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Regulation of lipid metabolism in the snow alga *Chlamydomonas nivalis* in response to NaCl stress: An integrated analysis by cytomic and lipidomic approaches

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ABSTRACT

The cultures of the snow alga *Chlamydomonas nivalis* in the exponential phage were stressed by NaCl (up to 1.5%) for 0~48 h, followed by Nile Red staining-based cytomic analysis (flow cytometry and confocal laser scanning microscopy). The fluorescent intensities of total lipids, and neutral and polar lipids increased to the maximum within 7 h in the NaCl stressed cells with the highest increase in total lipids by 2-fold (0.75%-NaCl for 7 h), the highest increase in neutral lipids by 68-fold (1%-NaCl for 7 h) and the highest increase in polar lipids by 10-fold (1.25%-NaCl for 5 h), respectively. Seven types and 22 kinds of polar lipid molecules were selected and identified as biomarkers by UPLC/Q-TOF-MS-based lipidomic analysis, which demonstrated differences in total lipids between the stress group (0.75%-NaCl for 7 h) and the control. The biological roles of the biomarkers in the alga under NaCl stress were discussed. The integrated approach based on "omics" technologies developed in the present work is validated as a powerful tool to successfully reveal the regulation of lipid metabolism in microalgae in response to stress stimulation.

1. Introduction

Lipids play vital roles in cellular structure and organization, signal transduction, trafficking, sorting of macromolecules, and are particularly essential to adaptation and tolerance to extreme environments in microorganisms, plants and alga [1]. Due to their complicate structures and multiple layers of diversities, the conventional methods for lipids extraction, separation and analysis are time consuming and require a large amount of lipids [2,3]. "Omics"-based novel technologies developed in recent years, including cytomic and lipidomic approaches [4,5], can provide powerful tools to decode molecular mechanisms involved complex lipid interactions in biological systems, and to identify biomarkers response to intrinsic and/or extrinsic stimuli.

Flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) are the two leading tools in cytomic analysis for rapid screening and quantification of intercellular lipids [5]. FCM has already been successfully applied in studying lipids of algal cells in different environments due to its rapid, simultaneous and quantitative lipids measurements related to Nile Red (a specific fluorescent probe of lipid molecules) staining [6–8]. As for CLSM, it can generate a sharp image with high resolution and minimal background noise by exciting the sample with a highly focused beam of laser light and only allowing the emitted fluorescent light [9]. Lipidomics is a new platform for biosystem-based study of all lipids and their functions within the cell. It only requires simply separating a small amount of lipid sample to identify and quantify lipid molecule species [4]. In this fast growing field, an advanced equipment of UPLC/Q-TOF-MS has been becoming a main powerful lipidomic tool due to its high resolution and sensitivity in obtaining multiparametric metabolite profiles from biofluids and/or plant cells [10,11]. However, metabolite data are hard to summarize and interpret without appropriate statistical and visualization tools. The use of multivariate statistical analysis, specifically, orthogonal

Abbreviations: CLSM, confocal laser scanning microscopy; DGDG, digalactosyldiacylglycerol; DGTS, l,2-diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine; DLU, degree of lipid unsaturation; ESI, electrospray ionization; FCM, flow cytometry; MGDG, monogalactosyl-diacylglycerol; OPLS-DA, orthogonal projection on latent structure discriminant analysis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositiol; SQDG, sulfoquinovosyl-diacylglycerol; UPLC/Q-TOF-MS, ultra performance liquid chromatography/quadrupole time of flight-mass spectrometry; VIP, very important in the projection.

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partial latent structures discriminant analysis (OPLS-DA) [12,13], is of great importance as an efficient and robust method for modeling, analysis, and interpretation of complex chemical and biological data.

The snow alga *Chlamydomonas nivalis* is a typical species of green microalgae distributed worldwide on the snowfield of polar region and other similar extreme environments, and is often used as a model species to investigate the mechanism of cell response and adaptation to stress [14,15]. Of the various hostile stress factors, *C. nivalis* has to tolerate salinities from seawater strength down to about 3‰ during the sea ice melt, while extreme hyperosmotic conditions may occur in winter with low temperature [16]. High levels of Fe, Ca, Cl, Mg, K, P and Al were found as the most abundant elements in the resting *C. nivalis* cells, and inorganic particles were found to be attached to the cell wall outside, indicating *C. nivalis* has the strong capability to enrich inorganic nutrients (trace elements) in cells over hundreds times than that in snow [17]. *C. nivalis* is highly resistant to NaCl stress and has a median lethal NaCl concentration of 1.2 M, which is three times higher than that for *C. reinhardtii* [18]. Obviously, salinity is one of the most important factors influencing algal survival and reproduction, and *C. nivalis* is the best species to investigate the regulatory mechanism of cell response and adaptation to salt stress. Generally, the regulation of lipid biosynthesis in microalgae is the most crucial strategy in resistant physiology to salt stress [19]. To date, most of previous investigations on snow algae focused on growth and metabolism with respect to low temperature and light intensity as limiting conditions [20–22], little involved in the effects of salinity stress on the growth and chemical composition in snow algae; no identification of lipid biomarkers as key physiological regulators in response to stress in *C. nivalis* was reported at lipidomic level except our recent work [23].

In the present work, to elucidate the regulation of intracellular lipid metabolism in *C. nivalis* in response to NaCl stress, Nile Red staining-based cytomic analysis (FCM and CLSM) and UPLC/Q-TOF-MS-based lipidomic analysis combined with multivariate statistical analysis (OPLS-DA) were developed as an integrated approach to rapidly screen and quantify the intercellular lipids as well as to identify lipid biomarkers. The biological roles of lipid biomarkers and the fatty acids in these molecules related to the rapid response to NaCl stress and adaptation were discussed.

2. Materials and methods

2.1. Reagents

HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), formic acid was purchased from Dikma (Lake Forest, CA, USA). BHT (butylated hydroxytoluene) and Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) were purchased from Sigma (St. Louis, MO, USA). The standards of glycolipids (MGDG, DGDG and SQDG) and phospholipids (PI, PG and PE) were purchased from Lipid Products (Redhill, UK). The other chemicals and solvents were of analytical grade. Distilled water was prepared in-house using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Algal culture, NaCl stress and biomass harvest

The snow alga *Chlamydomonas nivalis* UTEX LB2824 was purchased from the Culture Collection of Algae at University of Texas at Austin (UTEX), USA. Seed culture was grown in 100-mL Bold 1NV medium (UTEX formula) [24] in 250-mL flask under continuous illumination of 2000 ± 200 lx at 20 ± 0.5 °C for 6 days to reach the exponential phase (5×10^5 cells/mL). Air was bubbled into the medium after filtration through a 0.45-μm membrane filter (Millipore Corp., Bedford, MA, USA) at a flow rate of 1.5 L/min. The stock solution of 25% NaCl was added into each flask to final concentrations at 0% (control), 0.25%, 0.50%, 0.75%, 1.00%, 1.25% and 1.50%, respectively. All treatments were performed in quadruplicate for culture at the above conditions and sampled from each flask at 0 (control), 1, 3, 5, 7, 11, 15, 24 and 48 h, respectively. Biomass was harvested by centrifugation and washed twice by distilled water prior to freeze drying. All stuffs, stock solution and media were autoclaved at 121 °C for 30 min before use.

2.3. Confocal laser scanning microscopy

1-mL algal cultures ($(4–5) \times 10^5$ cells/mL) were stained with Nile Red solution in acetone at final concentration of 2 μg/mL by a modified protocol of Dempster and Sommerfeld [25]. Fluorescent images of stained cells were captured on a confocal laser scanning microscope (TCS SP5; Leica Microsystems CMS GmbH, Germany) using LAS AF (Leica Application Suite Advanced Fluorescence) 1.8.1 build 1390 software under HCX PL APO CS 100 × NA 1.4 oil-immersion with confocal pinhole set at Airy 1 and 3× zoom factor for improved resolution with eight bits. The laser available in this setup is He–Ne 543 nm, and the fluorescent signal of emission from the sample was scanned from 560 to 615 nm. Laser transmission and scan settings were constant in all scans. The size of the images obtained was 1024 × 1024 in 8-bit format. TIF images of the representative cells were selected for quantification of the average fluorescent intensity by using software LAS AF Lite.

2.4. Flow cytometry

Nile Red-stained cells above were immediately analyzed on a flow cytometer (Cell Lab Quanta SC™; Beckman Coulter®, USA) equipped with a 488-nm argon laser. Upon excitation at 488 nm, Nile Red exhibits yellow-gold and red fluorescence when it dissolved in neutral and polar lipids [6], which were detected in FL1 (530 ± 30 nm) and FL2 (575 ± 30 nm) channels, respectively [7].

2.5. Extraction, liquid chromatographic separation and mass spectrometry of total lipids

The methods for extraction, liquid chromatographic separation and mass spectrometry of total lipids in this study were identical to that reported in our recent study [23].

2.6. Multivariate statistical analysis

The three-dimensional datasets (m/z, retention time, ion intensity) from both of ESI modes were exported to SIMCA-P$^+$ V12.0 software (Umetrics AB, Umea, Sweden) for multivariate statistical analysis. OPLS-DA models were conducted, and permutation tests with 200 iterations were carried out in the software to investigate the predictability and possible over-fit of data in OPLS-DA model [26].

3. Results and discussion

3.1. CLSM analysis of changes in total lipid contents

Fluorescent images of Nile Red-stained cells in the control and NaCl stressed groups of *C. nivalis* and their quantified curves of mean fluorescent intensities referred as total lipid (excitation, 543 nm; emission, 560–615 nm) [27] by CLSM are shown in Fig. 1a and b. It can be seen that the fluorescent intensities increased to the maximum when the time of NaCl stress prolonged to 7 h at all NaCl concentrations except 1.50% that reached to the maximum at 5 h, then decreased with the longer stress time. Although the mechanism for the initial increase of total lipid content in NaCl stress cells is not clear, the increase of lipid content may correlate with the adaptive response to high NaCl concentration such as cell volume changes and glycerol production [28]. The phenomena that the fluorescent intensities did not constantly increase might be explained by that the growth of snow algae was remarkably inhibited by higher NaCl concentration or longer stress time due to high osmotic stress or reduced water potential [16], therefore, the fluorescent intensities or total lipid contents became lower. The stressed cells of *C. nivalis* under 0.75%-NaCl for 7 h had the maximal change rate of 2-fold in total lipids compared with the control (Fig. 1b), indicating this stress condition had induced the biggest change in lipid profiles in *C. nivalis* for stress adaptation.

3.2. FCM analysis of changes in neutral and polar lipid contents

The fluorescences of Nile Red-stained cells of *C. nivalis* were monitored by flow cytometry in FL1 (Fig. 2a) and FL2 channel (Fig. 2b) referred as the contents of neutral (free sterols, free fatty acids and triglycerides) and polar (phospholipids and glycolipids) lipids, respectively [7]. Both of the fluorescent intensities in FL1 and FL2 channel increased to the maximum within 5 or 7 h, and