Chlamydomonas: Triacylglycerol Accumulation

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Abstract The unicellular microalga *Chlamydomonas reinhardtii* exhibits immense metabolic flexibility, adjusting to changes in the environment and nutrient availability. One metabolic response under stress conditions is the synthesis of the neutral lipid triacylglycerol (TAG), accumulating as intracellular lipid droplets in the cytosol and chloroplast. With increased industrial interest in microalgal production of biofuels, feed, food, and chemicals, research on lipid metabolism using *C. reinhardtii* as a model system has accelerated in recent years. Conditions in which *C. reinhardtii* accumulates TAG have been identified, with nitrogen starvation as one of the most commonly used methods for induction. Genome, transcriptome, proteome, and lipidome analyses have provided information on the pathways involved in TAG synthesis and degradation. These studies have demonstrated that although a multitude of stress conditions induce TAG accumulation, there are differential response and regulatory mechanisms occurring under various induction conditions. Studies utilizing mutants have further led to the identification of pathways and regulatory components contributing to TAG synthesis and degradation. TAG metabolism is a multifaceted process in *C. reinhardtii*, and induction of TAG accumulation is accompanied by major reorganization of metabolic pathways, adjustments of photosynthetic complexes, membrane lipid recycling, and changes in carbon partitioning.

1 Introduction

Intracellular polymers serving as energy reserves are found in organisms across the tree of life. Among these, synthesis and accumulation of the neutral lipid triacylglycerol (TAG) in the form of lipid droplets are widespread in eukaryotic cells and are also found in prokaryotes (Gao and Goodman 2015; Waltermann et al. 2007). The unicellular microalga *Chlamydomonas reinhardtii* (hereafter Chlamydomonas) is no exception and accumulates TAGs, especially under nutrient

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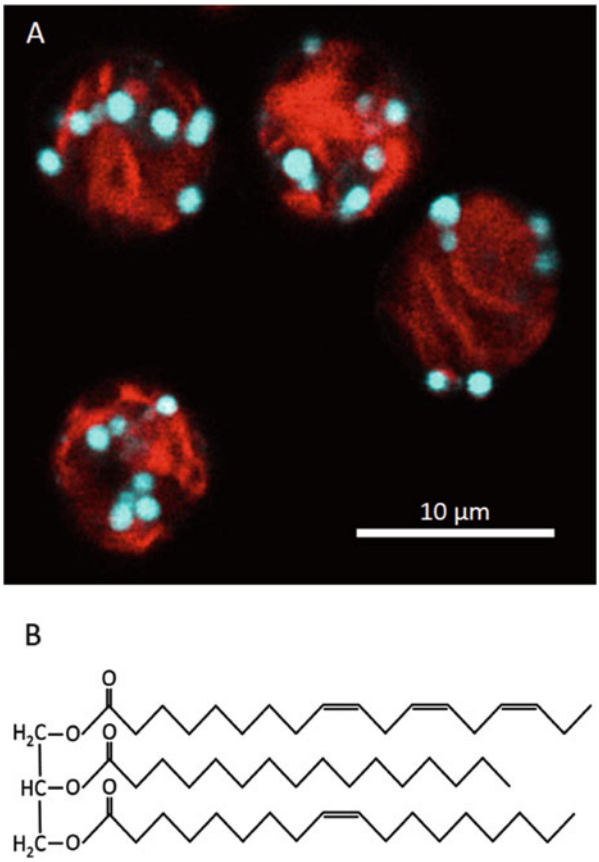

Fig. 1 *Chlamydomonas reinhardtii* accumulates triacylglycerol (TAG) in intracellular lipid droplets. (**a**) A false color image of Chlamydomonas cells after 4 days of nitrogen starvation and stained with nile red. *Cyan* represents fluorescence signal from nile red-stained lipid droplets, and *red* represents chlorophyll autofluorescence. Image was taken with a Leica SP5 confocal microscope using a 488 nm laser excitation and emission captured at 536–544 nm for nile red and 676–684 nm for chlorophyll. (**b**) An example of a triacylglycerol lipid species, TAG 18:3/16:0/18:1. Reference for protocol used for nile red staining and imaging: (Terashima et al. 2015)

deprivation or other environmental stress factors (Fig. 1a) (Merchant et al. 2012; Goold et al. 2015). Chlamydomonas has been studied in more detail compared to any other alga, resulting in the availability of molecular and genetic tools, annotated genome information, and an ever-increasing library of mapped mutants (Liu and Benning 2013; Blaby et al. 2014; Li et al. 2016b). For this reason, Chlamydomonas has become a model system to investigate microalgal lipid metabolism, and research in this field has particularly gained traction due to potential interest in the production of biofuels and high-grade lipids (Hu et al. 2008; Liu and Benning 2013; Goncalves et al. 2016).

TAG is a neutral lipid consisting of a glycerol backbone esterified with three fatty acid chains (Fig. 1b). TAGs allow for a considerable amount of metabolic energy to be stored due to its reduced and anhydrous nature. As TAGs are insoluble in water, they are also a suitable storage compound because they do not affect the aqueous substrate concentrations in the cells, allowing for storage of carbon compounds without affecting cellular metabolic flux (Flatt 1995). Additionally,

compared with free fatty acids, TAGs have low toxicity (Wältermann and Steinbüchel 2006).

Lipid droplets were once considered metabolically dormant storage organelles but have recently been recognized as important organelles for energy metabolism, playing a significant role in communication with other cellular organelles (Gao and Goodman 2015; Liu et al. 2013b). Therefore, understanding the physiological process and regulation of TAG accumulation merits investigation, both for basic science and for biotechnological applications.

2 Triacylglycerol Accumulation in Chlamydomonas

TAG accumulation in Chlamydomonas varies greatly depending on the strains and growth conditions. For wild type, 2 days of nitrogen starvation results in 2–15% dry weight TAG accumulation and 20–65% TAG accumulation reported for strains blocked in starch synthesis (Li et al. 2010a; Siaut et al. 2011; James et al. 2011). Numerous studies have been conducted to test different cultivation conditions of various wild-type and mutant strains. These types of growth tests and mutant characterizations in combination with omics analyses are beginning to reveal the complex metabolic pathways and regulation behind TAG accumulation in Chlamydomonas.

2.1 TAG Synthesis Is Triggered by Exposure to Stressful Growth Conditions

In Chlamydomonas, TAG accumulation is induced under stressful growth conditions. Among nutrient limitation stress, nitrogen starvation has the strongest induction of TAG synthesis, with TAG appearing in the form of lipid droplets already after 6 h following transfer to nitrogen-free, acetate-containing media (photoheterotrophic conditions), and increases exponentially up to 3 days, after which accumulation continues at a slower pace (Park et al. 2015; Siaut et al. 2011). TAG accumulation can be further enhanced under photoheterotrophic conditions by supplying the culture with extra acetate after 2 days of nitrogen starvation or even by growing cells in nitrogen-replete conditions but with extra acetate, effectively changing the carbon-to-nitrogen ratio in the media (Goodson et al. 2011; Goodenough et al. 2014; Fan et al. 2012). TAG synthesis also occurs under photoautotrophic nitrogen-depleted conditions but at a slower rate (Merchant et al. 2012; Davey et al. 2014). Additionally, when observed over a longer period, TAG initially accumulates over the first 2 days under photoautotrophic conditions but decreases rapidly back to baseline levels by day 6 (Schulz-Raffelt et al. 2016). However, another study found accumulation to steadily continue over 10 days

under photoautotrophic conditions with low nitrogen levels (tenfold lower than normal conditions) (Davey et al. 2014). Supplying minimal levels of nitrogen may sustain photoautotrophic TAG synthesis longer. Photosynthesis appears to be important for TAG synthesis, as TAG accumulation was significantly compromised under dark heterotrophic conditions and under photoheterotrophic conditions with photosynthesis blocked by the addition of a chemical inhibitor of photosystem II, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Fan et al. 2012).

Aside from nitrogen depletion, other growth conditions such as phosphate or sulfur deficiency, anaerobiosis, and salt or high light stress can induce TAG accumulation but to a lesser extent (Bajhaiya et al. 2016; Sato et al. 2014; Fan et al. 2011; Hemschemeier et al. 2013; Siaut et al. 2011). In nutrient-limiting conditions, TAG accumulation acts as a sink for carbon and photosynthetically generated reducing equivalents, a process that occurs over hours to days (Johnson and Alric 2013; Li et al. 2012b). There are also faster induction conditions that cause TAGs to accumulate already within an hour, such as heat stress and chemical treatments (Legeret et al. 2016; Kim et al. 2013, 2015). Heat stress and treatment with fungicide fenpropimorph, an inhibitor of sterol biosynthesis, induce chloroplast polar membrane lipids to be rapidly converted to TAGs (Legeret et al. 2016; Kim et al. 2015). Similarly, brefeldin A treatment causes ER stress, resulting in lipid droplet increase (Kim et al. 2013). Lipid droplets are believed to be in part synthesized in the ER, and disrupting vesicular transport in the ER by brefeldin A treatment may result in the accumulation of substrates of TAG synthesis, further enhancing TAG accumulation. Heat stress and chemical treatments that damage the cell can cause a rapid accumulation of unstable compounds such as unfolded proteins with exposed hydrophobic residues, and lipid droplets may provide a docking site for such unstable compounds and prevent further cellular damage (Kim et al. 2013; Welte 2007).

2.2 *TAG and Starch Are the Main Carbon Sinks in Chlamydomonas*

Under nitrogen starvation, growth is compromised with little cell density change observed after 24 h (Valledor et al. 2014; Park et al. 2015). Transcriptomic analyses after 48 h of nitrogen starvation indicate, as expected, that protein synthesis and glyoxylate and gluconeogenesis pathways are stalled and acetate is incorporated into fatty acids (Miller et al. 2010). Transcriptomics and proteomics starting from 0.5 to 24 h after switch to media without nitrogen show the metabolic transitioning leading to TAG accumulation (Park et al. 2015). Interestingly, glyoxylate cycle inhibition occurs very early on, resulting in a decrease of transcripts already 2 h and full protein reduction 24 h after switch to nitrogen-starved media. In contrast, gluconeogenesis is initially induced during the first couple of hours followed by reduction. TCA cycle transcripts were reduced during the first 24 h, but protein

levels remained stable. An enzyme of the TCA cycle, citrate synthase (CIS), had decreased transcripts after 2 days of nitrogen starvation and appears to affect TAG accumulation (Deng et al. 2013). RNA interference-based suppression of *CIS* gene expression led to increased TAG accumulation and overexpressing *CIS*-reduced cellular TAG levels, suggesting a link between TAG accumulation and cellular carbon flux via CIS. These data indicate that TAG accumulation is accompanied by major metabolic reorganization. In addition, proteomic analysis points to induction of ammonia uptake and assimilation enzymes and starch synthesis enzymes, while protein biosynthesis and amino acid degradation enzymes were decreased (Valledor et al. 2014). By storing excess carbon as starch and TAGs under nitrogen starvation, when nitrogen becomes readily available again, turnover of these stored fixed carbon reserves can allow for rapid synthesis of proteins for cell growth (Scott et al. 2010). As expected, lipid droplets formed during nitrogen starvation are rapidly consumed, with TAG content returning to basal levels within 2 days (Li et al. 2012a; Siaut et al. 2011).

Starch appears to be the initial carbon sink during nitrogen starvation, with rapid accumulation occurring during the first 24 h, while TAG accumulation is more delayed and lasts for several days (Siaut et al. 2011; Fan et al. 2012; Gardner et al. 2013; Krishnan et al. 2015). Accordingly, transcripts for enzymes involved in starch synthesis were induced already after 30 min following transfer to nitrogen-deprived medium (Park et al. 2015). Not surprisingly, mutants blocked in starch synthesis accumulate higher levels of TAG, and these "starchless" mutants are still among the highest TAG-accumulating strains in Chlamydomonas today, with high TAG content on a per-cell basis and by dry weight when measured during the early phase of TAG accumulation (~24 h) and during the later phases (24–96 h) (Ball et al. 1991; Zabawinski et al. 2001; Wang et al. 2009; Li et al. 2010b, a; Work et al. 2010; Goodson et al. 2011; Velmurugan et al. 2013). However, it is worth noting that various regularly used laboratory background strains of Chlamydomonas showed differing amounts of TAG accumulation, suggesting that a wide range of factors can affect carbon flux (Siaut et al. 2011). TAG accumulation is a multifaceted phenomenon with no single "on" and "off" switch, and comparison of mutants to its original background strain is crucial.

2.3 TAG Stems from Both Exogenous and Photosynthetically Fixed Carbon Sources

Fixed carbon via photosynthesis and acetate taken up from the media are both carbon sources for TAGs (Davey et al. 2014). Addition of exogenous lipids or fatty acids to the medium has also been reported to induce TAG accumulation (Grenier et al. 1991; Fan et al. 2011). Fatty acids that are esterified to the glycerol backbone to generate TAG are either synthesized de novo or derived from degraded membrane lipids (Liu and Benning 2013). Fatty acids are synthesized in the chloroplast

and get incorporated into TAGs either directly in the chloroplast or the free fatty acids are exported to the cytosol followed by import to the endoplasmic reticulum (ER) for TAG synthesis (Fan et al. 2011; Riekhof et al. 2005). Because changing the carbon-to-nitrogen ratio affects carbon flux in the cell, the highest TAG accumulation, albeit transient, has been achieved by adding external carbon sources, with increases observed both during photoheterotrophic conditions by additional acetate and during photoautotrophic conditions by induction in higher CO_2 levels (Goodson et al. 2011; Goodenough et al. 2014; Fan et al. 2012; Gardner et al. 2013; Goncalves et al. 2016).

3 TAG Synthesis: A Process Involving the Chloroplast and the ER

Microscopy images show lipid droplets in both the cytosol and the chloroplast, and TAG synthesis is thought to occur both in the ER and the chloroplast (Fan et al. 2011; Goodson et al. 2011). A complete set of genes predicted to be involved in TAG synthesis has been identified in the Chlamydomonas genome; however, most enzymes lack experimental evidence for subcellular localization (Riekhof et al. 2005; Merchant et al. 2012; Li-Beisson et al. 2015). Utilizing subcellular prediction programs, proteomic data, and comparison to higher plants have provided hypothesized localization of pathways involved in TAG biosynthesis (Tardif et al. 2012; Li-Beisson et al. 2015). A model for ER-localized and chloroplast TAG synthesis pathways is shown in Fig. 2. Under nitrogen starvation, the total amount of fatty acids increases, suggesting that a certain amount of TAGs are derived from de novo synthesis and not solely from membrane lipid recycling (Moellering and Benning 2010).

3.1 TAGs Are De Novo Synthesized or Generated via Membrane Lipid Recycling

For de novo synthesis, TAG is synthesized by the sequential acylation of glycerol-3-phosphate via the production of its precursor diacylglycerol (DAG), which also acts as a precursor for the synthesis of other membrane lipids (Riekhof et al. 2005; Li-Beisson et al. 2015). DAG is synthesized through three enzymatic steps from glycerol-3-phosphate (Fig. 2). Glycerol-3-phosphate is acylated at the sn-1 position by glycerol-3-phosphate acyltransferase (GPAT) followed by a second acylation at the sn-2 position by lysophosphatidic acid acyltransferase (LPAT). The phosphate group on the sn-3 position is removed by a phosphatidic acid phosphatase (PAP) to yield DAG. Next, DAG is converted to TAG by the esterification of the sn-3 position with an acyl group. Diacylglycerol acyltransferase (DGAT)

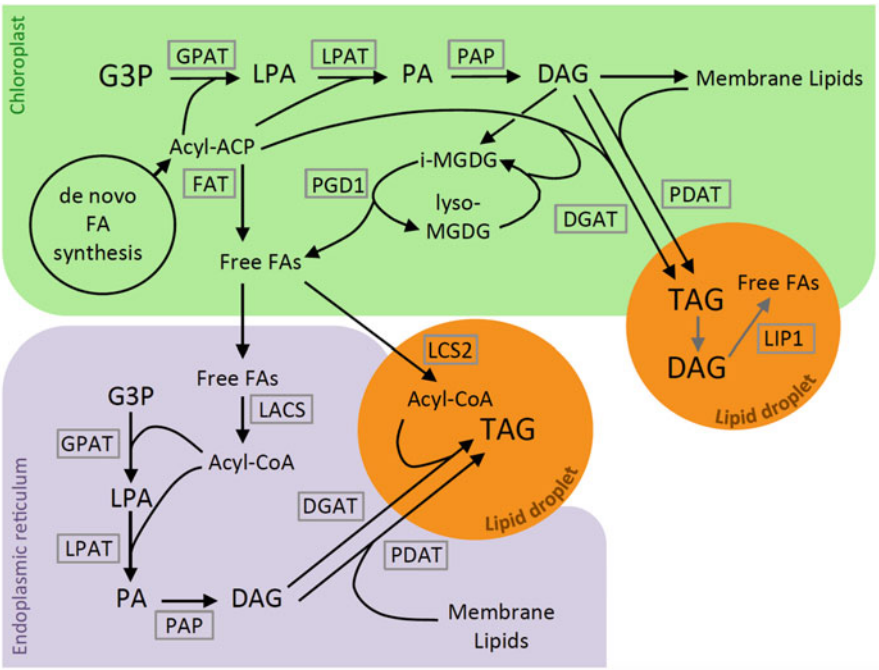

Fig. 2 Model for triacylglycerol (TAG) synthesis in *Chlamydomonas reinhardtii*. TAG is de novo synthesized via the acylation of glycerol-3-phosphate (G3P) with acyl chains derived from fatty acids synthesized in the chloroplast. Alternatively, acyl chains from membrane lipids are incorporated into TAGs. *ACP* acyl carrier protein, *DAG* diacylglycerol, *DGAT* diacylglycerol acyltransferase, *FA* fatty acid, *FAT* fatty acyl-ACP thioesterase, *GPAT* glycerol-3-phosphate acyltransferase, *i-MGDG* immature monogalactosyldiacylglycerol, *LACS* long-chain acyl-CoA synthetase, *LCS2* long-chain acyl-CoA synthetase 2, *LIP1* diacylglycerol lipase, *LPA* lysophosphatidic acid, *LPAT* lysophosphatidic acid acyltransferase, *PA* phosphatidic acid, *PAP* phosphatidic acid phosphatase, *PDAT* phospholipid:diacylglycerol acyltransferase, *PGD1* plastid galactoglycerolipid degradation 1. The localization of the enzymes should been seen as speculative, as experimental evidence for many are lacking or based on proteomic data, which does not rule out dual targeting (see text for details). Additionally, the mechanism of lipid transport between organelles is currently unknown, and the *arrows* are not indicative of any specific trafficking routes. *Green*, chloroplast; *purple*, endoplasmic reticulum; *orange*, lipid droplet. References for the figure: Riekhof et al. (2005), Moellering and Benning (2010), Nguyen et al. (2011), Fan et al. (2011), Goodson et al. (2011), Li et al. (2012a, b, 2016b), Boyle et al. (2012), Yoon et al. (2012), Liu and Benning (2013), Li-Beisson et al. (2015), Park et al. (2015), Goold et al. (2015), Goncalves et al. (2016)

catalyzes the reaction using acyl-CoA as a substrate (acyl-CoA-dependent TAG synthesis). Chlamydomonas has six genes encoding for DGAT (gene *DGAT1*, type I, and genes *DGTT1–DGTT5*, type II) (Miller et al. 2010). Heterologous complementation assays of Chlamydomonas DGTT in yeast mutants confirmed the functionality of DGTT1–DGTT3, but not DGTT4 (Hung et al. 2013). The reasons for so many isoforms of diacylglycerol transferases in Chlamydomonas

are unclear. However, recent research has shown distinct substrate specificities for each DGTT (Liu et al. 2016). DGTT1 favors unsaturated acyl-CoAs (especially polyunsaturated acyl-CoAs) and prefers shorter-chain acyl-CoAs. DGTT2 favors monounsaturated acyl-CoA, and DGTT3 prefers C16-containing acyl-CoA. Additionally, DGTT1 is partial for a DAG with C16 fatty acid in the sn-2 position, while DGTT2 and DGTT3 favor C18 at that position.

Aside from de novo synthesis, membrane lipids can be recycled directly into TAGs by replacing the head group with an acyl group or indirectly by transferring the acyl group from an acyl-lipid to DAG to generate TAG (Li-Beisson et al. 2015). The enzyme responsible for removing the head group from an acyl-lipid to yield DAG is currently unknown, although an abundant thylakoid membrane lipid monogalactosyldiacylglycerol (MGDG) appears to be the major contributing lipid source recycled to TAG via this mechanism (Legeret et al. 2016). Lipids that have been converted to DAG by removing the head group or de novo synthesized DAG can be subsequently acylated via an acyl-CoA-independent pathway by phospholipid:diacylglycerol acyltransferase (PDAT), which catalyzes the reaction using an acyl-lipid as an acyl donor (Fig. 2) (Boyle et al. 2012; Deng et al. 2012; La Russa et al. 2012; Hung et al. 2013). Chlamydomonas has one gene for PDAT, and in vitro assays revealed that it has a broad substrate specificity and can utilize phospholipids, galactolipids, DAG, and cholesteryl esters as acyl donors for TAG synthesis (Boyle et al. 2012; Yoon et al. 2012). Knockdown *pdat* lines accumulate higher levels of MGDG, sulfoquinovosyl diacylglycerol (SQDG), and phosphatidylglycerol (PG), suggesting that these lipids can act as substrates in vivo (Yoon et al. 2012).

As expected, nitrogen starvation RNA-seq resulted in the increase of three acyltransferases (*DGAT1*, *DGTT1*, *PDAT1*). *DGTT1* and *DGAT1* are also induced in other stress conditions (S, P, Zn, Fe) (Boyle et al. 2012; Hernandez-Torres et al. 2016). Knockdown lines for *DGTT1–DGTT3*, each with a single DGTT suppressed, resulted in a 20–35% decrease in TAG accumulation (Liu et al. 2016). Similarly, insertional mutants and artificial micro-RNA knockdown lines for *PDAT* showed up to 25% reduction in TAG accumulation (Boyle et al. 2012; Yoon et al. 2012). These knockdown line phenotypes indicate that both PDAT- and DGAT-dependent pathways contribute to TAG accumulation in Chlamydomonas.

Degradation of acyl-lipids by lipases also provides acyl chains for subsequent TAG generation. A galactoglycerolipid lipase, plastid galactoglycerolipid degradation 1 (PGD1), degrades immature MGDG, and the acyl chains are incorporated into TAG (Fig. 2) (Li et al. 2012b). Similarly, MGDG was found to be a source for fatty acids to generate TAG in a mutant *fdx5*, defective in a chloroplast ferredoxin (Yang et al. 2015). FDX5 was found to interact with two fatty acid desaturases important for the production of mature MGDG. In the *fdx5* knockout mutant, MGDG desaturation was compromised, and this immaturity likely promoted its degradation by lipases and subsequent incorporation into TAGs. Additionally, MGDG is likely not the sole recycled lipid as lipidomic analysis of cells accumulating TAGs in response to heat stress indicated that the sn-3 position of DAG is esterified from acyl groups derived from diacylglyceryl-trimethylhomoserine (DGTS) and phosphatidylethanolamine lipids (Legeret et al. 2016).

3.2 TAG Synthesis Requires Lipid Trafficking Across Organellar Membranes

TAG synthesis occurring outside of the chloroplast ("eukaryotic pathway") requires fatty acids to be transported out of the chloroplast where they were synthesized, a process that is not well understood (Riekhof et al. 2005; Li-Beisson et al. 2015; Li et al. 2016a). For the three enzymes synthesizing DAG from glycerol-3-phosphate, at least two isoforms exist for each enzyme, and based on protein localization prediction programs, it is highly plausible that DAG synthesis occurs in both the chloroplast and ER (Li-Beisson et al. 2015). Analysis of fatty acid components from TAGs revealed C16 fatty acids to be enriched at the sn-2 position, indicative of chloroplast-derived DAG precursor, as most extrachloroplastic membranes contain C18 fatty acids at this position (Fan et al. 2011). Furthermore, GPAT and PDAT have been identified in the chloroplast proteome, which points to a probable chloroplast localization of this pathway (Terashima et al. 2010, 2011; Yoon et al. 2012). Additionally, several proteins involved in DAG and TAG synthesis have been identified in the lipid proteome, suggesting parallel pathways occurring among various organelles (discussed in more detail in Sect. 6.2) (Moellering and Benning 2010; Nguyen et al. 2011).

Lipid precursors such as free fatty acids are likely imported into the ER from the chloroplast (Liu and Benning 2013). In *Arabidopsis thaliana*, several proteins have been identified that mediate fatty acid transport, which is thought to occur through membrane contact sites between the chloroplast and the ER (Block and Jouhet 2015). Components identified to play a role in fatty acid and lipid transport in higher plants, such as a transporter localized to the chloroplast inner envelope fatty acid export 1 (FAX1), lipid transfer proteins, and acyl-CoA binding proteins, have been identified in the Chlamydomonas genome but lack experimental evidence (Li et al. 2016a). The ATP-binding cassette (ABC) transporter that mediates lipid trafficking, consisting of trigalactosyldiacylglycerol proteins TGD1, TGD2, and TGD3, is localized to the inner envelope membrane of the chloroplast in higher plants (Benning 2009; Roston et al. 2012). The *TGD* genes are also present in Chlamydomonas, of which experimental evidence shows that the *TGD2* gene is necessary for phosphatidic acid trafficking from the ER to the chloroplast, indicating that lipid trafficking between these two organelles is not unidirectional (Warakanont et al. 2015).

Origins of fatty acids can be speculated based on the degree of saturation because fatty acids derived from de novo synthesis are more saturated than those derived from membrane lipids (Fan et al. 2011; Li et al. 2012b). During de novo TAG synthesis, the exported fatty acids are thought to be activated by long-chain acyl-CoA synthetase, resulting in acyl-CoA, allowing for incorporation during TAG synthesis (Li et al. 2016b). This conclusion is based on the presence of more unsaturated TAGs in a mutant in a long-chain acyl-CoA synthetase, *lcs2*, suggesting that in the absence of LCS2, production of TAGs from de novo

synthesized fatty acids is diminished, but not membrane-recycled TAGs. The *lcs2* mutant had 50% reduction in TAG abundance. Proteomic analysis localized LCS2 to the lipid droplets, which indicates direct synthesis of acyl-CoA from chloroplast-derived fatty acids on the surface of the lipid droplets (Moellering and Benning 2010). In addition to the export of fatty acids, membrane lipids must also be mobilized across organellar membranes so that they can be readily degraded and stored in the form of TAGs.

4 Regulation: Accumulation and Turnover of TAGs

Although the environmental stresses that stimulate TAG accumulation have been thoroughly documented, the regulation mechanisms that lead to the induction of TAG accumulation are not well understood. Transcriptomic studies have identified differentially expressed genes and pinpoint possible transcriptional regulators involved in a coordinated response of TAG accumulation (Miller et al. 2010; Boyle et al. 2012; Blaby et al. 2013; Lopez Garcia de Lomana et al. 2015; Schmollinger et al. 2014; Gargouri et al. 2015). Transcription factors are of particular interest because current induction conditions involve nutrient starvation, which ultimately limits cell growth and is not ideal for biofuel applications. Finding ways to turn on TAG accumulation while maintaining growth will maximize output and would have a major impact on biotechnological applications.

4.1 Transcription Factors Are Key Targets for Understanding TAG Synthesis Regulation

Comparison of transcriptome response between nitrogen, sulfur, and phosphorous starvation showed commonly up- or downregulated genes, but the majority of transcripts were differentially regulated between each of the stress conditions (Boyle et al. 2012; Schmollinger et al. 2014; Hernandez-Torres et al. 2016). Although various nutrient stresses can cause TAG accumulation, judging from the vastly different transcriptome responses between different nutrient starvation conditions, unique transcription factors may play a role in initiating TAG accumulation. mRNA of a putative transcription factor for TAG accumulation, *nitrogen response regulator 1* (*NRR1*), was found to be induced under nitrogen starvation (Boyle et al. 2012). NRR1 is presumed to be a transcription factor due to the presence of a *SQUAMOSA* promoter-binding protein domain at its N-terminus. *NRR1* has a similar transcript profile to *DGTT1* and ammonium transporter *AMT1D*, with induction observed within half an hour after transfer to media without nitrogen, and appears to be specific for nitrogen starvation. However, NRR1 is not the sole regulator for TAG accumulation under nitrogen starvation,

as the *nrr1* insertional mutant results in a 50% reduction of TAG accumulation. Additionally, several putative DNA-binding proteins were identified based on transcript accumulation under nitrogen starvation, and their low nitrogen content compared to the average of the proteome indicates easier protein induction under nitrogen limitation (Schmollinger et al. 2014). Overall, proteins high in nitrogen content were found to be reduced under nitrogen starvation, while proteins low in nitrogen were induced, indicating a survival strategy by cellular nitrogen redistribution. In another omics-based study, 70 putative transcription factors and transcriptional regulators were found to be co-regulated during nitrogen deprivation (Gargouri et al. 2015). Among them, the mRNA of the putative transcription factor *TAZ3* was induced over fivefold under nitrogen deficiency and showed a similar expression profile to *NRR1*.

Under phosphorous deficiency, *PSR1* was recently found to be a transcriptional regulator, where loss of function in the mutant *psr1* resulted in decreased starch and lipid accumulation (Bajhaiya et al. 2016). This further supports the notion of distinct response pathways for TAG induction under various conditions. These putative transcription factors showing transcript induction under nutrient-limiting conditions are targets for further studies to elucidate TAG accumulation response.

4.2 Putative Kinases Are Involved in TAG Accumulation

In addition to putative transcription factors and key enzymes in TAG synthesis and carbon partitioning, candidates for signaling pathways for sensing environmental stress and inducing starch and TAG accumulations have been identified (Park et al. 2015; Schmollinger et al. 2014). A mutant screen for strains with decreased TAG accumulation under sulfur deficiency led to the identification of *triacylglycerol accumulation regulator1* (*tar1*), which carried an insertion in a gene for a tyrosine phosphorylation-regulated kinase (Kajikawa et al. 2015). Diminished lipid accumulation in *tar1* was also observed in nitrogen starvation, and TAG accumulation could be restored to wild-type levels in the complemented strains, suggesting that TAR1 is a positive regulator for TAG accumulation under nitrogen and sulfur starvation, although its targets are currently unknown. Interestingly, *tar1* did not show the characteristic chlorotic phenotype accompanied by downregulation of photosynthesis that occurs during nitrogen starvation in the wild type and appears to be compensated by increasing chloroplast membrane lipid accumulation and genes involved in thylakoid maintenance, stress response, and viability.

Another kinase, which, unlike TAR1, appears to negatively regulate starch and TAG accumulation under photoautotrophy was recently identified (Schulz-Raffelt et al. 2016). This kinase is a dual-specificity tyrosine-phosphorylation-regulated kinase specific to plants (DYRKP), and a *dyrkp* mutant was initially identified as *starch degradation 1*, *std1*, due to higher intracellular starch reserves after nutrient resupply (Chochois et al. 2010). *Std1* accumulates increased starch and TAGs and maintains higher photosynthetic activity compared to the wild type under nitrogen-

starved photoautotrophic conditions (Schulz-Raffelt et al. 2016). Although the interaction partners of DYRKP are still unknown, DYRKP activity is necessary to inhibit starch and TAG accumulation under conditions of low cellular energy status, such as photoautotrophic conditions under low light.

4.3 *TAG Remobilization After the Return of Nitrogen Is a Rapid, Regulated Response*

On the flip side to TAG accumulation, when conditions become favorable for growth, the cells need to respond accordingly to degrade TAGs and resume growth. After nitrogen resupply in dark conditions, starch was found to be degraded initially, with 70% consumed within the first 20 h, followed by most of the TAGs being consumed during 20–24 h after nitrogen resupply along with the return of chlorophyll (Siaut et al. 2011). In general, numerous studies found lipid droplets to disappear within 36 h of nitrogen resupply (Cagnon et al. 2013; Li et al. 2012a; Valledor et al. 2014). Lipases act to hydrolyze TAGs for remobilization. In vitro analysis of PDAT, introduced earlier as an acyltransferase that synthesizes TAG (Sect. 3.1), revealed the enzyme to also act as a lipase that can degrade phospholipids, galactolipids, and TAGs to release free fatty acids (Yoon et al. 2012). However, judging from the induced *PDAT* transcripts under nitrogen starvation and the reduced accumulation of TAGs in *pdat* knockdown and mutant lines, PDAT is currently postulated to act as an acyltransferase rather than a lipase in vivo (Boyle et al. 2012; Yoon et al. 2012). Therefore, a lipase that uses TAG as a substrate and mediates TAG turnover in vivo has not yet been identified in Chlamydomonas, although transcriptomics and proteomics have identified candidate lipases (Miller et al. 2010; Goodenough et al. 2014; Nguyen et al. 2011). LIP1 lipase has been characterized in Chlamydomonas and was found to hydrolyze DAG, thereby assisting in TAG turnover by preventing DAG overaccumulation, but is unable to use TAG as a substrate (Li et al. 2012a).

In order to focus on TAG turnover, a mutant screen was performed to identify strain defect in mobilizing accumulated TAGs and initiating growth (i.e., exiting quiescence) even after 24 h following nitrogen resupply (Tsai et al. 2014). Eight mutants, named *compromised hydrolysis of triacylglycerols* (*cht*1–*cht*8), were isolated with *cht7* displaying the strongest phenotype, unable to mobilize TAGs, and resume growth under a variety of environmental signals, not just nitrogen resupply, although viability was not affected. *CHT7* encodes for a protein containing two cysteine-rich motifs, which could play a role in DNA binding, and GFP-tagged CHT7 complementation rescued the phenotype and localized the protein to the nucleus suggesting that CHT7 may act as a transcription factor required for quiescence exit (Tsai et al. 2014; Li et al. 2014).

5 Photosynthesis and TAG Accumulation

TAG accumulation is severely compromised in the dark, suggesting that photosynthesis is an important contributor for TAG synthesis (Fan et al. 2012). However, chlorosis is a signature phenotype of nitrogen starvation in Chlamydomonas, and, accordingly, photosynthetic efficiency decreases under nitrogen deprivation (Schmollinger et al. 2014). Chlorosis and downregulation of photosynthesis appear to be a mediated process and not just an inevitable result of nitrogen limitation, as the mutant *tar1* (see Sect. 4.2) does not turn chlorotic under nitrogen starvation and has high levels of photosynthetic activity compared to the wild type while maintaining similar viability. In Chlamydomonas wild-type cells, the accumulation of TAG and the concurrent decrease in chlorophyll and photosynthetic activity are closely linked response mechanisms, not just a passive consequence of stress.

5.1 Photosynthetic Electron Transport Chain Complexes Are Reduced Under Nitrogen Starvation

Upon nitrogen starvation, photosynthetic efficiency decreases due to reduction in light-harvesting complexes, photosystem I and II complexes, cytochrome b_6f, and ATP synthase, observed both in photoautotrophic and photoheterotrophic conditions (Plumley and Schmidt 1989; Peltier and Schmidt 1991; Schmollinger et al. 2014; Juergens et al. 2015). Interestingly, mitochondrial ATP synthase and cytochrome bc_1 complex and proteins of the TCA cycle are induced under photoheterotrophic conditions, which is also reflected by respiration rates remaining high compared to the drastic reduction of oxygen evolution (i.e., via photosynthesis) (Valledor et al. 2014; Schmollinger et al. 2014).

Downregulation of photosynthesis happens rapidly after the switch to nitrogen deficiency. Even after 6 h under nitrogen deprivation, CO_2 uptake was reduced, and after 24 h, linear electron flow decreased relative to cyclic electron flow (Juergens et al. 2015). Also, during the first few hours, transcripts, proteins, and pigments involved in non-photochemical quenching (NPQ), namely, photosystem II D1 subunit protein, subunit S transcript, LHCSR transcript, and zeaxanthin, are induced, although NPQ itself was not found to be elevated (Miller et al. 2010; Juergens et al. 2015). Components necessary for NPQ may be upregulated to prepare the cells for a sudden increase in light intensity, and the light intensity at 160 $\mu Em^{-2}\ S^{-1}$ used in the study by Juergens et al. may not have been enough to induce NPQ. A more in-depth analysis of the relationship between nutrient deprivation, TAG accumulation, and high light stress would be interesting. A gradual decrease in photosystem II was also observed under nitrogen starvation, which also resulted in photosynthetic hydrogen production after 3 days of nitrogen-deprived

photoheterotrophic conditions, although this effect was delayed compared to sulfur deprivation, which results in rapid degradation of photosystem II (Wykoff et al. 1998; Philipps et al. 2012).

As expected, under photoautotrophic conditions, TAG synthesis is much more linked to photosynthetic capacity. Acetyl-CoA is a key metabolite necessary for the synthesis of fatty acids, and multiple metabolic pathways can lead to its generation. Under photoautotrophic conditions, where acetate is not available to generate acetyl-CoA, the pyruvate dehydrogenase complex in the chloroplast is a major source of acetyl-CoA leading to TAG accumulation (Shtaida et al. 2014). Knockdown lines of the E1α subunit of the pyruvate dehydrogenase accumulate less TAGs compared to the wild type, only under photoautotrophic conditions.

5.2 TAG Accumulation Acts as Sink for Photosynthetically Generated Reducing Equivalents and Protects Against ROS Damage

The tight link between photosynthesis and TAG accumulation is further demonstrated through the analysis of the *pgd1* mutant (introduced in Sect. 3.1), which lacks a galactoglycerolipid lipase (Li et al. 2012b). TAG accumulation is compromised in this mutant, and this resulted in increased chlorosis and loss of viability after nitrogen deprivation, a phenotype that can be rescued by the addition of photosystem II inhibitor DCMU. This provided experimental evidence that TAG acts as an electron sink for the photosynthetic electron chain and compromising TAG accumulation, as seen in the PGD1 mutant, results in the generation of toxic reactive oxygen species. The observation that Chlamydomonas does not accumulate TAGs in the dark further indicates that TAG accumulation plays the role of sequestering photosynthetically derived reducing equivalents under nutrient starvation conditions, where other metabolic pathways cannot work fast enough to provide an outlet for these generated reducing powers.

6 Lipid Droplets: Morphology, Cellular Localization, and TAG Species

Lipid droplets consist of a TAG core surrounded by a polar lipid monolayer and associated proteins. Lipid droplets in Chlamydomonas are unique compared to their plant counterparts because substantial TAG synthesis appears to occur in the chloroplast, whereas TAG synthesis occurs in the ER in plants (Fan et al. 2011; Liu and Benning 2013). Although there are still many unknown aspects of lipid

droplet formation and its associated lipid species and proteins, lipidomic, proteomic, and microscopic analyses have revealed novel components of Chlamydomonas lipid droplets.

6.1 Lipid Droplets Contain a Wide Range of Lipid Species

The prominent polar lipids in Chlamydomonas lipid droplets are DGTS and digalactosyldiacylglycerol (DGDG), while phospholipids, which are primarily found in lipid droplets in yeast and animals, make up less than 25% of the polar lipids in lipid droplets in Chlamydomonas (Tsai et al. 2015). Interestingly, phosphatidylcholine, which is a major phospholipid found in plants and animals, is absent in Chlamydomonas (Yang et al. 2004). Instead, DGTS acts as the major polar lipid source for extrachloroplastic membranes in Chlamydomonas (Riekhof et al. 2005). On the lipid droplets, more than half of the polar lipids are galactolipids, such as DGDG, which are usually found in chloroplast membranes. However these lipid droplet-localized galactolipid species are more saturated, having increased abundance of 16:0 and 18:0 fatty acids compared to their chloroplast counterparts (Tsai et al. 2015). TAG profiling of nitrogen-starved Chlamydomonas identified 140 distinct species with most acyl groups consisting of 16- or 18-carbon chains (James et al. 2011; Liu et al. 2013a). Under nitrogen starvation and phosphate starvation, TAG species were particularly enriched with 16:0 and 18:1 fatty acids (Iwai et al. 2014). Aside from TAGs, the lipid droplet core contains some free fatty acids and carotenoids (Wang et al. 2009; Moellering and Benning 2010).

6.2 Lipid Droplet Proteome Consists of Structural and Metabolic Proteins

Proteomic analysis of lipid droplets identified a highly abundant protein, the major lipid droplet protein (MLDP) (Moellering and Benning 2010; Nguyen et al. 2011). MLDP is a green alga-specific 28 kDa protein, and suppression through RNAi resulted in larger lipid droplets, but without increase in total TAG accumulation per cell (Moellering and Benning 2010). In wild-type Chlamydomonas, MLDP abundance was shown to directly correlate with intracellular TAG abundance with localization to the lipid droplet surface (Tsai et al. 2014). In plants, oleosins are the main structural proteins present in the phospholipid monolayer that maintain lipid droplet integrity and prevent coalescence of the droplets (Huang 1992). MLDP is structurally not similar to oleosin, lacking a long hydrophobic polypeptide that inserts into the lipid droplet matrix to promote stable association. Furthermore, Chlamydomonas has a gene encoding for a protein similar to oleosin, but transcript

and protein levels were detected only in low amounts (Huang et al. 2013). Therefore, MLDP, instead of oleosin, appears to act as the key component for lipid droplets due to its sheer abundance and its ability to stabilize lipid droplet size by preventing coalescence (Tsai et al. 2015).

Aside from MLDP, other proteins are localized to the lipid droplets. Two independent proteomic studies identified 259 proteins and 248 proteins from lipid droplets isolated from nitrogen-starved photoheterotrophic and nitrogen-starved photoautotrophic cells, respectively (Moellering and Benning 2010; Nguyen et al. 2011). Among these, proteins involved in lipid metabolism include acyl-CoA synthetases (including LCS2 discussed earlier) and DGTS synthesis enzymes BTA1, GPAT, LPAT, and PDAT. The presence of BTA1 suggests direct synthesis of DGTS for the lipid monolayer of lipid droplets (Moellering and Benning 2010). The presence of key enzymes involved in TAG synthesis is indicative of TAG synthesis, in part, occurring directly at the lipid droplets. Interestingly, both proteomic studies did not identify DGAT, but a comparative study on the lipid droplet proteome versus other organellar proteomes must be conducted to determine the presence and localization of this protein. Furthermore, both studies isolated total lipid droplets from cell extracts, making localization of proteins between the chloroplast- and ER-derived lipid droplets difficult. Additionally, ER-chloroplast lipid trafficking proteins (TGD1–TGD3), discussed in more detail below, are also localized to the lipid droplets (Nguyen et al. 2011).

6.3 Biogenesis of Lipid Droplets Is an Elusive Process

Currently, not much is known about the biogenesis of lipid droplets. A widely used model for lipid droplet formation in non-photosynthetic eukaryotes, where lipid droplets accumulate exclusively in the cytosol, is via the accumulation of neutral lipid globules between leaflets of the ER membrane bilayer (Pol et al. 2014; Wilfling et al. 2014). These globules are thought to move laterally within the ER membrane, and as they increase in size, the bilayer leaflet separates and eventually forms a droplet. In yeast cells, lipid droplets still attached to the ER as well those detached have been observed (Jacquier et al. 2011; Wilfling et al. 2013). Electron microscopy images of Chlamydomonas cells also show close association of the cytoplasmic lipid droplets, usually around 1–2 μm in diameter, to the ER as well as the chloroplast outer membrane (Moellering and Benning 2010; Goodson et al. 2011). Chloroplast lipid droplets that are smaller (~60 nm) and often found attached to the thylakoid membranes are referred to as plastoglobules (Engel et al. 2015). Interestingly, a nitrogen-starved starchless mutant showed chloroplastic lipid droplets that are much larger than plastoglobules, suggesting that the plastoglobules could serve as a precursor for larger chloroplast lipid droplets (Goodson et al. 2011; Fan et al. 2011). Similarly, small lipid droplets (250–1000 nm) present under normal growth conditions could also act as a precursor for stress-induced cytoplasmic lipid droplets (Goodson et al. 2011).

7 Biotechnological Applications and Perspectives

Microalgae have been identified as a possible source for sustainable fuel, feed, food, and chemicals (Hu et al. 2008; Liu et al. 2012; Wijffels et al. 2013; Klok et al. 2014). Microalgae-based resources are not yet utilized at a global scale due to the high expense of cultivation, harvesting, and processing, especially when performed at a large scale (Slade and Bauen 2013). In the last decade, there has been an exponential increase in efforts to identify strains with altered TAG accumulation, especially in Chlamydomonas, in order to find clues in cultivation and genetic engineering strategies for biotechnological applications.

7.1 Mutants that Show Increased TAG Accumulation Has Been Identified

To date, only a handful of mutants relating to TAG metabolism have been characterized (for a list, refer to Goncalves et al. 2016). Many enzymes having a role in TAG metabolism or regulation have been identified by observing a decrease in TAG accumulation resulting from silencing or gene disruption via insertional mutagenesis, as seen for mutants in *pdat1*, *pgd1*, *nrr1*, and *tar1* discussed earlier (Boyle et al. 2012; Yoon et al. 2012; Li et al. 2012b; Kajikawa et al. 2015). Mutants with increased TAG accumulation have also been identified, with the starchless mutants being the most characterized (Li et al. 2010a; Wang et al. 2009; Work et al. 2010). Genetic engineering of strains with the aim of increasing TAG accumulation has had mixed outcomes. One clear target for increasing TAG accumulation is to overexpress DGAT, aiming to maximize DAG conversion to TAG. Individual overexpression of three type II DGATs (*DGTT1–DGTT3*) resulted in no changes in TAG accumulation when nitrogen-starved under photoheterotrophic conditions (La Russa et al. 2012). However, overexpression of *DGTT4* under the control of a promoter induced under phosphate starvation yielded increased TAG accumulation under photoheterotrophic conditions (Iwai et al. 2014). Overexpression of type II DGATs, *DGTT1* and *DGTT5*, was reported to show 27% and 48% increase in TAG accumulation, respectively (Deng et al. 2012).

7.2 Many Mutant Screens Have Been Developed with the Aim of Isolating Strains with Perturbed Lipid Content

Several mutant screens have been performed to isolate strains with altered TAG accumulation. To this end, lipophilic dyes such as nile red and BODIPY have been used as a readout for cellular TAG abundance (Greenspan et al. 1985; Moellering

and Benning 2010; Cirulis et al. 2012; Velmurugan et al. 2013). Various factors have to be addressed regarding fluorescence correlation with actual TAG accumulation, ensuring consistent dye penetration into the cells and choosing correct filters to separate chlorophyll autofluorescence from lipid dye fluorescence (Cagnon et al. 2013; Terashima et al. 2015). Arraying mutant strains in a 96-well format and screening for increased or diminished fluorescence signal can lead to the isolation of candidate mutants, which could be further analyzed by alternative methods to confirm the phenotype such as thin-layer chromatography or mass spectrometry (Li et al. 2012b; Yan et al. 2013). The mutant *pgd1* described earlier was isolated through this approach (Li et al. 2012b). Similarly, individual mutants in 96-well plate format have been analyzed by flow cytometry (Cagnon et al. 2013). Utilizing flow cytometry with cell sorting capabilities [i.e., fluorescence-activated cell sorting (FACS)] greatly increases the throughput of mutant analysis as 10,000 cells can be analyzed per second (Terashima et al. 2015). This allows for pooled cultivation, staining, and analysis, followed by individual mutant isolation after phenotype-based enrichment (Xie et al. 2014; Velmurugan et al. 2013; Terashima et al. 2015; Kajikawa et al. 2015). This approach has been successfully implemented in Chlamydomonas and in other microalgae as well (Montero et al. 2011; Doan and Obbard 2011, 2012; Manandhar-Shrestha and Hildebrand 2013).

Isolating a strain with altered lipid accumulation from a mutant pool generated via random insertional mutagenesis has been very successful. However, a major bottleneck has been in identifying the mutation site of a strain of interest. To address this issue, a large-scale mutant library with mapped insertion sites that cover much of the Chlamydomonas genome has been generated (Zhang et al. 2014; Li et al. 2016b). From a library of Chlamydomonas strains with mapped cassette insertion sites, hypothesis-driven characterization of a mutant can lead to subsequent identification of key components of TAG metabolism, as was the case for LCS2 discussed above (Li et al. 2016b). Furthermore, utilizing mutant pools with mapped insertion sites for high-throughput screening will be powerful as phenotypes can be matched to insertion sites rapidly.

8 Conclusions

An increased interest in microalgal TAG accumulation in the last decade has accelerated research in this field. Genome information has allowed prediction of pathways present in Chlamydomonas, and mutant analyses are revealing the enzymes contributing to TAG metabolism. Through numerous studies on transcriptomics, proteomics, and lipidomics, it has become clear that major metabolic shifts occur in Chlamydomonas under TAG-accumulating conditions, with TAG originating from both de novo synthesis and membrane recycling. The initiation of TAG synthesis and degradation appear to be controlled by transcription factors and kinases, involving a multitude of parallel and interconnected metabolic pathways. TAG is synthesized via a variety of carbon sources, and simply inhibiting

one component of TAG accumulation does not lead to complete inhibition of TAG synthesis. Although various stress conditions induce TAG accumulation, the response mechanisms are distinct with differential gene regulation observed that is unique to the applied stress.

While the main metabolic pathways in TAG metabolism are beginning to be elucidated, many aspects of the current model lack experimental evidence, especially regarding signaling cascades, the roles of various enzyme isoforms, protein localizations, lipid droplet biogenesis, and lipid trafficking mechanisms. The growing number of mapped mutants available to the research community will assist in elucidating TAG metabolism by identifying key genes or localizing proteins, such as by tagged protein complementation. Additionally, further understanding the link between TAG accumulation and photosynthesis will be of significance for industrial production of biofuels, food, feedstock, and high-grade oils in order to maximize sun energy capture into desirable products.

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