Enhanced cadmium accumulation in maize roots—the impact of organic acids

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Received: 20 July 2006 / Accepted: 16 October 2006 / Published online: 7 November 2006
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Abstract Low molecular weight organic acids are important components of root exudates and therefore, knowledge regarding the mechanisms of cadmium (Cd) uptake and distribution within plants under the influence of organic acids, is necessary for a better understanding of Cd behavior in the plant–soil system. In this study, acetic and malic acids increased the uptake of Cd by maize (*Zea mays* L. cv. TY2) roots and enhanced Cd accumulation in shoots under hydroponic conditions. Concentration-dependent net Cd influx in the presence and absence of organic acids could be resolved into linear and saturable components. The saturable component followed Michaelis–Menten kinetics, which indicated that Cd uptake across the plasma membrane was transporter-mediated. While the K_m values were similar, the V_{max} values in the presence of acetic and malic acids were respectively 6.0 and 3.0 times that of the control. Zinc

transporters were the most probable pathways for Cd accumulation. It was hypothesized that Cd(II)–organic acid complexes associated with the root zone, could decompose and liberate Cd^{2+} for subsequent absorption by maize roots; and that in the layer of the roots or within the root free space, depletion of Cd^{2+} was buffered by the presence of Cd(II)–organic acid complexes. Plant response to elevated Cd levels involved overproduction of organic acids in maize roots as a resistance mechanism to alleviate Cd toxicity.

Keywords Cadmium · Maize (*Zea mays*) · Organic acids · Transporter · Uptake

Introduction

In recent years, concerns over the possible health and ecosystem effects of heavy metals in soils and accumulation in plants have increased. Low molecular weight organic acids are important components of root exudates. Under a wide range of soil conditions organic acids are typically negatively charged anions capable of reacting strongly with metal ions in both the soil solution and solid phases (Jones and Darrah, 1994). Organic acids are important soil components for the dissolution of metals from insoluble mineral phases in soil, increasing metal mobility

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in the vicinity of roots and enhancing metal phytoavailability to plants (López-Bucio et al., 2000).

Many studies have concentrated on the important role that organic acids play at the plant–soil interface. Organic acids can increase desorption of heavy metals and rare earth elements from soil and consequently increase metal concentrations in the soil solution (Qin et al., 2004; Shan et al., 2002). In pot experiments, organic acids triggered uranium accumulation in Indian mustard and Chinese cabbage (Huang et al., 1998), increased chromium accumulation in tomato (Srivastava et al., 1999), and enhanced accumulation of light rare earth elements in natural perennial fern fronds (Shan et al., 2003). In addition, under hydroponic conditions, lanthanum uptake by barley, maize and wheat roots was enhanced in the presence of organic acids (Han et al., 2005; Wang et al., 2004a, b).

Among the various heavy metals, cadmium (Cd) is of particular health concern, because Cd is a toxic element easily taken up via plant roots. Therefore, the public health risk through food chain exposure to Cd containing foodstuffs will increase significantly (Dudka and Miller, 1999). Cadmium is a trace contaminant in many commercial phosphate fertilizers and continued application of phosphate fertilizers to crops has led to contamination of large areas of agricultural land in many countries throughout the world due to the constant anthropogenic release of Cd from these fertilizers to the soil. The bioavailability of Cd in the soil–plant system is considered to be a key factor controlling plant uptake, and several papers have proposed the involvement of organic acid chelation in the process. Krishnamurti et al. (1997) demonstrated that various organic acids were able to influence the rate of Cd release from different soils and increase the solubility of Cd from bulk soils. Cieśliński et al. (1998) found that the high Cd accumulating durum wheat cultivar Kyle had significantly higher amounts of organic acids in the rhizosphere soil than that of the low accumulating cultivar Arcola, regardless of soil type. Statistically significant increases were observed in Cd accumulation by wheat with increasing concentrations of organic acids in pot experi-

ments (Nigam et al., 2000). Under hydroponic conditions, Cd uptake and translocation in tomato plants increased after citric acid pre-incubation (Senden et al., 1995).

The enhancement of metal solubility in soils by organic acids and the subsequent metal uptake by plants has been mainly attributed to the metal chelating properties of the organic acids. One unresolved question is, in the vicinity of the root surface, how do organic acids exert their effects on plant uptake of metals? Are free metal ions or organic ligand-complexes taken up by plants? At the present time, further research is required to establish whether free metal ions are accumulated or if intact organo-metal complexes are transferred into the plant. In addition, plants vary in their ability to accumulate Cd (Hill et al., 2002), and differences in plant–soil interactions may affect the amounts of Cd entering the edible plant parts. For these reasons, it is essential to obtain knowledge of Cd uptake mechanisms and distribution processes of Cd in plants.

The aim of the present study was to investigate the mechanisms of enhanced Cd uptake by maize roots as influenced by organic acids. The cation channels and transporters, through which Cd was potentially absorbed into maize cytosol, were investigated and an improved understanding of the biological processes involved in Cd acquisition and distribution inside maize seedlings in the presence of organic acids was obtained.

Materials and methods

Plant cultivation

Maize (*Zea mays* L. cv. TY2) seeds were obtained from the Chinese Academy of Agricultural Sciences (Beijing, China). Seeds were first surface sterilized in 3% H_2O_2 , soaked in 2.8 mM $\text{Ca}(\text{NO}_3)_2$ for 4 h, then germinated on moist filter paper for 4 d in the dark and subsequently transferred into hydroponic solution. Maize seedlings were cultured in a nutrient solution consisting of major nutrients (one-third-strength of 1 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$, 0.5 mM K_2SO_4 , 1 mM MgSO_4 and 1.5 mM NH_4NO_3) and

micronutrients (full-strength of 75 μM EDTA-Fe, 46 μM H_3BO_3 , 9 μM MnSO_4 , 0.8 μM ZnSO_4 , 0.3 μM CuSO_4 and 0.8 μM Na_2MoO_4). ZnSO_4 was equilibrated with H_4EDTA before addition to the nutrient solution (Norvell and Welch, 1993). The solution was buffered to pH 5.8 with 2 mM Mes–Tris. Plants were grown in a plant growth chamber with operating conditions maintained at 16 h light/8 h darkness at 25/18°C, respectively, relative humidity between 50% and 60% and a light intensity of 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Aerated nutrient solutions were changed every 3 d. In all studies, 12-days-old uniform maize seedlings were used.

Bacterial growth in nutrient solutions was minimized by soaking and cleaning all glassware prior to use with sodium hypochlorite; autoclaving macronutrient stock solution and deionized water, and adding 30 mg l^{-1} of the antibiotic cefotaxime to all assay solutions (Bell et al., 2003).

Effect of molar concentration ratio (organic acids:Cd) on Cd uptake by maize roots

Uniform maize seedlings grown in nutrient solutions containing 5 μM Cd in the absence of organic acids were used as controls. Seven different molar concentration ratios (1:1, 2:1, 3:1, 5:1, 10:1, 50:1 and 100:1) of acetic or malic acid to Cd were examined. Acetic and malic acids were chosen as representative organic acids because they are ubiquitous and abundant in many plant exudates (Cieřliński et al., 1998; Jones, 1998). Maize seedlings were harvested 2 d after exposure to amended solutions. During this period fresh incubation solution was replaced every 12 h. Roots were desorbed with 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) for 15 min then gently blotted, weighed and digested for final determination of Cd. Experiments were performed in quadruplicate. The use of 5 mM ice-cold CaCl_2 for desorption was previously reported by Rauser (1987), who suggested that 5 mM CaCl_2 was effective in removing the Cd adsorbed on cell walls of maize roots. A similar desorption step was also used by Hart et al. (1998) and Lombi et al. (2002).

Time-dependent kinetics of Cd uptake by maize roots under the influence of organic acids

Uniform maize seedlings were transferred to a control nutrient solution containing 5 μM Cd without added organic acids, while in the other two uptake experiments 250 μM of either acetic or malic acids were added separately to control solutions. At increasing time intervals following exposure of seedlings to the incubation solution (5, 10, 20, 40, 60, 90 and 120 min, and 4, 6, 12, 24, 36 and 48 h) maize seedlings were harvested. During short-term uptake experiments (0–120 min), subsamples of roots were collected and total Cd determined without desorption, while the remaining roots were desorbed in 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) for 15 min prior to Cd determination. During long-term uptake experiments (0–2 d), the incubation solutions were replaced every 12 h, and all harvested maize roots were desorbed and digested for Cd determination. After 2 d exposure, maize shoots were also harvested and separated into stems and leaves. Roots, stems and leaves of maize seedlings were dried at 60°C for 2 d and finely ground. An aliquot of dried plant material (0.200 g) was digested for Cd determination. All experiments were performed in quadruplicate.

Concentration-dependent kinetics of Cd uptake by maize roots under the influence of organic acids

Three serial experiments were performed simultaneously to test root uptake of Cd at different Cd concentrations. Uniform maize seedlings were incubated with fresh nutrient solutions containing various concentrations of Cd (0, 0.25, 0.50, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 μM Cd). In two of these experiments, acetic and malic acids were added, separately, at a molar concentration ratio of 50:1 (organic acid:Cd). After incubation for 20 min, seedlings were harvested, and the roots desorbed in 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) for 15 min. The concentration of Cd in the maize roots was quantified following acid digestion. Each serial experiment was performed in quadruplicate.

Effects of various blockers of cation channels on Cd influx into maize roots

Uniform maize seedlings were incubated in nutrient solutions amended with either 0.50 mM verampamil (ACROS, Fisher Company, Pittsburgh, PA, USA), diethyl pyrocarbonate (DEPC, AVOCADO, Johnson Matthey Company, Ward Hill, MA, USA) or tetraethylammonium chloride (TEA-Cl, Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA). Unamended nutrient solution was used as a control. These three compounds have previously been identified as cation channel blockers by Essah et al. (2003) and Montague (1993), who also reported their appropriate application concentrations. Cd concentration was maintained at 5.0 μM during this experiment. Acetic and malic acids were applied at a molar concentration ratio of 50:1 (organic acid:Cd). Maize seedlings were harvested after 4 h incubation. Roots were desorbed and acid digested prior to final determination of Cd. All experiments were performed in quadruplicate.

Effect of Zn or Fe deficiency on Cd uptake by maize roots

Zn or Fe deficiency was induced in uniform maize seedlings by replacing a full nutrient solution with a nutrient solution without Zn or Fe for 6 d (Lombi et al., 2002). The seedlings were subsequently incubated in a 5.0 μM Cd uptake solution with and without organic acids. The molar concentration ratio was maintained at 50:1 (organic acid:Cd). After 20 min exposure, maize roots were harvested, desorbed, and digested prior to Cd determination. All experiments were performed in quadruplicate.

Effect of Zn concentration on Cd accumulation by maize roots

Uniform maize seedlings grown in nutrient solutions were transferred to treatment solutions containing 5.0 μM Cd without organic acids and increasing concentrations of Zn, supplied as $\text{Zn}(\text{NO}_3)_2$ (0, 2.5, 5.0, 10, 25, 50, 100, 175 and 250 μM Zn). Maize seedlings were also incubated in the presence of 250 μM organic acid. In these

treatment solutions the Zn concentration was maintained at 100 μM while the Cd concentration remained at 5.0 μM . Seedlings were harvested 20 min after incubation commenced and maize roots were desorbed and digested with acid prior to Cd determination. All experiments were performed in quadruplicate. The dose of Zn used in the present study is very low compared with the doses used in other studies or present in contaminated soils (Keller et al., 2003; Robinson et al., 1998; Schwartz et al., 2003; Zhao et al., 2003).

Root organic acid extraction and determination

Maize seedlings were incubated for 2 h in a number of treatment solutions, including a control nutrient solution or nutrient solution containing 5.0 μM Cd with and without organic acids. At the end of the experiments, roots were cut off, gently blotted, weighed and frozen with liquid N_2 (Pellet et al., 1995). The frozen roots were ground under the protection of liquid N_2 and mixed with 95% ethanol in a chilled mortar. The resulting mixture was centrifuged at 30,000 g for 30 min at 4°C (Centrifuge 5804R; Eppendorf, Hamburg, Germany). The supernatant was retained, and the pellet was resuspended in 95% ethanol and respun. This procedure was repeated four times for each sample and the combined supernatants, retained from the four extractions, evaporated to dryness under vacuum. The residue was redissolved in 15 ml of Milli-Q water (Easy-pure, Dubuque, IA, USA), and filtered through a disposable syringe filter (0.2 μm).

Organic acids in root homogenates were analyzed with a capillary electrophoresis (CE) system (P/ACE MDQ; Beckman Instruments, Fullerton, CA, USA) controlled by a Pentium II computer interfaced via PACE 1.2.1 software (Beckman Instruments) according to Piñeros et al. (2002) with little modification. Organic acids were separated in a 60-cm capillary (75 μm i.d.) with a constant separation voltage of -20 kV at 25°C. Peaks were detected with a UV absorbance detector at a wavelength of 214 nm, and were first identified on the basis of their migration time, with subsequent confirmation by spiking samples with organic acids standards.

Membrane permeability

Maize seedlings were incubated in different treatment solutions for 2 d. During this period fresh incubation solution was replaced every 12 h. Treatment solutions including control nutrient solutions or nutrient solutions amended with $5.0 \mu\text{M}$ Cd with and without organic acids. At the end of the experiments, roots were thoroughly washed twice with 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) to eliminate nutrient solution from the apoplast and then blotted on filter paper. Each sample consisted of one seedling which was then introduced to 25 ml of fresh 0.2 mM CaSO_4 solution. Seedlings were incubated for 4 h in a water bath at 25°C with gentle shaking (Llamas et al., 2000). Potassium (K^+) efflux to the external solution, as well as K^+ remaining in the roots, was measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Optima 2000DV, Perkin Elmer Co., USA; detection limit: 40 mg l^{-1}). In order to determine K^+ in roots, following incubation, roots were washed, homogenized in 10 ml Milli-Q water, boiled for 30 min and centrifuged at $5,000 \text{ g}$, and 20°C for 10 min. The supernatant was then analyzed using ICP-OES to determine the K^+ content. All experiments were performed in quadruplicate.

Determination of Cd

High Cd concentrations were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, detection limit of $4 \mu\text{g l}^{-1}$) (Optima 2000DV, Perkin–Elmer Co., Boston, MA, USA), while low Cd concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS, detection limit of 3.6 ng l^{-1}) (*X*-series, Thermo Electron, Waltham, MA, USA). Cd in plant materials was determined following acid digestion. Fresh plant material (0.25 g) was digested with 3 ml of $\text{HNO}_3\text{--HClO}_4$ (2:1, v/v) under high-pressure conditions and transferred quantitatively into a 10 ml volumetric flask and diluted to volume with 2% HNO_3 . The accuracy of both ICP-OES and ICP-MS was checked using a certified reference material peach leave (national first-class environmental standard

sample, GBW 08501, PR China), in which the Cd content is certified to be $0.018 \mu\text{g g}^{-1} \text{ d. wt.}$

Data analysis

Data analysis was performed using the commercially available statistical software, MICROCAL_ORIGIN_6.1 (Microcal Software, Northampton, MA, USA) or SPSS 12.0 for windows (SPSS, Chicago, IL, USA).

Speciation calculations

The chemical speciation of Cd in incubation solutions containing organic acids was predicted using the computer program MINEQL (Westall et al., 1976) and the stability constants of Smith et al. (1997). Full consideration was given to mole balances, relevant thermodynamic equilibrium constants and ionic strengths in performing these calculations.

Results

Influence of molar concentration ratio of organic acids to Cd on the uptake of Cd by maize roots

As can be seen from Fig. 1, both acetic and malic acids stimulated the uptake of Cd by maize roots, which is shown as a function of the molar concentration ratio of organic acid to Cd. At the concentration range of organic acids considered here, acetic and malic acids exerted a similar trend, with acetic acid showing a greater enhancement effect than that of malic acid. The content of Cd in the roots increased steadily from the ratio of 1:1 to 50:1. The maximum enhancement effect was observed at the ratio of 50:1. Under optimal conditions the Cd content of desorbed maize roots increased by 110 and 75% in the presence of acetic and malic acids, respectively, after 2 d of exposure, compared with the control ($P < 0.01$). The contents of Cd remaining in the roots at the ratio of 50:1 were 31 and 24% higher than those at the ratio of 2:1 due to the application of acetic and malic acid, respectively ($P < 0.05$). The Cd content of the roots decreased with a further

Fig. 1 Effect of molar concentration ratio (organic acid:Cd) on the uptake of Cd by maize (*Zea mays* L.) roots. Values are means ($\pm$ SE) of four replicates. The molar concentration ratio (0:1) indicates maize roots treated with Cd but without the application of organic acids. Cd concentration was maintained at 5.0 μ M. Uptake for 2 d, desorption in 5 mM ice-cold CaCl_2 (5 mM Mes-Tris, pH 6.0) for 15 min

increase in the organic acid to Cd ratio to 100:1. We used 50:1 (organic acid:Cd) as molar concentration ratio in all our subsequent studies.

Time dependent Cd uptake by maize roots under the influence of organic acids

The Cd uptake patterns were similar between control and organic acid treated solutions for both short and long-term experiments. However, in comparison to the control, Cd accumulation in maize roots was consistently greater in the presence of organic acids. For maize roots without CaCl_2 desorption (Fig. 2a), the Cd uptake was biphasic, exhibiting an initial 20 min rapid phase and a later slower linear phase 20–120 min. For maize roots with CaCl_2 desorption the dynamic processes of the three uptake experiments were also biphasic, but the slower linear phase of accumulation between 10 min and 120 min dominated the uptake curve. After 2 h exposure, total Cd in the desorbed maize roots was 3.0 and 2.2 times greater than that of the control ($P < 0.05$) under the influence of acetic and malic acid, respectively.

Long term exposure to Cd also showed biphasic uptake, a rapid uptake phase in the first 6 h, followed by a slow unsaturated phase up to

Fig. 2 Time-dependent Cd uptake by maize (*Zea mays* L.) roots for short term exposure (2 h) without (a) or with (b) desorption in 5 mM ice-cold CaCl_2 (5 mM Mes-Tris, pH 6.0) for 15 min, and for long term exposure (2 d) with desorption (c). Values represent means ($\pm$ SE) of four replicates. Cd concentration was maintained at 5.0 μ M. The molar concentration ratio (organic acid:Cd) was 50:1

48 h (Fig. 2c). The uptake did not reach saturation, because maize seedlings were still growing and no equilibrium was reached. Compared with

Fig. 3 Contents of Cd in dry matter of maize (*Zea mays* L.) roots, stems and leaves under the influence of organic acids. Values are means ($\pm$ SE) of four replicates. Uniform maize seedlings were harvested after 2 d incubation with Cd concentration maintained at $5.0 \mu\text{M}$. The molar concentration ratio (organic acids:Cd) was 50:1

Cd uptake in the absence of organic acids, Cd content in the desorbed maize roots increased by 113 and 61% for acetic and malic acid respectively when exposed for 48 h, (Fig. 3, $P < 0.01$). Treatment with organic acids not only increased the Cd absorption by maize roots, but also increased Cd concentrations in maize shoots. Acetic acid was more effective than malic acid in enhancing Cd uptake. After 48 h of exposure, acetic and malic acids had increased the content of Cd in maize stems by 85 and 48%, respectively (Fig. 3, $P < 0.01$), and Cd content in the leaves by 70 and 24% over that of the control (Fig. 3, $P < 0.05$).

Concentration-dependent kinetics of Cd uptake by maize roots under the influence of organic acids

The linear nature of short-term, time-dependent accumulation of Cd (Fig. 2b) suggests that unidirectional Cd influx into the root symplast occurs regardless of the application of organic acids. Thus, we chose a 20 min uptake period to investigate the concentration-dependent uptake kinetics of Cd. This relatively short uptake period allowed us to investigate uptake while minimizing the possibility of Cd efflux across the plasma membrane back into the external solution. The concentration-dependent uptake kinet-

Fig. 4 Concentration-dependent uptake kinetics of Cd into maize (*Zea mays* L.) roots. Roots were immersed into a solution containing different Cd levels. The molar concentration ratio (organic acid:Cd) was 50:1. Exposure for 20 min with desorption in 5 mM ice-cold CaCl_2 (5 mM Mes-Tris, pH 6.0) for 15 min: (a) Overall kinetics curves for Cd uptake influx. Data points and error bars represent means ($\pm$ SE) of four replicates; (b) Resolution of overall kinetics curves into saturable and linear components. Cd (.....), malic acid + Cd (—), acetic acid + Cd (---)

ics of Cd influx was characterized by smooth, nonsaturating curves (Fig. 4a). Organic acids increased Cd uptake over the whole range of uptake solution Cd concentrations. The uptake kinetics of Cd in the absence and presence of acetic and malic acids were similar (biphasic) and could be graphically resolved into saturable and linear components through a modified Michaelis-Menten kinetics model: $V = aC + V_{\max}C/(K_m + C)$ (Fig. 4b). This resolution showed that acetic and malic acid could increase $V_{\max}$ for Cd influx by 6.0 and 3.0 times, respectively, relative to the control ($P < 0.001$).

Table 1 Kinetic parameters of Cd uptake in maize (*Zea mays* L.) roots in the presence of acetic and malic acids

Treatment	$V_{\max}$ (nmol g ⁻¹ root f. wt h ⁻¹)	K_m (μM)
Cd	20.31 ± 3.30	0.20 ± 0.03
Malic acid + Cd	60.39 ± 12.14	0.16 ± 0.02
Acetic acid + Cd	122.6 ± 20.12	0.43 ± 0.03

Data are means ± SE ($n = 4$)

However, both organic acids had no significant effect on K_m (Table 1).

Contribution of Ca²⁺, Na⁺ and K⁺ cation channels to Cd influx

We selected three cation channel blockers: verampamil (a Ca²⁺ channel blocker), diethyl pyrocarbonate (DEPC, a Na⁺ channel blocker), and tetraethylammonium chloride (TEA-Cl, a K⁺ channel blocker) to study the contribution of various cation channels to Cd influx into maize roots under the influence of organic acids. All of these blockers had no statistically significant inhibition effect on Cd influx into all treated maize roots (Fig. 5). Therefore, our study does not support any Cd uptake through Ca²⁺, Na⁺ and K⁺ cation channels.

Fig. 5 Effects of 0.5 mM verampamil, tetraethylammonium chloride (TEA-Cl), diethyl pyrocarbonate (DEPC) on the influx of Cd into maize (*Zea mays* L.) roots under the influence of organic acids. Values represent means (± SE) of four replicates. Uniform maize roots were exposed for 4 h. Cd concentration was maintained at 5.0 μM. The molar concentration ratio (organic acid:Cd) was 50:1. Desorption in 5 mM ice-cold CaCl₂ (5 mM Mes-Tris, pH 6.0) for 15 min

Contribution of Zn²⁺ transporter to Cd influx

The influence of Zn or Fe deficiency on Cd uptake by maize roots was investigated to determine if Zn transporters potentially contributed to Cd influx. While Zn deficiency greatly enhanced Cd uptake, Fe deficiency had no statistically significant effect on Cd uptake relative to controls (Fig. 6). After inducing Zn deficiency, Cd accumulation in maize roots increased 3-fold when compared to maize roots incubated in full-nutrient solutions without the influence of organic acids ($P < 0.01$). With the application of acetic and malic acids Cd accumulation was also considerably enhanced by 4.0 and 3.6 times, respectively, in comparison with controls by inducing Zn deficiency ($P < 0.01$).

In addition, our results showed that without organic acids when Zn concentration in the incubation solution increased to 2.5 μM, Cd accumulation decreased to 87% of total Cd in maize roots incubated in full-nutrient solution ($P < 0.01$) (Fig. 7). As the Zn concentration was increased Cd uptake was inhibited, such that at 100 μM Zn, Cd uptake was markedly inhibited to 38% ($P < 0.01$). Further application of Zn to 250 μM inhibited Cd content in roots to 30% ($P < 0.01$). The application of acetic and malic acids to a hydroponics solution containing

Fig. 6 Influence of Zn or Fe deficiency on Cd uptake by maize (*Zea mays* L.) roots in the presence of organic acids. Values are means (± SE) of four replicates. Zn and Fe deficiency was induced for 6 d, and thereafter the plants were transferred to uptake solutions containing 5.0 μM Cd. The molar concentration ratio (organic acids:Cd) was 50:1. After 20 min uptake, uniform maize seedlings were harvested and roots desorbed in 5 mM ice-cold CaCl₂ (5 mM Mes-Tris, pH 6.0) for 15 min

Fig. 7 Effect of different levels of Zn on Cd uptake by maize (*Zea mays* L.) roots without the application of organic acids. Values are means ($\pm$ SE) of four replicates. Cd concentration was maintained as $5.0 \mu\text{M}$. Uniform maize seedlings were harvested after 20 min exposure and roots desorbed in 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) for 15 min

$100 \mu\text{M}$ Zn also decreased Cd accumulation in maize roots to 26 and 30%, respectively ($P < 0.01$) (Fig. 8).

Organic acids response to enhanced Cd accumulation in maize roots

The concentrations of various organic acids in undesorbed maize roots were determined by capillary electrophoresis, and the results are

Fig. 8 Effect of Zn on Cd uptake by maize (*Zea mays* L.) roots under the influence of organic acids. Values are means ($\pm$ SE) of four replicates. Uniform maize seedlings were incubated in uptake solutions containing $5.0 \mu\text{M}$ Cd and $100 \mu\text{M}$ Zn. The molar concentration ratio (organic acids:Cd) was 50:1. Seedlings were harvested after 20 min uptake and roots desorbed in 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) for 15 min

shown in Table 3. Enhanced Cd accumulation in maize roots due to the application of organic acids to the nutrient solution was accompanied by increased concentrations of acetic, formic, oxalate, malic, succinic, tartaric and citric acids in the maize roots. The enhanced organic acids were most probably produced in responses to excess Cd cation uptake, which could be a resistance strategy to Cd toxicity. Earlier studies have shown that, when plants are exposed to a heavy metal threat, organic acids may be produced in the plant cells as potential ligands for heavy metal complexation and therefore organic ligands play an important role in heavy metal tolerance and detoxification in plants (Rauser, 1999; Clemens, 2001).

Discussion

Enhanced Cd uptake by maize roots under the influence of organic acids

Our research showed that both acetic and malic acids could stimulate the uptake of Cd by maize roots. With a molar concentration ratio of 50:1 (organic acid:Cd), the Cd content of desorbed maize roots increased by 110 and 75% in the presence of acetic and malic acids respectively after 2 d of exposure compared with the control ($P < 0.01$) (Fig. 1).

The time dependent Cd uptake patterns were biphasic. For maize roots without CaCl_2 desorption (Fig. 2a), the initial rapid phase (within the first 20 min) was presumably due to diffusion of Cd into the free space and binding to the root-cell walls. The slower linear phase of Cd uptake over the subsequent 20–120 min may correspond to transport across the plasma membrane. For maize roots with CaCl_2 desorption the dynamic uptake process resembled the report of Lasat et al. (1996) (Fig. 2b). The initial rapid component (within the first 10 min) included cell-wall-associated Cd not removed by the desorption treatment. The slower linear phase of accumulation with exposure above 10 min was primarily due to Cd transport into the symplasm with a minor component due to undesorbed cell-wall-bound Cd.

The concentration-dependent uptake kinetics could be graphically resolved into saturable and linear components (modified Michaelis–Menten kinetics model: $V = aC + V_{\max}C/(K_m + C)$, Fig. 4b). We suggest that the linear component represents cell-wall-bound Cd that remained after desorption. The saturable component of uptake indicates a transporter-limited process. Similar concentration-dependent kinetics has been reported for several other divalent cations, including Zn (Lasat et al., 1996), putrescine (DiTomaso et al., 1992), and paraquat (Hart et al., 1992). Acetic and malic acid increased $V_{\max}$ for Cd influx by 6.0 and 3.0 times, respectively, relative to the control ($P < 0.001$) (Table 1). These results showed that in all treatments (with or without organic acid), Cd transported across the plasma membrane should be transporter-mediated, but the capacity of influx was much greater under the influence of organic acids.

How did Cd transport across the plasma membrane of maize roots under the influence of organic acids?

Till now the mechanisms of uptake, transport and accumulation of Cd in plants have not fully been understood. It is generally believed that Cd uptake by plants occurs mainly through cation channels or carriers. Lindberg et al. (2004) found that Cd uptake into the cytosol of wheat protoplasts takes place by channels permeable to Ca^{2+} and K^+ . Based mainly on uptake studies and growth effects, transporter proteins of the ZIP family were also proposed as possible transporters for Cd. Elevated *IRT1* transcription accompanied increased Cd accumulation in peas (Cohen et al., 1998). Higher accumulation of Cd was related to overexpressed *AtIRT1* in *Arabidopsis thaliana* (Connolly et al., 2002). An increase in the abundance of *TcIRT1-G* mRNA in the *Thlaspi caerulescens* ectotype Ganges led to enhanced uptake of Cd (Lombi et al., 2002). Similar uptake and transport systems for Cd and Zn may exist in the Cd hyperaccumulator of *Arabidopsis halleri* (Küpper et al., 2000). The Zn transporter (*ZNT1*) has also been recently cloned from *T. caerulescens* and shown to mediate

low-affinity uptake of Cd (Lasat et al., 2000; Pence et al., 2000).

Under the influence of organic acids, Cd transport across the root-cell plasma membrane of maize seedlings was also examined in this study. All of the blockers of Ca^{2+} , Na^+ and K^+ channels had no statistically significant inhibition effect on Cd influx into all treated maize roots (Fig. 5). Therefore, any Cd uptake through these cation channels was unfeasible. Iron deficiency had no statistically significant effect on Cd uptake, so Cd uptake via Fe transporters may not occur either. Only zinc deficiency exerted a stimulatory effect on Cd uptake in maize roots, which may be related to an up-regulation in the expression of genes encoding for Zn^{2+} uptake (Fig. 6). If similar uptake and transport systems for Cd and Zn exist in the roots of this maize cultivar, competitive transport interactions between these two ions may occur. Our experimental results demonstrated that the application of Zn could decrease Cd accumulation in maize roots in the absence (Fig. 7) or presence (Fig. 8) of acetic and malic acids. In addition, concentration-dependent net Cd influx in the presence and absence of organic acids could be resolved into linear and saturable components (Fig. 4b). The saturable component followed Michaelis–Menten kinetics, which indicated that Cd uptake across the plasma membrane should be transporter-mediated.

With the above results in mind, we conclude that Cd was most probably taken up into the maize roots by Zn transporter proteins. Cd and Zn are both metals from group IIb of the periodic table and exhibit numerous similar chemical properties and environmental behaviors. As Cd is not known to be an essential plant micronutrient, it is noteworthy that Cd uptake appears to occur via a carrier-mediated system. Previous studies reported the K_m values for root Zn uptake ranged from 2 μM to 8 μM (Chaudhry and Loneragan, 1972; Lasat et al., 1996; Mullins and Sommers, 1986; Veltrup, 1978). The K_m values from 0.16 μM to 0.43 μM for Cd uptake measured in this study are therefore comparatively lower (Table 1). Thus, if Zn and Cd shared a common influx pathway, the transporter appeared to have a greater affinity for Cd.

Table 2 Calculated Cd chemical species in the applied nutrient solutions at various organic acid:Cd molar concentration ratios (50:1, 5:1, 2:1)

Treatment	Cd speciation (%)			
	Complex constants	50:1	5:1	2:1
Acetic acid (Ac) + Cd	$[\text{Cd}(\text{Ac})_2]^0$ (3.15)	82.7	61.4	38.5
	$[\text{Cd}(\text{Ac})]^+$ (1.90)	14.7	32.7	42.2
	$[\text{Cd}]^{2+}$	2.6	5.9	19.3
Malic acid (Mal) + Cd	$[\text{Cd}(\text{Mal})]^0$ (5.36)	89.6	72.8	59.6
	$[\text{Cd}(\text{HMal})]^+$ (2.34)	8.2	23.4	28.7
	$[\text{Cd}]^{2+}$	2.2	3.8	11.7

Cd concentration was maintained at 5.0 μM

How did organic acids enhance Cd uptake by maize roots?

In this study, acetic and malic acids enhanced Cd uptake by maize roots in the order: acetic acid > malic acid. The complex capacity of acetic acid with Cd^{2+} is lower than that of malic acid with Cd^{2+} (Table 2). This phenomenon is similar to our previous findings (Han et al., 2005), which showed that the lower the stability constants of organo-metallic complexes the greater the metal uptake. In addition, speciation calculations indicated that in the incubation solutions containing

organic acid, Cd was mainly chelated with organic acid (> 80%, Table 2).

No nutrient carriers are thought to exist for organo-metallic complexes in the plasma membrane (Bell et al., 2003). Diffusion of charged or polar chelates across the plasma membrane at any significant rate is unlikely since synthetic chelates are too large and too polar to move through the plasmalemma lipid bilayer (Berne and Levy, 1998). On the basis of the size and polarity of the intact $\text{Cd}(\text{II})$ –organic acid complexes, absorption of intact $\text{Cd}(\text{II})$ –organic acid complexes was unlikely and free Cd ions were more easily available to maize roots than intact $\text{Cd}(\text{II})$ –organic acid complexes. Investigations on the influence of various molar concentration ratios of organic acids to Cd on Cd uptake by maize roots (Fig. 1) and relevant Cd chemical species calculations (Table 2) showed that the more Cd complexed with organic acids, the greater the enhanced Cd uptake. Nor and Cheng (1986) reported that plant roots were able to liberate trace metals from dissolved organo-metallic complexes once they were associated with the root zone.

Therefore, a possible mechanism to explain the observed effects of organic acids on Cd uptake was that, when $\text{Cd}(\text{II})$ –organic acid complexes

Table 3 Contents^a of organic acids in maize (*Zea mays* L.) roots

Organic acid	N ^b	N + malic acid	N + acetic acid	N + Cd	N + Cd + malic acid	N + Cd + acetic acid	Detection limit
Acetic acid	0.56 ± 0.04	0.49 ± 0.04	0.56 ± 0.04	0.53 ± 0.02	1.30 ± 0.08**	1.32 ± 0.09**	0.04
Formic acid	1.30 ± 0.08	1.28 ± 0.08	1.43 ± 0.08	1.25 ± 0.06	1.68 ± 0.09*	2.9 ± 0.1**	0.08
Lactic acid	0.171 ± 0.009	0.143 ± 0.008	0.155 ± 0.009	0.134 ± 0.006	0.18 ± 0.02	0.166 ± 0.004	0.05
Oxalic acid	nd	0.020 ± 0.002**	0.041 ± 0.004**	0.049 ± 0.005**	0.058 ± 0.005**	0.078 ± 0.005**	0.05
Malic acid	0.81 ± 0.04	1.10 ± 0.06*	1.19 ± 0.06*	1.07 ± 0.06*	1.9 ± 0.1**	3.6 ± 0.20**	0.06
Succinic acid	0.074 ± 0.004	0.089 ± 0.006	0.114 ± 0.006*	0.094 ± 0.006	0.144 ± 0.008**	0.26 ± 0.01**	0.04
Tartaric acid	0.173 ± 0.012	0.27 ± 0.02*	0.26 ± 0.02*	0.27 ± 0.02*	0.45 ± 0.02**	0.63 ± 0.04**	0.06
Citric acid	0.110 ± 0.006	0.14 ± 0.01*	0.16 ± 0.01*	0.143 ± 0.008*	0.27 ± 0.01**	0.51 ± 0.03**	0.04

Cd concentration was maintained as 5.0 μM . The molar concentration ratio (organic acid:Cd) was 50:1. Data are means ± SE ($n = 4$)

^a Units are $\mu\text{mol g}^{-1}$ fresh root; nd, not detectable; ^b N is nutrient solution; * $P < 0.05$; ** $P < 0.01$

became associated with the root zone, they could be decomposed and Cd^{2+} liberated. Subsequently free Cd (Cd^{2+}) could be readily absorbed by maize roots in the layer of the roots or within the root free space. Depletion of Cd^{2+} would be buffered by the presence of Cd(II)–organic acid complexes at higher solution organic acids concentrations. This mechanism also explained why acetic acid exerted greater effect than malic acid did, since acetic acid had a lower association constant for Cd and would form more labile complexes. Senden et al. (1995) reported a similar effect on the Cd uptake and distribution among tomato plant roots in the presence of citric acid.

In addition, K^+ efflux to the external solution and K^+ contents in the roots were similar regardless of treatment (Fig. 9). This demonstrated that membrane integrity was not adversely affected by the application of 250 μM organic acid and 5.0 μM Cd. Consequently, the enhancement of Cd uptake under the influence of organic acids was not due to toxicological action.

The major conclusions from this study, resulting from the influence of organic acids, were: (1) the maximum influx rate (V_{max}) for Cd uptake into maize root cells was enhanced; (2) dissolved Cd(II)–organic acid complexes could be decom-

posed and liberate Cd^{2+} when they were associated with the maize root surface. However, direct compelling evidence for this speculation needs further detailed study; (3) the enhanced influx of Cd into maize roots was most probably mediated by Zn transporters; (4) concentrations of natural organic acids in maize roots were greatly increased when Cd uptake was enhanced, and their complexation with Cd supposedly contributed to Cd detoxification in maize roots.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant 20237010 and 20177030). We thank Prof. Naibing Bai (Institute of Software, Chinese Academy of Sciences) for generous help in Cd speciation calculations.

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Fig. 9 Potassium efflux to the external solution and K^+ contents in maize (*Zea mays* L.) roots after treatment for 2 d with and without the presence of 250 μM organic acid and 5.0 μM Cd. Values are means ($\pm$ SE) of four replicates. N: nutrient solution

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