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Cadmium Accumulation in Relation to Organic Acids and Nonprotein Thiols in Leaves of the Recently Found Cd Hyperaccumulator *Rorippa globosa* and the Cd-accumulating Plant *Rorippa islandica*

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Abstract It has been proposed that organic acid and nonprotein thiol are involved in the hyperaccumulation of metals. In this study, Cd accumulation, organic acid, and nonprotein thiol production and their relationships in the leaves of Cd-hyperaccumulator *Rorippa globosa* were examined and compared with a closely related species, *Rorippa islandica*. The results showed that there was no reduction in biomass of *R. globosa* when treated with 25 μg Cd g^{-1} (T_2), despite Cd accumulation in the leaves was up to 158.2 μg g^{-1} DW. On the other hand, the growth of Cd-treated *R. islandica* was obviously inhibited as it accumulated more than 100 μg g^{-1} DW in the leaves. Therefore, *R. islandica* behaved as a Cd-accumulating plant. The Cd treatments could significantly induce the synthesis of acetic acid in both species, suggesting that acetic acid, as the most abundant organic acid, might be related to the Cd accumulation. Significant positive correlations between Cd concentrations and both tartaric and malic acid concentrations in the leaves of *R. globosa* were observed. There was a significant positive correlation between Cd concentrations and acetic acid concentrations in the leaves of *R. islandica*. This trend of tartaric and malic acids in the leaves of *R. globosa* and acetic acid in the leaves of *R. islandica* might be related to Cd accumulation. In addition, a quadratic relationship was obviously observed for NP-SH contents and total Cd concentrations in the leaves of *R. globosa*, indicating that NP-SH was significantly related to Cd accumulation and tolerance.

Keywords Cadmium · Hyperaccumulation · Nonprotein thiol · Organic acid · *Rorippa globosa* · *Rorippa islandica*

Introduction

As an important environmental contaminant, cadmium (Cd) is extremely toxic to living organisms. Because of its high mobility in soil-plant systems, Cd in excessive amounts is hazardous to human health and the functioning of ecosystems. Contamination of soils by Cd has become a serious worldwide problem (Cai and Braids 2002; Zhou and Song 2004). In recent years, there has been increasing interest in the development of phytoextraction techniques for the remediation of metal-contaminated soils (Chaney and others 1997; Garbisu and Alkorta 2001). The key to phytoextraction is the identification and selection of hyperaccumulating plants, which can accumulate exceptional concentrations of trace elements or heavy metals in their aerial parts without visible toxicity symptoms. So far, over 450 plant species have been identified as hyperaccumulators of trace metals, metalloids, and nonmetals, and the majority of them (75%) are Ni hyperaccumulators, whereas the Cd hyperaccumulators are relatively scarce (Reeves 2003; Verbruggen and others 2009). Using foliar concentration thresholds above 0.01% Cd, only about seven species qualify as evident Cd hyperaccumulators. In addition to *Thlaspi caerulescens*, *Arabidopsis halleri* from Europe (Brown and others 1994;

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Küpper and others 2000), and *Athyrium yokoscense* from Japan (Morishita and Boratynski 1992), several Cd hyperaccumulators such as *Sedum alfredii* (Yang and others 2004), *Viola baoshanensis* (Liu and others 2004), *Solanum nigrum* (Wei and others 2004), and *Rorippa globosa* (Wei and Zhou 2006) were found in China. However, Cd hyperaccumulation in higher plants is a rare phenomenon. In addition, the small amount of information on the mechanisms of Cd uptake, translocation, and detoxification in Cd hyperaccumulators hampers the optimization of phytoextraction techniques and further commercial application.

The Zn, Cd, or Ni in the cells of hyperaccumulators tends to be distributed in a variety of locations, including cell walls, cytoplasm, and vacuoles (Krämer and others 2000; Cosio and others 2005; Ma and others 2005). It is assumed that most of the hyperaccumulated metals are bound to ligands such as organic acids, amino acids, peptides, and proteins. Although some aspects of metal detoxification by ligands have been discovered, there is no complete picture of the different chelators involved in the different stages of plant internal transport and storage of metals in hyperaccumulators (Verbruggen and others 2009). In *T. caerulescens* plants, a significant fraction of Zn accumulation within the roots seemed to be associated with histidine (Salt and others 1999), whereas Boominathan and Doran (2003) found that most of the Cd accumulated in the hairy roots was localized in the cell walls, with approximately 13% of the total Cd and which was associated with organic acids. In the shoots, most Zn was associated with organic acids, mainly citrate (Salt and others 1999), whereas Ueno and others (2005) deduced that Cd in the shoots was bound to malate. In addition, Küpper and others (2004) found that in *T. caerulescens* a large fraction of the foliar Cd was bound by some weak oxygen ligands such as organic acids. With regard to other hyperaccumulators, in *Arabidopsis halleri* a large proportion of Zn in the shoots was associated with malate (Sarret and others 2002). Ni seems to be associated mainly with citrate in the shoots of *Alyssum* (Lee and others 1978) and *T. goesingense* (Krämer and others 2000).

Because of the low association constants of organic acids with metals, Callahan and others (2006) argued against a role for organic acids in the hyperaccumulation mechanism (such as long-distance transport), in spite of their constitutively elevated concentrations in hyperaccumulators (Lee and others 1978; Ueno and others 2005; Montargès-Pelletier and others 2008). So their role may be limited to vacuolar sequestration. As organic acids are located primarily in the vacuole (Ryan and Walker-Simmons 1983), the formation of metal–organic acid complexes is favored to occur in the acidic environment of the vacuole (Haydon and Cobbett 2007). The association between metals and organic acids would suggest that metal detoxification occurs by vacuolar sequestration.

The nonprotein thiol compounds are composed of several acid-soluble sulfhydryl components such as cysteine, γ-glutamylcysteine, glutathione (GSH), and phytochelatins (PCs) (De Vos and others 1992). GSH is a well-known antioxidant that plays a prominent role in the defense against oxidative stress (Szalai and others 2009). Complexation with PCs has been identified as an important mechanism in the detoxification from Cd, Pb, and Hg in various plant species (Gupta and others 1998; Cobbett 2000). The content of thiol is closely related to PCs because the latter are thiol-rich peptides that are synthesized with GSH as a building block. It has been shown that nonprotein thiols are known to play a pivotal role in terrestrial plants in their response to trace metals (Rauser 1999).

Based on our systematic investigation in northern China, the recently found Cd-hyperaccumulator *Rorippa globosa* (Turcz.) Thell. has been identified (Wei and Zhou 2006). The aims of this study were to determine Cd uptake and accumulation and its effect on the levels of organic acids and nonprotein thiols in the Cd hyperaccumulator *R. globosa* compared with a closely botanically related species *R. islandica*, and to assess the role of organic acids and nonprotein thiols in Cd accumulation and tolerance.

Materials and Methods

Soil Preparation and Plant Culture

Soil samples were collected from an agricultural field in the Shenyang Station (123°41′E and 41°31′N) of Experimental Ecology, Chinese Academy of Sciences. The fresh soil samples were air-dried, passed through a sieve of 4.0 mm, and thoroughly mixed with basal fertilizers. The tested soil was meadow burozem, and chemical analysis showed that organic matter, total N, pH, and Cd concentration in the soil were 1.52%, 0.11%, 6.50, and 0.20 mg kg^{-1}, respectively. The applied rate of basal fertilizers was 150 mg N kg^{-1} dry soil as urea and 60 mg P kg^{-1} and 80 mg K kg^{-1} as KH_2PO_4. There were five Cd-level treatments corresponding to each plant species: control (no Cd addition) and treatments T_1-T_4 at the concentrations of 10, 25, 50, and 100 μg g^{-1} air-dried soil, respectively, and each treatment was performed in triplicate. Cd was applied as $CdCl_2 \cdot 2.5H_2O$, mixed thoroughly with the soil samples, and equilibrated for 14 days before the pot-culture experiment.

Seeds of *R. globosa* and *R. islandica* were collected from a noncontaminated field in the Shenyang Station of Experimental Ecology, Chinese Academy of Sciences. Seeds were germinated on filter paper moistened with deionized water. After germination, 2-week-old seedlings were transferred to 9.0-cm-diameter pots that were 12.0 cm deep, each filled with 1.0 kg of soil sample. Four seedlings were planted per

pot. The pots were kept in a lookum with a glass roof (in the Shenyang Station of Experimental Ecology) to facilitate natural photoperiods and avoid the effects of rainfall. During cultivation, minimal and maximal temperatures were in the range of 11–20 and 23–32°C, respectively. Soil was watered to reach 60% of water-holding capacity and maintained at this level by daily watering throughout the cultivation. Seven weeks after transplanting, samples of both plants were collected and analyzed.

Plant Growth Measurements

Plants were harvested by cutting the shoots at the soil surface and the roots were carefully separated from the soil. The shoots and roots were rinsed with distilled water, wiped with tissue paper, and weighed. Divided leaves, stems, and roots were dried at 105°C for 30 min, then at 70°C for Cd determination.

Determination of Cd

Plant samples for Cd determination were ground using a ball mill and wet digested in $HNO_3/HClO_4$ (87:13 v/v). Water-soluble Cd in the leaves was measured with 0.2 g dried plant powder suspended in 50 ml deionized water following the method of Perronnet and others (2000). The supernatant was filtered on a 0.45-μm Millipore filter. The content of Cd was determined by atomic absorption spectrophotometry (Hitachi model 180-80).

Measurement of Organic Acids and Nonprotein Thiols

A total of 0.5 g fresh weight (FW) of leaves was frozen in liquid nitrogen. The freeze-dried leaves were ground to powder in liquid nitrogen. Then the dried powders were ground with 2 ml 0.5 M HCl solution using a mechanical agate mill and then extracted at 60°C for 1 h. The suspension was then filtered through a 0.45-μm Millipore filter. Samples were analyzed using a Waters 2695 Alliance HPLC equipped with a reverse phase C18 column (Waters, 4.6 × 250 mm). The column was operated at 35°C. The mobile phase was 18 mM potassium dihydrogen phosphate (pH 2.25) with a flow rate of 0.8 ml/min. Organic acids were determined using a Waters 996 photodiode array detector set at 210 nm.

The content of nonprotein thiols (NP-SH) was measured following the method of Ellman (1959). A total of 0.5 g fresh weight (FW) of leaves was frozen in liquid nitrogen, and dried powders were homogenized in 2 ml 5% sulfosalicylic acid using a mechanical agate mill. After centrifuging at 10,000 rpm for 15 min at 4°C, the content of NP-SH was measured in the supernatant by reaction with the Ellman reagent and absorbance was recorded at 412 nm.

Statistical Analysis

Analysis of variance (ANOVA) was performed for all data sets. The least significant difference (LSD) was used to compare the plant tissues. Pearson correlation coefficients and quadratic regression coefficients were calculated between the different plant parameters.

Results

Plant Growth and Cd Bioaccumulation

Dry weights of *R. globosa* shoots and roots were insignificantly ($p > 0.05$) affected by Cd treatments T_1 and T_2 compared with those of the control (Fig. 1). On the other hand, increased Cd in the soil reduced the dry biomasses of *R. globosa* shoots and roots by 12.1-66.0% and 45.5-70.5% in treatments T_3 and T_4, respectively. Compared with *R. globosa*, the growth of Cd-treated *R. islandica* was severely inhibited (Fig. 1). Dry biomasses of *R. islandica* shoots and roots decreased significantly ($p < 0.05$) with all the treatments. Dry biomass of *R. islandica* shoots under treatment T_1 had a slight (8.6%) but statistically significant ($p < 0.05$) decrease and major decreases (about 38.4–85.4%) for treatments T_2-T_4 compared with the control. Under the highest Cd treatment (T_4), dry root biomass of

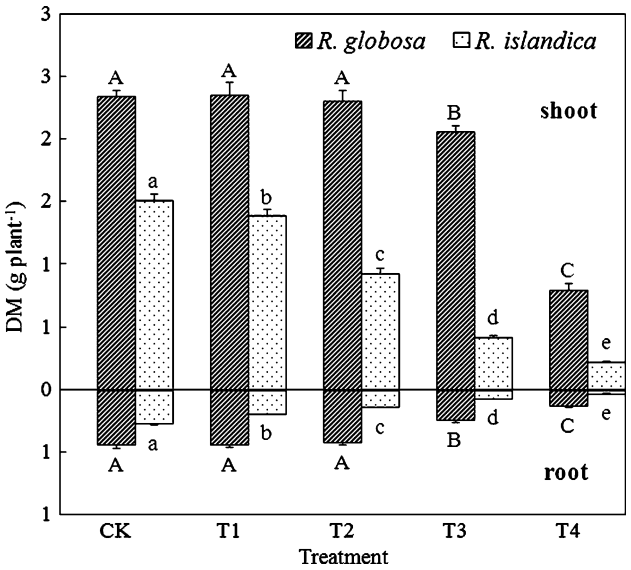

Fig. 1 Dry biomass of the shoots and roots of *Rorippa globosa* and *Rorippa islandica* grown under different Cd treatments. Bars denote standard deviation. Statistical analyses (1 factor: Cd treatments) were performed for each parameter on dry biomass in *R. globosa* (*capital letters*) and dry biomass in *R. islandica* (*lower-case letters*). The different letters were significantly different at the 5% risk level. CK, no Cd addition; treatments T_1-T_4, 10, 25, 50, and 100 μg Cd g^{-1} air-dried soil

R. islandica was severely inhibited by 90.0%. These data showed that *R. islandica* was considerably less tolerant of Cd than *R. globosa*.

The accumulation of Cd in the shoots and roots of the two species was proportional with the increasing concentration of Cd in the soils (Table 1). For all treatments, the concentration of Cd in the shoots of *R. globosa* was always higher than that in the roots, and the ratio of shoot Cd/root Cd concentrations varied between 1.9 and 3.0. Similarly, *R. islandica* had higher Cd concentrations in the shoots than in the roots, and shoot Cd/root Cd concentrations ratio varied between 1.1 and 1.8. On the whole, the concentration of Cd in the shoots of *R. globosa* was 1.0-1.5 times as much as that of *R. islandica*, and furthermore the shoot Cd/root Cd ratio of *R. globosa* was higher than that of *R. islandica*. These results indicated that *R. globosa* could be much more efficient in accumulating and translating Cd from the roots to the shoots than *R. islandica*.

The addition of Cd could induce an increase of water-soluble Cd concentration in the leaves of both *R. globosa* and *R. islandica* (Table 2). Similar to the concentration of total Cd in the shoots, the concentration of water-soluble Cd in *R. globosa* was 1.0-1.7 times higher than that in *R. islandica*. Furthermore, the percentage of water-soluble Cd in the leaves of *R. globosa* was higher than that of *R. islandica*, except for treatment T_2. In *R. globosa* leaves, this percentage increased from 23.5% in treatment T_1 to 36.5% in treatment T_4, whereas it increased from 13.9% in treatment T_1 to 35.2% in treatment T_2 and declined to 32.8% in the highest treatment (T_4) in *R. islandica* leaves.

Organic Acid Levels in the Leaves of *R. globosa*

The concentrations of organic acids in the leaves of *R. globosa* varied from 19.2 to 33.9, from 56.7 to 115.6, and from 107.2 to 193.2 μmol g^{-1} for tartaric, malic, and acetic acid, respectively (Fig. 2a). The application of Cd had significant effects on the induction of acetic acids. The level of acetic acid in the leaves of *R. globosa* treated with 25 μg Cd g^{-1} (T_2) reached the maximum value of 193.2 μmol g^{-1}, about 2.0 times as much as that in the control. On the other hand, the low Cd concentrations (T_1 or T_2) did not induce accumulation of tartaric acid and malic acid; however, the two acids trended higher with higher Cd concentrations (T_3 and T_4). As for oxalic acid, its concentration greatly decreased to 42.6% of the level in the control with increasing Cd concentrations in the soil. The total concentrations of tartaric, malic, acetic, and oxalic acids in the leaves of

Table 1 Accumulation of Cd in various tissues of *R. globosa* and *R. islandica* grown under different Cd treatments

Treatment	*R. globosa* (μg g^{-1} DW)			*R. islandica* (μg g^{-1} DW)		
	Leaf	Shoot	Root	Leaf	Shoot	Root
CK[a]	4.5 ± 0.3A	3.8 ± 0.2B	2.0 ± 0.1C	4.4 ± 0.4a	3.3 ± 0.3b	2.7 ± 0.3c
T_1[a]	109.9 ± 10.7A	91.1 ± 4.6B	32.1 ± 0.9C	132.0 ± 7.0a	88.8 ± 5.7b	49.8 ± 4.9c
T_2[a]	158.2 ± 10.3A	130.0 ± 6.1B	51.2 ± 2.8C	157.0 ± 2.6a	130 ± 6.1b	115.5 ± 12.7c
T_3[a]	276.3 ± 9.7A	259.3 ± 10.8A	85.4 ± 12.8C	204.3 ± 5.0a	171.2 ± 4.2b	162.4 ± 6.6c
T_4[a]	432.7 ± 22.8A	410.6 ± 23.1A	142.3 ± 4.1C	335.5 ± 24.7a	284.5 ± 18.1b	254.0 ± 9.5c

Data are expressed as mean ± standard deviation

CK no Cd addition; treatments T_1–T_4 = 10, 25, 50, and 100 μg Cd g^{-1} air-dried soil

[a] Statistical analyses (1 factor: type of tissue) were performed for each parameter on Cd concentration in *R. globosa* (capital letters) and Cd concentration in *R. islandica* (lower-case letters). Means followed by the different letters were significantly different at the 5% risk level

Table 2 Concentration of water-soluble Cd in the leaves of *R. globosa* and *R. islandica* in spiked soils and the percentage of water-soluble Cd to total Cd in the leaves

Treatment	Water-soluble Cd (μg g^{-1} DW leaves)		Water-soluble Cd/Total Cd (%)	
	R. globosa	*R. islandica*	*R. globosa*	*R. islandica*
CK	nd	nd		
T_1	25.8 ± 1.2	18.3 ± 1.1	23.5 ± 0.7	13.9 ± 0.5
T_2	54.1 ± 3.1	55.3 ± 2.6	34.2 ± 0.9	35.2 ± 1.3
T_3	97.3 ± 4.3	71.2 ± 4.1	35.2 ± 2.3	35.0 ± 2.1
T_4	157.8 ± 6.7	110.4 ± 3.7	36.5 ± 2.1	32.8 ± 1.1

Data are expressed as mean ± standard deviation

nd not detected, *CK* no Cd addition, treatments T_1-T_4 = 10, 25, 50, and 100 μg Cd g^{-1} air-dried soil

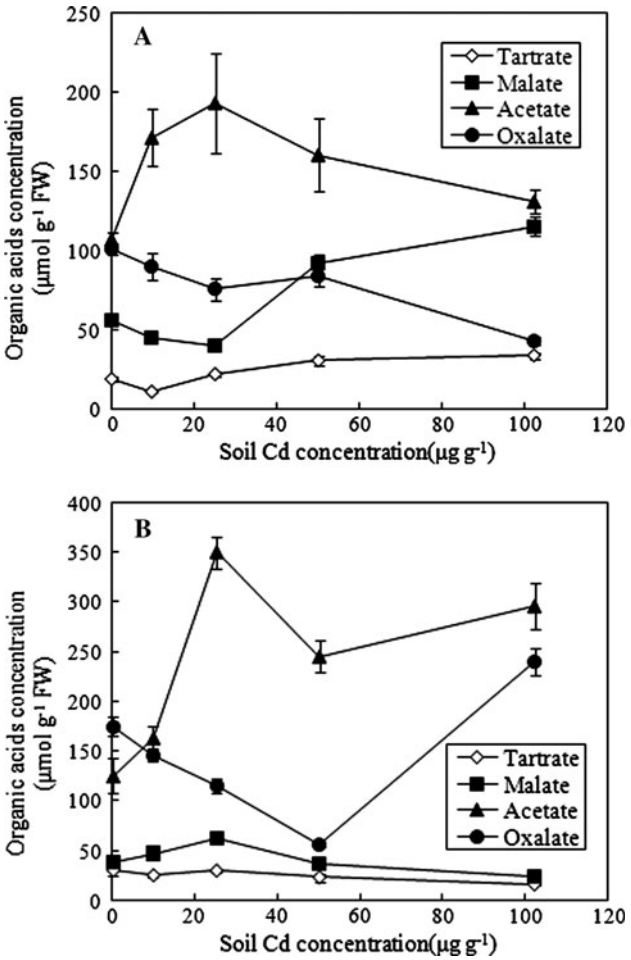

Fig. 2 The effect of various Cd treatments on the concentrations of organic acids in the leaves of *R. globosa* (**a**) and *R. islandica* (**b**). *Bars* denote standard deviation

R. globosa ranged from 284.0 to 367.7 μmol g^{-1}. Acetic acid was the most abundant organic acid, up to 107.2-193.2 μmol g^{-1}, whereas tartaric acid was less abundant. The application of Cd increased the total level of organic acids in the leaves of *R. globosa*.

Similar to *R. globosa*, the application of Cd induced the accumulation of acetic acid in the leaves of *R. islandica* (Fig. 2b). The level of acetic acid reached the maximum value (350.4 μmol g^{-1}) when the plants were treated with T_2, about 2.8 times as much as that in the control. In general, acetic acid was also the most abundant among the four organic acids in the leaves of *R. islandica*, varying between 125.9 and 350.4 μmol g^{-1}, whereas tartaric acid was less abundant; and, furthermore, the Cd treatments had a negligible effect on the level of tartaric acid compared with that in the control, except for treatment T_2. Contrary to *R. globosa*, the level of malic acid in the leaves of *R. islandica* was enhanced by treatments T_1 and T_2 and declined below the level of that of the control when the plants were treated with higher Cd concentrations (T_3 and T_4). The concentration of oxalic acid in *R. islandica* decreased by 16.6–67.6% with increasing Cd concentrations in the soil (T_1–T_3) and that the inhibition of level of oxalic acid was strongly enhanced with the highest Cd treatment (T_4). The total concentrations of tartaric, malic, acetic, and oxalic acids in the leaves of *R. islandica* ranged from 361.8 to 576.3 μmol g^{-1}, which was much higher than those in the leaves of *R. globosa*. The application of Cd kept unchanged or increased the total level of organic acids in the leaves of *R. islandica*.

Organic Acids in Relation to Cd Bioaccumulation

There were positive linear correlations between the concentrations of tartaric and malic acids and Cd concentrations in the leaves of *R. globosa* (Table 3), but no obvious relationship was observed for other organic acids tested. Correspondingly, positive correlations were found between tartaric acid and malic acid ($r = 0.852^{**}$), and between total Cd and water-soluble Cd ($r = 0.994^{**}$). The concentration of oxalic acid was negatively correlated with the concentrations of total Cd and water-soluble Cd in the leaves of *R. globosa*, and its correlation with water-soluble Cd ($r = -0.891^{**}$) was similar to its correlation with total Cd ($r = -0.889^{**}$). It can be concluded that Cd accumulation in the leaves of *R. globosa* was positively related to the accumulation of tartaric and malic acids.

In contrast with the results for *R. globosa*, there was a significant positive correlation between the concentration of acetic acid and the concentration of Cd, and a significant negative correlation between the concentration of tartaric acid and the concentration of Cd in the leaves of *R. islandica* (Table 3). No obvious relationship was observed for malic and oxalic acids. Similar to *R. globosa*, positive correlations were also found between tartaric acid and malic acid ($r = 0.685^{**}$) and between total Cd and water-soluble Cd ($r = 0.958^{**}$) in the leaves of *R. islandica*. These results indicated that acetic acid could contribute to the accumulation of Cd in the leaves of *R. islandica*.

Nonprotein Thiol Levels in the Leaves and its Relation with Cd Bioaccumulation

The application of Cd induced the accumulation of NP-SH in the leaves of *R. globosa* (Fig. 3). The level of NP-SH reached the maximum value (2.4 μmol g^{-1}), about 2.4 times as much as that in the control when the plants were treated with T_2, and declined to 1.9 μmol g^{-1} in the highest treatment (T_4), about 1.9 times as much as that in the control. Contrary to *R. globosa*, the level of NP-SH in the leaves of *R. islandica* was enhanced at the rate of 75.9% in treatment T_1 and declined to a level of less than

Table 3 Pearson correlation coefficients between organic acid concentrations and Cd accumulation in the leaves of *R. globosa* and *R. islandica* ($n = 15$)

		Tartaric conc.	Malic conc.	Acetic conc.	Oxalic conc.	Total Cd conc.	Water-soluble Cd conc.
R. globosa	Tartaric conc.	1					
	Malic conc.	0.852**	1				
	Acetic conc.	−0.214	−0.381	1			
	Oxalic conc.	−0.683**	−0.678**	−0.004	1		
	Total Cd conc.	0.821**	0.853**	0.039	−0.889**	1	
	Water-soluble Cd conc.	0.843**	0.884**	−0.028	−0.891**	0.994**	1
R. islandica	Tartaric conc.	1					
	Malic conc.	0.685**	1				
	Acetic conc.	−0.218	0.224	1			
	Oxalic conc.	−0.397	−0.491	−0.083	1		
	Total Cd conc.	−0.797**	−0.439	0.655**	0.249	1	
	Water-soluble Cd conc.	−0.725**	−0.430	0.748**	0.18	0.958**	1

* Significance at $p < 0.05$, ** significance at $p < 0.01$

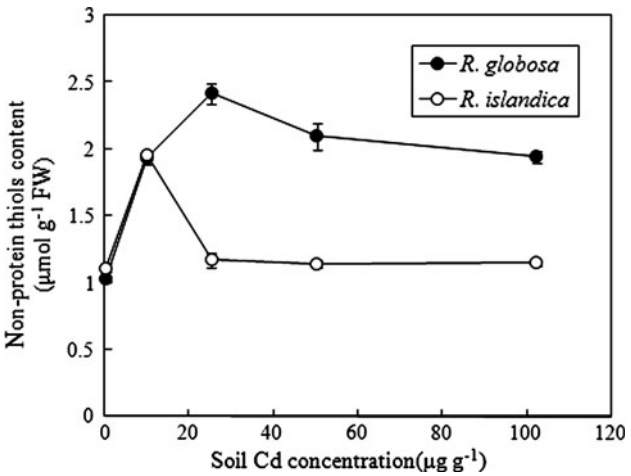

Fig. 3 The content of nonprotein thiols in the leaves of *R. globosa* (*filled circle*) and *R. islandica* (*open circle*) grown under different Cd treatments. *Bars* denote standard deviation

that of the control when the plants were treated with higher Cd concentrations (T_2–T_4). The concentration of NP-SH in the leaves of *R. globosa* was roughly 0.9-2.1 times as much as that of *R. islandica*.

No obviously linear correlation between NP-SH concentrations and total Cd concentrations was observed in the leaves of *R. globosa* and *R. islandica*. Strictly speaking, the relationship between NP-SH concentration and total Cd concentration in the leaves of *R. globosa* could be described best by the quadratic parabolic equation (Fig. 4a), and the F value was 29.1 ($p < 0.001$). The coefficient of determination (r^2) for the regression equation was 0.829 ($p < 0.001$). On the other hand, no obvious quadratic relationship was observed for NP-SH content and total Cd concentration in the leaves of *R. islandica* (Fig. 4b); furthermore, the coefficient of determination (r^2) for the regression equation was 0.187 ($p > 0.05$). These results indicate that Cd accumulation was significantly related to the synthesis of NP-SH in the leaves of *R. globosa*.

Discussion

This study further confirmed the previous finding that *R. globosa* possessed the ability to hyperaccumulate Cd, characterized by the accumulation of Cd in the stems or leaves of the plant that exceeds the critical standard (100 μg Cd g^{-1} DW) of a Cd hyperaccumulator, and by having a ratio of Cd in the shoots and roots greater than 1.0. The plant also displayed strong tolerance to Cd, with healthy growth when the soil had up to 25 μg Cd g^{-1} and an accumulation of 158.2 μg Cd g^{-1} DW in the leaves. On the other hand, Cd accumulation in the leaves of *R. islandica* was greater than 100 μg Cd g^{-1} DW in Cd treatment T_1 and the growth of the plant was obviously inhibited by Cd stress. Therefore, *R. islandica* does not possess the tolerant characteristics of Cd hyperaccumulators, but it does possess the ability to accumulate and translate Cd. So far, more than 450 plant species that hyperaccumulate various trace metals, metalloids, and nonmetals in their shoots are now known worldwide. Of these, almost 25% are Brassicaceae family members, including numerous *Alyssum*, *Brassica*, and *Thlaspi* species. *Rorippa* is a flowering plant genus in the Brassicaceae family that contains approximately 80 species that are native from Europe through central Asia, Africa, and North America. Our study confirmed that *R. globosa* and *R. islandica* had Cd hyperaccumulative characteristics,

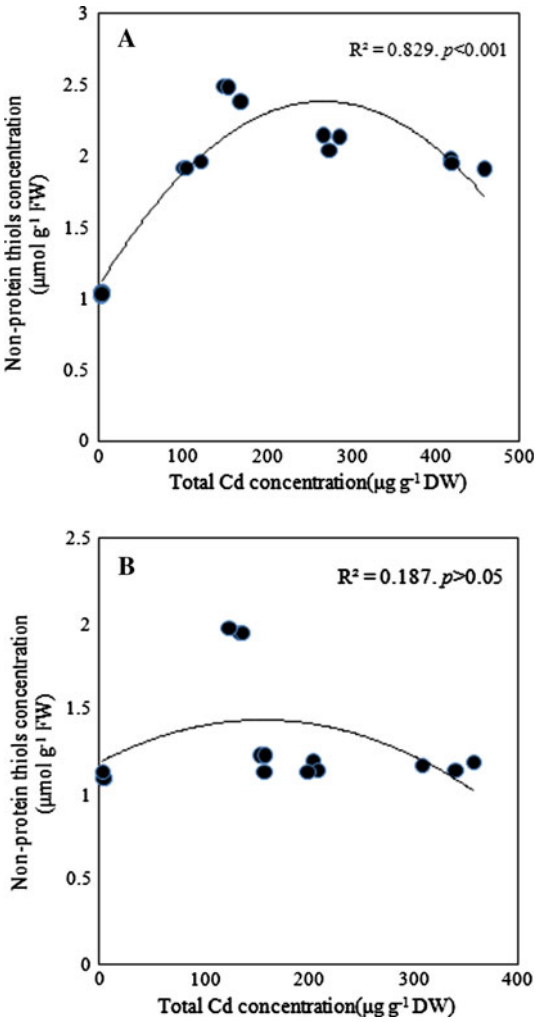

Fig. 4 The relationship between total Cd concentration and the content of NP-SH in the leaves of *R. globosa* (**a**) and *R. islandica* (**b**). The quadratic parabolic curves were drawn using the regression analysis tool of Excel 2007. The regression fits are determined by coefficients of determination (R^2); $n = 15$

implying that there is a need for further investigation to identify hyperaccumulators from *Rorippa* species.

Results showed that approximately 30% of Cd accumulation in the leaves of both *R. globosa* and *R. islandica* was readily extracted with water, suggesting that some of the Cd accumulation is associated with water-soluble compounds such as organic acids. For treatment T_1, the percentage of water-soluble Cd to total Cd was 1.7 times higher in the leaves of *R. globosa* than in the leaves of *R. islandica*. Correspondingly, no toxicity symptoms were observed in *R. globosa*, but the growth of *R. islandica* was inhibited. The results of this study suggest that the organic acids in the leaves of *R. globosa* might play an important role in Cd tolerance, at least in treatment T_1. However, the percentage of water-soluble Cd in the leaves of *R. globosa* was similar to that of *R. islandica* in other treatments, suggesting that other metabolic and cellular strategies may be used to detoxify *R. globosa* of Cd ions besides complexation with organic acids.

In *R. globosa* and *R. islandica* leaves for all samples, the organic acid/Cd molar ratios were far greater than 100, indicating that these ligands are concentrated enough to bind all Cd atoms present in the leaves. However, *R. islandica* was considerably less tolerant of and efficient at accumulating and translating Cd from the roots to the shoots than *R. globosa*. In addition, the total concentrations of tartaric, malic, acetic, and oxalic acids in the leaves of *R. islandica* were greater than those of *R. globosa*. Considered together, the high levels of organic acids are not the primary reason for Cd hyperaccumulation and hypertolerance, which coincides with our earlier study on the Cd hyperaccumulator *Solanum nigrum* (Sun and others 2006). However, the difference between *R. globosa* and *R. islandica* with respect to organic acids and their responses to different Cd levels may be responsible for the difference in ability to accumulate and tolerate Cd.

In general, hyperaccumulators exhibit high concentrations of organic acids, usually citrate and malate (Tolrà and others 1996; Boominathan and Doran 2003). Shen and others (1997) reported that malate and citrate in the leaves of *T. caerulescens* were constitutive, but citrate in the roots was induced upon Zn exposure. In our experiments only malic acid was constitutive in the leaves of *R. globosa* and *R. islandica*, but citric acid was undetectable in the leaves of *R. globosa* and *R. islandica*. This result is consistent with that of the study by Wójcik and others (2006), and it showed that only malate was constitutively present in the shoots and roots of *T. caerulescens* and that citrate accumulation was induced by Zn treatment but not by Cd treatment. In addition, there was a positive linear correlation between the concentration of malic acid and Cd concentration in the leaves of *R. globosa* but no significant relationship was observed in *R. islandica*. These results suggest that malic acid may be a biological marker of Cd tolerance and hyperaccumulation.

Early studies showed that acetate as root exudation is involved in heavy-metal bioavailability for uptake into roots (Ernst 1974; Fan and others 1997). Little is known about the complexation of acetate with heavy metals in the shoots of hyperaccumulators. In our work, acetic acid was the most abundant organic acid in the leaves of both *R. globosa* and *R. islandica*. Although no obviously linear correlation was found between the concentration of acetic acid and the concentration of Cd in the leaves of *R. globosa*, the application of Cd had significant effects on the induction of acetic acid. Similarly, Cd treatment induced the accumulation of acetic acid in the leaves of *R. islandica*. Moreover, there was a significant positive correlation between the concentration of acetic acid and the

concentration of Cd, suggesting that acetic acid could contribute to the accumulation of Cd in *R. globosa* and *R. islandica*.

Stability constants can be used to compare the stability of any two ligands. For similar complexes, the higher the value of the stability constant, the more stable the complex. In the present study, the order of stability constants for the detected organic acids was as follows: Cd-oxalate > Cd-malate > Cd-tartrate > Cd-acetate, that is, the Cd-oxalate complex is formed more easily than the others. However, it is clear from our results that the application of Cd reduced the level of oxalic acid in *R. globosa* and *R. islandica*. Oxalic acid had no connection with Cd accumulation and tolerance in *R. globosa* and *R. islandica* despite its central role in aluminum tolerance mechanisms (Ma and others 2001). Although this study suggests that tartaric, malic, and acetic acids in the leaves of *R. globosa* might play an important role in Cd tolerance and hyperaccumulation, further studies are still necessary to determine the accumulation forms of Cd and the cellular distribution in the leaves of *R. globosa*.

Nouairi and others (2009) reported that there was an increase in the content of NP-SH in leaves of the Cd hyperaccumulator *Brassica juncea* after Cd exposure due to its role against Cd-induced oxidative stress. The antioxidant property of NP-SH depends on the oxidation of the -SH group of the tripeptide to disulfides (Noctor and Foyer 1998; Sanita di Toppi and Gabbrielli 1999). Our results are consistent with those of Nouairi and others (2009). *R. globosa* exhibited high amounts of NP-SH synthesis by Cd treatments, indicating its ability to tolerate cellular metal loads. The increased level of NP-SH may result from the stimulation of the sulfate reduction pathway such as APS reductase and serine acetyltransferase (Noctor and Foyer 1998), whereas a slight decrease observed at higher Cd concentrations (T_3 and T_4) was possibly due to consumption for synthesis of GSH and PCs. However, it was shown by the regression equation that the content of NP-SH was significantly related to total Cd concentration in the leaves of *R. globosa*, indicating that Cd accumulation caused plant stress, resulting in thiol formation. This was similar to the results described for the As hyperaccumulator Chinese Brake fern (*Pteris vittata* L.) by Tu and others (2004), who reported that thiol formation might have contributed to the ability of Chinese Brake fern to hyperaccumulate As.

As indicated in the results, *R. globosa* could be much more efficient in accumulating and translating Cd from the roots to the shoots than *R. islandica*. Moreover, *R. globosa* had considerably higher tolerance to Cd than *R. islandica*. However, *R. islandica* did possess the ability to accumulate and translocate Cd and could be classified as a Cd-accumulating plant. Acetic acid as the most abundant organic acid in both species might be related to their Cd accumulation and tolerance. It is proposed that tartaric and malic acids in the leaves of *R. globosa* and acetic acid in the leaves of *R. islandica* might be indicators of Cd accumulation. In addition, there was a significant quadratic relationship between the content of NP-SH and total Cd concentration in the leaves of *R. globosa*, indicating that NP-SH was obviously related to Cd accumulation and tolerance.

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