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Systems-level analysis of metabolic mechanism following nitrogen limitation in benthic dinoflagellate *Prorocentrum lima*

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ABSTRACT

Prorocentrum lima is a typical diarrheic shellfish poisoning (DSP) toxin-producing benthic dinoflagellate usually found attached to or associated with macrophytes, floating detritus, debris, or other substrates. In this paper, to gain a deeper understanding of the molecular and cellular responses to N limitation in *P. lima*, transcriptome profiling was performed. We found that most genes related to photosynthesis, porphyrin and chlorophyll metabolism were down-regulated following N limitation, while some genes concerning fatty acid biosynthesis, starch synthesis and ubiquitin pathway were up-regulated. Meanwhile, ABC transports ABCB1 and ABCG2 and glutathione S-transferase were up-regulated under N-limited conditions. These transcriptomic data were further corroborated by the biochemical analyses which revealed that starch, lipid content was increased, while photosynthetic efficiency was decreased. In addition, TEM analysis demonstrated that chloroplasts appeared smaller and were less abundant, while a major fraction of the cell volume was occupied by lipid bodies and starch granules, and thylakoid was twisted in the cells. Taking together, we proposed that N limitation could induce the accumulation of neutral lipid and starch in *P. lima* cells for carbon fixation trough recycling chloroplast membranes by autophagy. ABCB and ABCG transporters might be involved in the transport of DSP toxins. Our findings might provide global information for the response or adaptation of *P. lima* to N limitation.

1. Introduction

Nutrients are indispensable components for growth and metabolism of phytoplankton and optimal provision of nutrient components is therefore pivotal. Given the significance of nutrient components, all the essential components must be available in appropriate concentration in the cultivation media which will enable the algae to grow without restriction. On the other hand, under nutrient limitations microalgae will undergo a series of metabolic acclimations, making for variations in cellular composition of macromolecules such as carbohydrates, lipids, proteins and second metabolites, etc. Therefore, it is important to learn the adaptation mechanism to changing ambient nutrient in phytoplankton species for understanding their ecological behavior, especially with regard to blooms [1, 2].

Among various nutrient supplements, macronutrient nitrogen has been considered as crucial component for growth and metabolism of algal cells as it is indispensable constituent for the synthesis of proteins, nucleic acids, and chlorophylls etc. Nitrogen accounts for 5 to 14% of cell dry weight in microalgae and uptake of nitrogen can be assimilated into active biochemical, which could be utilized intracellularly for various metabolic and physiological processes [3]. Availability of nitrogen nutrient affects the cellular metabolism, growth, biochemical composition, photosynthesis and other cellular activities, which in turn affects the cellular physiological activities. It is well known that N-limitation inhibits cell growth, changes pigment composition, decreases photosynthetic energy harvesting and photosynthetic efficiency [4]. Furthermore, N-deficiency might trigger some dinoflagellates to form cysts [5]. These facts have instigated various studies to analyze the metabolic response of marine phytoplankton such as diatoms, dinoflagellates and other microalgae species under nitrogen limitation [6]. Transcriptomic analysis of nitrogen deprived *Phaeodactylum tricornutum* revealed that expressions of key genes involved in TCA cycle, carbon fixation, and nitrogen assimilation were significantly increased [7]. N-deprivation enhanced the expression of genes of nitrate transport, signal transduction, amino acid metabolism, DNA repair and hemolysin manufacture has been observed [8]. Similarly, the N-depleted *Karenia brevis* cultures exhibited increase in the expression of transcripts involved in N-assimilation relative to nutrient replete cells [9]. Paralleled to the conspicuous decrease in photosynthetic performance in N-starved cultures, chloroplast-associated genes were also found to be down-

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regulated in the dinoflagellate *Alexandrium minutum* [10]. However, it is obvious that various microalgal species have different eco-physiological strategies and may respond differently to nitrogen and phosphorus limitation/depletion [1, 2].

Microphytobenthos, living on the sediment surface, make significant contribution to the total primary production of estuarine and other shallow water ecosystems [11], and they actually appear to be nutrient-limited in the overlying water in the sediment [12]. However, due to the assumption that microphytobenthos community is accessible to an inexhaustible nutrient supply from the sediment pore water, so far less attention has been paid on nutrient limitation in microphytobenthos [13]. Earlier studies have reported that benthic microalgal productivity in coral reef environment is limited by the availability of inorganic nitrogen [14, 15]. The dinoflagellate *Prorocentrum lima* is a cosmopolitan and commonly reported worldwide benthic species, which is most often associated with epiphytism on seaweeds, but is also free-living on sandy or muddy substrata as well as solid surfaces such as pilings and submerged lines, etc. As a known producer of diarrheic shellfish poisoning (DSP) toxins, *P. lima* has been associated to DSP episodes in different parts of the world. However, the synthetic pathway of DSP toxins remains unclear, and very few information about biosynthesis of okadaic acid (OA), one of the main components of DSP toxins, is available to date [16]. Despite tremendous progress in research on identifying and assessing toxic components from *P. lima*, assessment of their molecular responses under nutrient limited conditions remains under-investigated [17, 18]. Reports on molecular response of *P. lima* under sub-optimal nutrient conditions are currently lacking, which is mainly due to huge complex genome and high numbers of transcribed genes like other planktonic dinoflagellates [10]. In this study, we therefore set out to gain insight into the global regulation of metabolic pathways in response to N-limited condition and characterize the OA biosynthesis in *P. lima*. We investigated the effects of nitrogen limitation on transcriptome profile of *P. lima* for 38 days. In addition, changes in contents of neutral lipid, starch and OA, chloroplast structure, and photosynthetic parameters in *P. lima* under N-limited condition were also examined.

2. Materials and methods

2.1. Algal culture

P. lima (CCMP 2579) was kindly provided by the National Center for Marine Algae and Microbiota (formerly CCMP). The strain was grown as batch cultures in sterile Erlenmeyer flasks containing f/2 medium, which was filter-sterilized through 0.22-μm filters (Jin-jing, China). The cultures were grown at 20 °C in an artificial climate incubator with an irradiance of 58 μmol/(m^2 s) in a 12/12 h light/dark cycle. Cell density was determined by counting microalgae cells under an inverted microscope (CKX41, Olympus, Japan).

To obtain transcriptome profile under different nutrient conditions, *P. lima* in an initial cell density of 1.2×10^7 cell/L was grown under either N-limited conditions (with 17.7 μM NO_3^-) or nutrient-sufficient conditions (with 883.0 μM NO_3^-). Trace metals and EDTA were at levels corresponding to f/2 medium in all cultures. Algal cells were collected at the 38th day for transcriptome analysis, qPCR, morphologic observation and OA detection, etc.

2.2. Detection of OA

Algal cells were harvested (3000 ×*g* for 5 min at 4 °C) from 50 mL of *P. lima*, then broken by a ultrasonic liquid processor (MISONIX, China) in ice-bath. Subsequently, 2 mL of 80% methanol was added to the cell pellets twice for extracting toxins. The supernatant was collected by centrifugation (3000 ×*g*, 10 min) and diluted to 10 mL with 80% methanol for OA detection. OA was measured by ELISA according to the manufacturer's instruction of okadaic acid (DSP) ELISA test kit (Abraxis, USA) [19].

2.3. Transmission electron microscopy (TEM) analysis

P. lima cells were harvested (3000 ×*g* for 3 min at 4 °C) and washed with PBS buffer thrice and then fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 100 mM sodium cacodylate buffer-NaOH, pH 7.4, for 2.5 h at 4 °C. The supernatant was gently removed, and the cells were washed in 0.1 M NaH_2PO_4 buffer (pH 7.2) for four times. Samples were post-fixed with 1% osmium tetroxide in 100 mM sodium cacodylate buffer (pH 7.4) and rinsed with 0.1 M NaH_2PO_4 buffer for three times. Thereafter, the samples were dehydrated in a graded series of acetone (30%, 50%, 70%, 80%, and 90%). After infiltration through epoxy propane and mixture of epoxy propane and epoxy resin (1:1), samples were embedded in 100% Spurr's epoxy resin, and polymerized at 60 °C for 24 h. Ultra-thin sections were obtained on an 8800 Ultratome III (LKB Instruments) using a Diatome diamond knife, and stained with uranyl acetate and lead citrate. The stained sections were examined under a transmission electron microscope (TECNAI-10, Philips, Holland). Images were recorded on a film (JEM-1010, JEOL, Japan).

2.4. Confocal laser scanning microscope (CLSM) analysis

Morphology of lipid droplets in *P. lima* cells was observed under a laser-scanning confocal microscope (Zeiss LSM 510 Meta, Zeiss, Germany) using Nile red staining method as described in previous paper [7]. Briefly, *P. lima* cells were stained with Nile Red (0.1 mg/mL in acetone, ratio 1:100) and lucifugally incubated at room temperature. A laser-scanning confocal microscope with excitation wavelength of 488 nm and emission wavelength of 505–550 nm was employed to observe the morphology of the Nile-red stained cells. Pictures were captured randomly from at least 20 cells for each sample.

2.5. Lipids analysis

Neutral lipid content in *P. lima* cells was analyzed by Nile Red staining as described by Peng et al. [20]. The quantitative comparison of neutral lipid contents among different treatments was expressed by relative fluorescence intensity. Briefly, cell cultures of *P. lima* were pretreated with 20% DMSO for 20 min at room temperature, then 30 μL of Nile Red (0.1 mg/mL in acetone) was added to 3 mL aliquot of the treated cell cultures in triplicates. After mixed by rapid inversion, the cultures were incubated under darkness for 20 min at room temperature. Then the stained cells were transferred to a 96-well plate, and fluorescence intensity (excitation at 530 nm, emission at 580 nm) was determined using a F4600 microplate reader (Hitachi, Japan).

2.6. Detection of starch

Algal cells were collected by centrifugation (3000 ×*g* for 5 min at 4 °C) from 50 mL of *P. lima*. Starch was extracted from six replicates of the N-limited and control cells, and its content was detected with a plant starch content kit (Solarbio, China) according to the manufacturer's instructions.

2.7. Measurement of photosynthesis activity

The chlorophyll fluorescence parameter Fv/Fm is the maximum photochemical quantum yield of the photosystem II reaction center. As a verifiable index of photosynthetic performance and acclimation status, it represents the light energy conversion efficiency of the photosynthetic process. The Fv/Fm value was determined using a Phyto-PAM Phytoplankton Analyzer (Walz, Germany) as previously described [21].