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Metabolic profiling of cadmium-induced effects in one pioneer intertidal halophyte *Suaeda salsa* by NMR-based metabolomics

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Abstract Cadmium is a non-essential element to living organisms and has become the severe contaminant in both seawater and sediment in the intertidal zones of the Bohai Sea. The halophyte, *Suaeda salsa* is the pioneer plant in the intertidal zones of Bohai Sea and has been widely applied in environmental sciences. In this study, the dose- and time-dependent effects induced by environmentally relevant concentrations (2, 10 and 50 μg l^{-1}) of cadmium were characterized in *S. salsa* using NMR-based metabolomics. The levels of amino acids (valine, leucine, glutamate, tyrosine, etc.), carbohydrates (glucose, sucrose and fructose), intermediates of tricarboxylic acid cycle (succinate, citrate, etc.) and osmolyte (betaine) were altered in the *S. salsa* samples after cadmium exposures. These metabolic biomarkers indicated the elevated protein degradation and disturbances in the osmotic regulation and energy metabolism caused by cadmium in *S. salsa*. Overall, our results demonstrated the applicability of NMR-based metabolomics for the detection of metabolic biomarkers that could be used for the interpretation of toxicological effects induced by contaminants in the pioneer plant *S. salsa* in the intertidal zones. In addition, the metabolic biomarkers could be potentially useful for the bio-monitoring of contaminants in the intertidal zones.

Keywords Cadmium · *Suaeda salsa* · NMR · Metabolomics

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Introduction

Due to the accelerating industrial developments in recent decades, heavy metal pollution has become a serious threat to the marine and coast environments of the Bohai Sea because of the persistent nature, long distance transport, and adverse effects of heavy metals to marine and coastal organisms (Zhang 2001). Cadmium, a non-essential metal element for organisms but important industry material, naturally found as an impurity with some minerals such as Zn, which has been one of most serious heavy metal contaminants in the marine water body and coastal intertidal sediment along the Bohai Sea and has posed threat to the human health and coastal ecosystem (Qin et al. 2006). The Cd concentration has been up to 50 μg l^{-1} in the sea water and 273 mg Kg^{-1} in the intertidal sediment in the heavily polluted estuaries and coastal sediments along the Bohai Sea (Zhang 2001). Chronic oral Cd ingestion can cause the itai-itai disease that is the most severe form of chronic Cd poisoning, and Cd has been classified as a carcinogen by the International Agency for Research on Cancer (IARC) (Takaki et al. 2004; Bertin and Averbeck 2006). Cd can also induce oxidative stress and leads to cell death depending on the exposure time due to the increase of reactive oxygen species in cells (Souguir et al. 2011;

Benavides et al. 2005; Company et al. 2004). Therefore, it is necessary to assess the toxicological effects in the intertidal organisms induced by Cd using the biomarkers.

The traditional toxicological approaches are usually based on the measures the biochemical or physiological responses in organisms exposed to toxicants, such as the levels of enzymatic activities to test for the oxidative stresses involved in the biological oxidation (Dazy et al. 2009; Matozzo et al. 2005; Elbaz et al. 2010). Recent developments in biology have greatly expanded the single biomarker approaches to the global analysis of molecules in a biological system, which led to the occurrence of -omics techniques including genomics, proteomics, transcriptomics and metabolomics (Saavedra and Bachere 2006; Dondero et al. 2006a, b). Metabolomics, a system biology approach, has been defined as the global profiling of metabolites, especially the low molecular weight (<1,000 Da) organic metabolites contained in cells, tissues, biofluids and even whole organism (Lindon et al. 1999; Davis 2005). The components (metabolites) of the metabolome, as the end products of metabolisms represent the functional responses of a cell. The quantitative measurements of large numbers of endogenous metabolites thus provide a broad view of the biochemical status of an organism that can be used to monitor and assess the metabolic responses induced by exogenous factors, such as environmental stressors (Fiehn 2002; Pedras and Zheng 2010; Sun et al. 2010; Bailey et al. 2003; Park et al. 2009; Manetti et al. 2004). This technique has been widely applied in environmental sciences with the term environmental metabolomics (Bundy et al. 2004, 2009). Environmental metabolomics on the plant usually performs studies on the plant-environment interactions and for assessing plant function and health at the molecular level, and focuses on the metabolic responses to the environmental changes (drought, salinity, etc.), abiotic stresses (contaminates), and transgenic events (Aliferis et al. 2009; Bundy et al. 2009; Eisenreich and Bacher 2007; Sun et al. 2010; Bailey et al. 2003; Manetti et al. 2004; Park et al. 2009).

The Chenopodiaceae C3 halophyte, *Suaeda salsa* is a dominant native halophyte in saline soil and can grow in the intertidal zones of Yellow River Delta, where soil salt content is often higher than 3% (Wang et al. 2007). As a matter of fact, *S. salsa* is the pioneer plant in the intertidal zones of Yellow River Delta (Wang et al. 2007). Due to the high tolerance to salinity and immobility, *S. salsa* has exhibited the potential as a bioindicator for the environmental monitoring of intertidal zones and saline soil compared with nonhalophytes and animals. Therefore it has been of great virtues in environmental sciences and applied for the monitoring environmental stressors in the intertidal zones and phyto-remediation of degraded wetland with pollutions (heavy metals and oil) or increasing salinity (Zhu et al. 2005; Xu et al. 2007; Li et al. 2007). For example, Zhu has reported the distribution and bioavailability of heavy metals (Cu, Zn, Pb and Cd) in *S. salsa* and suggested that *S. salsa* was applicable for the phyto-remediation of heavy metal polluted soil (Zhu et al. 2005). As a pioneer halophyte in the intertidal zones of Bohai Sea, the physiological and molecular responses of *S. salsa* to salinity have been extensively studied and hence been suggested as a bioindicator of saline soils (Zhang et al. 2001; Li et al. 2002, 2004; Wang et al. 2001, 2004; Han et al. 2005). To our knowledge, no application of metabolomics on *S. salsa* has been carried out, which limits the understanding of the metabolic responses induced by environmental stressors including increased salinity, drought and contaminants.

In the present study, ^{1}H NMR-based metabolomics was applied to the halophyte *S. salsa* exposed to environmentally relevant concentrations of cadmium (2, 10 and 50 μg l^{-1}) to detect the metabolic responses (metabolite biomarkers), and then to examine the subsequent toxicological effects induced by cadmium. Also, the antioxidant status and bioaccumulation of cadmium in *S. salsa* were characterized using enzymatic activities and cadmium concentrations in plant tissues. Based on these combined biochemical parameters including the metabolic biomarkers, antioxidant status and bioaccumulation, it was expected to elucidate the effects of cadmium in the pioneer halophyte *S. salsa* in the intertidal zones of Bohai Sea.

Materials and methods

Plant cultivation and treatments

The seeds of *S. salsa* were collected from Yellow River Delta in November, 2009 and stored in a refrigerator at 4°C for 7 months. The seeds were surface sterilized using 0.5% $HgCl_2$ for 10 min, and then washed in sterilized double distilled water for three times. Eighty seeds with similar size were sown in the sterilized sands in four replicate plastic jugs with a diameter of 20 cm ($n = 20$, one control and three Cd-exposed groups). The sown *S. salsa* seeds were irrigated with Hoagland's nutrient solution containing extra 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 extra 0.1% NaCl and gradient concentrations of Cd. The dissolved Cd concentrations (2, 10 and 50 μg l^{-1}, prepared from $CdCl_2$) in the Hoagland's nutrient solution were environmentally relevant to the real situation of Cd pollution in the seawater around intertidal zone of Yellow River Delta (Zhang 2001). The culture condition was 28 ± 4°C, photoperiod 12 h light/12 h darkness, relative

humidity 70% and photo-synthetically active radiation 600 μmol m^{-2} s^{-1}. After exposure for 1, 2 and 3 weeks, the seedlings ($n = 5$) of *S. salsa* from both control and exposed groups were randomly harvested. After quick measure of the total length and weight of above-ground part of seedlings, all the plant samples were flash-frozen in liquid nitrogen and stored at −80°C prior to metabolite extraction, enzymatic assay and determination of Cd concentrations.

Metabolite extraction

Polar metabolites were extracted from the whole leaves and stems using the solvent system of methanol/water (1/1) (Kim and Verpoorte 2010; Huie 2002). Briefly, the tissue sample was ground in a liquid N_2-cooled mortar and pestle. The tissue powder (weighing from 250 to 300 mg per sample) 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 (3,000×*g*, 10 min, 4°C), the supernatant was removed and then lyophilized prior to NMR analysis. It was subsequently resuspended in 600 μl of 100 mM of phosphate buffer (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 3,000×*g* for 5 min at 4°C. The supernatant substance (550 μl) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

NMR analysis

Extracts of *S. salsa* tissue were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K). One dimensional (1D) ^{1}H NMR spectra were obtained using a 11.9 μs pulse, 6009.6 Hz spectral width, mixing time 0.1, 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 ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were assigned following tabulated chemical shifts (Fan 1996) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada). Some of the metabolites were confirmed by the 2D NMR method, J-resolved NMR spectroscopy that was acquired using eight scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5,000 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). The relaxation delay of 1.5 s was used. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to Fourier transformation, and tilted by 45°, symmetrized about F1, and then calibrated, using TopSpin (version 2.1, Bruker).

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 0.005 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. All the NMR spectra were generalized log transformed (glog) with a transformation parameter $\lambda = 1.0 \times 10^{-9}$ (Purohit et al. 2004; Parsons et al. 2007) to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks.

Principal components analysis (PCA) was used in this work for the separation of control with various concentrations of Cd-exposed groups. PCA is an exploratory unsupervised pattern recognition method since it calculates inherent variation within the data sets without use of the class membership. The algorithm of PCA calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of variance. For each model built, the loading vectors for the PCs can be used for the identification of the contributive metabolites (metabolic biomarkers) for the clusters (Xu 2004).

Measurement of antioxidant enzymatic activities

Catalase (CAT, EC 1.11.1.6) activity was measured in terms of the decomposition of hydrogen peroxide, which was monitored directly by the decrease in absorbance at 240 nm (Beers and Sizer 1952). The activity of Glutathione peroxidase activity (GPx, EC 1.11.1.9) was measured according to Drotar et al. (1985) using glutathione as substrate. The activity of glutathione *S*-transferases (GST, E.C. 2.5.1.18) was determined according to the method of Habig et al. (1974) by evaluating the conjugation of GSH with the standard model substrate 1-chloro-2,4-dinitrobenzene. Protein concentration was determined by the Coomassic brilliant blue G-250 dye-binding method, using

bovine serum albumin as standard (Bradford 1976). All the enzyme activities were expressed as U mg^{-1} protein.

Cadmium concentrations in plant tissues

The homogeneously ground samples ($n = 5$) of above-ground part of *S. salsa* from 1, 2 and 3 weeks of exposure were dried at 80°C to the constant weights. The dried tissue was digested in concentrated nitric acid (70%, Fisher Scientific) using a microwave digestion system (CEM, MAR5). The samples were heated in the microwave oven (program: heating at 15 min to 200°C and holding at 200°C for 15 min). All completely digested samples were diluted appropriately with ultra pure water for the quantification of Cd using ICP-MS (Agilent 7500i, Agilent Technologies Co. Ltd, USA).

Results

Effects of Cd exposure on the growth of *S. salsa*

Table 1 illustrates the weight of above-ground part and total length of *S. salsa* exposed to various concentrations (2, 10 and 50 μg l^{-1}) of Cd for 1, 2 and 3 weeks. No significant differences were observed from either the weight of above-ground part or total length of *S. salsa* between the control and exposed groups after Cd exposure for either 1 or 3 weeks. However, the total length of *S. salsa* seedling from each Cd-exposed group after 2 weeks of exposure was shorter than that of control group. Especially, the average total length of 10 μg l^{-1} Cd-treated group was significantly ($p < 0.05$) decreased compared with that of control group.

Responses of antioxidant enzymes to Cd exposures

After exposure for 1 week, the significant elevation ($p < 0.05$) of CAT activities was found in both 10 and 50 μg l^{-1} Cd-treated groups (Table 2). However, there were no significant differences in both GST and GPx activities between the control and Cd-exposed groups. For either 2 or 3 weeks of Cd exposures, no significant responses in both CAT and GPx activities were observed from all the Cd-exposed groups compared with that of control group. The GST activity was significantly decreased ($p < 0.05$) in the *S. salsa* samples from 10 μg l^{-1} Cd-treated group for 2 weeks of exposure and 50 μg l^{-1} Cd-treated group for 3 weeks of exposure.

Cadmium concentrations in above-ground part of tissue of *S. salsa*

Table 3 shows the accumulation of Cd in the homogenous above-ground part of *S. salsa* tissues after 1, 2 and 3 weeks of exposure. The Cd concentrations are presented as

Table 1 The growth (weight of above-ground part and length of whole seedling) of *S. salsa* after exposure to Cd for 1, 2 and 3 weeks

Treatment group	1 week		2 weeks		3 weeks	
	Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)
Control	2.1 ± 0.6	21.3 ± 2.8	5.5 ± 2.0	30.1 ± 2.4	6.4 ± 1.4	34.5 ± 3.2
2 μg/l Cd	2.3 ± 0.6	23.7 ± 2.8	4.9 ± 3.1	29.8 ± 3.4	7.8 ± 2.8	36.7 ± 3.2
10 μg/l Cd	3.2 ± 0.9	21.5 ± 1.6	4.4 ± 1.5	25.8 ± 1.3*	6.3 ± 3.1	31.6 ± 5.1
50 μg/l Cd	2.2 ± 0.5	23.3 ± 2.4	5.2 ± 1.8	28.2 ± 2.5	6.3 ± 1.3	34.8 ± 3.6

Values are presented as the mean ± standard deviation

Significant difference among groups was tested by one-way analysis of variance ($p < 0.05$) and indicated by * ($p < 0.05$)

Table 2 The activities (U mg^{-1} protein) of CAT, GPx and GST in the above-ground part of seedlings from both control and Cd-exposed *S. salsa* samples

Treatment group	1 week			2 weeks			3 weeks		
	CAT	GPx	GST	CAT	GPx	GST	CAT	GPx	GST
Control	0.74 ± 0.72	1.14 ± 0.60	0.75 ± 0.44	2.43 ± 1.10	0.61 ± 0.73	0.52 ± 0.08	2.65 ± 2.40	0.44 ± 0.19	0.27 ± 0.08
2 μg/l Cd	1.07 ± 1.59	1.05 ± 0.80	0.92 ± 0.22	3.44 ± 1.78	0.93 ± 1.02	0.50 ± 0.20	1.85 ± 2.11	0.50 ± 0.49	0.19 ± 0.02
10 μg/l Cd	3.31 ± 1.71*	1.15 ± 1.24	0.79 ± 0.06	2.43 ± 0.73	0.66 ± 0.60	0.36 ± 0.06**	1.13 ± 0.94	0.53 ± 0.21	0.22 ± 0.05
50 μg/l Cd	1.95 ± 0.80*	1.63 ± 1.05	0.85 ± 0.05	2.84 ± 1.54	0.72 ± 0.73	0.42 ± 0.13	1.66 ± 1.15	0.47 ± 0.27	0.17 ± 0.05*

Values are presented as the mean ± standard deviation

Significant difference among groups was tested by one-way analysis of variance ($p < 0.05$) and indicated by * ($p < 0.05$) and ** ($p < 0.01$)

Table 3 The accumulated concentrations (μg g^{-1}) of cadmium in the above-ground part of seedlings of *S. salsa* from both control and Cd-exposed groups

Treatment group	1 week	2 weeks	3 weeks
Control	0.040 ± 0.012	0.048 ± 0.014	0.080 ± 0.042
2 μg/l Cd	0.174 ± 0.022***	0.214 ± 0.070***	0.200 ± 0.044***
10 μg/l Cd	0.814 ± 0.209***	0.852 ± 0.310***	0.735 ± 0.201***
50 μg/l Cd	3.041 ± 0.354***	2.691 ± 0.505***	2.722 ± 0.481***

Values are presented as the mean ± standard deviation

Significant difference among groups was tested by one-way analysis of variance ($p < 0.05$) and indicated by *** ($p < 0.001$)

mean ± standard deviation. The average concentrations of Cd in the three Cd-exposed groups were highly significantly elevated ($p < 0.001$) compared with that of control groups at all the three exposure times. The clear dose-dependent accumulations were also observed between Cd-exposed groups. However, it was interestingly found that the Cd concentrations in each exposed group with various exposure times were not significantly different ($p > 0.05$), which indicated that there were no time-dependent effects of Cd accumulation within 3 weeks of exposure time.

^{1}H NMR spectroscopy of above-ground part of tissue extracts from *S. salsa*

Figure 1 presents a representative ^{1}H NMR spectrum of tissue extracts from the homogenous above-ground part of *S. salsa*. Several metabolite classes were observed, including amino acids (branched chain amino acids: valine, leucine and isoleucine, alanine, glutamate, aspartate, tyrosine, etc.), energy storage compounds (sucrose, fructose and glucose), organic osmolytes (betaine and dimethylglycine), and intermediates in the tricarboxylic acid cycle (succinate, citrate, malate, etc.). Overall, the original NMR spectrum (Fig. 1a) was dominated by one of the organic osmolytes, betaine (3.25 and 3.91 ppm) that is a secondary metabolite to maintain the osmotic balance in *S. salsa* and was *approx.* 10–100 times higher than other metabolites in the ^{1}H NMR spectral intensities.

PCA of ^{1}H NMR spectra from above-ground part of *S. salsa* tissue extracts

PCA was conducted on the ^{1}H NMR spectral data sets generated from the control and three Cd-exposed groups of *S. salsa* after exposures for 1, 2 and 3 weeks, respectively (Figs. 2a, 3a, 4a). After Cd exposure for 1 week, the control (red inverted triangles) and 10 μg l^{-1} Cd-treated (dark blue squares) group was obviously separated along PC2 axis from the PC scores plots ($p < 0.05$) (Fig. 2a). The samples

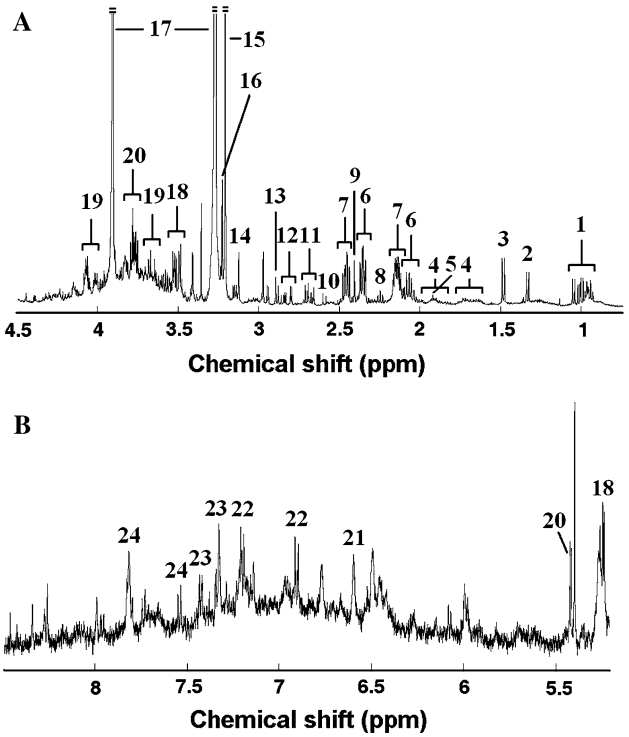

Fig. 1 A representative 1-dimensional 500 MHz ^{1}H NMR spectrum of tissue extracts from *S. salsa* using extraction solvent system of methanol/water (1/1) (**a**) and vertical expansion of the aromatic region (**b**). Metabolite assignments: *1* branched chain amino acids: leucine; isoleucine and valine, *2* lactate, *3* alanine, *4* arginine, *5* acetate, *6* glutamate, *7* glutamine, *8* γ-aminobutyric acid, *9* succinate, *10* citrate, *11* malate, *12* aspartate, *13* dimethylglycine, *14* malonate, *15* choline, *16* phosphocholine, *17* betaine, *18* glucose, *19* fructose, *20* sucrose, *21* fumarate, *22* tyrosine, *23* phenylalanine and *24* hippurate

of high concentration (50 μg l^{-1}) of Cd-treated group (blue pentagrams) were apparently classified along positive PC1 from the controls. However, no significant separation ($p > 0.05$) between control and 2 μg l^{-1} Cd-treated groups was found. From the corresponding PC1 loading plot, the metabolic profiles of plant tissue extracts showed clear increase in branched chain amino acids (valine, leucine and isoleucine), lactate, arginine, glutamate, glutamine, citrate, succinate, malate, choline, glucose, fructose, tyrosine and phenylalanine and decrease in betaine, sucrose and fumarate in 50 μg l^{-1} Cd-treated *S. salsa* tissues (Fig. 2b). The distinguishable metabolic changes caused by Cd exposure in 10 μg l^{-1} Cd-treated *S. salsa* tissues included the increased branched chain amino acids, succinate, citrate, glucose, fructose, fumarate and tryptophan, together with the decreased malate, betaine and sucrose (Fig. 2c).

For the samples with 2 weeks of Cd exposures, the clear separations ($p < 0.05$) between control and exposed groups were found along PC1 axis. However, the 10 and 50 μg l^{-1} Cd-treated *S. salsa* samples were clustered tightly ($p > 0.05$), which indicated no significant metabolic differences between these two Cd-treated groups. The dose-

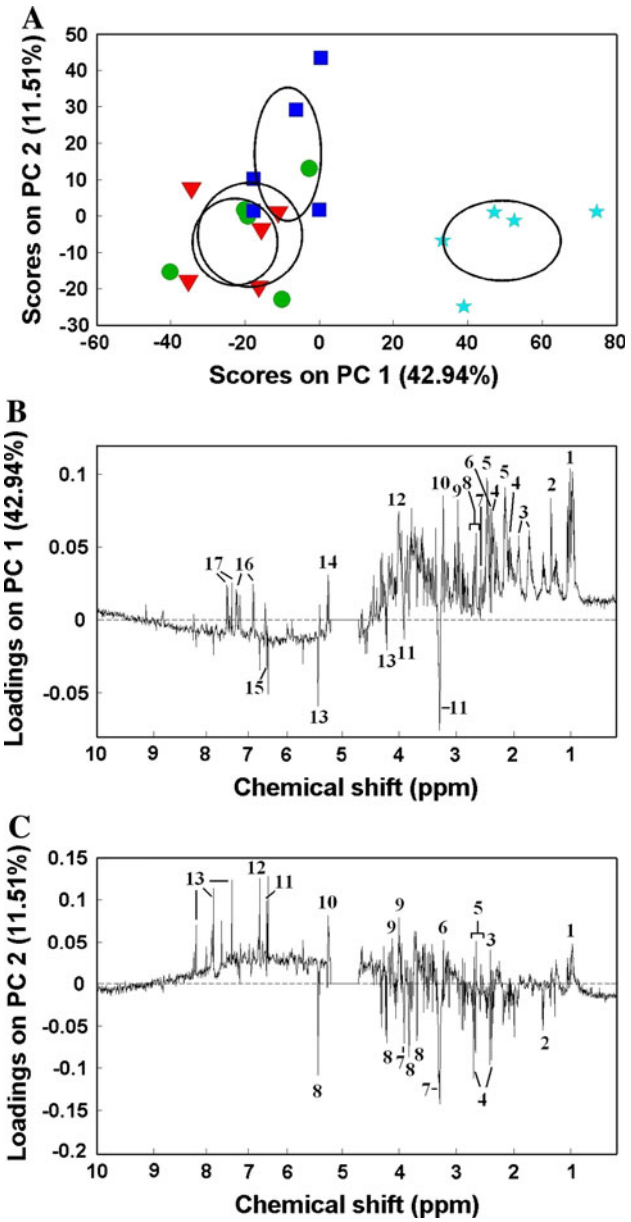

Fig. 2 PCA scores plot (**a**), PC1 loadings plot (**b**) and PC2 loadings plot (**c**) from the analysis of the 1D ^{1}H NMR spectra of the *S. salsa* from control (*red inverted triangles*), 2 μg l^{-1} Cd-treated (*green circles*), 10 μg l^{-1} Cd-treated (*dark blue squares*) and 50 μg l^{-1} Cd-treated (*blue pentagrams*) groups with 1 week of exposure. Ellipses represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Metabolite assignments: *1* branched chain amino acids: leucine; isoleucine and valine, *2* lactate, *3* arginine, *4* glutamate, *5* glutamine, *6* succinate, *7* citrate, *8* malate, *9* unknown (2.97 ppm), *10* choline, *11* betaine, *12* fructose, *13* sucrose, *14* α-glucose, *15* fumarate, *16* tyrosine and *17* phenylalanine (Color figure online)

dependent metabolic changes included the elevation in the branched chain amino acids, arginine, succinate, citrate and tryptophan as well as the reduced malate and glucose (Fig. 3b). Although the ^{1}H NMR spectra from both control and low dose (2 μg l^{-1}) of Cd-treated groups were clustered together, the *S. salsa* samples treated with higher

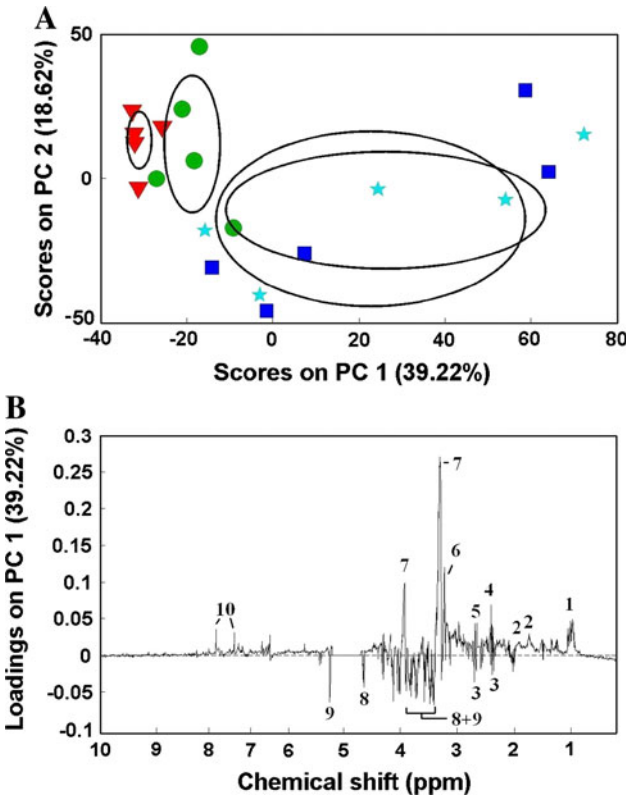

Fig. 3 PCA scores plot (**a**) and corresponding PC1 loadings plot (**b**) from the analysis of the 1D ^{1}H NMR spectra of the *S. salsa* from control (*red inverted triangles*), 2 μg l^{-1} Cd-treated (*green circles*), 10 μg l^{-1} Cd-treated (*dark blue squares*) and 50 μg l^{-1} Cd-treated (*blue pentagrams*) groups with 2 weeks of exposure. Ellipses represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Metabolite assignments: *1* branched chain amino acids: leucine; isoleucine and valine, *2* arginine, *3* malate, *4* succinate, *5* citrate, *6* choline, *7* betaine, *8* β-glucose, *9* α-glucose and *10* tryptophan (Color figure online)

doses (10 and 50 μg l^{-1}) of Cd were classified along PC1 axis with positive PC1 scores (Fig. 4a). The metabolic profiles from higher doses of Cd-exposed samples exhibited the significant increases in alanine, malate, choline, sucrose and an unknown metabolite (2.97 ppm), as well as the decreases in betaine, fructose, glucose and fumarate (Fig. 4b).

Discussion

As a pioneer halophyte in the intertidal zones of Yellow River Delta, *S. salsa* has been intriguing to researchers and applied across multiple areas (Zhang et al. 2001; Cui et al. 2008; Song et al. 2009; Wang et al. 2007). The tolerance to the salinity has been extensively studied by Zhang's and Wang's groups (Zhang et al. 2001; Cui et al. 2008; Wang et al. 2001, 2004). The expressed sequence tags from NaCl-treated *S. salsa* cDNA library have been published and

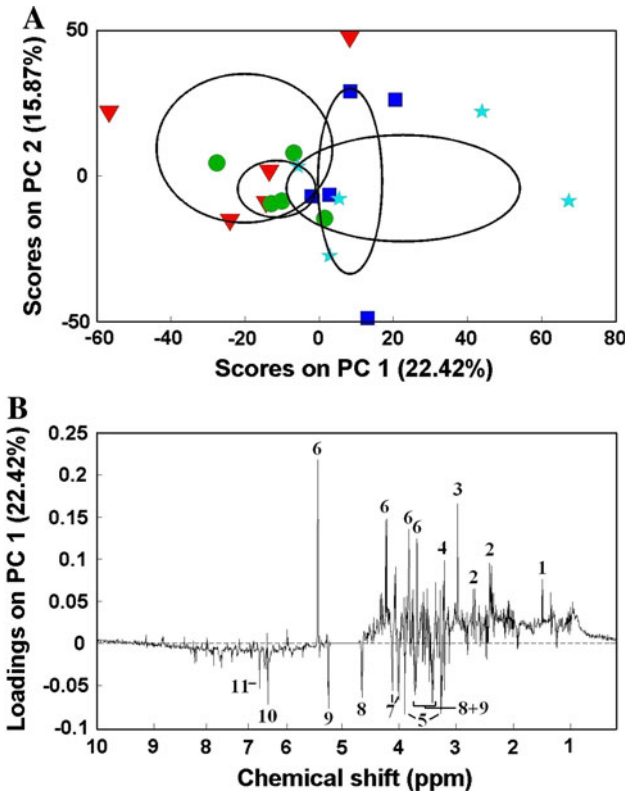

Fig. 4 PCA scores plot (**a**) and corresponding PC1 loadings plot (**b**) from the analysis of the 1D ^{1}H NMR spectra of the *S. salsa* from control (*red inverted triangles*), 2 μg l^{-1} Cd-treated (*green circles*), 10 μg l^{-1} Cd-treated (*dark blue squares*) and 50 μg l^{-1} Cd-treated (*blue pentagrams*) groups with 3 weeks of exposure. Ellipses represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Metabolite assignments: *1* alanine, *2* malate, *3* unknown 1 (2.97 ppm), *4* choline, *5* betaine, *6* sucrose, *7* fructose, *8* *β*-glucose, *9* *α*-glucose, *10* fumarate and *11* unknown 2 (6.75 ppm) (Color figure online)

uploaded to the public database of National Center for Biotechnology Information (NCBI) (Zhang et al. 2001). However, the metabolic profile of *S. salsa* is of a lack. Hence it is necessary to carry out the investigation on the metabolic profile as well as its application in the environmental sciences.

All the ^{1}H NMR spectra were dominated by betaine that is a main organic osmolyte to maintain the osmotic balance and was ~10–100 times higher than other metabolites in the NMR spectral intensities (Fig. 1a). Betaine can be synthesized or taken up from the environment by cells for the protection against osmotic stress, drought, high salinity or high temperature in biological systems including both plants and animals (especially marine invertebrates) (Moghaieb et al. 2004). The pathway of betaine synthesis is short and straightforward: choline monooxygenase converts choline (a detectable metabolite in *S. salsa*, Fig. 1) to betaine aldehyde, and betaine aldehyde dehydrogenase converts this product to betaine (Greenway and Osmond 1972; Lee et al. 2004; Peel et al. 2010). In higher plants such as *S. salsa*, betaine is an important secondary metabolite of alkaloid for the protection of osmotic stresses (Moghaieb et al. 2004; Lee et al. 2004). As a halophyte, *S. salsa* is native to the saline soil containing a high saline up to 3% salinity, therefore the organic osmolyte, betaine plays important physiological roles in osmotic regulation and hence was detected at a high level in *S. salsa* tissues.

Although there was no significant difference on the growth (Table 1) between the control and Cd-exposed seedlings of *S. salsa*, the significant increases ($p < 0.05$) of CAT activities in *S. salsa* tissues exposed to higher doses (10 and 50 μg l^{-1}) of Cd were observed together with the highly significant ($p < 0.01$) accumulation of Cd in all the three Cd-treated samples with 1 week of exposure. It implied that these treatments (2, 10 and 50 μg l^{-1}) of Cd didn't affect the normal growth of *S. salsa*, however, the higher doses (10 and 50 μg l^{-1}) of Cd induced oxidant stress in the *S. salsa* samples. In the PCA analysis, the 10 and 50 μg l^{-1} Cd-treated groups were respectively separated along positive PC2 and positive PC1 axes from both control and 2 μg l^{-1} Cd-treated groups (Fig. 2a), which indicated the metabolic responses induced by 10 and 50 μg l^{-1} Cd exposures in *S. salsa* seedlings.

After 2 weeks of exposure, the average weight of aboveground part and total length of *S. salsa* seedlings exposed to Cd were lower than that of control group, which showed the inhibition of the growth of *S. salsa* to Cd exposures. From the PCA scores plot, the clear separation between control and the low dose (2 μg l^{-1}) of Cd-exposed groups was discovered (Fig. 3a). It apparently revealed the metabolic responses induced by this dose of Cd after 2 weeks of exposure, which was different from the PCA results of the samples with 1 week of exposure. However, the samples of low dose (2 μg l^{-1}) of Cd-exposed group were clustered with the control samples after exposed to Cd for 3 weeks (Fig. 4a). It might be accounted for the recovery of metabolic responses due to the adaptation of *S. salsa* to this Cd treatment (2 μg l^{-1}). Interestingly, the Cd concentrations in each exposed group with various exposure times were not significantly different ($p > 0.05$), which indicated that there were no time-dependent effects of Cd accumulation within 3 weeks of exposure time. It seemed there were constant accumulations of Cd in *S. salsa* depending on the exposed concentrations of Cd. Ma has reported that *S. salsa* is an efficient accumulator for Cd and suggested that *S. salsa* is uniquely applicable as a plant for the phytoremediation of Cd pollution in the intertidal zones (Ma 2004). However, there is no report on the temporal accumulation of Cd in *S. salsa*. Therefore the further study is necessary to elucidate the detailed mechanisms of Cd accumulation in *S. salsa*.

Commonly, the higher doses (10 and 50 μg l^{-1}) of Cd induced the abundance of branched chain amino acids (valine, leucine and isoleucine), succinate, citrate, glucose, fructose and depletion of betaine and sucrose after 1 week of exposure. The increase in the branched chain amino acids is intriguing, which has also been observed in the *Arabidopsis thaliana* under dark condition and in the water-stressed tomato (Ishizaki et al. 2005; Semel et al. 2007). In the highest dose (50 μg l^{-1}) of Cd-exposed samples, the other amino acids including glutamate, glutamine, arginine, tyrosine and phenylalanine were elevated as well, whilst only tryptophan was increased in the 10 μg l^{-1} Cd-treated samples. The significantly increased amino acids suggested that protein degradation was elevated in *S. salsa* induced by the Cd exposures. Succinate, citrate, malate and fumarate are key intermediates in the tricarboxylic acid (TCA) cycle. Therefore the changes in these metabolites indicated the disturbances in the TCA cycle that was related to the energy metabolisms induced by Cd exposures in *S. salsa*. In plant, sucrose can be commonly converted to glucose and fructose. The elevated glucose and fructose and depleted sucrose were discovered in both 10 and 50 μg l^{-1} Cd-treated samples herein, indicating the elevation of conversion of sucrose to glucose and fructose induced by Cd exposures. As the key secondary metabolite, betaine plays an important role in the osmotic regulation. The osmolytes can be actively accumulated in high salinity environments and released when the salinity decreases. In our case, the levels of betaine were commonly reduced in the higher doses of Cd-treated samples after exposure for 1 and 3 weeks, suggesting the osmotic stress induced by Cd in *S. salsa*. In addition, the elevation of choline was detected in the highest dose (50 μg l^{-1}) of Cd-exposed groups. Since betaine is synthesized from choline, the elevated choline should be related to the depleted betaine.

After exposure for 2 weeks, the levels of citrate, glutamate, glutamine, fructose, tyrosine and phenylalanine in the 50 μg l^{-1} Cd-exposed groups recovered to the control levels. It might imply the biological acclimation of *S. salsa* to the Cd exposure. However, the increased glucose and fructose were found in the 50 μg l^{-1} Cd-exposed sample after 3 weeks of exposure as well as the decreased sucrose, which was completely different from the metabolic responses induced by this dose of Cd in the plant samples after 1 week of exposure. The mechanisms were unclear and needed further study.

Conclusions

In this study, the dose- and time-dependent metabolic responses induced by environmentally relevant concentrations (2, 10 and 50 μg l^{-1}) of cadmium were characterized in the homogenous above-ground part of halophyte *S. salsa* using NMR-based metabolomics. The significant metabolic differences included the amino acids (valine, leucine, glutamate, tyrosine, etc.), carbohydrates (glucose, sucrose and fructose), intermediates of tricarboxylic acid cycle (succinate, citrate, etc.) and osmolyte (betaine) in the *S. salsa* samples induced by cadmium. These metabolic biomarkers indicated the elevated protein degradation, disturbances in the osmotic regulation and energy metabolisms. Overall, this work demonstrated that NMR-based metabolomics was useful for the detection of metabolic biomarkers induced by contaminants in the pioneer plant *S. salsa* in intertidal zones.

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