2008; Siaut et al., 2011). Thus, it was hypothesized that carbon partitioning could be affected by blocking the pathway of starch synthesis, leading to the diversion of carbon into TAGs and, hence, increased TAG yields. Indeed, increases in oil accumulation in a starch-less mutants have been observed for Chlamydomonas (Li et al., 2010a; Li et al., 2010b; Work et al., 2010) and Chlorella (Ramazanov and Ramazanov, 2006). Recently, a more detailed analysis (Fan et al., 2012) showed that in wild-type Chlamydomonas, TAG only accumulated after the maximum amount of starch was reached. In the starch-less Chlamydomonas mutant, TAG accumulation in response to N deprivation was initiated earlier and continued to a higher level than in the wild-type strain. Thus, photosynthate can be directly channeled into TAG biosynthesis in green algae as, perhaps, is already the case in Nannochloropsis, which does not accumulate starch. Thus, one viable approach towards the engineering of high TAG yielding algal strains is the elimination of competing carbon utilization pathways to maximize channeling of photosynthate into lipid biosynthesis.

1.8. Omics studies provide insights into algal metabolism

The development of next generation sequencing has facilitated genome sequencing and genome-wide analysis of gene expression in all organisms, including microalgae, and the availability of algal genome and transcriptome data is rapidly increasing. Comparison of transcriptome information for wild-type and mutant algal strains, or a single strain, grown in different conditions, provides a platform for in-depth gene annotation. Although mRNA levels are not always a direct predictor of the activity of the respective gene product, tentative correlations between gene-expression and metabolism can become apparent leading to useful hypotheses that can be corroborated by further experimentation. Ultimately, transcriptome-based studies have the promise of leading to the identification of regulatory principles such as transcription factors or protein kinases, which ultimately can become targets for the engineering of oil yield in algae. For example, to explore the mechanisms underlying the induction of TAG biosynthesis in microalgae, a Chlamydomonas transcriptome study was performed comparing global gene expression in N-replete and N-deprived Chlamydomonas cells (Miller et al., 2010). Generally, genes involved in TAG biosynthesis, such as DGATs, were increased in expression in N-deprived cells. Most notably, putative lipase-encoding genes were highly regulated following N deprivation. Among 130 putative lipase-encoding genes, 27% increased and 8.5% decreased in transcript levels by 2-fold or more following N deprivation. Presumably, the expression level of genes encoding lipases involved in degrading TAG should decrease following N deprivation, while membrane lipid lipase encoding genes may be up-regulated in order to allow conversion of membrane lipids to precursors for TAG synthesis or remodeling of the photosynthetic membrane in response to nutrient stress. The same study (Miller et al., 2010) also showed that following N deprivation, Chlamydomonas cells change their metabolism from converting acetate to glucose to a more direct incorporation of acetate into fatty acids by down-regulating glyoxylate cycle

activity and gluconeogenesis. The direct analysis of metabolite fluxes was consistent with the observed changes in expression of genes encoding key enzymes of the glyoxylate cycle and gluconeogenesis, validating the general predictive power of global transcriptome studies with regard to changes in metabolism. Typically, Chlamydomonas in the laboratory is grown photoheterotrophically in the presence of acetate as in this study, and information on photoautotrophic conditions mimicking more closely an oil production situation is still very limited (Tevatia et al., 2012).

One advantage of the increasing number of algal transcriptome studies is that one can compare the co-expression of a group of genes under different conditions. This approach was successful in identifying a potentially novel N response regulator, NRR1 (Boyle et al., 2012). The *NRR1* gene encodes a protein with a conserved DNA binding domain, and NRR1, therefore, is predicted to be a transcription factor. The expression of *NRR1* was activated following N deprivation. In addition, the expression patterns of *NRR1*, *DGTT1*, and *AMT1D*, encoding an ammonium transporter, were highly similar. Based on this evidence it was suggested that *NRR1* is a regulatory gene involved in N assimilation and TAG accumulation. Consistent with this, the respective Chlamydomonas *nrr1* mutant accumulated only half of the amount of TAG found in the corresponding parental strain following N deprivation (Boyle et al., 2012).

For Chlamydomonas, transcriptome information for various genetic backgrounds and different conditions is available (Boyle et al., 2012; Castruita et al., 2011; Fang et al., 2012; Gonzalez-Ballester et al., 2010; Miller et al., 2010) and steadily increasing, and an analysis tool to compare expression similarity among different conditions and genetic backgrounds has become available (Lopez et al., 2011). In addition, transcriptome studies in other algal species are being completed as well, allowing comparisons across species. For example, the metabolic

pathways of the green alga *Dunaliella tertiolecta*, which is also considered for biofuel production, were recently reconstructed based on RNA-seq data (Rismani-Yazdi et al., 2011). In this microalga, a full set of genes encoding essential enzymes involved in fatty acid synthesis, TAG synthesis and other metabolic pathways were identified. Interestingly, many transcripts annotated in this study had no orthologs in Chlamydomonas. In summary, aside from more focused genetic and biochemical approaches to investigate lipid metabolism in algae, the application of transcriptome analysis is increasingly aiding the identification of genes relevant to lipid accumulation and particularly the regulation of and integration of lipid metabolism in algae.

1.9. Conclusion

The advantages and limitations of algae as feedstocks for biofuel production have recently been evaluated (Jones and Mayfield, 2012). While screening algae species for high lipid productivity is important for improving biodiesel production (Griffiths and Harrison, 2009), understanding the basic principles of lipid biosynthesis and metabolism is critical for developing genetic engineering approaches and cultivation methods to boost algal lipid production. Several aspects of acyl lipid metabolism are different in microalgae and plants, which necessitates basic research into algal lipid metabolism. It seems likely that some of the established paradigms for plant lipid metabolism will not be directly applicable to lipid metabolism in microalgae. It also seems possible that research in microalgae may one day help close existing gaps in our knowledge of lipid metabolism in plants. Towards this end, research focused on intracellular lipid trafficking in microalgae, i.e. the import and export of lipid precursors by plastids, might be particularly rewarding. In this context it is revealing that Chlamydomonas lacks PtdCho, a

central metabolite of lipid metabolism in plants, yet can synthesize all the necessary lipids, including TAGs. The lack of PtdCho may have required adjustments of lipid metabolism in Chlamydomonas or this may be a unique property of the laboratory strain(s) of Chlamydomonas currently widely used and, thus, one might wonder whether Chlamydomonas is indeed the best representative green microalga for studies of lipid metabolism. The mechanistic studies of lipid metabolism in other green algae and oleogenic algae from different clades underway will determine the role of DGTS and PtdCho and will provide a fascinating picture of the diversity and adaptability of lipid metabolism in photosynthetic organisms.

The regulation of TAG synthesis and break down is a stress response rather than a developmental phenomenon in microalgae and, thus, the mechanisms are likely different in microalgae and developing plant seeds, where TAG biosynthesis has been most intensively studied. However, it should be noted that green tissues in plants accumulate oils when stressed as well (Moellering et al., 2010), and a better understanding of the regulation of lipid metabolism in algae may motivate new hypothesis-driven studies of stress-induced TAG accumulation in plants. As the least characterized organelle, the mechanisms of lipid droplet formation and turnover are poorly understood for any organisms or cell type. It seems likely that new discoveries in the field of microalgal lipid metabolism will fill gaps in our understanding of lipid metabolism in general and demonstrate the evolutionary diversity of lipid metabolism in algae. Thus, basic studies of lipid metabolism in algae will not only benefit the future engineering of sustainable feedstocks for fuels, food and specialized chemicals, but also provide insights into lipid droplet formation and turnover in general.

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CHAPTER 2

Triacylglycerol profiling of microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* using ultrahigh performance liquid chromatography-mass spectrometry[2]

[2]Data in this chapter has been published in: Liu, B., Vieler, A., Li, C., Jones, A.D., Benning, C. (2013) Triacylglycerol profiling of microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica*. *Bioresource Technology* 146: 310-316. I performed all experiments and wrote the draft of the paper.

2.1. Abstract

Triacylglycerols (TAGs) from microalgae can serve as a feedstock for the production of biofuels. To gain a comprehensive understanding of TAG metabolism in algae through genetic and molecular approaches, and to improve algal biofuel production, efficient and quantitative phenotyping methods focusing on TAGs are required. Towards this end, a facile ultrahigh performance liquid chromatography–mass spectrometry protocol was developed for TAG profiling, achieving identification and quantification of intact TAG molecular species in two algae. TAG profiling was performed in *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* grown in nitrogen (N)-replete or N-depleted medium. For the quantification of algal TAGs and fatty acids, two sets of internal standards were developed by taking advantage of the presence of pheophytin and specific fatty acids in algal samples. Comparison of algal TAG levels was simplified by using these internal standards for TAG analysis, paving the way for high-throughput mutant screening.

2.2. Introduction

Triacylglycerol (TAG) is a high energy storage compound that accumulates in animals, plants, and algae (Merchant et al., 2012), and considerable cellular resources are devoted to its synthesis and degradation (Liu and Benning, 2013). Recently, the ability of microalgae to accumulate TAG has attracted scientific and public interest due to their potential to provide biofuel feed stocks (Hu et al., 2008). The choice of unicellular microalgae as models has opened up the manipulation of TAG biosynthesis and turnover in a more facile way than is currently possible for animal and plant cells. Consequently, obtaining accurate quantitative TAG composition data and fatty acyl group profiles of TAGs in microalgae are of particular interest.

Chlamydomonas reinhardtii is the most investigated microalgal species as it has a sequenced genome (Merchant et al., 2007), an increasing number of transcriptome data sets (Lopez et al., 2011), and an extensive set of molecular tools available (Liu and Benning, 2013). Forward (Li et al., 2012b) and reverse genetics strategies have been successfully developed and applied, for instance (Li et al., 2012a; Yoon et al., 2012), to characterize the function of genes involved in TAG production in Chlamydomonas. Furthermore, as the possibility of producing biofuel directly from Chlamydomonas has been discussed (James et al., 2011), findings in Chlamydomonas pointing to unique mechanisms involved in TAG metabolism (Li et al., 2012b) have become particularly relevant.

Microalgae from the heterokont genus Nannochloropsis have been specifically considered for biofuel production because of their naturally high TAG content. In Nannochloropsis, a starch synthesis pathway is absent, leading to the accumulation of TAGs as a major product of photosynthetic carbon fixation (Vieler et al., 2012a). To date, genome

sequences are available for two species, *Nannochloropsis gaditana* (Radakovits et al., 2012) and *Nannochloropsis oceanica* (Pan et al., 2011; Vieler et al., 2012b). In addition, nuclear transformation (Vieler et al., 2012b) and targeted gene replacement by homologous recombination (Kilian et al., 2011) have recently been reported for *Nannochloropsis sp.*, which opens up Nannochloropsis to molecular approaches for gene functional studies and engineering.

From among several possible stress conditions, N deprivation is commonly used to induce TAG accumulation in microalgae. Quantitative analyses of TAG and membrane lipids during the time course of N deprivation have been reported (Boyle et al., 2012). Moreover, in Chlamydomonas, global gene expression has been compared between N-replete and N-deprived cells in at least two transcriptomic studies (Boyle et al., 2012; Miller et al., 2010), showing that genes encoding enzymes involved in TAG biosynthesis, such as DGAT1 and PDAT1, are regulated during N deprivation. In addition, formation of lipid droplets during N deprivation has been extensively studied at the cell biological and physiological levels (Wang et al., 2009) and also by applying proteomics and metabolomics analyses (Moellering and Benning, 2010; Nguyen et al., 2011; Xiao et al., 2013). Thus, as N deprivation-induced TAG accumulation in microalgae is increasingly probed at many levels, there is a need for accurate and expeditious methods for TAG profiling in algal reference species such as Chlamydomonas and Nannochloropsis.

With regard to the analysis of TAGs in algae, a frequently used, quantitative approach is based on gas chromatography-flame ionization detection (GC-FID) following conversion of TAGs to fatty acid methyl esters (FAMEs). However, TAG molecules are disassembled during transesterification, leaving the actual molecular species composition of the TAG pool in the cell unresolved. Detailed molecular analysis of intact TAG species is useful for drawing conclusions

regarding the biochemical origin of TAG molecules. Even though the absolute positional analysis of fatty acyl groups attached to the glyceryl backbone is beyond common mass spectrometry practice, applying a mass spectrometry-based TAG profiling approach that can distinguish different molecular species can provide information regarding their biochemical origin. Recent analytical efforts demonstrated the application of TAG profiling to algal lipid extracts by direct mass spectrometry (MS) (Danielewicz et al., 2011) and liquid chromatography-mass spectrometry (LC-MS) (Kind et al., 2012; MacDougall et al., 2011). During direct MS analysis, chlorophylls were removed by solid phase extraction prior to MS to avoid signal interference (Danielewicz et al., 2011). It was determined that without any separation, oxidation of polyunsaturated fatty acyl groups in algal TAGs would be minimized during the direct MS analysis. On the other hand, using LC-MS approaches, TAGs and membrane lipids were separated and identified from several microalgal species, providing information on lipid profiles for the identification of algal strains suitable for biofuel applications (Kind et al., 2012; MacDougall et al., 2011). However, two reference species *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* have not yet been directly compared using TAG profiling techniques. In addition, robust quantification of algal TAGs by LC-MS has not yet been reported, but would be desirable, in particular in a high-throughput mode for mutant identification.

Here the focus is on the microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* CCMP1779 to develop a comprehensive TAG profiling approach by ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-MS), by which all TAG species accumulating in the algae can be identified. TAG quantification using different approaches was compared. Furthermore, pheophytin and the ratio of two fatty acids are explored

as simple internal standards suitable for quantitative high throughput mutant screen strategies for model algae species.

2.3. Materials and Methods

2.3.1. Algae strains and culture conditions

Chlamydomonas reinhardtii cell wall-less strain dw15-1 (cw15, nit1, mt+) was obtained from Arthur Grossman and is referred to as wild type. The Chlamydomonas *pgd1* mutant strain was generated during a previous mutant screen project using dw15-1 as the parental strain (Li et al., 2012b). *Nannochloropsis oceanica* CCMP1779 was originally obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (https://ncma.bigelow.org/). Chlamydomonas cells were grown in Tris-acetate-phosphate (TAP) medium (Harris, 2009). Nannochloropsis cells were grown in f/2 medium with 2.5 mM nitrate medium (Vieler et al., 2012a). N deprivation was used to elicit TAG accumulation in both algae by omitting N-containing salts from the medium. Algal cells during log-phase growth were collected by centrifugation (3000 x g, 4 ℃, 5 min), washed with sterilized water and resuspended in the corresponding medium without N (TAP -N or f/2 -N) for further growth. Both Chlamydomonas and Nannochloropsis were grown in a shaker at 100 rpm under continuous light (80 μmol m^{-2} s^{-1}) at 22 ℃. While harvesting algal cultures, cell densities were determined using a Z2 Coulter Counter (Beckman-Coulter, https://www.beckmancoulter.com) with a 100 mm aperture according to the manufacturer instruction. For the purpose of total fatty acid measurement, 1 mL cell culture was harvested by filtration through a circular glass filter (GF/A, 25 mm, Whatman,

http://www.whatman.com). Quantification of chlorophylls in Chlamydomonas was performed as previously described (Li et al., 2012b).

2.3.2. Dry weight determination

A procedure modified from (Zhu and Lee, 1997) was used to determine dry weight of algal cells. Briefly, circular glass filters (GF/F, 25mm, Whatman) were pre-dried in a 120 ℃ oven overnight and their weights were determined. Algal cells (10 mL) were loaded on the center of the filter and the medium was removed under vacuum. Then, 20 mL washing buffer (0.5 M NH_4HCO_2) were applied to the filter. Filtered cells were lyophilized overnight and weighed again. Biomass dry weight was calculated as the difference between the two weight measurements.

2.3.3. Lipid extraction

For Chlamydomonas and Nannochloropsis, cell pellets were collected from at least 10 mL mid-log-phase N-replete or N-deprived algal cultures by centrifugation (3000 x *g*, 5 min) and resuspended in 1 mL lipid extraction solvent (methanol: chloroform: formic acid, 2:1:0.1). Following the addition of 0.5 mL extraction buffer (1 M KCl, 0.2 M H_3PO_4) the solution was vigorously mixed. Cell debris was removed by centrifugation for 3 min at 13,780 x *g*. The lower organic phase constituted the lipid extract and was further subject to lipid analysis.

2.3.4. TAG fatty acyl group analysis by gas chromatography

Lipid extraction, TAG separation and fatty acyl transesterification were performed according to (Moellering and Benning, 2010). Briefly, algal lipid extract was loaded onto Si60 silica TLC plates (EMD Millipore, http://www.emdmillipore.com) and developed with 100 mL solvent (petroleum ether: diethyl ether: acetic acid, 8:2:0.1). After separation, TAGs were subjected to transmethylation. Then, FAMEs were extracted, dried and redissolved for GC analysis. GC-FID was modified from a previous study (Rossak et al., 1997). Briefly, a capillary DB-23 column (length 30 m, diameter 0.25 mm, film thickness 0.25 μm; Agilent Technologies, http://www.agilent.com) was heated as follows: initial temperature 140 ℃, increased by 25 ℃/min to 160 ℃, then by 8 ℃/min to 250 ℃, and held at 250 ℃ for 4 min.

2.3.5. TAG profiling by UHPLC-MS

Algal lipid extracts were diluted with methanol: chloroform (1:1) and mixed with 2 μM of the TAG standard glyceryl triheptadecanoate (51:0, number of carbons in fatty acids: number of double bonds, 17:0/17:0/17:0, Sigma-Aldrich, http://www.sigmaaldrich.com), before separation by UHPLC. Samples were analyzed on a QTRAP™ 3200 mass spectrometer from Applied Biosystems/MDS Sciex (Concord, ON, Canada) coupled to Shimadzu LC-20AD pumps and SIL-HTc autosampler (http://www.shimadzu.com). Chromatographic separations employed a Supelco Ascentis Express C18 column (5 cm x 2.1 mm, 2.7 μm, Supelco, http://www.sigmaaldrich.com/Supelco) held at 30 ℃. The mobile phase consisted of Solvent A: 10 mM ammonium acetate in water: acetonitrile (10:90 v/v) and Solvent B: 20 mM ammonium acetate in isopropanol. Separation was conducted using a flow rate of 0.4 mL/min, with gradient

elution as follows: 15% B from 0-5 min, followed by a linear increase to 55% B at 42.5 min, followed by a sudden step to 100% B and a hold at 100% B for 2.5 min. At 45 min, the solvent returned to the initial conditions. For each analysis, a volume of 5 μL was injected. The mass spectrometer was operated in positive ion mode for monitoring $[M+NH_4]^+$ ions using a TurboIonSpray ion source. Enhanced Product Ion (EPI) scanning was employed for the identification of algal TAG species. A collision potential of 40 V was used for EPI scans to generate product ions for structure elucidation. Other settings used were (arbitrary units if not specified): curtain gas, 10; ion spray voltage, 4000 V; temperature, 500 ℃; gas 1, 15; gas 2, 15; declustering potential, 60 V; entrance potential, 10 V; and CAD gas, HIGH. Q1 Multiple Ion Monitoring was used for quantitative analysis. The HPLC system and mass spectrometer were controlled by Analyst 1.4.2 software (AB SCIEX).

2.4. Results and Discussion

2.4.1. TAG profiling of Chlamydomonas and Nannochloropsis

In this study, we attempted to identify all TAG species in algal lipid samples by UHPLC-MS/MS. In addition, a UHPLC-MS approach was developed for TAG quantification, confirmed by quantitative comparisons with GC-FID measurements.

2.4.1.1. Identification of algal TAG species by UHPLC-MS/MS

Lipids extracted from algae were separated by UHPLC followed by MS detection. Membrane lipids and other more polar compounds in the lipid extracts eluted during the first 10

minutes and all TAG species between 10 and 40 minutes. It is anticipated that TAGs with the same equivalent carbon number (ECN) elute at similar retention times while TAGs with larger ECN elute later in reverse phase liquid chromatography (Buchgraber et al., 2004). UHPLC gradients were optimized using the ECN as guidance. Based on the ECN, non-TAG compounds were ruled out, which had the same mass, but different retention time as specific TAG molecular species. Several mass spectrometry scan types were used to identify all TAG species from the two algae. First, enhanced mass spectra (ion trap) scanning was employed during the optimization of liquid chromatography conditions, as well as to obtain general information about TAG retention times and signal abundances (peak areas). Next, tandem mass spectrometry (MS/MS) enhanced product ion spectra and constant neutral loss scans were generated to confirm diagnostic TAG fragments indicative of acyl group length and degree of unsaturation. In this step, the elution time of UHPLC was shortened to expedite proceedings. Applying these approaches, the TAG molecular species in the extracts were annotated.

A total of 140 TAG species were identified in N-deprived Chlamydomonas cells (Table 2.1). It should be noted that additional TAG species might have been present in algal extracts, but were below the current method limit of detection. However, TAG molecular species summarized in Table 2.1 represent a complete list of TAGs in Chlamydomonas following N deprivation as annotated in this study. Most of the acyl groups attached to the glycerol backbone in TAGs were 16-carbon (C16) and 18-carbon (C18) acyl groups, consistent with a previous analysis of bulk lipid FAMEs (Li et al., 2012b). Interestingly, tandem mass spectrometry identified short chain (C14) and long chain (C20) acyl groups in TAGs as well as odd-numbered chain acyl groups (C15 and C19). Those acyl groups might be too low in abundance to be detected by GC-FID.

In Nannochloropsis, 102 TAG species were found by UHPLC-MS/MS analysis (Table 2.2). Acyl groups 12:0, 20:0, 20:1, 20:2 and 20:3 were identified in addition to those previously detected by GC-FID of FAMEs (Vieler et al., 2012b). In Nannochloropsis, a high degree of unsaturation was mainly observed in 20-carbon acyl groups, for instance, 20:4 and 20:5. On the other hand, shorter chain acyl groups were relatively more saturated in Nannochloropsis compared to Chlamydomonas. These results are interpreted to suggest that the long chain acyl groups with lower desaturation, for instance, 20:0, 20:1, 20:2 and 20:3, are intermediates in the fatty acid desaturation pathway, which generally involves desaturation of acyl groups attached to membrane lipids prior to TAG assembly. The presence of these low abundant more highly saturated fatty acids implies that they may be derived from alternative desaturation and elongation pathways other than the recently proposed main pathway involving the elongation of partially desaturated 18-carbon fatty acids only (Vieler et al., 2012b). Similar to Chlamydomonas, TAGs containing odd numbered acyl groups were detected in Nannochloropsis, but with higher abundance. About 5% of total TAGs contained odd numbered acyl groups, consistent with a previous report (Gelin et al., 1997).

2.4.1.2. Quantification of algal TAGs by UHPLC-MS

One challenge of mass spectrometry-based lipidomics is achievement of accurate quantification of the different TAG molecular species, since authentic and certified reference standards of all TAGs are not commercially available. Despite similar structures, TAG molecules with different numbers of carbons or double bonds per chain have different ionization efficiencies. In a related study (MacDougall et al., 2011), authors suggested that it was not

possible to quantify all TAG molecular species in parallel in a sample by LC-MS because response factors for different TAG standards varied by up to 50%. The same conclusion was reached in this study, by including eight different commercially available TAG standards. In addition, the signals from different TAG molecular species can interfere with each other, especially when chromatographic overlap results in suppression of ionization. However, for practical purposes only one external TAG standard, glyceryl triheptadecanoate, was included for quantification purposes on a weight basis accepting the introduction of an error based on differences in the response factors which, however, may average out across the different TAG species. Using a relatively long elution time, TAGs with different ECNs were sufficiently separated to avoid signal interference. All TAG isomers with the same number of carbons and double bonds were combined during quantification, as non-TAG compounds were excluded due to their lack of chromatographic overlap. Finally, to confirm the accuracy of TAG quantification by UHPLC-MS, the results were compared with a GC-FID measurement. For this purpose, Chlamydomonas cells were harvested 0, 12, 24, and 48 hours after N deprivation, followed by lipid extraction. Lipid extracts were subjected to both UHPLC-MS and GC-FID analyses. Comparison of the two quantification methods is summarized in Figure 2.1. Chlamydomonas displayed a negligible amount of TAGs during growth on N-replete medium (Moellering and Benning, 2010), and began to accumulate TAGs only following N deprivation. Both methods provided comparable TAG quantifications and the differences between the two methods decreased over time as the total TAG content increased. N-replete and 40-hours-N-deprived Nannochloropsis cells were subjected to TAG quantification as well. Quantifications by UHPLC-MS gave lower values (e.g. by 38% for the 40 h time point in Figure 2.1B) compared to those done by GC-FID, which is consistent with the results obtained for the Chlamydomonas

samples (e.g. for the 24 h time point in Figure 2.1A, quantification by UHPLC-MS showed 40% lower amounts than that done by GC-FID). The above results suggest that TAG quantification by UHPLC-MS is internally robust and comparable, within reason, to the quantification by GC-FID. The consistent differences in values between the two quantification approaches could be due to the different ionization efficiencies of TAGs during UHPLC-MS that were not accounted for as mentioned above. In addition, it is possible that a higher fraction of nonspecific background signals were misclassified as FAMEs by GC-FID compared with UHPLC-MS. As a result, earlier time points of N deprivation with generally lower TAG levels showed greater relative difference between GC-FID and UHPLC-MS. Lastly, sample treatment, reference standard and data processing are distinct between the two approaches, contributing to the difference in absolute amounts obtained by the two different methods.

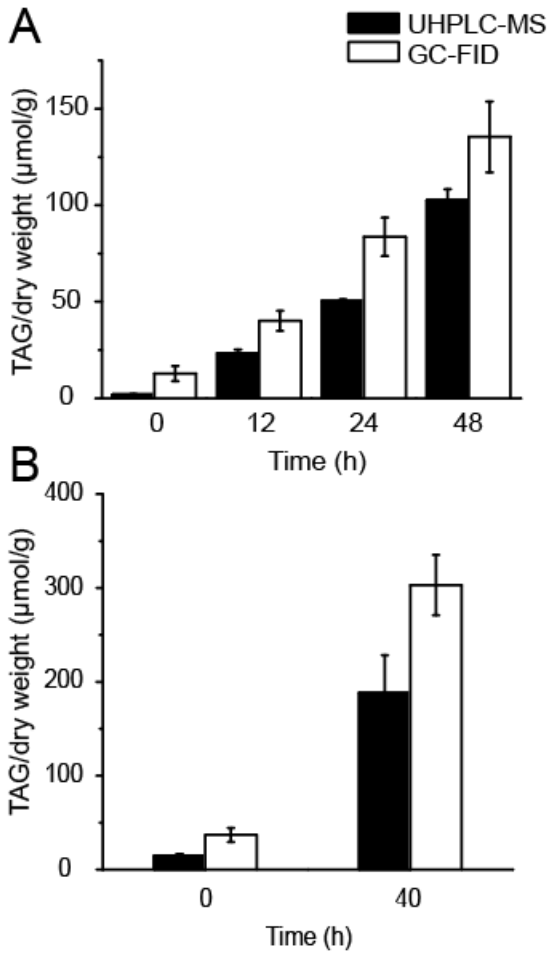

Figure 2.1. Comparison of algal TAG quantifications using UHPLC–MS and GC–FID. N deprived Chlamydomonas (A) and Nannochloropsis (B) were harvested during the time course and subjected to TAG quantification by both UHPLC–MS and GC–FID. Quantification results are normalized based on cell dry weight. The average of at least three measurements and standard deviation are shown.

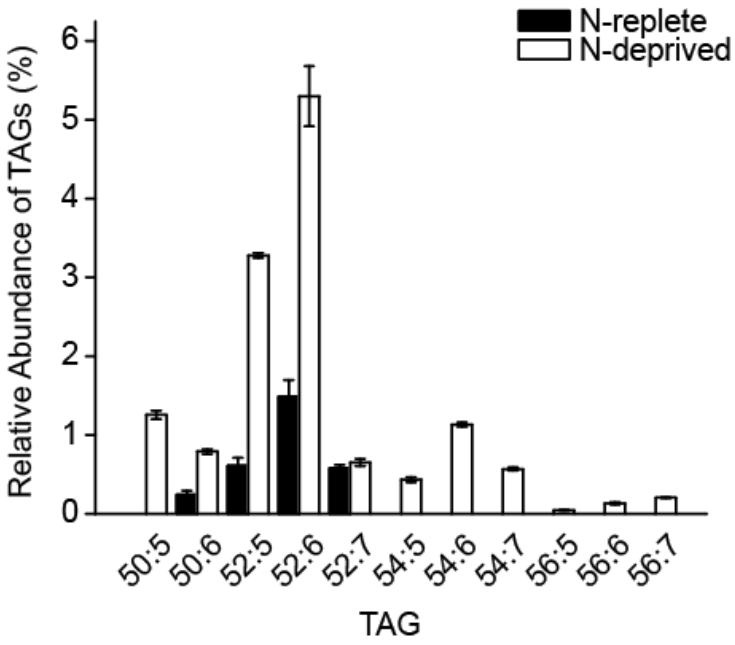

Figure 2.2. Relative abundance of polyunsaturated TAG molecular species in Nannochloropsis. Based on UHPLC–MS quantification, the ratios of TAGs with at least five double bonds over total TAGs are compared between N-replete and 40-h-N deprived Nannochloropsis cells. Each TAG represents the sum of all isomers with the same number of carbons and double bonds. The average of at least three measurements and standard deviation are shown.

Not being able to accumulate starch, Nannochloropsis cells contained a substantial amount of TAGs during favorable growth conditions likely to store energy reserves in non-carbohydrate form (Liu and Benning, 2013; Vieler et al., 2012b). On the other hand, Nannochloropsis increasingly accumulated TAG following N deprivation in a similar manner as Chlamydomonas. Comparisons were drawn between N-replete and N-deprived Nannochloropsis cells for both TAG molecular species detected by UHPLC-MS, as well as the TAG acyl group profiles obtained by GC-FID of FAMEs. As Table 2.3 shows, long chain polyunsaturated fatty acyl groups (LCPUFAs) were not present in the TAG fraction as determined by GC-FID when Nannochloropsis was grown in N-replete medium, while 20:4 and 20:5 were detected in TAGs isolated from N-deprived cells. A similar conclusion can be drawn based on TAG molecular species profiles (Figure 2.2). TAG molecular species with a total of more than five acyl double bonds in the molecule are clearly more abundant in N-deprived Nannochloropsis cells. These results suggest that while LCPUFAs were not present in *de novo* synthesized TAGs as energy reserve during normal growth, LCPUFAs entered the TAG pool in response to N deprivation, perhaps by channeling acyl groups from membrane lipids that are rich in LCPUFAs into TAGs.

2.4.2. Internal standards for high-throughput TAG profiling in Chlamydomonas.

2.4.2.1. Pheophytin as a normalization standard in UHPLC-MS

As the key pigment of photosynthesis, chlorophyll *a* is present in green algae such as Chlamydomonas. During lipid extraction as used in this study, the magnesium ion in the chlorophyll *a* molecule was released due to the acidification by formic acid present in the extraction solvent, giving rise to pheophytin *a* (Figure 2.3). Due to its similar polarity and

molecular weight as algal TAG, pheophytin is abundant in lipid extracts of Chlamydomonas subjected to UHPLC-MS (Figure 2.4). Similarly, a recent study showed pheophytin *a* and other chlorophyll derivatives were observed in algal crude oils samples by mass spectrometry during lipid profiling (Lee et al., 2013). Assuming that the amount of chlorophyll is relatively independent of TAG biosynthesis and stable in Chlamydomonas cells, the pheophytin peak in UHPLC-MS can serve as a normalization standard to compare the relative amount of TAG in algae, for example, when comparing different mutant and wild-type strains. To test this idea, wild-type Chlamydomonas and the low-TAG *pgd1* mutant strain (Li et al., 2012b) were subjected to 48 hours N deprivation, followed by lipid extraction and UHPLC-MS analysis. Figure 2.4 shows the signals for representative TAGs as well as pheophytin for the two strains. The ratio of the TAG signals over the pheophytin signal can serve as a measure to compare relative differences in TAG content in the two strains. To confirm the observations made by UHPLC-MS analysis, chlorophyll measurements and TAG quantification by GC-FID were performed. Consistent with published data (Li et al., 2012b), GC-FID results showed that the *pgd1* mutant contained less than 50% TAG of wild-type Chlamydomonas after 48 h of N deprivation, while the amount of chlorophyll *a* in wild-type (0.41 $\pm$ 0.05 pg/cell) and *pgd1* (0.48 $\pm$ 0.03 pg/cell) strains were similar after 48 h of N deprivation. This TAG/pheophytin *a* ratio provides a robust parameter for the high-throughput UHPLC-MS screening of lipid samples from a mutagenized population. In such a mutant screen, sample preparation will be simplified by bypassing the requirement for using comparable cell numbers or by omitting the accurate addition of external standards. Moreover, for screening purposes the elution time can be shortened to increase throughput. Compared with current mutant screen strategies, the UHPLC-MS-based screening approach provides additional phenotyping information such as TAG

composition. Chlorophyll content declines during N deprivation (Li et al., 2012b). Thus, a drawback to this approach is that TAG mutants cannot be identified when they are also affected differently in the synthesis or turnover of the photosynthetic membrane relative to the wild type during N deprivation, leading to differences in chlorophyll *a* and, therefore, pheophytin *a* content between the wild type and the respective mutant.

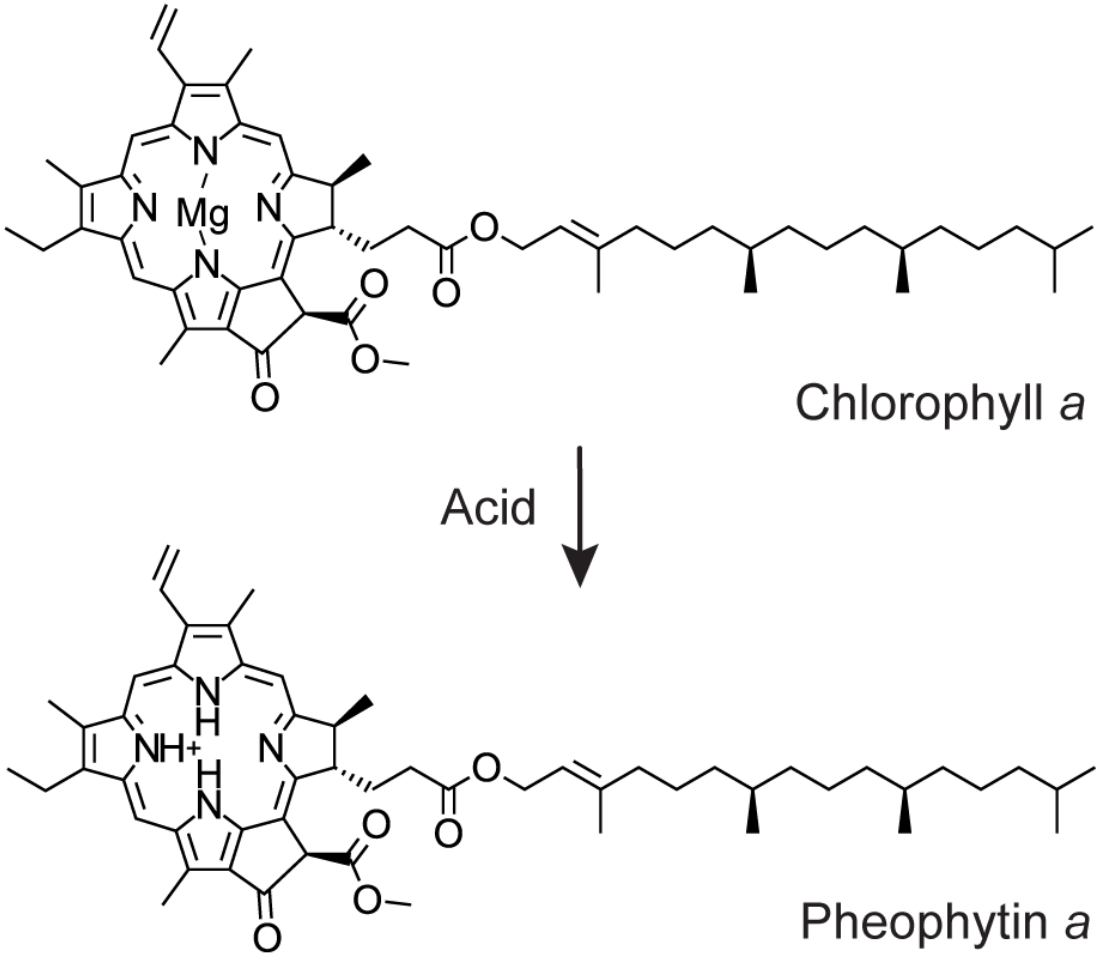

Figure 2.3. Transformation of chlorophyll *a* to pheophytin *a* in an acidic buffer. Pheophytin *a* is single positively charged with a mass-to-charge ratio (*m/z*) of 871.6. Pheophytin *a* with one heavy isotope (predominantly ^{13}C) distributed through the molecule (isotopologue abundance = 40% of the monoisotopic composition), was detected in the same Q1 Scan as TAG 52:4, which has an *m/z* of 872.8.

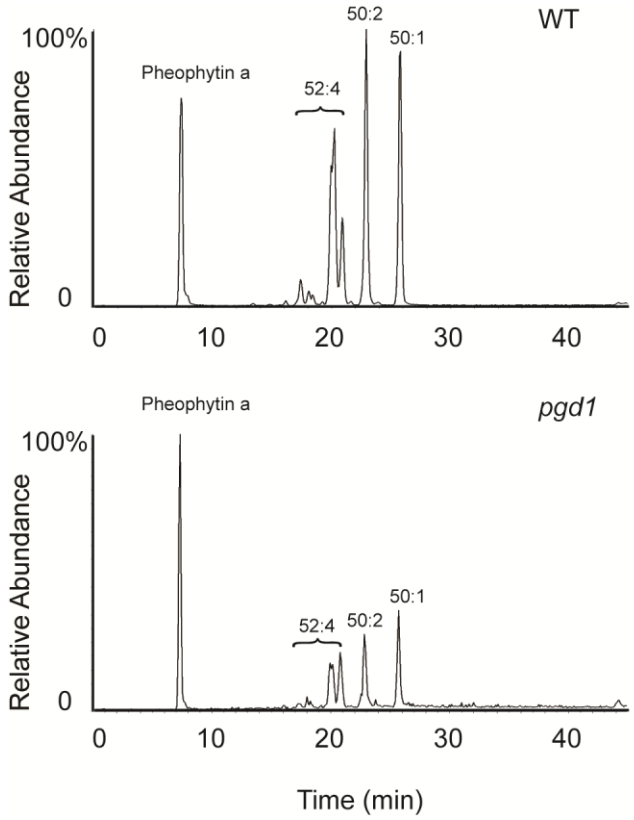

Figure 2.4. Pheophytin *a* as an internal standard in UHPLC–MS. Q1 Multiple Ion Monitoring chromatograms are compared for extracts of 48-h-N-deprived wild-type Chlamydomonas and the *pgd1* strain, a low TAG mutant. Three representative TAGs were selected and their chromatograms were overlaid, including 50:1, 50:2 and 52:4.

2.4.2.2. The ratio of two fatty acyl groups as an internal standard in GC-FID

GC-based FAME analysis has been widely used for the quantification of TAGs or the isolation of membrane lipid or TAG biosynthesis mutants in Chlamydomonas (Li et al., 2012b). Recently, it was reported that algal lipids could be quantified after one step *in situ* transesterification of wet algal materials (Patil et al., 2013). However, to quantify TAG, lipids are usually extracted and separated by TLC prior to FAME preparation and analysis, which can be time consuming. Thus, an improved and simplified high-throughput GC-FID-based analysis is desirable for screening mutant algal strains with abnormal TAG levels.

TAG accumulation is usually accompanied by increases in the *de novo* fatty acid synthesis in microalgae (Miller et al., 2010; Vieler et al., 2012b). It has been reported that under stress conditions such as N deprivation, a substantial amount of newly synthesized fatty acids in Chlamydomonas cells contributes to the increase in TAG content (Fan et al., 2011; Li et al., 2012b). Consequently, the relative abundance of 16:0, one of the newly synthesized fatty acids, increases in both TAG and the total lipid fraction under these conditions (Boyle et al., 2012). Alternatively, newly synthesized fatty acids enter the membrane lipid assembly pathway and undergo fatty acid desaturation. In Chlamydomonas, 16:4 is one of the fatty acid desaturation products, exclusively attached in the *sn*-2 position of monogalactosyldiacylglycerol under N-replete conditions (Zäuner et al., 2012). This lipid is primarily found in the photosynthetic membrane and due to its physiological importance, the amount of 16:4 is stable in Chlamydomonas cells following N deprivation, when a small fraction is also found in TAG (Boyle et al., 2012). Thus, the ratio of two fatty acids, 16:0 over 16:4, in total Chlamydomonas fatty acids reflects, within limitations, the ratio of acyl groups in TAGs over total fatty acids in the extract. This idea was tested during a time course following N-deprivation of wild-type

Chlamydomonas. A tight correlation (r^2 = 0.986) between the 16:0-to-16:4 ratio in FAMEs derived directly from algal cells and the TAG-to-total acyl group ratio was observed (Figure 2.5). The dataset represents the time points following N deprivation. Therefore, to estimate the TAG amount in Chlamydomonas by GC-FID of FAMEs, it is no longer necessary to isolate TAG from lipid extracts during sample preparation, as the 16:0-to-16:4 ratio can be obtained by total FAMEs analysis. This principle can be easily applied to a large scale screen for TAG mutants of Chlamydomonas. Similar to the UHPLC-MS-based high throughput strategy described above, it is not necessary to know the algal cell concentration or to add an external standard. This method can be applied to other microalgae to estimate changes in TAG accumulation. For instance, the 16:0-to-20:5 acyl ratio in Nannochloropsis lipid extracts could be easily determined for mutant screening purposes.

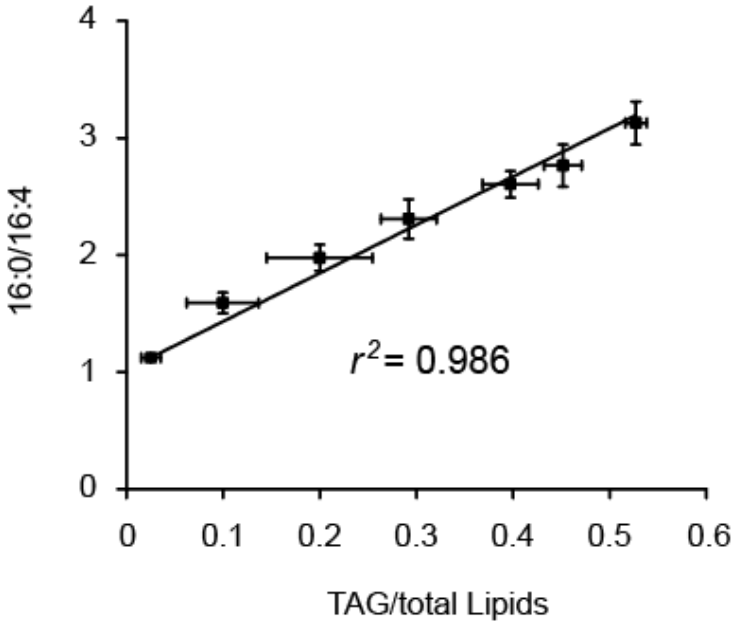

Figure 2.5. Correlation between the Chlamydomonas TAG-to-total-lipid ratio and the 16:0-to-16:4 fatty acid methyl ester ratio. To generate the TAG-to-total-lipid ratio, TAG was isolated from lipid extracts by TLC separation and both TAG and total lipid were subjected to the transesterification reaction and then quantified by GC–FID. The TAG-to-total-lipid ratio was calculated on a weight basis. To calculate the 16:0-to-16:4 ratio, algal cells were directly subjected to the transesterification reaction, followed by GC–FID to quantify both 16:0 and 16:4, generating the mole ratio of the two fatty acid methyl esters. Data points from left to right represent the wild-type samples collected from nitrogen deprivation at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h, respectively. The average of three measurements and standard deviation are shown.

2.5. Conclusion

Complete TAG species profiling was achieved by UHPLC-MS, identifying all TAG species from Chlamydomonas and Nannochloropsis. Algal TAG profiling provides not only information on TAG molecular species composition, but also clues regarding the pathway(s) of fatty acid synthesis and TAG metabolism during stress conditions. It was also demonstrated that UHPLC-MS and GC-FID-based FAME analyses provide a similar quantification of TAG content of lipid extracts. In addition, two sets of internal standards for monitoring relative TAG levels were described, allowing simpler processing for large scale sample analysis during UHPLC-MS and GC-FID-based approaches.

Table 2.1. TAG species in N-deprived Chlamydomonas reinhardtii detected using UHPLC-MS and UHPLC-MS/MS.

TAG	Fatty Acids	TAG	Fatty Acids	TAG	Fatty Acids
54:1	20:0/18:1/16:0	52:5	18:0/18:1/16:4	50:5	18:1/16:1/16:2
54:1	20:1/18:0/16:0	52:5	18:1/18:1/16:3	50:5	18:2/18:3/14:0
54:2	20:0/18:1/16:1	52:5	18:1/18:2/16:2	50:5	18:2/16:0/16:3
54:2	20:0/18:2/16:0	52:5	18:1/18:3/16:1	50:5	18:3/16:0/16:4
54:2	20:1/18:1/16:0	52:5	18:1/18:4/16:0	50:6	18:1/16:1/16:4
54:3	18:1/18:1/18:1	52:5	18:2/18:2/16:1	50:6	18:2/16:0/16:4
54:3	20:0/18:3/16:0	52:5	18:2/18:3/16:0	50:6	18:3/18:3/14:0
54:3	20:1/18:2/16:0	52:6	18:0/18:2/16:4	50:6	18:3/16:0/16:3
54:3	18:0/18:0/18:3	52:6	18:0/18:4/16:2	50:6	18:3/16:1/16:2
54:4	20:1/18:3/16:0	52:6	18:1/18:1/16:4	50:7	18:1/16:3/16:3
54:4	18:0/18:1/18:3	52:6	18:1/18:2/16:3	50:7	18:2/16:1/16:4
54:4	18:1/18:1/18:2	52:6	18:2/18:2/16:2	50:7	18:2/16:2/16:3
54:5	20:0/18:1/16:4	52:6	18:2/18:3/16:1	50:7	18:3/16:0/16:4
54:5	18:0/18:1/18:4	52:6	18:2/18:4/16:0	50:7	18:3/16:1/16:3
54:5	18:1/18:1/18:3	52:6	18:3/18:3/16:0	50:8	18:1/16:3/16:4
54:5	18:1/18:2/18:2	52:7	18:0/18:3/16:4	50:8	18:2/16:2/16:4
54:6	20:1/18:1/16:4	52:7	18:1/18:2/16:4	50:8	18:3/16:1/16:4
54:6	18:0/18:3/18:3	52:7	18:1/18:3/16:3	50:8	18:4/16:0/16:4
54:6	18:1/18:1/18:4	52:7	18:2/18:2/16:3	50:9	18:1/16:4/16:4
54:6	18:1/18:2/18:3	52:7	18:2/18:3/16:2	50:9	18:2/16:3/16:4
54:6	18:2/18:2/18:2	52:7	18:3/18:3/16:1	50:9	18:3/16:2/16:4
54:7	20:1/18:2/16:4	52:7	18:3/18:4/16:0	50:10	18:2/16:4/16:4
54:7	20:2/18:1/16:4	52:8	18:1/18:3/16:4	50:10	18:3/16:3/16:4
54:7	18:0/18:3/18:4	52:8	18:2/18:3/16:3	50:11	18:3/16:4/16:4
54:7	18:1/18:3/18:3	52:8	18:3/18:3/16:2	48:0	16:0/16:0/16:0
54:7	18:2/18:2/18:3	52:9	18:1/18:4/16:4	48:1	18:1/16:0/14:1
54:8	20:1/18:3/16:4	52:9	18:2/18:3/16:4	48:1	16:0/16:0/16:1
54:8	18:1/18:3/18:4	52:9	18:3/18:3/16:3	48:2	18:1/16:1/14:0
54:8	18:2/18:3/18:3	52:10	18:2/18:4/16:4	48:2	18:2/16:0/14:0
54:9	18:2/18:3/18:4	52:10	18:3/18:3/16:4	48:3	18:1/16:2/14:0
54:9	18:3/18:3/18:3	52:11	18:3/18:4/16:4	48:3	18:2/16:1/14:0
54:10	18:3/18:3/18:4	50:0	18:0/16:0/16:0	48:3	18:3/16:0/14:0
52:0	20:0/18:0/16:0	50:1	18:1/16:0/16:0	48:4	18:1/16:3/14:0

Table 2.1. (cont'd)

TAG	Fatty Acids	TAG	Fatty Acids	TAG	Fatty Acids
52:1	20:1/16:0/16:0	50:2	18:1/16:0/16:1	48:4	18:2/16:2/14:0
52:1	18:0/18:1/16:0	50:2	18:2/16:0/16:0	48:4	18:3/16:1/14:0
52:2	18:0/18:1/16:1	50:3	18:1/18:2/14:0	48:4	18:4/16:0/14:0
52:2	18:0/18:2/16:0	50:3	18:1/16:0/16:2	48:4	16:0/16:0/16:4
52:2	18:1/18:1/16:0	50:3	18:1/16:1/16:1	48:5	18:1/16:4/14:0
52:3	18:0/18:1/16:2	50:3	18:2/16:0/16:1	48:5	18:2/16:3/14:0
52:3	18:0/18:3/16:0	50:3	18:3/16:0/16:0	48:5	18:3/16:2/14:0
52:3	18:1/18:1/16:1	50:4	18:1/16:0/16:3	48:6	18:2/16:4/14:0
52:3	18:1/18:2/16:0	50:4	18:1/16:1/16:2	48:7	18:3/16:4/14:0
52:4	18:0/18:1/16:3	50:4	18:2/18:2/14:0		
52:4	18:1/18:1/16:2	50:4	18:2/16:1/16:1	53:1	19:0/18:1/16:0
52:4	18:1/18:2/16:1	50:4	18:3/16:0/16:1	53:2	19:1/18:1/16:0
52:4	18:1/18:3/16:0	50:4	18:4/16:0/16:0	39:1	18:1/16:0/15:0
52:4	18:2/18:2/16:0	50:5	18:1/16:0/16:4	39:2	18:1/16:0/15:1

Table 2.2. TAG species identified in *Nannochloropsis oceanica* using UHPLC-MS and MS/MS.

TAG	Fatty Acids	TAG	Fatty Acids	TAG	Fatty Acids
56:1	24:0/16:0/16:1	52:3	20:3/16:0/16:0	46:0	16:0/16:0/14:0
56:6	20:4/18:1/18:1	52:3	18:1/18:1/16:1	46:1	18:1/14:0/14:0
56:6	20:5/18:0/18:1	52:3	18:1/18:2/16:0	46:1	16:0/16:1/14:0
56:7	20:3/18:2/18:2	52:4	20:3/16:0/16:1	46:2	16:1/16:1/14:0
56:7	20:4/18:1/18:2	52:4	20:4/16:0/16:0	44:1	16:0/16:1/12:0
56:7	20:5/18:1/18:1	52:4	18:1/18:3/16:0	44:1	16:1/14:0/14:0
54:1	22:0/16:0/16:1	52:4	18:2/18:2/16:0	51:2	19:1/16:0/16:1
54:1	20:0/18:0/16:1	52:5	20:3/16:1/16:1	51:2	18:1/18:1/15:0
54:1	20:0/18:1/16:0	52:5	20:4/16:0/16:1	51:2	18:1/17:0/16:1
54:1	18:0/18:0/18:1	52:5	20:5/16:0/16:0	51:2	18:1/17:1/16:0
54:2	18:0/18:1/18:1	52:6	20:5/18:1/14:0	51:3	20:3/16:0/15:0
54:3	18:1/18:1/18:1	52:6	20:5/16:0/16:1	51:3	19:2/16:0/16:1
54:4	20:3/18:0/16:1	52:7	20:5/16:1/16:1	51:3	18:1/18:1/15:1
54:4	20:3/18:1/16:0	50:0	20:0/16:0/14:0	51:3	18:1/18:2/15:0
54:4	20:4/18:0/16:0	50:0	18:0/16:0/16:0	51:3	18:1/17:1/16:1
54:4	18:1/18:1/18:2	50:1	18:0/16:0/16:1	49:0	18:0/16:0/15:0
54:5	20:3/18:2/16:0	50:1	18:1/16:0/16:0	49:0	17:0/16:0/16:0
54:5	20:4/18:1/16:0	50:2	18:1/16:0/16:1	49:1	18:0/16:1/15:0
54:5	20:5/18:0/16:0	50:3	20:3/16:0/14:0	49:1	18:1/16:0/15:0
54:5	18:1/18:2/18:2	50:3	18:1/16:1/16:1	49:1	17:0/16:0/16:1
54:6	22:6/16:0/16:0	50:3	18:2/16:0/16:1	49:1	17:1/16:0/16:0
54:6	20:5/18:1/16:0	50:4	20:3/16:1/14:0	49:2	18:1/16:1/15:0
54:6	18:1/18:2/18:3	50:4	20:4/16:0/14:0	49:2	17:0/16:1/16:1
54:6	18:2/18:2/18:2	50:4	18:2/16:1/16:1	49:2	17:1/16:0/16:1
52:0	20:0/16:0/16:0	50:4	18:3/16:1/16:0	47:0	18:0/15:0/14:0
52:0	18:0/18:0/16:0	48:0	18:0/16:0/14:0	47:0	17:0/16:0/14:0
52:1	20:0/16:0/16:1	48:0	16:0/16:0/16:0	47:0	16:0/16:0/15:0
52:1	18:0/18:1/16:0	48:1	18:1/16:0/14:0	47:1	18:1/15:0/14:0
52:2	20:0/16:1/16:1	48:1	16:1/16:0/16:0	47:1	17:1/16:0/14:0
52:2	20:1/16:0/16:1	48:2	16:0/16:1/16:1	47:1	16:0/16:0/15:1
52:2	20:2/16:0/16:0	48:2	18:1/16:1/14:0	47:1	16:0/16:1/15:0
52:2	18:0/18:1/16:1	48:3	18:2/16:1/14:0	47:2	16:0/16:1/15:1
52:2	18:1/18:1/16:0	48:3	16:0/16:1/16:2	47:2	16:1/16:1/15:0
52:3	20:2/16:0/16:1	48:3	16:1/16:1/16:1	45:0	16:0/15:0/14:0

Table 2.3. TAG fatty acyl group content of N-replete Nannochloropsis compared to 40-hours-N-deprived Nannochloropsis. Data was generated by GC-FID (n.d., not detected).

Fatty Acyl	+N (%)	-N (%)
14:0	8.8 ± 0.5	6.7 ± 0.3
16:0	47.3 ± 0.6	51.6 ± 1.3
16:1	32.7 ± 2.9	32.3 ± 0.7
18:0	9.9 ± 1.1	2.6 ± 0.3
$18:1\Delta^9$	1.3 ± 2.2	5.2 ± 0.3
18:2	n.d.	0.5 ± 0.1
20:4	n.d.	0.4 ± 0.1
20:5	n.d.	0.8 ± 0.3

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LITERATURE CITED

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CHAPTER 3

Triacylglycerol Accumulation involves Turnover of Membrane Lipids in Dark Hypoxia-Stressed *Chlamydomonas reinhardtii*

3.1. Abstract

Photosynthetic microalgae produce triacylglycerols (TAGs) under abiotic stress conditions such as nutrient deprivation, a process exploited for biofuel production and other applications. The mechanisms of TAG biosynthesis and its relationship to general lipid metabolism in microalgae are not yet fully understood. Here we investigate the mechanism of TAG accumulation in *Chlamydomonas reinhardtii* under hypoxia in the dark, conditions encountered by dense natural algal populations at night, when energy is derived heterotrophically. Under those conditions, membrane lipid degradation provides unsaturated fatty acids, which are used for TAG assembly. Thus membrane lipid turnover is a key contributor to TAG accumulation in dark hypoxia-stressed algae. Analyses of algal strains with mutations in genes encoding different enzymes of lipid metabolism indicate that assembly of TAGs in dark hypoxic microalgae involves specific enzymes, different from those used during *de novo* synthesis of TAGs in the light. Metabolization of diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine, a betaine lipid of microalgae, was shown to be relevant under dark hypoxic conditions.

3.2. Introduction

Lipid metabolism in microalgae has become an intense research focus during recent years because lipids from microalgae can serve as precursors of biofuels (Hu et al., 2008), and give rise to high value compounds in the form of poly unsaturated fatty acids (PUFAs) with benefits to human health (Jump et al., 2012). Moreover, experimental manipulation of triacylglycerol (TAG) biosynthesis and degradation is relatively facile in microalgae, which makes them suitable unicellular models for the general study of storage lipid biochemistry and its pathological dysfunction in eukaryotic cells including potential mammalian cells (Liu and Benning, 2013).

Chlamydomonas reinhardtii is a unicellular green alga, as well as an established model organism for biological research (Merchant et al., 2007). To date, the algal research community has established a full set of molecular tools, paving the way for genetic and molecular analysis of *C. reinhardtii* (Fuhrmann, 2002; Liu and Benning, 2013). In addition, a high quality draft genome sequence (Merchant et al., 2007) and extensive transcriptome analyses (Lopez et al., 2011) of *C. reinhardtii* have become available, facilitating further in-depth molecular and biochemical research efforts.

Lipid metabolism, especially TAG-related metabolism in *C. reinhardtii*, has been extensively investigated (Merchant et al., 2012; Radakovits et al., 2010). While *C. reinhardtii* maintains a minimum amount of TAG during regular growth, it accumulates TAG upon exposure to abiotic stresses such as nutrient depletion (Boyle et al., 2012). Among those stresses, nitrogen (N) deprivation is the most studied scenario and has become a standard treatment for initiating TAG biosynthesis in algae. Fan et al. (Fan et al., 2011) proposed that during N

deprivation in the light, most TAGs are assembled *de novo* by the chloroplast pathway, distinct from the well known mechanism in land plants, in which TAG biosynthesis is a function of the endoplasmic reticulum (ER) pathway (Li-Beisson et al., 2013). Characterization of the *C. reinhardtii* mutant *pgd1* (Li et al., 2012b), which is deficient in TAG accumulation during N deprivation in the light, led to the conclusion that fatty acid *de novo* synthesis in the plastid followed by a galactolipase-mediated process for fatty acyl export from the plastid substantially contributes to TAG biosynthesis under photoheterotrophic growth conditions. These emerging details indicate that the mechanisms of TAG-related lipid metabolism in *C. reinhardtii* likely differ from those in land plants and, therefore, require further study to gain a complete insight. Besides N deprivation, other stress treatments can also stimulate TAG accumulation in *C. reinhardtii* but may do so in different ways.

Hypoxia is a common condition microalgae encounter, under which the O_2 partial pressure in the medium cannot meet the need for respiration (Quinn et al., 2002). This condition occurs in dense algal cultures such as production ponds during the night when photosynthesis cannot occur and, therefore, plays an essential role in the diurnal cycle of algal biomass production. Hypoxia initiates fermentation making available metabolic energy, a process which has been extensively studied (Catalanotti et al., 2013). Interestingly, accumulation of TAGs, which are energy-dense compounds with a high number of fully reduced carbons, was observed in *C. reinhardtii* under dark hypoxia stress (Hemschemeier et al., 2013; Matthew et al., 2009). While lipid metabolism under hypoxia conditions has been interpreted at the gene expression level (Hemschemeier et al., 2013; Toepel et al., 2013), detailed biochemical analyses are required to understand the mechanisms of TAG metabolism in *C. reinhardtii* under these conditions. Here we explore the biochemical evidence for the mechanism of TAG assembly

under dark hypoxia conditions. TAG biosynthesis-related algal mutant strains grown under dark hypoxia conditions were employed to probe contributions of different pathways to TAG accumulation.

3.3. Materials and Methods

3.3.1. Strains, growth conditions and physiological analysis

C. reinhardtii strains D66^{+} (*nit2 cw15 mt*$^{+}$), *pdat1-1* and *pdat1-2 (pdat1 nit2 cw15 mt*$^{+}$) (Boyle et al., 2012) and dw15-1 (*nit1 cw15 mt*$^{+}$) and *pgd1* (*pgd1 nit1 cw15 mt*$^{+}$) (Li et al., 2012b) were grown photoheterotrophically in Tris Acetate Phosphate (TAP) medium under a continuous light (80 μmol m^{-2} s^{-1}) at 22 ℃ on a shaker at 100 rpm. Cell concentration was determined with a Z2 Coulter Counter (Beckman-Coulter) using a 100 mm aperture according to the manufacturer's instruction. The concentration of dissolved oxygen in algal cultures was determined by a fiber optic oxygen sensor system (NeoFox, Ocean Optics).

3.3.2. Hypoxia Stress Treatment of *C. reinhardtii*

The establishment of algal hypoxia stress condition was modified from a previous approach (Hemschemeier et al., 2013). Briefly, mid-log-phase algae were transferred to a 50 mL black tube (Argos Technologies), filling the head space as much as possible. Then, the tube cap was closed tightly to generate a dark and air-tight system. The algal culture was vigorously shaken (200 rpm) to initiate the hypoxia stress. As a control of hypoxia stress treatment, a dark-

aerobic condition was established. Fifty mL of mid-log-phase algal culture was transferred to 250 mL flask, which was loosely covered by aluminum foil. This algal culture was shaken at 100 rpm. Both treatments were performed at 22 ℃ and *C. reinhardtii* cells were harvested at 48 hour after the onset of treatment.

3.3.3. Lipid Analysis and TAG Positional Analysis

Lipid extraction, separation by TLC and fatty acyl transesterification were performed according to previous reports (Liu et al., 2013; Moellering and Benning, 2010). Positional analysis of TAG was performed with *Rhizopus arrhizus* lipase (Sigma) as previously described (Li et al., 2012b) with modifications. Briefly, 200 nmol of TAG was suspended in 900 μL of phosphate buffered saline (PBS) and subjected to sonication (Sonicator 3000 with microprobe; Misonix) for 6 x 10 s (power setting 1.5) on ice. Then, 200 μg *R. arrhizus* lipase was added to the TAG-buffer system to initiate the digestion at ambient temperature. The reaction was stopped after 15 min and subjected to lipid extraction and TLC separation. In terms of lipid bands on the TLC plate, free fatty acids were original fatty acyl groups attached at *sn*-1 and *sn*-3 positions of TAG, and monoacylglycerol contained the remaining fatty acyl group at the *sn*-2 position. Those two classes of lipids were subjected to transesterification and gas chromatography quantification, as previously described (Liu et al., 2013).

3.3.4. Pulse-Chase Labeling Analysis of Lipids

C. reinhardtii cells were grown to early-log phase (~3 x 10^6 cells mL^{-1}) in TAP medium and transferred to autotrophic medium (TP) lacking acetate (Terauchi et al., 2010). Then, [^{14}C-U]-acetate (specific activity 45-60 mCi/mmol; Perkin Elmer) was added to the algal culture, reaching the final concentration of radioactivity at 0.2 μCi/mL. After about 12-hour growth, about 70% of the labeled acetate was incorporated into *C. reinhardtii* cells, which was determined by liquid scintillation counting. Radiolabeled algal cells were transferred to non-radiolabeled TAP medium and allowed to recover for about 6 hours under regular growth conditions in the light. Then, the algal culture was subjected to dark hypoxia treatment and harvested at these time points after the onset of stress: 0h, 12h, 24h, 36h and 48h. Lipid extracts were separated by TLC. Isolated lipids including TAG, a mixture of DAG and free fatty acid, MGDG, DGTS, DGDG, mixture of a PtdEtn and PtdGro, SQDG and phosphatidylinositol, were subjected to liquid scintillation counting. The radioactivity of all lipid fractions were summed, and percentages for each lipid fraction were calculated.

3.3.5. Yeast Microsomal Assay for CrPDAT1 activity

In a previous study (Boyle et al., 2012), codon-optimized *CrPDAT1* was inserted into the vector pGPD417-LNK to generate plasmid pIKB507 for expression of CrPDAT1 in *S. cerevisiae*. Plasmids pIKB507 and pGPD417-LNK were transformed into YPD1078, a yeast strain lacking acyltransferase activity as described (Boyle et al., 2012). To prepare microsomes, yeast cells from a single colony were grown in liquid casamino acids medium minus uracil (Li et al., 2012a) to reach mid-log phase. After centrifugation (1500 x *g*, 4 ℃, 5 min), the yeast cell pellet was resuspended in 3 mL of extraction buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 1mM

DTT, 300 mM $(NH_4)_2SO_4$, 5% glycerol) on ice. About 2 mL of acid-washed glass beads (0.45 mm; Sigma-Aldrich) were added into the yeast cell solution, which was then vortexed vigorously (1 min x 5). The solution was incubated on ice for 1 min between each vertex. The solution was centrifuged (13000 x *g*, 4 ℃, 5 min) and then the supernatant was ultracentrifuged (100,000 x *g*, 4 ℃, 30 min). The pellet of microsomes was resuspended in PBS buffer on ice, and the protein concentration was determined by the Bradford assay.

C. reinhardtii was labeled by [^{14}C-U]-acetate as described above and the ^{14}C-labeled lipid DGTS was collected by TLC separation. ^{14}C-DGTS (35 nmol with total radioactivities of 10,000 to 40,000 dpm) and 2 μg DAG (16:0/18:1, Avanti) were loaded in a tube and dried under a stream of nitrogen. One hundred μL of PBS (with 0.426 mM sodium deoxycholate) was added, followed by sonication (6 x 10 s, power setting 1.5) on ice. One hundred μg protein equivalent of yeast microsome was added to the reaction, which was incubated at room temperature overnight and stopped by adding organic solvent for lipid extraction. The lipid extracts were developed by TLC and radioactivity of TAG and substrates were determined by liquid scintillation counting.

3.4. Results

3.4.1. Lipid related algal cell metabolism during hypoxia stress

Hypoxia was induced by filling black, tightly closed culturing tubes to the top with medium and letting the algae consume oxygen through respiration over time. To confirm that hypoxia was reached in this self-contained oxygen-consuming system, dissolved oxygen was measured with an oxygen sensitive probe at the end of 48-hour stress treatment. For control

purposes, *C. reinhardtii* cultures were maintained in flasks loosely covered with aluminum foil, which resembles the dark environment, but allows air exchange. After 48 hours, the concentration of dissolved oxygen was indistinguishable from that in ambient water which was the concentration read at time zero (Figure 3.1). In dark hypoxia-stressed algal cultures, the apparent concentration of dissolved oxygen was near zero after rapid equilibration of the probe (Figure 3.1). The above results confirm that *C. reinhardtii* becomes hypoxic during the course of the treatment, as used in the subsequent experiments.

It was previously reported that the lipid profiles between photoheterophically grown and darkness-treated *C. reinhardtii* were similar (Hemschemeier et al., 2013), indicating that short-term darkness has a minor effect on algal lipid metabolism. However, lipid profiles changed considerably when algal cells were subjected to dark hypoxia stress (Hemschemeier et al., 2013). As shown in Figure 3.2A, TAG accumulates when overall lipid content decreases under dark hypoxia stress. The change is pronounced during the early phase of the time course and TAG accumulation stabilizes towards the end of hypoxia treatment, consistent with a previous study which showed TAG accumulation mainly occurred during the first 24 hours of hypoxia induced following sulfur depletion (Matthew et al., 2009). The apparent changes in total lipid and TAG contents over time (Figure 3.2A) indicate that *C. reinhardtii* actively synthesizes TAG while net lipid biosynthesis decreases under dark hypoxia stress.

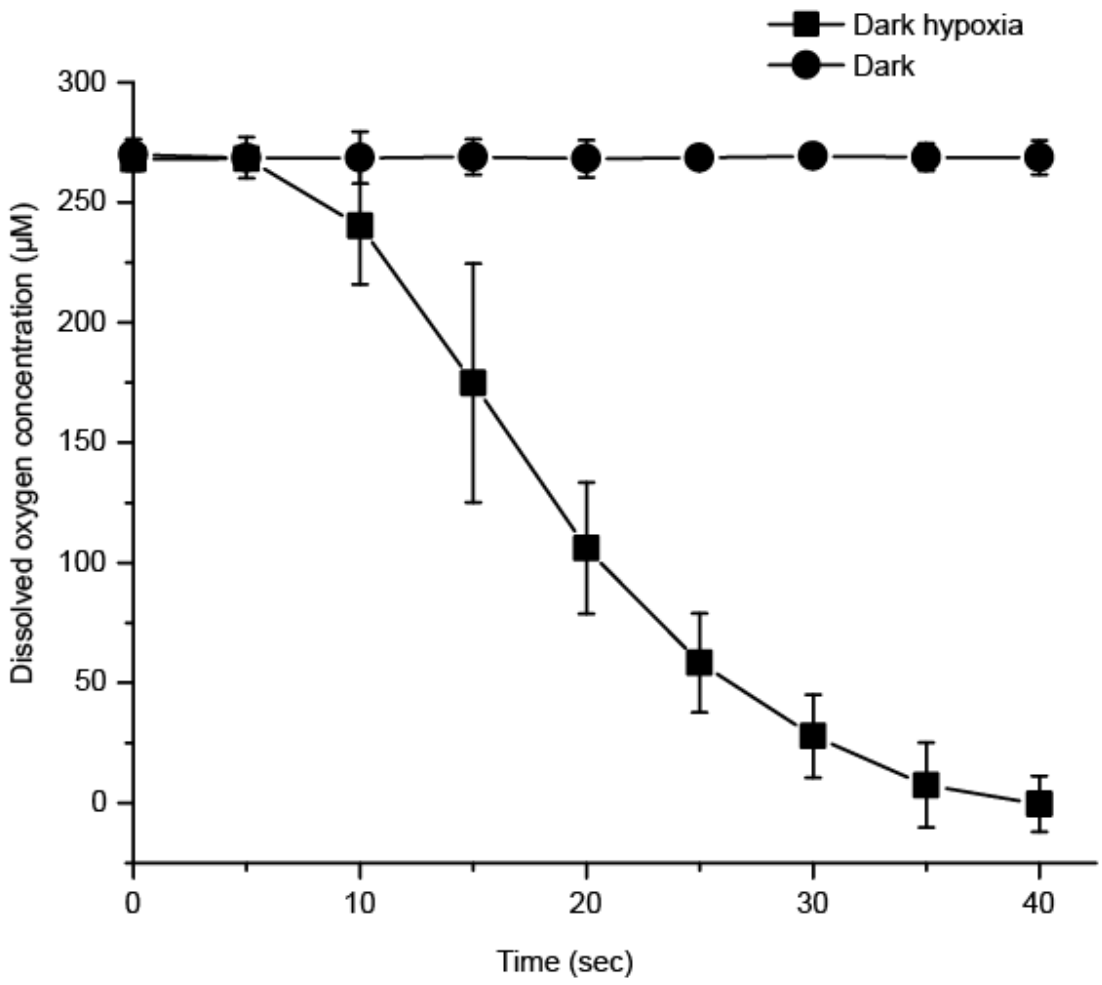

Figure 3.1. Measurement of concentration of dissolved oxygen in 48-hour dark hypoxia stressed *C. reinhardtii* D66^{+} cultures. Data points represent the real-time readouts from the oxygen sensor after the probe was inserted into the algal culture. Error bars represent the standard deviation from three biological replicates.

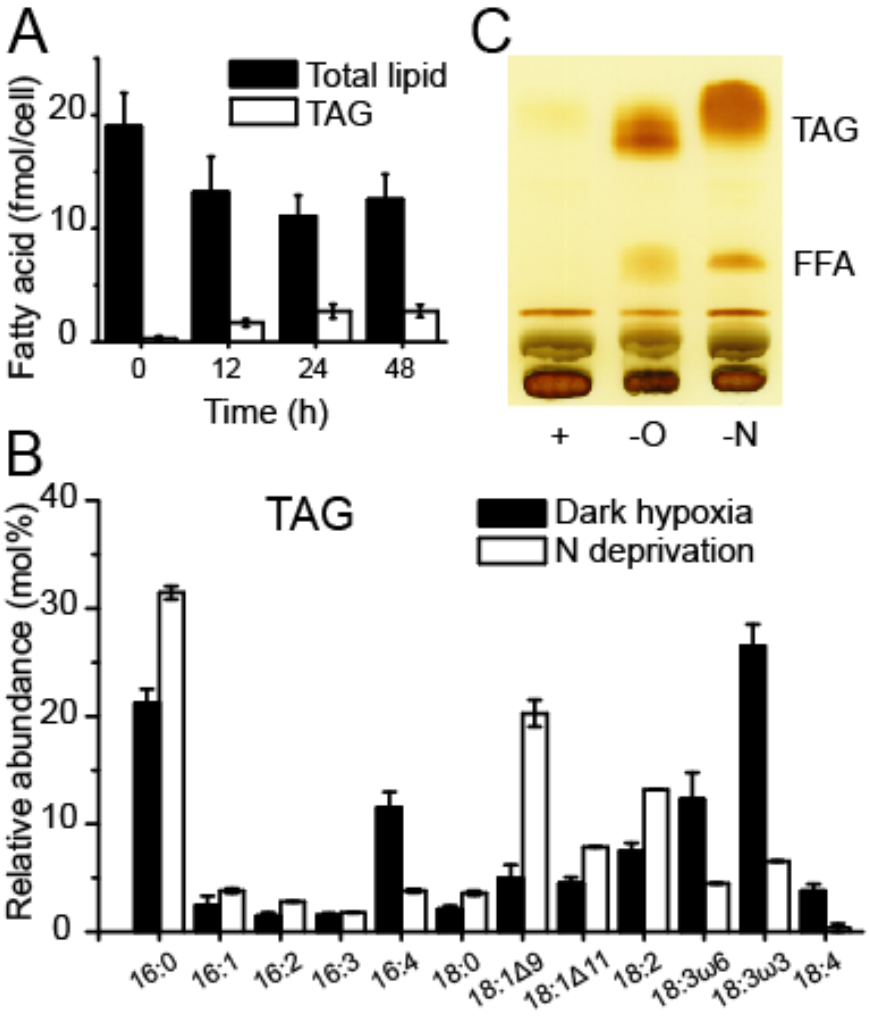

Figure 3.2. TAG accumulation in *C. reinhardtii* D66^{+} under dark hypoxia stress. (A) Quantification of fatty acids from total lipid and TAG during the time course of hypoxia treatment. (B) Comparison of TAG fatty acid profiles between 48-hour dark hypoxia stressed and N deprived *C. reinhardtii*. Data were generated by gas chromatography measurements and error bars represent standard deviation from three biological replicates. (C) TLC separation of TAGs from algae under different conditions. The solvent used for TLC was petroleum ether: diethyl ether: acetic acid, 8:2:0.1.

3.4.2. Fatty acids from membrane lipids are reused for TAG biosynthesis under hypoxia stress

As *C. reinhardtii* contains a negligible amount of TAG during normal photoheterotrophic growth (Moellering and Benning, 2010), dark hypoxia stress-induced TAGs accumulation was compared with that induced by N deprivation in the light, a more commonly used condition for the induction of algal TAG biosynthesis (Boyle et al., 2012; Miller et al., 2010). Fatty acid profiles of TAGs of 48-hour dark hypoxia stressed and 48 hour N-deprived algae kept in the light were compared. During N deprivation, *de novo* fatty acid synthesis is activated, largely contributing to TAG assembly (Li et al., 2012b). As a result, 16:0 and $18{:}1\Delta^{9}$ were abundant in TAG from N-deprived *C. reinhardtii* (Figure 3.2B). On the other hand, algal TAG fatty acids were more unsaturated under dark hypoxia stress. PUFAs, such as 16:4, 18:3 and 18:4 were more enriched in TAG produced under dark hypoxia stress (Figure 3.2B). It should be noted that when *C. reinhardtii* is grown under optimal photoheterotrophic conditions, 16:4 is exclusively present at the *sn*-2 position of monogalactosyldiacylglycerol (MGDG), while $18{:}3\omega^{6}$ and 18:4 are mainly found at the *sn*-2 position of diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine (DGTS) and phosphatidylethanolamine (PtdEtn), both of which are ER pathway derived lipids (Giroud et al., 1988). As TAG fatty acids are more unsaturated under dark hypoxia stress than following N deprivation, the chromatographic migration of TAGs from the different lipid extracts analyzed by thin layer chromatography (TLC) differed. Figure 3.2C shows iodine vapor stained lipids on a developed TLC plate. The TAG mixture from dark hypoxia-stressed algae did not migrate as far as TAG produced following N deprivation, because lipid with unsaturated fatty acids migrated slower than lipid with saturated fatty acids in the TLC system used in this study.

It is commonly assumed that the origin of glycerolipids from one of the two major acyl lipid assembly pathways, at the ER or the plastid envelopes, in photosynthetic organisms can be distinguished by the fatty acyl group at the *sn*-2 position of the glycerolipid (Giroud et al., 1988), although, whether this assumption holds true for *C. reinhardtii*, has not yet been rigorously tested by comparing the substrate specificities of the involved acyltransferases. About 80% of fatty acids at the *sn*-2 position of TAGs are composed of 16-carbon fatty acids under hypoxia stress, similar to those produced following N deprivation (Figure 3.3A). This suggests that TAG biosynthesis occurs mostly through the chloroplast pathway in *C. reinhardtii* under dark hypoxia stress, while the ER pathway produces the other 20% of TAGs, in which the *sn*-2 position carries 18-carbon fatty acyl groups. In terms of the profile of TAG *sn*-2 fatty acids, 16:4 was higher under dark hypoxia stress (Figure 3.3B) than observed for N-deprived cells. Meanwhile, 16:4 was almost absent at the *sn*-1 or *sn*-3 positions of TAGs (Figure 3.3C), suggesting that 16:4 remains at the *sn*-2 position of lipids, regardless of MGDG or TAG, under dark hypoxia stress.

Pulse-chase labeling experiments were performed to explore the dynamic change of lipid metabolism and understand the relationship between different lipid species in dark hypoxia-stressed *C. reinhardtii*. Cells were labeled with [^{14}C]-acetate in the light, and labeled algal cells were then transferred to non-radioactive medium to start the chase period. After a 6 h recovery period in the light, the algal culture was subjected to dark hypoxia stress. The fraction (in percent) of radioactivity incorporated into major lipids of *C. reinhardtii* was recorded during the time course of hypoxia stress. The relative amount of radioactivity in TAG increased (Figure 3.4A), consistent with overall TAG accumulation under dark hypoxia. Similarly, an increase was observed in the relative amount of radioactivity in the mixture of diacylglycerol (DAG) and free fatty acids, with DAG being the immediate precursors for TAG biosynthesis. These DAGs and

free fatty acids are likely the products derived from the degradation of membrane lipids rather than from *de novo* synthesis because during the chase period, only non-radioactive carbons in the medium are available for *de novo* biosynthesis of fatty acids. The relative amount of radioactivity of the three major membrane lipids, MGDG, digalactosyldiacylglycerol, (DGDG) and DGTS decreased under dark hypoxia stress (Figure 3.4B), consistent with general lipid degradation in *C. reinhardtii* (Figure 3.2A). Overall, the pulse-chase labeling results link the two events, membrane lipid degradation and TAG accumulation, suggesting that fatty acids are released from membrane lipids and then channeled into TAG assembly.

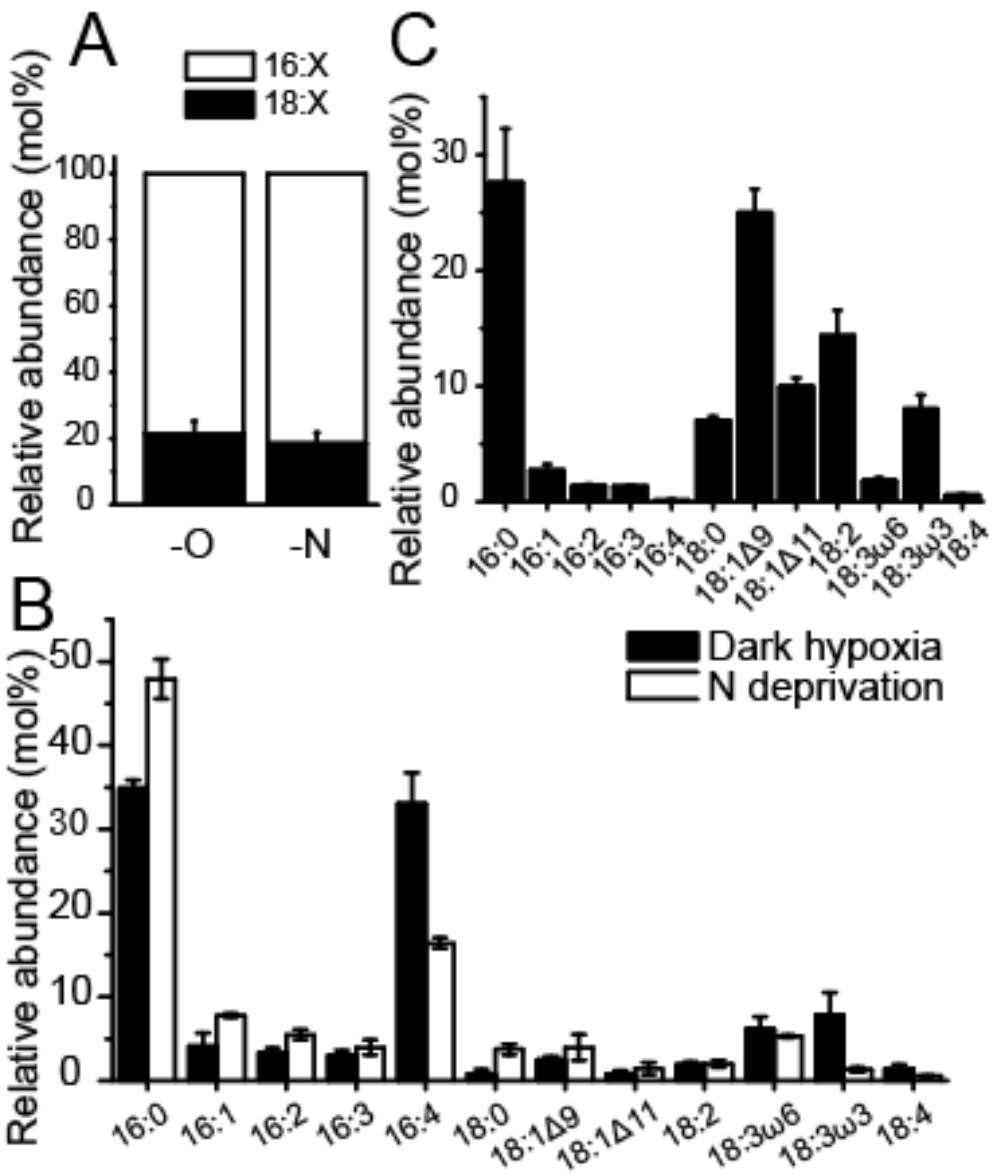

Figure 3.3. Positional analyses of TAG accumulated in dark hypoxia-stressed *C. reinhardtii* D66^{+}. Comparison of carbon number (A) and profiles (B) of fatty acid at the *sn*-2 position of TAGs accumulated in 48-hour dark hypoxia and N deprivation. (C) Profile of fatty acid at the *sn*-1 and *sn*-3 positions of TAG from 48-hour dark hypoxia stressed *C. reinhardtii*. Error bars represent standard deviation from three biological replicates.

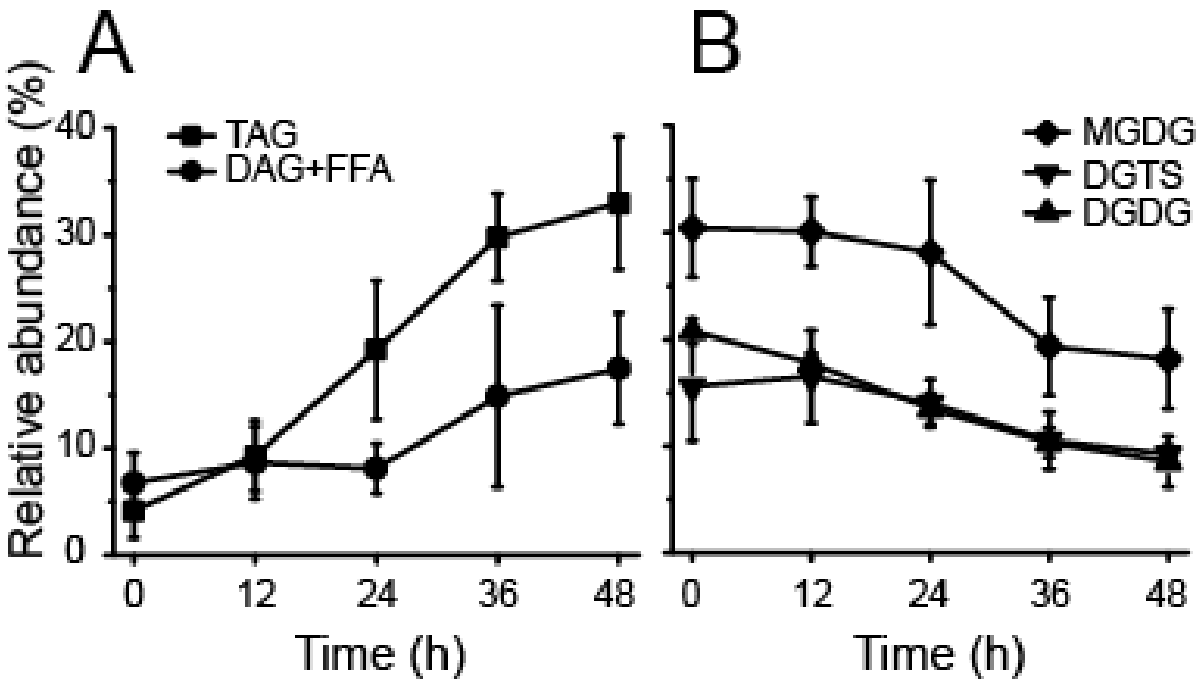

Figure 3.4. Pulse-chase labeling of lipids in *C. reinhardtii* D66^{+} under dark hypoxia. Relative amount of radioactivity incorporated into (A) TAG and mixture of DAG and free fatty acid, and (B) MGDG, DGTS and DGDG during the chase period. Time 0 is the onset of dark hypoxia treatment. Error bars represent standard deviation from three biological replicates.

3.4.3. PGD1 is only a minor contributor to TAG accumulation under dark hypoxia stress

The contribution of a recently characterized *C. reinhardtii* protein PGD1, which is involved in TAG accumulation during N deprivation (Li et al., 2012b), was tested under dark hypoxia stress. The respective *pgd1* mutant and its parental strain dw15-1 were subjected to dark hypoxia treatment. Under these conditions, the lipid profile of both, total lipid and TAG content, were similar between *pgd1* and dw15-1 (Figure 3.5A). This was contrary to observations following N deprivation, when the biosynthesis of TAG in *pgd1* was reduced to about half of that in dw15-1, as the *pgd1* mutant is likely impaired in the transfer of newly incorporated fatty acids prior to desaturation from MGDG to TAG (Li et al., 2012b). Another notable *pgd1* phenotype following N deprivation is that the relative amount of $18{:}1\Delta^9$ in TAG is lower in the mutant (Li et al., 2012b), which was not observed under dark hypoxia stress. The TAG fatty acid profiles of *pgd1* and dw15-1 were similar for all fatty acid species (Figure 3.5B). To conclude, while lipase PGD1 is involved in algal TAG biosynthesis following N deprivation, it makes little contribution to TAG accumulation under dark hypoxia stress, as no lipid phenotype was observed in the *pgd1* mutant. Thus, the main pathways of TAG assembly under dark hypoxia must be different from that following N deprivation.

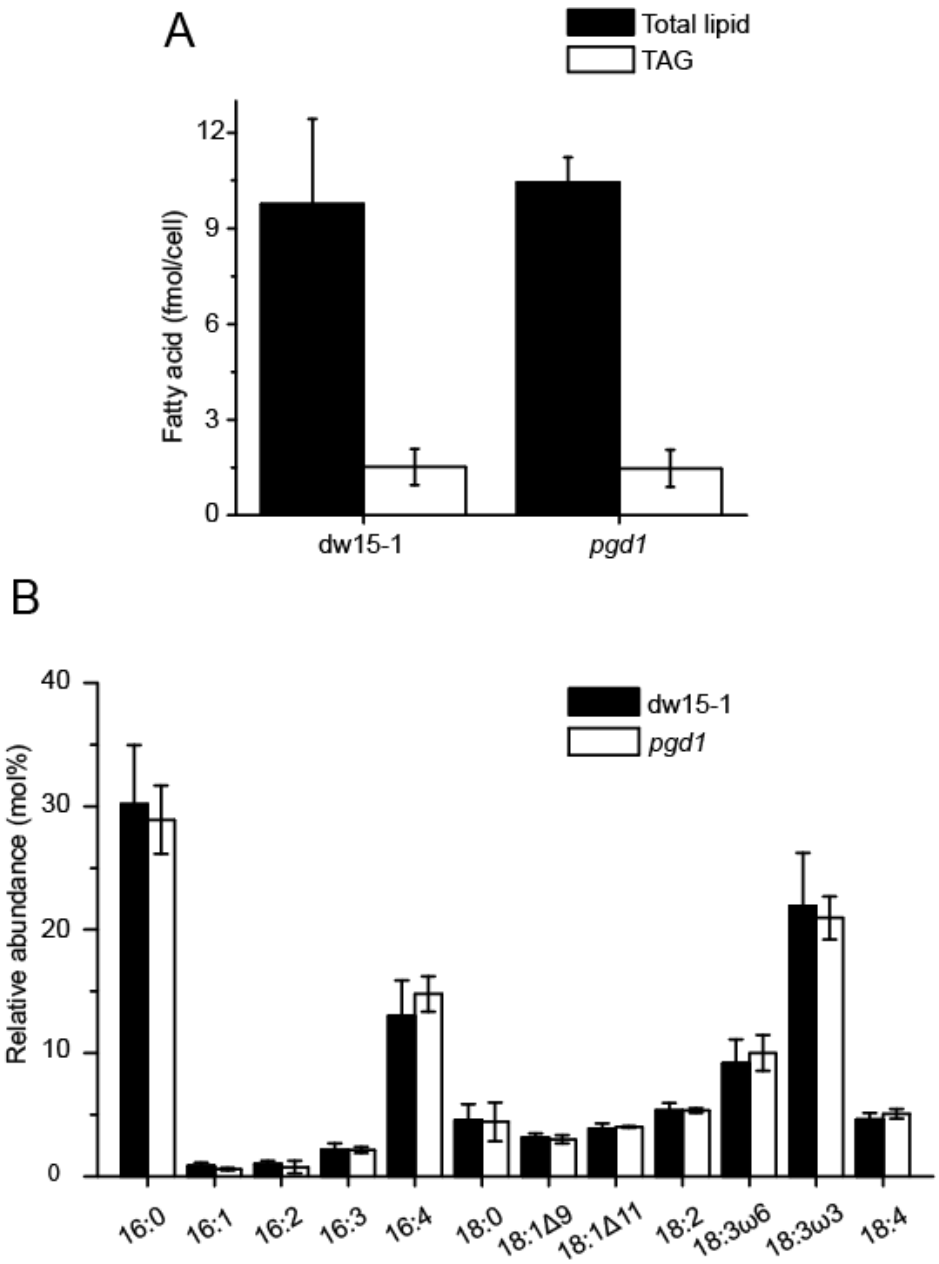

Figure 3.5. Lipid profile of the *pgd1* mutant under dark hypoxia stress. (A) Comparison of total fatty acid and TAG fatty acid contents from 48-hour dark hypoxia-stressed *pgd1* and dw15-1. (B) Comparison of TAG fatty acid profiles between 48-hour dark hypoxia-stressed *pgd1* and dw15-1. Error bars represent standard deviation from three biological replicates.

3.4.4. PDAT1 is involved in TAG assembly under dark hypoxia stress

The likely *C. reinhardtii* protein ortholog of plant Phospholipid: Diacylglycerol Acyltransferase (PDAT) was identified in a proteomics study of N-deprived algae (Nguyen et al., 2011). Later, two independent *C. reinhardtii* mutant strains with mutations in the *PDAT1* gene were generated and characterized following N deprivation (Hemschemeier et al., 2013). These two *pdat1* mutants were subjected to dark hypoxia stress to explore the contribution of PDAT1 to TAG biosynthesis under these conditions. A decreased TAG amount was observed in both mutant strains after dark hypoxia treatment, compared with their parental strain $D66^{+}$ (Figure 3.6A), indicating that TAG biosynthesis was partially disrupted in the *pdat1* mutants under dark hypoxia stress. Furthermore, the TAG fatty acid profiles showed that relative amounts of 16:4, $18{:}3\omega^{6}$ and 18:4 were lower in the *pdat1* mutants (Figure 3.6B). As 16:4, $18{:}3\omega^{6}$ and 18:4 are exclusively present at the *sn*-2 position of specific membrane lipids during normal growth in the light, the TAG fatty acid profile in the *pdat1* mutants was in agreement with the proposed function of PDAT1 in *C. reinhardtii* (Nguyen et al., 2011; Yoon et al., 2012), the removal of fatty acyl groups from the *sn*-2 position of membrane lipids and transfer to DAG for TAG assembly. As $18{:}3\omega^{6}$ and 18:4 are exclusively present at the *sn*-2 position of DGTS and PtdEtn (Giroud et al., 1988), the amount of DGTS and its fatty acid profile were tested in hypoxia-stressed algae. No statistically significant difference was observed in DGTS amounts between wild type and *pdat1* mutants (Figure 3.6C). However, comparison of the fatty acid profile of DGTS with wild type revealed that the *pdat1* mutants retained a higher fraction of $18{:}3\omega^{6}$ and 18:4 (Figure 3.6D), suggesting that *pdat1* mutants are deficient in transferring those fatty acyl groups from the *sn*-2 position of DGTS to DAG for TAG biosynthesis under dark hypoxia stress.

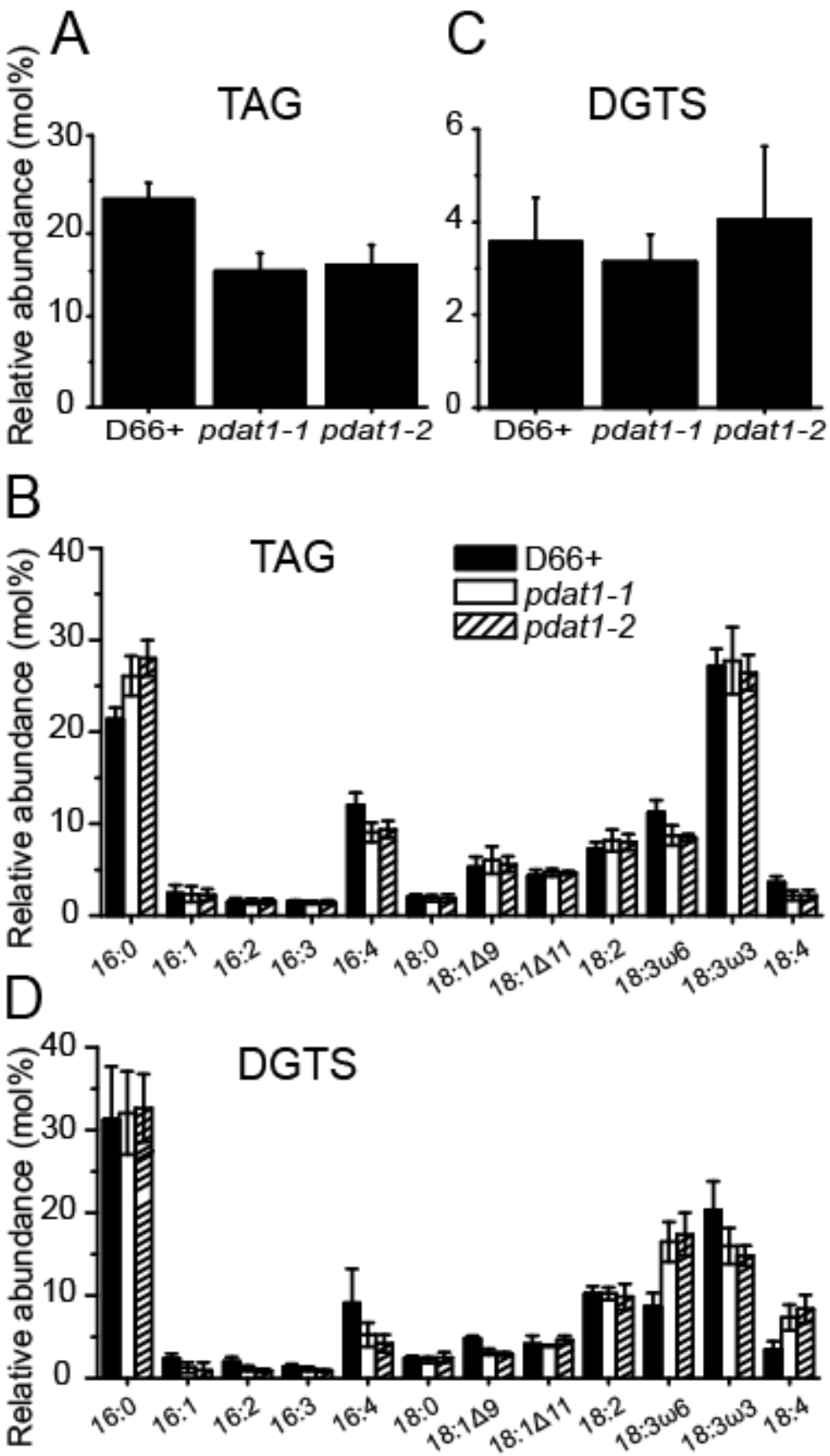
A
TAG
Relative abundance (mol%)
30
20
10
0
D66+
pdat1-1
pdat1-2
C
DGTS
6
4
2
0
D66+
pdat1-1
pdat1-2
B
TAG
D66+
pdat1-1
pdat1-2
Relative abundance (mol%)
40
30
20
10
0
16:0
16:1
16:2
16:3
16:4
18:0
18:1Δ9
18:1Δ11
18:2
18:3ω6
18:3ω3
18:4
D
DGTS
Relative abundance (mol%)
40
30
20
10
0
16:0
16:1
16:2
16:3
16:4
18:0
18:1Δ9
18:1Δ11
18:2
18:3ω6
18:3ω3
18:4

Figure 3.6. Lipid analyses of *pdat1* mutant strains. Comparison of relative abundance (A) and fatty acid profile (B) of TAG accumulated during 48-hour dark hypoxia stress between $D66^{+}$ and *pdat1* mutants. Comparison of relative abundance (C) and fatty acid profile (D) of DGTS in 48-hour dark hypoxia stressed $D66^{+}$ and *pdat1* mutants. Error bars represent standard deviation from three biological replicates.

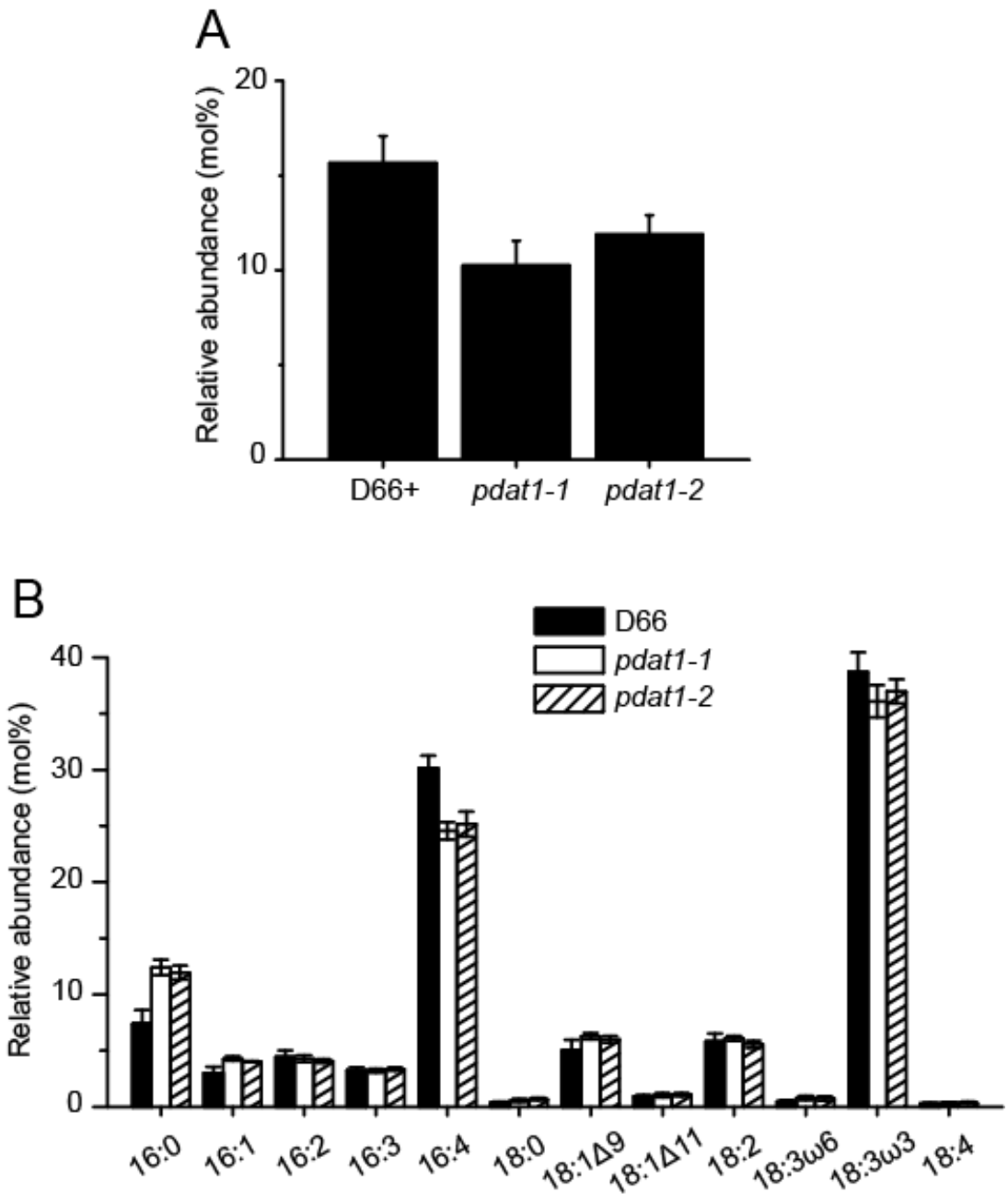

Figure 3.7. MGDG of *pdat1* mutants under dark hypoxia stress. (A) Comparison of MGDG fatty acid contents from 48-hour dark hypoxia-stressed *pdat1* mutants and D66^{+}. (B) Comparison of MGDG fatty acid profiles between 48-hour dark hypoxia stressed *pdat1* mutants and D66^{+}. Error bars represent the standard deviation from three biological replicates.

Overall, lipid analyses suggested that DGTS is a substrate of PDAT1, at least under dark hypoxia stress. Since the *sn*-2 position of MGDG is always occupied by 16:4 during regular growth, and 16:4 is lower in mutant TAG (Figure 3.6B), MGDG content and its fatty acid profile were analyzed. Surprisingly, the *pdat1* mutants had a lower total amount of MGDG compared to the wild type (Figure 3.7A), and also a lower amount of 16:4 in MGDG (Figure 3.7B). The above phenotypes would be consistent with a higher rate of MGDG degradation in *pdat1* mutants under dark hypoxia stress and may point to an alternate MGDG degradation process activated in the *pdat1* mutant.

3.4.5. Yeast microsomal assays confirmed DGTS as substrate of *Cr*PDAT1

CrPDAT1 expression was achieved in yeast lacking acyltransferase activity similar as previously described (Boyle et al., 2012) and a microsomal assay was employed to test *Cr*PDAT1 activity on labeled DGTS, which was isolated from [^{14}C]-acetate-fed *C. reinhardtii* cells. Presumably, *Cr*PDAT1 transfers fatty acyl groups from [^{14}C]-DGTSs to DAGs, forming [^{14}C]-TAGs. After the reaction, the relative radioactivity of TAG over total radioactivity was compared between samples using microsomes harboring *Cr*PDAT1 and an empty vector control (Figure 3.8). While no labeled TAG was detected in the control group, more than 7% radioactivity was observed in TAG produced in the *Cr*PDAT1 containing microsome fraction. This result suggested that *Cr*PDAT1 is able to utilize DGTS and DAG as substrates to produce TAG.

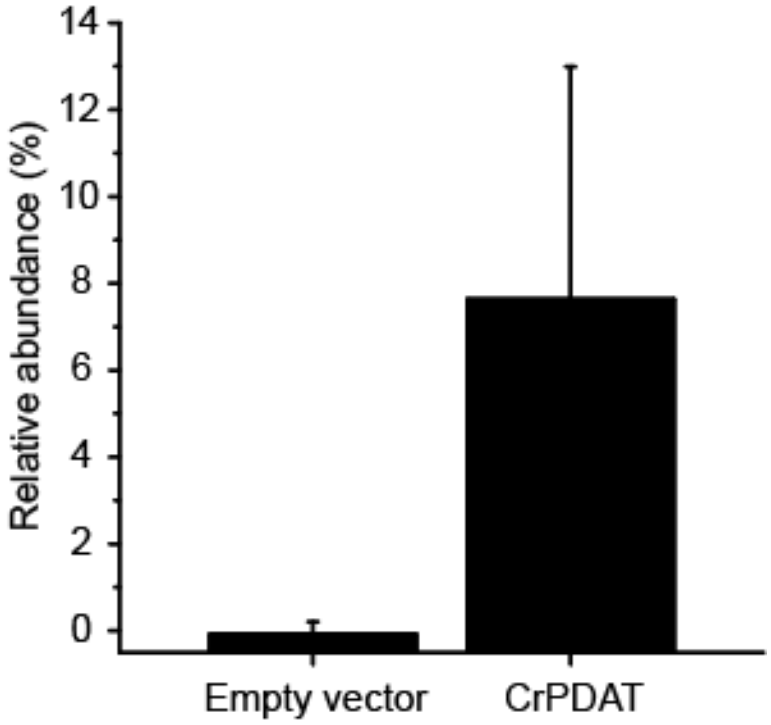

Figure 3.8. DGTS is the substrate of *Cr*PDAT1 for TAG synthesis. Relative abundance of radioactivity incorporated into TAG, the expected product of the reaction, is compared between yeast microsome harboring either a plasmid expressing *CrPDAT1* or empty vector. Error bars represent the standard deviation from three biological replicates.

3.5. Discussion

An intriguing question is why microalgae increase TAG biosynthesis in response to a broad range of stresses. One external factor is the availability of the carbon precursor present in the algal culture. Acetate is supplied in the algal medium for photoheterotrophic growth of *C. reinhardtii* in most published TAG accumulation studies, and it has been shown that different concentrations of acetate correlate with the levels of TAG accumulated following N deprivation (Fan et al., 2012). Carbon dioxide fixation by photosynthesis is another contributor to TAG accumulation during specific stresses. Following N deprivation, the reducing power generated by photosynthetic electron transport chain is dissipated in part through *de novo* fatty acid synthesis and TAG assembly, which is a safe and recyclable way to sequester reduced carbon in lipid droplets (Li et al., 2012b) under conditions when cellular processes such as protein synthesis and cell division is suspended (Miller et al., 2010) but photosynthesis is still continuing. If TAG synthesis is disrupted as in the *pgd1* mutant, reduction of the electron transport chain increases, leading to increased production of cell damaging reactive oxygen species at photosystem I and eventually cell death (Li et al., 2012b).

To date, most of the *C. reinhardtii*-focused TAG accumulation studies have been performed in the light following N deprivation that involve the factors mentioned above, and it has been determined that *de novo* fatty acid synthesis contributes to TAG assembly during N deprivation (Fan et al., 2011; Li et al., 2012b). However, there is at least one other proposed pathway contributing to TAG biosynthesis in microalgae, which is lipid turnover through the degradation of membrane lipids to generate precursors such as diacylglycerol or acyl groups for TAG assembly (Liu and Benning, 2013). In plants, lipid remodeling in response to freezing stress involves diacylglycerol transfer from membrane lipids to TAG (Moellering and Benning,

2011). This process might also occur under other stress conditions, for instance, N deprivation, under which membrane lipid degradation was observed in several algal species (Fan et al., 2011; Simionato et al., 2013). Membrane lipid remodeling, the change in membrane lipid acyl group composition and the formation of TAGs, has also been observed following iron deficiency (Simionato et al., 2013; Urzica et al., 2013). In contrast, the study described here provides more direct biochemical evidence demonstrating that the transfer of acyl groups from membrane lipids can contribute to TAG biosynthesis.

Under dark hypoxia treatment, oxygenic photosynthesis cannot occur, but *C. reinhardtii* is still able to accumulate highly unsaturated TAGs, which is distinct from N deprivation (Figure 3.2B). Because oxygen is not available, beta oxidation of fatty acids should not be able to contribute to the degradation of acyl groups. Thus, membrane lipid degradation is attenuated, as hypoxia becomes more severe during the time course (Figure 3.2A). The distinct lipid phenotypes of *pgd1* and *pdat1* mutants under dark hypoxia stress suggest that membrane lipid turnover, rather than *de novo* fatty acid synthesis, makes a major contribution to TAG biosynthesis. Moreover, DGTS, a betaine lipid, was shown to be a substrate of PDAT1, at least under dark hypoxia stress (Figure 3.6C, D, and 3.8). DGTS is not a phospholipid, and because it has also been previously demonstrated that galactolipids are a substrate of PDAT1 in *C. reinhardtii* (Yoon et al., 2012), the current name implying the transfer of acyl groups only from phospholipids for this enzyme is misleading and should be revised to PDAT-like.

Overall, the mechanism of TAG biosynthesis in *C. reinhardtii* appears to be different depending on the nature of the encountered stress, with different responses to dark hypoxia stress or N deprivation and likely other stresses. Under dark hypoxia stress, the fatty acids released from membrane lipids primarily channel to TAG assembly, while the contribution of *de novo*

fatty acid synthesis to TAG accumulation is reduced under these non-photosynthetic conditions. Assuming membrane lipid turnover and *de novo* fatty acid synthesis independently contribute to TAG biosynthesis, future studies on lipid metabolism in microalgae under dark hypoxia stress will provide a simple approach to reveal new aspects of TAG metabolism of *C. reinhardtii* not readily apparent under photoheterotrophic conditions.

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LITERATURE CITED

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CHAPTER 4

Lipid profiling of *Chlamydomonas reinhardtii* grown under different stress conditions[3]

[3]Data in this chapter have been published in: Boyle, N.R., Page, M.D., Liu, B., et al., Benning, C., Pellegrini, M., Grossman, A., Merchant, S.S. (2012) Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. *J. Biol. Chem.* 287: 15811-25. Hemschemeier, A., Caseroc, D., Liu, B., Benning, C., Pellegrinic, M., Happea, T., Merchant, S.S. (2013) COPPER RESPONSE REGULATOR1–Dependent and –Independent Responses of the Chlamydomonas reinhardtii Transcriptome to Dark Anoxia. *The Plant Cell* 25:3186-211; and Blaby, I.K., Glaesener, A.G., Mettler, T., Fitz-Gibbon, S.T., Gallaher, S.D., Liu, B., Boyle, N.R., Kropat, J., Stitt, M., Johnson, S., Benning, C., Pellegrini, M., Casero, D., Merchant, S.S. (2013) Systems-Level Analysis of Nitrogen Starvation-Induced Modifications of Carbon Metabolism in a Chlamydomonas reinhardtii Starchless Mutant. *The Plant Cell* 25:4305-23. I performed lipid analyses and participated in writing the figure legends, methods, and results sections of all three manuscripts.

4.1. Abstract

The microalga *Chlamydomonas reinhardtii* accumulates triacylglycerol (TAG) upon stresses, including nutrient depletion. TAG biosynthesis and related lipid metabolism during stresses are still being investigated because of their potential importance to humans as a future source of renewable energy. Extensive efforts on transcriptome analyses of *C. reinhardtii* wild-type and mutant strains have been made to understand the physiological events that take place during stress conditions. Lipid profiling was performed on nitrogen-deprived and hypoxia-stressed algae to assist in the interpretation of gene expression profiles. Functions of *PDAT1*, *NRR1*, *CRR1* and *STA6* were revealed by lipid analyses of corresponding algal mutant strains under specific stress conditions.

4.2. Introduction

Many microalgae are capable of accumulating triacylglycerol (TAG) under certain circumstances (Liu and Benning, 2013). TAGs produced from algae could serve as precursors of biofuels (Merchant et al., 2012) and polyunsaturated fatty acids (PUFAs) for human consumption (Lenihan-Geels et al., 2013). In addition, manipulation of algal lipid metabolism is relatively easy (Liu and Benning, 2013), especially in the model microalga *Chlamydomonas reinhardtii*, making this organism a good model for studying TAG related events in multi-cellular organisms.

Chlamydomonas reinhardtii is the most investigated single-cellular green alga species (Harris, 2009). Since its genome was sequenced (Merchant et al., 2007), scientists in the community have contributed to transcriptome analyses of *C. reinhardtii* with different genetic backgrounds and under a variety of growth conditions (Blaby et al., 2013; Fang et al., 2012; Lopez et al., 2011). In terms of next generation sequencing, as techniques become mature and the cost continues to drop, there will be more transcriptome data sets available in the future. Such progress has been facilitating in-depth research on algal physiology and biochemistry. In addition, a full set of molecular tools has been developed in *C. reinhardtii* during the past decade (Fuhrmann, 2002; Liu and Benning, 2013), including nuclear transformation and the availability of selectable markers, paving the way for its genetic manipulation.

As with many other microalgae, *C. reinhardtii* accumulates TAG after stress induction, for instance, nitrogen (N) deprivation (Boyle et al., 2012; Miller et al., 2010). Lipid metabolism relating to TAG biosynthesis under stresses has been actively investigated in *C. reinhardtii*. One strategy is to perform comprehensive RNA-seq analyses during a timecourse of applying stresses

which are capable of initiating TAG accumulation in *C. reinhardtii*. Another strategy is to employ mutagenesis approaches to explore functions associated with genes of interest which contribute to the process of TAG accumulation in microalgae.

RNA-seq analyses have been performed for *C. reinhardtii* during the timecourse of a series of stress conditions by scientists in Dr. Merchant's group at UCLA. The analyzed strains included wild type, newly identified mutants with TAG biosynthesis defects and known mutants with less characterization of lipid metabolism. Here, detailed lipid profiling, including quantification of individual lipid classes by gas chromatography-flame ionization detection (GC-FID), was used to help interpretation of changes in gene expression and to explore new directions of algal lipid metabolism research.

4.3. Materials and Methods

C. reinhardtii samples were prepared by scientists in the Merchant laboratory and shipped on dry ice to East Lansing for lipid analyses. Along with algal cell samples for lipid analyses, the number of cells in each sample was counted and calculated by our collaborators. Such data was later utilized for calculating lipid content on a per cell basis.

Quantification of fatty acid content of algal lipid samples was described previously (Boyle et al., 2012; Hemschemeier et al., 2013). Briefly, for quantitative total fatty acid analysis, 5 ml of cell culture was collected on Whatman GF/A 25-mm circular glass filters, frozen in liquid nitrogen, and lyophilized. The filters (with cells) were transferred to reaction tubes and subjected to fatty acid methyl transesterification reactions and gas chromatography as described below. For TAG content, 20 ml of cell culture was collected by centrifugation (2170 x *g*, 2 min),

washed in 1 ml of water, collected by centrifugation (13,780 x *g*, 5 min) in a 1.5-ml screw cap micro-centrifuge tube, and frozen in liquid nitrogen. Prior to analysis, frozen cells were resuspended in 1 ml of methanol/chloroform/formic acid (2:1:0.1 (v/v/v)). Extraction solution (1 M KCl, 0.2 M H_3PO_4; 0.5 ml) was added and mixed by vortexing. Cell debris was removed by centrifugation for 3 min at 13,780 x *g*. Lipids were separated by thin layer chromatography using petroleum ether/diethyl ether/acetic acid (8:2:0.1 (v/v/v)). Samples were run on baked Si60 silica plates (EMD Chemicals) for 5 min. After separation, lipids were reversibly stained by exposure to iodine vapor. The TAG band was located below pigments but above free fatty acid and DAG bands. TAG bands were quantitatively recovered by scraping the plate with a razor blade and transferring the material to a fatty acid methyl ester reaction tube. HCl in methanol (1 N, 1 ml) was added to each tube, and 5 μg of an internal standard (fatty acids 15:0, working concentration 50 g/ml in methanol) was added. The head space in the tube was purged with nitrogen (N_2) and the cap tightly sealed. Samples were incubated at 80 ℃ for 25 min and allowed to cool to room temperature. Aqueous NaCl (0.9%, 1 ml) and *n*-hexane (1 ml) were added, and the samples were shaken vigorously. After centrifugation at 1690 x *g* for 3 min, the hexane layer was removed, transferred to a new tube, and dried under N_2 gas stream. The resulting fatty acid methyl esters were dissolved in 100 μl of hexane. The fatty acid content and composition of the extracts were determined by gas chromatography with flame ionization detection, as described previously (Rossak et al., 1997). The capillary DB-23 column (Agilent) was operated as follows: initial temperature 140 ℃, increased by 25 ℃/min to 160 ℃, then by 8 ℃/min to 250 ℃, and held at 250 ℃ for 4 min.

4.4. Results and Discussion

4.4.1 Analyses of algal TAG accumulation during nutrient depletion

N deprivation conditions were applied to *C. reinhardtii* as such treatment is arguably the most effective inducer of TAG accumulation. To identify suitable time points for transcriptome analysis, TAG accumulation of wild-type algae during a 48 hour timecourse of N deprivation was monitored. Fatty acid quantification results show that the relative amount of TAG continues to increase during the time course of N deprivation (Figure 4.1). These results are consistent with observations of increasing signal from stained lipid droplets throughout the time course of N deprivation (Boyle et al., 2012). Statistical analysis using the student's *t* test indicates that 8-, 12-, 24-, and 48-h samples are significantly different from the 0-h sample, suggesting algae start to synthesize TAG actively several hours after the onset of N deprivation, likely because a minor amount of N source is still present inside the algal cell or because the replacement of N-replete by N-free medium does not remove N completely. The above results were used to determine when algae would be subjected to transcriptome analysis, assuming changes in mRNA abundance are relevant to changes in TAG accumulation. As a result, three time course experiments were subsequently performed: 0–1 h, 0–8 h, and 0–48 h following transfer of algal cells to N deplete medium.

Fatty acid profiles of total lipids were analyzed throughout the timecourse of N deprivation. The relative abundance of the fatty acids 16:0 and 18:1 Δ^9 increase during N deprivation (Figure 4.2). As 16:0 and 18:1Δ^9 are both nascent fatty acids without further desaturation or elongation, this result suggests that the fatty acid *de novo* synthesis is activated in N deprivation. As both the content of TAG and total lipids increase during N deprivation

(Moellering and Benning, 2010), it is straightforward to hypothesize that fatty acid *de novo* synthesis contributes to TAG accumulation, as well as to the overall increase of lipid content during N deprivation.

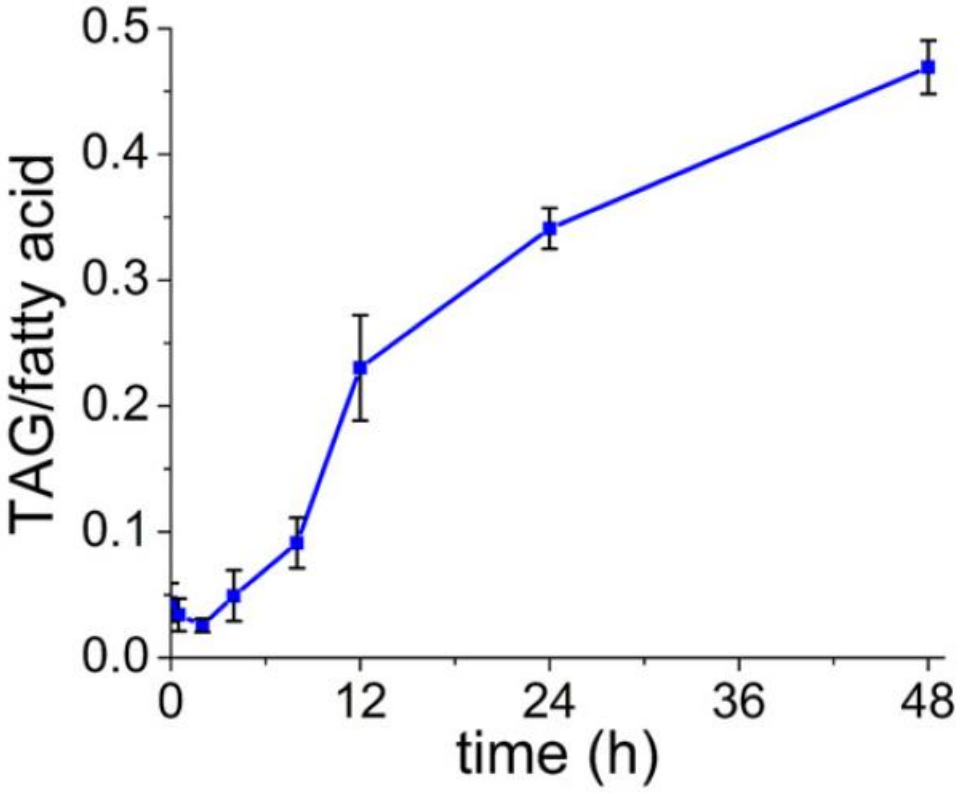

Figure 4.1. Time course of increased TAG accumulation in nitrogen-starved *C. reinhardtii* wild type cells. Fraction of fatty acids in TAG in nitrogen-starved cells during a 48-h time course of nitrogen starvation on a mol/mol basis. *Error bars* represent the standard deviation from three biological replicates. Two-tailed Student's *t* test on experimental triplicates for each time point indicates that 8-, 12-, 24-, and 48-h samples are statistically different from the 0-h sample at 98% confidence.

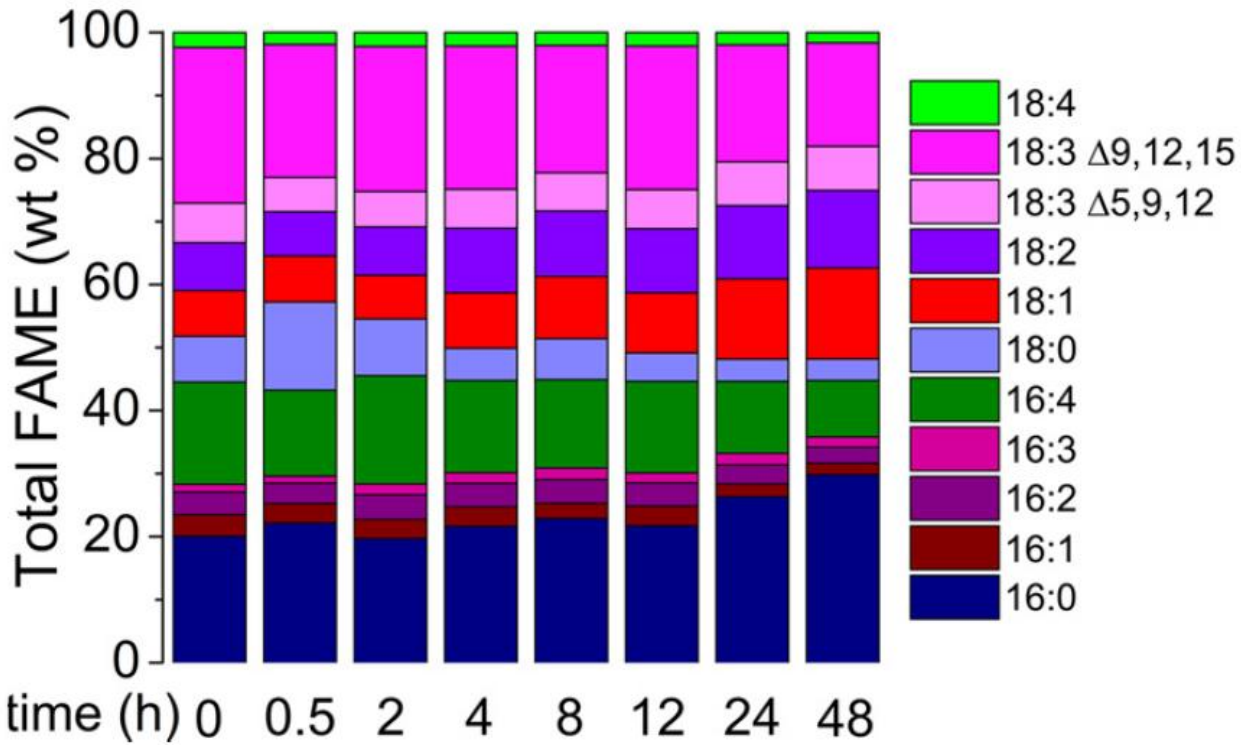

Figure 4.2. Time course of TAG fatty acid profiles in nitrogen-starved *C. reinhardtii* wild type cells. Fatty acid composition of lipids at each time point was measured by gas chromatography. The average of three experimental replicates is shown.

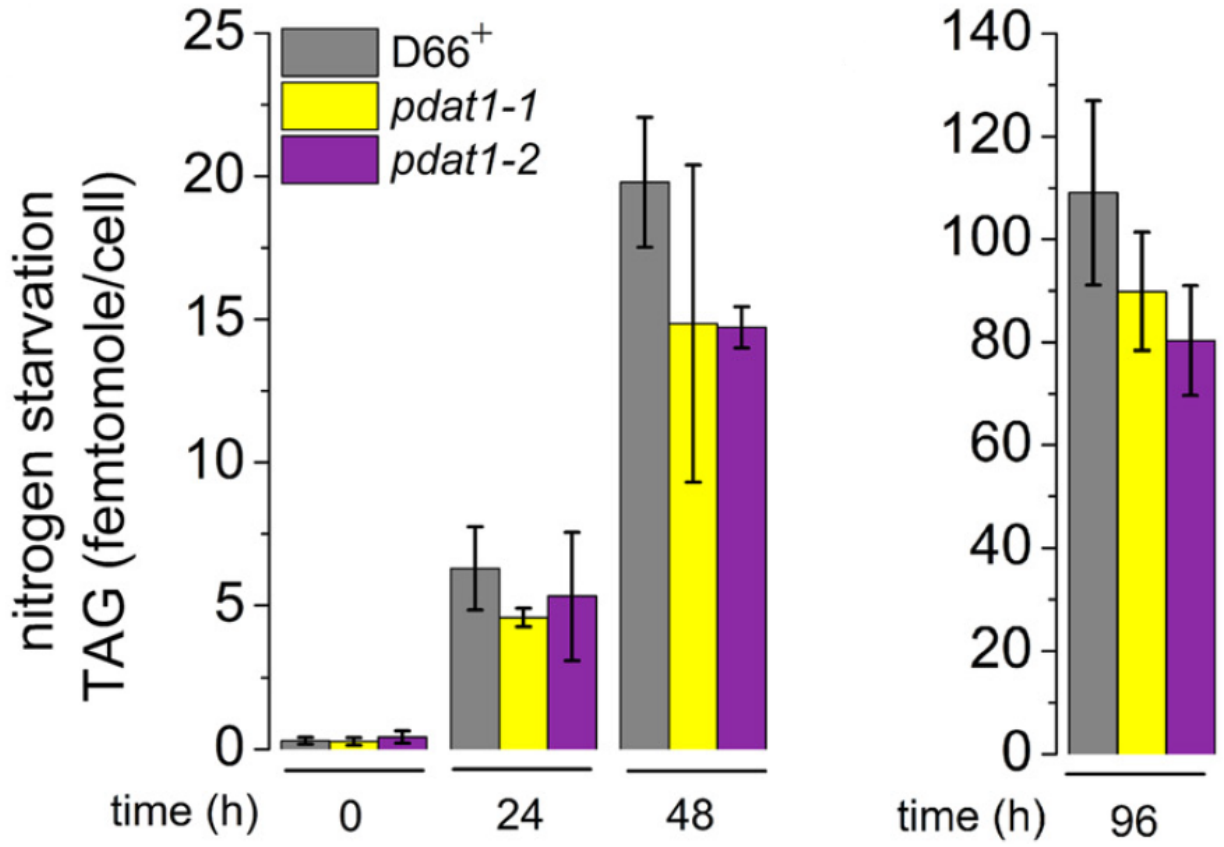

Figure 4.3. TAG Phenotype of *C. reinhardtii* mutant strains *pdat1-1* and *pdat1-2* in N deprivation. TAG content of *pdat1-1* (*yellow*), *pdat1-2* (*purple*), and the parent strain D66^{+} (*gray*) measured at 0, 24, 48, and 96 h after nitrogen starvation by quantitative gas chromatography. Error bars represent the standard deviation from at least three biological replicates.

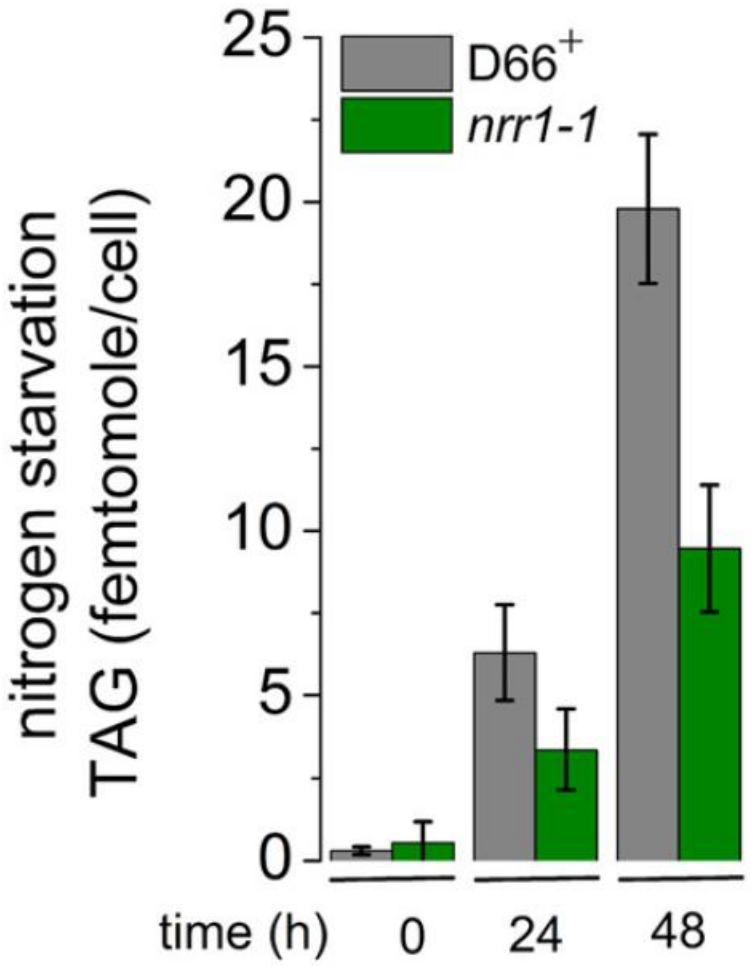

Figure 4.4. TAG Phenotype of *C. reinhardtii nrr1* mutant strain under N deprivation. TAG content of *nrr1* (*green*) and the parent strain D66^{+} (*gray*) measured at 0, 24, and 48 h after nitrogen starvation. Error bars represent the standard deviation from three biological replicates.

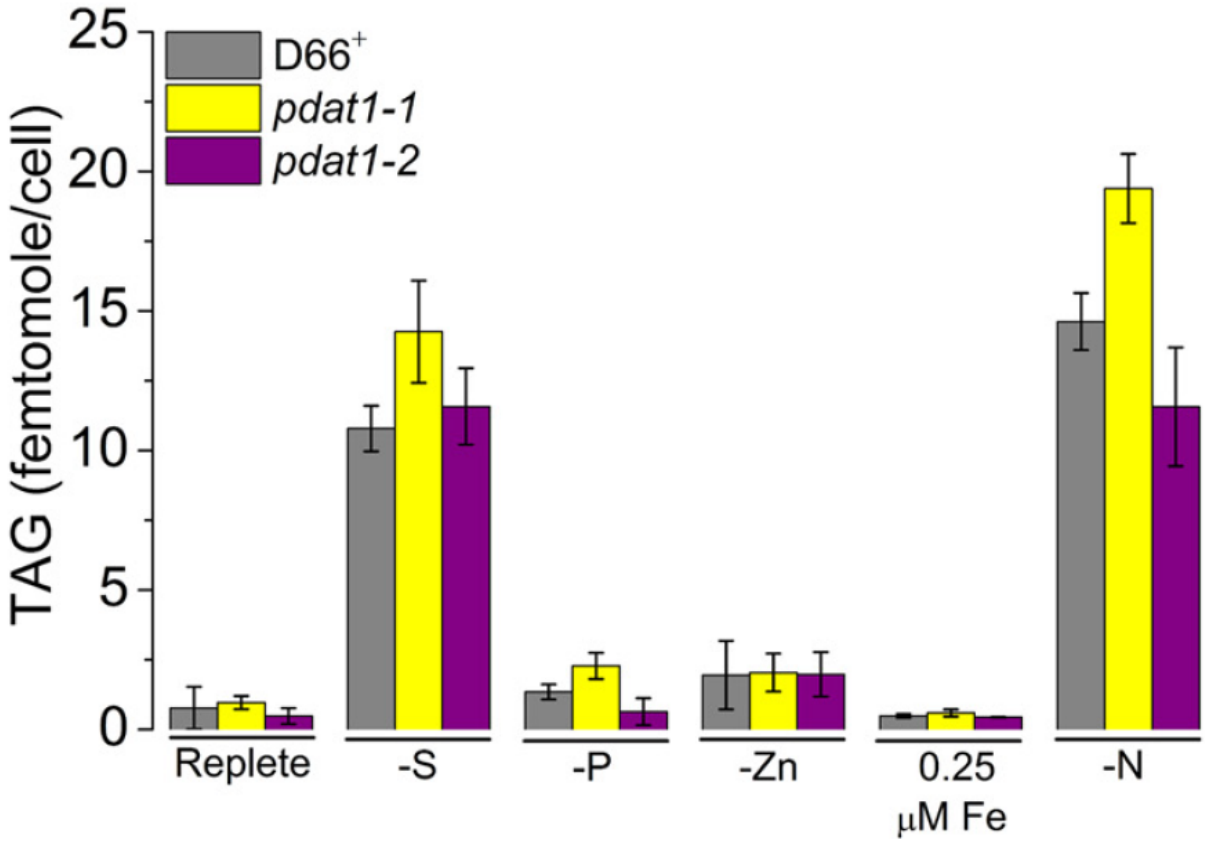

Figure 4.5. TAG Phenotype of *C. reinhardtii* mutant strains *pdat1-1* and *pdat1-2* under stresses. The corresponding stress conditions are -S, -P, -Zn, 0.25 μM Fe, and -N. Error bars represent the standard deviation from three biological replicates.

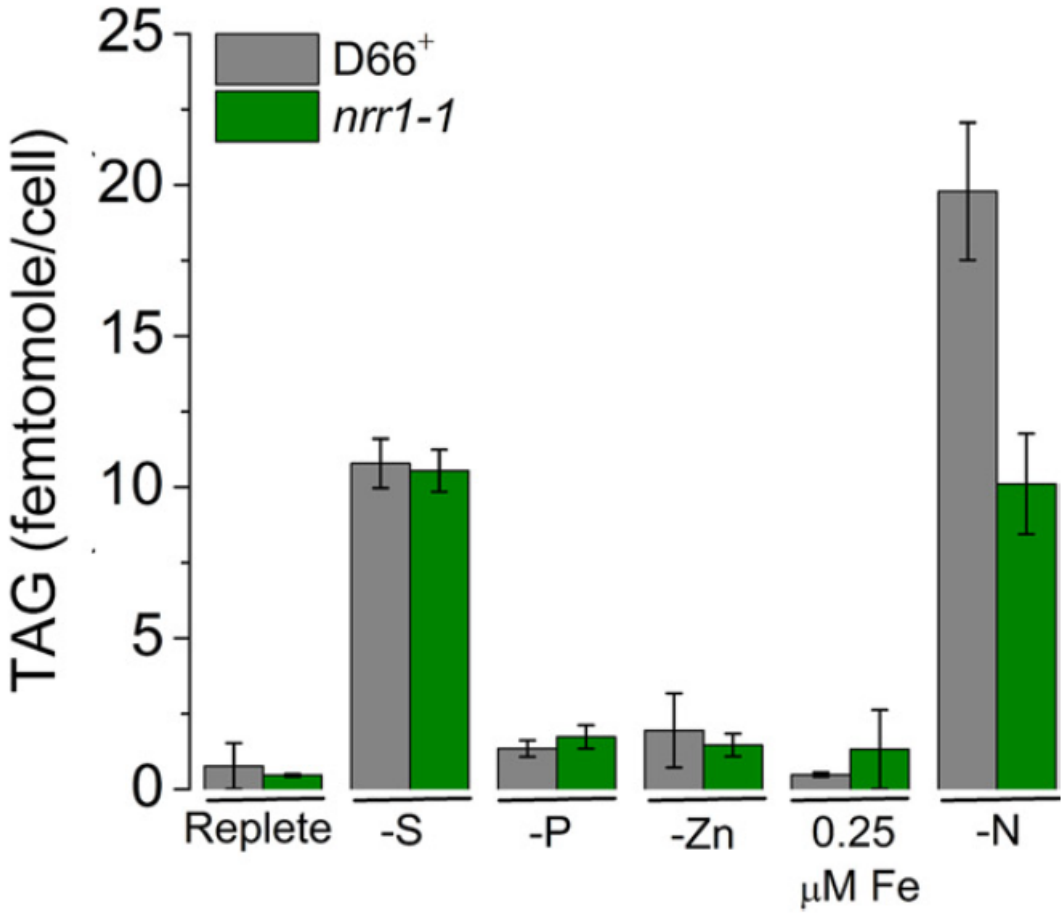

Figure 4.6. TAG Phenotype of *C. reinhardtii* mutant strain *nrr1* under stresses. The corresponding stress conditions are -S, -P, -Zn, 0.25 μM Fe, and -N. Error bars represent the standard deviation from three biological replicates.

C. reinhardtii strains with a mutation or disruption in genes which would presumably be involved in TAG biosynthesis were isolated and subjected to N deprivation and followed by lipid analyses. Those strains include two independent mutants *pdat1-1* and *pdat1-2*, which are deficient in the gene encoding Phospholipid:Diacylglycerol AcylTransferase 1 (PDAT1), and a transcription factor mutant *nrr1* with deficiency in *Nitrogen Response Regulator 1* (*NRR1*). Comparison of TAG contents following N deprivation between wild type and *pdat1* mutants (Figure 4.3) suggests *PDAT1* makes at least a partial contribution to TAG accumulation during N deprivation, as both mutants accumulate about 25% less TAG compared with wild-type cells. These results are in agreement with those of the transcriptomics study which showed that the expression of the *PDAT1* gene was activated during N deprivation (Boyle et al., 2012), confirming the function of PDAT1 in TAG accumulation. Similarly, lower TAG levels were observed in the *nrr1* mutant during N deprivation (Figure 4.4), suggesting that this transcription factor plays a regulatory role in TAG biosynthesis during N deprivation.

All three mutant strains isolated in the same study were subjected to deprivation of other nutrients, including sulfate, phosphate and zinc, and low concentration of iron (0.25 μM), to study the function of the corresponding genes in TAG accumulation in those stresses. As shown in Figure 4.5 and Figure 4.6, different stresses have an impact on algal TAG accumulations at different levels. While sulfate deprivation is the second strongest stress to induce TAG accumulation besides N deprivation, phosphate deprivation and zinc deprivation also impact TAG accumulation, and low iron concentrations had little or no impact on TAG biosynthesis. Interestingly, N deprivation is the only stress in which the *nrr1* mutant produces less TAG than wild type (Figure 4.6), suggesting the mutated transcription factor in *nrr1* is specifically responsible for TAG accumulation during N deprivation.

4.4.2 Analyses of TAG accumulation in a *starchless* mutant during N deprivation

In addition to TAG, starch is another type of energy reserve for microalgae. It has been repeatedly reported that wild-type *C. reinhardtii* accumulates both TAG and starch under stresses (Liu and Benning, 2013). The starchless *C. reinhardtii* mutant *sta6* was subjected to N deprivation, in comparison with its parental strain, to understand the relationship of TAG and starch biosynthesis pathways. During a timecourse of N deprivation, comprehensive transcriptome analyses were performed for *sta6* and the wild-type strain. Moreover, metabolite profiling, including lipid analysis, has been pursued to consolidate the interpretations from RNA-seq data. TAG contents were measured for 96-hour N deprived wild type, *sta6* and mutant complementation strains. Figure 4.7 shows that the *sta6* mutant accumulates more TAG than wild type during N deprivation, while the complementation lines restore the TAG phenotype to some extent. The TAG contents are consistent with the signal intensity of the Major Lipid Droplet Protein 1, MLDP1 detected by Western Blot at the same time points during N deprivation, suggesting the starchless *C. reinhardtii* mutant deposits more energy and carbon into TAG upon stress treatment.

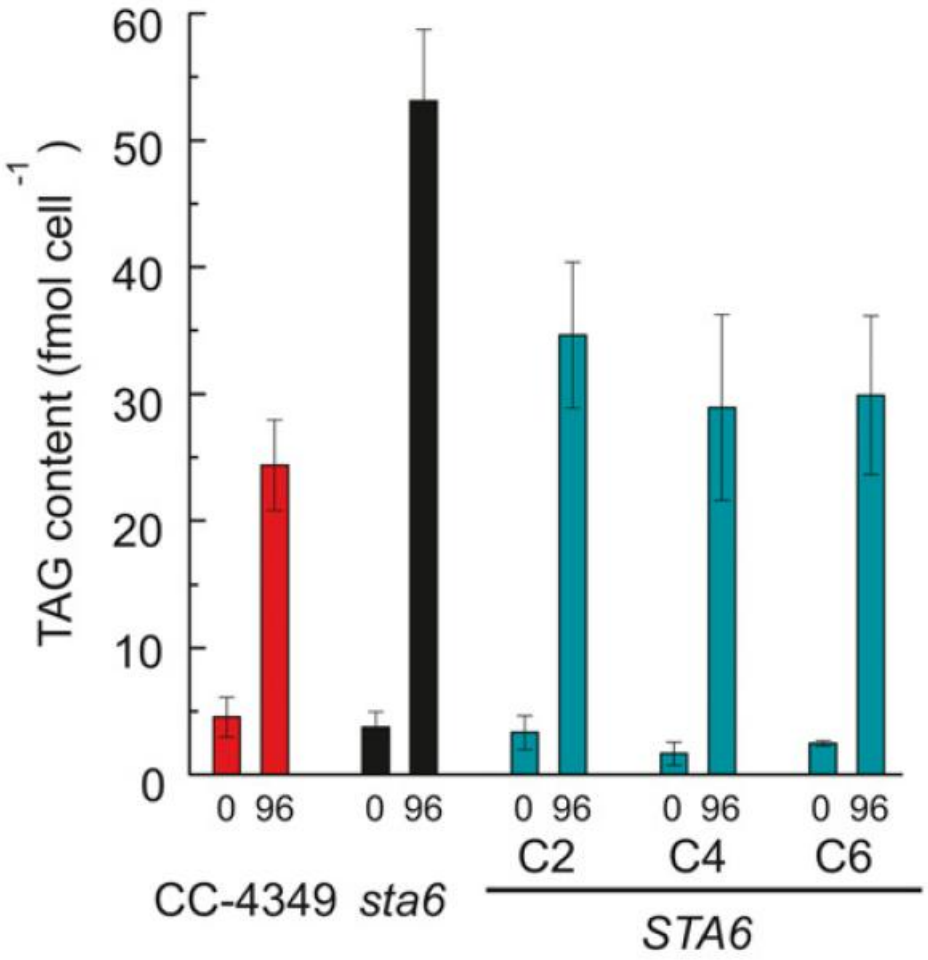

Figure 4.7. Increased TAG in the *sta6* mutant. TAG per cell for the wild type is shown in red, *sta6* in black, and three strains complemented for *STA6* (*STA6*-C2, *STA*6-C4, and *STA6*-C6) in blue. Error bars represent the standard deviation calculated from three biological replicates.

4.4.3 Lipid profiling of hypoxia stressed *C. Reinhardtii*

Anaerobiosis occurs when algae undergo hypoxia stress. Upon such conditions, cellular fermentation processes are activated, which has been extensively studied in *C. reinhardtii* (Catalanotti et al., 2013). Interestingly, TAG accumulation was observed in hypoxia stressed *C. reinhardtii* (Matthew et al., 2009). Transcriptome analysis was performed for a wild type and a *crr1 C. reinhardtii* mutant with a deficiency in the gene *Copper Response Regulator1* (*CRR1*) to study the regulation during hypoxia stress by the transcription factor *CRR1*. In addition, comprehensive lipid profiling was pursued for hypoxia stressed wild-type and mutant algae to understand the relationship with gene expression and lipid metabolism during anaerobiosis. Lipid data demonstrates that the content total fatty acid decreases under hypoxia stress, especially for wild type *C. reinhardtii*, compared with both control groups, algae grown in non-stress conditions and in the dark aerobically (Figure 4.8A). For both wild type and the *crr1* mutant, the content of TAG increases while the level of most membrane lipids decreases during hypoxia stress (Figure 4.8B). Especially, galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are more than 50% degraded compared with unstressed control groups. Moreover, 16:4 and $18{:}3\Delta^{9}$, the two signature fatty acids of MGDG, are enriched in TAG accumulated during hypoxia stress (Figure 4.8C), suggesting that membrane lipids might be degraded and fatty acids released for TAG assembly. Additionally, when the mutant is considered in terms of lipid contents and lipid fatty acid profiles, *crr1* exhibits less difference between hypoxia-stress and non-stress conditions (Figure 4.8, 4.9), confirming a regulatory function of *CRR1* in lipid metabolism.

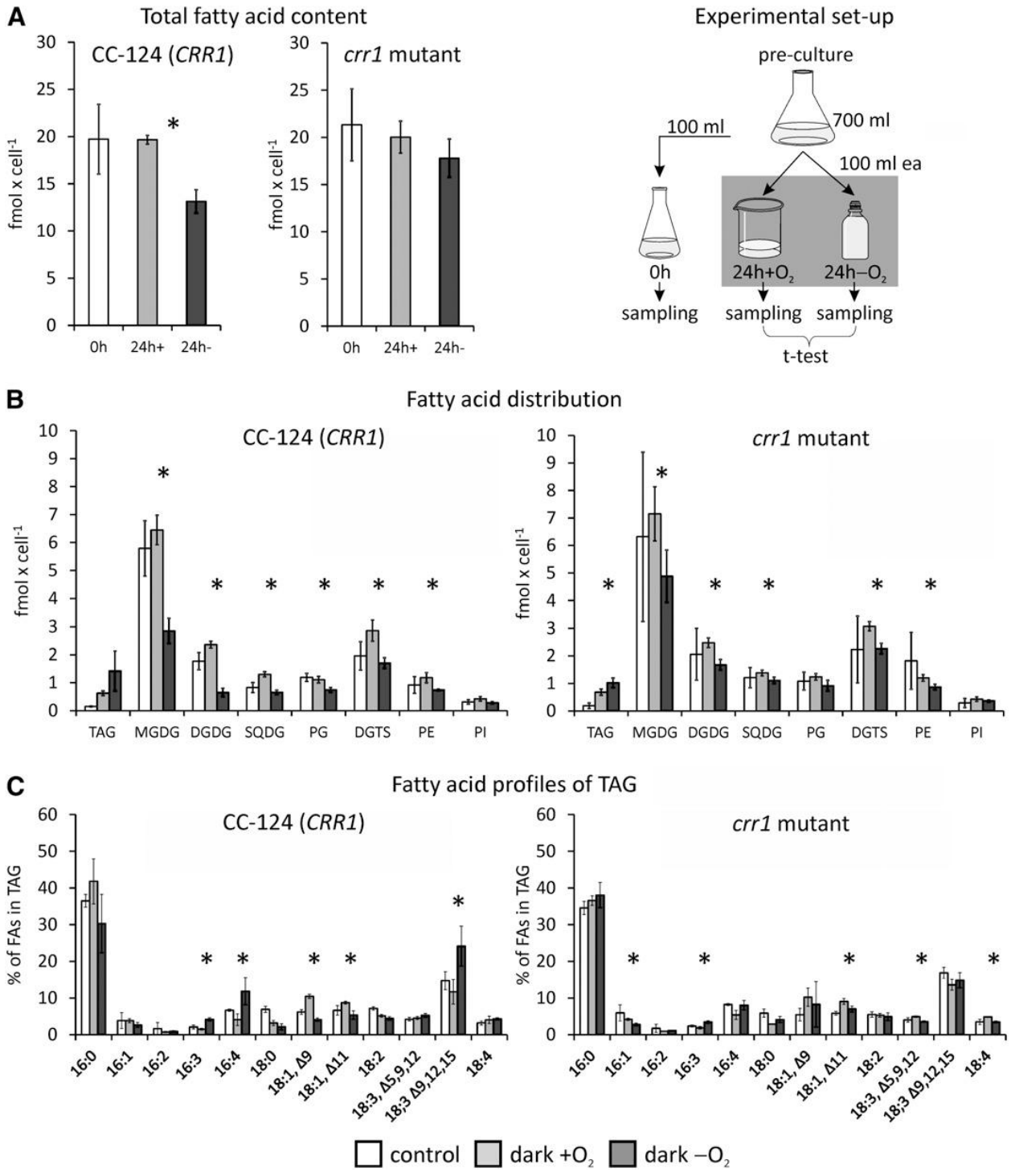

Figure 4.8. Profiles of fatty acids and lipids under hypoxia stress. The total content of fatty acids (A), the amounts of fatty acids associated with TAG or membrane lipids (B), and profile of fatty acid species

Figure 4.8. (cont'd)

associated with TAG (C) were analyzed in *C. reinhardtii* wild-type CC-124 (*CRR1*) and *crr1* mutant cells grown aerated in the light (white bars) and then transferred to open beakers (light gray bars, dark + O_2) or sealed flasks (dark gray bars, dark - O_2) in the dark for 24 h. Values shown are the averages of biological triplicates. Error bars indicate the standard deviation. Asterisks indicate that the difference between samples incubated anaerobically versus aerobically in the dark was significant (P value <0.05). SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; DGTS, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

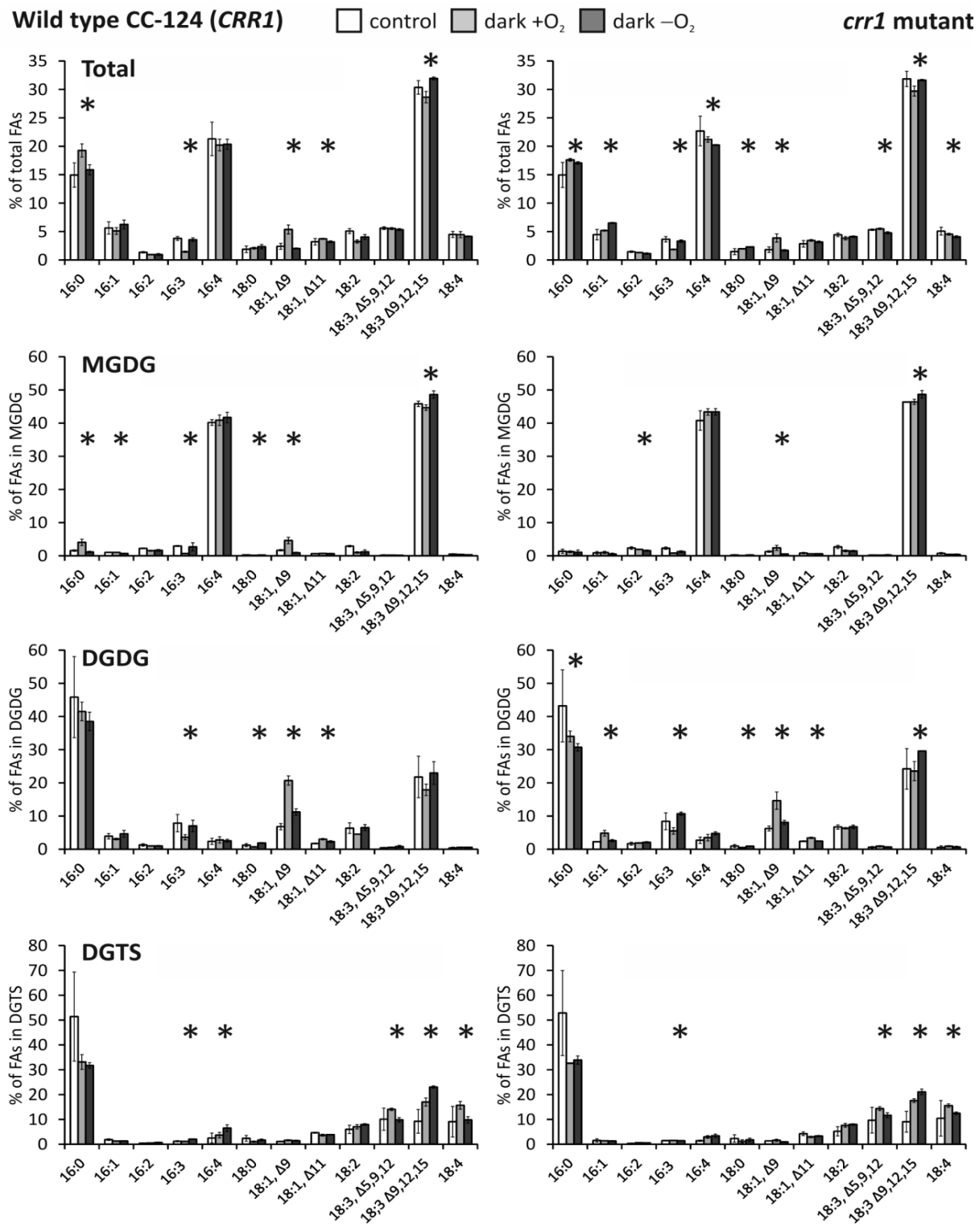

Figure 4.9. Fatty acid profiles of dark incubated *C. reinhardtii* cultures. Wild type CC-124 and mutant strain *crr1* were grown under standard conditions in the light and then transferred to

Figure 4.9. (cont'd)

open beakers in the dark (light gray bars, dark + O_2) or sealed flasks (dark gray bars, dark – O_2). Cell samples were withdrawn just prior to the transfer from the preculture (white bars, control) and after 24h of dark-incubation. Total fatty acid profiles as well as the association of individual fatty acid species with a certain lipid class were analyzed. Values shown are averages of biological triplicates. Error bars indicate the standard deviation. Asterisks indicate that the difference between samples incubated anaerobically *versus* aerobically in the dark was significant (P value <0.05). MGDG: monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, DGTS: diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine.

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CHAPTER 5

Conclusions and perspectives

The storage lipid in microalgae, triacylglycerol (TAG), has recently garnered increased interest from both public and research communities because of its potential for biofuel applications (Merchant et al., 2012). Investigations employing biochemical and genetic approaches have been increasingly used to understand basic TAG metabolism in microalgae. In addition, algal strain selection, yield optimization and metabolic engineering have been pursued to improve the value of algal TAGs in industrial applications (Georgianna and Mayfield, 2012). Before this work, our knowledge about TAG biosynthesis and degradation in microalgae was mostly adapted from that in Arabidopsis, a model plant which has been extensively investigated for its lipid metabolism. In this post-genomics era, functional annotation of genes related to algal TAG metabolism has been performed by comparison of algal and plant genomes (Merchant et al., 2007). However, experimental evidence is still needed to confirm the predicted function of algal genes in most cases. Moreover, TAG metabolism in microalgae can be different from that in plants (Liu and Benning, 2013), highlighting the importance of pursuing wet lab experiments to understand the biochemistry of TAG in microalgae.

The model microalga *Chlamydomonas reinhardtii* accumulates TAG upon stresses, for instance, nutrient depletion. Transcriptomic analysis has been performed for *C. reinhardtii* under conditions which induce TAG accumulation, including nitrogen (N) deprivation (Boyle et al., 2012; Miller et al., 2010) and dark hypoxia stress (Hemschemeier et al., 2013). Therefore, the expression profile of genes involved in TAG biosynthesis is available and can be compared between different conditions (Lopez et al., 2011). In addition, forward genetics approaches have been conducted in the Benning lab to generate and characterize *C. reinhardtii* mutant strains with abnormal phenotypes in TAG content. For instance, a novel gene *Plastid Galactolipid Degradation 1* (*PGD1*) has been characterized to be functional in TAG biosynthesis during N

deprivation (Li et al., 2012). The focus of this dissertation was to study several aspects of the biochemistry of algal TAG metabolism. First of all, a liquid chromatography–mass spectrometry (LC-MS)-based TAG profiling approach was developed for algal TAG identification and quantification (Chapter 2). Secondly, biochemical characterization of TAG biosynthesis in dark hypoxia stressed *C. reinhardtii* was conducted (Chapter 3). The third project was quantitative analysis of TAG and membrane lipids from various algal samples produced in collaborative projects (Chapter 4). And lastly, forward and reverse genetics approaches were attempted to understand algal TAG biosynthesis during N deprivation (Appendix).

5.1. Algal TAG profiling by ultrahigh performance liquid chromatography–mass spectrometry

The project described in Chapter 2 aimed to establish an ultrahigh performance liquid chromatography–mass spectrometry (UHPLC-MS) based platform to identify and quantify TAGs in microalgae. The conventional method of quantitative lipid analysis is to perform fatty acid methyl esterification for acyl lipids, including TAG, followed by gas chromatography (GC) analysis of derived fatty acid methyl esters (FAMEs). This method is straightforward and robust, and it has been used in the Benning lab for more than 15 years (Rossak et al., 1997). However, fatty acyl groups disassemble from the glyceryl group during the FAME reaction, leading to the loss of molecular integrity of the acyl lipids. In the newly developed LC-MS approach, which was developed by us, algal lipid extracts were separated by LC so that TAGs and membrane lipids were intact prior to MS detection. Thus, the molecular species of algal TAG can be identified by LC-MS.

Two microalgae, *C. reinhardtii* and *Nannochloropsis oceanica* have been subjected to the TAG profiling strategy. In both algal species, we were able to identify more than one hundred TAG molecular species by LC-MS approaches. The composition of major TAG species was different between the two algae: while 48:1 (number of carbons in fatty acids: number of double bonds) is the dominant TAG species in *N. oceanica*, nine different TAGs are the major TAGs in *C. reinhardtii*. In addition, we identified new fatty acid species in both microalgae, including short chain fatty acids 12:0 and 14:0 and long chain fatty acids 20:0, 20:1, 20:2 and 20:3. The amount of those fatty acyl groups in algal TAG were very low, and could not be detected by the GC method.

We confirmed that quantification of algal TAG by LC-MS was relatively accurate and robust, in comparison with GC measurements. As the LC-MS approach was able to maintain the integrity of the whole TAG molecule, quantitative TAG measurement suggested that polyunsaturated TAGs became enriched when *N. oceanica* was subjected to N deprivation, consistent with the fact that GC analysis could only detect long chain polyunsaturated fatty acids 20:4 and 20:5 in N deprived *N. oceanica*.

Interestingly, we developed two sets of internal standards by taking advantage of the presence of pheophytin and specific fatty acids in algal samples. This first internal standard was the pheophytin *a* peak in the LC-MS chromatogram. Pheophytin *a* is the derivative of chlorophyll *a*, which is relatively stable and independent from TAG biosynthesis in *C. reinhardtii*, so that the peak of pheophytin *a* was able to serve as a reference to estimate the TAG level in LC-MS. The second internal standard was the ratio of saturated fatty acid 16:0 to polyunsaturated fatty acid 16:4, as determined by GC analysis. The ratio of these two fatty acids was proven to correlate with the TAG content in N deprived *C. reinhardtii*. As an external

standard can be omitted, both internal standards have the potential to be applied in the analysis of a large number of samples, as would be encountered during high throughput phenotypic screening.

In the future, a bioanalytical approach for in-depth TAG structural analysis can be developed. For instance, lipase digestion is still the major method to distinguish the fatty acyl groups at the *sn*-2 position of TAG from those at the *sn*-1 or *sn*-3 position. A rapid and reliable analytical approach, such as LC-tandem MS, which is able to determine the fatty acyl groups at the *sn*-2 position of TAGs will be beneficial the study of biosynthesis of TAG in microalgae.

5.2. Algal TAG accumulation through membrane lipid remodeling during hypoxia stress

Following N deprivation, *C. reinhardtii* accumulates TAG through *de novo* fatty acid synthesis (Li et al., 2012). In Chapter 3, another scenario of algal TAG accumulation upon stress induction was described and characterized. When *C. reinhardtii* was subjected to dark hypoxia stress, fatty acyl groups were removed from membrane lipids, and then reutilized for TAG biosynthesis.

It has been reported that *C. reinhardtii* accumulates TAG during hypoxia stress (Matthew et al., 2009). Transcriptomic analyses have been performed to understand the gene expression profile under this stress condition (Hemschemeier et al., 2013). In this collaborative project, quantitative analysis of algal TAG has been conducted, showing that TAGs accumulating in the hypoxia stressed *C. reinhardtii* were more unsaturated than those under N deprivation. Follow-up experiments demonstrated that under hypoxia stress, *C. reinhardtii* accumulated TAG at the

cost of total lipids. The above evidence led to the hypothesis that microalgae accumulate TAG through membrane lipid remodeling during dark hypoxia conditions.

I performed radioactive pulse-chase labeling experiments to understand the relationship between the degradation of membrane lipids and TAG accumulation. The result confirmed that in hypoxia stressed *C. reinhardtii*, radiolabeled ^{14}C moved from membrane lipids to TAG, suggesting that by membrane lipid remodeling, fatty acyl groups were released for TAG biosynthesis. Then, TAG biosynthesis-affected *C. reinhardtii* mutant strains were subjected to dark hypoxia stress. A mutant *pgd1*, with a gene deficiency in the *de novo* fatty acid synthesis pathway, accumulated less TAG during N deprivation. However, no abnormal TAG phenotype was observed in the hypoxia stressed *pgd1* mutant strain, suggesting that membrane lipid remodeling, the major TAG biosynthesis pathway, is relatively independent of the *de novo* fatty acid synthesis pathway. In addition, lipid analysis of *pdat1* mutants, which were deficient in the so called *Phospholipid: Diacylglycerol Acyltransferase 1* (*PDAT1*) gene, demonstrated that TAG accumulation was affected in both *pdat1* mutants, while membrane lipids DGTS and MGDG were also abnormal under the hypoxia condition. These results suggested that the enzyme PDAT1, which should be renamed as PDAT-like, was active during hypoxia stress, transferring fatty acids from membrane lipids to TAG.

In summary, we characterized algal TAG accumulation through membrane lipid remodeling as summarized in Chapter 3. As one of two known pathways for TAG biosynthesis, membrane lipid remodeling was detected in hypoxia stressed *C. reinhardtii*, while the other pathway, *de novo* fatty acid synthesis, has been repeatedly studied during N deprivation. In this study, 80% of the fatty acyl groups at the *sn*-2 position of algal TAG that accumulated under

hypoxia stress were 16-carbon fatty acids, which is similar to those under N deprivation. These results suggested 80% of TAGs were derived from chloroplast lipid biosynthesis pathway. Thus, the subcellular location where the reactions of membrane lipid remodeling occur will be of interest in future investigations. Localization of PDAT1, PGD1 and other algal TAG biosynthesis related proteins should be investigated to understand the compartmentalization of TAG synthesis in *C. reinhardtii* upon different stresses. Another interesting question is whether different TAG biosynthesis related enzymes and regulators work in hypoxia stress or N deprivation. For instance, three types of acyl-CoA: diacylglycerol acyltransferases (DGAT) were indentified while there are at least five type two DGAT genes in the *C. reinhardtii* genome. It will be intriguing to understand the specific function of each DGAT in *C. reinhardtii*.

5.3. Lipid profiling of *C. reinhardtii* strains in different growth conditions

Chapter 4 summarized collaborative projects in which I conducted lipid profiling for *C. reinhardtii* wild-type and mutant strains under various stress conditions. Led by Dr. Merchant, transcriptomic analysis has been performed for stress treated *C. reinhardtii*. Lipid analysis of algae under the stress conditions is beneficial when interpreting the gene expression profile. Three independent sets of lipid analyses were performed for *C. reinhardtii* samples. First, quantitative TAG analysis was performed for wild type and mutant strains *pdat1-1*, *padat1-2* and *nitrogen response regulator 1* (*nrr1*) in a timecourse of N deprivation. The second project was comparing TAG content in N deprived wild type and starchless mutant, *sta6*. Lastly, a comprehensive lipid analysis, including quantification of TAG and membrane lipids, was conducted for a *C. reinhardtii* wild type and *copper response regulator1* (*crr1*) mutant.

Two independent *pdat1* mutants were originally generated from the first project, providing the *C. reinhardtii* strains used in the project described in Chapter 3. In addition, the results of above lipid profiling projects led to the idea of the work described in Chapter 3 that membrane remodeling contributes to algal TAG accumulation under hypoxia stress. In the future, lipid analysis should be conducted along with the transcriptomic analyses of new *C. reinhardtii* cell samples. The lipid profile can be combined with the transcriptomic profile, providing insights into the relationship between algal lipid metabolism and gene regulation.

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APPENDIX

APPENDIX A: Investigation of TAG biosynthesis in *Chlamydomonas reinhardtii* by genetics approaches[4]

A.1. Abstract

Three *C. reinhardtii* mutant strains with abnormal triacylglycerol (TAG) levels during nitrogen deprivation have been identified by forward genetics approaches. Detailed lipid analysis confirmed the TAG phenotypes and site-finding PCR was applied to identify the location of mutated genes in these mutants. Furthermore, gene complementation was attempted to rescue the TAG phenotype by introduction of corresponding gene in *C. reinhardtii* mutants. In addition, two mutant strains with point mutations in the *BTA1* gene were generated by a reverse genetics approach, namely targeting induced local lesions in genomes (TILLING). Characterization of *bta1* TILLING mutants was pursued to understand the function of BTA1, the algal betaine lipid synthase, during the process of TAG accumulation.

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[4] *Chlamydomonas reinhardtii* mutant strains B2 and A10 were indentified from a mutant pool which has been generated by Eric Moellering. Generation and earlier characterization of mutant strain Ligend was performed by Chia-Hong Tsai. Barb Sears performed crosses and genetic assays. *BTA1* point mutations and relevant backcrosses were performed by Setsuko Wakao from Krishna Niyogi lab. Three undergraduate students contributed to this project: Christiane Iserman indentified the location of mutated genes in *C. reinhardtii* mutant strain A10; Nicholas Pokorzynski characterized mutant strain B2 and Milton Akeem Williams complemented mutated genes in strain A10. I performed genetic analyses of mutant strains B2, A10 and Ligend, as well as characterization of *bta1* mutant strains.

A.2. Introduction

To understand the mechanism of algal TAG biosynthesis during stress conditions and the regulation of such process, different mutant screen strategies were developed to identify novel *C. reinhardtii* genes which are involved in TAG accumulation in the Benning lab. In the first mutant screen which was led by Eric Moellering, fluorescence intensity of Nile-Red stained lipid droplet has been compared with that from chlorophyll in algal cells. By such primary screen, a much smaller group of mutant candidates with high or low TAG levels were selected for further characterization. As a result, a mutant strain *pgd1* has been identified with low TAG phenotype during N deprivation and the gene *PGD1*, encoding a galactolipid lipase in *C. reinhardtii*, has been functionally characterized (Li et al., 2012). Besides *pgd1*, other insertional mutant strains from the primary screen with potential high or low TAG phenotype are available to be confirmed and characterized. In the second mutant screen project which has been led by Chia-Hong Tsai, a *C. reinhardtii* mutant, namely Ligend, has been identified with low TAG phenotype during N deprivation. The plasmid insertion in the genome of Ligend has been located and genetic complementation and further characterization of this mutant strain is required to understand the mechanism of TAG accumulation in *C. reinhardtii.*

In addition, reverse genetics approaches were applied to confirm and further understand the predicted function of specific genes and proteins during algal TAG accumulation. For instance, the *BTA1* gene has shown to encode a betaine lipid synthase, the only protein with such activity in *C. reinhardtii.* To date, the activity of BTA1 has been characterized *in vitro*, confirming the predicted function (Riekhof et al., 2005). In *C. reinhardtii*, BTA1 catalyzes a two-step reaction, synthesizing betaine lipid from substrate diacylglycerol (DAG). The betaine lipid, diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine (DGTS), is the end product of this reaction.

Interestingly, DGTS shares structural similarity with phosphatidylcholine (PtdCho) (Figure A.1), which is the major membrane phospholipid in plants but absent from *C. reinhardtii*. As PtdCho has been shown to be an intermediate during lipid biosynthesis in plants, it has been hypothesized that DGTS functionally substitutes PtdCho in microalgae (Liu and Benning, 2013). However, a recent study demonstrated that monogalactosyldiacylglycerol (MGDG) served as a 'hub' to transfer fatty acids for TAG biosynthesis in *C. reinhardtii* (Li et al., 2012), suggesting MGDG might have a partially similar role in microalgae as PtdCho in plants. However, whether DGTS plays additional roles besides membrane structural roles is still unclear. Moreover, the function of DGTS in *C. reinhardtii*, especially during the process of TAG accumulation, awaits further characterization.

Here, identification and characterization of *C. reinhardtii* high or low TAG mutant strains is performed and updated. In addition, as two independent TILLING mutants of *BTA1* gene had been generated by our collaborators, characterization of these two mutants has been pursued in N deprived *C. reinhardtii*.

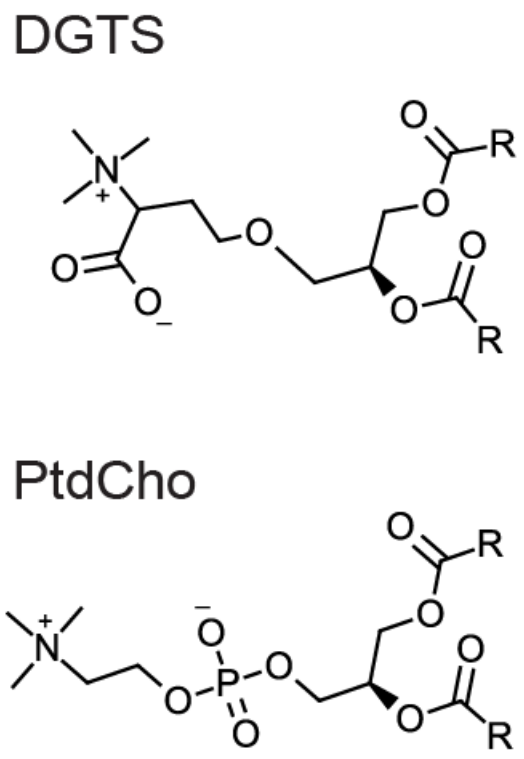

Figure A.1. Molecular structures of DGTS and PtdCho. R represents a hydrocarbon chain.

A.3. Materials and Methods

A.3.1. Strains and growth conditions

Chlamydomonas reinhardtii cell wall-less strain dw15-1 (cw15, nit1, mt+) was obtained from Arthur Grossman and is referred to as wild type. The *C. reinhardtii* mutant strains B2 and A10 were generated during a previous mutant screen project using dw15-1 as the parental strain (Li et al., 2012). Mutant strain Ligend was generated by another not yet published mutant screen strategy developed by Chia-Hong Tsai and Benning. Mutant strains were crossed with CC-198 (er-u-37, str-u-2-60, mt-; Chlamydomonas Resource Center; http://www.chlamycollection.org) for genetic analysis. In another project, *C. reinhardtii* strains 4A+ and 4A- were obtained from Krishna Niyogi. S18F and P206L, which are two mutants generated from 4A+ background were generated by Krishna Niyogi laboratory. *C. reinhardtii* cells were grown in Tris-acetate-phosphate (TAP) medium (Harris, 2009), on a shaker at 100 rpm under continuous light (80 μmol m^{-2} s^{-1}) at 22 ℃. N deprivation was used to induce TAG accumulation in algae by omitting N-containing salts from the medium. Algal cells during log-phase growth were collected by centrifugation (3000 x g, 4 ℃, 5 min), washed with sterilized water and resuspended in the TAP medium without N (TAP -N) for further growth. While harvesting algal cultures, cell densities were determined using a Z2 Coulter Counter (Beckman-Coulter, https://www.beckmancoulter.com) with a 100 mm aperture according to the manufacturer instruction. For the purpose of total fatty acid measurement, 1 mL cell culture was harvested by filtration through a circular glass filter (GF/A, 25 mm, Whatman, http://www.whatman.com). Quantification of chlorophylls in Chlamydomonas was performed as previously described (Li et al., 2012).

A.3.2. Mutant screen and genetic analysis

For forward genetics approaches, all three *C. reinhardtii* mutants described in this section were generated by plasmid disruption. The primary mutant screen strategy for generating strains B2 and A10 has been previously described (Li et al., 2012). Briefly, a linearized pHyg3 plasmid conferring resistance to hygromycin B (Berthold et al., 2002) was inserted into the genome of wild type *C. reinhardtii* using glass bead transformation (Kindle, 1990). Insertional mutant strains were subjected to N deprivation and followed by Nile-Red staining (Sigma-Aldrich, http://www.sigmaaldrich.com). The fluorescence intensity of Nile-Red stained neutral lipids was compared with an internal standard, the chlorophyll fluorescence from the algal chloroplast. As a result, a group of outlier mutant strains with high or low level of TAG were identified from the primary screen. This group of mutants, including *pgd1*, B2 and A10, was further subjected to lipid separation and isolation, and TAG quantification by gas chromatography to identify mutant strains with robust and significant TAG phenotypes.

To perform Southern Blot, *C. reinhardtii* genomic DNA was extracted according to (Newman et al., 1990) and digested with restriction endonucleases (New England Biolabs, https://www.neb.com). Digested genomic DNA was resolved by agarose gel electrophoresis, followed by transferring to a nylon membrane (Amersham Hybond N+, GE Healthcare, http://www.gelifesciences.com), and fixation under ultraviolet light. A digoxigenin (DIG) labeled probe for Southern Blot was generated by PCR amplification. The probe was designed to hybridize with a 234 bp region of pHyg3 DNA encoding hygromycin B resistance. The sequence of PCR primers (SF and SR) as well as other nucleotides described in this section is shown in

Table A.1. DNA hybridization with DIG labeled probe and signal detection were performed according manufacturer's instructions (http://www.roche.com).

SiteFinding PCR was performed for genotyping B2 and A10 according to (Tan et al., 2005). SiteFinder1, SiteFinder3 and SiteFinder6 as well as SFP1 and SFP2 were used for both B2 and A10 to localize plasmid insertions. Various GSPs, which are modified from (Tan et al., 2005) according the sequence of pHyg3, have been used in SiteFinding PCR experiments. Specifically, GSP3-1, GSP3-3 and GSP3-4 were used for B2 insertion localization and GSP5-4, GSP5-6, GSP6-1 and GSP6-3 were used for A10 insertion localization. The sequence of above mentioned oligonucleotides are listed in Table A.1.

To complement mutated genes in mutant strain B2 and A10, a co-transformation strategy was conducted to introduce the genome sequence of a specific gene into corresponding mutant background. A plasmid pMN24 (Fernandez et al., 1989) containing *C. reinhardtii* nitrate reductase gene *NIT1* was selected as a transformation selection marker. Bacterial artificial chromosomes (BACs) containing *C. reinhardtii* genomic DNA (Clemson University Genomics Institute, http://www.genome.clemson.edu) was used to obtain genes mutated in B2 and A10. A piece of DNA containing complete specific gene and flanking regions was digested from BAC and isolated. Meanwhile, plasmid pMN24 was linearized by *Bam*HI. The two pieces of DNAs were co-transformed into *C. reinhardtii* wild-type and mutant strains to complement corresponding mutated gene. TAP plates containing nitrate instead of ammonium were used for selection of the presence of *NIT1* gene. Later, the transformants which formed colony on selection plate were tested by PCR to detect whether BAC fragment was co-transformed into *C. reinhardtii* genome.

A.3.3. Lipid analysis

Lipid extraction, separation and fatty acyl transesterification were performed according to (Moellering and Benning, 2010). Briefly, algal lipid extract was loaded onto Si60 silica then layer chromatography (TLC) plates (EMD Millipore, http://www.emdmillipore.com) and developed with 100 mL solvent, which was determined by the lipid species to be isolated. To separate neutral lipids, including TAG, DAG and free fatty acid, solvent system petroleum ether-diethyl ether-acetic acid (8:2:0.1 by volume) was used. To separate polar lipids, solvent system chloroform-methanol-acetic acid- H_2O (75:13:9:3 by volume) was used. After separation, lipids were isolated from TLC plate and subjected to transmethylation. Then, FAMEs were extracted, dried and redissolved for gas chromatography (GC) analysis. Gas chromatography-flame ionization detection was modified from a previous study (Rossak et al., 1997). Briefly, a capillary DB-23 column (length 30 m, diameter 0.25 mm, film thickness 0.25 μm; Agilent Technologies, http://www.agilent.com) was heated as follows: an initial temperature at 140 ℃ was used, this temperature was increased by 25 ℃/min to 160 ℃, then by 8 ℃/min to 250 ℃, and held at 250 ℃ for 4 min.

A.3.4. Isolation of *C. reinhardtii* lipid droplets

Isolation of lipid droplets from N-deprived *C. reinhardtii* cells has been described in (Moellering and Benning, 2010). Briefly, a *C. reinhardtii* cell pellet was suspended in 5 mL isolation buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM KCl, 0.5 M sucrose, 1 mM 2,2'-Dipyridyl and 1 mM PMSF) with protease inhibitor (Roche, one tablet for every 10 ml of buffer) on ice.

The cell suspension was treated with a Potter homogenizer (20 strokes) followed by sonication (Sonicator 3000 with a Misonix microprobe) for 2min at power level 4. The suspension was centrifuged at 4 ℃ (4696 *g* X 10 min). After centrifugation, a Teflon tip was used to pick the layer of lipid droplets which floated on the top of suspension. Lipid droplets were transferred to 1.2 mL washing buffer (150 mM KCl instead of that in isolation buffer). Then 1 mL of buffer 2 (isolation buffer without sucrose) was loaded carefully on the top of lipid droplet suspension. Suspension was ultra-centrifuged at 4 ℃ (100,000 *g* X 30 min). Lipid droplets were transferred from the top of suspension and subjected to lipid extraction and further lipid analysis procedure.

A.4. Results and Discussion

A.4.1. Characterization of *C. reinhardtii* mutant strain B2

The *C. reinhardtii* mutant strain B2, which accumulates higher TAG amounts than wild type during N deprivation, was identified during quantitative fatty acid analysis from a pool of mutant candidates. After 48-hour N deprivation, B2 contains about 60% TAG in total lipid, compared with 45% of that in wild type (Figure A.2A). Moreover, the fatty acid content in B2 is more than 60% higher than that in wild type on cell basis (Figure A.2B), suggesting that the hyper-accumulation of TAG in B2 was not at the cost of reducing cellular membrane lipids.

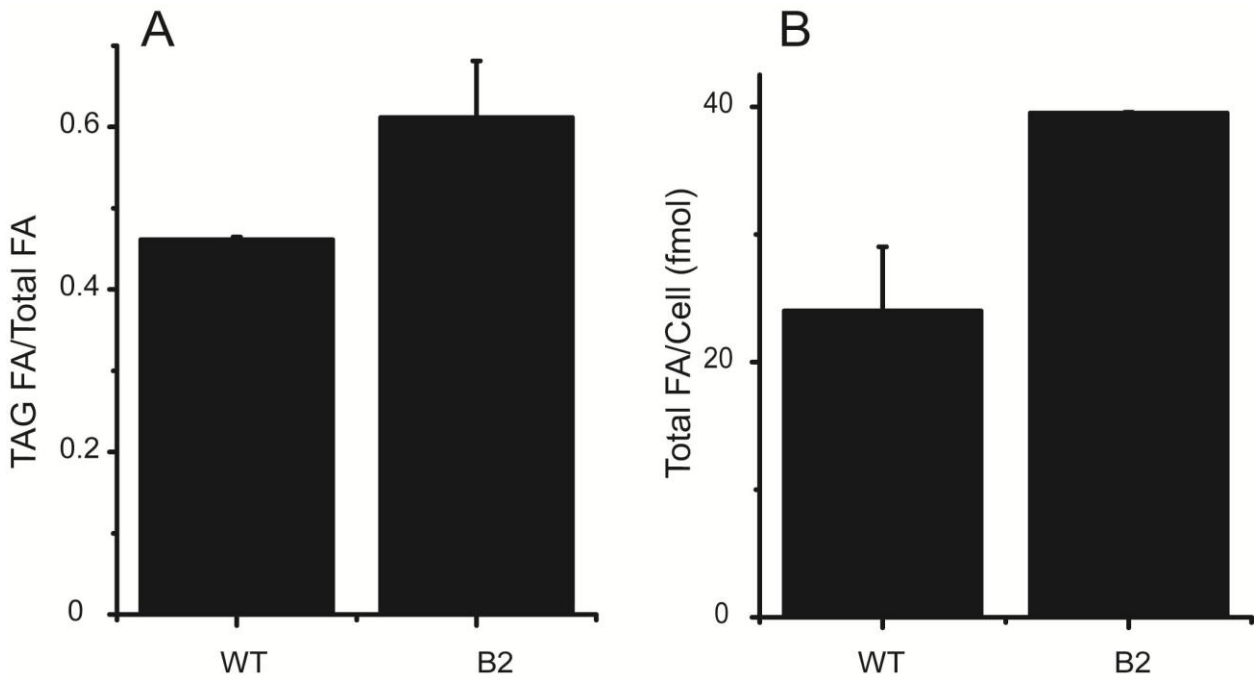

Figure A.2. High TAG accumulation in *C. reinhardtii* mutant strain B2. N deprived wild type and B2 mutant were subjected to lipid quantitation by GC analysis. The ratios of TAG fatty acid over total fatty acid (A) and total fatty acid contents per cell (B) are compared between wild type and B2 mutant. The average of at least three measurements and standard deviation are shown.

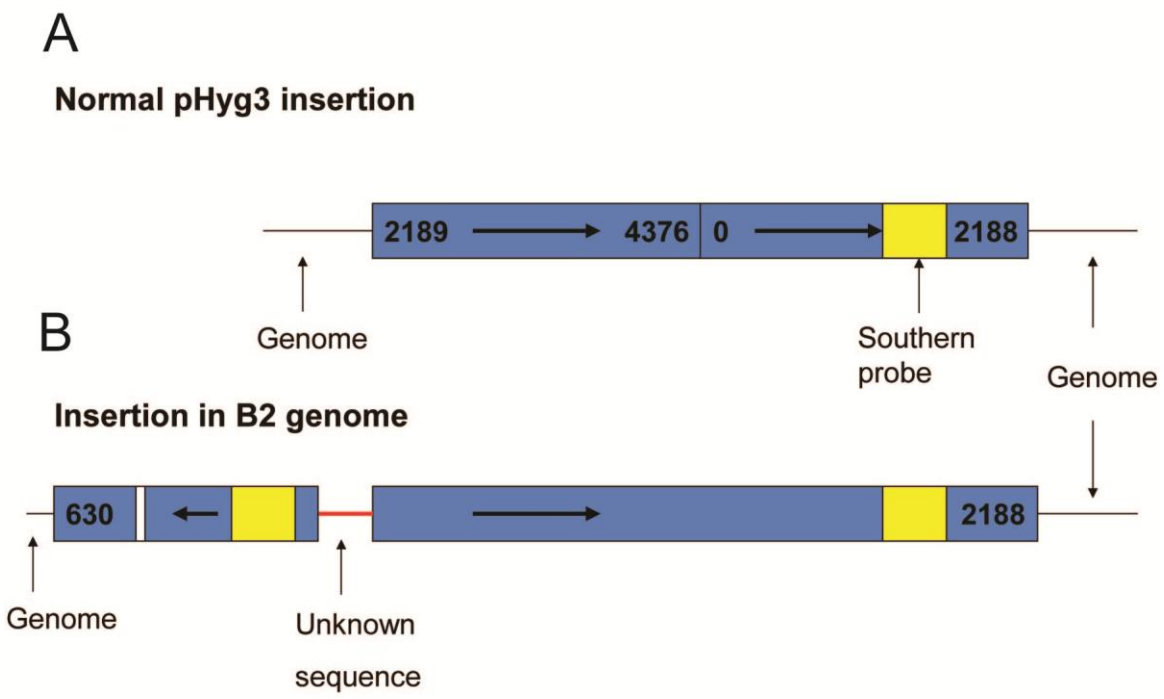

Figure A.3. Model of plasmid insertion in the B2 mutant. Integration of plasmid pHyg3 into the genome of *C. reinhardtii* mutant B2 (B) is compared with that in a single insertion scenario (A). Plasmid pHyg3 is shown in blue color. Yellow regions represent the DNA sequence hybridizing with probe of Southern Blot. The genome sequence flanking the plasmid insertion is shown in black line. Red line represents a piece of DNA with unknown sequence inside the insertion of B2.

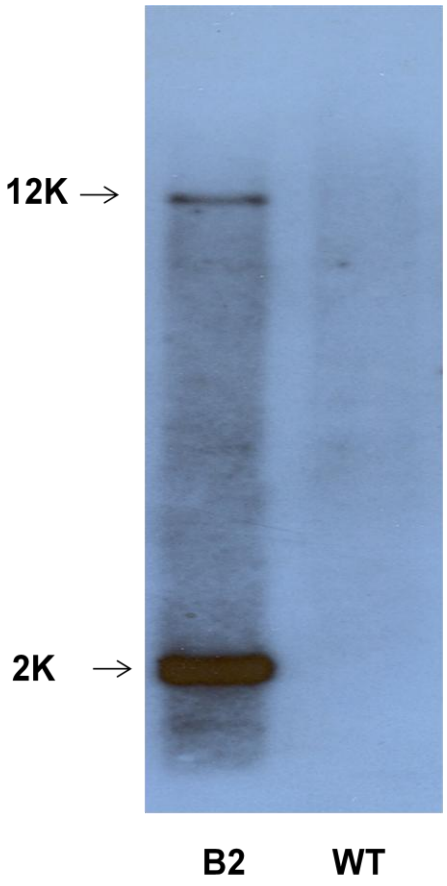

Figure A.4. Southern Blot of the pHyg3 insertion in B2 mutant. Genomic DNAs of *C. reinhardtii* strain B2 and wild type (WT) were digested with *Sac*I and hybridized with a DIG probe targeting at pHyg3 plasmid. The size of bands was estimated based on the DNA ladder control in the gel electrophoresis.

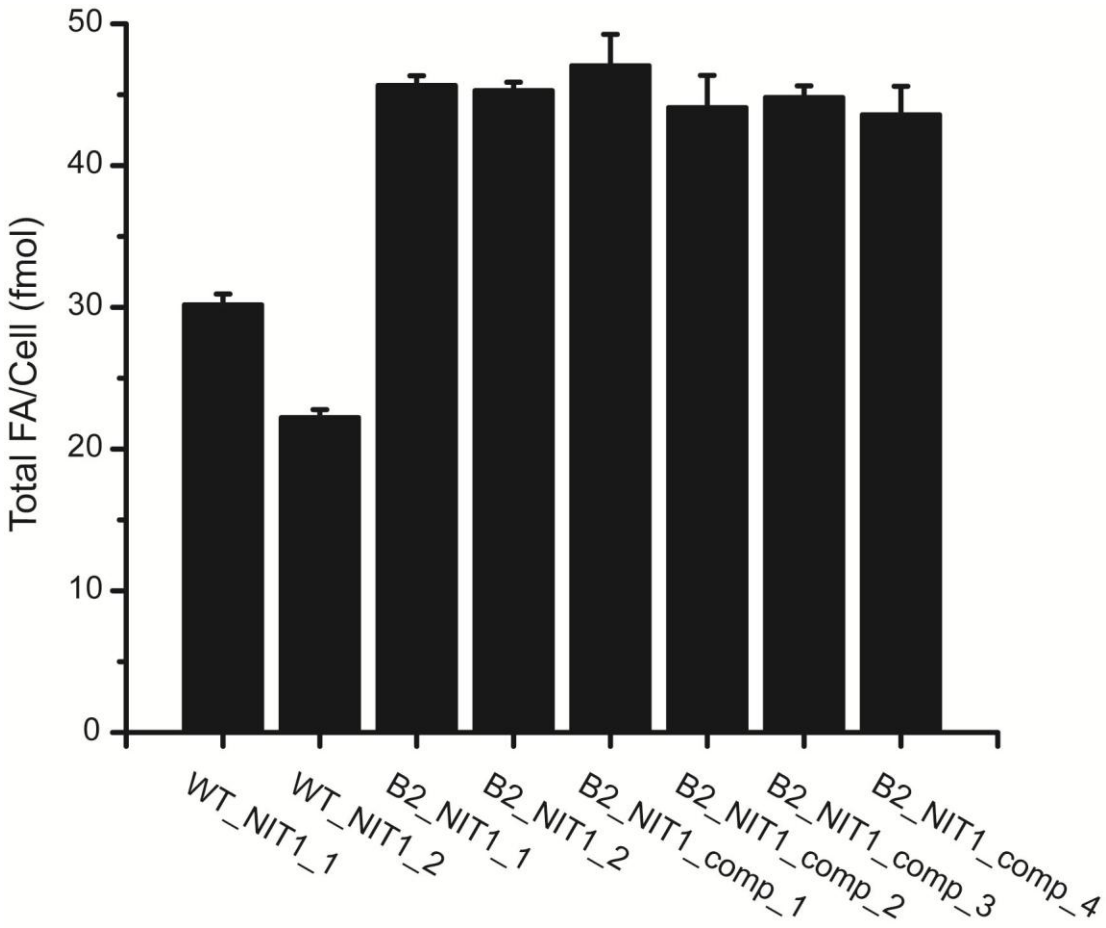

Figure A.5. Genetic complementation of lipid phenotype in B2 with wild-type genomic DNA. Total fatty acid contents of wild type, B2 and complemented lines are compared. For a co-transformation control purpose, *NIT1* gene is present in the genome of all strains. The average of at least three measurements and standard deviation are shown.

Molecular analyses have been conducted to understand the genetic background of *C. reinhardtii* mutant strain B2. By applying SiteFinding PCR approach, the location of a pHyg3 plasmid insertion was indentified in the B2 genome. This insertion was integrated into the 5' UTR region of a putative protein kinase gene (Cre05.g244550) and a short piece of genome DNA (14 bp) was deleted during the integration event. PCR data suggested that there are more than one copies of pHgy3 at this insertion site and the plasmid insertion is complex, as illustrated in Figure A.3. In this insertion, one end was confirmed to be a complete linearized pHyg3 plasmid while the other end is a piece of truncated pHgy3 DNA in the opposite direction. However, the DNA sequence of the linkage between the two ends has not yet been identified. Knowing the sequence of both ends of such an insertion in B2, there are at least two regions of DNA containing the same sequence which hybridize to the probe of Southern Blot (Figure A.3). Assuming the unknown linkage region in the insertion was digested by restriction endonuclease, *Sac*I in this case, two bands were observed in the radiograph of the Southern Blot (Figure A.4). However, these results are not sufficient to prove that there is only one insertion site in the B2 genome background. Further confirmations by molecular analysis are still required to support such interpretation.

To demonstrate whether the disruption of the Cre05.g244550 gene leads to the high TAG phenotype in *C. reinhardtii* mutant strain B2, gene complementation has been performed. BACs containing the Cre05.g244550 gene were digested, releasing a piece of DNA fragment containing complete sequence of the Cre05.g244550 gene with more than 500 bp flanking sequence at both ends. This DNA fragment was then co-transformed with the linearized pMN24 plasmid. The successful transformants were selected by PCR and subjected to lipid analysis. Lipid analysis (Figure A.5) shows that the complementation strains have similar level of total

fatty acids as B2 mutant after N deprivation. Although there is still suspicion that the integration of *NIT1* gene might change lipid content in all transformants, such results suggest that the Cre05.g244550 gene in B2 mutant might not rescue the abnormal TAG phenotype and hence another mutations in a different gene might be responsible.

In summary, the high TAG phenotype has been discovered and confirmed in *C. reinhardtii* mutant strain B2. In the B2 genome, one insertion site has been identified, which disrupted a putative protein kinase gene, Cre05.g244550. To identify whether there is the only insertion site in B2 genome, Southern Blot and back-cross experiments have been conducted. However, the current results are still not conclusive. The fact that the Cre05.g244550 gene did not rescue the abnormal TAG phenotype in B2 mutant, suggested that the hyper TAG accumulation in B2 strain during N deprivation might be attributed to another gene or unknown factors. It should be noted that whole genome sequencing has been conducted for a group of *C. reinhardtii* strains, including B2 mutant. The primary bioinformatics analysis suggested that there is only one plasmid insertion in the genome of B2. However, this does not rule out other secondary point mutations that are difficult to identify by the re-sequencing strategy. In addition, lipid analysis performed on the progeny lines which were generated from a cross between B2 and CC-198 showed 3 out of 32 progeny lines gave ambiguous results, suggesting the Hygromycin B resistance marker may not to be closely linked to the mutation causing the lipid phenotype.

A.4.2. Characterization of *C. reinhardtii* mutant strain A10

Another *C. reinhardtii* mutant strain, namely A10, with a low TAG phenotype during N deprivation, has been identified in the same mutant pool as mutant B2. After 48-hour N deprivation, A10 accumulates less TAG compared with wild type (Figure A.6A). The total lipid content of A10 following N deprivation was lower as well (Figure A.6B). Further phenotypic characterizations of A10 demonstrated that this mutant strain grows slower than wild type (Figure A.7). Other experimental evidence indicated that mutant A10 may have a smaller cell size and contains an abnormal amount of chlorophylls and carotenoid compared with wild type.

Southern Blot analysis was conducted to confirm that a single plasmid insertion is present in the A10 genome background (Figure A.8). Meanwhile, SiteFinding PCR was performed to locate the pHyg3 insertion. The results suggested that a single copy of pHyg3 with 632bp deletion is inserted into chromosome 12, leading a 7.6 Kb deletion of genomic DNA in A10 mutant. As a result, three continuous genes are affected, including Cre12.g560250, Cre12.g560300 and Cre12.g560350.

Complementation with individual genomic fragments for all three affected genes has been attempted in *C. reinhardtii* mutant strain A10. Similar to B2, co-transformation approaches were performed for A10 complementation. Difficulty was met in obtaining the DNA fragment containing the complete Cre12.g560350 gene sequence from BACs. Thus, only Cre12.g560250 and Cre12.g560300 genes were individually transferred into A10. Lipid analysis of complemented lines showed that for both genes, transgenic strains still have lower amounts of total fatty acid, similar to the original A10 mutant (Figure A.9). The results suggested that none of the Cre12.g560250 and Cre12.g560300 genes rescues the low TAG phenotype in A10 during N deprivation.

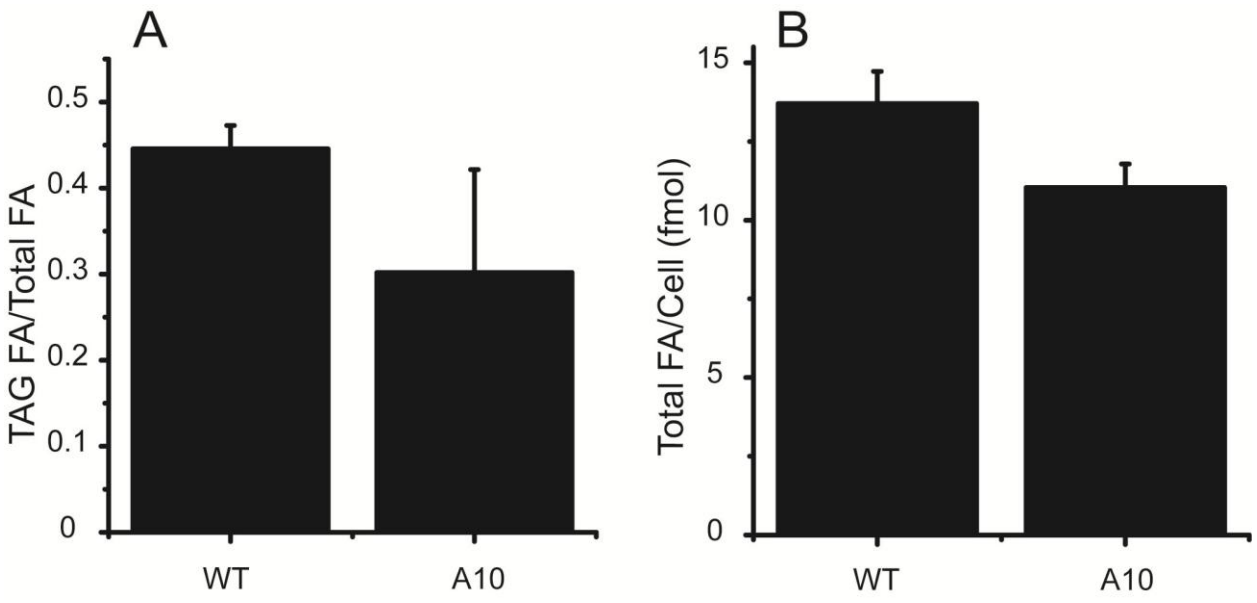

Figure A.6. Low TAG phenotype in *C. reinhardtii* mutant strain A10. N deprived wild type and A10 mutant were subjected to lipid quantitation by GC analysis. The ratios of TAG fatty acid over total fatty acid (A) and total fatty acid contents per cell (B) are compared between wild type and A10 mutant. The average of at least three measurements and standard deviation are shown.

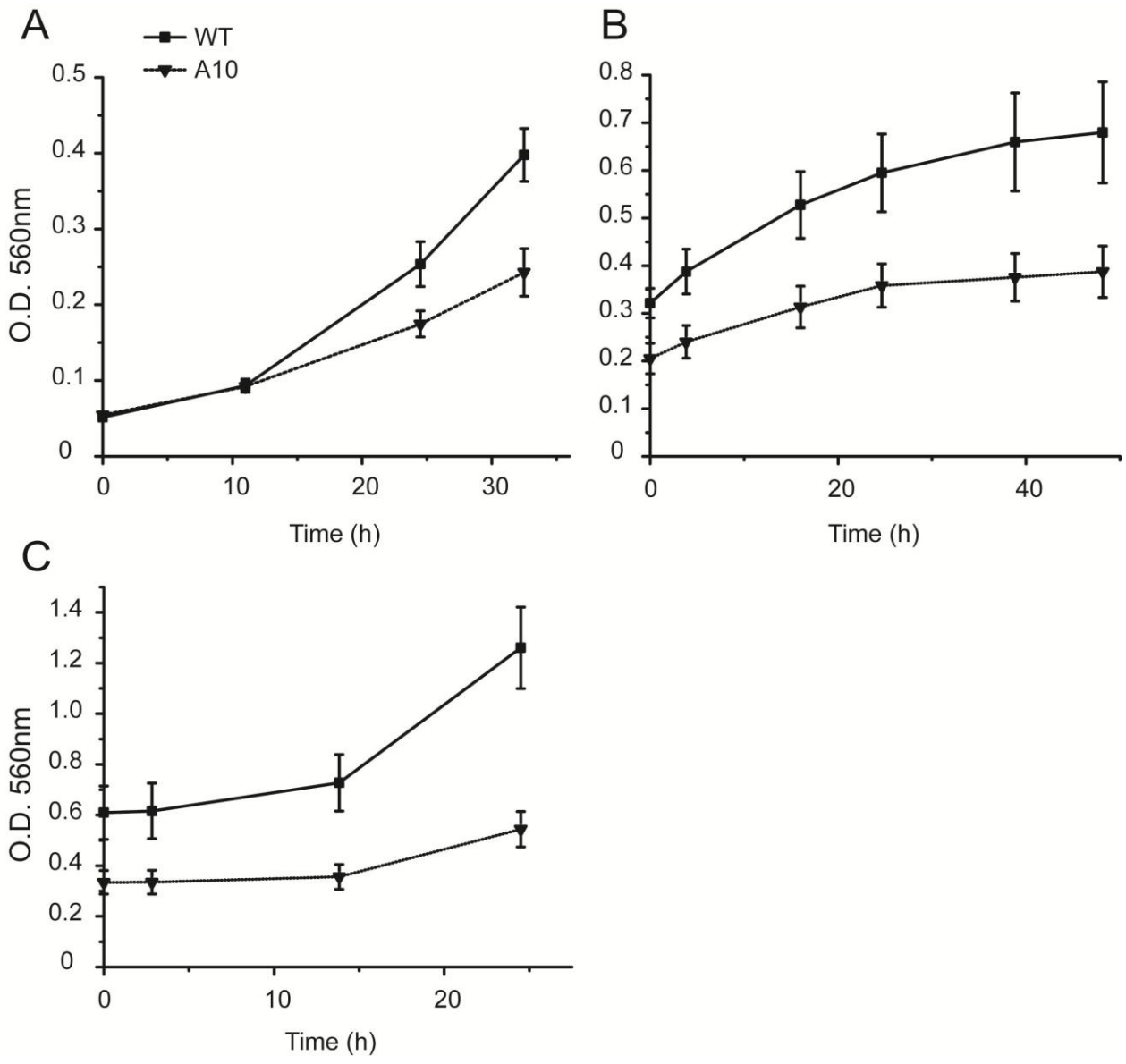

Figure A.7. Slow growth phenotype of A10 mutant. Growth rates during N replete (A), 48-hour N deprived (B) and N recover (C) conditions are compared between wild type *C. reinhardtii* and A10 mutant. The average of at least three measurements and standard deviation are shown.

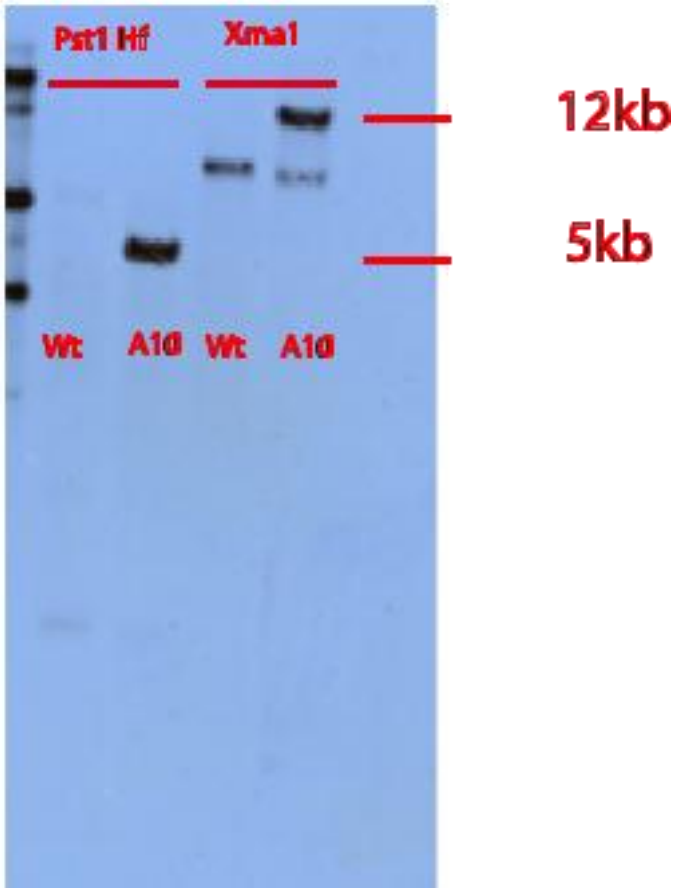

Figure A.8. Southern Blot of the pHyg3 insertion in A10 mutant. Genomic DNAs of *C. reinhardtii* strain B2 and wild type (WT) were digested with *Pst*I (left) or *Xma*I (right) and hybridized with a DIG probe targeting at pHyg3 plasmid. The size of bands were estimated based on the DNA ladder control in the gel electrophoresis.

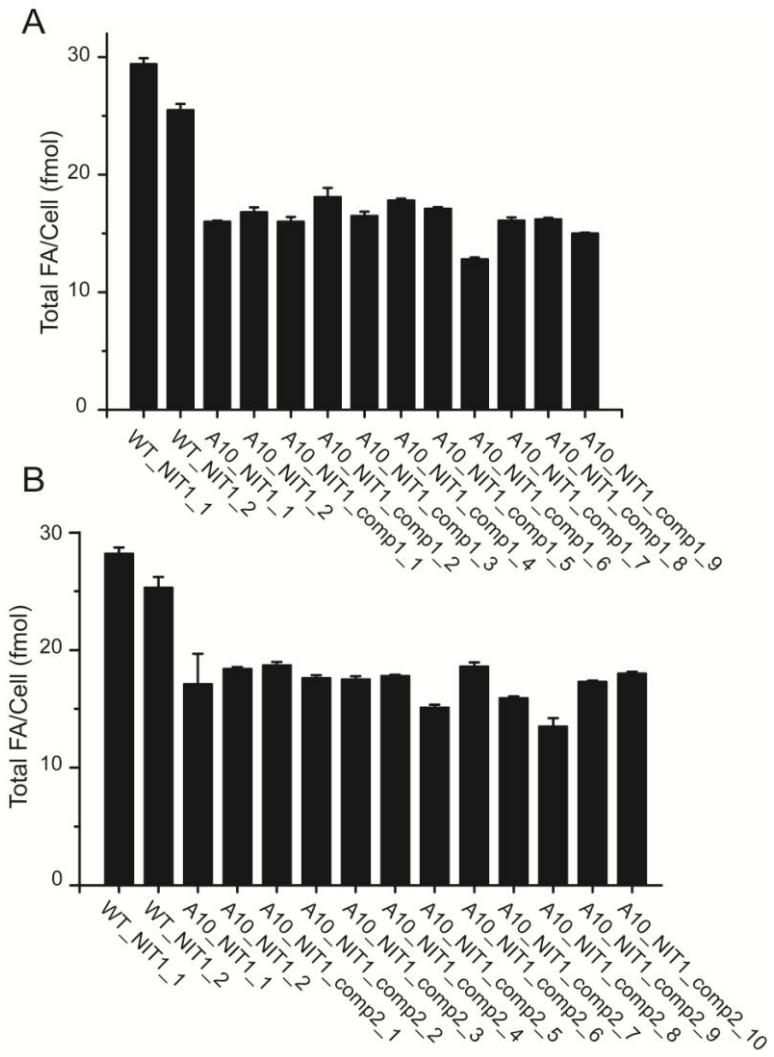

Figure A.9. Genetic complementation of lipid phenotype in A10 with wild-type genomic DNA. Comparison of total fatty acid content is made among wild type, mutant A10 and complemented lines with gene Cre12.g560250 (A) or Cre12.g560300 (B). For a co-transformation control purpose, *NIT1* gene is present in the genome of all strains. The average of at least three measurements and standard deviation are shown.

In summary, a low TAG phenotype has been discovered and confirmed in *C. reinhardtii* mutant strain A10. In the A10 genome, a single plasmid insertion has been confirmed by Southern Blot. In addition, the insertion site has been localized. As a result, three genes were disrupted in A10 genome. Out of these three genes, Cre12.g560250 and Cre12.g560300 have been complemented in the A10 genome. However, the TAG phenotype has not been rescued. Complementation of the third gene Cre12.g560350 to the A10 mutant has not been finished by co-transformation of BAC fragment approach. An alternative method should be pursued to demonstrate whether Cre12.g560350 is the key gene leading to low TAG accumulation. Interestingly, another *C. reinhardtii* mutant strain with disruption of the Cre12.g560350 gene has been identified recently (Meslet-Cladiere and Vallon, 2012) but with minimum molecular characterization. This mutant and its parental strain might be utilized for further study on TAG accumulation in *C. reinhardtii*.

A.4.3. Complementation of *C. reinhardtii* Mutant Strain Ligend

Using a different mutant screen strategy from A10 and B2 screens, a low TAG *C. reinhardtii* mutant Ligend has been identified in 48-hour N deprivation. Prior characterization of Ligend strain by Chia-Hong Tsai demonstrated that a single plasmid insertion is present in the genome of the mutant, leading to the deletion of a 20 Kb genomic DNA. As a result, a total of five genes in Ligend, including Cre01.g034350, Cre01.g034380, Cre01.g034400, Cre01.g034450 and Cre01.g034500, were disrupted.

Complementation analysis using four out of five affected genes in Ligend has been conducted. Lipid analysis has been performed for complemented strains. Figure A.10 shows the

comparison of ratios of TAG fatty acids to total fatty acid in N deprived *C. reinhardtii*. The results demonstrated that the low TAG phenotype has not been rescue in the transgenic lines, as their TAG levels were similar with mutant Ligend. The complementation with gene Cre01.g034350 has been conducted, which also suggested such gene cannot rescue the low TAG phenotype. Complementation of gene Cre01.g034500 has not been done yet.

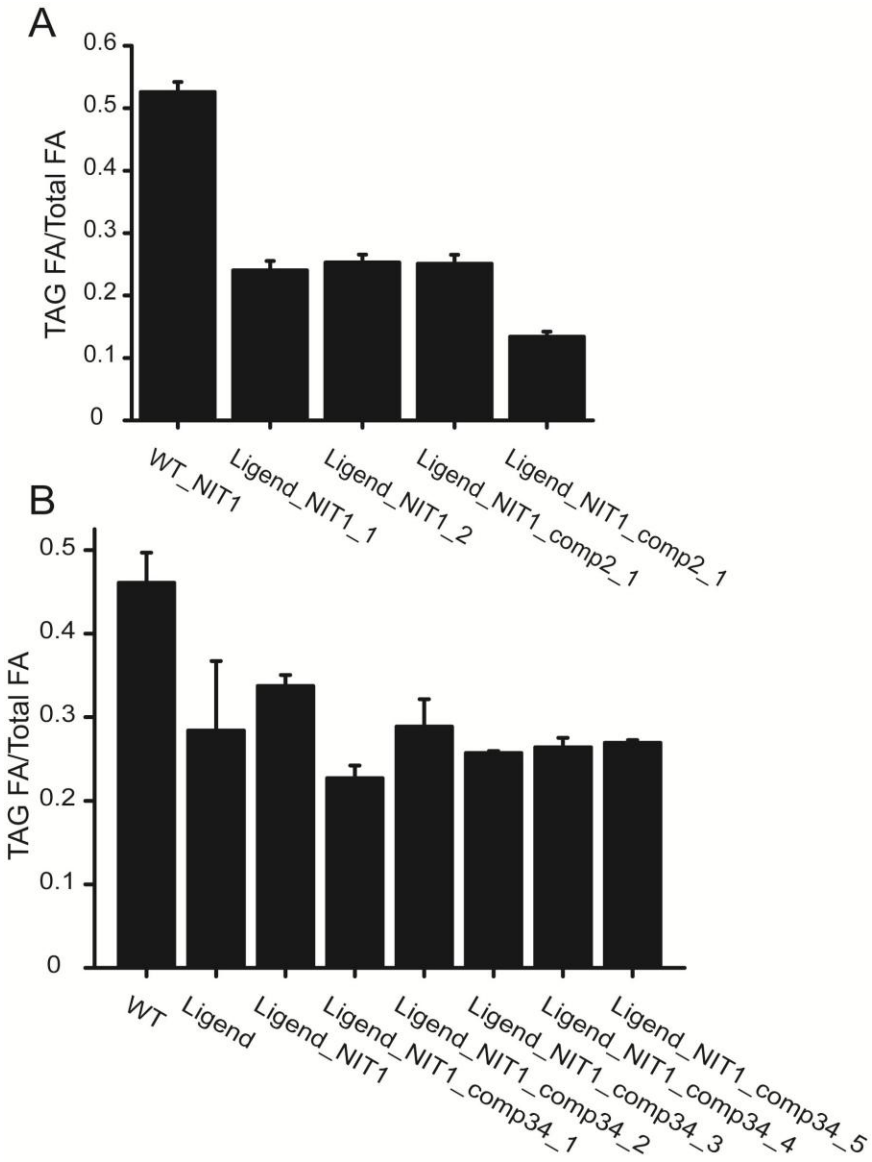

Figure A.10. Genetic complementation of lipid phenotype in Ligend with wild-type genomic DNA. Comparison of total fatty acid contents is made among wild type, mutant A10 and complemented lines with gene Cre01.g034380 (A) or Cre01.g034400 and Cre01.g034450 (B). For a co-transformation control purpose, *NIT1* gene is present in the genome of all strains. The average of at least three measurements and standard deviation are shown.

A.4.4. Characterization of *bta1* TILLING Mutants

In *C. reinhardtii*, BTA1 is the only enzyme catalyzing the biosynthesis of DGTS (Riekhof et al., 2005), which has been considered as the substitute of PtdCho in microalgae and prokaryotic organisms (Klug and Benning, 2001). Thus, the betaine lipid synthase BTA1 and its enzyme reaction product, DGTS, appear to be critical in lipid metabolism in *C. reinhardtii*. Reverse genetics approaches have been pursued to understand the role of the *BTA1* gene and its corresponding protein product. Attempts of knocking down the gene expression of *BTA1* by an artificial microRNA silencing approach led to reduced growth. However, no abnormal lipid phenotype, or a change in the DGTS content, has been observed in *BTA1* knock-down lines.

In collaboration with Dr. Krishna Niyogi's group, two independent TILLING mutant *C. reinhardtii* strains with point mutation in the *BTA1* gene have been generated. Since the BTA1 protein contains two functional groups, which catalyze the two-step reaction of DGTS biosynthesis, the initial plan was to generate mutants with dysfunction in the second step of the reaction. As a result, both TILLING mutants, namely S18F and P206L, contain a point mutation in the region encoding the BtaB-like domain of *BTA1* gene. The BtaB-like domain is proposed to be responsible for adding methyl groups to diacylglycerolhomoserine (DGHS), forming the final product DGTS (Riekhof et al., 2005).

Characterization of *bta1* TILLING mutants revealed no difference compared to wild type in terms of lipid profile during regular growth condition. Then, algae were subjected to N deprivation followed by lipid analysis to inquire whether *bta1* has abnormal lipid metabolism during the process of TAG accumulation. Figure A.11 shows the comparison of TAG contents among S18F and P206L and wild type after 48-hour N deprivation. Both *bta1* mutants produced less TAG than the wild type, suggesting the dysfunction of BTA1 had an impact on TAG

biosynthesis during N deprivation. Comparison of major membrane lipids of wild type and *bta1* TILLING mutants has been made under both N replete and N deprived conditions (Figure A.12). Following N deprivation, the relative amount of MGDG and DGTS decreased in *C. reinhardtii*, because of general membrane degradation and relative amount of TAG increased. However, in both *bta1* mutants, the relative amount of MGDG and DGTS are higher than those in the wild type (Figure A.12A and B), suggesting membrane lipid remolding might be slower in the *bta1* mutants. However, it should be mentioned that during N deprivation, the higher relative amount of MGDG and DGTS in the mutants might have contributed to the observed lower TAG accumulation. Further analysis is required to clarify the impact of lipid metabolism in *bta1* TILLING mutants. Besides, no difference in DGDG content between wild type and mutants has been detected (Figure. A.12).

It has been hypothesized that two types of lipid droplet exist in *C. reinhardtii* (Liu and Benning, 2013), which originates from different lipid biosynthesis routes – chloroplast pathway and endoplasmic reticulum (ER) pathway. If so, the two types of lipid droplets might have distinct monolayer membrane composition. It was reported that in lipid droplets, TAG is covered by one type of membrane lipid, which has not been classified yet (Nguyen et al., 2011). Purification of lipid droplets from N deprived *C. reinhardtii* has been attempted, followed by TLC separation to determine the lipid compositions in this organelle. Figure A.13 shows the TLC separation of lipids isolated from the lipid droplet. The result suggested that DGTS and DGDG are the two major membrane lipid components in lipid droplet, though contamination by other subcellular membrane systems may not be avoided during the purification procedure.

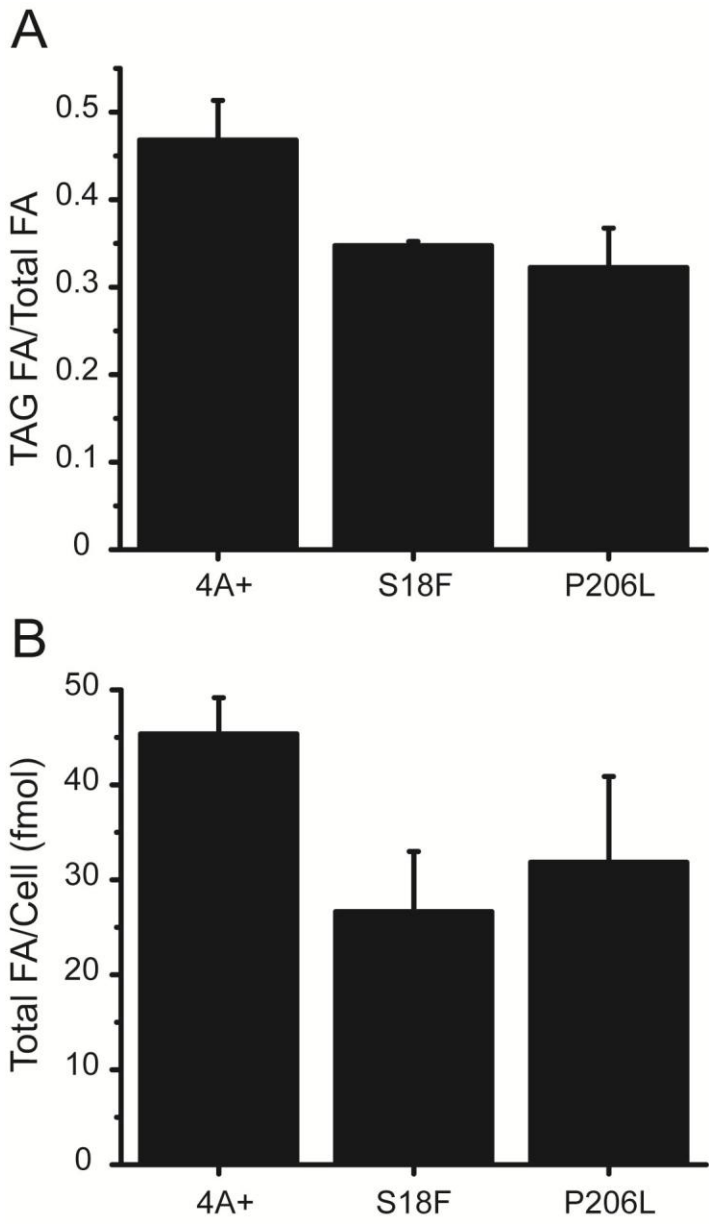

Figure A.11. Abnormal TAG accumulation in *bta1* TILLING *C. reinhardtii* mutant strains. N deprived wild type and *bta1* TILLING mutants were subjected to lipid quantitation by GC analysis. The ratios of TAG fatty acid over total fatty acid (A) and total fatty acid contents per cell (B) are compared between wild type mutant strains S18F and P206L. The average of at least three measurements and standard deviation are shown.

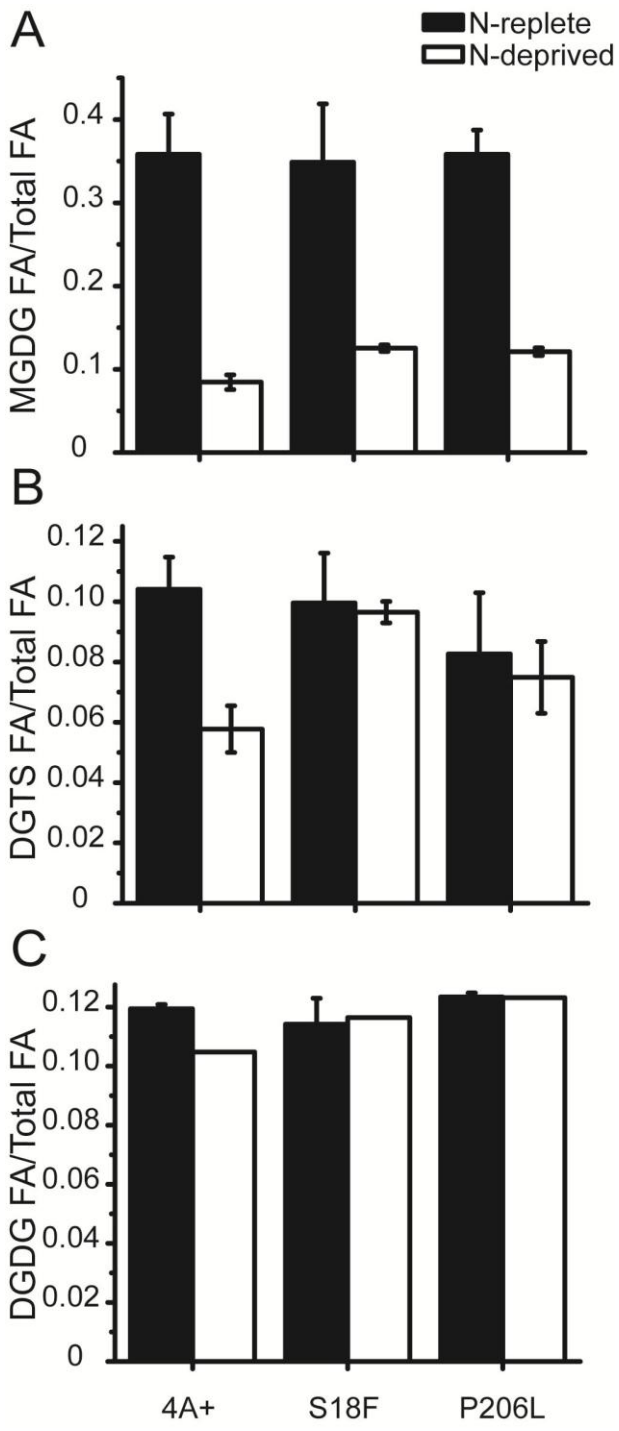

Figure. A.12. Change in the relative amount of membrane lipids after N deprivation. Ratios of fatty acid of individual membrane lipid including MGDG (A), DGTS (B) and DGDG (C) to

Figure A.12. (cont'd)

total fatty acids are compared between wild type and *bta1* TILLING mutants S18F and P206L in both N replete and N deprived conditions. The average of at least three measurements and standard deviation are shown.

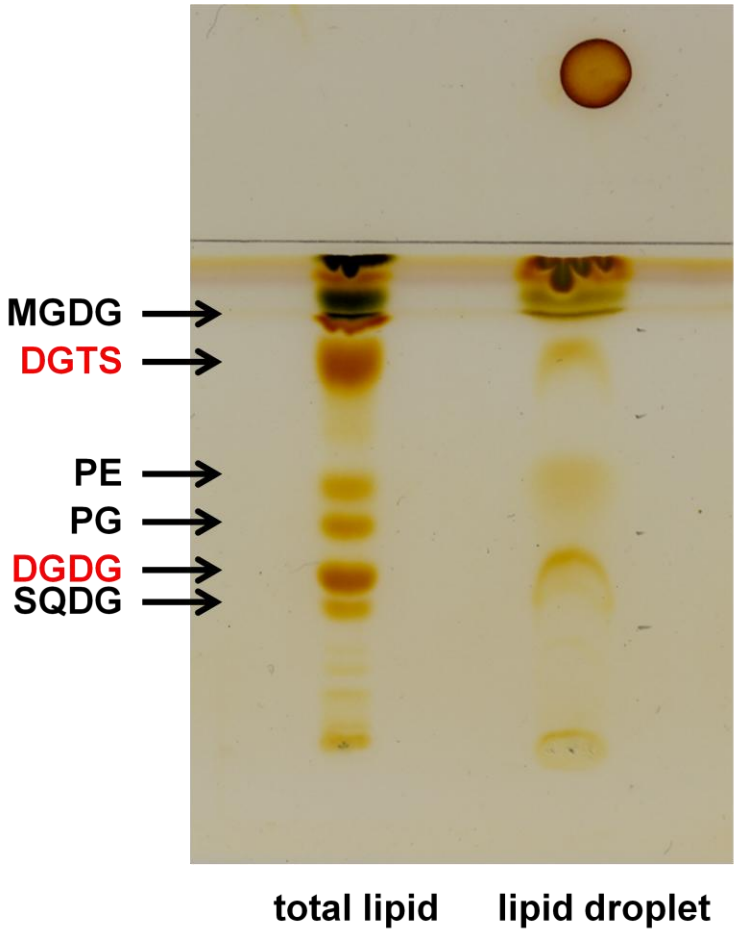

Figure A.13. Membrane lipid separation of *C. reinhardtii* lipid droplet. Purified lipid droplet from 48-hour N deprived wild type algae was resolved by TLC separation. Membrane lipids are shown on the plate. The left lane represents sample of total lipid from 48-hour N deprived *C. reinhardtii*..

Betaine lipids are widely present in eukaryotic algae as well as prokaryotic organisms. This class of lipid is under-investigated, even though it might play critical roles in organelle structuring and lipid metabolism. For *C. reinhardtii*, in which PtdCho is absent, DGTS is the major ER originated membrane lipid (Giroud et al., 1988). Thus, the biosynthesis and metabolization of DGTS has been targeted and investigated by reverse genetic approaches. Previously, RNA interference silencing of *BTA1*, the only known gene which encodes DGTS synthase, has been attempted in Benning lab. The *BTA1* knock-down strains showed only slower growth phenotype compared with control group, but no lipid phenotype. Similar phenomenon has been observed in gene silencing on the same gene by artificial microRNA approaches. The above results were interpreted to mean that the function of DGTS is critical for *C. reinhardtii* because the cell needs a certain level of DGTS before performing cell division. In this case, an alternative strategy should be pursued to understand the function of *BTA1* by its end product DGTS. Point mutation in of *BTA1* gene may have a moderate effect on *C. reinhardtii* so that no lipid or physiological phenotype has been observed in both *bta1* TILLING mutants during regular growth. Predicted but not detected in mutant strains, DGHS is the intermediate of DGTS. However, further efforts are worthwhile to identify possibly low amounts of DGHS in the *bta1* TILLING mutants by using a more sensitive method. Interestingly, a relatively lower amount of TAG and higher levels of DGTS and MGDG was observed in *bta1* TILLING mutants during N deprivation, suggesting that BTA1 is functional in TAG accumulation. It should be noted that lipid metabolism changes rapidly during the TAG accumulation process, which occurs immediately after the onset of N deprivation. The abnormal lipid phenotype in *bta1* mutants might be only observed in rapid dynamic change in lipid metabolism, rather than during normal growth of the microalgae. Additional experimental efforts suggested that DGTS and DGDG

might be the membrane lipids on the surface of the *C. reinhardtii* lipid droplet. Such a result can be linked with the possible function of BTA1 in TAG accumulation. Analysis of lipid droplets in *bta1* TILLING mutants will provide new information between the relationship between DGTS and TAG biosynthesis and further shine light on general algal lipid metabolism in stress conditions.

Table A.1. Oligonucleotide primers used in this section. All primer sequences are written in 5' to 3' direction. N indicates a random nucleotide.

Name	Sequence
SF	ACCAACATCTTCGTGGACCT
SR	CTCCTCGAACACCTCGAAGT
SiteFinder1	CACGACACGCTACTCAACACACCACCTCGCACAGCGTCCTCAAGCGG CCGCNNNNNNGCCT
SiteFinder3	CACGACACGCTACTCAACACACCACCTCGCACAGCGTCCTCAAGCGG CCGCNNNNNNGCCG
SiteFinder6	CACGACACGCTACTCAACACACCACCTCGCACAGCGTCCTCAAGCGG CCGCNNNNNNGCAT
SFP1	CACGACACGCTACTCAACAC
SFP2	ACTCAACACACCACCTCGCACAGC
GSP3-1	GGGACCAACATCTTCGTGGA
GSP3-3	ACTGCTCGCCTTCACCTTCC
GSP3-4	CTGGATCTCTCCGGCTTCAC
GSP5-4	CAGCTGGCGTAATAGCGAAGA
GSP5-6	CGCCTGATGCGGTATTTTCT
GSP6-1	TTGCTACAGGCATCGTGGTG
GSP6-3	TCCTCCGATCGTTGTCAGAA

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