BBA - Molecular and Cell Biology of Lipids 1863 (2018) 1478–1487

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

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

Triacylglycerol accumulates exclusively outside the chloroplast in short-term nitrogen-deprived *Chlamydomonas reinhardtii*

Miao Yang[a,b,c], Yingying Meng[a,1], Yadong Chu[a], Yan Fan[a,b], Xupeng Cao[a], Song Xue[a,*], Zhanyou Chi[c]

[a] Marine Bioengineering Group, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China

ARTICLE INFO

Keywords:
Chlamydomonas
Chloroplast
Extra-chloroplastidic TAG accumulation
Membrane glycerolipids
Short-term nitrogen starvation
Subcellular glycerolipidomics

ABSTRACT

In microalgae, triacylglycerol (TAG) biosynthesis occurs by parallel pathways involving both the chloroplast and endoplasmic reticulum. A better understanding of contribution of each pathway to TAG assembly facilitates enhanced TAG production via rational genetic engineering of microalgae. Here, using a UPLC-MS(/MS) coupled with TLC-GC-based lipidomic platform, the early response of the major glycerolipids to nitrogen stress was analyzed at both the cellular and chloroplastidic levels in the model green alga *Chlamydomonas reinhardtii*. Subcellular lipidomic analysis demonstrated that TAG was accumulated exclusively outside the chloroplast, and remained unaltered inside the chloroplast after 4 h of nitrogen starvation. This study ascertained the existence of the glycolipid, digalactosyldiacylglycerol (DGDG), outside the chloroplast and the betaine lipid, diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS), inside the chloroplast. The newly synthesized DGDG and DGTS prominently increased at the extra-chloroplastidic compartments and served as the major precursors for TAG biosynthesis. In particular, DGDG contributed to the extra-chloroplastidic TAG assembly in form of diacylglycerol (DAG) and DGTS in form of acyl groups. The chloroplastidic membrane lipid, monogalactosyldiacylglycerol (MGDG), was proposed to primarily offer DAG for TAG formation outside the chloroplast. This study provides valuable insights into the subcellular glycerolipidomics and unveils the acyl flux into the extra-chloroplastidic TAG in microalgae.

1. Introduction

As promising renewable feedstocks for production of biofuels, triacylglycerols (TAGs) can be substantially produced under various stress conditions in microalgae. Nitrogen deficiency has been considered the most efficient manner to induce TAG biosynthesis and accumulation [47]. Deciphering the mechanism of TAG accumulation induced by nitrogen stress is indispensable to enhance TAG production via targeted genetic engineering in microalgae.

Nitrogen stress response generated by microalgae has been extensively investigated, mainly at the gene, transcription and protein levels [1–3]. Moreover, liquid chromatography-mass spectrometry (LC-MS) assisted lipidomic analyses have facilitated our understanding of the intricate lipid metabolism, particularly TAG accumulation, in microalgae [2,4]. The nitrogen stress-induced TAG accumulation commonly encompasses gradual chloroplast degradation and remodeling of the lipidome. As the largest fraction of lipidome [5], glycerolipids, which consist of glycolipids, phospholipids, betaine lipids and neutral lipids, play essential roles in maintaining lipid homeostasis via coordinated actions under nitrogen depletion in microalgae. The remodeling of membrane glycerolipids serves as a crucial strategy for microalgae to respond and adapt to a varied environment. To date,

Abbreviations: chlorophyll, Chl; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; ER, endoplasmic reticulum; ESI, electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; MGDG, monogalactosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulphoquinovosyldiacylglycerol; TAG, triacylglycerol; TEM, transmission electron microscopy; TLC-GC, thin layer chromatography-gas chromatography; UPLC-MS, ultra performance LC-MS

* Corresponding author.
E-mail addresses: yangmiao0314@dicp.ac.cn (M. Yang), yadongchu@dicp.ac.cn (Y. Chu), fany1993@dicp.ac.cn (Y. Fan), c_x_p@dicp.ac.cn (X. Cao), xuesong@dicp.ac.cn (S. Xue), chizhy@dlut.edu.cn (Z. Chi).
[1] Current Address: College of Chemistry, Chemical Engineering and Materials Science, Shandong Normal University, Jinan 250014, China.

https://doi.org/10.1016/j.bbalip.2018.09.009
Received 17 March 2018; Received in revised form 25 August 2018; Accepted 23 September 2018
Available online 25 September 2018
1388-1981/

studies on LC-MS-based glycerolipid response induced by nitrogen stress have been reported in several microalgae, such as *Phaeodactylum tricornutum* [6], *Chlamydomonas reinhardtii* [7] and *Nannochloropsis oceanica* [8], but most studies were experiments at the cellular level.

The microscopic and biochemical analyses have extended knowledge of the subcellular organization of TAG accumulation in the oleaginous microalgae. Freeze-fracture electron microscopy together with transmission electron microscopy (TEM) observation [9,10] and the subcellular localizations of acyltransferases involved in TAG biosynthesis [11–13] have verified the chloroplast and endoplasmic reticulum (ER) pathways for TAG accumulation in microalgae. More recently, Moriyama et al. [14] have unveiled the absence of chloroplast lipid droplet (LDs) based on the electron microscopic observation and 3D reconstruction by confocal fluorescence microscopy in *C. reinhardtii*. The source analyses of TAG acyl components [15] together with proteins (structural and functional proteins) and polar lipids constituting the monolayer on the surface of LDs [7,16–18] have further provided evidences for involvement of the chloroplast and ER in TAG assembly. Thus, it is necessary to perform subcellular studies on TAG accumulation mechanism in microalgae. Nevertheless, the mechanism of TAG accumulation at different subcellular compartments remains largely unknown in microalgae due to the technical difficulties in obtainment of available biomaterials, mainly the available chloroplast or ER fractions [19].

Studies on the transcriptional regulatory network have demonstrated that *C. reinhardtii* coordinates transcriptional downregulation of 57 metabolic enzymes and induces an increase in TAG amounts within 4 h of nitrogen deprivation [20]. A study of lipidomic and transcriptomic analyses of heat stress-treated *C. reinhardtii* detected a three-fold increase in TAG levels and proposed an alternative pathway for assembly of polyunsaturated TAG that involves a direct turnover from membrane lipids after 1 h of heat stress [2]. However, the response mechanism involving changes in the glycerolipidome for TAG accumulation remains mostly unclear in short-term nitrogen-deprived *C. reinhardtii*.

Here, to characterize the mechanism of TAG accumulation induced by short-term nitrogen starvation, we focus on the early responses of the major glycerolipids at both the cellular and chloroplastidic levels in the model green alga, *C. reinhardtii*. Our previous work has showed that the nitrogen-stressed available chloroplasts could be successfully isolated from the photoautotrophically grown *C. reinhardtii* [21]. Using an ultra performance LC-MS (UPLC-MS) coupled with thin layer chromatography-gas chromatography (TLC-GC)-based lipidomic platform, the subcellular lipidomic analyses unveil the origin of extra-chloroplastidic TAG accumulation from both the chloroplastidic and extra-chloroplastidic compartments. This study provides valuable insights into the subcellular lipid remodeling triggered by nitrogen stress at the molecular level and discloses the acyl flux into extra-chloroplastidic TAG in green microalgae.

2. Material and methods

2.1. Microalgal strain and culture conditions

The cell wall-deficient mutant of *C. reinhardtii* strain CC4326 (http://www.chlamycollection.org/) was used for this study. This strain was first cultured under nitrogen replete conditions for 4 d and 4 h, and then under nitrogen deprivation for the next 4 h, as previously reported [21]. The nitrogen-deprived cells for 0 h were used to indicate nitrogen-replete cells. Briefly, algal cultures were photoautotrophically grown in minimal medium in photobioreactors (4.5 cm in diameter, 45 cm in height, 600 ml of culture volume), bubbled with air (120 ml min^{-1}) containing 4% (v/v) CO_2. The irradiance was set as 100 μmol m^{-2} s^{-1}, illuminated from one side by cool white fluorescent tubes when subjected to nitrogen stress. Before and after nitrogen starvation, a portion of algal cultures was immediately used for chloroplast isolation, and the residual was divided into aliquots of 30 ml each (10–15 mg of dry biomass), followed by centrifugation (3000 × *g*, 5 min, 4 °C) and storage at −80 °C for the subsequent lipidomic analyses.

2.2. Chloroplast isolation from C. reinhardtii and TEM observation under nitrogen-replete and -deprived conditions

Isolation of chloroplast fractions from nitrogen-replete and -starved *C. reinhardtii* was performed as previously described [21]. Briefly, the harvested cells were first gently broken using a custom-made cell disruptor under 0.55 MPa of nitrogen pressure. The pressates were centrifuged (750 × *g*, 2 min, 4 °C) and the chloroplasts were purified on a Percoll step gradient (20%/45%/65%). After centrifugation at 4200 × *g* for 15 min at 4 °C, the intact chloroplasts were obtained between the 45% and 65% layers. The pelleted chloroplasts were promptly frozen and stored at −80 °C for the succeeding lipid analyses.

The cells and chloroplasts of *C. reinhardtii* were fixed as previously reported [21] and images were taken with a JEM-1200EX electron microscope (JEOL Ltd., Tokyo, Japan) using an ANT camera system.

2.3. Chlorophyll (Chl) quantification and growth measurement

Two milliliters of cell cultures or the equivalent chloroplast fraction were pelleted and sonicated in 2 ml of ethanol on ice. The pigment extracts were centrifuged at 12,000 rpm for 2 min. The Chl concentrations of algal cultures and chloroplast fraction were determined using method of Jespersen and Christoffersen [22] and calculated as Chl = 18.08 OD_{649} + 6.63 OD_{665}, where OD_{649} and OD_{665} are the absorption value of the supernatant at 649 and 665 nm, respectively. Cultures (~5 ml) were filtered onto a pre-dried Whatman GF/C filter (47 mm diameter) and dried to a constant weight with a net biomass of > 2 mg at 60 °C. The dry weight of algal cells was the difference value between the final weight and the weight of the filter. The mass of the chloroplast fraction was converted to the original cellular biomass based on the Chl amount and the calculation formula was as follows:

$$M_{cell-cp} = M_{chl} \times k \quad (1)$$

$$k = D/C \quad (2)$$

where $M_{cell-cp}$ was the equivalent cellular biomass (mg) of the isolated chloroplasts. M_{chl} was the mass (μg) of Chl derived from the isolated chloroplasts. The coefficient "*k*" was the cellular biomass per unit mass of Chl. D was the concentration (mg ml^{-1}) of dry weight of algal cells. C was the concentration (μg ml^{-1}) of Chl derived from algal cell cultures.

2.4. Fatty acid composition analysis

One-step acid-catalyzed direct transesterification was used to determine the content of fatty acids [23]. Triheptadecanoin (TAG 51:0, 17:0/17:0/17:0, Sigma-Aldrich, USA) was added as an internal standard. Fresh cells of ~10 mg of dry biomass were mixed with 5 ml of 2% H_2SO_4-methanol (v/v, H_2SO_4/methanol) in a 10-ml flask, followed by stirring at 70 °C for 1 h with refluxing. The generated fatty acid methyl ester mixtures were extracted into hexane and quantified using an Agilent GC 7890A equipped with a flame ionization detector and a DB-23 column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies, USA). The content of fatty acids was standardized to nmol of fatty acids per mg of cellular dry biomass.

2.5. Lipid extraction

The total lipids were extracted as described by Bligh and Dyer [24] before quantification of TAG and polar lipids for fresh algal cells and chloroplast fractions. The extraction solvent was chloroform:methanol:water (1:1:0.9, v/v/v). The samples were first added