Saturating light induces sustained accumulation of oil in plastidal lipid droplets in *Chlamydomonas reinhardtii*

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Short title: High light induces reserve accumulation in Chlamydomonas

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Saturating Light Induces Sustained Accumulation of Oil Primarily Stored in Lipid Droplets of Plastidial Origin in *Chlamydomonas reinhardtii*

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One sentence summary

Saturating light induces oil storage in *Chlamydomonas*, and the newly formed oil accumulate in lipid droplets distinct in protein and lipid compositions from those induced by nitrogen starvation.

AUTHOR CONTRIBUTIONS

H.G., G.P., B.J., F.B., Y. L-B designed research; H.G., S.C., B.L., Y.L., H.J., S.B., P.A. performed research; H.G., M.T., B. L., Y.L., H.J., M.T., F.B., G.P., Y. L-B analysed data; H.G., F.B., M.T., G.P., Y. L-B wrote the paper. All authors agreed on the manuscript.

ABSTRACT

Enriching algal biomass in energy density is an important goal in algal biotechnology. Nitrogen (N) starvation is considered the most potent trigger of oil accumulation in microalgae and has been thoroughly investigated. However, N-starvation causes slow down and eventually arrest of biomass growth. In this study, we show that exposing a *Chlamydomonas reinhardtii* culture to saturating light (SL) under a non-limiting CO_2 concentration in turbidostatic photobioreactors induces a sustained accumulation of lipid droplets (LDs) without compromising growth, which results in much higher oil productivity than N starvation. We also show that the polar membrane lipid fraction of SL-induced LDs are rich in plastidial lipids (~70%), in contrast to N-starvation induced LDs which contain ~60% lipids of ER origin. Proteomic analysis of LDs isolated from SL-exposed cells identified >200 proteins, including known proteins of lipid metabolism, as well as 74 proteins uniquely present in SL-induced LDs. LDs induced by SL and N-depletion thus differ in protein and lipid contents. Taken together, lipidomic and proteomic data thus show that a large part of the sustained oil accumulation occurring under SL is likely due to the formation of plastidial LDs. We discuss our data in relation to the different metabolic routes used by microalgae to accumulate oil reserves depending on cultivation conditions. Finally, we propose a model in which oil accumulation is governed by an imbalance between photosynthesis and growth, which can be achieved by impairing growth or by boosting photosynthetic carbon fixation, with the latter resulting in higher oil productivity.

Key Words: Microalgae, Biomass, High light, Lipidomics, Productivity, Energy imbalance

INTRODUCTION

Neutral lipid accumulation by microalgae has recently regained intensive interest because these organisms are considered promising as a feedstock for the production of renewable fuels and fatty acid (FA)-derivatives (Rosenberg et al., 2008; Wijffels and Barbosa, 2010; Khozin-Goldberg and Cohen, 2011). Most microalgal species do not accumulate large amounts of neutral lipids (i.e. triacylglycerols TAGs) when grown under optimal conditions (Sheehan, 1998). Neutral lipid accumulation can, however, be induced by exposing cells to unfavourable culture conditions, for example: removing nutritional elements (nitrogen, sulphur, iron, phosphate etc) from the media; increasing salinity or growth temperature (Moellering and Benning, 2010; Siaut et al., 2011; Urzica et al., 2013; Abida et al., 2015; Légeret et al., 2016); or exposing cells to small chemically active molecules (Kato et al., 2013; Kim et al., 2013; Kim et al., 2015). Most of the current understanding of TAG metabolism in *C. reinhardtii* has been gained through the study of molecular mechanisms occurring during N starvation response (Fan et al., 2011; Goodson et al., 2011; Siaut et al., 2011; Tsai et al., 2014; Tsai et al., 2015). It is uncertain if the mechanisms of TAG accumulation upon N starvation are generally applicable or whether different mechanisms are employed under other types of conditions. A major limitation in the use of microalgae to produce oil is the fact that nitrogen (N) deprivation, as well as most other TAG-inducing conditions, provoke impairment in protein synthesis and cell division, thus limiting productivity (Hu et al., 2008; Scott et al., 2010).

Biomass productivity is the result of highly co-ordinated cellular processes starting with the capture of light by photosystems, fixation of CO_2 through the Calvin-Benson Cycle, to cell growth and division. Light is one of the most variable environmental parameters during the growth of photoautotrophs in natural environments. In non-saturating light, CO_2 fixation and biomass productivity increase linearly as a function of light intensity. Above a certain threshold, light saturation occurs. A considerable body of work has documented the effects of high light on photosynthesis, including the effects on the pigment content (Bonente et al., 2012), on the induction of dissipation or protection mechanisms (Peers et al., 2009), and on the production of reactive oxygen species (ROS) (Fischer et al., 2006; Forster et al., 2006; Erickson et al., 2015; Sato et al., 2015). The effect of light intensity on carbon allocation and reserve formation has also been explored (Pal et al., 2011; Fan et al., 2012; Klok et al., 2013; He et al., 2015). For example, increasing light intensity has been shown to increase the cellular neutral lipid content in a number of microalgal species, including

Haematococcus pluvialis (Zhekisheva et al., 2002), *Tichocarpus crinitus* (Khotimchenko and Yakovleva, 2005) and in *Chlamydomonas reinhardtii* (Mettler et al., 2014). Molecular factors involved in TAG storage under high light are still to be uncovered.

Oil accumulation is associated with the formation of lipophilic droplets, called lipid droplet (LDs) (or oil bodies, or oleosomes) (Jolivet et al., 2013). LDs are specialized intracellular organelles made of a neutral lipid core surrounded by a membrane lipid coat in which proteins are embedded (Huang, 1996). LDs serve as a temporary storage site for neutral lipids, and also participate in the active synthesis and metabolisms of these non-membrane forming lipids (Goodman, 2008; Farese Jr and Walther, 2009; Chapman et al., 2012; Goold et al., 2014; Tsai et al., 2015). The current model of LD biogenesis suggests that these lipid-rich subcellular structures arise from membrane 'budding' or 'blistering", thus the lipid molecules present in the LD lipid coat infer its origin of biogenesis. For example, oil bodies in the oilseed are coated by a mono-layer of lipids of mostly ER-origin (Huang, 1992; Tzen and Huang, 1992) whereas plastoglobules are covered by lipids usually found as part of the thylakoid membranes (Austin et al., 2006). Thus, protein and lipid composition of LDs can shed light on the likely subcellular location of TAG synthesis and LD biogenesis. Compared to oilseeds, the unicellular microalga *C. reinhardtii* offers an excellent platform to study LD formation due to the ease with which researchers can induce lipid droplet biogenesis or degradation by simply changing the culture media (Goodson et al., 2011; Goold et al., 2014; Tsai et al., 2015).

Here, we have used a turbidostatic continuous cultivation system to study the effect of light intensity on oil accumulation and productivity in *C. reinhardtii* cells. We report that exposure to a saturating light (SL) leads to increased cellular oil content and to much higher oil productivity than observed in response to N-depletion. A general model in which oil accumulation is a result of an imbalance between photosynthetic activity and demand for growth is discussed. We also provide insights into the mechanism of oil accumulation under SL by reporting the proteomic and lipidomic analyses of SL-induced lipid droplets.

RESULTS

Experimental Setup and Choice of Light Intensity

To investigate the formation of carbon reserves in response to high light under photoautotrophic conditions, it is paramount that light supply and penetrance is kept uniform all throughout culture stages. When cultivating algae in batch cultures under current laboratory conditions, the biomass increase leads to a decrease of light perceived per cell due to shading in dense cultures. To achieve constant light perception, we cultivated *C. reinhardtii* in continuous culture in photobioreactors (PBRs) monitored as turbidostats. This allows for the cultures to be maintained at constant biomass levels (i.e. under a defined physiological condition) throughout the course of the experiment with minimal differences between cultures in terms of light exposure, and other parameters such as pH, nutrient or CO_2 supply.

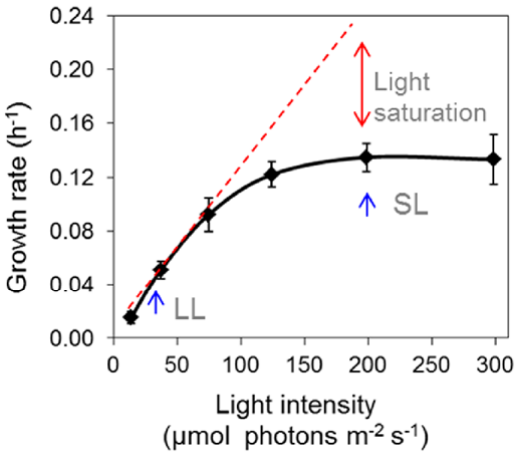
We first measured biomass productivity as a function of photon flux density (PFD) (**Figure 1**). Non-limiting CO_2 condition was achieved by bubbling air containing 1.8% CO_2. The growth rate increased progressively with rising PFD in the range of 0-100 μmol m^{-2} s^{-1} but when light reached >120 μmol m^{-2} s^{-1}, a plateau in biomass growth was observed. Light intensities above that threshold are thus saturating lights (SL). To evaluate the effects of light intensity on carbon reserve formation, based on this first experiment, cells of *Chlamydomonas* were cultivated first at a sub-saturating low light (LL, 37 μmol m^{-2} s^{-1}) then exposed to a saturating light of 200 μmol m^{-2} s^{-1} for 48 h (indicated as blue arrows in **Figure 1**). It is worth noting that the light intensity at which saturation of photosynthesis and growth was obtained under this experimental set up (around 200 μmol m^{-2} s^{-1}) may appear low compared to what is generally used in most laboratory conditions (i.e. shake flasks with mono-directional illumination). Such a low light saturation level resulted from the use of a relatively low-density culture ($OD_{880\ nm}$ = 0.4) and radial illumination (creating a homogenous light inside the PBR). Since high light is a relative term, to avoid ambiguity, in this study, we have instead used the term 'saturating light (SL)' throughout.

To dissect mechanistic differences in carbon reserve formation between cells exposed to SL and those cultivated under N-starvation conditions, we setup four 1-L PBRs, two for SL experiments, and two for the N-depletion experiments. To reflect real production conditions, we employed a gradual N-depletion process rather than an abrupt N-starvation. The experimental setups used and the parameters analysed in the rest of the work are outlined in **Figure 2**.

Figure 1. Growth rate of biomass as a function of photon flux density in turbidostatic photobioreactors.

Strain CC-124 was cultivated in photobioreactors (PBRs), with a supply of 1.8% CO_2 in air. Growth rate was calculated as a rate of injection of fresh media to an existing culture while maintaining OD_{880nm} at 0.4 (equivalent to ~2 million cells mL^{-1}). The light intensities used in the rest of this study are indicated by blue arrows. Low light (LL) refers to 37 μmol m^{-2} s^{-1} and saturating light (SL) refers to 200 μmol m^{-2} s^{-1}.

Data are means of two biological replicates and two technical replicates each. Error bars represent standard deviations.



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167 **Exposure to Saturating Light (SL) Increases Intracellular Oil and Starch Contents**

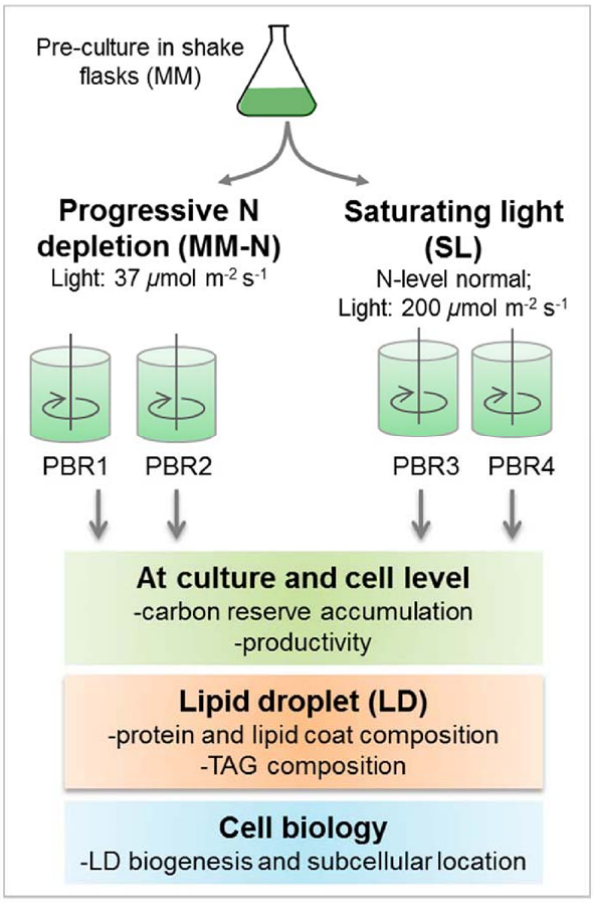
168 Quantification of intracellular oil and starch showed that SL-exposed cells accumulated both

169 starch and TAGs in a parallel manner (**Figure 3A**). Oil and starch accumulation was also

Figure 2. Experimental setup for the comparison of nitrogen starvation and saturating light conditions.

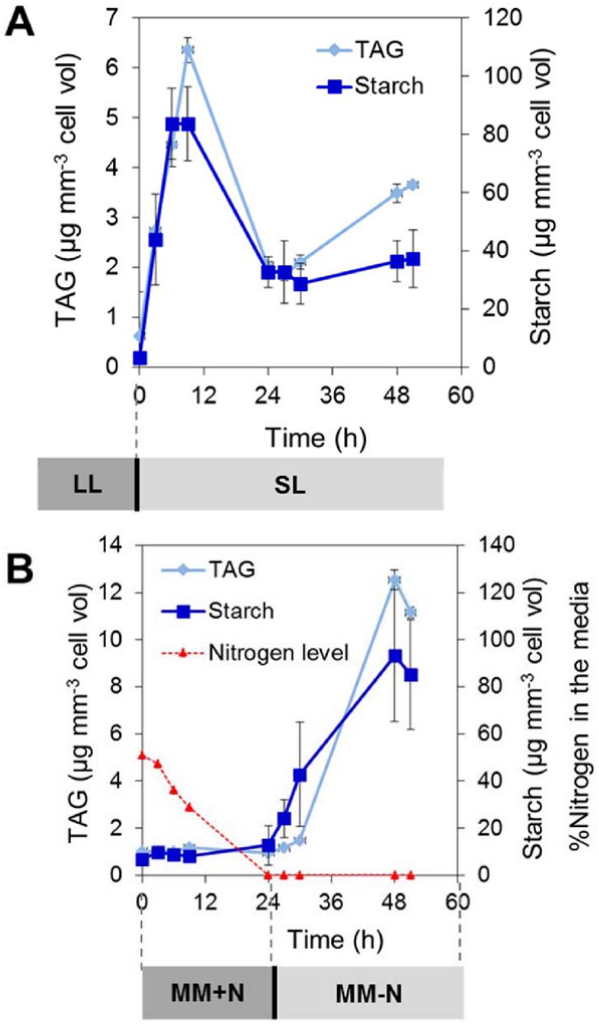
Strain CC-124 was maintained at a constant OD_{880nm} of 0.4 (equivalent to ~2 million cells mL^{-1}). SL: saturating light refers to a light intensity of 200 μmol $m^{-2}s^{-1}$. Nitrogen starvation is achieved through progressive limitation and culture nitrogen level was measured.

Abbreviations: MM, minimal media; N, nitrogen; SL, saturating light; LD, lipid droplet; PBR, photobioreactor; OD, optical density; TAG, triacylglycerols.



170 observed in N-starved cells under photoautotrophic PBR conditions (**Figure 3B**). Maximal
171 oil accumulation measured in SL-exposed cells (~6 µg mm^{-3} at 9 h) reached almost 50% of
172 the level determined for N-starved cells (~12 µg mm^{-3} at 24 h after N-depletion) and maximal
173 starch accumulation was similar (~80 µg mm^{-3} cells) under both conditions. A transient peak

Figure 3. Exposure to saturating light triggers oil and starch accumulation.
Cells were cultivated in photobioreactors in photoautotrophic conditions. Time zero in the SL and the N starvation experiment correspond to low light (LL) and +N controls, respectively.
Data are means of two biological replicates with two technical replicates. Error bars represent standard deviations.
Abbreviations: MM, minimal media; N, nitrogen; TAG, triacylglycerols. SL, saturating light; LL, low light.



174 of TAG/starch accumulation was seen around 9 h upon SL exposure, followed by a drop and
175 stabilization upon 24 h. The stabilized level of reserves was still much higher than before SL
176 exposure, but lower than the maximal level observed under N-depletion. Moreover, an
177 increase in total fatty acids and also in carbon/nitrogen (C/N) ratio (from 4.8 to 5.7) were

observed in cells exposed to SL compared to those cultivated under LL (Supplemental **Figure S1**), supporting the TAG quantification data (Figure 3).

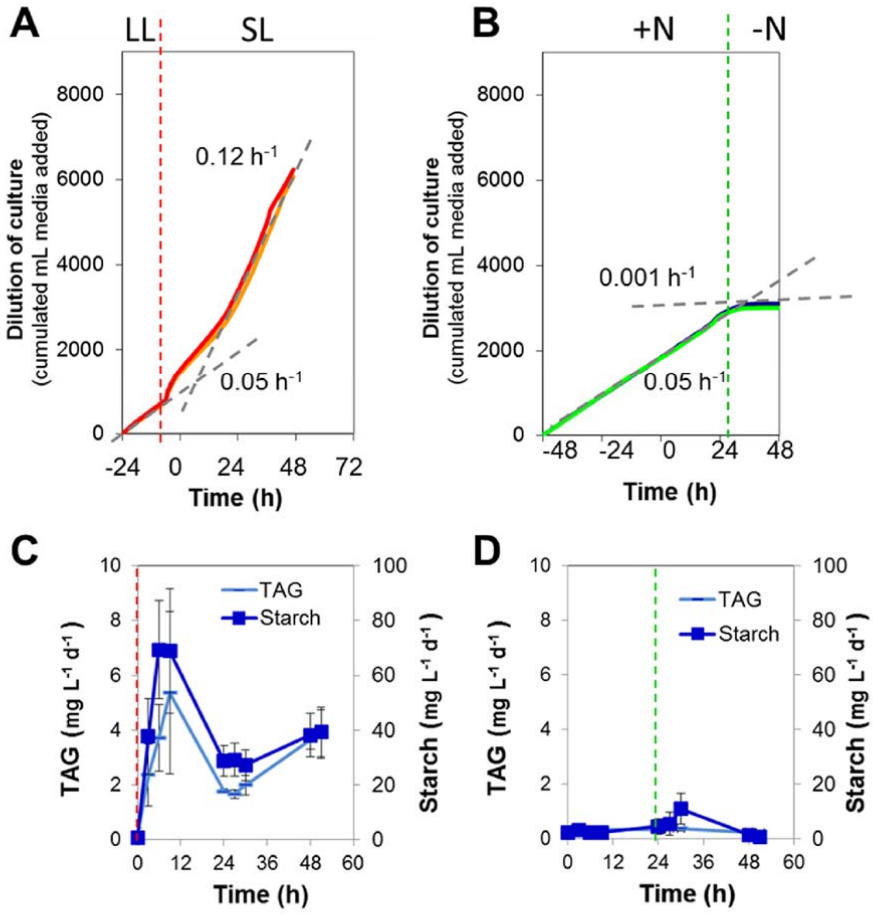
Oil and Starch Productivities are Higher in SL-Exposed Cultures than in N-Starved Cultures

Upon SL exposure, the specific growth rate of the *C. reinhardtii* culture grown in PBRs progressively increased from 0.05 h^{-1} to a new steady state, which was reached at 0.12 h^{-1} (**Figure 4A**). In sharp contrast, N depletion led to a progressive decrease in the growth rate until it halted completely 6 h after full N-depletion (**Figure 4B**). Calculation of oil and starch productivity by using specific growth rates with cellular oil and starch contents revealed that compared to N starvation, SL-exposed cells achieved a 15 times higher productivity in TAG (~ 6 mg L^{-1} d^{-1} vs. ~ 0.4 mg L^{-1} d^{-1}) and a 7 times higher productivity in starch (~ 70 mg L^{-1} d^{-1} vs. 10 mg L^{-1} d^{-1}) (**Figure 4C, D**). These results therefore show clearly that sustained productivity of oil and starch could be achieved in PBRs under SL.

SL-Exposed Cells Accumulate Oil in Lipid Droplets (LDs)

Oil accumulation under SL exposure was found to form distinct LDs that were visible after Nile red staining (**Figure 5A, B**). Interestingly, a significant portion of LDs in SL-exposed cells were smaller than LDs under N starvation. This contrasted to those formed under N-starvation, which were bigger and more abundant (**Figure 5C**). It is known that N-starved *Chlamydomonas* WT cells accumulate LDs mostly at the junction of endoplasmic reticulum and plastid envelops (the so-called β-cyto-LDs) (Goodson et al., 2011). Here, we thus only focused on the subcellular location of the LDs present in the SL-treated cells. This was achieved by obtaining confocal microscopy images of several focal planes. For example, when moving the focal plane from the top all the way down to the bottom of the cell, we observed the presence or absence of LDs (**Figure 5D, E, F**). This contrasted with the detection of chlorophyll autofluorescence. These observations suggested that at least some of the LDs formed were likely present inside the plastid (**Figure 5D**: white arrows point to one example of this LD type). LDs that appeared to be cytosolic or associated to plastid membranes in the same cell could also be seen. To confirm these observations, we further examined the difference in the nature of LD populations under SL and N-starvation by analysing the protein and lipid composition of isolated LDs.

Figure 4. Growth rates, oil and starch productivity under saturating light or nitrogen starvation.
A. Growth rates under saturating light (SL).
B. Growth rates under nitrogen (N) starvation.
C. Productivity of oil and starch under SL.
D. Productivity of oil and starch under N starvation.
Photobioreactors were first stabilized for 4 days, then exposed either to saturating light irradiance, or to nitrogen depletion. The growth rate was calculated based on the cumulated medium added to the photobioreactor culture to maintain a constant OD during cell culture. Red dotted lines indicate the moment SL is switched on, and green dotted lines indicate when nitrogen is completely depleted from the media. Data are means of two biological replicates and two technical replicates. Error bars denote standard deviations.
Abbreviations: MM, minimal media; N, nitrogen; SL, saturating light; LL, low light. L: liters of culture



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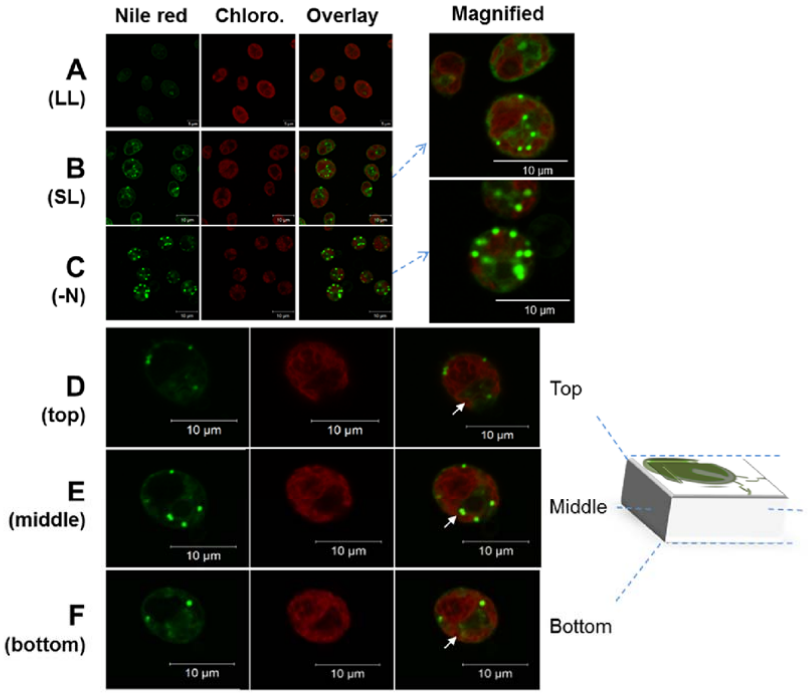
LDs in SL-Exposed Cells Contain TAGs and Polar Lipids of Mostly Plastidial Origin

To determine lipid and proteins present in LDs, we isolated LDs from both SL-exposed cells and N-starved cells cultivated in PBRs following 9 h SL-exposure (SL 9h), or 24 h N-starvation (MM-N 24h), respectively. These time points were chosen because they

Figure 5. Visualization of lipid droplets by Nile red staining and microscopy.
A. Chlamydomonas cells cultivated under normal conditions (MM+N/LL)
B. Chamydomonas cells after 9 h of saturating light (SL+N).
C. *Chlamydomonas* cells after 24 h of N depletion (MM-N/LL).
D. The 'top' focal plane of an image of Nile red stained SL-exposed cell
E. The 'middle' focal plane of an image of Nile red stained SL-exposed cell
F. The 'bottom' focal plane of an image of Nile red stained SL-exposed cell
LD, lipid droplet; SL, saturating light; N, nitrogen.



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correspond to the moment when the maximal cellular oil content was reached. After lipids were extracted from isolated LDs, polar and neutral fractions were separated by solid-phase extractions (SPE), and the proportion of each fraction was quantified after being converted to their fatty acid methyl esters (FAMEs). LDs of N-starved cells contained 97% of neutral

Figure 6. Lipid composition of lipid droplets (LDs) isolated from SL-exposed cells and N-starved cells.

A. Proportion of membrane lipids versus triacylglycerols (TAG) in isolated LDs. Lipid amounts were determined by quantification of total fatty acids in each lipid fraction.

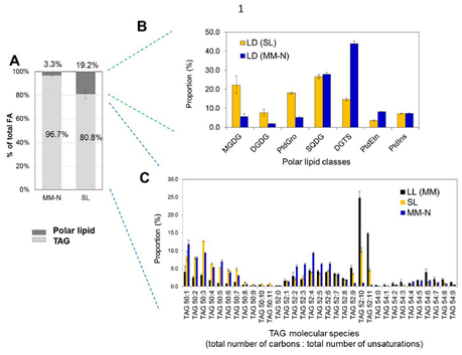
B. Proportion of major membrane lipid classes in isolated LDs. Content for each lipid class was determined by TLC.

C. Distribution of triacylglycerol (TAG) molecular species in isolated LDs.

Lipid droplets were isolated from cells being N-starved for 24 h (corresponding to 48 h in **Figure 3**) or from cells exposed to saturating light (200 μmol m^{-2} s^{-1}) for 9 h. These time points were chosen because they represent the peak of oil accumulation under either treatment.

Data are means of two biological and two technical replicates, with standard deviations shown.

Abbreviations: FA, fatty acid; TAG, triacylglycerol; MM-N, minimal media under nitrogen starvation; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PtdGro, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; DGTS, diacylglycerol *N,N,N*-trimethylhomoserine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; LD, lipid droplet; ER, endoplasmic reticulum.



lipids and 3% polar lipids whereas those of SL-exposed cells contained only 81% neutral lipids and 19% polar lipids (**Figure 6A**). In a typical LD topology, neutral lipids are covered by a polar membrane shell (Jolivet et al., 2013). Since smaller LDs contain a higher proportion of polar membrane lipids than larger LDs, this suggests that the LDs in SL-

exposed cells are smaller than those induced by N-starvation, which was consistent with microscopic examinations (**Figure 5**).

The polar lipids of LDs isolated from SL-exposed cells showed a high content in MGDG and SQDG, followed by PtdGro and DGTS, then DGDG, PtdIns and PtdEtn (**Figure 6B**). This composition clearly contrasts with the LDs of N-starved cells, where DGTS and SQDG are the major polar lipid species (**Figure 6B**), which corroborates previous observations (Wang et al., 2009). With the exception of PtdIns, molecular species within a particular lipid class also differed between these two LD types (**Supplemental Figure S2**). LDs induced by SL contained a high proportion (~70%) of lipids unique to photosynthetic membranes (i.e. MGDG, DGDG, SQDG), whereas LDs of cells in the N-starvation condition contained ~60% of extra-plastidial lipids (DGTS and PtdEtn).

TAG molecular species present in the two LD types were also found to differ. Compared to TAG species present in cells under optimal growth condition (i.e. low light and nutrient replete condition; LL), SL-induced LDs contained significant higher proportion of all TAG50 species, yet with significant reductions in TAG species rich in polyunsaturated fatty acids (TAG52:10 and TAG52:11) (**Figure 6C**). Interestingly, the above polyunsaturated TAG species are present in higher proportions in LDs induced by SL than those triggered by N starvation (**Figure 6C**).

LDs of SL-Exposed Cells Contained Higher Proportions of Proteins of Plastidial Origin

In order to gain further insights in LD formation under SL, we performed a proteomic analysis to compare the protein compositions of LDs accumulating in SL to those isolated from N-starvation conditions. Total proteins associated to LDs isolated under SL or -N conditions were loaded and separated on a SDS-PAGE. Protein extracts were loaded based on an equal amount of total lipids. LDs formed under SL showed a distinct protein composition compared to those of N-induced LDs (**Figure 7A**). Proteomic analyses by LC-MS/MS identified 222 proteins associated to LDs in SL-exposed cells, and 303 proteins in LDs formed under N-starvation (**Figure 7B; Supplemental Table S1A, B**). To be stringent, we kept only those proteins for which ≥2 unique peptides and ≥2 specific spectral counts (SSC) were identified. Proteins were ranked according to their estimated relative abundance within each LD biogenesis condition. Comparisons between the two proteomes showed that 148 proteins are identified in both proteomes (**supplemental Table S1C)**, whereas 74 unique proteins were identified in SL-induced LDs (**supplemental Table S1D**), a large portion of which (31/74) were of plastidial origin based on a prediction using the green algae protein-

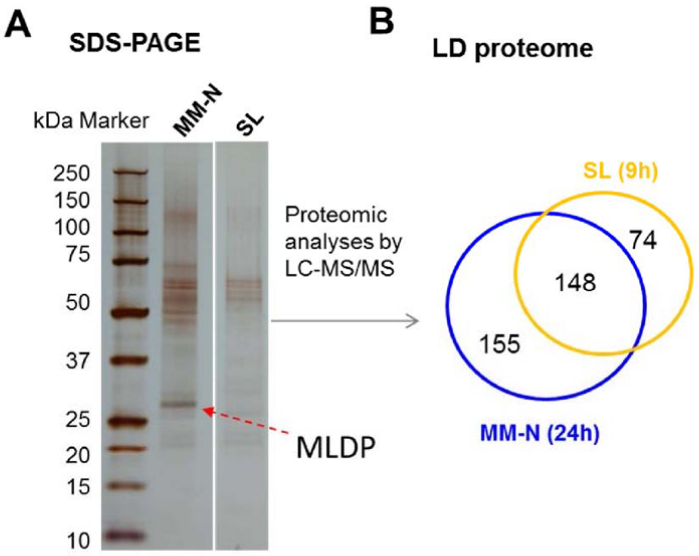
Figure 7. Comparison of proteomic composition of lipid droplets (LDs) isolated from cells subjected to saturating light (SL) or N-starvation.

A. Silver nitrate-stained SDS-PAGE of total proteins extracted from purified LDs.

B. Venn diagram showing the total number of proteins associated to LDs under each condition. Only proteins with ≥2 peptides and ≥2 specific spectra counts were retained for analyses.

LDs were purified from cultures either after 24 h nitrogen depletion, or from cultures after being exposed to saturating light for 9 h.

Abbreviations: LD, lipid droplet; SL, saturating light; kDa, kilo daltons; LC-MS/MS: liquid chromatography – tandem mass spectrometry; MM, minimal media; N, nitrogen.



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2 adapted algorithm PredAlgo (Tardif et al., 2012). We detected significant amount of 'plastidial' proteins in both LD preparations, but we could not tell if these proteins are contaminations or are they integral part of the LD proteome. These questions should be addressed in the future through studies of protein subcellular localizations.

The identity and annotation for the top 30 ranked proteins associated to LDs isolated from SL-exposed cells are listed in **Table 1**. Proteins common to both LD isolates include the major lipid droplet protein (MLDP), the diacylglyceryl-*N,N,N*-trimethylhomoserine synthesis protein (BTA1), the cyclopropane-fatty-acyl-phospholipid synthase (CFA2), an α/β hydrolase, a glycosyl hydrolase (GHL1), a long chain acyl-CoA synthetase (LCS2), a β-tubulin 1 (TUB1), a lysophospholipid acyltransferase (LPLAT), and one of the components of the plastid lipid reimport machinery (TGD2) (**Supplemental Table S1C; Table 2**). These proteins have been found to be present in all three previously reported LD proteomics studies (Moellering and Benning, 2010; Nguyen et al., 2011; Tsai et al., 2015). With the exception of TGD2, all of the above-mentioned top-ranked proteins in N-induced LDs are not the same major proteins in SL-LDs, i.e. their ranks in SL-LDs being relatively inferior to those in N-induced LDs (**Table 2**). These rankings were calculated based on a computed calculation of total number of SSCs and values of exponentially modified protein abundance index (emPAI), and can be used to evaluate the relative abundance of each protein in a sample.

Among the 74 proteins found only in LDs of SL-origin, of particular note is the presence of relatively large proportion (~45%) of proteins of plastidial origin, including the Proton Gradient Regulation Like1 (PGRL1), the Plastid Lipid Associated Protein 8 (PLP8 Cre14.g611700), and also a 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone (MPBQ/MSBQ) methyltransferase (VTE3) (**Supplemental Table S1D**).

DISCUSSION

In this study, we showed that saturating light (SL) can increase cellular oil content while maintaining cell turnover and biomass growth, thus leading to a sustainable production of carbon reserves. This clearly contrasts with the well-characterized N starvation-induced oil accumulation, where oil productivity remained low despite achieving a higher cellular TAG content. We further showed that SL induces formation of smaller LDs than those formed under N starvation. The SL-induced LDs had a lipid and protein coat enriched in components of plastidial origin, thus LDs formed under SL are distinct from those formed under N-starvation. This serves as the first report on LD composition beyond the model of N-starvation-induced LDs in *C. reinhardtii*.

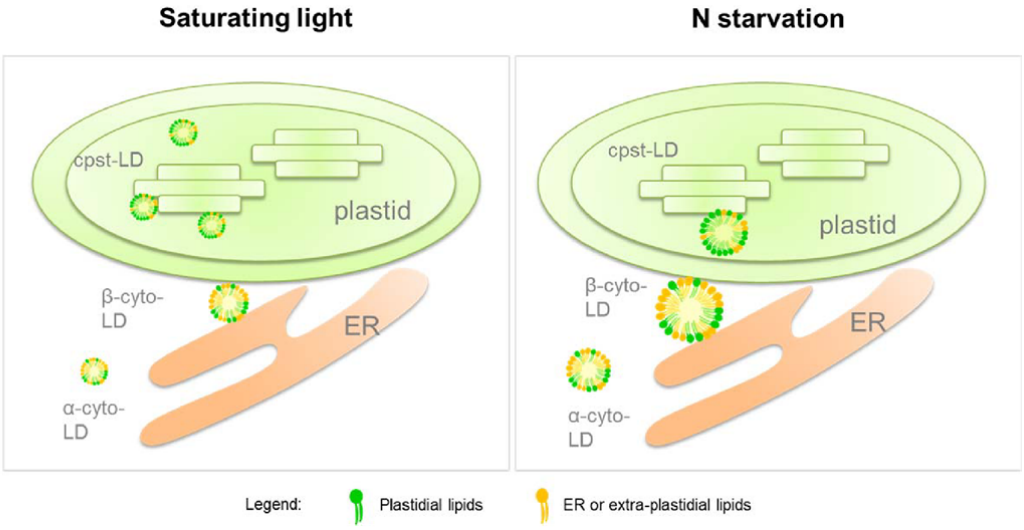
Origin of TAGs under Saturating Light

An increase in total fatty acids was seen together with an increase in C/N ratio in cells exposed to SL (**supplemental Figure S1**). The positive correlation between TAG levels and C/N ratio has also been observed previously in the seeds of Arabidopsis when the plant is grown under increasing irradiance (Li et al., 2006). Thus, after being exposed to SL, the cells re-adjust their metabolism to store the extra carbon and energy as energy-dense materials i.e. starch and oil. These storage compounds provide a temporary reservoir to accommodate the extra fluxes of ATP and reducing powers thereby ensuring cell energetic homeostasis. This increased de novo fatty acid synthesis is further reflected in the changes in total fatty acid compositions before and after SL exposure (**Figure S1D;** and **Figure 6C**), i.e. increased proportion of saturated C16 fatty acid and a reduction in polyunsaturated fatty acid species. Increased C flow to TAG synthesis under high light have also already been recently reported in the heterokont alga Nannochloropsis, in which activities of acetyl-CoA carboxylase (ACCase) and diacylglycerol acyltransferase (DGAT) are significantly increased following a transition from low to high light cultivation conditions (Ma et al., 2016).

In addition to the increased C flow to fatty acid synthesis, the composition of total polar lipids was altered following exposure to SL. SL-treated cells contained a reduced proportion of monogalactosyldiacylglycerol (MGDG) and increased proportion of digalactosyldiacylglycerol (DGDG) and diacylglycerol *N,N,N*–trimethylhomoserine (DGTS), with the other major lipids remaining constant (**Supplemental Figure S1C**). Taken together, these data suggest that under SL, both *de novo* fatty acid synthesis and membrane lipid

316 remodelling contribute to TAG formation, a phenomenon similar to what is occurring in cells
317 starved for nitrogen.
318
319 **Biogenesis and Physiological Functions of Lipid Droplets under Saturating Light**
320 Most of our knowledge of LD biology in *C. reinhardtii* is gained from studies of LDs
321 induced by N starvation. Three types of LDs (α-cyto-LDs, β-cyto-LDs, cpst-LDs) have been
322 distinguished in *C. reinhardtii* upon N starvation; the types and relative abundance of each
323 LD population differs depending on the cultivation condition and on the genotype (Fan et al.,
324 2011; Goodson et al., 2011). For example, it has been observed that optimally growing cells
325 contain a few α-cyto-LDs (i.e. free cytosolic LDs), whereas upon N starvation, wild-type
326 strains of *C. reinhardtii* store oil mostly in β-cyto-LDs (i.e. LDs formed at the junction
327 between ER and plastid envelop), whereas the starch-less mutant *bafJ5* accumulates cpst-LDs
328 (i.e. chloroplast-located LDs) in addition to β-cyto-LDs (Goodson et al., 2011). Here we
329 observed that SL can also trigger LD formation. Part of the LDs seem to be inside the plastids
330 (cpst-LDs), and part of them being outside the plastid but in most cases seem to be associated
331 to the plastid envelops (β-cyto-LDs)(**Figure 8**). Lipidomics analyses showed that LDs
332 formed under SL exposure are made of a mixture of saturated and polyunsaturated TAGs,
333 and covered by protein- and lipid-coat rich in components of plastidial origin. The presence
334 of a plastidial TAG biosynthesis pathway is not surprising. It has previously been observed in
335 the N-starved starch-less mutant *bafJ5* (Fan et al., 2011; Goodson et al., 2011), and can also
336 be deduced by the subcellular targeting of key TAG enzymes in the plastid (Li-Beisson et al.,
337 2015).
338 The protein and lipid composition of the LD coat further demonstrated that SL-
339 induced LDs consist of components of mostly plastidial origin. For example, besides
340 common proteins, SL-induced LDs were found to contain a significant subset of plastid-
341 resident proteins including PGRL1, PLP8 and VTE3. PGRL1 is a major component of cyclic
342 electron flow (CEF) in *C. reinhardtii*, and has been proposed to supply additional ATP for
343 photosynthesis, particularly in conditions of high ATP demand (Tolleter et al., 2011; Dang et
344 al., 2014). The PGRL1 deficient mutant (*pgrl1*) has been recently reported to accumulate less
345 neutral lipids under N starvation, and it was suggested that CEF supplies ATP for lipid
346 biosynthesis during N-starvation (Chen et al., 2015). The association of PGRL1 with LDs of
347 SL-exposed cells expands its role in supplying ATP for TAG synthesis under SL conditions.
348 Interestingly, homologs of the two other proteins (PLP8 and VTE3) in the model plant
349 *Arabidopsis thaliana* and have been found to be associated with plastoglobules (PGs), a type

Figure 8. A model on LD biogenesis in cells exposed to saturating light or N-starvation.
Three types of LDs were drawn, partly based on observations made in this study, and partly based on that Goodson et al (Goodson et al., 2011).
Cpst: chloroplast; cyto: cytoplasm



350 of LDs specific to plant plastids (Kessler and Vidi, 2007). PLP8 is known to play a structural
351 role, whereas VTE3 is required for vitamin E synthesis and recycling (Martinis et al., 2013).
352 Vitamin E synthesis is essential for photo-adaptation to survive in higher irradiance. The

353 presence of VTE3 in isolated SL-LDs supports the view that LDs play a photo-protective
354 roles via synthesizing oil-soluble vitamins.
355 We conclude that a significant sub-population of SL-induced LDs is associated to the
356 chloroplast (cpst-LDs and β-cyto-LDs), consistent with our microscopic observations. Under
357 SL exposure, wild-type strains of *Chlamydomonas* would mostly form cpst-LDs. The
358 formation of cpst-LDs have previously been observed in the starch-less mutant *bafJ5* during
359 N starvation (Goodson et al., 2011). Therefore, cpst-LDs are formed either in the absence of
360 the 'starch' (*bafJ5* mutant), or under a high fluence rate, extra photosynthetic products being
361 diverted to the formation of LDs inside the plastid. Both situations create an imbalance in the
362 'supply and demand' of reduced carbon (discussed in the next section) and favour the
363 formation of plastidial LDs, a convenient and local solution. However, in the absence of the
364 protein/lipid composition data for cpst-LDs of the starch-less mutant, we cannot assert that
365 the two cpst-LDs are of the same nature, and this question should be investigated in the
366 future.
367 It should bear in mind that LD isolates in each condition most likely represent a
368 mixture of LDs of chloroplast-LDs (cpst-LDs) and cytosolic-LDs (cyto-LDs). The difference
369 in protein relative abundance (i.e. as shown by rankings)(**Table 2**) could be because these
370 proteins are indeed present in much less concentration in all sub-population of LDs induced
371 by SL than those triggered by N-starvation, or alternatively, it could be that these proteins
372 were only associated with a specific sub-population of LDs in the SL-exposed cells. This and
373 other possibilities can only be answered once the subcellular localization information is
374 available for these proteins under the different culture conditions.
375
376 **Imbalance Between Photosynthesis and Growth Governs Reserve Accumulation**
377 Previous studies have shown that oil accumulation is triggered by conditions which impair
378 cell growth (Merchant et al., 2012; Liu and Benning, 2013). Increasing light intensity has also
379 been reported as a means to increase oil content when combined with N starvation (Klok et
380 al., 2013; Kandilian et al., 2014). The latter two studies have shown that in conditions of N
381 starvation, high light participates to increase the energy imbalance, resulting in a higher TAG
382 storage in the heterokont *Nannochloropsis oculata* and the green microalga *Neochloris*
383 *oleoabundans*. The current study provides a detailed report on the effect of light on reserve
384 accumulation under optimal growth conditions for the model microalga *C. reinhardtii*. We
385 found that SL-exposed cells accommodate an excess of light energy by storing reduced
386 carbon in the forms of starch and neutral lipids.

The impact of high light on storage reserve accumulation is sometimes considered as a consequence of a 'light stress' effect. We observed a 'burst' in oil/starch content following SL-exposure. This suggests that cells respond to SL in two phases: an initial strong response, followed by an acclimated phase. This initial burst is most likely due to a combined stress response with an increased C flux toward reserve formation at the beginning, followed by a metabolic adaptation to the increased irradiance. As cultivation under SL proceeded, acclimated cells synthesized higher amounts of carbon reserves simultaneous to an increased growth rate. Thus saturating light combined with a non-limiting CO_2 supply induces significant oil accumulation in *C. reinhardtii* in conditions that supports maximal growth. We therefore conclude that oil accumulation is not necessarily linked to a stress effect inducing growth impairment, but may occur in conditions of maximal growth.

Upon steady-state SL exposure, the photosynthetic capacity may exceed the cell division and growth capacity, thus creating an imbalance between the formation of photosynthetic products and the capacity for their direct consumption. The cellular metabolism would thus be re-adjusted to store the extra energy as energy-dense materials i.e. starch and oil. These storage compounds provide a temporary reservoir to accommodate the extra fluxes of ATP and reducing power thereby ensuring cell energetic homeostasis. Based on these data, we propose a more general model in which starch and oil accumulations are triggered when an imbalance between photosynthetic CO_2 fixation and growth occurs (**Figure 9**). Such an imbalance may occur in conditions where growth is primarily affected (such as nutrient starvation, heat, salinity or chemical treatment…), but may also occur in conditions of SL where photosynthetic CO_2 fixation exceeds the ability of algae to grow and replicate. The large increase in neutral lipid and starch productivity achieved under SL clearly points out the biotechnological advantage to increase reserve accumulation under conditions not compromising growth in green microalgae.

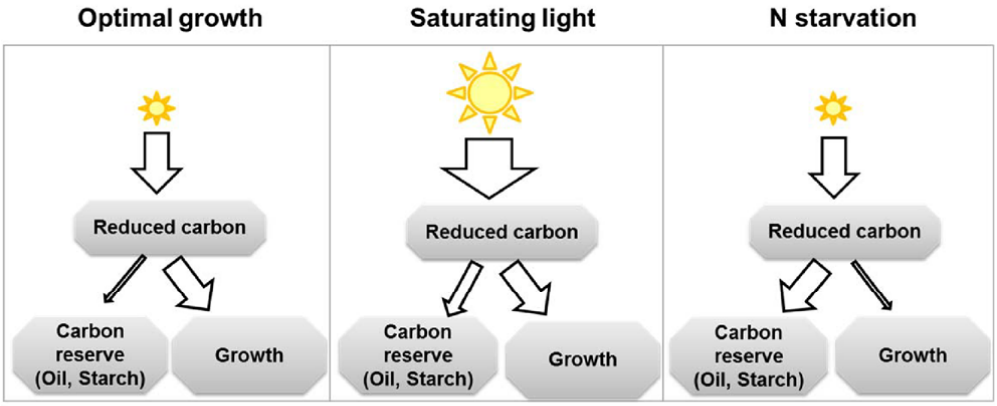
MATERIALS AND METHODS

C. reinhardtii Strains and Pre-Cultures

The wild-type *C. reinhardtii* strain CC-124 (*mt- nit1 nit2*) was used throughout this study. Cells were usually maintained in Tris-Acetate-Phosphate (TAP) medium (Harris, 2001) in Erlenmeyer flasks at 25°C with shaking at 120 rpm under constant illumination (150 μmol photons m^{-2} s^{-1}). For photoautotrophic growth, cultures grown in minimal medium (MM) were kept under identical conditions with additional supply of 1.8% CO_2 in air (Cagnon et al.,

Figure 9. Carbon reserve formation in *Chlamydomonas* in response to energy imbalance: a model

During normal growth (low light, nutrient-replete conditions), *Chlamydomonas* cells convert most of the solar energy they have captured through the photosystems and fixed as organic compounds, to support biomass growth (cell division); only a small fraction is used to make temporary carbon reserves (starch and oil). Creating an energy imbalance either through an increased input of energy (saturating light with respect to biomass growth), or a decrease in energy use (through blocking growth via nitrogen starvation) results in re-allocation of the excess of energy to the synthesis of reserve compounds (oil and starch).



421 2013). Cell numbers were counted using a Beckman-Coulter multisizer (Beckman Coulter).
422 Cellular concentration was determined as number of cells per mL of culture, or total cellular
423 volume (μm^3) per mL of culture.
424

Cultivation in Photobioreactors (PBRs)

PBR cultures were cultivated in MM media in automated Biostat A Plus PBR systems (Sartorius Stedium) as described previously (Dang et al., 2014). Cells were maintained at a constant OD_{880nm} (= 0.4) throughout experimentation. Specific growth rates were determined from the measurement of fresh medium added to the turbidostat to maintain a constant biomass concentration. All the PBRs were stirred at a constant 300 rpm.

Quantification of Starch

Starch were isolated and quantified based on the protocol described in (Klein and Betz, 1978). Cells were harvested bycentrifugation of *Chlamydomonas* cells at 1000 *g* for 3 min. Cellular pellets were resuspended in 1 mL methanol, and centrifuged again for 3 min at 1000 *g* to remove all chlorophyll. The methanol washed pellet was air-dried under a fume-hood, and subsequently resuspended in 400 µL of distilled water, samples were then autoclaved for 20 min at 121°C. Amyloglucosidase (0.2 U - Roche) was added and samples were incubated for 1 h at 60°C. Samples were then centrifuged to pellet particulate matter. Glucose was measured with an YSI2700 select sugar analyser (YSI Life Sciences) using commercial glucose as a standard.

Isolation of Lipid Droplets (LDs)

In order to isolate low-abundant LDs, a previously established *Chlamydomonas* LD isolation protocol (Nguyen et al., 2011) was used with the following minor changes. A desktop ultra-centrifuge replaced the standard ultracentrifuge, and smaller 5-mL ultracentrifuge tubes were used to decrease the surface area of the lipid pad, and increase the ease with which the lipid pad is completely recovered. Additionally, to avoid loss of material the hexane wash was omitted and during the final wash a sucrose-free buffer was used to replace the 0.4 M sucrose buffer. In the final step, a sucrose free buffer floats above all other buffers and allows for easier isolation of the final washed LDs.

Lipid Extractions, Quantification of Lipid Classes by Thin Layer Chromatography (TLC) and Lipid Molecular Species Analysis Using an Ultra-Performance Liquid Chromatography – Tandem Mass Spectrometry (UPLC-MS/MS)

A modified Bligh and Dyer method (Bligh and Dyer, 1959) was used to extract lipids. Samples were initially dissolved by vortexing in 1 mL quenching solution (1 mM EDTA, 0.15 M acetic acid) in an 8-mL glass tube with a Teflon screw-cap. Three mL of

methanol:chloroform (2:1 v/v) was added, if samples were to be used for LC-MS/MS at this stage internal standards TAG51:0 (comprised of 17:0/17:0/17:0 fatty acids) and PtdEtn34:0 (comprised of 17:0/17:0 fatty acids) were added. Samples were then vortexed for 10 min, 1 mL of chloroform and 0.8 mL of KCl (0.8% w/v) was added, and samples were vortexed again for 10 min. Phases were separated by centrifugation at 1000 *g* for 2 min at 4°C. The lower phase was then isolated and transferred to a new clean glass tube. To the remaining phase, 1 mL of hexane was added to extract again the remaining lipids. Phases were then separated by centrifugation at 1000 *g* for 2 min at 4°C. The upper phase containing lipids was then transferred to the chloroform extract using a glass Pasteur pipette. The combined chloroform and hexane extracts were then dried under a flow of nitrogen and re-suspended in a mixture of chloroform:methanol (2:1 v/v) for subsequent analyses.

To analyse TAG content in cells and major polar lipid species, total lipids extracted by the above protocol were processed using an automated "High-Performance Thin-Layer Chromatography" platform (HP-TLC, CAMAG). The detailed TLC method has been described in (Siaut et al., 2011). Lipid molecular species were analysed by LC-MS/MS and conditions were detailed in (Légeret et al., 2016).

Generation of Fatty Acid Methyl Esters (FAMEs) and Quantification by Gas Chromatography-Flame Ionization Detector (GC-FID)

Conversion of total lipids to their fatty acid methyl esters were carried out as described previously (Siaut et al., 2011). A fraction of extract was mixed in an 8-mL glass tube with 1 mL of methanol containing 5% H_2SO_4 v/v, to which 10 µL butyrated hydroxyl-toluene (BHT) (1% v/v) and 5-25 µg of TAG(17:0/17:0/17:0) standard were added. Each sample was vortexed for 30 sec, and heated to 85°C for 90 min. After cooling down to room temperature, 1.5 mL 0.9% NaCl (w/v) was added together with 1 mL hexane. Samples were vortexed for 10 min, and centrifuged at 1000 *g* for 2 min at 4°C. The upper (organic) phase was then isolated with a clean glass Pasteur pipette, and evaporated to dryness under a stream of nitrogen. Samples were finally resuspended in hexane. Fatty acid methyl ester species were separated by the use of a 'TRACE GC Ultra' gas chromatograph (Thermo Fisher Scientific, USA) using a polar TR-WAX column (Thermo Fisher Scientific, 30m x 0.25mm). Detailed GC conditions have been described in (Nguyen et al., 2013).

Protein Extraction, SDS-PAGE and Proteomic Analysis

Proteins were extracted from isolated LDs by addition of cold acetone (80% v/v) and SDS (0.3% v/v). Extracted proteins were separated on SDS-PAGE following standard protocols (Sambrook and Russell, 2001). Proteins were then stained with a silver nitrate solution for 30 min. For proteomics analysis, samples in biological duplicates were concentrated at the top of a NuPage precasted gel (Invitrogen) ("stacking" migration). Bands were cut and submitted to LC-MS/MS analysis following the same procedure as reported in (Nguyen et al., 2011) except for the following: the LC-MS/MS experiment were performed on a LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) coupled to an Ultimate 3000 LC system. The *C. reinhardtii* protein database used for Mascot (v2.4, 4, Matrix Science) searches was constituted of the models downloaded from Phytozome (release 169_v4.3, http://phytozome.jgi.doe.gov/), plus mitochondrion and plastid-encoded proteins from NCBI (total of 17191 entries). Acetyl (protein *N*-α-acetylation) and methionine oxidation were set as variable modifications and carbamidomethyl cysteine as fixed modification. Two mis-cleavages were allowed. Mascot search results were automatically filtered as previously described (Nguyen et al., 2011) with the IRMa 1.31.1 version software (Dupierris et al., 2009).

Microscopy

Samples were stained first with Nile red (Sigma) at a final concentration of 1 µg mL^{-1} (from a stock solution of 1 mg mL^{-1} in methanol) for 10 min in the dark, then imaged with a Confocal microscope TCS SP2 using an 63xoil immersion objective (Leica; Germany). The Nile red signal was captured using a laser excitation line at 488 nm; and emission was collected between 554-599 nm. Chlorophyll autofluorescence was measured by excitation at 635 nm and emission captured between 650-714 nm. Pseudo colours for images were obtained using the ZEN (Carl Zeiss) software.

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Tables

Table 1. Identity and abundance of the top 30 proteins found to be associated to LDs isolated from cells exposed to saturating light (SL) for 9 h.

\N: no annotation is available.

Abbreviations: SL, saturating light; SSC, specific spectra count; emPAI: exponentially modified protein abundance index. The rankings were calculated based on a computed calculation of total number of SSCs and values of exponentially modified protein abundance index (emPAI), and can be used to evaluate the relative abundance of each protein in a sample.

ID (Phytozome or JGI)	Symbol	Defline	SSC	emPAI	Rank
Cre61.g792450.t1.2	\N	\N	48.5	0.765	1
Cre07.g324200.t1.1	BTA1	Diacylglyceryl-*N,N,N*-trimethylhomoserine synthesis protein	40.5	2.071	2
Cre01.g013700.t1.1	\N	\N	24.5	7.566	3
gi|41179057|ref|NP_958414.1|	\N	ATP synthase CF1 beta subunit [Chloroplast encoded]	24	1.978	4
gi|41179032|ref|NP_958388.1|	\N	Photosystem II 47 kDa protein [Chloroplast encoded]	24	1.452	5
Cre17.g698000.t1.1	ATP2	Mitochondrial F1F0 ATP synthase, beta subunit	21	1.683	6
Cre12.g540550.t1.2	α/β hydrolase	\N	21	1.523	7
Cre05.g241950.t1.1	\N	\N	20.5	6.462	8
Cre41.g786600.t1.1	AAA1	Plastidic ADP/ATP translocase	20.5	1.239	9
gi|41179063|ref|NP_958420.1|	\N	Photosystem II protein D2 [Chloroplast encoded]	20	1.713	10

Cre04.g229300.t1.2	RCA1	Rubisco activase	19	2.027	11
Cre12.g498600.t1.1	\N	Eukaryotic translation elongation factor 1 alpha	18	1.612	12
gi|41179065|ref|NP_958422.1|	\N	Photosystem II 44 kDa protein [Chloroplast encoded]	17.5	1.394	13
Cre09.g405500.t1.2	MLDP	Major lipid droplet protein	17	4.425	14
gi|41179003|ref|NP_958358.1|	\N	Cytochrome f [Chloroplast encoded]	16.5	2.316	15
Cre09.g398700.t1.1	CFA2	Cyclopropane-fatty-acyl-phospholipid synthase	16	1.365	16
Cre09.g386650.t1.1	ANT1	ADP/ATP carrier protein, mitochondrial	14.5	2.047	17
Cre16.g672650.t1.1	\N	Mitochondrial substrate carrier protein, possible 2-oxoglutarate/malate carrier	14	2.618	18
Cre02.g081050.t1.1	FAP24	Flagellar Associated Protein	14	0.965	19
Cre29.g778950.t1.1	FMG1-B	Flagella membrane glycoprotein, major form	14	0.104	20
Cre03.g171050.t1.2	GHL1	Glycosyl hydrolase	13	0.943	21
Cre02.g130650.t1.1	\N	\N	13	0.583	22
Cre01.g002500.t1.1	COP2	Chlamyopsin, light-gated proton channel rhodopsin	12.5	2.709	23
Cre17.g738050.t1.1	AGG4	Flagellar membrane protein, paralog of AGG2	12	2.590	24
Cre17.g734300.t1.2	\N	\N	12	0.411	25
Cre15.g638500.t1.1	CYC1	Ubiquinol:cytochrome c oxidoreductase cytochrome c1	11.5	1.561	26
Cre03.g172300.t1.1	\N	Mitochondrial phosphate carrier protein	10.5	0.901	27
Cre01.g025350.t1.1	FAP235	Flagellar Associated Protein	10	2.777	28
Cre03.g196900.t1.1	\N	\N	10	1.813	29
Cre23.g766250.t1.1	LHCBM1	Chlorophyll a/b binding protein of LHCII	10	1.396	30

Table 2. A short list of key proteins consistently found in all 3 previously published proteomic studies of LDs isolated from *C. reinhardtii*.

Note: these proteins have demonstrated roles related to LD biology, and the cited previous proteomics studies are (Moellering and Benning, 2010; Nguyen et al., 2011; Tsai et al., 2015).

552 Abbreviations: MM, minimal medium; N, Nitrogen; SL, saturating light; Score, Mascot score
553 (average from two replicates); SSC, specific spectra count (average from two replicates).

Synonym	Defline	LDs (MM-N)				LDs (SL)			
		Peptides	SSC	Score	Rank	Peptides	SSC	Score	Rank
BTA1	Diacylglyceryl-*N,N,N*-trimethylhomoserine synthesis protein	41	94	2120	1	24	41	1198	2
MLDP	Major lipid droplet protein	19	75	1467	2	13	17	982	14
α/β hydrolase	alpha beta hydrolase	28	63	1832	3	13	21	970	7
LCS2	Long chain acyl-CoA synthetase	32	54	1646	4	7	7	323	59
GHL1	Glycosyl hydrolase	23	46	1268	5	11	13	597	21
CFA2	Cyclopropane-fatty-acyl-phospholipid synthase	19	34	1147	6	10	16	597	16
LPLAT	Lysophospholipid acyltransferase	20	33	966	7	4	4	213	129
TUB1	Beta tubulin 1	15	16	673	23	6	6	272	85
TGD2	Permease-like component of an ABC transporter	3	3	152	252	5	5	270	98

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