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A chloroplast pathway for the de novo biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii*

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ABSTRACT

Neutral lipid metabolism has been extensively studied in yeast, plants and mammals. In contrast, little information is available regarding the biochemical pathway, enzymes and regulatory factors involved in the biosynthesis of triacylglycerol (TAG) in microalgae. In the conventional TAG biosynthetic pathway widely accepted for yeast, plants and mammals, TAG is assembled in the endoplasmic reticulum (ER) from its immediate precursor diacylglycerol (DAG) made by ER-specific acyltransferases, and is deposited exclusively in lipid droplets in the cytosol. Here, we demonstrated that the unicellular microalga *Chlamydomonas reinhardtii* employs a distinct pathway that uses DAG derived almost exclusively from the chloroplast to produce TAG. This unique TAG biosynthesis pathway is largely dependent on de novo fatty acid synthesis, and the TAG formed in this pathway is stored in lipid droplets in both the chloroplast and the cytosol. These findings have wide implications for understanding TAG biosynthesis and storage and other areas of lipid metabolism in microalgae and other organisms.

1. Introduction

Triacylglycerols (TAGs) are depots for fatty acids, the most concentrated form of energy available in eukaryotic cells [1]. Many microalgae are known to be capable of accumulating large amounts of TAGs, particularly under N-starved conditions [2], which are increasingly discussed as a source of renewable alternatives to petroleum fuels [2–4]. Recent years have seen major advances in our understanding of lipid metabolism and its regulation in plants, particularly in the model plant Arabidopsis [5]; however, the biochemical pathways, and the enzymes and the regulatory factors involved in TAG accumulation in microalgae are as yet poorly defined [2,6].

The unicellular green alga *Chlamydomonas reinhardtii* is a model organism for the study of many fundamental biological processes [7] including lipid metabolism [8]. As in higher plants, the biosynthesis of fatty acids, the building blocks for TAG and membrane lipids, occurs in the chloroplast in *C. reinhardtii* [9], and is catalyzed by two large, evolutionarily conserved enzymes: acetyl-CoA carboxylase (ACCase) and type-2 fatty acid synthase [8,10,11]. The resulting fatty acids can be used directly in the chloroplast to sequentially acylate glycerol-3-phosphate (G-3-P) by chloroplast-resident acyltransferases to produce lysophosphatidic acid (LysoPA) and phosphatidic acid (PA). The PA and its dephosphorylated product diacylglycerol (DAG) generated in the chloroplast serve primarily as precursors for structural lipids of the photosynthetic membrane system [12,13]. Alternatively, fatty acids can be exported into the cytosol and used to sequentially acylate G-3-P in the ER by ER-resident acyltransferase isoforms. The resultant PA can be dephosphorylated to produce DAG that, in contrast to chloroplasts, can be used to synthesize both membrane lipids and storage TAG [12,13]. Due to the stringent substrate specificity of LysoPA acyltransferases present either in the chloroplast or ER, lipids made by the chloroplast or ER pathway are characterized by the presence of a 16- or 18-carbon fatty acid at the *sn*-2 position of glycerol backbone, respectively [14,15].

The biosynthesis of TAG has long been known to occur in microsomal membranes on the basis of subcellular fractionation studies [16–18] with the TAG being deposited in ER-derived lipid droplets in the cytosol [19,20]. In support of the ER localization of TAG biosynthetic activities, DAG acyltransferases (DGAT), the major enzymes involved in the acylation of DAG with acyl-CoA to produce TAG in plants [21] and mammals [22,23] have been shown to localize primarily to the ER network, and TAG in the storage organs of many plants are characterized by the exclusive presence of 18-carbon (C18) fatty acids at the *sn*-2 position of glycerol backbone [24].

As many other microalgae, *Chlamydomonas* cells accumulate TAG under N [25,26] or P [27] limiting conditions or in response to high salinity [28]. Despite these reports, the biosynthetic origin of TAG in *Chlamydomonas* has remained unclear. Here, we present biochemical and ultrastructural evidence that *Chlamydomonas* employs a chloroplast pathway for the de novo synthesis of TAG. The

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discovery of the chloroplast pathway of TAG biosynthesis will greatly aid in research directed at understanding lipid metabolism and its regulation in microalgae and possibly other organisms.

2. Materials and methods

2.1. Strains and growth conditions

The *C. reinhardtii* strains used were the cell wall-less *cw15* (*cw15 nitl mt*$^+$) [29] and the starchless mutant BAFJ5 (*cw15 sta6*) [30]. The cells were grown in Tris–acetate–phosphate (TAP) [31] liquid culture medium under constant illumination (50 μmol m^{-2} s^{-1}) at 22 °C on an orbital shaker. To induce N or P starvation, cells at the late-logarithmic phase of growth were pelleted at 2000×*g* for 5 min, washed once with and resuspended in TAP lacking the corresponding nutrient elements. Cell numbers were determined using a hematocytometer. Cell viability was assessed by plating cell culture on TAP agar medium and counting colonies after 10 d of growth.

2.2. Chloroplast isolation

Intact chloroplasts were isolated from the synchronized cell cultures maintained at 22 °C in a 12-h photoperiod. Cells were broken by passing them through a 27-gauge syringe needles. Chloroplasts were separated by centrifugation using a discontinuous Percoll gradient consisting of 5 ml of 65% and 6 ml 45% Percoll as described [32].

2.3. Lipid analysis

Total lipids were extracted from algal cells into methanol–chloroform–formic acid (2:1:0.1, by volume) followed by phase separation by the addition of 1 M KCl and 0.2 M H_3PO_4. Polar lipids (MGDG, PG, DGDG, DGTS) were separated on ammonium sulfate-impregnated silica plates (Si250 with preadsorbant layer; Mallinckrodt Baker) by thin layer chromatography using a solvent system of acetone–toluene–water (91:30:3, by volume). PI and SL were separated with a solvent system consisting of methyl acetate–isopropanol–chloroform–methanol–KCl (0.25%) in a ratio of 25:25:25:10:4 (by volume). Neutral lipids were separated on untreated silica plates with hexane–diethyl ether–acetic acid (70:30:1, by volume) and visualized by sulfuric acid by charring. For some samples, polar and neutral lipids were separated on a single plate using a double development (2/3 in acetone–toluene–water (91:30:3, by volume), then fully in hexane–diethyl ether–acetic acid (70:30:1, by volume)). The positional distribution of fatty acids in individual lipids was determined using *Rhizopus lipase* (Sigma) as described [33]. For quantitative analysis, lipids were visualized by brief iodine staining. Individual lipids were scraped from the plates and used to prepare fatty acid methyl esters by acid-catalyzed transmethylation. Separation and identification of the fatty acid methyl esters (FAMEs) was performed on an HP5975 gas chromatography (GC)-mass spectrometer (MS) (Hewlett-Packard, Palo Alto, CA) fitted with 60 m × 250 μm SP-2340 capillary column (Supelco, Bellefonte, PA) with helium as a carrier gas. The oven temperature was increased from 100 to 180 °C at a rate of 25 °C min^{-1} then to 240 °C at 8 °C min^{-1} and held at this temperature for 3 min. FAMEs were quantified essentially as described [33] and the amounts of lipids were calculated based on the content of fatty acids derived from GC using heptadecanoic acid as an internal standard.

2.4. Transmission electron microscopy

Cells were fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2, followed by a secondary fixation in 1% (w/v) osmium tetroxide in the same buffer. After this double fixation, samples were dehydrated in a graded series of acetone, embedded in Spurr's epoxy resin (Electron Microscopy Sciences) and sectioned with a diamond knife in an ultramicrotome. The thin sections were stained with uranyl acetate and lead citrate. Micrographs were taken with a FEI BioTwinG^2 Transmission Electron Microscope.

3. Results

3.1. TAG accumulation depends on de novo fatty acid synthesis in the chloroplast

In short-term experiments, we found that N starvation was most effective in inducing TAG accumulation, followed in effectiveness by salt stress. A small amount of TAG was also accumulated in cells exposed to high light, whereas P starvation failed to increase the amount of TAG significantly, even after extended treatment times up to 6 d (data not shown). Consequently, we focused our subsequent analysis primarily on TAG biosynthesis in response to N starvation.

TAG accumulation in *Chlamydomonas* cells depleted of N was reported to be accompanied by an increase in the amount of total

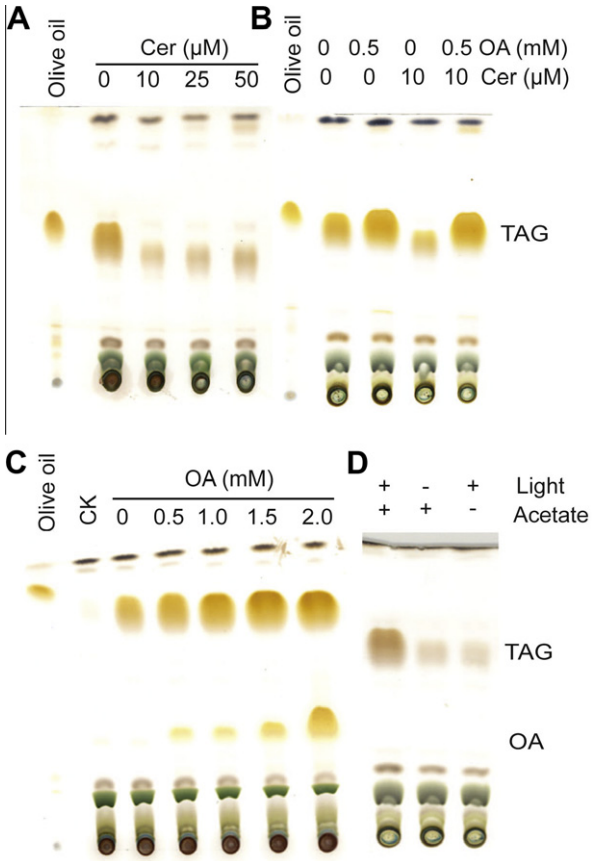

Fig. 1. TAG accumulation in Chlamydomonas is dependent on de novo fatty acid synthesis. (A and B) Inhibition of TAG synthesis by cerulenin (Cer) and reversal of its inhibitory effect by oleic acid (OA). Cells were grown for 2 d in medium lacking N in the presence of various amounts of cerulenin (A) or in the presence of 10 μM cerulenin and 0.5 mM OA (B). (C) Effect of exogenous OA on TAG accumulation. Cells were cultured in medium without N in the presence of various amounts of OA for 2 d. Control (CK) cells were grown in complete medium. (D) Dependence of TAG accumulation on acetate and light. Cells were starved for N in the dark or in the light without acetate for 2 d. Thin-layer chromatograms of neutral lipids are shown. Lipids were loaded on an equal-cell-number basis and visualized with sulfuric acid by charring.

cellular fatty acids [25]. It has therefore been postulated that the biosynthesis of TAG under N starvation requires de novo fatty acid synthesis. To examine the metabolic origin of fatty acids in TAG in more detail, we treated *Chlamydomonas* with various concentrations of cerulenin, a specific inhibitor of the β-keto-acyl-ACP synthase component of the type-2 fatty acid synthase [34–36]. We found that treatment with cerulenin strongly inhibited TAG accumulation induced by N starvation (Fig. 1A), with the maximal inhibition observed at 10 μM. In these conditions, 10 μM cerulenin inhibited TAG accumulation by 79% from 94.83 ± 4.50 to 19.89 ± 2.72 μg/10^5 cells over a 2-d treatment, whereas cell viability was only reduced slightly by 12% in comparison with cells starved for N in the absence of cerulenin. The inhibitory effect of cerulenin on oil accumulation was completely reversed upon addition of 0.5 mM of oleic acid (Fig. 1B), confirming the specificity of cerulenin's effect on fatty acid production. It is noteworthy that the addition of 0.5 mM oleic acid alone substantially increased TAG accumulation in N-starved cells (Fig. 1B), and the amount of TAG increased proportional to the concentration of oleic acid up to 1.5 mM in the medium (Fig. 1C). Inversely, N-starved cells grown in the dark or in the light without acetate as a carbon source produced only limited amounts of TAG (Fig. 1D). These results suggest that TAG accumulation in *C. reinhardtii* under N starvation is largely attributable to the de novo synthesis of fatty acids in the chloroplast and that this process is limiting for overall TAG production.

The accumulation of significant amounts of TAG in the presence of cerulenin as shown in Fig 1A might suggest a role of pre-existing membrane lipids in TAG synthesis. To test this possibility, we followed the time course of changes in individual membrane lipids and TAG in cells starved for N. As shown in Fig. 2A, TAG started to accumulate 6 h after N withdrawal, and then steadily increased over time and reached up to 42% of total cellular fatty acids after 48 h. During this time period, the most

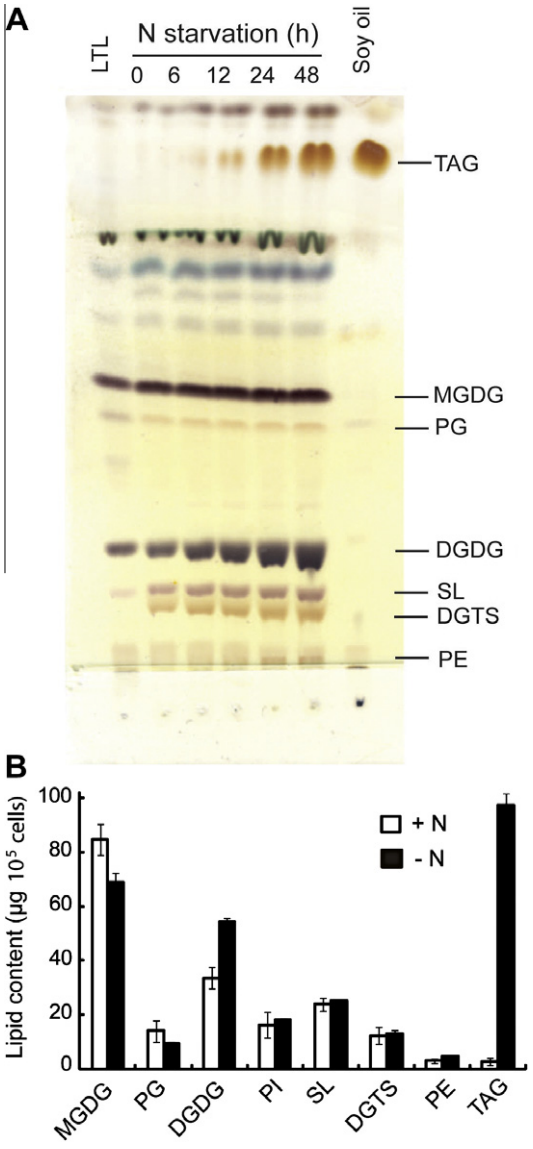

Fig. 2. Alterations in membrane lipids and TAG in response to N starvation. (A) Lipids were extracted from an equal volume of cell culture at various time points as indicated following N withdrawal and separated by a single silica gel plate as described in Materials and Methods. Polar lipids were visualized with a-naphthol and neutral lipids with sulfuric acid by charring. Total lipids extracted from Arabidopsis leaves (LTL) were used as a control showing the presence of DGTS in *Chlamydomonas*. (B) Lipids were extracted from cells cultured in medium lacking N for 2 d. Values are means and standard deviation of three independent experiments. DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N*,*N*,*N*-trimethylhomoserine; MGDG, monogalactosyldiacylglycerol; PE, phosphatidylethanoalamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SL, sulfoquinovosyldiacylglycerol.

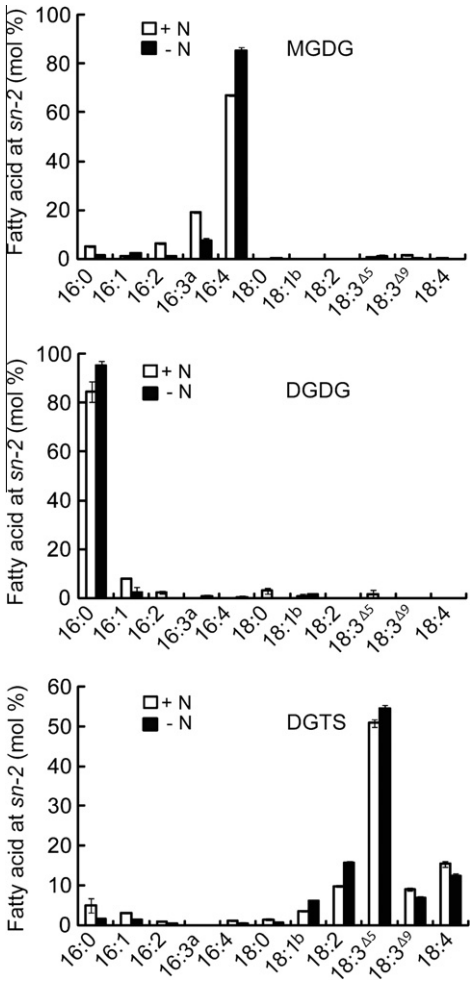

Fig. 3. N starvation does not alter the fatty acid distribution patterns of membrane lipids. Fatty acid compositions exclusively at the *sn*-2 position of MGDG, DGDG and DGTS isolated from *cw15* cells cultured in complete medium (+N) or cells shifted to medium lacking N (−N) for 2 d are shown. Data are presented as the means and standard deviation of three replicates. Only the first double bond counting from the carboxyl end for 18:3 is indicated by superscript to distinguish its two isomers $18:3^{\Delta5,9,12}$ and $18:3^{\Delta9,12,15}$. [a]The sum of $16:3^{\Delta4,7,10}$ and $16:3^{\Delta7,10,13}$; [b]The sum of $18:1^{\Delta9}$ and $18:1^{\Delta11}$.

pronounced changes in membrane lipids occurred in the chloroplast lipid digalactosyldiacylglycerol (DGDG), which closely mirrored the increase in TAG content. At 48 h after N withdrawal, the amount of DGDG increased by 62%, whereas the most abundant chloroplast-specific lipid monogalactosyldiacylglycerol (MGDG) decreased by 19% (Fig. 2B). There was also a 33% decrease in phosphatidylglycerol (PG), whereas the third major chloroplast lipid sulfoquinovosyldiacylglycerol (SL) remained largely unaltered, as did the two major extrachloroplastidic membrane lipids diacylglycerol-*N*,*N*,*N*-trimethylhomoserine (DGTS) and phosphatidylethanoalamine (PE). When expressed on a per cell basis, the combined amount of MGDG and PG decreased from 98.7 ± 9.6 to 69.1 ± 3.1 μg/10^5 cells following 2 d of N starvation, during which the amount of TAG increased from 2.7 ± 1.2 to 97.3 ± 4.6 and that of DGDG from 33.6 ± 3.9 to 54.3 ± 1.6 μg/10^5 cells. Assuming that the decreased amounts of MGDG and PG are entirely metabolized to TAG, this would account for only about 30% of TAG accumulated in cells N-starved for 2 d. These results, together with the inhibition of TAG accumulation by cerulenin (Fig. 1A) strongly suggest that TAG accumulation in *Chlamydomonas* under N starvation is largely a result of de novo fatty acid synthesis.

3.2. The DAG moiety of TAG is of chloroplast origin

Unlike higher plants, the chloroplast of *Chlamydomonas* is autonomous with regard to thylakoid lipid biosynthesis [8]. Consequently, all the thylakoid lipids including MGDG, DGDG and SL in *Chlamydomonas* contain exclusively C16 fatty acids at the *sn*-2 position of glycerol backbone as opposed to the lipids exclusively present in extrachloroplastidic membranes such as PE and DGTS that contain mostly C18 fatty acids as the same position [15]. Such typical fatty acid distribution patterns of MGDG, DGDG and DGTS remain largely unaltered in response to N starvation (Fig. 3).

To determine the metabolic origin of the DAG moiety of TAG, we analyzed the positional distribution of esterified fatty acids in TAG isolated from N-starved cells. Quite unexpectedly, we observed that in approximately 90% of the molecular species of TAG, the *sn*-2 position was esterified with C16 fatty acids (Fig. 4A). Almost all of the unsaturated C16 fatty acids were present at the *sn*-2 position of TAG and the *sn*-1 and/or *sn*-3 positions were occupied predominantly by C18 acyl groups and C16 saturated fatty acids (Fig. 4B). The fatty acid composition at the *sn*-2 position of glycerolipids is believed to be controlled primarily by the substrate specificities of LysoPA acyltransferases [14,37], but fatty acid availability may also play a role [38]. However, because the C18 fatty acids (70%) were more abundantly present at the *sn*-1 + 3 positions than C16 acyl chains (30%), the possibility that the enrichment of C16 acyl chains at the *sn*-2 position of TAG is due to limited availability of C18 fatty acids for G-3-P acylation reaction catalyzed by the acyltransferase in the ER is rather unlikely. The presence of both C16 and C18 fatty acids at the *sn*-1 + 3 positions is consistent with the limited substrate specificity of acyltransferases responsible for acylating the *sn*-1 and *sn*-3 position of TAG observed in plants [14]. We thus conclude that TAG is assembled from DAG made by chloroplast-specific acyltransferases in *Chlamydomonas* under N starvation conditions.

To test whether or not this fatty acid distribution pattern is specific to TAG isolated from N-starved cells, we performed positional analysis of TAGs isolated from cells subjected to high light treatment or salt stress. As shown in Fig. 5, the positional distribution of fatty acids in TAGs isolated from cells subjected to high

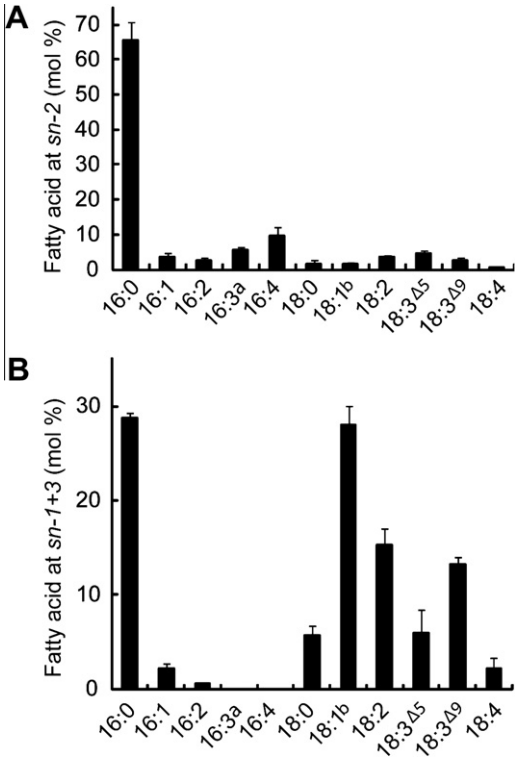

Fig. 4. The *sn*-2 position of TAG is highly enriched in C16 fatty acids. Fatty acid compositions exclusively at the *sn*-2 (A) or *sn*-1 + 3 (B) positions of TAG isolated from *cw*15 cells cultured in medium lacking N for 2 d are shown. Data are presented as the means and standard deviation of three replicates. Only the first double bond counting from the carboxyl end for 18:3 is indicated by superscript to distinguish its two isomers 18:$3^{\Delta5,9,12}$ and 18:$3^{\Delta9,12,15}$. [a]The sum of 16:$3^{\Delta4,7,10}$ and 16:$3^{\Delta7,10,13}$; [b]the sum of 18:$1^{\Delta9}$ and 18:$1^{\Delta11}$.

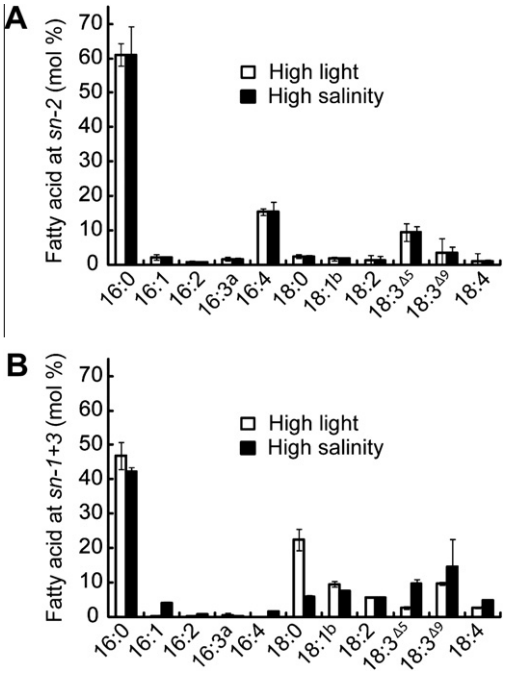

Fig. 5. Positional analysis of TAG isolated from cells exposed to high salinity or high light. Fatty acid compositions exclusively at the *sn*-2 (A) or *sn*-1 + 3(B) positions of TAG isolated from *cw*15 cells cultured in complete medium with addition of 100 mM NaCl or under a light intensity of 500 μmol^{-2} photons m^{-2} s^{-1} for 2 d are shown. Data are presented as the means and standard deviation of three replicates. Only the first double bond counting from the carboxyl end for 18:3 is indicated by superscript to distinguish its two isomers 18:$3^{\Delta5,9,12}$ and 18:$3^{\Delta9,12,15}$. [a]The sum of 16:$3^{\Delta4,7,10}$ and 16:$3^{\Delta7,10,13}$; [b]The sum of 18:$1^{\Delta9}$ and 18:$1^{\Delta11}$.

light treatment or salt stress was very similar to their distribution in TAG isolated from N-starved cells, implying the operation of the chloroplast TAG biosynthetic pathway under all three stress conditions.

3.3. TAG is deposited in lipid droplets in both the chloroplast and the cytosol

Wild type Chlamydomonas cells accumulated large starch granules in chloroplasts, particularly under N starvation conditions, which often distorted and obscured chloroplast membrane structures [25,39], thus making difficult the study of the intracellular distribution of lipid droplets by ultrastructural analysis. To test whether the TAG accumulated in response to N withdrawal is in fact deposited in the chloroplast, we took advantage of available starchless mutants of *C. reinhardtii* [40]. Lipid analysis revealed that the positional distribution of TAG isolated from the N-starved starchless mutant was similar to that of TAG from its parental strain maintained under the same conditions (data not shown), suggesting the inactivation of starch biosynthesis in the starchless mutant does not result in a modification of the TAG biosynthetic pathway in *C. reinhardtii*.Under short-term N starvation conditions, the chloroplasts of starchless mutant were able to retain extensive thylakoid membrane systems; albeit with less stacking than in the control cells maintained under optimal growth conditions (Fig. 6). Strikingly, they contained lipid droplets of similar size, shape and osmiophilicity as those seen in the cytosol (Fig. 6B and C). In a survey of 100 cell cross sections using electron microscopy, 168 (45%) lipid droplets were found inside the chloroplast and 207 (55%) in the cytosol. In most instances, the cytosolic lipid droplets were found to be partially enclosed by the envelope membranes of chloroplasts (Fig. 6D), a situation reminiscent of ER and lipid droplet interactions observed in mammalian cells [41], suggesting that the chloroplast envelope membranes may be the sites of lipid droplet biogenesis in *Chlamydomonas*. Chloroplasts purified from N-starved cells by Percoll gradient centrifugation also contained TAG (data not shown). Positional analysis revealed that the TAG isolated from purified chloroplasts exhibited the same fatty acid distribution pattern characterized by an enrichment of C16 fatty acids at the *sn*-2 position as that of TAG from the whole cells (data not shown). These observations point to a common origin of lipid droplets in both the chloroplast and the cytosol.

4. Discussion

By analogy to higher plants, it has commonly been assumed that TAG in microalgae is synthesized in the ER catalyzed by ER-specific acyltransferases and deposited in ER- derived lipid droplets in the cytosol [2,6,8,10,42,43]. In disagreement with this assumption, we found in this study that the model alga *Chlamydomonas* synthesizes TAG from its precursor DAG containing almost exclusively C16 fatty acids at the *sn*-2 position typical of glycerolipids assembled by chloroplast-specific acyltransferases and the TAG accumulated in this alga is stored in lipid droplets in the chloroplast, in addition to

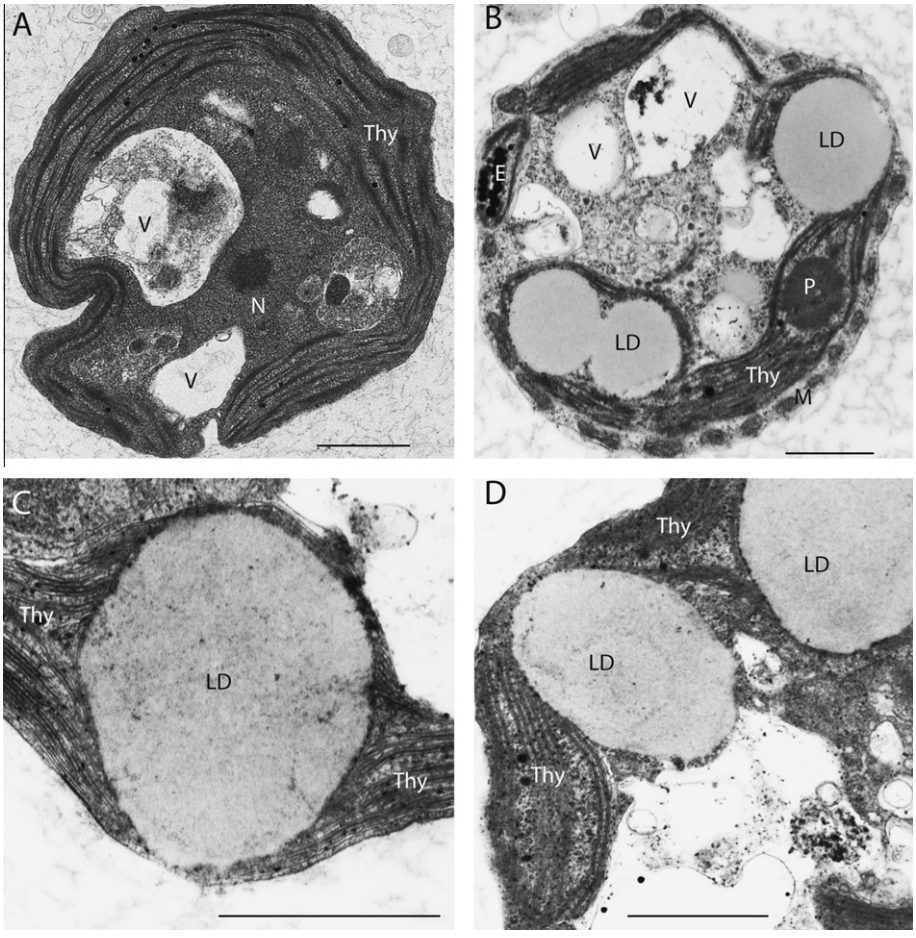

Fig. 6. Lipid droplets are present in the chloroplast and the cytosol. Representative electron micrographs of the starchless mutant BAJF5 cells grown in complete medium (A) or cells shifted to medium lacking N for 2 d (B–D) are shown. (B) The presence of lipid droplets in the chloroplast and the cytosol in a single cell. (C) High magnification electron micrograph of a single lipid droplet inside the chloroplast. (D) The close association of cytosolic lipid droplets with the chloroplast. Scale bars represent 2 μm. E, eyespot; LD, lipid droplets; M, mitochondria; N, nucleus; P, pyranoid; Thy, thylakoid membranes; V, vacuoles.

cytosol. The synthesis of TAG of chloroplast origin has been reported in plants [44,45] and it is attributed to the combined action of galactolipid:galactolipid galactosyltransferase (GGGT) and the TAG biosynthetic enzymes [44,45], which produce TAG of the same fatty acid composition at the *sn*-2 position as that of MGDG [46]. This is unlikely to be the case for *Chlamydomonas*, because the fatty acid composition at the *sn*-2 position of TAG (Fig. 4) is distinct from that of MGDG (Fig. 3). In addition, the envelope membranes of *Chlamydomonas*chloroplasts were reported to lack the GGGT activity [47] and genes with significant sequence similarity to GGGT have not been found in the complete genome sequence of *C. reinhardtii* [48]. Furthermore, our results clearly demonstrated that N-starvation-induced TAG accumulation is dependent on de novo fatty acid synthesis. In summary, the results from the present study support a chloroplast pathway for the biosynthesis of TAG from its immediate precursor DAG assembled de novo by chloroplast-specific acyltransferases in *Chlamydomonas*.

Our findings in the study raise intriguing questions about the subcellular site of TAG synthesis and the origin of lipid droplets in *Chlamydomonas*. Both acyl-CoA-dependent and acyl-CoA-independent mechanisms contribute to TAG synthesis in plants [49] and yeast [50]. A similar situation seems to hold true for *Chlamydomonas* [6,43], but their relative contribution to TAG assembly remains to be determined. Adding yet another level of complexity is the observation that the key TAG assembly enzyme in mammalian cells has been shown to be dynamically associated with the ER, lipid droplets and mitochondria-associated ER membranes [51]. Likewise, although TAG biosynthetic activities [16–18,52] and DGAT enzymes [21] in plants were reported to reside primarily in microsomal membranes, they have been shown to be associated with chloroplast envelope membranes in spinach and Arabidopsis leaves [53,54]. TAG synthetic activities in or associated with chloroplasts would provide the key components in the proposed chloroplast TAG biosynthetic pathway in *Chlamydomonas* and may explain the presence of lipid droplets in both chloroplast and the cytosol. Further studies are apparently needed to test these and other possibilities. Nevertheless, our findings thus far have wide implications for understanding TAG biosynthesis and lipid droplet biogenesis in microalgae and other organisms and for exploring microalgae as biochemical factories for the production of nutritional lipids and biofuels.

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