Origin of β-carotene-rich Plastoglobuli in *Dunaliella bardawil*

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The halotolerant microalgae *Dunaliella bardawil* accumulates under nitrogen deprivation two types of lipid droplets: plastoglobuli rich in β-carotene (βC-plastoglobuli) and cytoplasmatic lipid droplets (CLD). We describe the isolation, composition and origin of these lipid droplets. Plastoglobuli contain β-carotene, phytoene and galactolipids, missing in CLD. The two preparations contain different lipid-associated proteins: major lipid droplet protein (MLDP) in CLD, and the proline-rich carotene globule protein (CGP) in βC-plastoglobuli. The compositions of triglycerides (TAG) molecular species, total fatty acids and sn-1+3 and sn-2 positions in the two lipid pools are similar, except for a small increase in palmitic acid in plastoglobuli, suggesting a common origin. The formation of CLD TAG precedes that of βC-plastoglobuli, reaching a maximum after 48h of N deprivation and then decreasing. Palmitic acid incorporation kinetics indicated that at early stages of N deprivation CLD TAG is synthesized mostly from newly formed fatty acids, whereas in βC-plastoglobuli large part of TAG is produced from fatty acids of pre-formed membrane lipids. Electron microscopic analyses revealed that CLD adhere to chloroplast envelope membranes concomitant with appearance of small βC- plastoglobuli within the chloroplast. Based on these results we propose that CLD in *D. bardawil* are produced in the endoplasmatic reticulum (ER) whereas βC-plastoglobuli are made in part from hydrolysis of chloroplast membrane lipids and in part by a continual transfer of TAG or of fatty acids derived from CLD.
INTRODUCTION

Eukaryotic cells accumulate neutral lipids in different tissues mainly in the form of lipid droplets (Murphy 2012). Most lipid droplets consist of a core of triglycerides (TAG) and/or sterol esters coated by a phospholipids monolayer and embedded with proteins (Zweytick et al. 2000). Plants accumulate triglycerides (TAG) in different tissues, primarily in seeds, but also in fruit, such as in palm oil, in flowers and in leaves. The best characterized system for TAG metabolism are oil seeds, in which TAG serves as the major carbon and energy reservoir to be utilized during germination (Huang 1992, Huang 1996). Recent studies show that lipid droplets are not just static pools of lipids, but have diverse metabolic functions (Farese Jr and Walther 2009). In addition, plants also contain plastoglobuli, small chloroplastic lipid droplets consisting primarily of storage lipids and pigments. Proteome analyses of plastoglobuli suggest that they are involved in synthesis and degradation of lipids, pigments and coenzymes (Ytterberg et al. 2006, Lundquist et al. 2012). It has been shown that plant plastoglobuli are associated with thylakoid membranes (Austin et al. 2006, Ytterberg et al. 2006).

It is not entirely clear where are TAG synthesized in the plant cell. Until recently it has been assumed that most TAG is made in the ER from fatty acids which are mostly synthesized in the chloroplast and imported to the cytoplasm (Joyard et al. 2010). However, the recent identification of the enzyme diacylglycerol acyl transferase (DGAT) in plant plastoglobuli (Lundquist et al. 2012), suggests that TAG may be synthetized directly in chloroplasts, although direct evidence for this is missing. TAG may be synthesized also from galactolipid fatty acids during stress or senescence by phytol ester synthases, which catalyze acyl trans-esterification from galactolipids to TAG (Lippold et al. 2012). Phosphatidyl choline (PC) plays a major role in acyl transfer of newly synthesized fatty acids from the chloroplast into TAG at the ER in plants (Bates et al. 2009). An indication for the origin of glycerolipids in plants is the identity of the fatty acids at the sn-2 position: if it originates in the chloroplast, it is mostly C16:0 whereas if it was made in the ER it is mostly C:18 (Heinz and Roughan 1983).

Many species of unicellular microalgae can accumulate large amounts of TAG under growth-limiting conditions such as nitrogen deprivation (Shifrin and Chisholm 1981,
Roessler 1990, Avron and Ben-Amotz 1992, Thompson 1996). In green microalgae, TAG are usually synthesized and accumulated in CLD (Murphy 2012), although in some cases, such as in *C. reinhardtii* starch-less mutants, they accumulate also in chloroplasts (Fan et al. 2011, Goodson et al. 2011). Recent studies indicate that the CLD are closely associated with ER membranes and possibly also with chloroplast envelope membranes (Goodson et al. 2011, Peled et al. 2012).

Green microalgae also contain two distinct types of chloroplastic lipid droplets. The first are plastoglobuli, similar in morphology to higher plants plastoglobuli (Bréhélin et al. 2007, Kessler and Vidi 2007). The second is the eyespot (stigma), part of the visual system in microalgae. The eyespot is composed of a cluster of β-carotene containing lipid droplets, organized in several layers, between grana membranes in the chloroplast (Häder and Lebert 2009, Kreimer 2009). Recent proteomic analysis of algal eyespot proteins revealed that they contain diverse structural proteins, lipid and carotenoid metabolizing enzymes, transporters and signal transduction components (Schmidt et al. 2006).

The origin of TAG in microalgae is still not clear. In *C. reinhardtii* it was found that the major fatty acids in the sn-2 position are 16:0 which according to the plant dogma is made in the chloroplast (Fan et al. 2011). In Chlamydomonas which lacks PC, MGDG was proposed to replace PC in the mobilization of fatty acids from plastidal galactoglycerolipids into TAG, based on mutation of a galactoglycerolipid lipase (Li et al. 2012). Based on these and other results, it has been proposed that in *Chlamydomonas* triglycerides are primarily produced in the chloroplast or in combination with ER (Li et al. 2012, Liu and Benning 2013).

Plants and algae lipid droplets contain structural major proteins, localized at the lipid droplet periphery, whose major function appears to be stabilization and prevention of fusion (Huang 1992, Katz et al. 1995, Huang 1996, Frandsen et al. 2001, Liu et al. 2009). In plant seed oils the major classes of lipid droplet proteins are oleosins and caleosins which have a characteristic hydrophobic loop with a conserved three proline domain (Hsieh and Huang 2004, Capuano et al. 2007, Purkrtova et al. 2008, Tzen 2012). Oleosin and caleosin analogous were recently identified also in some green microalgal species (Lin et al. 2012, Vieler et al. 2012, Huang et al. 2013). However, the most abundant lipid
droplets proteins in Volvocales order green algae are a new family of major lipid droplet proteins (MLDP), structurally distinct from plant oleosins and caleosins (Moellering and Benning 2010, Peled et al. 2011, Davidi et al. 2012). Plastoglobules have different major lipid-associated proteins termed PAP-fibrillins, which form a distinct protein family with no sequence or structural similarities to oleosins (Kim and Huang 2003). We have previously identified in the βC-plastoglobuli a lipid-associated protein, termed carotene globule protein (CGP), whose degradation destabilized the lipid droplets (Katz et al. 1995). The proteome of Chlamydomonas lipid droplet indicates that algal CLD also contain several enzymes suggesting that they are involved in lipid metabolism (Nguyen et al. 2011).

The halotolerant green algae *Dunaliella bardawil* and *D. salina* Teodoresco, are unique in that they accumulate under high light stress or nitrogen deprivation large amounts of plastidic lipid droplets (βC-plastoglobuli), which consist of TAG and of two isomers of β-carotene, *all-trans* and 9-*cis* (Ben-Amotz et al. 1982, Ben-Amotz et al. 1988). *D. bardawil* also accumulates under the same stress conditions CLD, similar to other green algae (Davidi et al. 2012). It has been demonstrated that the function of βC-plastoglobuli is to protect the photosynthetic system against photoinhibition (Ben-Amotz et al. 1989). The enzymatic pathway for β-carotene synthesis in *D. bardawil* and *D. salina* has been partly identified, but the sub-cellular localization of β-carotene biosynthesis is not known (Jin and Polle 2009). The synthesis of β-carotene depends on TAG biosynthesis (Rabbani et al. 1998), however the origin of βC-plastoglobuli is not known. Are they formed within the chloroplast or are they made in the cytoplasm? Is the TAG in βC-plastoglobuli and in CLD identical or different and where is it formed?

*D. bardawil* is an excellent model organism for isolation of lipid droplet for several reasons: First, *D. bardawil* contains large amount of both CLD and βC-plastoglobuli (Ben-Amotz et al. 1982, Fried et al. 1982), making it possible to obtain sufficient amounts of proteins and lipids from the two types of lipid pools for detailed analyses. Second, Dunaliella does not have a rigid cell wall and can be lysed by a gentle osmotic shock, which does not rupture the chloroplast. Therefore it is possible to sequentially release pure CLD and βC-plastoglobuli by a two-step lysis (Katz et al. 1995). Third, *D.
*bardawil* seems to lack the eyespot structure, which can be clearly observed in other Dunaliella species even in a light microscope or by electron microscopy, but has never been observed in *D. bardawil* by us. This avoids the risk of cross-contamination of βC-plastoglobuli with eyespot proteins. Finally, the availability of protein markers for the major lipid droplet associated proteins, CGP and MLDP, enabled both good immunolocalization and careful monitoring of the purity of the preparations by Western analysis.

In this work we describe the purification, lipid compositions and protein profile of two lipid pools from *D. bardawil*: CLD and plastidic βC-plastoglobuli. A detailed proteomic analysis of these lipid droplets will be described in another manuscript. Combined with detailed EM studies, these results led to surprising conclusions regarding the origin of the plastidic βC-plastoglobuli.
RESULTS

Isolation and lipid composition of CLD and plastidic βC-plastoglobuli from D. bardawil

CLD and βC-plastoglobuli were isolated from D. bardawil essentially as described earlier (Katz et al. 1995), with one modification. In brief, cells incubated for 48 h in N-depleted medium at high light (DB-N), were washed and lysed by a gentle osmotic shock. We noticed that the osmotic shock does not release most of the CLD from the lysed cells, and therefore introduced a syringe treatment after osmotic cell disruption, which released the majority of the CLD as shown by the protein SDS-PAGE profile and by the level of TAG in the preparation (Fig. S1). Chloroplasts were washed and lysed by sonication. CLD and βC-plastoglobuli were isolated by flotation centrifugation through sucrose density layers. Control cells grown in N-sufficient medium (DB+N) were also analyzed.

The two populations of purified lipid droplets, CLD and βC-plastoglobuli, were analyzed by Nile red and TLC. In the Nile red fluorescence staining (Fig. 1A), both CLD and βC-plastoglobuli show the characteristic staining of neutral lipids at 580–590 nm, which is apparent also in intact N-deprived cells. No chlorophyll fluorescence emission was observed at 685 nm, indicating that the two lipid pools are practically clean of contamination by chloroplast membranes. TLC analysis of neutral lipid composition (Fig. 1B) reveals that CLD contain mostly TAG, whereas βC-plastoglobuli also contain high amount of β-carotene and another minor carotenoid. Both preparations also contain small amounts of polar lipids and no detectable chlorophyll. In order to further analyze the trace polar lipids, larger amounts of total lipid extracts were resolved on TLC, and the samples were compared to thylakoid lipid extracts (Fig. 1C). The polar lipid compositions differ between the two preparations: whereas βC-plastoglobuli polar lipids are almost identical to thylakoid in lipid composition, predominated by monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), as confirmed by galactololipid staining (Fig. 1C, right), the CLD contain two different polar lipid components which are not galactolipids.
In order to compare the TAG molecular species of the two droplet populations, we analyzed lipid extracts by reverse-phase HPLC using a Halo-C8 column. The chromatogram of CLD and of βC-plastoglobuli lipid extracts is shown in Fig. 2. The two chromatograms are very similar, except for three peaks which appear only in the latter. Two major peaks (at 5 min and 8 min), were identified as β-carotene and phytoene (a β-carotene precursor), respectively, based on their absorption spectra, as determined by a diode-array detector. The third peak (MS-9, at 33.3 min, Fig. 2), is a unique TAG species that does not appear in other Dunaliella species. Of the major TAG peaks (between 9 to 28 min.), each composed of 2-3 individual components, the minor smaller peaks (between 9-17 min), show slight differences in relative peaks distributions, whereas the 3 major peaks (between 18-28 min) are almost identical in both fractions. TAG peaks were identified by reference to TAG standards.

Fatty acid composition analysis of D. bardawil cells and the two types of lipid droplets was conducted by GC analysis of the fatty acid methyl esters (FAMEs) (Fig. 3A). The two population of lipid droplets were highly enriched in the fatty acid 18:1ω9 in comparison to whole cell extracts, reaching about 40% of the fatty acid content of isolated lipid droplets. In contrast, both populations were relatively deprived of fatty acids 18:3ω3 and 16:4ω3, which are components of the chloroplast thylakoid membrane (Evans et al. 1982). These results are in agreement with our previous study in D. salina (Davidi et al. 2012) and studies in C. reinhardtii which showed elevated levels of 18:1ω9, under N-deprivation (Wang et al. 2009, James et al. 2011, Siaut et al. 2011).

As noted above, the identity of the fatty acids at the sn-2 position in higher plants is considered as an indicator for its origin: 18C when made in the ER or 16C when made in the chloroplast. In order to test the applicability of this criterion to Dunaliella lipids, we first analyzed the fatty acid compositions at the sn-2 and sn-1 positions of cytoplasmatic membrane polar lipids (microsomal fraction) and of thylakoid membrane galactolipids, as controls for ER-made and for chloroplast-made lipids. Since we found that microsomal lipid extracts contain significant amounts of galactolipids, indicating chloroplast membrane contamination, we performed the positional analysis on isolated PC, which is a component in microsomal membranes, but is excluded from chloroplast membranes.
(Table S1). The analysis was made by exposure of the former to phospholipase A2, which specifically cleaves fatty acids at the sn-2 position, and of the latter to Rhizopus lipase, which specifically cleaves fatty acids at sn-1+3 positions. As expected, galactolipids sn-2 fatty acids are mostly 16C in length predominated by 16:4, consistent with a chloroplast origin (see also (Cho and Thompson 1987a, Cho and Thompson 1987b)). Conversely, the microsomal sn-2 fatty acids of PC are slightly enriched in 18C fatty acids, consistent with previous a report in another Dunaliella species (Ha and Thompson 1991). Conversely, the chloroplast membranes sn-1 fatty acids are composed of a mixture of 16C and 18C fatty acids, whereas PC sn-1 shows a clear 16C bias, consistent with previous reports (Ha and Thompson 1991). Next, we analyzed the sn-1+3 and sn-2 positions of TAG in CLD and in βC-plastoglobuli (Fig. 3B,C) by exposure of isolated TAG to Rhizopus lipase. As shown in Fig. 3C, the fatty acids compositions at the sn-2 positions in both lipid pools contains a similar mixture of 16C and of 18C fatty acids, with a slight 18C bias for CLD TAG, clearly differing from chloroplast galactolipids (16C/18C =20, Table 1), but resembling microsomal polar lipids. The sn-1+3 position fatty acids in both TAG pools are enriched in 18C fatty acids but differ in the higher level of palmitic acid (16:0) in βC-plastoglobuli.

**Time-course of formation of cytoplasmic and chloroplastic lipid droplets**

In order to learn if the formation of CLD and of chloroplastic βC-plastoglobuli occur simultaneously or if one preceeds the other, we performed a physical seperation and quantitation of the TAG contents of the two lipid populations at different times after exposure to N-deprivation.

As shown in Fig. 4A, the rate of TAG accumulation in the two lipid populations is very different: whereas CLD increase in level until 48 h and then decrease, chloroplastic plastoglobuli increase mostly after 3-6 days. These results suggest that the formation of CLD precedes that of chloroplastic βC-plastoglobuli.

Pulse-labeling experiments with 14C-palmitic acid (14C-PA) were designed to answer 3 questions: Are TAG in the two lipid pools synthesized de novo, from newly-incorporated fatty acids? Are TAG produced from degradation of membrane polar lipids in the
chloroplast or in microsomal cytoplasmic membranes? Are TAG in βC-plastoglobuli made from pre-formed TAG in CLD? 14C-PA was chosen because we found that this fatty acid, which is a major component in all polar and neutral glycerolipid fractions in D. bardawil, is rapidly incorporated into both cytoplasmic and chloroplastic membrane lipids, as shown below. To answer the 1st and 3rd questions, N-deprived cells were labeled for 4h with 14C-PA, than all free 14C-PA was removed by washing and supplementation of 250-fold excess unlabelled PA. After different periods of incubation in N-deprived medium, cells were disrupted and 14C contents in CLD, βC-plastoglobuli, cytoplasmatic microsomal membranes and in chloroplast membranes were analyzed. If βC-plastoglobuli TAG are produced from CLD TAG, then we expected to find an increased labeling in βC-plastoglobuli correlated with a decreased labeling in CLD.

The 14C distribution pattern (Fig. 4B, Table S1) showed fast incorporation into both microsomal and chloroplast membranes, followed by decrease with time, indicating that there is no permeability barrier for PA into the chloroplast. The decrease with time in 14C content in the two membrane fractions differ: whereas in microsomal membranes the decrease continues for 72h, reaching about 15% from the point of PA dilution, in the chloroplast membranes 14C decrease levels off after 24h, and it is roughly correlated with the decrease in chlorophyll (Fig. 4C), suggesting that in the microsomal membranes, the decrease is mostly due to degradation and to resynthesis of new lipids (turnover), whereas in chloroplast membranes the decrease results from net degradation of about 60% of the membranes and no de novo synthesis.

The time-courses of 14C incorporation into the two polar lipid droplets greatly differ: whereas in CLD there is a 7-fold increase in labeling in the first 24h, followed by a subsequent small decrease, in βC-plastoglobuli there is a small but progressive increase in labeling during 72h (Fig. 4B). The large increase in incorporation into CLD in the first 24h, is correlated with the major decrease in labeling of both chloroplast and microsomal membrane lipids, which could indicate acyl editing, namely, that PA is first incorporated into specific polar membrane lipids and next transacylated into TAG (Bates et al. 2009, Li et al. 2012). The subsequent decrease in labeling in CLD between 40h and 88h of N
deprivation is roughly correlated with the increase in labeling in βC-plastoglobuli (53,000cpm and 41,000cpm, respectively).

In order to test if βC-plastoglobuli TAG may be synthesized from degradation of pre-formed chloroplast (or other membrane) polar lipids, we designed another 14C-PA pulse-labeling experiment, in which D. bardawil cells were incubated with 14C-PA in N-sufficient (complete) growth medium before entering N-deprivation, and then residual 14C-PA was removed by washing and dilution with access unlabeled PA. Samples of cells taken at 0h or 48h after entering N-deprivation, were disrupted and fractionated into membrane and lipid droplets and their 14C contents were analyzed.

As shown in Fig. 5 and in Table 2, the 14C distribution in CLD and in βC-plastoglobuli is very different from the de novo synthesis experiment (Fig. 4B). Over a third of the total 14C in all lipid fractions was recovered in the βC-plastoglobuli fraction, compared to 5% in the de novo synthesis after 40h, whereas the CLD fraction contained about 20% of the total 14C, compared to over 80% in the de novo synthesis experiment. The number of counts recovered from the βC-plastoglobuli fraction was similar to the decrease in counts in the chloroplast membrane fraction (285,000cpm compared to 260,000cpm, respectively), constituting about 50% of the total 16C fatty acids in polar chloroplasmalast membrane lipids. Also the recovery of 14C labeled TAG in CLD was close to 40% of the microsomal 14C contents at the onset of N deprivation, suggesting significant utilization of 16C fatty acids for production of TAG from degradation of polar membrane lipids in both fractions, particularly of βC-plastoglobuli. The larger decrease in microsomal 14C contents during 48h of N-deprivation, is consistent with the faster lipid turnover in microsomal membranes.

**Protein composition**

Protein analysis of the purified droplets was conducted by SDS-PAGE (Fig. 6A). The analysis revealed different protein patterns of the two lipid droplet pools. The cytoplasmatic fraction was enriched with a 30 kDa protein which was identified in our previous study on Dunalilla (Davidi et al. 2012) and termed major lipid droplet protein (MLDP). The chloroplastic fraction was enriched with a 36-38 kDa protein/s, composed
of at least two components, which was previously characterized in our group (Katz et al. 1995) and termed carotene globule protein (CGP).

Western blot analysis of the purified CLD and βC-plastoglobuli with anti-MLDP and anti-CGP antibodies was conducted (Fig. 6B). For this purpose anti-MLDP and anti-CGP specific rabbits polyclonal antibodies were utilized (Katz et al. 1995, Davidi et al. 2012). The results demonstrate the specificity of anti-MLDP antibodies to the CLD and of anti-CGP antibodies to the chloroplast βC-plastoglobuli. No cross-reaction with anti-Rubisco antibodies, a common chloroplast contaminant, could be detected in the purified lipid droplets proteins (Fig. 6B, upper lane) strengthening the conclusion that the two lipid pools are pure.

**CGP structure**

CGP has been isolated and cloned in our lab (Shoham 1995) and found to be a nuclear encoded 378 amino acid protein with 42.5 kDa. The best prediction for the cleavage site of the transit peptide is after Ala67, leaving a mature protein of a calculated MW of about 36 kDa, close to the estimated MW from SDS-PAGE. Sequence analysis of CGP and MLDP revealed that these two proteins share only very limited sequence homology. The MLDP is part of new group of green algal proteins that do not resemble any other known protein (Davidi et al. 2012). CGP is a proline-rich protein with 37 proline-residues (Fig. 7A). A comprehensive BLAST search that was carried out in all EST databases of *D. salina* Teodoresco (GenBank and JGI), identified at least four different CGP genes (Fig. 7B). A BLAST search to CGP in the National Center for Biological Information database of non-redundant proteins identified a low level of homology to SOUL-domain-containing protein, also called SOUL heme-binding proteins. Multiple alignment of CGP with ortholog proteins is available in Fig. S2. A Phylogenetic tree of algal and Arabidopsis SOUL heme-binding proteins, including CGP is presented in Fig. 7C. MLDP was added to the tree for reference. Notably, SOUL heme-binding proteins are also present in the *C. reinhardtii* eyespot (Schmidt et al. 2006, Wagner et al. 2008) and where also detected in Arabidopsis plastoglobules (Ytterberg et al. 2006, Lundquist et al. 2012). Kyte and Doolittle (1982) hydropathy plots and Grand average of hydropathy values (GRAVY) (Fig. S3A) revealed that CGP and MLDP differ also in their secondary structure: CGP is
more hydrophilic (-0.58), in comparison to MLDP (-0.053). The secondary structure analysis (Guermeur et al. 1999) (Fig. S3B) shows that MLDP is highly structured protein, consisting mostly of α-helices, whereas CGP is mostly unstructured protein, probably due to its high content of proline residues.

Using software for prediction of post-translational modifications revealed no putative conserved sites for palmitoylation, prenylation, myristoylation or GPI-anchor for the D. bardawil MLDP (Davidi et al. 2012), ruling out the possibility of a post-translational lipid anchor. However, CGP has one predicted palmitoylation site on Cys13, which probably localizes in the transit peptide.

**Cellular localization of MLDP and CGP**

Immunoelectron microscopy was used in order to determine the cellular localization of MLDP and CGP in D. bardawil cells. For the EM analysis, cells were fixed, cryo-protected in sucrose, frozen, cryosectioned, and immunolabeled first with anti-MLDP or anti-CGP antibody, respectively, followed by gold-conjugated goat anti-rabbit IgG. A comparison of control cells (Fig. 8A,D) with cells deprived of N for 6 days, shows large accumulation of starch granules and also of lipid droplets in the cytoplasm and chloroplast (Fig. 8B,E). As shown in Fig. 8C (and Fig. S4), MLDP is concentrated at the periphery of the CLD, whereas CGP was localized at the periphery of the chloroplast βC-plastoglobuli (Fig. 8F). Interestingly, MLDP seems to be excluded from the contact area with the chloroplast membrane (Fig. 8C, Fig. S4-B, see also Fig. 12 and discussion below). No label could be observed in other cellular compartments or in control cells. Also, we could not observe labeling of MLDP on βC-plastoglobuli or CGP on the CLD. These results and the Western blot analysis of purified lipid droplets described in Fig. 8B, justify the utilization of MLDP and CGP as specific markers for localization of CLD and βC-plastoglobuli, respectively.

**Time course of TAG, β-carotene, CGP and MLDP formation**

In order to clarify if the inductions of the proteins CGP and MLDP are correlated with the accumulation of TAG and of β-carotene, we followed in parallel the mRNA and protein levels of CGP and MLDP, TAG and β-carotene levels, during 7-10 days of nitrogen
deprivation. *D. bardawil* cells were grown in complete or in N-deprived media for 10 days and samples for RNA, protein extraction, TAG and β-carotene were collected at indicated times. Semi-quantitative PCR of MLDP and CGP mRNA expression (Fig. 9A), revealed a faster initial expression of MLDP during the first 24 h and maximal protein levels after 4 and 7 days of N deprivation for MLDP and CGP, respectively (Fig. 9B).

The increase in MLDP and CGP are roughly correlated with the increase in TAG and β-carotene levels, respectively: The accumulation of TAG, determined by TLC analysis, reached maximal level after about 6 days (Fig. 10A), whereas the level of β-carotene increased in two kinetic phases, first between 0-5 days and second between 6-10 days (Fig. 10B). In contrast, control cells did not accumulate either TAG or β-carotene. The chlorophyll content of N-deprived cells decreased by more than 50% after 4 days of N-deprivation in comparison to control cells (Fig. 10C).

**Origin of chloroplastic βC-plastoglobuli, as visualized by Electron Microscopy**

Control and N-deprived *D. bardawil* cells were visualized using different electron microscopy methods. Cryo-SEM of control cells (Fig. 11A) reveals oval cell with cup-shaped chloroplast and small numbers of CLD. After 2 days of N-deprivation, *D. bardawil* cells increase in size and the number and diameter of CLD significantly increase (Fig. 11B-D). Within the chloroplast, arrays of small βC-plastoglobuli (less than 200 µm in diameter) can also be observed (indicated by white arrows, Fig. 11C,D).

In order to monitor the dynamics of CLD and βC-plastoglobuli accumulation during N-deprivation, TEM images of *D. barawil* cells were taken at 0, 1, 3 and 7 days of N-deprivation (Figs. S5-S7). In order to obtain a clearer view of the initial stage of chloroplast βC-plastoglobuli formation, electron tomography imaging was performed on *D. bardawil* cell after 1 day of N-deprivation (Fig. 12B-D). Control cells have a highly ordered cup-shaped chloroplast, that occupies most of the cell volume, with small cytoplasmatic intrusions. Hardly any starch or lipid droplets are visible (Fig. 12A). Already after one day of N-deprivation, there is a radical change in cell morphology: the chloroplast is filled with starch granules and has fewer thylakoid membranes and the cytoplasm contains several large lipid droplets, of about 500 µm in diameter (Figs. 12B,
Notably, many CLD seem to be closely associated with the outer chloroplast surface and in some cases appear almost engulfed by the chloroplast (Figs. S5E,F). Within the chloroplast appear arrays of smaller droplets (of about 100 µm, marked by red arrows), bordered by the chloroplast envelope membranes and by the outermost thylakoid membranes. In some cases, these small droplets appear tightly squeezed to the large CLD, separated by a nonuniformly-stained chloroplast envelope membrane (Fig. 12D). A movie of the tomography reconstruction that best represents our findings is available on Movie S1. This morphology was observed in dozens of cells and was also observed in cells after 2 and 3 days of N-deprivation, although at a lower frequency (Fig. S6). After 7 days of deprivation, the cells appear swollen, the cytoplasm contains only a few lipid droplets whereas the chloroplast contains large amounts of starch granules and large number of βC-plastoglobuli, ranging in size from 200-500 µm, and localized mostly at the outer periphery (Fig. S7A,B). Some blank spaces and tears to chloroplast membranes in the vicinity of starch granules can be seen, possibly resulting from damage by starch granules during centrifugation.
DISCUSSION

In this work we tried to clarify the interrelations between two neutral lipid droplets in *Dunaliella bardawil*, CLD and βC-plastoglobuli, the latter being unique to this species. This was achieved by improved isolation of the two lipid pools combined with lipid composition analyses, biochemical analyses of lipid biosynthesis and electron microscopy.

The isolated βC-plastoglobuli and CLD have similar TAG molecular species and fatty acid compositions (in both total fatty acid, fatty acid at *sn*-1+3 and *sn*-2 positions), but differ in the presence or absence of β-carotene, in their polar lipid compositions and in their major lipid-associated proteins, as will be detailed below.

A major question that concerned us in this study is the origin of TAG in CLD and in βC-plastoglobuli in *D. bardawil*. The finding that TAG *sn*-2 fatty acids in both CLD and in βC-plastoglobuli are a mixture of 16C and 18C, give no clear indication for a cytoplasmatic origin at the ER, which shows a clear 18C bias both in higher plants (Heinz and Roughan 1983) and in algae including Dunaliella (Ha and Thompson 1991), nor for a chloroplastic origin, which has a clear 16C bias ((Cho and Thompson 1987a), Table S1), although the cytoplasmatic TAG fatty acid composition is much closer to that of PC than to chloroplast membrane lipids (Table 1). A similar *sn*-2 fatty acid composition was reported previously for both cytoplasmatic and chloroplast associated TAG in *D. salina* (Ha and Thompson 1991). These results contrast the finding in *C. reinhardtii*, were TAG *sn*-2 fatty acids are mostly 16C, indicating that they are made in the chloroplast (Fan et al. 2011). The applicability of the *sn*-2 fatty acids criterion for the origin of TAG is questionable, because it is now well established that the transfer of fatty acid acyl groups into TAG proceeds through acyl editing by specific enzymes with different fatty acid specificities. For example, in *C. reinhardtii*, fatty acid acyl transfer from MGDG to TAG, which has a preference for *sn*-1 18:1 fatty acids (Li et al. 2012), may not alter *sn*-2 fatty acid in TAG, whereas PC editing shows a different fatty acid specificity (Bates et al. 2009) and PES1 and PES2, which catalyze hydrolysis of fatty acids from chloroplast membrane lipids and their transacylation to TAG (Lippold et al. 2012), show poor specificity for fatty acids, and may scramble *sn*-2 fatty acid composition in TAG. The
finding that PA incorporation into polar lipids precedes the incorporation into TAG (Fig. 4B) suggests that acyl editing is probably part of TAG biosynthesis also in *D. bardawil*.

The origin of TAG in *D. bardawil* lipid droplets is not clear. The very similar fatty acid composition (Fig. 3) and the similar TAG molecular species (Fig. 2), strongly suggest a common origin. In contrast, according to the $^{14}$C-PA pulse-labeling experiments, the two TAG pools seem to have different origins: In the first 40-48h of N deprivation, most CLD TAG seem to be produced by direct incorporation of newly-synthetized fatty acids (Fig. 4B) and a smaller part from pre-formed polar lipids (Fig. 5), whereas for βC-plastoglobuli TAG, significant part of fatty acids, amounting to 50% of 16C fatty acids in chloroplast membranes, seem to be released from degraded chloroplast membrane polar lipids (Figs. 5, Table 2). However, chloroplast membrane lipids degradation cannot provide most TAG fatty acids simply because the amount of total membrane fatty acid contents in *D. bardawil* is only around 20% of that of the fatty acid TAG content (see for example Fig. 1B, lane 2).

The only difference in fatty acid composition between the two TAG pools is the higher contents of 16:0 FA in βC-plastoglobules, particularly at the *sn1+3* position (Fig. 3). Also the only significant differences in the TAG molecular species compositions in the two lipid pools are in the low molecular weight species, which are enriched in 16C fatty acids (peaks between 9-17min, Fig. 2). The reason for these differences may be the higher contribution of 16C fatty acids derived from degradation of chloroplast membrane lipids and incorporated into TAG in plastoglobules, consistent with the $^{14}$C-palmitic acid pulse-labeling experiments (Fig. 5, Table 2) during the first 48h of N-deprivation.

The time-course of TAG accumulation in the two lipid pools, and the $^{14}$C-PA pulse-labeling experiments (Fig. 4A,B) suggest that the major biosynthesis of CLD takes place in the first 48h of N deprivation/high light stress, whereas the accumulation of βC-plastoglobuli continues for 7 days. Similarly, EM pictures revealed a progressive increase in plastoglobuli and a decrease in CLD from day 1 to day 7 of N deprivation (Figs. S5-S7). The correlation between the increase in βC-plastoglobuli TAG and the decrease in CLD TAG after 48h of stress, combined with their similar TAG composition, is
consistent with the idea of lipid transfer from the cytoplasm into the chloroplast for the formation of βC-plastoglobuli.

A possible mechanism that may lead to a similar TAG composition of the cytoplasmatic and chloroplastic lipid droplets is a dynamic transfer of fatty acids or of TAG molecules from the CLD to the βC-plastoglobules through the chloroplast envelope membranes. This possibility is consistent with the close proximity of CLD and of βC-plastoglobules to chloroplast envelope membranes observed by electron microscopy (Figs S5-S7, Fig. 12). At present we do not have any biochemical evidence for such a fatty acid or TAG transfer, but we have identified in a proteomic analysis lipases and acyl transferases in the CLD proteome, and of three phytyl ester synthases (PES) homologs in the βC-plastoglobuli proteome, which could be involved in such a mechanism (Davidi et al, unpublished results). Such a dynamic lipid transfer may explain also why at later stages of N deprivation/high light stress, as β-carotene biosynthesis proceeds within the chloroplast, increasing part of TAG is mobilized from the cytoplasm into the chloroplast to generate more plastoglobules for incorporation of the pigment.

In summary, we propose that at the early stages of stress, CLD TAG is produced at the ER, mostly by incorporation of new-synthetized fatty acids at the ER and the process may involve fatty acid shuttling through PC or another polar membrane lipid. Subsequently, plastoglobuli start to be created within the chloroplast from TAG made in part from fatty acids hydrolyzed from chloroplast membrane lipids and in part from fatty acids or TAG molecules derived from cytoplasmic droplets.

It is interesting to compare the similarities and differences between D. bardawil and between C. reinhardtii: starchless mutants of C. reinhardtii also accumulate lipid droplets both in the cytoplasm and in the chloroplast, but the chloroplastic droplets resemble in size those in the cytoplasm, do not contain β-carotene and they have not been characterized. Interestingly, the cytoplasmatic lipid droplets in these mutants were observed to adhere to the outer chloroplast envelope (Goodson et al. 2011) similar to the observation in D. bardawil. Moreover, as already discussed, the fatty acids at the sn-2 position in C. reinhardtii TAG is primarily C16, suggesting that it is made in the chloroplast by the same enzyme that produces chloroplast membrane lipids whereas in D.
bardawil it is a mixed C16/C18 composition, differing from chloroplast polar lipids, indicating that most TAG are not produced in the chloroplast.

Nevertheless, there are several clear biochemical and physiological differences between these species which could explain this difference: First, Dunaliella is a strict photoautotroph, in contrast to C. reinhardtii which is a heterotroph, and its response to N-deprivation is less severe than reported in C. reinhardtii (Moellering and Benning 2010, Cakmak et al. 2012, Msanne et al. 2012), and the N-depleted Dunaliella cells maintain high photosynthetic activity (50-100% of the activity of control cells after 48h of N-deprivation on a per-cell basis). Second, Dunaliella contains phosphatidyl choline (PC) (Evans et al. 1982, Fried et al. 1982, Evans and Kates 1984, Al-Hasan et al. 1987, Peeler et al. 1989) whereas Chlamydomonas lacks PC (Giroud et al. 1988). Since PC is an essential component in the transfer of fatty acids between the cytoplasm and the chloroplast (Li-Beisson et al. 2010), its absence in Chlamydomonas may result in a different subcellular trafficking of lipids than in plants and other algae (Li et al. 2012). Third, Chlamydomonas does not synthesize massive amounts of β-carotene, which has to be deposited in TAG droplets as is the case in D. bardawil under N deprivation. It is possible, therefore, that D. bardawil evolved a unique mechanism for TAG accumulation in the chloroplast to enable the deposition of β-carotene in this lipid droplets.

The finding that the polar lipid compositions in CLD and in βC-plastoglobuli differ (Fig. 1C), suggests that the origins of the polar lipid monolayers of these lipid droplets are different. CLD polar lipids most probably originate in the ER. In contrast, the similarity of βC-plastoglobuli polar lipids to thylakoid/stroma membrane galactolipids (Fig. 1C), suggest that they originate from chloroplast membranes. Indeed, our electron microscopy images, indicate close contacts between βC-plastoglobuli and thylakoid/inner envelope membranes (Figs. 12,S5-S7), consistent with a dynamic exchange of polar lipids between these organelles. Similar close contacts between plant plastoglobuli and chloroplast envelope and thylakoid membranes have been previously reported (Austin et al. 2006). This finding is consistent with the suggested function of lipid droplets in inter-organellar lipid transfer (Liu et al. 2007, Zehmer et al. 2009).
The βC-plastoglobuli and the CLD are characterized by different major lipid-associated proteins (Figs. 6,9), CGP (Katz et al. 1995) and MLDP (Davidi et al. 2012) in βC-plastoglobuli and in CLD, respectively. The CGP protein, which has been previously characterized at the protein level (Katz et al. 1995), differs in sequence from green algae MLDPs suggesting that it has a different origin.

We have shown in this work that formation of two lipid bodies in Dunaliella bardawil is a complex event that involves both hydrolysis of membrane lipids and de-novo synthesis and probably also of trans-membrane lipid transfer into the chloroplast. Even though the molecular details of this mechanism are still not clear, we believe that the present study and our following proteomic analysis of these lipid droplets, will contribute to the presently poor understanding of the biogenesis of lipid droplet in plants and in microalgae.
METHODS

Strain and growth condition

*Dunaliella bardawil* is an isolated species (Ben-Amotz et al. 1989) deposited at the American Type Culture Collection (ATCC), Rockwille, MD, USA, No. 30861. Culturing conditions and growth media were as previously described (Ben-Amotz et al. 1989). The media were supplemented with 2 M NaCl. To induce N limitation, mid-log phase cells were centrifuged for 5 min at 5,000 g, washed once with growth medium lacking KNO$_3$ (-N medium), and resuspended in -N medium. The light intensity was raised from 150 to 300 $\mu$Em$^{-2}$ s$^{-1}$ for growth in complete and in N-deprived media, respectively. Cells were counted with the automated cell counter Cellometer (Nexelom Bioscience LLC, Lawrence, MA, USA).

Lipid droplets isolation

Isolation of lipid droplets was performed essentially as previously described with some modifications (Jiménez and Pick 1994, Katz et al. 1995). In brief, algae after 2 days of N-deprivation were washed, osmotically-lyzed and centrifuged at low speed for separation of the cytoplasmatic and chloroplastic fractions. Chloroplasts were passed twice through a 25 ml syringe (1.5 inches, gauge 21) and centrifuged for 15 min at 5000 g. This treatment was found to release the majority of CLD. Chloroplasts were pelleted by centrifugation, washed twice and lyzed by sonication. Purification of CLD and of $\beta$C-plastoglobuli was performed by floatation on a discontinuous sucrose gradient consisting of three layers (30% sucrose containing the droplet fraction; 15% sucrose and 5% sucrose, all containing 10 mM Tris–HC1, pH 8) and centrifugation at 75,000 g for 2 h. The crude lipid droplets, recovered from the top fraction, were collected and re-purified by floatation on a second sucrose gradient. The purified lipid droplets were collected from the top and kept frozen in liquid nitrogen.

Cloning the CGP gene

This part of the work was performed during 1993-95 by T. Shoham and I. Gokhman (Shoham 1995). CGP (NCBI accession no. JX646677) was cloned from a cDNA library
of high light-induced *D. bardawil* cells, with the aid of anti-CGP polyclonal antibodies. In brief, *D. bardawil* culture in complete growth medium (Fisher et al. 1994) was exposed for 36 h to high light intensity of 1,600 μE m⁻² s⁻¹ in a Warburg temperature-controlled shaker. RNA was isolated using Tri Reagent. cDNA to poly(A⁺) mRNA was synthesized and cloned into the gamma Uni-ZAP XR expression vector (Promega, Madison, WI) as described (Fisher et al. 1996). A phage clone was isolated and a screen with anti-CGP antibodies (Katz et al. 1995) was subsequently shown to include a partial 3’ end of the CGP gene. The full-length cDNA was isolated by the 5’-RACE procedure (CLONTECH Laboratories, Palo Alto, CA). To obtain the corresponding genomic DNA sequences, primers based on cDNA sequences were used with templates of genomic DNA digested by Sau3A, HaeII, TaqI or MspI, followed by fragment circularization by ligase, in several consecutive steps of inverted PCR amplification and subsequent cloning.

**Generation of anti-MLDP and anti-CGP antibodies**

Polyclonal antibodies against CGP and against MLDP were raised in rabbits as described before (Katz et al. 1995, Davidi et al. 2012).

**Gel electrophoresis and Western analysis**

Protein extracts from total cells were generated from pellets of culture samples containing 2x10⁶ cells resuspended in 0.1 ml of bursting solution (5 mM HEPES, pH7.5, 5 mM γ-caproic acid, 1 mM benzamidine, 1 mM PMSF), followed by the addition of 50 µl of sodium dodecyl sulfate (SDS) sample buffer. Proteins from isolated lipid droplets and pure chloroplasts (purified as described in section “Cytoplasmatic and chloroplast TAG pools analysis” for chloroplast fraction at 0 time point) were precipitated in 80% acetone (Davidi et al. 2012). Proteins were analyzed by 12% SDS–PAGE and stained with Coomassie blue or immunoblotted with anti-MLDP, anti-CGP or anti-Rubisco (a gift from Prof. M. Edelmann, The Weizmann Institute of Science) antibodies. Dilution factors of anti-MLDP, anti-CGP and anti-Rubisco were 1:1,000, 1:2,000 and 1:20,000, respectively.

**cDNA preparation**
Cells pre-cultured for 48h in complete growth medium were collected by centrifugation, washed once and cultured in N-deficient medium. After 0, 6, 12, 24, 32, 48, 72, 96, 168 h, samples of 10ml containing 1-2x10^7 cells, were taken for RNA isolation. The cells were collected by centrifugation and immediately flash-frozen in liquid nitrogen and stored at -80°C for further use. Total RNA was isolated using tri-reagent procedure according to manufacture protocol (Molecular Research Center, Cincinnati, OH, USA). Independent RNA isolations were conducted for each growth period. Template cDNA was synthesized using 0.1 µg total RNA in a total volume of 20 µl, using Superscript kit (Invitrogen, Carlsbad, CA, USA). MLDP and CGP gene expression of N-deprived cells was examined using MLDP gene primers (F: 5’-GAAGCCATACTCAGGGAAC, R: 5’-CAGACTTTTTTAAGCAACG), CGP primers (F: 5’-CCCCAGCAGCTGACGCCC, R: 5’-TGGTCAGGCACCACGGG) and was compared with the expression of 18S control gene.

**Bioinformatics analysis**

Sequences were routinely searched using BLAST (Altschul et al. 1997). Sequences were aligned using the CLUSTALW multiple sequence alignment program (Thompson et al. 1994) at [http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/). Hydropathy plots were generated employing the Kyte–Doolittle algorithm (Kyte and Doolittle 1982), using the Prot Scale program at [http://www.expasy.ch/tools/protscale.html](http://www.expasy.ch/tools/protscale.html). The value G in each graph is the grand average of hydropathy value (GRAVY) for each protein and was calculated using the GRAVY calculator program at [http://www.gravy.laborfrust.de/](http://www.gravy.laborfrust.de/). Post-translational modifications were screened using online software: Palmitoylation: [http://csspalm.biocuckoo.org/online.php](http://csspalm.biocuckoo.org/online.php), prenylation: [http://mendel.imp.ac.at/sat/PrePS/index.html](http://mendel.imp.ac.at/sat/PrePS/index.html), N-myristoylation: [http://mendel.imp.ac.at/myristate/SUPLpredictor.htm](http://mendel.imp.ac.at/myristate/SUPLpredictor.htm), GPI-anchor: [http://gpi.unibe.ch/](http://gpi.unibe.ch/).

Secondary structure predictions were obtained using secondary structure consensus prediction program [http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html](http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html).

**Lipid analysis**
For TAG quantification two methods were used: Nile red fluorescence enhancement in live cells and thin-layer chromatography (TLC) in cell extracts. Nile red was added to live cells (5x10^5 cells/ml) in the fresh growth medium or purified lipid droplets (normalized to cell concentration of 5x10^5 cells/ml), at a final concentration of 1 µM. Nile red fluorescence emission was measured after 3–10 min, when the signal reached maximal and steady level. The parameters of Nile red fluorescence measurement: excitation at 488 nm, emission maxima at 580-590 nm. The increase in fluorescence emission was utilized to express the relative increase in TAG level. For the TLC analysis, lipids were extracted from 10^7 cells as previously described (Khozin-Goldberg et al. 2005), separated on TLC Silica gel 60 aluminum sheets (Merck, Darmstadt, Germany) in n-hexane: diethyl-ether: acetate (85:15:1) solution. Phosphatidyl choline (PC) was isolated from microsomal membrane lipid extracts by separation on TLC plates developed vertically in chlorophorm:methanol:water (65:25:4) and horizontally in chlorophorm:methanol:isopropylamine: ammonium hydroxide (65:35:0.5:5). Finally lipid detection was performed by 5 min exposure to iodine vapor. For quantification, densitometric analysis of spots was compared with standards of 0.5–2 µg triolein. For polar lipids detection TLC was performed as described with exception that lipids were separated on chloroform: methanol: DDW (65:25:4) and with 1 µg diacylglycerol (DAG) as standard. For glactolipid detection TLC plates were sprayed first with 0.5% α-naphtol in 50% methanol (in DDW) next with 95% sulfuric acid and heated for 20 min at 110°C. Galactolipids appear as purple spots while other polar lipids appear as yellow spots.

Chlorophyll and carotenoid contents of *D. bardawil* control and N-deprived cells or of purified lipid droplets were determined in lipid extracts from the absorbance at 663 and 480 nm, respectively (Shaish et al. 1990). For GC/MS analysis of fatty acid composition and total lipid contents, lipids were extracted from 50 ml cells (5X10^6 cells/ml) and 2 ml of purified droplets. Total fatty acid methyl esters (FAME) were prepared through acid hydrolysis in methanol and then analyzed by GC/MS (Bigogno et al. 2002) performed by Dr. Inna Khozin-Goldberg, Prof. Zvi Cohen’s lab in Ben-Gurion University). Three replicates or each sample were examined.
The $sn$-1+3 and $sn$-2 distributions of fatty acids in TAG from purified lipid droplets were determined using Rhizopus lipase (Sigma) as previously described (Fischer et al. 1973) with minor modifications. The TAG were extracted and separated on TLC as described above. The band containing the TAG was scrapped off, dissolved in n-hexane and dried. Tris-HCl buffer (40 mM, pH 7.2) (0.1 mL) containing 50 mM sodium borate (to reduce positional migration of fatty acids) was added to the dried lipid sample and the mixture was sonicated for 10 min. One hundred µL of lipase (100 units) was added to the mixture and incubated at room temperature for 10 min with continuous shaking. The reaction was terminated by addition of 1 mL of methanol, and lipids were extracted with methanol:chloroform:DDW (1:1:1). The lipids were resolved into free fatty acids and monoacylglycerol (MAG) by TLC in n-hexane:diethylether (20:80) and after visualizing the lipids with Iodine the free fatty acid and MAG bands were scraped off and extracted with chloroform:methanol (1:1, v/v). Fatty acid methyl esters (FAME) were prepared through acid hydrolysis in methanol and then analyzed by GC/MS (performed by Dr. Inna Khozin-Goldberg, Prof. Zvi Cohen’s lab in Ben-Gurion University). Three replicates of each sample were examined.

The $sn$-1 and $sn$-2 of microsomal and chloroplast membrane lipids were determined by exposure to Rhizopus lipase or to phospholipase A2 (Sigma) and analysis of release compared to total fatty acids. Microsomal and chloroplast membranes were isolated as previously described (Jiménez and Pick 1994, Bates et al. 2009). Tris-HCl buffer (40 mM, pH 7.2) (900µl) and diethylether (1.5 ml) were added to the samples together with twenty µl of enzyme for 30 min incubation with shaking in room temperature. The reaction ended by evaporation of the diethylether. Lipids were extracted, separated and fatty acids were analyzed as described above.

Cytoplasmatic and chloroplast TAG pools analysis

For TAG pools analysis *D. bardawil* cells were grown at high light in N-deficient medium and samples were collected at 0, 12, 24, 48, 72, 144 h. The cells were washed and lysed osmotically followed by syringe treatment and centrifugation to separate cytoplasmatic and βC-plastoglobuli lipid droplets. The sup was separated as cytoplasmatic fraction. The chloroplasts were washed and collected as chloroplast...
fraction. Both fractions were subjected to lipid extraction and the lipids were analyzed by TLC as described above. Three repeats were made for each sample.

**Incorporation of 14C-palmitic acid into *D. bardawil* lipids**

To estimate the distribution of 14C in the different fractions two protocols were used: In one, 5 µCi of 14C-PA (1 µM final concentration) was added to 300ml *D. bardawil* culture that has been induced for 12 h in high light at N-deficient medium. After 4h, the cells were washed, diluted into fresh N-deprived medium supplemented with 250 µM unlabeled palmitic acid. Samples were collected after 0, 24 h, 48 h or 72h (total 16, 40h, 64h or 88h of N deprivation, respectively). Cytoplasmatic and chloroplastic TAG was separated as described above. In the second, cells were labeled for 5h in complete (N sufficient) medium before transfer to N deprivation. The incubation with 14C-PA was terminated by washing and dilution with 250-fold unlabeled PA and the cell were transferred for 48h to N deprivation at high light. Cell samples were taken before and 48h after N deprivation for lysis and fractionation into cytoplasmic membranes, chloroplast membranes, cytoplasmic droplets and βC-plastoglobuli as described above. 14C in each fraction was counted. The extracted lipids from each fraction were also separated on TLC (as described above) and the TAG bands were scraped off and dissolved in 1 ml n-hexane and 10 ml scintillation solution. The 14C radiolabelling was measured using a Tri-carb liquid scintillation counter (PerkinElmer, U.S.A.).

**Lipid analysis by High-Performance Liquid Chromatography (HPLC)**

The lipid profile of *D. bardawil* purified lipid droplets was determined by Reverse phase HPLC – Waters e2695 (Waters Corporation, MA, USA), using a 2.7 µm Halo C8 4.6x150 mm column (Advanced Materials Technology, Wilmington, DE, USA). For Tag analysis The lipid extracts were dissolved in acetonitrile:isopropanol:hexane (2:2:1) and were seperated with a mobile phase of acetonitrile:isopropanol gradient (0-30%), at a flow rate of 1.2ml/min, at 40ºC. For fatty acid analysis the lipid extracts were dissolved in chloroform:methanol (1:1) and were separated with a mobile phase methanol:water:acetic acid (75:25:1) to acetonitrile:methanol:tetrahidrofuran:acetic acid (50:37.5:12.5:1) gradient (0-30%), at flow rate of 0.8 ml/min at 40ºC.
The lipids were detected using Corona Charged Aerosol Detector (CAD) (ESA Biosciences Inc., Chelmsford, MA, USA). TAG were identified and quantified by comparison to TAG standards: glyceryltrimyristilate (C14:0), glyceryltripalmitin (C16:0), glyceryltripalmitolein (C16:1), glyceryltristearate (C18:0), glyceryltriblend (C18:1) (Sigma-Aldrich, St. Louis, MO, USA). β-carotene and phytoene were detected using same system with Photodiode-array detector (Waters Corp., Milford, MA, USA) and were identified by their absorbance spectra at 488 nm and 286 nm, respectively.

**Electron Microscopic Techniques**

**1. Gold immunolabeling**

Samples were prepared according to the Tokuyasu method (Tokuyasu 1973). In brief: Algae cultured for 0, 1 or 6 days in N-deprived medium, were fixed in 2% glutaraldehyde and 0.1% acroleine in growth medium for 1 h. Fixed cells were washed in growth medium and embedded in 10% gelatin in water. The gelatin was hardened at 4ºC, post fixed overnight with the fixation medium described above, washed in cacodylate buffer and cut into 0.5 mm pieces. The embedded cell pieces were then incubated overnight in 2.3 M sucrose, frozen in liquid nitrogen and cut into 80-90 nm slices with an EM FC6 cryo-ultramicrotome (Leica Microsystems, Vienna, Austria). After 30 minutes incubation in blocking solution (0.5% gelatin, 0.5% BSA, 0.2% glycine, 0.1% Tween-20 in PBS), slices were incubated for 2 hours with anti-MLDP or anti-CGP polyclonal rabbit antibodies in blocking solution (1:150 dilution). Slices were washed with PBS containing 0.2% glycine, incubated for 30 minutes in 10 nm colloidal-gold-conjugated goat anti-rabbit antibodies (Electron Microscopy Sciences, Hatfield, PA, USA) diluted 1:20 in blocking solution and washed in PBS and bi-distilled water. Labeled sections were stained with 2% uranyl acetate, embedded in methylcellulose and observed in a Tecnai Spirit Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV. Images were recorded using an Eagle 2K x 2K CCD camera (FE, Eindhoven, The Netherlands).

**2. Cryo-SEM**
Algae cultured either in complete or in N-deprived media for 48 h, were centrifuged and a drop of pellet was sandwiched between two aluminum platelets with depth of 25 µm each. The sample was then high-pressure frozen in utilizing a HPM010 high-pressure freezing machine (Bal-Tec, Liechtenstein). The frozen samples were mounted on a holder and transferred to a BAF 60 freeze-fracture device (Leica Microsystems, Vienna, Austria) using a VCT 100 Vacuum Cryo-Transfer device (Leica Microsystems). After fracturing and etching at a temperature of -105°C for 5 minutes, samples were coated with 4 nm Pt/C by double-axis rotary shadowing. Samples were transferred to an Ultra 55 SEM (Zeiss, Germany) using a VCT 100 and were observed using a secondary electrons in-lens detector at an acceleration voltage of 2 kV at a temperature of -120°C.

3. TEM observation and STEM tomography

Algae cultured in complete or in N-deprived media for 1, 2, 3 or 7 days were fixed in 2% glutaraldehyde and 0.1% acrolein in cacodylate buffer for 1h at room temperature and for 15 h at 4°C. The fixed samples were centrifuges and the pellet was embedded in 3.4% agarose (BD, Franklin Lakes, NJ USA), dehydrated in ethanol and infiltrated in a series of increasing concentration of Epon (Electron Microscopy Sciences, Hatfield, PA, USA). After polymerization at 60°C, 60-80 nm sections were stained with uranyl acetate and lead citrate and examined in a Tecnai T12 electron microscope (FEI, Eindhoven, the Netherlands) operating at 120 kV. Images were recorded with a F224HD 2k x 2k CCD camera (TVIPS, Gauting, Germany).

For STEM tomography, blocks were prepared as above and sectioned to a thickness of approximately 470 nm, double-stained with uranyl acetate and lead citrate, decorated on both sides with 12nm colloidal gold markers. Sections were imaged in a scanning-transmission mode using a bright-field detector (GatanInc, Pleasanton, CA, USA) in a Tecnai F20 transmission electron microscope (FEI, Eindhoven, The Netherlands). Dual-axis tilt series were acquired at 1.5° intervals over a range of ± 64°, using Explore3D tomography acquisition software (FEI, Eindhoven, The Netherlands). Alignment and 3D reconstruction were performed with IMOD image-processing package (Kremer et al. 1996).
Figures legend:

**Figure 1.** TLC and Nile red analysis of isolated lipid droplets.
(A) Nile red Fluorescence emission spectra of *D. bardawil* control cells (DB + N), N-deprived cells (DB – N) and purified CLD and βC-plastoglobuli.
(B) TLC analysis of neutral lipids in lipid extracts from *D. bardawil* control cells (lane 1), N-deprived cells (lane 2), purified CLD (lane 3), purified βC-plastoglobuli (lane 4) and 1µg of triolein standard (lane 5).
(C) TLC analysis of polar lipids in extracts from *D. bardawil* chloroplasts (lane 1), 1µg of diacylglyceride standard (lane 2), purified βC-plastoglobuli (lane 3) and purified CLD (lane 4) after iodine staining (left) and galactolipid staining (right). The major polar lipids of CLD and βC-plastoglobuli are marked in arrowheads.

TLC and Nile red analysis were conducted in samples normalized to equal cell number.

**Figure 2.** HPLC chromatogram of lipid extracts from cytoplasmic droplets and βC-plastoglobuli. The chromatogram represents lipid extraction fractions from cytoplasmic droplets (blue) and βC-plastoglobuli (black) and was compared to TAG standards. βC: β-Carotene, MS-9: TAG peak which was collected and send to MS and FAME analysis.

**Figure 3.** Fatty acid composition and positional analysis of *D. bardawil* isolated lipid droplets. (A) Total fatty acid composition analysis of *D. bardawil* control (DB+N) and N-deprived (-N) cells together with isolated lipid droplets (CLD and βC-plastoglobuli). Fatty acid compositions of TAG sn-1+3 (B) or sn-2 (C) positions from CLD and βC-plastoglobuli are shown. The values shown represent means and SD of 3-5 replicates.

**Figure 4.** Time course of formation of CLD and of βC-plastoglobuli TAG.
(A) Total TAG contents, *D. bardawil* cells were grown in N-deficient media. At each indicated time the cytoplasm and chloroplast fractions were separated and TAG were extracted from each fraction. The values shown represent means and SD of three replicates.
(B) Incorporation of ⁰¹⁴C-PA during N deprivation into cytoplasmic and chloroplast membranes and into lipid droplets. Cells were labeled with ⁰¹⁴C-PA for 4h starting 12h after the onset of N deprivation. Cell samples taken at 0, 24h,48h or 72h after labeling were fractionated and the ¹⁴C in each fraction was determined. Numbers represent averages of 3 repeats.
(C) Turnover of polar lipids cytoplasmic and chloroplastic membranes. ¹⁴C contents in cytoplasmic and chloroplastic membrane fractions are expressed as % from 0 time, at the end of labeling with ¹⁴C-PA. The chloroplast corrected plot shows the ratio of
counts divided by chlorophyll contents in the fraction also expressed as % from 0 time.

**Figure 5.** Changes in distribution of $^{14}$C labeled lipids during N deprivation. Cells were labeled with $^{14}$C-PA before the onset of N deprivation and then deprived of N for 48h in the absence of $^{14}$C-PA. Black and grey bars represent the levels of $^{14}$C in the different lipid fractions before and after N deprivation, respectively.

**Figure 6.** Protein profiles and western blot analysis of major lipid-associated proteins in CLD and in βC-plastoglobuli.

(A) Protein analysis of CLD and βC-plastoglobuli by SDS-PAGE. Lane 1- MW standards. Lane 2- proteins from βC-plastoglobuli. Lane 3- proteins from CLD. Twenty µg proteins were loaded on each lane. The results shown are representative of three experiments.

(B) Western blot on purified CLD (1), βC-plastoglobuli (2) and pure chloroplasts (3) proteins with anti-Rubisco (dilution 1:20,000), anti-MLDP (dilution 1:1,000), anti-CGP (dilution 1:2,000) antibodies.

**Figure 7.** CGP amino acid sequence, paralogs and phylogenetic tree.

(A) CGP amino acid sequence. Proline residues are shadowed yellow. Proline-rich basic domains are marked by lines 1-3. The arrow indicates putative cleavage site of the transit peptide.

(B) Multiple alignment of CGP with three paralogs.

(C) Phylogenic tree of CGP with SOUL-domain proteins from algae and Arabidopsis. The alignment was generated by the CLUSTAL W program and the phylogram was constructed by the neighbor-joining method using MEGA5 software (Tamura et al. 2011). CGP and orthologs (followed by NCBI accession numbers in parentheses): SOUL-domain-containing protein [Coccomyxa subellipsoidea] (EIE18519.1), hypothetical protein [Chlamydomonas reinhardtii] (XP_001691398.1), hypothetical protein [Volvox carteri] (XP_002947474.1), predicted protein [Ostreococcus lucimarinus] (XP_001418356.1), SOUL heme-binding protein [Arabidopsis thaliana] (NP_001190345.1), hypothetical protein [Chlorella variabilis] (EFN56543.1), SOUL-domain-containing protein in plastoglobules [Arabidopsis thaliana] (ABG48434.1), major lipid droplet protein (MLDP) [Dunaliella bardawil] (AEW43285.1).

**Figure 8.** Localization of MLDP and CGP by Gold immunolabeling.
Algae were cultured in complete (DB+N) or N-deficient media (DB-N) for 6 days. Cryo-sections of fixed cells were treated with anti-MLDP or with anti-CGP polyclonal rabbit antibodies followed by incubation with 10 nm gold-conjugated goat anti-rabbit antibodies.

(A) DB+N cells treated with anti-MLDP antibodies.
(B) DB-N cells treated with anti-MLDP antibodies.
(C) Enlarged view of (B) showing high concentration of gold particles (white arrows) in the periphery of the CLD but not in the contact area of the chloroplast.
(D) DB+N cells treated with anti-CGP antibodies.
(E) DB-N cells treated with anti-CGP antibodies.
(F) Enlarged view of (E) showing gold particles in the periphery of the βC-plastoglobuli (white arrows).
N- nucleus, CLD- cytoplasmatic droplets, St- starch.
Bars: 2 µm (A,B,D,E), 200 nm (C,E).

Figure 9. Time courses of mRNA and protein expression of MLDP and CGP.
(A) mRNA expression of MLDP and CGP. PCR was conducted on cDNA extracted from N-deprived cells at the indicated times, with MLDP and CGP complete gene-specific primers, respectively, and 18S primers as control.
(B) Protein expression of MLDP and CGP was conducted by western blot analysis. D. bardawil were grown in N-deficient media during 7 days of culturing. Each lane contains proteins extracted from 2 x 10^6 cells. Dilution of anti-MLDP antibody was 1:1,000, and anti-CGP antibody was 1:2,000.
The results shown are representative of three experiments.

Figure 10. Rates of accumulation of TAG and β-carotene and decrease in chlorophyll during N-deprivation in D. bardawil.
D. bardawil cells were cultured in complete (DB + N) or in N-deprived (DB – N) media for 10 days.
(A) TAG content of DB + N and DB-N cells. Quantification of TAG by TLC analysis of lipid extracts (Fig. 1B) was by reference to 1 µg trilein standard.
(B) β-carotene content of DB + N and DB-N cells. β-carotene content was calculated from the absorbance of the cell lipid extracts at 480 nm.
(C) Chlorophyll content of DB + N and DB-N cells. Chlorophyll content was calculated from the absorbance of the cell lipid extracts at 663 nm.
The values shown represent means and SD of three replicates.

Figure 11. Cryo-SEM images of D. bardawil control and N-deprived cells.
(A) D. bardawil cell grown in complete medium (control cell).
(B) D. bardawil cells after 2 days of N-deprivation.
(C) Enlarged view of (B) showing CLD in the cytoplasm and high cluster of βC-plastoglobuli in the chloroplast (white arrows).
(D) Section of chloroplast showing an array of βC-plastoglobuli (white arrows).
N- nucleus, Thyl- thylakoid membranes, Chl- chloroplast, CLD- cytoplasmatic droplets.

**Figure 12.** Tomographic reconstruction of D. bardawil cell after 1 day of N-deprivation.

(A) TEM image of control cell grown in N-sufficient medium.
(B) TEM image of cell grown in N-deficient medium for 1 day. It should be noted that this thick (470 nm) slice was used for tomographic analysis (C,D) compared to the standard 70 nm in Fig. 12A.
(C) Tomographic view of a cell section containing four CLD and βC-plastoglobuli.
(D) Enlarged view of (B) showing two CLD surrounded by a nonuniformly-stained chloroplast envelope membrane (arrow heads) and adjacent βC-plastoglobuli (red arrows).

N- nucleus, P- pyranoid, St- starch, M- mitochondrion
Bars: 1 µm (A), 2 µm (B), 500 nm (C), 200 nm (D).

**Table 1.** sn-2 16C/18C FA ratios in different lipid fractions.
The 16C/18C fatty acid ratios were calculated from the fatty acid contents in Table S1 and in Figs 3B, 3C.

**Table 2.** Comparison of 14C distribution between lipid fractions labeled during N deprivation or from preformed polar lipids.
A comparison of 14C distribution in lipid fractions in cells labeled during N- deprivation and extracted 24h after termination of labeling (de-novo synthesis, total time at N deprivation 40h, see Fig. 4B), or before N deprivation (synthesis from preformed polar lipids, Fig. 5). The values in the table represent the 14C distribution in each lipid fraction expressed as % of total.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** Improvement of CLD purification.
CLD were isolated with or without syringe treatment following the osmotic shock.
(A) SDS-PAGE of CLD protein before (1) and after (2) syringe treatment, together with protein marker (3).
(B) TLC of lipid extracted from CLD before (1) and after (2) syringe treatment, together with triolein standard (3).
Figure S2. Multiple sequence alignment of CGP and SOUL heme-binding protein orthologs.
The alignment was constructed by CLUSTALW algorithm. CGP and orthologs (followed by NCBI accession numbers in parentheses): *Chlamydomonas reinhardtii* (XP_006691398.1) *Volvox carteri* (XP_002947474.1), *Chlorella variabilis* (EFN56543.1), *Micromonas* sp. RCC299 (XP_002502391.1).

Figure S3. Hydropathy plot and secondary structure prediction of MLDP and CGP.
(A) Hydropathy plot of MLDP and CGP were generated employing the Kyte-Doolittle algorithm (Kyte and Doolittle 1982), using the Prot Scale program at http://www.expasy.ch/tools/protscale.html. The G value in each graph is the GRAVY for each protein calculated using the GRAVY calculator program at http://www.gravy-calculator.de/.
(B) Secondary structure prediction of MLDP and CGP was obtained by consensus secondary prediction program http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html. Blue lines represent α-helices, red lines represent extended strand, and purple lines represent unstructured domains.

Figure S4. Localization of MLDP and CGP by Gold immunolabeling after 1 day of N-deprivation.
Algae were cultured in complete (DB+N) or N-deficient media (DB-N) for 1 days. Cryo-sections of fixed cells were treated with anti-MLDP or with anti-CGP polyclonal rabbit antibodies followed by incubation with 10 nm gold-conjugated goat anti-rabbit antibodies.
(A) Image of DB-N cell whole cell.
(B) Enlarged view of DB-N cell treated with anti-MLDP antibodies.
(C) Enlarged view of DB-N cell treated with anti-CGP antibodies.
Chl- chloroplast, CLD- cytoplasmatic droplets.
Bars: 2 µm (A), 200 nm (B), 100 nm (C).

Figure S5. TEM imaged of *D. bardawil* control and 1 day N-deprived cells.
(A) *D. bardawil* cell grown in complete medium (control cell).
(B) *D. bardawil* cell grown N-deprived medium for 1 day.
(C)-(H) Enlarged views of *D. bardawil* cells after 1 day of N-deprivation showing CLD adjacent to small βC-plastoglobuli (red arrows) separated by nonuniformly-stained chloroplast envelope membranes.
Bars: 1 µm (A,B), 500 nm (C-F).
**Figure S6.** TEM imaged of *D. bardawil* N-deprived cells after 3 days.

(A) *D. bardawil* cell grown in N-deprived medium for 3 days (DB-N 3d).

(B) Enlarged view of (B) showing cytoplasmatic droplet and small βC-plastoglobuli (red arrows).

Bars: 1 µm (A,B), 500 nm (D), 300 nm (C).

**Figure S7.** TEM imaged of *D. bardawil* N-deprived cells after 7 days.

(A) *D. bardawil* cell grown in N-deprived medium for 7 days sowing a lot of starch (black bodies), a large CLD in the cytoplasm and bigger βC-plastoglobuli in the chloroplast.

(B) Enlarged view of chloroplast showing an array of βC-plastoglobuli.

Bars: 1 µm (A), 500 nm (B).

**Movie S1.** Tomographic reconstruction movie of *D. bardawil* cell after 1 day of N-deprivation.

The movie shows sequence tomographic slices throughout a tomographic reconstructed volume. It can be seen that the two CLD are surrounded by a distorted chloroplast envelope membrane and that several small βC-plastoglobuli are formed and accumulate in the adjacent chloroplast. Each slice in the movie is an average of 20 tomographic slices, 2 nm in thickness each. The full frame width is 4 µm.

**Table S1.** Fatty acid *sn*-2 and *sn*-1 positional analysis of membrane lipids.

Chloroplastic and cytoplasmatic membranes were isolated, lipids were extracted, PC from cytoplasmatic membranes was isolated from two-dimensional TLC plates, and then lipids were treated with Rhizopus lipase or with phospholipase A2. Released and total fatty acid compositions were determined and expressed as % of total. Data represent averages of 3 repeats.
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**Table 1:** sn-2 16C/18C FA ratios in different lipid fractions

| Lipid class | Cytoplasm | Chloroplast |
|-------------|-----------|-------------|
| TAG         | 0.75      | 1.00        |
| Polar lipids| 0.69      | 17.6        |

The 16C/18C fatty acid ratios were calculated from the fatty acid contents in Table S1 and in Figs 3B, 3C.
Table 2: Comparison of $^{14}$C distribution between lipid fractions labeled during N deprivation or from preformed polar lipids

| Labeling stage       | N deprivation | Pre-labeling | $^{14}$C content (% of total) |
|----------------------|---------------|--------------|------------------------------|
| CLD                  | 70.8±1.1      | 20.8±1.5     |                              |
| βC plastoglobuli     | 4.8±0.6       | 33.9±1.0     |                              |
| Cytoplasmic membranes| 9.4±0.3       | 7.8±0.8      |                              |
| Chloroplast membranes| 15.0±0.7      | 37.5±0.4     |                              |

A comparison of $^{14}$C distribution in lipid fractions in cells labeled during N-deprivation and extracted 24h after termination of labeling (de-novo synthesis, total time at N deprivation 40h, see Fig. 4B), or before N deprivation (synthesis from preformed polar lipids, Fig. 5). The values in the table represent the $^{14}$C distribution in each lipid fraction expressed as % of total.
Figure 1. TLC and Nile red analysis of isolated lipid droplets.

(A) Nile red Fluorescence emission spectra of D. bardawil control cells (DB + N), N-deprived cells (DB – N) and purified CLD and βC-plastoglobuli.

(B) TLC analysis of neutral lipids in lipid extracts from D. bardawil control cells (lane 1), N-deprived cells (lane 2), purified CLD (lane 3), purified βC-plastoglobuli (lane 4) and 1 μg of triolein standard (lane 5).

(C) TLC analysis of polar lipids in extracts from D. bardawil chloroplasts (lane 1), 1 μg of diacylglyceride standard (lane 2), purified βC-plastoglobuli (lane 3) and purified CLD (lane 4) after iodine staining (left) and galactolipid staining (right). The major polar lipids of cytoplasm droplets and βC-plastoglobuli are marked in arrowheads.

TLC and Nile red analysis were conducted in samples normalized to equal cell number.
Figure 2. HPLC chromatogram of lipid extracts from CLD and βC-plastoglobuli. The chromatogram represents lipid extraction fractions from CLD (blue) and βC-plastoglobuli (black) and was compared to TAG standards. βC: β-Carotene, MS-9: TAG peak which was collected and send to MS and FAME analysis.
Figure 3. Fatty acid composition and positional analysis of *D. bardawil* isolated lipid droplets. (A) Total fatty acid composition analysis of *D. bardawil* control (DB+N) and N-deprived (-N) cells together with isolated lipid droplets (CLD and βC-plastoglobuli). Fatty acid compositions of TAG in sn-1,3 (B) or sn-2 (C) positions from CLD and βC-plastoglobuli are shown. The values shown represent means and SD of 3-5 replicates.
Figure 4. Time course of formation of CLD and of βC-plastoglobulin TAG.

(A) Total TAG contents. D. bardawil cells were grown in N-deficient media. At each indicated time the cytoplasm and chloroplast fractions were separated and TAG were extracted from each fraction. The values shown represent means and SD of three replicates.

(B) Incorporation of 14C-PA during N-deprivation into cytoplasmic and chloroplast membranes and into lipid droplets. Cells were labeled with 14C-PA for 4h starting 12h after the onset of N-deprivation. Cell samples taken at 0, 24, 48 or 72h after labeling were fractionated and the 14C in each fraction was determined. Numbers represent averages of 3 repeats.

(C) Turnover of polar lipids in cytoplasmic and chloroplastic membranes. 14C contents in cytoplasmic and chloroplastic membrane fractions are expressed as % from 0 time, at the end of labeling with 14C-PA. The chloroplast corrected contents are defined as 100%, and chlorophyll contents in the fraction also expressed as % from 0 time.
Figure 5. Changes in distribution of $^{14}$C labeled lipids during N-deprivation. Cells were labeled with $^{14}$C-PA before the onset of N-deprivation and then deprived of N for 48h in the absence of $^{14}$C-PA. Black and grey bars represent the levels of $^{14}$C in the different lipid fractions before and after N deprivation, respectively.
Figure 6. Protein profiles and western blot analysis of major lipid-associated proteins in CLD and in βC-plastoglobuli.

(A) Analysis by SDS-PAGE. Lane 1- MW standards. Lane 2- proteins from βC-plastoglobuli. Lane 3- proteins from CLD.

(B) Western blot on purified CLD (1), βC-plastoglobuli (2) and pure chloroplasts (3) proteins with anti-Rubisco (dilution 1:20,000), anti-MLDP (dilution 1:1,000), anti-CGP (dilution 1:2,000) antibodies.

Twenty μg proteins were loaded on each lane. The results shown are representative of three experiments.
Figure 7. CGP amino acid sequence, paralogs and phylogenetic tree.

(A) CGP amino acid sequence. Proline residues are shadowed yellow. Proline-rich basic domains are marked by lines 1-3. The arrow indicates putative cleavage site of the transit peptide.

(B) Multiple alignment of CGP with three paralogs.

(C) Phylogenetic tree of CGP with SOUL-domain proteins from algae and Arabidopsis. The alignment was generated by the CLUSTAL W program and the phylogram was constructed by the neighbor-joining method using MEGA5 software (Tamura et al. 2011). CGP and orthologs (followed by NCBI accession numbers in parentheses): SOUL-domain-containing protein [Coccomyxa subellipsoideae] (EIE18519.1), hypothetical protein [Chlamydomonas reinhardtii] (XP_001691398.1), hypothetical protein [Volvox carteri] (XP_002947474.1), predicted protein [Ostreococcus lucimarinus] (XP_00148356.1), SOUL heme-binding protein [Arabidopsis thaliana] (NP_01910345.1), hypothetical protein [Chlorella variabilis] (EFN56543.1), SOUL-domain-containing protein in plastoglobules [Arabidopsis thaliana] (ABD3434.4), major lipid droplet protein (MLDP) [Dunaliella bardawill] (AEW28285.1).
Figure 8. Localization of MLDP and CGP by Gold immunolabeling.
Algae were cultured in complete (DB+N) or N-deficient media (DB-N) for 6 days. Cryo-sections of fixed cells were treated with anti-MLDP or with anti-CGP polyclonal rabbit antibodies followed by incubation with 10 nm gold-conjugated goat anti-rabbit antibodies.

(A) DB+N cells treated with anti-MLDP antibodies.
(B) DB-N cells treated with anti-MLDP antibodies.
(C) Enlarged view of (B) showing high concentration of gold particles (white arrows) in the periphery of the CLD but not in the contact area of the chloroplast.
(D) DB+N cells treated with anti-CGP antibodies.
(E) DB-N cells treated with anti-CGP antibodies.
(F) Enlarged view of (E) showing gold particles in the periphery of the βC-plastoglobuli (white arrows).

Bars: 2 μm (A,B,D,E), 200 nm (C,E).
Figure 9. Time courses of mRNA and protein expression of MLDP and CGP. (A) mRNA expression of MLDP and CGP. PCR was conducted on cDNA extracted from N-deprived cells at the indicated times, with MLDP and CGP complete gene-specific primers, respectively, and 18S primers as control. 

(B) Protein expression of MLDP and CGP was conducted by western blot analysis. D. bardawil were grown in N-deficient media during 7 days of culturing. Each lane contains proteins extracted from 2 x 10^6 cells. Dilution of anti-MLDP antibody was 1:1,000, and anti-CGP antibody was 1:2,000 and anti-actin, for control 1:250. The results shown are representative of three experiments.
Figure 10. Rates of accumulation of TAG and β-carotene and decrease in chlorophyll during N-deprivation in *D. bardawil*.
*D. bardawil* cells were cultured in complete (DB + N) or in N-deprived (DB – N) media for 10 days.

(A) TAG content of DB + N and DB-N cells. Quantification of TAG by TLC analysis of lipid extracts (Fig. 1B) was by reference to 1 μg triolein standard.

(B) β-carotene content of DB + N and DB-N cells. β-carotene content was calculated from the absorbance of the cell lipid extracts at 480 nm.

(C) Chlorophyll content of DB + N and DB-N cells. Chlorophyll content was calculated from the absorbance of the cell lipid extracts at 663 nm. The values shown represent means and SD of three replicates.
Figure 11. Cryo-SEM images of *D. bardawil* control and N-deprived cells.

(A) *D. bardawil* cell grown in complete medium (control cell).

(B) *D. bardawil* cells after 2 days of N-deprivation.

(C) Enlarged view of (B) showing CLD in the cytoplasm and high cluster of βC-plastoglobuli in the chloroplast (white arrows).

(D) Section of chloroplast showing an array of βC-plastoglobuli (white arrows).

N- nucleus, Thyl- thylakoid membranes, Chl- chloroplast, CLD- cytoplasmic droplets.
Figure 12. Tomographic reconstruction of D. bardawil cell after 1 day of N-deprivation.

(A) TEM image of control cell grown in N-sufficient medium.

(B) TEM image of cell grown in N-deficient medium for 1 day. It should be noted that this thick (470 nm) slice was used for tomographic analysis (C,D) compared to the standard 70 nm in Fig. 12A.

(C) Tomographic view of a cell section containing four CLD and βC-plastoglobuli.

(D) Enlarged view of (B) showing two CLD surrounded by a nonuniformly-stained chloroplast envelope membrane (arrow heads) and adjacent βC-plastoglobuli (red arrows).

N- nucleus, P- pyranoid, St- starch, M- mitochondrion
Bars: 1 μm (A), 2 μm (B), 500 nm (C), 200 nm (D).