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Triacylglycerol synthesis during nitrogen stress involves the prokaryotic lipid synthesis pathway and acyl chain remodeling in the microalgae *Coccomyxa subellipsoidea*

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ABSTRACT

Triglyceride (TAG) synthesis during nitrogen starvation and recovery was addressed using *Coccomyxa subellipsoidea* by analyzing acyl-chain composition and redistribution using a bioreactor-controlled time course. Galactolipids, phospholipids and TAGs were profiled using liquid chromatography tandem mass spectroscopy (LC–MS/MS). TAG levels increased linearly through 10 days of N starvation to a final concentration of 12.6% dry weight (DW), while chloroplast membrane lipids decreased from 5% to 1.5% DW. The relative quantities of TAG molecular species, differing in acyl chain length and glycerol backbone position, remained unchanged from 3 to 10 days of N starvation. Six TAG species comprised approximately half the TAG pool. An average of 16.5% of the acyl chains had two or more double bonds consistent with their specific transfer from membrane lipids to TAGs during N starvation. The addition of nitrate following 10 days of N starvation resulted in a dramatic shift from chloroplast-derived to endoplasmic reticulum-derived galactolipids (from <12% to >40%). A model for TAG synthesis in *C. subellipsoidea* was developed based on the acquired data and known plant pathways and data presented.

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1. Introduction

Eukaryotic green algae accumulate triglyceride (TAG) at the expense of cellular reproduction while under abiotic stress (e.g., nitrogen, sulfur and iron deprivation) [1,2]. Recent studies using continuous cultures support this correlation by demonstrating a progression between reproductive growth and lipogenesis depending on the extent of the stress applied [3,4]. This correlation is not as obvious during less extreme stress conditions. In the case of reduced N deprivation, several reports in some algal species have shown an increased biomass [4,5]. These studies are consistent with a specific dissociation between growth and lipogenesis. It is precisely this dissociation that supports the generation and/or selection of an algal TAG production strain, which is essential and a key priority in algal biofuel research. In order to achieve this goal, it is first essential to gain a comprehensive understanding of metabolic changes leading to TAG synthesis in microalgae.

Genomic and transcriptional profiling show a number of algal species contain TAG synthesis, membrane lipid remodeling, and acyl chain editing genes that are homologous to known plant genes and further demonstrate differential expression patterns under conditions promoting TAG synthesis [6–10]. Phosphatidylcholine:diacylglycerol acyltransferase (PDAT) and the type-1 and type-2 diacylglycerol acyltransferases (DGATs), for example, are up-regulated under N starvation in *Chlamydomonas reinhardtii*. The role of PDAT in TAG synthesis has been confirmed by insertional mutagenesis [7]. While there are some changes in gene expression, the expectation of significant differential gene expression during N starvation has not been confirmed in proteomic studies. The levels of most lipid metabolic enzymes remain relatively constant during N stress [11–13] suggesting changes in their activities are likely the result of post-translational regulation as opposed to significant de novo protein synthesis. Metabolomic approaches, likewise, have been unable to completely address the specific metabolic processes and their relative importance leading to increased TAG synthesis [14–16].

Comprehensive studies addressing the metabolic processes underlying TAG synthesis in algae have lagged behind the phenotypic data showing oil accumulation during abiotic stress. In plants, TAG synthesis occurs through a complex and interwoven set of metabolic steps, which are organellar- and tissue-specific, many of which occur at specific

Abbreviations: LC–MS/MS, liquid chromatography tandem mass spectroscopy; GC–MS, gas chromatography mass spectroscopy; TAG, triglyceride; MRM, multiple reaction monitoring; DAG, diacylglyceride; MGDG, monogalactosyl-diacylglyceride; DGDG, digalactosyl-diacylglyceride; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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points during development [17]. Chloroplast- and ER-specific isoforms of lysophosphatidic acid acyltransferase (LPAT) catalyze the addition of palmitoyl-CoA or oleoyl-CoA, respectively, at the *sn*-2 position of the glycerol backbone to form phosphatidic acid, which is subsequently dephosphorylated by phosphatidic acid phosphatase (PAP) to form diacylglycerol (DAG) leading to distinctive pools of complex lipids [18]. TAG synthesis occurs in the ER from DAG and acyl-CoA, which is catalyzed by one of several isoforms of DGAT [19–21]. The Kennedy Pathway coexists with phosphatidylcholine:diacylglycerol acyltransferase (PDAT), which catalyzes the coenzyme A (CoA) independent acylation of DAG using phosphatidylcholine (PC) as the acyl donor, resulting in TAG and lyso-PC [22]. In developing soybeans, the primary acceptor of de novo synthesized fatty acid is lyso-PC resulting in PC, which in turn functions as the acyl donor for TAG synthesis [23]. This pathway provides the bulk of TAG synthesis in developing leaves, while DGAT is more active during leaf senescence [24]. A third enzyme catalyzing the acylation of phytol during leaf senescence or stress in *Arabidopsis thaliana*, phytyl ester synthase (PES), was recently characterized with DGAT bi-functionality ex vivo, making PES the only known chloroplastic DGAT [25]. Adding to the complexity of TAG synthesis are acyl-editing reactions and remodeling of membrane lipids [26,27].

Recently, we discovered a divergence in activities of the prokaryotic (chloroplastic) and eukaryotic (ER) TAG metabolic pathways between algae of the classes *Chlamydomonadales*, *Chlorophyceae* and *Trebouxiophyceae* [28]. This exemplifies that, while much is currently known about the pathways of TAG synthesis in *C. reinhardtii*, there is a scarcity of information in other algae. This research presented here represents the first steps in determining the mechanism of TAG synthesis in *Coccomyxa subellipsoidea* C-169. This strain was isolated from Marble Point, Antarctica and may offer adaptive strategies, particularly at low temperatures, for TAG and other lipid production and thus serve as an ideal feedstock for biofuel production. The work detailed in this study represents the first steps required to address the metabolic changes leading to increased TAG synthesis in *C. subellipsoidea* C-169. Following ten days of N starvation, as expected, there was increased TAG synthesis with a commensurate loss of chloroplast membrane lipids. A key finding from this work was that the increased levels of TAG largely come from de novo fatty acid synthesis and, as with plants, a significant number of acyl groups were transferred from membrane lipid pools. With the re-addition of N, the TAG pool is rapidly degraded and the resultant acyl chains serve as substrates for the synthesis of galactolipids. These results suggest a distinct metabolic program is induced by N stress and is defined by changes in fatty acid synthesis, the relative contributions of membrane lipid acyl chain recycling, and the relative activities of the chloroplast and endoplasmic reticulum LPATs.

2. Materials and methods

2.1. Bioreactor growth conditions

C. subellipsoidea C169 was a gift from Dr. James VanEtten (University of Nebraska-Lincoln). The initial inoculum was grown for one week in 50 mL cultures. These cultures were centrifuged, cell pellets re-suspended in 200 mL of fresh Bolds basal medium (BBM), and added to a sterile, round, water jacketed 3 L glass bioreactor (Applikon Biotechnology, Foster City, CA) to a final volume of 2 L. The culture was grown photoautotrophically with 200 μmol photons m^{-2} s^{-1} at 25 °C and mixed using an impeller with two Rushton turbine blades and three baffles. A thermocirculator was used to maintain a constant temperature in the bioreactor by circulating water through the jacket. Bubbling compressed air through a 0.2 mm polytetrafluoroethylene (PTFE) filter provided 330 ppm CO_2 at 1 L min^{-1}; the pH was maintained at 6.6 using 0.1 M KOH and 0.1 M HCl. The pH was monitored using an autoclavable pH electrode (Applikon Biotechnology Inc., USA). Air input, temperature and pH were continuously recorded using the BioXpert software from Applikon and never varied. The culture was collected after five days of growth by centrifugation and suspended in 200 mL of BBM media with no N source added (N-BBM), which was added to 1.8 L of N-BBM in the bioreactor. The bioreactor was slightly pressurized from the air input, and opening an external valve allowed for the sterile removal of samples by displacement through a stainless steel tube with an open end 2 in. from the bottom. 80 mL aliquots were collected at 24 h intervals, chilled immediately on ice, subdivided, centrifuged, and the cell pellets stored at −80 °C until use as detailed below. Three pellets from each time point were lyophilized and the dry weights (DW) determined. As is standard for bioreactor experiments with continuous monitoring [29], the data presented is from one representative experiment, of three biological replicates. The data presented represent the mean ± standard error of the mean (SEM) or standard deviation (SD) as noted with an n = 3–5.

2.2. Confocal microscopy using Nile Red and chlorophyll determination

The development and degradation of lipid droplets was visualized using the lipophilic fluorophore Nile Red (Sigma) according to Chen et al. [30] with minor changes. Briefly, algal pellets corresponding to 1 mL of cells with an optical density of 2.0 at 550 nm were suspended in 0.5 mL of 50 μM Nile Red in 3:1 aqueous DMSO and stained for 30 min at 37 °C in a shaking incubator. Fluorescence images were collected using a laser scanning confocal microscope (Olympus FluoView 500). Chlorophyll concentration was measured during the bioreactor time course as a proxy for changes in photosynthetic capacity and chloroplast integrity. Briefly, bioreactor culture was collected and cell density measured by absorbance at 550 nm. Chlorophyll was extracted immediately from 1 mL of culture into 1 mL of methanol after homogenization and cellular debris removed by centrifugation. The sample was measured by UV in a quartz cuvette using the following equations to determine chlorophyll a (Chl a) and chlorophyll b (Chl b) concentrations:

$$\text{Chl a} = 16.29\ A^{(665.2-750.0)} - 8.54\ A^{(652.0-750.0)}$$

$$\text{Chl b} = 30.66\ A^{(652.0-750.0)} - 13.58\ A^{(665.2-750.0)}.$$

2.3. Lipid standards and extraction

Internal standards used in quantifying lipids were as follows: 1,2,3-triheptadecanoyl-*sn*-glycerol (Nu-Chek Prep, Elysian, MN), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine, and 1,2-diheptadecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (Avanti Lipids, Alabaster, AL). Monogalactosyl-diacylglyceride (MGDG) and digalactosyl-diacylglyceride (DGDG) standards were quantified using internal standards with known stearoyl and stearoyl/palmitoyl compositions (Matreya LLC, Pleasant Gap, PA). Endogenous saturated galactolipid content was determined to be negligible during unlimited and N starved growth. Triplicate samples were extracted with methanol/chloroform (2:1) containing 0.01% butylated hydroxytoluene (BHA; Sigma-Aldrich) using a bead mill (Qiagen TissueLyser LT, Qiagen, Valencia, CA) set to 50 Hz for 5 min. A slightly modified method of the Bligh and Dyer [31] technique for total lipid extraction was employed. Briefly, bead mill disrupted cells in chloroform/methanol (2:1) were incubated at room temperature for 1 h on a multi-vial vortexer. Lipids were partitioned by adding 1 mL of chloroform and 2 mL of 0.8% aqueous potassium chloride solution followed by a brief vortexing step and centrifugation for 5 min at 2520 ×*g*. The organic phase was removed to a fresh vial and evaporated under nitrogen gas. The sample was finally dissolved in into methanol/chloroform (1:1).

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