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Response of *Gracilaria lemaneiformis* to nitrogen deprivation

Yang Wang, Yinqi Feng, Xiaojuan Liu, Mingqi Zhong, Weizhou Chen, Fan Wang, Hong Du*

Guangdong Provincial Key Laboratory of Marine Biotechnology and STU-UNIVPM Joint Algal Research Center, Department of Biology, College of Sciences, Shantou University, Shantou 515063, Guangdong, China

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ABSTRACT

The rhodophyte *Gracilaria lemaneiformis* plays a significant role in nitrogen (N) utilization as an economic macroalgae. However, very little is known about how *G. lemaneiformis* adapts to N deprivation. In this study, *G. lemaneiformis* were pre-cultured in artificial seawater with 50 μM inorganic N for 4 days and two groups were set after the pre-culture, N-deprived treatment with 0 μM N nutrient and N-replete control with 50 μM N nutrient. The response of *G. lemaneiformis* to N deprivation was analyzed at physiological, transcriptomic and proteomic levels. Physiologically, our data revealed that N depletion resulted in the increase of C/N ratio in a sustained period of N deprivation. Nitrogenous compounds were available in N depletion, including amino acids, phycoerythrin and soluble protein, but not DNA and chlorophyll-a. Furthermore, transcriptomic analysis underscored two major responses of *G. lemaneiformis* to N deprivation: 1) N assimilation pathway, PSII and PSI initially responded to N deprivation in 4 days, but declined sharply during the N deprivation; 2) Carbon fixation and glycolysis metabolism both rapidly recovered during a sustained period of N deprivation from strong repression of the first 2 days. Proteomics data indicated that electron-transfer proteins in the photosynthesis of *G. lemaneiformis* increased dramatically, but phycoerythrin protein decreased under N deprivation. However, GS protein and ribulose-bisphosphate carboxylase (RubisCO) protein remained stable. Therefore, this investigation revealed that the C-N metabolism was essential to survival under N deprivation.

1. Introduction

Elementary nitrogen is a necessary component to be incorporated into structural and physiological compounds that are vital for seaweed growth and development [1]. Three types of inorganic N in seawater are available for seaweed utilization (nitrate, nitrite, and ammonium). Nitrate and nitrite must be transformed to ammonium to be assimilated into seaweed cells. Seaweed efficiently employs N assimilation to fix excess inorganic N into N-containing compounds for growth as well as N storage [2]. However, N deprivation can significantly influence seaweed growth and productivity in mariculture systems [3]. *G. lemaneiformis* (Bory de Saint-Vincent) Greville is a major agar source in seaweed aquaculture and is widely distributed along the Chinese coast [4]. In recent years, the productivity of *G. lemaneiformis* from some cultivation fields in China has been influenced by N-deprived seawater during culture periods due to high density of seaweed biomass that inhibited the N supplementation of seawater [5]. Moreover, strong seasonal fluctuations in the N supplementation also influenced the production of *G. lemaneiformis*, isolating the culture from the supply of dissolved nutrients in seawater [6]. If inorganic N shortage was maintained over a long period, then *G. lemaneiformis* growth would be severely limited and disease outbreaks would be likely to occur in the seaweed population, especially in the Nan'ao Island cultivation field (116.5°E, 23.3°N) in Shantou, Guangdong, China. Therefore, there is considerable interest to investigate N assimilation and the N-use strategy of *G. lemaneiformis* to understand *G. lemaneiformis* adaptations under N deprivation.

As an ecological adaption to N deprivation, stored N can be remobilized to support seaweed survival without ongoing inorganic N supply. In macroalgae species, the growth rates are reduced when the total N content of the thallus is < 2% of dry weight [7]. Severe N limitation in seaweed is considered as N content at below 1.7% of dry weight [8]. Consequently, the synthesis of N-containing molecules, such as proteins and nucleic acids, will be impacted by N deprivation below the critical concentration. However, *Gracilaria (Gracilariaceae, Rhodophyta)* has an N-storage capability that can support the growth in *G. tikvahiae* species under N-deprived conditions [9, 10]. Moreover, Wang et al. demonstrated that *G. lemaneiformis* showed a high capacity to absorb inorganic N to replenish phycoerythrin when *G. lemaneiformis* was transferred from N-deprived treatment to a N-repleted culture [11]. However, little is known about how *G. lemaneiformis* employs N-containing molecules to support algae survival under N-deprived

* Corresponding author.
E-mail address: hdu@stu.edu.cn (H. Du).

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conditions.

In *G. lemaneiformis*, important enzymes were found to be involved in the N-C metabolism processes while free ammonium was released from nitrogenous compounds. These include glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), and asparagine synthetase (AS) [12]. Free ammonium was associated with glutamate to yield glutamine via the GS/GOGAT pathway [13]. Essentially, glutamine and glutamate served as initial N carriers for the synthesis of organic N compounds, such as amino acids, nucleotides, chlorophylls, and phycoerythrin [14]. Furthermore, a major proportion of the total N in a photosynthetic organism was invested in the proteins required to perform photosynthesis [15]. Nevertheless, carbon skeletons were produced via carbon fixation and glycolysis [16]. Carbon skeletons were critical to combine with N for amino acids and proteins biosynthesis [17]. Therefore, a shortage in the supply of N typically impacts the proteins for N metabolism and photosynthesis. However, few studies have examined the interaction of the N metabolism with other metabolisms in N-deprived *G. lemaneiformis*. Thus, there is a lack of important information that could be obtained by monitoring proteins and gene expressions during the C-N metabolism.

To investigate the *G. lemaneiformis* N-utilization strategy after transferring this seaweed to N-deprived condition in a systematic fashion and out of an N-repleted condition, the proteome and transcriptome were combined to study the responses of gene and protein expressions to N deprivation in a non-model organism (*G. lemaneiformis*). Proteomic analysis was employed to investigate the response of red seaweed (*Gracilaria gracilis*) to N limitation [18]. The transcriptome can provide a comprehensive understanding for *G. lemaneiformis* environmental stress response studies [19, 20]. In this study, the expressions of proteins and genes were associated with culture physiology, and this physiology was evaluated via parallel measurements of dry weight, photosynthetic efficiency, C and N contents, key enzyme activities, and various nitrogenous compounds compared to the N-repleted culture. This study could potentially serve as a relevant reference for economical *G. lemaneiformis* production.

2. Materials and methods

2.1. Algal growth and treatments

The *G. lemaneiformis* used for this study was obtained from the Nan'ao Island cultivation field (116.5°E, 23.3°N) in Shantou, Guangdong, China. *G. lemaneiformis* culture was cultivated with a 50 μM (NH_4^+: NO_3^- = 2: 1) N source in artificial seawater for four days before the onset of the N deprivation. The cultures were divided into two treatments using 7 g of *G. lemaneiformis*: N-repleted treatment (50 μM) and N-deprived treatment (0 μM). The light level and temperature were maintained at 60 μmol $m^{-2} s^{-1}$ and 21 ± 1 °C for 20 days. During the experiment, artificial seawater was refreshed every two days. The samples that were detached from cultures were branches, and they were used for the following indexes.

2.2. Measurements of dry weights, C, and N elements

Aliquots (0.5 g) of samples were cut into small segments every two days, and dry weights (g) were measured. The dried material was ground into powder to analyze the amounts of N and C elements in a Vario EL cube Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

2.3. Measurement of F_v/F_m

The chlorophyll fluorescence of all treatment samples was measured with a pulse amplitude-modulated (PAM) fluorometer (Imaging-PAM; Walz, Effeltrich, Germany). The F_v/F_m (maximum effective quantum yield of photosystem II) was then determined as follow: $F_v = (F_m - F_0)$. F_0 was measured after the culture adapt to the dark for 15 min [21]. Saturating actinic light pulses were employed to identify maximum fluorescence (F_m) in the dark-adapted culture.

2.4. Measurement of amino acids

Fresh samples (0.5 g from cultivar of branches) were finely powdered in liquid nitrogen using a mortar and resuspended in PBS buffer (0.01 M, pH 7.4). Samples were shaken at 4 °C for 10 min before ultrasonication for 5 min at 4 °C (a Sonic disruption in 3 s and interval in 3 s), followed by centrifugation for 30 min at 11,000*g* at 4 °C, and the upper aqueous phase was taken. Methanol was added into the upper aqueous phase at 1 mL to keep the mixed solution static overnight. Samples were then centrifuged for 30 min at 14,000*g* at 4 °C, and the upper aqueous phase was collected and filtered through 0.45 μm hydrophobic membranes. Standard samples of Asp, Glu and Gln were prepared in stored concentration 8×10^{-3} M and diluted into 64 μM mixed standard solution. The filtered samples were mixed with derivatization (1:10) by five times and stated for 1 min. There was 10 mL of the final solution injected into a C18 chromatographic column HPLC apparatus set as 337 nm wavelength of incidence fluorescence and 454 nm emission wavelength detector, 37 °C. The values were calculated as intercellular concentration (μM). All data were calculated with three biological replicates and reported as the means ± SD.

2.5. Measurement of phycoerythrin, soluble protein and chlorophyll-a

Fresh samples (0.1 g of *G. lemaneiformis*) were finely powdered in liquid nitrogen using a mortar. These powdered samples were resuspended in 1.5 mL of 0.1 M PBS buffer (pH 6.8) and shaken at 4 °C for 1 min before centrifugation for 30 min at 11,000 × *g* at 4 °C. Subsequently, the upper aqueous phase was collected and diluted. The concentration of phycoerythrin (PE) was calculated as PE (mg/mL) = 0.1247 $[(A_{564} - A_{730}) - 0.4583(A_{618} - A_{730})]$ N, where N indicates the dilution times [22]. The supernatants from the above phycobiliprotein extractions were used to determine the soluble protein concentrations with Coomassie brilliant blue kit (Jiancheng Biotech Company, Nanjing, China). A 50 μL aliquot of the supernatant was measured via UV-VIS spectrophotometry at 595 nm. The precipitations from the centrifugation step were obtained, and 5 mL DMF was added to extract chlorophyll-a overnight. The upper phase was obtained following centrifugation for 10 min at 6000 *g* at 4 °C, and the concentrations of chlorophyll-a were calculated as chlorophyll-a (mg/g) = [12.65(A664 − A750) − 2.99(A647 − A750) − 0.04(A625 − A750)] Ve / (1000·I·W), where Ve is the volume of DMF, I is the optical path in cuvette (cm), and W is the fresh culture weight.

2.6. Measurements of DNA

The young and fresh branches (0.15 g per sample) were ground into a fine powder in a pre-cooled mortar with liquid nitrogen. The following procedure was performed according to the manufacturer's guidelines for DNA extraction (Plant Genomic DNA Kit, Tiangen Biotech Co., Ltd., Beijing, China). The DNA contents were determined by absorbance at 260 nm in a Nanodrop 2000 c Spectrophotometer (Thermo Fisher Scientific Inc.).

2.7. Extraction of RNA

The young and fresh branches (0.15 g per sample) were ground into a fine powder in a pre-cooled mortar with liquid nitrogen. The total RNA from each sample was isolated with a Trizol Kit according to manufacturer's instructions (Promega, USA). Total RNA was treated with RNase-free DNase I (Takara Bio, Japan) for 30 min at 37 °C to remove residual DNA. RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) as well as RNase