Journal of Plant Physiology **166** (2009) 579—587

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JOURNAL OF PLANT PHYSIOLOGY

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Cadmium uptake and xylem loading are active processes in the hyperaccumulator *Sedum alfredii*

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Received 3 May 2008; received in revised form 18 August 2008; accepted 2 September 2008

KEYWORDS
Cadmium;
Sedum alfredii;
Symplastic pathway;
Translocation;
Uptake

Summary

Sedum alfredii is a well known cadmium (Cd) hyperaccumulator native to China; however, the mechanism behind its hyperaccumulation of Cd is not fully understood. Through several hydroponic experiments, characteristics of Cd uptake and translocation were investigated in the hyperaccumulating ecotype (HE) of *S. alfredii* in comparison with its non-hyperaccumulating ecotype (NHE). The results showed that at Cd level of 10 μM measured Cd uptake in HE was 3–4 times higher than the implied Cd uptake calculated from transpiration rate. Furthermore, inhibition of transpiration rate in the HE has no essential effect on Cd accumulation in shoots of the plants. Low temperature treatment (4 °C) significantly inhibited Cd uptake and reduced upward translocation of Cd to shoots for 9 times in HE plants, whereas no such effect was observed in NHE. Cadmium concentration was 3–4-fold higher in xylem sap of HE, as compared with that in external uptake solution, whereas opposite results were obtained for NHE. Cadmium concentration in xylem sap of HE was significantly reduced by the addition of metabolic inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP), in the uptake solutions, whereas no such effect was noted in NHE. These results suggest that Cd uptake and translocation is an active process in plants of HE *S. alfredii*, symplastic pathway rather than apoplastic bypass contributes greatly to root uptake, xylem loading and translocation of Cd to the shoots of HE, in comparison with the NHE plants.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; HE, hyperaccumulating ecotype; NHE, non-hyperaccumulating ecotype; NS, nutrient solution; US, uptake solution.

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doi:10.1016/j.jplph.2008.09.001

Introduction

In the past decades, phytoremediation has gained acceptance as a technology and has been acknowledged as an area of research (Pilon-Smits, 2005). Natural hyperaccumulator plant species can be effective in phytomining or phytoextraction of the heavy metals from contaminated or mineralized soils (Raskin et al., 1997). Hundreds of hyperaccumulators have been identified in the past 20 years, and frequently researched for the mechanisms that underlie their uptake, transport, and detoxification of pollutants (e.g., Krämer et al., 1996; Lombi et al., 2002; Hanikenne et al., 2008). However, potential use of hyperaccumulators in phytoremediation is limited by a lack of knowledge of many basic plant processes (Pilon-Smits, 2005). In general, two processes other than bioavailability determine metal accumulation in plants: uptake activity and efficiency of translocation (Clemens, 2006). A better understanding of the mechanisms controlling metal entry into root and translocation to the shoot in hyperaccumulators may contribute to both genetic modification and conventional breeding strategies of plants for phytoremediation (McGrath and Zhao, 2003).

Metals can be absorbed by plant roots both passively and actively. Lead uptake is generally considered to be passive, apoplastic binding of Cd has also been reported in roots of wheat cultivars (Hart et al., 1998) and Cd hyperaccumulator, *Thlaspi caerulescens* (Lombi et al., 2001). As a non-essential element, Cd was suggested to enter into plant cells actively via uptake systems for essential elements, especially Zn (Pence et al., 2000), Ca (Perfus-Barbeoch et al., 2002) or Fe (Lombi et al., 2002). However, in the Cd hyperaccumulator *Thlaspi caerulescens*, a specific mechanism of Cd uptake mediated by high-affinity Cd transporters was suggested (Zhao et al., 2002). Root-to-shoot translocation of Cd generally occured via the xylem and is driven by transpiration from the leaves (Salt et al., 1995; Hart et al., 1998; Ueno et al., 2008). This indicated that Cd transfer from the root medium to the xylem in the hyperaccmulator *Arabidopsis halleri* was an energy-dependent process. However, the relative contribution of the symplastic and apoplastic pathways to the delivery of cations to xylem is still rarely known (White, 2001). For improving potential use of hyperaccumulators in phytoremediation, different mechanisms of metal uptake and translocation in plants imply different strategies. Targets of the symplastic pathway are the transporters in cell membranes, while targets of the apoplastic pathway may be diverse (White et al., 2002).

The aim of this study is to identify the relative contribution of symplastic pathway and apoplastic bypass in a newly identified Cd hyperaccumulator native to China. The hyperaccumulating ecotype (HE) of *Sedum alfredii*, *Crassulaceae*, originally grew in a Pb/Zn mined area of South China, is the first non-*Brassicaceae* Cd hyperaccumulator identified so far (Yang et al., 2004). The other two Cd hyperaccumulators *Thlaspi caerulescens* (Zhao et al., 2002) and *Arabidopsis halleri* (Zhao et al., 2006) are both *Brassicaceae*. Plants of HE *S. alfredii* can accumulate more than 6000 $\mu g\,g^{-1}$ Cd in shoots (Yang et al., 2004), whereas its contrasting non-hyperaccumulating ecotype (NHE) showed neither tolerance nor hyperaccumulation ability to Cd (Xiong et al., 2004). Although *S. alfredii* has been recently studied considerably, little information is available on its possible mechanisms of Cd uptake (Yang et al., 2005; Sun et al., 2007). Our previous research suggests that rapid root-to-shoot translocation involving reduced root cell sequestration or enhanced xylem loading, probably by symplastic pathway, may be a crucial process in hyperaccumulation of Cd by HE *S. alfredii* (Lu et al., 2008). In this study, the exact role of symplastic and apoplastic pathway in Cd uptake and translocation is compared in the two ecotypes of *S. alfredii* differing in Cd accumulation, as to better understand Cd uptake and accumulation characteristics of the hyperaccumulator HE *S. alfredii*, for further developing strategies of improved phytoremediation by using this plant species.

Materials and methods

Plant materials and growth conditions

Seedlings of two contrasting ecotypes of *Sedum alfredii* Hance were cultivated according to Yang et al. (2005). The hyperaccumulating ecotype (HE) of *S. alfredii* was originally obtained from an old Pb/Zn mine area in Zhejiang Province, China, and the non-hyperaccumulating ecotype (NHE) of *S. alfredii* from a tea garden of Hangzhou in Zhejiang Province. Plants were chosen to grow in non-contaminated soil for several generations to minimize the internal metal contents, then uniform and healthy shoots were selected and cultivated in the basal nutrient solution containing 2 mM Ca^{2+}, 4 mM NO_3^+, 1.6 mM K^+, 0.1 mM $H_2PO_4^-$, 0.5 mM Mg^{2+}, 1.2 mM SO_4^{2-}, 0.1 mM Cl^-, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe–EDTA. Nutrient solution pH was adjusted daily to 5.5–5.8 with 0.1 M NaOH or HCl. Plants were grown under glasshouse conditions with natural light, day/night temperature of 26/20 °C and day/night humidity of

70/85%. The nutrient solution was continuously aerated and renewed every 3 days.

Cd uptake measurement and calculation

The 2-week-old seedlings of two *S. alfredii* ecotypes were placed in 500 mL uptake solution (2 mM MES–Tris (pH = 5.8), 0.5 mM $CaCl_2$) (US) or basal nutrient solution (NS) with supply of 10 μM or 100 μM $CdCl_2$. Each treatment was replicated 4 times. Cadmium concentrations in the solutions were analyzed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Agilent 7500a, USA) before uptake experiments. Plants were harvested after 2 days and Cd concentrations were measured using ICP-MS (Agilent 7500a, USA), and Cd uptake was calculated based on the dry weight of root biomass. Water loss, resulting from transpiration, was also recorded by weight at the onset and end of the experiments.

Transpiration inhibitor experiment

After 2 weeks of pre-cultivation, 2.0% transpiration inhibitor (87% paraffin base petroleum oil and 13% surfactants) was sprayed upon the leaves of half of the seedlings of HE *S. alfredii*. Thereafter, all plants were transferred to custom-built vessels containing 500 mL basal nutrient solution with supply of 50 μM Cd. Plants were re-sprayed with the transpiration inhibitor solution once a day. At each time interval (1, 3, 5, and 7 d), plants were harvested, and water depletion in the vessels was measured by the weight difference method. Evaporative loss from vessels with no plants was measured to eliminate errors. Roots and shoots of the plants were separated, oven-dried and weighed. Cadmium concentrations in the plant tissues were analyzed by ICP-MS (Agilent 7500a, USA).

Low temperature experiment

After 2 weeks of pre-cultivation, seedlings of HE or NHE *S. alfredii* were placed in aerated uptake solution containing 2 mM MES–Tris (pH = 5.8), 0.5 mM $CaCl_2$ and 10 μM $CdCl_2$, either in low temperature (4 °C) or room temperature (25 °C) condition. For the 4 °C treatment, plants were transferred to ice-cold pretreatment solution 30 min prior to the uptake, and uptake containers were placed in an ice bath and shaded from light. At each time interval (0, 2, 4, 8, 16, 24, 48, 72 h), three seedlings in one pot were harvested and a 2.0 mL aliquot of the uptake solution was taken from each pot for the determination of Cd concentrations by ICP-MS (Agilent 7500a, USA), each treatment was replicated 4 times. Plants were rinsed, separated into roots and shoots, oven-dried and weighed. Cadmium in plant tissues were analyzed by ICP-MS (Agilent 7500a, USA) after digestion with HNO_3–$HClO_4$.

Xylem sap collection and analysis

Plants of HE and NHE *S. alfredii* grown hydroponically for 8 weeks were used for xylem sap collection. Plants were decapitated 3–4 cm above the roots after treatment with 10 μM Cd for 4 h in the uptake solution (2 mM MES–Tris (pH = 5.8), 0.5 mM $CaCl_2$). Treatments were replicated 4 times, and six plants in the same pot were treated as one replicate. Immediately after de-topping, each stem was rinsed with deionized water and blotted with absorbent paper to remove contaminants from cut cells. Sap flowing from the tubing was collected in sterile vials at time-points indicated in Figure 3. At the onset of each xylem sap collection, a 1.0 mL aliquot of the uptake solution was taken from each pot for Cd determination. For xylem sap samples, a subsample of 0.5 mL was mixed with 2.5 mL of 2% (w/v) nitric acid. Cadmium concentrations in all samples were determined by ICP-MS (Agilent 7500a, USA).

In a separate experiment, plants of the two ecotypes were placed in the same uptake solutions (2 mM MES–Tris (pH = 5.8), 0.5 mM $CaCl_2$) with different treatments including: control, 100 μM Cd, 100 μM Cd plus 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 100 μM Cd plus 10 μM 2,4-dinitrophenol (DNP). Xylem sap from the plants was collected 4 h after treatments as described above. Total volume of xylem sap collected within 24 h was measured for each replicate, and Cd concentration in the xylem sap was analyzed as mentioned above.

Statistical analysis of data

All data were statistically analyzed using the SPSS package (Version# 11.0), analysis of variance (ANOVA) was performed on the data sets, with the mean and SE of each treatment calculated.

Results

Cadmium uptake and transpiration

Despite the same Cd supply levels (10 or 100 μM), the activity of Cd^{2+} in the nutrient solution (NS) was much lower than that in the uptake solution (US), as a result of the presence of large amount of ions in the NS (Figure 1). The results showed that measured Cd uptake by both ecotypes of *S. alfredii* in the US was much higher than that in the NS, regardless of the treatments (Figure 1). More importantly, measured Cd uptake by HE was 5-fold and/or 3-fold ($P<0.01$) higher than that calculated from transpiration rate at either low (10 μM) or high (100 μM) Cd exposure level in the US (Figure 1a, b). In the NS, significantly higher Cd uptake ($P<0.01$) by HE was also marked at low Cd supply level, as compared with the calculated value (Figure 1a).

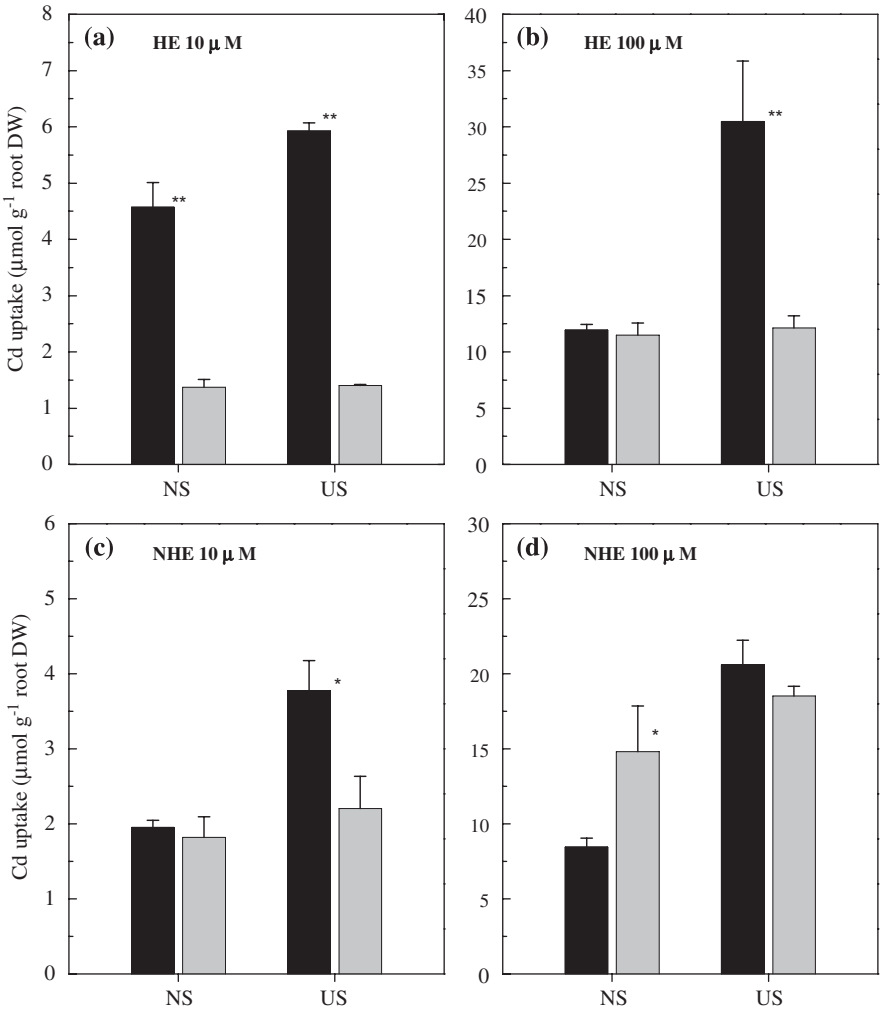

Figure 1. Comparison of measured cadmium (Cd) and calculated Cd uptake by HE (a, b) and NHE (c, d) *Sedum alfredii*. Plants were grown in nutrient solution (NS) or uptake solution (US) with supply of 10 μM (a, c) or 100 μM (b, d) Cd for 2 d. Means marked with one or two asterisks indicate significant difference between measured Cd uptake (black columns) and that calculated from transpiration rate (gray columns) at $P<0.05$ or $P<0.01$, respectively. Data points and error bars represent means and SEs of four replicates.

Cadmium uptake by NHE was lower than that by HE on average, and no consistent difference between measured Cd uptake and that calculated from transpiration rate was observed for the NHE plants (Figure 1c, d). Measured Cd uptake by the NHE plants exposed to 10 μM Cd in the US was around 2-fold ($P<0.05$) higher than that calculated from transpiration rate (Figure 1c); however, it was significantly lower ($P<0.05$) than the calculated value when plants were exposed to 100 μM Cd in the NS (Figure 1d).

Transpiration of the HE plants was largely prevented by the application of the transpiration inhibitor on the leaves (Figure 2a). After treatment with the inhibitor for more than 5 days, transpiration rate of the plants were reduced by 53% as compared with the control ($P<0.01$) (Figure 5a). However, no significant effect of the treatment on the Cd accumulation was noted in either roots or shoots of the plants (Figure 2b, c). Cadmium concentrations in shoots of the HE plants were linearly increased with time, regardless of the treatment of inhibitor (Figure 2c). More than 3 days of 50 μM Cd treatment resulted in root death of the NHE plants.

Effect of low temperature on Cd uptake and translocation

The data in Figure 3 indicated that inhibition of Cd uptake by the low temperature (4 °C) was much more pronounced in the HE than that in the NHE. Time-course Cd concentration in the uptake solution depleted dramatically by the HE plants and was extremely low (less than 0.2 μM) after 72 h, while reduction of Cd concentration by the NHE

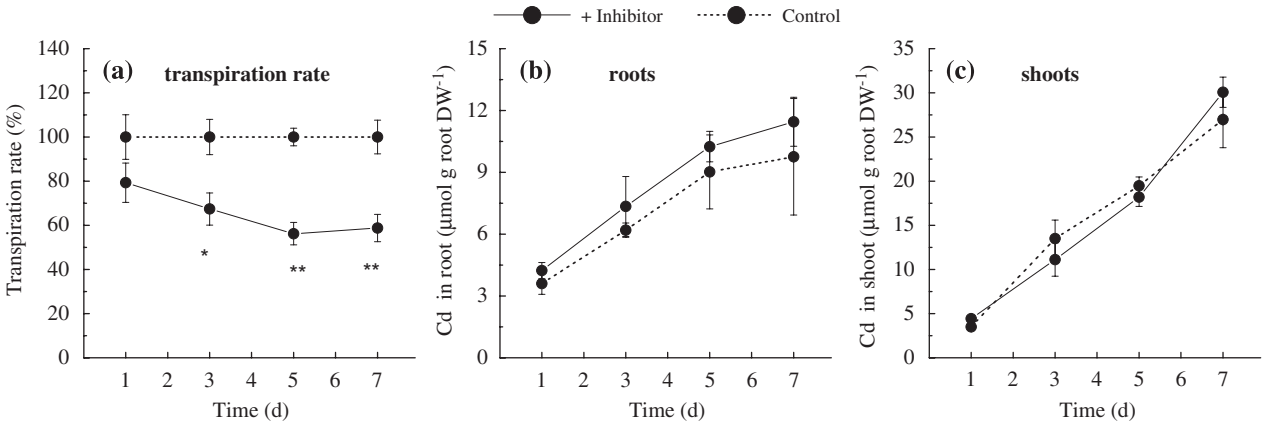

Figure 2. Effect of transpiration inhibitor on transpiration rate (a) and Cd accumulation in shoots (b) of HE *Sedum alfredii*. Plants were applied with (solid line) or without (dotted line) transpiration inhibitor, and afterwards placed in the 500 mL nutrient solution with supply of 50 μM Cd for 1, 3, 5, and 7 d, respectively. One or two asterisks indicate significant difference between the control and the treatment at $P<0.05$ or $P<0.01$, respectively. Data points and error bars represent means and SEs of four replicates.

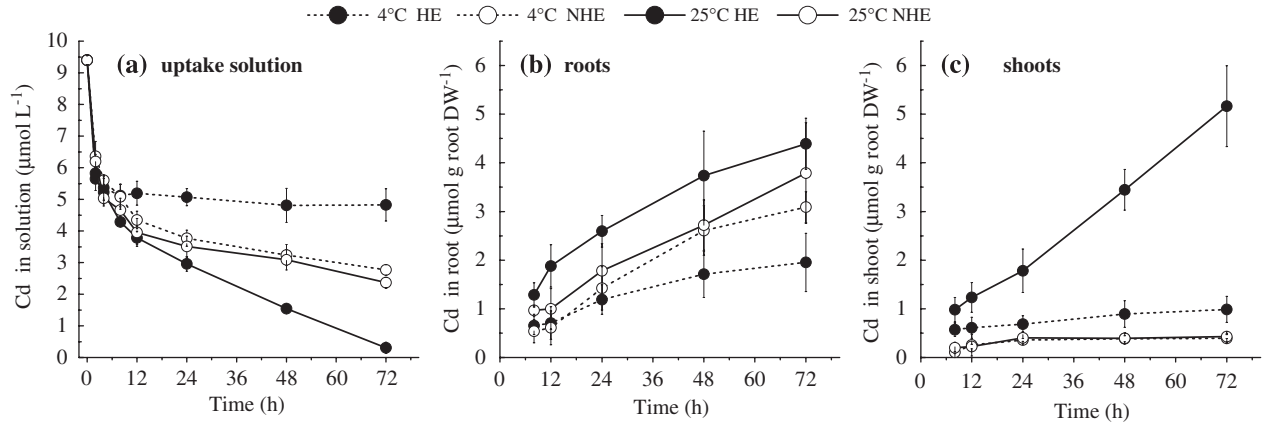

Figure 3. Time-course Cd concentration in uptake solution (a), roots (b) and shoots (c) of HE (black circle) and NHE (white circle) *Sedum alfredii* with treatments of 10 μM Cd, as affected by low temperature (4 °C, dotted line; 25 °C, solid line). Data points and error bars represent means and SEs of four replicates.

plants became less marked after 12 h absorption (Figure 3a). Cadmium uptake by the HE plants was significantly ($P<0.01$) inhibited by the low temperature, whereas no such effect occurred in the NHE plants (Figure 3a). Determination of Cd content in plant tissues further supported the above results (Figure 3b, c). Cadmium concentration in roots of HE was 2–3 times higher when the plant were placed in room temperature (25 °C) than in low temperature (4 °C), whereas no difference in root Cd concentrations of NHE was found between the treatments of low (4°C) and room (25 °C) temperatures (Figure 3b). The effect of low temperature on decreasing shoot Cd concentration was more pronounced ($P<0.01$) in HE, Cadmium content in shoots of HE increased time-dependently at least within 72 h under room temperature (25 °C), whereas no increase of Cd was not observed in shoots of HE after 12 h when the plants were placed in 4 °C uptake solution (Figure 3c). Cadmium in shoots of the HE reached 10-fold higher under 25 °C than under 4 °C at the uptake time of 72 h. However, similar Cd concentrations in shoots of NHE were observed under both low and room temperature (Figure 3c).

Cd transport in xylem

Time-dependent variation of Cd concentration in the xylem sap of the plants and the uptake solution was showed in Figure 4 for both HE and NHE *S. alfredii*. No significant variation of Cd concentrations in xylem sap of both ecotypes occurred within 48 h, whereas Cd in the uptake solution decreased gradually, especially in the uptake

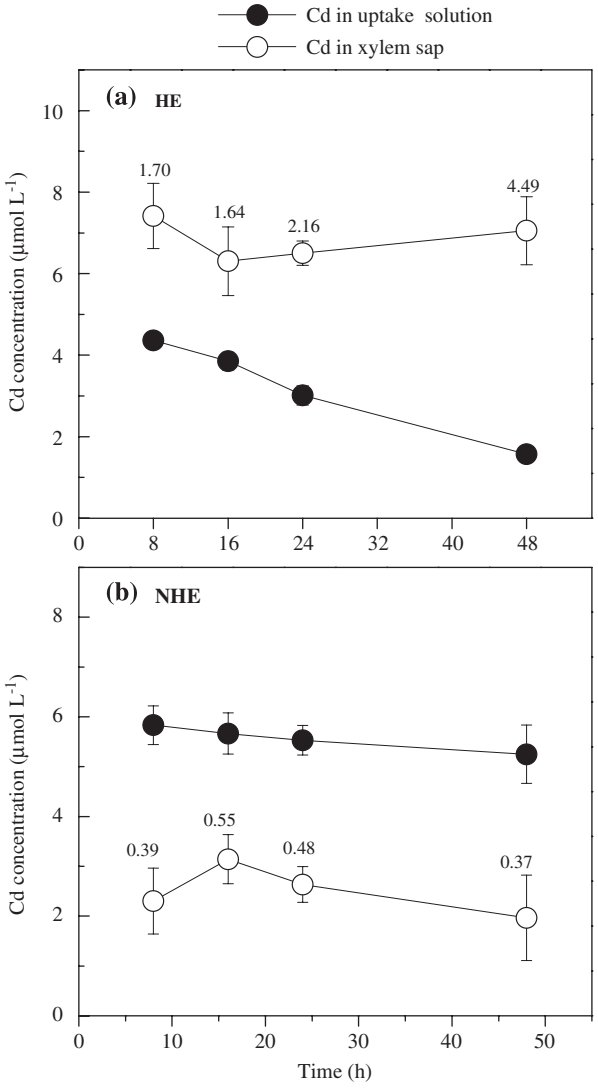

Figure 4. Time-course Cd concentration in the uptake solution (black circle) and xylem sap (white circle) of HE (a) and NHE (b) *Sedum alfredii* with treatments of 10 μM Cd. The numbers inside figures indicate ratios of Cd in xylem sap to Cd in external solutions. Six plants in the same pot were treated as one replicate. Data points and error bars represent means and SEs of four replicates.

solutions where the HE plants were placed (Figure 4). Cadmium concentration was constantly higher in the xylem sap of HE than that in the uptake solution, and the ratio of Cd in xylem sap to Cd in the uptake solution reached 4.49 high at 48 h after decapitation (Figure 4a). In contrast, the ratio of Cd in the xylem sap of NHE to Cd in the uptake solution was constantly below 1.0 at all times examined (Figure 4b). Meanwhile, Cd concentration in the xylem sap of the HE plants was approximately 4-fold higher than that of NHE on average (Figure 4).

Cadmium exposure (100 μM) decreased water transport significantly ($P<0.05$) in xylem of NHE plants, while having no essential effect on that of HE (Figure 5a). After treatments with 100 μM Cd, the volume of xylem sap collected from the HE plants was 2-fold greater ($P<0.05$) (Figure 5a), and the concentration of Cd in the sap was 4-fold higher ($P<0.01$) (Figure 5b) as compared with that of NHE. Treatments with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 10 μM 2,4-dinitrophenol (DNP), two metabolic inhibitors, resulted in considerable reduction of xylem transport of Cd in both ecotypes of *S. alfredii*. Total volume of xylem sap collected from HE plants within 24 h was significantly decreased by CCCP or DNP ($P<0.05$), and no xylem sap was collected from NHE plants after CCCP treatment (Figure 5a). Meanwhile, Cd concentration in the xylem sap of the HE plants was markedly reduced by CCCP ($P<0.05$) (Figure 5b). Treatment of DNP significantly inhibited the xylem transport of Cd in HE plants ($P<0.05$), while no such reduction of Cd levels in the xylem sap of the NHE plants was observed (Figure 5b).

Discussion

Passive (apoplastic) uptake involves diffusion of ions in the soil solution into the root endodermis along a chemical potential (concentration) gradient, while active ion uptake takes place against the concentration gradient with high selectivity of ions and energy-consuming mechanism (Marschner, 1995). Cadmium uptake by both HE and NHE *S. alfredii* was not entirely concentration-dependent, as higher Cd uptake by the plants was indicated from the US, which contained same concentrations but higher activities of Cd when compared with the NS (Figure 1). This indicates that Cd uptake in both HE and NHE plants is not solely driven by apoplastic bypass. The independent variation of Cd uptake and transpiration rate between the two ecotypes (Figure 1) also supports that apoplastic bypass dose not account for the ecotypic variation of *S. alfredii* in Cd accumulation. Furthermore, the results from this study convincingly demonstrate that Cd uptake by the HE exceeds water uptake greatly, this was characterized by both a constant decline in Cd concentration in the solution with time (Figure 3a) and significantly higher measured Cd uptake as compared with that calculated from transpiration rate (Figure 1a, b). This suggests the presence of symplastic pathway of Cd uptake in HE. Great inhibition of Cd uptake in HE by low temperature, further supports that Cd uptake by the plants is an energy-dependent active process. However, in the

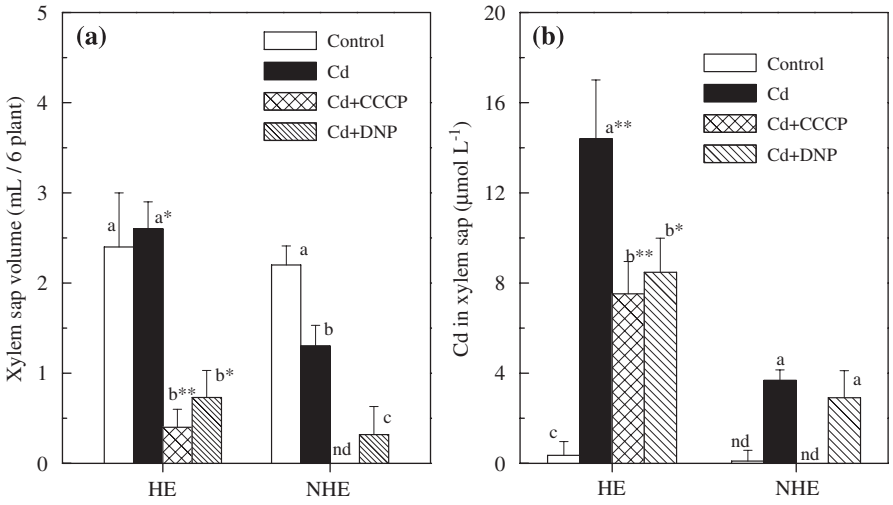

Figure 5. Total volume of xylem sap (a) and Cd concentration in the xylem sap (b) collected from HE and NHE *Sedum alfredii* within 24 h after decapitation. Plants were grown in uptake solutions (2 mM MES–Tris (pH = 5.8), 0.5 mM $CaCl_2$) with treatments including: control, 100 μM Cd, 100 μM Cd plus 100 μM CCCP, and 100 μM Cd plus 10 μM DNP. After 4 h exposure, plants were decapitated 3–4 cm above the roots for collection of xylem sap. Six plants in the same pot were treated as one replicate. One or two asterisks indicate significant difference between HE and NHE at $P<0.05$ or $P<0.01$, respectively. Different letters indicate signicant difference between different treatment at $P<0.05$. Data points and error bars represent means and SEs of four replicates.

NHE plants, the evidence for active process in Cd uptake and translocation is less obvious. Although Cd uptake by NHE was significantly higher than the calculated value at low Cd supply level, the contrast effect was observed when the plants were treated with high Cd level. Liang et al. (2006) suggested the coexistence of ''passive uptake'' and ''active uptake'' mechanisms in Si uptake and transport in the same plant species, depending much upon plant species and external Si supply, this may also possibly be true here for Cd uptake in NHE. However, the absence of obvious inhibition of Cd uptake by low temperature in NHE indicates that a passive uptake mechanism seems to prevail in roots of the plants.

It is generally assumed that ions reach the xylem by symplastic pathway in plants as the apoplast of cortex and stele of roots are hydraulically separated by the Casparian band. However, a recent technique provided clear evidences for the permeability of Casparian bands to ions in young roots of corn and rice (Ranathunge et al., 2005). Apoplastic pathway was likely to contribute to the delivery of Ca and Zn to the xylem (White, 2001; White et al., 2002), and Cd was also possible to enter into plants and translocate to shoots passively after the breakdown of some physiological barrier in the plant roots (Salt et al., 1995). In this study, we believe that root-to-shoot translocation of Cd in the hyperaccumulator, HE *S. alfredii*, is an energy-dependent process, as indicated by the significant inhibition of shoot Cd concentration by low temperature treatment. Meanwhile, Cd accumulation in shoots of the HE plants was increased despite the significantly reduced transpiration rate, suggesting root-to-shoot translocation of Cd in the HE is not mainly dominated by the apoplastic bypass. The higher ratios of Cd in xylem sap to Cd in external solutions suggest that Cd entry into xylem involves a symplastic pathway, and supporting evidence for this conclusion is the significant suppression of Cd concentration in xylem sap by the metabolic inhibitors, CCCP or DNP. Similarly, a dominant role of symplastic pathway in the transport of Cd to xylem was suggested in the other two Cd hyperaccumulators, *Thlaspi caerulescens* (Xing et al., 2008) and *Arabidopsis halleri* (Ueno et al., 2008). In contrast, no significant role of symplastic pathway is indicated in the Cd entry to xylem and translocation to shoots of NHE, owing to the absence of the obvious effect of DNP and low temperature on xylem transport and root-to-shoot translocation of Cd, respectively.

In conclusion, the present study points out that apoplastic bypass makes a very small contribution to the hyperaccumulation of Cd in shoots of the HE *S. alfredii*, and the majority of Cd follows a symplastic pathway into the stele of the root and subsequently translocates to shoots, and thus cellular influx and efflux of Cd would be the main control points for Cd transport within the plants. The great difference between the two ecotypes of *S. alfredii* was observed in their efficiency of root-to-shoot Cd translocation, indicated that the HE

plants equipped with enhanced transport capacity of Cd to shoots similar to other reported hyperaccumulators (Baker et al., 1994; Zhao et al., 2006). Our previous research showed that more efficient Cd symplastic uptake by roots of the HE, and preferential Cd localization within the root cylinder of the HE in comparison with the NHE (Lu et al., 2008). Thus, we suggest that efficient transport of Cd into the root symplasm, and efflux into xylem vessels, may play an important role in Cd hyperaccumulation in HE *S. alfredii*. A P-type ATPase, *HMA4*, has been suggested to play an important role in efflux metals from the root symplasm into the xylem vessels necessary for shoot hyperaccumulation in both the Cd hyperaccumulators, *Thlaspi caerulescens* (Papoyan and Kochian, 2004) and *Arabidopsis halleri* (Courbot et al., 2007; Hanikenne et al., 2008). Therefore, in HE *S. alfredii*, further investigation of specific transporters in root or leaf cell plasma membrane is necessary to unravel the possible mechanisms underlying the trait of Cd hyperaccumulation in this plant species. In addition, as a *Crassulaceae* plant, *Sedum alfredii* is able to keep stomata closed during the hottest and driest part of the day reduces the loss of water through evapotranspiration, and thus to grow in environments that would otherwise be far too dry. Therefore, the absence of positive relation of transpiration rate and Cd hyperaccumulation in shoots of the HE *S. alfredii* make it an excellent plant material in phytoremediation of the contaminated soils, especially in arid environment. This also helps to reduce the possibly secondary pollution of Cd in the environment caused by irrigation during the phytoremediation.

Acknowledgments

The authors thank the financial supports by Key Project from the National Natural Science Foundation of China (30630046), Program for Changjiang Scholars and Innovative Research Team in University (IRT0536), and by "973" Project from the Science and Technology Ministry of China (2002CB410804). The authors are grateful to Huagang Huang, Kai Wang, Xing-wen Cao and Jiang Zhu for their kind assistance with our experiments, and to Mr. Xiang Hu for his help with ICP-MS analysis.

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