New Phytologist

Research

Comparative physiological responses of *Solanum nigrum* and *Solanum torvum* to cadmium stress

Jin Xu[1,2], Yiyong Zhu[3], Qing Ge[1,2], Yulong Li[1,2], Jianhang Sun[1], Yuan Zhang[1,2] and Xiaojing Liu[1,2]

[1]Key Laboratory of Agricultural Water Resources, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 286 Huaizhong RD, Shijiazhuang 050021, China; [2]Hebei Province Engineering Laboratory for Plant Breeding and Germplasm Enhancement of Stress-Tolerant Plants, 286 Huaizhong RD, Shijiazhuang 050021, China; [3]College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

Author for correspondence:
Jin Xu
Tel: +86 311 85871745
Email: xujin@sjziam.ac.cn

Received: *29 April 2012*
Accepted: *3 June 2012*

New Phytologist (2012)
doi: 10.1111/j.1469-8137.2012.04236.x

Key words: accumulation, amino acid, cadmium (Cd), organic acid, *Solanum nigrum*, *Solanum torvum*, tolerance.

Summary

- Under cadmium (Cd) stress, *Solanum nigrum* accumulated threefold more Cd in its leaves and was tolerant to Cd, whereas its low Cd-accumulating relative, *Solanum torvum*, suffered reduced growth and marked oxidative damage. However, the physiological mechanisms that are responsible for differential Cd accumulation and tolerance between the two *Solanum* species are largely unknown.
- Here, the involvement of antioxidative capacity and the accumulation of organic and amino acids in response to Cd stress in the two *Solanum* species were assessed.
- *Solanum nigrum* contains higher antioxidative capacity than does *S. torvum* under Cd toxicity. Metabolomics analysis indicated that Cd treatment also markedly increased the production of several organic and amino acids in *S. nigrum*. Pretreatment with proline and histidine increased Cd accumulation; moreover, pretreatment with citric acid increased Cd accumulation in leaves but decreased Cd accumulation in roots, which indicates that its biosynthesis could be linked to Cd long-distance transport and accumulation in leaves.
- Our data provide novel metabolite evidence regarding the enhancement of citric acid and amino acid biosynthesis in Cd-treated *S. nigrum*, support the role of these metabolites in improving Cd tolerance and accumulation, and may help to provide a better understanding of stress adaptation in other *Solanum* species.

Introduction

Cadmium (Cd) is one of the most toxic heavy metal elements, which negatively affects plant growth and development (Macek *et al.*, 2002). Many studies have attempted to clarify the mechanism of Cd toxicity in plants (Scandalios, 2005; Küpper *et al.*, 2007; Xu *et al.*, 2009; Gao *et al.*, 2010). Although Cd is toxic to most plants, several species can accumulate high amounts of Cd without any sign of toxicity (Van de Mortel *et al.*, 2008). These species are called hyperaccumulators (Baker & Brooks, 1989). By contrast, to prevent Cd accumulation in shoot tissues, several low Cd-accumulating species have evolved various mechanisms to restrict the entry of Cd to the xylem (Van de Mortel *et al.*, 2008; Yamaguchi *et al.*, 2010).

A typical symptom of Cd toxicity is oxidative damage. Cd toxicity leads to the generation of reactive oxygen species (ROS) by displacing Fe from proteins and inhibiting the electron transport chain in the chloroplast and mitochondria in plants. The excessive ROS react with lipids, proteins and pigments, which results in membrane damage and enzyme inactivation (Scandalios, 2005). Several studies have suggested that the inhibition of antioxidative systems by Cd also promotes ROS production (Schutzendubel *et al.*, 2001; Cho & Seo, 2005; Wang *et al.*, 2008). Research has indicated that the inhibition of photosynthesis is a primary damage mechanism in plants exposed to Cd even at lower concentrations and in Cd hyperaccumulators (Zhou & Qiu, 2005; Küpper *et al.*, 2007). Cd toxicity inhibited photosynthetic electron transport and the activity of photosystem II (PSII), as shown by a decreased variable fluorescence, and thereby induced oxidative stress (McCarthy *et al.*, 2001; Romero-Puertas *et al.*, 2004). Cd does not participate in Fenton-type reactions; therefore, it can only indirectly lead to oxidative stress (Stoch & Bagchi, 1995; Olmos *et al.*, 2003; Romero-Puertas *et al.*, 2004; Garnier *et al.*, 2006). Thus, it is much more likely that Cd-related oxidative stress is a consequence of inhibition of photosynthesis, especially in leaves.

One of the important mechanisms that plants use to cope with Cd toxicity is the production of metal-binding peptides, known as phytochelatins (PCs) (Cobbett & Goldsbrough, 2002; Brunetti *et al.*, 2011). PCs are cysteine-rich polypeptides with the general structure of (γ-Glu-Cys)n Gly (n = 2–11) that may play a role in heavy metal detoxification and accumulation in plants (Cobbett, 2000). PCs combine Cd with high affinity. AtABCC1 and AtABCC2 are two important PC transporters for

vacuolar sequestration of Cd in *Arabidopsis*, thereby reducing Cd toxicity (Park *et al.*, 2012). Cd toxicity also affects metabolite concentrations in plants. The Cd-induced production of organic acids, such as malic acid, L-tartaric acid, acetic acid and citric acids, could desorb heavy metals from the soil matrix into the soil solution and facilitate metal transport into the xylem. This process may therefore promote the translocation of heavy metals from the roots to the shoots (Wenger *et al.*, 2003; Quartacci *et al.*, 2005; Gao *et al.*, 2010).

Amino acids are the precursors to and constituents of proteins, and they play an important role in metabolism and development. Plants that were exposed to toxic metals have also been shown to accumulate specific amino acids, which may have beneficial functions (Bassi & Sharma, 1993; Costa & Morel, 1994; Shah & Dubey, 1997; Chen *et al.*, 2003; Sharma & Dietz, 2006). The amino acids that accumulate under heavy metal stress play various roles in plants, including acting as signaling molecules, acting as osmolytes, regulating ion transport and facilitating detoxification (Smirnoff & Stewart, 1987). Krammer *et al.* (1996) reported that histidine (His) accumulation is responsible for nickel (Ni) hyperaccumulation in *Alyssum*. Salt *et al.* (1999) found that His plays an important role in Zn homeostasis in the roots, whereas organic acids are involved in xylem transport and Zn storage in the shoots of *Thlaspi caerulescens*. These researchers also observed the presence of a Ni-histidine complex in the xylem sap of these plants. A number of studies confirmed that Pro, which accumulates under heavy metal stress, may function as a protein-compatible hydrotrope (Srinivas & Balasubramanian, 1995). This could alleviate cytoplasmic acidosis and maintain an appropriate $NADP^+$/NADPH ratio that is compatible with metabolism. When the stress is overcome, the breakdown of Pro provides sufficient reducing agents to support mitochondrial oxidative phosphorylation and generation of ATP, which facilitate growth recovery in the plants (Hare & Cress, 1997). Smirnoff & Stewart (1987) reported that asparagine formed an intracellular complex with Zn, which thereby decreased its toxicity in *Deschampsia cespitosa*. Bottari & Festa (1996) reported that asparagine can bind to Cd, lead (Pb), and Zn. Several studies indicate that the complexation of heavy metals by metabolites (viz. organic acids and amino acids) may play an important role in metal detoxification, transport and accumulation (Rai, 2002; Xu *et al.*, 2009; Gao *et al.*, 2010). However, a broad understanding of the changes in metabolites in Cd-treated plants remains unclear. Further, as in most of the earlier studies, the measurement of only a subset of compounds makes it difficult to understand the full metabolic profile of the amino acid pools as they change in response to Cd stress.

Solanum nigrum is a Cd accumulator (Sun *et al.*, 2006). By contrast, *S. torvum* cv Torubamubiga is a low Cd-accumulating plant (Arao *et al.*, 2008). In a previous study, we found that several amino acid biosynthesis-related genes and several citrate and malate biosynthesis-related genes were more highly expressed in *S. nigrum* roots than in *S. torvum* roots. In the present study, we measured the concentrations of these metabolites in the roots of both species grown with or without Cd. We also compared Cd tolerance and accumulation between *S. nigrum* and *S. torvum*. We provide novel metabolite evidence regarding the enhancement of citric acid and amino acid biosynthesis and support the observation that these metabolites accumulate in Cd-treated *S. nigrum*. These findings provide a basis for the further elucidation of the molecular mechanisms that are associated with Cd accumulation and root-to-shoot long-distance transport and may help to provide a better understanding of stress adaptation in other *Solanum* species.

Materials and Methods

Plant materials and growth conditions

The seeds of *S. nigrum* L. and *S. torvum* Sw. were kindly provided by the Germplasm Bank of Wild Species in Southwest China. To obtain seedlings, the seeds were sown under sterile conditions in Petri dishes that contained MS medium (Murashige & Skoog, 1962) and solidified with 0.8% (w/v) agar (Sigma). Seven-day-old seedlings were transferred into Hoagland solution (Hoagland & Arnon, 1950) and grown in a sterilized, pathogen-free glasshouse. The cultures were maintained at 22–25°C under a 16 h photoperiod. The treatment with $CdCl_2$ was applied to 4-wk-old seedlings that were grown in Hoagland solution. The culture solution was replaced every 3 d.

Detection of ROS

To measure the O_2^- content, the treated plant materials (0.5 g) were ground in liquid nitrogen. The obtained powder was suspended in 50 mM phosphate-buffered saline (PBS) buffer (pH 7.8). After centrifugation (15 min, 12 000 ***g***), the supernatant was used for O_2^- content measurements as previously described (Verma & Mishra, 2005).

For H_2O_2 content determination, the tissue powder was suspended in 100 mM PBS buffer (pH 7.8) that contained 1% (w/v) polyvinylpyrrolidone (PVP). After centrifugation (20 min, 12 000 ***g***), the supernatant was used for H_2O_2 content measurements as described by Verma & Mishra (2005).

The amount of O_2^- in the leaves and roots was monitored by incubation and infiltration under vacuum for 3 h and 20 min, respectively, in a solution of 2 mM nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich) in 20 mM phosphate buffer (pH 6.1) containing 10 mM NaN_3. The reaction was stopped by transferring the seedlings into distilled water. For localizing the H_2O_2 that was produced by the leaves, the treated leaves were immersed and infiltrated under vacuum with 1 mg ml^{-1} of 3,3′ diaminobenzidine (DAB) (D5637, Sigma-Aldrich) (pH 3.8) for 3 h and cleared by boiling in alcohol (95%, v/v) for 5 min. No coloration was observed when infiltration was carried out in the presence of ascorbic acid (AsA), thus confirming the H_2O_2 specificity of DAB staining, in accordance with previous reports (Thordal-Christensen *et al.*, 1997; Lee *et al.*, 2002; Dutilleul *et al.*, 2003). Photos were taken using a Carl Zeiss Imaging System. The Cd-induced H_2O_2 accumulation in the root tips was monitored using the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA). The treated roots were incubated and

infiltrated in the dark under vacuum with 10 μM DCFH-DA (Beyotime, Beijing, China) in PBS buffer for 30 min at 37°C, washed three times in PBS buffer and then viewed under a Leica laser scanning confocal microscope (excitation, 488 nm; emission, 530 nm).

Measurement of oxidative damage

The Cd-induced oxidative damage (membrane liquid peroxidation) was estimated by measuring the malondialdehyde (MDA) concentrations. Fresh plant tissues were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) solution. After centrifugation (15 min, 12 000 ***g***), an aliquot of the supernatant was added to 0.5% thiobarbituric acid (TBA) in 20% TCA and heated at 90°C for 30 min. After cooling on ice, the mixture was centrifuged at 8000 ***g*** for 5 min. The absorbance was recorded at 532 and 600 nm. The MDA concentration was calculated from the difference between the absorbance values at 532 and 600 nm (Ben Amor *et al.*, 2005).

To assess the amount of cell death in the root tips exposed to Cd stress, the roots were immersed for 1 min in 3 μg ml^{-1} propidium iodide (PI) that was dissolved in distilled water. After they were washed, the samples were examined under a fluorescence microscope using an excitation wavelength of 546 nm. For each treatment, nine roots were analyzed using a compound microscope (Zeiss Axioskop).

Antioxidant analyses

To measure the reduced glutathione (GSH) and oxidized glutathione (GSSG) content, 200 mg of fresh material was extracted in 0.1 M HCl, and the extract was incubated on ice for 30 min. After centrifugation at 20 000 ***g*** for 10 min, the supernatant was used to measure the glutathione content by high-performance liquid chromatography (HPLC) using the monobromobimane derivatization method; GSSG was analyzed by treatment with dithiothreitol (DTT) and was determined as total GSH as described by Bechtold *et al.* (2004).

The AsA contents were measured using an HPLC method as described by Esparaza Rivera *et al.* (2006). The fresh plant materials were immediately frozen and ground in liquid nitrogen. AsA was extracted in ice-cold 5% (w/v) metaphosphoric acid that contained 1% (w/v) DTT by vortexing; the solution was oscillated at 4°C for 30 min and then centrifuged at 8000 ***g*** for 15 min. The supernatant was filtered through a 0.45 μm Millipore filter. The samples were analyzed by HPLC using an Inertsil C4 column that was run with a phosphoric acid/methanol gradient, and the absorbance was recorded at 254 nm.

To measure the antioxidative enzyme activity, the treated plant materials (0.5 g) were ground in liquid nitrogen to extract the total protein. The obtained powder was suspended in 3 ml of extraction buffer that contained 50 mM sodium phosphate buffer (pH 7.5), 1% (w/v) PVP and 0.1 mM EDTA. To determine the ascorbate peroxidase (APX) activity, the tissue powder was suspended in 50 mM Hepes buffer (pH 7.5) containing 1% (w/v) PVP and 0.5 mM ascorbate. After centrifugation (30 min, 12 000 ***g***), the supernatant was used for enzyme activity measurements. The enzyme activities of superoxide dismutase (SOD), catalase (CAT), and APX were measured as previously described (Xu *et al.*, 2010). The guaiacol peroxidase (POD) activity was determined by measuring the increase in the absorbance at 470 nm as a result of the oxidation of guaiacol. The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.05% guaiacol, 1 mM H_2O_2, and 50 μl enzyme in a volume of 3 ml. The activity was estimated with an extinction coefficient of 26.6 mM^{-1} cm^{-1} (Zhu *et al.*, 2004). The glutathione reductase (GR) activity was estimated by measuring the decrease of the absorbance at 340 nm as a result of NADPH oxidation (Zhu *et al.*, 2004). The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM GSSG, 0.12 mM NADPH, and 150 μl enzyme in a volume of 3 ml. The activity was estimated with an extinction coefficient of 6.2×10^3 μM^{-1} cm^{-1}. One unit of GR was defined as 1×10^3 μM GSSG reduced min^{-1}.

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis

Four-week-old plants grown in Hoagland solution were treated with 50 μM $CdCl_2$ for 12 h, or 1, 3, or 5 d. The treated roots were immersed in a solution that contained 1 mM EDTA for 2 h and then thoroughly rinsed with distilled water. The samples were oven-dried at 75°C for 48 h. The dried plant tissues were ground and digested in concentrated nitric acid for 2–3 d at room temperature. The samples were then boiled for 1–2 h until they were completely digested. After adding 4 ml of Millipore-filtered deionized water and a brief centrifugation, the contents of Cd, Zn, Fe, Mn and Cu were determined using ICP-MS. Each experiment was repeated at least five times.

Measurement of Cd^{2+} flux with the scanning ion-selective electrode technique (SIET)

The net Cd^{2+} flux was measured noninvasively using SIET (BIO-001A; Younger USA Sci. & Tech. Corp., Beijing, China). Ion-selective microelectrodes with an external tip diameter of *c.* 3 μm were manufactured and silanized with tributylchlorosilane, and the tips were backfilled with a commercially available ionselective cocktail (Cd Ionophore I, 20909, Fluka, Switzerland). The microelectrodes were calibrated in 50 and 500 μM Cd^{2+} before the flux measurement. Only the electrodes with Nernstian slopes > 25 mV per decade were used (Ma *et al.*, 2010). After exposure to 50 μM $CdCl_2$ for 24 h, the root segments were sampled for the Cd^{2+} flux measurement. The measuring solution contained 50 μM $CdCl_2$, 100 μM KCl, 20 μM $CaCl_2$, 20 μM $MgCl_2$, 500 μM NaCl, 100 μM Na_2SO_4 and 300 μM 2(N-morpholino) ethane sulfonic acid (MES), pH 5.7. The Cd^{2+} flux data were recorded for a period of 10–15 min. The flux data were obtained with the ASET software, which is part of the SIET system. The three-dimensional ionic fluxes were calculated using MageFlux (http://xuyue.net/mageflux). The negative values in the figure represent the cation influx or anion efflux and vice versa.

Organic acid content

The treated roots (0.5 g) were ground in liquid nitrogen and then transferred to 2 ml of a 0.5 M HCl solution for extraction at 60°C for 1 h. The suspension was centrifuged at 8000 ***g*** for 10 min. After it was filtered through a 0.45 μm Millipore filter, the malic acid and citric acid contents were determined by HPLC using a reverse-phase C18 column and a Waters 996 Photodiode array detector that was set at 210 nm. The mobile phase was 20 mM potassium dihydrogen phosphate (pH 2.25) with a flow rate of 0.8 ml min^{-1} (Sun *et al.*, 2006).

Amino acid quantification by LC-MS/MS Q-TRAP

Frozen tissue powder (100 mg) was added to 500 μl of cold (–20°C) metabolite extraction solution (85% (v/v) HPLC-grade methanol, 15% (v/v) MilliQ water, and 100 mg l^{-1} ribitol), and it was then vortexed briefly and shaken at 130 ***g*** for 20 min at 65°C. After a 10 min centrifugation at 15 000 ***g***, the supernatant (40 μl) was labeled with iTRAQ reagents (AA 45/32 kit, Applied Biosystems) as recommended by the manufacturer and analyzed on an Applied Biosystems 3200 Q TRAP LC-MS/MS system that was equipped with a RP-C18 column (150 mm length, 4.6 mm diameter, 5 mm particle size) (Spitzner *et al.*, 2008).

Feeding assay

To study the effects of pretreatment with organic and amino acids on Cd accumulation in plants, the seedlings were pretreated with 0.5 mM malic acid, citric acid, glycine, proline, histidine, alanine, or serine for 1 h before they were transferred to fresh Hoagland solution containing 50 μM $CdCl_2$. After 1, 3, and 5 d, the seedlings of both species were harvested and dried before Cd analyses.

Statistical analysis

For each treatment, at least eight plants were analyzed; all the experiments were repeated at least three times. The results are presented as means ± SD. For statistical analysis, we used ANOVA and Tukey's test with the SPSS 16.0 software package (SPSS, Chicago, Illinois, USA). Differences between the treatments were tested by the least significant difference (LSD) test at a 0.05 probability level.

Results

Effect of Cd on the photosynthetic efficiency and oxidative damage

For comparison of Cd tolerance in the two species, the effect of 50 μM Cd on the photosynthetic efficiency of *S. nigrum* and *S. torvum* was analyzed. As shown in Fig. 1, *S. nigrum* has a higher photosynthetic efficiency than *S. torvum*. Cd treatment inhibited the light-dependent electron transport rate, and the inhibitory effect was more obvious in *S. torvum* than in *S. nigrum*. The lowest photosynthetic rate was observed for *S. torvum* with Cd.

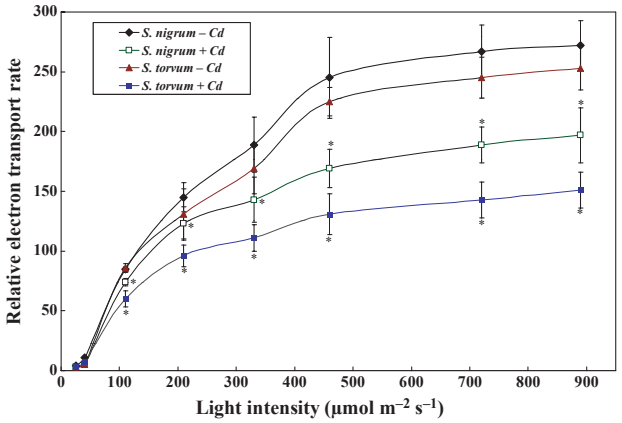

Fig. 1 Light intensity-dependent electron transport rate. The relative electron transport rate was calculated from the product of Φ_{PSII} (for electron flux through PSII) and light intensity (μmol m^{-2} s^{-1}) for *Solanum nigrum* and *Solanum torvum*. Values are means ± SD.The asterisks indicate the values that were significantly different from their appropriate controls (without Cd) ($P < 0.05$).

Reactive oxygen species are formed when stress or malfunctions impede electron flow in the photosynthetic electron transport chain (Freeman *et al.*, 2010). Cd-induced ROS accumulation and the resulting oxidative damage have been broadly reported in plants, and it has been used to indicate the degree of damage (Rodriguez-Serrano *et al.*, 2009; Xu *et al.*, 2009). To further compare the Cd tolerance in the two species, the effects of Cd toxicity on the production of ROS were analyzed. In plants treated with 50 μM Cd for 1 and 5 d, the O_2^- accumulation in *S. nigrum* leaves and roots was lower than that in *S. torvum* (Fig. 2a,b). Similarly, the H_2O_2 accumulation in *S. nigrum* leaves and roots was lower than in *S. torvum* grown with Cd (Fig. 2c,d).

The production of ROS in the two *Solanum* species was also analyzed *in situ* using stains that were sensitive to superoxide and hydrogen peroxide (Fig. 3a,b). The DCFH-DA fluorescent probe, DAB, and NBT staining techniques have been successfully used for H_2O_2 and O_2^- detection in plants (Ramel *et al.*, 2009; Xu *et al.*, 2010). Several studies described the limitations in the use of ROS probes and reagents with excised leaves or whole plantlets (Hideg *et al.*, 2002; Ramel *et al.*, 2009). However, vacuum infiltration has been successfully used on excised leaves under various experimental conditions (Hideg *et al.*, 2002; Xie *et al.*, 2008; Freeman *et al.*, 2010). Moreover, under the conditions of the present work, whatever the dye used (and therefore the ROS detected), the nonstressed or stressed plants presented expected responses related to ROS production (Fig. 3). As shown in Fig. 3(a,b), the leaves and roots of *S. torvum* displayed higher Cd-induced H_2O_2 and O_2^- accumulation than those of *S. nigrum*.

We next examined the degree of oxidative damage in the leaves and roots by determining the amount of lipid peroxidation, which was estimated from the MDA content (Barclay & McKersie, 1994). As shown in Fig. 4(a), treatment with Cd markedly increased the MDA concentrations by 2.38- and 2.36-fold in the leaves, and 1.56- and 2.34-fold in the roots of

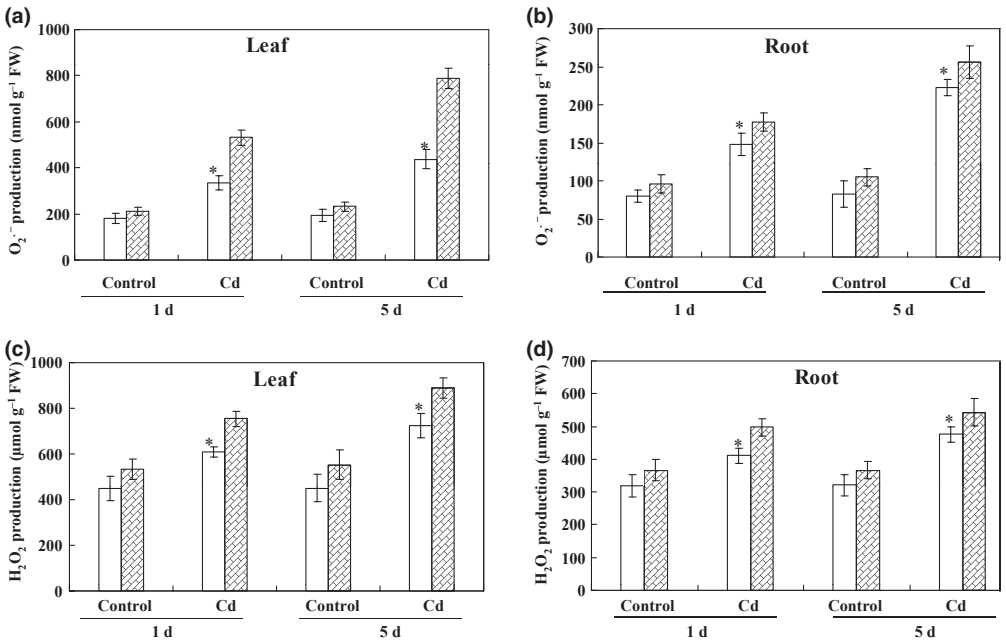

Fig. 2 Cadmium (Cd)-induced production of superoxide (O_2^-; a, b) and hydrogen peroxide (H_2O_2; c, d) in 4-wk-old *Solanum nigrum* (open bars) and *Solanum torvum* (hatched bars) plants that were exposed to 50 μM $CdCl_2$ for 1 and 5 d. Values are means ± SD. The asterisks indicate the values that were significantly different from those of *S. torvum* ($P < 0.05$).

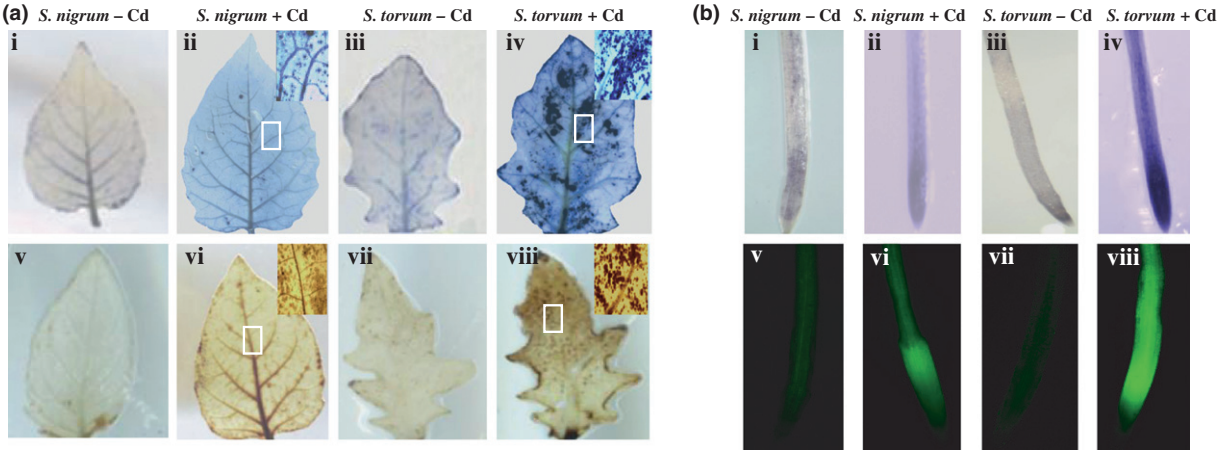

Fig. 3 Cadmium (Cd)-induced superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as visualized by *in situ* reactive oxygen species (ROS) staining in the leaves (a) and roots (b) of 4-wk-old plants that were exposed to 50 μM $CdCl_2$ for 5 d. (a) The O_2^- accumulation was measured in the leaves of *Solanum nigrum* (i, ii) and *Solanum torvum* (iii, iv) using nitroblue tetrazolium (NBT) staining. The H_2O_2 accumulation was measured in the leaves of *S. nigrum* (v, vi) and *S. torvum* (vii, viii) using DAB staining. (b) The O_2^- accumulation was measured in the roots of *S. nigrum* (i, ii) and *S. torvum* (iii, iv) using NBT staining. The H_2O_2 accumulation was measured in the roots of *S. nigrum* (v, vi) and *S. torvum* (vii, viii) using the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA).

S. nigrum and *S. torvum*, respectively, when compared with the controls. After Cd treatment, the MDA concentrations in *S. torvum* were higher in the leaves and roots than in those in *S. nigrum*. The integrity of the plasma membrane (PM) was also examined using PI staining. PI is a membrane-impermeable dye that binds to nucleotides; it is generally excluded from living cells. A PI-positive nucleus strongly indicates a loss of membrane integrity (De Cnodder *et al.*, 2005). Consistent with the phenomenon of ROS accumulation in the roots, the results from the PI staining showed that the exposure to Cd led to obvious cell death in the root tips after 5 d of treatment, and *S. torvum* showed a stronger red fluorescence in the roots compared with *S. nigrum* (Fig. 4b). These results indicate that *S. nigrum* plants experienced less oxidative damage than *S. torvum* plants.

Quantification of antioxidants

The differences observed between *S. nigrum* and *S. torvum* regarding the Cd-induced ROS and cell death led us to investigate the amounts of antioxidants that were present in the roots and leaves of both species. Several important nonenzymatic and enzymatic antioxidants were examined in this study (Fig. 5).

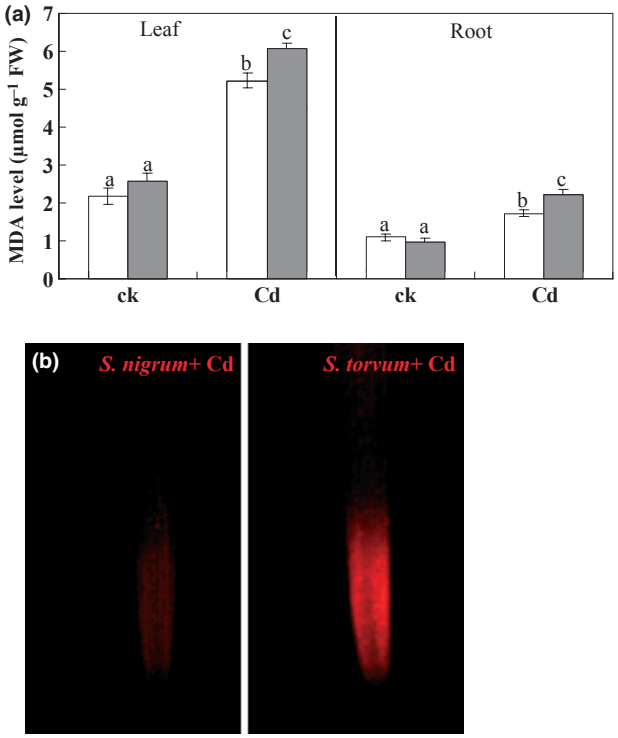

Fig. 4 (a) Malondialdehyde (MDA) concentrations in the leaves and roots of 4-wk-old *Solanum nigrum* (open bars) and *Solanum torvum* (closed bars) plants that were grown in Hoagland solution containing 50 μM $CdCl_2$ for 5 d. Values are means ± SD. The columns that are labeled with different letters were significantly different at $P < 0.05$. (b) The effect of cadmium (Cd) stress on the plasma membrane (PM) integrity. The roots of *S. nigrum* and *S. torvum* were excised and then stained with 3 μg ml^{-1} of propidium iodide (PI) for 1 min as described in the Materials and Methods section. ck, untreated control; Cd, 50 μM $CdCl_2$.

Compared with *S. torvum*, *S. nigrum* contained more GSH and GSSG when it was grown without Cd. After 1 d of Cd treatment and compared with *S. torvum*, *S. nigrum* accumulated higher concentrations of GSH. The glutathione redox state (GSH : GSSG ratio) in the plants that were grown without Cd was 5.64 in leaves and 6 in the roots for *S. nigrum*, and 7.33 in the leaves and 8.67 in the roots for *S. torvum*; the plants that were grown with Cd showed a ratio of 5.92 in the leaves and 4.78 in the roots of *S. nigrum*, and 3.75 in the leaves and 1.83 in the roots of *S. torvum* (Supporting Information, Fig. S1). Therefore, although the concentrations of the GSH : GSSG ratio in *S. torvum* were higher than those in *S. nigrum* when the plants were grown in normal conditions, the GSH : GSSG ratios dropped more severely (1.95-fold reduction in the leaves and 4.74-fold reduction in the roots) in *S. torvum* than in *S. nigrum* (1.05-fold increase in the leaves and 1.25-fold reduction in roots) after 24 h of Cd treatment (Figs 5a,b, S1).

We also measured AsA, which is another important antioxidant molecule in plants. As shown in Fig. 5(c), the AsA concentrations in the *S. nigrum* leaves were higher than those in *S. torvum* with or without Cd treatment. Treatment with Cd reduced the accumulation of AsA in *S. nigrum* roots; however, there was no significant effect on the AsA concentration in *S. torvum* roots.

We next examined the activities of several antioxidative enzymes (Fig. 5d–h). Compared with *S. torvum*, the activities of superoxide dismutase (SOD), CAT, APX and GR were higher in *S. nigrum*, whereas the POD activity was lower after 24 h of exposure to 50 μM $CdCl_2$.

Metabolic analysis of organic and amino acids

Because the transcriptome analyses showed differences in the constitutive (−Cd) and inducible (+Cd) expression levels of genes that were associated with the biosynthesis and metabolic processes of organic and amino acids, we measured the concentrations of these metabolites in the roots of both species grown with or without Cd. Organic acid compounds are often associated with heavy metal stresses and long-distance metal transport, and they are considered to be a good indicator of Cd accumulation. We found that in the plants grown without Cd, the concentrations of malic acid and citric acid in *S. nigrum* were 1.57-fold and 1.88-fold higher than in *S. torvum*, respectively, and in the plants grown with Cd, the malic acid and citric acid concentrations in *S. nigrum* were 2.03-fold and 5.1-fold higher than in *S. torvum*, respectively (Table 1).

An analysis of the whole amino acid metabolite pools revealed a greater response to Cd in *S. nigrum* roots than in the roots of *S. torvum*. Twenty-six amino acids and their derivatives were detected in the roots of *S. nigrum* and/or *S. torvum* (Table 1, Fig. 6). In the roots of plants that were grown without Cd, six amino acids or derivatives were more highly accumulated in the *S. nigrum* roots (Ala, Gly, Tyr, EtN, Asp, and Glu), whereas another six amino acids or derivatives were more highly accumulated in the *S. torvum* roots (Cit, Ser, Thr, Orn, bAib, and GABA). After 24 h of Cd treatment, 67% of these amino acids or derivatives (18 amino acids or derivatives) showed an elevated accumulation in the *S. nigrum* roots compared with the untreated control, whereas only 11% of these amino acids or derivatives (three amino acids or derivatives) showed an elevated accumulation in the *S. torvum* roots. By contrast, 63% of these amino acids or derivatives (17 amino acids or derivatives) showed a reduced accumulation in the *S. torvum* roots, whereas only 22% of these amino acids or derivatives (six amino acids) showed a reduced accumulation in the *S. nigrum* roots (Table 1, Fig. 6). Two amino acids (Abu and bAla) showed similar concentrations in both plants and were markedly reduced after Cd treatment. In the Cd-supplied plants, only Hyp, bAib and GABA showed a higher accumulation in the *S. torvum* roots than in *S. nigrum*. Therefore, in comparison with the low Cd accumulator *S. torvum*, the Cd accumulator *S. nigrum* tended to contain higher concentrations of organic acids and amino acids, especially when it was grown in the presence of Cd.

Physiological effects of organic and amino acids on Cd tolerance and accumulation

Transcriptome analyses revealed differences in the expression levels of genes that were associated with the biosynthesis and metabolic processes of organic and amino acids. Furthermore,

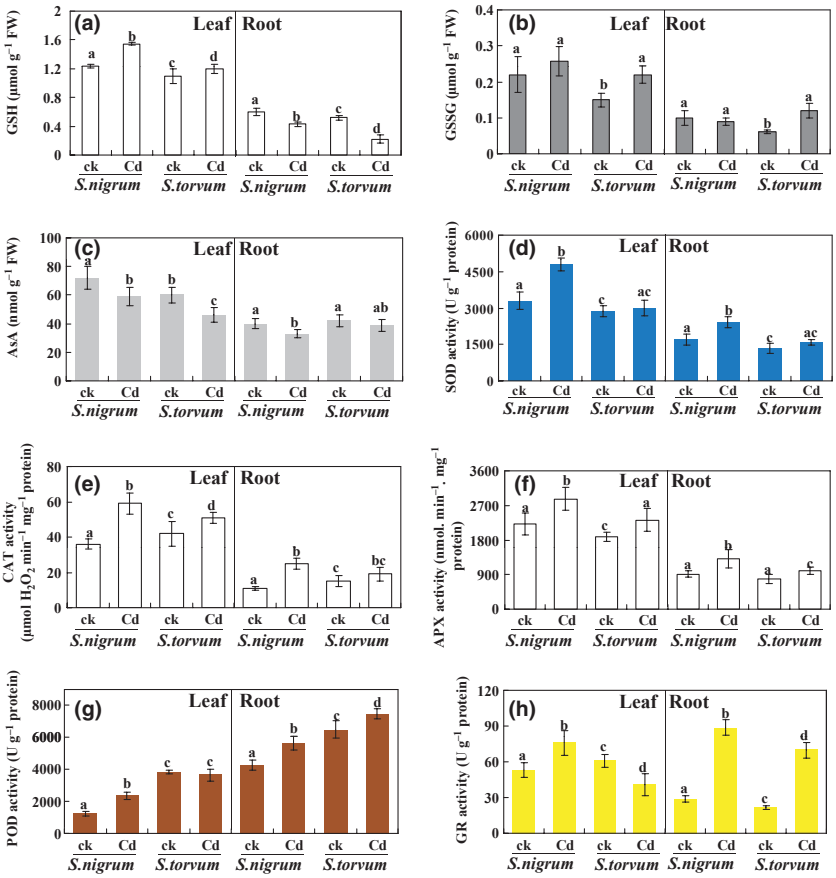

Fig. 5 Antioxidant content and antioxidative enzyme activity in *Solanum nigrum* and *Solanum torvum* plants grown with or without 50 μM $CdCl_2$ for 1 d. (a) Reduced glutathione (GSH) concentration, (b) oxidized glutathione (GSSG) concentration, (c) ascorbic acid (AsA) concentrations, (d) superoxide dismutase (SOD) activity, (e) catalase (CAT) activity, (f) ascorbate peroxidase (APX) activity, (g) peroxidase (POD) activity, (h) glutathione reductases (GR) activity. Values are means ± SD. The different letters indicate the values that were significantly different at $P < 0.05$. ck, untreated control; Cd, 50 μM $CdCl_2$.

our metabolic analysis indicated that *S. nigrum* contains higher concentrations of organic acids and amino acids than *S. torvum*. These observations indicated that these organic and amino acids may be involved in Cd tolerance and accumulation. Several reports also highlighted that the exogenous addition of organic and amino acids can enhance heavy metal tolerance in plants (Srinivas & Balasubramanian, 1995; Hare & Cress, 1997; Salt *et al.*, 1999; Wenger *et al.*, 2003). To test whether the differential accumulation of these organic and amino acids contributed to the Cd tolerance and accumulation in *S. nigrum* and *S. torvum*, we supplemented the plants with these organic and amino acids and assessed plant performance with MDA, PM integrity, and Cd accumulation in the roots. We applied these compounds as pretreatments to avoid the pH changes that they may cause in hydroponic solutions (Xu *et al.*, 2009). Application of these compounds alone did not affect the MDA concentration and PM integrity in seedlings without Cd treatment (data not shown). Pretreatment with 0.5 mM malic acid or citric acid effectively reduced the MDA concentrations in both *S. nigrum* and *S. torvum* plants (Table 2), supporting the positive effect of these organic acids on reducing the amount of lipid peroxidation in Cd-treated seedlings. Similarly, supplementation with Gly, Pro, His, Ala, and Ser reduced the MDA concentrations in both species, which suggests that these amino acids positively affect Cd tolerance in plants by reducing oxidative damage. Because exogenous organic and amino acids reduced the amount of lipid peroxidation in the two species exposed to Cd stress, we examined the effect of exogenous organic and amino acids on cell survival by two different methods, Evans blue staining and electrolyte leakage analysis, which both measure PM integrity. These tests showed similar results: all of these supplemented metabolites improved PM integrity of the two species under Cd stress (Table 2). However, supplementation with Phe, Thr, Met, Asn, Leu, Val, Ile, and Tyr did not show significant effect on Cd tolerance in the two species (data not shown).

Organic acid- and amino acid-mediated ion uptake in plants has been previously reported (Quartacci *et al.*, 2005; Gao *et al.*, 2010). To investigate whether the Cd-induced production of these compounds is related to Cd accumulation in plants, we measured the Cd content in seedlings of both species. After pretreatment with 0.5 mM malic acid, citric acid, Gly, Pro, His, Ala, or Ser for 1 h, the plants were subsequently transferred to fresh Hoagland solution containing 50 μM $CdCl_2$ for 5 d, and the Cd content was measured by ICP-MS. As shown in Fig. 7, compared with the Cd treatment alone, a significant increase in the Cd content was observed in the leaves of both species pretreated with citric acid, Pro, or His. However, the Cd contents were not significantly altered in the leaves of the two species that were pretreated with malic acid, Gly, Ala, or Ser.

To complement our observation that pretreatment with citric acid, Pro, or His increased Cd accumulation in *S. nigrum* and *S. torvum* leaves, we also measured the Cd accumulation in the

Table 1 Metabolomic analysis of the *Solanum nigrum* and *Solanum torvum* roots in response to cadmium (Cd)

Metabolites	Abbr.	Snck (μg g^{-1} FW)	Sntr (μg g^{-1} FW)	Stck (μg g^{-1} FW)	Sttr (μg g^{-1} FW)
L-tyrosine	Tyr	0.5 ± 0.15b	5.23 ± 0.8a	UD	0.58 ± 0.12b
L-tryptophan	Trp	UD	5.5 ± 1.15a	UD	0.24 ± 0.03b
L-phenylalanine	Phe	0.34 ± 0.04b	3.46 ± 0.445a	0.34 ± 0.04b	0.18 ± 0.03c
L-leucine	Leu	1.37 ± 0.38b	3.92 ± 0.445a	1.06 ± 0.17b	0.26 ± 0.035c
L-citrulline	Cit	0.64 ± 0.045c	2.41 ± 0.34a	1.36 ± 0.2b	0.32 ± 0.045d
L-alanine	Ala	1.78 ± 0.215b	12.5 ± 1.55a	1.18 ± 0.14c	0.73 ± 0.16d
L-isoleucine	Ile	0.35 ± 0.06b	4.01 ± 0.38a	0.3 ± 0.035b	0.19 ± 0.035c
L-valine	Val	0.44 ± 0.075b	5.21 ± 0.45a	0.36 ± 0.04b	0.24 ± 0.03c
L-serine	Ser	1.73 ± 0.23c	52.9 ± 5.5a	2.8 ± 0.35b	1.62 ± 0.135c
L-proline	Pro	2.2 ± 0.55b	3.04 ± 0.35a	2.76 ± 0.335b	0.64 ± 0.17c
L-threonine	Thr	UD	8.05 ± 1.6a	4.94 ± 0.65b	1.65 ± 0.39c
L-ornithine	Orn	UD	28.8 ± 4.15a	1.44 ± 0.175b	UD
Glycine	Gly	2.74 ± 0.18b	24.8 ± 3.15a	1 ± 0.25c	0.8 ± 0.225c
L-lysine	Lys	UD	5.83 ± 1.15	UD	UD
L-methionine	Met	UD	1.64 ± 0.205	UD	UD
L-asparagine	Asn	UD	2.17 ± 0.25	UD	UD
L-histidine	His	UD	9.76 ± 1.6	UD	UD
citric acid	CA	15 ± 1.4b	37 ± 4.2a	8 ± 0.56c	7.3 ± 0.34c
D,L-β-amino-isobutyric acid	bAib	0.33 ± 0.045b	0.03 ± 0.015d	0.63 ± 0.16a	0.05 ± 0.03c
β-alanine	bAla	4.33 ± 0.5a	1.11 ± 0.15b	3.8 ± 0.655a	0.8 ± 0.25b
L-α-amino-n-butyric acid	Abu	39.5 ± 4.5a	10.3 ± 2b	36.4 ± 4.3a	12.4 ± 2.15b
γ-amino-n-butyric acid	GABA	0.56 ± 0.04c	0.34 ± 0.044d	1.3 ± 0.225a	0.78 ± 0.175b
L-aspartic acid	Asp	9.39 ± 1a	7.6 ± 1.6b	1.68 ± 0.25c	0.81 ± 0.115d
Ethanolamine	EtN	0.28 ± 0.085a	0.19 ± 0.01b	0.17 ± 0.025b	0.2 ± 0.017ab
hydroxy-L-proline	Hyp	UD	UD	UD	0.48 ± 0.035
L-glutamine	Gln	3.99 ± 0.335a	3.97 ± 0.5a	4.27 ± 0.5a	4.63 ± 1.05a
L-glutamic acid	Glu	3.67 ± 0.415a	3.58 ± 0.7a	2.31 ± 0.33b	1.95 ± 0.225b
L-cysteine	Cys	UD	UD	UD	UD
Malic acid	MA	72 ± 8.5a	79 ± 5.0a	46 ± 3.2b	39 ± 7.5b

Snck, control for *S. nigrum* roots; Stck, control for *S. torvum* roots; Sntr, Cd-treated *S. nigrum* roots; Sttr, Cd-treated *S. torvum* roots. The different letters within each line indicate significant differences at $P < 0.05$. UD, undeterminable.
Orange, up-regulated amino acids compared with the control; green, down-regulated amino acids compared with the control; gray, the changes on amino acid abundance are insignificant or unchanged.

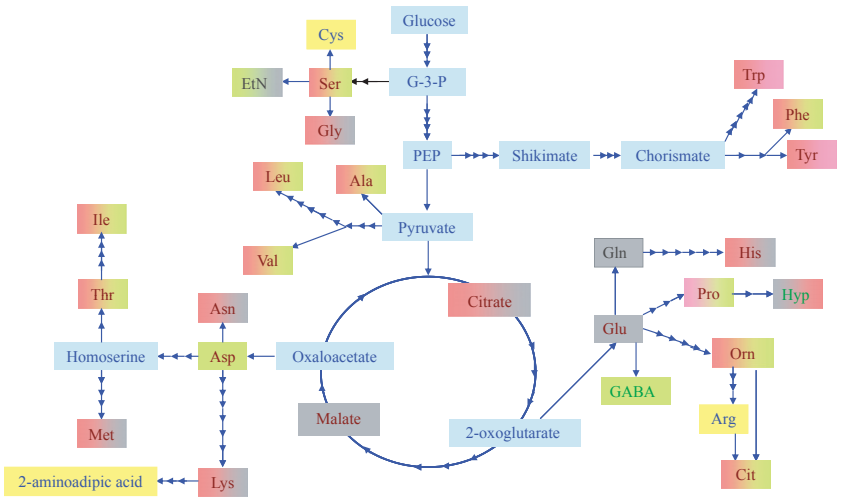

Fig. 6 Effect of cadmium (Cd) stress on metabolite concentrations in *Solanum nigrum* and *Solanum torvum* roots. Metabolites that have significantly higher or lower abundance in Cd-treated roots of *S. nigrum* (left) or *S. torvum* (right) compared with their untreated control ($P < 0.05$) are represented by red (higher) and green (lower) boxes. The gray boxes represent metabolites whose abundance is unchanged. Red and green letters represent metabolites that are significantly more abundant in Cd-treated roots of *S. nigrum* or *S. torvum*, respectively ($P < 0.05$). The gray letters represent metabolites whose abundance is similar between the two species after Cd treatment. The yellow and blue boxes represent metabolite concentrations that are undeterminable or not determined, respectively. All data were extracted from Table 1and the abbreviations are described there. PEP, phosphoenolpyruvate; G-3-P, glyceraldehyde-3-phosphate.

Table 2 Effects of exogenous organic and amino acids feeding on cadmium (Cd) tolerance in *Solanum nigrum* and *S. torvum* seedlings

	MDA concentration (μmol g^{-1} FW)				Evans Blue uptake (OD600)				Electrolyte leakage (%)			
	S. nigrum		*S. torvum*		*S. nigrum*		*S. torvum*		*S. nigrum*		*S. torvum*	
Treatment	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
ck	2.1 ± 0.3d	1.1 ± 0.07e	2.55 ± 0.34d	0.95 ± 0.4f	0.1 ± 0.04d	0.17 ± 0.06e	0.21 ± 0.03e	0.31 ± 0.04e	12.1 ± 1.3e	8.4 ± 1.1e	19.3 ± 1.1d	10.2 ± 1.2e
Cd	5.2 ± 0.29a	1.72 ± 0.12a	5.88 ± 0.33a	2.32 ± 0.15a	0.38 ± 0.04a	0.5 ± 0.06a	0.52 ± 0.05a	0.78 ± 0.07a	17.1 ± 1.8a	14.3 ± 0.9a	25 ± 3.3a	19.1 ± 2.1a
Malic acid + Cd	4.5 ± 0.34b	1.5 ± 0.12b	5.0 ± 0.3b	2.1 ± 0.05b	0.32 ± 0.01b	0.44 ± 0.03b	0.41 ± 0.06bc	0.68 ± 0.04b	15 ± 1.3c	12.2 ± 1.0b	23.7 ± 1.2b	17.1 ± 1.2bc
Citric acid + Cd	4 ± 0.41c	1.47 ± 0.1bc	4 ± 0.39c	1.7 ± 0.14c	0.28 ± 0.02bc	0.38 ± 0.04c	0.38 ± 0.06c	0.56 ± 0.04c	14.3 ± 1.5cd	10.7 ± 0.8cd	21.8 ± 0.9c	16.4 ± 0.8c
Gly + Cd	4.6 ± 0.34b	1.22 ± 0.1d	4.8 ± 0.32b	1.89 ± 0.13bc	0.3 ± 0.02bc	0.39 ± 0.03c	0.37 ± 0.04cd	0.62 ± 0.02c	16 ± 0.7b	12.8 ± 1.1b	23.1 ± 0.7b	18.3 ± 0.5b
Pro + Cd	3.8 ± 0.29	1.43 ± 0.11bc	3.95 ± 0.3	1.37 ± 0.08e	0.25 ± 0.03c	0.28 ± 0.02d	0.34 ± 0.04d	0.5 ± 0.03d	14 ± 0.9d	9.8 ± .05d	21.9 ± 0.7c	14.5 ± 0.7d
His + Cd	4.7 ± 0.34b	1.44 ± 0.12bc	4.5 ± 0.34bc	1.52 ± 0.13d	0.31 ± 0.04bc	0.31 ± 0.05d	0.36 ± 0.05cd	0.48 ± 0.08d	14.1 ± 1.1d	10.3 ± 1.1d	21.2 ± 1.0c	15 ± 1.1cd
Gln + Cd	4.6 ± 0.3b	1.5 ± 0.07b	4.2 ± 0.29c	1.7 ± 0.1c	0.31 ± 0.03b	0.42 ± 0.04b	0.47 ± 0.08b	0.7 ± 0.05b	14.8 ± 1.2c	11.3 ± 1.2c	23.4 ± 0.5b	17.8 ± 0.7b
Asn + Cd	4.41 ± 0.34c	1.4 ± 0.03c	4.68 ± 0.34b	1.78 ± 0.12c	0.33 ± 0.04b	0.4 ± 0.03bc	0.4 ± 0.02c	0.69 ± 0.04b	15.5 ± 1.3bc	12.9 ± 0.9b	22.1 ± 0.8bc	17.9 ± 0.6b
Ala + Cd	4.48 ± 0.33bc	1.34 ± 0.12c	4.67 ± 0.3b	1.73 ± 0.1c	0.34 ± 0.02b	0.44 ± 0.06b	0.44 ± 0.05b	0.61 ± 0.02c	15.1 ± 1.2c	12.1 ± 1.1b	23.6 ± 0.7b	16.7 ± 0.7c
Ser + Cd	4.3 ± 0.32c	1.42 ± 0.11bc	4.6 ± 0.4bc	1.78 ± 0.13c	0.29 ± 0.04bc	0.4 ± 0.03bc	0.42 ± 0.06bc	0.62 ± 0.03c	15.1 ± 1.4c	12.5 ± 0.8b	21.7 ± 1.8c	18 ± 0.4b

After pretreatment with 0.5 mM malic acid, citric acid, Gly, Pro, His, Ala, or Ser for 1 h, the plants were subsequently transferred to fresh Hoagland solution that containing 50 μM $CdCl_2$ for 5 d.
The different letters indicate the values that were significantly different at $P < 0.05$.
MDA, malondialdehyde; Ala, L-alanine; Asn, L-asparagine; Gln, L-glutamine; Gly, Glycine; His, L-histidine; Pro, L-proline; Ser, L-serine.

roots of both species. After pretreatment with citric acid, Pro, or His, the Cd contents in the leaves markedly increased (Fig. 8a,d); however, the root Cd contents decreased in both species following pretreatment with citric acid (Fig. 8b). We also compared the Cd leaf : root ratios in the Cd-treated plants with or without pretreatment. As shown in Fig. 8(c,f), the citric acid pretreatment markedly increased the Cd leaf : root ratios; however, there were no significant differences between the two amino acid-pretreated plants.

These results demonstrate that, although the citric acid, Pro, and His pretreatments improved Cd tolerance and accumulation, these metabolites play a different role in the Cd distribution in plants. Therefore, we wondered whether these metabolites affected the root Cd fluxes. As a result, we used SIET to detect the effects of pretreatment on Cd uptake. The Pro and His pretreatments increased Cd absorption in the roots of both species; however, the Cd influxes in the two species that were pretreated with citric acid were not affected (Fig. 9).

Discussion

In this study, we analyzed the differential responses of antioxidative content and organic and amino acid accumulation in *S. nigrum* and *S. torvum* seedlings to Cd toxicity at physiological and metabolomic concentrations. The Cd accumulator *S. nigrum* was more tolerant to Cd stress than *S. torvum*; this was determined based on photosynthetic rate, ROS accumulation, MDA concentration, electrolyte leakage, and PM integrity. Previous studies have indicated that Cd accumulation is threefold higher in *S. nigrum* leaves than in *S. torvum* leaves, which demonstrates the high Cd detoxification capability of *S. nigrum*. Our findings are consistent with previous reports that *S. nigrum* seedlings are much more tolerant to Cd toxicity than nonaccumulators and that this difference involves an adaptive response (Sun *et al.*, 2006; Xu *et al.*, 2009). These results also suggest that changes in metabolic pathways via the modulation of organic and amino acid concentrations are important for Cd tolerance in plants.

An important mechanism contributing to *S. nigrum*'s Cd tolerance may be its capacity to reduce Cd-associated oxidative stress. Electrons lost from inefficient photosynthetic electron transport can react with molecular oxygen, forming O_2^-, which then is converted to H_2O_2 and other free radical intermediates (Freeman *et al.*, 2010). Such negative effects of Cd on photosynmay be further magnified by a subsequent increase in ROS generation. Excess exposure to ROS may cause cell death in *S. torvum* leaves. *S. nigrum* contained higher concentrations of the ROS-scavenging metabolites GSH and AsA, a higher GSH : GSSG ratio, and higher antioxidative enzyme (SOD, CAT, APX, and GR) activities, which suggested that *S. nigrum* has a high free radical-scavenging capacity; this result may explain the lower ROS concentrations that were observed in *S. nigrum* in the presence of Cd (Figs 2, 3). Consistent with the observed ROS accumulation, the amounts of lipid peroxidation, which were estimated from the MDA content, the electrolyte leakage, and cell death were higher in *S. torvum*.

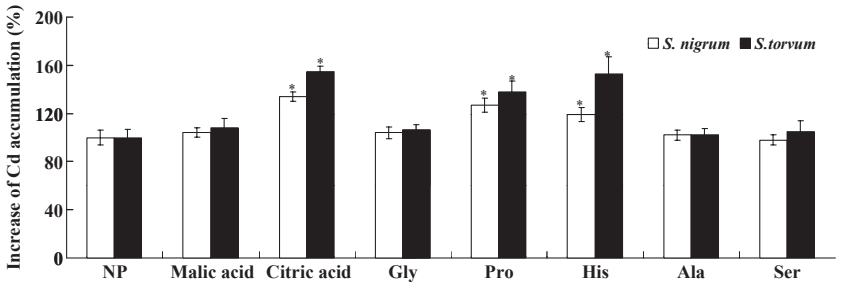

Fig. 7 Effects of exogenous organic acid and amino acid pretreatments on cadmium (Cd) accumulation in *Solanum nigrum* and *Solanum torvum* plants. After 1 d of treatment, the increase in the Cd accumulation was calculated by dividing the Cd content in the pretreated seedlings by its corresponding value in the unpretreated seedlings. Values are means ± SD. Asterisks indicate values that are significantly different from those of the plants treated with Cd alone (without pretreatment, NP) ($P < 0.05$). NP, no pretreatment; Gly, Glycine; Pro, L-proline; His, L-histidine; Ala, L-alanine; Ser, L-serine.

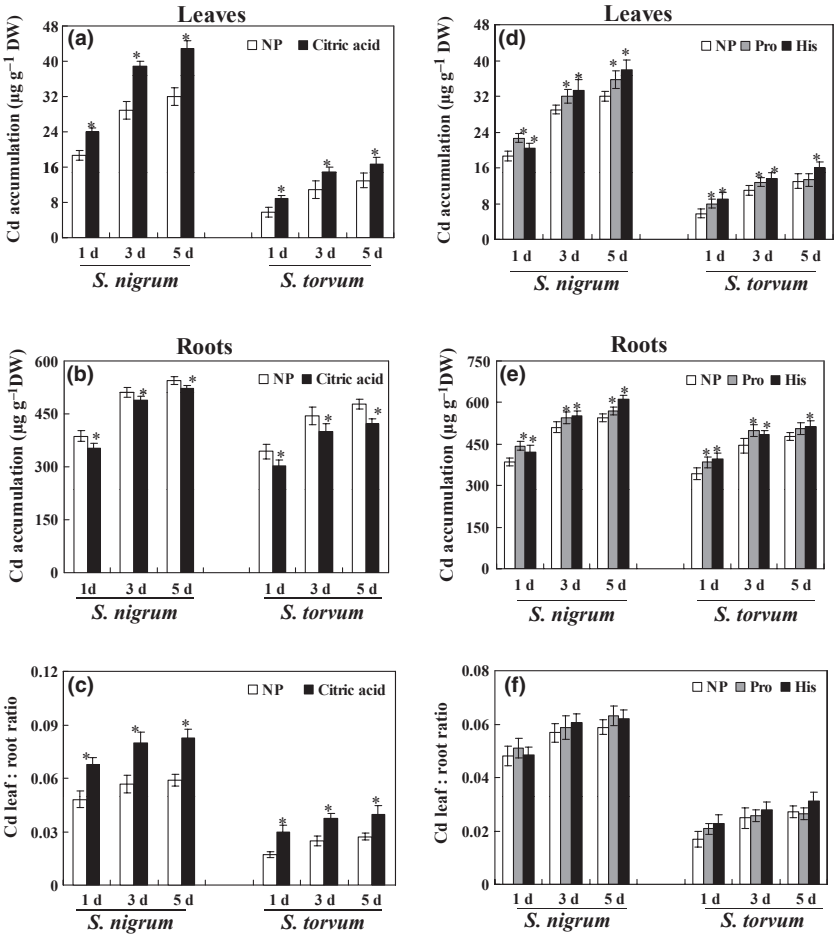

Fig. 8 Effects of exogenous organic acid (a–c) and amino acid (d–f) pretreatments on cadmium (Cd) accumulation in *Solanum nigrum* and *Solanum torvum* plants. Cd content in the leaves (a, d), roots (b, e), and the Cd leaf : root ratios (c, f) of the plants were measured. Values are means ± SD. Asterisks indicate values that are significantly different from those of the plants treated with Cd alone (without pretreatment, NP) ($P < 0.05$). NP, no pretreatment. His, L-histidine; Pro, L-proline.

The malic acid and citric acid concentrations in *S. nigrum* were higher than those in *S. torvum*, especially following Cd treatment. Previous transcriptome analysis has revealed that two citrate synthase genes, *CSY* and *CLA*, and two malate metabolism-related genes, *MDH* and *mMDH*, are more highly expressed in *S. nigrum*. Several studies have indicated that organic acids may play a role in heavy metal hyperaccumulation. The hyperaccumulator *T. caerulescens* contains high concentrations of malic acid and citric acid (Tolra *et al.*, 1996; Salt *et al.*, 1999). Sarret *et al.* (2002) found that the hyperaccumulator *A. halleri* contained constitutively high concentrations of malic acid, and Zn was predominantly complexed to malic acid in the shoots. These chelates may bind metal ions and facilitate metal transport into the xylem, which may thereby promote the root-to-shoot translocation of heavy metals (Wenger *et al.*, 2003; Quartacci *et al.*, 2005; Gao *et al.*, 2010).

Cadmium toxicity also perturbed amino acid metabolism in plants. The metabolomic analysis of amino acids revealed a greater Cd response in the roots of *S. nigrum* than in those of *S. torvum*. Using the LC-MS/MS Q-TRAP technique, we found that the accumulation of many amino acids was higher in the roots of *S. nigrum* than in the roots of *S. torvum*. Only Hyp,

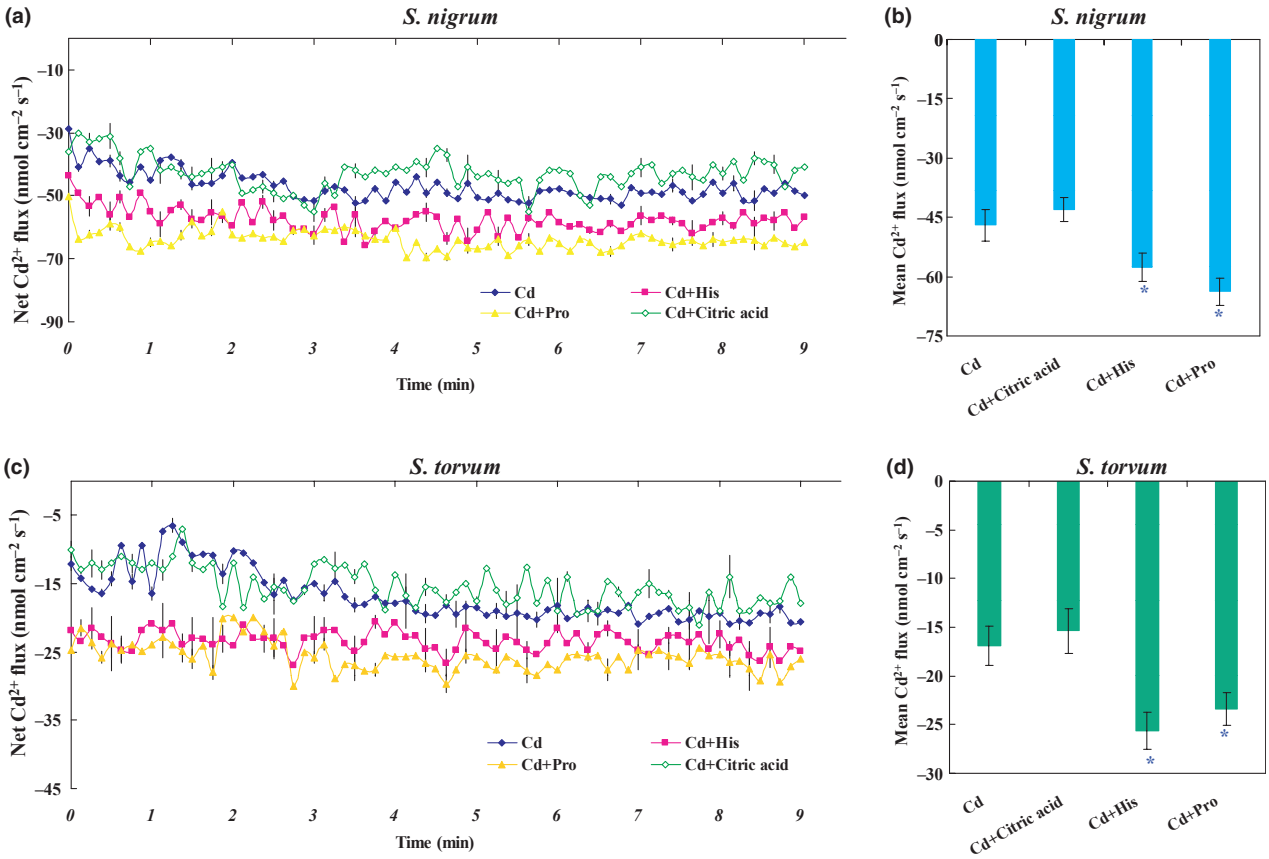

Fig. 9 Effects of exogenous citric acid, His, or Pro pretreatments on the net fluxes (a, c) and the mean fluxes (b, d) of Cd^{2+} in *Solanum nigrum* and *Solanum torvum* roots. Values are means ± SD. Asterisks indicate values that are significantly different from those of the roots treated with Cd alone (without pretreatment) ($P < 0.05$). His, L-histidine; Pro, L-proline.

bAib and GABA showed a higher accumulation in the Cd-treated *S. torvum* roots than in the *S. nigrum* roots. GABA and Pro, which are well known to be two stress markers (Narayan & Nair, 1990; Bown & Shelp, 1997), exhibited opposite abundance trends between the two *Solanum* species under Cd stress, which suggests that two different stress defense pathways exist in the two species. Pro is a precursor in the Hyp biosynthesis pathway (Fig. 6). Thus, increased consumption of Pro for Hyp biosynthesis during Cd stress could be the reason for the observed decrease in Pro content in *S. torvum* roots. Pro is an important osmoprotectant when plants are exposed to abiotic stresses. Our previous study indicated that Pro played a role in the alleviation of Cd toxicity by detoxifying ROS, thereby increasing the glutathione concentration and protecting antioxidative enzyme activities in *S. nigrum* seedlings (Xu *et al.*, 2009). A higher accumulation of Pro in *S. nigrum* supports the observed higher Cd tolerance in *S. nigrum* than in *S. torvum*. Hyp is an important component of the Casparian band. A high accumulation of Hyp in *S. torvum* roots may play a protective role in preventing Cd translocation from the roots to the aerial parts of the plant. We also observed that Glu content is not significantly affected by Cd stress, and Asp is markedly decreased in *S. nigrum* roots. This situation may be a consequence of buffering effects through the modulation of Pro/His/Orn and Asn/Lys/Thr/Ile/Met, which are enhanced in *S. nigrum*. Indeed, these amino acids are products of Glu and Asp metabolism, respectively. This result is also consistent with our previous transcriptome analysis, which revealed that an Asp biosynthesis-related gene, *ASP*, showed higher expression in *S. nigrum*. A previous transcriptome analysis also revealed that a cysteine desulfurase gene, *NFS*, which can catalyze Cys to Ala, showed higher expression in *S. nigrum*. Supporting that result, the Ala content was increased in *S. nigrum*. By contrast, Cys was decreased to undeterminable concentration. The enhanced expression of the aromatic amino acid biosynthesis-related gene *MEE* supports the observed accumulation of Trp, Phe, and Tyr in *S. nigrum* roots. Cd toxicity decreased auxin biosynthesis and thereby inhibited plant growth and development. Trp is an essential amino acid required not only for protein synthesis but also for the production of many plant metabolites, including the hormone auxin (Jing *et al.*, 2009). Thus, the accumulation of Trp could improve auxin concentrations in *S. nigrum* exposed to Cd stress. This result is consistent with the observation that *S. nigrum* grew better than *S. torvum* under Cd stress. Chaffei *et al.* (2004) suggested that an increase in the proportion of high N : C amino acids, such as Asn, Pro, and Arg in roots, is a protective strategy in plants for preserving roots as a nutritional safeguard organ to ensure future recovery. Consistent with this hypothesis, our metabolic analysis indicated the accumulation of

a large amount of amino acids in the roots of *S. nigrum*, which indicated high Cd accumulation and tolerance. The amino acid accumulation in *S. nigrum* roots also suggested that these Cd-chelating molecules are highly active in *S. nigrum* roots and that upon binding Cd, they may form a complex that can be translocated from the roots to the shoots (Couturier *et al.*, 2010). Therefore, a range of possible explanations exist for the beneficial effects of amino acid accumulation on Cd tolerance and accumulation through the retention of nutrition state, the modification of biosynthetic processes and the promotion of the long-distance transport or redistribution of Cd in plants.

The finding that Cd toxicity markedly increased the production of several organic and amino acids in *S. nigrum* and the findings that the exogenous application of these organic acids improved Cd phytoextraction in *S. nigrum* (Sun *et al.*, 2006; Gao *et al.*, 2010) suggest that these metabolites may be related to Cd-toxicity adaptation in plants. However, the physiological mechanisms of these chelates on Cd distribution and accumulation in plants were largely unclear. To better understand the effects of these metabolites on Cd accumulation and to determine whether the differential accumulation of these organic and amino acids may be involved in plant Cd tolerance and accumulation in *S. nigrum* and *S. torvum*, we performed an exogenous feeding assay and assessed plant performance with MDA and PM integrity and Cd accumulation in the leaves and roots of *S. nigrum* and *S. torvum*. Our study revealed that malic acid and citric acid reduced MDA concentrations and increased PM integrity in both *S. nigrum* and *S. torvum* plants, which supports the positive effect of these organic acids on Cd tolerance in plants. Similarly, the pretreatment with Gly, Pro, His, Ala, and Ser effectively reduced MDA concentrations and increased PM integrity in both species, which suggests that these amino acids improved Cd tolerance in plants.

Although all of these examined metabolites improved Cd tolerance, only citric acid, Pro, and His effectively increased the leaf Cd accumulation in the two species, which suggests that the constitutive up-regulation of these metabolites may be an important underlying molecular mechanism for *S. nigrum*'s Cd tolerance and accumulation. Interestingly, although citric acid increased the leaf Cd accumulation, it markedly reduced the root Cd contents. A comparison analysis of the Cd leaf : root ratios suggested that the pretreatment with citric acid increases the long-distance root-to-shoot transport of Cd. The detection of root Cd fluxes showed that citric acid did not affect the Cd influx in the two species' roots. These results indicated that the high production of citric acid in *S. nigrum* largely contributes to the long-distance root-to-shoot Cd transport and Cd accumulation in leaves.

In contrast to citric acid, although the pretreatments with Pro and His increased the Cd accumulation in the leaves and roots, they did not significantly change the Cd leaf : root ratio during the recording periods (from 1 to 5 d). A noninvasive ion flux analysis by SIET showed that Pro and His increased Cd influx in the roots. Therefore, the high accumulation of Pro and His in *S. nigrum* promoted Cd uptake and improved root-to-shoot Cd transport, which thereby increased leaf Cd accumulation.

In this study, we provide novel metabolite evidence regarding the enhancement of citric acid and amino acid biosynthesis in Cd-treated *S. nigrum* and support the role of these metabolites in improving Cd tolerance and accumulation. We also show that the exogenous application of citric acid, Pro, and His benefit *S. nigrum* and *S. torvum*, and we highlight a range of mechanisms that may be responsible for conferring Cd tolerance and accumulation. The breeding and cultivation of Cd accumulators or low Cd accumulators will benefit from an understanding of these mechanisms.

Acknowledgements

The authors would like to thank the Germplasm Bank of Wild Species in Southwest China for providing the *S. nigrum* and *S. torvum* seeds. This work was supported by the China National Natural Sciences Foundation (31170228), the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-Q-25), and the National Major Special Project on New Varieties Cultivation for Transgenic Organisms (grant no. 2009ZX08009-130B).

References

Arao T, Takeda H, Nishihara E. 2008. Reduction of cadmium translocation from roots to shoots in eggplant (*Solanum melongena*) by grafting onto *Solanum torvum* rootstock. *Soil Science and Plant Nutrition* **54**: 555–559.

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements: a review of their distribution, ecology, and phytochemistry. *Biorecovery* **1**: 81–126.

Barclay KD, McKersie BD. 1994. Peroxidation reactions in plant membranes: effects of free fatty acids. *Lipids* **29**: 877–883.

Bassi R, Sharma SS. 1993. Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. *Annals of Botany* **72**: 151–154.

Bechtold U, Murphy DJ, Mullineaux PM. 2004. *Arabidopsis* Peptide methionine sulfoxide reductase 2 prevents cellular qxidative damage in long nights. *Plant Cell* **16**: 908–919.

Ben Amor N, Hamed KB, Debez A, Grignon C, Abdelly C. 2005. Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity. *Plant Science* **168**: 889–899.

Bottari F, Festa MR. 1996. Asparagine as a ligand for cadmium (II). Lead (II) and zinc (II). *Chemical Speciation and Bioavailability* **8**: 75–83.

Bown AW, Shelp BJ. 1997. The metabolism and functions of γ-aminobutyric acid. *Plant Physiology* **115**: 1–5.

Brunetti P, Zanella L, Proia A, De Paolis A, Falasca G, Altamura MM, Sanità di Toppi L, Costantino P, Cardarelli M. 2011. Cadmium tolerance and phytochelatin content of *Arabidopsis* seedlings over-expressing the phytochelatin synthase gene *AtPCS1*. *Journal of Experimental Botany* **62**: 5509–5519.

Chaffei C, Pageau K, Suzuki A, Gouia H, Ghorbel MH, Masclaux-Daubresse C. 2004. Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. *Plant and Cell Physiology* **45**: 1681–1693.

Chen YX, He YF, Luo YM, Yu YL, Lin Q, Wong MH. 2003. Physiological mechanism of plant roots exposed to cadmium. *Chemosphere* **50**: 789–793.

Cho UH, Seo NH. 2005. Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Science* **168**: 113–120.

Cobbett CS. 2000. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology* **123**: 825–832.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53**: 159–182.

Costa G, Morel JL. 1994. Water relations, gas exchange and amino acid content in Cd-treated lettuce. *Plant Physiology and Biochemistry* **32**: 561–570.

Couturier J, de Faÿ E, Fitz M, Wipf D, Blaudez D, Chalot M. 2010. PtAAP11, a high affinity amino acid transporter specifically expressed in differentiating xylem cells of poplar. *Journal of Experimental Botany* **61**: 1671–1682.

De Cnodder T, Vissenberg K, Van Der Straeten D, Verbelen JP. 2005. Regulation of cell length in the *Arabidopsis thaliana* root by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid: a matter of apoplastic reactions. *New Phytologist* **168**: 541–550.

Dutilleul C, Garmier M, Noctor G, Mathieu C, Chetrit P, Foyer CH, de Paepe R. 2003. Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. *Plant Cell* **15**: 1212–1226.

Esparaza Rivera JR, Stone MB, Stushnoff C, Pilon-Smits E, Kendall PA. 2006. Effects of ascorbic acid applied by two hydrocooling methods on physical and chemical properties of green leaf lettuce stored at 5°C. *Journal of Food Science* **71**: 270–276.

Freeman JL, Tamaoki M, Stushnoff C, Quinn CF, Cappa JJ, Devonshire J, Fakra SC, Marcus MA, McGrath SP, Van Hoewyk D *et al.* 2010. Molecular mechanisms of selenium tolerance and hyperaccumulation in *Stanleya pinnata*. *Plant Physiology* **153**: 1630–1652.

Gao Y, Miao CY, Mao L, Zhou P, Jin ZG, Shi WJ. 2010. Improvement of phytoextraction and antioxidative defense in *Solanum nigrum* L. under cadmium stress by application of cadmium-resistant strain and citric acid. *Journal of Hazardous Materials* **181**: 771–777.

Garnier L, Simon-Plas F, Thuleau P, Agnel JP, Blein JP, Ranjeva R, Montillet JL. 2006. Cd affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant, Cell & Environment* **29**: 1956–1969.

Hare PD, Cress WA. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* **21**: 79–102.

Hideg E, Barta C, Kalai T, Vass I, Hideg K, Asada K. 2002. Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photoinhibition or UV radiation. *Plant and Cell Physiology* **43**: 1154–1164.

Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. *California Agriculture Experiment Station Circular* **347**, Berkeley, CA. USA..

Jing YJ, Cui DY, Bao F, Hu ZB, Qin ZX, Hu YX. 2009. Tryptophan deficiency affects organ growth by retarding cell expansion in *Arabidopsis*. *The Plant Journal* **57**: 511–521.

Krammer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC. 1996. Free histidine as a metal chelator in plant that accumulate nickel. *Nature* **379**: 635–638.

Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Šetlík I. 2007. Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. *New Phytoloist* **175**: 655–674.

Lee BH, Lee H, Xiong L, Zhu JK. 2002. A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. *Plant Cell* **14**: 1235–1251.

Ma WW, Xu WZ, Xu H, Chen YS, He ZY, Ma M. 2010. Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. *Planta* **232**: 325–335.

Macek T, Mackova M, Pavlikova D, Szakova J, Truksa M, Singh Cundy A, Kotrba P, Yancey N, Scouten WH. 2002. Accumulation of cadmium by transgenic tobacco. *Acta Biotechnologica* **22**: 101–106.

McCarthy I, Romero-Puertas MC, Palma JM, Sandalio LM, Corpas FJ, Gómez M, Del Río LA. 2001. Cadmium induces senescence symptoms in leaf peroxisomes of pea plants. *Plant, Cell & Environment* **24**: 1065–1073.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–497.

Narayan VS, Nair PN. 1990. Metabolism enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochemistry* **29**: 367–375.

Olmos E, Martinez-Solano JR, Piqueras A, Hellin E. 2003. Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). *Journal of Experimental Botany* **54**: 291–301.

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y. 2012. The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. *The Plant Journal* **69**: 278–288.

Quartacci MF, Baker AJM, Navari-Izzo F. 2005. Nitrilotriacetate- and citric acid-assisted phytoextraction of cadmium by Indian mustard (*Brassica juncea* (L.) Czernj, Brassicaceae). *Chemosphere* **59**: 1249–1255.

Rai VK. 2002. Role of amino acids in plant responses to stresses. *Biologia Plantarum* **45**: 481–487.

Ramel F, Sulmon C, Bogard M, Couee I, Gouesbet G. 2009. Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. *BMC Plant Biology* **9**: 28–45.

Rodriguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, del Río LA, Sandalio LM. 2009. Cellular response of pea plants to cadmium toxicity: cross-talk between reactive oxygen species, nitric oxide and calcium. *Plant Physiology* **150**: 229–243.

Romero-Puertas MC, Rodríguez-Serrano M, Corpas FJ, Gómez M, del Río LA, Sandalio LM. 2004. Cd-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant, Cell & Environment* **27**: 1122–1134.

Salt DE, Prince RC, Baker AJM, Raskin I, Pickering IJ. 1999. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environmental Science & Technology* **33**: 713–717.

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MA, Manceau A. 2002. Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **130**: 1815–1826.

Scandalios JG. 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research* **38**: 995–1014.

Schutzendubel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Godbold DL, Polle A. 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots Pine roots. *Plant Physiology* **127**: 887–898.

Shah K, Dubey RS. 1997. Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant. *Biologia Plantarum* **40**: 121–130.

Sharma SS, Dietz KJ. 2006. The significance of amino acids and amino-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany* **57**: 711–726.

Smirnoff N, Stewart GR. 1987. Nitrogen assimilation and zinc toxicity to zinc-tolerant and non-tolerant clones of *Deschampia caespitosa* (L) Beau. *New Phytologist* **107**: 671–680.

Spitzner A, Perzlmaier AF, Geillinger KE, Reihl P, Stolz J. 2008. The proline-dependent transcription factor Put3 regulates the expression of the riboflavin transporter MCH5 in *Saccharomyces cerevisiae*. *Genetics* **180**: 2007–2017.

Srinivas V, Balasubramanian D. 1995. Proline is a protein-compatible hydrotrope. *Langmuir* **11**: 2830–2833.

Stoch SJ, Bagchi D. 1995. Oxidative mechanism in the toxicity of metal ions. *Free Radical Biology and Medicine* **18**: 321–336.

Sun RI, Zhou QX, Jin CX. 2006. Cadmium accumulation in relation to organic acids in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. *Plant and Soil* **285**: 125–134.

Thordal-Christensen H, Yangdou Wei ZZ, Collinge DB. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant Journal* **11**: 1187–1194.

Tolra RP, Poschenrieder C, Barcelo J. 1996. Zinc hyperaccumulation in *Thlaspi caerulescens*. II. Influence on organic acids. *Journal of Plant Nutrition* **19**: 1541–1550.

Van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, Van der Ent S, Blankestijn H, Ghandilyan A, Tsiatsiani S, Aarts MGM. 2008. Expression differences for genes involved in lignin, glutathione and sulfate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **31**: 301–324.

Verma S, Mishra SN. 2005. Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. *Journal of Plant Physiology* **162**: 669–677.

Wang Z, Zhang YX, Huang ZB, Huang L. 2008. Antioxidative response of metal-accumulator and non-accumulator plants under cadmium stress. *Plant and Soil* **310**: 137–149.

Wenger K, Gupta SK, Furrer G, Schulin R. 2003. The role of nitrilotriacetate in copper uptake by tobacco. *Journal of Environmental Quality* **32**: 1669–1676.

Xie ZX, Duan LS, Tian XL, Wang BM, Eneji AE, Li ZH. 2008. Coronatine alleviates salinity stress in cotton by improving the antioxidative defense system and radical-scavenging activity. *Journal of Plant Physiology* **165**: 375–384.

Xu J, Yin HX, Li X. 2009. Protective effects of proline against cadmium toxicity in micropropagated hyperaccumulator, *Solanum nigrum* L. *Plant Cell Reports* **28**: 325–333.

Xu J, Yin HX, Li YL, Liu XJ. 2010. Nitric oxide is associated with long-term zinc tolerance in *Solanum nigrum*. *Plant Physiology* **154**: 1319–1334.

Yamaguchi H, Fukuoka H, Arao T, Ohyama A, Nunome T, Miyatake K, Negoro S. 2010. Gene expression analysis in cadmium-stressed roots of a low cadmium-accumulating solanaceous plant, *Solanum torvum*. *Journal of Experimental Botany* **61**: 423–437.

Zhou WB, Qiu BS. 2005. Effects of cadmium hyperaccumulation on physiological characteristics of *Sedum alfredii* Hance (Crassulaceae). *Plant Science* **169**: 737–745.

Zhu ZJ, Wei GQ, Li J, Qian QQ, Yu JQ. 2004. Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). *Plant Science* **167**: 527–533.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 GSH : GSSG ratio.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.