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Root adaptations to cadmium-induced oxidative stress contribute to Cd tolerance in the hyperaccumulator *Sedum alfredii*

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Abstract

Short-term responses of *Sedum alfredii* roots to Cd exposure was compared in Cd hyperaccumulator (HE) and non-hyperaccumulating ecotype (NHE). Cadmium exposure significantly inhibited root elongation and induced loss of plasma membrane integrity and lipid peroxidation of roots tips in the NHE, whereas these effects were much less pronounced in the HE plants. A strong accumulation of reactive oxygen species with increasing Cd concentration was noted in the NHE root tips, but not in HE. After Cd exposure, a dose-dependent decrease in oxidized glutathione and marked increase in reduced glutathione and non-protein thiols were observed in root tips of HE, but were not seen in the NHE plants. These results suggest that the HE tolerates high Cd in the environment through the differential adaptations against Cd-induced oxidative stress.

Additional key words: glutathione, hydrogen peroxide, lipid peroxidation, membrane integrity, reactive oxygen species.

Introduction

Cadmium enters the environment mainly from industrial processes and phosphate fertilizers and might be toxic to plants resulting in root tip damage, reduced photosynthesis and growth inhibition (Das *et al.* 1997). Cadmium causes formation of reactive oxygen species (ROS; Rodriguez-Serrano *et al.* 2006), induces antioxidant responses in all plant organs and interferes with the redox status of cells (Sandalio *et al.* 2001, Schutzendubel *et al.* 2001, Bertin and Averbeck 2006). In most plants, visible effects of exposure to high Cd doses are growth inhibition and leaf chlorosis (Das *et al.* 1997). In some plant species including *Thlaspi caerulescens* (Boominathan and Doran 2003) and *Brassica juncea* (Pietrini *et al.* 2003), Cd can accumulate to substantial concentrations without any toxic effects. The tolerance of Cd by these species involves a complex network of homeostatic mechanisms that control the uptake, accumulation, trafficking, and detoxification of the metal (Clemens 2001, Das *et al.* 1997). Once uptake and translocation of Cd have occurred, a number of mechanisms are involved that regulate the concentrations of free Cd ions among different plant organelles and hence minimize the damage to metabolism from exposure to excess Cd (Clemens 2001, Hall 2002). Compartmentation of Cd to vacuoles and cell walls (Kramer *et al.* 1996, Bidwell *et al.* 2004), and its chelation (Kramer *et al.* 1996, Salt *et al.* 1999, Ueno *et al.* 2005) have been suggested to play a dominant role in metal detoxification by hyperaccumulators (Clemens 2001). Metal detoxification, however, is frequently incomplete and protection against Cd-induced oxidative damage is necessarily involved (Boominathan and Doran 2002, 2003, Freeman *et al.* 2004). Glutathione (GSH) has been suggested to play an important role in detoxification

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Abbreviations: CAT - catalase; DHE - dihydroethidium; DTNB - 5,5'-dithiobis-2-nitrobenzoic acid; GSH - reduced glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; NPT - non-protein thiols; ROS - reactive oxygen species; TBARS - thiobarbituric acid reactive substances; TCA - trichloroacetic acid; TMP - 2,2,6,6-tetramethyl-4-(methylsulfonyloxy)-1-piperidinooxy.
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of heavy metals in plants (Han *et al.* 2008). GSH-mediated Ni tolerance has been previously observed in *Thlaspi* hyperaccumulators (Freeman *et al.* 2004), and Cd tolerance/accumulation was reported to be associated with GSH biosynthesis in the accumulator plant *Brassica juncea* (Zhu *et al.* 1999a,b).

Sedum alfredii is a recently identified Cd hyperaccumulator (Yang *et al.* 2004) of increasing interest for its potential use in phytoextraction (Li *et al.* 2007, Tian *et al.* 2009). Plants of the hyperaccumulating ecotype (HE) of *S. alfredii* grow naturally in a Pb/Zn mine area, where Cd concentration is up to 400 mg kg^{-1}(soil). Yang *et al.* (2004) reported that this ecotype of *S. alfredii* grew hydroponically at Cd up to concentration 200 μM, whereas its nonhyperaccumulating ecotype (NHE) can not survive at 50 μM Cd (Xiong *et al.* 2004). Like many other hyperaccumulators, *S. alfredii* has an enhanced ability to translocate most of absorbed Cd from roots to shoots (Lu *et al.* 2008), to protect the roots from excess of Cd. However, roots must also possess an extraordinary ability to tolerate Cd, as the toxic metal enters the roots first. Roots of the Cd hyperaccumulator, *Thlaspi caerulescens* have been suggested to possess mechanisms for enhanced metal tolerance independent of the shoots (Nedelkoska and Doran 2000) and antioxidative metabolism might play an important role in countering the toxic effects of Cd in hairy roots of this species (Boominathan and Doran 2003). Previous studies on the hyperaccumulator *S. alfredii* indicated that there was no relationship between antioxidative defense mechanisms namely superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase activities as well as ascorbic acid contents, and Cd tolerance in *S. alfredii* (Jin *et al.* 2008b). Jin *et al.* (2008a), however, did not provide a clear explanation for the apparent ability of roots to tolerate high cellular Cd contents.

Root tips, which comprise meristematic, elongating and differentiating cells within a short distance, are a very active zone of metabolism, and extremely sensitive to biotic or abiotic stresses and thus represent an ideal tissue to examine the tolerance mechanisms and early responses to Cd. The objective of this study was to determine Cd effects on root elongation, ROS (H_2O_2 and $O_2^{\cdot-}$) formation, glutathione metabolism, lipid peroxidation and plasma membrane integrity in hyperaccumulating and non-hyperaccumulating ecotypes of *S. alfredii*.

Materials and methods

Seedlings of two contrasting ecotypes of *Sedum alfredii* Hance were cultivated hydroponically. The hyperaccumulating ecotype (HE) of *S. alfredii* was obtained from an old Pb/Zn mine area in Zhejiang Province, China, and the non-hyperaccumulating ecotype (NHE) of *S. alfredii* was obtained from a tea plantation in Hangzhou in Zhejiang Province. Plants were grown in non-contaminated soil for several generations. Uniform and healthy shoots were then selected, rooted and cultivated in the basal nutrient solution containing: 2 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.1 mM KCl, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6$ Mo_7O_{24}, 100 μM Fe-EDTA. Nutrient solution pH was adjusted daily to 5.8 with 0.1 M NaOH or 0.1 M HCl. Plants were grown in glasshouse with natural irradiance, day/night temperature of 26/20 °C and air humidity of 70/85 %. The nutrient solution was aerated continuously and renewed every three days.

After growing for 14 d, plants were treated with 0, 10, 25, 100, 400 μM Cd^{2+}, added as $CdCl_2$, in 1/4-strength basal nutrient solution. The pH for both culture and treatment solutions was adjusted to 5.8. In all following experiments, three replicate plants were used and analyzed independently.

Root length measurements were performed according to Schutzendubel *et al* .(2001). Roots were marked 5 mm behind the tips with water-resistant ink two days before Cd treatments. Root lengths were measured on 20 plants per treatment until harvest after 24 d.

At harvest, intact roots were soaked in 20 mM Na_2-EDTA for 15 min to desorb putatively adsorbed Cd^{2+} on root surfaces. After rinsing and blotting dry, root tips were cut 10 mm behind the apex, weighed, and digested with HNO_3-$HClO_4$. Concentrations of Cd in the filtrates were analysed using inductively coupled plasma mass spectroscopy (ICP-MS; *Agilent 7500a*, CA, USA).

Plasma membrane integrity was measured spectrophotometrically as Evans blue uptake (Yamamoto *et al.* 2001). After Cd treatment, thirty root tips (10 mm) were incubated in Evans blue solution [0.025 % (m/v) Evans blue in 100 μM $CaCl_2$ (pH 5.6)] for 30 min. After washing the roots for 15 min with water, the trapped Evans blue was released from the roots by homogenizing root tips in 50 % (v/v) methanol and 1 % (m/v) sodium-dodecyl suphate (SDS). The homogenate was incubated for 15 min in a water bath at 50 °C, centrifuged at 14 000 *g* for 15 min and absorbance of the supernatant was determined at 600 nm using spectrophotometer (*Lambda 35*, *Perkin Elmer*, Singapore).

The lipid peroxidation in root tips was determined in terms of malondialdehyde (MDA) content, measured according to the method of Dixit *et al*. (2001) using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

After treatments, intact roots were rinsed several times with 0.5 mM $CaCl_2$ (pH 4.5), dried with filter papers, and immediately immersed into the following specific reagents. Histochemical detection of loss of

plasma membrane integrity and lipid peroxidation in root apices was performed as described by Yamamoto *et al*. (2001). For plasma membrane integrity determination, roots were incubated in Evans blue solutions for 30 min. For lipid peroxidation analysis, roots were incubated in Schiff's reagent for 60 min, rinsed with a solution containing 0.5 % (m/v) $K_2S_2O_5$ (prepared in 0.05 M HCl) until the root color became light red. The stained roots were washed three times with sufficient volume of distilled water and were observed under a light microscope (model *SZH-ILLD, Nikon*, Tokyo, Japan). Superoxide radicals were detected by staining with 10 μM dihydroethidium (DHE) (Yamamoto *et al.* 2002), and H_2O_2 was localized using 10 μM carboxy-H_2DCFDA (*Molecular Probes*, *Eugene*) for 15 min and then rinsed (Freeman *et al.* 2004). Then, the segments were washed twice in the same buffer for 15 min each and were then embedded in 30 % polyacrylamide blocks (Rodriguez-Serrano *et al.* 2006). As negative controls, the root segments from control and Cd-treated plants of approximately 20 mm from the apex were incubated for 30 min with $O_2^{\cdot-}$ scavenger 1.0 mM 2,2,6,6-tetramethyl-4-(methylsulfonyloxy)-1-piperidinooxy (TMP) or H_2O_2 scavenger 1.0 mM ascorbate (ASC) prior to staining with the fluorescence dyes (Rodríguez-Serrano *et al*, 2006). A *Nikon Eclipse 3000* epifluorescent microscope (Melville, NY, USA) equipped with a green fluorescent protein filter (excitation 450 to 490 nm, emission 500 to 530 nm) was used for epifluorescence images. Autofluorescence was not observed in unstained controls at the exposure time used. Images were captured with a *SPOT* camera (*Nikon*).

The content of H_2O_2 was measured according to the method of Jin *et al*. (2008a) with the following modification: root tips were ground in 50 mM K-phosphate buffer (pH 7.8). To the homogenate, 5 % trichloacetic acid (TCA) was added. The mixture was centrifuged at 10 000 *g* for 10 min. The supernatant (1 cm^3) was added to 1 cm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 2 cm^3 of 1 M KI. H_2O_2 content was estimated based on the absorbance of the supernatant at 390 nm. Determination of $O_2^{\cdot-}$ production was done as described by Huang *et al*. (2008). Root tips samples were ground with ice-cold sodium phosphate buffer (pH 7.8, 50 mM) in an ice bath. The extract was centrifuged at 13 000 *g* for 20 min at 4 °C, and the supernatant (0.5 cm^3) was incubated at 25 °C for 60 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture was then incubated with 1 cm^3 of 17 mM anhydrous *p*-aminobenzene sulphonic acid and 1 cm^3 of 7 mM α-naphthylamine at 25 °C for 30 min. The absorbance was measured at 530 nm. A calibration curve was established using sodium nitrite.

Non-protein thiols (NPT) were extracted by homogenizing approximately thirty root tips in 2 cm^3 ice-cold 5 % (m/v) sulfosalicylic acid solution. After centrifugation at 10 000 *g* at 4 °C for 30 min, NPT in the supernatant was measured with Ellman's reagent (De Vos *et al.* 1992). Briefly, 0.3 cm^3 of the supernatant was added to 1.2 cm^3 0.1 M K-phosphate buffer (pH 7.6). After obtaining a stable absorbance at 412 nm, 0.025 cm^3 of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) solution was added and the increase in absorbance at 412 nm was read. GSH and GSSG were assayed by the GSSG-recycling method according to De Vos *et al.* (1992).

All data were statistically analyzed using *SPSS* (version *11.0*). Differences between treatments were determined by the least significant difference (LSD, $P \leq 0.05$) from the analysis of variance (*ANOVA*). Differences between the two ecotypes were tested by a paired *t*-test ($P \leq 0.05$ or 0.01).

Results

The content of Cd in selected 10 mm root tips increased in response to the external Cd concentration with no significant differences between the two ecotypes (Table 1). The first visual symptom of Cd phytotoxicity was the inhibition of root elongation in both ecotypes. Root elongation inhibition was observed at a Cd concentration of 10 μM and inhibition increases up to at least 100 μM in roots of NHE plants, while in case of HE, the inhibition of root elongation was much smaller, and a stimulation of root elongation was observed when HE plants were exposed to 10 μM Cd (Table 1).

Evans blue uptake in root tips of NHE, especially into surface cells, was clearly observed at all Cd treatments (10 - 100 μM), while in root tips of HE, Evans blue uptake was rarely observed (Fig. 1*A*). Histochemical analysis by staining with Schiff's reagent indicated no significant lipid peroxidation in roots of HE plants under Cd stress, while significant peroxidation was observed in roots of NHE at all concentrations of Cd (Fig. 1*B*). Severe distortion of root tips was observed in roots of NHE growing in 100 μM Cd (Fig. 1*B*). Accumulation of Evans blue and MDA indicated the loss of plasma membrane integrity and lipid peroxidation in root tips of NHE, whereas these effects were much less pronounced in root tips of HE when the plants were exposed to 0 - 100 μM Cd (Table 1).

Contents of both H_2O_2 and $O_2^{\cdot-}$ in root tips of NHE plants increased linearly with the external Cd supply (Table 1). In HE plants, H_2O_2, was not significantly increased under 100 μM Cd exposure, while $O_2^{\cdot-}$ content increased once Cd concentrations exceeded 100 μM (Table 1). The detection of ROS in roots *in vivo* was carried out using fluorescence probes, carboxy-H_2DCFDA and dihydro-ethidium (DHE), respectively.

The specificity of the two probes was checked by using specific ROS scavengers, ASC for H_2O_2 (Fig. 1C), and TMP for $O_2^{\cdot-}$ (Fig. 1*D*). Roots of HE did not vary in H_2O_2 accumulation until plants were exposed to 100 μM Cd. By contrast, 10 μM Cd exposure resulted in an accumulation of H_2O_2 in roots of NHE, that increased with increasing Cd concentrations (Fig. 1*C*). A strong dose dependent accumulation of $O_2^{\cdot-}$ with increasing Cd was observed in roots of NHE, while only slight fluorescence was found in the epidermis of roots of HE at all Cd concentrations (Fig. 1*D*).

Regardless of Cd treatments, the glutathione pool (GSH + oxidized glutathione) was consistently higher in root tips of HE than that of NHE (Table 1). Higher contents of non-protein thiols (NPT) and the GSH/GSSG ratio were also observed in most root tips of HE at 10 μM Cd and higher concentrations. In response to Cd exposure (10 - 100 μM), the content of GSH increased in root tips of HE plants, but no significant change of GSH was observed in NHE plants. A does-dependent decrease in GSSG and an increase in NPT caused by Cd were marked in root tips of HE plants, but were not seen in NHE plants. A Cd-induced increase in the GSH/GSSG ratio also occurred in roots tips of HE up to 400 μM Cd, while in NHE, a decrease of GSH/GSSG ratio was observed.

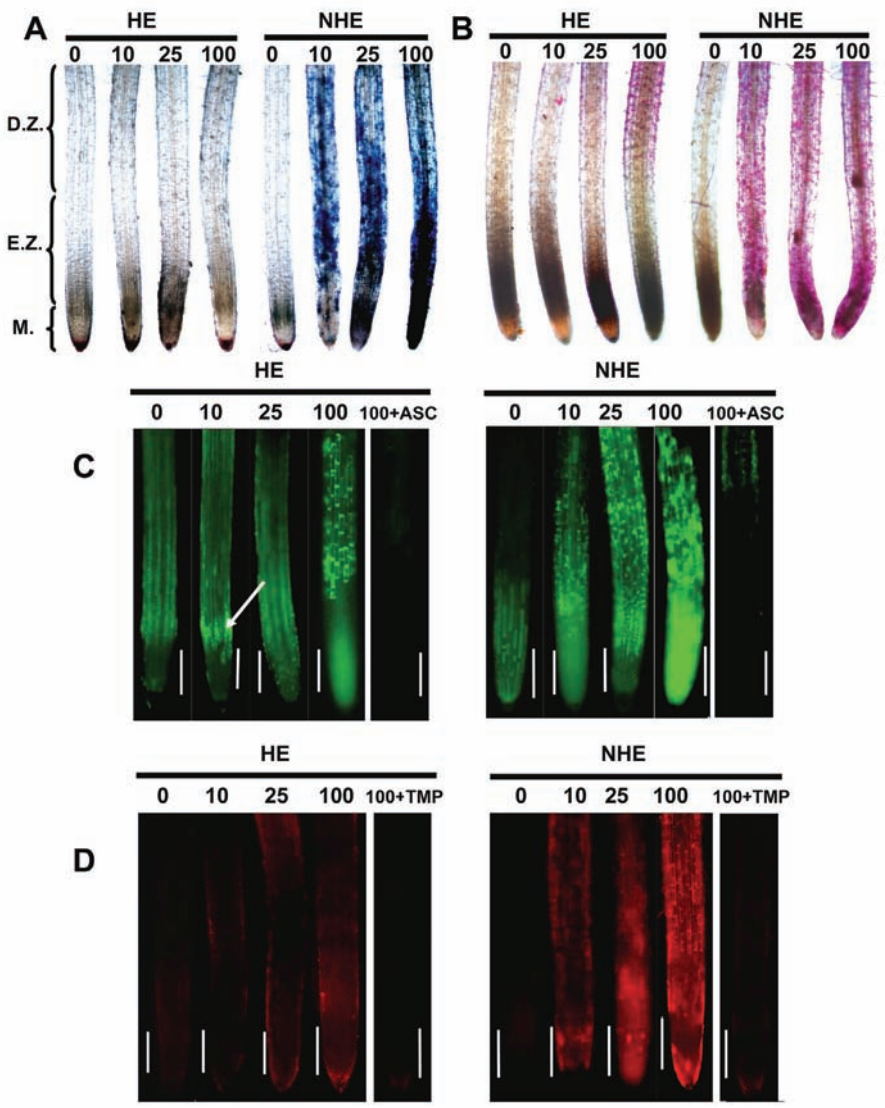

Fig. 1. Microscope images of Evans blue uptake (*A*), lipid peroxidation (*B*), contents of H_2O_2 (*C*), and $O_2^{\cdot-}$ (*D*) in roots of HE and NHE *S. alfredii*. Seedlings were treated with different Cd concentrations (0, 10, 25 and 100 μM) for 24 h. Histochemical analysis of the loss of plasma membrane integrity and lipid peroxidation were conducted with Evans Blue and Schiff's reagent, respectively. H_2O_2-dependent and $O_2^{\cdot-}$-dependent fluorescence were investigated by carboxy-H_2DCFDA and DHE labeling, respectively. As negative controls, roots from 100 μM Cd-treated plants of both ecotypes were incubated with 1.0 mM ASC or 1.0 mM TMP, which acts as H_2O_2 and $O_2^{\cdot-}$ scavengers, respectively. *Bar* = 1 mm, *arrow* in C indicates the Cd-induced H_2O_2 enhancement in the expansion zone of the root.

Table 1. Response of Cd contents [μg g^{-1}(f.m.)], root elongation [% of control], Evans Blue uptake [% of control], contents of MDA [nmol g^{-1}(f.m.)], H_2O_2 [μmol g^{-1}(f.m.)], $O_2^{\cdot-}$ [μmol g^{-1}(f.m.)], GSH [nmol g^{-1}(f.m.)], GSSG [nmol g^{-1}(f.m.)], GSH/GSSG ratio and NPT [nmol g^{-1}(f.m.)] content in roots tips of two *S. alfredii* ecotypes under 0, 10, 25, and 100 μM Cd for 24 h. Means ± SE of three replicates of individual plants. Different letters indicate significant difference between treatments at $P < 0.05$. One or two asterisks indicate significant difference between the two ecotypes at $P < 0.05$ or $P < 0.01$, respectively.

Cd supply	Cd contents	Root elongation	Evans Blue uptake	MDA	H_2O_2	$O_2^{\cdot-}$	GSH	GSSG	GSH/GSSG	NPT
HE										
0	0.1±0.0c	100±0b	100±13a	10.6±0.8a	4.92±0.48a	11.6±0.2b	232±27b	189±36a	1.23±0.54d	594±78d
10	33.0±2.2b	105±0a	104±10a	10.8±0.7a	4.76±0.33a	12.2±1.5b	371±46a	131±21b	2.84±0.25c	867±59c
25	50.4±4.0a	95±1b	114±18a	10.2±1.4a	4.62±0.26a	11.8±1.2b	384±96a	99±44b	3.89±0.19b	1099±43b
100	53.1±4.7a	90±1c	123±15a	11.6±1.4a	5.26±0.40a	17.0±1.1a	406±43a	62± 9c	6.60±0.68a	1288±112a
NHE										
0	0.1±0.0c	100±0a	100±10c	8.7±1.0b	4.44±0.27b	12.9±1.0c	156±32a	48±10a*	3.28±0.38a*	522±44a
10	39.2±2.9b	87±1b**	187±14b**	14.4±1.9a*	5.05±0.14b	16.6±1.3b*	120±56a*	49± 9a*	2.18±0.39b	581±55a*
25	42.5±3.6b	69±3c**	263±28a**	15.3±1.5a*	6.46±0.74a*	21.3±1.9a**	143±30a*	61± 8a	2.36±0.29b*	652±59a**
100	56.2±7.5a	53±5d**	296±31a**	16.2±2.1a*	7.43±0.69a**	22.7±2.8a*	116±38a*	53±10a	2.17±0.17b**	687±78a**

Discussion

Inhibition of root elongation is one of the most distinct symptoms of Cd phytotoxicity (Schutzendubel *et al.* 2001). In this study, we observed an approximately 50 % inhibition of root elongation in roots of NHE plants grown in 100 μM Cd solution within 24 h. In contrast, roots of HE plants were much more tolerant to Cd exposure and even exhibited modest stimulation of root elongation by 10 μM Cd, which was probably caused by Cd enhanced H_2O_2 accumulation in the expansion zone of the roots (Fig. 1*C*). A similar result was also observed in growing, unstressed maize roots (Liszkay *et al.* 2004). H_2O_2 located in elongation zone is thought to be converted to $OH^{\cdot}$, which has a loosening effect on cell wall (Liszkay *et al.* 2004) and therefore stimulates cell elongation (Foreman *et al.* 2003, Dunand *et al.* 2007).

The H_2O_2 observed in root tips from non-Cd-treated plants (Fig. 1*C*) could be associated with normal cell differentiation and elongation, however, when exposed to excess Cd, an excess of ROS accumulated because of an imbalance of redox systems (Schutzendubel *et al.* 2001). Hence plant control of ROS build-up is essential to prevent oxidative damage to membranes and proteins. Here, the oxidative burst of H_2O_2 and $O_2^{\cdot-}$ occurred in root tips of NHE plants even under low Cd treatments (10 μM, Fig. 1*C*,*D*). These ROS may cause the death of plants by damaging membrane lipids, proteins and nucleic acids (Bertin and Averbeck 2006). In contrast, the ROS content of HE root tips was essentially unaffected by Cd supply lower than 100 μM demonstrating the high detoxification capacity of HE roots (Fig. 1*C*,*D*, Table 1). The ability of HE roots to maintain ROS contents at low levels represent an important feature of this hyper-accumulator species. This differs from the Ni hyperaccumulator, *Alyssum bertolonii*, which possesses detoxification mechanisms that allow unimpeded growth of roots even in the presence of elevated concentrations of H_2O_2 (Boominathan and Doran 2002).

High contents of ROS triggers oxidative stress and/or programmed cell death (Gechev and Hille 2005). Lipids in general and unsaturated lipids in particular are also sensitive to oxidation by ROS generated under stress conditions The differential response patterns in Cd-induced lipid peroxidation and the loss of plasma membrane integrity between two ecotypes of *S. alfredii* was highly consistent with over production of ROS. Increased lipid peroxidation caused by Cd was frequently reported in plants (Sandalio *et al.* 2001, Metwally *et al.* 2005), even in the Cd hyperaccumulator, *T. caerulescens* (Boominathan and Doran 2003). In our study, however, the tolerance ability of HE *S. alfredii* acts by preventing peroxidation of lipids and loss of root plasma membrane integrity, while roots of NHE exhibited enhanced Cd-induced lipid peroxidation and subsequent loss of plasma membrane integrity, especially on the root surface.

GSH is well known as an important antioxidant (Clemens 2001, Wojcik and Tukiendorf 2011). The rapid induction of GSH biosynthesis in root exposed to Cd may be involved in Cd tolerance in the hyperaccumulators. In the present study, HE roots contained consistently higher GSH content than NHE (Table 1), and exhibited a marked induction of GSH production at low Cd concentration (10 μM), whereas no effect of Cd addition on GSH production was noted in NHE plants. These results imply that Cd may directly activate GSH synthesis in the HE, but not in NHE. The GSH/GSSG ratio also remained high and even increased under Cd stress in roots of HE plants, implying that the role of GSH was not simply providing

reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide and lipid hydroperoxides. The reduction of GSSG caused by Cd observed in the roots of HE plants may be related to the elevated Cd-induced glutathione reductase (GR) activity as reported by Jin *et al.* (2008a). Moreover, the induction of additional sinks for reduced sulfur was indicated since NPT in roots of the HE plants positively responded to increasing Cd concentration. This suggests that some other SH-compounds, but not phytochelatins (Sun *et al.* 2007), in addition to GSH, were possibly involved in the Cd detoxification in the hyperaccumulator *S. alfredii*.

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