Title:

Expression of *Chlamydomonas reinhardtii* chloroplast diacylglycerol acyltransferase-3 is activated by light in concert with triacylglycerol accumulation.

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Short title:

Light-activated diacylglycerol acyltransferase 3 in *Chlamydomonas*

26 **Abstract**

27 Considerable progress has been made towards the understanding of triacylglycerol (TAG)
 28 accumulation in algae. One key aspect is finding conditions that trigger TAG production without
 29 reducing cell division. Previously, we identified a soluble diacylglycerol acyltransferase (DGAT),
 30 related to plant DGAT3, with heterologous DGAT activity. In this work, we demonstrate that
 31 *Chlamydomonas reinhardtii* DGAT3 localizes to the chloroplast and its expression is activated by
 32 light, in correspondence with TAG accumulation. *Dgat3* mRNAs and TAGs increased in both wild
 33 type and starch-deficient cells grown with acetate upon transferring them from dark or low light to
 34 higher light. Light-activated DGAT3 expression and TAG accumulation depended on the
 35 preexisting levels of TAGs, suggesting the existence of a regulatory loop. These results indicate that
 36 DGAT3 could be responsible, at least in part, for light-dependent TAG accumulation. Moreover, our
 37 results present DGAT3 as a promising target of future studies oriented to the industrial applications
 38 of TAGs.

40 **Keywords**

41 *Chlamydomonas*/ chloroplast/ diacylglycerol acyltransferase/ light activation/ triacylglycerols.

43 **Introduction**

44 Current interest in the development of sustainable sources of liquid fuels and other industrial
 45 compounds has produced a remarkable expansion in the study of eukaryotic microalgae as
 46 feedstock for triacylglycerols (TAGs) (Banerjee et al., 2016; Li-Beisson et al., 2019; Sato et al.,
 47 2017; Xu et al., 2016). One of the most remarkable contributions to this field was the finding that
 48 neutral lipid generation in algae could be induced by stress and nutrient depletion (Allen et al.,
 49 2018; Pal et al., 2011; Rearte et al., 2018; Sharma et al., 2012; Yang et al., 2015). The best
 50 characterized condition is nitrogen deprivation, which stimulates TAG production in the majority of

the algal species studied, independently of their taxonomic classification and lifestyles (Blaby et al., 2013; Martin et al., 2014; Msanne et al., 2012; Weng et al., 2014). Likewise, most of the enzymes that participate in TAG synthesis investigated to date are those that respond to a lack of nitrogen (Banerjee et al., 2017; Boyle et al., 2012; Chen et al., 2015; Guo et al., 2017; Xu et al., 2018). These enzymes are mostly homologs of proteins characterized in animals, yeast and plants that are involved in the conventional pathway carried out in the endoplasmic reticulum (ER)/cytosol (Bhatt-Wessel et al., 2018; Liu et al., 2011; Lung and Weselake, 2006; Shockey et al., 2006). Despite these advances, the drawback of any production scheme based on nitrogen deprivation is that TAG accumulation occurs in parallel to a decrease in algal cell division, which reduces total lipid productivity (Takeuchi and Benning, 2019). Hence, finding conditions that yield large amounts of TAGs during optimal growth is one of the big conundrums of this research area.

With this challenge in mind, we set out to search for novel enzymes that could participate in TAG synthesis in algae of diverse evolutionary origins. Using a HMMER iterative approach (Bagnato et al., 2017a), we identified a soluble diacylglycerol acyltransferase (DGAT) exclusive to green algae and moderately related to plant DGAT3 (Bagnato et al., 2017b). DGATs catalyze the formation of TAG by performing an acylation of a fatty acid (FA), most frequently activated as an acyl-CoA, onto the sn-3 position of a molecule of diacylglycerol (DAG) (Yen et al., 2008). Phylogenetic analysis showed that the DGAT3 clade shares a most recent ancestor with a group of uncharacterized proteins from cyanobacteria, suggesting a prokaryotic origin, whereas the canonical DGATs, DGAT1 and DGAT2, are only related to proteins from eukaryotes. In addition, most of the algal sequences within the DGAT3 group have predicted chloroplast localization (Bagnato et al., 2017b). In plants, DGAT3 has been characterized in only a few species, including *Arabidopsis thaliana*, *Vernicia fordii* (tung tree) and *Arachis hypogaea* (peanut) (Aymé et al., 2018; Cao et al., 2013; Chi et al., 2014; Hernández et al., 2012; Saha et al., 2006). DGAT3 protein sequences show very distinctive features, particularly in comparison with DGAT1 and DGAT2 (Cao, 2011; Liu et

al., 2012; Turchetto-Zolet et al., 2011). The most conspicuous difference between DGAT3 and its counterparts is the fact that its sequence exhibits a carboxy-terminal iron-sulfur (2Fe-2S) cluster-binding domain that has a fold similar to that present in thioredoxins (Aymé et al., 2018; Bagnato et al., 2017b). Although the role of this domain in DGAT3 remains unknown, the majority of the homologs that have been studied to date act as low-potential electron carriers (Fukuyama, 2004; Zu et al., 2002).

The acylation activities of DGAT1 and DGAT2 have been widely described and reviewed (Yen et al., 2008). The conserved amino acids of these two protein families have been extensively studied in animals, yeast and plants and some of the residues that are important for catalysis have been determined in mutagenesis experiments (Liu et al., 2011; Lopes et al., 2014; Stone et al., 2006). In contrast, DGAT3 activity has only been demonstrated *in vitro* for the *A. thaliana*, *A. hypogaea* and *Chlamydomonas reinhardtii* proteins (Aymé et al., 2018; Bagnato et al., 2017b; Chi et al., 2014; Hernández et al., 2012; Saha et al., 2006) and little is known about the identity or location of the acylation domains. We previously reported that *C. reinhardtii* DGAT3 expressed in *Escherichia coli* produced and increase in TAGs in the presence of oleate (Bagnato et al., 2017b). In DGAT3 protein sequence, we identified three histidine-containing motifs similar to those that perform acylation in glycerol-3-phosphate acyltransferase (GPAT) and acylglycerol-3-phosphate acyltransferase (AGPAT) (Takeuchi and Reue, 2009; Yamashita et al., 2014), but those motifs have not been functionally analyzed.

The content of hydrophobic regions in DGAT3 is considerably lower than those of DGAT1 and DGAT2. Additionally, many of the hydrophobic regions of the latter two proteins translate into transmembrane segments, whereas DGAT3 appears to have no transmembrane domains. Most remarkably, in DGAT1 and DGAT2, the catalytic motifs either flank or are partially embedded in hydrophobic regions, whereas the putative catalytic motifs of *C. reinhardtii* DGAT3 are flanked by hydrophilic regions. All these observations led us to hypothesize that DGAT3 participates in a

soluble TAG synthesis pathway in the chloroplast, where it could act as an electron carrier in addition to performing an acylation reaction (Bagnato et al., 2017b).

In this work, we demonstrate empirically that *C. reinhardtii* DGAT3 is localized to the chloroplast and advance into its metabolic regulation. We demonstrate that DGAT3 expression is induced by light in the presence of acetate and that this induction occurs in correspondence with light-dependent TAG accumulation. Both DGAT3 expression and TAG levels increase upon shifting cells from dark or low light to higher light, albeit affected by the particularities of each strain. Altogether, our results suggest that light-dependent TAG accumulation could be carried out, at least in part, by the action of DGAT3. In addition to providing insights into the possible enzymatic components of light-activated TAG production, our results further advance into the relationship between light and carbon in regards to TAG metabolism.

112

113 Results

Figure 1A shows a schematic representation of *C. reinhardtii* DGAT3 full-length protein. This polypeptide has a transit peptide of approximately 70 amino acids, as predicted by both PredAlgo and TargetP (Bagnato et al., 2017b). Contiguous to this fragment, the sequence features a region of about 223 amino acids with no apparent structure. In this disordered portion, we previously predicted at least two putative acyltransferase motifs that include the conserved histidine that most frequently acts as catalytic residue (Bagnato et al., 2017b). To the carboxy end, DGAT3 has a conserved 2Fe-2S cluster-binding domain, which we previously modeled on the thioredoxin-like ferredoxin of *Aquifex aeolicus* (Yeh et al., 2000), followed by a carboxy end with no significant similarity to any characterized motif.

In order to confirm the chloroplast targeting prediction, we isolated *C. reinhardtii* chloroplasts from two cell wall-less strains, cc-5155 and cc-4348 (*sta6*) and analyzed the presence of DGAT3 by western blot analysis. Figure 1B shows that DGAT3 protein was present in both total

126 protein and chloroplast protein samples from both strains. As expected, the chloroplast polyprotein
127 of elongation factor Ts (PETs) (Beligni et al., 2004) was also detected in both fractions, whereas
128 only traces of beta tubulin, a cytosolic marker, were observed in chloroplasts. These results indicate
129 that *C. reinhardtii* DGAT3 is indeed a chloroplast protein.

130 All proteins containing 2Fe-2S cluster-binding domains characterized so far act as low
131 potential electron carriers (Fukuyama, 2004; Zu et al., 2002). This prompted us to hypothesize that
132 DGAT3 might accomplish the synthesis of TAGs in conjunction with the photosynthetic machinery
133 and, hence, respond to light. TAGs were previously shown to increase in *C. reinhardtii* cc-124 cells
134 after transferring them from low light to saturating light (Goold et al., 2016), but the enzymes
135 responsible for this accumulation remain unknown. With the purpose of analyzing a potential
136 connection between DGAT3 and light, we evaluated the effect of shifting cells from dark to light in
137 classical light-activation experiments (Beligni et al., 2004; Coragliotti et al., 2011). Cells were
138 cultured in Tris-Acetate-Phosphate (TAP) media under continuous light of 4,500 lux until they
139 reached a cell density of 3×10^6 cells/ml, transferred to the dark for 16 h and then shifted back to
140 the initial light condition (4,500 lux). This experiment was done with both wild type cc-125 and cc-
141 4348 cells. The cc-4348 strain is cell wall-less and has a deletion in the *STA6* gene (encoding a
142 subunit of ADP-glucose pyrophosphorylase), which renders this strain deficient in starch synthesis
143 (Zabawinski et al., 2001). Expression was determined by RT-qPCR. Two genes were evaluated as
144 potential endogenous controls, *actin* and *gblp* (Table 1). According to the analysis of RT-qPCR data,
145 *gblp* levels resulted stable in the experiments tested (Supplementary Data, Tables S1 and S2),
146 whereas *actin* was only relatively stable in cc-4348 experiments and fluctuated considerably upon
147 transferring cc-125 cells from dark to light (data not shown). By virtue of these results, the
148 expression levels of all target genes were subsequently normalized to *gblp* levels.

149 Figure 2A shows that, in the cc-4348 strain, *dgat3* mRNAs increased 2-fold as early as 1 h
150 into the light period, peaked at about 12 h (an average value of about 4-fold) and remained elevated

151 until the end of the experiment (3-fold). In cc-125 cells, no significant variations in *dgat3* mRNAs
152 were observed between the end of the dark and the light period (Figure 2A, Table S3). In the same
153 assays, TAGs accumulated in cc-4348 upon transferring the cells to light, reaching a maximum
154 intensity at 6 h into the light period and decreasing considerably at 24 h (Figure 2B). In contrast, in
155 cc-125 cells, no remarkable variations in TAG content were observed during the first 12 h of light
156 compared to the end of the dark, and only a decline at 24 h could be observed (Figure 2B, Figure
157 S1). These results show that DGAT3 expression and TAG content behave similarly upon shifting
158 TAP-cultured cells from dark to moderate light conditions. While both *dgat3* mRNA levels and
159 TAGs increased within the first 12 h into the light period in the cc-4348 strain, they remained fairly
160 stable in the wild type strain.

161 To determine the response to light of other enzymes that perform TAG synthesis, we
162 analyzed the mRNA levels of DGAT1 and phospholipid diacylglycerol acyltransferase (PDAT). We
163 selected those two enzymes since both have the potential to carry out TAG synthesis in the
164 chloroplast, according to previous evidence (Bagnato et al., 2017b; Yoon et al., 2012). Quality
165 control of RT-qPCR data indicated that the results obtained for *dgat1* mRNAs were sound, whereas
166 *pdat* RT-qPCRs were only reliable for cc-4348. For this last strain, although the high counts (Cts)
167 obtained suggested that the levels of *pdat* mRNAs were considerably low, the melting curves and
168 agarose gels obtained showed that a single, pure amplicon was being amplified (Tables S1 and S2).
169 For cc-125 *pdat* mRNAs, Cts were even higher and the melting curves contained multiple peaks,
170 turning the analysis unreliable (data not shown).

171 Figure 2A shows that both *dgat1* and *pdat* mRNAs decreased moderately in TAP-grown cc-
172 4348 cells 1 h after shifting the cells from dark to light and returned to the initial levels between 6
173 and 24 h into the light period. In cc-125 cells, no significant variations in *dgat1* mRNAs were
174 observed between the end of the dark and the light period (Figure 2A, Table S3). In general, these

175 results suggest that TAG accumulation induced by moderate light in cc-4348 cells cannot be
176 explained by the pattern of *dgat1* and *pdat* mRNAs in our experimental conditions.

177 TAG accumulation in the chloroplast has been reported to occur only in the presence of
178 acetate (Fan et al., 2011; Goodenough et al., 2014; Goodson et al., 2011). With the aim of analyzing
179 if this pattern also held true for DGAT3 induction in cc-4348 cells, we analyzed *dgat3* mRNAs and
180 TAG accumulation in this strain cultured in minimal media (without acetate) and transferred from
181 dark to light as in the previous experiment. Figure 3A shows that *dgat3* mRNAs were reduced
182 significantly upon transferring the cells from dark to light in minimal media. In the same conditions,
183 TAG content remained relatively stable for the initial hours after shifting the cells to light and
184 decreased mildly at 24 h (Figure 3B, Figure S1). Hence, we concluded that both light-activated
185 DGAT3 expression and TAG accumulation depend upon the presence of acetate in the media in cc-
186 4348 cells, in our experimental conditions.

187 The differences in the accumulation of *dgat3* mRNAs and TAGs between cc-125 and cc-
188 4348 cells shown in Figure 2 are most likely due to cc-4348 deficiency in starch accumulation.
189 Light provides the means to generate reducing power that needs to be harnessed properly in order to
190 protect the photosystems. The relatively high concentration of acetate in TAP media imposes the
191 necessity to store the excess of carbon. In cc-4348, TAGs are the main storage molecules for both
192 carbon and reducing power and, hence, it is not surprising that their levels increase upon
193 transferring the cells from dark to moderate light (4,500 lux). We hypothesized that we could
194 achieve a similar effect in cc-125 cells by increasing the light intensity, in order to subject the cells
195 to a condition in which the excess of reducing power could not be effectively stored solely into
196 starch. To this end, we measured *dgat3* mRNA levels and TAG accumulation in cc-125 cells
197 cultured in TAP media at 4,500 lux, transferred to the dark for 16 h and then shifted to high light
198 (15,000 lux). Figure 4A shows that, unexpectedly, *dgat3* mRNAs declined upon transferring cc-125
199 cells from dark to high light. In the same conditions, TAGs remained relatively stable for the first 12

h, and declined at 24 h into the light (Figure 4B), similarly to the experiment in Figure 2B. When analyzing each sample into detail, we realized that the intensity of the TAG signals in the ED samples relative to the samples harvested in the light, at both moderate and high light, was substantially higher for cc-125 (Figures 2B and 4B) than for the cc-4348 strain (Figure 2B). Since the levels of TAGs after a prolonged period at 4,500 lux were relatively low in the cc-125 strain (Figures 2B and 4B, 24HL samples), we assumed that the high levels of TAGs at the ED were originated from dark accumulation, probably due to a reduction of O₂ in non-aerated, batch cultures, as reported previously (Hemschemeier et al., 2013). In agreement with this assumption, we observed that TAG levels increased during a 48 h-dark period and decreased slowly during the subsequent light period in cc-125 cells (Figure S1).

In order to evaluate the response to light without subjecting cc-125 cells to a dark period, we evaluated DGAT3 and TAG content by replacing the dark interval with a low light (1,500 lux) period of the same length, after which cells were transferred to high light. Figure 5A shows that *dgat3* mRNAs increased 1 h after transferring the cells to high light, peaked at 6 h into the high light period (ca. 5-fold) and declined at longer times, remaining at about 2.5-fold until the end of the experiment. In this case, *dgat1* mRNAs also showed a moderate but steady increase of approximately 2-fold between 1 and 12 h into the high light period, decreasing again to the initial levels at the end of the experiment. Figure 5B shows that, at the end of the low-light period (ELL), TAG levels were relatively low and increased 1 h into the high light period, returning to basal levels afterwards. Altogether, these results show that induction of DGAT3 expression and TAGs by light also occurs in the cc-125 strain.

221

222 Discussion

223 In this work, we report that light-induced TAG accumulation in *C. reinhardtii* occurs in agreement
224 with the expression of DGAT3, a soluble, chloroplast-localized DGAT. In the conditions

investigated, TAG production could not be fully explained by changes in the expression of DGAT1 or PDAT, two enzymes that could potentially synthesize TAGs on the chloroplast envelope. These results point to DGAT3 as a feasible and exciting candidate for light-activated TAG synthesis in the chloroplast, as shown in the model of Figure 6.

In algae, TAG accumulation is mostly associated with the canonical ER/cytosol pathway (Goold et al., 2016; Tsai et al., 2015; Weng et al., 2014). However, under certain conditions, TAGs have also been proposed to accumulate in association with the chloroplast (Fan et al., 2011; Goodson et al., 2011). Goold et al (2016) reported that saturating light triggers TAG synthesis in *C. reinhardtii*, both inside and outside the plastid, mostly associated with the chloroplast envelope. In this work, we report that DGAT3 expression and TAG accumulation do not only respond to high light, but undergo light-dependent activation in a broad sense, since their levels also increase at moderate light intensities, as shown in Figure 6A. True light activation is expected to operate to some extent at all light intensities, including limiting light conditions, in which the rate of light absorption is well matched with the rate of photosynthesis (Björkman and Demmig, 1987). In our case, an increment in the content of TAGs after the transition from dark to limiting light (4,500 lux) occurred in the *sta6* mutant and matched DGAT3 induction. Neither of the other two enzymes evaluated (DGAT1 and PDAT) could explain the boost of TAGs in limiting light in the *sta6* mutant. The evaluation of those two enzymes was not circumstantial. PDAT is targeted to the chloroplast in *C. reinhardtii* and can synthesize TAGs from several substrates (Yoon et al., 2012). DGAT1, though frequently assumed to be localized to the ER, has a predicted chloroplast transit peptide and could be anchored to the chloroplast envelope (Bagnato et al., 2017b). Hence, although we cannot rule out that either post-translational activation or the expression of other enzymes (e.g. DGAT2, phytol ester synthases) participate in moderate light-activated TAG production, the strong agreement between TAG levels and DGAT3 expression provides the appealing possibility that the accumulation of TAGs in response to limiting light is catalyzed by DGAT3. Unfortunately, there are no DGAT3

250 mutants available in the Chlamydomonas Library Project (CLiP), but future work will be aimed at
251 following a reverse genetics approach to interrogate the function of the *dgat3* gene.

252 The effect of high light, although related, has significant differences with light activation. In
253 high light, it has been demonstrated that the rate of photosynthesis becomes saturated, but light in
254 excess is still absorbed and can damage the photosystems and other macromolecules within the cell
255 (Niyogi, 1999). Hence, the response to high light is probably a combination of light activation and
256 stimulation of photoprotective mechanisms. In our case, TAG accumulation in wild type cc-125
257 cells at high light (15,000 lux) was accompanied by an up-regulation of both *dgat3* and *dgat1*
258 mRNAs. Even though not all enzymes that can synthesize TAGs have been evaluated in our high
259 light experiments, one could hypothesize that a more general response to stress is activated in these
260 conditions compared to moderate light. Recently, it was demonstrated that high light induces ROS,
261 which leads to TAG accumulation, in agreement with an increase in DGAT2A and PDAT (Chouhan
262 et al., 2020). All these results suggest that light activation and light stress operate via related, but not
263 necessarily identical, pathways. We propose that, while limiting light might only activate DGAT3
264 expression and TAG accumulation in the chloroplast (Figure 6A), high light might also activate the
265 expression of other enzymes that participate either in the chloroplast or the ER, as shown in Figure
266 6B and as also proposed previously (Chouhan et al., 2020; Goold et al., 2016). In addition, while
267 DGAT3 expression might be a target of the light signal transduction cascade, other enzymes
268 involved in TAG synthesis might be targets of stress/ROS signaling cascades (Figures 6A and 6B).
269 It is noteworthy that, in both limiting light and high light, the increase in *dgat3* mRNAs and TAG
270 levels occurred as a pulse shortly after transferring the cells to light, consistent with light activation,
271 as has been shown for other targets (Beligni et al., 2004; Gray et al., 2003; Idoine et al., 2014;
272 Salvador et al., 1993). DGAT3 mRNAs were previously shown to also respond to nitrogen
273 deprivation, but this occurred only at long periods after imposing this stress (Boyle et al., 2012;
274 Goodenough et al., 2014; Liu et al., 2016). In the same N deplete conditions, DGAT1, PDAT and a

few of the DGAT2 isoforms had a more rapid response. Altogether, these results suggest that DGAT1, DGAT2 and PDAT are mostly related to stress as already proposed, whereas DGAT3 might be mainly regulated by light and, perhaps indirectly or circumstantially, moderately responsive to sources of stress such as nitrogen deprivation.

The idea that algae accumulate TAGs as a sink for excess photosynthetic energy and reductant in order to prevent photochemical damage was proposed long ago (Roessler, 1990). In this widely accepted hypothesis (Du and Benning, 2016; Hu et al., 2008; Klok et al., 2014; Li et al., 2012; Solovchenko, 2012), photosynthetic carbon and energy assimilation that can no longer be directed to growth result in the storage of excess reduced carbon (overflow products), both in the form of starch and TAGs. In this scenario, the differences in the response of DGAT3 and TAGs to light between the wild type and *sta6* strains could be explained by the fact that *sta6* cells cannot synthesize starch and have, hence, an increased pressure for the storage of reduced carbon in the form of TAGs. In this work, we provide evidence that advances further into the differences between the two types of metabolism.

Our TLCs showed that, in wild type cells, TAGs increased in the dark in batch cultures and remained high until the end of this period, whereas *sta6* cells had low levels of TAGs at the end of the dark. As Hemschemeier et al (2013) previously reported, TAGs accumulate in *Chlamydomonas* during extended darkness (24 h) in TAP media, due to the reduction of oxygen concentration in batch cultures. In our experiments, wild type cells incubated in the dark most likely generated energy and reducing power from acetate consumption and starch degradation, with TAG levels rising due to hypoxia. In contrast, in *sta6* cells, the effect of oxygen reduction on TAG accumulation was evidently counteracted by the need to catabolize TAGs for energy and reducing power in the dark. We propose that the differences in light activation between wild type and *sta6* cells are related to this. After a 16-h period of darkness, neither DGAT3 expression nor TAGs increased upon transferring wild type cells to light, and we hypothesize that this is related to the high levels of

300 TAGs at the end of the dark, since the situation changed when darkness was replaced by low light.
 301 At the end of a 16 h low-light period, the levels of TAGs were low, more similar to the levels in the
 302 *sta6* strain at the end of the dark. In this condition, a pulse increase in DGAT3 and TAGs could be
 303 observed after shifting the cells to high light, evidencing that DGAT3 and TAGs do respond to light
 304 in the wild type. More broadly, our results suggest that light-activated DGAT3 expression and,
 305 hence, TAG accumulation are tied to the preexisting levels of TAGs. In other words, light-activated
 306 DGAT3 expression might be subjected to negative feedback inhibition by the end product TAG, as
 307 shown in the model of Figure 6. The existence of regulatory loops for TAG biosynthesis in *C.*
 308 *reinhardtii* has already been proposed (Liu et al., 2016). Since the over-accumulation of TAGs could
 309 lead to cell obesity, growth arrest and cell death, at least during nitrogen deprivation (Goodson et
 310 al., 2011), maintaining TAG levels at bay could ensure that the cell can properly store overflow
 311 products in a sustainable manner during the growth phase.

312 It was previously reported that the production of TAGs that occurs under nitrogen
 313 deprivation was dependent on exogenous acetate for both *C. reinhardtii* *cw15* and *sta6* strains
 314 (Goodenough et al., 2014; Goodson et al., 2011). In this work, the increase in TAGs and DGAT3
 315 levels also depended on the presence of acetate after transferring *sta6* cells from dark to light. In
 316 minimal media, *dgat3* mRNA levels markedly decreased in the light, whereas TAG levels remained
 317 relatively stable for the first 12 h into the light period and decreased moderately at 24 h. These
 318 results extend the observations presented above and suggest that DGAT3 regulation, and eventually
 319 TAG synthesis, are at a crossroads between the provision of reducing power by light and carbon
 320 supply. The sequence features of DGAT3 are ideal for this type of control. The presence of a 2Fe-2S
 321 cluster-binding domain indicates that this protein could accept electrons, directly or indirectly, from
 322 the photosynthetic machinery. We could hypothesize that, as part of DGAT3 regulation, these
 323 electrons are further funneled towards the synthesis of fatty acids and their acylation onto molecules

of DAG (Bagnato et al., 2017b). In this scenario, it is not surprising that the expression of this enzyme is regulated by the two key components of this process: light and carbon.

Although we propose that DGAT3 can synthesize TAGs in the chloroplast, we cannot conclude from our results the final destination of those TAGs. Chloroplast TAGs have been connected to chloroplast lipid droplets (LDs) (Goodenough et al., 2014; Goodson et al., 2011; Goold et al., 2016), but the existence of that entity has recently been questioned (Moriyama et al., 2018). Beyond this controversy, several reports have proposed the appealing idea that a direct metabolic flow occurs between the chloroplast envelope and cytosolic LDs, without the essential intervention of the ER (Moriyama et al., 2018; Goodenough et al., 2014; Liu and Benning, 2013). In addition, chloroplast TAGs could be stored into plastoglobules, lipo-protein structures intimately related to the thylakoid membranes that contain TAGs, among other lipophilic compounds (Steinmüller and Tevini, 1985; Tevini and Steinmüller, 1985). If we consider all these possibilities, DGAT3-dependent TAG synthesis could result in the accumulation of TAGs stored into either plastoglobules or LDs, the latter either in the chloroplast or the cytosol. Future research will surely shed light into the participation of this unique enzyme in the complex metabolism of TAGs in green algae.

Materials and Methods

Chlamydomonas reinhardtii strains and culture conditions

The wild type *C. reinhardtii* strain cc-125 (137c, *mt+* *nit1 nit2*), the cell wall-less strain cc-5155 (*mt+* *cw15*) and the cell wall-less, starch deficient strain cc-4348 *sta6* (*mt+* *sta6-1*), which contains a deletion of the gene encoding an ADP-glucose pyrophosphorylase subunit and, hence, is incapable of starch formation (Zabawinski et al., 2001) were obtained from the *Chlamydomonas* Resource Center, University of Minnesota, MN, USA. Cells were maintained on Tris-Acetate-Phosphate (TAP) (Gorman and Levine, 1965) agar plates. Prior to experiments, cells were inoculated at 10^4

cells mL⁻¹ in 100 ml Erlenmeyer flasks containing either liquid TAP or Tris-minimal media (Gorman and Levine, 1965) and grown on a rotary shaker at 23°C and continuous white light (6,400K) at 4,500 lux (approximately 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). TAP and Tris-minimal media have identical recipes, with the exception that TAP contains 18.3 mM acetate, while Tris-minimal is acetate-free. All experiments were started when cell densities were approximately 3×10^6 cells mL⁻¹. Unless specified, in dark-light experiments cells were transferred to either dark (D) or low light (LL; 1,500 lux, approx. 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) conditions for 16 h. Next, cultures were transferred to light of either 4,500 lux or 15,000 lux (high light; approx. 210 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Samples were taken at 1 h, 6 h, 12 h and 24 h into the light period. Since we wished to analyze the effect of light on mRNA and lipid accumulation, the cultures were grown in batch mode until the end of the dark period, but were switched to semi-continuous mode after transferring them to the light, in order to maintain cell densities constant and avoid an effect of differential cell shading between samples. Previously (Coragliotti et al., 2011), we had done this by centrifuging cells from all time points and resuspending them in fresh TAP to avoid a possible effect of nutrients coming from different volumes of fresh medium between samples. However, we modified this procedure since it was recently reported that centrifugation induces lipid production (Kato et al., 2019). The modified protocol consisted in adding, every hour, the volume of fresh medium necessary to revert the cell density to the value at the end of the dark, which we had previously determined that did not impact the accumulation of the selected target molecules at the times points analyzed (unpublished results). In addition to the maintenance of cell densities, each sample harvested accounted for 10% or less of the total culture volume, in order to avoid drastic changes in culture volume that could affect light penetration. At the end of the experiments, cells were harvested by centrifugation at 2,000 x g for 10 min, supernatants were discarded and pellets were flash-frozen in liquid N₂ and stored at -80°C until further use. At least three independent, non simultaneous, experiments were done for each type of experimental design.

374

375 **Chloroplast isolation**

376 Cells were harvested at 2×10^6 cells mL⁻¹, pelleted at 1,000 x g for 10 min and resuspended in
 377 chloroplast isolation buffer (0.3 M sorbitol, 5 mM MgCl₂, 5 mM EGTA, 5 mM EDTA, 20 mM
 378 Hepes-KOH pH 8.0, 40 mM NaHCO₃). Cells were re-pelleted and washed in chloroplast isolation
 379 buffer two more times. The final suspension was pipetted into an ice-cold N₂ decompression
 380 chamber (Parr Model 4639, Parr Instrument Company, illinois, USA). The chamber was pressurized
 381 at 150 psi for 2 min and de-pressurized at about 3 mL min⁻¹. The cells collected at the release tube
 382 were loaded onto a 20/45/65 % volumetric 3-step Percoll gradient. In such a gradient, intact cells
 383 will sediment at the bottom of the tube, whereas intact chloroplasts will sediment at the interphase
 384 between the 45 and 65 % layers and cell debris and cell contents will sediment in or on top of the 20
 385 % layer. Percoll step solutions were prepared from a 95 % Percoll stock solution (95 % (v/v)
 386 Percoll, 3 % (w/v) PEG 6,000, 1 % (w/v) Ficoll and 1 % (w/v) BSA) diluted into a gradient mixture
 387 (25 mM Hepes-KOH pH 8.0, 10 mM EDTA, 5 % sorbitol). Gradients were centrifuged in a Sorvall
 388 HB-6 swinging bucket rotor at 6,000 x g for 20 min at 4 °C (with the brake off). Intact chloroplasts
 389 were carefully pipetted from the interphase, washed twice in chloroplast isolation buffer and
 390 resuspended in total protein lysis buffer for further analysis.

391

392 **RNA isolation and RT-qPCR**

393 MIQE guidelines (Bustin et al., 2009) were taken into account for experimental design, sample
 394 manipulation and data analyses. RNAs were isolated as described previously (Coragliotti et al.,
 395 2011) with some modifications. The volumes equivalent to 10⁷ cells were harvested per sample and
 396 lysis was done in 1 mL final volume of 70 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM EDTA, pH 8
 397 and 2.7 % SDS at room temperature for 30 minutes. Homogenates were extracted twice with
 398 phenol: chloroform: isoamyl alcohol (25: 24: 1). The upper aqueous phases were precipitated with 1

399 volume of isopropyl alcohol overnight at -20°C. Pellets were washed with consecutive 70% and
400 100% ethanol washes and resuspended in diethyl pyrocarbonate (DEPC)-treated H₂O once dry.
401 RNAs were quantified in a Nanodrop (Thermo Scientific™ NanoDrop™ OneC Microvolume UV-
402 Vis Spectrophotometer) by determining the Abs_{260nm} and purity was estimated with the Abs₂₆₀/Abs₂₈₀
403 and the Abs₂₆₀/Abs₃₂₀ ratios: only samples whose values were approximately 2.0 and 1.34,
404 respectively, were used. In addition, RNA integrity was visualized in 0.9% agarose gels stained with
405 Sybr™ Safe (Thermo Fisher Sci. Cat. #S33102). One microgram of total RNAs from each sample
406 was treated with DNase (Thermo Fisher Sci. Cat. #EN0521) and used for the synthesis of first
407 strand cDNAs using MMLV (Thermo Fisher Sci. Cat. #28025013), both procedures according to
408 the manufacturers' instructions. Copy DNAs were stored at -20°C until further use. For RT-qPCR,
409 *dgat3*, *dgat1*, *pdat*, *gblp* and *actin* mRNAs were analyzed. Details on GeneBank accession
410 numbers, oligo sequences and length and position of the amplicons on the coding sequence of the
411 mRNAs are shown in Table 1. The optimal temperature and dynamic range of each pair of oligos
412 for each type of experiment were determined. We worked within template concentrations in which
413 acceptable linearity (R² ≥0.980) and efficiencies were observed. In addition, only comparisons
414 between genes whose efficiencies differed by less than 10% were made (all between 100 and 109%
415 efficiencies). Sybr-Green-based technology was used for RT-qPCR reactions; mixtures consisted in
416 2 µl of diluted template and 8 µl of FastStart Universal SYBR Green Master (Rox) (Cat. #
417 04913914001, Sigma-Aldrich Argentina). Reactions were set in technical duplicates or triplicates in
418 MicroAmp® Fast Optical 48-well 0.1 mL reaction plates (Applied Biosystems, Cat. #4375816,
419 through Invitrogen Argentina), covered with 48-well optical adhesive film (Applied Biosystems,
420 Cat. #4375323, through Invitrogen Argentina) and run in an Applied Biosystems StepOne™ Real-
421 Time PCR System with the following program: Holding stage: 95 °C for 10 min; cycling stage: 40
422 cycles of 95 °C for 15 sec (melting) and 60 °C for 1 min (annealing and extension), with an
423 additional cycle for the determination of melting curves. Only conditions in which the reaction

melting curve showed a single peak were analyzed, single amplicons were confirmed in agarose gels for selected samples. Data was analyzed using LinReg PCR (Ruijter et al., 2014) (Tables S1 and S2) to determine baselines and calculate individual PCR efficiencies for each sample. Starting concentrations for each sample, expressed in arbitrary fluorescence units (N0s) were used to calculate technical averages and to normalize target genes to reference genes and experimental conditions to control conditions.

430

431 **Protein extraction and western blot analyses**

C. reinhardtii proteins were extracted in 50 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1 % SDS, 1 mM PMSF, 0.5 mM Leupeptin. Protein concentration in homogenates was determined with the bicinchoninic acid reagent using a bovine seroalbumin (BSA) standard curve. Samples were heated for 3 min at 95°C in 60 mM Tris-HCl pH 6.8, 12.5 mM EDTA, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol and 0.02% bromophenol blue and separated in 12% SDS-polyacrilamide gels. PageRuler™ Plus prestained protein ladder (Thermo Fisher Sci. Cat. #26619, Invitrogen Argentina) was used as a size standard. Gels were either stained with 0.25% coomassie brilliant blue R-250 or transferred onto nitrocellulose for western blot analyses. Blots were blocked in 1x TSBT with 5% skimmed milk and incubated with primary antibodies overnight at 4°C on a rotary shaker. The primary antibodies used were: anti-*C. reinhardtii* DGAT3 (1:500 dilution of rabbit polyclonal antisera), anti-*C. reinhardtii* polyprotein of elongation factor Ts (PETs, 1:1000 dilution of rabbit polyclonal antisera) and mouse monoclonal anti-*Drosophila* beta-tubulin (E7 antibody, used at 0.5 µg/ml, obtained from the Developmental Studies Hybridoma Bank). E7 was deposited to the DSHB by Klymkowsky, M. (DSHB Hybridoma Product E7). Blots were washed in 1 x TBST and the corresponding secondary antibodies coupled to alkaline phosphatase were incubated at a 1:15,000 dilution for 1 h at room temperature. Blots were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Argentina).

449

450 **Lipid extraction and thin layer chromatography**

451 Lipid extraction was done according to Bligh and Dyer (1959). Briefly, cells were resuspended in
 452 3.8 ml of chloroform: methanol: H₂O (1: 2: 0.8), vortexed for 20 sec and incubated at room
 453 temperature for 1 h, with occasional vortexing. Total lipid extraction was evidenced by the white
 454 color of the protein solid phase, which indicated that chlorophyll had completely partitioned to the
 455 chloroform phase. For phase partitioning, 1 mL chloroform and 1 mL H₂O were added and tubes
 456 were centrifuged at 6,000 rpm for 2 min at 4 °C. The lower chloroform phase was transferred to
 457 another tube and extracts were dried for 1 h at 48 °C in a rotational vacuum concentrator (Martin
 458 Christ model RVC 2-18). Dried extracts were resuspended in a known volume of chloroform and
 459 spotted on 500 µm silica gel G-60 thin layer chromatography (TLC) glass plates. A mixture of
 460 hexane: sulphuric ether: formic acid (80: 20: 2, v/v) was used as running solvent system. Plates
 461 were run until the solvent front was 1 cm from the top of the plates (approx. 40 min) and dried for 1
 462 h. Lipid spots were located after spraying the TLC plates with 3% CuSO₄ (w/v), 8% H₃PO₄ (v/v)
 463 and exposing them to 180°C for 10 min (Marsh and Weinstein, 1966). Lipid classes were identified
 464 by comparison to standards spotted along with the samples. Olive oil was used as a standard of
 465 TAGs. Oleic acid was used as free fatty acid standard and was kindly provided by Materia Hnos
 466 SACIF, Mar del Plata, Argentina (Product code MATSOL 101 OD). Biological replicates of TLCs
 467 shown in the main figures are shown in Figure S1.

468

469 **Data and statistical analyses**

470 Graphs were done in RStudio, using the dplyr and ggplot2 packages (RStudio Team, 2020). For a
 471 better visualization of changes induced by light, RT-qPCR graphs were done using fold changes
 472 between the light conditions and the corresponding ED or ELL condition. For statistical analyses,
 473 dN0 values were used. These values are equivalent to dCt values, but corrected by LinRegPCR

(Ruijter et al., 2014) for individual sample PCR efficiencies. Correlation between the data from each experiment was evaluated using Pearson's test. Residuals were calculated and used to test for normal distribution (<https://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Regression.htm>). In the absence of evidence against normal distribution, one-way ANOVA was performed in RStudio. Significant differences between light conditions and the corresponding controls (ED or ELL) were evaluated using the post-hoc Dunnett's test, using the RStudio DescTools package. A two-sided alternative hypothesis was used. Significant differences at p-values < 0.001, 0.01, 0.05 and 0.1 were reported. All the results of statistical analyses are shown in Table S3.

482

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489

490 **Competing Interests**

491 The authors declare no conflict of interests.

492

493 **References**

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Table 1. Summary of oligonucleotides used for RT-qPCR and amplicon position and length for each of the genes analyzed.

Name	Accession	Amplicon length (nt)	Oligo names	Oligo Sequence	Start	Stop	Tm	GC%
<i>DGAT3</i>	XM_001691290.1	107	DGAT3Forward	5' AAATGCTCGGCTCGTTCTC 3'	52	71	62	52.6
			DGAT3Reverse	5' TGTGTTGGCATGTGAGAGG 3'	140	159	62	52.6
<i>GBLP</i>	XM_001698013.1	87	GBLPForward	5' ACACCAAGGATGTGCTGTC 3'	314	333	62	52.6
			GBLPReverse	5' TCCACAGCTTGATGGTCTTG 3'	381	401	62	50
<i>DGAT1</i>	PNW88823.1	104	DGAT1Forward	5' CCTTTGCAGGCATCATGTTTC 3'	788	808	62	50
			DGAT1Reverse	5' CGCGATCCAGAAGATGTAGTT 3'	871	892	62	47.6
<i>PDAT</i>	JN815265.1	98	PDATForward	5' CTGGAGGCGGTTGACTATTT 3'	976	996	62	50
			PDATReverse	5' CTGACTAGGCTGTTGCTGTC 3'	1054	1074	62	55
<i>PDAT</i>	JN815265.1	76	PDAT2Forward	5' GCGATTGCGGACACAATTAAG 3'	2051	2072	62	47.6
			PDAT2Reverse	5' TGACATGGTCGCTGTTGAG 3'	2108	2127	62	52.6
<i>Actin</i>	XP_001699068.1	125	ActinForward	5' CGCAAGGACCTCTACAACAA 3'	874	894	62	50
			ActinReverse	5' GCCACCACCTTGATCTTCAT 3'	979	999	62	50

Figure 1. *Chlamydomonas reinhardtii* DGAT3 localizes to the chloroplast. A) Schematic representation of DGAT3 protein showing the predicted chloroplast transit peptide (cTP), the amino terminal unstructured region (UR), the 2Fe-2S cluster-binding domain (2Fe-2S) and the non-homologous carboxy end (CE). The numbers below are the predicted amino acid positions of each domain junction. **B)** Western blot analysis of total protein (TP) and chloroplast protein (CP) fractions from *C. reinhardtii* strains cc-5155 and cc-4348. Five micrograms of proteins per lane were separated in 12% SDS-polyacrilamide gels and either stained with Coomassie Brilliant Blue (CB) or analyzed by western blot using anti-DGAT3, anti-tubulin (Tub) and anti-PETs antibodies. Arrowheads indicate approximate molecular weights, estimated by comparison with molecular size standards.

Figure 2. Exposure to moderate light triggers accumulation of DGAT3 mRNAs and TAGs in *sta6* cells in the presence of acetate. *C. reinhardtii* cells from wild type (cc-125) and the *sta6* mutant (cc-4348) were grown in TAP media under continuous light (4,500 lux) to approximately 3×10^6 cells/ml, transferred to dark conditions for 16 h and re-transferred to 4,500 lux of light. Samples were harvested at the end of the dark period (ED) and 1 h, 6 h, 12 h and 24 h after shifting the cells to light (1HL, 6HL, 12HL and 24HL, respectively). **A**) Box plots showing the expression of *dgat3*, *dgat1* and *pdat* mRNAs analyzed by RT-qPCR. Results are shown as the fold change of each gene normalized to the endogenous control (*gblp*) in the same condition and to the ED sample. Values for independent biological triplicates are shown, vertical bars indicate minimum and maximum values, horizontal black strips indicate median values. Statistical analyses were done using dN0 values by means of one-way ANOVA and Dunnett's multiple comparisons test; significant differences of samples against the ED sample are shown, ** P < 0.01, * P < 0.05, ▲ P < 0.1. **B**) Thin layer chromatography (TLC) of lipids extracted using the Bligh and Dyer method, separated in silicagel G-60 plates using hexane:sulphuric ether:formic acid (80:20:2, v/v) as solvent system and developed by charring. Sample volumes equivalent to 12 million cells were loaded on each lane. Arrowheads show the spots of triacylglycerol (TAG) and free fatty acid (FFA) standards. The black box represents the dark period, the yellow box represents moderate light. A representative image of three independent biological replicates is shown.

Figure 3. Response of DGAT3 mRNAs and TAG levels in *sta6* cells grown in minimal media.

Cells from the *sta6* mutant (cc-4348) were grown in Tris-minimal media under continuous light (4,500 lux) to approximately 3×10^6 cells/ml, transferred to dark conditions for 16 h and re-transferred to 4,500 lux of light. Samples were harvested at the end of the dark period (ED) and 1 h, 6 h and 24 h after shifting cells to light (1HL, 6HL and 24HL, respectively). **A**) Dot plot showing the expression of *dgat3* mRNAs analyzed by RT-qPCR. Results are expressed as the fold change of *dgat3* normalized to the endogenous control (*gblp*) in the same condition and to the ED sample. Green circular dots show the values of independent biological replicates. Vertical bars indicate standard deviations, black diamond dots show median values. Statistical analyses were done using dN0 values by means of one-way ANOVA and Dunnett's multiple comparisons test; significant differences against the ED sample are shown, ** $P < 0.01$, * $P < 0.05$. **B**) Thin layer chromatography (TLC) of lipids extracted using the Bligh and Dyer method, separated in silicagel G-60 plates using hexane:sulphuric ether:formic acid (80:20:2, v/v) as solvent system and developed by charring. Sample volumes equivalent to 30 million cells were loaded on each lane. Arrowheads show the spots of triacylglycerol (TAG) and free fatty acid (FFA) standards. The black box represents the dark period, the yellow box represents moderate light. A representative image of three independent biological replicates is shown.

Figure 4. DGAT3 mRNAs and TAGs decrease in the wild type strain after shifting cells from darkness to high light. Cells from cc-125 were grown in TAP media under continuous light (4,500 lux) to approximately 3×10^6 cells/ml, transferred to dark conditions for 16 h and then shifted to 15,000 lux of light. Samples were harvested at the end of the dark period (ED) and 1 h, 6 h, 12 h and 24 h after shifting cells to light (1HL, 6HL, 12HL and 24HL, respectively). **A**) Box plots showing the expression of *dgat3* and *dgat1* mRNAs analyzed by RT-qPCR. Results are expressed as the fold change of each gene normalized to the endogenous control (*gblp*) in the same condition and to the ED sample. Values for independent biological triplicates are shown, vertical bars indicate minimum and maximum values, horizontal black strips indicate median values. Statistical analyses were done using dN0 values by means of one-way ANOVA and Dunnett's multiple comparisons test; significant differences against the ED sample are shown, * $P < 0.05$, ▲ $P < 0.1$. **B**) Thin layer chromatography (TLC) of lipids extracted using the Bligh and Dyer method, separated in silicagel G-60 plates using hexane:sulphuric ether:formic acid (80:20:2, v/v) as solvent system and developed by charring. Sample volumes equivalent to 10 million cells were loaded on each lane. Arrowheads show the spots of triacylglycerol (TAG) and free fatty acid (FFA) standards. The black box represents the dark period, the orange box represents high light. A representative image of three independent biological replicates is shown.

Figure 5. DGAT3 mRNAs and TAGs accumulate in the wild type strain after shifting cells from low light to high light. Cells from the wild type cc-125 strain were grown in TAP media under continuous light (4,500 lux) to approximately 3×10^6 cells/ml, transferred to 1,500 lux for 16 h and then switched to 15,000 lux of light for 24 h. Samples were harvested at the end of the low light period (ELL) and 1 h, 6 h, 12 h and 24 h after shifting cells to high light (1HL, 6HL, 12HL and 24HL, respectively). **A**) Box plots showing the expression of *dgat3* and *dgat1* mRNAs analyzed by RT-qPCR. Results are expressed as the fold change of each gene in each condition compared to the endogenous control (gblp) in the same condition. Vertical bars indicate minimum and maximum values, horizontal black strips indicate median values. Statistical analyses were done using dN0 values by means of one-way ANOVA and Dunnett's multiple comparisons test; significant differences against the ED sample are shown, *** P < 0.001, ** P < 0.01, * P < 0.05, ▲ P < 0.1. **B**) Thin layer chromatography (TLC) of lipids extracted using the Bligh and Dyer method, separated in silicagel G-60 plates using hexane:sulphuric ether:formic acid (80:20:2, v/v) as solvent system and developed by charring. Sample volumes equivalent to 10 million cells were loaded on each lane. Arrowheads show the spots of triacylglycerol (TAG) and free fatty acid (FFA) standards. The black box represents the dark period, the orange box represents high light. A representative image of three independent biological replicates is shown.

Figure 6. Schematic model of light-induced TAG accumulation in *C. reinhardtii*. **A)** When cells are transferred from dark to limiting light conditions in the presence of acetate, a pulse increase in DGAT3 expression occurs (upward black arrow by DGAT3). We hypothesize that reducing power provided by light and organic carbon supplied in the form of acetate are directed, as part of the regulation of this enzyme, towards the synthesis of TAGs in the chloroplast, which also increase with moderate light (upward black arrow by TAG). When the levels of TAGs reach a certain level, we propose that a regulatory loop inhibits further DGAT3 expression and TAG synthesis (purple dashed line). This mechanism could prevent light activation in cc-125 cells that come from a hypoxic dark period and, thus, have elevated pre-existing TAG levels. **B)** When cells are transferred to high light, we hypothesize that DGAT3-mediated TAG accumulation is not enough to harness the electron excess furnished by light. Hence, the expression of stress/ROS-related enzymes, such as DGAT1 (this work), PDAT and DGAT2 (Chouhan et al., 2020) is activated. We cannot conclude from our results what the relative contribution of each enzyme under high light is or whether DGAT3 expression is activated at irradiances other than those evaluated in this work, but we propose that the responses shown in B) are activated gradually as more photons that are absorbed cannot be stored by the mechanisms in A). In addition, while light response to limiting light might only involve the chloroplast, the response to high light might occur in both the plastid and the ER/cytosol, as proposed previously (Chouhan et al., 2020; Goold et al., 2016). Dashed lines correspond to processes yet to be fully demonstrated. Only the synthesis of TAGs is shown, without addressing their final storage.