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Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*

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Regular Paper

Microalgal oils have attracted much interest as potential feedstocks for renewable fuels, yet our understanding of the regulatory mechanisms controlling oil biosynthesis and storage in microalgae is rather limited. Using *Chlamydomonas reinhardtii* as a model system, we show here that starch, rather than oil, is the dominant storage sink for reduced carbon under a wide variety of conditions. In short-term treatments, significant amounts of oil were found to be accumulated concomitantly with starch only under conditions of N starvation, as expected, or in cells cultured with high acetate in otherwise standard growth medium. Time-course analysis revealed that oil accumulation under N starvation lags behind that of starch and rapid oil synthesis occurs only when carbon supply exceeds the capacity of starch synthesis. In the starchless mutant BAFJ5, blocking starch synthesis results in significant increases in the extent and rate of oil accumulation. In the parental strain, but not the starchless mutant, oil accumulation under N starvation was strictly dependent on the available external acetate supply and the amount of oil increased steadily as the acetate concentration increased to the levels several-fold higher than that of the standard growth medium. Additionally, oil accumulation under N starvation is saturated at low light intensities and appears to be largely independent of *de novo* protein synthesis. Collectively, our results suggest that carbon availability is a key metabolic factor controlling oil biosynthesis and carbon partitioning between starch and oil in *Chlamydomonas*.

Keywords: *Chlamydomonas reinhardtii* • Fatty acids • Starch • Triacylglycerol.

Abbreviations: DAG, diacylglycerol; CAP, chloramphenicol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DGAT, diacylglycerol: acyl-CoA acyltransferase; CHX, cycloheximide; PA, phosphatidic acid; TAG, triacylglycerol; TAP, Tris-acetate phosphate.

Introduction

Oil in the form of triacylglycerol (TAG) is the major storage compound for fatty acids; the most energy-dense storage form of reduced carbon in the majority of living organisms (Durrett et al. 2008). Many microalgae are capable of accumulating large quantities of oil, which has been widely regarded as potential feedstocks for the production of biodiesel as a renewable alternative to petroleum fuels (Hu et al. 2008, Wijffels and Barbosa 2010). Realizing this potential, however, demands in-depth knowledge of algal biology in general, and TAG metabolism and its regulation in particular.

Major progress has been made in recent years in our understanding of lipid metabolism at the biochemical and molecular levels, especially in model organisms such as Arabidopsis (Wallis and Browse 2010). In contrast, most of our knowledge about the biochemical pathways, the enzymes and the regulatory factors involved in the biosynthesis of membrane lipids and storage TAG in microalgae is largely inferred from genome databases (Sato and Moriyama 2007, Rismani-Yazdi et al. 2011), genome-wide transcriptional analysis (Miller et al. 2010) and proteomic profiling (Moellering and Benning 2010, Nguyen et al. 2011).

By analogy to higher plants, the biosynthesis of TAG and membrane lipids in microalgae begins with de novo fatty acid synthesis in the chloroplast (Sirevag and Levine 1972, Moellering et al. 2009, Miller et al. 2010). The resultant fatty acids can be used either directly in the chloroplast or, following export into the cytosol in the endoplasmic reticulum (ER), to sequentially acylate glycerol-3-phosphate to produce phosphatidic acid (PA). Dephosphorylation of PA generates diacylglycerol (DAG), which serves as an immediate precursor for the synthesis of both membrane lipids and storage TAG. As the final and only committed step in the pathway of TAG biosynthesis, the conversion of DAG to TAG is catalyzed by diacylglycerol: acyl-CoA acyltransferase (DGAT) and phospholipid:

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diacylglycerol acyltransferase in microalgae (Miller et al. 2010, Khozin-Goldberg and Cohen 2011).

Chlamydomonas reinhardtii has recently emerged as a model organism for studying TAG biosynthesis and storage (Merchant et al. 2011). As with many other microalgae, *Chlamydomonas* cells accumulate TAG in oil droplets in response to N starvation (Wang et al. 2009, Moellering and Benning 2010, Siaut et al. 2011, Msanne et al. 2012). By analogy to higher plants, it is generally assumed that TAGs in microalgae are synthesized in the ER from DAG assembled by ER-specific acyltransferases and the TAG made by this pathway is stored in ER-derived lipid droplets in the cytosol (Riekhof et al. 2005, Hu et al. 2008, Moellering et al. 2009, Miller et al. 2010, Khozin-Goldberg and Cohen 2011). Recent results, however, are inconsistent with an ER pathway of TAG biosynthesis, because the DAG moiety of TAG is of chloroplast origin (Fan et al. 2011) and oil droplets were accumulated in both the chloroplast and the cytosol in a starchless mutant of *Chlamydomonas* (Fan et al. 2011, Goodson et al. 2011).

The present study was undertaken to investigate the regulatory factors controlling oil biosynthesis with an emphasis on the metabolic interconnection between starch and oil in *Chlamydomonas*. The starch biosynthetic pathway has been particularly well characterized at the molecular genetic level in *Chlamydomonas* (Ball and Morell 2003), and the metabolic interaction between starch and oil synthetic pathways has been a subject of much investigation in this alga (Wang et al. 2009, Li et al. 2010, Work et al. 2010, Siaut et al. 2011), but the role of starch in TAG accumulation is still controversial and fundamental questions remain regarding the nature of their interaction and the factors controlling carbon partitioning between these two storage products. The results from the present study demonstrate that starch and oil synthesis are two competing pathways of carbon storage, with starch biosynthesis dominating over oil accumulation. Consequently, high rates of TAG synthesis occur only when carbon supply exceeds the capacity of starch synthesis or in a mutant devoid of starch. Our results indicate that carbon availability is the key metabolic factor controlling the rate and extent of oil accumulation and the flux of carbon between starch and oil in *Chlamydomonas*.

Results

Starch is the dominant sink for carbon storage in *Chlamydomonas*

Many abiotic stress factors have been reported to increase TAG accumulation in a wide variety of algal species (Hu et al. 2008). We observed that when growing cells of *C. reinhardtii* strain *dw15* were exposed to salt, osmotic stresses or cultured in Tris-acetate phosphate (TAP) medium lacking P, S or Mg for 3 d, they accumulated significant amounts of starch but limited amounts of TAG (**Fig. 1A**). Similarly, treatments of growing cells with a high light intensity of 400 μmol m^{-2} s^{-1} or suboptimal temperatures of 7 or 37°C for 2 d caused only slight increases in

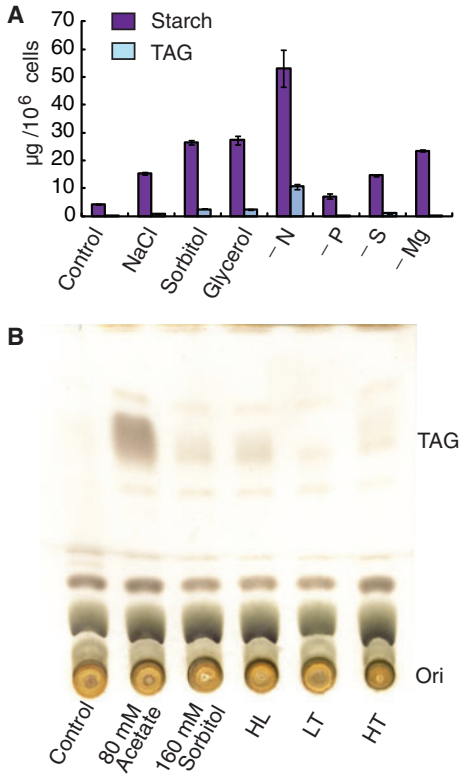

Fig. 1 Starch is the primary storage product in *Chlamydomonas* under stress conditions. (A) Starch and TAG content in *dw15* cells cultured in TAP medium lacking N, P, S or Mg, or in TAP medium containing 100 mM NaCl, 200 mM glycerol or 200 mM sorbitol for 3 d. (B) Thin-layer chromatograph of neutral lipids isolated from *dw15* cells cultured in TAP medium under a light intensity of 400 μmol m^{-2} s^{-1} (HL), at a suboptimal temperature of 7°C (LT) or 37°C (HT), or in TAP medium containing 80 mM acetate or 160 mM sorbitol for 2 d. Control cells were maintained in TAP medium under a light intensity of 50 μmol m^{-2} s^{-1} at 24°C. Lipids were visualized by H_2SO_4 and charring. Values in (A) are the means and standard deviation of three independent experiments.

TAG (**Fig. 1B**) but marked increases in starch from 4.3 ± 0.3 to 22.1 ± 0.3, 15.7 ± 6.2 and 20.3 ± 0.7 μg per 10^6 cells, respectively. Cells starved for N resulted in large increases in both starch and TAG, as expected from recent studies (Siaut et al. 2011, Li et al. 2010), but the amount of starch per cell was >5-fold higher than that of TAG under our growth conditions (**Fig. 1A**).

Besides N starvation, excess acetate supply also resulted in a significant accumulation of both starch and TAG, but again starch was the dominant storage product (**Fig. 2**). Increasing concentrations of acetate from 20 to 60 mM caused significant increases in starch from 8.0 ± 1.0 to 25.5 ± 5.7 μg per 10^6 cells (**Fig. 2A**) and TAG from 0.1 ± 0.1 to 1.3 ± 0.2 μg per 10^6 cells (**Fig. 2B**). A further increase in acetate to 80 mM resulted in an additional 32% increase in the starch content, but a >200% rise in TAG. Treatment with sorbitol at an equiosmolar concentration did not lead to a significant increase in TAG (**Fig. 1A, B**), suggesting that TAG accumulation in cells grown under high acetate is not due to osmotic stress caused by acetate addition.

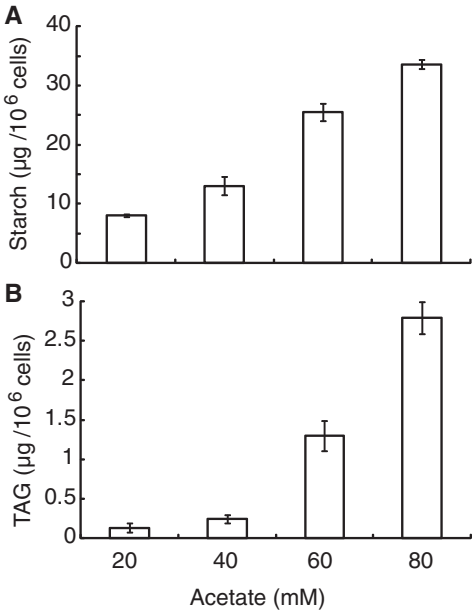

Fig. 2 Changes in starch (A) and TAG (B) content in response to high acetate under N-replete conditions. Cells of *dw15* strain were first grown in standard growth medium until early stationary phase, and then switched to TAP medium containing various amounts of acetate and further cultured for 2 d. Values are the means and standard deviation of three independent experiments.

Increasing acetate supply enhances the accumulation of oil but not starch

To gain insight into the metabolic relationship between starch and oil biosynthesis, we monitored the time course of changes in oil and starch in response to N starvation. Following transfer of *dw15* cells to TAP medium lacking N, the starch levels increased rapidly within the initial 24 h, reaching a maximum level at 48 h, with no significant change between 48 and 72 h (**Fig. 3A**). The accumulation of TAG lagged behind that of starch and the rapid increase in TAG occurred after 24 h of N starvation (**Fig. 3B**) when starch accumulation slowed down (**Fig. 3A**). Afterwards, the amount of TAG continued to significantly increase until the end of the time course. Importantly, the *Chlamydomonas* starchless mutant BAFJ5, which is defective in ADP-glucose pyrophosphorylase (Zabawinski et al. 2001) and therefore devoid of starch, accumulated TAG at a much faster initial rate under N starvation in comparison with its complemented strain *sta6*-C6 (Li et al. 2010) and *dw15* (**Fig. 3C**). When cells of *dw15* were starved for N in the absence of acetate as an external carbon source, they accumulated predominantly starch as the storage product (**Fig. 3A, B**), albeit at a reduced rate in comparison with the rate observed in cells starved for N in TAP medium, which contains 17 mM acetate (Harris 2009). On the other hand, increasing the concentration of acetate to 60 mM in TAP medium lacking N greatly enhanced the rate and extent of TAG accumulation (**Fig. 3B**), but only slightly affected the level of starch (**Fig. 3A**) in comparison with cells starved for N in the presence of 17 mM acetate. Note that cells remained intact following 72 h of N starvation in the presence or absence of acetate supply (**Supplementary Fig. 1**). As expected, oil droplets, as revealed by Nile red staining, were much less abundant in cells starved for N in the absence of added acetate compared with cells starved for N in the presence of 17 mM acetate (**Fig. 3D, E**). In addition, there was an increase in the size and number of oil droplets as the concentration of acetate was increased from 17 to 60 mM (**Fig. 3E, F**). Using ^{14}C-acetate, we found that acetate was rapidly incorporated into TAG and membrane lipids under both N-depleted and N-replete conditions, and more label was accumulated in TAG under N-depleted conditions, as expected (**Supplementary Fig. 2**). Taken together, these results establish that starch synthesis is the primary sink for carbon and energy in *Chlamydomonas* and that rapid oil accumulation occurs only when there is an excess of reduced carbon beyond the demand for starch synthesis.

Increased oil accumulation in the starchless mutant is to a large extent attributable to increased carbon availability

The influence of acetate on TAG accumulation in response to N starvation in *dw15*, BAFJ5 and *sta6*-C6 was investigated in more detail. When growing cells were transferred to media lacking N but containing various levels of acetate and further cultured for 48 h, during which time very limited amounts of TAG were accumulated in the absence of acetate supply (**Fig. 3B**), the amount of TAG in *dw15* and *sta6*-C6 increased steadily with increasing acetate supply up to 60 mM, and declined thereafter (**Fig. 4**). In the starchless mutant BAFJ5, the amount of TAG increased more rapidly, reaching a maximum level three times higher than that observed in *dw15* and *sta6*-C6 at 20 mM acetate, and remaining unchanged with further increasing the acetate concentration to 60 mM before declining towards the 100 mM concentration. Note that the starchless mutant accumulated >20-fold more oil than *dw15* and *sta6*-C6 when N starved in the absence of acetate supply (**Fig. 4**). The differences, however, became much smaller with increasing acetate concentrations in the medium, so that at 60 mM acetate the amount of oil was only about 0.5-fold higher in the starchless mutant in comparison with *dw15* and *sta6*-C6. Together, these results suggest that TAG accumulation in *Chlamydomonas* under N starvation is to a large extent limited by the carbon supply and that increased TAG accumulation in the starchless mutant is mostly attributed to increased carbon availability in the absence of starch synthesis. However, the maximal level of oil reached in the starchless mutant is still 56% higher than in *dw15* and the complemented strain *sta6*-C6, suggesting that factors additional to carbon availability are involved in determining the upper limits of oil accumulation in *Chlamydomonas*.

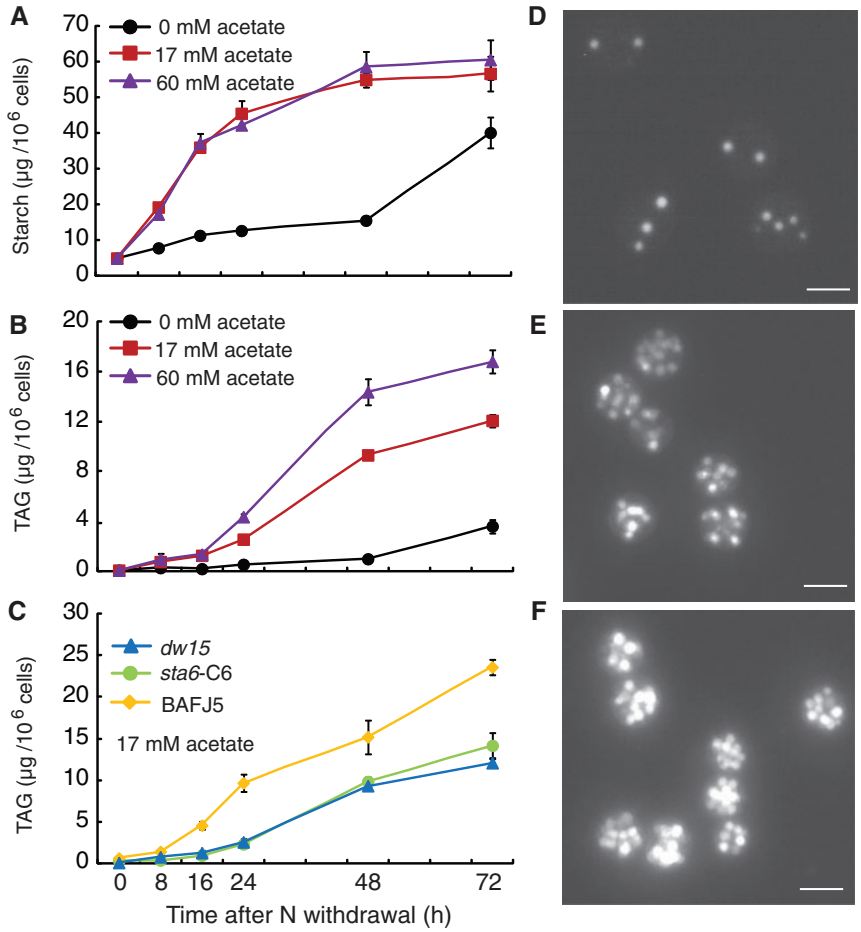

Fig. 3 TAG and starch accumulation in *Chlamydomonas* in response to N starvation. Cells were first grown in standard growth medium until the early stationary phase, and then switched to medium lacking N and further cultured for 3 d. (A and B) Time course of starch (A) and TAG (B) accumulation in *dw15* cells starved for N in the presence of various amounts of acetate. (C) Time course of TAG accumulation in cells of *dw15* (triangle), *sta6*-C6 (circle) and BAFJ5 (diamond) cultured in standard TAP medium lacking N. Values are the means and standard deviation of three independent experiments. (D, E and F) Fluorescence images of Nile red-stained cells cultured in TAP medium lacking N in the presence of 0 (D), 17 (E) or 60 (F) mM acetate for 3 d. Scale bar = 10 μm.

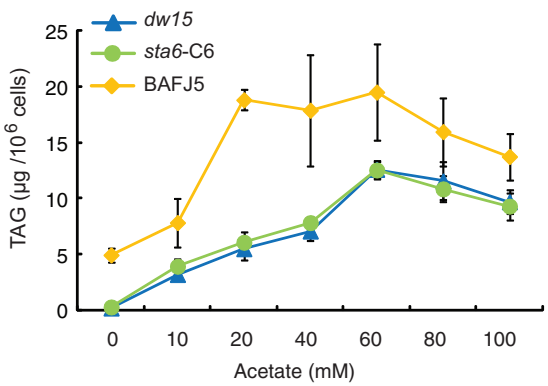

Fig. 4 TAG accumulation under N starvation as a function of acetate concentration. Cells of *dw15* (triangle), *sta6*-C6 (circle) and BAFJ5 (diamond) were first grown in standard growth medium until early stationary phase, and then switched to medium lacking N and further cultured for 2 d in the presence of various amounts of acetate. Values are the means and standard deviation of three independent experiments.

TAG accumulation is largely independent of *de novo* protein synthesis

The results shown above suggest that TAG accumulation is controlled by carbon availability rather than the enzymes involved in fatty acid synthesis and TAG assembly. To further test this hypothesis, we examined the effects of the protein synthesis inhibitors cycloheximide (CHX) and chloramphenicol (CAP), which inhibit cytoplasmic and plastidic protein synthesis, respectively, on TAG accumulation in response to N starvation. For this purpose, growing cells were first pre-incubated with 10 μg ml^{-1} CHX or 200 μg ml^{-1} CAP for 30 min and then shifted to TAP lacking N. CHX and CAP at these concentrations were previously shown to very effectively inhibit protein synthesis in *Chlamydomonas* (Roessler and Lien 1984, Satoh et al. 2004, Yildiz et al. 1994, Crespo et al. 2005). To eliminate the possible effect of protein synthesis inhibitors on acetate availability for fatty acid synthesis, cells were N starved in the presence of 60 mM acetate. As shown in **Fig. 5A**, treatments with

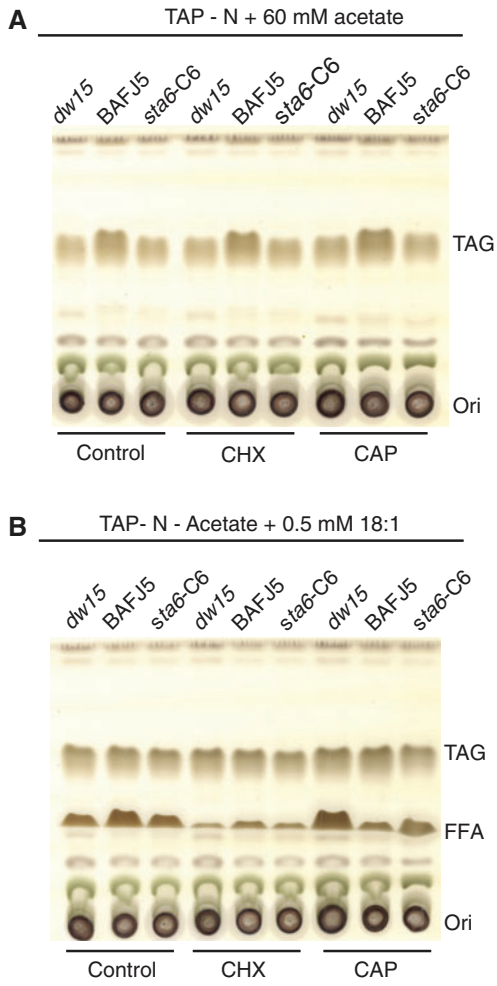

Fig. 5 TAG accumulation is largely independent of *de novo* protein synthesis. (A and B) Thin-layer chromatographs of neutral lipids isolated from cells incubated in TAP medium lacking N but containing 60 mM acetate (A) or in TAP medium lacking both N and acetate but containing 0.5 mM 18:1 (B) for 16 h. Early stationary phase cells were pretreated with 10 μg ml^{-1} CHX or 200 μg ml^{-1} CAP for 30 min and then shifted to TAP lacking N. Control cells were N starved in TAP medium containing 60 mM acetate (A) or in TAP medium lacking acetate but containing 0.5 mM 18:1 (B). Lipids were spotted on an equal cell number basis corresponding to 10^7 cells, and visualized by H_2SO_4 and charring.

CHX or CAP did not significantly affect the amounts of TAG accumulated in cells starved for N for 16 h. In addition, TAG accumulation in cells starved for N in TAP medium lacking acetate but containing 0.5 mM oleic acid (18:1) was completely insensitive to protein synthesis inhibitors (**Fig. 5B**). Note that treatments with protein synthesis inhibitors did not significantly change the difference in ability to accumulate TAG between BAFJ5 and *dw15* or *sta6*-C6 in response to N starvation in the presence of 60 mM acetate (**Fig. 5A**), suggesting that *de novo* protein synthesis is not responsible for increased TAG synthesis in the starchless mutant.

It is worth noting that whereas there was a marked difference in the amounts of TAG accumulated in N-starved cells in the presence of 60 mM acetate between BAFJ5 and *dw15* or *sta6*-C6 (**Fig. 5A**), this difference disappeared when cells were N starved in medium lacking acetate but containing 0.5 mM 18:1 (**Fig. 5B**). A likely explanation for these observations is that exogenous free fatty acids are exclusively channeled into TAG but not used for the synthesis of starch, whereas acetate is used for the synthesis of both starch and TAG. In support of this possibility, quantification of starch contents showed that the addition of 0.5 mM 18:1 did not significantly affect starch content when *dw15* cells were N starved in the absence of exogenous acetate (**Supplementary Fig. 3**), whereas the addition of 60 mM acetate caused a drastic increase in the amount of both starch and TAG (**Fig. 3 A, B**). The lack of difference in the TAG content between BAFJ5 and *dw15* or *sta6*-C6 in response to N starvation in the absence of acetate but in the presence of 0.5 mM 18:1 also suggests that there is no major difference in the activity of TAG assembly enzymes between *dw15* and BAJF5. Together, these results reinforce the ideas that TAG accumulation under N starvation is, to a major extent, independent of *de novo* protein synthesis and that increased ability to accumulate TAG in starchless mutants is largely a consequence of increased carbon availability for fatty acid synthesis.

The biosynthesis of starch and oil both are dependent upon active photosynthetic electron transport

The biosynthesis of fatty acids, the building blocks for membrane lipids and storage TAG, is an energy-demanding process. In green tissues of higher plants, photosynthetic electron transport is the primary source of ATP and NADPH for fatty acid synthesis, whereas in non-photosynthetic tissues these cofactors are provided by carbon oxidation or metabolite shuttles (Rawsthorne 2002). Similarly, starch synthesis in leaf chloroplasts is fueled by photophosphorylation at the thylakoid membrane (Geigenberger 2011). To determine the role of photosynthesis in the formation of storage reserves in *Chlamydomonas*, we monitored starch and oil accumulation in response to N starvation in the dark. Consistent with our previous report (Fan et al. 2011), cells starved for N in the dark accumulated negligible amounts of TAG after 2 d of N withdrawal (**Fig. 6A**). Light is known to stimulate acetate uptake in *Chlamydomonas* (Eppley et al. 1963). To test if TAG accumulation in the dark is limited by acetate availability, growing cells were starved for N in the presence of 60 mM acetate and their TAG content quantified. Similar to cells starved for N in the light (**Fig. 3A**), high acetate increased TAG content in cells starved for N in the dark, but only to 32% of that observed in cells starved for N in TAP medium containing 17 mM acetate in the light (**Fig. 6A**). Further increasing the acetate concentration from 60 to 80 mM did not lead to a significant increase in the level of TAG. Similar to TAG, starch accumulation in response to N starvation was also strongly inhibited in the dark (**Fig. 6B**) and increasing acetate supply only resulted in moderate

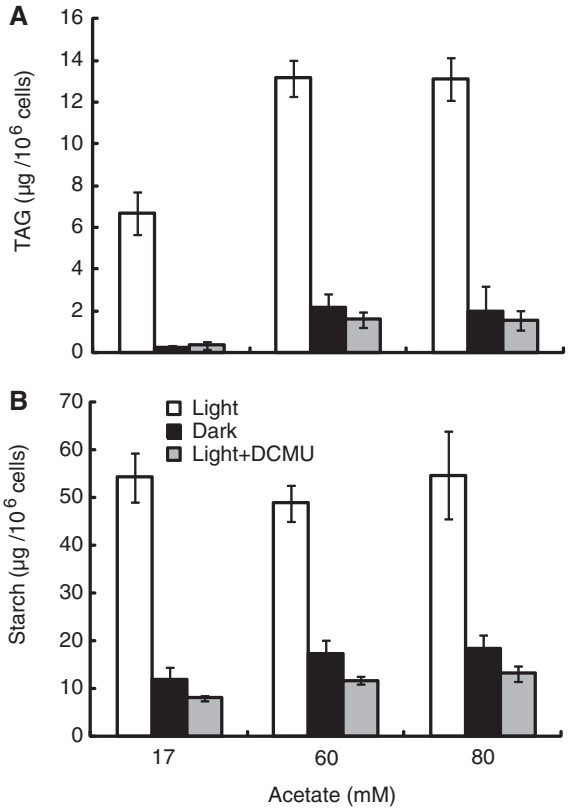

Fig. 6 TAG (A) and starch (B) accumulation in response to N starvation is dependent on active photosynthetic electron transport. Cells of *dw15* strain were first grown in standard growth medium until early stationary phase, and then switched to medium lacking N in the presence of various amounts of acetate and further cultured for 2 d in the light (white bars), dark (black bars) or in the light with the addition of 10 μM DCMU (gray bars). Values are the means and standard deviation of three independent experiments.

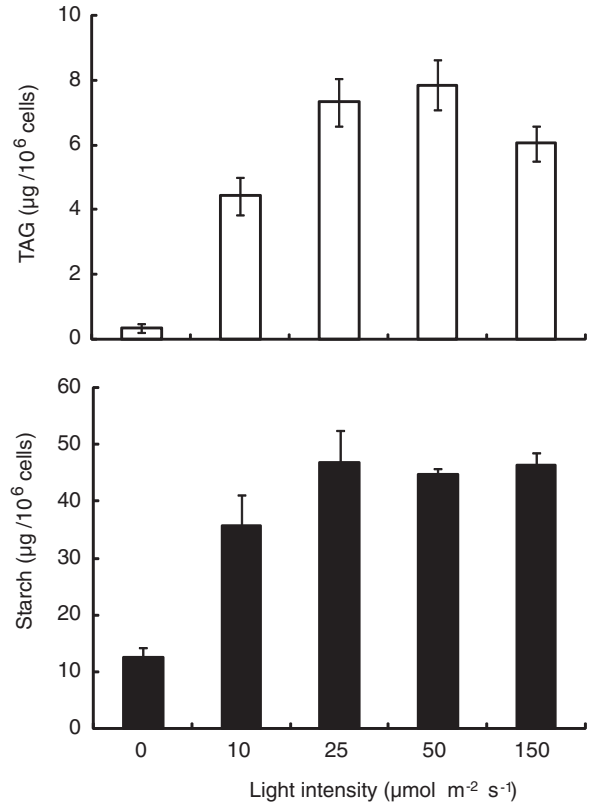

Fig. 7 The effect of light irradiance on TAG (white bars) and starch (black bars) accumulation in *Chlamydomonas* in response to N starvation. Cells of *dw15* strain were first grown in standard growth medium until early stationary phase, and then switched to medium lacking N and further cultured for 2 d under different light levels. Values are the means and standard deviation of three independent experiments.

increases in the amount of starch. These results suggest that the limited accumulation of starch and TAG in the dark is not solely due to the restricted acetate uptake, but rather may reflect the dependency of storage product synthesis on the supply of ATP and/or NADPH through light reactions of photosynthesis.

To further test the role of the photosynthetic electron transport in storage product accumulation in response to N starvation, we applied the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). As shown in **Fig. 6**, addition of 10 μM DCMU led to an almost complete inhibition of the accumulation of both starch and oil. Again, the inhibitory effect of DCMU on storage product accumulation could only be partially relieved by increasing the acetate concentration to 80 mM. Taken together, these results support the notion that the light reactions of photosynthesis are the major source of free energy and reductant for the synthesis of starch and TAG in *Chlamydomonas* under conditions of N starvation.

Energy is not a limiting factor in starch and oil biosynthesis

To further understand the role of light in the formation of storage reserves, we determined the amounts of starch and TAG in cells starved for N under four light intensities representing 20%, 50%, 100% or 300% of the growth light. As shown in **Fig. 7**, cells starved for N in the dark accumulated very limited amounts of TAG and small amounts of starch. When illuminated with a low light level of 10 μmol m^{-2} s^{-1}, starch amounted to 80% and TAG to 56% of the levels found in cells under growth light conditions (50 μmol m^{-2} s^{-1}). Maximum levels of both starch and oil were found at 25 μmol m^{-2} s^{-1}, which was the equivalent of 50% of growth light intensity. Further increases in light levels from 25 to 150 μmol m^{-2} s^{-1} did not cause increases in either starch or TAG. Thus, although photosynthetic light reactions are the major source of cofactors for starch and TAG synthesis, energy is not a limitation in storage product synthesis in N-starved cells under growth light conditions.

Discussion

Carbon availability is the key metabolic factor controlling oil biosynthesis

The results of the present study show that starch is the preferred path for carbon and energy storage in *Chlamydomonas*. Substantial accumulation of TAG occurs only when (i) there is an adequate supply of metabolic cofactors through photosynthetic electron transport; (ii) starch biosynthesis is saturated for the available carbon or is blocked; and (iii) the carbon source is in excess over that required for N metabolism. N assimilation and metabolism in growing cells constitutes a major sink for carbon (Huppe and Turpin 1994), and N deprivation increases carbon availability through at least two mechanisms: (i) by restricting the use of carbon in N metabolism and cell growth, and (ii) by stimulating the hydrolytic release of carbon skeletons from proteins and other cellular compounds (Msanne et al. 2012). Thus N limitation has long been recognized as the most critical biological constraint in inducing oil accumulation in microalgae (Roessler 1990). N starvation has also been shown to induce TAG accumulation in Arabidopsis seedlings (Gaude et al. 2007), which was reported to be enhanced by sugar feeding (Yang et al. 2011). Induction of storage lipid accumulation by soluble carbohydrates has also been noted in *Chlorella protothecoides* (Heredia-Arroyo et al. 2010, Xiong et al. 2010), cell suspension cultures of oilseed rape (Weselake et al. 1998) and in Arabidopsis seedlings overexpressing *WRI1* (Cernac and Benning 2004, Sanjaya et al. 2011). In mammals, sugar-rich diets induce hypertriacylglycerolemia via *de novo* lipogenesis (Chong et al. 2007). Thus, excess carbon availability relative to metabolic demand represents a widespread signal triggering oil synthesis, and thus links nutritional status with storage functions in microalgae, plants and humans.

Oil biosynthesis is limited by carbon precursor supply

On the basis of biochemical and genetic analyses, various reactions and metabolic factors have been implicated in controlling oil biosynthesis in plants. These include the enzyme catalyzing the committed step in the pathway of fatty acid synthesis, such as plastidic acetyl-CoA carboxylase (Ohlrogge and Jaworski 1997), the enzymes involved in fatty acid utilization, such as DGAT (Jako et al. 2001) and lysophosphatidic acid acyltransferase (Zou et al. 1997), the provision of glycerol-3-phosphate for TAG assembly (Vigeolas et al. 2007), oxygen supply for storage metabolism (Vigeolas et al. 2003) and fatty acid supply (Bao and Ohlrogge 1999). In addition, quantitative information regarding the control of oil synthesis was obtained using metabolic control analysis for several oil crops. In oil palm and olive, fatty acid synthesis has been shown to exert much higher control of carbon flux to oil than the fatty acid utilization reactions (Ramli et al. 2002). In support of this notion, recent comparative transcriptome and metabolite analyses revealed that a major control over oil biosynthesis in oil palm resides in reactions associated with fatty acid synthesis and its immediate substrate supply, rather than with TAG assembly (Bourgis et al. 2011).

Similar to oil palm, several lines of evidence suggest that carbon supply for fatty acid synthesis rather than fatty acid utilization is the limiting step in oil accumulation in *Chlamydomonas*. First, early studies showed that the addition of exogenous complex lipids led to a 10-fold increase in TAG content in cells under normal growth conditions (Grenier et al. 1991). We recently found that the amounts of TAG in cells under N starvation increased proportionally with respect to the amount of oleic acid added to the growth medium (Fan et al. 2011). These results imply that fatty acid production is limiting in the pathway of oil biosynthesis and that the enzymes responsible for TAG assembly are present in excess with respect to fatty acid supply under both normal and N starvation conditions in *Chlamydomonas*. Second, we showed in this study that oil accumulation in N-starved cells is strongly dependent on carbon supply and the amounts of oil increased with increasing acetate concentration in the medium, suggesting that carbon supply rather than the enzymes involved in fatty acid synthesis is the limiting factor in oil synthesis under N starvation conditions. Third, data from this work and other studies (Wang et al. 2009, Li et al. 2010, Work et al. 2010) have demonstrated that blocking starch synthesis, the competing pathway of oil accumulation, results in several-fold increases in oil content in *Chlamydomonas* starchless mutants. Importantly, we showed in this study that the magnitude of the differences in oil content between the starchless mutant and the wild type became much smaller when acetate concentrations in the medium were increased, which is likely due to the saturation of starch biosynthesis in the wild-type strain. This result is consistent with the hypothesis that increased oil accumulation in starchless mutants is, to a large extent, the result of increased carbon availability for oil synthesis.

The relationship between starch and oil biosynthesis

Our findings indicate a close reciprocal relationship between the pathways for the biosynthesis of starch and oil in *Chlamydomonas*. This is consistent with several reports indicating that genetic inactivation of starch synthesis results in substantial increases in oil content in *Chlamydomonas*, as mentioned above, and in *Chlorella* (Ramazanov and Ramazanov 2006), but contrasts with findings in developing oilseeds of plants showing that inhibition of starch synthesis causes a decrease (Periappuram et al. 2000) or no change (Vigeolas et al. 2004) in the final seed oil content. The reasons for this difference are not presently understood. However, starch and oil syntheses in developing oilseeds are not only temporally but also spatially separated processes (Focks and Benning 1998, Smith et al. 2010). Early studies suggested that seed starch might serve as a source of carbon and reductant for the synthesis of oil (Norton and Harris 1975). However, embryo-specific inhibition of starch synthesis did not lead to

significant changes in the oil content in mature seeds (Vigeolas et al. 2004). An alternative hypothesis is that the early build-up of starch contributes to establishing embryos as a sink organ for carbon, rather than playing a direct role in oil synthesis (da Silva et al. 1997, Vigeolas et al. 2004). A more recent study, however, has linked seed starch metabolism to cell division and differentiation rather than to storage functions specific to embryos of oilseeds (Smith et al. 2010).

Unlike oilseeds, a close interconnection between the pathways for the biosynthesis of storage starch and oil is expected in the unicellular green alga *Chlamydomonas* for the following reasons. First, this work and our previous studies (Fan et al. 2011) showed that oil accumulation in response to N starvation or high acetate is dependent on *de novo* fatty acid synthesis in the chloroplast, rather than on conversion of pre-existing membrane lipids. Thus, the pathways for the biosynthesis of starch and oil compete for the same carbon source. Second, although energy is not limiting for the synthesis of storage products under growth irradiance, rapid starch and oil synthesis are both dependent on active photosynthetic electron transport. Third, we have shown in previous studies (Fan et al. 2011) that TAG is stored in oil droplets in the chloroplast, in addition to the cytosol. Thus, the pathways for the biosynthesis of starch and oil compete not only for common carbon precursors and metabolic energy, but also for space in chloroplasts for the storage of end products. In *Chlamydomonas* the chloroplast occupies 40% of the cell volume (Harris 2009), which in wild-type strains is packed with starch granules under stress conditions such as N starvation (Goodson et al. 2011), likely because starch accumulation precedes that of oil, as demonstrated in this study. In starchless mutants there is a 40% increase in the storage space for oil due to the absence of starch granules. This may explain why the maximal oil content was found to be about 50% higher in the starchless mutant than in the parental strains. In support of this possibility, it has recently been shown that the increased oil storage in the starchless mutant is related to its ability to accumulate oil droplets in chloroplasts (Goodson et al. 2011).

In conclusion, we have demonstrated that carbon precursor supply is the major limiting factor in TAG biosynthesis in *Chlamydomonas*. This implies that the enzymatic machinery associated with fatty acid synthesis to TAG assembly is present in excess of, or readily inducible in response to, the metabolic needs of cells. Thus, genetic engineering approaches aimed at boosting the oil content through overexpression of the rate-limiting enzymes involved in fatty acid synthesis or TAG assembly may not be fruitful. This notion is supported by previous attempts to overexpress acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis (Ohlrogge and Jaworski 1997), which in fact failed to increase oil accumulation in a diatom (Dunahay et al. 1996). Our findings therefore have broad implications not only for advancing our understanding of the regulation of oil biosynthesis and carbon partitioning, but also for physiological and metabolic engineering strategies to develop microalgae-based biofuel production systems.

Materials and Methods

Strains and growth conditions

The *C. reinhardtii* strains used in this study were the cell wall-less *dw15* (*cw15 nit1 mt*$^+$) (Riekhof et al. 2003) and the starchless mutant BAFJ5 (*cw15 sta6*) lacking the small subunit of ADP-glucose pyrophosphorylase (Zabawinski et al. 2001) and its complemented strain *sta6*-C6 (Li et al. 2010). These strains were grown on plates containing TAP (Harris 2009) solidified with 1.5% agar. In routine experiments, pre-cultures were made by inoculating 50 ml of TAP liquid medium with a small pellet of cells taken from plates and growing under constant illumination (50 μmol m^{-2} s^{-1}) at 24°C on an orbital shaker for 3 d. The experimental cultures were started by inoculating 100 ml of liquid TAP with 1 ml of the pre-cultures and growing until they reached stationary phase at about 1–2 × 10^7 cells ml^{-1}. To induce N, P, S or Mg starvation, cells were pelleted at 2,000 × *g* for 5 min, and washed once with and resuspended in TAP lacking corresponding nutrient elements. To inhibit *de novo* protein synthesis, early stationary phase cells were pre-incubated with 10 μg ml^{-1} CHX in water or 200 μg ml^{-1} CAP in ethanol for 30 min and then shifted to TAP lacking N. For 18:1 and DCMU treatments, the compounds (both in ethanol) were added directly to the growth media. Control cells were treated with either water or ethanol alone. CHX, CAP, DCMU and 18:1 were all purchased from Sigma–Aldrich. Cells were counted with a hematocytometer or an automated cell counter (TC10^{TM}, Bio-Rad).

Starch analysis

Cells were collected by centrifugation at 13,000 × *g* for 5 min, and the pellets were washed with 1 ml of 100% methanol for pigment extraction and centrifuged again. Following methanol extraction, the pellets were dried at room temperature and then resuspended in 0.2 N KOH. The suspension was incubated at 95°C for 1 h for starch solubilization. After neutralization with 1 M acetic acid and centrifugation for 5 min at 13,000 × *g*, the supernatants were used for starch quantification using a commercial starch assay kit (SA20, Sigma–Aldrich).

Lipid analysis

Total lipids were extracted as previously described (Fan et al. 2011). Neutral lipids were separated on silica plates (Si250 with a pre-adsorbant layer; Mallinckrodt Baker) by thin layer chromatography using a solvent system of hexane–diethyl ether–acetic acid (70:30:1, by volume). For quantitative analysis, lipids were visualized by brief iodine staining and identified by co-chromatography with lipid extracts of known composition. The TAG band was scraped from the plate and used to prepare fatty acid methyl esters. The separation and identification of the fatty acid methyl esters was performed on an HP5975 gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) fitted with a 60 m × 250 μm SP-2340 capillary

column (Supelco, Bellefonte, PA, USA) with helium as a carrier gas. The methyl esters were quantified using heptadecanoic acid as the internal standard, as described previously (Fan et al. 2011).

Fluorescence microscopy

For microscopy analyses, *Chlamydomonas* cells were stained with a fluorescent dye, Nile red (Sigma–Aldrich), at a final concentration of 1 μg ml^{-1} for 10 min in the dark and observed under a Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany).

Supplementary data

Supplementary data are available at PCP online.

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