the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **82,** 504–522 doi: 10.1111/tpj.12787

SI CHLAMYDOMONAS

Metabolism of acyl-lipids in *Chlamydomonas reinhardtii*

Yonghua Li-Beisson[1,2,3,*], Fred Beisson[1,2,3] and Wayne Riekhof[4]

[1]*Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Institut de Biologie Environnementale et Biotechnologie, CEA Cadarache, 13108 Saint-Paul-lez-Durance, France,*

[2]*Centre National de la Recherche Scientifique (CNRS), 13108 Saint-Paul-lez-Durance, France,*

[3]*Aix-Marseille Université, UMR 7265, 13284 Marseille, France, and*

[4]*School of Biological Sciences and Center for Biological Chemistry, University of Nebraska – Lincoln, Lincoln, NE 68588, USA*

Received 14 December 2014; revised 24 January 2015; accepted 2 February 2015; published online 7 February 2015.
*For correspondence (e-mail yonghua.li@cea.fr).

SUMMARY

Microalgae are emerging platforms for production of a suite of compounds targeting several markets, including food, nutraceuticals, green chemicals, and biofuels. Many of these products, such as biodiesel or polyunsaturated fatty acids (PUFAs), derive from lipid metabolism. A general picture of lipid metabolism in microalgae has been deduced from well characterized pathways of fungi and land plants, but recent advances in molecular and genetic analyses of microalgae have uncovered unique features, pointing out the necessity to study lipid metabolism in microalgae themselves. In the past 10 years, in addition to its traditional role as a model for photosynthetic and flagellar motility processes, *Chlamydomonas reinhardtii* has emerged as a model organism to study lipid metabolism in green microalgae. Here, after summarizing data on total fatty acid composition, distribution of acyl-lipid classes, and major acyl-lipid molecular species found in *C. reinhardtii*, we review the current knowledge on the known or putative steps for fatty acid synthesis, glycerolipid desaturation and assembly, membrane lipid turnover, and oil remobilization. A list of characterized or putative enzymes for the major steps of acyl-lipid metabolism in *C. reinhardtii* is included, and subcellular localizations and phenotypes of associated mutants are discussed. Biogenesis and composition of *Chlamydomonas* lipid droplets and the potential importance of lipolytic processes in increasing cellular oil content are also highlighted.

Keywords: *Chlamydomonas reinhardtii*, green microalgae, membrane lipids, lipid droplets, desaturases, acyltransferases, lipases, lipid mutants, microalgal oil, biofuels.

INTRODUCTION

The term 'lipid' traditionally encompasses several groups of hydrophobic or amphipathic compounds that are structurally and functionally unrelated, but have in common their solubility in organic solvents and often poor solubility in water. These compounds play a variety of important biological functions in cells, including as basic components of biological membranes (e.g. phosphoglycerolipids, galactoglycerolipids, sterols, sphingolipids), reserve compounds (triacylglycerols or TAGs) and signaling molecules (e.g. phosphoinositides, oxylipins) (Somerville *et al.*, 2000).

Lipids are usually classified based either on chemical composition and structure (Harwood and Scrimgeour, 2007), self-assembly properties in aqueous systems (Small, 1968) or biosynthetic origin (Fahy *et al.*, 2005, 2009). From a metabolic point of view, a useful distinction is to be made between lipids derived from fatty acids (FAs), which are often referred to as acyl-lipids and represent the vast majority of lipids found in cells (mostly glycerolipids and sphingolipids) and lipids that have other biosynthetic origins (e.g. sterols, prenols, and polyketides). Most acyl-lipids are glycerolipids, with a glycerol backbone esterified by two FAs and a head group (or three fatty acids in the case of TAGs). The variations in head groups, as well as the nature of acyl chains and their stereospecific positions on the glycerol molecule expand the number of possible glycerolipid species to hundreds or thousands of distinct

molecules. Many sphingolipid structures also exist, which differ by their sphingoid base backbone, the *N*-linked fatty acid, and their *O*-linked head group (Sperling and Heinz, 2003). Using modern lipidomic tools, 100–300 different molecular species of acyl-lipids can be currently detected in a typical photosynthetic eukaryote (Welti and Wang, 2004; Liu *et al.*, 2013; Nguyen *et al.*, 2013; Vu *et al.*, 2014).

Acyl-lipid classes come in different proportions in the various cell membranes. In photosynthetic organisms the synthesis of FAs occurs in chloroplasts and the assembly of many lipid classes is restricted to specific cell compartments and membranes (Li-Beisson *et al.*, 2013). The study of the trafficking of FAs and acyl-lipids between membranes is therefore an important aspect of plant and microalgal cell biology (Jouhet *et al.*, 2007; Benning, 2009).

Besides their biological functions, lipids are a major component of animal feed and human diet, an important source of food supplements and nutraceuticals, a major feedstock for chemical industries, and a promising renewable alternative to petroleum-based materials and fuels (Durrett *et al.*, 2008; Lu *et al.*, 2011). Among all lipid sources, microalgae have attracted biotechnologists because they are primary producers of very long-chain ω-3 polyunsaturated FAs (VLC-PUFAs), which are important for human nutrition (Riediger *et al.*, 2009). Microalgal strains have thus been used either as a producer of these nutritional VLC-PUFAs or as a source for isolation of unusual fatty acid desaturases (Guschina and Harwood, 2006; Venegas-Caleron *et al.*, 2010; Khozin-Goldberg *et al.*, 2011; Ruiz-Lopez *et al.*, 2012). More recently, the ability of microalgae to accumulate high amounts of oil, and their high biomass productivity, have made them one of the most promising oil producers for biodiesel (Chisti, 2007; Hu *et al.*, 2008; Wijffels and Barbosa, 2010; Liu and Benning, 2013). However, yields and fatty acid composition of microalgal oils need to be improved for biofuel or food applications (Hu *et al.*, 2008), which require genetic modifications and a deeper understanding of acyl-lipid metabolism in model microalgal species (Merchant *et al.*, 2012; Liu and Benning, 2013).

Historically, the green model microalga *Chlamydomonas reinhardtii* has not been the focus of many studies on fatty acid metabolism because it does not synthesize VLC-PUFAs and was considered as 'non-oleaginous'. It was thus excluded from the 'Aquatic Species Program' screening effort led by the US Department of Energy in the 1970s (Sheehan *et al.*, 1998). However, in the last 10 years, given the renewed interest in microalgal oil, *Chlamydomonas* has emerged as a model for dissecting the molecular mechanism of oil accumulation (Merchant *et al.*, 2012; Liu and Benning, 2013). Compared with other microalgal species, *Chlamydomonas* has a well understood physiology, a fully sequenced genome, and the most developed molecular genetic and genomic tools, thus allowing genetic engineering of metabolic pathways (Rochaix, 2002; Merchant *et al.*, 2007; Day and Goldschmidt-Clermont, 2011; Michelet *et al.*, 2011). *Chlamydomonas* has a high capacity for synthesizing lipids, for example, lipids comprise around 20% of dry biomass of vegetative cells (Figure 1). In this review, we summarize 40 years of research on acyl-lipid metabolism in *C. reinhardtii*, from the early biochemical works of the 1970s, to the first isolation of lipid mutants, to the many genetic and cell biological studies that have followed the sequencing of the nuclear genome in 2007 (Merchant *et al.*, 2007). For simplicity, we use '*Chlamydomonas*' in this review to refer to *C. reinhardtii.*

A SHORT HISTORY OF LIPID RESEARCH IN *C. REINHARDTII*

Due to its unicellular nature, high growth rate, remarkable metabolic flexibility under distinct environmental and nutrient conditions, *C. reinhardtii*, compared with land plants, offered some unique opportunities to address basic

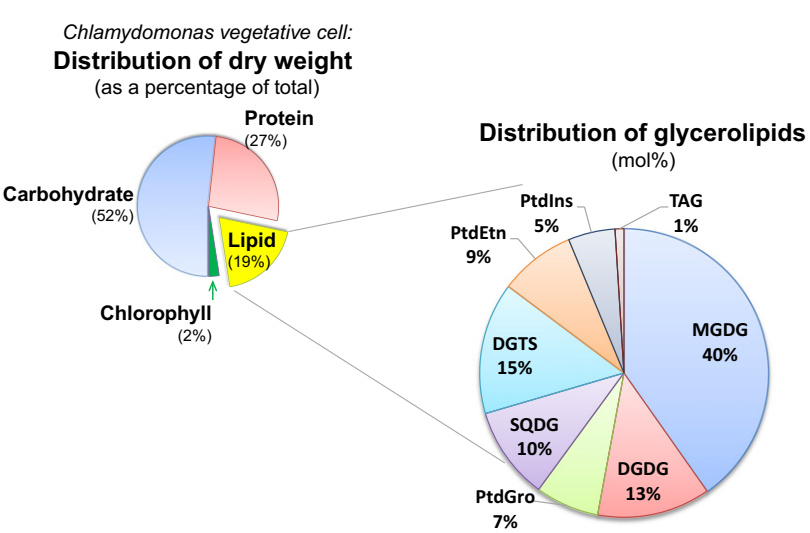

Figure 1. Distribution of dry weight and glycerolipids in *Chlamydomonas*.
The data are based on that of (Boyle and Morgan, 2009) and (Giroud *et al.*, 1988). These compositions were those of vegetative cells cultivated under photoautotrophic conditions.
MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; SQDG, sulfoquinovosyldiacylglycerol; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TAG, triacylglycerol.

questions in lipid metabolism. The first report on lipids of *Chlamydomonas* can be dated back to 1972 when Sirevag and Levin (Sirevåg and Levine, 1972) demonstrate that cell-free extracts of *C. reinhardtii* catalyzed the incorporation of acetyl-CoA and malonyl-CoA into long-chain FAs. This paper was followed by a series of studies in the 1970s to 1980s focusing on biochemical analyses of fatty acid composition and lipid molecular species, and the site of their biosynthesis (Eichenberger, 1976; Eichenberger and Boschetti, 1978; Schlapfer and Eichenberger, 1983; Giroud and Eichenberger, 1988, 1989; Giroud *et al.*, 1988). Many of these works are still useful references for current genetic dissection of lipid metabolism in *Chlamydomonas*.

The first *Chlamydomonas* mutant defective in a gene implicated in lipid metabolism was isolated based on screening of chlorophyll fluorescence (Seras *et al.*, 1989). Two large scale mutant screens were done subsequently, both were based on the screening of modifications in photosynthetic fluorescence (Sato *et al.*, 1995; El Maanni *et al.*, 1998; Pineau *et al.*, 2004). Direct screening of *Chlamydomonas* mutants for a defect in lipid compositions have first been carried out in the group of Professor Christoph Benning (Michigan State University), and this led to the isolation of a mutant deficient in the synthesis of sulfoquinovosyl diacylglycerol (SQDG) (Riekhof *et al.*, 2003). Since then, other screening efforts have been launched in several laboratories and have led to the identification of three other proteins of lipid metabolism (Li *et al.*, 2012a; Nguyen *et al.*, 2013; Tsai *et al.*, 2014). In parallel to these efforts, reverse genetic approaches based on multiplex PCR-based screening have also led to the isolation of null mutants, *pdat1-1*(*pdat1-2)* (defective in the phospholipid: diacylglycerol acyltransferase, PDAT) and *nrr1* (*n*itrogen-*r*esponsive *r*egulator 1) defected in a protein with a DNA-binding domain thus likely to be a transcription factor (Gonzalez-Ballester *et al.*, 2011; Boyle *et al.*, 2012). These and all other known *Chlamydomonas* lipid mutants are summarized in Table 1.

A gene annotation effort focusing on glycerolipid metabolism was undertaken by Riekhof and collaborators before the release of the *Chlamydomonas* genome sequences (Riekhof *et al.*, 2005). In total, 48 proteins orthologous to known plant and yeast lipid proteins were found and metabolic maps for key glycerolipid synthesis and fatty acid desaturations were constructed.

Current research on *Chlamydomonas* lipids and their metabolism is multifaceted and powered by the development of sophisticated genetic, genomic, transcriptomic, proteomic and lipidomic tools (Rolland *et al.*, 2009; Miller *et al.*, 2010; Lopez *et al.*, 2011; Dutcher *et al.*, 2012; Liu *et al.*, 2013; Nguyen *et al.*, 2013; Schmollinger *et al.*, 2014; Zhang *et al.*, 2014). These have led to discoveries of novel aspects of lipid metabolism in *C. reinhardtii*, which will be discussed below.

BASIC FEATURES OF *CHLAMYDOMONAS* LIPIDS

Fatty acid composition

The fatty acid composition of *Chlamydomonas* has been determined and reported numerous times recently (Zauner *et al.*, 2012; Nguyen *et al.*, 2013; Pflaster *et al.*, 2014), and the composition and biosynthesis of FA in this organism was determined initially and investigated experimentally in the late 1980s (Giroud and Eichenberger, 1988; Giroud *et al.*, 1988). *Chlamydomonas* FA compositions share features typical of higher plants such as Arabidopsis, in that essentially all FA esterified to the polar glycerolipids are of 16 or 18 carbons. With the exception of a significant amount of palmitic acid (16:0) esterified in the *sn*-2 position of the chloroplastic DGDG and SQDG polar lipid classes, nearly all of the FAs are polyunsaturated, as discussed below. The most divergent feature of *Chlamydomonas* fatty acid composition, relative to higher plants and most other chlorophyte algae, is the presence of Δ4 and Δ5 unsaturated PUFA, which are synthesized by front-end desaturases in the chloroplast (Zauner *et al.*, 2012) and ER (Kajikawa *et al.*, 2006), respectively. The chloroplast-localized desaturase, denoted as CrΔ4FAD (Zauner *et al.*, 2012) acts upon monogalactosyldiacylglycerol (MGDG) to generate the novel PUFA 16:4$\Delta^{4,7,10,13}$, a component of the predominant MGDG molecular species (Figure 2), while the ER-localized Δ5 desaturase acts on linoleic (18:2$\Delta^{9,12}$) and α-linolenic (18:3$\Delta^{5,9,12}$) acids esterified to diacylglycerol-*N*,*N*,*N*-trimethylhomoserine (DGTS) and phosphatidylethanolamine (PtdEtn), and forms the *bis*-methylene interrupted FAs pinolenic acid (PA, 18:3$\Delta^{5,9,12}$) and coniferonic acid (CA, 18:4$\Delta^{5,9,12,15}$). A recent study (Pflaster *et al.*, 2014) has also identified *Chlamydomonas*-like strains of the Volvocales algal class which make Δ6 unsaturated FA, which are esterified to DGTS and presumably PtdEtn, analogous to the Δ5 unsaturated species made by the standard laboratory isolates of *C. reinhardtii.* This discovery calls into question the functional significance of the specificity and regioselectivity of front-end desaturases in the genus. The functions of these highly unsaturated FA in extrachloroplastic membranes of *Chlamydomonas* remain uncharacterized, and would be prime candidates for genetic dissection and functional analysis of mutants lacking extrachloroplastic front-end desaturase activity.

Composition of polar glycerolipid classes

The composition of polar glycerolipid headgroups has been originally determined in the previously cited works of Giroud and Eichenberger (Giroud *et al.*, 1988) and subsequent studies have largely confirmed these early determinations. Two key and defining features of polar glycerolipid compositions in *Chlamydomonas* relative to higher plants are: (i) the apparent absence of the otherwise ubiquitous phospholipids, phosphatidylcholine (PtdCho)

Table 1 A summary of *Chlamydomonas* mutants defected in lipid metabolism

Mutant	Target(s) of the mutation	Type of mutagenesis	Major phenotypes	References
mf1	Δt3 and Δ9 chloroplast desaturases (putative)	UV radiation	0% PtdGro-16:1(3t); 30% wild-type level of PtdGro; 0% of PSII activity remaining	Pineau *et al.* (2004), Trémolières *et al.* (1991)
mf2	Δt3 and Δ9 chloroplast desaturases (putative)	UV radiation	0% PtdGro-16:1(3t); 30% wild-type level of PtdGro; zero of PSII activity remaining	
pmf1	Δt3 and Δ9 chloroplast desaturases (putative)	UV radiation	10% PtdGro-16:1(3t); 35% wild-type level of PtdGro; 10% of PSII activity remaining	El Maanni *et al.* (1998)
pmf2	Δt3 and Δ9 chloroplast desaturases (putative)	UV radiation	10% PtdGro-16:1(3t); 35% wild-type level of PtdGro; 10% of PSII activity remaining	
hf-2	SQDG synthesis	UV radiation	5% of wild-type level of SQDG; 60% of PSII activity remaining	Sato *et al.* (1995)
hf-9 (fad6)	Chloroplast omega-6 fatty acid desaturase	UV radiation	Trace amount of chloroplast PUFA level; 10% of PSII activity remaining	
lpb1/ugp3	UDP-glucose pyrophosphorylase	Insertional mutagenesis	Dies more rapidly under P-starvation than WT	Chang *et al.* (2005)
sqd1	UDP-sulfoquinovose synthase	Insertional mutagenesis	0% of SQDG and ASQD remaining; reduced growth under P-starvation	Riekhof *et al.* (2003)
crfad7/ CC-620	ω-3 fatty acid desaturase	Insertional mutagenesis/ natural mutation	<30% ω-3 fatty acids remaining in the *crfad7* mutant; and 0% in the strain CC-620	Nguyen *et al.* (2013), Pflaster *et al.* (2014)
pdat1-1/ *pdat1-2*	Phospholipids:diacylglycerol acyltransferase	Insertional mutagenesis	Insertional mutants, *pdat1-1* and *pdat1-2*, accumulate 25% less TAG compared with the parent strain	Boyle *et al.* (2012)
nrr1-1	Nitrogen regulator 1	Insertional mutagenesis	*nrr1-1* accumulates only 50% of the TAG compared with the parental strain in nitrogen-starvation conditions	
pgd1	Galactolipid lipase	Insertional mutagenesis	reduced TAG content, altered TAG composition, and reduced galactoglycerolipid turnover	Li *et al.* (2012a)
cht7	Defected in a DNA-binding protein	Insertional mutagenesis	Unable to use oils stored in the droplet following nitrogen recovery, thus severely impaired in regrowth	Tsai *et al.* (2014)

ASQD, 2′-*O*-acyl-sulfoquinovosyldiacylglycerol; *cht*, compromised hydrolysis of triacylglycerols; *hf*, high fluorescence; *lpb*, low phosphate bleaching; *mf*, minimum fluorescence; PSII, photosystem II; *pmf*, photosynthesis minimum fluorescence; *pgd*, plastid galactoglycerolipid degradation; PtdGro, phosphatidylglycerol; PUFA, polyunsaturated fatty acids; UV, ultraviolet; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; WT, wild-type.
This figure is partly based on that of Trémolières (1998).

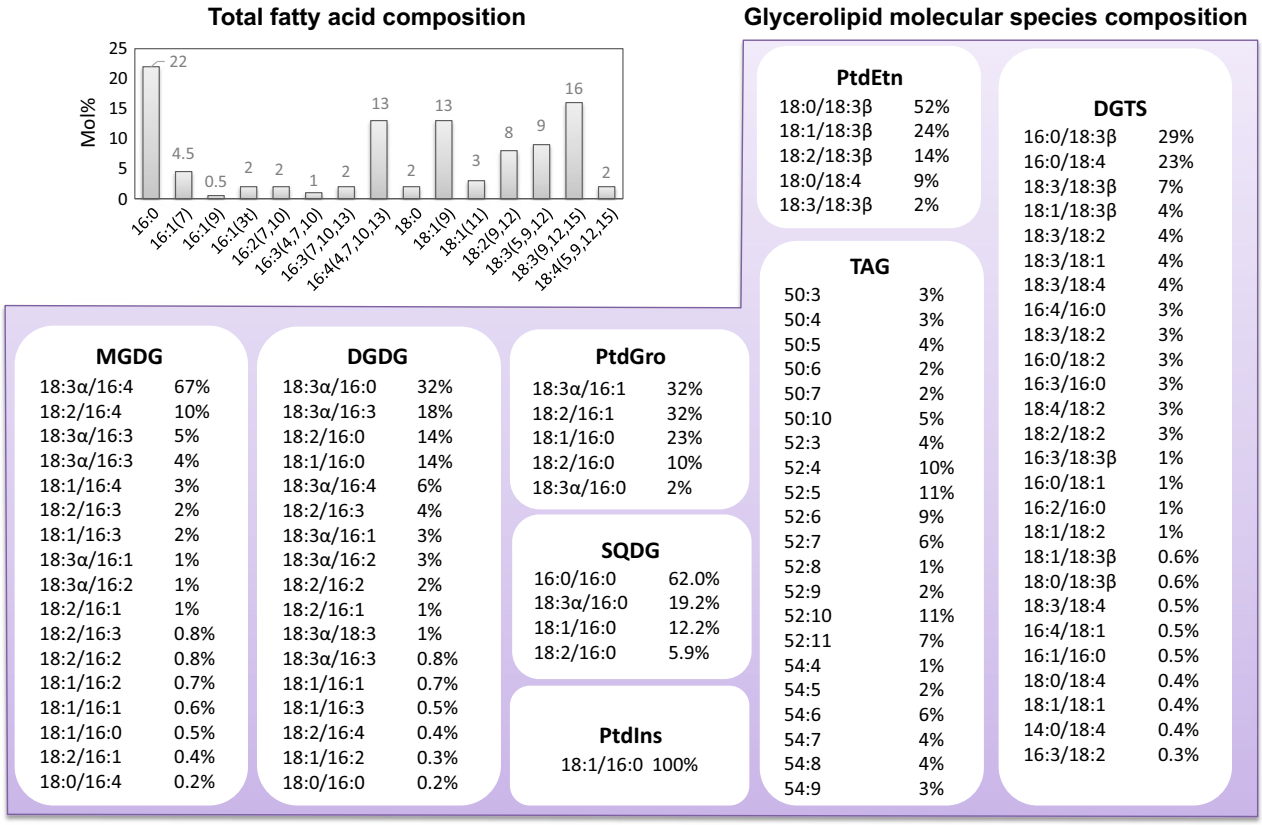

Figure 2. Fatty acid and glycerolipid molecular species distribution in *Chlamydomonas*.
Whole-cell fatty acid composition are from (Siaut *et al.*, 2011) and relative abundance for each molecular species of glycerolipid from (Nguyen *et al.*, 2013) and 18:3 isomer distribution is partly based on (Trémolières, 1998). Molecular species of membrane lipids are written as lipid class (*sn*-1 FA/*sn*-2 FA); and for TAGs, only total carbon: total number of double bonds are shown. The percentage is the relative abundance of this lipid molecule within that particular lipid class. 18:3α refers to 18:3$\Delta^{9,12,15}$ and 18:3β refers to 18:3$\Delta^{5,9,12}$. When nothing is noted, suggested that it could be either of above fatty acids.
FA, fatty acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; SQDG, sulfoquinovosyldiacylglycerol; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TAG, triacylglycerol.

and phosphatidylserine (PtdSer) (Giroud *et al.*, 1988; Moellering *et al.*, 2010); and (ii) presence of the non-phosphorous betaine lipid, DGTS. As indicated in Figures 1 and 2, using data from (Siaut *et al.*, 2011; Nguyen *et al.*, 2013), the polar headgroup composition of wild-type *Chlamydomonas* chloroplasts growing in nutrient replete culture is dominated by galactolipids, sulfolipids, and phosphatidylglycerol, and these species almost exclusively contain 16:X FA at the *sn*-2 position, which is characteristic of the 'prokaryotic' type lipid biosynthetic machinery of the chloroplast. Conversely, in the extrachloroplastic space, PtdEtn and DGTS predominate, and have a reversal of *sn*-2 specificity, being almost exclusively esterified with 18:X FA. This almost complete separation of *sn*-2 specificity between ER and chloroplast lipids mimics that which would be predicted from higher plants in which the 'eukaryotic' pathway of plastid glycerolipid synthesis has been inactivated, e.g. by inactivating the TGD complex that mediates lipid transfer between the ER and the chloroplast (Hurlock *et al.*, 2014).

FATTY ACID SYNTHESIS

Analogous to higher plants, synthesis of C16 and C18 FAs in *Chlamydomonas* is presumed to occur exclusively in the chloroplast using a multi-subunit bacterial-type acetyl-CoA carboxylase and type II fatty acid synthase, as outlined in Figure 3. However multiple components of the multipartite type II FAS enzyme system were predicted to be dual targeted to both the mitochondrion and chloroplast (Riekhof *et al.*, 2005). This proposition was based on three pieces of evidence:

1. Mitochondria in plants are known to synthesize short-chain FA as precursors for lipoic acid biosynthesis (Wada *et al.*, 1997).

2. The genes encoding components of the type II FAS are predicted to be dual targeted to chloroplasts and mitochondria by TargetP analysis.

3. The genes encoding the type II FAS components are present as single copies in the *Chlamydomonas* genome, while they are encoded by multiple genes in higher plants.

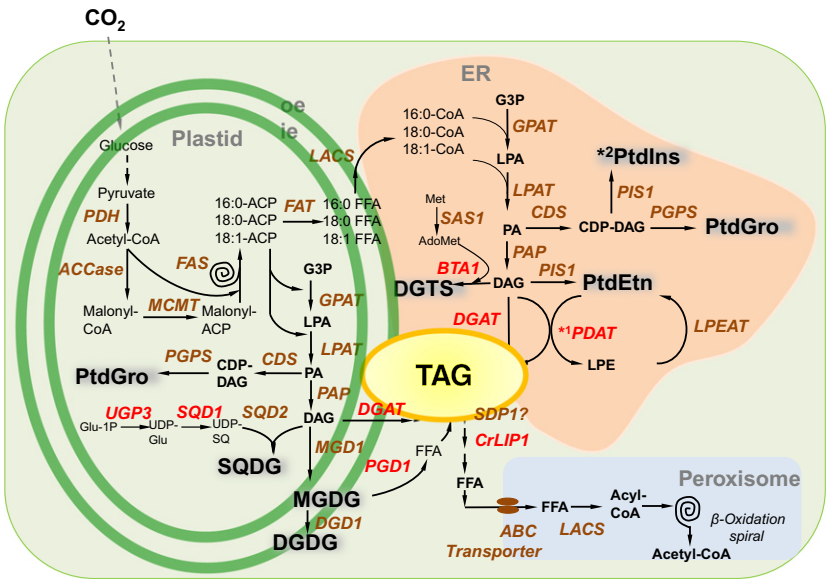

Figure 3. Reactions known or thought to be involved in glycerolipid metabolism in *Chlamydomonas*.
End products are in bold; Enzymes are in brown and italic. *[1]: PDAT has been shown to use multiple lipids as substrate, and in this figure, for simplicity reasons, it is drawn next to a PtdEtn. *[2]: For simplicity reason, PtdIns synthesis is drawn here in the ER, but so far evidence for location of this reaction is still lacking. In red are the enzymes that have been characterized.
ABC, ATP-binding cassette; ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; AdoMet, *S*-adenosylmethionine; BTA1, betaine lipid synthase; CDS, mitochondrial half-size ABC transporter; CoA, coenzyme A; CDP, cytidine 5′-diphosphate; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGD1, digalactosyldiacylglycerol synthase; ER, endoplasmic reticulum; FAT, fatty acyl-ACP thioesterase; FAS, fatty acid synthase; FFA, free fatty acid; G3P, glycerol 3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; LACS, long chain acyl-CoA synthetase; LPA, lysophosphatidic acid; LPAT, lysophosphatidic acid acyltransferase; LPE, lysophosphatidylethanolamine; MCMT, malonyl-CoA:acyl carrier protein malonyltransferase; MGD1, monogalactosyldiacylglycerol synthase; PA, phosphatidic acid; PDAT, phospholipid:diacylglycerol acyltransferase; PDH, pyruvate dehydrogenase; PAP, phosphatidic acid phosphatase; PGPS, phosphatidylglycerolphosphate synthase; PIS1, phosphatidylinositol synthase; SAS1, *S*-adenosylmethionine synthetase; SDP1, sugar dependent 1; SQD1, UDP-sulfoquinovose synthase; SQD2, sulfoquinovosyldiacylglycerol synthase; TAG, triacylglycerol; UDP, uridine 5′-diphosphate; UGP3, UDP-glucose pyrophosphorylase.

Table 2 presents a comprehensive list of gene designations that are predicted to encode components of the fatty acid synthesis machinery, as well as the thioesterases which are predicted to release FA from acyl carrier protein (ACP) for export from the chloroplast and incorporation into glycerolipids in the ER.

MEMBRANE GLYCEROLIPID BIOSYNTHESIS

The major pathways of diacylglycerol (DAG) assembly in both chloroplasts and ER are predicted to be conserved between *Chlamydomonas* and higher plants based on the presence of genes homologous to the glycerol-3-phosphate acyltransferases (GPAT) and lyso-phosphatidate acyltransferases (LPAT) in higher plants (Table 2). Likewise, the major pathways of galactolipid, sulfolipid, and phosphatidylglycerol (PtdGro) synthesis are apparently conserved between *Chlamydomonas* and Arabidopsis (Figure 4), indicating that these core lipid metabolic pathways have remained unchanged throughout the course of plant evolution. One possible exception could be that of synthesis of phosphatidylinositol (PtdIns) because there is only one molecular species occur in *Chlamydomonas* (see Figure 2) and its *sn*-2 position is esterified with 16:0, usually suggesting a plastidial-based LPAT. Nevertheless, none of the LPAT specificities has been characterized so far for *Chlamydomonas*, thus we are not sure if PtdIns synthesis occur in the ER as in most higher plants, or if it occurs in the plastid.

The novel lipid composition of *Chlamydomonas* discussed above can be accounted for by identification of genes that are present in *Chlamydomonas* but not in higher plants, or those that are apparently missing from *Chlamydomonas*, relative to Arabidopsis or yeast. First, PtdCho is absent in *Chlamydomonas*, and is apparently replaced by the non-phosphorous betaine lipid DGTS. Synthesis of DGTS is carried out by the BTA1 enzyme (Riekhof *et al.*, 2005), which is a multi-domain protein that carries out all four enzymatic reactions of DGTS synthesis; transfer of the homoserine carbon skeleton of *S*-adenosylmethionine (SAM) to DAG, forming the ether-linked intermediate diacylglycerylhomoserine (DGHS). This is followed by *N*-trimethylation of the intermediate by three additional SAM, forming DGTS. This system for synthesis of DGTS is also present in fungi, and is regulated by phosphate starvation (Riekhof *et al.*, 2014), resulting in the replacement of PtdCho with DGTS in many fungal species encountering phosphate deprivation.

The absence of phosphatidylserine (PtdSer) is consistent with an apparent lack of both CDP-DAG-dependent and

Table 2 A summary of predicted genes and proteins involved in major steps of acyl-lipid metabolism in *Chlamydomonas reinhardtii*

Pathway	JGI v5.5 (Augustus u111.6) ID	Gene name abbreviations	Description	Subcellular localization	References
FA biosynthesis	Cre12.g519100.t1.2	ACX1 (α-CT)	α-Carboxyltransferase (ACCase complex)	C	n.a.
	Cre12.g484000.t1.2	BCX1 (β-CT)	β-Carboxyltransferase (ACCase complex)	C	n.a.
	Cre17.g715250.t1.2	BCC1	Acetyl-CoA biotin carboxyl carrier protein (ACCase complex)	C	n.a.
	Cre01.g037850.t1.1	BCC2	Acetyl-CoA biotin carboxyl carrier protein (ACCase complex)	C	n.a.
	Cre08.g359350.t1.2	BCR1	Biotin carboxylase (ACCase complex)	C	n.a.
	Cre16.g673109.t1.1	ACP1	Acyl carrier protein	M	n.a.
	Cre13.g577100.t1.2	ACP2	Acyl carrier protein	C	n.a.
	Cre14.g621650.t1.1	MCT1	Malonyl-CoA: ACP transacylase	C	n.a.
	Cre02.g088250.t1.2	MCT2	Malonyl-CoA: ACP transacylase	O; C	n.a.
	Cre11.g467723.t1.1, Cre11.g467723.t2.1	KAS1	3-Ketoacyl-CoA-synthase (FAS complex)	C	n.a.
	Cre07.g335300.t1.2	KAS2	3-Ketoacyl-ACP-synthase (FAS complex)	C	n.a.
	Cre04.g216950.t1.2	KAS3	Putative β-ketoacyl synthase (FAS complex)	C	n.a.
	Cre03.g208050.t1.2	HAD1	3-Hydroxyacyl-ACP dehydratase (FAS complex)	C	n.a.
	Cre03.g172000.t1.2	KAR1	3-Oxoacy-[acyl carrier protein] reductase (FAS complex)	C	n.a.
	Cre06.g294950.t1.1	ENR1	Enoyl-[acyl carrier protein] reductase (FAS complex)	C	n.a.
	Cre08.g373050.t1.1	ACC1	Homomeric ACCase 1, predicted to be mitochondria	M	n.a.
FA activation and export	Cre06.g256750.t1.2	FAT1/FATA (FatA/FatB)	Acyl carrier protein thioesterase	C	n.a.
	Cre03.g182050.t1.2	LCS1 (LACS3/LACS4)	Long-chain acyl-CoA synthetase	O; LD	Nguyen *et al.* (2011)
	Cre13.g566650.t2.1, Cre13.g566650.t1.2	LCS2 (LACS5/LACS8)	AMP dependent synthetase/ligase	O; LD	Nguyen *et al.* (2011)
FA desaturations	Cre17.g701700.t2.1, Cre17.g701700.t1.2	CrSAD/FAB2	Plastid acyl-ACP desaturase, Δ-9 stearate desaturase	C	Hwangbo *et al.* (2014)
	Cre17.g711150.t1.2	CrFAD2	ω-6 fatty acid desaturase, Δ-12	O	Chi *et al.* (2008)
	Cre16.g673001.t2.1	CrFAD4	Δ-3 palmitate desaturase	O	n.a.
	Cre09.g397250.t1.2	CrFAD5	MGDG-specific palmitate Δ-7 desaturase	C	n.a.
	Cre04.g217945.t2.1, Cre04.g217945.t1.1, Cre04.g217945.t3.1	CrFAD5 like	FAD5-like protein	C	n.a.
	Cre04.g217919.t1.1	CrFAD5 like	FAD5-like protein	M	n.a.
	Cre04.g217939.t1.1	CrFAD5 like	FAD5-like protein	O	n.a.
	Cre13.g590500.t1.1	CrFAD6 (CrDES6)	ω-6 fatty acid desaturase	C	Sato *et al.* (1997)
	Cre06.g288650.t1.2	CrFAD6a	ω-6 fatty acid desaturase-like protein	C	n.a.
	Cre01.g038600.t1.2	CrFAD7 (FAD3/FAD8)	Chloroplast glycerolipid ω-3-fatty acid desaturase	C[a]	Nguyen *et al.* (2013), Pflaster *et al.* (2014)
	Cre01.g037700.t1.2	CrΔ4FAD	MGDG-specific Δ-4 fatty acid desaturase	C[a]	Zauner *et al.* (2012)
	Cre10.g453600.t1.2, Cre10.g453600.t2.1	CrDES	A front-end ω-13 desaturase	O	Kajikawa *et al.* (2006)
From glycerol to DAG	Cre02.g143000.t1.2	GPA1 (ATS1/ACT1)	Plastidial glycerol-3-phosphate O-acyltransferase	C	n.a.
	Cre06.g273250.t1.2	GPAT (GPAT9)	Glycerol-3-phosphate phosphate acyltransferase, contains PlsC domain	C	n.a.

(continued)

Table 2. (continued)

Pathway	JGI v5.5 (Augustus u111.6) ID	Gene name abbreviations	Description	Subcellular localization	References
	Cre09.g398289.t1.1	LPAT (LPAT1/ATS2)	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase	C	n.a.
	Cre17.g707300.t1.2	LPAT	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase	SP	n.a.
	Cre12.g506600.t1.2	PAH1 (PAH1/PAH2)	Phosphatidic acid phosphatase, PP1 type, lipin	C	n.a.
	Cre05.g230900.t1.1	PAP1	Phosphatidate phosphatase	SP	n.a.
	Cre05.g240000.t1.2	PAP2	Phosphatidate phosphatase	SP	Deng *et al.* (2013)
	Cre03.g150050.t1.1	PAP2-like	Phosphatidate phosphatase family	C	n.a.
Galactolipid synthesis	Cre13.g585301.t1.1	MGD1	Monogalactosyldiacylglycerol synthase	SP	n.a.
	Cre13.g583600.t1.2	DGD1	Digalactosyldiacylglycerol synthase	O	n.a.
Phospholipid synthesis	Cre12.g561550.t1.2	CDS1	Mitochondrial half-size ABC transporter (PtdGro synthesis)	SP	n.a.
	Cre02.g095106.t1.1	PGPS1	Phosphatidylglycerolphosphate synthase (PtdGro synthesis)	O	n.a.
	Cre13.g604700.t1.1	PGPS2	Phosphatidylglycerolphosphate synthase (PtdGro synthesis)	C	n.a.
	Cre03.g162601.t1.1	PGPS3	Phosphatidylglycerolphosphate synthase (PtdGro synthesis)	M	n.a.
	Cre03.g180250.t1.2	INO1	*myo*-Inositol-1-phosphate synthase (PtdIns synthesis)	O	n.a.
	Cre10.g419800.t1.2	PIS1	Phosphatidylinositol synthase (PtdIns synthesis)	SP	n.a.
	Cre01.g038250.t1.2	SDC1	Serine decarboxylase (PtdEtn synthesis)	C	n.a.
	Cre03.g186200.t1.2	PCT1	Phosphatidate cytidylyltransferase	O	Yang *et al.* (2004b)
	Cre12.g489050.t1.2	PCT2	Phosphatidate cytidylyltransferase	O	n.a.
	Cre12.g539000.t1.2	ECT1	CDP-ethanolamine synthase	SP	Yang *et al.* (2004a)
	Cre06.g291600.t1.1, Cre06.g291600.t2.1	ETK1	Ethanolamine kinase (PtdEtn synthesis)	O	n.a.
Sulfolipid synthesis	Cre12.g554250.t1.1	UGP3 (LPB1)	UDP-glucose pyrophosphorylase	C	Chang *et al.* (2005)
	Cre16.g656400.t1.2	SQD1	UDP-sulfoquinovose synthase	C	Riekhof *et al.* (2003)
	Cre01.g038550.t1.1	SQD2	Sulfoquinovosyldiacylglycerol synthase	C	n.a.
	Cre16.g689150.t1.2	SQD3	Sulfoquinovosyldiacylglycerol synthase	C	n.a.
DGTS synthesis	Cre06.g250200.t1.2	SAS1	S-adenosylmethionine synthetase	O	n.a.
	Cre07.g324200.t1.2	BTA1	Diacylglyceryl-*N*,*'N*,*'N'*-trimethylhomoserine synthase	O	Riekhof *et al.* (2005)
TAG synthesis	Cre01.g045903.t1.1	CrDGAT1	Diacylglycerol acyltransferase, DGAT Type 1	C	Boyle *et al.* (2012), Deng *et al.* (2012), Goodenough *et al.* (2014), Huang *et al.* (2013), La Russa *et al.* (2012), Sanjaya *et al.* (2013)
	Cre12.g557750.t1.1	CrDGTT1	Diacylglycerol acyltransferase, DGAT Type 2	SP	
	Cre09.g386912.t1.1	CrDGTT2	Diacylglycerol acyltransferase, DGAT Type 2	SP	
	Cre06.g299050.t1.2	CrDGTT3	Diacylglycerol acyltransferase, DGAT Type 2	O	
	Cre03.g205050.t1.2	CrDGTT4	Diacylglycerol acyltransferase, DGAT Type 2	SP	
	Cre02.g079050.t1.1	CrDGTT5	Diacylglycerol acyltransferase, DGAT Type 2	M	
	Cre02.g106400.t1.1	CrPDAT1/LCA1	Phospholipid diacylglycerol acyltransferase	C	Yoon *et al.* (2012), Boyle *et al.* (2012)
LD structural proteins	Cre09.g405500.t1.1	MLDP	Major lipid droplet protein	O; LD	James *et al.* (2013), Moellering and Benning (2010), Nguyen *et al.* (2011)
	Cre01.g039550.t1.1	PLP1 (PLAP1)	Plastid-lipid associated protein PAP/fibrillin family protein	C	Schmidt *et al.* (2006)
	Cre03.g188650.t1.2	PLAP2	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre14.g618050.t1.2	PLAP3	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre12.g502250.t1.2	PLAP4	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre03.g176350.t1.2, Cre03.g176350.t2.1	PLAP5	Plastid-lipid associated protein PAP/fibrillin family protein	C	

(continued)

Table 2. (continued)

Pathway	JGI v5.5 (Augustus u111.6) ID	Gene name abbreviations	Description	Subcellular localization	References
	Cre03.g188700.t1.2	PLAP6	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre14.g611450.t1.1	PLAP7	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre14.g611700.t1.1	PLAP8	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre02.g143667.t1.1	PLAP9	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre03.g189300.t1.1	PLAP10	Plastid-lipid associated protein PAP/fibrillin family protein	C	
	Cre11.g478850.t1.2	PLAP11	Plastid-lipid associated protein PAP/fibrillin family protein	C	
Lipid trafficking	Cre06.g268200.t1.2	TGD1	Lipid transfer machine permease	M; LD	n.a.
	Cre16.g694400.t1.2	TGD2	Permease-like component of an ABC transporter	O; LD	n.a.
	Cre16.g658526.t1.1	TGD3	Putative ABC transport system ATP-binding protein	C; LD	n.a.
	Cre13.g583550.t1.2	VIP1 (VIPP1)	Vesicle inducing protein in plastids 1	C	Nordhues *et al.* (2012)
	Cre11.g468050.t1.2	VIP2 (VIPP1)	Vesicle inducing protein in plastids 1 like	C	n.a.
	Cre12.g536050.t1.2	ALA1	P-type ATPase; putative phospholipid-transporting ATPase	O	n.a.
	Cre12.g536000.t1.2	ALA2	Phospholipid-translocating P-type ATPase, flippase	O	n.a.
	Cre16.g656500.t1.1, Cre16.g656500.t2.1	ALA3	ATPase, phospholipid transporter	O	n.a.
Lipases	Cre03.g193500.t1.2	PGD1/CGLD15	Galactolipid lipase	O	Li *et al.* (2012a)
	Cre09.g390615.t1.1	LIP1/FAP12	Likely a DAG lipase	O	Li *et al.* (2012b)
	Cre17.g699100.t1.1	SDP1/TGL20	Patatin-like TAG lipase	M	n.a.
	g13764	Hydrolase	a/β Hydrolase: soluble epoxide hydrolase	LD[a]	Nguyen *et al.* (2011)
β-oxidation pathway	Cre15.g637761.t3.1,	CTS	Peroxisomal long-chain acyl-CoA transporter, ABC superfamily	M	n.a.
	Cre12.g507400.t1.2	LCS3 (LACS6/LACS7)	Long-chain acyl-CoA synthetase	M	n.a.
	Cre16.g689050.t1.1	ACX1 (ACX1/ACX5)	Acyl-CoA oxidase	C	n.a.
	Cre05.g232002.t2.1, Cre05.g232002.t1.1	ACX2 (ACX2)	Acyl-CoA oxidase	O	n.a.
	Cre16.g687350.t1.2	ACX3 (ACX3/ACX6)	Acyl-CoA oxidase	M	n.a.
	Cre16.g695100.t1.1	ACX4 (ACX4)	Acyl-CoA oxidase	M	n.a.
	Cre16.g695050.t1.2	ECH1 (MFP2/AIM1)	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	SP	n.a.
	Cre06.g308100.t1.2	ECH2	3-Hydroxyacyl-CoA dehydrogenase	O	n.a.
	Cre10.g463150.t1.1	DCI1	Enoyl-CoA hydratase/isomerase family	O	n.a.
	Cre17.g723650.t1.2	ATO1 (KAT1/KAT2/PKT1)	3-Oxoacyl CoA thiolase/acetyl-CoA acyltransferase 1 (ATO1)	M	n.a.
	Cre17.g731850.t1.2	RED	Peroxisomal 2,4-dienoyl-CoA reductase	M	n.a.
	Cre06.g278215.t1.1	HIBCH	3-Hydroxyisobutyryl-CoA hydrolase	O	n.a.
Regulatory proteins	Cre16.g673250.t1.1	NRR1	Putative nitrogen specific regulator/DNA-binding protein	O	Boyle *et al.* (2012)
	Cre11.g481800.t1.1	CHT7	Compromised hydrolysis of triacylglycerols 7	N[a]	Tsai *et al.* (2014)

ACP, acyl-carrier protein; C, Chloroplast; FA, fatty acid; FAS, fatty acid synthase; FAD, fatty acid desaturase; LD, lipid droplet; n.a., not available; M, mitochondria; N, nuclear; O, other; SP, secretory pathway.

[a]Where experimental evidence of a subcellular localization is available. Subcellular localization is predicted using the PredAlgo programme (https://giavap-genomes.ibpc.fr/cgi-bin/predalgodb.perl?page=main) (Tardif *et al.*, 2012). This table is made partly based on Merchant *et al.* (2012) and Riekhof *et al.* (2005).

base-exchange PtdSer synthase enzymes from the genome (Riekhof *et al.*, 2005), and *Chlamydomonas* also lacks obvious phosphatidylserine decarboxylases, indicating that the synthesis of PtdEtn in this organism is solely the responsibility of the cytidine diphosphate-ethanolamine dependent Kennedy pathway. In this pathway, serine is directly decarboxylated to form ethanolamine via serine decarboxylase, and then phosphorylated, converted to CDP-ethanolamine, and then the ethanolaminephosphate moiety transferred to diacylglycerol to form PtdEtn. Two components of this pathway have been experimentally verified (Yang *et al.*, 2004a,b). The lack of PtdCho in *Chlamydomonas* can be accounted for by a lack of phosphatidylethanolamine methyltransferase and phosphoethanolamine methyltransferase homologs, however the cytidylyltransferase which makes CDP-ethanolamine is capable of using choline as a substrate (Yang *et al.*, 2004b). This raises the prospect that provision of choline to *Chlamydomonas* could lead to the accumulation of PtdCho, though this has not been experimentally verified.

FATTY ACID DESATURATIONS

Chlamydomonas synthesizes a mixture of C16 and C18 FAs with up to four unsaturations (Figure 2) (Giroud and Eichenberger, 1988; Moellering and Benning, 2010; Siaut *et al.*, 2011). Desaturation reactions are catalyzed by enzymes called fatty acid desaturases (FAD) that convert a single bond between two carbon atoms (C–C) to a double bond (C=C) at specific positions of a fatty acyl chain (Los and Murata, 1998; Shanklin and Cahoon, 1998). Two nomenclatures are usually used to define the specific site of desaturation by reference to the carboxyl terminus (Δ-position) or the methyl terminus (ω-position). Fatty acids are abbreviated by number of carbons: number of double bonds (positions in the acyl chain according to Δ nomenclature). For example linoleic acid is $18{:}2\Delta^{9,12}$.

Depending on substrates, three types of FAD can be distinguished: soluble acyl-CoA (Coenzyme A) desaturases, soluble acyl-ACP desaturases, and membrane-bound lipid desaturases (Los and Murata, 1998). In the green lineage, the first desaturation reaction is catalyzed by the soluble stearoyl-ACP desaturase (SAD/FAB2), which converts an 18:0-ACP to an $18{:}1\Delta^{9}$-ACP in the plastid lumen. The *CrFAB2* has been cloned and its overexpression in the native host *Chlamydomonas* resulted in 2.4-fold higher amount of oleic acid (18:1) (Hwangbo *et al.*, 2014). Interestingly, the *CrFAB2* overexpressing lines also produced higher amount of linoleic acid (18:2) and palmitate (16:0), leading to an >28% total increase in FAs in the overexpressor as compared to the control strain.

Earlier biochemical studies carried out in *Chlamydomonas* have suggested that further desaturations downstream of SAD/FAB2 occurred on membrane lipids (Giroud and Eichenberger, 1988). For each step of desaturation needed to synthesize *Chlamydomonas* membrane lipids, at least one homolog corresponding to known plant membrane-bound lipid desaturase is encoded in the *Chlamydomonas* genome (Figure 4 and Table 2). Experimental evidence has been provided for CrFAD2 (Chi *et al.*, 2008), CrFAD6 (Sato *et al.*, 1997), CrFAD7 (Nguyen *et al.*, 2013; Pflaster *et al.*, 2014), CrΔ4FAD (Zauner *et al.*, 2012), and CrDES (Kajikawa *et al.*, 2006). Functions of the homologs to the Arabidopsis PtdGro-specific Δ3 palmitate desaturase (FAD4) (Gao *et al.*, 2009) and the MGDG-specific palmitate Δ7 desaturase (FAD5) (Heilmann *et al.*, 2004) remain to be demonstrated. The protein(s) required for the synthesis of $18{:}1\Delta^{11}$ remain elusive, although several routes could be possible (Sakurai *et al.*, 2014).

ω-6 fatty acid desaturases: endoplasmic CrFAD2 and plastidial CrFAD6

ω-6 fatty acid desaturase catalyzes the respective formation of $16{:}2\Delta^{7,10}$ and $18{:}2\Delta^{9,12}$ from $16{:}1\Delta^{7}$ and $18{:}1\Delta^{9}$, thus providing diunsaturated fatty acid substrate for subsequent desaturases. One mutant (*hf-9*, *h*igh *f*luorescence 9) deficient in an ω-6 fatty acid desaturase has been isolated from *Chlamydomonas* based on screening a ultraviolet (UV) mutagenized population for variations in chlorophyll fluorescence (Sato *et al.*, 1995). The *hf-9* mutant showed a large reduction (>60 mol%) in all FAs containing ≥2 double bonds, besides this, it also showed reduced photosynthetic O_2 evolution as well as an altered chloroplast structure (Sato *et al.*, 1995). The corresponding increase in the monoenoic acids ($16{:}1\Delta^{7}$ and $18{:}1\Delta^{9}$) suggested a precursor-product relationship; cloning and expression of the *CrFAD6* complemented the fatty acid phenotype. However the photosynthetic defects persisted in the complemented lines, suggesting other mutations occur in the *hf-9* strain. The presence of >50 mol% dienoic acids in the *hf-9* mutant indicates the existence of other ω-6 desaturases; indeed *Chlamydomonas* genome contains two other genes (*CrFAD2* and *CrFAD6a*) homologous to *CrFAD6* (Table 2). Both proteins possess typical features of membrane-bound desaturases, i.e. the presence of three histidine boxes and membrane-spanning regions (Shanklin and Cahoon, 1998). Heterologous expression of *CrFAD2* in the yeast *Saccharomyces cerevisiae* led to production of 18:2 but not that of *CrFAD6*, consistent with its respective subcellular locations because the microsomal CrFAD2 would find its electron donor cytochrome b_5 in the heterologous yeast host, but the activity of plastidial CrFAD6 is limited due to the absence of ferredoxin, a plastid-located electron donor (Chi *et al.*, 2008).

A single plastidial ω-3 fatty acid desaturase in *C. reinhardtii*

The ω-3 PUFAs are the major fatty acid molecules present in all lipid classes of *Chlamydomonas*, be it plastidial or

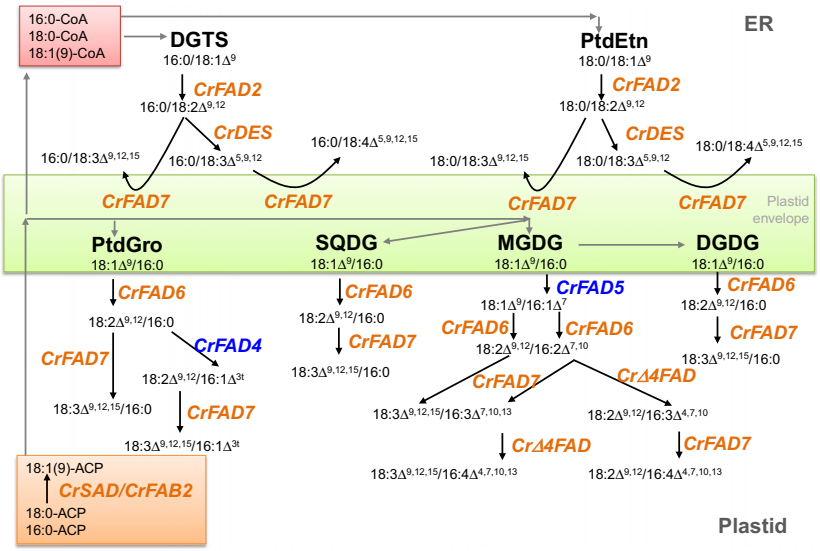

Figure 4. Major desaturation steps and their desaturases in *Chlamydomonas*.
Examples of molecular species from each lipid class are given, and noted as lipid class (*sn*-1 FA/*sn*-2 FA). In italic are enzymes, among which the uncharacterized desaturases are in blue. The figure is modified with permission from Figure 2 in Riekhof *et al.* (2005).
ACP, acyl carrier protein; CoA, Coenzyme A; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N*,*N*,*N*-trimethylhomoserine; ER, endoplasmic reticulum; FAD, fatty acid desaturase; IE, inner plastid envelope; MGDG, monogalactosyldiacylglycerol; OE: outer plastid envelope; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

ER. The ω-3 FAs in *Chlamydomonas* are 16:3$\Delta^{7,10,13}$, 16:4$\Delta^{4,7,10,13}$, 18:3$\Delta^{9,12,15}$ and 18:4$\Delta^{5,9,12,15}$ (Giroud and Eichenberger, 1988; Fan *et al.*, 2011; Zauner *et al.*, 2012). These FAs are synthesized by ω-3 desaturases, which introduce a double bond between the third and fourth carbon from the methyl end of a dienoic acid. In higher plants, plastidial or ER ω-3 desaturation reactions are catalyzed by distinct enzymes located in the plastid and ER compartments (Wallis *et al.*, 2002). Interestingly, *Chlamydomonas* has only one enzyme responsible for the formation of ω-3 FAs (Nguyen *et al.*, 2013; Pflaster *et al.*, 2014). This enzyme was named as CrFAD7 because of higher sequence similarity to the plant plastidial-located FAD7 than to the ER-located FAD3 as well as its demonstrated plastidial location (Riekhof *et al.*, 2005; Nguyen *et al.*, 2013; Pflaster *et al.*, 2014). The presence of a single plastid-located ω-3 fatty acid desaturase related to the cyanobacterial desB ω-3 fatty acid desaturase seems common among Chlorophyceae microalgae (Nguyen *et al.*, 2013). Phylogenetic analyses suggest the ER-located FAD3 desaturase of plants has evolved later (Sperling *et al.*, 2003). A most likely scenario would be that CrFAD7 is located in the plastid envelope allowing it to act on ER lipids at ER-plastid contact points and on plastidial lipids synthesized in the envelope such as MGDG and digalactosyldiacylglycerol (DGDG) (Figure 4). This is consistent with the notion that the plastid envelope is the major site for the assembly of plastidial lipids and also a site where extensive acyl-exchanges occur between different lipid classes (Douce and Joyard, 1990; Joyard *et al.*, 1998; Benning, 2009; Rolland *et al.*, 2012).

The MGDG-specific Δ-4 fatty acid desaturase: an unusual plastid-located cytochrome b_5 fusion protein

One of the most abundant lipid molecules in an actively growing culture of *Chlamydomonas* is the MGDG18:3-$\Delta^{9,12,15}$/16:4$\Delta^{4,7,10,13}$ (*sn*-1/*sn*-2), which makes up >60% of all MGDG molecular species (Figure 2) (Nguyen *et al.*, 2013). Based on a phylogenetic comparison, Benning and co-workers (Zauner *et al.*, 2012) have identified the Δ4 desaturase responsible for synthesis of 16:4$\Delta^{4,7,10,13}$. Additionally, the authors showed that by altering the expression level of CrΔ4FAD led to changes in MGDG amount, and this suggests that lipid amount and desaturations are tightly regulated. Although plastid-located, the CrΔ4FAD has a functional N-terminal cytochrome b_5 domain, distinguishing it from other plastidial desaturases. This raises an interesting question because the desaturation of FAs is known to require two electrons and one molecule of oxygen (Shanklin and Cahoon, 1998). Ferredoxin is the electron donor for plastid-located desaturation reactions; whereas cytochrome b_5 provides electrons for ER-based desaturases (Los and Murata, 1998). As no evidence for the presence of a cytochrome b_5 oxidoreductase is available in the plastid of *Chlamydomonas*, the electron donor cytochrome b_5 could possibly receive electrons from ferredoxin rather than NAD(P)H. One of the other possibilities could be that CrΔ4FAD is located in the plastid envelope thus has access to electron donor present in the cytosol. This, again, puts CrΔ4FAD in the same subcellular compartment as that of CrFAD7. Taken together, these two studies (CrFAD7 and

CrΔ4FAD) point out the central role plastid envelop plays in the synthesis and homeostasis of lipids in the single-celled alga. This is not surprising given the fact that *Chlamydomonas* contains only one single large plastid which takes up over 70% cellular space (Harris, 2001). The plastid envelope thus offers a vast stage for cellular metabolism and interactions (Joyard *et al.*, 1998; Mehrshahi *et al.*, 2014).

The front-end ω-13 desaturase

For as yet unknown reasons, most unsaturated double bonds present in algae and plants are separated from each other by one single methylene (–CH_2–) group, but exceptions do occur. *Chlamydomonas* cells synthesize significant amounts (~10 mol%) of pinolenic acid (PA; 18:3$\Delta^{5,9,12}$) and coniferonic acid (CA; 18:4$\Delta^{5,9,12,15}$) (Giroud and Eichenberger, 1988), the bis-methylene-interrupted FAs which are common in pine seed oil (Wolff *et al.*, 2000). Both PA and CA are found to be present specifically at the *sn*-2 position of microsomal membrane lipids DGTS and PtdEtn (Giroud *et al.*, 1988). The biological functions of these FAs in *Chlamydomonas* or any other organisms containing them are poorly understood; and this could be answered in the future with the isolation of null mutant or via gene targeted suppression using amiRNA silencing technology.

Based on sequence homology and using a heterologous expression system, Kajikawa and co-workers have isolated and characterized the Δ-5 desaturase (CrDES) responsible for the synthesis of PA and CA (Kajikawa *et al.*, 2006). As all currently known 'front-end' desaturases (Meesapyodsuk and Qiu, 2012), CrDES contained a cytochrome b_5 domain at the N-terminus. Heterologous expression of *CrDES* in methylotrophic yeast *Pichia pastoris* and in transgenic tobacco plants led to the production of both FAs (>40%), with corresponding decrease in linoleic acid and α-linolenic acid, respectively. This study thus not only demonstrated that Cre10.g453600 encodes a functional ω-13 fatty acid desaturase, but also suggested its substrate as linoleic acid and α-linolenic acid. Identification of the protein responsible for PA/CA synthesis offers the opportunity to produce such FAs in transgenic plants for large scale productions. Indeed, PA has been demonstrated to confer a number of beneficial effects for example, it has been shown to lower TAG intake in rat (Asset *et al.*, 1999) and has also been shown to inhibit human breast cancer cell metastasis *in vitro* (Chen *et al.*, 2011).

BIOSYNTHESIS, ACCUMULATION AND TURNOVER OF TRIACYLGLYCEROLS

Triacylglycerols (TAGs) are the major constituents of most fats and oils. Their metabolism is being intensively researched because TAGs are major energy storage compounds in eukaryotic cells, and have a high nutritional value and versatile utility in industrial applications. In this section, we summarize current knowledge on the formation of lipid droplets and the major reactions that are required for TAG synthesis and turnover in cells of *Chlamydomonas*.

Induction of oil accumulation

Unlike in oilseeds in which oil accumulation is under tight developmental control (Baud and Lepiniec, 2009), accumulation of intracellular lipid droplets in *Chlamydomonas* is a response to environmental cues. The most potent inducer of oil accumulation is depletion of nitrogen from the culture medium, but other triggers have been reported, such as high light, iron, sulfur and phosphorus depletion, increase in salinity, and treatment by the ER stress chemical brefeldin A (Wang *et al.*, 2009; Fan *et al.*, 2011; Siaut *et al.*, 2011; Boyle *et al.*, 2012; Hemschemeier *et al.*, 2013; Kim *et al.*, 2013; Urzica *et al.*, 2013). In order to gain insights into the mechanisms underlying N removal-induced oil accumulation, transcriptomic studies have been used (Miller *et al.*, 2010). These authors showed that under mixotrophic conditions (i.e. with CO_2 and acetate as carbon sources), N deprivation induces a downregulation of photosynthesis and protein synthesis. In addition, acetate is channeled to fatty acid synthesis and away from neoglucogenesis.

NRR1, standing for nitrogen-responsive regulator, has been identified as a lipid 'trigger' based on comparative transcriptomic studies of oil accumulation processes in response to nitrogen starvation. Knockout of NRR1 led to an over 50% reduction in oil content under nitrogen starvation (Boyle *et al.*, 2012). This is the only known transcriptional regulator of oil accumulation reported so far.

Biosynthesis of triacylglycerols

Overview of the pathway. In *Chlamydomonas*, TAG biosynthesis is thought to occur through the same enzymatic steps as in plants (Figure 3; Ohlrogge and Browse, 1995). In this scheme, the first reactions are common to the synthesis of membrane lipids and lead to the formation of DAG, a central intermediate in the synthesis of membrane glycerolipids. None of the GPAT and LPAT of *Chlamydomonas* homologs has been characterized and shown to be important for membrane and storage lipids however. The genome of *Chlamydomonas* encodes three homologous genes to the plant PAP enzymes (Table 2). The CrPAP2 has been shown to be upregulated under nitrogen starvation, possess PAP activities, and also play a role in regulating TAG levels, because down- or upregulation of the transcript level of CrPAP2 leads to reduced or increased oil content, respectively (Deng *et al.*, 2013).

The final step of the pathway converts DAG into TAG and is the only one committed to TAG synthesis in plants. It consists in the esterification of the *sn*-3 position of a

DAG molecule by an acyl-group. Depending on the acyl-donor (acyl-CoA or acyl-lipid), this reaction can be catalyzed either by diacylglycerol acyltransferase (DGAT) or phospholipid:diacylglycerol acyltransferase (PDAT). These are often named as the 'Acyl-CoA dependent' pathway and 'Acyl-CoA independent' (or 'transacylation') pathway, respectively. Homologs of both types of acyltransferases have been identified in *Chlamydomonas* (Table 2) but only knockout mutants of CrPDAT are available. Possible subcellular localization and substrate specificities of the *Chlamydomonas* TAG biosynthetic enzymes are discussed below.

Formation of DAG. For each of the three enzymatic steps needed to go from glycerol-3-phosphate to DAG, a putative plastidial and a putative ER enzyme exist (Table 2). Existence of a plastidial- and an ER-localized pathways of TAG synthesis would be consistent with observed location of lipid droplets (LDs) in the starchless mutant BAFJ5 (*cw15sta6*) (Fan *et al.*, 2011; Goodson *et al.*, 2011). However, in BAFJ5, it has also been shown that 90% of the TAG molecules extracted from nitrogen-deprived whole cells had a C16 fatty acid in the *sn*-2 position of the glycerol backbone, similar to those of isolated chloroplasts (Fan *et al.*, 2011). This structure is similar to the plastidial membrane lipids MGDG and DGDG and different from the ER membrane lipid DGTS, which has mostly C18 FAs in the *sn*-2 position. These results thus strongly suggest that the DAG backbone used for TAG synthesis originates from a chloroplastic set of DAG-forming enzymes.

Conversion of DAG to TAG by CrPDAT. PDAT activity was first discovered in some plant species accumulating high amount of unusual FAs, and the corresponding genes were identified and characterized in these plants and in yeast (Dahlqvist *et al.*, 2000). PDAT enzymes act through transacylation to the *sn*-3 position of a DAG of a fatty acid present in the *sn*-2 position of a membrane lipid.

The genome of *Chlamydomonas* encodes a single PDAT, named CrPDAT. CrPDAT has been detected in the LD-proteome (Nguyen *et al.*, 2011) and reduced TAG amount has been found in insertional mutants (*pdat1-1*, *pdat1-2*) (Boyle *et al.*, 2012) and also in artificial miRNA silenced PDAT strains (Yoon *et al.*, 2012) under both nitrogen deplete and nitrogen-replete conditions. However the reduced but not abolished TAG content in the CrPDAT null mutants (Yoon *et al.*, 2012) suggest an overlapping contribution of some DGAT homologs to oil synthesis, as occurs in plants and yeast (Petschnigg *et al.*, 2009; Zhang *et al.*, 2009).

In vitro biochemical studies have shown that the CrPDAT possesses transacylase or acyl-hydrolase activities toward a broad range of lipid substrates including TAGs, phospholipids, galactolipids and cholesteryl esters (Yoon *et al.*, 2012). Since the plant and yeast PDATs have been shown to use mainly PtdCho as an acyl-donor, the absence of this lipid in *Chlamydomonas* raises the question of whether DGTS, which is structurally similar to PtdCho, serves as the major substrate for CrPDAT. This is highly likely considering that enzymes known to use DGTS as substrate, for example the CrFAD2 or CrDES, have been shown to be able to use PtdCho as substrate in heterologous hosts when DGTS is absent (Kajikawa *et al.*, 2006; Chi *et al.*, 2008). Nevertheless, the activity of CrPDAT toward DGTS remains to be tested.

Role of the six DGAT isoforms of Chlamydomonas. DGAT catalyzes the acyl-esterification of DAG from an acyl-CoA. The DGAT activity is catalyzed by two structurally unrelated enzymes in higher plants, i.e. type I (DGAT1) and type II (DGAT2) (Durrett *et al.*, 2008; Chapman and Ohlrogge, 2012). *Chlamydomonas* contains one type I (annotated as CrDGAT1) and five type II DGAT (annotated as DGTT1-5) (Table 2) (Miller *et al.*, 2010; Boyle *et al.*, 2012; Merchant *et al.*, 2012). Several subcellular localizations (one plastid, one mitochondrion, three secretory pathways and one other) have been predicted for these DGATs based on a program specifically adapted for algal proteins (Table 2) (Tardif *et al.*, 2012).

The function of CrDGAT1 in TAG synthesis in *Chlamydomonas* has been inferred from its transcriptomic response to nitrogen starvation (Boyle *et al.*, 2012). Some studies have focused on elucidating the functions of various *Chlamydomonas* type II DGTT isoforms (Boyle *et al.*, 2012; Deng *et al.*, 2012; Hung *et al.*, 2013). Three of the five type 2 DGTT genes are known to be upregulated following nitrogen starvation (Miller *et al.*, 2010; Boyle *et al.*, 2012) and differential expression of DGTT isozymes has been observed under other TAG-inducing conditions, such as sulfur or iron deprivation (Boyle *et al.*, 2012; Urzica *et al.*, 2013). Downregulation of *DGTT1* and *DGTT5* transcription have resulted in a decrease in lipid content; and their overexpression increased lipid content in *Chlamydomonas* (Deng *et al.*, 2012). DGTT1, 2 and 3 have been shown to complement a yeast mutant defective in TAG synthesis (Hung *et al.*, 2013). The Arabidopsis plants overexpressing *DGTT2* produced >20-fold more oil in their leaves than wild-type plants (Sanjaya *et al.*, 2013). Intriguingly, silencing of *DGTT4* caused an unexpected increase in lipid content, and its expression in the yeast TAG deficient mutant background failed to restore the mutant phenotype (Hung *et al.*, 2013).

Why *Chlamydomonas* possesses such a relatively large number of DGAT (six in *Chlamydomonas* versus two in Arabidopsis) is not clear. This is particularly remarkable considering that gene families of lipid metabolism are generally smaller than in Arabidopsis (Riekhof *et al.*, 2005). The unusually high number of *Chlamydomonas* DGAT

genes might be related to different acyl-CoA specificities, or to different subcellular locations, or to the need for DGAT isoforms adapted to specific effectors of enzyme activity that may be produced under the various TAG-inducing stresses.

Subcellular localization of TAG-synthesizing activities. Whether the final step of TAG synthesis occurs inside and/or outside the chloroplast remains unclear, but microcopy data showed that in the BAFJ5 starchless mutant LDs are present inside the chloroplast, and also in the cytosol in close association with the chloroplast envelop membrane (Fan *et al.*, 2011; Goodson *et al.*, 2011). Presence of a TAG-synthesizing enzyme on the chloroplast envelope seems therefore likely, which is supported by protein subcellular localization analyses (Table 2) suggesting that several major TAG-synthesizing activities (CrDGAT1 and CrPDAT) are putatively plastid-located.

Source of acyl chains for TAG accumulation: de novo synthesis or acyl-recycling. In response to nitrogen starvation, TAG accumulation was found to be accompanied by an increase in the total amount of cellular FAs, indicating the importance of *de novo* fatty acid synthesis (Moellering and Benning, 2010; Work *et al.*, 2010). The addition of the inhibitor cerulenin, a specific inhibitor of the β-keto-acyl-ACP synthase, to cell cultures of *Chlamydomonas* prohibited TAG accumulation by 80% than control cells after 2-day nitrogen starvation, thus highlighted such a contribution (Fan *et al.*, 2011). Exogenously supplied acetate can boost oil accumulation further under nitrogen starvation serves as another proof (Goodson *et al.*, 2011; Fan *et al.*, 2012). Acyl chains recycled from membrane lipids have also been postulated to contribute to TAG synthesis, because major membrane lipids were reduced concurrent to oil accumulation (Siaut *et al.*, 2011). Transfer of an acyl chain from one lipid to another can be achieved by two means: via the reaction of a transacylase such as CrPDAT, or by the lipase-catalyzed release of a free fatty acid and reactivation to acyl-CoA or acyl-ACP. Contribution of the transacylation pathway to oil synthesis has been demonstrated through study of the CrPDAT (Boyle *et al.*, 2012; Yoon *et al.*, 2012). A lipase-mediated supply of acyl chains for TAG synthesis has recently been shown via the isolation of a mutant defective in a galactoglycerolipid lipase, named CrPGD1 (for Plastid Galactoglycerolipid Degradation 1) (Li *et al.*, 2012a). The *pgd1* mutant accumulated reduced amount of TAGs than wild-type following nitrogen starvation (Li *et al.*, 2012a). Furthermore, the recombinant protein produced in *E. coli* can hydrolyze MGDG to produce free FAs and lyso-MGDG (Li *et al.*, 2012a). Taken together, these evidence demonstrate that TAG synthesized under nitrogen starvation partly are coming from *de novo* synthesis and partly are from acyl chains already present in membrane lipids. It should be noted that the relative contribution of one or the other route is often determined by culture conditions (photoautotrophic versus mixotrophic), or by the type of stress applied (light, temperature or nutrient etc.).

Structure and composition of TAG-filled lipid droplets

Lipid droplets are the major sites for neutral lipid storage in eukaryotic cells (Huang, 1992; Murphy, 1993; Goodman, 2008; Murphy *et al.*, 2009). These structures have a neutral lipid core surrounded by a polar lipid monolayer decorated with proteins. Studies carried out on LDs isolated from multiple model organisms ranging from yeasts to higher plants to humans have indicated that LDs are not only an important storage site for TAGs, but also participate actively in several subcellular mechanisms, including lipid synthesis, degradation, trafficking, signaling and lipid homeostasis. Current data suggest that a similar range of functions can be attributed to algal LDs (Goold *et al.*, 2014). In plants, two types of LDs are distinguished based on their subcellular locations and on their compositional differences. LDs usually refer to those that are largely present in the cytosol and are rich in TAGs. Those present in the plastid are called plastoglobules and contain mainly prenylquinones and carotenoids and a lower amount of TAGs (Kessler and Vidi, 2007; Brehelin and Kessler, 2008). Both types of LDs occur in wild-type strains of *Chlamydomonas*. Genes encoding plastid-lipid associated PAP/fibrillin family proteins, the structural proteins of plant plastoglobules, are present in the *Chlamydomonas* genome (Table 2), but nothing is known about the proteomic and lipidomic content or compositions of *Chlamydomonas* plastoglobules.

A proteomics study of isolated LDs from two strains of *Chlamydomonas* (*dw15* or BAFJ3 *i.e. cw15sta1-2*) cultivated under nitrogen-starvation conditions revealed the diversity of proteins associated to LDs, among which over 30 proteins were known to be involved in lipid metabolism (Moellering and Benning, 2010; Nguyen *et al.*, 2011). Both studies also identified a novel and previously unknown protein as the most abundant protein present in isolated LDs, and this protein is named MLDP, for major lipid droplet protein. MLDP or its close homologs have since been found to be present in some other Chlorophyta algae but not in mosses nor in vascular plants (Moellering and Benning, 2010; Davidi *et al.*, 2012; Goold *et al.*, 2014).

Besides the typical cytosolic LDs and plastidial plastoglobules, the starchless mutant BAFJ5 synthesized a third type, termed chloroplast LDs (Fan *et al.*, 2011; Goodson *et al.*, 2011). Both cell biological and biochemical data support this observation, however its biogenesis is not yet fully understood. TAGs contained in the chloroplastic LDs could be formed via the activity of CrDGAT1, a potentially

chloroplast-localized enzyme (Table 2). Proteomic and lipidomic analyses of the different sub-populations of LDs should aid in the understanding of their biogenesis. Subcellular lipidomic analyses of specialized regions of tissues have recently been demonstrated to be possible with the aid of a matrix assisted laser desorption/ionization–MS imaging (MALDI-MSI) approach (Horn and Chapman, 2012; Horn *et al.*, 2012); and the use of such a tool for microalgae should yield important insights onto lipid compositional differences between different types of LDs.

In addition to LDs (cytosolic and chloroplast) and plastoglobules, *Chlamydomonas* possesses another type, located in the eyespot. The eyespot apparatus is present universally in flagellated green algae, and allows the cell to swim toward or away from light (phototaxis). Under the electron microscope, eyespots appear as a region of electron-dense granules located just inside the chloroplast envelop (Harris, 2001). The eyespot is enriched in carotenoid pigments and also in TAGs (25%) (Moellering and Benning, 2010; Goodson *et al.*, 2011). Indeed, proteomic study of the isolated eyespot from *Chlamydomonas* revealed the presence of over 3.5% of total eyespot proteome (202 proteins; ≥2 peptides) as lipid metabolism-related proteins (i.e. 7 with ≥2 peptides) and also several proteins with PAP/fibrillin domain (Schmidt *et al.*, 2006). Similar to other LD-proteomes, the eyespot proteins include the betaine lipid synthase (BTA1), LPAT, LACS, and DGAT, suggesting active TAG synthesis in the oil globules of eyespot.

TAG lipolysis and fatty acid degradation

Induction of lipolysis in Chlamydomonas. TAG degradation can be induced by simply adding back nitrogen to a N-starved culture (Siaut *et al.*, 2011; Li *et al.*, 2012b). This causes disappearance of LDs within hours and usually a 'greening' process i.e. the resynthesis of plastidial membranes (Siaut *et al.*, 2011; Li *et al.*, 2012b; Cagnon *et al.*, 2013). Oil degradation is catalyzed by TAG lipases, which release free FAs and DAG molecules. TAG lipases may further degrade DAGs to MAGs and even go up to glycerol, but DAG lipases and MAG lipases could also be involved. No TAG lipase has yet been characterized in *Chlamydomonas* but many putative lipases (>130) are encoded in the *Chlamydomonas* genome (Miller *et al.*, 2010). One of the strongest candidates for a TAG lipase is a protein showing 45% identity to the known Arabidopsis TAG lipase SDP1 (sugar dependent 1) (Eastmond, 2006; Table 2).

The only acylglycerol lipase characterized in *Chlamydomonas* is CrLIP1 (for Lipase 1), which has been identified because its transcription is negatively correlated to oil content (Li *et al.*, 2012b). Detailed biochemical characterization as well as sequence homology searches indicated that CrLIP1 likely acts as a DAG lipase. Silencing of CrLIP1 led to a decrease in TAG degradation following nitrogen resupply. Furthermore heterologous expression of *CrLIP1* in yeast restored the phenotypes associated with the *tgl3/tgl4* mutant.

Increasing evidence suggests that TAG hydrolysis is not only induced by a particular culture condition (e.g. nitrogen resupply), but is also a part of a continuous balance between oil synthesis and degradation. For example, under active TAG synthesis (nitrogen-depletion conditions), the expression of genes encoding putative lipases is induced by N starvation (Miller *et al.*, 2010; Merchant *et al.*, 2012) and putative lipases have been detected in proteomic studies of LDs (Moellering and Benning, 2010; Nguyen *et al.*, 2011). Conversely, under nitrogen-replete conditions, blockage of fatty acid degradation by brefeldin A or arrest of TAG hydrolysis by silencing of CrLIP1 lipase increases oil content (Li *et al.*, 2012b; Kato *et al.*, 2013). Increases in oil reserves as a result of downregulation of lipolytic enzymes have also been observed in yeast (Daum *et al.*, 2007; Ducharme and Bickel, 2008), in the diatom *Thalassiosira pseudonana* (Trentacoste *et al.*, 2013) and also in higher plants (Slocombe *et al.*, 2009; James *et al.*, 2010; Fan *et al.*, 2014).

To identify proteins involved in TAG turnover, two genetic screens have been performed to isolate mutants defective in oil remobilization following nitrogen resupply. The first one was based on the measurement of Nile red fluorescence of cell cultures following nitrogen resupply (Cagnon *et al.*, 2013). In a second screen the amount of MLDP was measured using anti-MLDP antibodies (Tsai *et al.*, 2014). The latter screen has allowed the isolation of a series of mutants '*c*ompromised in *h*ydrolysis of *T*AGs (*cht).* One of these mutants, *cht7*, has been shown to contain 10-fold higher TAG levels than wild-type after lipolysis induced by nitrogen resupply and also to be severely impaired in regrowth (Tsai *et al.*, 2014). Molecular genetic analyses identified that the genetic locus underlying the *cht7* phenotype encodes a protein similar to plant and mammalian DNA-binding proteins and evidence was provided that CHT7 is a negative regulator of cellular quiescence.

β-oxidation and the 'elusive' peroxisome in Chlamydomonas. After being cleaved off the glycerol backbone, FAs are further metabolized via beta-oxidation reactions (Figure 3). Candidate genes encoding proteins homologous to known plant proteins essential to fatty acid transport across membranes, fatty acid activation and the activities required for two carbon degradation reactions of the fatty acid beta-oxidation are encoded in the genome of *Chlamydomonas* (Table 2). None has so far been experimentally characterized. Interestingly, many of the candidate genes showed an overall reduction in transcription following nitrogen removal (Miller *et al.*, 2010). This lends additional support to the idea that fatty acid turnover is constitutive, and that downregulation of fatty acid β-oxidation could potentially boost oil accumulation.

In animals, fatty acid beta-oxidation occurs in the mitochondrion and the peroxisome, whereas in plants it is almost exclusively in the peroxisome (Poirier *et al.*, 2006; Graham, 2008). In *Chlamydomonas* it is not yet clear if both mitochondria and peroxisomes are involved as in mammalian cells. It has long been thought that peroxisomes are absent in *Chlamydomonas*. This conclusion was drawn based on the absence of a crystalloid core, a typical feature of plant- or animal-type peroxisomes apparent under the electron microscope and caused by the presence of large amounts of catalase inside peroxisomes (Beevers, 1979). However in *Chlamydomonas*, the catalase is located in mitochondria (Kato *et al.*, 1997), which might explain the lack of crystalloid core, and thus the widespread use of the term 'microbody' instead of peroxisomes in *Chlamydomonas.*

One of the most conserved features of peroxisome is the employment of peroxisomal targeting signal (PTS) to import their proteins. This has been observed to be conserved from mammalian cells to yeast and to higher plants. Some *Chlamydomonas* proteins were also found to contain functional PTS sequences, thus giving evidence that *Chlamydomonas* cells possess peroxisomes (Shinozaki *et al.*, 2009). Further experimental evidence demonstrated that some microbodies from *Chlamydomonas* employ a targeting mechanism based on PTSs (Hayashi and Shinozaki, 2012). These studies also provided for the first time a method to track *in vivo* the microbodies/peroxisomes in live cells of *Chlamydomonas*.

CONCLUSION AND UNANSWERED QUESTIONS

In the nearly 70 years since isolation of the wild-type strains, *C. reinhardtii* has served as a model organism for studying a number of important processes, recently including lipid metabolism (Merchant *et al.*, 2012; Liu and Benning, 2013). Lipid research on *Chlamydomonas* flourished with the sequence of its genome in 2007 and with the pressing need for alternative fuels. Although most of the recent works have focused on the understanding of storage lipid accumulation in response to nitrogen stress, progress has also been made in the understanding of fatty acid desaturation and membrane lipid assembly in *Chlamydomonas*. The resulting current picture of the metabolic steps required for synthesis of major membrane/neutral lipids and desaturations of their FAs indicates that lipid metabolism in this seemingly simple unicellular alga is complex, and distinct in several aspects from the well characterized lipid metabolic pathways in vascular plants. Despite this progress, many gaps remain in our understanding of lipid metabolism in *Chlamydomonas*. For example, although one protein involved in the trigger of neutral lipid accumulation has been identified (Boyle *et al.*, 2012), the molecular mechanisms and elements of the signaling pathway downstream and upstream of this protein remain to be elucidated. Also, we still know relatively little information about the biogenesis of LDs in *Chlamydomonas*, and the exact biochemical and molecular mechanisms involved for their cytosolic and plastidial accumulations. Understanding of the trafficking of FAs and lipids in algal cells is also very limited. Many efforts directed toward understanding basic lipid metabolism in microalgal cells are therefore still needed in order to harness algal lipid metabolism for efficient production of fatty acid-derived biofuels and high value compounds.

ACKNOWLEDGEMENTS

Y.L-B. and F.B. acknowledge the financial support from the Agence Nationale de la Recherche (ANR-12-BIME-0001-02 Diesalg and ANR-13-JSV5-0005 MUsCA). Algal research in the Riekhof laboratory is supported by a grant from the US National Science Foundation (EPS-1004094), and by the University of Nebraska–Lincoln Research Council.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

Asset, G., Staels, B., Wolff, R.L., Bauge, E., Madj, Z., Fruchart, J.C. and Dallongeville, J. (1999) Effects of *Pinus pinaster* and *Pinus koraiensis* seed oil supplementation on lipoprotein metabolism in the rat. *Lipids*, **34**, 39–44.

Baud, S. and Lepiniec, L. (2009) Regulation of *de novo* fatty acid synthesis in maturing oilseeds of Arabidopsis. *Plant Physiol. Biochem.* **47**, 448–455.

Beevers, H. (1979) Microbodies in higher-plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **30**, 159–193.

Benning, C. (2009) Mechanisms of lipid transport involved in organelle biogenesis in plant cells. *Annu. Rev. Cell Dev. Biol.* **25**, 71–91.

Boyle, N. and Morgan, J. (2009) Flux balance analysis of primary metabolism in *Chlamydomonas reinhardtii*. *BMC Syst. Biol.* **3**, 4.

Boyle, N.R., Page, M.D., Liu, B. *et al.* (2012) Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*. *J. Biol. Chem.* **287**, 15811–15825.

Brehelin, C. and Kessler, F. (2008) The plastoglobule: a bag full of lipid biochemistry tricks. *Photochem. Photobiol.* **84**, 1388–1394.

Cagnon, C., Mirabella, B., Nguyen, H.M., Beyly-Adriano, A., Bouvet, S., Cuine, S., Beisson, F., Peltier, G. and Li-Beisson, Y. (2013) Development of a forward genetic screen to isolate oil mutants in the green microalga *Chlamydomonas reinhardtii*. *Biotechnol. Biofuels*, **6**, 178.

Chang, C.W., Moseley, J.L., Wykoff, D. and Grossman, A.R. (2005) The LPB1 gene is important for acclimation of *Chlamydomonas reinhardtii* to phosphorus and sulfur deprivation. *Plant Physiol.* **138**, 319–329.

Chapman, K.D. and Ohlrogge, J.B. (2012) Compartmentation of triacylglycerol accumulation in plants. *J. Biol. Chem.* **287**, 2288–2294.

Chen, S.J., Hsu, C.P., Li, C.W., Lu, J.H. and Chuang, L.T. (2011) Pinolenic acid inhibits human breast cancer MDA-MB-231 cell metastasis *in vitro*. *Food Chem.* **126**, 1708–1715.

Chi, X.Y., Zhang, X.W., Guan, X.Y., Ding, L., Li, Y.X., Wang, M.Q., Lin, H.Z. and Qin, S. (2008) Fatty acid biosynthesis in eukaryotic photosynthetic microalgae: identification of a microsomal delta 12 desaturase in *Chlamydomonas reinhardtii*. *J. Microbiol.* **46**, 189–201.

Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294–306.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and Stymne, H. (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc. Natl Acad. Sci. USA*, **97**, 6487–6492.

Daum, G., Wagner, A., Czabany, T. and Athenstaedt, K. (2007) Dynamics of neutral lipid storage and mobilization in yeast. *Biochimie*, **89**, 243–248.

Davidi, L., Katz, A. and Pick, U. (2012) Characterization of major lipid droplet proteins from *Dunaliella*. *Planta*, **236**, 19–33.

Day, A. and Goldschmidt-Clermont, M. (2011) The chloroplast transformation toolbox: selectable markers and marker removal. *Plant Biotech. J.* **9**, 540–553.

Deng, X.D., Gu, B., Li, Y.J., Hu, X.W., Guo, J.C. and Fei, X.W. (2012) The roles of acyl-CoA: diacylglycerol acyltransferase 2 genes in the biosynthesis of triacylglycerols by the green algae *Chlamydomonas reinhardtii*. *Mol. Plant*, **5**, 945–947.

Deng, X.-D., Cai, J.-J. and Fei, X.-W. (2013) Involvement of phosphatidate phosphatase in the biosynthesis of triacylglycerols in *Chlamydomonas reinhardtii*. *J. Zhejiang Univ. Sci. B*, **14**, 1121–1131.

Douce, R. and Joyard, J. (1990) Biochemistry and function of the plastid envelope. *Ann. Rev. Cell Biol.* **6**, 173–216.

Ducharme, N.A. and Bickel, P.E. (2008) Minireview: lipid droplets in lipogenesis and lipolysis. *Endocrinology*, **149**, 942–949.

Durrett, T.P., Benning, C. and Ohlrogge, J. (2008) Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J.* **54**, 593–607.

Dutcher, S.K., Li, L., Lin, H., Meyer, L., Giddings, T.H., Kwan, A.L. and Lewis, B.L. (2012) Whole-genome sequencing to identify mutants and polymorphisms in *Chlamydomonas reinhardtii*. *G3*, **2**, 15–22.

Eastmond, P.J. (2006) Sugar-dependent1 encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating Arabidopsis seeds. *Plant Cell*, **18**, 665–675.

Eichenberger, W. (1976) Lipids of *Chlamydomonas reinhardtii* under different growth conditions. *Phytochemistry*, **15**, 459–463.

Eichenberger, W. and Boschetti, A. (1978) Occurrence of 1(3),2-diacylglyceryl-(3)-O-4′-(*N*,*N*,*N*-trimethyl)-homoserine in *Chlamydomonas reinhardtii*. *FEBS Lett.* **88**, 201–204.

El Maanni, A., Dubertret, G., Delrieu, M.J., Roche, O. and Tremolieres, A. (1998) Mutants of *Chlamydomonas reinhardtii* affected in phosphatidylglycerol metabolism and thylakoid biogenesis. *Plant Physiol. Biochem.* **36**, 609–619.

Fahy, E., Subramaniam, S., Brown, H.A. *et al.* (2005) A comprehensive classification system for lipids. *J. Lipid Res.* **46**, 839–862.

Fahy, E., Subramaniam, S., Murphy, R.C., Nishijima, M., Raetz, C.R.H., Shimizu, T., Spener, F., van Meer, G., Wakelam, M.J.O. and Dennis, E.A. (2009) Update of the LIPID MAPS comprehensive classification system for lipids. *J. Lipid Res.* **50**, S9–S14.

Fan, J.L., Andre, C. and Xu, C.C. (2011) A chloroplast pathway for the *de novo* biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii*. *FEBS Lett.* **585**, 1985–1991.

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

Fan, J., Yan, C., Roston, R., Shanklin, J. and Xu, C. (2014) Arabidopsis lipids, PDAT1 acyltransferase, and SDP1 triacylglycerol lipase synergistically direct fatty acids toward β-oxidation, thereby maintaining membrane lipid homeostasis. *Plant Cell*, **26**, 4119–4134.

Gao, J., Ajjawi, I., Manoli, A., Sawin, A., Xu, C., Froehlich, J.E., Last, R.L. and Benning, C. (2009) Fatty acid desaturase4 of Arabidopsis encodes a protein distinct from characterized fatty acid desaturases. *Plant J.* **60**, 832–839.

Giroud, C. and Eichenberger, W. (1988) Fatty-acids of *Chlamydomonas reinhardtii* – structure, positional distribution and biosynthesis. *Biol. Chem. H-S*, **369**, 18–19.

Giroud, C. and Eichenberger, W. (1989) Lipids of *Chlamydomonas reinhardtii* – incorporation of C-14 acetate, C-14 palmitate and C-14 oleate into different lipids and evidence for lipid linked desaturation of fatty acids. *Plant Cell Physiol.* **30**, 121–128.

Giroud, C., Gerber, A. and Eichenberger, W. (1988) Lipids of *Chlamydomonas reinhardtii* – analysis of molecular-species and intracellular site(s) of biosynthesis. *Plant Cell Physiol.* **29**, 587–595.

Gonzalez-Ballester, D., Pootakham, W., Mus, F. *et al.* (2011) Reverse genetics in *Chlamydomonas:* a platform for isolating insertional mutants. *Plant Methods*, **7**, 24.

Goodenough, U., Blaby, I., Casero, D. *et al.* (2014) The path to triacylglyceride obesity in the *sta6* strain of *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **13**, 591–613.

Goodman, J.M. (2008) The gregarious lipid droplet. *J. Biol. Chem.* **283**, 28005–28009.

Goodson, C., Roth, R., Wang, Z.T. and Goodenough, U. (2011) Structural correlates of cytoplasmic and chloroplast lipid body synthesis in *Chlamydomonas reinhardtii* and stimulation of lipid body production with acetate boost. *Eukaryot. Cell*, **10**, 1592–1606.

Goold, H., Beisson, F., Peltier, G. and Li-Beisson, Y. (2014) Microalgal lipid droplets: composition, diversity, biogenesis and functions. *Plant Cell Rep.* **34**, 545–555.

Graham, I.A. (2008) Seed storage oil mobilization. *Ann. Rev. Plant Biol.* **59**, 115–142.

Guschina, I.A. and Harwood, J.L. (2006) Lipids and lipid metabolism in eukaryotic algae. *Prog. Lipid Res.* **45**, 160–186.

Harris, E. (2001) *Chlamydomonas* as a model organism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 363–406.

Harwood, J.L. and Scrimgeour, C.M. (2007) Fatty acid and lipid structure. In *The Lipid Handbook*, 3rd edn (Gunstone, F.D., Dijkstra, A.J. ed.). Boca Raton: Taylor & Francis Group, CRC Press, pp. 1–36.

Hayashi, Y. and Shinozaki, A. (2012) Visualization of microbodies in *Chlamydomonas reinhardtii*. *J. Plant. Res.* **125**, 579–586.

Heilmann, I., Mekhedov, S., King, B., Browse, J. and Shanklin, J. (2004) Identification of the Arabidopsis palmitoyl-monogalactosyldiacylglycerol Δ7-desaturase gene FAD5, and effects of plastidial retargeting of Arabidopsis desaturases on the *fad5* mutant phenotype. *Plant Physiol.* **136**, 4237–4245.

Hemschemeier, A., Casero, D., Liu, B., Benning, C., Pellegrini, M., Happe, T. and Merchant, S.S. (2013) Copper response regulator1–dependent and–independent responses of the *Chlamydomonas reinhardtii* transcriptome to dark anoxia. *Plant Cell*, **25**, 3186–3211.

Horn, P.J. and Chapman, K.D. (2012) Lipidomics in tissues, cells and subcellular compartments. *Plant J.* **70**, 69–80.

Horn, P.J., Korte, A.R., Neogi, P.B., Love, E., Fuchs, J., Strupat, K., Borisjuk, L., Shulaev, V., Lee, Y.J. and Chapman, K.D. (2012) Spatial mapping of lipids at cellular resolution in embryos of cotton. *Plant Cell*, **24**, 622–636.

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

Huang, A.H.C. (1992) Oil bodies and oleosins in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 177–200.

Huang, N.-L., Huang, M.-D., Chen, T.-L.L. and Huang, A.H.C. (2013) Oleosin of subcellular lipid droplets evolved in green algae. *Plant Physiol.* **161**, 1862–1874.

Hung, C.-H., Ho, M.-Y., Kanehara, K. and Nakamura, Y. (2013) Functional study of diacylglycerol acyltransferase type 2 family in *Chlamydomonas reinhardtii*. *FEBS Lett.* **587**, 2364–2370.

Hurlock, A.K., Roston, R.L., Wang, K. and Benning, C. (2014) Lipid trafficking in plant cells. *Traffic*, **15**, 915–932.

Hwangbo, K., Ahn, J.-W., Lim, J.-M., Park, Y.-I., Liu, J. and Jeong, W.-J. (2014) Overexpression of stearoyl-ACP desaturase enhances accumulations of oleic acid in the green alga *Chlamydomonas reinhardtii*. *Plant Biotechnol. Rep.* **8**, 135–142.

James, C.N., Horn, P.J., Case, C.R., Gidda, S.K., Zhang, D.Y., Mullen, R.T., Dyer, J.M., Anderson, R.G.W. and Chapman, K.D. (2010) Disruption of the Arabidopsis CGI-58 homologue produces Chanarin-Dorfman-like lipid droplet accumulation in plants. *Proc. Natl Acad. Sci. USA*, **107**, 17833–17838.

James, G.O., Hocart, C.H., Hillier, W., Price, G.D. and Djordjevic, M.A. (2013) Temperature modulation of fatty acid profiles for biofuel production in nitrogen-deprived *Chlamydomonas reinhardtii*. *Bioresour. Technol.* **127**, 441–447.

Jouhet, J., Maréchal, E. and Block, M.A. (2007) Glycerolipid transfer for the building of membranes in plant cells. *Prog. Lipid Res.* **46**, 37–55.

Joyard, J., Teyssier, E., Miege, C., Berny-Seigneurin, D., Marechal, E., Block, M.A., Dorne, A.J., Rolland, N., Ajlani, G. and Douce, R. (1998) The biochemical machinery of plastid envelope membranes. *Plant Physiol.* **118**, 715–723.

Kajikawa, M., Yamato, K.T., Kohzu, Y., Shoji, S., Matsui, K., Tanaka, Y., Sakai, Y. and Fukuzawa, H. (2006) A front-end desaturase from *Chlamydomonas reinhardtii* produces pinolenic and coniferonic acids by omega 13 desaturation in methylotrophic yeast and tobacco. *Plant Cell Physiol.* **47**, 64–73.

Kato, J., Yamahara, T., Tanaka, K., Takio, S. and Satoh, T. (1997) Characterization of catalase from green algae *Chlamydomonas reinhardtii*. *J. Plant Physiol.* **151**, 262–268.

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

Kessler, F. and Vidi, P.A. (2007) Plastoglobule lipid bodies: their functions in chloroplasts and their potential for applications. In *Green Gene Technology: Research in an Area of Social Conflict* (Fiechter, A. and Sautter, C. eds). Berlin, Heidelberg: Springer, pp. 153–172.

Khozin-Goldberg, I., Iskandarov, U. and Cohen, Z. (2011) LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. *Appl. Microbiol. Biotechnol.* **91**, 905–915.

Kim, S., Kim, H., Ko, D. *et al.* (2013) Rapid induction of lipid droplets in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* by Brefeldin A. *PLoS ONE*, **8**, e81978.

La Russa, M., Bogen, C., Uhmeyer, A., Doebbe, A., Filippone, E., Kruse, O. and Mussgnug, J.H. (2012) Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. *J. Biotechnol.* **162**, 13–20.

Li, X., Moellering, E.R., Liu, B., Johnny, C., Fedewa, M., Sears, B.B., Kuo, M.-H. and Benning, C. (2012a) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in *Chlamydomonas reinhardtii*. *Plant Cell*, **24**, 4670–4686.

Li, X.B., Benning, C. and Kuo, M.H. (2012b) Rapid triacylglycerol turnover in *Chlamydomonas reinhardtii* requires a lipase with broad substrate specificity. *Eukaryot. Cell*, **11**, 1451–1462.

Li-Beisson, Y., Shorrosh, B., Beisson, F. *et al.* (2013) Acyl-lipid metabolism. *The Arabidopsis Book*. USA: The American Society of Plant Biologist, e0161.

Liu, B. and Benning, C. (2013) Lipid metabolism in microalgae distinguishes itself. *Curr. Opin. Biotechnol.* **24**, 300–309.

Liu, B., Vieler, A., Li, C., Daniel Jones, A. and Benning, C. (2013) Triacylglycerol profiling of microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica*. *Bioresour. Technol.* **146**, 310–316.

Lopez, D., Casero, D., Cokus, S.J., Merchant, S.S. and Pellegrini, M. (2011) Algal functional annotation tool: a web-based analysis suite to functionally interpret large gene lists using integrated annotation and expression data. *BMC Bioinform.* **12**, 282.

Los, D.A. and Murata, N. (1998) Structure and expression of fatty acid desaturases. *BBA-Lipid Lipid Met.* **1394**, 3–15.

Lu, C.F., Napier, J.A., Clemente, T.E. and Cahoon, E.B. (2011) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Curr. Opin. Biotech.* **22**, 252–259.

Meesapyodsuk, D. and Qiu, X. (2012) The front-end desaturase: structure, function, evolution and biotechnological use. *Lipids*, **47**, 227–237.

Mehrshahi, P., Johnny, C. and DellaPenna, D. (2014) Redefining the metabolic continuity of chloroplasts and ER. *Trends Plant Sci.* **19**, 501–507.

Merchant, S.S., Prochnik, S.E., Vallon, O. *et al.* (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*, **318**, 245–250.

Merchant, S.S., Kropat, J., Liu, B., Shaw, J. and Warakanont, J. (2012) TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. *Curr. Opin. Biotechnol.* **23**, 352–363.

Michelet, L., Lefebvre-Legendre, L., Burr, S.E., Rochaix, J.D. and Goldschmidt-Clermont, M. (2011) Enhanced chloroplast transgene expression in a nuclear mutant of *Chlamydomonas*. *Plant Biotech. J.* **9**, 565–574.

Miller, R., Wu, G.X., Deshpande, R.R. *et al.* (2010) Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. *Plant Physiol.* **154**, 1737–1752.

Moellering, E.R. and Benning, C. (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **9**, 97–106.

Moellering, E.R., Miller, R. and Benning, C. (2010) Molecular genetics of lipid metabolism in the model green alga *Chlamydomonas reinhardtii*. In *Lipids in Photosynthesis* (Wada, H. and Murata, N., eds). Netherlands: Springer, pp. 139–155.

Murphy, D.J. (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Prog. Lipid Res.* **32**, 247–280.

Murphy, S., Martin, S. and Parton, R.G. (2009) Lipid droplet-organelle interactions; sharing the fats. *BBA - Mol. Cell Biol. Lip.* **1791**, 441–447.

Nguyen, H.M., Baudet, M., Cuiné, S. *et al.* (2011) Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii:* with focus on proteins involved in lipid metabolism. *Proteomics*, **11**, 4266–4273.

Nguyen, H.M., Cuiné, S., Beyly-Adriano, A., Légeret, B., Billon, E., Auroy, P., Beisson, F., Peltier, G. and Li-Beisson, Y. (2013) The green microalga *Chlamydomonas reinhardtii* has a single ω-3 fatty acid desaturase that localizes to the chloroplast and impacts both plastidic and extraplastidic membrane lipids. *Plant Physiol.* **163**, 914–928.

Nordhues, A., Schottler, M.A., Unger, A.K. *et al.* (2012) Evidence for a role of VIPP1 in the structural organization of the photosynthetic apparatus in *Chlamydomonas*. *Plant Cell*, **24**, 637–659.

Ohlrogge, J. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell*, **7**, 957–970.

Petschnigg, J., Wolinski, H., Kolb, D., Zellnig, G., Kurat, C.F., Natter, K. and Kohlwein, S.D. (2009) Good fat, essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. *J. Biol. Chem.* **284**, 30981–30993.

Pflaster, E.L., Schwabe, M.J., Becker, J., Wilkinson, M.S., Parmer, A., Clemente, T.E., Cahoon, E.B. and Riekhof, W.R. (2014) A high-throughput fatty acid profiling screen reveals novel variations in fatty acid biosynthesis in *Chlamydomonas reinhardtii* and related algae. *Eukaryot. Cell*, **13**, 1431–1438.

Pineau, B., Girard-Bascou, J., Eberhard, S., Choquet, Y., Tremolieres, A., Gerard-Hirne, C., Bennardo-Connan, A., Decottignies, P., Gillet, S. and Wollman, F.A. (2004) A single mutation that causes phosphatidylglycerol deficiency impairs synthesis of photosystem II cores in *Chlamydomonas reinhardtii*. *Euro. J. Biochem.***271**, 329–338.

Poirier, Y., Antonenkov, V.D., Glumoff, T. and Hiltunen, J.K. (2006) Peroxisomal beta-oxidation – A metabolic pathway with multiple functions. *BBA-Mol. Cell Res.* **1763**, 1413–1426.

Riediger, N.D., Othman, R.A., Suh, M. and Moghadasian, M.H. (2009) A systemic review of the roles of n-3 fatty acids in health and disease. *J. Am. Diet. Assoc.* **109**, 668–679.

Riekhof, W.R., Ruckle, M.E., Lydic, T.A., Sears, B.B. and Benning, C. (2003) The sulfolipids 2′-O-acyl-sulfoquinovosyldiacylglycerol and sulfoquinovosyldiacylglycerol are absent from a *Chlamydomonas reinhardtii* mutant deleted in SQD1. *Plant Physiol.* **133**, 864–874.

Riekhof, W.R., Sears, B.B. and Benning, C. (2005) Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii:* discovery of the betaine lipid synthase BTA1(Cr). *Eukaryot. Cell*, **4**, 242–252.

Riekhof, W.R., Naik, S., Bertrand, H., Benning, C. and Voelker, D.R. (2014) Phosphate starvation in fungi induces the replacement of phosphatidylcholine with the phosphorus-free betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine. *Eukaryot. Cell*, **13**, 749–757.

Rochaix, J.D. (2002) The three genomes of *Chlamydomonas*. *Photosynth. Res.* **73**, 285–293.

Rolland, N., Atteia, A., Decottignies, P., Garin, J., Hippler, M., Kreimer, G., Lemaire, S.D., Mittag, M. and Wagner, V. (2009) *Chlamydomonas* proteomics. *Curr. Opin. Microbiol.* **12**, 285–291.

Rolland, N., Curien, G., Finazzi, G., Kuntz, M., Maréchal, E., Matringe, M., Ravanel, S. and Seigneurin-Berny, D. (2012) The biosynthetic capacities of the plastids and integration between cytoplasmic and chloroplast processes. *Annu. Rev. Genet.* **46**, 233–264.

Ruiz-Lopez, N., Sayanova, O., Napier, J.A. and Haslam, R.P. (2012) Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants. *J. Exp. Bot.* **63**, 2397–2410.

Sakurai, K., Moriyama, T. and Sato, N. (2014) Detailed identification of fatty acid isomers sheds light on the probable precursors of triacylglycerol accumulation in photoautotrophically grown *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **13**, 256–266.

Sanjaya, Miller, R., Durrett, T.P. *et al.* (2013) Altered lipid composition and enhanced nutritional value of Arabidopsis leaves following introduction of an algal diacylglycerol acyltransferase 2. *Plant Cell*, **25**, 677–693.

Sato, N., Tsuzuki, M., Matsuda, Y., Ehara, T., Osafune, T. and Kawaguchi, A. (1995) Isolation and characterization of mutants affected in lipid metabolism of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **230**, 987–993.

Sato, N., Fujiwara, S., Kawaguchi, A. and Tsuzuki, M. (1997) Cloning of a gene for chloroplast omega 6 desaturase of a green alga, *Chlamydomonas reinhardtii*. *J. Biochem.* **122**, 1224–1232.

Schlapfer, P. and Eichenberger, W. (1983) Evidence for the involvement of diacylglyceryl (*N, N, N*) homoserine in the desaturation of oleic and linoleic acids in *Chlamydomonas reinhardtii* (Chlorophyceae). *Plant Sci. Lett.* **32**, 243–252.

Schmidt, M., Gessner, G., Matthias, L. *et al.* (2006) Proteomic analysis of the eyespot of *Chlamydomonas reinhardtii* provides novel insights into its components and tactic movements. *Plant Cell*, **18**, 1908–1930.

Schmollinger, S., Muhlhaus, T., Boyle, N.R. *et al.* (2014) Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism. *Plant Cell*, **26**, 1410–1435.

Seras, M., Garnier, J., Tremolieres, A. and Guyon, D. (1989) Lipid biosynthesis in cells of the wild-type and of 2 photosynthesis mutants of *Chlamydomonas reinhardtii*. *Plant Physiol. Biochem.* **27**, 393–399.

Shanklin, J. and Cahoon, E.B. (1998) Desaturation and related modifications of fatty acids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 611–641.

Sheehan, J., Dunahay, T., Benemann, J. and Roessler, P.G. (1998) *A Look Back at the US Department of Energy's Aquatic Species Program–Biodiesel from Algae*. Golden, CO: National Renewable Energy Laboratory.: US Department of Energy's Office of Fuels Development.

Shinozaki, A., Sato, N. and Hayashi, Y. (2009) Peroxisomal targeting signals in green algae. *Protoplasma*, **235**, 57–66.

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

Sirevåg, R. and Levine, R.P. (1972) Fatty acid synthetase from *Chlamydomonas reinhardti:* sites of transcription and translation. *J. Biol. Chem.* **247**, 2586–2591.

Slocombe, S., Cornah, J., Pinfield-Wells, H., Soady, K., Zhang, Q., Gilday, A., Dyer, J. and Graham, I. (2009) Oil accumulation in leaves directed by modification of fatty acid breakdown and lipid synthesis pathways. *Plant Biotechnol. J.* **7**, 694–703.

Small, D. (1968) A classification of biologic lipids based upon their interaction in aqueous systems. *J. Am. Oil Chem. Soc.* **45**, 108–119.

Somerville, C.R., Browse, J., Jaworski, J. and Ohlrogge, J. (2000) Lipids. In *Biochemistry and Molecular Biology of Plants* (Buchanan, B.B., Gruissem, W. and Jones, R.L. eds). Rockville, MD: American Society of Plant Physiologists, pp. Chap 10.

Sperling, P. and Heinz, E. (2003) Plant sphingolipids: structural diversity, biosynthesis, first genes and functions. *BBA-Mol. Cell Biol. L.* **1632**, 1–15.

Sperling, P., Ternes, P., Zank, T.K. and Heinz, E. (2003) The evolution of desaturases. *Prostag. Leukotr. E. S. S.* **68**, 73–95.

Tardif, M., Atteia, A., Specht, M. *et al.* (2012) PredAlgo, a new subcellular localization prediction tool dedicated to green algae. *Mol. Biol. Evol.* **29**, 3625–3639.

Trémolières, A. (1998) Glycerolipids: composition, biosynthesis and function in *Chlamydomonas*. In *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas* (Rochaix, J.D., Goldschmidt-Clermont, M. and Merchant, S. eds). Netherlands: Springer, pp. 415–431.

Trémolières, A., Roche, O., Dubertret, G., Guyon, D. and Garnier, J. (1991) Restoration of thylakoid appression by Δ3-trans-hexadecenoic acid-containing phosphatidylglycerol in a mutant of *Chlamydomonas reinhardtii*. Relationships with the regulation of excitation energy distribution. *BBA-Bioenergetics*, **1059**, 286–292.

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

Tsai, C.-H., Warakanont, J., Takeuchi, T., Sears, B.B., Moellering, E.R. and Benning, C. (2014) The protein compromised hydrolysis of triacylglycerols 7 (CHT7) acts as a repressor of cellular quiescence in *Chlamydomonas*. *Proc. Natl Acad. Sci. USA*, **111**, 15833–15838.

Urzica, E.I., Vieler, A., Hong-Hermesdorf, A., Page, M.D., Casero, D., Gallaher, S.D., Kropat, J., Pellegrini, M., Benning, C. and Merchant, S.S. (2013) Remodeling of membrane lipids in iron-starved *Chlamydomonas*. *J. Biol. Chem.* **288**, 30246–30258.

Venegas-Caleron, M., Sayanova, O. and Napier, J.A. (2010) An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. *Prog. Lipid Res.* **49**, 108–119.

Vu, H.S., Shiva, S., Roth, M.R. *et al.* (2014) Lipid changes after leaf wounding in *Arabidopsis thaliana:* expanded lipidomic data form the basis for lipid co-occurrence analysis. *Plant J.* **80**, 728–743.

Wada, H., Shintani, D. and Ohlrogge, J. (1997) Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proc. Natl Acad. Sci. USA*, **94**, 1591–1596.

Wallis, J.G., Watts, J.L. and Browse, J. (2002) Polyunsaturated fatty acid synthesis: what will they think of next? *Trends Biochem. Sci.* **27**, 467–473.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S. and Goodenough, U. (2009) Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **8**, 1856–1868.

Welti, R. and Wang, X.M. (2004) Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. *Curr. Opin. Plant Biol.* **7**, 337–344.

Wijffels, R.H. and Barbosa, M.J. (2010) An outlook on microalgal biofuels. *Science*, **329**, 796–799.

Wolff, R.L., Pedrono, F., Pasquier, E. and Marpeau, A.M. (2000) General characteristics of *Pinus* spp. seed fatty acid compositions, and importance of Delta 5-olefinic acids in the taxonomy and phylogeny of the genus. *Lipids*, **35**, 1–22.

Work, V.H., Radakovits, R., Jinkerson, R.E., Meuser, J.E., Elliott, L.G., Vinyard, D.J., Laurens, L.M.L., Dismukes, G.C. and Posewitz, M.C. (2010) Increased lipid accumulation in the *Chlamydomonas reinhardtii sta7-10* starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. *Eukaryot. Cell*, **9**, 1251–1261.

Yang, W., Moroney, J.V. and Moore, T.S. (2004a) Membrane lipid biosynthesis in *Chlamydomonas reinhardtii:* ethanolaminephosphotransferase is capable of synthesizing both phosphatidylcholine and phosphatidylethanolamine. *Arch. Biochem. Biophys.* **430**, 198–209.

Yang, W.Y., Mason, C.B., Pollock, S.V., Lavezzi, T., Moroney, J.V. and Moore, T.S. (2004b) Membrane lipid biosynthesis in *Chlamydomonas reinhardtii:* expression and characterization of CTP: phosphoethanolamine cytidylyltransferase. *Biochem. J.* **382**, 51–57.

Yoon, K., Han, D., Li, Y., Sommerfeld, M. and Hu, Q. (2012) Phospholipid: diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. *Plant Cell*, **24**, 3708–3724.

Zauner, S., Jochum, W., Bigorowski, T. and Benning, C. (2012) A cytochrome b(5)-containing plastid-located fatty acid desaturase from *Chlamydomonas reinhardtii*. *Eukaryot. Cell*, **11**, 856–863.

Zhang, M., Fan, J., Taylor, D.C. and Ohlrogge, J.B. (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. *Plant Cell*, **21**, 3885–3901.

Zhang, R., Patena, W., Armbruster, U., Gang, S.S., Blum, S.R. and Jonikas, M.C. (2014) High-throughput genotyping of green algal mutants reveals random distribution of mutagenic insertion sites and endonucleolytic cleavage of transforming DNA. *Plant Cell*, **26**, 1398–1409.