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Proteome responses of *Gracilaria lemaneiformis* exposed to lead stress

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ABSTRACT

Proteome response of plants is an important process that enables them to cope with environmental stress including metal stress. In this study, the proteome of *Gracilaria lemaneiformis* exposed to lead was investigated. Two-dimensional gel electrophoresis analysis revealed 123 protein spots, among which 14 proteins were significantly differentially expressed and identified using MALDI-TOF MS. Two of the up-regulated proteins were identified and predicted to be involved in photosynthesis and signal transduction, while eleven down-regulated proteins were functionally grouped into five classes including photosynthesis, energy metabolism, protein metabolism, carbohydrate transport and metabolism, and antioxidation proteins. There was also an up-regulation in superoxide dismutase, peroxidase, glutathione s-transferase, and heat-shock protein 70 upon Pb exposure. Proteomic studies provide a better picture of protein networks and metabolic pathways primarily involved in intracellular detoxification and defense mechanisms.

1. Introduction

Metal contamination has become one of the major worldwide environmental problems affecting water, soil, plant, animal and human health (Moreno-Jiménez et al., 2016; Xie et al., 2015). Generally, metals can be classified into two categories, viz., beneficial and harmful or potentially harmful metals. For instance, metals such as Cu, Zn, Fe, Mn, Mo, Ni and Co are known for their beneficial involvement in different biological processes. Copper for example, plays an important role in electron transport chain and lipid peroxidation (Muralidharan et al., 2012), while zinc is a cofactor of some enzymes, with the zinc-enzyme complex involved in protein binding, the regulation of enzyme activity, as well as regulation of transcription and translation (Broadley et al., 2007). However, some metals including Cd, Pb and Hg are non-essential elements during the growth of algae, but are mostly toxic at low concentrations. Cd exposure might not only result in damage of photosynthetic apparatus, degeneration of mitochondria and the abnormal mitosis, but also cause the accumulation of ROS in active photosynthetic tissues (Lin and Aarts, 2012).

Among all metals, lead (Pb) is one of the most serious pollutants, with massive amounts released into the environment by human activities. Contamination of the food chain by Pb is the main entry route into humans (Ali et al., 2013). In recent years, many studies on the effects of Pb ions in high plants and microalgae have been carried out, such as Pb accumulation and detoxification in different plants, and its effect on the amount of protein and proline (Amanifar et al., 2014; Karimi et al., 2012; Shahid et al., 2014). Additionally, toxic metal ions, including Pb, have been shown to interfere with the folding and aggregation of nascent or non-native proteins, thereby profoundly affecting cellular protein homeostasis (Hasan et al., 2017). However, very few studies have explored the effects of Pb on macroalgae, especially the proteome response of macroalgae to Pb. The red alga, *Gracilaria lemaneiformis* is an important economic macroalga, not only as a source of food for human and fish, but also a major alga for the remediation of water eutrophication, for which reason it is widely cultivated along the northern and southern coast of China (Liu et al., 2018; Xu et al., 2017). The complete genome sequence of *G. lemaneiformis* is currently unavailable; hence, research at the molecular level is hampered. Thus, given that Pb is a major pollutant in the Nan'ao Island area in Guangdong Province of China, we anticipate that proteomic study of *G. lemaneiformis* under Pb exposure will provide useful insights into the molecular mechanisms of this alga. The findings from this study will also provide fundamental data for the prevention and detection of metal contamination in the aquatic ecosystem.

2. Materials and methods

2.1. Material and treatments

The *G. lemaneiformis* used in this study was collected from Nan'ao

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Island cultivation field (116.5°E, 23.3°N), Shantou, Guangdong, China. The algae were acclimatized for one week in a light incubator maintained at 20 ± 1 °C under 12:12 h L: D photoperiod with light intensity at 50 μmol photons $m^{-2} s^{-1}$. The *G. lemaneiformis* was then transferred into triangular flasks containing fresh artificial seawater. Three experimental groups were setup: a control group and groups exposed to $PbCl_2$ at a final concentration of 0.05 mg L^{-1}, 0.5 mg L^{-1}, 2.5 mg L^{-1}, 5 mg L^{-1}, and 10 mg L^{-1}. Each group was composed of three biological replicates, and each replicate contained 5 g of *G. lemaneiformis*. After 72 h of exposure, samples were collected for analysis.

2.2. Basic parameters analysis

The growth rate = $[(w_t / w_0)^{1/t} - 1] \times 100\%$, where w_0 is the fresh weight of algae at the beginning (g), w_t is the fresh weight of algae at time t (g), t is the time interval between two detection points (date). The pigments and soluble proteins were analyzed as described in references (Duke et al., 1989; Moran, 1982). Superoxide dismutase, peroxidase and glutathione s-transferase activities were detected according to the instructions on the kits (Nanjing Jiancheng Bioengineering Institute, China).

2.3. Total protein extraction

Each sample (2 g) was ground finely into powder in liquid nitrogen with mortar and pestle, and homogenized with 5 mL extraction buffer Trizol (Takara, Japan) by vortexing for 1 min before ultrasonication 3 s/3 s for 5 min at 0 °C on ice. Subsequently, 1 mL chloroform was added into each ultrasonicated sample, and the mixture vortexed for 1 min before being centrifuged at 12,000 rpm for 15 min, 4 °C. The centrifuge 5415 D was bought from Eppendorf, Hamburg, Germany. The supernatant was transferred into a new Eppendorf (EP) tube, 400 μL ethanol added, vortexed and centrifuged (12,000 rpm, 5 min, 4 °C). Next the supernatant was transferred to an EP tube, 2 mL isopropanol added, and incubated for 40 min at 4 °C. Centrifugation (12,000 rpm, 5 min, 4 °C) was carried out to precipitate the proteins. Finally, proteins were rinsed three times with 95% ethanol and dried at room temperature. Protein concentration was determined using the 2D-Quant Kit (GE Healthcare).

2.4. Western blotting

Protein samples (200 μg) were separated on 12% polyacrylamide gel, transferred onto nitrocellulose membranes, blocked with 5% skimmed milk powder in PBS, and probed with primary antibody. Anti-HSP70 and anti-actin antibodies (Roche) were used at a dilution of 1:3000 in 5% skimmed milk powder dissolved in PBS. Membranes were washed in PBS and probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5000) in 5% skimmed milk powder dissolved in PBS.

2.5. Two-dimensional gel electrophoresis (2-DE) and image analysis

The protein samples (700 μg) from the total protein extracts were resolved in IEF buffer (Bio-Rad, Hercules, CA, USA). The IPG strips (11 cm, linear pH 4–7, Bio-Rad Ready-Strip, Bio-Rad) were rehydrated overnight with 460 μL IEF buffer containing protein samples. Proteins were focused using a Protean IEF (Bio-Rad) at 20 °C, applied for 4 h at 250 V, 1 h at 1000 V, 10 h at 6000 V, 12 h at 6000 V. After focusing, proteins were equilibrated with solution (50 mmol L^{-1} Tris–HCl, pH 8.8, 6 mol L^{-1} urea 30% glycerol, 2% SDS) containing 1% DTT for 10 min, and then with solution containing 2.5% iodoacetamide. In the second dimension, proteins were separated on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Gel images were scanned with a HP laser scanner and analyzed with the software PDQuest version 8.0.1 (Bio-Rad). For each sample, three replications were performed. The size of each spot was normalized to a relative size, and the mean values were calculated from triplicate data. Quantitative analysis for each data set was created between each control group and Pb-treated group. Student's *t*-test ($p < 0.05$) was used to determine the statistically significant difference in protein fold changes relative to control. A 2.0-fold change in normalized spot densities was considered indicative of a differentially expressed component.

2.6. Protein identification using MALDI-TOF MS

The protein spots of interest were excised from Coomassie stained gels. Excised protein spots were in-gel digested with 25 μL of 7 ng/μL trypsin in 50 mM ammonium bicarbonate buffer and digested at 37 °C for 18–20 h (Ellias et al., 2012). Peptide mass fingerprinting was carried out on a Voyager-DE STR mass spectrometer equipped with a 337 nm N_2 UV laser, and mass spectra was obtained in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 65%, a guide wire voltage of 0.010%, and a delay time of 150 ns. Data was acquired by summing 128 laser pulses and subjected to three-point internal calibration using trypsin autodigestion peaks (842.5099 and 2211.1046) and an angiotensin I peak (1296.6853). Monoisotopic peptide masses were analyzed with MoverZ (www.proteometric.com), and the NCBI non-redundant protein database was searched using Mascot software (www.matrixscience.com). The following parameters were used for database searches: mass tolerance of 50 ppm, one missed cleavage, oxidation of methionine, and cysteine modified by iodoacetamide. Protein identification was accepted with a MASCOT score higher than 60, and with more than five matched peptides. The MASCOT protein search was performed via all plants' database.

3. Results and discussion

The *G. lemaneiformis* cultures were grown for 3 and 6 days in media containing 0, 0.05, 0.5, 2.5, 5 and 10 mg L^{-1} $PbCl_2$, respectively. Growth rate is shown in Fig. 1a. It can be seen that the growth rate was slightly increased in the presence of 0.05 mg L^{-1} lead. The difference between treated and untreated cultures was confirmed by a slightly higher Chl-a, phycoerythrin, a light-harvesting pigment and soluble proteins in 0.05 mg L^{-1} lead treatment compared to the control (Fig. 1b–d). However, the presence of higher lead (> 0.05 mg L^{-1}) in the culture medium seemed to slow down the growth rate and inhibit the accumulation of pigment as well as soluble proteins. When the concentration of lead was 10 mg L^{-1}, the algae went into negative growth rate. These results indicated that low $PbCl_2$ (0.05 mg L^{-1}) exposure could stimulate algal growth with accumulation of pigments and soluble proteins, while high $PbCl_2$ (> 0.05 mg L^{-1}) exposure inhibited algal growth and increased the level of pigments and soluble proteins. This observation could be related to the fact that the cell wall being the first barrier, might have prevented the assimilation of metals at low concentrations (Macfie and Welbourn, 2000). However, at higher concentration the cell wall could not bind all the metal ions, thus some were able to enter the cell to cause damage (Jamers et al., 2013).

Previous studies have shown that plants response to metals (termed metal homeostasis) is regulated at the molecular level (Lin and Aarts, 2012), with this process also regulating the generation of metal-induced reactive oxygen species (ROS) (Smeets et al., 2008). The “oxidative burst” defense mechanism is conserved and important across different plants, animals and algae (Zozaya-Valdés et al., 2017). Macroalgae are capable of releasing superoxide dismutase (SOD), peroxidase (POD) and other oxidases to degrade ROS and hence protect themselves. In this study, when algae were exposed to different Pb concentrations, increased concentration of Pb induced the accumulation of antioxidant enzymes (Fig. 2a, b). A dramatic decrease in antioxidant enzymes at 10 mg L^{-1} Pb was observed, with one possible reason being that the high Pb concentration in the culture was beyond the tolerance of the