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RESEARCH ARTICLE

Nitrate facilitates cadmium uptake, transport and accumulation in the hyperaccumulator *Sedum plumbizincicola*

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Abstract The aims of this study are to investigate whether and how the nitrogen form (nitrate (NO_3^-) versus ammonium (NH_4^+)) influences cadmium (Cd) uptake and translocation and subsequent Cd phytoextraction by the hyperaccumulator species *Sedum plumbizincicola*. Plants were grown hydroponically with N supplied as either NO_3^- or NH_4^+. Short-term (36 h) Cd uptake and translocation were determined innovatively and quantitatively using a positron-emitting ^{107}Cd tracer and positron-emitting tracer imaging system. The results show that the rates of Cd uptake by roots and transport to the shoots in the NO_3^- treatment were more rapid than in the NH_4^+ treatment. After uptake for 36 h, 5.6 (0.056 μM) and 29.0 % (0.290 μM) of total Cd in the solution was non-absorbable in the NO_3^- and NH_4^+ treatments, respectively. The local velocity of Cd transport was approximately 1.5-fold higher in roots (3.30 cm h^{-1}) and 3.7-fold higher in shoots (10.10 cm h^{-1}) of NO_3^-- than NH_4^+-fed plants. Autoradiographic analysis of ^{109}Cd reveals that NO_3^- nutrition enhanced Cd transportation from the main stem to branches and young leaves. Moreover, NO_3^- treatment increased Cd, Ca and K concentrations but inhibited Fe and P in the xylem sap. In a 21-day hydroponic culture, shoot biomass and Cd concentration were 1.51 and 2.63 times higher in NO_3^-- than in NH_4^+-fed plants. We conclude that compared with NH_4^+, NO_3^- promoted the major steps in the transport route followed by Cd from solution to shoots in *S. plumbizincicola*, namely its uptake by roots, xylem loading, root-to-shoot translocation in the xylem and uploading to the leaves. *S. plumbizincicola* prefers NO_3^- nutrition to NH_4^+ for Cd phytoextraction.

Keywords Ammonium · Cadmium · Nitrate · Positron-emitting tracer imaging system (PETIS) · *Sedum plumbizincicola* · Transport · Uptake · Xylem sap

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Introduction

Cadmium (Cd) is one of the most hazardous and ubiquitous inorganic contaminants in soils and waters. Dietary intake of food from crops grown in Cd-contaminated soils poses risks

to human health. Among the various remediation techniques available, phytoextraction, the use of metal hyperaccumulator plants to remove pollutants from soils, is considered to be a cost-efficient and environmentally friendly method, although it still faces many challenges (McGrath et al. 2006). The mechanisms of hypertolerance and hyperaccumulation by plants have been studied extensively in recent years. There are various physiological steps in the transport route taken by Cd in hyperaccumulators from the rhizosphere to the shoots which are of great importance for phytoextraction, such as root uptake, xylem loading, root-to-shoot translocation, unloading in the shoots and sequestration in the shoots. The rate of Cd transportation at each step may show high dependency on the activities of transporter proteins located in the plasma membrane or tonoplast membrane. Cd uptake in hyperaccumulators appeared to occur partly through other cation transporters such as iron (Fe) (Lombi et al. 2002) or zinc (Zn) (Zhao et al. 2006) or calcium (Ca) channels (Lu et al. 2008). The root-to-shoot translocation of Cd may be affected by the activity of transporter proteins such as *HMA4* contributing to xylem loading and unloading (Papoyan and Kochian 2004; Courbot et al. 2007). Cd hypertolerance is achieved mainly through vacuolar sequestration and complexation with ligands (Kupper et al. 2004; Ma et al. 2005; Ueno et al. 2005).

Nitrogen (N) is an essential macronutrient for the biosynthesis of amino acids, proteins, and enzymes in plants. Nitrate (NO_3^-) and ammonium (NH_4^+) are the main inorganic N sources for plants in most agricultural systems. Since N accounts for 80 % of the total nutrients taken up by roots, the chemical form in which it is absorbed strongly influences the cellular charge balance. Physiologically, the uptake of NO_3^- and NH_4^+ induces a net release of OH^-/HCO_3^- and H^+ ions, respectively. Therefore, when NH_4^+ is supplied the decrease in rhizosphere pH results in an increase in metal bioavailability and consequently an increase in metal accumulation by the plant (Zaccheo et al. 2006). However, it was reported that NO_3^- transport across the plasma membrane results in the membrane potential being temporarily depolarised but it is then hyperpolarised (McClure et al. 1990; Miller et al. 2001) and this may enhance the membrane transport of cations. In contrast, uptake of NH_4^+ depolarises the membrane potential and thus inhibits cation uptake (Crawford and Glass 1998). Some studies have reported that fertilisation with NO_3^- (but not NH_4^+) increases shoot Cd and Zn content and shoot biomass of *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) and thus enhances Cd and Zn hyperaccumulation (Schwartz et al. 2003; Monsant et al. 2008; Xie et al. 2009). Similar enhancement of Cd accumulation has also been found in other plant species such as camomile (Kovacik et al. 2011) and tomato (Luo et al. 2012).

In general, radioisotope tracers such as ^{109}Cd are useful tools for analysing Cd uptake kinetics based on temporal changes in the amount of a substance in the plant body (Zhao et al. 2002; Lu et al. 2008). ^{109}Cd has also been widely used to visualise Cd distribution within plant tissues by autoradiography (Cosio et al. 2005). However, only the static distribution of Cd at a given moment can be obtained by autoradiography. In recent years, the positron-emitting tracer imaging system (PETIS) has been employed to study the uptake and translocation of metals using the positron-emitting tracers ^{52}Mn (Tsukamoto et al. 2006), ^{62}Zn (Suzuki et al. 2008), ^{52}Fe (Tsukamoto et al. 2009) and ^{107}Cd (Fujimaki et al. 2010) in intact living plants. This system enables monitoring of the real-time movement of the tracer in living plants as a video, and also quantitative analysis of the movement of substances by freely selecting a region of interest (ROI) on the image data obtained. Recently, a real-time imaging system for Cd using positron-emitting ^{107}Cd tracer and PETIS was established (Fujimaki et al. 2010). Ishikawa et al. (2011) improved the design of the root box and realised direct observation of Cd uptake by the roots in solution culture, and characterised clearly the differences in Cd dynamics from the culture solution to the grains between high- and low-Cd accumulating rice cultivars. So far, there has been no application of PETIS to investigate Cd uptake by any hyperaccumulator species.

Sedum plumbizincicola, a species in the family Crassulaceae (Wu et al. 2006, 2013a), has been reported to be a Cd hyperaccumulator exhibiting fast growth, large biomass, asexual reproduction and perennial habit (Wu et al. 2007, 2008) and showing remarkable potential in the phytoextraction of Cd from polluted soils (Jiang et al. 2010; Liu et al. 2011; Wu et al. 2013b). In mining areas, Cd concentrations in the shoots reached 241 mg kg^{-1} (unpublished data). In a pot experiment, the Cd concentrations in stems and leaves reached 424 and 285 mg kg^{-1} when soil Cd was 15.3 mg kg^{-1} (Wu et al. 2007). In a field plot experiment, the shoot dry biomass reached 12 t ha^{-1} and Cd uptake reached 0.65 kg ha^{-1} when the plants were grown in soils contaminated with 3 mg kg^{-1} Cd (Liu et al. 2009, 2011). There is much interest in increasing Cd extraction capacity and biomass yields of *S. plumbizincicola* to increase remediation efficiency (Wu et al. 2012). It is not known whether and how the form of N influences the growth, Cd uptake and translocation by this species. In soil conditions, NH_4^+ is readily oxidised to nitrite and NO_3^- by the soil-nitrifying bacteria and the soil pH is influenced by biological processes (Sarathchandra 1978; Zaccheo et al. 2006). The effects of N form can be compared in a solution culture experiment in which the solution pH and N form are maintained constant. Thus, in the present study the effects of N form on plant growth, Cd uptake and translocation in *S. plumbizincicola* were investigated

hydroponically to help enhance phytoremediation efficiency. Moreover, real-time imaging and quantitative analysis of Cd movement in living plants were conducted using ^{107}Cd tracer and PETIS.

Materials and methods

Plant materials and culture

Shoots of *S. plumbizincicola* were collected from an old lead/zinc mine area in the suburbs of Hangzhou city, Zhejiang province, east China. Shoots were cultured in Hoagland solution which was aerated continuously and replaced once a week. After 2–4 weeks, healthy plants of uniform size were selected for the subsequent experiments.

At the start of the experiments seedlings were cultured in fresh nutrient solution containing (in micromolars) 50 KH_2PO_4, 600 K_2SO_4, 200 $MgSO_4$, 100 $CaCl_2$, 10 FeEDDHA, 5 $ZnSO_4$, 5 H_3BO_3, 1 $MnSO_4$, 0.2 $CuSO_4$, 0.03 Na_2MoO_4 and 1,000 2-(*N*-morpholino)ethanesulphonic acid (MES). Two N-source treatments, i.e. 1 mM NO_3^- and 1 mM NH_4^+, were supplied as 0.5 mM $Ca(NO_3)_2$ and 0.5 mM $(NH_4)_2SO_4$, respectively. To provide the same concentration of Ca (0.6 mM), 0.5 mM $CaCl_2$ was supplied in the NH_4^+ treatment. Cd treatments of 1 or 30 μM were used depending on the experiments described below. The solution pH was adjusted to 5.9 with 10 mM Tris. The solution culture experiments were conducted in a controlled environment greenhouse with day/night temperatures of 30/25 °C, a relative humidity of 70 % and natural sunlight without supplementary illumination.

Experimental design

Experiment 1: PETIS analysis

In this experiment, each seedling was acclimatised in 0.7-L fresh solution containing 1 mM NO_3^- or 1 mM NH_4^+ for 3 days before the start of the ^{107}Cd supplementation experiment.

Experiment 2: xylem sap analysis

For xylem sap analysis each treatment had four replicate pots and each pot contained 0.7-L solution and 1 plant. Seedlings were pre-treated with 1 mM NO_3^- or NH_4^+ for 3 days. Then 1 or 30 μM Cd (supplied as $CdSO_4$) was added. Xylem sap collection started 2 h after Cd addition according to the methods of Uraguchi et al. (2009). Shoots were cut 3 cm above the shoot base with fresh razor blades. The shoot incision was quickly washed with distilled water and dried with absorbent paper to remove broken cells from the sap. The shoot stumps were covered with 1.5-mL plastic vials containing a small piece of cotton. Xylem sap (50–100 μL/plant) absorbed in the cotton was collected by centrifuging at 3,000 rpm for 30 s. Elemental concentrations in the xylem sap were determined by ICP-AES (VISTA-PRO, Varian, Palo Alto, CA).

Experiment 3: long-term solution culture

In long-term solution culture seedlings treated with one of the two N forms were supplied with 30 μM $CdSO_4$. Each treatment had four replicate pots, and each pot contained 1 L solution and two plants. The solution was aerated continuously and renewed every 3 days. Plants were harvested after 6 and 21 days of N and Cd treatment. Shoots and roots were quickly rinsed with deionised water. Plants were oven-dried at 80 °C and were digested with HNO_3–$HClO_4$ (3:2, *v*/*v*). Elemental concentrations in the digests were determined using a flame atomic absorption spectrophotometer (Varian SpectrAA 220 FS, Varian, Palo Alto, CA).

^{107}Cd tracer and PETIS imaging

The PETIS imaging experiments were conducted following the methods of Fujimaki et al. (2010) and Ishikawa et al. (2011) with some modifications. ^{107}Cd isotope was produced by bombarding a silver foil for 48 min with a 17-MeV energy proton beam at a current of 2 μA delivered from a cyclotron at the Takasaki Ion Accelerator Facility for Advanced Radiation Application at the Japanese Atomic Energy Agency. The irradiated target was dissolved in concentrated nitric acid and the ^{107}Cd was purified by an $AgCl_2$ precipitation reaction after the addition of 2 M HCl. Finally, 22.0 MBq of ^{107}Cd was fed to each test plant.

Seedlings pre-treated with the different N forms were transferred into 50-mL syringe tubes containing the same solution. Before the imaging experiment, the solution was replaced with fresh solution containing 1 mM NO_3^- or NH_4^+. Purified ^{107}Cd and nonradioactive Cd at a total concentration of 1 μM were simultaneously injected into the tube. The solution was stirred continuously with gentle aeration in order to maintain a uniform composition. The surface level of the solution in the tube was continuously maintained by supplying fresh solution (without Cd) with an automatic solution supply system. Simultaneously, the rate of water uptake by the plants was continuously monitored by weight. Two opposing detector pairs of the PETIS apparatus (a modified PPIS-4800; Hamamatsu Photonics, Hamamatsu, Japan) were used to image ^{107}Cd tracer in solution and plants which were placed in the mid-plane between the two detectors. A positron emitted from a tracer immediately undergoes annihilation by collision with an electron of an

adjacent atom in the plant tissue. A pair of annihilation γ-rays emitted from the decaying positron was detected by the pair of detectors at the same moment. The emission point was then determined as the midpoint of the two incident points. Repeated determinations of the emission points reconstructed a static image of the tracer distribution. One frame, which is the unit of time required to obtain one static image of sufficient quality, was set to 5 min in this study. Consequently, 432 frames were collected over 36 h to yield serial time-course imaging. The typical size of the field of view in the detector head was 12 cm in width and 19 cm in height and the spatial resolution was approximately 2 mm. All PETIS experiments were conducted in a growth chamber at 25 °C and 70 % humidity with continuous light at a density of 400 μmol m^{-2} s^{-1}.

Qualitative and quantitative analyses of PETIS data

The dataset obtained from the PETIS apparatus was transferred to a personal computer for analysis using NIH Image J 1.45 s software to determine Cd dynamics in the solution and plant tissue both qualitatively and quantitatively. Time courses of the radioactivity of ^{107}Cd over time within each ROI were generated through manual selection of ROIs on the image data. A time-course curve of Cd accumulation within the ROI indicated the amounts of total Cd consisting of the sums of radioactive and nonradioactive Cd. Data plotting and fitting analysis of the time-activity curves were performed using Gnuplot version 4.2 software (http://www.gnuplot.info/).

Autoradiography

Test plants were dissected and placed on imaging plates (Fujifilm, Tokyo, Japan) in cassettes after the PETIS imaging and sufficient decay of ^{107}Cd. After several days of exposure the imaging plates were scanned using a bioimaging analyser (Typhoon FLA 7000, GE Healthcare, Tokyo, Japan) to obtain the autoradiographic images of ^{109}Cd in the plant bodies. ^{109}Cd, with a longer half-life (461 days) than ^{107}Cd (6.5 h), was also produced at a minor ratio (approximately 1/3,000) in the production process of ^{107}Cd. This was absorbed by the plants during the PETIS experiments but not detected by the PETIS apparatus because it is not a positron emitter.

Statistics

All statistical analysis was conducted with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Mean values were compared by independent-sample t test or one-way ANOVA followed by Fisher's least significant difference (LSD) test at $P<0.05$.

Results

Real time ^{107}Cd uptake

Real-time ^{107}Cd uptake and transport by *S. plumbizincicola* fed with equal concentrations of cations (e.g. Ca^{2+}, K^+ and Mg^{2+}) but different N sources (NO_3^- vs. NH_4^+) was imaged using PETIS. Plants were supplied with 1 μM Cd (Cd amount was around 45 nmol) including radioactive ^{107}Cd and subjected to PETIS for 36 h. Figure 1 and Supplementary video S1 show representative results of PETIS imaging of Cd uptake and transport. It was clear that in both N treatments, Cd was accumulated by the roots immediately after the Cd was supplied and the Cd intensity in the solution decreased simultaneously. Cd appeared in the shoots after around 1 h. However, the decreasing rates of Cd concentration in the solution and Cd uptake by the roots and transport into the shoots were faster in the NO_3^- treatment than in the NH_4^+ treatment.

Qualitative and quantitative analyses of PETIS data of Cd dynamics were conducted. Time courses of the radioactivity of ^{107}Cd over time within each ROI were generated through manual selection of ROI on the image data. Representative curves of Cd dynamics in the solution, Cd uptake into the roots and movement into the shoots are shown in Figs. 2 and 3. Because the volume of the solution was automatically maintained by supplying a fresh solution without Cd during the experiment, only absorption by the roots would have decreased the amount of Cd in the culture solution. Therefore, the curve of Cd dynamics in the solution directly reflected root absorption (Fig. 2b). It was clear that the Cd concentration in the solution (ROI A and B) decreased rapidly at the beginning and levelled off at about 18 h in both N treatments. However, the rate of decrease in Cd in the NO_3^- treatment was faster than that in the NH_4^+ treatment. After 36 h of uptake, 5.6 (0.056 μM) and 29.0 % (0.290 μM) of the total Cd added in the solution remained non-absorbable in NO_3^- and NH_4^+ treatments, respectively.

To pursue this model, it was assumed that the Cd absorption, which is likely mediated by transporters, follows Michaelis–Menten kinetics in which the reaction rate is approximately proportional to the concentration of the substance in the range below the K_m value. Thus, the curve of Cd dynamics in solution from 0.5 to 18 h (Fig. 2b) was fitted by the following equation (Fujimaki et al. 2010):

$$c_{ct}(t) = c_0 e^{-kt} + c_u \quad (1)$$

Where $c_{ct}(t)$ denotes total Cd level (in micormolars) at time t (h); c_0 denotes the initial absorbable Cd level (in micromolars); k denotes the coefficient of the proportionality between the absorption rate and the concentration of Cd in solution (in hours); and c_u denotes the non-absorbable Cd

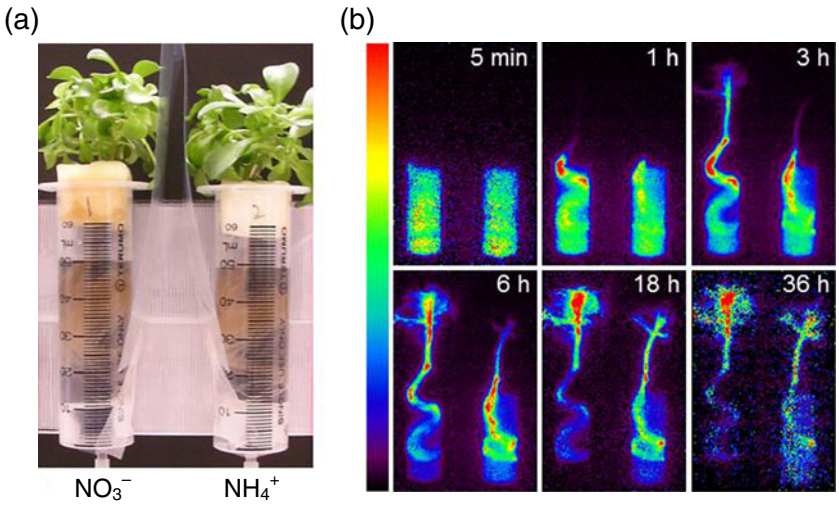

Fig. 1 Serial images of ^{107}Cd movements in *S. plumbizincicola* fed with NO_3^- or NH_4^+

level (in micromolars). The parameters that provided the best fit (Fig. 2b) in the NO_3^- treatment were estimated to be c_0=0.450 μM, c_u=0.089 μM and k=0.505 h^{-1}. By comparison, the parameters in the NH_4^+ treatment were estimated to be c_0=0.414 μM, c_u=0.306 μM and k=0.487 h^{-1}. Nevertheless, it was observed that the Cd curve in solution within the first 0.5 h was complex and decreased faster than the first order reaction kinetics, especially in the NO_3^- treatment (Fig. 2b).

Cd transport in the plants

Figure 2b, d shows the time courses of the amounts of Cd in whole plant roots and shoots. In roots of both NO_3^- and NH_4^+ treatments, Cd accumulation by the roots was observed just after the ^{107}Cd was supplied (Fig. 2b). In the NO_3^- treatment, the amount of Cd in the roots increased more rapidly over time than in the NH_4^+ treatment. The total amount of Cd in the roots of the NO_3^- treatment reached a peak of 15.7 nmol at 1.4 h and then decreased rapidly. The total amount of Cd in the roots of the NH_4^+ treatment increased slowly and reached a plateau of approximately 14.1 nmol from approximately 5.0 to 7.5 h and then decreased slowly. The time-course curves of Cd in shoots showed opposite trends to solution values (Fig. 2d). Cd in the shoots of the NO_3^- treatment increased more rapidly and reached a plateau of approximately 31.2 nmol at 21 h after feeding, while Cd in the shoots of the NH_4^+ treatment increased slowly and reached a plateau of approximately 16.4 nmol at 32 h after feeding. It was estimated that at the end of the PETIS experiment 74.8

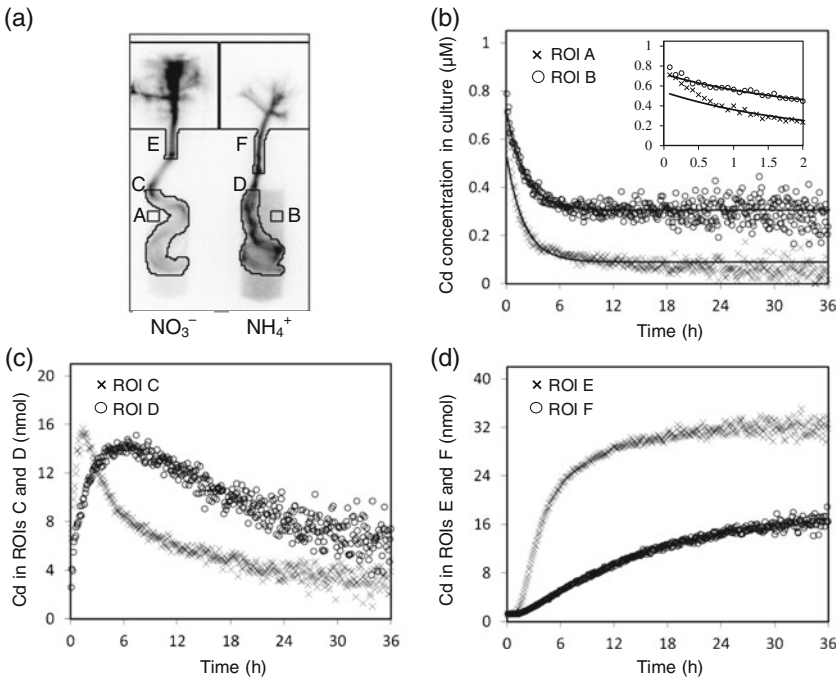

Fig. 2 Time course of Cd concentration in solution and amounts of Cd in roots and shoots of *S. plumbizincicola* fed with NO_3^- or NH_4^+. *ROI* region of interest, *ROIs A* and *B* solution, *ROIs C* and *D* roots, *ROIs E* and *F* shoots. The curve fitted for Cd dynamics in solution is indicated

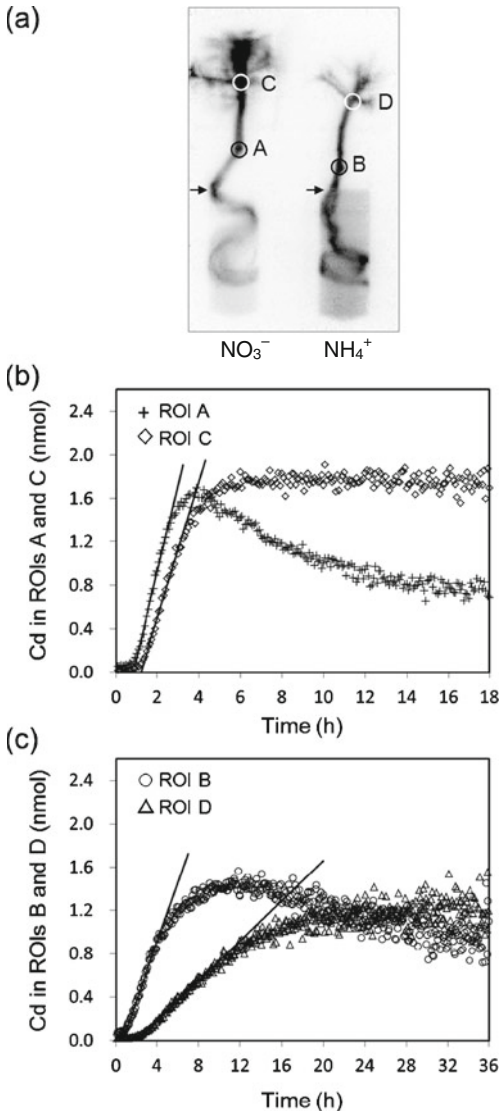

Fig. 3 Time course of amount of Cd in selected regions of *S. plumbizincicola* fed with NO_3^- or NH_4^+. *ROI* region of interest, *ROIs A* and *B* shoot base, *ROIs C* and *D* shoots; *arrows* indicate solution surface. *Fitted lines* are indicated to estimate the arrival times

and 51.4 % of Cd absorbed by the roots was transported into the shoots in the NO_3^-- and NH_4^+-fed plants, respectively.

The timing and velocities of Cd transport from the nutrient solution to the shoots were analysed. ROIs A to D in Fig. 3a were for analysis of Cd transport velocity in roots and shoots shown in Fig. 3b, c. Initial slopes of the curves generated from the PETIS data were fitted to the lines and the X intercepts of the fitted lines were used as the arrival times of Cd at the respective ROIs (Fig. 3b, c). It was estimated that Cd arrived at the shoot base (ROIs A and B) at 0.83 and 0.65 h in NO_3^-- and NH_4^+-treated plants, respectively. Based on the distance from the solution surface to the shoot base, it was estimated that the local velocity of Cd transport in the roots (above the solution surface) was 3.30 and 2.20 cm h^{-1} in NO_3^-- and NH_4^+-treated plants, respectively. Moreover, Cd arrived at the selected shoot area ROIs C and D after 1.21 and 2.04 h, respectively. Consequently, the local velocity of Cd transport in the shoots was estimated to be 10.10 and 2.70 cm h^{-1} in NO_3^-- and NH_4^+-treated plants. Clearly, the transportation of Cd in xylem sap of roots and stem in NO_3^--treated plant was much more rapid than in NH_4^+-treated plant. The rates of water uptake by the plants were monitored continuously and were shown to be highly constant during the experiment, with an average of 0.069 and 0.054 g g^{-1} plant h^{-1} (fitted from 0.5 to 36 h) for NO_3^- and NH_4^+ treatments (Fig. 4).

Cd distribution by autoradiography

After 36 h of PETIS experiments, the test plants were dissected and subjected to autoradiography of ^{109}Cd. This provided the static distribution of Cd at individual leaf, stem and root, which is supplementary analysis above PETIS analysis at the whole shoot level (Fig. 5). Both test plants showed similar morphology with nine branches on the main stem. Strong accumulation of ^{109}Cd was observed in the main stems and roots of both NO_3^-- and NH_4^+-fed plants. Moreover, Cd accumulation in branches and young leaves (smaller leaves that linked to or near branch in Fig. 5) was higher than in mature leaves (larger leaves) of both plants. This indicates that Cd in the main stems was readily transported to the branch and young leaves other than mature leaves irrespective of N form. However, it was found that Cd accumulation in branch and young leaves of the NO_3^--fed plant was stronger than in the NH_4^+-fed plant although the Cd signal in the main stems of these two plants appeared to be similar. This indicates that Cd transportation from the main stem to branches and young leaves in the NO_3^--fed plant was promoted compared with the NH_4^+-fed plant.

Cd concentrations in xylem sap

Xylem sap was collected 2 h after Cd addition from *S. plumbizincicola* seedlings pre-fed with 1 mM NO_3^- or

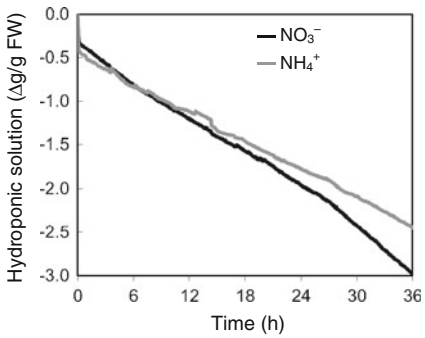

Fig. 4 Time course of water uptake by *S. plumbizincicola* fed with NO_3^- or NH_4^+

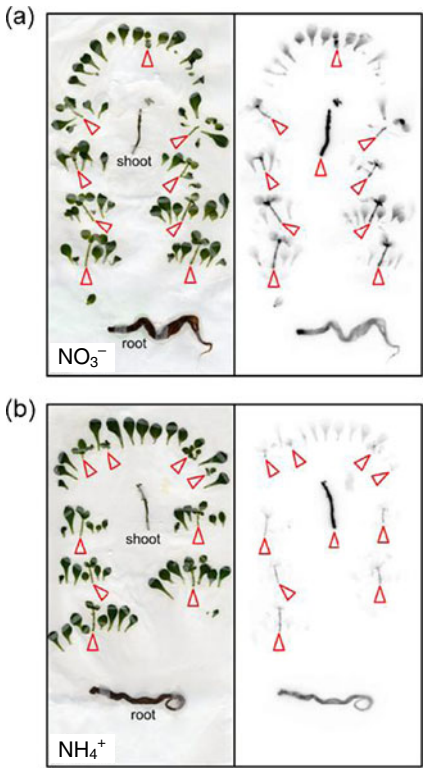

Fig. 5 Autoradiography of static ^{109}Cd in tissues of *S. plumbizincicola* pre-treated with NO_3^- or NH_4^+ for 3 days and then supplied with Cd for 36 h. *Arrows* indicate the main stem and branches

NH_4^+ for 3 days (Table 1). The concentrations of Cd and other ions in the xylem sap were not significantly different between the two rates of Cd addition (1 and 30 μM). The Cd concentration in the xylem sap of NO_3^- pre-treated plants was 2.1 to 2.9 times higher than in NH_4^+ pre-treated plants. The Cd concentration in the xylem sap of the NO_3^- treatment was approximately 110-fold higher than in the external solution (1 μM) while in the NH_4^+ treatment, it was approximately 50-fold higher than in the external solution. Moreover, Ca and K concentrations in the xylem sap of NO_3^- pre-treated plants were around 1.5 times higher than in NH_4^+ pre-treated plants. However, Fe in the former was significantly lower than in the latter and P in the former was 1.5 times lower than in the latter. There was no significant difference in Zn or Mg concentrations in the xylem sap between NO_3^- and NH_4^+ pre-treated plants.

Plant growth and Cd accumulation in long-term solution culture conditions

In the present hydroponic experiment *S. plumbizincicola* was supplied with 30 μM Cd and 1 mM NO_3^- or NH_4^+ (Table 2). There was no significant difference in dry biomass of shoots on the 6th day. However, there was 51.4 and 88.2 % more shoot and root biomass, respectively, in the NO_3^- treatment compared with the NH_4^+ treatment on the 21st day. Shoot Cd concentrations were 1.88 and 2.63 times higher in the NO_3^- than the NH_4^+ treatment on days 6 and 21, but root Cd concentrations showed no difference. Consequently, shoot Cd accumulation was significantly promoted by NO_3^- treatment compared with NH_4^+ because of the enhancement of both plant biomass and Cd transport to the shoots on the 21st day. It was observed that Cd accumulation in NO_3^--fed shoots reached 1.86 mg pot^{-1} after 21 days of culture, 4.23 times higher than in NH_4^+-fed shoots.

Discussion

This is the first reported study to visualise Cd movement non-invasively in an intact Cd hyperaccumulator, *S. plumbizincicola*, using ^{107}Cd tracer and improved PETIS which enabled direct and simultaneous observation of the radiotracer-treated culture solution and plants (Fujimaki et al. 2010; Ishikawa et al. 2011). The imaging data were applied for quantitative analysis of the dynamics and kinetics of Cd uptake and transport in the whole plant supplied with two different inorganic N sources, NO_3^- and NH_4^+. Physiologically, the uptake of NH_4^+ will decrease the rhizosphere pH, which results in an increase in metal bioavailability and consequently an increase in metal accumulation by plants. Considering this, the solution in the present experiment was buffered with 1 mM MES-Tris to eliminate the effects of pH changes on Cd uptake. Moreover, concentrations of cation in solution, such as Ca^{2+}, K^+ and Mg^{2+} were balanced between the N treatments to eliminate their competitive effects on Cd uptake (Lu et al. 2008; Zhu et al. 2011). Therefore, the different Cd dynamics in this study were clearly atributable to the effects of the different forms of N.

Cd uptake by roots

It is well known that Cd uptake by plant roots consists of apoplastic binding and symplastic uptake (Cataldo et al. 1983; Hart et al. 1998). Zhao et al. (2002) found that root apoplastic binding of Cd in the hyperaccumulator *N. caerulescens* reached a plateau after approximately 45 min and then symplastic uptake dominated the dynamics. In the present PETIS experiment, a drastic drop in the Cd absorption curve in solution was observed after the first few hours in both N treatments (Fig. 2b). A complex shape, which decreased more rapidly than first order reaction kinetics, appeared within the first 0.5 h, especially in the NO_3^- treatment (Fig. 2b). A similar phenomenon was observed in previous studies of Cd uptake dynamics in rice (Fujimaki et al. 2010; Ishikawa et al. 2011). This may be explained in terms of the complicated combination of both apoplastic binding and symplastic uptake of Cd by the roots. After

Table 1 Elemental concentrations in the xylem sap of *Sedum plumbizincicola* pre-treated with 1 mM NO_3^- or NH_4^+ for 3 days and then supplied with different concentrations of Cd for 2 h

Treatment	Cd (μM)	Ca (mM)	K (mM)	Mg (mM)	Fe (μM)	Zn (μM)	P (mM)
1 mM NO_3^-+1 μM Cd	111±6 a	5.47±0.52 a	4.07±0.45 a	1.08±0.07 a	0.90±0.58 b	89.9±9.9 a	0.77±0.15 b
1 mM NH_4^++1 μM Cd	38.0±26.0 b	3.80±0.26 c	2.64±0.28 b	1.10±0.15 a	26.1±8.0 a	86.2±23.6 a	1.18±0.15 a
1 mM NO_3^-+30 μM Cd	107±2 a	4.49±0.41 b	4.52±0.35 a	1.03±0.08 a	1.55±1.13 b	89.2±40.2 a	0.77±0.13 b
1 mM NH_4^++30 μM Cd	50.0±3.0 b	3.30±0.36 c	2.77±0.37 b	0.91±0.15 a	22.2±5.0 a	98.2±25.3 a	1.15±0.41 a

Values are means±SD (n=4); different letters in the same column indicate significant differences based on one-way ANOVA followed by LSD test (P<0.05)

0.5 h, the dynamics of Cd uptake by the roots in both N treatments were fitted well by the first order reaction kinetics (Fig. 2b; Eq. 1), indicating that transporter-mediated symplastic Cd uptake and transportation dominated the dynamics.

The time course of Cd dynamics in both solution and roots (Fig. 2b, c) indicates that the Cd absorption rate was always higher in the NO_3^- treatment than the NH_4^+ treatment. The higher Cd absorption rate by the roots of NO_3^- might be attributed to NO_3^--enhanced up-regulation of Cd transporters involved in symplastic uptake and transportation of Cd in the roots. It is known that in addition to its nutrient function NO_3^- also acts as a signal molecule that controls many aspects of plant metabolism and development (Crawford 1995; Stitt 1999; Krouk et al. 2010). It is estimated that NO_3^- responsive genes may account for up to 10 % of the transcriptome (Krouk et al. 2010). Luo et al. (2012) reported that compared with NH_4^+, NO_3^- application directly enhances Cd uptake of tomato, which they attributed to the up-regulation of the Fe uptake systems in the roots. In the present study, considering the depressed Fe but elevated K and Ca concentrations in the xylem sap of the NO_3^- treatment (Table 1), it appears that Cd is taken up by the roots instead of Fe. Moreover, it would be reasonable that NO_3^- might up-regulate the expression of K transporters or Ca channels which can also be involved in low-affinity Cd uptake and translocation in the roots. Furthermore, NO_3^- transport across the plasma membrane results in the membrane potential being temporarily depolarised and then being hyperpolarised (McClure et al. 1990; Miller et al. 2001) and this may enhance the membrane transport of cations. In contrast, uptake of NH_4^+ depolarises the membrane potential and thus inhibits cation uptake (Crawford and Glass 1998). On the other hand, it was also observed that a higher concentration of Cd (0.290 μM) in the culture solution still remained unabsorbed after the plateau in the NH_4^+ treatments, while it was depleted to a much lower level (0.056 μM) in the NO_3^- treatments (Fig. 2b). It seems that, compared with NO_3^-, NH_4^+ competed with Cd uptake by the root. Nevertheless, another possible explanation is that NO_3^- induced the expression of some high-affinity Cd transporters in the roots of *S. plumbizincicola* which operated efficiently at a low external Cd concentration.

Cd translocation from root to shoot

In general, the root-to-shoot translocation of heavy metals is theoretically dependent on the following processes: (1) symplastic uptake by the roots, (2) root retention, (3) xylem loading, and (4) xylem unloading and uptake by leaf cells. Low vacuolar sequestration in roots and rapid xylem loading are critical characters for hyperaccumulator plants (Baker et al. 1994; Lasat et al. 1996, 1998; Zhao et al. 2006). In the present study, the Cd dynamics in the root reflect the combination of Cd uptake from the solution and the transfer from root to shoot, including retention within vacuoles and

Table 2 Dry weights of shoots and roots, Cd concentration and accumulation of *Sedum plumbizincicola* hydroponically fed with 30 μM Cd and 1 mM NO_3^- or NH_4^+ for 6 or 21 days

Plant part	Nitrogen form	Dry weight (mg pot^{-1})		Cd concentration (mg kg^{-1})		Plant Cd accumulation (mg pot^{-1})	
		6 days	21 days	6 days	21 days	6 days	21 days
Shoots	NO_3^-	237±32 a	504±78 a	1799±626 a	3262±852 a	0.41±0.11 a	1.86±0.43 a
	NH_4^+	208±33 a	333±58 b	959±187 a	1241±281 b	0.20±0.02 b	0.44±0.08 b
Roots	NO_3^-	29±2 a	64±17 a	2469±94 a	3511±517 a	0.07±0.01 a	0.22±0.05 a
	NH_4^+	19±3 b	34±6 b	2524±299 a	2941±537 a	0.05±0.01 a	0.10±0.01 b

Values are means±SD (n=4). For each trait, means in columns followed by different letters indicate significant differences from each other according to independent-sample t test (P<0.05)

xylem loading (Fig. 2c). The drastic drop after 1.4 h in the NO_3^--fed plant may be attributed to the rapid depletion of the Cd supply from solution to root, low vacuolar sequestration and vigorous xylem loading. In contrast, the gentle saturation and slow decline curves in the NH_4^+-treated plant indicate stronger retention within vacuoles and relatively low xylem loading. Generally, compared with NO_3^-, NH_4^+ is assimilated into some amino acids and consequently some classes of proteins in roots, which might facilitate the vacuolar sequestration of Cd in the roots but decrease xylem loading and translocation to the shoots (Yang et al. 2006). Moreover, xylem sap analysis revealed that the Cd concentration in NO_3^- and NH_4^+ treatments was around 110- and 50-fold higher than that in the external solution (1 μM), respectively (Table 1). Though there was a risk that the xylem sap sample in micro tubes was concentrated as water evaporated during sap collection, it can be concluded that xylem loading of Cd was more efficient in the NO_3^--fed plant than in the NH_4^+-fed plant. This is also consistent with the Cd dynamics in the shoots (Fig. 2d). Recently, numerous studies have demonstrated that P_{1B}-type heavy metal ATPase, *HMA4*, localised at the plasma membranes and expressed predominantly in the root vascular tissues, likely plays an important role in the xylem loading of Cd and Zn in the hyperaccumulators *N. caerulescens* and *Arabidopsis halleri* (Becher et al. 2004; Papoyan and Kochian 2004; Talke et al. 2006). Thus, compared with NH_4^+, NO_3^- may also induce the up-regulated expression of Cd transporters involved in Cd xylem loading in addition to Cd uptake in *S. plumbizincicola*.

The present study shows that the local velocity of Cd transport was approximately 1.5- and 3.7-fold higher in the roots and shoots of NO_3^--fed plants than NH_4^+-fed plants, respectively (Fig. 3). Moreover, at the end of the PETIS experiment a larger proportion of the Cd absorbed by the roots was transported into the shoots in the NO_3^--fed plants (74.8 %) than the NH_4^+-fed plants (51.4 %). Autoradiography of ^{109}Cd after PETIS also reveals that Cd transportation from the main stem to branches and young leaves in NO_3^--fed plants was promoted compared with NH_4^+-fed plants (Fig. 5). Generally, root-to-shoot translocation of Cd occurs via the xylem and is driven by transpiration from the leaves. In the present study, the rates of water uptake by NO_3^--fed plants (0.069 g g^{-1} plant h^{-1}) were slightly higher (1.3-fold) than NH_4^+-treated plants (0.054 g g^{-1} plant h^{-1}) (Fig. 4). However, the differences in water uptake cannot fully explain the differences in local velocity of Cd transport (3.7-fold) and the differences in Cd distribution between these two N treatments. Comparatively, it is suggested that unlike NH_4^+ which is mainly assimilated or stored in the roots, NO_3^- is translocated as a hydrated anion in the xylem where cationic charges are required for charge balance (Monsant et al. 2010). Meanwhile, NO_3^- fertiliser enhanced the accumulation of organic anions, such as glutamate, malate and oxalate in the shoots and this was accompanied by an increase in the concentrations of cations (K^+, Ca^{2+}, Mg^{2+} and Na^+) (Arnozis and Findenegg 1986; Turan and Sevimli 2005). This is consistent with our results showing that concentrations of the cations Ca and K in the xylem sap increased but the anion P was inhibited in the NO_3^- treatment compared with the NH_4^+ treatment (Table 1). Moreover, it has been demonstrated that Cd in the xylem sap of the hyperaccumulator *A. halleri* was mainly in aqueous free ionic form and with little Cd-citrate complexation (Ueno et al. 2008). Moreover, stimulated production of valine and other responsive amino acids may be involved in Zn unloading into leaf cells of the hyperaccumulator *S. alfredii* (Yang et al. 2006). Therefore, the charge balance from the anion NO_3^- and organic acids in the xylem sap may be responsible for the higher transport velocity of Cd. Meanwhile some organic acids may facilitate Cd unloading into leaf cells in NO_3^--treated plants compared with NH_4^+-treated plants. These effects together enhance Cd transportation from root to shoot in *S. plumbizincicola*. Further physiological and molecular research is required to confirm these hypotheses.

Plant biomass and Cd accumulation

In the present long-term hydroponics experiments, plant growth was also significantly promoted by NO_3^- nutrition compared with NH_4^+. As a result, Cd hyperaccumulation in *S. plumbizincicola* was largely enhanced by NO_3^- supplementation rather than NH_4^+ (Table 2). This is consistent with previous studies showing that NO_3^- fertilisation led to both higher biomass production and higher Cd and Zn extraction than NH_4^+ in *N. caerulescens* (Schwartz et al. 2003; Monsant et al. 2008, 2010, 2011; Xie et al. 2009), camomile (Kovacik et al. 2011) and tomato (Luo et al. 2012). The stimulated uptake of nutrient elements such as Ca and K may be partly responsible for the stimulation of plant growth. The ameliorated plant metabolism and development process may in turn improve Cd tolerance and detoxification resulting in a further increase in Cd accumulation in *S. plumbizincicola*. We suggest that *S. plumbizincicola* is usually grown in dry fields where N tends to be predominantly in the NO_3^- form. This adaptability thus makes the hyperaccumulator prefer NO_3^- nutrition to NH_4^+.

Conclusions

This is the first report of successful visualisation and quantification of Cd uptake and root-to-shoot translocation in intact plants of the hyperaccumulator species *S. plumbizincicola* supplied with different forms of inorganic N using PETIS, a real-time imaging method. The results provide clear physiological evidence that compared with NH_4^+, NO_3^- promoted

plant growth and the major steps in the transport route of Cd from solution to shoots including its uptake by roots, xylem loading, root-to-shoot translocation in the xylem, and unloading to leaves. As a result, Cd accumulation in the shoots was largely enhanced. Thus, in the present hydroponic conditions NO_3^- fertilisation rather than NH_4^+ is the preferred choice for Cd phytoextraction by *S. plumbizincicola* but further research is required to investigate the effects in soil conditions where the chemical and physiological processes involved will be more complicated than in nutrient solution conditions.

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