CHARACTERIZATION OF LIPASES INVOLVED IN TRIACYLGLYCEROL METABOLISM IN *CHLAMYDOMONAS REINHARDTII*

By

Xiaobo Li

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ABSTRACT

CHARACTERIZATION OF LIPASES INVOLVED IN TRIACYLGLYCEROL METABOLISM IN CHLAMYDOMONAS REINHARDTI

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Triacylglycerol (TAG) is the major storage lipid for eukaryotic organisms, including animals, fungi, land plants and algae. With a higher energy density than carbohydrates and proteins, TAG from plant sources serves as an ideal feedstock for biofuel production. By transesterification, TAGs can be converted to fatty acid methylesters (FAMEs), which are similar to components of diesel derived from petroleum in carbon chain length and viscosity. Microalgae grow faster and have a larger potential for industrial biofuel production than land plants. In microalgae TAG production can be induced by nutrient stresses such as nitrogen (N) deprivation. Insights into the mechanism of oil production are required for further optimization of the biofuel production process. As an experimental organism for studying lipid metabolism, Chlamydomonas reinhardtii represents the most studied alga with multiple genetic and molecular biological tools available. In a transcript-profiling study to compare transcript abundance of genes in Chlamydomonas under normal growth conditions and N deprived conditions, a large number of predicted lipase-encoding genes appeared to be differentially regulated under the two conditions. This observation suggests that lipases play an important role in TAG metabolism of Chlamydomonas.

Conceivably, TAG production can be enhanced by suppressing TAG degradation enzymes, such as TAG lipases. One avenue of research pursued a reverse genetic screen of several lipase candidates by heterologous expression in a yeast mutant deficient in TAG lipases. Over-expression of one of the candidate genes, named CrLIP1, rescued the yeast mutant.
Recombinant CrLIP1 protein exhibited lipolytic activity on diacylglycerol (DAG) and polar lipids, but not TAG. RNA suppression of the *CrLIP1* gene led to delayed TAG degradation in *Chlamydomonas*, possibly through reduced capacity to degrade DAG, which is an intermediate in TAG turnover. It is also possible that CrLIP1 acts on TAG but requires an unknown cofactor which was missing in the lipase assays.

To complement the transcript profiling and reverse genetic approaches, an unbiased forward genetic screen was performed to obtain mutants with abnormal TAG amounts. Focus was given to a low TAG mutant disrupted in a gene designated Plastid Galactolipid Degradation 1 (PGD1), which was identified as a lipase-encoding gene. In the above-mentioned transcriptomic study, PGD1 was up-regulated following N deprivation. The *pgd1* mutant is decreased in acyl fluxes from galactolipids to TAG. Recombinant PGD1 protein exhibited lipase activity on the substrate monogalactosyldiacylglycerol (MGDG). Multiple lines of evidence suggest PGD1 takes part in an acylation/deacylation cycle to export *de novo* synthesized fatty acids from the plastid.

The low TAG mutant *pgd1* provides a tool for understanding the consequences of reduced TAG production. The *pgd1* mutant exhibited chlorosis, accumulation of oxidative damage and loss of viability, which was reversed by the exposure to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a specific inhibitor for photosynthetic electron transport. This indicates that cells synthesize fatty acids and TAG to relieve the strain of excess electron transport and prevent the accompanying oxidative stress. These studies demonstrate the physiological significance of the accumulation of TAG under stress conditions and will impact how scientists approach the improvement of the production of biofuels from algae.
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In August 2007 I left the Department of Bioengineering, Xi’an Jiaotong University and started my Ph.D. in the DOE-Plant Research Lab at Michigan State University. The degree-granting program for me is the Department of Plant Biology. After three lab rotations, in April 2008, I decided to work with Dr. Christoph Benning on algal lipid metabolism. Christoph asked his colleague Min-Hao Kuo to join the project because he knew I need to use yeast as a tool for certain experiments. This co-advising strategy proved successful in providing guidance from different perspectives and training me a broad set of skills.

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Chapter 1

Algal triacylglycerol metabolism and lipases-a literature review.
Introduction.

Triacylglycerol (TAG) is composed of a glycerol backbone with three esterified fatty acyl chains. The acyl chains esterified to glycerol are usually stereospecifically numbered as sn-1, 2 and 3 (Figure 1.1). In contrast to lipids contained in cell membranes such as phospholipids, TAG lacks a polar headgroup. Therefore, TAG is classified as a “neutral lipids” while membrane lipids are commonly called “polar lipids”. TAG is synthesized universally by animals, fungi, land plants and algae to store carbon and energy. Through transesterification with methanol, a TAG molecule can be converted into three fatty acid methylesters (FAMEs, see Figure 1.1) which can be blended into petroleum diesel and used by current diesel engines (Durrett et al., 2008). Microalgae produce a substantial amount of TAGs which provide a potential feedstock for biofuel production to relieve the widely-recognized energy crisis (Hu et al., 2008). To further improve the yield and lower the cost of algal biofuel production, a more detailed understanding of algal lipid metabolism pathways is urgently required. Algal lipid metabolism is relatively understudied compared to that of land plants. Most of the knowledge of algal lipid metabolism is based on in silico annotation with information derived from seed plants, especially the model plant Arabidopsis thaliana. In the model alga Chlamydomonas reinhardtii, orthologs for most genes involved in glycerolipid biosynthesis can be identified in the genome (Riekhof et al., 2005). However, plants produce TAGs in the seeds while the unicellular algae such as Chlamydomonas accumulate TAGs under nutrient deprivation (Weers and Gulati, 1997). Hence regulation of related enzymes is expected to be different between seed plants and algae. Transcript profiling and reverse genetics have been performed recently in Chlamydomonas to reveal genes central to TAG biosynthesis (Miller et al., 2010; Boyle et al., 2012). This chapter
will summarize current understanding and remaining questions for algal lipid metabolism with a focus on the metabolism of TAG.

*Chlamydomonas reinhardtii* as a model system

*Chlamydomonas reinhardtii* is a unicellular green alga with a cell diameter of about 10 µm (Fang et al., 2006). Chlamydomonas strains have been isolated from the soil and fresh water. Studies on Chlamydomonas have been recorded since the 19th century, initially on sexual reproduction by J.N. Goroshankin in 1875, and then on multiple processes such as cell motility, phototaxis and photosynthesis (Harris, 1989).

Chlamydomonas haploid cells grow vegetatively through mitotic divisions but can be induced to enter a sexual cycle through nitrogen starvation and subsequent mixing of cells of two mating types ($mt^+$ and $mt^-$). The diploid zygote forms and undergoes meiosis to produce four progeny spores (Goodenough et al., 1995). The predominance of the haploid stage has important implications for genetic research on Chlamydomonas. A mutation in a haploid strain will have an immediate effect while in diploid organisms, it can be compensated by the wild-type allele. With the sexual cycle, strains can be crossed to combine different traits. Additionally, a fraction of diploid cells obtained from mating can be maintained vegetatively (Ebersold, 1967), facilitating a complementation test for different mutations or maintenance of a mutation that is lethal in a haploid background.

The flagella of a Chlamydomonas cell share the 9+2 microtubule doublet structure with human cilia (Silflow and Lefebvre, 2001). Thus, research on the structure and function of Chlamydomonas flagella have yielded principles that have been applied to human health. The primary cause of the mouse autosomal-recessive polycystic kidney disease proved to be a defect
in a protein which has been discovered in the intraflagellar transport particles in Chlamydomonas (Rosenbaum and Witman, 2002). Studies of mutants with abnormal flagellar size or function (McVittie, 1972) and characterization of the flagellar proteome (Pazour et al., 2005) revealed numerous genes involved in flagellar biogenesis and movement.

Chlamydomonas has a single chloroplast that is conserved between green algae and land plants but different from the rhodoplasts in red algae in ultrastructure and composition of pigments (Keeling, 2004; Wise and Hoober, 2006). Chlamydomonas can grow in the dark on organic carbon sources such as acetate as well as photoautotrophically with CO₂ as the sole carbon source (Grossman et al., 2003). As a result, mutants that are completely blocked in photosynthesis can be maintained by supplementing acetate in the growth medium. Studies of Chlamydomonas photosynthesis have yielded knowledge in key processes such as dynamics of state transitions and cyclic photosynthetic electron transport (Finazzi, 2005). In the recent years, the mechanism of photoprotection has been elucidated to a large extent through characterizing mutants deficient in non-photochemical quenching (Niyogi, 1999).

Studies of carbon storage in Chlamydomonas have focused on starch biosynthesis and turnover. Several mutants deficient in starch biosynthesis have been isolated and the mutated loci identified (Zabawinski et al., 2001). Seminal research on Chlamydomonas lipid metabolism has dealt with the biosynthesis and function of membrane lipids such as sulfoquinovosyl diacylglycerol (Riekhof et al., 2003), but studies on algal triacylglycerol (TAG) metabolism are preliminary. Chlamydomonas is unrivaled among algae in the molecular genetic toolset developed, including the random insertional mutagenesis (Kindle, 1990), RNA suppression technique (Molnar et al., 2009) and the use of GFP expression for protein localization (Neupert et al., 2009). Genetic characterization of TAG metabolism pathways in
Chlamydomonas will reveal genes that can be engineered for optimization of TAG production as biofuel feedstock.

**Comparison between Arabidopsis and Chlamydomonas lipid metabolism pathways**

Figure 1.2 outlines the major pathways for membrane glycerolipid biosynthesis in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* based on biochemical studies and genome annotation (Riekhof et al., 2005). The pathways of TAG metabolism will be discussed later. In seed plants such as Arabidopsis, fatty acids are synthesized in the stroma of the chloroplast (Ohlrogge et al., 1979). The assembly of glycerolipids occurs through two pathways in the plastid and at the endoplasmic reticulum (ER) respectively (Roughan and Slack, 1982). In the chloroplast, two fatty acids attached to the acyl-carrier protein (ACP) are incorporated into the backbone of glycerol-3-phosphate sequentially, by the glycerol-3-phosphate acyltransferase (GPAT, Figure 1.2, arrow 1) and lyso-phosphatidic acid acyltransferase (LPAT, Figure 1.2, arrow 2) enzymes respectively, to produce phosphatidic acid (PtdOH). PtdOH can be converted into diacylglycerol (DAG) through the removal of the phosphate group by the PtdOH phosphatase (PAP, Figure 1.2, arrow 3). Alternatively, fatty acids can be exported to the ER and activated into acyl-coenzyme A (acyl-CoA, Figure 1.2 arrows 10 and 11), the substrate for the GPAT (Figure 1.2, arrow 12) and LPAT (Figure 1.2, arrow 13) enzymes associated with the ER (Ohlrogge and Browse, 1995). Biochemical evidence indicates that in the plastid, PtdOH is the precursor for phosphatidylglycerol (PtdGro, Figure 1.2, arrows 7, 8, 9) (Xu et al., 2006) while monogalactosyldiacylglycerol (MGDG, Figure 1.2, arrow 5) and sulfoquinovosyldiacylglycerol (SQDG, Figure 1.2, arrow 4) are derived from DAG with UDP-galactose and UDP-sulfoquinovose as the headgroup donors (Benning and Somerville, 1992a, b; Yu et al., 2002).
Digalactosyldiacylglycerol (DGDG) is formed upon the transfer of another galactose to MGDG (Figure 1.2, arrow 6) (Dormann et al., 1999). In plants, phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) are the major glycerolipids that are synthesized in the ER.

Previous radiolabeling experiments in plants showed that DAG backbones of glycerolipid assembled at the ER can cycle back to the plastid for plastidic lipid synthesis (Roughan et al., 1980). The two sources of plastid lipids are referred as the “eukaryotic” (ER) and “prokaryotic” (plastid) pathways (Roughan and Slack, 1982). DAG backbones from these two pathways could be distinguished based on the length of fatty acids at the sn-2 position; the LPAT in the chloroplast prefers C16 fatty acids as opposed to the LPAT of the ER, which uses C18 acyl-CoAs (Frentzen, 1998). In Chlamydomonas, positional analysis showed that DAG backbones of the plastidic lipids were almost exclusively from the prokaryotic pathway (Giroud et al., 1988). This suggests that Chlamydomonas lacks the lipid trafficking from the ER back to the chloroplast, which has been extensively studied in Arabidopsis (Figure 1.2, arrow 18) (Xu et al., 2003; Awai et al., 2006; Lu et al., 2007; Xu et al., 2008).

For pathways that are common to Arabidopsis and Chlamydomonas, the latter is usually less redundant. One prominent example is the galactolipid biosynthesis pathway. In Arabidopsis MGD1 and DGD1 catalyze the synthesis of MGDG and DGDG respectively under normal growth conditions, while MGD2, MGD3 and DGD2 are induced under phosphate deprivation to produce more galactolipids as functional replacements to phosphatidylcholine (Benning and Ohta, 2005). Additionally, a processive enzyme, galactolipid:galactolipid galactosyltransferase (GGGT, a.k.a. SFR2) which uses MGDG instead of UDP-galactose as the galactose donor is induced under freezing stress, generating trigalactosyldiacylglycerol (TGDG) (Moellering et al., 2010). Among the above-mentioned glycosyltransferases, a BLAST (Altschul et al., 1997)
search of the Chlamydomonas genome (Riekhof et al., 2005) yielded orthologs for MGD1 and DGD1 only. The lack of alternative galactolipid synthesis pathways may arise from the fact that Chlamydomonas does not have phosphatidylcholine (Giroud et al., 1988) as discussed below and phosphate deprivation would be a relatively mild stress.

**Phosphatidylcholine (PtdCho) is absent in Chlamydomonas**

In terms of lipid composition, the most striking difference between Chlamydomonas and Arabidopsis is the lack of PtdCho but presence of a betaine lipid diacylglycerol-N,N,N,-trimethylhomoserine (DGTS) in Chlamydomonas (Giroud et al., 1988). In plants, PtdCho plays a central role in lipid metabolism. The fatty acids exported from the plastid were proposed to be incorporated into PtdCho first (Bates et al., 2007), possibly by the action of an acyl-CoA:lyso-phosphatidylcholine acyltransferase (Stahl et al., 2008), then liberated by an unidentified phospholipase (arrows 19 and 20 in Figure 1.2). PtdCho is also a major substrate for multiple desaturases that produce polyunsaturated fatty acids. Through PtdCho synthesis and degradation, the DAG backbone containing polyunsaturated fatty acids can be incorporated into TAG, a pathway important for the industrial value of plant oil (Vogel and Browse, 1996; Bates et al., 2012). Thus, the absence of PtdCho will result in substantial differences in Chlamydomonas TAG metabolism from that of plants.

DGTS is a lipid which has been found in some algal species (Eichenberger, 1982), a photosynthetic bacterium *Rhodobacter sphaeroides* (Benning et al., 1995), and non-seed plants such as ferns (Eichenberger, 1993). Despite the lack of phosphorus, the headgroup of DGTS resembles that of PtdCho (Figure 1.3). Furthermore, DGTS and PtdCho have similar biophysical properties (Sato and Murata, 1991). It has been hypothesized that DGTS replaces PtdCho in its
structural role of as the major extraplastidic membrane lipid. The pathway of DGTS biosynthesis was initially elucidated in *Rhodobacter sphaeroides*. BtaA encodes the enzyme that transfers the 3-amino 3-carboxylpropyl residue from S-adenosylmethionine to DAG while the BtaB protein conducts the successive methylation of the N atom in the amino group (Klug and Benning, 2001). Interestingly, these two activities are harbored by a single protein named BTA1 in Chlamydomonas. The protein sequence at the N-terminus of BTA1 is similar to that of the bacterial BtaB while the C-terminus is similar to that of BtaA (Riekhof et al., 2005).

**A general overview of triacylglycerol (TAG) biosynthesis in Chlamydomonas**

Triacylglycerol is composed of a glycerol backbone and three fatty acyl chains which usually contain 16-20 carbon atoms (Figure 1.1). TAG can be derived from DAG by one of the two alternative routes: diacylglycerol acyltransferase (DGAT) or phospholipid:diacylglycerol acyltransferase (PDAT). DGAT was initially discovered in human and mammals, with two isoforms-DGAT1 and DGAT2. The enzymes transfer an acyl group from acyl-Coenzyme A to a DAG backbone (Figure 1.4A). These two isoforms both catalyze the acylation of DAG but do not share sequence similarity (Oelkers et al., 1998). In the seed plant Arabidopsis, DGAT1 but not DGAT2 was found to contribute to seed oil biosynthesis (Zhang et al., 2009). More recently, a novel protein conferring DGAT activity was found in developing peanut cotyledons; it has sequence similarity to proteins of neither the DGAT1 nor the DGAT2 family. This protein was thus designated DGAT3. An important difference is that DGAT3 is soluble while DGAT1 and DGAT2 are membrane-bound (Saha et al., 2006; Rani et al., 2010).
PDAT activity was demonstrated for yeast and plants not longer after the discovery of the first DGAT (Dahlqvist et al., 2000). The major difference between PDAT and DGAT is that PDAT utilizes a phospholipid molecule instead of acyl-CoA as the acyl donor (Figure 1.4B).

For algae in various lineages, nutrient deprivation, especially nitrogen (N) deprivation, is a common trigger for TAG accumulation. Examples include green algae such as Neochloris oleobundans (Pruvost et al., 2009) or the eustigmatophyte Nannochloropsis sp. (Suen et al., 1987). For diatoms, which contain silicates as their cell wall components, limitation of silicon also induces TAG accumulation (Roessler, 1988). In addition to the nutrient availabilities, light intensity and other factors such as temperature also play a role in regulating algal TAG biosynthesis. For example, in Nannochloropsis salina, high light stress has been reported to increase TAG content (Van Wagenen et al., 2012). Thus, besides the obvious role of storing energy, TAG has been proposed to serve as an electron sink to prevent the formation of reactive oxygen species from photosynthesis (Hu et al., 2008).

As a model green alga, Chlamydomonas has been repeatedly observed to accumulate TAG under N-deprivation (Weers and Gulati, 1997; Wang et al., 2009; Moellering and Benning, 2010). Many studies on Chlamydomonas TAG metabolism have been performed on cells grown photoheterotrophically, with acetate in the medium (Tris-acetate-phosphate, TAP) and constant light. Under this condition, both acetate and light seem to be required for TAG production (Fan et al., 2011; Goodson et al., 2011). Increasing the concentration of acetate from 17 mM to 60 mM greatly enhances TAG production (Fan et al., 2012). However, a parallel study was not performed in which CO₂ was passed through the medium and it remains unclear whether it is the carbon source or the energy contained in acetate that contributes to the increase of TAG content.
As mentioned above, starch is another major energy reservoir in Chlamydomonas that is stored upon N-deprivation and importantly, the starchless mutants were found to over-accumulate TAG (Work et al., 2010). This finding has significant value for industrial biofuel production if it holds true for promising algal production species. Additionally, characterizations of the starchless mutants will help elucidate the mechanism through which TAG synthesis is induced.

Inhibition of fatty acid synthesis by cerulenin substantially decreased the amount of TAG that accumulates under N-deprivation (Fan et al., 2011), indicating that de novo synthesized fatty acids are important building blocks of TAG molecules. Indeed, the most abundant fatty acids sequestered into TAG include the de novo synthesized fatty acids-16:0 and 18:1 (Fan et al., 2011). However, polyunsaturated fatty acids such as 18:3 and 16:4 were also observed (Moellering and Benning, 2010). Since most desaturases act on the membrane glycerolipids instead of free fatty acids or acyl-ACPs (Napier et al., 1999), the polyunsaturated fatty acids are most likely obtained from the degradation of membrane lipids. This deduction is consistent with the observation that the chloroplast of Chlamydomonas degenerates during N-deprivation (Moellering and Benning, 2010).

The fact that approximately equal amounts of C16 and C18 fatty acids were contained in TAG of Chlamydomonas (Fan et al., 2011) was initially surprising. In seed plants, TAG is exclusively from extraplastidic origins. As a result, TAG contains only C18 fatty acids at the sn-2 position and more than half C18 fatty acids in total (Lu et al., 2009). In contrast, positional analysis showed that TAG in Chlamydomonas has mostly C16 fatty acids at sn-2, which indicates a chloroplast origin for the DAG backbones used in TAG biosynthesis and explains the abundance of C16 fatty acids in TAG (Fan et al., 2011).
Recent progress in understanding TAG metabolism through the genomics, genetics and cell biology of Chlamydomonas

TAG is stored in globular organelles commonly referred to as lipid droplets (Farese and Walther, 2009). In order to exist in the aqueous cytoplasm, the surface of lipid droplets is covered with a monolayer of polar lipids, containing mostly phosphatidylcholine (Krahmer et al., 2011). Since membranes are usually defined as bilayers of polar lipids, the monolayer of polar lipids has been called a “hemi-membrane” (Tauchi-Sato et al., 2002). As discussed above, PtdCho is lacking in Chlamydomonas and probably some other polar lipids function in its place to coat the surface of lipid droplets.

Chlamydomonas also deviates from seed plants in the locations and protein composition of lipid droplets. The major lipid droplet proteins differ between mammals (“PAT” family of proteins) and plants (oleosins for seed lipid droplets) while for baker’s yeast, a major structural protein has not yet been identified (Athenstaedt et al., 1999; Jolivet et al., 2004; Walther and Farese, 2009). In Chlamydomonas, the most abundant protein in the lipid droplet proteome was designated MLDP (Major Lipid Droplet Protein). Bioinformatic investigations showed that orthologs of MLDP can only be found in green algal species (Moellering and Benning, 2010). Despite the lack of sequence similarity between MLDP and Arabidopsis oleosin, RNA suppression lines of MLDP gene in Chlamydomonas resembled the Arabidopsis oleosin mutants in that lipid droplets are enlarged (Moellering and Benning, 2010). This suggests a similar function between Chlamydomonas MLDP and plant oleosin in maintaining the surface structure of lipid droplets.

In plant seeds, lipid droplets are cytosolic (Huang, 1996) but in senescent leaves, small lipid droplets called plastoglobuli have been observed within the chloroplasts (Martin and
Wilson, 1984). In the unicellular alga Chlamydomonas, when a starchless mutant was used for ultrastructural analysis of lipid droplets, surprisingly, two independent groups observed a substantial number of lipid droplets which are similar to the cytosolic lipid droplets in size but located in the plastid or connected to the plastidic envelopes (Fan et al., 2011; Goodson et al., 2011). This might have to do with the above-mentioned finding that TAG in Chlamydomonas is mostly derived from the DAG backbones of chloroplast origin (Fan et al., 2011). Localization studies of the enzymes catalyzing TAG biosynthesis (discussed below) will be required to clarify the process of lipid droplet biogenesis.

The Next-Generation Sequencing technique is revolutionizing molecular genetic research for all organisms. The frequency of detection of a transcript by sequencing can serve as an indicator of its abundance. Solexa sequencing (Illumina Inc.) combined with 454 sequencing (454 Life Sciences) were carried out by Miller et al. (2010) to reveal genes differentially regulated by N-deprivation. The transcript abundance of multiple genes encoding metabolic enzymes or transcription factors increased or decreased. This study was followed by a time course analysis of transcript abundance with time points ranging from minutes to days of N-deprivation (Boyle et al., 2012). Both investigations obtained similar results regarding the enzymes predicted to be involved in TAG metabolism. One example is that the DGTT1 gene predicted to encode a type two diacylglycerol acyltransferase is increased more than 50 fold in its transcript abundance after 48 h of N-deprivation. Specific genes such as the PDAT1 have been selected from the databases for reverse genetic studies (Boyle et al., 2012).

One important discovery from both of the transcriptomic investigations is that a large number of predicted lipase-encoding genes were observed to be differentially regulated by N-deprivation. Among the 130 candidate lipase genes in Chlamydomonas, 35 (27%) are up-
regulated and 11 (8.5%) are down-regulated while the numbers for all of the genes in the genome average are 13% and 11% respectively. Possible functions of these putative lipases are discussed below.

**Potential roles of lipases in triacylglycerol metabolism**

The term “lipase” was originally used for the enzymes that degrade TAG (Feinberg, 1991). The enzymes that hydrolyze phospholipids are referred to as phospholipases, which can be further divided into phospholipase A\(_1\) (PLA\(_1\)), phospholipase A\(_2\) (PLA\(_2\)), phospholipase B (PLB), phospholipase C (PLC) and phospholipase D (PLD). PLC and PLD digest the phosphodiester bonds on the glycerol backbone side and the polar headgroup side respectively while PLA\(_1\) and PLA\(_2\) remove the acyl chains at the sn-1 and sn-2 positions respectively (Vandeene and Dehaas, 1966). If a phospholipase sequentially cleaves off the two acyl groups, it is designated as PLB (Wang, 2001). Both TAG lipases and PLA\(_1\)/PLA\(_2\) catalyze the hydrolysis of the carboxyl ester bonds between the acyl chains and their glycerol backbone. In fact, multiple proteins were found to possess lipolytic activity against both TAG and phospholipids (Kuusi et al., 1982). In the recent years, the term “lipase” has been used to denote many proteins with acyl hydrolase activity against polar lipids (phospholipids, galactolipids, etc.) instead of triacylglycerol (Ellinger et al., 2010; Bonaventure et al., 2011). In this dissertation, “putative lipase” will also be used to describe a protein predicted to have an acyl hydrolase motif but lacking information about its substrate specificity.

TAG metabolism can be affected by lipases in different ways. Intuitively, a TAG molecule can be hydrolyzed by a TAG lipase (reaction 1 in Figure 1.5), DAG lipase and
monoacylglycerol (MAG) lipase consecutively. In Baker’s yeast, deletion of genes encoding TAG lipases from the genome has been shown to double the amount of TAG accumulated (Athenstaedt and Daum, 2005; Kurat et al., 2006). The system in which TAG degradation has been most intensively studied is the mammalian adipose tissue. In human beings, the hormone sensitive lipase (HSL) was first found to liberate fatty acids from fat in the adipose tissue (Vaughan et al., 1964) and was thought to be the major TAG lipase until the recent finding of the adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004). Characterizations of mutants deficient in these two enzymes and in vitro kinetic studies indicate that ATGL is the major TAG lipase while HSL mostly degrades the DAG generated by ATGL activity to form MAG, a substrate for the third lipase-monoacylglycerol lipase (MAG) (Zechner et al., 2009).

Activities of the above-mentioned lipases are regulated at multiple levels. Insulin inhibits phosphorylation of HSL, which facilitates the attachment of cytosolically localized HSL to the surface of lipid droplets (Slavin et al., 1994). An adipogenic transcription factor PPARgamma was reported to transcriptionally activate mouse ATGL (Kim et al., 2006). In the recent years, CGI-58, a protein which shares sequence similarities to lipases but with the serine catalytic center replaced during evolution, emerged as an important regulator for fat mobilization. Binding to CGI-58 is required for ATGL to exhibit full TAG hydrolysis activity (Zimmermann et al., 2009).

During the past decade, major TAG lipases have been identified in Arabidopsis (Eastmond, 2006), fruit fly (Gronke et al., 2005) and the budding yeast (Athenstaedt and Daum, 2003, 2005; Kurat et al., 2006). These lipases as well as the mammalian ATGLs belong to the family of patatin-like lipases (Zimmermann et al., 2004) while many members of this family exhibit lipolytic activity against phospholipids instead of TAG (Banerji and Flieger, 2004).
Lipases that attack polar lipids can also affect the metabolism of TAG. The acyl group generated by polar lipid hydrolysis can be used a substrate for the DGAT reaction (reaction 2, Figure 1.5). In plants, it has been proposed that the fatty acid exported from the chloroplast to cytosol, after being activated into acyl-CoA, is first incorporated into PtdCho through an acyltransferase. A phospholipase then removes this acyl group to the cytosolic acyl-CoA pool, which can be used for the biosynthesis of TAG and other extraplastidic lipids (Bates et al., 2007). In Chlamydomonas, PtdCho is absent but it remains possible that another lipid serves this role.

Besides the trafficking of fatty acids through the acyl-editing mechanism, breakdown of membrane components in Chlamydomonas may also require lipases. Under N-deprivation, the fatty acids contained in TAG include polyunsaturated species such as 16:4 and 18:3, which could only be obtained by membrane lipid degradation (Moellering and Benning, 2010). The lipases involved in this process are likely encoded by those genes up-regulated by N-deprivation (Miller et al., 2010).

Last but not least, lipases may affect cell metabolism by producing or removing lipid signaling molecules. In plants, upon pathogen infection, two galactolipases, DGL and DAD, remove the 18:3 fatty acids from plastidic lipids, which are then utilized by the oxylipin pathway to produce the phytohormone jasmonic acid (JA) (Hyun et al., 2008; Ellinger et al., 2010). While the presence of JA in Chlamydomonas is not reported, there are probably other lipid molecules playing signaling roles.
**Aims of the thesis research**

This thesis aims to identify novel genes involved in TAG metabolism in Chlamydomonas. Three investigations have been performed: Chapter 2 describes reverse genetics on several candidate TAG lipases. One lipase designated CrLIP1 was discovered and characterized. Chapter 3 reports a forward genetic screen for mutants with abnormal amounts of TAG. A mutant deficient in TAG accumulation was characterized extensively. Chapter 4 describes further the low-TAG mutant, which was used as a tool to explore the physiological significance of TAG accumulation.
Figure 1.1. Structure of triacylglycerol and transesterification of triacylglycerol to produce fatty acid methyl esters. $R_1$, $R_2$ and $R_3$ represent hydrocarbon chains.
Figure 1.2 Comparison of major membrane glycerolipid biosynthesis pathways between Arabidopsis and Chlamydomonas. Regular arrows indicate processes that can be found in both organisms. Dashed arrows represent processes unique to Arabidopsis while the bold arrow is unique to Chlamydomonas. Only major substrates or products are shown for each reaction. Phosphatidylserine and phosphatidylinositol are less relevant for discussions in this dissertation and are skipped. Phosphatidylglycerol is also partly synthesized in extraplastidic membranes, but in trace amounts compared to the plastic pathway and is thus skipped. Abbreviations: ACP, acyl carrier protein; AdoMet, S-adenosyl methionine; CDP-Cho, cytidine diphosphate-choline; CDP-DAG, cytidine diphosphate-diacylglycerol; CDP-Etn, cytidine diphosphate-ethanolamine; CTP,
cytidine triphosphate; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-N,N,N-trimethylhomoserine; FAS, fatty acid synthesis; G-3-P, glycerol-3-phosphate; lyso-PtdOH, lyso-phosphatidic acid; MGDG, monogalactosyldiacylglycerol; PGP, phosphatidylglycerol-phosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdOH, phosphatidic acid; SQDG sulfoquinovosyldiacylglycerol; UDP-Gal; UDP-SQ, uridine diphosphate-sulfoquinovose. Names of enzymes or processes for each arrow: 1, plastid glycerol-3-phosphate acyltransferase; 2, plastid lyso-phosphatidic acid acyltransferase (chloroplast); 3, plastid phosphatidic acid phosphatase; 4, SQDG synthase; 5, MGDG synthase; 6, DGDG synthase; 7, CDP-DAG synthase; 8, PGP synthase; 9, PGP phosphatase; 10, thioesterase; 11, long-chain acyl-CoA synthetase; 12, ER associated glycerol-3-phosphate acyltransferase; 13, ER-associated lyso-phosphatidic acid acyltransferase; 14, ER associated phosphatidic acid phosphatase; 15, CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase; 16, betaine lipid synthase; 17, CDP-choline:diacylglycerol cholinephosphotransferase; 18, lipid trafficking from ER to plastid, catalyzed by multiple protein components; 19, acyl-CoA:lyso-PtdCho acyltransferase 20, phospholipase.
Figure 1.3. Structures of PtdCho and DGTS. R represents a hydrocarbon chain.
Figure 1.4. TAG synthesis catalyzed by DGAT and PDAT activities. X represents an organic group that varies between different phospholipids.
Figure 1.5. Lipases affect TAG content by degrading TAG or providing acyl groups for TAG synthesis. X represents a polar headgroup that varies between different polar lipids.
Chapter 2

Rapid triacylglycerol turnover in *Chlamydomonas reinhardtii* requires a lipase with broad substrate specificity\(^1\)

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ABSTRACT

When deprived of nitrogen (N), the photosynthetic microalga *Chlamydomonas reinhardtii* accumulates large quantities of triacylglycerols (TAG), affording a promising source of biofuel. Prominent transcriptional changes have been found associated with conditions leading to TAG accumulation, suggesting that key enzymes for TAG metabolism might be among those fluctuating in their expression during TAG synthesis and breakdown. Using a *Saccharomyces cerevisiae* lipase null mutant strain for functional complementation, I identified the *CrLIP1* gene from Chlamydomonas based on its ability to suppress the lipase deficiency-related phenotypes of the yeast mutant. In Chlamydomonas, an inverse correlation was found between the *CrLIP1* relative transcript level and TAG abundance when Chlamydomonas cultures were reversibly N-deprived. *CrLIP1* protein expressed and purified from *E. coli* exhibited lipolytic activity against diacylglycerol (DAG) and polar lipids. The lipase domain of *CrLIP1* is most similar to two human DAG lipases, DAGLα and DAGLβ. The involvement of *CrLIP1* in Chlamydomonas TAG hydrolysis was corroborated by reducing the abundance of the *CrLIP1* transcript with an artificial microRNA, which resulted in an apparent delay in TAG lipolysis when N was re-supplied. Together, these data suggest that *CrLIP1* facilitates TAG turnover in Chlamydomonas primarily by degrading DAG presumably generated from TAG hydrolysis.
INTRODUCTION

Plants, animals and fungi accumulate triacylglycerols (TAGs) to store excessive carbon and energy. For industrial biofuel production, triacylglycerol can be converted into fatty acid methyl esters (FAMEs) through transesterification, the major components of biodiesel (Durrett et al., 2008). TAG harvested from cells can thus be a source of biofuel. Thanks to the faster growth rate and higher oil content, microalgae can in theory produce TAG at yields surpassing those of plants (Luo et al., 2010b). However, details of lipid metabolism in algae are poorly understood.

*Chlamydomonas reinhardtii* has a long history of serving as a model system to study cell motility and photosynthesis (Rochaix, 1995; Merchant et al., 2007; Harris et al., 2009). The recent development of genetic and molecular biological tools such as nuclear genome transformation (Kindle, 1990) and gene silencing with artificial microRNA (Molnar et al., 2009) has also made Chlamydomonas an excellent model alga for a wider spectrum of research topics, including lipid metabolism and biofuel production. In Chlamydomonas the glycerolipid composition was explored (Giroud et al., 1988) and the lipid metabolism pathways have been annotated *in silico* (Riekhof et al., 2005). Certain enzymes for TAG metabolism have also been characterized experimentally (Boyle et al., 2012). Similar to many other algae, Chlamydomonas accumulates TAG under nitrogen deprivation (Wang et al., 2009; Moellering and Benning, 2010).

In plants, TAG biosynthesis starts with plastid production of fatty acids, which are then esterified to glycerol-3-phosphate, forming phosphatidic acid (PtdOH). The endoplasmic reticulum (ER) is another site for lipid assembly and fatty acid modification (Roughan and Slack, 1982). Diacylglycerol is produced after the removal of the phosphate by a PtdOH phosphatase. Diacylglycerol acyltransferases (DGAT) and phospholipid:diacylglycerol acyltransferases (PDAT) then transfer an acyl chain from acyl
coenzyme A or a phospholipid to DAG to synthesize TAG (Zhang et al., 2009), which can be considered to be a vehicle for carbon storage. TAG turnover (lipolysis) is initiated by the action of TAG lipases, which generate DAG and free fatty acids (Eastmond, 2006). DAG can be further hydrolyzed into monoacylglycerol and glycerol. Certain lipases hydrolyze all acyl chains from the glycerol backbone, while others may act specifically on one kind of glycerolipid (Utsugi et al., 2009). To engineer TAG content, both TAG biosynthetic and hydrolytic enzymes have to be considered. For example, overexpressing an Arabidopsis DGAT increases seed oil (Jako et al., 2001), whereas deleting the major TAG lipases in the budding yeast \textit{Saccharomyces cerevisiae} results in enhanced accumulation of TAG in cells (Athenstaedt and Daum, 2003, 2005; Kurat et al., 2006). In \textit{Chlamydomonas}, functions of two DGATs and a PDAT have been described (Boyle et al., 2012; La Russa et al., 2012a, b). Lipases for the TAG turnover chain reactions are yet to be identified.

Like many other microalgae, \textit{Chlamydomonas reinhardtii} cells do not maintain a large TAG pool during vegetative growth, but do so following nutrient deprivation, most notably nitrogen (N). Seventy-two hours after N removal, TAG accounts for 50% of all cellular fatty acids, which results from both increased fatty acid biosynthesis and membrane remodeling (Miller et al., 2010). These metabolic flux changes are accompanied by genome-wide adaptation of transcription, including a large number of putative lipase-encoding genes that are up- or down-regulated (Miller et al., 2010). I reasoned that the repressed genes during TAG accumulation may encode lipases that degrade TAG or its initial breakdown products (i.e., diacylglycerol and monoacylglycerol). The N-deprivation-induced lipase genes, on the other hand, might be involved in shuffling fatty acids from membrane lipids to TAG. Here, I describe the identification of a lipase-encoding gene required for rapid TAG turnover in \textit{Chlamydomonas}. 

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MATERIALS AND METHODS

Yeast methods

Yeast cells were grown according to standard procedures (Sherman, 1991). Yeast transformation was performed using the lithium acetate method as previously described (Gietz et al., 1992). Diameters of yeast cells were determined using the MicroSuite Basic software (Olympus) after conducting microscopy. A uniform lower limit of 4.1 µm long-axis cell length was arbitrarily set to exclude young daughter cells. All yeast strains (Table 1) were derived from theyMK839 (Kuo et al., 1998) background, a derivative of S288C. To delete TGL4, PCR was done with primers 4DF and 4DR (all primer sequences are shown in Table 2.3) to amplify Kluyveromyces lactis TRP1 from pBS1479 (Rigaut et al., 1999). The PCR product was then transformed into yMK839 for tryptophan prototroph selection to obtain yXL001. The deletion was verified by genomic PCR. To delete TGL3 from yXL001, PCR was performed to amplify KanMX6 from pFA6a-KanMX6 (Wach et al., 1994). The PCR product was then gel purified and used as the template for another PCR with primers 3DF and 3DR. The new PCR product was transformed to yXL001 and cells were plated onto YPD with 200 μg/ml G418 for selection, creating yXL005 after genomic PCR verification of the tgl3Δ::KanMX allele. In some cases, yeast cells were grown in casamino acids (CAA) medium minus uracil (CAA –ura) that was essentially identical to the synthetic complete medium (Sherman, 1991) except the supplement of 0.5% casamino acids and uracil dropout.

To create a cDNA clone for CrLIP1 expression in yeast, Chlamydomonas RNA was prepared with the RNeasy Plant Mini Kit (Qiagen). Reverse transcription was conducted with Superscript II reverse transcriptase (Invitrogen) to obtain cDNA. CrLIP1 coding sequence was
amplified using Phusion polymerase (NEB) and primers 595F and 595R. The PCR product was co-transformed into yeast cells together with a NotI linearized yeast expression vector pMK595 (Luo et al., 2010a) by homologous recombination (Ma et al., 1987). A single clone of pMK595 with \textit{CrLIP1} integrated (pMK595CrLIP1) was obtained through transformation of the yeast crude DNA into \textit{E. coli} and sequenced to be correct (Table 2.2). pMK595 and pMK595CrLIP1 were transformed into yMK839 to obtain yXL023 (referred to as \textit{WT-vect} in Figures) and yXL026 (\textit{WT-CrLIP1}), and into yXL005 to obtain yXL077 (\textit{tgl3Atgl4Δ-vect}) and yXL080 (\textit{tgl3Atgl4Δ-CrLIP1}) respectively.

\textbf{Chlamydomonas strains and growth conditions}

The cell wall-less strain dw15-1 (cw15, nit1, \textit{mt} \textsuperscript{+}) obtained from Arthur Grossman (Carnegie Institution, Stanford) was used for all experiments performed on Chlamydomonas. Liquid and solid cultures were grown in Tris-acetate-phosphate (TAP) medium as described before (Moellering and Benning, 2010). For N-deprivation or re-supply, cells were collected by centrifugation at 3000 x g (4°C, 3 min), washed twice and re-suspended in TAP-N (TAP medium with NH\textsubscript{4}Cl omitted) or TAP respectively.

\textbf{PCR}

Quantitative real-time PCR on \textit{CrLIP1} was performed using primers qRT-F and qRT-R with the Applied Biosystems 7500 Fast Real-time PCR system. Data were normalized to the commonly used \textit{RACK1} gene using primers RACK1-F and RACK1-R (Chang et al., 2005). The \textit{2(−ΔΔC(T))} method (Livak and Schmittgen, 2001) was employed for data analysis. RT-PCR for
the agarose gel electrophoresis was performed using the same primers with the real-time PCR for \textit{RACK1} and \textit{CrLIP1}. For \textit{DGTT1}, DGTT1-F and DGTT1-R were used.

\textbf{Western blotting}

To examine the expression of the \textit{CrLIP1} cDNA in yeast, log-phase yeast cells were collected by centrifugation (3000 x g, 5 min) and then re-suspended in 2X SDS-PAGE loading dye (0.12 M Tris-HCl, pH 6.8, 0.04% bromophenol blue, 4% SDS, 20% glycerol, 5.6% β-mercaptoethanol) with the same volume of acid-washed glass beads (0.45 mm, Sigma-Aldrich). The mixture was boiled in a water bath for 5 min followed by 5 min of vortexing. After an additional cycle of boiling and vortexing, the lysates were centrifuged at 21,000 x g for 1 min and the supernatant was analyzed by SDS-PAGE. To examine the subcellular localization of recombinant CrLIP1, yeast cell pellets were suspended in 0.1 M PBS (pH 7.4) containing the Complete protease inhibitor cocktail (Roche, one tablet for every 10 ml of buffer). Glass beads (0.45 mm) were added, followed by vigorous agitation in a Mini-Bead Beater (BioSpec) for 45 sec and 1-minute ice incubation. The beating was repeated 3 times. After the last bead-beating, lysates were collected by spinning through a pinhole punched at the bottom of the tube (1,000 x g, 5 min, 4°C). The supernatant of the low-speed spinning was fractionated by 100,000 x g centrifugation (90 min, 4°C). The secondary supernatant was defined as the soluble fraction in Figure 2.1. The membrane-enriched pellet was suspended in PBS containing the protease inhibitor tablet. For immunoblotting, the 12CA5 monoclonal antibody (Roche) was used to probe for proteins with an HA epitope tag and the His monoclonal antibody (H1029, Sigma-Aldrich) was used for the hexahistidine-tagged protein in \textit{E. coli}. 

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Lipid analysis

To extract lipids, Chlamydomonas and *E. coli* cells were harvested and re-suspended in the lipid extraction solvent containing methanol, chloroform, and formic acid (88%) (2:1:0.1 by volume). In addition, the bacterial cells expressing *CrLIP1* were shaken at 26°C for 6 hours after overnight IPTG (isopropyl-β-D-thiogalactopyranoside) induction before cells were collected (described below). I found that this extra step allowed better lipase action in *E. coli*. For lipid extraction, Chlamydomonas and *E. coli* cells were vortexed for 30 sec in the extraction solvent for cell lysis. For yeast, cells were re-suspended in the solvent; and glass beads (0.45 mm) were added. Yeast lipids were extracted by using a Mini-Bead Beater (BioSpec) as described above for protein preparation, except that 0.5 volume of lipid extraction buffer (1M KCl, 0.2M H₃PO₄) was added to the organic phase after its collection through spinning through a pinhole at the bottom of the tube. For Thin-layer chromatography (TLC), lipids were loaded onto Silica Gel 60 plates (EMD chemicals, #5721-7) and developed in petroleum ether-diethyl ether-acetic acid (80:20:1 by volume, to separate neutral lipids) or chloroform-methanol-acetic acid-H₂O (75:13:9:3 by volume, for separation of polar lipids). Lipids were visualized by exposure to iodine vapor. For quantification of fatty acid methylesters derived from a certain lipid species on the TLC plate, corresponding spots were scraped and subjected to transesterification and GLC as previously described (Rossak et al., 1997).

Yeast metabolic labeling

To measure the rate of lipid synthesis, yeast cells were grown in CAA –ura medium for 48 h at 26°C to early stationary phase. To each 5-ml culture 20μCi [¹⁴C] acetate (specific activity
45-60 mCi/mmol; Perkin Elmer) was added. Cultures were then continually shaken (app. 150 rpm in a Lab-Line floor incubator) at 26°C and were harvested 25 min, 40 min, 65 min, 100 min and 140 min after acetate supplementation. Total lipids were extracted (see above) and resolved by TLC resolution (petroleum ether:diethylether:acetic acid = 80:20:1 by volume) that separated neutral lipids. TAG, DAG, and TLC origins (containing a mixture of polar lipids) were isolated for radioactivity measurement by liquid scintillation counting. For yeast cellular TAG hydrolysis analysis, 50μCi (0.83-1.11 μmol) [14C]-acetate was added to 5 ml cultures grown for 48 h at 26°C. After 4 h of labeling, cells were collected by centrifugation, washed with fresh medium and diluted 28 fold with fresh CAA –ura medium to an optical density (at 600 nm) of 0.4-0.6. The cells were shaken for another 5 h with aliquots frozen at each time point. The growth curves were determined with cells grown under exactly the same conditions except that 1 μmol non-labeled acetate was used. Lipid analysis was as above for lipid synthesis rate analysis.

**Recombinant protein production**

The CrLIP1 coding sequence was amplified using primers LICF and LICR and the Phusion polymerase. The PCR product was then integrated into an expression vector pMK1006 through ligation-independent cloning (Aslanidis and de Jong, 1990). BL21 CodonPlus (Stratagene) transformed with empty pMK1006 vector or pMK1006CrLIP1 were grown to the log phase at 37°C and protein production was induced by addition of IPTG to a final concentration of 0.5 mM. After 16 h of shaking at 220 rpm at 16°C, cells were harvested by centrifugation (6,000 x g, 5 min, 4°C). For protein preparation, cells were collected and suspended in the lysis buffer (20 mM Tris-HCl, pH 7.9, 10% glycerol, 150 mMNaCl, 1 mM dithiothreitol) and subjected to three freeze-thaw cycles, followed by sonication on ice (90 times
of alternating 1-sec pulse and 0.3-sec rest, with 1 minute ice-chilling after every 15 cycles; Branson digital sonicator 250 with 25% energy output was used). Cell-free extracts were obtained through centrifugation at 21,000 x g for 15 min and applied to a Ni-NTA affinity purification column (Qiagen). CrLIP1 was eluted with the lysis buffer supplemented with 200 mM imidazole. A spectrophotometric assay with the Bio-Rad Protein Assay Dye Reagent Concentrate (BioRad #500-006 EDU)(Bradford, 1976) was used to determine protein concentrations.

Lipase assay

For the lipase assay with various substrates, lipids dissolved in organic solvents were dried under a stream of nitrogen gas. The dried lipid was then dissolved in 350 μL of 0.1 M phosphate saline buffer (PBS, pH 7.4) containing varying concentrations of Triton X-100 (see below) by sonication (Sonicator 3000 with a Misonix microprobe) for 6 x 10 sec (power setting 1.5). Purified CrLIP1 protein or the same volume of protein storage buffer was added. Dithiothreitol (DTT) was added fresh to the final concentration of 2 mM. The mixture was then vortexed and incubated at room temperature.

For the TAG lipase assay, 10 μCitriolein (specific activity 30-120 Ci/mmol; Perkin Elmer) and 5.6nmol olive oil was dried and re-suspended in 350 μL PBS containing 0.11 mM Triton X-100. The mixture was split in halves and 18μg of purified CrLIP1 in 20 μL protein storage buffer was added to one of the aliquots. The mixture was then vortexed vigorously for 5 sec and incubated at room temperature for 12 hr. I tried multiple other conditions, with varied amounts of olive oil (0-250 nmol), DTT or supplementation of CaCl₂. Emulsifiers including phosphatidylcholine/phosphatidylinositol mix and BSA were also used to replace Triton X-100.
without an observable effect on the result. For the DAG lipase assay, 150 nmoldiolein (Avanti
Polar Lipids) was dried and sonicated as described for TAG, except that 0.53 mM instead of 0.11
mM Triton X-100 was used. Purified CrLIP1, 18µg in 50 µL protein storage buffer, was added.
The mixture was then vortexed briefly and incubated at room temperature for 16 hr.

For assays containing radioactive polar lipids, Chlamydomonas cells were grown to log
phase in TAP medium, harvested and suspended in medium with 30 µCi of $^{14}$C acetate
mentioned above (final concentration 20 - 23.3 µM). Cells were harvested after another 4 h of
standard growth (see above). Total lipids were extracted and resolved by TLC developed in
chloroform-methanol-acetic acid-H$_2$O (75:13:9:3 by volume). Individual lipids were visualized
by radiography and then scraped off the plate before extraction with chloroform-methanol (1:1
by volume) from silica gel. Lipids (35 nmol with total radioactivity of 10,000-40,000 dpm) were
dried and dissolved as described for TAG. For lipase assays, the emulsified lipid substrates were
divided into halves, with 18 µg purified CrLIP1 added to one set and the same volume of protein
storage buffer added to the other set. The mixture was incubated for 12 h at room temperature.

To prepare radioactive steryl esters for in vitro lipolysis reactions, 1 ml of early stationary-phase
tgl3Atgl4Δ-vect yeast cells were diluted to 25 ml fresh CAA –ura medium with additional 30 µCi
of $^{14}$C-acetate and grown for 48 h at 26°C. Total lipids were extracted and resolved by TLC as
described above. Steryl esters were then isoalted from the TLC plate. Assay conditions were the
same as lipids derived from Chlamydomonas cells, with 35 nmol labeled steryl esters (10,000 dpm)
as the substrates. For assays with PtdCho, 60 nmol 18:1$^9$/16:0 PtdCho (Sigma-Aldrich) or 18:1$^9$/16:0 PtdCho (Avanti Polar Lipids) was dried and dissolved as described for
TAG. CrLIP1 (36 µg) was added and aliquots were flash frozen at 0, 6 and 16 h. The 0 h control
was used to verify the intactness of PtdCho. The mixture was incubated for 12 h at room temperature. Background levels of fatty acids carried over with purified CrLIP1 protein were estimated in a control reaction without substrate lipid supplied, and subtracted from the free fatty acids data obtained with substrate.

All the lipase reactions were quenched by adding two volumes of lipid extraction solvent. Lipid products were then analyzed by TLC as described above. For *Rhizopus arrhizus* lipase (Sigma-Aldrich) digestion, 200 µg of the enzyme preparation was used and the incubation time was shortened to 20 min.

**AmiRNA construct**

The artificial microRNA construct to silence *CrLIP1* expression was generated according to Molnar et al. (2009). Briefly, four primers were designed using the MicroRNA Designer protocols ([http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Help](http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Help)). The primers were annealed by 5 min of boiling and overnight cooling to form a double-stranded fragment with *CrLIP1* targeting sequences and overhangs compatible with the *SpeI* digestion site. This fragment was then integrated into *SpeI* linearized pChlamiRNA3int vector (Molnar et al., 2009) through ligation and transformation. To generate *CrLIP1* knock-down lines, the artificial microRNA construct (pChlamiRNA3intCrLIP1) or the empty vector was digested with *KpnI* (NEB) and transformed into the dw15-1Chlamydomonas strain using the glass bead transformation method (Kindle, 1990). TAP agar containing 10 µg/ml paromomycin was used for selection. Real-time PCR described above was used to screen for lines with reduced mRNA abundance of *CrLIP1*. 
Bioinformatics

A BLAST search (Altschul et al., 1997) of CrLIP1 protein sequence against human genome was performed on National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/). Protein sequences were aligned with the ClustalW2 program (Larkin et al., 2007).

RESULTS

Heterologous expression of \textit{CrLIP1} complemented a yeast mutant deficient in TAG lipase activity

To identify the Chlamydomonas lipases involved in TAG turnover, I used a yeast mutant deficient in TAG lipase for functional complementation. In yeast, the major TAG lipases are encoded by \textit{TGL3} and \textit{TGL4} (Athenstaedt and Daum, 2003, 2005; Kurat et al., 2006). Their deletion results in hyper-accumulation of TAG in stationary-phase cells. This phenotype can be rescued by ectopic expression of a mouse adipose triglyceride lipase (Kurat et al., 2006). To choose Chlamydomonas candidate genes for a lipase that suppresses the yeast lipid overstocking phenotype, putative lipases based on Gene Ontology of the JGI Chlamydomonas 3.0 database were cross-referenced to our transcriptomic studies of N-replete and depleted cells (Miller et al., 2010), and to the data from a lipid droplet proteomics study (Moellering and Benning, 2010). Eight genes were selected for initial studies. The respective cDNAs were placed under the control of the constitutive \textit{ADH1} promoter from a yeast high-copy plasmid. An N-terminal triple HA tag was added to the coding sequence. These genes were tentatively named \textit{CrLIP1} through \textit{CrLIP8} (summarized in Table 2.4). Candidates \textit{CrLIP1}, \textit{CrLIP3}, \textit{CrLIP5}, \textit{CrLIP6} and \textit{CrLIP8} were successfully cloned and expressed in the \textit{tgl3A tgl4A} double knockout mutant. Of
the five genes tested, CrLIP1 (protein ID 184308 and 519543, JGI *Chlamydomonas* 4.0; a.k.a. FAP12 (Pazour et al., 2005), see Discussion) consistently showed reduction of TAG and changes in cell growth (see below). For the remainder of this work, I focused on CrLIP1 for its role in lipolysis.

I first examined the expression of CrLIP1 in yeast. As expected for a lipase, the majority of recombinant CrLIP1 was associated with the membrane-enriched fraction that also included lipid droplets and TAG (Figures 2.1A-C). Importantly, the TAG over-accumulation phenotype of the tgl3Δtgl4Δ mutant (Athenstaedt and Daum, 2005) was partially suppressed by CrLIP1 overexpression (Figure 2.1D). There was no discernible change in the steady-state levels of DAG or major phospholipids, initially suggesting that CrLIP1 primarily affected the TAG accumulation or hydrolysis in yeast. Although CrLIP1 did not decrease the TAG content in wild-type background to a statistically significant level (Figure 2.1D), it caused consistent differences in the fatty acid compositions of TAG in both wild-type and the tgl3Δtgl4Δ mutant, with an increase in the relative amount of 16:1 and a decrease in 16:0 (Figure 2.1E). This might be due to substrate specificity of CrLIP1 if it is a *bona fide* lipase. Besides these lipid phenotypes, I also noticed that the tgl3Δtgl4Δ mutant grew to a higher optical density (OD) in the stationary phase (Figure 2.1F). Overexpressing CrLIP1 prevented this OD increase. One of the underlying reasons for this phenomenon can be ascribed to the changes in cell size, as revealed by the measurement of cellular diameter under a light microscope (Figure 2.1G). Overall, the double lipase knockout strain had a higher number of large cells than did the wild-type strain. Larger particles should absorb more light, resulting in higher OD. Over-expressing CrLIP1 in the tgl3Δtgl4Δ background caused the overall cell size distribution to approach that of the wild-type
cells. Together, these results demonstrated that the Chlamydomonas CrLIP1 protein was able to functionally complement the yeast TAG lipases Tgl3 and Tgl4.

**Heterologous expression of CrLIPI lowers the rate of TAG biosynthesis in yeast**

To explore the role of CrLIP1 in yeast TAG metabolism in a more direct way, metabolic labeling experiments were performed. Stationary phase cells were chosen as TAG accumulates when cells enter stationary phase (Larkin et al., 2007). Additionally, from 48 h to 60 h, the increase in cell number was within 4 % for all strains tested (Figure 2.1F), thus the cell number during the 2-h labeling period was regarded as constant, hence permitting us to normalize changes in lipid labeling to cell numbers. The radioactivity incorporated into TAG, DAG, and polar lipids (mainly phospholipids in yeast) was measured (Figure 2.2A-C). Consistent with the steady state lipid analysis results (Figure 2.1D), CrLIP1 lowered the rate of TAG accumulation (Figure 2.2A) while the effect on polar lipid was relatively minor (Figure 2.2B). Interestingly, labeling of DAG increased in the first 90 min after the addition of radioactive acetate, and dropped afterwards. Figure 2.2C showed that the incorporation of label into DAG was faster than that of TAG (Figure 2.2A), consistent with DAG being the precursor for TAG biosynthesis. The expression of CrLIP1 did not affect the initial DAG labeling, but rather accelerated its reduction. As the immediate precursor of TAG, the reduction in DAG labeling likely reflected its conversion to TAG. The faster appearance of label in DAG than in TAG and the decrease of label in DAG after 90 min are consistent with a substrate product relationship of DAG and TAG, respectively. The faster decline of DAG label in the presence of CrLIP1 would be consistent with CrLIP1 being a DAG lipase, which could affect the synthesis of TAGs as observed. Indeed, *in vitro* experiments (below) confirmed that CrLIP1 has DAG lipase activity.
When growing out of the stationary phase, yeast degrades TAG to produce precursors supporting multiple cellular functions, including membrane polar lipid biosynthesis and energy consumption (Larkin et al., 2007). To shed light on the function of CrLIP1 in yeast, I employed metabolic pulse-chase labeling. Toward this end, stationary phase yeast cells were labeled with radioactive acetate and then transferred to fresh medium without the label. Radioactivity in TAG and total polar lipids was quantified. During the course of the chasing period, all strains showed varying degrees (up to about 30%) of increase in the optical density (Figure 2.2F), a result of cells undergoing mitosis. To accommodate the differences in cell density, I measured the radioactivity of TAG and polar lipids over a unit volume (per ml) of all strains, and normalized it to the starting counts. During the outgrowth, the label in cellular polar lipids increased whereas TAG labeling remained constant in wild-type cells (open squares, Figures 2.2D and 2.2E). Labeling of DAG was very low at the times tested, consistent with very rapid turnover of the DAG pool following the onset of the chase phase of the experiment. The increase of total radioactivity contained in polar lipids likely resulted from the incorporation of persisting radiolabeled acetate and fatty acids. On the other hand, the total amount of TAG remained steady, which I suggest to be a result of balanced TAG synthesis and hydrolysis. Indeed, in the \( \text{tgl3}\Delta \text{tgl4}\Delta \) double lipase knockout strain (open triangles, Figures 2.2D and 2.2E), the total TAG amount increased drastically. Importantly, consistent with a role in lipolysis, expression of \( \text{CrLIP1} \) in both wild-type and yeast lipase null strains caused a reduction in TAG labeling levels when cells emerged out of the stationary phase (close triangles and squares, Figures 2.2D). In addition, labeling of polar lipids also showed a clear decrease in cells expressing the algal \( \text{CrLIP1} \) protein (Figure 2.2E). Together, these results strongly suggest a lipolytic function of \( \text{CrLIP1} \).
Recombinant CrLIP1 hydrolyzes diacylglycerol but not triacylglycerol \textit{in vitro}

To examine the lipase activity of CrLIP1 biochemically, the cDNA of \textit{CrLIP1} was cloned into an \textit{E. coli} expression vector. A hexahistidine-tagged CrLIP1 protein was produced, purified (Figure 2.3A), and subjected to TAG hydrolysis assays (Figure 2.3B). Purified CrLIP1 ran as a doublet on SDS-PAGE (Figure 2.3A). Although the underlying reason for this phenomenon remains unclear, the fact that both bands reacted with anti-His antibody (Figure 2.4) indicated that these two bands were isoforms of CrLIP1, not co-purified contaminants. Radioactive triolein supplemented with olive oil was incubated with the purified CrLIP1 protein before TLC analysis of the reaction products. Neither iodine vapor stain (not shown) nor autoradiography detected production of free fatty acids by CrLIP1 (Figure 2.3B). Multiple attempts with varying reaction conditions all failed to detect TAG lipase activity of CrLIP1. I also tried another neutral lipid-steryl esters extracted from yeast cells as the substrate and detected no lipolytic activity either (Figure 2.5).

I had noted that BLAST searches against non-redundant databases revealed considerable similarity between CrLIP1 and the human \textit{sn}-1-specific diacylglycerol lipases DAGL$\alpha$ and DAGL$\beta$ (Bisogno et al., 2003) (identity scores of 22 with DAGL$\alpha$ and 26 with DAGL$\beta$) (see Figure 2.6). This prompted us to test the activity of CrLIP1 against DAG. Commercial radio-labeled DAG was not available. To choose a DAG substrate capable of differentiating the reaction products from bacterial fatty acids co-purified with the recombinant CrLIP1 protein, I took advantage of the fact that \textit{E. coli} does not synthesize oleic acids (18:1$\Delta^9$) (Kito et al., 1972), and thus used diolein [1,2-Di(cis-9-octadecenoyl)glycerol] as the substrate for \textit{in vitro} lipolysis.
assays. Reaction mixtures were resolved by TLC, followed by gas-liquid chromatography (GLC) of FAMEs to identify the fatty acid products. At the position of free fatty acids, there was no visible staining in the absence of CrLIP1 (Figure 2.7A), demonstrating that diolein by itself remained intact through the entire reaction. In the presence of CrLIP1, on the other hand, iodine staining clearly revealed the production of free fatty acids. Indeed, GLC analysis of the free fatty acids isolated from the TLC plate determined unequivocally the presence of oleic acid when CrLIP1 was present in the reaction (Figure 2.7C). I thus concluded that CrLIP1 possesses DAG lipase activity.

**Recombinant CrLIP1 degrades membrane lipids of *E. coli***

Using crude lysates of *E. coli* for the initial testing of the CrLIP1-dependent lipase activity, I noticed a prominent signal of free fatty acids prior to the addition of an exogenous substrate, suggesting that CrLIP1 may have had already acted on an abundant bacterial lipid species. To explore this possibility further, I isolated bacterial lipids for TLC analysis, and observed the presence of substantial amounts of free fatty acids in the CrLIP1-containing extracts (Figure 2.8A). In *E. coli*, phosphatidylethanolamine (PtdEtn) is the most abundant glycerolipid (Rietveld et al., 1993) and I suspected that the free fatty acids were at least partly derived from PtdEtn hydrolysis. If so, one of the PtdEtn hydrolysis intermediates, lyso-PtdEtn, might be detectable in the bacterial lipid extracts when CrLIP1 was overproduced. Indeed, when the sn-1-specific *Rhizopus arrhizus* lipase (Fischer et al., 1973) was used to generate a standard for lyso-PtdEtn, lyso-PtdEtn increased along with free fatty acids (Figure 2.8B). GLC analysis provided the quantitative support for both free fatty acids and lyso-PtdEtn increase and PtdEtn reduction in the CrLIP1-overexpressing sample (Figure 2.8C). These results strongly suggest that CrLIP1 is
capable of hydrolyzing glycerophospholipids such as PtdEtn that are the major components of biological membranes. Intriguingly, in the neutral TLC (Figure 2.8A) on lipids extracted from *E. coli* cells, a new compound (indicated by the arrow) was detected upon *CrLIP1* over-expression. I do not yet know the identity and origin of this species. While this phenomenon is likely related to the biochemical activity of *CrLIP1*, definitive identification of this bacterial lipid species will require additional efforts beyond the scope of the current work.

**Recombinant CrLIP1 hydrolyzes polar lipids**

Many lipases such as the human ATGL (Jenkins et al., 2004) and yeast Tgl4 (Rajakumari and Daum, 2010) act on a broad spectrum of glycerolipid substrates. The apparent *CrLIP1*-dependent lipolysis of PtdEtn in *E. coli* prompted us to explore what other membrane lipids might be substrates for *CrLIP1*. I focused on abundant Chlamydomonas polar lipids that each constituted greater than 10% of the membrane lipids: MGDG (monogalactosyldiacylglycerol), DGTS (diacylglycerol-N,N,N-trimethylhomoserine), PtdEtn, PtdGro (phosphatidylglycerol) and DGDG (digalactosyldiacylglycerol). Radio-labeled polar lipids were prepared by growing cells in [14C]-acetate containing medium since acetate predominantly labels acyl chains rather than the glycerol backbone (Schneider and Roessler, 1994). These substrates would therefore allow us to follow the lipase activity by tracking the production of free fatty acids. Lipolysis reaction products were resolved by TLC and detected by autoradiography. For the five substrates used, lipolysis was documented by the apparent production of radioactive free fatty acids when *CrLIP1* was present (Figures 2.9A and B). While *CrLIP1* appeared to be more active on phospholipids (i.e., PtdEtn and PtdGro) under the assay conditions, it was difficult to quantitatively determine the substrate preference for these polar lipids, because they were mixtures of molecular species.
with varying specific activities due to the preparation of the substrates by pulse-labeling of Chlamydomonas cells. Nonetheless, as shown in Figures 2.7 to 2.9, I was able to demonstrate the lipase activity of CrLIP1 against DAG and polar lipids.

**Recombinant CrLIP1 hydrolyzes both acyl chains of phosphatidylcholine (PtdCho) with a preference for the sn-1 position**

Diacylglycerol and polar lipids harbor two acyl chains at the sn-1 and sn-2 positions of the glycerol backbone. Many lipases prefer one position over the other. For example, the lipase from *Rhizopus arrhizus* strongly prefers the sn-1 ester bond (Fischer et al., 1973). The human DAGLs also prefer sn-1 over sn-2 although both activities were detectable (Bisogno et al., 2003). To explore the position preference of CrLIP1, I chose phosphatidylcholine (PtdCho) as the substrate. PtdCho is not present in Chlamydomonas, but gave us an opportunity to test a number of commercially available molecular species. Specifically, I used 18:1\(^9\)\(\Delta_9\)/16:0 (sn-1/sn-2) and 16:0/18:1\(^9\)\(\Delta_9\) PtdCho (Figure 2.10). By following the time-dependent liberation of fatty acids from each substrate, the preference (or the lack of which) for position and/or saturation was observable. Lipase reactions were sampled at 0, 6, and 16 h, and PtdCho, lyso-PtdCho, and free fatty acids were extracted from the corresponding spots on TLC plates for GLC analysis. Only negligible amounts of free fatty acids and lyso-PtdCho were present at 0 h (Figures 2.10C and D). After 6 h of incubation, both free fatty acids and lyso-PtdCho emerged along with a decrease in PtdCho (Figures 2.10E and F). For both PtdCho species, fatty acids were mainly, but not exclusively released from the sn-1 position, regardless of the acyl chain. From 6 to 16 h (Figures 2.10G and H), the amount of PtdCho remained largely unchanged, whereas the level of lyso-
PtdCho decreased, suggesting CrLIP1 was also able to hydrolyze lyso-PtdCho. Based on these results, I can conclude that CrLIP1 prefers the \( sn-1 \) position, but can also hydrolyze the acyl chain at the \( sn-2 \) position. Moreover, the \( sn-1 \) oleate and palmitate were mobilized equally well by CrLIP1, suggesting that both oleate and palmitate are comparable substrates for CrLIP1.

**Involvement of CrLIP1 in Chlamydomonas TAG metabolism**

Although I have yet to directly observe TAG lipolysis by using a purified recombinant CrLIP1 protein, it is possible that CrLIP1 is indirectly involved in cellular TAG metabolism. This is because remodeling of lipids, membrane and storage, is one of the responses to N-deprivation that leads to TAG accumulation (Moellering and Benning 2010). In addition, DAG is both a precursor and a product (intermediate) for TAG and membrane lipid metabolism (Miege and Marechal, 1999). The lipase action of CrLIP1 as shown in Figures 7 to 10 may impact the flux of DAG and polar lipids, which could eventually affect TAG biosynthesis and breakdown. I took several approaches to test this hypothesis. First, I examined the relationship between changes in *CrLIP1* transcript and TAG abundance following N-deprivation and N resupply. If CrLIP1 plays a role in storage lipid metabolism in Chlamydomonas cells, it was expected that the expression of the *CrLIP1* gene would also respond to N fluctuation. Chlamydomonas cells started to accumulate TAG shortly after N removal and continued to increase their TAG content (Figure 2.11A)(Wang et al., 2009; Moellering and Benning, 2010; Siaut et al., 2011). N resupply triggered fast TAG mobilization. Consistent with a role in lipolysis, the transcript of *CrLIP1* declined steadily when TAG was accumulating (Figure 2.11B). Notably, there was a surge of *CrLIP1* transcript that coincided with the N resupply-triggered TAG mobilization. The obvious
inverse correlation between CrLIP1 transcript level and TAG abundance strongly suggests that CrLIP1 exerts a catabolic function in Chlamydomonas TAG metabolism.

To further link CrLIP1 expression to TAG metabolism, I used an artificial microRNA (AmiRNA) (Molnar et al., 2009) to reduce the expression of CrLIP1. Sixty independent transformants were screened for a reduction of CrLIP1 mRNA level, and two lines (1-44 and 1-56) exhibited 70-80% reduction (Figure 2.12A). These two lines and a vector-only control were subjected to N-deprivation and TAG quantification. I only observed a slight increase in the TAG levels for the 1-56 line 48 and 72 hours into N-deprivation (Figure 2.12B). The lack of a significant change in the TAG pool size in CrLIP1 knockdown lines seemed to be in agreement with the N-deprivation-dependent decline of CrLIP1 mRNA, that is, the CrLIP1 lipase activity was likely to be down-regulated when TAG synthesis prevailed in the overall energy flux. Artificial knockdown of CrLIP1 transcript level thus caused a minimal effect on the total TAG level. On the other hand, when ammonium was added to N-deprived cultures to trigger TAG breakdown, knocking down CrLIP1 expression resulted in a delay of TAG mobilization. The amount of TAG normalized to that of total lipids was significantly higher in the CrLIP1 knockdown lines than that in the empty vector control (Figure 2.12C). This phenomenon held true when the TAG content was measured as an absolute amount per cell at 24 h after the re-supply of N (Figure 2.12D). This observation conforms to a lipolytic role of CrLIP1in vivo, and is reminiscent of the slower TAG turnover during post-germination growth of the sdp1 lipase mutant of Arabidopsis thaliana (Eastmond, 2006). Intriguingly, the maximal TAG amount in the sdp1 mutant seeds is also indistinguishable from the wild-type seeds (Eastmond, 2006). I thus conclude that the Chlamydomonas CrLIP1 plays a critical role in ensuring fast TAG turnover when cells emerge from N-deprivation.
DISCUSSION

**CrLIP1 affects TAG content possibly by degrading DAG**

From the data presented above, I suggest that CrLIP1 affects Chlamydomonas and yeast TAG metabolism in different ways. *In vitro*, the recombinant CrLIP1 protein clearly can hydrolyze DAG and phospholipids, but not TAG under the standard test conditions. On the other hand, metabolism of TAG in yeast and in Chlamydomonas is affected by changes in the amount of CrLIP1. Given that DAG is an immediate precursor for TAG synthesis and an intermediate of TAG degradation, changes in DAG pool sizes will presumably impact TAG abundance in different directions. Accordingly, as shown in Figure 2.13, I propose that CrLIP1 participates in TAG turnover in Chlamydomonas by facilitating DAG removal during TAG mobilization (“reaction 1”, Figure 2.13), hence contributing to expeditious conversion of TAG into cell building blocks and metabolic energy following the re-introduction of N. Repression of *CrLIP1* expression by artificial microRNA may transiently increase the DAG pool, which then delays TAG turnover by product inhibition through feedback control. The overall rate of TAG hydrolysis is thus reduced. However, possibly due to functional redundancy, or as a result of metabolism of DAG through other routes such as conversion to phosphatidic acid (Arisz et al., 2009), TAG that accumulates following N-deprivation eventually will be turned over quantitatively (Figure 2.12B). In yeast, the *tgl3Δ tgl4Δ* double mutant has no detectable TAG lipase activity (“reaction 2”, Figure 2.13)(Kurat et al., 2006). This argues against the notion that CrLIP1 suppresses the TAG accumulation phenotype solely by expediting DAG removal following the first TAG hydrolysis step, as the lipolytic route of DAG production is blocked by
the deletion of TAG lipases. Rather, I suggest two hypotheses. First, yeast gradually accumulates TAG when cells enter the stationary phase (Clausen et al., 1974; Leber et al., 1994). The introduction of a heterologous DAG lipase CrLIP1 may result in a shortage of DAG supply for TAG biosynthesis (“reaction 3”, Figure 2.13), hence partially preventing the TAG accumulating phenotype seen in the lipase knockout strains. Secondly, due to the pleiotropic metabolic as well as signaling functions of DAG, the steady-state DAG amount has to be tightly controlled (Shen et al., 2011). The action of CrLIP1 in yeast may cause alterations in TAG metabolism by an indirect route involving DAG signaling. Nevertheless, I cannot exclude the possibility that CrLIP1 is indeed a TAG lipase in vivo but requires an unknown co-factor that was absent in our assay mixtures or a post-translational modification of the protein which could not be performed in E. coli cells.

In addition to phenotypes of TAG metabolism, overexpressing CrLIP1 in yeast also affected the rate of polar lipid accumulation in cells emerging from stationary phase. The observed phospholipid lipase activity (Figures 2.8-2.10) can account for the above phenotype, although the stationary-phase cells appear to be relatively insensitive to CrLIP1 overexpression (Figures 2.1D and 2.2B). Alternatively, as DAG can be converted to phosphatidic acid and other phospholipids including PtdEtn and PtdCho (Weyer et al., 2010), CrLIP1 may simply hydrolyze and remove DAG from the pool for polar lipid biosynthesis, thus creating a futile cycle hampering the rate of polar lipid accumulation.

Is there another function of CrLIP1?

In Chlamydomonas, CrLIP1 appears to be actively expressed during vegetative growth when cells maintain a negligible amount of steady-state TAG (see time 0, Figure 2.11). The
qRT-PCR results in Figure 2.11 are consistent with our earlier work, in which global RNAseq analysis was employed to examine transcriptomic changes during N-deprivation. The *CrLIP1* transcript hits were 2921 ± 687 under N-replete conditions, and were down to 1335 ± 149 following 48 hours of N-deprivation (Miller et al., 2010). Comparatively, the average transcript hits of all genes were only 570 during vegetative growth. qRT-PCR verified the abundant *CrLIP1* mRNA when compared with a constitutively expressed *RACK1* gene (Figure 2.14). These data demonstrate the active expression of *CrLIP1* during vegetative growth. Assuming that transcript abundance correlates with the protein level, these expression data suggest that CrLIP1 may be a relatively abundant protein during vegetative growth. Given that many Chlamydomonas major membrane lipids such as MGDG and DGDG are derived from DAG (Miller et al., 2010), and that CrLIP1 is capable of hydrolyzing DAG and these major Chlamydomonas polar lipids, it seems possible that CrLIP1 may be involved in DAG and membrane lipid turnover during mitotic growth when active membrane synthesis and remodeling take place. In contrast, following N-deprivation, DAG is synthesized but then channeled into TAG production by DGATs (Boyle et al., 2012). A DAG lipase activity of CrLIP1 would be at odds with the need for cells to accumulate TAG, hence the observed decline of *CrLIP1* transcript following N-deprivation. To delineate the potential additional function of CrLIP1 in vegetative cells, the localization of CrLIP1 may have provided some useful information. Unfortunately, our attempts to express a CrLIP1-GFP fusion protein have not been successful. Intriguingly, CrLIP1 was detected in a flagellum proteomic survey, and was hence designated FAP12 (Flagella Associated Protein 12) (Pazour et al., 2005). Thus far, I have not observed a discernible flagellar phenotype associated with either CrLIP1 knock-down lines characterized above (data not shown). One possibility is that the *CrLIP1* repression in the AmiRNA lines was not sufficient to bring about an apparent
flagellar phenotype. On the other hand, given the similarity between CrLIP1 and the human DAG lipases DAGL\(\alpha\) and DAGL\(\beta\) that are localized in the plasma membrane (Bisogno et al., 2003), it is possible that part of the flagella-associated CrLIP1 population may have derived from the plasma membrane as the flagellar membrane is confluent with the plasma membrane (Rosenbaum and Witman, 2002). Thus, the presence of CrLIP1 in flagella does not contradict our hypothesis of CrLIP1 involvement in TAG metabolism.

CrLIP1 did not exhibit a TAG lipase activity under our test conditions, and repressing CrLIP1 expression delayed but did not prevent TAG turnover. Therefore, other TAG lipases in *Chlamydomonas reinhardtii* remain to be identified.
Figure 2.1. Expression of CrLIP1 rescues a yeast mutant deficient in TAG lipases. (A) Western blotting for HA-tagged CrLIP1 protein expressed in yeast. Whole cell extracts from tgl3Δtgl4Δ yeast cells bearing either the empty pMK595 vector (vect) or pMK595CrLIP1 were centrifuged to obtain a membrane-enriched pellet (P). 10 µg of proteins from both the soluble (S) and pellet fractions were loaded for SDS-PAGE before western blotting (panel A) or Coomassie brilliant blue staining (panel B). (C) Thin-layer chromatogram of lipids extracted from
membrane-enriched fractions containing 10 µg of proteins. Lipids were visualized by exposing the plate to iodine vapor. (D) Cellular contents of fatty acid methylesters derived from major glycerolipids. Lipids were extracted from 2-day old stationary-phase yeast cells. Lipid abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol. (E) Fatty acid compositions of TAG. Fatty acids are designated as chain length:number of double bonds. For D and E, averages of three replicates are shown with errors bars indicating SD (n=3). (F) Growth curves of yeast strains in uracil dropout medium supplemented with 0.5% casamino acids (CAA-ura). Stationary phase cells were diluted to an optical density (at 600 nm) of 0.1 to start the time course. Representative results from more than three independent experiments are shown here. Data are presented as averages from three measurements of the same culture. Error bars indicate standard deviation. G, Distribution of yeast cell diameters in µm (long axis). Cells were grown in medium without uracil for 48 hr. For each strain, two populations from two single colonies were analyzed. More than 200 cells were measured for each population. Percentages of cells within each size range are shown.
Figure 2.2. Metabolic labeling and chase studies of TAG and phospholipid synthesis in yeast strains over-expressing CrLIP1. \(^{14}\text{C}\) acetate was added to stationary phase yeast cultures. Samples were taken at time points indicated, with TAG (panel A), polar lipids (panel B), and DAG (panel C) extracted and quantified. The radioactivity was normalized to the cell number.
Figure 2.2. (cont’d)

Data are averages ± SD (n=3). (D and E) Chase experiments. Stationary phase yeast cells were labeled, briefly washed and then diluted to fresh, non-labeled medium. Cells were collected at four time points, with the radioactivity TAG (panel D) and polar lipids (panel E) quantified, and normalize to the starting counts. (F) Quantification of cell numbers over the course of chase experiments. The optical density at 600 nm was measured. For panels D, E and F, data are averages of two technical replicates with the error bars indicating standard deviations.
Figure 2.3. Recombinant CrLIP1 fails to hydrolyze triacylglycerol in vitro. (A) SDS-PAGE of bacterial whole cell lysates (WCL, 6 µg) and purified CrLIP1 protein. Arrows point to two potential isoforms of recombinant CrLIP1. The unlabeled lane shows molecular mass markers. (B) Thin-layer chromatogram of lipids extracted from triacylglycerol lipase assay mixtures with radioactive triolein and olive oil as the substrate. An autoradiogram of TLC is shown. For the buffer-only control lane, same volume of CrLIP1 storage buffer was used instead of the purified CrLIP1 protein. Rhizopus lipase (RL) digested triolein was loaded as the marker for free fatty acids.
Figure 2.4. Western blotting for hexahistidine-tagged CrLIP1 protein purified from *E. coli* cells. Two µg purified protein was loaded for immunodetection. Note the doublets of CrLIP1 isoforms.
**Figure 2.5. Recombinant CrLIP1 fails to degrade steryl esters in vitro.** Thin-layer chromatographic resolution of lipids extracted from lipase assay mixtures with radioactive steryl esters derived from yeast cells as the substrate. TLC was developed in petroleum ether-diethyl ether-H2O (80:20:1 in volume). An autoradiograph is shown. The position of free fatty acids was marked by running oleic acids in the TLC and aligning the film to the TLC plate. Appearance of radioactive free fatty acids is considered as evidence for esterase activity on steryl esters.
Figure 2.6. **CrLIP1 shares sequence similarity with two human DAG lipases DAGLα and DAGLβ.** Shown are Clustal W2 results using the lipase domains of each of the three proteins. The “GXSXG” hydrolase motif is boxed. Part of the sequence for each protein was used as indicated by the residue numbers on the right side. Asterisk (*), colon (:) and period (.) denote conserved residues, conservative substitutions and semiconservative substitutions respectively.
Figure 2.7. Recombinant CrLIP1 degrades diacylglycerol in vitro. (A) Thin-layer
chromatogram of lipids extracted from diacylglycerol lipase assay mixtures with diolein as the substrate for CrLIP1. Lane 1, control reaction with diolein and CrLIP1 storage buffer; lane 2, reaction with diolein and purified CrLIP1; lane 3, control reaction with purified CrLIP1 without diolein to show fatty acids contained in the protein prep. Diolein and oleic acid (18:1Δ⁹) were loaded as markers. The deep staining at the origins might be from ingredients of the reaction buffer such as dithiothreitol or glycerol. (B-D) Gas-liquid chromatograms of fatty acid methyl esters (FAMEs) derived from free fatty acids scraped from the TLC plate shown in panel A. Arrows point to the location or expected location of oleic FAME.
Figure 2.8 Recombinant CrLIP1 hydrolyzes phosphatidylethanolamine, the major membrane lipid of *E. coli*. (A) Thin-layer chromatograms of total lipids extracted from *E. coli* cells. Note the CrLIP1-dependent production of a significant increase of free fatty acids in the *E. coli* lysate. The arrow points to an unidentified lipid. (B) TLC of in vitro PtdEtn hydrolysis.
Figure 2.8 (cont’d)

reactions. CrLIP1 causes the reduction of the PtdEtn substrate, and produces free fatty acids and lyso-PtdEtn. Markers include PtdEtn and oleic acid from a commercial source, and lyso-PtdEtn generated by the Rhizopus lipase (RL). (C) Quantitative analysis of CrLIP1-dependent PtdEtn hydrolysis in *E. coli*. Data are presented as the ratio of fatty acids in each lipid to fatty acids in the total lipid extract. Averages and standard deviations are shown (n=3).
Figure 2.9. *Recombinant CrLIP1 degrades major polar lipids of Chlamydomonas in vitro.* A, a thin-layer chromatogram of CrLIP1-catalyzed polar lipid hydrolysis. Each polar lipid was isolated from Chlamydomonas cells metabolically labeled with $^{14}$C acetate. Positions of the polar lipid substrates are labeled on the left with arrows near the corresponding substrates. Lipid abbreviations: MGDG, monogalactosyldiacylglycerol; DGTS, diacylglycerol-N,N,N-trimethylhomoserine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; DGDG, digalactosyldiacylglycerol. Chloroform-methanol-acetic acid-H$_2$O (75:13:9:3 in volume) was used to develop the TLC. B, for a better resolution between MGDG and free fatty acids, MGDG assay mixtures were loaded to another TLC developed in petroleum ether-diethyl ether-H$_2$O (80:20:1 in volume). MGDG and DGDG digested with Rhizopus lipase (RL) were loaded to provide markers for free fatty acids. X-ray films exposed to TLC plates are shown.
Figure 2.10. Recombinant CrLIP1 cleaves both acyl groups of phosphatidylcholine with a preference for sn-1 position. (A and B) Schematic representations of the two phosphatidylcholine (PtdCho) substrate. Circled letter “P” denotes phosphate. (C-H) Quantitative analyses of PtdCho lipolysis products. Each lipid species was scraped off the TLC plate, transesterified to FAMEs, and quantified by GLC. Panels C, E, and G represent products derived from 18:1Δ⁹/16:0 PtdCho, whereas panels D, F, and H are from the 16:0/18:1Δ⁹ PtdCho reactions. Averages and standard deviations are shown (n=3).
Figure 2.11. Inverse correlation between \textit{CrLIP1} transcription and TAG abundance in \textit{Chlamydomonas}. (A) TAG abundance through N deprivation and resupply. Data are presented as the molar ratio of fatty acids contained in TAG to total fatty acids in the lipid extract. Log-phase cells were N-deprived for 72 hr. After N re-supply, cells were grown for another 36 hr. Portions of cultures at each time point were harvested for lipid and mRNA extraction. (B) \textit{CrLIP1} mRNA abundance from the same cultures examined in panel A. The mRNA was quantified by qRT-qPCR. \textit{CrLIP1} transcripts were normalized to the constitutively expressed RACK1, and the \textit{CrLIP1/RACK1} ratio at the time of N-deprivation initiation was set as 1. In both A and B, data are shown as averages ± SD (n=3).
Figure 2.12. Suppression of *CrLIP1* expression delays TAG turnover in Chlamydomonas.

(A) Expression of CrLIP1 in two AmiRNA lines compared to an empty-vector control. mRNA quantification was expressed as CrLIP1/RACK1 ratio, and then normalized to the empty-vector control. (B) TAG accumulation at 48 and 72 hr of nitrogen deprivation. (C) TAG degradation
Figure 2.12. (cont’d)

after nitrogen re-supply described in Figure 7. (D) TAG amount normalized by cell number 24 h after N re-supply to N deprived cells as described for panel C. Data are shown as averages ± SD (A, n=3; B, n=4; D, n=3).*per t-test statistically different at p< 0.05; **per t-test statistically different at p< 0.01.
Figure 2.13. A model for how CrLIP1 might affect TAG metabolism. The DAG lipase activity of CrLIP1 may affect TAG biogenesis and hydrolysis in yeast and Chlamydomonas, respectively. Enzymes are italicized. Note that the model stresses components directly related to CrLIP1 over-expression or knock-down. Not all enzymes or metabolites are shown. Abbreviations for enzymes and lipids: ACP, acyl carrier protein; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; DGK, diacylglycerol kinase; DGTS, diacylglycerol-N,N,N-trimethylhomoserine; FA, fatty acid; G-3-P, glycerol-3-phosphate; MGDG, monogalactosyldiacylglycerol; PLC, phospholipase C; PLD, phospholipase D; PtdOH, phosphatidic acid; TAG, triacylglycerol; TAGL, triacylglycerol lipase.
Figure 2.14. RT-PCR quantitative comparison of RACK1, CrLIP1 and DGTT1 transcripts in vegetatively growing cells. No template control was performed for each gene. Calculated sizes for PCR products are: 139 bp for RACK1, 138 bp for CrLIP1 and 98 bp for DGTT1.
Table 2.1. Yeast strains used in this study.

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Table 2.3. Oligonucleotide primers used in this study. All primer sequences are written in 5’ to 3’ direction. N represents a random nucleotide.

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Chapter 3

A galactoglycerolipid lipase is required for triacylglycerol accumulation following nitrogen deprivation in *Chlamydomonas reinhardtii*¹

¹This project was done in collaboration with several colleagues and is part of a paper in press at *The Plant Cell* in Xiaobo Li, Eric R. Moellering, Bensheng Liu, Cassandra Johnny, Marie Fedewa, Barbara B. Sears, Min-Hao Kuo, Christoph Benning. (2012) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in *Chlamydomonas reinhardtii*. Eric R. Moellering initiated the mutant screen. Marie Fedewa participated in the screen. Bensheng Liu contributed Figure 3.3B. Barbara B. Sears performed crossing between the mutant and another strain, providing progeny that were analyzed by me for lipid content (Figure 3.1B).
ABSTRACT

Following nitrogen (N) deprivation microalgae accumulate triacylglycerols. To gain mechanistic insights into this phenomenon, I identified mutants with reduced TAG content following N deprivation in the model alga *Chlamydomonas reinhardtii*. In one of the mutants the disruption of a galactoglycerolipid lipase-encoding gene, tentatively designated Plastid Galactoglycerolipid Degradation 1 (PGD1), was responsible for the primary phenotype: reduced TAG content, altered TAG composition, and reduced galactoglycerolipid turnover. The recombinant PGD1 protein, which was purified from *E. coli* extracts, hydrolyzed monogalactosyldiacylglycerol into its lyso-lipid derivative. *In vivo* pulse chase labeling identified galactoglycerolipid pools as a major source of fatty acids esterified in triacylglycerols following N deprivation. Moreover the fatty acid flux from plastid lipids to triacylglycerol was decreased in the pgd1 mutant. Apparently, *de novo* synthesized fatty acids in Chlamydomonas are, at least partially, first incorporated into plastid lipids before they enter triacylglycerol synthesis.
INTRODUCTION

Triacylglycerol (TAG) is a universal storage lipid in plants, algae, fungi, and animals. TAG is composed of a glycerol backbone to which three fatty acyl chains are esterified. By transesterification with methanol TAG can be converted into fatty acid methyl esters (FAMEs) commonly referred to as biodiesel (Durrett et al. 2008). Microalgae have been considered as sustainable feedstock for the production of biofuels because they accumulate substantial amounts of TAG following nutrient deprivation. Theoretical calculations suggest that microalgae can surpass crop plants in their TAG yield per land area used (Weyer et al. 2010). Despite the recent interest in microalgae, this phylogenetically diverse group of photosynthetic organisms is not well understood at the molecular and biochemical levels, and the mechanistic basis of algal lipid metabolism and of TAG accumulation still needs to be explored in detail. Much of the current molecular understanding of photosynthetic lipid biosynthesis is based on work with Arabidopsis thaliana and other land plant models, providing paradigms that may not be directly transferable given their evolutionary divergence from microalgae. Indeed, our current information on lipid metabolism in the green algal model Chlamydomonas reinhardtii, which is mostly based on genome annotation (Riekhof et al. 2005) or early labeling and lipid profiling experiments (Giroud et al. 1988, Giroud and Eichenberger 1989), suggests that lipid metabolism in this organism is distinct in crucial aspects from that of land plants. Most strikingly, Chlamydomonas lacks phosphatidylcholine (PtdCho), but instead contains the betaine lipid diacylglycerol-N,N,N-trimethylhomoserine (DGTS).

Seed plants typically have two assembly pathways for glycerolipids (Roughan and Slack 1982). Fatty acids are synthesized de novo in the plastid while attached to acyl carrier proteins (ACPs) (Ohlrogge et al. 1979). Acyltransferases at the inner chloroplast envelope membrane
transfer acyl groups from acyl-ACPs to glycerol 3-phosphate leading to the formation of
phosphatidic acid (PtdOH), the precursor of glycerolipids of the thylakoid membrane.
Alternatively, fatty acids are exported from the plastid for assembly of extraplastidic
glycerolipids including TAGs at the endoplasmic reticulum (ER). Because the acyltransferases
associated with the inner plastid envelope membrane and the ER have different acyl group
preferences, glycerolipids assembled by the two pathways can be distinguished based on their
acyl group composition (Heinz and Roughan 1983). In Chlamydomonas, the analysis of the acyl
groups in the glyceryl backbone of the galactoglycerolipids, which are the predominant lipids in
the thylakoid membranes, suggests that their assembly is completely dependent on the plastid
pathway (Giroud et al. 1988). In contrast, in seed plants such as Arabidopsis the
galactoglycerolipid molecular species are nearly equally derived from the ER and the plastid
assembly pathway (Browse et al. 1986), thus requiring an elaborate system of lipid transfer
between the ER and the plastid envelopes (Benning 2009).

In particular, the lack of phosphatidylcholine (PtdCho) in Chlamydomonas is expected to
affect other aspects of glycerolipid metabolism. For example, isotope labeling of cytosolic lipids
in pea leaves indicated that most of the acyl groups synthesized de novo in the plastid are first
incorporated into PtdCho instead of PtdOH (Bates et al. 2007). Thus, it was proposed that acyl
editing of PtdCho is an important aspect of fatty acid export from the plastid, cycling acyl groups
through PtdCho before they enter the cytosolic acyl-CoA pool, which ultimately provides acyl
groups for glycerolipid assembly at the ER. The lack of PtdCho in Chlamydomonas raises
several questions, particularly whether an alternative mechanism of acyl editing, possibly
involving DGTS or another lipid, or a mechanism completely independent of acyl editing exists,
which is involved in the export of fatty acids from the plastid. These apparent differences in the
cellular organization of basic membrane glycerolipid metabolism in Chlamydomonas may also extend to differences in the formation of TAGs and their storage in lipid droplets (Liu and Benning 2012). Typically, lipid droplets are formed at the ER in all eukaryotic cells. However, recent reports on TAG a starch-less mutant of Chlamydomonas with increased accumulation in Chlamydomonas of oil suggest that TAG-containing lipid droplets are present in plastids (Fan et al. 2011, Goodson et al. 2011), raising the possibility that TAG is either directly assembled in plastids, or imported into them.

Aside from the basic mechanisms of glycerolipid assembly in Chlamydomonas, the details of the regulation of TAG synthesis are unclear as well. Like other microalgae, Chlamydomonas produces lipid droplets filled with TAGs following nutrient deprivation (Moellering and Benning 2010, Wang et al. 2009), conditions that involve genome-wide transcriptional changes (Castruita et al. 2011, Miller et al. 2010). Intriguingly, among the genes up-regulated or down-regulated by N deprivation was a large number of genes annotated to encode lipases (Miller et al. 2010). One of the down-regulated genes was recently shown to encode a lipase involved in TAG turnover in Chlamydomonas (Chapter 2). As a complement to transcript profiling in revealing genes involved in TAG metabolism or its regulation, Eric Moellering and I developed a genetic screen for mutants with abnormal TAG levels following N deprivation. Here I describe the identification of a low-TAG mutant with a lesion in a galactoglycerolipid lipase-encoding gene. This gene was among the up-regulated lipase-encoding genes following N deprivation (Miller et al. 2010), consistent with a role for acyl editing or turnover of galactoglycerolipids during TAG formation in Chlamydomonas.
MATERIALS AND METHODS

Strains and growth conditions

The cell wall-less dw15-1 (cw15, nit1, mt⁺) strain of *Chlamydomonas reinhardtii* was obtained from A. Grossman and is referred to as the wild-type (with regard to *PGD1*) parental strain throughout. This strain was crossed to CC-198 (er-u-37, str-u-2-60, mt-; Chlamydomonas Resource Center; http://www.chlamycollection.org) for genetic analysis. Cells were grown in Tris-acetate-phosphate (TAP) medium (20 mM Tris, 0.1 g/L MgSO_4 7H_2O (0.4 mM), 0.05 g/L CaCl_2 2H_2O (0.34 mM), 10 mL/L glacial acetic acid, 10 mM NH_4Cl, 1 mM phosphate and trace elements (Harris 1989)) under continuous light (70-80 μmol m⁻² s⁻¹) at 22°C or ambient room temperature (~22°C) for solid media, which contained 1.5 % agar. Ammonium chloride was omitted from N-depleted (TAP-N) medium. To induce TAG biosynthesis, cells were collected by centrifugation (3000 x g, 4°C, 3 min), washed twice with TAP-N and finally resuspended in TAP-N of the same volume. For spotting on TAP-N agar, approximately 10⁶ cells from a log-phase culture were concentrated in 5 μL.

Primary mutant screen

Plasmid disruption was used to generate mutants of the wild-type parental strain dw15-1. Transformation using glass beads was performed as previously described (Kindle 1990) using the pHyg3 plasmid conferring resistance to Hygromycin B (Berthold et al. 2002). The plasmid was linearized with *NdeI* (all the restriction endonucleases were purchased from New England Biolabs, http://www.neb.com). After 8 hrs of recovery, cells were spread onto agar-solidified
TAP medium containing 10 μg/mL Hygromycin B. Colonies were picked into 96-well plates with 1.1 mL TAP medium and grown for three days. For N deprivation and induction of TAG biosynthesis, small culture droplets (~3 μL) were transferred with a 48-pin replicator to inoculate a new 96-well plate containing TAP medium containing 0.5 mM ammonium chloride and grown for 6-7 days under continuous light (70-80 μmol m\(^{-2}\) s\(^{-1}\)) at ambient room temperature (~22°C).

For normalization within a 96-well plate, chlorophyll fluorescence was used. For this purpose 100 μL of N-deprived cells were transferred to a black 96-well plate (Black Flat Bottom Polystyrene NBS™ Microplate 3991, Corning, http://www.corning.com) and read at 455 nm excitation with an emission filter cut off >685 nm using a FLUOstar Optima 96-well plate reader (BMG Labtech, http://www.bmglabtech.com). In the same plate to visualize neutral lipids, 100 μL Nile-Red (Sigma-Aldrich; http://www.sigmaaldrich.com) stock solution (5 μg/mL in 10% methanol containing 0.04 % Triton X-100) was added. The wavelength settings for Nile-Red fluorescence were 455 nm for excitation and 550-560 nm for emission. A background reading for this filter set was obtained prior to the addition of Nile-Red (cells only) and subtracted. The neutral lipid specific signal was calculated as [(Nile-Red fluorescence – background fluorescence)/chlorophyll fluorescence]. To identify outliers in individual 96 plate sets, the Median Absolute Deviation (MAD) was determined as [1.482 X median X |individual value-median|] according to (Rousseeuw and Croux 1993) and the z-score was calculated as [(individual value-median)/MAD] as previously described for another mutant screen (Lu et al. 2008). The threshold for the z-score was set at +/- 3.

**Genetic analysis**
In preparation for crossing, the *pgdl* mutant in the dw15-1 background and CC-198 were separately grown for five days on TAP with 4 g/L yeast extract, transferred with a sterile loop to TAP agar with 10 % the normal concentration of N for two days, and then suspended at high density in test tubes with sterile water and placed on a shaker overnight. On the following day, the two cell types were combined using 0.5 mL aliquots, removed after 2-3 h of mating and plated onto TAP medium completely lacking N and solidified with 4% agar. After one day in the light, the zygote plates were moved to the dark. Five days after mating, zygospores were sampled as described by Harris (Harris 1989). On the day following transfer of the zygospores to normal TAP solidified with 2% agar, meiotic progeny were identified under a dissecting microscope and separated with a glass needle. After 7 days, the colonies were sufficiently large to transfer to non-selective media for subsequent replica-plating.

**DNA and RNA techniques**

Genomic DNA of Chlamydomonas was prepared according to (Newman et al. 1990). For Southern blotting, genomic DNA was digested with *Bam*HI and resolved by agarose gel electrophoresis (10 μg DNA per lane). DNA was transferred to a nylon membrane (Amersham Hybond N+, GE Healthcare, http://www.gelifesciences.com) and fixed under ultraviolet light. Digoxigenin (DIG) labeling of the probe, DNA transfer, and signal detection were performed using a kit from Roche (http://www.roche.com) following the manufacturer’s instructions. The probe was generated through PCR amplification of a 234 bp region within the hygromycin B resistance cassette with primers SF and SR (all primer sequences can be found in Supplemental Table 1).
For genotyping and “SiteFinding” PCR (Tan, G. H. et al. 2005), Taq polymerase (Invitrogen, http://www.invitrogen.com) was used. For genotyping, the PCR conditions were according to the protocol provided by Invitrogen with primers F1, S2-1, and R. SiteFinding PCR was conducted according to (Tan, G. H. et al. 2005) with minor modifications and with primers optimized for the pHyg3 plasmid. The primers used for finding the insertion in \textit{PGD1} were: SiteFinder6 in combination with S1-1 and S1-2, SiteFinder8 in combination with S2-1 and S2-2. In addition, nested primers SFP1 and SFP2 were used for both combinations.

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com) according to the manufacturer’s instructions. To obtain cDNA as the template for RT-PCR, RNA was subjected to reverse transcription with Superscript II reverse transcriptase (Invitrogen). For real-time PCR, the commonly used reference gene \textit{RACK1} was employed for normalization using previously reported primers (Chang et al. 2005). Primers used for \textit{PGD1} were qF and qR. Data were analyzed with the 2(-ΔΔC(T)) method (Livak and Schmittgen 2001).

For expression of \textit{PGD1} in \textit{E. coli}, cDNA was originally amplified with primers CF1 and CR1 using the Failsafe PCR Kit from Epicentre (http://www.epibio.com). The PCR product was then integrated into the NotI-linearized yeast vector pMK595 (Luo et al. 2010) by homologous recombination in \textit{Saccharomyces cerevisiae}(Ma et al. 1987). The fusion plasmid was recovered by transforming \textit{E. coli} with yeast DNA extract and designated pXL1238. This plasmid was then used as a template for PCR using primers CF2 and CR2 and Phusion polymerase (New England Biolabs) to generate a fragment with \textit{BamHI} and \textit{SalI} sites. The PCR product was ligated into pCR-Blunt (Invitrogen) and cut out with \textit{BamHI} and \textit{SalI}. This fragment was ligated into pLW01-DsRed (Lu and Benning 2009) to generate plasmid pXL1256. pXL1256 was sequenced and mutations were found. The mutated region was removed by restriction digestion and the
remaining backbone was ligated with digested RT-PCR product of that region to obtain plasmid pXL1262. Another PCR was performed to amplify PGD1 cDNA from pXL1262 using primers CF3 and CR3. PCR product and an expression vector pMK1006 (provided by M.-H. Kuo) were combined using a ligation independent cloning procedure (Aslanidis and de Jong 1990) and sequenced for confirmation. In this plasmid, PGD1 expression was under the control of a T7 promoter and the resulting fusion protein was N-terminally tagged with poly-histidine.

**Mutant complementation**

A co-transformation protocol was used to introduce wild-type sequences into the pgd1 mutant in the dw15-1 (nit-) background. Plasmid pMN24 (Fernandez et al. 1989) containing the *Chlamydomonas* nitrate reductase gene NIT1 as selection marker was digested with BamHI and used for glass bead transformation of the pgd1 mutant. A bacterial artificial chromosome (BAC) 5E6 containing wild-type genomic DNA was obtained from the Clemson University Genomics Institute (http://www.genome.clemson.edu), and was digested with KpnI and Asel to excise a 9.5 kb fragment containing the PGD1 genomic DNA. In each transformation, 0.25 μg linearized pMN24 and 0.3 μg gel purified BAC fragment were used. TAP plates containing 0.5 mM nitrate instead of 10 mM ammonium were used for selection. The nitrate served initially as the nitrogen source and the low concentration led eventually to conditions of N deprivation and chlorosis of the pgd1 mutant but not the complemented lines nor the wild-type parental control. After transformation of pgd1 with pMN24, colonies from non-complemented lines formed and bleached within approximately three weeks when grown under continuous light (70-80 μmol m\(^{-2}\) s\(^{-1}\)) at ambient room temperature (~22°C). Complemented lines forming green colonies were re-
streaked and maintained on agar solidified TAP medium with 10 mM nitrate as the sole N source to avoid growth of potentially contaminating non-transformed cells.

**Lipid analysis and pulse-chase labeling**

Lipid extraction, thin-layer chromatography (TLC) of neutral lipids, transesterification and gas-liquid chromatography were done according to Moellering and Benning (2010). Briefly, lipids were extracted from cell pellets with methanol, chloroform, 88% formic acid (2:1:0.1 by volume). To the extract 0.5 volume of 1 M KCl, 0.2 M H₃PO₄ was added, mixed and phases were separated by low speed centrifugation. For TAG quantification, lipids were resolved by TLC on Silica G60 plates (EMD chemicals, #5721-7, http://www.emdchemicals.com) developed in petroleum ether-diethyl ether-acetic acid (80:20:1 by volume). Polar lipids were separated on the same plate using chloroform-methanol-acetic acid-H₂O (75:13:9:3 by volume) as solvent. To analyze lyso-glycolipids during for the PGD1 assay, acetone-toluene-H₂O (91:30:7.5 by volume) was used, instead. Brief exposure to iodine vapor was employed for visualization of lipids. Transesterification of each lipid and separation of fatty acid methyl esters by GLC were as previously described (Rossak et al. 1997). Transesterification was conducted on pellets with a known number of cells to determine the cellular total fatty acid content. Staining with α-naphtol (Benning et al. 1995) was used for the PGD1 assay to detect galactoglycerolipids.

For pulse-chase labeling experiments, cells were grown to log phase in TAP medium and either used directly (Figure 3.8), or transferred to TAP-N medium and grown for 12 h to induce N deprivation. Cells were harvested and resuspended at a concentration of 3-8 X 10⁸ per mL either in modified TAP (Figure 3.8) or TAP-N medium (Figure 3.9 and Figure 3.10) containing 6
mM acetate (normal TAP contains 17.5 mM). To these cultures $[^{14}C\text{-U}]$-acetate (specific activity 45-60 mCi/mmol; Perkin Elmer, http://www.perkinelmer.com) was added to provide 1 μCi/mL. In a typical experiment after 1-4 h of incubation in the light 20-40% of the labeled acetate was incorporated as determined by liquid scintillation counting. At the end of the pulse labeling phase, cells were centrifuged and washed to remove the labeled acetate, and cells were resuspended in TAP-N containing the normal amount of acetate to initiate the chase phase. Lipid extracts were prepared as described above, split in half, and analyzed for polar lipids DGTS, PtdEtn, MGDG, DGDG, PtdGro, and a mixture of SQDG and PtdIns, which could not be individually analyzed due to their low total amount in this experiment. Material at the origin of the TLC was also analyzed and included. The other half of the sample was analyzed for non-polar lipids DAG and TAG. Lipids were isolated from the TLC plates and incorporation of label into each lipid was quantified by scintillation counting. These lipid fractions were summed up and percentages for each lipid fraction were calculated.

**Recombinant protein production and PGD1 assay**

BL21 (codon+) *E. coli* strains harboring the empty pMK1006 vector or the pMK1006-\textit{PGD1} construct were grown to log phase at 37°C. Isopropyl-β-D-thiogalactopyranoside was added to the final concentration of 0.5 mM to induce protein production. Cells were harvested after 3 h of induction. To extract proteins, cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.9, 10% glycerol, 150 mM NaCl, 1 mM dithiothreitol). The mixture was then frozen in liquid nitrogen and thawed at room temperature for three cycles and sonicated to lyse cells. Lysates were centrifuged at 21,000xg for 15 min to obtain inclusion bodies. Inclusion bodies were washed with 5 mL/g wash buffer (4 M urea, 0.5 M NaCl, 1 mM EDTA, 1 mg/ml sodium
deoxycholate, 50 mM Tris-Cl pH 8.0) twice and denatured with solubilization buffer (8 M urea, 50 mM Tris-Cl pH 8.0, 10 mM dithiothreitol) by incubation at 50°C for 20 min. The supernatant was collected after centrifugation at 21,000 X g for 30 min and subjected to Ni-NTA affinity purification as described before (Lu and Benning 2009). His-tagged PGD1 protein was eluted with solubilization buffer containing 200 mM imidazole. Aliquots of purified proteins were diluted in 15 different buffers of the QuickFold Kit (AthenaES http://www.athenaes.com/), assayed for lipase activity, and 40 X dilution into protein refolding buffer (50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine-HCl, 0.05% polyethylene glycol 3350, 1 mM dithiothreitol) was found to be optimal for PGD1. After 1 h incubation at 4°C, proteins were aliquoted and kept frozen at -80°C. Protein concentration was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (http://www.bio-rad.com) according to the manufacturer’s instructions.

To prepare lipid substrates from Chlamydomonas or *E. coli* cells, lipids were extracted from 48 h N-deprived Chlamydomonas cells or IPTG induced *E. coli* cells expressing cucumber MGDG synthase (Shimojima et al. 1997) and resolved by polar TLC. Corresponding bands were isolated and lipids were recovered from silica gel by extraction with chloroform-methanol (1:1 by volume). For each PGD1 reaction, 75 nmol lipid substrates extracted from Chlamydomonas, or *E. coli* cells expressing the cucumber MGDG synthase were used. The organic solvent was removed under an N₂ stream and the lipids were resuspended in 350 μL 0.1 M phosphate saline buffer (PBS; pH 7.4) with 4.28 mM Triton X-100 and dispersed by sonication (Sonicator 3000 with microprobe, Misonix, http://www.misonix.com) for 6 X 10 s (power setting 1.5) on ice. Then 100 μL additional PBS was added. Per assay 10 μg refolded PGD1 protein (quantified as stated above) in 50 μL protein refolding buffer were added. As a negative control, 50 μL protein
refolding buffer only were added. The PGD1 refolding buffer inhibited Rhizopus lipase (Sigma-Aldrich). Therefore, 10 μg lipase dissolved in 50 μL PBS instead of protein refolding buffer was used unless otherwise noticed. Dithiothreitol was added to a final concentration of 1 mM from a freshly prepared stock solution. The mixture was sonicated again for 5 s and incubated at ambient temperature (~22°C). After 6 h incubation (3 h for Rhizopus lipase to prevent potential loss of lyso-lipid standards), reactions were stopped by the addition of 1 mL solvent used for lipid extraction, and lipid extracts were analyzed by TLC described above. For gas chromatograms on free fatty acids and lyso-MGDG generated by PGD1, 9 h was used to obtain more prominent signals. To measure the velocity of MGDG hydrolysis, reactions were quenched after 3 h of incubation.

**Positional analysis of TAG**

Positional analysis of TAG was performed with Rhizopus lipase using a similar procedure as described above. Briefly, lipids were extracted from 48 h N-deprived Chlamydomonas cells and resolved by neutral TLC. TAG was extracted from silica gel with chloroform-methanol (1:1 by volume) as above. Approximately 10 μg was dried under an N₂ stream and resuspended in PBS containing Triton X-100 as above. Rhizopus lipase was dissolved in PBS and 20 μg was added to the emulsified TAG preparation. Lipids were extracted from the reaction mixtures and resolved by neutral lipid TLC (described above). Free fatty acids and monoacylglycerol spots were scraped for transesterification as above. Background levels of fatty acids carried over with Rhizopus lipase were estimated in a control reaction without substrate lipid supplied, and subtracted from the free fatty acids data obtained with substrate.
RESULTS

Isolation of TAG mutants

To generate *Chlamydomonas* mutants with altered TAG content, random, insertional gene-disruption was conducted by introducing a linearized pHyg3 plasmid (Berthold et al. 2002) into the cell wall-less Chlamydomonas strain dw15-1.1, which is referred to as the parental wild-type strain (because it is wild-type (with regard to its lipid content and synthesis). Hygromycin B-resistant transgenic lines were picked into a 96-well plate and induced for TAG accumulation by transfer to low-N medium. During the primary screen, Nile-Red fluorescence-staining of neutral lipids (Chen et al. 2011, Kimura et al. 2004) was used to monitor neutral lipids in a high-throughput mode using a 96-well plate reader. Putative mutants differing in Nile-Red fluorescence from the wild-type parental strain based on statistical criteria as defined under Material and Methods were reanalyzed by extracting lipids, separating them by thin-layer chromatography (TLC), followed by quantification of TAG-derived fatty acid methylesters by gas liquid chromatography (GLC). Of 34,000 independent transgenic lines generated, 80 were initially found to exhibit an altered Nile-Red fluorescence intensity, of which six mutants with robust and reproducible changes in TAG levels were eventually isolated. The focus here is on the characterization of one of the low-TAG mutants, initially designated line E12. After in-depth analysis it was renamed *plastid galactoglycerolipid degradation 1* (*pgd1*), the designation used from here on.

The *pgd1* mutant has reduced TAG and becomes chlorotic following N deprivation
Over the course of three days following N deprivation the \textit{pgd1} mutant showed an approximately 50\% reduction in the ratio of fatty acids in TAG over total fatty acids in the lipid extract, a parameter that allows a robust comparison of relative TAG content between different lines, in this case \textit{pgd1} and the wild-type parental strain dw15-1 (Figure 3.1A). Because non-homologous integration of linearized plasmids into the Chlamydomonas genome can potentially occur multiple times in a single line, genetic linkage of Hygromycin B resistance and the lipid phenotype were examined to confirm insertional tagging of the gene responsible for the lipid phenotype, a prerequisite for subsequent gene identification. Towards this end, the \textit{pgd1} mutant was crossed with CC-198, a cell-walled strain (mating type\textsuperscript{−}) and close relative of dw15-1, which is mating type\textsuperscript{+} and the wild-type parental strain of \textit{pgd1}. Strains CC-198 and dw15-1 were compared for their lipid composition, but did not show major differences in TAG content. After 48 h of N deprivation, the ratio of fatty acids in TAG over total fatty acids in extracts was 0.46 ± 0.04 for dw15-1 and 0.51 ± 0.04 for CC-198. A total of 83 meiotic progeny lines were analyzed, of which 40 were resistant and 43 sensitive to Hygromycin B. The observed ratio approached the hypothetical 2:2 segregation ratio suggesting a single plasmid insertion in the genome, although the statistical limitations of the experiment would allow for multiple, but very tightly linked plasmid insertions. Lipid analysis was performed on 14 progeny lines (Figure 3.1B) and the results were compared to the wild-type parental strain and \textit{pgd1}. The TAG fatty acid to total fatty acid ratio of the eight Hygromycin B sensitive lines was similar to that of the parental strain, while the six resistant lines showed a ratio similar to that of the original \textit{pgd1} mutant. Thus, the Hygromycin B resistance marker appeared to be closely linked to the mutation causing the lipid phenotype.
In addition to TAG deficiency, *pgd1* cells gradually developed chlorosis and fully bleached over the course of 12 days following N-depletion (Figure 3.1C), which was accompanied by reduced cell viability (see below). However, in N-replete medium there was no discernible difference in growth between the wild-type parental strain and *pgd1* (Figure 3.2). Thus, the ability to produce or maintain TAG seems to be required for the long-term viability of the cells following N deprivation, which provides a clue towards a physiological role of TAG accumulation under nutrient stress that will be further explored below.

The *pgd1* lipid phenotype is caused by disruption of a putative lipase gene

To identify the plasmid insertion site, “SiteFinding” PCR (polymerase chain reaction) (Tan et al. 2005) was employed. Random primers combined with primers annealing to the positive strand of the Hygromycin B resistance gene (*aph7") on the pHyg3 plasmid (Figure 3.3A, primer S1) generated two partial pHyg3 plasmid sequences present in opposite orientations as depicted in Figure 3.3A, but no *bona fide* genomic flanking DNA. Probing a Southern blot of BamHI-digested *pgd1* genomic DNA with a pHyg3 fragment as indicated in Figure 3.3A, two signals were observed (Figure 3.3B), while only one would be expected for a single insertion due to the presence of only a single BamHI site in pHyg3. However, probing *pgd1* genomic DNA double-digested with PstI (no site in pHyg3) and XhoI (single site in pHyg3) with the same probe, a single band was present (Figure 3.3B). Together these data suggested that two pHyg3 fragments were present in opposite orientations at the *pgd1* locus. No true signal was obtained from genomic DNA of the wild-type parental strain (Figure 3.3B).

Through “SiteFinding” PCR with plasmid-specific, nested primers S2-1 and S2-2 complementary to the other end of pHyg3 (Figure 3.3A, S2), a flanking genomic DNA (to the
right side of the insertion as shown in Figure 3.3A) was amplified. Sequencing indicated that one end of the insertion bordered sequences within the predicted untranslated region of a gene previously annotated as CGLD15 (Conserved in Green Lineage and Diatoms15; Chlamydomonas v5.3 genome in the Phytozome database, http://www.phytozome.net/) on chromosome 3 (position 6320421 - 6327099; gene locus Cre03.g193500) (Merchant et al. 2007), which I designated PGD1 based on functional analysis presented below. A conserved catalytic triad of Ser-Asp-His was predicted for the translated protein sequence of this gene, which is a typical motif for hydrolases such as lipases (acyl hydrolases). The flanking genomic sequence on the left side of the insertion (refer to Figure 3.3A) was obtained by PCR with primers F1 and S3 (Figure 3.3A). Sequence analysis of this fragment showed that the insertion was accompanied by the deletion of 14 bp of genomic sequence that is unlikely to affect the neighboring gene 5’ to PGD1. Based on these analyses, PGD1 was considered to be the most likely affected gene in the pgd1 mutant and responsible for the observed lipid phenotype.

Insertions into the promoter or untranslated region of PGD1 were expected to affect gene expression. Quantifying PGD1 transcript levels by real-time PCR (Figure 3.3C) showed greatly reduced expression of this gene in the pgd1 mutant. The real time PCR results also confirmed the increased expression of the PGD1 gene in the wild-type parental strain following N deprivation previously observed during global transcript analysis (Miller et al. 2010). The up-regulation of PGD1 expression following N deprivation in parallel with TAG accumulation suggested that the gene product might play a role in TAG biosynthesis.

To independently confirm that the phenotypes of the pgd1 mutant were indeed caused by the insertion into PGD1 described above, complementation analysis with a PGD1-containing fragment from the bacterial artificial chromosome (BAC) clone, 5E6 (Grossman et al. 2003), was
conducted. The fragment used for transformation contained 2 kb 5’ and 1 kb 3’ of the predicted\textit{PGD1} gene and was devoid of other predicted open reading frames. The pMN24 plasmid (Fernandez et al. 1989) containing the \textit{NIT1} gene encoding nitrate reductase was used in a co-transformation experiment for selection on agar plates with nitrate as the N source. (Note, the parental wild-type strain dw15-1 as well as \textit{pgd1} carry a mutation in the genomic \textit{NIT1} gene). To screen for DNA fragments rescuing the observed chlorosis phenotype of \textit{pgd1} on N-limited medium, I developed a “Single Step N Deprivation-Colony Color Screen” method. Agar plates containing 0.5 mM instead of 10 mM nitrate were used for selection allowing colonies to form, which then became N-deprived as nitrate was depleted. Under these conditions, \textit{pgd1} mutant colonies turned from green to white within three weeks while colonies of the wild-type parental strain or \textit{pgd1} colonies harboring an introduced wild-type copy of the \textit{PGD1} gene were expected to remain green (Figure 3.4A). When the \textit{PGD1} genomic fragment was co-transformed with the \textit{NIT1} marker, approximately 5-10 \% colonies remained green. This frequency is at the lower end of the range for previously reported co-transformation efficiencies (Kindle 1990). Eight colonies scored as green and another eight colonies scored as white were chosen and the phenotype was confirmed by spotting cells onto -N agar plates (Figure 3.4B). Genotyping was performed on the junction of the plasmid insertion to confirm the presence of the gene disruption typical for the \textit{pgd1} mutant (Figure 3.4C). Primers F1 and R were expected to give a signal specific for \textit{PGD1} either in the genome or introduced through the fragment, while primers S2-1 and R were expected to give a signal specific for \textit{pgd1}. According to this reasoning, seven of the eight green lines (G1-G7) and one of the white lines (W4) contained the wild-type \textit{PGD1} gene (Figure 3.4C). The presence of a signal from a combination of S2-1 and R indicative of the presence of the \textit{pgd1} background ruled out contamination by the parental strain. It seems likely that in outlier
G8 a secondary mutation caused the observed suppressor phenotype and in outlier line W4 the introduced \textit{PGD1} gene was either mutated, or not adequately expressed, or silenced. Quantitative lipid analysis of three green colony-forming lines (G1-3) showed that they regained their ability to accumulate TAGs to similar levels as the parental strain (Figure 3.4D).

**Extraplastidic lipids of \textit{pgd1} are affected in a consistent way**

The fact that disrupting \textit{PGD1} led to lower TAG content argues against its gene product’s role as a TAG lipase, because decreased TAG lipase activity in the mutant would be expected to increase TAG content. An alternative hypothesis was that PGD1 releases acyl groups from membrane lipids. The activation of the released fatty acids by formation of acyl-CoAs would then make them available for TAG synthesis. To identify the lipid substrates for such a presumed lipase, the abundant membrane lipids DGTS, phosphatidylethanolamine (PtdEtn), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PtdGro), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylinositol (PtdIns) were analyzed in the wild-type parental strain and \textit{pgd1} grown on N-replete and N-depleted medium for 48 h (Figure 3.5A). The relative fraction of DGDG increased following N deprivation as recently reported (Fan et al. 2011). However, no statistically significant difference between the relative amounts of the respective membrane lipid classes for the wild-type parental strain and the \textit{pgd1} mutant was observed.

Although relative amounts of membrane lipid classes were not altered under the growth conditions used, it seemed possible that specific molecular species within each lipid class represented by differences in the respective acyl group substituents were altered in the mutant. For example, changes in fatty acid profiles of glycerolipids have been diagnostic in determining
whether the ER or plastid pathways of lipid assembly were affected in the respective mutants of Arabidopsis (Kunst et al. 1988, Xu et al. 2003). Overall the decreased TAG content in pgd1 was reflected by a reduced total amount of fatty acids per cell (Figure 3.5C) raising the question whether specific TAG molecular species were missing in pgd1 consistent with the disruption of one of several hypothetical TAG assembly pathways. Indeed, the total fatty acid profile of pdg1 was altered. Most prominently the relative fraction of oleate (18:1\textsuperscript{\Delta 9}; number of carbons : number of double bonds and position of double bonds from the carboxyl end) was reduced (Figure 3.5B). Following N deprivation the wild-type parental strain showed an increase in the relative amount of oleate that was not observed for pgd1, while the acyl composition of pgd1 was indistinguishable from that of the parental strain under N-replete growth conditions (Figure 3.5B). When the fatty acyl group profile of individual lipids following N deprivation was examined, a decrease in oleate was observed for pgd1 not only in TAG (Figure 3.5D), but also in DGTS (Figure 3.6A) and PtdEtn (Figure 3.6B). The latter two are presumed to be extraplastidic membrane lipids (Giroud et al. 1988, Giroud and Eichenberger 1989), while the exclusive location of TAG in cytosolic lipid droplets has recently been questioned (Fan et al. 2011, Goodson et al. 2011). Oleate accounts for approximately 25% of the acyl groups in TAG, but only up to 10% in DGTS or PtdEtn explaining why a loss of a specific molecular species containing this fatty acid has more drastic effects on overall TAG content than on that of DGTS and PtdEtn. The plastid lipids MGDG (Figure 3.6C), DGDG (Figure 3.6D), and PtdGro (Figure 3.6E), were not altered in their acyl composition. As apparently only extraplastidic lipids are affected in the pgd1 mutant, it seems possible that PGD1 activity affects the export of acyl groups
from the plastid or the assembly of extraplastidic lipids, assuming that the fraction of TAG missing in \textit{pgd1} is extraplastidic.

\textbf{Oleate is decreased in the sn-1 and sn-3 position of TAGs in pgd1}

To gain more information on the origin of the diacylglycerol moiety for TAG biosynthesis and possible role of oleate (18:1$^{\Delta 9}$) in limiting TAG biosynthesis in \textit{pgd1}, positional analysis of TAG acyl groups was conducted with \textit{Rhizopus arrhizus} lipase. \textit{Rhizopus} lipase specifically hydrolyzes the \textit{sn-1} position of membrane glycerolipids or the \textit{sn-1/sn-3} positions of TAG and is frequently used for the positional analysis of acyl groups in glycerolipids (Fischer et al. 1973, Siebertz and Heinz 1977). Consistent with previous observations (Fan et al. 2011), the \textit{sn-2} position of TAG is mostly composed of C16 acyl groups while \textit{sn-1/sn-3} positions contain both C16 and C18 acyl groups (Figure 3.7). While a decrease in oleate in the \textit{sn-1} or \textit{sn-3} position was obvious, the method did not allow us to distinguish between the two positions. For \textit{sn-1/sn-3}, the relative content of 18:4, 18:3$^{\omega 6}$ and 18:1$^{\Delta 11}$ were 2-fold higher in the \textit{pgd1} mutant than in the wild-type parental strain (Figure 3.7). This was also seen in the total composition of all TAG acyl groups (Figure 3.5D). Interestingly, 18:4 and 18:3$^{\omega 6}$ are mostly found in the extraplastidic lipids DGTS (Figure 3.6A) and PtdEtn (Figure 3.6B). Vaccenic acid (18:1$^{\Delta 11}$) is produced through elongation of 16:1$^{\Delta 9}$, at least in plants (Nguyen et al. 2010), and is presumed to be extraplastidic. In view of an approximate 50\% reduction of TAG in the \textit{pgd1} mutant, the 2-fold relative increase in these three fatty acids suggests that the supply of TAG precursors from extraplastidic lipid turnover was not affected.
Precursor fluxes from plastid lipids to TAG are reduced in \textit{pgd1}

The analysis described above can only provide a static picture of lipid composition. However, the defect in a putative lipase encoding gene in the \textit{pgd1} mutant suggested that lipid remodeling or turnover might play a role in TAG accumulation in Chlamydomonas following N deprivation. To observe possible changes in the dynamics of lipid metabolism in the \textit{pgd1} mutant, I employed pulse-chase labeling of membrane lipids using $[^{14}\text{C}]$-acetate which can be converted easily to precursors of fatty acid biosynthesis in plastids. The labeling pulse was provided either before (150 min pulse duration, Figure 3.8) or during N deprivation (200 min pulse duration initiated 12 h after the start of N deprivation, Figure 3.9 and Figure 3.10). Lipids were extracted as indicated and fractions of label incorporation into major lipids during the chase stage were calculated. The difference in the incorporation of label into TAG between the wild-type parental strain and \textit{pgd1} was more prominent when the labeling pulse was applied during N deprivation (Figure 3.9) compared to its application prior to N deprivation (Figure 3.8). This observation suggested that exchange of \textit{de novo} synthesized acyl groups through the membrane lipid pool into TAGs during N deprivation might involve PGD1, rather than the conversion of preexisting membrane lipids formed under N-replete conditions to TAGs. When the pulse was applied following N deprivation, plastid lipids, especially galactoglycerolipids, were rapidly labeled (Figure 3.9). Conditions were chosen such that the total label in the lipid extract during the chase phase remained approximately the same. In the wild-type parental strain (Figure 3.9A) an increase of label in TAG was observed in parallel with a decrease of label in membrane lipids suggesting the incorporation of acyl or diacylglycerol groups derived from membrane lipids into TAGs. After 25 h of chase the label remaining in membrane lipids was lower than that in TAGs.
This situation was reversed in the *pgd1* mutant (Figure 3.9B). In particular the fraction of label in the two galactoglycerolipids DGDG and MGDG remained much higher in *pgd1*. Because MGDG is its precursor, DGDG was labeled with some delay. In fact, it was the most highly labeled lipid in the *pgd1* mutant extracts presumably because the transfer of labeled acyl or diacylglycerol groups from MGDG into TAGs was disrupted in the mutant. When the pulse was applied prior to N deprivation (Figure 3.8), MGDG was the most highly labeled lipid reflecting the fact that it is also the most abundant lipid under N-replete conditions, when TAG biosynthesis is repressed. Apparently, following N deprivation *de novo* synthesized acyl groups in the plastid are incorporated first into plastid membrane lipids, in particular MGDG, prior to becoming incorporated into TAGs, and the incorporation of acyl groups into TAG seems to require PGD1. Thus MGDG serves as precursor for a fraction of acyl or diacylglycerol groups, those containing oleic acid (see Figure 3.5), incorporated into TAGs following N deprivation. This process is disrupted in the mutant and more MGDG is converted to DGDG instead of TAG. While PtdGro was rapidly turned over, the rates of turnover remained approximately the same in the *pgd1* mutant and the wild type (Figure 3.10). Based on these data I concluded that PGD1 is likely to be a galactoglycerolipid lipase prompting me to tentatively name the gene *Plastid Galactoglycerolipid Degradation 1* (*PGD1*).

**PGD1 hydrolyzes acyl groups of MGDG with a preference for the sn-1 position**

To more directly determine the biochemical activity of PGD1, I produced the recombinant protein in *E. coli*. The recombinant protein was affinity-purified from denatured inclusion bodies (Figure 3.11A), renatured, and offered various substrate lipids in a lipase activity assay. To control for spontaneous lipid hydrolysis or lyso-lipid contamination I assayed
the protein refolding buffer without proteins. As a positive control I assayed Rhizopus lipase. I employed MGDG of different molecular composition as substrates to determine the enzyme’s preference. “Mature” MGDG was isolated from Chlamydomonas cells, which predominantly contain molecular species 18:3/16:4 (sn-1/sn-2). Using this substrate, the lyso-MGDG product band that was generated was rather faint (Figure 3.11B). By analyzing the different fractions, I found that in the remaining MGDG after PGD1 hydrolysis, the 16:0 and 18:1<sup>Δ9</sup> acyl groups selectively disappeared while the major acyl groups 16:4 and 18:3 remained (Figure 3.12A and 5B). This suggested that PGD1 prefers to hydrolyze de novo synthesized MGDG (18:1<sup>Δ9</sup>/16:0) and the reaction stops when 18:1<sup>Δ9</sup>/16:0 is depleted in the assay mixture. Hypothesizing that PGD1 only hydrolyzes de novo-formed MGDG to release 18:1<sup>Δ9</sup> for further TAG biosynthesis, reduction of 18:1<sup>Δ9</sup> in TAG of the pgd1 mutant can be explained. The lyso-MGDG obtained using MGDG purified from Chlamydomonas exclusively contained 16:0 (Figure 3.12C), suggesting that PGD1 prefers the sn-1 position. However, the low amount of free fatty acids generated (Figure 3.12D) made it difficult to draw a firm conclusion, considering that there may be lipids or fatty acids co-purified with the PGD1 protein.

To confirm that PGD1 prefers less desaturated molecular species, MGDG synthesized in <i>E. coli</i> by the activity of recombinant cucumber MGDG synthase (Shimojima et al. 1997) was used. In the buffer control lane, presumably no hydrolysis occurred as no generation of lyso-MGDG was seen (Figure 3.11 B). MGDG synthesized in <i>E. coli</i> contains a combination of 18:1<sup>Δ11</sup>, 16:0 or 18:1<sup>Δ9</sup> acyl groups and is similar to the newly assembled MGDG in Chlamydomonas. As indicated in Figure 3.11B by the intensity of the lyso-MGDG band, PGD1
was more active on the MGDG species produced in *E. coli* using the cucumber MGDG synthase than the mature, mostly unsaturated MGDG from Chlamydomonas.

To obtain more information on the substrate preference of PGD1, I compared the acyl composition of the remaining MGDG substrate, lyso-MGDG and free fatty acid products after incubation with the corresponding fractions obtained from Rhizopus lipase hydrolysis. Rhizopus lipase was inhibited by the buffer used for PGD1 refolding (Figure 3.11B). To generate lyso-lipid (including lyso-MGDG) standard, PBS instead of protein refolding buffer was used to dissolve Rhizopus lipase for all the reactions mentioned below. The *E. coli* derived MGDG contains 16:0, 16:1\(^{\Delta9}\) and 18:1\(^{\Delta11}\) (Figure 3.11D, untreated sample) with 16:0 and 18:1\(^{\Delta11}\) present in the *sn*-1 (Figure 3.11F), and 16:1\(^{\Delta9}\) and 18:1\(^{\Delta11}\) in the *sn*-2 position (Figure 3.11E), as determined by Rhizopus lipase digestion. After PGD1 hydrolysis, the substrate MGDG was partly remaining (Figure 3.11B). However, all the three major fatty acids were decreased to a similar extent (Figure 3.11G). Thus, the remaining MGDG was due to limited enzyme activity instead of the preference between different molecular species within *E. coli*-derived MGDG. Lyso-MGDG (Figure 3.11H) and free fatty acids (Figure 3.11I) generated by PGD1 resembled the corresponding fractions following Rhizopus lipase digestion in fatty acid compositions, indicating that PGD1 prefers acyl groups at the *sn*-1 position similar to Rhizopus lipase.

I also explored the kinetics of PGD1 mediated hydrolysis of *E. coli*-derived MGDG. Lyso-MGDG was detectable in 30 min and continuously increased within 4.5 h of incubation (Figure 3.13A). At 4.5 h, the bulk of MGDG substrate still remained and I chose a 3 h incubation time to test the relationship between reaction velocity and substrate MGDG availability. It should be noted that MGDG is not soluble and, therefore, classic enzyme kinetics is not directly
applicable in this case. It should also be cautioned that the purified PGD1 enzyme went through a denaturation process, and the lipase activity may not be completely regained during refolding for all molecules present. Lyso-MGDG instead of free fatty acids was quantified as free fatty acids can be either derived from MGDG or lyso-MGDG. The hydrolysis of MGDG exhibited saturation as expected for an enzyme catalyzed reaction, with a linear increase in reaction velocity up to an apparent MGDG concentration of 300 μM (Figure 3.13B).

**PGD1 does not act on DGDG**

During the labeling experiment shown in Figure 3.9, labeling of DGDG increased to a greater extent in the *pdg1* mutant than did MGDG, suggesting it might be a possible substrate of PGD1. To test this possibility, DGDG extracted from Chlamydomonas was used as a substrate in the PGD1 assay. However, no formation of lyso-DGDG was detected by sugar-specific staining (Figure 3.11C). When *E. coli*-derived MGDG and Chlamydomonas derived-DGDG were offered in equal amounts, only lyso-MGDG was formed, showing that PGD1 used MGDG but not DGDG in this competition experiment, which might reflect the *in vivo* situation in which both lipids are present in the same membrane. While a single MGDG molecular species 18:3/16:4 predominates in Chlamydomonas (Figure 3.6C), DGDG is represented by a greater variety of molecular species with mostly 16:0, at the sn-2 position, and considerable amounts of 18:1Δ9, 18:2 and 18:3 acyl groups present at the sn-1 position of the glycerol moiety (Giroud et al. 1988), (Figure 3.6D). Apparently, none of these DGDG molecular species is hydrolyzed by PGD1 under the conditions used. In addition, I tested PGD1 activity on other major membrane lipids prepared from Chlamydomonas extracts. PGD1-dependent generation of lyso-DGTS and lyso-
PtdEtn were detectable by iodine staining, but much lower than those generated by Rhizopus lipase (Figure 3.14A and 7B). Lyso-SQDG hydrolysis was not detectable by sugar-specific staining (Figure 3.14D). Repeated trials of PtdGro hydrolysis by Rhizopus lipase failed to yield an obvious lyso-PtdGro band (Figure 3.14), which is expected to run slower than PtdGro on TLC plates. This might be due to the fact that the \( sn-2 \) position of PtdGro is composed of 16:0 and 16:1\(^{\Delta3} \) only (Giroud et al. 1988) while iodine stains lipids by binding to double bonds. Nevertheless, a major decrease in the lipid substrate after PGD1 hydrolysis was visible for \textit{E. coli} MGDG (Figure 3.11B) but not PtdGro nor DGTS, PtdEtn and SQDG. At this time, synthetic molecular species are not available for lipids such as MGDG and DGTS so I could not compare lipids with exactly the same acyl compositions but different head groups.

**DISCUSSION**

The primary phenotype through which the \textit{pgd1} mutant was identified, is a reduction in the accumulation of TAGs following N deprivation (Figure 3.1). Because the affected gene (Figure 3.3), \textit{PGD1}, encodes a galactoglycerolipid lipase with preference for the \( sn-1 \) position of the respective glyceryl backbone (Figure 3.11), I had to reconsider how TAGs are synthesized in Chlamydomonas. A mechanistic model that accommodates my current findings on the \textit{pgd1} mutant and the PGD1 protein, and that places PGD1 into the context of overall cellular lipid metabolism is shown in Figure 3.15. Because the \textit{pgd1} mutant still accumulates considerable amounts of TAGs, additional pathways not involving PGD1 are contributing to TAG accumulation in N-deprived cells. For simplification purposes, a single lipid droplet is shown receiving TAG assembled at the ER or the plastid envelopes.
An MGDG deacylation/acylation cycle involved in TAG biosynthesis of Chlamydomonas

Figure 3.15 summarizes my current working hypothesis of PGD1 function in TAG metabolism. I have placed the galactoglycerolipids, in particular MGDG, at the center of the plastid envelope pathway as my pulse-chase labeling experiment (Figure 3.9) suggested that acyl groups or entire DAG moieties move through the membrane lipid fraction of the chloroplast prior to incorporation into TAGs. While the labeling of galactoglycerolipids in the pgd1 mutant was increased, the relative amounts of the membrane lipid classes did not change in the mutant. Apparently, the pool size of MGDG and other membrane lipids is strictly controlled to maintain functional photosynthetic membranes.

The importance of galactoglycerolipids in TAG metabolism in Chlamydomonas may arise from the fact that this alga lacks PtdCho (Giroud et al. 1988). PtdCho is one of the most rapidly labeled and metabolized membrane lipids in seed plants and acyl exchange involving PtdCho has been suggested to play a role in the export of fatty acids relevant for extraplastidic membrane lipid biosynthesis including that of TAGs (Bates et al. 2007, Bates et al. 2009). Chlamydomonas membranes contain the betaine lipid DGTS which has been widely assumed to play at least some of the roles of PtdCho in Chlamydomonas due to similarities in structure and biophysical properties of the two lipids (Sato and Murata 1991). However, my labeling and lipid analysis data showed no differences for DGTS between the parental strain and the pgd1 mutant and suggested it is not involved, at least in the aspect of TAG biosynthesis that is affected in the pgd1 mutant. Although DGTS and PtdEtn molecular species isolated from Chlamydomonas were hydrolyzed by recombinant PGD1 to a limited extent (Figure 3.14A and 7B), DGTS and PtdEtn showed the same change in molecular species composition in the mutant, i.e., the reduction of
oleic acid containing species (Figure 3.5 and Figure 3.6), as was seen also for TAGs. It should be noted that DGTS and PtdEtn are extraplastidic membrane lipids. Oleate (18:1<sup>Δ9</sup>) and palmitate (16:0) typically are the de novo synthesized acyl groups incorporated into the glyceryl backbone. Thus the reduction of oleate in TAG in pgd1 suggests that the TAG affected in this mutant is derived from glyceryl moieties containing these de novo synthesized acyl groups. Pulse-chase labeling data obtained when labeled acetate was added prior to N deprivation showed few differences between the mutant and the parental strain (Figure 3.8). However, stark differences were observed, when the pulse was given following N deprivation (Figure 3.9) suggesting that the fraction of TAG requiring PGD1 activity indeed involves de novo fatty acid biosynthesis.

As DGTS and PtdEtn in the pgd1 mutant are likely down-stream products of PGD1 activity just like TAG as discussed above, it seems probable that a plastid lipid serves as the true substrate for PGD1 and that PGD1 may be involved in cycling newly synthesized fatty acids through the plastid polar lipid pool. DGDG is not likely a major substrate for PGD1 in cells as it is also not a substrate for PGD1 <i>in vitro</i>, even though it is highly labeled during pulse-chase experiments in the pgd1 mutant (Figure 3.9). The delay in labeling of DGDG as compared to MGDG is consistent with biosynthesis of DGDG by galactosylation of MGDG. Thus if cycling of acyl groups into TAG through the MGDG pool is reduced in pgd1, the reduced flux from MGDG to TAG allows for greater availability of MGDG for DGDG biosynthesis explaining increased labeling of this lipid in the mutant.

<i>In vitro</i> lipase assays suggested that PGD1 prefers MGDG over DGDG, with a preference for MGDG molecular species with fewer double bonds over 18:3/16:4 species, and a preference for acyl groups at the sn-1 position over sn-2. To explain these observations, I propose an acyl-
editing cycle (Figure 3.15, process 1) involving MGDG assembled from de novo synthesized fatty acids (18:1\(^{\Delta 9}\)/16:0). One function of such a cycle involving a transient MGDG pool might be the transfer of fatty acids synthesized in the plastid through the plastid envelope membranes effectively accomplishing a net export of 18:1\(^{\Delta 9}\) acyl groups. As 18:1\(^{\Delta 9}\) is a major fatty acid in TAG (Figure 3.5D), but not in DGTS or PtdEtn (Figure 3.6A and 2B), TAG is the most affected of the extraplastidic lipids in the pgd1 mutant. Interestingly, MGDG in the pgd1 mutant did not accumulate 18:1\(^{\Delta 9}\) (Figure 3.6C), but apparently continued to become desaturated to its mature 18:3/16:4 molecular species. Alternatively, it seems likely that MGDG assembly from newly synthesized acyl groups is feedback inhibited, adjusting the flow through the pathway to the demands of the cell. Thus, the total amount of fatty acids in the pgd1 mutant is lower than in the wild-type parental strain (Figure 3.5C) and the relative amount of 18:1\(^{\Delta 9}\) in the total lipid extracts was reduced (Figure 3.5D).

Mature MGDG found in thylakoid membranes is predominantly composed of the 18:3/16:4 species (Figure 3.6C). Perhaps the presence of the unusual 16:4 acyl group, or other highly unsaturated acyl groups, protects MGDG degradation by PGD1, because it is necessary building block of the photosynthetic membrane, while allowing cycling of de novo synthesized acyl groups through the MGDG pool. This process is not perfect as 18:3 and 16:4 acyl groups were present in TAGs of the wild-type parental strain and the pgd1 mutant indicating some turnover of mature MGDG (Figure 3.15, process 2). It is likely that following severe N deprivation photosynthetic membranes containing mature MGDG species are degraded to some extent, perhaps involving lipases different from PGD1, and that these acyl or diacylglycerol moieties find their way into the TAG fraction. But the bulk of the membrane has to be
maintained for a rapid recovery when nitrogen is re-supplied. Thus, the resistance of mature Chlamydomonas MGDG to PGD1-catalyzed hydrolysis supports the hypothesis of de novo synthesized acyl group cycling through a specific MGDG subpool. For this hypothesis to be valid, an acyl-ACP:lyso-MGDG acyltransferase activity is required for acylation of lyso-MGDG. Such an enzyme activity with a preference for the sn-1 position has been described for a cyanobacterium (Chen et al. 1988). I assume that Chlamydomonas has an ortholog, although the identity of the gene is not yet known.

One possible shortcoming of the proposed hypothesis is that no chloroplast transit peptide was predicted for PGD1 suggesting it to be extraplastidic. However, a cytosolic location would give PGD1 access to MGDG in the outer leaflet of the outer envelope membrane, where this lipid has been shown to be present (Joyard et al. 1991). Analogously, the outer envelope protein, SFR2, of Arabidopsis (Moellering et al. 2010) acts on MGDG. While I attempted to resolve this issue, subcellular localization of PGD1 using GFP fusion constructs was unsuccessful. Alternative localization approaches such as immunolocalization of PGD1 will have to await the availability of antibodies.

**How does oleate availability affect TAG biosynthesis?**

The fatty acid composition of mutant TAG (Figure 3.5D and Figure 3.7) showed besides a lack of 18:1\(^\Delta^9\) a higher relative abundance of 18:1\(^\Delta^{11}\), 18:3\(^\omega^6\) and 18:4 acyl groups. To explain these observations, I considered the different sources for acyl groups that might be present in TAGs: 1. de novo synthesis (Figure 3.15, process1), for which 18:1\(^\Delta^9\) is the diagnostic fatty acid most increased following N deprivation (Figure 3.5C); 2. plastid membrane lipid degradation
(Figure 3.15, process 2) indicated by the presence of 16:4 and 18:3\(^{\omega 3}\) in TAGs derived from mature MGDG; and 3. extraplastidic membrane lipid modification and turnover (Figure 3.15; process 3) characterized by the presence of 18:1\(^{\Delta 11}\), 18:3\(^{\omega 6}\), and 18:4 acyl groups in TAG. Fatty acids such as 16:0 can be derived from multiple processes and are less diagnostic. It has been demonstrated that the DAG backbone for TAG species mostly originates from the chloroplast pathway since the \(sn\)-2 position of TAGs of Chlamydomonas contains up to 80% 16-carbon fatty acids (Figure 3.7) and (Fan et al. 2011). I suggest that the plastid DAG pool primarily contributes to TAG biosynthesis. Plastid DAG can derive from both, \textit{de novo} assembly and plastid membrane lipid degradation (Figure 3.15, process 2). Turnover of \textit{de novo} synthesized MGDG (Figure 3.15, process 1) will contribute to the cytosolic acyl-CoA pool which provides acyl groups for the \(sn\)-3 position in TAGs. Similarly, lipid modification and turnover at the ER (Figure 3.15, process 3) likely provide acyl-CoA substrate for the diacylglycerol acyltransferase (DGAT). The absence of PGD1 impairs the export of 18:1\(^{\Delta 9}\) with two consequences: 1. decrease of 18:1\(^{\Delta 9}\)-CoA as substrate for TAG biosynthesis (Figure 3.5D, arrow 1); and 2. decrease of 18:1\(^{\Delta 9}\) in the DAG backbones of DGTS and PtdEtn as shown in Figure 3.6A and 2B (Arrows 2 and 3).

The relative amounts of fatty acids from extraplastidic membrane lipid turnover (18:1\(^{\Delta 11}\), 18:3\(^{\omega 6}\), and 18:4) were doubled in TAG of the \textit{pgd1} mutant (Figure 3.5D and Figure 3.7). Considering the approximately 2-fold decrease in TAG content in the \textit{pgd1} mutant, this can be
explained by the fact that extraplastidic membrane lipid turnover (Figure 3.15, process 3) was not affected by the *pgd1* mutation, while the total TAG amount was decreased 2-fold.

In contrast, the relative amounts of fatty acids provided by mature plastid membrane lipid turnover (16:4 and 18:3 \(\omega_3\)) remained the same or only slightly increased (Figure 3.5D and Figure 3.7). As a consequence, the absolute amounts of these two fatty acids in TAGs were decreased.

**CONCLUSIONS**

The isolation of the *pgd1* mutant led to the discovery of a galactolipid lipase that plays a role in TAG accumulation following N deprivation in Chlamydomonas. This finding was not predicted based on our current knowledge of lipid metabolism in seed plants. However, Chlamydomonas lacks PtdCho, which is the polar lipid in plants on which the modification of acyl groups followed by acyl exchange happens. Thus, one might wonder whether the TAG assembly pathway presented here is specific to Chlamydomonas. A cursory check suggests that there are possible orthologs of PGD1 in plants and other algae and that the hypothesis outlined in Figure 3.15 may, therefore, also have some relevance to TAG biosynthesis in plants and other algae, at least under certain growth conditions and perhaps with modifications.
Figure 3.1. Phenotypes of the *pgd1* mutant compared to the wild-type parental strain (WT).

(A) Time course of triacylglycerol (TAG) accumulation following N deprivation and (B) phenotypic analysis of progenies from a cross between *pgd1* and CC-198. Hs and Hr indicate Hygromycin B sensitive and resistant lines respectively. (A, B) The ratio of fatty acids (FA) in TAGs over total fatty acids in the lipid extracts is shown. Averages of three independent measurements are provided. Error bars indicate standard deviation. (C) Appearance of the same patches of N-deprived cells palced on agar-solidified TAP-N medium, 0-12 days after plating. (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.)
Figure 3.2. Growth curves of wild-type parental strain (WT) and pgd1 mutant in regular TAP medium. Cells were grown to stationary phase and inoculated into fresh TAP medium to an optical density (at 750 nm) of 0.04. This experiment was repeated more than three times with two biological replicates each time. A representative result is shown here. Each data point is the average from three technical replicates with relative standard deviations smaller than 3 %.
Figure 3.3. Molecular characterization the pgd1 mutant. (A) A schematic representation of the pHyg3 insert into the genome of the pgd1 mutant. The triangle represents the insert. Thick arrows indicate the orientation of the positive strand of the aph7'' gene conferring resistance to Hygromycin B. The PGD1 gene model is shown in a 5’ to 3’ direction from left to right, with exons and introns represented by black boxes and connecting lines, respectively. 5’ and 3’ untranslated regions are shown as white boxes. Crosshatched boxes indicate the position of the Southern probe used in (B). PCR primer
Figure 3.3. (cont’d)

sites are indicated by small arrows, which are not drawn to scale. The binding sites for the “SiteFinding” primers and nested primers for the two ends of the insertion are shown as single arrows S1 and S2, respectively. Sequences for all primers can be found in Supplemental Table 1. (B) Southern blot of the pHyg3 insertion and surrounding genomic DNA. Genomic DNA was digested with BamHI or PstI and XhoI and probed with the fragment (cross-hatched box) as shown in (A) PstI cuts outside the insert and sites are not shown in (A). (C) Reverse transcription-quantitative PCR of the PGD1 transcript in the wild-type parental strain (WT) and the pgd1 mutant grown for 48 h in TAP (+N) or TAP-N (-N) medium. The abundance of PGD1 mRNA was normalized to RACK1. Data are presented as average ± SD (n=3).
Figure 3.4. Genetic complementation of *pgdI* phenotypes with wild-type genomic DNA.
Figure 3.4. (cont’d)

(A) Section of an agar plate with $pgd1$ mutant colonies 20 days after transformation with a wild-type $PGD1$ containing fragment. Green colonies (arrows) are presumed to be complemented lines; white colonies show the $pgd1$ characteristic chlorosis phenotype. (B) Confirmation of the phenotypes of lines which form green (G1-8) and white colonies (W1-8) following restreaking and 10 d of growth on TAP-N. The $pgd1$ mutant as well as the wild-type parental strain (WT) are included. (C) Genotyping of the different lines. A scheme depicting the insertion site shows the primer locations with arrows; sections of DNA gels with PCR products obtained with PCR primers as indicated are shown below. Primer sizes are not to scale. (D) Quantitative analysis of TAG of three lines rescued with $PGD1$ genomic DNA after 48 h of growth in TAP-N medium. The ratio of fatty acids (FA) in TAGs over total fatty acids in the lipid extracts is shown. Averages of three independent measurements are provided. Error bars indicate standard deviation.
Figure 3.5. Detailed lipid analysis of the wild-type parental strain (WT) and pgd1 mutant in N-replete medium (+N) and 48 h after transfer to N-depleted medium (-N). (A) Relative...
abundance of major polar lipid classes. (B) Relative fatty acid (FA) composition and (C) cellular contents of total cellular fatty acids. (D) Composition of fatty acids esterified to TAG. Averages of three replicates are shown with errors bars indicating SD. Lipid abbreviations: DGTS, diacylglycerol-\(N,N,N\)-trimethylhomoserine; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol. Fatty acids are designated as chain length: number of double bonds. Positions of double bonds are indicated with \(\Delta\) (counting from carboxyl group) or \(\omega\) (counting from the methyl group). In (B) 16:2 is composed of 16:2\(^\Delta7,10\) and 16:2\(^\Delta10,13\).
Figure 3.6. Fatty acid compositions of DGTS, PtdEtn, MGDG, DGDG, PtdGro of the wild-type parental strain (WT) and pgd1 mutant in N-replete medium (+N) and 48 h after transfer to N-depleted medium (-N). Lipid abbreviations are as defined for Figure 3.5. Averages of three replicates are shown with error bars indicating SD. Fatty acids are designated as chain length: number of double bonds. Positions of double bonds are indicated with Δ.
Figure 3.6. (cont’d)

(counting from carboxyl group) or \( \omega \) (counting from the methyl group).
Figure 3.7. Positional analysis of TAG acyl groups of the wild-type parental strain (WT) and *pgd1* mutant 48 h after transfer to N-depleted medium (-N). Purified TAG from Chlamydomonas cells were hydrolyzed by Rhizopus lipase. Free fatty acids were presumed to be derived from *sn-1/sn-3* position of the glyceryl backbone of TAG (A) while monoacylglycerol contains the *sn-2* position acyl groups (B). Averages of three replicates are provided with error bars indicating standard deviation.
Figure 3.8. *In vivo* pulse-chase acetate labeling of lipids in the wild-type parental strain (WT) and the *pgd1* mutant before N deprivation. Labeled acetate was added prior to the transfer to TAP-N medium. The length of the $^{14}$C acetate labeling pulse was 150 min after which the cells were transferred to TAP-N medium lacking labeled acetate. Cells were collected at the times indicated and lipid extracts were prepared and analyzed. The fraction of label in all analyzed lipids is given; only lipids containing the bulk of the label or those most relevant for the discussion are shown. Lipid abbreviations are as defined for Figure 3.5. The data are from one representative experiment of a series of independent experiments.
Figure 3.9. *In vivo* pulse-chase acetate labeling of lipids in the wild-type parental strain (WT) and the *pgd1* mutant. Labeled acetate was added 12 h following transfer of cells to TAP-N medium. The length of the $^{14}$C acetate labeling pulse was 200 min after which the cells were transferred to TAP-N medium lacking labeled acetate. Cells were collected at the times indicated and lipid extracts were prepared and analyzed. The fraction of label in all analyzed lipids is given; lipids containing the bulk of the label or those most relevant for the discussion are shown in this figure. Fractions of label in other lipids are shown in Figure 3.10. Lipid abbreviations are as defined for Figure 4. The data are from one representative experiment of a series of independent experiments.
Figure 3.10. *In vivo* pulse-chase acetate labeling of PtdEtn, PtdGro, SQDG and PtdIns in the wild-type parental strain (WT) and the *pgd1* mutant. The data are from the same set of experiments as shown in Figure 3.9. Lipid abbreviations are as described in Figure 3.5. SQDG, PtdIns and TLC origin were scraped together as one fraction (SPO).
Figure 3.11. Activity of the recombinant PGD1 protein on MGDG and DGDG. (A) SDS-PAGE of purified PGD1 protein and whole cell lysates (WCL) from *E. coli* cells expressing the *PGD1* open reading frame and the empty vector control. Protein loading was 6 μg per lane for whole cell lysates. Purified PGD1 protein loaded was 1 μg (Quantified as in Methods; possibly biased by components in refolding buffer). Proteins were stained by Coomassie Brilliant Blue. The arrow points towards the PGD1 protein. (B) A thin-layer chromatogram of polar lipids from the lipase assay mixtures to which either mature MGDG extracted from *Chlamydomonas*, or MGDG extracted from the *E. coli* (*E. coli*) strain over-expressing cucumber MGDG synthase were added as substrates. (C) A thin-layer chromatogram of polar lipids from the lipase assay mixtures to which either DGDG extracted from *Chlamydomonas* alone or mixed in with *E. coli* derived MGDG at a 1:1 molar ratio were added as substrates. Glycolipids were visualized with α-naphtol reagent. Reaction products obtained with refolded PGD1 protein, blank refolding buffer and *Rhizopus arrhizus* lipase dissolved in protein refolding buffer (RaLip-R) or PBS.
(RaLip-P) were analyzed. (D)-(I) Gas liquid chromatograms of methyl esters derived from a buffer control reaction containing *E. coli*-derived MGDG (D) or different fractions after lipase reaction with *E. coli*-derived MGDG as discussed in details in the text. As an internal standard, 15:0 was used.
Figure 3.12. Hydrolysis of Chlamydomonas-derived MGDG by PGD1. Gas liquid
Figure 3.12. (cont’d)

chromatograms of methyl esters derived from different fractions after lipase reaction are shown as discussed in detail in the text. As an internal standard 15:0 was used. (A) input Chlamydomonas MGDG; (B) remaining MGDG after PGD1 hydrolysis; (C) and (D) lyso-MGDG and free fatty acids generated from PGD1 hydrolysis, respectively.
Figure 3.13. Quantitative hydrolysis of E. coli derived MGDG by PGD1. (A) A thin-layer
Figure 3.13. (cont’d)

chromatogram of polar lipids from the PGD1 assay mixtures to which MGDG extracted from the
E. coli strain over-expressing cucumber MGDG synthase was added as substrate. Glycolipids
were visualized with α-naphtol reagent. One aliquot of the same volume was extracted and
loaded onto the TLC for each time point. (B) Dependence of MGDG hydrolysis on MGDG
concentration. Reaction velocity was quantified as the amount of lyso-MGDG generated per min
per mg purified PGD1 protein. A representative result is shown for each panel.
Figure 3.14. Activity of the recombinant PGD1 protein on DGTS, PtdEtn, PtdGro and SQDG. Thin-layer chromatograms of polar lipids from the PGD1 assay mixtures to which
Figure 3.14. (cont’d)

Chlamydomonas-derived membrane lipids were added as substrates. (A), diacylglycerol-\(N,N,N\)-trimethylhomoserine (DGTS); (B) PhosphatidylEthanolamine (PtdEtn); (C) Phosphatidyglycerol (PtdGro); (D) Sulfoquinovosyldiacylglycerol (SQDG). Exposure to iodine vapor was used to visualize lipids for reactions on DGTS, PtdEtn and PtdGro. SQDG and lyso-SQDG were stained by \(\alpha\)-naphtol reagent. Substrates treated with Rhizopus lipase was used to generate the lyso-lipid standards. A representative result is shown for each panel.
Figure 3.15. Hypothesis placing PGD1 into the context of overall cellular lipid metabolism explaining its role in TAG biosynthesis. For simplicity a single lipid droplet (LD) is shown forming at the endoplasmic reticulum (ER) or the chloroplast envelope (Cp Env). Thylakoid membranes harboring the two photosystems have been omitted. Three lipid turnover processes discussed in the text are indicated by numbers: 1. Turnover of newly synthesized MGDG; 2. acyl group modification and lipid turnover at the ER; 3 turnover of mature MGDG and other thylakoid lipids at the plastid envelopes. Enzymes, protein complexes and processes are italicized: DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase complex; Fd, ferredoxin; FNR, ferredoxin: NADP⁺ reductase; PSI and II, Photosystem I and II. Substrates and products: DAG, diacylglycerol; e-, electron; FA, fatty acid (carbons: double bonds); MGDG, monogalactosyldiacylglycerol; PL, polar lipids; TAG, triacylglycerol; ROS, reactive oxygen species. Not all intermediates or reactions involved are shown.
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Chapter 4

Chlamydomonas shunts electrons into triacylglycerols to avoid photooxidative stress during nutrient deprivation

1 Two undergraduates worked on this project under my supervision. Fabian Brandenburg contributed data for Figure 4.3. Cassandra Johnny contributed part of data for Figures 4.1, 4.4, 4.5 and 4.6. I provided supervision, summarized the data and wrote this chapter. Figures 4.1 and 4.2 are part of a paper in press at The Plant Cell in Xiaobo Li, Eric R. Moellering, Bensheng Liu, Cassandra Johnny, Marie Fedewa, Barbara B. Sears, Min-Hao Kuo, Christoph Benning. (2012) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in Chlamydomonas reinhardtii.
ABSTRACT

A mutant in *Chlamydomonas reinhardtii* was previously found to be deficient in triacylglycerol (TAG) biosynthesis under nitrogen (N) deprivation and designated *pgd1* (Chapter 3). In this study, the *pgd1* mutant is used to explore the physiological significance for TAG production in microalgae. The *pgd1* mutant exhibited chlorosis, accumulated reactive oxygen species (ROS) and lost viability under N deprivation. Blocking photosynthetic electron transport by 3-(3,4-dichlorophenyl)-1,1-dimethylurea reversed these phenotypes. This suggests that TAG accumulation serves as an electron sink under N deprivation to prevent the generation of reactive oxygen species through reduction of molecular oxygen (Mehler reaction). A suppressor screen was conducted in the *pgd1* mutant background for suppression of the chlorosis phenotype and several suppressor mutants were obtained successfully. Biochemical, physiological and genetic characterizations of these suppressors are in progress.
INTRODUCTION

Under nitrogen (N) deprivation, algae such as *Chlamydomonas reinhardtii* produce substantial amount of triacylglycerols (TAGs) (Wang et al., 2009; Moellering and Benning, 2010). The physiological significance of this process has not been extensively explored. Understanding of this process will have implications on how algae can be modified to produce more TAG for large-scale biofuel production. In microalgae, besides N deprivation, other stresses such as high salinity also increase TAG content substantially (Weers and Gulati, 1997; Hu et al., 2008). Therefore, instead of being just carbon and energy storage, TAG accumulation is likely a mechanism conferring stress resistance. Hu et al. hypothesized that under stress conditions, electrons accumulate in the photosynthetic electron transport chain. Over-reduction of the photosynthetic electron transport chain can lead to a burst of reactive oxygen species (ROS) (Mehler, 1951; Asada, 1994), which may damage membrane lipids, proteins and nucleic acids. By channeling electrons into the production of NADPH, which can be efficiently consumed by fatty acid synthesis, the oxidative stress can be relieved (Hu et al., 2008). This hypothesis is supported by the observation that TAG is increased in *Nannochloropsis salina* cells receiving light above the saturating light intensity (Van Wagenen et al., 2012). However, it could not be directly tested without a mutant strain that is impaired in TAG accumulation.

In a previous screen for mutants with altered TAG metabolism, a mutant disrupted in the Plastid Galactolipid Degradation 1 (*PGD1*) gene was found to be 50 % decreased in the TAG content. *PGD1* encodes a lipase that facilitates an acylation/deacylation cycle on *de novo* synthesized monogalactosyldiacylglycerol (MGDG) and provides 18:1\(\Delta^9\) for extraplastidic lipid biosynthesis (Chapter 3). Importantly, the *pgd1* mutant was specifically affected in the amount of
TAG but not the major membrane lipids (Figure 3.5) and thus provides an excellent tool to explore the consequences that occur when TAG is less abundant.

In this research, the pgd1 mutant is characterized in its survival rate, production of reactive oxygen species, and response to the inhibition of the photosynthetic electron transport under N deprivation. A suppressor screen in the pgd1 mutant background was also performed to test the relationship between TAG production and redox control.

MATERIALS AND METHODS

Strains and growth conditions

The pgd1 mutant strain was derived from dw15-1 (cw15, nit1, mt+) strain of Chlamydomonas reinhardtii obtained from the Arthur Grossman Lab as described in Chapter 3. The dw15.1 strain is thus termed the wild-type parental strain (WT). Conditions for photoheterotrophic growth in Tris-acetate-phoshate (TAP) medium (Harris, 1989) and N deprivation were as described before (Moellering and Benning, 2010). For photoautotrophic growth, HCl was used instead of glacial acetic acid for neutralization and the medium is termed Tris-minimal-phoshate (TMP). For spotting on TAP-N agar, log-phase cells were condensed to 2 X10^7 cells/ml and 5 μL was loaded for each strain.

Chlorophyll, viability and TBARS analyses

Chlorophylls were extracted from fresh cell pellets with 80% acetone and concentrations were calculated from the absorbance values at 647 nm and 664 nm according to (Zieger and Egele, 1965). To assess cell viability, cells were grown in liquid cultures of TAP or TAP-N. On days 0, 2, 4, and 7 a set volume of culture was diluted and spread onto agar-solidified TAP.
medium supplemented with 0.4 % yeast extract. To assess cell viability, colony forming units were counted one week later. Cells from a second aliquot were fixed in 3.7% formaldehyde (in water) and counted using a hemocytometer under a microscope. Viability percentages (colonies formed per total cells counted each day) were normalized to the values on day 0. TBARS were prepared by extraction with thiobarbituric acid/trichloroacetic acid solution (0.3 % and 3.9 % respectively) and determined by measuring absorbance at 532 nm as previously described (Baroli et al., 2003). The extinction coefficient used was $155 \text{ mM}^{-1}\text{cm}^{-1}$.

**Primary and secondary suppressor screen**

Mutants were generated by plasmid insertion in the *pgd1* mutant background through glass bead transformation (Kindle, 1990). The selectable marker used was the NIT1 gene which encodes the nitrate reductase and confers the ability to grow on medium with nitrate instead of ammonium. The NIT1 DNA was obtained through *BamHI* and *SalI* digestion of the pMN24 (Fernandez et al., 1989), followed by gel purification (all the restriction endonucleases were purchased from New England Biolabs, [http://www.neb.com](http://www.neb.com)). For each transformation 250 ng DNA was used. The transformants were then inoculated in TAP and grown in the dark on a shaker for 8h, and spread on low nitrate plates (TAP with 0.5 mM nitrate) as described in Chapter 3. Typically colonies were visible in a week and after another week, most colonies turned white. The colonies that remained green were restreaked and maintained on solid TAP medium with 10 mM nitrate.

For a second screen, the putative suppressors were grown in regular TAP together with dw15-1 and the *pgd1* mutant to log-phase. Cells were then spotted to a TAP-N agar plate and
incubated under continuous light (70-80 μmol m$^{-2}$ s$^{-1}$). After 10 days, the appearance of the putative suppressor was compared to that of the pgd1 mutant and dw15-1 by the naked eyes.

**DNA and RNA techniques**

Genomic DNA of Chlamydomonas strains was prepared according to (Newman et al., 1990). SiteFinding PCR was conducted according to (Tan et al., 2005) to identify the insertion locus of the NIT1 gene, with minor modifications in the PCR conditions and with primers optimized for the NIT1 marker. The primers used for insertion identification in pgd1fb12 were: SiteFinder5 (CACGACACGCTACTCAACACACCACCTCGCACAGCGTCTCAAGCGGCAGCGCNGNNGCTA, 5’-3’, N represents a random nucleotide) in combination with L2 (ATGTTC-ACGGAGCACCTCTTT) and L1 (GACGCAGAGATCCAGTTCTA). In addition, nested primers SFP1 (CACGACACGCTACTCAACAC) and SFP2 (ACTCAACACACCACCTCGCACAGC) were used. For reverse transcriptase-PCR (RT-PCR), pgd1fb12qRTF (TGCTTCAAGGAGGAGACGTT) and pgd1fb12qRTR (CCCAGGTCACGTGTGTAGAA) were used to amplify a region of cDNA in gene 525683 (see Results and Discussion). RACK1-F (GTCATCCACTGCCTGTGCTT) and RACK1-R (CCTTCTTGCTGGTGATGTTG) were used to amplify the RACK1 cDNA.

**Lipid analysis**

Lipid extraction, thin-layer chromatography (TLC) for lipid separation and gas-liquid chromatography (GLC) were performed as previously described (Moellering and Benning, 2010). Briefly, cell pellets was resuspended and vortexed in methanol, chloroform, 88% formic acid (2:1:0.1 by volume). To the mixture, 0.5 volume of lipid extraction buffer (1 M KCl, 0.2 M H$_3$PO$_4$) was added and vortexed, followed by low speed centrifugation for phase separation. For
TAG quantification and fatty acid profile analysis, lipids were loaded to TLC on Silica G60 plates (EMD chemicals, #5721-7, http://www.emdchemicals.com) and developed in petroleum ether-diethyl ether-acetic acid (80:20:1 by volume). Spots were scraped for transesterification and GLC after brief exposure to iodine vapor.

RESULTS AND DISCUSSION

The \textit{pgd1} mutant loses viability following N deprivation

In contrast to the wild-type parental strain, the \textit{pgd1} mutant liquid cultures and colonies on agar solidified medium became chlorotic 5-9 days following N deprivation (Figure 4.1A). I took advantage of this observation during the complementation analysis described in Chapter 3 (Figure 3.4). The increasing chlorosis correlated well with the decrease in the chlorophyll content (Figure 4.1B), as well as the decline in the viability of the \textit{pgd1} strain following N deprivation (Figure 4.1C). Following N deprivation Chlamydomonas typically ceases cell growth after approximately one cell cycle (James et al., 2011). However, these cells continue to capture light with their photosynthetic light harvesting complexes. If electron acceptors become limiting due to the cessation of growth under these conditions, photosynthetic electron transport chain components may become overreduced. Indeed, it has been hypothesized that enhanced fatty acid synthesis and sequestration of acyl groups in TAG provide an electron sink, because acyl groups are among the most reduced carbon compounds that algae can produce (Hu et al., 2008). A potential consequence of TAG deficiency is the increase in the the NADPH/NADP$^+$ ratio. This is because NADPH is a major reductant in fatty acid synthesis. With decreasing availability of NADP$^+$, molecular oxygen may become an alternative electron acceptor for photosystem I. Thus when photosynthetic electron transport exceeds the capacity of the NADP$^+$ pool to accept
electrons, in the *pgd1* mutant due to decreased TAG synthesis, superoxide may be generated and further converted to H$_2$O$_2$ and hydroxyl radicals (Mehler, 1951). Overproduction of these highly cytotoxic reactive oxygen species (ROS) may lead to cell death. To begin to test this hypothesis, I took advantage of the herbicide DCMU, which specifically inhibits photosynthetic electron transfer at the acceptor side of PS II (Trebst et al., 1970). DCMU treatment is well known to decrease the generation of superoxide and other reactive oxygen species from PS I (Davies et al., 1996; Wen et al., 2008; Robert et al., 2009). When DCMU was added to N-deprived cultures, chlorosis and loss of viability were suppressed (Figure 4.1A, B, and C). To further verify this hypothesis, I analyzed thiobarbituric acid reactive substances (TBARS), a product of reactive oxygen species (Baroli et al. 2003), and observed a burst of TBARS in the *pgd1* mutant, which was also reversed by DCMU (Figure 4.1D). As expected, DCMU did not rescue the *pgd1* TAG phenotype, but did decrease TAG levels of the wild-type parental strain (Figure 4.1E) because of the decrease in electrons provided for NADPH generation as recently observed by others (Fan et al., 2012). A model explaining how cells shunt electrons to TAG biosynthesis to prevent the generation of ROS is shown in Figure 4.2.

The assay employed to detect thiobarbituric acid reactive substances (TBARS) is commonly used to measure the consequences of oxidative stress in Chlamydomonas (Baroli et al., 2003; Fischer et al., 2007). As products of lipid peroxidation, TBARS are easier to detect than ROS themselves which are short-lived (Shulaev and Oliver, 2006). I observed a strong accumulation of TBARS in the *pgd1* mutant on day 7 of N deprivation (Figure 4.1D). However, this effect was preceded by the loss of ability to form colonies indicating a loss in cell viability (Figure 4.1C). Similarly, Baroli et al. tested the ability to form colonies and TBARS accumulation in a Chlamydomonas mutant sensitive to high light and also observed a similar lag
in TBARS formation (Baroli et al., 2004). It is likely that lower amounts of ROS can cause loss of colony forming ability, while the formation and accumulation of detectable levels of TBARS requires more time. However, we cannot exclude the alternate explanation that cell death itself is the cause of TBARS accumulation in the \textit{pgd1} mutant.

If the proposed hypothesis that TAG biosynthesis mitigates ROS formation at PSI following N deprivation is correct, I expect to identify more mutants deficient in TAG accumulation that lose viability following N deprivation. In fact, the essentiality of TAG accumulation opens new opportunities for additional forward genetic screens of mutants compromised in genes required for TAG biosynthesis and its regulation, or even photosynthetic electron transport.

\textbf{Suppressor mutants were successfully obtained}

To further understand the physiological significance of TAG accumulation, a suppressor screen was conducted in the background of the \textit{pgd1} mutant, for suppression of the chlorosis phenotype. The \textit{pgd1} mutant was derived from the dw15-1 strain which was disrupted in the nitrate reductase gene. Taking advantage of that, insertion mutagenesis was performed with the NIT1 marker and colonies were selected on agar solidified medium containing nitrate instead of ammonium as the nitrogen source. The concentration of nitrate used was 0.5 mM, which is lower than the regular concentration of 10 mM. As described in Chapter 3, the low concentration allowed colonies to form and after two weeks, most colonies show the phenotype of the \textit{pgd1} as the nitrate is depleted.

Among 10,000 colonies obtained from transformation, approximately 25 colonies remained green (Figure 4.3A). These 25 strains were then grown up to log-phase and spotted to
an agar plate without nitrogen source for a confirmation of phenotype. Figure 4.3B shows examples of a confirmed suppressor and a false positive strain. Two confirmed suppressors, \textit{pgdlfb12} and \textit{pgdlfb16}, have been further characterized as described below.

**No suppressor mutant is rescued in the amount or fatty acid composition of TAG**

If the \textit{pgdl} mutant loses viability due to the overreduced photosynthetic electron transport chain caused by decreased fatty acid synthesis as hypothesized in Figure 4.2, increased fatty acid synthesis will rescue the mutant. TAG amount and fatty acid composition was analyzed in all the suppressor mutants. The \textit{pgdl} mutant showed low amount of TAG and low $18:1^\Delta_9$ abundance in TAGs. However, in most of the suppressor mutants the amount of TAG or the percentage of $18:1^\Delta_9$ in TAG were the same as in the \textit{pgdl} mutant. The \textit{pgdlfb12} and \textit{pgdlfb16} are shown in Figure 4.4. Interestingly, the fraction of $18:1^\Delta_9$ in the TAG was even lower in mutant \textit{pgdlfb12} than in the original \textit{pgdl} mutant (Figure 4.4).

Why wasn’t a mutant isolated with a rescued TAG content? It is possible that more genes are involved in photosynthetic electron transport or redox control than those involved in TAG biosynthesis and that the suppressor screen is not yet saturated. Another possibility is that the \textit{pgdl} mutation serves as a bottleneck for fatty acid export and mutations upstream or downstream cannot compensate for this defect.

**Mutant \textit{pgdlfb16} exhibited slower growth**

To investigate whether any of the suppressor mutants is blocked in photosynthetic electron transport, three mutants (\textit{pgdlfb3}, \textit{pgdlfb12} and \textit{pgdlfb16}) were grown in parallel with
the \textit{pgd1} mutant and its wild-type parental strain (WT) in TMP or TAP under constant light. Hypothetically, a mutation that decreases photosynthetic electron transport would lower the phototrophic growth. While \textit{pgd1fb3} and \textit{pgd1fb12} did not exhibit a difference from the \textit{pgd1} mutant and WT, \textit{pgd1fb16} had a slower growth rate (Figure 4.5) in both TMP and TAP. Photoheterotrophic growth in TAP under constant light is dependent on both photosynthesis and the organic carbon source-acetate for energy. At this time, it remains elusive whether the slower growth phenotype of \textit{pgd1fb16} is due to a defect in photosynthesis or other processes such as cell cycle progression. To differentiate between these two possibilities, growth will be tested in TAP medium in the dark. If the growth of \textit{pgd1fb16} is similar to that of the \textit{pgd1} mutant, \textit{pgd1fb16} is probably affected in photosynthesis. Otherwise, the low growth rate in TMP and in TAP under light should be attributed to other reasons.

\textit{pgd1fb12} is disrupted in a putative aldehyde dehydrogenase gene and possibly several other genes

SiteFinding PCR (Tan et al., 2005) was performed on several mutants to identify the insertion loci of NIT1 marker with success on \textit{pgd1fb12} and \textit{pgd1fb16} (Figure 4.6A). Band 1 was amplified from every strain, including the \textit{pgd1} mutant. Since the mutation in the endogenous NIT1 gene in the dw15-1 strain was not a deletion, this band is likely to be from amplification of that locus. Bands 2 and 3 were specific for \textit{pgd1fb12} and \textit{pgd1fb16} respectively and have been sequenced and BLASTed (Altschul et al., 1997) against the Chlamydomonas genome (\url{http://genome.jgi-psf.org/Chlre4/Chlre4.home.html}). For \textit{pgd1fb12}, the insertion is mapped to 5 bp above the 5’ UTR region of a gene predicted to encode an aldehyde dehydrogenase (protein ID: 525683, \url{chromosome_8;3499179-3508643}). A diagram of the
insertion is shown in Figure 4.6B. For pgd1fb16, BLAST (Altschul et al., 1997) yielded multiple hits in the genome and further experiments are required to elucidate the insertion locus.

RT-PCR was done to confirm that the insertion affected the expression of 525683. Under both N replete (+N) and N-deprived (-N) conditions, the transcript of 525683 is missing while that of a positive control gene-RACK1 can be detected (Figure 4.6C). We concluded that in mutant pgd1fb12, 525683 is abolished in the transcription. However, whether the mutation in 525683 is responsible for the phenotype will have to await the identification of the other junction of the plasmid and complementation experiments.
Figure 4.1. Biochemical and physiological characterization of wild-type parental strain (WT) and the *pgd1* mutant following N deprivation. (A) Appearance of cultures grown in TAP-N for the number of days indicated. The electron transport chain inhibitor DCMU dissolved in dimethyl sulfoxide was present at a final concentration of 2 μM as indicated. Two
Figure 4.1. (cont’d)

representative cultures per line are shown. (B) Time course of total cellular chlorophyll (Chl) content. (C) Time course of cell viability relative to day 0, the start of N deprivation following transfer to TAP-N medium. (D) Time course of cellular thiobarbituric acid reactive substances (TBARS) content. (E) TAG accumulation presented as ratio of fatty acids (FA) in TAGs over total fatty acids in the lipid extracts after 2 d of N deprivation. For all quantitative data, three replicates were averaged with SD indicated by the error bars.
Figure 4.2. A scheme on the role of TAG production in redox control. Enzymes, protein complexes and processes are italicized. Abbreviations: FAS, fatty acid synthase complex; Fd, ferredoxin; FNR, ferredoxin: NADP$^+$ reductase; PSI and II, Photosystem I and II; TAG, triacylglycerol; ROS, reactive oxygen species. Not all intermediates or reactions involved are shown. The dashed arrow indicates multiple steps.
Figure 4.3. Examples of primary and secondary screen for \textit{pgd1} suppressors. WT represents \textit{dw 15.1}, the wild-type parental strain for \textit{pgd1}. A, primary screen. Transformants were spread to TAP media with 0.5 mM nitrate as the nitrogen source. Typically it takes one week for colonies to form and after another week most colonies will turn white. The colonies that remain green (as pointed to by the arrow) will be picked and maintained as putative suppressors. These strains will be confirmed in the phenotype by spotting same number of log-phase cells to TAP-N which completely lacks ammonium or nitrate. After 10 days, \textit{pgd1} mutant can be told from its wild-type parental strain in appearance and the candidate strains will be visually scored to be suppressed or not. One example of positive and negative result is shown.
Figure 4.4. Analysis of TAG amount and fatty acid compositions in *pgd1fb12* and *pgd1fb16*. WT indicates dw 15.1, the wild-type parental strain for *pgd1*. A, amount of TAG accumulated after 48 h of growth in N depleted medium. Data are presented as the ratios of fatty acids esterified to TAG versus the total fatty acids in the lipid extract. B, percentages of each fatty acid contained in TAG. Fatty acids are shown as number of carbon: number of double bonds with Δ (counting from carboxyl group) or ω (the first double bond counting from the methyl group) indicating the position of double bonds. Trace amount of 16:1 and 16:2 constitute around 1% of TAG fatty acids and are skipped in the graph. Data shown are averages from two biological replicates with error bars representing standard deviation.
Figure 4.5. Growth curves of *pgd1fb16*, *pgd1* mutant and *dw15.1* (WT) in TAP (A) and TMP media (B). Cells were grown to saturation and inoculated into fresh medium to an optical density (at 750 nm) of 0.04. Each data point is the average from three technical replicates with relative standard deviations smaller than 3%.
Figure 4.6. Molecular characterization *pgd1fb12*. A, Products of SiteFinding PCR with genomic DNA of several mutants resolved on agarose gel. B, a gene model (5'-3') of obtained from the Chlamydomonas genome database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). Red boxes and blue boxes represent exons and the untranslated regions (UTRs) respectively. C, Products of RT-PCR with RNA obtained from cells grown with nitrogen or cells nitrogen deprived for 48 h as the templates.
Chapter 5.

Conclusions and perspectives.
The field of algal lipid metabolism has been growing rapidly during the past several years. While algae of the Nannochloropsis genus produce triacylglycerol up to 50% of their dry weight (Radakovits et al., 2012), Chlamydomonas has long been used as a model system for cell motility and photosynthesis studies (Grossman et al., 2003). Genetic techniques such as nuclear transformation (Kindle, 1990) and RNA suppression (Molnar et al., 2009) are well developed.

TAG accumulation under nitrogen (N) deprivation was documented in Chlamydomonas more than a decade ago (Weers and Gulati, 1997) and was recently confirmed by several independent groups (Wang et al., 2009; Moellering and Benning, 2010). N-deprivation was more effective than high salinity and high light for induction of TAG production (Fan et al., 2011). To understand the molecular mechanisms involved in TAG accumulation, several research groups performed transcript profiling to reveal genes up- or down-regulated by N-deprivation. Many genes predicted to encode lipid metabolism-related enzymes have been shown to be differentially regulated (Miller et al., 2010; Boyle et al., 2012). Among these genes were a large number that encode lipases (Miller et al., 2010). During my Ph.D research, two projects were conducted on lipase candidates which affect the turnover and biosynthesis of TAG respectively.

**CrLIP1 is involved in the regulation of TAG turnover in Chlamydomonas.**

The research described in Chapter 2 aimed to discover a lipase involved in TAG degradation. When N is supplied to N-deprived Chlamydomonas cells, the stored TAG is degraded, providing precursors for membrane lipid biosynthesis or energy for cell growth (Nguyen et al., 2011). In yeast, inhibition of TAG turnover by deleting lipases has been demonstrated to increase TAG content (Athenstaedt and Daum, 2003, 2005) and I was curious whether this approach could be applied to algae to increase their TAG production.
Several putative lipase-encoding genes were heterologously expressed in the yeast lipase deficient mutant *tgl3Δtgl4Δ* (Athenstaedt and Daum, 2005; Kurat et al., 2006) for functional complementation. The candidates were chosen based on the transcriptional regulation by N-deprivation. Down-regulated candidate genes were thought to be more likely to encode TAG lipases as lipolysis is expected to be inhibited under N-deprivation. Several up-regulated candidates were also considered, as it is possible that a lipase protein is produced along with TAG so it can be rapidly activated through post-translational modifications once N is re-supplied. Among five candidate genes screened, over-expression of a down-regulated candidate, *CrLIP1*, reversed the hyper-accumulation of TAG in the yeast mutant. RNA suppression of the *CrLIP1* gene in Chlamydomonas delayed the TAG turnover after N re-supply to N-deprived cultures. However, the TAG accumulation was largely unaffected. This suggests a difference in the physiology of TAG metabolism between yeast and Chlamydomonas. In yeast, TAG increases when cells leave log phase and enter stationary phase (Athenstaedt and Daum, 2003). The fact that *tgl3Δtgl4Δ* accumulates more TAG than wild-type strains (Athenstaedt and Daum, 2005; Kurat et al., 2006) indicates that TAG synthesis and degradation probably occur together with synthesis surpassing degradation. In Chlamydomonas, during N-deprivation, TAG degradation might be negligible compared with its synthesis. As a result, *CrLIP1* knock down lines did not over-accumulate TAG during N-deprivation but exhibited delayed TAG turnover.

Recombinant CrLIP1 protein was produced and purified from *E. coli* cells for *in vitro* lipase assays. CrLIP1 degraded diacylglycerol (DAG) and polar lipids. However, no TAG hydrolysis was observed upon CrLIP1 treatment under various reaction conditions. That observation led to a major unsolved question: is CrLIP1 a DAG lipase or a TAG lipase? As over-expression of *CrLIP1* in yeast decreased cellular content of TAG but not DAG, it is possible that
CrLIP1 uses TAG as the major substrate but the activity requires an unknown cofactor which was missing in the in vitro system. Alternatively, the CrLIP1 protein produced in yeast may have undergone a post-translational modification (e.g. phosphorylation) which did not occur in E. coli due to the absence of the corresponding mechanism. Future assays should include common cofactors such as ADP or NAD or should be performed with CrLIP1 protein produced in yeast cells. To overcome the low abundance of the HA (hemagglutinin) tagged CrLIP1 protein in yeast strains (not detectible on SDS-PAGE by Coomassie Brilliant Blue staining), it can be enriched by immunoprecipitation with an anti-HA antibody.

Some other evidence seems to suggest that CrLIP1 might be mainly a DAG lipase as it was detected in the flagellar proteome of Chlamydomonas (Pazour et al., 2005). The flagellar membrane is physically connected to the plasma membrane, and is the location for human DAG lipases (Bisogno et al., 2003). In contrast, TAG lipases in yeast and Arabidopsis were found to be localized on lipid droplets (Athenstaedt and Daum, 2003, 2005; Eastmond, 2006; Kurat et al., 2006). For the yeast strain over-expressing CrLIP1, localization can be accomplished by immunofluorescence by taking advantage of the HA tag. In Chlamydomonas, production of a GFP fusion protein failed so production of a protein with an epitope tag or generation of an antibody against CrLIP1 with the recombinant protein from E. coli will be favored.

Even if CrLIP1 is shown to have in vitro TAG lipase activity, it is not likely the major TAG lipase in Chlamydomonas because in the AmiRNA lines the TAG turnover was slightly decreased but not abolished. After 36 h of growth in N-containing medium, the TAG contents in both the knock down lines and the empty vector control were below 3 % of total lipids. If CrLIP1 is not responsible, then which gene in Chlamydomonas is encoding the major TAG lipase? In the model seed plant Arabidopsis thaliana, mutation in the lipase-encoding SDP1 gene
blocked seed oil liberation and consequently, resulted in slower post-germinative growth (Eastmond, 2006). A BLAST (Altschul et al., 1997) search of the Chlamydomonas genome (http://genome.jgi-psf.org/pages/blast.jsf?db=Chlre4) with SDP1 protein sequence showed a candidate with an E-Value of 2.97E-98. The cDNA of this candidate gene was recently cloned (Jaruswan Warakanont, unpublished) and future work should focus on biochemical characterization of this protein and phenotypic analysis of its RNA suppression lines.

**Oleate can be exported from the plastid through an acylation/deacylation cycle mediated by PGD1.**

To complement the reverse genetic approach, a large-scale forward genetic screen was performed to obtain mutants with abnormal TAG amounts. This led to the identification of the PGD1 gene (described in Chapter 3) which was actually one of the putative lipase-encoding genes up-regulated under N-deprivation (Miller et al., 2010).

Disruption of the *PGD1* gene lowered the relative amount of oleate (18:1\(^\Delta 9\)) in several glycerolipids, including the storage lipid TAG and the membrane lipids diacylglycerol-\(N,N,N,\)-trimethylhomoserine (DGTS) and phosphatidylethanolamine (PtdEtn). All three of these are likely to be located outside of the plastid. In seed plants PtdEtn has been shown to be absent from the plastid (Jouhet et al., 2007) and DGTS is presumed to extraplastidic (Giroud and Eichenberger, 1988). TAG is at least partly extraplastidic in Chlamydomonas as some lipid droplets clearly exist in the cytosol despite the finding of plastid-located lipid droplets in starchless mutants of Chlamydomonas (Fan et al., 2011; Goodson et al., 2011). The fact that these three lipids lack the same fatty acid strongly suggest that export of 18:1\(^\Delta 9\) from the plastid
is blocked by the \textit{pgd1} mutation. Pulse-chase labeling showed that upon the switch to N-deprivation, \textit{de novo} synthesized acyl groups go through the most abundant plastid lipid monogalactosyldiacylglycerol (MGDG) before being incorporated into TAG. \textit{In vitro} assays proved PGD1 to be a lipase which digests the \textit{sn-1} position of MGDG, with a preference for \textit{de novo} synthesized species (18:1$^{\Delta 9}$/16:0). These data support the presence of an acylation/deacylation cycle acting on lyso-MGDG/MGDG to export 18:1$^{\Delta 9}$.

This discovery is an important advance in understanding how the fatty acids are exported from the plastid for the green lineage organisms. However, due to the complexity of the fatty acid export process, several major questions remain unsolved. The first question is: is there another mechanism for exporting fatty acids? There are two major reasons that I suspect other mechanisms exist to export fatty acids from the plastid. First of all, the acylation/deacylation cycle proposed applies only to 18:1$^{\Delta 9}$ while 16:0 is another major fatty acid exported to the cytosol (Bates et al., 2007). The activity of PGD1 on the \textit{sn-1} position of \textit{de novo} synthesized MGDG does not directly facilitate the trafficking of 16:0. Secondly, the \textit{PGD1} gene is up-regulated by approximately 10 fold under N-deprivation while the vegetative growth under N replete conditions should also require the export of 18:1$^{\Delta 9}$. Probably there is a basal mechanism for the export while the acylation/deacylation cycle of MGDG is induced upon stress conditions that require a higher capacity of fatty acid export. This interpretation also explains the unaffected vegetative growth of \textit{pgd1} mutant.

Details of the acylation/deacylation mechanism also require further study. One immediate question is where the PGD1 protein is localized. No transit peptide was identified for PGD1 with
the TargetP program (http://www.cbs.dtu.dk/services/TargetP/). However, even if PGD1 is cytosolically localized, it would have access to the MGDG pool as MGDG is present on the outer envelope, inner envelope as well the thylakoids of a chloroplast (Jouhet et al., 2007). Similar to the case of CrLIP1, I was unable to create a successful PGD1 cDNA-GFP fusion protein was not successfully produced. This might be due to a transcriptional silencing mechanism which is hypothesized to be present in Chlamydomonas to repress exogenous genes (Neupert et al., 2009). Antibody against PGD1 is being raised with a synthetic PGD1 fragment and will be tested by my co-workers in the future.

If the proposed model is true, an acyltransferase which uses lyso-MGDG as the acyl acceptor, acyl-ACP (acyl carrier protein) as the acyl donor, and prefers to acylate at the sn-1 position is required to complete the cycle. Such an acyltransferease has been discovered in a cyanobacterium (Chen et al., 1988). Given the widely accepted endosymbiosis hypothesis that plastids evolved from an engulfed cyanobacterium (Howe et al., 2008), I expect this acyltransferase exists in Chlamydomonas as well. However, this enzyme was not purified to homogeneity and the protein sequence is not currently available. Identification of this acyltransferase can be accomplished if more mutants with phenotypes similar to those of pgd1 are obtained. Alternatively, this acyltransferase can be identified through biochemical characterizations of multiple candidate acyltransferases.

Last but not least, it remains unclear where the exported 18:1Δ9 is incorporated into the DAG backbone for TAG biosynthesis. Different from seed plants, the sn-2 position of TAGs in Chlamydomonas is almost exclusively C16 fatty acids, which indicates a chloroplast origin of DAG backbones for TAG synthesis by the diacylglycerol acyltransferase (DGAT) activity (Fan
et al., 2011). However, in a starchless mutant, lipid droplets were observed both inside and outside of the plastid (Fan et al., 2011; Goodson et al., 2011) while in its parental strain only cytosolic lipid droplets were observed despite that they are also associated with the plastid envelopes as well as the ER membrane (Goodson et al., 2011). The exact location of TAG synthesis will await the identifications of the major diacylglycerol acyltransferases.

This work has important implications for the lipid metabolism of land plants. In the model seed plant Arabidopsis thaliana, people have proposed a similar acylation/deacylation cycle, with phosphatidylcholine (PtdCho) as the intermediate (Bates et al., 2007). Recombinant PGD1 protein exhibited activity on both MGDG and PtdCho (unpublished). BLAST (Altschul et al., 1997) of the PGD1 protein sequence against the Arabidopsis genome (http://arabidopsis.org/) indicated three genes with high similarity which are possibly involved in the acylation/deacylation cycle on PtdCho. While PtdCho is absent in Chlamydomonas (Riekhof et al., 2005) both lipids are present in bulk in Arabidopsis (Jouhet et al., 2007). Expressing the proteins from these three genes in vitro and testing their substrate preference is necessary to establish their functions. Phenotypic analysis of mutants disrupted in one or more of these three genes would be a complementary approach to reveal whether they are involved in fatty acid export in Arabidopsis. Algae accumulate TAG under certain stresses while plants produce oil in certain tissues such as seeds and pollen (Zhang et al., 2009). Tissue-specific expression databases will provide important clues about the roles of these genes.

Triacylglycerol accumulation in Chlamydomonas reinhardtii prevents oxidative stress under N-deprivation
TAG accumulation under N-deprivation has been repeatedly reported without extensive studies of the physiological significance of this phenomenon. The low TAG mutant \textit{pgd1} was used as a tool to understand the consequences of lower TAG accumulation as described in Chapter 4.

The \textit{pgd1} mutant exhibited chlorosis, accumulated thiobarbituric acid reactive substances (TBARS) and lost viability after 7 days of N-deprivation. These phenotypes could not be ascribed to altered membrane lipid metabolism as the \textit{pgd1} mutant is specifically altered in the amount of TAG without a major difference from the wild-type parental strain in membrane lipid composition. Inhibition of photosynthetic electron transport by exposure to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Trebst et al., 1970) in the medium reversed the phenotypes of the \textit{pgd1} mutant. The following model was proposed to explain the observations above: under N-deprivation, cells stop dividing, and consequently the NADPH generated from photosynthetic electron transport might be in excess compared to N-replete conditions. This can lead to the reduction of alternative electron acceptors including molecular oxygen (Asada, 1994). By synthesizing fatty acids which are highly reduced compared to carbohydrates and proteins, NADPH can be sequestered to prevent the overreduction of the photosynthetic electron transport chain.

While the above model is supported by the avoidance of chlorosis, TBARS accumulation and loss of viability following treatment with DCMU, it can be further corroborated if multiple independent mutants deficient in TAG production exhibit similar phenotypes. A suppressor screen has been performed in the \textit{pgd1} mutant background for suppression of the chlorosis phenotype (Chapter 4). Several suppressors that result from second site disruption have been isolated. Further molecular and physiological characterization of two suppressors is underway. I
expect some of the mutants to be affected in aspects of the generation/detoxification of reactive oxygen species or photosynthesis.

Under N deprivation, Chlamydomonas cells also accumulate a considerable amount of starch (Work et al., 2010), which might function as another sink for the photosynthetic electron flux. In mutants that were disabled in starch biosynthesis, \textit{sta6} and \textit{sta7}, TAG was over-accumulated (Fan et al., 2012; Work et al., 2010). Apparently TAG biosynthesis served to consume the NADPH which could not be allocated to starch biosynthesis. With a low-TAG mutant \textit{pgd1}, it is possible that starch accumulation will be enhanced to partly relieve the overreduction of the photosynthetic electron transport chain, and \textit{pgd1} still loses viability because starch synthesis consumes NADPH less efficiently than fatty acid synthesis on a carbon number base (Hu et al., 2008). If this hypothesis is correct, a double mutant with both the \textit{pgd1} mutation and the \textit{sta6} or \textit{sta7} mutation will lose viability earlier than the \textit{pgd1} mutant.

This project also provides clues about how TAG synthesis can be induced. Conceivably, instead of being triggered by the nutrient deprivation, the overreduction of the photosynthetic electron transport chain may be the signal to activate the lipid biosynthesis machinery. These possibilities can be tested by further pharmacological manipulations of photosynthesis or forward genetic screens for abnormal regulation on important marker genes such as the \textit{DGAT} genes. Answers to these questions will contribute to the development of algae as important sources of bioenergy.
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