Plant Physiology and Biochemistry 104 (2016) 71–80

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

iTRAQ-based proteomic analysis reveals the mechanisms of silicon-mediated cadmium tolerance in rice (*Oryza sativa*) cells

Jie Ma, Huachun Sheng, Xiuli Li, Lijun Wang*

College of Resources and Environment, Huazhong Agricultural University, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 31 January 2016
Received in revised form
17 March 2016
Accepted 17 March 2016
Available online 19 March 2016

Keywords:
Silicon (Si)
Cadmium (Cd)
iTRAQ
Proteome
Rice (*Oryza sativa*) cells

ABSTRACT

Silicon (Si) can alleviate cadmium (Cd) stress in rice (*Oryza sativa*) plants, however, the understanding of the molecular mechanisms at the single-cell level remains limited. To address these questions, we investigated suspension cells of rice cultured in the dark environment in the absence and presence of Si with either short- (12 h) or long-term (5 d) Cd treatments using a combination of isobaric tags for relative and absolute quantitation (iTRAQ), fluorescent staining, and inductively coupled plasma mass spectroscopy (ICP-MS). We identified 100 proteins differentially regulated by Si under the short- or long-term Cd stress. 70% of these proteins were down-regulated, suggesting that Si may improve protein use efficiency by maintaining cells in the normal physiological status. Furthermore, we showed two different mechanisms for Si-mediated Cd tolerance. Under the short-term Cd stress, the Si-modified cell walls inhibited the uptake of Cd ions into cells and consequently reduced the expressions of glycosidase, cell surface non-specific lipid-transfer proteins (nsLTPs), and several stress-related proteins. Under the long-term Cd stress, the amount of Cd in the cytoplasm in Si-accumulating (+Si) cells was decreased by compartmentation of Cd into vacuoles, thus leading to a lower expression of glutathione S-transferases (GST). These results provide protein-level insights into the Si-mediated Cd detoxification in rice single cells.

1. Introduction

Due to the use of phosphate fertilizers and irrigation of heavy metal contaminated water, high cadmium (Cd) levels have been observed in various agricultural soils (Sebastian and Prasad, 2014), consequently, Cd is accumulated in crop plant tissues (Lima et al., 2006). Cd toxicity in plants includes the induction of reactive oxygen species (ROS), disruption of cellular processes/structures and the growth inhibition (Sanita di Toppi and Gabbrielli, 1999). The mechanisms for plants to alleviate Cd stress vary from exclusion, compartmentation, and the synthesis of stress-related proteins for resistance or complexation (Clemens, 2006; Adrees et al., 2015). The sequestration of Cd by phytochelatins (PCs) might play a central role in Cd detoxification (Cobbett and Goldsbrough, 2002).

Rice (*Oryza sativa*) provide grains as food for billions of people, Cd contamination not only results in biomass decreasing in plants but also leads to diseases in humans (Nawrot et al., 2010). High level of Silicon (Si) is accumulated by rice and can alleviate heavy metal Cd toxicity (Ma, 2004; Vaculík et al., 2015) and improve plant growth (Epstein, 2009). Wang et al. (2000) demonstrated that the silica deposited in rice cell walls can prevent the uptake of Cd. Very recently, evidence at the single cell level has suggested that Si can form complexations with Al (Prabagar et al., 2011) and codepositions with Cd in cell walls (Liu et al., 2013) by an interaction between Si and hemicelluloses of cell walls (Ma et al., 2015). Moreover, Si can alleviate abiotic stress by increasing the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) and increasing the concentration of GSH (Liang et al., 2003), and activating *OsLsi* and *OsHMA3* genes (Kim et al., 2014), suggesting that Si may have an effect on protein expression. Nwugo and Huerta (2011) investigated proteomic changes of rice leaves to identify 60 proteins which were regulated by Cd and/or Si treatments, showing that Si plays a more active role in physiological processes than previously proposed.

Despite advances made in elucidating the importance of Si in plant biology at the whole-plant level, the molecular mechanisms of Si-induced Cd stress tolerance at the single cell level remain unclear. Therefore, we investigated rice individual cells with the same developmental stages and functions by a proteomic approach

* Corresponding author.
E-mail address: ljwang@mail.hzau.edu.cn (L. Wang).

http://dx.doi.org/10.1016/j.plaphy.2016.03.024
0981-9428/

of isobaric tags for relative and absolute quantitation (iTRAQ) to analyze the protein expression profiles of single cells suspension-cultured in the absence and presence of Si following a short- or long-term of Cd exposure. The aim of this study is to provide possible molecular mechanisms of Si-induced alleviation of Cd stress in rice single cells.

Our results highlight the roles of the cell wall bound form of Si in preventing Cd uptake into cells and emphasize the roles of the cell wall- and cell membrane-related proteins in delaying the initiation of the toxic effects of Cd in Si-accumulating cells. Meanwhile, maintaining the normal GSH system in Si-accumulating cells at a short and long time period of Cd exposure leads to the alleviation of Cd toxicity. Furthermore, the down-regulation of a large proportion of proteins reveals that Si plays a key role in delaying the Cd toxicity. To our knowledge, this is the first single-cell level proteomic study to provide molecular mechanisms of the Si-induced alleviation of Cd stress.

2. Materials and methods

2.1. Cell culture and Si/Cd treatments

Rice suspension cells (*O. sativa* L. cv Zhonghua 11) were cultured by the methods we used previously (Chu et al., 1975; Thomas et al., 1989; Liu et al., 2013). Briefly, cells were cultivated in the absence (−Si) or presence (+Si) of 1 mM silicic acid (Na_2SiO_3) in the AA nutrient solution for 3 months and the medium was renewed every 5 days. Then 30 μM of cadmium (Cd) was added for 12 h (short-term) or 5 days (long-term) prior to further experiments and analyses. The concentration of Si in nutrient solution was determined based on the physiological concentrations of rice, and the concentration of 30 μM of Cd was selected because our previous studies have demonstrated the maximum Cd uptake ability of the cells (Ma et al., 2015).

2.2. Protein extraction

About 3 g of cells were collected in each treatment (+Si/+Cd 12 h; −Si/+Cd 12 h; +Si/+Cd 5 d; −Si/+Cd 5 d) and washed with Milli-Q water 5 times by repeated centrifugation at 10 °C, 1700*g* for 5 min, then stored at −80 °C. Protein mining was performed by grounding the frozen cell samples into powders in a mortar with liquid nitrogen and homogenized in extraction SDT buffer (4% sodium dodecyl sulphate (SDS), 1 mM dithiothreitol (DTT), 150 mM Tris−HCl, pH 8.0), vortexed briefly, and heated in a boiling water bath for 5 min. After ultrasonicating 10 times (80 w, 10 s ultrasonication with 15 s pause per time), the sample was heated 5 min in boiling water bath again, and then cleared by centrifuging at 14,000*g* for 45 min at 25 °C. The supernatant were extracted and stored at −80 °C for use. The final protein concentrations were measured by the bicinchoninic acid (BCA) method.

2.3. Quantitative proteomic analysis

Protein digestion was performed by Filter aided sample preparation (FASP) method (Wisniewski et al., 2009) and the resulting peptide mixtures were labeled with iTRAQ isobaric tagging reagents according to the manufacturer's instructions (Applied Biosystems, CA, USA). Briefly, mixture of extracted proteins of the eight samples (4 treatments × 2 biological replicate samples, 50 μg for each) was used as reference (REF). DTT (100 mM) was added into 400 μg of REF and each protein group, respectively. Then the treatments were heated in a boiling water bath for 5 min. After cooling, 200 μl UA buffer (8 M urea, 150 mM Tris−HCl, pH 8.0) was mixed in and centrifuged at 14,000*g* for 30 min to remove the DTT and other low-molecular-weight components. Then 100 μl IAA (50 mM iodoacetamide in UA buffer) was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μl UA buffer three times, and then washed with 100 μl DS buffer (50 mM triethylammoniumbicarbonate, pH 8.5) twice. Finally, the protein suspensions were digested with 3 μg trypsin (Promega, Madison, Wis. USA) in 40 μl DS buffer for 18 h at 37 °C, resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm.[25] iTRAQ reagent labels were dissolved in a final concentration of 70% v/v ethanol and added to the respective peptide mixture. The samples were labeled using 8-plex iTRAQ kit (Supporting File 1: Table S1). After labeling and being multiplexed, samples were dried in a vacuum centrifuge and then stored at −20 °C.

Experiments were carried out with an Easy nLC (Thermo Fisher Scientific, Waltham, MA, USA) that was coupled to Q Exactive mass spectrometer (MS) (Thermo Finnigan, San Jose, CA, USA). 5 μg of each peptide mixture samples was loaded onto a Thermo scientific EASY column (2 cm × 100 μm, 5 μm-C18) packed in-house with Thermo scientific EASY column (75 μm × 100 mm, 3 μm-C18). Samples injection was followed by an initial wash step with 0.1% Formic acid (buffer A) and eluted with a linear gradient of 84% acetonitrile and 0.1% Formic acid (buffer B) at a flow rate of 250 nL/min controlled by IntelliFlow technology over 120 min. The column eluate was directed into the Q Exactive mass spectrometer, and the instrument was run with peptide recognition mode. MS data was acquired using a data-dependent top10 method, a TOF MS survey was acquired (300−1800 m/z, 0.5 s) for High-energy collisional dissociation (HCD) fragmentation. Determination of the target value is based on predictive Automatic Gain Control (pAGC). Dynamic exclusion set to 40 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%, which specifies the minimum percentage of the target value likely to be reached at maximum fill time.

2.4. iTRAQ data analysis

The Q Exactive mass spectrometer output RAW files were analyzed by MASCOT engine (Matrix Science, London, UK; version 2.2) for protein identification, and Proteome Discoverer (Thermo, version 1.3) was employed for quantitation. Data were searched against a nonredundant International Protein Index *O. sativa* sequence database (144,512 sequences, February 2013) from uniprotOryzasativa.fasta. For protein identification, the following search parameters were selected, Enzyme: Trypsin, Mass Values: Monoisotopic, Max Missed cleavage: 2, Fixed modification: Carbamidomethyl (C), iTRAQ 8-plex (N-term), iTRAQ 8plex (K), Variable modification: Oxidation (M), iTRAQ 8-plex (Y), Peptide mass tolerance: 20 ppm, Fragment Mass Tolerance: 0.1 Da, Decoy database pattern: Reverse.

The MASCOT search results were further processed using the programs BuildSummary, Isobaric Labeling Multiple File Distiller and Identified Protein iTRAQ Statistic Builder within the ProteomicsTools (version 3.05, Information can be accessed from Research Center for Proteome Analysis http://www.proteomics.ac.cn/). BuildSummary program was used for assembling protein identifications based on a target-decoy search in shotgun proteomics. All reported data were at the 99% confidence level for protein identification as determined by false discovery rate (FDR) of 1% (Sandberg et al., 2012).

The program Isobaric Labeling Multiple File Distiller and Identified Protein iTRAQ Statistic Builder were used to calculate the