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Transcriptional responses to phosphorus stress in the marine diatom, *Chaetoceros affinis*, reveal characteristic genes and expression patterns in phosphorus uptake and intracellular recycling

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ABSTRACT

Chaetoceros spp. are ecologically important ocean diatoms, and it is hypothesized that their genetic adaptations to phosphorus (P) stress may be different from the adaptations of model species with fully sequenced genomes. To investigate how phosphorus availability affects gene expression at the mRNA level, next-generation sequencing (NGS) was used to construct transcriptomes for *Chaetoceros affinis* (CCMP 160) cultures grown under low-phosphate and nutrient-replete conditions. This operation generated 29,285 scaffolds, and subsequent sequence comparisons resulted in the identification of 10,972 expressed genes, of which 503 were up-regulated and 553 were down-regulated by P stress. The differential expression patterns of 41 of these genes were confirmed by performing quantitative reverse-transcription polymerase chain reactions. These results demonstrated that genes related to nutrient acquisition are up-regulated in *C. affinis* under P deficiency. Moreover, *C. affinis* is unique in that it possesses a "classical" alkaline phosphatase and two type II Na^+/Pi cotransporters. Three genes homologous to the ones involved in the higher plant regulatory circuit for P uptake were also identified. P deficiency resulted in enhanced intracellular recycling of P in *C. affinis* via the upregulation of several ribonuclease genes. However, the expression of a gene involved in sulfolipid production was unaffected. Regarding photochemical reactions, the high transcription levels of light-harvesting complex genes, but low maximum quantum efficiency (F_v/F_m), implied that excess energy was dissipated under P deficiency. Furthermore, the NAD-malic enzyme was up-regulated to generate pyruvate via an alternative pathway that is less dependent on P. In low-P cultures, decreased rates of cell proliferation were found during the early stationary phase, consistent with the significant decrease in transcription of genes encoding cell division control protein 45 and ribonucleoside-diphosphate reductase.

1. Introduction

Diatoms are the dominant phytoplankton species in coastal seas and offshore upwelling regions and are responsible for approximately 40% of primary production in the ocean (Nelson et al., 1995). The carbon and energy generated via photosynthesis are stored in the form of lipids and carbohydrates (Valenzuela et al., 2012). Diatoms tend to sink in the water column, either in the form of individual cells, or in the form of fecal pellets after being grazed by zooplankton. Thus, diatom production significantly contributes to carbon deposition in the deep sea and has important effects on the global carbon cycle (Karl et al., 2012).

In natural environments, diatom growth is often limited by the availability of one or more types of nutrients. Although nitrogen and iron are frequently reported as limiting nutrients, there is increasing evidence of phosphorus (P) limitation at specific locations (Girault et al., 2013; Hamada et al., 2011). P is an essential nutrient for all organisms. It is used in the biosynthesis of various cellular components, including nucleic acids, sugars, proteins, and lipids. Phospholipids are the major constituent of cell membranes, and phosphate-containing nucleotides are the building blocks of nucleic acids. P deficiency not only inhibits the synthesis of the cell membrane and nucleotides but also affects signal transduction and several other metabolic pathways (Moseley et al., 2006).

The transcriptome and proteome studies of two model diatoms, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, revealed that P deficiency caused a series of intracellular changes (Dyhrman et al., 2012; Yang et al., 2014). Cells attempted to increase P uptake by producing more phosphate transporters and tried to utilize dissolved organic P

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by increasing the production of alkaline phosphatase. Other changes included the increasing production of polyphosphate and the modified cell membrane containing sulfolipids. These observations revealed that both species have evolved biochemical pathways that are essential to their survival in environments with variable P availability. In *P. tricornutum*, a separate transcriptome study indicated that the depletion of exogenous phosphate might be an early trigger for lipid accumulation (Valenzuela et al., 2012). In addition, certain phosphate-responding cyclins may function in linking nutritional status and the start of cell division (Huysman et al., 2010).

The aforementioned transcriptome studies on *T. pseudonana* and *P. tricornutum* provided new insights into the metabolic networks of diatoms. However, the information obtained from these two species is only a small subsample of the true genomic diversity of all diatoms. Diatom species that differ in their cell volumes are known to have different metabolic rates, intrinsic growth rates, photosynthetic capacities, and respiratory rates (Connolly et al., 2008). In addition, individual species may use different strategies to relieve P deficiency. For example, the diatom *Chaetoceros ceratosporus* utilizes phosphate diesters by expressing diesterase under conditions of P deficiency, but the same response is not observed in another diatom, *Skeletonema costatum* (Yamaguchi, 2005).

In marine planktonic diatoms, *Chaetoceros* is probably the largest genus, containing approximately 400 described species (Pai et al., 2013). These species are widely distributed in saline environments such as oceans and salt lakes, and several species are important bloom formers in upwelling and coastal habitats (Horner et al., 1997). They are also one of the main biomass contributors among microplankton in polar regions (Jung et al., 2007). The reason for their broad distribution and adaptation to diverse environments may be related to unique regulatory pathways in nutrient utilization. In this genus, *Chaetoceros affinis* is a cosmopolitan species commonly found in coastal waters (Kang et al., 2006; Sunesen et al., 2008). To obtain an overall view of the metabolic pathways of an ecologically important diatom under P deficiency, next-generation sequencing (NGS) technique was employed to construct three transcriptomes of cultured *C. affinis* (CCMP 160). As a result, a functionally annotated set of *C. affinis* sequences expressed under replete and low-P conditions has been generated, which illuminates the metabolic responses of this diatom to P availability. Metabolic responses commonly observed in other diatoms and responses unique to *C. affinis* are discussed.

2. Material and methods

2.1. Culture conditions

The diatom *C. affinis* (clone CCMP 160) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) and was grown in f/2 medium (Guillard, 1975) prepared with GF/F filtered and autoclaved natural seawater. The unialgal culture was maintained at 24 °C and illuminated at an irradiance level of 115 μmol photons m^{-2} s^{-1} under a 12:12 h light/dark cycle.

Phosphate manipulation experiments were conducted in either f/2 replete or f/2 low-P ($[PO_4^{3-}] = 3$ μM) medium prepared with 0.22-μm filtered artificial seawater (Goldman and McCarthy, 1978). Before the beginning of individual incubations, *C. affinis* was acclimated to the relevant growth conditions for several days, with daily monitoring of the maximum quantum efficiency of photosystem II (F_v/F_m). When the F_v/F_m of the low P culture decreased to 0.4, the replete and low-P cultures under acclimation were separately transferred to full strength f/2 and low-P fresh media, respectively. These established treatments and resulting transcriptomes were given the names of Replete and LowP_A, respectively. At a later time, a second low-P treatment was established following the same procedure, and named LowP_B. Algal cells were grown in 2 flasks with a volume of 2.5 L each for Replete and in 2 carboys with a volume of 15 L each for LowP_A and LowP_B. During the incubation period, a 1-mL sample was harvested daily from individual cultures and preserved with acidic Lugol's solution (Smayda, 1974) for the determination of cell abundance. A separate 6-mL sample was used for F_v/F_m measurement, and another 12-mL sample was used for the measurement of alkaline phosphatase activity.

2.2. Cell enumeration

Cell abundance was determined by placing 1 mL of Lugol's fixed sample into a Sedgewick-Rafter counting cell (Wildlife Supply Company, Yulee, FL, USA). Cells were counted using a light microscope (Optiphot-2, Nikon, Tokyo, Japan) at a magnification of 100×.

2.3. F_v/F_m measurements

Triplicate samples of 2 mL algal culture were placed in quartz cuvettes (NSG Precision Cells, New York, NY, USA) and adapted in the dark for 15 min. The cuvettes were then placed into a Fluorescence Induction and Relaxation fluorometer (FIRe, Satlantic, Halifax, Canada). The level of F_v/F_m was measured before and after the application of a single-turnover flash at a wavelength of 450 ± 15 nm with a duration of 80 μs (Campbell et al., 1998).

2.4. Alkaline phosphatase activity (APA) measurements

An artificial AP substrate, 3-O-methylfluorescein phosphate (Sigma-Aldrich, St. Louis, MO), was added to an algal sample at a final concentration of 0.55 μM. The reaction mixture was then incubated in the dark at 25 °C for 30 min. The fluorescence intensity of 3-O-methylfluorescein generated by AP digestion was detected using a fluorometer (10-AU, Turner Designs, Sunnyvale, CA, USA) equipped with a band-pass excitation filter of 390 to 500 nm and a band-pass emission filter of 510 to 700 nm (Liu et al., 2010; Perry, 1976). The measured fluorescence intensities at the beginning and the end of the incubation period were converted to moles phosphate generated by AP through a calibration curve, and APA was expressed as fmol PO_4^{3-} $cell^{-1}$ min^{-1}.

2.5. RNA isolation

When the F_v/F_m readings of the two LowP cultures dropped below 0.4, the cells of all three cultures were harvested by filtration through a 20-μm mesh screen. To extract total RNA, the harvested cells were subjected to supersonic disruption (VCX600, Sonics & Materials Inc., Newtown, CT, USA) on ice. RNA was isolated from the crude extract using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNase free DNaseI set (Qiagen, Valencia, CA, USA) was applied for the on-column digestion of residual DNA. The extracted RNA was eluted in diethyl-pyrocarbonate-treated water, and the concentration was determined using a spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA) at wavelengths of 260 and 280 nm.

2.6. Construction of a 454 library and sequencing

Poly(A) RNA and cDNA were generated from the total RNA of both Replete and LowP_A samples using the Creator SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA), with modified original primers 5′ AAGCAGTGGTATCAACGCAGAGTGGCCATTACGG CCGGG 3′ and 5′ AAGCAGTGGTATCAACGCAGAGTGCAGTTTTTTTTTTT TTT TTTTTT 3′. Poly(A) RNA isolation of the LowP_B culture was accomplished using a Dynabeads oligo(dT) system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality of the poly(A) RNA was examined using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The resulting cDNA was used to construct a 454 library in accordance with the supplier's instructions (454 Life Sciences, Branford, Connecticut, USA).