Algal Research 7 (2015) 66–77

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Molecular mechanisms for photosynthetic carbon partitioning into storage neutral lipids in *Nannochloropsis oceanica* under nitrogen-depletion conditions

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ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 8 November 2014
Accepted 16 November 2014
Available online 17 December 2014

Keywords:
Nannochloropsis
Carbon partitioning
Storage carbohydrates
Lipidomes
Membrane lipids

ABSTRACT

Polysaccharides are a major carbon/energy-reservoir in microalgae, yet their relationship with another form of carbon/energy storage, triacylglycerol (TAG), is poorly understood. Here employing oleaginous microalga *Nannochloropsis oceanica* as a model, we probed the crosstalk between carbohydrate metabolism and TAG accumulation by tracking the temporal dynamics of lipidomes, monosaccharides and polysaccharides and transcripts of selected genes over 14 days under nitrogen-depleted (N−) and nitrogen-replete (N+) conditions. Glucose, galactose and mannitol were the main monosaccharides in IMET1, and laminarin may be the storage polysaccharide that competes for carbon precursors with TAG. Transcriptional expression analysis revealed that the β-1,3-glucan degradation and pyruvate dehydrogenases pathways were the main regulatory components involved in driving carbon flow to TAG synthesis. Furthermore, temporal changes of lipidomes and transcripts of glycerolipid metabolism genes were indicative of possible conversion of membrane lipids to TAG, especially under an early stage of nitrogen deprivation conditions. A carbon partitioning model for *N. oceanica* was proposed, in which β-1,3-glucan metabolism, acetyl–CoA synthesis and membrane lipid turnover/degradation, in addition to *de novo* fatty acid synthesis, all contributed to TAG synthesis.

1. Introduction

Under stress conditions such as nitrogen (N) deprivation, microalgae can store photosynthetically fixed carbon in the form of oils, i.e., energy-dense neutral lipids (e.g., triacylglycerols; TAG), which can be converted to biofuels along with other potential applications [1–3]. However a key biological hurdle for an economically viable microalgal oil industry is the lack of desirable industrial strains capable of high oil productivity in outdoor large-scale cultivation [4,5].

In many microalgae, multiple forms of storage carbon products derived from the photosynthetically fixed carbon can be found (such as water-soluble polysaccharides, starch and TAG [6–8]). As biosynthesis of the multiple forms of storage compounds may require the same carbon precursors (e.g. glucose) and reducing power [9,10], and thus, understanding and tuning the partitioning of carbon precursors into the various forms of carbon storage are of key interest for rational metabolic engineering of industrial microalgal strains for improved oil production.

Polysaccharides such as starch are one abundant form of carbon in many microalgae. For example, we and others showed that the model microalga *Chlamydomonas reinhardtii* can accumulate starch as much as 40% of cell dry weight under high light and N deprivation conditions [11–13]. Moreover, genetically blocking the starch synthesis pathway in *Chlamydomonas* cells led to over 10-fold increase in TAG accumulation [11,13,14]. It was further revealed that the carbon metabolism of the starchless mutant featured an increased carbon flow towards hexose-phosphate by up-regulation of the glyoxylate pathway and gluconeogenesis [15]. In the model diatom *Phaeodactylum tricornutum*, it is not starch but chrysolaminarin that is the main form of storage polysaccharide and can make up 20%–30% of cell dry weight [16], and like starch biosynthesis in *C. reinhardtii*, the chrysolaminarin synthesis pathway competes for carbon with the pathways for lipid biosynthesis [17]. These results suggested that the manipulation of carbohydrate metabolism might potentially enhance TAG production. However, most studies that probed the role of carbohydrates in lipid accumulation have been

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http://dx.doi.org/10.1016/j.algal.2014.11.005
2211-9264/

conducted in laboratory model organisms such as *C. reinhardtii* and *P. tricornutum* [15,17]. For oleaginous microalgae with demonstrated capability for large-scale cultivation, experimental evidence for the dynamic relationship between carbohydrates and neutral lipids during the oleaginous process has been sparse, largely due to the poor knowledge about identity, profiles and dynamics of storage carbohydrates in these non-model organisms.

Another abundant form of carbon in microalgal cells is membrane lipid, which makes up 5 to 20% of cell dry weight [18–20]. The conversion of membrane lipids into TAG may occur in microalgae, though potential contribution of this pathway to overall TAG biosynthesis has yet to be determined. In *C. reinhardtii*, it was suggested that the acyl moiety of membrane glycerolipids can be transferred to a diacylglycerol molecule by a phospholipid:diacylglycerol acyltransferase (PDAT) to produce TAG, or it can be cleaved by a lipase and then activated to form acyl-CoA for *de novo* TAG biosynthesis [21,22]. In *Nannochloropsis gaditana* [23] and *N. oceanica* [24,25], it was shown that a small amount of accumulated TAG under N-deprivation was postulated to be linked to degradation and recycling of membrane lipids. This was further supported by the up-regulation of the genes encoding *PDAT* and specific lipases during the first 48 hours (h) of N deprivation [24]. However, it is not known whether or how the membrane lipids and TAG homeostasis was sustained beyond such initial phase of TAG accumulation under N deprivation.

Nannochloropsis spp. are a group of unicellular photosynthetic heterokonts distributed widely in sea, fresh and brackish waters. They are of industrial interest due to their ability to grow rapidly, synthesize large amounts of TAGs and high-value polyunsaturated fatty acids (PUFAs) (e.g., eicosapentaenoic acid) and tolerate broad environmental and culture conditions [26,27]. The genomes of several *Nannochloropsis* species have been sequenced [28–33], and the transcriptome and lipidome of one of the species *N. oceanica* IMET1 were simultaneously characterized for the first 48 h of N depletion [24,34]. However, the role of carbohydrates and the link between carbohydrates and TAG in the TAG-accumulating process are not clear. Considering that the cellular content of lipids can reach over 50% of cell dry weight over an extended period of time under N deprivation [25,35], elucidation of longer-term responses beyond the first 48 h of N depletion should be crucial.

In this study, employing oleaginous microalga *N. oceanica* IMET1 as a model, we probed the crosstalk between carbohydrate metabolism and accumulation of TAG, by generating time-course profiles of neutral sugars and glycerolipids in *N. oceanica* IMET1 cells grown under N-replete (N+) and N-depleted (N−) conditions for 14 days, which shed light on carbon partitioning dynamics in *N. oceanica* IMET1. In parallel, transcripts levels of the key genes in glycerolipids and carbohydrate metabolisms were tracked, which further revealed the potential contribution of membrane lipids and storage carbohydrates to TAG biosynthesis. Finally, a carbon partitioning model for *N. oceanica* was proposed. These findings pave the way for understanding and exploiting the links between carbohydrate and lipid metabolisms for enhanced oil production in this and related microalgae.

2. Materials and methods

2.1. Algal strains, culture conditions and nitrate measurements

N. oceanica strain IMET1 was cultured in a modified f/2 medium containing 1000 mg L^{-1} $NaNO_3$ [24]. The final pH was adjusted to 7.8. For the preparation of inoculum, microalgal cultures were grown in nitrogen-replete medium in 1 L columns at 22 °C with 24 h cool white fluorescent illumination (55 μmol m^{-2} s^{-1}) bubbled with 1.5% (w/w) CO_2, with the initial cell density as 3×10^7 cells mL^{-1}. At the log phase (cell density of ~2×10^8 cells mL^{-1}), cells were harvested by centrifugation (3500 *g* at 20 °C for 5 min). The harvested cells were resuspended to 2×10^8 cells mL^{-1} in nitrogen-replete medium (modified f/2 1000 mg L^{-1} $NaNO_3$; N+) or nitrogen-depleted medium (modified f/2 without $NaNO_3$; N−). The cells were cultivated under conditions identical to that of inoculum preparation. The cultures were sampled at 1, 2, 3, 4, 8 and 14 days after the start of nitrogen depletion or repletion. Cells were pelleted by centrifugation (3500 *g* at 20 °C for 5 min). Aliquots for RNA preparation were frozen by liquid nitrogen and then stored at −80 °C, while those for lipidome analysis were washed with water and freeze-dried. Algal culture was centrifuged at 3500 *g* at 20 °C for 5 min and the supernatants were used to measure the nitrate concentration by using a QuikChem 8500 (Lachat Instruments, Loveland, CO, USA) following the manufacturer's instructions.

2.2. Analysis of total lipid, total carbohydrate and total protein contents

Thirty milligrams lyophilized algal powder was loaded to Dionex ASE350 (Thermo Scientific) and extracted by methanol:dimethyl sulfoxide (DMSO) (9:1, v/v) once and by hexane:ethyl ether (1:1, v/v) twice. The extraction temperature and pressure were 125 °C and 1500 psi respectively. The extracts were centrifuged at 1000 *g* for 10 min after being mixed with 15 mL water and then the upper organic layer was transferred to a new labeled vial. The centrifugation and transfer procedure were repeated until the lipid was completely eluted. In the end, the organic layer was evaporated under the protection of nitrogen gas and then freeze-dried overnight. Total lipid content was calculated as net lipid weight divided by net algal biomass weight.

Ten milligrams of lyophilized algal powder was used for the analysis of total carbohydrate content [36]. Briefly the algal powder was incubated with 0.5 mL acetic acid at 80 °C for 20 min and then 10 mL acetone was added, followed by centrifugation at 3500 *g* for 10 min. The supernatant was discarded. The pellet was resuspended in 2.5 mL 4 M trifluoroacetic acid (TFA) and then boiled for 4 h. The suspension was cooled and then centrifuged at 10,000 *g* for 3 minutes. Then 20 μL supernatant was mixed with 900 μL sulfuric acid (15 mL):H_2O (7.5 mL):phenol (0.15 g) and boiled for 20 min prior to reading the optical density at 490 nm (OD_{490}). To quantify total carbohydrate content, glucose was used to establish the standard curve.

Total protein content was determined as previously described [37]. Briefly, 10 mg freeze-dried algal powder was hydrolyzed in 100 μL of 1 M sodium hydroxide (NaOH) and then incubated at water bath at 80 °C for 10 min. Then 900 μL H_2O was added to the hydrolysate to bring the volume to 1 mL. The mixture was centrifuged at 12,000 *g* for 30 min and the supernatant was transferred to a new tube. This extraction procedure was repeated twice, and all the resulted supernatants were pooled together. Then the protein concentration was measured by Bio-Rad Protein assay kit (cat no. 500-0002). Bovine serum albumin (BSA) was used as standard.

2.3. Quantification of intracellular neutral sugars

Freeze-dried biomass (100 mg) was dissolved in 25 mL H_2O, and 10 μL DNase (2 U μL^{-1}) was used to remove DNA. Then the sample was processed in French Pressure to break cell wall. The pressure was set as 20,000 psi and samples were treated for four paths. Efficiency of the disruption was monitored by microscopic observation and centrifugation. Then the ruptured cells were centrifuged at 27,000 *g* at 4 °C for 20 min. The supernatant was boiled at 100 °C for 10 min and then freeze-dried. The resulted samples were resuspended in 5 mL sterilized H_2O and distributed into five aliquots: three were treated with 2 M TFA at 100 °C for 6 h to release glucose in polymeric form, while the remaining two were treated under sterilized H_2O at 100 °C for 6 h. Then the aliquots were evaporated in vacuum under nitrogen gas to remove TFA. The pellet was resuspended in H_2O and the suspension was treated with a 0.22 μm filter. Finally, the suspension was diluted and analyzed with DIONEX ICS-5000 PA10 column using 1 M potassium hydroxide as eluent.