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Metabolic and Gene Expression Changes Triggered by Nitrogen Deprivation in the Photoautotrophically Grown Microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169

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Abstract

Microalgae are emerging as suitable feedstocks for renewable biofuel production. Characterizing the metabolic pathways involved in the biosynthesis of energy-rich compounds, such as lipids and carbohydrates, and the environmental factors influencing their accumulation is necessary to realize the full potential of these organisms as energy resources. The model green alga *Chlamydomonas reinhardtii* accumulates significant amounts of triacylglycerols (TAGs) under nitrogen starvation or salt stress

in medium containing acetate. However, since cultivation of microalgae for biofuel production may need to rely on sunlight as the main source of energy for biomass synthesis, metabolic and gene expression changes occurring in *Chlamydomonas* and *Coccomyxa* subjected to nitrogen deprivation were examined under strictly photoautotrophic conditions. Interestingly, nutrient depletion triggered a similar pattern of early synthesis of starch followed by substantial TAG accumulation in both of these fairly divergent green microalgae. A marked decrease in chlorophyll and protein contents was also observed, including reduction in ribosomal polypeptides and some key enzymes for CO_2 assimilation like ribulose-1,5-bisphosphate carboxylase/oxygenase. These results suggest that turnover of nitrogen-rich compounds such as proteins may provide carbon/energy for TAG biosynthesis in the nutrient deprived cells. In *Chlamydomonas*, several genes coding for diacylglycerol:acyl-CoA acyltransferases, catalyzing the acylation of diacylglycerol to TAG, displayed increased transcript abundance under nitrogen depletion but, counterintuitively, genes encoding enzymes for *de novo* fatty acid synthesis, such as 3-ketoacyl-ACP synthase I, were down-regulated. Understanding the interdependence of these anabolic and catabolic processes and their regulation may allow the engineering of algal strains with improved capacity to convert their biomass into useful biofuel precursors.

Keywords: chlorophytes, starch, triacylglycerols, diacylglycerol:acyl-CoA acyltransferase, 3-Ketoacyl-ACP synthase, biomass, biofuels

1. Introduction

Algae are a diverse group of eukaryotic organisms with important roles in marine, freshwater, and even terrestrial ecosystems. For instance, 30–50% of the planetary net photosynthetic productivity (the difference between autotrophic gross photosynthesis and respiration) is of marine origin and dependent on phytoplankton biomass (Field et al., 1998; Boyce et al., 2010). Recently, the great potential of algal species as feedstocks for renewable biofuel production has also gained recognition (Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010). Unicellular microalgae are capable of harnessing sunlight and CO_2 to produce energy-rich chemical compounds, such as lipids and carbohydrates, which can be converted into fuels (Hu et al., 2008; Rodolfi et al., 2009; Wijffels and Barbosa, 2010). However, the commercial production of algal biofuels is currently hindered by limitations in the biological productivity of strains, culture systems, and harvesting/extraction processes (Sheehan et al., 1998; Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010). As recently proposed, a multidisciplinary approach, including advances in basic biology and metabolic engineering of algal strains as well as in culture systems, bioprocessing, and integrated biorefinery design, may be required to realize the full potential of microalgae as sustainable biofuel sources (Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010).

Desirable strain characteristics include rapid growth rate, high product content, tolerance to variable environmental conditions, resistance to predators and viruses and ease of harvest and extraction (Griffiths and Harrison, 2009; Rodolfi et al., 2009; Radakovits et al., 2010). An additional algal feature for biodiesel production is the suitability of the lipid composition, with triacylglycerols (TAGs) being the preferred substrate (Schenk et al., 2008; Radakovits et al., 2010). Algae synthesize, under optimal growth conditions, fatty acids predominantly for esterification into glycerol-based membrane lipids (Guschina and

Harwood, 2006; Hu et al., 2008; Khozin-Goldberg and Cohen, 2011). However, factors such as temperature, irradiance, and nutrient availability affect both lipid composition and lipid content in many algal species (Guschina and Harwood, 2006; Hu et al., 2008; Khozin-Goldberg and Cohen, 2011; Siaut et al., 2011). Upon certain environmental stresses (particularly nutrient shortage), various algae accumulate energy-rich storage compounds such as starch and TAGs (Guschina and Harwood, 2006; Hu et al., 2008; Rodolfi et al., 2009; Wang et al., 2009; Dean et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; Siaut et al., 2011). Fatty acids are the common precursors for the formation of both membrane lipids (required for growth) and TAGs (involved in energy storage and fatty acid homeostasis), but it remains to be elucidated how algal cells coordinate the distribution of these precursors to distinct destinations in response to environmental stimuli (Sheehan et al., 1998; Guschina and Harwood, 2006; Hu et al., 2008; Radakovits et al., 2010). Interestingly, biomass productivity and lipid content appear to be inversely correlated in many algae (Sheehan et al., 1998; Hu et al., 2008; Rodolfi et al., 2009) and nutrient limitation stimulates TAG accumulation but at the expense of growth (Rodolfi et al., 2009; Li et al., 2011).

Given the outlined constraints, improving algal strain performance will require a greater understanding of carbon allocation between biosynthetic pathways and of the regulatory mechanisms controlling this distribution, particularly in response to environmental stresses. This need is also emphasized by the limited success in increasing total oil content in higher plants and algae by the direct engineering of single lipid biosynthesis components (Dunahay et al., 1996; Durrett et al., 2008; Li et al., 2010; Radakovits et al., 2010; Work et al., 2010). The unicellular green alga *Chlamydomonas reinhardtii* has emerged as a model system for studying algal physiology, photosynthesis, metabolism, and flagellar structure and function (Harris, 2001; Merchant et al., 2007). Its nuclear, plastid, and mitochondrial genomes have been sequenced and a set of genomic, molecular, and genetic tools is also available for this organism (Harris, 2001; Grossman et al., 2007; Merchant et al., 2007). *Chlamydomonas* has been shown to accumulate significant amounts of TAGs under nitrogen starvation or salt stress (Wang et al., 2009; Dean et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; James et al., 2011; Siaut et al., 2011). However, most experiments characterizing storage lipid synthesis in *C. reinhardtii* have been carried out under photoheterotrophic conditions, in acetate-containing medium (Wang et al., 2009; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; James et al., 2011; Siaut et al., 2011).

Large-scale cultivation of microalgae for biofuel production may need to be based on sunlight, captured by photosynthesis, as the main source of energy (Hu et al., 2008; Griffiths and Harrison, 2009; Rodolfi et al., 2009; Scott et al., 2010; Wijffels and Barbosa, 2010). Thus, metabolic and gene expression changes occurring in *Chlamydomonas* subject to nitrogen deprivation under strictly photoautotrophic conditions were examined. TAG and starch syntheses were also analyzed, under analogous environmental conditions, in another alga with sequenced nuclear, plastid, and mitochondrial genomes, *Coccomyxa* sp. C-169 (Smith et al., 2011). This microalga belongs to the class Trebouxiophyceae, within the division Chlorophyta (Palmqvist et al., 1997; Zoller and Lutzoni, 2003), and it is very divergent in phylogeny and habitat from *C. reinhardtii* (see below). Interestingly, nitrogen depletion

triggered a similar pattern of early synthesis of starch followed by significant TAG accumulation in these fairly different green microalgae. The results also suggest that turnover of nitrogen-rich compounds in the nutrient-starved cells may provide carbon/energy for TAG biosynthesis. Understanding the interdependence of these anabolic and catabolic pathways and their regulation may be key for the metabolic engineering of algal strains with improved biofuel productivity.

2. Results

2.1. Cell growth and triacylglycerol accumulation in C. reinhardtii subject to nitrogen deprivation

To analyze the effect of nitrogen (N) depletion on *Chlamydomonas* grown under photoautotrophic conditions, the wild-type CC-125 strain was precultured to middle logarithmic phase in high salt (HS) minimal medium (Harris, 1989). Cells were then centrifuged, resuspended in either HS or HS–N medium at a density of 0.5×10^6 cells mL^{-1} and grown under photoautotrophic conditions for 6 days. Cultures were sampled daily for specific analyses. In nitrogen replete medium, cell density increased ~8-fold during the examined period whereas in medium lacking nitrogen cells approximately doubled in number within the first 48 h followed by an arrest in cell division (Fig. 1A). As reported for *Chlamydomonas* grown in medium containing acetate (Work et al., 2010), it was also noticed in this study that the average cell size increased during acclimation to nitrogen starvation (Fig. 1B), presumably as a consequence of the greater accumulation of carbon storage compounds (see below).

To assess lipid accumulation during the growth period, cells were examined by fluorescence microscopy after staining with the nonpolar lipid fluorophore Nile Red (Greenspan et al., 1985; Chen et al., 2009). Lipid body formation, as revealed by Nile Red fluorescence, increased significantly in nitrogen-stressed cells relative to those cultured in nutrient-replete medium (Fig. 1B). The levels of lipid-derived fatty acid methyl esters (FAMEs) were also analyzed, by gas chromatography–flame ionization detection (GC–FID), to evaluate if the detected increase in nonpolar lipids during nitrogen deprivation corresponded to TAGs. Total cellular lipids were extracted and either derivatized and quantified by GC–FID or separated by thin layer chromatography (TLC). The TAG fraction, identified by co-migration with a purified TAG standard, was recovered from the TLC plate and its fatty acid content and composition also analyzed by GC–FID. Total fatty acid (FA) content per cell remained relatively constant, and similar to that of cells grown in nutrient replete medium, during the first 2 days of nitrogen starvation (Fig. 2A). However, FA levels showed an increase after 72 h in nutrient-depleted medium and doubled after 6 days of nitrogen deprivation. The proportion of FAs in TAGs also increased significantly in nitrogen-stressed cells (Fig. 2B), representing ~70% of the total fatty acids in the cells by the end of the examined period (Fig. 2C).

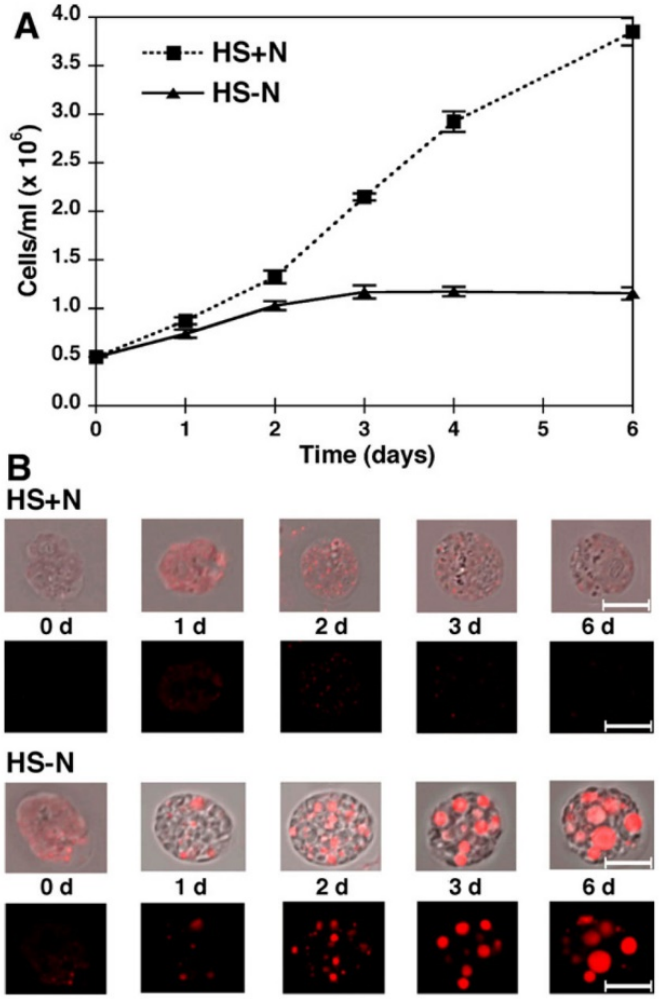

Figure 1. Growth and nonpolar lipid accumulation of *Chlamydomonas reinhardtii* CC-125 subject to nitrogen deprivation. Cells were cultured photoautotrophically, for the indicated times, in high salt medium (HS + N) or in the same medium lacking nitrogen (HS – N). (A) Growth curves displaying changes in cell density over time. Each data point represents the average of three independent experiments (± SE). (B) Fluorescence microscopy detection of nonpolar lipid accumulation by Nile Red staining. The images shown are representative of typical cells at the different time points. In each medium series, the upper panels correspond to merged transmitted light and fluorescent images, whereas the lower panels correspond to fluorescent images. Scale bars equal 10 μm.

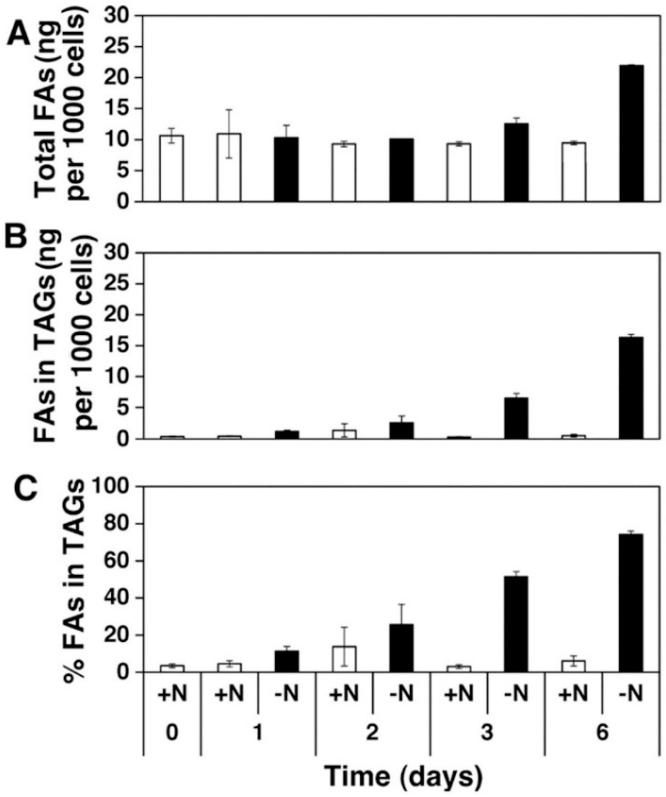

Figure 2. Total fatty acid and TAG accumulation in *Chlamydomonas* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were incubated for the indicated times in HS medium, either nutrient replete (+N) or nitrogen depleted (–N). Values indicate the mean of three independent experiments (± SE). (A) Total fatty acid content expressed as nanograms per 1000 cells. (B) Fatty acids in TAGs expressed as nanograms per 1000 cells. (C) Fatty acids in the TAG fraction expressed as percentage of the total FAs in a cell.

The fatty acid composition of *C. reinhardtii* subject to nitrogen starvation for 6 days was similar to that of the predominant TAG fraction (Table 1 and Supplementary Table 1). Cells grown in nutrient-replete medium were rich in polyunsaturated FA species characteristic of membrane lipids, in particular 16:4Δ4,7,10,13 and 18:3Δ9,12,15 (α-linolenic acid) (Table 1). In contrast, nitrogen-starved cells showed an increased abundance of saturated fatty acids and of those with lower degree of unsaturation such as 16:0 (palmitic acid), 18:1D9 (oleic acid), and 18:2D9,12 (linoleic acid) (Table 1). This compositional bias, similar to that of many plant oils (Cahoon and Schmid, 2008), was even more pronounced in the TAG fraction of cells starved for nitrogen during 6 days (Table 1 and Supplementary Table 1).

Table 1. Fatty acid composition (wt.% of total FAs ± SD, $n = 3$) of the total lipid extract (TLE) or purified triacylglycerols (TAG) from *C. reinhardtii* cells cultured in HS – N medium for the indicated times

	16:0	16:1	16:3	16:4 Δ4,7,10,13	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ5,9,12	18:3 9,12,15	18:4 Δ5,9,12,15	Other
0 day (TLE)	17.0 ± 0.2	3.4 ± 0.2	3.1 ± 0.1	20.2 ± 0.3	2.2 ± 0.	2.7 ± 0.1	3.8 ± 0.2	5.3 ± 0.7	5.5 ± 0.2	31.9 ± 0.4	3.7 ± 0.3	1.1
1 day (TLE)	20.4 ± 1.3	2.9 ± 0.4	2.9 ± 0.1	17.3 ± 0.5	2.1 ± 0.1	6.5 ± 0.9	3.7 ± 0.2	6.6 ± 0.5	5.8 ± 0.7	27.3 ± 0.8	3.3 ± 0.4	1.2
2 day (TLE)	21.9 ± 3.4	2.7 ± 0.5	3.1 ± 0.2	14.8 ± 3.3	2.3 ± 0.2	8.1 ± 2.2	3.9 ± 0.4	7.9 ± 1.1	5.9 ± 0.1	24.9 ± 3.0	3.0 ± 0.3	1.5
3 day (TLE)	27.5 ± 0.3	1.8 ± 0.2	2.9 ± 0.1	9.7 ± 0.8	2.6 ± 0.1	10.6 ± 1.3	4.5 ± 0.3	9.7 ± 0.4	6.8 ± 0.4	19.9 ± 1.1	2.4 ± 0.2	1.5
6 day (TLE)	30.6 ± 0.6	1.7 ± 0.1	2.7 ± 0.1	6.9 ± 0.2	2.9 ± 0.1	12.1 ± 0.5	4.4 ± 0.3	12.3 ± 0.2	6.6 ± 0.2	16.1 ± 0.6	2.1 ± 0.2	1.6
6 day (TAG)	31.6 ± 0.5	1.9 ± 0.1	2.5 ± 0.1	5.6 ± 0.2	2.7 ± 0.1	15.6 ± 0.6	4.3 ± 0.3	13.3 ± 0.2	6.0 ± 0.2	12.9 ± 0.5	1.9 ± 0.2	1.8

2.2. Changes in starch, chlorophyll, and protein content in C. reinhardtii *subject to nitrogen deprivation*

To characterize further the metabolic changes triggered by nitrogen shortage under photoautotrophic conditions, starch, chlorophyll, and protein levels in nutrient-stressed cells were also analyzed. Starch accumulation occurred very rapidly upon incubation in nitrogen-depleted medium, increasing by ~14-fold after 2 days of nutrient deprivation and reaching maximal level 1 day later, at ~18-fold the amount of starch measured in cells cultured in regular HS medium (Fig. 3A). Interestingly, the majority of starch synthesis appears to take place prior to the most significant accumulation of TAGs (cf. Figs. 3A and 2B).

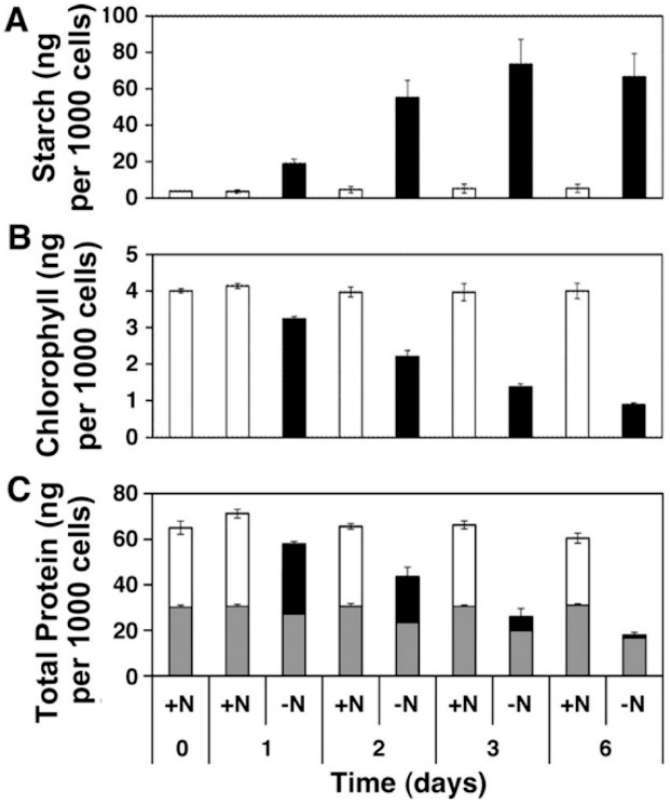

Figure 3. Starch, chlorophyll, and protein contents in *Chlamydomonas* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were cultured for the indicated times in HS medium, either nutrient replete (+N) or nitrogen depleted (–N). Values indicate the mean of three independent experiments (± SE). (A) Starch content expressed as nanograms per 1000 cells. (B) Chlorophyll amount expressed as nanograms per 1000 cells. (C) Total protein content expressed as nanograms per 1000 cells. The hatched bars indicate soluble protein amounts.

Nitrogen-starved cells had a yellowish appearance compared to those grown under nutrient-replete conditions, resulting from a marked decrease in chlorophyll content with time of exposure to nitrogen depleted medium (Fig. 3B). Indeed, cells incubated in nitrogen-

free medium for 6 days had ~20% the chlorophyll amount measured in cells cultured in standard HS medium. Similarly, the content of total and soluble proteins also decreased substantially in nutrient-deprived cells (Fig. 3C). *Chlamydomonas* cells subjected to nitrogen deprivation for 6 days had ~26% of the total protein amount in cells grown under nutrient replete conditions. Immunoblot assays also indicated a pronounced reduction, triggered by nitrogen shortage, in specific proteins such as the chloroplast located large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (involved in CO_2 fixation) and tryptophan synthase β subunit (involved in tryptophan biosynthesis) as well as in subunits of the cytosolic ribosome, like ribosomal protein S16 (Fig. 4). These three polypeptides decreased to between 25% and 30% of their normal levels after 6 days of nutrient depletion. In contrast, nitrogen starvation only caused a minor reduction in the steady-state amount of other proteins, such as histone H3 (Fig. 4). These observations, taken together, are consistent with progressive loss of certain plastidial functions, such as photosynthesis and amino acid biosynthesis, and of cytosolic protein translation capabilities triggered by nitrogen depletion. However, histone proteins, that may be critical for preserving chromatin organization, appear to be much less affected by nutrient deprivation.

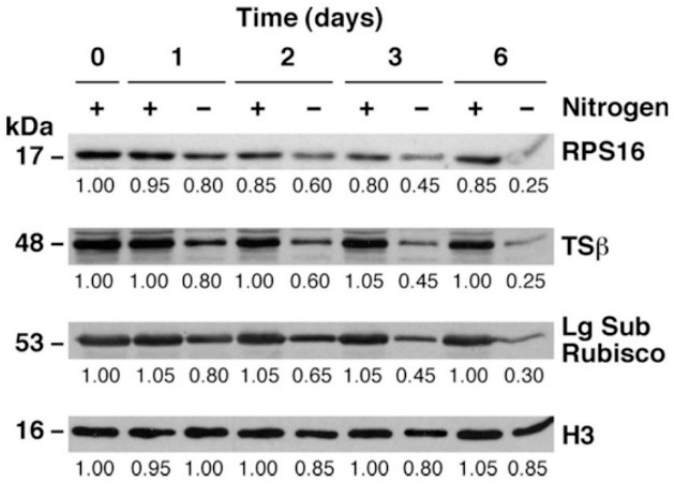

Figure 4. Immunoblot analysis of specific polypeptides in *C. reinhardtii* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in HS medium with (+) or without (–) nitrogen. Whole cell protein extracts were separated by SDS–PAGE, transferred to nitrocellulose and probed with antibodies raised against ribosomal protein S16 (RPS16), tryptophan synthase β subunit (TSβ), the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Lg Sub Rubisco) or histone H3 (H3). The panels show representative images from one out of three independent experiments. Numbers below the blots indicate the relative abundance of the examined proteins.

2.3. Triacylglycerol and starch accumulation in Coccomyxa sp. C-169 subject to nitrogen deprivation

To begin assessing the representativeness of *Chlamydomonas* as a model system for studying metabolic processes triggered by nutrient starvation in green microalgae, the lipid and starch contents in cells of *Coccomyxa* sp. C-169 experiencing nitrogen depletion were also investigated. *Coccomyxa* belongs to the class Trebouxiophyceae (together with species of the genus *Chlorella*) within the green algae, and it is quite divergent phylogenetically from *Chlamydomonas* which groups with members of the class Chlorophyceae (Palmqvist et al., 1997; Zoller and Lutzoni, 2003; Smith et al., 2011). In addition, strain C-169 originated from Marble Point Antarctica (Holm-Hasen, 1964) and is representative of algae adapted to an extreme environment as opposed to *Chlamydomonas* CC-125 which was reportedly collected from a potato field in Amherst, Massachusetts (Harris, 1989). Thus, it was reasoned that if similar metabolic responses to nitrogen deprivation are observed in *Coccomyxa* and *Chlamydomonas*, they might be indicative of processes conserved in a wide spectrum of green microalgae. However, *Coccomyxa* cells are smaller and grow slower, photoautotrophically under ambient levels of CO_2, than those of *Chlamydomonas* (data not shown). This limitation, which among other species-specific differences may be related to the lack of a carbon concentrating mechanism in *Coccomyxa* (Palmqvist et al., 1997), required longer incubation times in nitrogen-free medium to assess metabolic changes triggered by nutrient depletion.

As observed in *Chlamydomonas*, *Coccomyxa* cells examined by fluorescence microscopy, after staining with the nonpolar lipid fluorophore Nile Red, also showed a significant increase in lipid body formation when subjected to nitrogen deprivation (Fig. 5A). The analysis of FAMEs by GC–FID established that total fatty acid content per cell remained relatively constant during the first 4 days of nitrogen starvation but did rise markedly after that, attaining an ~80% increase by the end of the examined period (Fig. 5B). The proportion of FAs in TAGs also augmented considerably in nitrogen-stressed cells (Fig. 5C), representing ~70% of the total fatty acids in the cells after 11 days of nutrient deprivation (Fig. 5D).

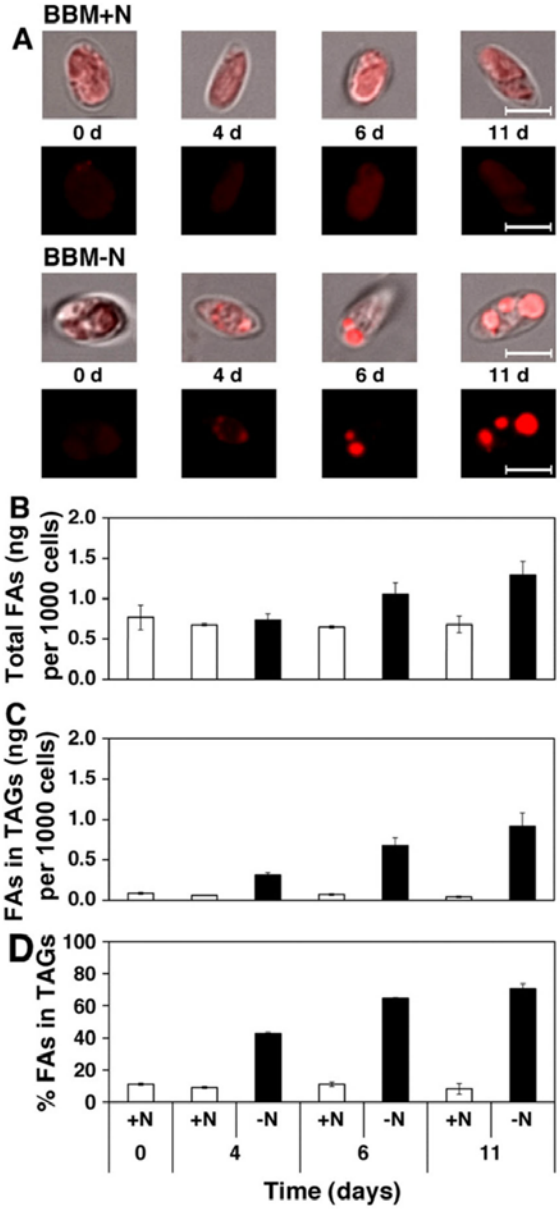

Figure 5. Lipid accumulation in *Coccomyxa* sp. C-169 subject to nitrogen deprivation under photoautotrophic conditions. Cells were incubated for the indicated times in BBM medium, either nutrient replete (+N) or nitrogen depleted (–N). (A) Fluorescence microscopy detection of nonpolar lipid accumulation by Nile Red staining. The images shown are representative of typical cells at the different time points. In each medium series, the upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bars equal 2.5 μm. (B) Total fatty acid content expressed as nanograms per 1000 cells. (C) Fatty acids in TAGs expressed as nanograms per 1000 cells. (D) Fatty acids in the TAG fraction expressed as percentage of the total FAs in a cell. Values indicate the mean of three independent experiments (± SE).

Coccomyxa cells grown in nutrient replete medium were rich in polyunsaturated FA species, in particular 18:2 and 18:3 (Table 2 and Supplementary Table 2). In contrast, nitrogen starved cells showed a marked increase in the abundance of the monounsaturated fatty acid 18:1 (Table 2 and Supplementary Table 2). These changes in FA composition were similar in trend to those occurring in *Chlamydomonas* under nitrogen deprivation, that is an increase in fatty acids with lower degree of unsaturation (cf. Tables 1 and 2). However, there were also species-specific differences. In *Chlamydomonas*, palmitic acid became the predominant FA in cells subject to nitrogen starvation whereas in *Coccomyxa* oleic acid showed the greatest abundance.

Table 2. Fatty acid composition (wt.% of total FAs ± SD, $n = 3$) of the total lipid extract (TLE) or purified triacylglycerols (TAG) from *Coccomyxa* sp. cells cultured in BBM – N medium for the indicated times

	16:0	16:1	16:2	16:3	18:1	18:2	18:3	Other
0 day (TLE)	17.5 ± 0.7	8.7 ± 1.0	7.8 ± 0.5	10.8 ± 0.8	7.5 ± 0.1	20.8 ± 0.3	23.6 ± 2.4	3.3
4 day (TLE)	18.1 ± 0.7	1.1 ± 0.5	2.9 ± 0.1	5.6 ± 0.1	30.4 ± 0.2	20.2 ± 0.3	17.4 ± 0.5	4.3
6 day (TLE)	17.0 ± 0.1	0.8 ± 0.1	1.7 ± 0.3	4.6 ± 0.3	40.1 ± 2.6	16.6 ± 1.1	14.8 ± 0.	4.5
11 day (TLE)	16.1 ± 0.4	1.3 ± 0.2	1.5 ± 0.0	3.9 ± 0.1	44.3 ± 1.7	15.1 ± 0.2	13.1 ± 0.1	4.7
11 day (TAG)	11.4 ± 0.3	0.4 ± 0.0	0.7 ± 0.1	2.5 ± 0.1	57.7 ± 0.3	13.7 ± 0.2	10.3 ± 0.3	3.2

Starch and chlorophyll contents in nitrogen-stressed *Coccomyxa* cells were also analyzed. Starch synthesis occurred much more rapidly than TAG accumulation, reaching a peak after 4 days of nitrogen deprivation (Fig. 6A). However, in relative terms, the amount of synthesized starch was much lower in *Coccomyxa* than in *Chlamydomonas* cells (cf. Figs. 3A and 6A). Nutrient shortage also resulted in a significant decrease in chlorophyll content in *Coccomyxa*. Cells incubated in nitrogen-free medium for 11 days had ~25% of the chlorophyll amount measured in cells cultured in standard medium (Fig. 6B). One caveat with the *Coccomyxa* experiments is that the small cell size and solid cell wall made it difficult to break the cells and extract metabolites, reducing the accuracy of starch measurements and making the analysis of protein content unreliable (data not shown). Nonetheless, the observations made herein suggest that TAG and starch accumulation, triggered by nitrogen depletion, followed similar overall patterns in *Chlamydomonas* and *Coccomyxa* grown photoautotrophically.

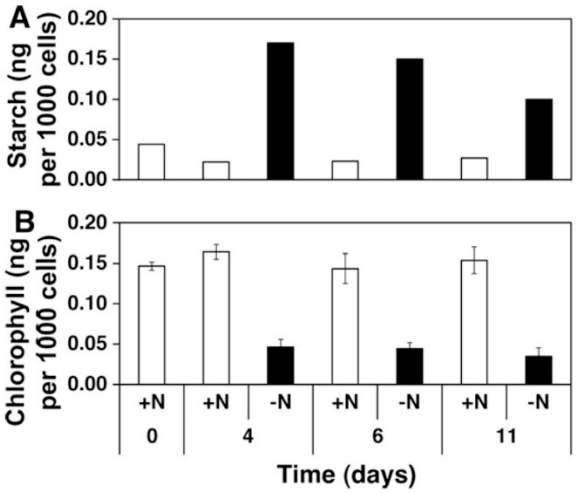

Figure 6. Starch and chlorophyll contents in *Coccomyxa* sp. C-169 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in BBM medium, either nutrient replete (+N) or nitrogen depleted (–N). (A) Starch content expressed as nanograms per 1000 cells. Values correspond to the average of two independent experiments. (B) Chlorophyll amount expressed as nanograms per 1000 cells. Values indicate the mean of three independent experiments (± SE).

2.4. Expression of lipid biosynthesis genes in C. reinhardtii *subject to nitrogen deprivation*

Homologs of many proteins involved in eukaryotic lipid metabolic pathways are encoded in the *C. reinhardtii* genome (Riekhof et al., 2005; Merchant et al., 2007; Khozin-Goldberg and Cohen, 2011). However, their expression under nitrogen depletion is poorly understood, as is the case in most microalgae (Hu et al., 2008; Miller et al., 2010; Khozin-Goldberg and Cohen, 2011). Thus, steady-state transcript levels for a few lipid biosynthesis genes were examined to assess whether their expression was consistent with the relatively late pattern of TAG accumulation under nitrogen stress (Fig. 2B). The *Chlamydomonas* genome contains six genes coding for diacylglycerol:acyl-CoA acyltransferases (DGATs), which catalyze the last step in the Kennedy pathway of TAG biosynthesis, the acylation of diacylglycerol to TAG (Weiss and Kennedy, 1956; Coleman and Lee, 2004; Courchesne et al., 2009). Based on primary sequence homology to functionally characterized eukaryotic enzymes, five genes encode DGAT2-like enzymes whereas the remaining one codes for a DGAT1 type isoform (Merchant et al., 2007; Khozin-Goldberg and Cohen, 2011; Turchetto-Zolet et al., 2011). However, no transcripts were detected, by reverse transcription-polymerase chain reaction (RT-PCR) assays, for one of the *DGAT2* genes (*DGTT5*; Prot ID: 536379) (data not shown). The remaining *DGAT2*-like genes—*DGTT1* (Prot ID: 536226), *DGTT2* (Prot ID: 519435), *DGTT3* (Prot ID: 523869), and *DGTT4* (Prot ID: 190539) as well as the unique *DGAT1* gene (Prot ID: 536378)—were tested for expression during the nitrogen-deprivation period by semiquantitative RT-PCR (Fig. 7).

While this manuscript was being prepared, Miller et al. (2010) reported a transcriptome study of *C. reinhardtii* subject to nitrogen deprivation under photoheterotrophic conditions. Their analysis focused predominantly on changes in transcript abundance occurring at 48 h

after nitrogen depletion in medium containing acetate. Under these conditions, *DGTT1* showed a large increase in steady-state transcript levels whereas the other *DGAT* genes displayed only minor or no change in expression (Miller et al., 2010). In the current study, it was also observed, under strictly photoautotrophic conditions, an early and substantial up-regulation of *DGTT1* in cells subject to nitrogen starvation (Fig. 7). Additionally, *DGTT3* and *DGTT4* were also expressed at significantly higher levels, in particular after 6 days of nitrogen deprivation (Fig. 7). These observations suggest that *DGTT1* may contribute to TAG synthesis early during nitrogen starvation but several other *DGAT* genes may also play a role during the period of maximal TAG accumulation under photoautotrophic conditions. Intriguingly, DGAT1-like enzymes are primarily involved in TAG biosynthesis in seeds of *Arabidopsis thaliana* (Zhang et al., 2009), but the only gene encoding this type of isoform in *Chlamydomonas* was expressed at relatively low levels and not affected by nitrogen depletion (Fig. 7).

The transcript abundance for two genes involved in *de novo* fatty acid synthesis was also examined. KASI, 3-ketoacyl-ACP Synthase I, is a component of the multimeric fatty acid synthase II (FASII) and catalyzes the acyl-acyl carrier protein (acyl-ACP) dependent elongation steps from C4 to C16 in higher plants (Baud and Lepiniec, 2010; Miller et al., 2010). The only gene in the *Chlamydomonas* genome coding for KASI (KASI; Prot ID: 205887) was found to decrease in expression, under photoautotrophic conditions, in cells subject to nitrogen depletion (Fig. 7). KAR, 3-ketoacyl-ACP reductase, is also part of the FASII complex and reduces the carbon 3 ketone to a hydroxyl group during FA synthesis (Baud and Lepiniec, 2010). This enzyme is also encoded by a single gene in the *Chlamydomonas* genome (*KAR*; Prot ID: 335991), and its transcript abundance decreased slightly in nitrogen-deprived cells (Fig. 7). These results were somewhat surprising since doubling of the FA content in cells starved for nitrogen (Fig. 2A) is strongly suggestive of *de novo* fatty acid synthesis. Moreover, experiments with cerulenin, a specific inhibitor of the 3-ketoacyl-ACP synthase of FASII, have also implicated *de novo* FA synthesis in TAG accumulation in nitrogen-deprived *Chlamydomonas*, albeit under photoheterotrophic conditions (Fan et al., 2011). Interestingly, in the transcriptome analysis of Miller et al. (2010) the *KASI* mRNA increased by ~2-fold after 48 h of nitrogen deprivation in acetate-containing medium. As discussed below, FASII enzymatic activity may not be limiting for TAG accumulation under our experimental conditions, but differences in substrate availability under photoautotrophic or photoheterotrophic conditions may cause variations in the response of algal metabolic pathways to nitrogen depletion.

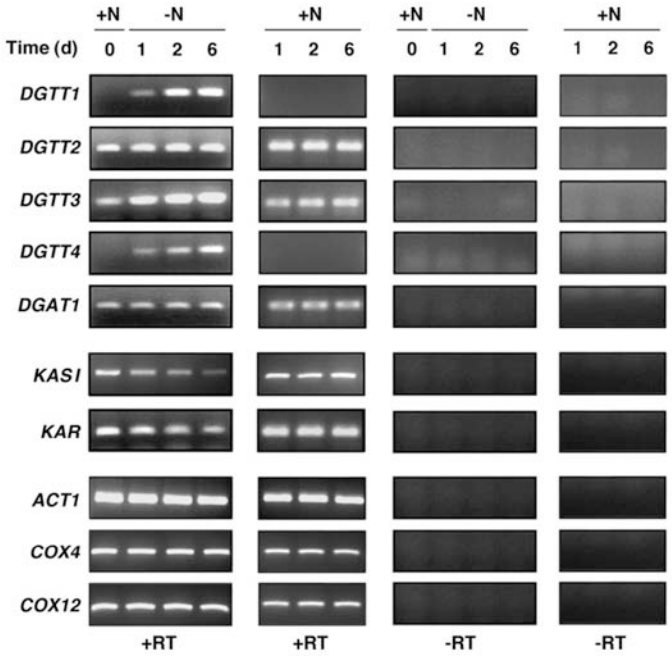

Figure 7. Expression of lipid biosynthesis genes in *Chlamydomonas reinhardtii* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in HS medium with (+N) or without (–N) nitrogen. Transcript abundance corresponding to specific genes involved in lipid synthesis was analyzed by semiquantitative RT-PCR. Reactions were performed as described under experimental procedures in the presence (+RT) or absence (–RT) of reverse transcriptase. The panels show representative images of agarose resolved RT-PCR products stained with ethidium bromide. Examined genes included those encoding diacylglycerol:acyl-CoA acyltransferases type 2 (*DGTT1*, *DGTT2*, *DGTT3*, and *DGTT4*) and type 1 (*DGAT1*), 3-ketoacyl-ACP synthase I (*KASI*) and 3-ketoacyl-ACP reductase (*KAR*). Amplification of the mRNAs corresponding to ACT1 (encoding actin), *COX4* (encoding mitochondrial cytochrome c oxidase subunit Vb), and *COX12* (encoding mitochondrial cytochrome c oxidase subunit VIb) were used as controls for equal amounts of input RNA and for the efficiency of the RT-PCRs.

3. Discussion

C. reinhardtii accumulates both starch and TAGs when subject to a number of stresses such as nitrogen deprivation, high salinity, sulfur depletion, or when exposed to high light (Klein, 1987; Ball et al., 1990; Matthew et al., 2009; Wang et al., 2009; Dean et al., 2010; Doebbe et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; Kropat et al., 2011; James et al., 2011; Siaut et al., 2011). Several groups have begun characterizing the metabolic pathways involved in TAG synthesis in nutrient-stressed cells of this organism (Wang et al., 2009; Dean et al., 2010; Li et al., 2010; Miller et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; Siaut et al., 2011). However, most studies have been performed under photoheterotrophic conditions (Wang et al., 2009; Li et al., 2010; Miller et al., 2010; Moellering and Benning, 2010; Work et al., 2010;

Fan et al., 2011; James et al., 2011; Siaut et al., 2011). In this case, acetate in the medium appears to contribute substantially to the accumulated TAGs (Fan et al., 2011, and data not shown) and, possibly, to the synthesized starch (Ball et al., 1990; Work et al., 2010). *Coccomyxa* species that participate, as lichen photobionts, in symbiotic associations with fungi are also capable of accumulating TAGs, but the effect of environmental factors on lipid metabolism is poorly understood in these algae (Guschina et al., 2003).

Microalgae are considered a promising source of renewable biofuels. Yet, for the production of algal biofuels to become economically feasible, various studies have underlined the necessity of low-cost culture systems, using sunlight as the sole or main energy source for biomass synthesis (Hu et al., 2008; Griffiths and Harrison, 2009; Rodolfi et al., 2009; Scott et al., 2010; Wijffels and Barbosa, 2010). Thus, metabolite accumulation in *Chlamydomonas* and *Coccomyxa* cells subject to nitrogen deprivation was examined under strictly photoautotrophic conditions, so that the information gained may be useful for understanding the biology of production strains (Morowvat et al., 2010). Nutrient depletion led to an arrest in cell division and an increase in starch and TAG content. In the absence of the nitrogen necessary for protein synthesis and cell growth, excess carbon from photosynthesis appears to be channeled into storage molecules, such as starch and TAGs. However, a detailed time course analysis of the metabolic changes triggered by nitrogen depletion suggests that turnover of nonlipid cellular components may also play a role in TAG accumulation. The involvement of two processes, photosynthesis and recycling of previously assimilated carbon, in the synthesis of lipids has also been proposed for the diatom *Cyclotella cryptica* subjected to silicon deficiency (Roessler, 1988).

Upon nitrogen deprivation, *Chlamydomonas* cells initially accumulate starch, which increases its content ~14-fold during the first 2 days of stress (Fig. 3A). Starch biosynthesis likely involves newly fixed carbon through photosynthesis since chlorophyll and protein contents remain relatively high at the beginning of the nutrient deprivation (Figs. 3B, C and 4). In contrast, there is virtually no change in total fatty acid levels on a per cell basis during this period (Fig. 2A). Nonetheless, since the cells almost double in number in the first 48 h of stress (Fig. 1A), maintaining a steady-state amount of fatty acids per cell implies the occurrence of *de novo* synthesis. The majority of the FAs are likely devoted to preserving membrane homeostasis, but a small fraction may also be used for incipient TAG biosynthesis (Fig. 2B). Although, our data cannot discriminate whether FAs employed in early TAG production are synthesized *de novo* and/or recycled from preexisting lipids such as those of the plastid membranes (Wang et al., 2009; Siaut et al., 2011).

At latter time points, *Chlamydomonas* cells show clear accumulation of total fatty acids (Fig. 2A) and a significant increase in TAG content (Fig. 2B and C). Conversely, starch levels display a slight decline after prolonged incubation in nitrogen-deprived medium, which becomes more obvious after 10 days of nutrient stress (data not shown). These observations suggest considerable *de novo* fatty acid synthesis in cells depleted of nitrogen for several days, but the synthesized lipids are unlikely to derive exclusively from newly (photosynthetically) assimilated carbon since chlorophyll and enzymes like Rubisco, that are essential for CO_2 fixation, are greatly diminished at these time points (Figs. 3B and 4). Indeed, a marked decrease in photosynthesis and overall anabolic processes has been re-

ported for nitrogen starved *Chlamydomonas* cells under both photoautotrophic and photoheterotrophic conditions (Martin and Goodenough, 1975; Bulte and Wollman, 1992; Li et al., 2010; Miller et al., 2010; Work et al., 2010). Thus, in *C. reinhardtii* cultured under photoautotrophic conditions in nitrogen-depleted medium, fatty acids for TAG biosynthesis may be partly obtained at the expense of the carbon/energy assimilated in other cellular components, in particular proteins which decrease substantially in content (Figs. 3C and 4), ribosomal RNAs recycled as a consequence of ribosome degradation (Martin and Goodenough, 1975; Martin et al., 1976; data not shown) and, to some degree, starch and chlorophyll (Fig. 3A and B). Conversion of previously assimilated carbon in the form of starch to nonpolar lipids, under nitrogen-limited conditions, has also been proposed for the oleaginous green alga *Pseudochlorococcum* sp. (Li et al., 2011).

Coccomyxa cells cultured in nitrogen-deprived medium accumulated less starch, in relative terms, than *Chlamydomonas* but a similar proportion of TAGs, representing ~70% of the total fatty acids in the cells after 11 days of nutrient deprivation (Figs. 5 and 6). The predominant fatty acid in *Coccomyxa* triacylglycerols was oleic acid, whereas palmitic acid showed the greatest abundance in *Chlamydomonas*. Interestingly, despite these species-specific differences, both *Coccomyxa* sp. C-169 and *C. reinhardtii* CC-125 displayed similar trends in the accumulation of starch and TAGs and in the reduction of chlorophyll content triggered by nitrogen deprivation. Given the substantial divergence in habitat and phylogeny between these algal species (see Section 2.3), these observations suggest that certain metabolic responses to N shortage may be shared by a broad range of green microalgae.

TAG biosynthesis can occur by several enzymatic mechanisms in eukaryotes (Hu et al., 2008; Baud and Lepiniec, 2010; Khozin-Goldberg and Cohen, 2011). In the Kennedy pathway, DGATs catalyze the acylation of diacylglycerol to TAGs (Weiss and Kennedy, 1956; Coleman and Lee, 2004; Courchesne et al., 2009). Although the specific mechanisms of TAG synthesis in microalgae are poorly characterized, increased abundance of the transcripts for several DGAT homologs (this work and Miller et al., 2010), particularly at later time points during nitrogen starvation, suggests that these enzymes may play a role in *Chlamydomonas*. In contrast, transcripts for KASI and KAR, subunits of the fatty acid synthase II complex (Baud and Lepiniec, 2010), appear to decrease as cells are incubated photoautotrophically in nitrogen-depleted medium. These results are counterintuitive since doubling of the FA content in nitrogen-starved *Chlamydomonas* cells is strongly suggestive of *de novo* fatty acid synthesis (Fig. 2A) and experiments with cerulenin, an inhibitor of KASI, have implicated *de novo* FA synthesis in TAG accumulation in medium containing acetate (Fan et al., 2011). Additionally, *KASI* appears to be up-regulated when cells are subject to nitrogen depletion in the presence of acetate (Miller et al., 2010). With the caveat that mRNA levels do not necessarily reflect protein amounts or enzymatic activities, we hypothesize that expression of (some) genes encoding FASII enzymes may be modulated by substrate availability. In this context, normal FASII activity may not be limiting for TAG accumulation under photoautotrophic conditions, as precursors for fatty acid synthesis may be scarce, likely derived at least in part from the recycling of previously assimilated carbon in proteins, ribosomal RNAs, chlorophyll, and possibly starch. However, KASI upregulation may be necessary when exogenous acetate provides an abundant precursor for lipid synthesis (Fan et al., 2011). This interpretation is consistent with the ~35% greater

accumulation of TAGs in cells subject to nitrogen deprivation for 6 days in acetate containing medium in comparison with those cultured in minimal medium (data not shown).

The primary product of CO_2 fixation, 3-phosphoglycerate, feeds directly into the starch biosynthesis pathway, but it can also be used as a precursor of acetyl-CoA for FA synthesis and of the glycerol backbone of TAGs (Hu et al., 2008; Radakovits et al., 2010). This prompted several groups to investigate whether biosynthesis of starch and TAGs compete with each other and whether *Chlamydomonas* mutants defective in starch synthesis would accumulate higher levels of lipids under nitrogen starvation (Wang et al., 2009; Li et al., 2010; Work et al., 2010; Siaut et al., 2011). Despite some promising results (Wang et al., 2009; Li et al., 2010; Work et al., 2010) the relationship between these two metabolic pathways appears to be more complex than mere competition (Work et al., 2010; Li et al., 2011; Siaut et al., 2011), emphasizing our incomplete understanding of the interdependence and regulation of metabolic processes in microalgae. Additionally, the results presented here suggest that recycling of other nonlipid cellular components may also contribute carbon/energy for TAG synthesis under nitrogen depletion.

4. Concluding remarks

Nutrient deprivation is one of the common stresses encountered by microorganisms in nature (Elser et al., 2007). Under nutrient-replete conditions, growth is promoted via increased transcription and translation, processes that require large amounts of anabolic structural components such as ribosomes (Warner, 1999). Indeed, proteins are the dominant fraction in the biomass of fast-growing photosynthetic microorganisms (Langner et al., 2009; Huo et al., 2011). In contrast, under nutrient depletion, growth is inhibited and most of the anabolic machinery becomes superfluous (Acquisti et al., 2009). In this context, anabolic structural components, such as ribosomes and chlorophyll, may be targeted for degradation and nutrient recycling (Kraft et al., 2008). When exogenous nitrogen is depleted, selective autophagic processes may function to make available endogenous nitrogen for limited *de novo* protein synthesis so that cells can adapt and change their fate, including in the case of *Chlamydomonas* differentiation into sexual gametes and acquisition of the ability to survive a prolonged nutrient stress (Martin and Goodenough, 1975; Ball et al., 1990; Wang et al., 2009). The recycled excess carbon likely provides substrates/energy for the synthesis of nitrogen-poor storage compounds such as TAGs. A greater understanding of these metabolic processes may allow the genetic engineering of algal strains with increased capacity to convert their biomass into useful biofuel precursors.

5. Experimental

5.1. Strains and culture conditions

C. reinhardtii CC-125 and *Coccomyxa* sp. C-169 were used in all the reported experiments. Unless stated otherwise, cultures were incubated under continuous illumination (180 μmol m^{-2} s^{-1} photosynthetically active radiation) on an orbital shaker (190 rpm) at 25°C and ambient levels of CO_2. Cells were initially grown photoautotrophically to the middle of the logarithmic phase in nitrogen replete high salt (HS) medium (Sueoka, 1960), in the case of

Chlamydomonas, or Bold's basal medium (BBM) (Bold, 1949), in the case of *Coccomyxa*. These precultured cells were collected by centrifugation and resuspended at a density of 0.5–1.0 × 10^6 cells mL^{-1} in regular HS or BBM media or in the same media lacking nitrogen (HS – N or BBM – N). Samples for analysis were taken immediately after resuspension (0 day) and at the times indicated in the figures and tables. Culture growth was monitored by counting cells with a hemocytometer (Harris, 1989).

5.2. Fluorescence microscopy

To assess the effect of nitrogen deprivation on nonpolar lipid accumulation, cells were stained with the lipophilic fluorophore Nile Red (Greenspan et al., 1985) as previously described (Chen et al., 2009). Images were acquired with a laser scanning confocal microscope (Olympus Fluoview 500), using an 100× oil immersion lens, and analyzed with the Fluoview (v4.3) software. Laser excitation was at an emission wavelength of 443 nm, and Nile Red fluorescence was detected between 560 and 590 nm using band-pass filtering. In the presence of nonpolar lipids, Nile Red emits a yellow-gold fluorescence (λ_{max} = 580 nm) (Greenspan et al., 1985), which is shown as red pseudo coloring in Figs. 1B and 5A.

5.3. Lipid analyses

Total lipids were extracted with a Bligh and Dyer (1959) procedure, transmethylated and quantified as fatty acid methyl esters (FAMEs) by gas chromatography with flame ionization detection (GC–FID) as described previously (Cahoon et al., 2006). Briefly, ~1.5 x 10^8 cells were collected by centrifugation in a 13 × 100 mm glass test tube with Teflon-lined screw cap. After removal of culture media by aspiration, triheptadecanoin (300 μg) (Nu-Chek Prep) was added as an internal standard from a stock solution at a concentration of 10 mg mL^{-1} in toluene. Lipids were then extracted from the cell pellet using a modification of the method described by Bligh and Dyer (1959). MeOH:$CHCl_3$ (3 mL, 2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene were added to the cell pellet and incubated at 25°C for 30 min. After addition of $CHCl_3$ (1 mL) and H_2O (1.8 mL), the tube was shaken vigorously and the content partitioned into two phases by centrifugation at 1000g. The upper phase was discarded and the lower organic phase, containing the extracted lipids, was transferred to a new glass tube. Extracted lipids were dried under a stream of N_2 and resuspended in $CHCl_3$:MeOH (0.5 mL, 6:1, v/v). One fourth of this extract was used for measuring total fatty acid content, and the remainder was used for measuring TAGs (see below). For analysis of the content and composition of fatty acids an aliquot of the lipid extract (125 μL) was transferred to another glass screw cap test tube, dried under N_2, and resuspended in toluene (250 μL) and H_2SO_4 in MeOH (1 mL, 2.5%, v/v). The tube was capped under N_2 and heated at 90°C for 30 min. Upon cooling, H_2O (0.5 mL) and heptane (0.7 mL) were added. The tube was shaken vigorously, and the contents were separated into two phases by centrifugation. The upper heptane layer containing FAMEs was analyzed using an Agilent 7890 gas chromatograph (Agilent Technologies) fitted with an Agilent INNOWax column (0.25 mm inner diameter × 30 cm length). The oven temperature was programmed from 185°C (1 min hold) to 235°C (2.5 min hold) at a rate of 7°C/min with hydrogen as the carrier gas. FAMEs levels were quantified relative to the methyl heptadecanoate from the internal standard. Different fatty acid methyl esters were identified by mobility relative to

standards as well as by gas chromatography–mass spectrometry using an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass selective detector. Chromatography conditions in the latter case were the same as described above, except for the use of helium as the carrier gas.

For analysis of TAGs, the remainder of the total lipid extract was dried under N_2 and resuspended in $CHCl_3$:MeOH (100 μL, 6:1, v/v). This extract was applied to a silica 60 thin layer chromatography plate (Sigma-Aldrich) and neutral lipids were resolved using a solvent system of 70:30:1 (v/v/v) heptane:Et_2O:AcOH. The TAG band was identified by co-migration with a TAG standard, stained lightly with iodine vapors, in an adjacent lane. The TAG fraction was then recovered from the plate and lipids resuspended in 0.4 mL of toluene and H_2SO_4 in MeOH (1.5 mL, 2.5%, v/v). Fatty acid methyl esters were prepared, extracted, and analyzed by gas chromatography as described above.

Double bond positions of unsaturated fatty acids were confirmed by GC–MS of derivatives generated from fatty acid methyl esters. Pyrrolidine derivatives were prepared and analyzed for double bond positions of polyunsaturated fatty acids (Andersson et al., 1975), and dimethyl disulfide derivatives were prepared and analyzed for double bond positions of monounsaturated fatty acids (Cahoon et al., 1994). Analyses of fatty acid derivatives was conducted with an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass selective detector fitted with a DB-1MS column (Agilent; 30 m length × 0.25 mm inner diameter × 0.25 μM film thickness). The GC oven was programmed from 185°C (1 min hold) at 10°C/min to 320°C (10 min hold).

5.4. Starch assays

Starch measurements were performed using an EtOH-washed chlorophyll-free cell pellet (Ball et al., 1990). In brief, ~2.0×10^7 cells were harvested by centrifugation, resuspended in an ethanol solution for chlorophyll extraction and then centrifuged again. Pellets were resuspended in distilled H_2O and boiled for 10 min to solubilize the starch. In the case of *Coccomyxa*, resuspended pellets were autoclaved for 15 min at 120°C for starch solubilization. Total starch was quantified using a commercial kit (Starch Assay Kit SA-20; Sigma-Aldrich), based on amyloglucosidase digestion to convert starch to glucose, according to the manufacturer's instructions.

5.5. Chlorophyll measurements

Chlorophyll content was determined using EtOH extraction (Arnon, 1949). An aliquot (1 mL) of culture, at a concentration of 1.0 × 107 cells mL^{-1}, was centrifuged and the pellet resuspended in 96% ethanol and vortexed to extract pigments. Cellular debris was pelleted by centrifugation and chlorophyll *a* and *b* levels were determined spectrophotometrically, in the supernatant, by measuring optical absorbance at 645 and 663 nm. Calculations of total chlorophyll (μg mL^{-1}) were performed as previously described (Arnon, 1949; Harris, 1989).

5.6. Protein determination

The concentration of solubilized proteins extracted from ~5.0×10^6 *Chlamydomonas* cells was determined using the Bio-Rad Protein Assay, by measuring absorbance at 595 nm with

a microplate reader. Briefly, cells were sonicated for 20 s (3×) in lysis buffer (250 μL, 50 mM Tris-HCl, pH 8.0, 20% guanidine hydrochloride, 10 mM EDTA, 10 mM DTT, 0.4 μM PMSF) and, after addition of 0.05% Triton X-100, centrifuged at 12,000g for 10 min. The supernatant was used for protein determination by the Bradford assay (Bradford, 1976) following the manufacturer's instructions (Bio-Rad). Dilutions of bovine serum albumin were used to prepare a series of protein standards. Total protein was measured by Lowry analysis with a commercially available kit (Sigma-Aldrich).

5.7. Immunoblot analyses

The steady-state level of selected proteins in *Chlamydomonas* was examined by Western blotting as previously described (van Dijk et al., 2005). Histone H3 and Ribosomal Protein S16 were detected with commercially available antibodies (ab1791 and ab26159, respectively; Abcam). The antibody against the large subunit of Rubisco was a generous gift of Robert Spreitzer (University of Nebraska–Lincoln) whereas the antibody against tryptophan synthase β subunit was kindly provided by Thomas McKnight (Texas A&M University).

5.8. Semiquantitative RT-PCR assays

Total RNA was isolated from *Chlamydomonas* cells with TRI reagent (Molecular Research Center), in accordance with the manufacturer's instructions, and treated with DNase I (Ambion) to remove contaminating DNA. Reverse transcription reactions were carried out as previously described (Carninci et al., 1998), and the synthesized cDNAs were then used as template in standard PCR reactions (Sambrook and Russell, 2001). The numbers of cycles showing a linear relationship between input RNA and the final product were determined in preliminary experiments. Most primers were designed to match exonic sequences flanking one or more introns to distinguish contaminating PCR products possibly generated by amplification of any remaining DNA. Controls also included the use as template of reactions without reverse transcriptase and verification of PCR products by hybridization with specific probes (data not shown). The PCR conditions for amplification of most templates were 30 cycles at 94°C for 30 s, at 56°C for 30 s and at 72°C for 20 s. Aliquots (5 μl) of each RT-PCR were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. The primer sequences were as follows: for *DGAT*1 511566-RT-F1, 50-ACTGGTGGAATGCGGCTAC-3′ and 511566-RT-R1 5′-TAGCAGCTCGTGGAACACAG-3′; for *DGTT1* 285889-RT-F1 5′-GAAGCAGGTGTTTGGCTTCT-3′ and 285889-RT-R1 5′-CAG TGCCTCCGTGTAGGTCT-3′; for *DGTT2* 184281-RT-F1 5′-GCGCCGCAACATTTACA TGG-3′ and 184281-RT-R1 5′-CAGCCGTACTCGGTCTTGTG-3′; for *DGTT3* 188937-RT-F1 5′-GTCAGAGCCAAGTGCTGGAC-3′ and 188937-RT-R1 5′-TCCACCTCCTTGTCGAAC TC-3′; for *DGTT4* 190539-RT-F1 5′-GCATGTTTGGGCAGTACGGC-3′ and 190539-RT-R1 5′-GCCTTGTGCTTGTCGTACAG-3′; for *DGTT5* 141301-RT-F1 5′-AGTCACTGCAGCAGC TGTCG-3′ and 141301-RT-R1 5′-GCCCACACACATCATGAGCG-3′; for *KASI* 205887-RT-F1 5′-CAGTGTGCTGCGGAATGC-3′ and 205887-RT-R1 5′-GGTCACACAAACACACATTTGA-3′; for *KAR* 335991-RT-F1 5′-GTCATCGGCCTGACCAAG-3′ and 335991-RT-R1 5′-ATGCCCT TGAGGATGGTCTC-3′; for *ACT1* ACT-Cod-F 5′-GACATCCGCAAGGACCTCTAC-3′ and ACT-Cod-R 5′-GATCCACATTTGCTGGAAGGT-3′; for *COX4* 187882-RT-F1 5′-GGATAA

GTTCGGCACTGAGG-3′ and 187882-RT-R1 5′-CGCCGACCTTCTTGATGTAC-3′ and for *COX12* 195712-RT-F1 5′-CACTGCTTTGTGCGATTCAA-3′ and 195712-RT-R1 5′-TTAGTA CTTGCCGGTCCACA-3′.

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Supplementary Table 1

Content of fatty acids in whole cells (WC) or in purified TAGs (TAG) from *C. reinhardtii* maintained in HS-N medium for the indicated times. Values shown are expressed as ng fatty acids/1000 cells ± s.d. (n = 3).

	16:0	16:1	16:3	16:4 Δ4,7,10,13	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ5,9,12	18:3 Δ9,12,15	18:4 Δ5,9,12,15	Other
0d (WC)	1.82±0.22	0.36±0.02	0.33±0.04	2.14±0.22	0.23±0.02	0.29±0.03	0.40±0.02	0.58±0.14	0.59±0.09	3.38±0.34	0.39±0.01	0.13
1d (WC)	2.35±0.56	0.34±0.08	0.33±0.07	1.97±0.40	0.23±0.03	0.72±0.14	0.41±0.05	0.74±0.12	0.64±0.10	3.04±0.45	0.36±0.04	0.13
2d (WC)	2.20±0.35	0.28±0.05	0.32±0.02	1.49±0.33	0.23±0.02	0.82±0.22	0.39±0.04	0.79±0.11	0.60±0.01	2.51±0.29	0.30±0.03	0.15
3d (WC)	3.46±0.31	0.23±0.04	0.36±0.05	1.20±0.09	0.32±0.02	1.35±0.27	0.57±0.07	1.22±0.13	0.85±0.04	2.48±0.10	0.30±0.01	0.20
6d (WC)	6.71±0.18	0.38±0.02	0.59±0.01	1.51±0.05	0.64±0.01	2.65±0.12	0.96±0.05	2.71±0.06	1.45±0.04	3.53±0.13	0.45±0.04	0.35
6d (TAG)	5.16±0.23	0.30±0.01	0.41±0.02	0.92±0.03	0.44±0.01	2.54±0.13	0.70±0.02	2.18±0.10	0.98±0.04	2.09±0.02	0.31±0.02	0.29

Supplementary Table 2

Content of fatty acids in whole cells (WC) or in purified TAGs (TAG) from *Coccomyxa* sp. maintained in BBM-N medium for the indicated times. Values shown are expressed as ng fatty acids/1000 cells ± s.d. (n=3).

	16:0	16:1	16:2	16:3	18:1	18:2	18:3	Other
0d (WC)	0.14±0.03	0.07±0.01	0.06±0.01	0.08±0.01	0.06±0.01	0.16±0.03	0.19±0.06	0.03
4d (WC)	0.13±0.01	0.01±0.01	0.02±0.00	0.04±0.01	0.22±0.03	0.15±0.02	0.13±0.02	0.03
6d (WC)	0.18±0.03	0.01±0.01	0.02±0.00	0.05±0.01	0.42±0.09	0.17±0.01	0.15±0.01	0.05
11d (WC)	0.21±0.02	0.02±0.00	0.02±0.00	0.05±0.01	0.57±0.10	0.19±0.02	0.17±0.02	0.06
11d (TAG)	0.10±0.02	0.01±0.00	0.01±0.00	0.02±0.00	0.53±0.09	0.13±0.02	0.10±0.01	0.03