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Toxicological responses in halophyte *Suaeda salsa* to mercury under environmentally relevant salinity

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ABSTRACT

Mercury is a highly risky heavy metal contaminant in intertidal zones in the Yellow River Delta (YRD). *Suaeda salsa* is a native halophyte in the YRD. In this work, we investigated the toxicological effects of mercury (20 μg L^{-1}) in *S. salsa* under environmentally relevant salinity (500 mM). The metabolic responses included the increased amino acids and decreased succinate, fructose, glucose, fumarate and ferulate in above-ground part of *S. salsa* exposed to mercury. The expression levels of INPS, CMO, BADH, CAT and GPx were elevated in above-ground part of *S. salsa* after combined Hg and salinity exposure. Increased activities of antioxidant enzymes including SOD, POD and CAT were uniquely observed in salinity-treated samples. Our results indicated potential oxidative stresses and disturbances in protein bio-degradation and energy metabolism induced by mercury in *S. salsa*. Additionally, both synergistic and antagonistic effects were observed in *S. salsa* exposed to combined mercury and salinity.

1. Introduction

Due to the anthropogenic activities in recent decades, heavy metal pollution has become a serious threat to water and soil ecosystems because of the persistent nature, long distance transport, and adverse effects of heavy metals to organisms (Carrasco et al., 2008; Ikingura and Akagi, 1996; Rainbow and Furness, 1990; Wang and Song, 2009). Among the heavy metals, mercury is non-essential to organisms and has posed great risk on both marine and coastal ecosystems due to its high toxicity to the organisms (Zhang, 2001). It has been reported that the mercury concentration has been up to 66 mg kg^{-1} in the intertidal sediment from some extremely heavily polluted sites along the Bohai Sea (Zhang, 2001). In soils and waters, the predominant form is Hg (II) that is highly water-soluble and can be readily accumulated in high plants (Clarkson, 1997). In addition, mercury is strongly phytotoxic and can induce severe injuries and physiological disorders due to its reactive properties (Chen et al., 2009; Elbaz et al., 2010). Mercury is often found in cells and tissues bound with thiol-containing proteins and small-molecular weight thiols such as cysteine and glutathione (GSH). In addition, mercury can induce oxidative stresses and hence lipid, protein, and DNA damages by generating production of reactive oxygen species (ROS) and lipid peroxides in organisms (Clarkson, 1997; Lund et al., 1993).

The Chenopodiaceae C3 halophyte *Suaeda salsa* is a native plant in the intertidal zones in the Yellow River Delta (YRD) and widely consumed as a favorable vegetable due to its sufficient nutrient components including vitamins, essential amino acids and trace elements and other antioxidant ingredients (Wang et al., 2007; Zhang et al., 2008; Zhao, 1998; Zhao et al., 2010). As a matter of fact, *S. salsa* is the pioneer plant in the intertidal zones of the YRD (Zhao, 1998). Due to the high tolerance to salinity and immobility, *S. salsa* has exhibited its potential as a bioindicator for the environmental monitoring of intertidal zones and saline soil compared with nonhalophytes and animals (Liu et al., 2011a). Therefore it has been of virtues in environmental sciences and applied for the monitoring of environmental stressors in the intertidal zones and phyto-remediation of degraded wetland with pollutions (heavy metals and oil) or increasing salinity (Li et al., 2007; Xu et al., 2007; Zhu et al., 2005). The toxicological effects of environmentally relevant cadmium in the above-ground part of *S. salsa* have been reported previously using NMR-based metabolomics (Liu et al., 2011a). However, salinity is a common environmental stressor to plants in the YRD. Therefore, it is necessary and meaningful to investigate the toxicological effects of heavy metal contaminants under salinity stress. To date, no application of combined metabolic profiling and gene

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expression quantification on *S. salsa* has been performed on molecular (metabolites and genes) responses induced by cooperative environmental stressors such as heavy metal contaminants and salinity.

In this study, metabolic differences between the control, mercury-, salinity- and mixed mercury and salinity-exposed *S. salsa* were determined by NMR-based metabolomics that has been widely applied in plant science (Bhalla et al., 2005). The genes expression levels of key enzymes involved in osmotic regulation, antioxidation and photosynthesis were measured using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique. Among the selected enzymes, *Myo*-inositol-1-phosphate synthase (INPS, BE644574) is the key enzyme of *Myo*-inositol (Ins) biosynthesis. It has been confirmed that INPS regulates the pathway of Ins biosynthesis (Nelson et al., 1998), which is a specific pathway in salt tolerance in the common ice plant (Vernon and Bohnert, 1992), and its expression can be feedback inhibited by *myo*-inositol. In higher plants, betaine is synthesized by oxidation of choline: choline→betaine aldehyde→betaine. The first step is catalyzed by choline monooxygenase (CMO) and the second step by betaine aldehyde dehydrogenase (BADH). Catalase (CAT) and glutathione peroxidase (GPx) are common antioxidant enzymes which protect cells from oxidative stresses. Although $NADP^+$-malate dehydrogenase (MDH) is one of the key enzymes in photosynthesis in C4 plants, it was found in *Suaeda salsa* as well (Zhang et al., 2001).

We have characterized the molecular responses induced by environmentally relevant mercury in halophyte *S. salsa* with or without environmental salinity at molecular levels on the basis of metabolic profiles, gene expressions and antioxidant enzymes. We have tried to answer two questions in our study. Firstly, what effects can be induced by mercury exposure in *S. salsa* on the basis of metabolic profiles, gene expressions and antioxidant enzyme activities? Secondly, what synergistic or/and antagonistic effects can be produced in *S. salsa* exposed to environmentally relevant mercury and salinity?

2. Materials and methods

2.1. Chemicals

Sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and mercury chloride ($HgCl_2$) were all in analytical grade and purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Deuterium oxide (D_2O, 99.9% in D) and sodium 3-trimethlysilyl [2,2,3,3-D4] propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, FL). The antioxidant enzyme kits were purchased from Nanjing Jiancheng Bioengneering Institute (Nanjing, China).

2.2. Cultivation of S. salsa under mercury, salinity and combined mercury and salinity exposures

The seeds of *S. salsa* were collected from the YRD in November, 2009 and stored in a refrigerator at 4 °C for 7 months. After surface sterilization in 0.5% $HgCl_2$, forty seeds were washed in sterilized double distilled water for three times and sown in the sterilized sands in 4 replicate plastic jugs with a diameter of 20 cm ($n=10$, one control and three chemical-exposed groups). The sown *S. salsa* seeds were irrigated with Hoagland's nutrient solution containing 0.1% NaCl. After sown in the plastic jugs for 4 weeks, all the seedlings of exposed groups were irrigated with the Hoagland's nutrient solution containing 20 $\mu g\,L^{-1}$ mercury, 500 mM NaCl and mixed 20 $\mu g\,L^{-1}$ mercury and 500 mM NaCl. The actual concentrations of mercury in Hoagland's solutions were determined using an atomic fluorescence spectrometer (SK-2002 A, Jinsuokun Ltd., Beijing, China). The concentration range of total mercury in Hoagland solutions was between 18.8 and 20.4 $\mu g\,L^{-1}$ (data not shown). The nominal concentration of mercury and salinity in the Hoagland's nutrient solution were environmentally relevant to the real situation along the heavily polluted intertidal zones in the YRD (Li et al., 2001). The culture condition was 28 ± 4 °C, photoperiod 12 h light/12 h darkness, relative humidity 70% and photo-synthetically active radiation 600 $\mu mol\,m^{-2}\,s^{-1}$. After exposure for 15 days, the seedlings ($n=6$) of *S. salsa* from both control and exposed groups were randomly harvested. After quick measure of the total length and fresh weight of the above-ground part of seedlings, all the plant samples including the tissues of roots and above-ground part of seedlings were flash-frozen in liquid nitrogen and stored at −80 °C prior to metabolite extraction, mRNA extraction and enzymatic assay.

2.3. Metabolite extraction

Polar metabolites were extracted from the plant tissues (root and above-ground part of seedling) using the solvent system of methanol/water (1/1) as described previously (Huie, 2002; Kim and Verpoorte, 2010; Liu et al., 2011b). Briefly, the tissue sample was ground in a liquid N_2-cooled mortar and pestle. The tissue powder was then transferred to a tube containing ~50 ceramic beads with 1 mm diameter, and then thoroughly homogenized in 3.33 ml g^{-1} methanol/water (1/1) using a high throughput homogenizer, Precellys 24 (Bertin, France). After homogenization, the sample was transferred to an Eppendorf tube and vortexed for 15 s three times. Following centrifugation (3000g, 10 min, 4 °C), the supernatant was removed and then lyophilized. It was subsequently re-dissolved in 600 μL phosphate buffer (0.1 M Na_2HPO_4 and NaH_2PO_4, including 0.5 mM TSP, pH 7.0) in D_2O. The mixture was vortexed and then centrifuged at 3000g for 5 min at 4 °C. The supernatant substance (550 μL) was pipetted into a 5 mm NMR tube before NMR analysis.

2.4. NMR spectroscopy

Extracts of *S. salsa* tissue were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K) (Liu et al., 2011b). One dimensional (1D) ^1H NMR spectra were obtained using a 11.9 μs pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, with 128 transients collected into 16,384 data points. Datasets were zero-filled to 32,768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All ^1H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were identified following tabulated chemical shifts (Fan, 1996) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada).

2.5. NMR spectral pre-processing and multivariate data analysis

One dimensional proton NMR spectra were converted to a format for multivariate analysis using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA). Each spectrum was segmented into 0.01 ppm bins between 0.2 and 10.0 ppm with bins from 4.70 to 5.20 ppm (water) excluded from all the NMR spectra. Bins between 8.32 and 8.35 ppm, between 8.26 and 8.28 ppm, between 8.24 and 8.26 ppm, between 8.18 and 8.20 ppm, between 7.97 and 8.01 ppm, between 7.70 and 7.85 ppm, between 7.52 and 7.56 ppm, and between 6.53 and 6.58 ppm containing pH-sensitive NMR peaks were compressed into single bins. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. The NMR spectra from the above-ground part of seedlings and root tissues were generalized log transformed (glog) with transformation parameters $\lambda = 1.0 \times 10^{-8}$ and 1.0×10^{-7}, respectively. The λ values were optimized using ProMetab software to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks (Parsons et al., 2007; Purohit et al., 2004).

PCA is an unsupervised pattern recognition method that was used to reduce the dimensionality of data and summarize the similarities and differences between multiple NMR spectra (Xu, 2004). The principal component score plots were used to visualize general clusters between various groups of samples. One way analysis of variance (ANOVA) was conducted on PC scores from each group to test statistical significance ($p < 0.05$) of separations. Chenomx software (Evaluation version, Chenomx Inc., Canada) was then applied to identify and quantify potentially significant metabolites between various groups (Hines et al., 2007). Metabolite concentrations were normalized to the mass of *S. salsa* tissue by calculating the concentration of metabolites in each NMR tube.

2.6. Total RNA extraction and gene quantification

Total RNA from the above-ground part of seedlings of *S. salsa* was isolated using the TRIzol reagent (Invitrogen). Gene-specific primers for *myo*-inositol 1-phosphate synthase (INPS, BE644574), choline monooxygenase (CMO, AW991015), betaine aldehyde dehydrogenase (BADH, DQ641924), catalase (CAT, AW990998), glutathione peroxidase (GPx, AW991114), malate dehydrogenase precursor (MDH, AW991100) and the internal control actin (BE231408) were used to amplify amplicons specific for *S. salsa*. The sequences of primers and the length of amplicons were shown in Table 1. The fluorescent real-time quantitative PCR amplifications were carried out in triplicate in a total volume of 20 μl containing 10 μl of 2 × SYBR Premix Ex Taq™ (TaKaRa), 0.4 μl of 50 × ROX Reference DYE II,