Enhancement of extraplastidic oil synthesis in Chlamydomonas reinhardtii using a type-2 diacylglycerol acyltransferase with a phosphorus starvation–inducible promoter

Masako Iwai¹,², Keiko Ikeda³, Mie Shimojima¹ and Hiroyuki Ohta¹,²,4, *

¹Center for Biological Resources and Informatics, Tokyo Institute of Technology, Midori-ku, Yokohama, Japan
²Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Chiyoda-ku, Tokyo, Japan
³Biomaterial Analysis Center, Technical Department, Tokyo Institute of Technology, Midori-ku, Yokohama, Japan
⁴Earth-Life Science Institute, Tokyo Institute of Technology, Meguro-ku, Tokyo, Japan

Summary

When cultivated under stress conditions, many plants and algae accumulate oil. The unicellular green microalga Chlamydomonas reinhardtii accumulates neutral lipids (triacylglycerols, TAGs) during nutrient stress conditions. Temporal changes in TAG levels in nitrogen (N)- and phosphorus (P)-starved cells were examined to compare the effects of nutrient depletion on TAG accumulation in C. reinhardtii. TAG accumulation and fatty acid composition were substantially changed depending on the cultivation stage before nutrient starvation. Profiles of TAG accumulation also differed between N and P starvation. Logarithmic-growth-phase cells diluted into fresh medium showed substantial TAG accumulation with both N and P deprivation. N deprivation induced formation of oil droplets concomitant with the breakdown of thylakoid membranes. In contrast, P deprivation substantially induced accumulation of oil droplets in the cytosol and maintaining thylakoid membranes. As a consequence, P limitation accumulated more TAG both per cell and per culture medium under these conditions. To enhance oil accumulation under P deprivation, we constructed a P deprivation–dependent overexpressor of a Chlamydomonas type-2 diacylglycerol acyl-CoA acyltransferase (DGTT4) using a sulphoquinovosyldiacylglycerol 2 (SQD2) promoter, which was up-regulated during P starvation. The transformant strongly enhanced TAG accumulation with a slight increase in 18 : 1 content, which is a preferred substrate of DGTT4. These results demonstrated enhanced TAG accumulation using a P starvation–inducible promoter.

Introduction

Photosynthetic organisms such as land plants, algae and various bacteria convert sunlight and CO₂ into chemical energy in the form of reduced carbon molecules including lipids. Triacylglycerols (TAGs) are major storage lipids that accumulate in developing seeds, fruits and leaves of several plant species (Somerville et al., 2001). Many microalgal species accumulate large amounts of TAGs, the major feedstock for biodiesel production, under unfavourable environmental conditions. Biodiesel from algae is usually composed of methyl esters of long-chain fatty acids. Recently, algae production has been an area of considerable interest because the photosynthetic productivity of algae is estimated to be higher than that of land plants, and does not require high-quality agricultural land to grow the biomass (Hu et al., 2008; Scott et al., 2010).

Nutrient deficiency causes a wide variety of cellular response mechanisms in living organisms. Chlamydomonas reinhardtii, also called the ‘green yeast’, is a tractable model organism because of its facile genetics, relatively small genome, and our ability to manipulate its nuclear, chloroplast and mitochondrial genetic material (Boyle et al., 2012; Dent et al., 2001; Kindle, 1990; Randolph-Anderson et al., 1993). Chlamydomonas reinhardtii has been used to study the regulation of nutrient stress responses (Grossman, 2000; Zhang et al., 2004). Different C. reinhardtii mutants increase lipid accumulation during nitrogen (N) limitation and increase anaerobic H₂ production with sulphur deprivation (Cakmak et al., 2012; Dean et al., 2010; Morzy, 2011). The most dramatic responses occur in N-starved cells. N-deficient C. reinhardtii can accumulate TAGs, especially in strains with impaired starch accumulation (Li et al., 2010a; Wang et al., 2009; Work et al., 2010). However, N deprivation causes both TAG accumulation and chloroplast breakdown, which almost completely prevents cell growth during TAG accumulation. TAGs also accumulate in C. reinhardtii after phosphorus (P) limitation (Weers and Gulati, 1997). However, to our knowledge, a comparison of the effects of N and P starvation on TAG accumulation in microalgae has not yet been made in detail.

Diacylglycerol acyltransferase (DGAT) transfers an acyl moiety from acyl-CoA to sn-1,2 diacylglycerol (DAG), thus contributing to TAG synthesis (Lung and Weselake, 2006; Rajakumari et al., 2008). In land plants, several studies have explored the effects of overexpression of lipid synthesis enzymes on TAG production (Burgal et al., 2008; Durrett et al., 2008; Jako et al., 2001; Shockey et al., 2006). There are four types of DGATs. The two types of membrane-associated DGATs are (i) the DGAT1 family...
enzymes, which are related to acyltransferases such as Saccharomyces cerevisiae Afp2p and (ii) the DGAT2 family enzymes, which are related to S. cerevisiae Dga1p. A more recently isolated type-3 DGAT is found in Arabidopsis thaliana and peanut and encodes a soluble cytosolic enzyme that contributes to TAG biosynthesis (Hernandez et al., 2012, Saha et al., 2006). A plastoglobule proteome study suggested that there is a fourth type of DGAT in Arabidopsis (Lundquist et al., 2012). Another type of acyltransferase is a phospholipid : diacylglycerol acyltransferase (PDAT), which transfers the acyl moiety at the sn-2 position of phospholipids to DAG, thereby producing TAG in an acyl-CoA-independent manner (Dahlqvist et al., 2000, Zhang et al., 2009).

The genome sequence revealed that Chlamydomonas has one type 1 DGAT (DGAT1), 5 type 2 DGATs (DGTT1–5) and one PDAT (PDAT1). During N nutrient starvation in C. reinhardtii, DGAT1 and DGTT1 transcripts are up-regulated, DGTT2 and DGTT3 transcripts are expressed at moderate levels, DGTT4 mRNA abundance is low and DGTT5 expression has not been detected (Boyle et al., 2012; Miller et al., 2010). Overexpression of DGAT2 candidate genes DGTT1–3 does not increase the lipid content or alter the lipid composition in C. reinhardtii (La Russa et al., 2012). Expression of DGTT2 in A. thaliana increased leaf TAG content, with some molecular species containing very-long-chain fatty acids (Li et al., 2012a). DGTT1–3, but not DGTT4, complements the phenotype of a yeast Adga1Δ/Δrr1 mutant lacking both DGAT and PDAT activity, increasing TAG levels (Hung et al., 2013).

In this study, we compared the temporal changes of TAGs in C. reinhardtii with N or P nutrient deficiency. To increase intracellular TAG accumulation, we used the promoter of SQD2, which encodes the sulfoquinovosyltransferase catalyzing the second step of sulpholipid biosynthesis and is up-regulated during P starvation, to successfully overexpress DGTT2–4 in the green microalga C. reinhardtii under P starvation. Detailed characterization of the ultrastructural changes and lipid profiles were performed in the overexpression strains, which showed that DGTT4 overexpression enhanced TAG accumulation.

**Results**

**Cell growth and morphology in cells precultured to logarithmic phase prior to P starvation**

Like many microalgae, C. reinhardtii accumulates TAG when cultivated in N-depleted medium (Dean et al., 2010; Fan et al., 2011; Li et al., 2010b; Moellinger and Benning, 2010; Wang et al., 2009; Work et al., 2010). To examine the physiological acclimation mechanisms in N- or P-starved cells, wild-type C. reinhardtii (CC-408) was precultured to the stationary or logarithmic phase in Tris-acetate-phosphate (TAP) medium. Cultures were then centrifuged and resuspended in fresh TAP, N-depleted (TAP–N) or P-depleted (TAP–P) medium. The stationary-phase cells were inoculated at high cell densities (7–8 × 10^6 cells/mL). The number of cells in cultures derived from stationary-phase cells did not increase even in fresh TAP medium (Figure 1a).

The cultures derived from logarithmic-phase cultures were inoculated at low cell densities (4–6 × 10^4 cells/mL); cell numbers increased rapidly when cells from logarithmic-phase cultures were transferred to fresh TAP medium (Figure 1b, c) but showed no increase in cell number shortly after transfer into N-deficient medium (Figure 1b). Under N-deprivation conditions, the normal ellipsoid-shaped C. reinhardtii cells were replaced with larger spherical cells that were chlorotic and nonmotile (Figure 1c, d). Vegetative cells in C. reinhardtii develop into gametes under N starvation (Rochaix, 1995). Cell numbers increased more in TAP–P medium than in TAP–N medium (Figure 1b). The cells exposed to P starvation were a paler shade of green (from chloroplasts) compared with those grown in TAP medium, had larger spherical-shaped cells similar to those in TAP–N medium and were still motile (Figure 1c, d). Indeed, photosynthetic activity has been previously observed under P stress conditions (Wykoff et al., 1998).

Living cells were stained with Nile Red, a reagent that yields brilliant fluorescence in a neutral lipid environment (Cooksey et al., 1987; Greenspan et al., 1985; Kimura et al., 2004). Figure 2 shows confocal images of CC-408 cells after 8 or 23 days of N or P starvation. After 8 days, large cup-shaped chloroplasts were observed in cells grown in TAP medium, whereas the chloroplasts of the nutrient-starved cells varied in shape. The cells under P starvation retained more thylakoid membranes than did cells under N starvation. After 23 days of culture, large cup-shaped chloroplasts were still observed in cells grown in TAP medium (similar to the results at 8 days), and chloroplasts were still (TAP–P) or rarely (TAP–N) observed under stress conditions. Lipid droplets were abundant in both the N-starved and P-starved cells.

**Differences in TAG accumulation and fatty acid composition under N or P deprivation**

Because lipid droplets were observed in P-starved cells, we investigated whether TAG was differentially accumulated in the nutrient-starved cells by quantifying lipid-derived fatty acid methyl esters (FAMES) using gas chromatography. TAG was extracted from liquid cell cultures at the indicated times after N or P deprivation (Figures 3–5).

N-starved cells derived from the precultured stationary-phase cells contained ~fivefold (3 days) and ~10-fold (8 days) more TAG per cell than the corresponding cells maintained in complete medium (Figure 3a). This is consistent with recent reports showing increased lipid synthesis in N-starved cells relative to the cells maintained under optimal growth conditions (Fan et al., 2011; James et al., 2011; Moellinger and Benning, 2010; Wang et al., 2009). We observed only a slight increase in the TAG levels under P-deprived conditions, similar to results using TAP medium (Figure 3a). The major TAG fatty acids in the cells cultured in either TAP or TAP–N medium (Figure 4) were 16 : 0, 18 : 1, 18 : 2 and 18 : 3, which are consistent with previously reported results (Moellinger and Benning, 2010; Siaut et al., 2011). The types of major TAG fatty acids in the cells cultured in TAP–P medium were similar to those in the cells cultured in TAP–N medium, although the fatty acid profiles were different between the two nutrient stress conditions (Figure 4). Levels of 18 : 3 (9,12 and 15) and 16 : 4 in TAGs increased with N deprivation between 3 and 8 days. The levels of these fatty acids decreased following P deprivation, whereas the level of 18 : 3 (5,9 and 12) transiently increased at 3 days.

Cells precultured to logarithmic phase had substantially increased TAG levels with both N and P deprivation (Figure 3b). Compared with cells grown in complete medium, the N-starved cells contained ~130- and ~20-fold and the P-starved cells contained ~110- and ~50-fold more TAG per cell after 8 and 13 days, respectively. In particular, cells had ~14.3 pg TAG/cell after 13 days in TAP–P medium. The levels of 18 : 3 (9,12,15)
and 16 : 4 in TAGs hardly increased in TAP, TAP–P or TAP–N medium, whereas 18 : 1 and 16 : 0 fatty acids were abundant in TAGs throughout the nutrient deprivation (Figure 5). This may reflect de novo-synthesized fatty acids that were mostly utilized for TAG synthesis. The levels of 18 : 2 increased moderately only under P deprivation, concomitant with the decrease in 16 : 0 and 18 : 1 (Figure 5). This result indicates that fatty acid profiles of TAGs were different between N and P.

Figure 1 Growth of Chlamydomonas reinhardtii CC-408 under the standard growth condition, N starvation or P starvation. C. reinhardtii CC-408 cells precultured to stationary phase (a) or logarithmic phase (b, c and d) in TAP medium were then inoculated into TAP medium or TAP lacking N (TAP–N) or P (TAP–P). (a and b) circle = TAP medium, triangle = TAP–P and square = TAP–N. Arrows in (a) and (b) indicate the time point of cell transfer into nutrient-depleted medium. Values represent the mean ± SD of three independent replicates. Cells cultured 5 days (c) or 13 days (d) after transfer to TAP, TAP–N or TAP–P medium. Bars = 10 μm.

Figure 2 Detection of neutral lipid in CC-408 using Nile Red staining. Logarithmic-phase cells were transferred to standard growth medium (TAP), N-starvation medium (TAP–N) or P-starvation medium (TAP–P) medium and cultured for 8 days (a) or 23 days (b). The green fluorescence observed in the presence of Nile Red indicates the presence of neutral lipids, whereas the red fluorescence corresponds to chlorophyll autofluorescence. Bars = 10 μm.
deprivation, although the origin of 18:2 in *C. reinhardtii* remains uncertain.

### Lipid droplet formation under N- or P-depleted conditions

A time course of lipid droplet formation in N- or P-deprived *C. reinhardtii* cells is shown in Figure 6. The lipid droplets were observed after 1 day of N deprivation and increased in size and number after 2 days (Figure S1). N-deprived cells were able to retain their thylakoid membranes, but there was less thylakoid stacking compared with P-deprived cells (as noted in Figure 2), and N-deprived cells accumulated starch granules. Lipid droplets were observed in the cytosol after 1 and 2 days of N deprivation. After 3 days of N deprivation, lipid droplets were observed in the cytosol and the chloroplast, as reported by Goodson *et al.*, 2011 (Figure S2). In P-deprived *C. reinhardtii* cells, lipid droplets were observed only in the cytosol (Figure 6).

### Construction and identification of CrDGTT2, 3 and 4 overexpression lines using the promoter of a gene up-regulated during P starvation

We attempted to increase the productivity of TAG using overexpression of genes that are important for TAG accumulation. We wanted to identify promoters of genes specifically up-regulated during P starvation that would enhance TAG accumulation within growing cells but not in the chloroplasts. To obtain cDNA, *C. reinhardtii* cells (precultured to logarithmic phase) grown in nutrient-depleted medium were harvested after 5 days, when TAG accumulation had already commenced (Figure 3b). We expected that the expression of the genes encoding UDP-glucose pyrophosphorylase 3 (UGP3) and sulphoquinovosyldiacylglycerol 2 (SQD2) would be induced with P depletion, because UGP3 and SQD2 are involved in sulpholipid biosynthesis, and the transcription levels of these genes are increased by P starvation in *C. reinhardtii* and *Arabidopsis* (Chang *et al.*, 2005; Okazaki *et al.*, 2009; Yu *et al.*, 2002). The expression of SQD2 and UGP3 in *C. reinhardtii* is shown in Figure 7. Expression of UGP3 and especially SQD2 was induced after P deprivation in *C. reinhardtii*, whereas SQD2 transcription was not up-regulated after N deprivation, which is consistent with previous work (Boyle *et al.*, 2012). Hence, the SQD2 promoter was suitable for inducing expression of transgenes to increase TAG production in *C. reinhardtii* with P depletion.
DGAT enzymes are important for TAG accumulation. To identify DGAT genes in *C. reinhardtii*, we searched the genomic database Phytozome (www.phytozome.net) with BLAST to identify amino acid sequences with similarity to *Arabidopsis* DGAT1 and DGAT2. The BLAST search identified one type-1 DGAT (DAGT1) and five type-2 DGATs (DGTT1 to 5). In agreement with previous reports (Boyle et al., 2012; Miller et al., 2010; Sanjaya et al., 2013), DGTT2 and DGTT3 were expressed at moderate levels, which remained constant for the most part after N deprivation (Figure 7). DGTT1 and DGTT4 mRNA abundance was low in TAP medium, but both showed a large increase in abundance after N deprivation. DGTT5 expression was not detected (data not shown). DGAT1 expression was up-regulated after P or N deprivation. DGTT1 to DGTT4 transcript abundance increased after P deprivation. During N nutrient starvation, DGTT1 transcripts are known to be up-regulated, DGTT2 and DGTT3 transcripts are expressed at moderate levels and DGTT4 mRNA abundance is low (Boyle et al., 2012; Miller et al., 2010). We expected that overexpression of DGTT2 to DGTT4 may enhance TAG accumulation with P depletion, because DGTT1 is already increased strongly and was validated by heterologous complementation in yeast (Boyle et al., 2012; Miller et al., 2010). We, therefore, generated overexpression cell lines for these three genes.

**Figure 5** Comparison of the fatty acid composition in the TAG fractions. The TAG fractions were isolated from cells cultured in TAP, TAP-P or TAP-N. CC-408 cells precultured to logarithmic phase in TAP medium were subsequently inoculated into TAP medium or TAP lacking N (TAP-N) or P (TAP-P) for the indicated number of days. Each level is shown as a percentage of the total. Values represent the mean ± SD of three independent replicates.

**Figure 6** Transmission electron micrographs of *Chlamydomonas reinhardtii* CC-408 during N or P limitation. CC-408 cells precultured to logarithmic phase in TAP medium were then inoculated into (i) TAP-N medium and grown for 1 (N1) or 2 (N2) days or (ii) TAP-P medium and grown for 3 (P3) or 5 (P5) days. Bars = 500 nm. LD, lipid droplet; M, mitochondrion; N, nucleus; S, starch granule; T, thylakoid membranes. The images on the left and right from are two individual cells.
The full length of the three CrDGAT2 candidate genes (DGTT2, DGTT3 and DGTT4) was amplified from a cDNA library, fused to the SQD2 promoter (pCrSQD2) and confirmed by sequencing. The three constructs (pCrSQD2-DGTT2 to -DGTT4) were transformed into CC-400 cells, a strain that lacks the cell wall. For each of the three CrDGAT2 genes, seven overexpression mutants were selected, and their relative levels of overexpression were measured by PCR (Figure S3) and qPCR (Figure S4). Three transformants showing high expression levels for each gene were then selected for the subsequent experiments.

Enhanced TAG accumulation in a CrDGTT4-overexpressing line after P deprivation

To confirm the effect of CrDGAT2 candidate gene overexpression, we compared the lipid content and fatty acid composition of the control and overexpression strains. The transformants were precultured to logarithmic phase prior to P depletion, as was done in the previous experiments. DGTT2- and DGTT3-overexpressing lines showed TAG accumulation similar to the control cell lines in TAP or P-depleted medium (Figure S5). This result may be due to the variable baseline expression of the endogenous genes, with DGTT2 and DGTT3 showing easily detectable expression levels even before nutrient starvation (Figure 7). In contrast, TAG accumulation increased in DGTT4-overexpressing lines compared with the control lines in P-depleted medium (Figure S5 and S6). Total TAG levels per cell were ~1.5- and ~2.5-fold greater in DGTT4-overexpressing lines #21 and #18, respectively, compared with the control line (Figure 8a). The total TAG accumulation in TAP-P was more rapid in the DGTT4-overexpressing line #18 than in the control line (Figure S7), reaching ~25 pg/cell at day 13, which was ~29-fold more than that in stationary culture-derived CC-408 cells after 8 days of N starvation (Figure 3a). Enhancement of TAG accumulation in DGTT4-overexpressing lines was also evident on a culture volume basis (Figure 8b). This agrees well with the confocal fluorescence microscopy results, which showed enhancement of Nile Red staining in the DGTT4-overexpressing line compared with the control line in TAP-P medium (Figure 9). The DGTT4-overexpressing cells retained the thylakoid membrane system similar to wild-type cells in TAP-P (Figures 6 and 10).

Levels of 16 : 0, 18 : 1 and 18 : 2 in TAGs increased, whereas the levels of 18 : 3 (5,9 and 12), 18 : 3 (9,12 and 15), 18 : 4 and 16 : 4 decreased in both the DGTT4-overexpressing cell line and the control line under P starvation (Figure 11). It is interesting to
The TAG synthesis pathways differ in cells under N and P deprivation

The fatty acid profiles were different between the two nutrient stress conditions. 16 : 4 and 18 : 3 (9,12 and 15) increased with N deprivation between 3 and 8 days in the cells precultured to stationary phase (Figure 4). Interestingly, 18 : 3 (5,9 and 12) transiently increased with P deprivation in conjunction with a decrease in 16 : 4 and 18 : 3 (9,12 and 15). Most of the 16 : 4 and 18 : 3 (9,12 and 15) is found in monogalactosyldiacylglycerol, which is synthesized exclusively by the chloroplast (Giroud et al., 1988; Sato, 1989). In contrast, 18 : 3 (5,9 and 12) is only attached to diacylglycerol-N,N,N-trimethylhomoserine (DGTS) and phosphatidylethanolamine (PE), which are extraplastidic membrane lipids. Fatty acid profiles shown in Figure 4 suggest that TAG accumulation in Chlamydomonas cells transferred from stationary-phase cultures to nutrient-depleted media was largely attributable to fatty acids from pre-existing membrane lipids.

The results also demonstrate that TAG synthesis pathways differ in cells under N and P deprivation. The levels of 18 : 3 (9,12 and 15) and 16 : 4 in TAGs in the cells precultured to logarithmic phase hardly increased in TAP, TAP–P or TAP–N medium, whereas 18 : 1 and 16 : 0 fatty acids were abundant in TAGs throughout nutrient deprivation (Figure 5). This may reflect de novo-synthesized fatty acids that were mostly utilized for TAG synthesis. The PGD1 lipase-dependent pathway also supplies de novo-synthesized acyl groups 18 : 1 (9) and 16 : 0 for TAG synthesis (Li et al., 2012b). The levels of 18 : 2 increased gradually only under P deprivation, concomitant with the decrease in 16 : 0 and 18 : 1. This result also indicates that fatty acid profiles of TAGs were different between N and P deprivation.

As shown in Figures 4 and 5, the fatty acid composition of TAG molecules was largely dependent on the growth phase when cells were nutrient-deprived. TAG molecules were mostly composed of fatty acids presumably synthesized during cell growth in nutrient-deprived cells that were precultured to logarthmic phase. This idea is supported by the substantial differences in fatty acid composition for the two preculture conditions. Indeed, 18 : 1 and 16 : 0 fatty acids, which generally constitute a major fatty acid pool in plastids, were mainly enriched in TAG molecules throughout cell growth. In plant tissues, the major glycerolipids are first synthesized using only 16 : 0 and 18 : 1 acyl groups, which are typically de novo-synthesized and incorporated into the glycerol backbone (Li et al., 2012b; Ohlrogge and Browse, 1995; Post-Beittenmiller et al., 1991; Salas and Ohlrogge, 2002). Modification of de novo fatty acid synthesis under specific conditions that could directly change a large portion of the fatty acid molecules in the TAG backbone would be advantageous for manipulating TAG molecular species. The accumulated cell TAG level per litre of culture after N deprivation of cells precultured at the logarithmic phase was reduced to 20% compared with cells precultured at the stationary phase prior to N deprivation (Figure 3c, d), because of inhibited cell growth following N deprivation (Figure 1b). In contrast, cells in TAP–P grew better than those in TAP–N (Figure 1b). The accumulated TAG levels following P deprivation of cells precultured at the logarithmic phase were similar to those in precultured stationary-phase cells following N deprivation (Figure 3c, d).

Enhanced TAG accumulation in CrDGTT4-overexpressing lines after P deprivation

The C. reinhardtii genome sequence revealed one type-1 DGAT (DGAT1), five type-2 DGATs (DGTT1–5) and one PDAT (PDAT1).
PDAT1 associates with oil bodies (Nguyen et al., 2011), and its silencing alters lipid composition (Boyle et al., 2012; Yoon et al., 2012). PDAT1 possesses broad substrate specificity for phospholipids, galactolipids, TAG and cholesteryl esters (Yoon et al., 2012). Catalyzing the final step of TAG biosynthesis, DGAT is one of the most intensively studied enzymes in the entire acyl lipid metabolism. Our assay showed that transcript abundance markedly (DGTT1-3) or slightly (DGTT4) increased after P deprivation (Figure 7). DGTT1 is suppressed under N-replete conditions and undergoes an increase in transcript abundance following N deprivation (Miller et al., 2010). DGTT1 is highly induced after 1 day of N starvation (Msanne et al., 2012) and is highly responsive at the level of mRNA abundance to both N concentration and time after removal of the N source (Boyle et al., 2012). Our results agreed well with these previous reports.

A recent study has shown that overexpression of DGAT candidate genes DGTT1–3 does not increase the lipid content or alter the lipid composition in C. reinhardtii (La Russa et al., 2012). In that study, the Chlamydomonas PsaD promoter and 3′ sequence were used for expression constructs in C. reinhardtii. The overexpression cell lines assessed during standard growth

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**Figure 9** Detection of neutral lipid in the pCrSQD2-DGTT4-18 transformant using Nile Red staining. Logarithmic-phase cells were transferred to TAP or TAP-P medium and cultured for 7 days. The green fluorescence observed in the presence of Nile Red indicates the presence of neutral lipids, whereas the red fluorescence corresponds to chlorophyll autofluorescence. (a and b) pSDGTT4-18; (c and d) empty vector control; (a and c) TAP, 7 days; (b and d) TAP–P, 7 days. Bars = 25 μm.

**Figure 10** Transmission electron micrographs of the pCrSQD2-DGTT4-18 transformant during P limitation. The pCrSQD2-DGTT4-18 transformant precultured to logarithmic phase in TAP medium was inoculated into TAP–P medium and grown for 5 days. Bars = 500 nm. LD, lipid droplet; M, mitochondrion; S, starch granule; and T, thylakoid membranes. Three different cells are shown in the three images.
Experimental procedures

Materials and culture conditions

Chlamydomonas reinhardtii reference strain CC-408 (C9, wild type mt−) and cell-wall-less strain CC-400 (cw15 mt+) were obtained from the Chlamydomonas Center (Duke University, Durham, NC). Liquid cultures were grown in continuous white light (20–40 μmol photons m⁻² s⁻¹). Standard TAP medium (Gorman and Levine, 1965) was used for standard growth. Nutrient deficiency was induced by centrifuging the cells (5 min at 2000 × g), washing them twice with the respective medium and subsequently resuspending them in TAP medium or TAP medium without N (TAP-N) or P (TAP-P). NH₄Cl was omitted in TAP-N. Potassium phosphate was replaced with 1.5 mM KCl in TAP-P (Quisel et al., 1996).

Analysis of differential gene expression

Differential expression of DGAT genes, UGP3 and SQD2 in C. reinhardtii strain CC-408 cultured in TAP, TAP-P or TAP-N medium was determined using RT-PCR, gel electrophoresis and qPCR. RNA was extracted from TAP, TAP-P or TAP-N cultures by the phenol/chloroform method. Total RNA (500 ng) was used for the synthesis of cDNA using oligo(dt)18 primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The respective cDNA was amplified by LA-Taq (Takara, Otsu, Japan) and separated by agarose gel electrophoresis. cDNA samples were diluted to equal concentrations by normalizing according to amplification of CBLP using 18 PCR cycles. CBLP served as an internal control (Chang et al., 2005). PCR cycle conditions were 3 min of initial denaturation at 94 °C and then 18–34 cycles of 30 s of denaturation, 30 s of annealing at 54 °C and 1 min of elongation at 72 °C. Primers are listed in Table S1. For qPCR, the respective cDNA was amplified by SYBR Premix Ex Taq II (Takara). Thermal Cycler Dice Real Time System and Multiplicate RQ software (Takara) were used for the analysis. The DGAT genes, UGP3 and SQD2 expression levels were normalized to CBLP mRNA expression. Primers are listed in Table S1.

Construction of CrDGAT2-overexpressing cell lines

To create cDNA clones for CrDGAT2 expression, RNA was prepared by the phenol/chloroform method from C. reinhardtii strain CC-408. Reverse transcription was conducted with SuperScript II reverse transcriptase (Invitrogen) to obtain cDNA. The proteins designated DGTT1–5 correspond to the following NCBI accession numbers: DGTT1 (JN815266.1), DGTT2 (XM_001694852.1), DGTT3 (XM_001691395.1), DGTT4 (XM_001694852.1) and DGTT5 (XM_001701615.1). The three putative type-2 DGAT2 coding sequences (DGTT2, DGTT3 and DGTT4) were amplified by PCR from cDNA using LA-Taq (Takara) and the primers DGTT2F and DGTT2R, DGTT3F and DGTT3R, and DGTT4F and DGTT4R, respectively (Table S2). The PCR products were cloned into pMD20 (Takara). The region of the promoter upstream of the start ATG of SQD2 (NCBI accession number: XM_001689610.1) was amplified by PCR from the Chlamydomonas genome using Pyrobest DNA Polymerase (Takara) and the primer pair CrSQD2f and CrSQD2r (Table S2). The PCR products were cloned into pZErO-2 (Invitrogen). Following DNA sequencing, the fusion of the promoter with DGTT2–4 was accomplished using a two-step PCR process. Primers are listed in Table S3. Primer type A contained an EcoRV restriction site. Primers type B and C were perfectly complement-
tary to each other. The first round of PCR involved pairing of primers A with B and C with D in separate reactions. The pairing of primers A and D in the second round of PCR, using the mixture of first-round PCR products as a template, resulted in the generation of PCR products 4–4, each of which was cloned into pMD20. The hygromycin-resistance cassette from pHyg3 (Berthold et al., 2002) was inserted into the introduced EcoRV restriction sites.

The vector constructs were then transformed via electroporation (Shimogawara et al., 1998) into the nuclear genome of CC-400 cells with 0.2 μg of vector DNA used per transformation. The hygromycin-resistance cassette was cloned into pMD20 as the vector control. Selection of transformants was performed on TAP medium containing 10 μg/mL hygromycin.

**Identification of DGGT2–4 overexpressing CC-400 cell lines by PCR analyses**

Approximately 200 positive transformants for each construct were identified by growth on the selection medium (TAP containing 10 μg/mL hygromycin). Seven transformants per construct were then cultured in liquid medium. Their levels of overexpression were evaluated by RT-PCR (Figure S3). Primers are listed in Table S1. Three transformants showing high expression levels for each gene were then selected for the TAG accumulation experiments (Figure S4).

**Confocal imaging of live cells**

Stock solutions of Nile Red (200 μg/mL) in acetone were prepared and stored in the dark as described (Greenspan et al., 1985). Cells were stained with Nile Red (0.1 g/mL final concentration). The Nile Red signal and chlorophyll autofluorescence were determined by confocal laser-scanning microscopy (TCS SE; Leica, http://www.confocal-microscopy.com) using an argon laser for excitation at 488 nm, a 556- to 580-nm filter for detection of the Nile Red signal and a 718- to 749-nm filter for detection of chlorophyll fluorescence. Images were merged and pseudo-coloured using Leica confocal software.

**Lipid extraction and fatty acid determination**

Total lipids were extracted from C. reinhardtii cells as described (Bligh and Dyer, 1959) and then separated by thin-layer chromatography (TLC) using the solvent system hexane/diethyl ether/acetic acid, 160 : 40 : 4. TAG was visualized under UV light after spraying the TLC plate with 0.001% (w/v) primuline in 80% (v/v) acetone and was isolated from the TLC plate.

FAMEs were obtained by incubation of lipids for 1 h at 85 °C in the presence of 5% (w/v) hydrogen chloride methanol solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Kobayashi et al., 2006; Yamaryo et al., 2003). FAMEs were extracted using hexane and were determined by gas chromatography: GC-2014 gas chromatograph (Shimadzu Corporation, Kyoto, Japan); HR-SS-10 capillary column, 25 m (length) × 0.25 mm (i.d.) (Shinwa Chemical Industries, Ltd., Kyoto, Japan); SGE PCWAX10 capillary column, 30 m (length) × 0.25 mm (i.d.) × 0.25 μm (d) (Sigma-Aldrich, St Louis, MO). The fatty acid profile was determined by comparison with a standard reference mix composed of fatty acid methyl esters (Supelco® 18917-1AMP, 18913-1AMP, CRM478851; Sigma-Aldrich). Fatty acid quantification was performed by comparison with the FAME derived from an internal standard pentadecanoic acid.

**Transmission electron microscopy**

The cells were collected by centrifugation. Samples were fixed with 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Samples were then washed six times in the buffer for 10 min each at room temperature. They were post-fixed with 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were dehydrated in a graded ethanol series and embedded in epoxy resin mixture (Quetol 651 mixture; Nissin EM). Ultrathin (75 nm) sections were cut with a diamond knife on a Leica Ultracut UCT ultramicrotome and were transferred onto copper grids. The sections were stained with 2% (w/v) uranyl acetate for 15 min followed by 0.4% (w/v) lead citrate for 5 min at room temperature. The specimens were observed on a Hitachi H-7500 transmission electron microscope at an accelerating voltage of 80 kV.

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**References**

Berthold, P., Schmitt, R. and Mages, W. (2002) An engineered Streptomyces hygroscopicus aph 7b gene mediates dominant resistance against hygromycin B in Chlamydomonas reinhardtii. Protist, 153, 401–412.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Boyle, N.R., Page, M.D., Lu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S.J., Hong-Hermesdorf, A., Shaw, J., Karpowicz, J.S., Gallagher, S.D., Johnson, S., Benning, C., Pellegrini, M., Grosoman, A. and Merchant, S.S. (2012) Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. J. Biol. Chem. 287, 15811–15825.

Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. and Browse, J. (2008) Metabolic engineering of hydroxy fatty acid production in plants: RDCGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnol. J. 6, 819–831.

Cakmak, T., Argun, P., Demiray, Y.E., Ozkan, A.D., Elibol, Z. and Tekinay, T. (2012) Differential effects of nitrogen and sulfur deprivation on growth and biodiesel feedstock production of Chlamydomonas reinhardtii. Biotechnol. Bioeng. 109, 1947–1957.

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.

Cooksey, K.E., Guckert, J.B., Williams, S.A. and Callis, P.R. (1987) Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbial. Methods, 6, 333–345.

Dahlqvist, A., Stahl, U., Lernman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and Styrne, S. (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.

Dean, A.P., Sagie, D.C., Estrada, B. and Pittman, J.K. (2010) Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioreourc. Technol. 101, 4499–4507.

Dent, R.M., Han, M. and Niyogi, K.K. (2001) Functional genomics of plant photosynthesis in the fast lane using Chlamydomonas reinhardtii. Trends Plant Sci. 6, 364–371.

Durrett, T.P., Benning, C. and Ohirogge, J. (2008) Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. 54, 593–607.
Fan, J., Andre, C. and Xu, C. (2011) A chloroplast pathway for the de novo biosynthesis of triacylglycerol in Chlamydomonas reinhardtii. FEBS Lett. 585, 1985–1991.

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.

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.

Grossman, D.S. and Levine, R.P. (1965) Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA. 54, 1665–1669.

Greenspan, P., Mayer, E.P. and Fowler, S.D. (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965–973.

Grossman, A. (2000) Acclimation of Chlamydomonas reinhardtii to its nutrient environment. Prost. 151, 201–224.

Hernandez, M.L., Whitehead, L., He, Z., Gazda, V., Gilday, A., Kozhevnikova, E., Vastig, F.E., Larson, T.R. and Graham, I.A. (2012) A cytosolic acyltransferase contributes to triacylglycerol synthesis in sucrose-rescued Arabidopsis seed oil catabolism mutants. Plant Physiol. 160, 215–225.

Hu, Q., Sommerfeld, M., Janis, 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.

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. 578, 2364–2370.

Jako, C., Kumar, A., Wei, Y., Zou, J., Barton, D.L., Giblin, E.M., Covello, P.S. and Taylor, D.C. (2001) Seed-specific over-expression of an oil body lipid associated protein in sinapis alba. Plant Physiol. 126, 861–874.

James, G.O., Hocart, C.H., Miller, W., Chen, K., Kordbacheh, F., Price, G.D. and Djordjevic, M.A. (2011) Fatty acid profiling of Chlamydomonas reinhardtii under nitrogen deprivation. Bioresour. Technol. 102, 3343–3351.

Kimura, K., Yamaoka, M. and Kamasaka, Y. (2004) Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. J. Microbiol. Methods, 56, 331–338.

Kindle, K.L. (1990) High-frequency nuclear transformation of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA, 87, 1228–1232.

Kobayashi, K., Masuda, T., Takamiya, K. and Ohta, H. (2006) Membrane lipid alteration during phosphate starvation is regulated by phosphate signaling and auxin/ cytokinin cross-talk. Plant J. 47, 238–248.

La Russa, M., Bogen, C., Uhmeyer, A., Doebele, A., Filippone, E., Kruse, O. and Grossman, A. (2000) Acclimation of Chlamydomonas reinhardtii under nitrogen deprivation. Photochem. Photobiol. 71, 426–427.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J. and Browse, J. (1995) Lipid biosynthesis. Plant Cell, 7, 957–970.

Sanjaya, Miller, R., Durrett, T.P., Kosma, D.K., Lydic, T.A., Muthan, B., Koo, A.J., Bykman, Y.V., Reid, G.E., Howe, G.A., Ohlrogge, J. and Benning, C. (1993) Characterization of the respiratory deficient dam-1 mutation of Chlamydomonas reinhardtii and its use as a recipient for mitochondrial transformation. Mol. Genet. Genomics. 236, 235–244.

Siaut, M., Cuine, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Brey, C., Beisson, F., Peltier, G., Ferro, M. and Le-Beisson, Y. (2011) Proteomic profiling of oil bodies isolated from the unicellular green microalga Chlamydomonas reinhardtii with focus on proteins involved in lipid metabolism. Proteomics, 11, 4266–4273.

Sato, N. (1989) Modulation of lipid and fatty acid content by carbon dioxide in Chlamydomonas reinhardtii. Plant J. 11, 1357–1366.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J. and Smith, A.G. (2010) Biodiesel from algae: challenges and prospects. Curr. Opin. Biotechnol. 21, 277–286.

Suh, Y., Han, D., Hu, G., Dauvillee, D., Sommerfeld, M., Ball, S. and Hu, Q. (2010b) Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii. Plant Biotechnology Journal, 8, 387–391.

Suh, Y., Han, D., Hu, G., Sommerfeld, M. and Xu, C. (2011) Synthesis and turnover of phospholipid biosynthesis in the green microalga Chlamydomonas reinhardtii. J. Oil. Sci. 56, 1–9.

Taylor, D.C. (2001) Seed-specific over-expression of an oil body lipid associated protein in sinapis alba. Plant Physiol. 126, 861–874.

Turco, Y., Kordbacheh, F., Price, G.D. and Djordjevic, M.A. (2011) Fatty acid profiling of Chlamydomonas reinhardtii under nitrogen deprivation. Bioresour. Technol. 102, 3343–3351.

Usuda, H., Shimogawara, K., Fujiwara, S., Grossman, A. and Cerutti, H. (2012) Lipid biosynthesis. Plant Physiol. 154, 1737–1752.

Morsy, F.M. (2011) Acetate versus sulfur deprivation role in creating anaerobiosis in light for hydrogen production by Chlamydomonas reinhardtii and Spirulina platensis: two different organisms and two different mechanisms. Photochem. Photobiol. 87, 137–142.

Morsy, F.M. (2011) Acetate versus sulfur deprivation role in creating anaerobiosis in light for hydrogen production by Chlamydomonas reinhardtii and Spirulina platensis: two different organisms and two different mechanisms. Photochem. Photobiol. 87, 137–142.
characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol. 11, 7.

Somerville, C., Browe, J., Jaworski, J. and Ohlrogge, J. (2001) Lipids. In Biochemistry and Molecular Biology of Plants. Rockville, MD: American Society of Plant Physiologists.

Wang, Z.T., Ulrich, 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.

Weers, P.M.M. and Gulati, R.D. (1997) Growth and reproduction of Daphnia galeata in response to changes in fatty acids, phosphorus, and nitrogen in Chlamydomonas reinhardtii. Limnol. Oceanogr. 42, 1584–1589.

Work, V.H., Radakovits, R., Jinkerson, R.E., Meuser, J.E., Elliott, L.G., Vinyard, D.J., Laurens, L.M., 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.

Wykoff, D.D., Davies, J.P., Melis, A. and Grossman, A.R. (1998) The regulation of photosynthetic electron transport during nutrient deprivation in Chlamydomonas reinhardtii. Plant Physiol. 117, 129–139.

Yamaryo, Y., Kanai, D., Awai, K., Shimojima, M., Masuda, T., Shimada, H., Takamiya, K. and Ohta, H. (2003) Light and cytokinin play a co-operative role in MGDG synthesis in greening cucumber cotyledons. Plant Cell Physiol. 44, 844–855.

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.

Yu, B., Xu, C. and Benning, C. (2002) Arabidopsis disrupted in SQD2 encoding sulfolipid synthase is impaired in phosphate-limited growth. Proc. Natl. Acad. Sci. USA, 99, 5732–5737.

Zhang, Z., Shrager, J., Jain, M., Chang, C.W., Vallon, O. and Grossman, A.R. (2004) Insights into the survival of Chlamydomonas reinhardtii during sulfur starvation based on microarray analysis of gene expression. Eukaryot. Cell, 3, 1331–1348.

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.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Size and number of lipid droplets (LDs) scored in a given sample. Wild-type CC-408 cells N-starved for 1 day (N1) and 2 days (N2). (a) The number of LBs per cell. (b) The number of LBs for each size.

Figure S2 Transmission electron micrographs of C. reinhardtii CC-400 during N deprivation. Cells N-starved for 3 days.

Figure S3 RT-PCR analysis showing DGTT2–DGTT4 expression in the overexpression lines after P deprivation. Cells cultured for 5 days. Seven transformants are shown for each gene (a, b and c).

Figure S4 Quantitative real-time PCR (qPCR) showing induction of DGTT4 gene expression in DGTT4-overexpressing cells after P deprivation. Cells cultured for 5 days. Three transformants and the empty vector control are shown.

Figure S5 Changes in TAG content of DGTT2- to DGTT4-overexpressing cells in response to P depletion. Cells cultured for 8 days. Three transformants are shown for each gene.

Figure S6 Changes in TAG content of DGTT4-overexpressing cells in response to P depletion. Cells cultured for 5 days. (a) total TAG per cell, (b) total TAG per litre of culture. Three transformants and the empty vector control are shown.

Figure S7 Changes in TAG content of pCrSQD2-DGTT4-18 and the empty vector control-24 in response to P depletion. Cells cultured for 0, 7 or 13 days.

Table S1 Primer sequences for RT-PCR and qPCR.

Table S2 Primers used for amplification of DGAT2 from C. reinhardtii cDNA and the SQD2 promoter from Chlamydomonas genomic DNA.

Table S3 Primers used for two-step PCR process.