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Physiological and molecular responses of *Prorocentrum donghaiense* to dissolved inorganic phosphorus limitation

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ABSTRACT

Prorocentrum donghaiense is an important dinoflagellate as it frequently forms harmful algal blooms that cause serious damage to marine ecosystems and fisheries in the coast of East China Sea. Previous studies showed that phosphorus acquisition (especially inorganic phosphorus) was the limiting factor for *P. donghaiense* growth. However, the responsive mechanism of this microalga under dissolved inorganic phosphorus (DIP) limitation is poorly understood. In this work, the physiological parameters and differentially expressed genes in *P. donghaiense* response to DIP limitation were comparatively analyzed. DIP-depleted *P. donghaiense* displayed decreased growth rate, enlarged cell size, decreased cellular phosphorus content, and high AP activities. A forward suppression subtractive hybridization (SSH) library representing differentially upregulated genes in *P. donghaiense* under DIP-depleted conditions was constructed, and 134 ESTs were finally identified, with a significant identity (E values $< 1 \times 10^{-4}$) to the deposited genes (proteins) in the corresponding databases. Five representative genes, namely, NAD-dependent deacetylase, phosphoglycolate phosphatase, heat shock protein (*HSP*) 90, rhodopsin, and *HSP40* were investigated through real-time quantitative PCR to verify the effectiveness of the established SSH library. Results showed that all the selected genes were differentially expressed and thus indicated that the established SSH library generally represented differentially expressed genes. These genes were classified into 11 categories according to their gene ontology annotations of biological processes. The members involved in functional responses such as cell defense/homeostasis, phosphorus metabolism, and cellular cycles were specially discussed. This study is the first to perform a global analysis of differentially expressed functional genes in *P. donghaiense* under DIP-depleted condition. It provided new insights into the molecular adaptive mechanisms of dinoflagellate in response to phosphorous limitation and elucidating the formation mechanism of algal blooms.

1. Introduction

Harmful algal blooms (HABs) frequently occur possibly because of marine eutrophication caused by excessive discharge of large amounts of nutrients, mainly nitrogen and phosphorus, from increasingly intensive human activities (Heisler et al., 2008; Anderson et al., 2012). Therefore, an overall and comprehensive understanding of molecular nutrient physiology is key to revealing the mechanisms of the formation and eradication of HABs (Dyhrman, 2008).

Phosphorus, which plays important regulatory roles in the initiation, duration, and toxin production of HABs, is a limiting factor of marine primary productivity (Lin et al., 2012; Hadjadji et al., 2014). Oceanic phosphorus mainly exists in two forms, namely, dissolved inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP). In general, phytoplankton preferentially utilizes DIP during their growth. However, the content of DIP in aquatic systems, including marine environments, is generally low (Meseck et al., 2009; Lin et al., 2012). As such, DIP commonly becomes the first limiting factor of phytoplankton growth. In coastal water areas, including the Changjiang Estuary and Pearl River Estuary of China where high nitrogen contents are caused by anthropogenic activities, DIP can be considered a remarkable stoichiometrical limiting factor of phytoplankton growth (Huang et al., 2005; Zhang et al., 2007a, 2007b). Under DIP-depleted conditions, marine microalgae exhibit a series of physiological responses, including a significant decrease in the total content of cellular phosphorus, retarded growth, enlarged cell size, and change in

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nutrition patterns (Burkholder et al., 2008; Hardison et al., 2013). High toxin production can be detected in toxic microalgae, including *Alexandrium tamarense*, *Dinophysis acuminata*, *Karlodinium veneficum*, *Pseudonitzschia multiseries*, and *Karenia brevis* (Anderson et al., 2012; John and Flynn, 2008; Fu et al., 2010; Hardison et al., 2013). Therefore, knowledge about the molecular adaptive mechanism of harmful algae under DIP-depleted condition is key to understanding their physiological responses and advantages over other marine phytoplankton and the occurrence mechanism of HABs.

Prorocentrum donghaiense is a common bloom-forming species commonly distributed along the East China Sea. This organism often causes large-scale coverage (over 1000 km^2) of HABs in the Changjiang Estuary in late spring and early summer. About 120 *P. donghaiense* blooms were recorded from 2000 to 2006 (Chen et al., 2013). In 2013 alone, 44 HABs were recorded in the Chinese coast; of these HABs, 33 were caused by dinoflagellates and 16 were triggered by *P. donghaiense*. HABs caused by *P. donghaiense* are large scale and persistent, and these HABs cause serious damages to marine ecosystems and fisheries. Therefore, *P. donghaiense* has been considered a notorious microalga in Chinese scientific community since 1995.

Phosphorus is a limiting factor of phytoplankton growth in East China Sea, specifically in the Changjiang Estuary. Unexpectedly, the DIP content in this water area is relatively low during blooms compared to high concentration of nitrogen (Han et al., 2003; Zhang et al., 2004; Huang et al., 2013). Nevertheless, *P. donghaiense* blooms last long, particularly under low phosphorus concentration. *P. donghaiense* exhibits a stronger interspecific competitive ability than other microalgae do under phosphorus limitation (Wang and Huang, 2003); *P. donghaiense* blooms can also occur in phosphorus-limited eutrophic and oligotrophic seas (Li, 2009). Moreover, phosphorus-limited eutrophication may be directly beneficial for *P. donghaiense* blooms (Li, 2009; Hadjadji et al., 2014). DIP concentration influences the population dynamics of *Prorocentrum* (Li et al., 2011). Thus, the adaptive ability and related molecular mechanism of the algal species under DIP limitation should be investigated to understand this ecological phenomenon.

Due to the extraordinarily high number of genes that are inferred from the giant genome, and the generally low transcriptional regulation level, the identification of differentially expressed genes from dinoflagellates using next-generation sequencing and DNA-chip is difficult and inefficient (Lin, 2011; Morey et al., 2011; Zhang et al., 2014). In contrast, suppression subtractive hybridization (SSH) is proved to be promising for gene regulation research (Zhang et al., 2014). In this study, *P. donghaiense* was cultured under DIP-replete and DIP-depleted conditions, and its physiological responses were subsequently investigated. A forward SSH library representing the upregulated genes in response to DIP-depleted condition was constructed with *P. donghaiense* cells cultured under two different DIP conditions. A certain amount of positive colonies of the library was randomly selected and sequenced to reveal the genes involved in the responses of *P. donghaiense* to DIP limitation. The effectiveness of the library representing the differentially expressed genes was further verified through real-time quantitative PCR (RT-qPCR). The cellular pathways and genes essential for such responses of *P. donghaiense* were also identified on the basis of existing knowledge on the role of these genes. The aim is to further understand the molecular mechanism behind –P stress in *P. donghaiense*.

2. Materials and methods

2.1. Algal culture

The clonal culture of *P. donghaiense* used in this study was established from the seawater samples collected from the East China Sea by micropipette isolation. The algal culture was maintained in 250 mL flasks supplied with 100 mL of sterile-filtered f/2 seawater medium (Guillard, 1975) at a salinity of 36 psu. The culture was grown at 20–22 °C under a 12 h light:dark cycle with a light intensity of 50–100 mmol photons $m^{-2} s^{-1}$. The culture was stirred manually daily, and the nutrient medium replaced monthly.

2.2. DIP limitation treatment

First, 100 mL of algal culture of *P. donghaiense* at exponential phase (approximately 10^5 cells/mL) was inoculated in triplicate to 1000 mL of f/2 seawater medium and cultured under the conditions described above. When the algal cells reached the exponential phase, each culture was equally divided into two parts; each ca. 500 mL. All 500 mL of algal cultures were filtered using nylon banderoles with 10 μm mesh and rinsed twice with 100 mL of sterile-filtered seawater. Afterward, the algal cells were transferred by washing into DIP-replete (DIP-replete group) and DIP-depleted media (DIP-depleted group) with a total volume of 500 mL. Both the DIP-replete and DIP-depleted media were same to the f/2 seawater medium described above, except that the initial concentration of phosphate was supplied at 36.2 μM and 0 μM (without addition of phosphate), respectively. Both groups were grown under the conditions described above. The whole culture experiment lasted for nine days, accompanied with following physiological measurements and cell collection for RNA isolation.

2.3. Physiological measurements

Physiological parameters, including cell density, cell size, cellular phosphorus content, and alkaline phosphatase (AP) activity of *P. donghaiense* cells, were measured for both DIP-replete and DIP-depleted groups. The cell densities were determined by counting cell number under a light microcopy with a hemocytometer. The average cell size (n = 20) was determined as longitudinal length by using a micrometer under a light microscopy. Cellular phosphorus was measured using the persulfate digestion method (Wetzel and Likens, 1995; Lampman et al., 2001). Briefly, algal cells were collected and washed twice with deionized water by centrifugation at 6000*g* for 8 min at 4 °C. Subsequently, these cells were ground with liquid nitrogen. The cell debris was digested by potassium persulfate and reacted with vitamin C and molybdate solution. Finally, the absorbance of the reaction mixture was determined spectrophotometrically using a UV-752 spectrophotometer (Shanghai Jinggong Industrial Co., Ltd., Shanghai, China) under a wavelength of 700 nm. The final cellular phosphorus content standardized by cell density is represented as cellular P-quota (pg/cell). For the measurement of AP and total soluble protein concentration, the algal cells were collected, and the same methods described above were performed. The algal debris was dissolved in extraction buffer containing 0.05 M Tris-HCl (pH 9.0). The AP activity assay was performed using the detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) mainly based on the methods described by González et al. (1994). The total soluble protein concentrations were measured using a protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China), following the method described by Bradford (1976). The final enzyme activities (specific activities) are represented by the value of actual enzyme activities divided by the protein concentration (AP unit/g protein). All the measurements at each time point were performed in triplicate for both DIP-replete and DIP-depleted groups.

2.4. RNA isolation

Algal samples (50 mL) were obtained from both groups at 6, 12, 24, and 48 h post DIP-replete and DIP-depleted treatments. The samples at each time point were centrifuged at 4000*g* for 8 min at 4 °C to collect the algal cells. The obtained algal cells were snap-frozen in liquid nitrogen before usage for RNA isolation. At the end of the DIP limitation culture experiment, all the algal cells from the DIP-depleted and DIP-replete groups sampled at different time points were combined as “tester” and “driver” samples of SSH, respectively. Total RNA was