Marine Genomics xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Marine Genomics

journal homepage: www.elsevier.com/locate/margen

Cellular metabolic responses of the marine diatom *Pseudo-nitzschia multiseries* associated with cell wall formation

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ARTICLE INFO

Article history:
Received 20 October 2013
Received in revised form 18 December 2013
Accepted 18 December 2013
Available online xxxx

Keywords:
Diatom
Pseudo-nitzschia multiseries
Cellular metabolism
Biosilicification
Comparative proteomics

ABSTRACT

In this study a comparative proteomics approach involving a mass spectrometric analysis of synchronized cells was employed to investigate the cellular-level metabolic mechanisms associated with siliceous cell wall formation in the pennate diatom *Pseudo-nitzschia multiseries*. Cultures of *P. multiseries* were synchronized using the silicate limitation method. Approximately 75% of cells were arrested at the G2 + M phase of the cell cycle after 48 h of silicate starvation. The majority of cells progressed to new valve synthesis within 5 h of silicon replenishment. We compared the proteome of *P. multiseries* at 0, 4, 5, and 6 h of synchronization progress upon silicon replenishment using two-dimensional gel electrophoresis. Forty-eight differentially expressed protein spots were identified in abundance (greater than two-fold change; $P < 0.005$), some of which are predicted to be involved in intracellular trafficking, cytoskeleton, photosynthesis, lipid metabolism, and protein biosynthesis. Cytoskeleton proteins and clathrin coat components were also hypothesized to play potential roles in cell wall formation. The proteomic profile analysis suggests that *P. multiseries* most likely employs multiple synergistic biochemical mechanisms for cell wall formation. These results improve our understanding of the molecular mechanisms underlying silicon cell wall formation and enhance our understanding of the important role played by diatoms in silicon biogeochemical cycling.

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1. Introduction

Diatoms represent one of the predominant types of eukaryotic unicellular microalgae in aquatic environments, and they play a major role in the global cycling of carbon and silicon (Nelson et al., 1995; Tréguer et al., 1995). These organisms require uptake of silicic acid from the environment to synthesize their siliceous cell walls. Diatoms are consequently the most significant contributors to the formation of biosilica in the oceans, producing an estimated of 240×10^{12} mol per year (Tréguer et al., 1995). As this characteristic is unique to these organisms, the mechanism of silicon metabolism by diatoms has garnered significant research interest in recent decades.

Valve and girdle bands constitute the main structure of the siliceous cell wall in diatoms. Processes related to silica structure formation occur in the silica deposition vesicle (SDV) (Pickett-Heaps et al., 1990). Some of the key components involved in silicon transport and precipitation have been successively described. These include, for example, the silicon transporters (SITs) (Hildebrand et al., 1998), silaffins and long chain polyamines (Kröger et al., 1999, 2000, 2001, 2002), silacidins (Wenzl et al., 2008), pleuralins (Kröger et al., 1997; Kroger and Wetherbee, 2000), cingulins (Kröger et al., 1994; Scheffel et al., 2011), and frustulins (Kröger et al., 1994, 1996). Recently, transcriptomics and proteomics approaches have revealed numerous new candidate proteins that may be involved in silica formation (Frigeri et al., 2006; Mock et al., 2008; Guillaume et al., 2009; Shrestha et al., 2012). However, many details of the biosilicification process remain unknown.

Given the complexity of diatom silica structure, multiple cellular metabolic pathways may be involved in formation of the cell wall. Some physiological and biochemical processes related to bio-silicification have been described. For example, a previous study has indicated that silicon transport and silicification processes are energized by oxidative phosphorylation (Blank and Sullivan, 1979; Blank et al., 1986). Silicon uptake and silicification of the cell wall have also been found to be closely coupled to the diatom cell cycle (Hildebrand et al., 2007), and a close relationship has been shown between the cytoskeleton and formation of diatom microscale structures (Pickett-Heaps et al., 1990; Van de Meene and Pickett-Heaps, 2002, 2004). Some studies clearly demonstrate the involvement of the cytoskeleton (actin and microtubules) in forming the shape of the SDV, as evidenced by direct observations and application of cytoskeletal inhibitors (Tesson and Hildebrand, 2010; Van de Meene and Pickett-Heaps, 2002, 2004). Recent work conducted

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http://dx.doi.org/10.1016/j.margen.2013.12.005

Please cite this article as: Xu, B., et al., Cellular metabolic responses of the marine diatom *Pseudo-nitzschia multiseries* associated with cell wall formation, Mar. Genomics (2013), http://dx.doi.org/10.1016/j.margen.2013.12.005

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by Shrestha et al. (2012) provided a large-scale picture of cellular processes associated with bio-silicification in the centric diatom *Thalassiosira pseudonana*. However, the cellular metabolic response of pennate diatoms to bio-silicification has not been fully elucidated to date. A clear understanding of the relationship between silicon metabolism and other aspects of cellular function needs to be uncovered.

Recent advances in synchronizing diatom cultures and the use of comparative proteomics technology can offer promising tools for the discovery of potential proteins of interest at time points when cultures are enriched with cells undergoing cell wall synthesis (Hildebrand et al., 2007; Frigeri et al., 2006). In the present study, a comparative proteomic investigation was carried out in the pennate diatom *Pseudo-nitzschia multiseries* to gain insight into the cellular processes that respond to bio-silicification. *P. multiseries* is an attractive model species for this purpose because it is a pennate species, and because work is in progress to sequence its whole genome. Four samples were collected at 0, 4, 5, and 6 h after silicon replenishment during synchronous growth for two-dimensional electrophoresis (2-DE) comparative proteomic and MS/MS analysis. Proteins with differential expression were detected and identified. The potential functions of these proteins, and their involvement in bio-silicification are also discussed. The results should provide the framework for further functional studies of each member of this network in the metabolic mechanisms associated with bio-silicification processes in diatoms.

2. Materials and methods

2.1. Culture methods

P. multiseries (CLN-18) was kindly provided by Dr. Don M. Anderson (Woods Hole Oceanographic Institution, Woods Hole, MA, USA). The cultures were maintained in f/2 enriched seawater medium with added silicon at 20 °C under a 12:12 light:dark (L:D) cycle with a light intensity of approximately 25–30 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

2.2. Synchronized cell growth

Synchronized cell growth was achieved through silicon limitation. Cultures were grown in triplicate 1 L polycarbonate carboys. Exponential-phase cultures of *P. multiseries* were harvested by centrifugation and washed with silicon-free f/2 medium. Cells were resuspended in silicon-free f/2 medium at 1.0×10^5–1.2×10^5 cells/mL and incubated under continuous light with aeration in a polycarbonate bottle. After 48 h, sodium silicate was added to the remaining culture at a final concentration of 200 μM to initiate synchronized progression through the cell cycle.

2.3. Flow cytometry

Flow cytometry was performed using EPICS XL (Beckman Coulter, USA). Samples for cell-cycle stage determination, approximately 1×10^6 cells of *P. multiseries*, were preserved in 1 mL methanol overnight. Cells were pelleted at 3000 g for 5 min, re-extracted in 0.8 mL methanol for 10 min, pelleted, and then washed with 1 mL PBS (20 mM phosphate buffer, pH 7.4, 150 mM NaCl) until the supernatant became colorless. Cells were re-extracted in 0.3% Triton-100 for 40 min and washed once with 1 mL PBS. DNase-free RNase A was added to achieve a concentration of 100 μg/mL, and cells were incubated for 1 h at 37 °C. Cells were then pelleted, washed twice in 1 mL PBS and resuspended in 25 μL PBS. To this suspension was added 10 μL of PI solution (2.5 mg/mL) and tubes were incubated at room temperature in the dark for 10 min. Fluorescence of cells was monitored in channel FL-3 (520 nm max. emission). The relative percentage of cells in G1, S, and G2 + M phases was calculated using MultiCycle software (Beckman Coulter, USA).

To monitor Rhodamine 123 (R123) incorporation, frozen cell pellets harvested from synchronous culture were resuspended in 100 μL PBS. To this sample was added 1 mL methanol and the sample was placed on a rotator for 15 min. Cells were pelleted, resuspended in 1 mL methanol, and incubated at −20 °C overnight. Cells were then pelleted, washed, resuspended in 1 mL PBS, and treated with RNase for 1 h as described above. Cells were finally pelleted, washed in 1 mL PBS, and resuspended in 0.7 mL PBS for analysis. Fluorescence was monitored in the FL-1 channel (525 nm emission max.). Samples containing 5000 cells were analyzed.

2.4. Silicic acid determinations

The concentration of silicic acid was measured using the molybdate method described by Strickland and Parsons (Strickland and Parsons, 1968). Aliquots of cells (12 mL) were harvested using 0.22 filter membranes. A 1-mL aliquot of supernatant was removed to a new tube and stored at −20 °C. This sample was used to measure silicic acid in the medium. Intracellular soluble-silicon pools and cell-wall silica levels were measured using the method described by Hildebrand (Hildebrand et al., 2007).

2.5. Fluorescence microscopy

R123 was added at 2 μg/mL to the culture 5 min prior to silicate addition, and fluorescence microscopy (Olympus BX-41) was performed as described (Frigeri et al., 2006).

2.6. Protein sample preparation

Whole cell proteins were extracted from synchronous cultures. Each experiment was performed three times in triplicate. Total proteins were extracted according to the method described by Lee (Lee and Lo, 2008). In protein extractions using Trizol (Roche, Switzerland), preparations were performed according to the manufacturer's instructions with some modifications. Briefly, 1 mL Trizol reagent was added to the cell pellet and the mixture subjected to sonication on ice for a total of 12 min, using short pulses of 5–10 s. Cell lysis was confirmed using light microscopy. Subsequently, 200 μL of chloroform was added to the cell lysate and the mixture shaken vigorously for 15 s. The mixture was allowed to stand for 5 min at room temperature before being centrifuged at 12000 ×*g* for 15 min at 4 °C. The top pale-yellow or colorless layer was removed. The reddish bottom layer was resuspended in 300 μL of ethanol and the mixture was centrifuged at 2000 ×*g* for 5 min at 4 °C. The supernatant was transferred to a new tube and 1.5 mL of isopropanol was added. The mixture was allowed to stand for at least 20 min to allow precipitation of proteins at room temperature. The mixture was then centrifuged at 14000 ×*g* for 10 min at 4 °C. The resulting pellet was briefly washed with 95% ethanol before being allowed to dry in air. Lysis buffer (500 μL) was added to solubilize the protein pellet before it was loaded onto the first dimension IEF.

2.7. Two-dimensional electrophoresis

First-dimension IEF was performed by loading 450 μl of rehydration buffer (8 M urea, 2% w/v CHAPS, 15 mM DTT, 0.5% v/v IPG buffer) containing 1.5 mg of protein onto IPG strips (24 cm) at pH 4–7 (GE Healthcare, USA). The proteins were focused using the Ettan IPGphor 3 system (GE Healthcare, USA) at ≤50 μA/strip at 20 °C. The voltage and duration used were as follows: 30 V for 12 h (active rehydration), 150 V for 1 h, 300 V for 2 h, 1000 V for 2 h, 4000 V for 2 h, 8000 V for 1 h, and 8000 V for approximately 10 h, until 80,000 Vhs was achieved. Prior to the second dimension separation, IPG strips were equilibrated with gentle shaking in equilibration buffer (6 M urea, 75 mM Tris–HCl [pH 8.8], 29.3% glycerol, 2% SDS, 1% bromophenol blue) supplemented with 1% [w/v] DTT for 15 min, and subsequently in the equilibration

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