Plant Cd^{2+} and Zn^{2+} status effects on root and shoot heavy metal accumulation in *Thlaspi caerulescens*

Ashot Papoyan, Miguel Piñeros and Leon V. Kochian

US Plant, Soil and Nutrition Laboratory, United States Department of Agriculture, Agricultural Research Service, Cornell University, Ithaca, New York 14853, USA

Author for correspondence:
Leon V. Kochian
Tel: +1 607 255-2454
Fax: +1 607 255-2459
Email: LVK1@cornell.edu

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Summary

- In this study we address the impact of changes in plant heavy metal, (i.e. zinc (Zn) and cadmium (Cd)) status on metal accumulation in the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*.
- *Thlaspi caerulescens* plants were grown hydroponically on both high and low Zn and Cd regimes and whole-shoot and -root metal accumulation, and root $^{109}Cd^{2+}$ influx were determined.
- High-Zn-grown (500 μM Zn) plants were found to be more Cd-tolerant than plants grown in standard Zn conditions (1 μM Zn). Furthermore, shoot Cd accumulation was significantly greater in the high-Zn-grown plants. A positive correlation was also found between shoot Zn accumulation and increased plant Cd status. Radiotracer ^{109}Cd root flux experiments demonstrated that high-Zn-grown plants maintained significantly higher root Cd^{2+} influx than plants grown on 1 μM Zn. It was also found that both nickel (Ni) and copper (Cu) shoot accumulation were stimulated by high plant Zn status, while manganese (Mn) accumulation was not affected.
- A speculative model is presented to explain these findings, suggesting that xylem loading may be one of the key sites responsible for the hyperaccumulation of Zn and Cd accumulation in *Thlaspi caerulescens*.

Key words: Cd (cadmium), heavy metals, hyperaccumulation, *Thlaspi caerulescens*, xylem loading, Zn (zinc).

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Introduction

Heavy metal uptake, translocation, and sequestration are key aspects of a plant's ability to accumulate and cope with high concentrations of heavy metals. Although essential micronutrients such as Zn, Ni, Cu, and Mn play an important role in different aspects of a plant's metabolism, they are also potentially toxic heavy metals. Likewise, other nonessential toxic heavy metals, such as Cd and lead (Pb), with similar physicochemical properties but lacking a biological function, compete with and enter the plant via the transport systems operating for micronutrient acquisition. Heavy metal hyperaccumulator plants such as *Thlaspi caerulescens* have evolved mechanisms for extreme heavy metal accumulation and detoxification. Elucidation of the mechanisms underlying this phenotype requires an understanding on how metal transport and accumulation processes differ between normal and hyperaccumulator plants, in particular with regard to how essential and nonessential heavy metals interact at various key metal transport sites in the plant. 'Normal' nonhyperaccumulator plants tend to store the absorbed heavy metals in the roots, whereas hyperaccumulator plants are capable of transporting most of the accumulated heavy metals to the shoots (Lasat *et al.*, 1998). Hyperaccumulator plants show a stronger influx of heavy metals into the roots than do nonaccumulator species (Lasat *et al.*, 1996). In addition, Zn concentrations in the xylem sap of *T. caerulescens* (a hyperaccumulator) have been shown to be several-fold higher than that found in the related nonaccumulator *Thlaspi arvense*. These observations emphasize the differences that exist at the

various steps involved in the transport of heavy metals into and across the root, and to the shoot between hyperaccumulating and nonaccumulator plant species. Studies examining the competitive effects between Zn and Cd transport in *T. caerulescens* have suggested that, at least in the leaf, the heavy metal Cd is transported via cellular Zn transporters (Cosio *et al.*, 2004). Similar hypotheses were proposed in a study that compared the heavy metal transport characteristics in roots from the *T. caerulescens* ecotypes, Prayon and Ganges (Lombi *et al.*, 2001). Circumstantial evidence led the authors to propose that while Zn and Cd were being transported via the same transporter in the case of the Prayon ecotype, Cd transport in the Ganges ecotype (which hyperaccumulates Cd to a higher degree) took place via a separate transporter. In contrast, in nonaccumulator plants, Zn and Cd uptake and accumulation are negatively correlated (Hart *et al.*, 2002; Wu *et al.*, 2003). Using ^{65}Zn and ^{109}Cd radiotracer flux techniques in the roots of bread and durum wheats, Hart *et al.* (2002) showed competition between root Zn and Cd uptake, such that high concentrations of Zn inhibit Cd uptake, and vice versa. These interactions can be explained by simple ionic competition between Zn and Cd for the heavy metal binding site in the transport proteins.

The ability of *T. caerulescens* to accumulate high concentrations of both Zn and Cd even from nutrient solutions or soils that contain high concentrations of both metals suggests that different or altered competitive ionic interactions may occur in hyperaccumulator species from those in nonaccumulators. The present study addressed these interactions by examining the effect of low and high plant Zn and Cd status on Zn and Cd accumulation, as well as their effects on the accumulation of the essential micronutrients Cu, Ni, and Mn.

Materials and Methods

Plant growth conditions

Thlaspi caerulescens J & C Presl. (ecotype Prayon) seedlings were grown on a modified Johnson's solution that had a macronutrient composition of (in mM) 1.2 KNO_3, 0.8 $Ca(NO_3)_2$, 0.2 $NH_4H_2PO_4$ and 0.2 $MgSO_4$ and a micronutrient composition (in μM) of 50 KCl, 12.5 H_3BO_4, 1 $MnSO_4$, 1 $ZnSO_4$, 0.5 $CuSO_4$, 0.1 Na_2MoO_4, 0.1 $NiSO_4$, and 7.5 Fe-EDDHA (N, N′-ethylenediamine-di(O-hydroxyphenylacetic acid). The solution was buffered at a pH of 5.5 with 1 mM MES (2-[N-morpholino]-ethanesulfonic acid) buffer. *Thlaspi* seeds were placed in a drop of 0.7% (w/v) low-temperature gelling agarose on nylon mesh circles (1 mm mesh openings) which, in turn, were positioned on a coarser mesh support sealed to the bottom of black plastic cups. The cups and seeds were fitted into holes cut into black plastic lids covering 5 l black plastic pots. Seedlings were grown in a growth chamber at 25 : 15°C (light : dark, 16 : 8 h) under a light intensity of 300 μmol photons m^{-2} s^{-1} for 2 wk. To ensure that there was no precipitation of Cd at the higher Cd concentrations used, particularly when the solution also contained 500 μM Zn, two different tests were conducted. First, all of the Cd- and Zn-containing nutrient solutions were centrifuged at 100 000 ***g*** to pellet any precipitating material, and the supernatant was analyzed via inductively coupled, plasma trace analyzer emission spectroscopy. This analysis indicated there was no precipitation of Cd or Zn from the nutrient solutions. Second, the solutions were speciated using the GHEOCHEM-PC speciation software (Parker *et al.*, 1995), and the predictions from this analysis indicated that no precipitation would occur, even at the highest Cd and Zn concentrations.

Heavy metal treatment and tissue metal analysis

After germination and growth in Johnson's nutrient solution for 2 wk, the seedlings were transferred into growth containers containing identical nutrient solution supplemented with specific concentrations of Zn (1 or 500 μM) and/or Cd (1, 5, 20, 40, 60, 80, 100 or 200 μM). The treatments continued for 2 months, with the treatment solutions being refreshed weekly. After 2 months, plants were harvested, and the root and shoot tissues were separated and oven-dried for 10 d at 65°C. Subsequently, dry weights for each sample were obtained and then the samples were digested in concentrated HNO_3 to dryness. Dried samples were then resuspended in 5% HNO_3 and the concentrations of specific micronutrients and heavy metals for each sample were determined using an inductively coupled, plasma trace analyzer emission spectrometer (Model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA, USA). For the determinations of root heavy metal content, the roots were first transferred into a desorption solution containing 5 mM $CaCl_2$ for 20 min, to desorb the heavy metal bound to the root cell wall.

^{109}Cd radiotracer flux experiments

Radiotracer (^{109}Cd) flux methodologies were used to determine Cd^{2+} influx into *T. caerulescens* roots using methods previously developed in our laboratory and described in Hart *et al.* (1998, 2002). Plants were grown on nutrient solution containing either 1 or 500 μM Zn for 2 months, after which the radiotracer experiments were conducted. Plants were placed for 15 min into a nonradioactive uptake solution of identical composition to that used for the radiolabeled uptake experiment, with the objective of acclimating the plants to mechanical stresses associated with transfer to the uptake apparatus. Following this pretreatment, root radiotracer uptake experiments were initiated by the addition of ^{109}Cd to the Cd uptake solutions (containing Cd at different concentrations and no Zn) for a 20 min uptake period. To terminate the Cd uptake experiment, the roots were transferred into desorption solution that contained a 10-fold excess of nonradioactive Cd to desorb ^{109}Cd bound to the cell walls, using a 20 min

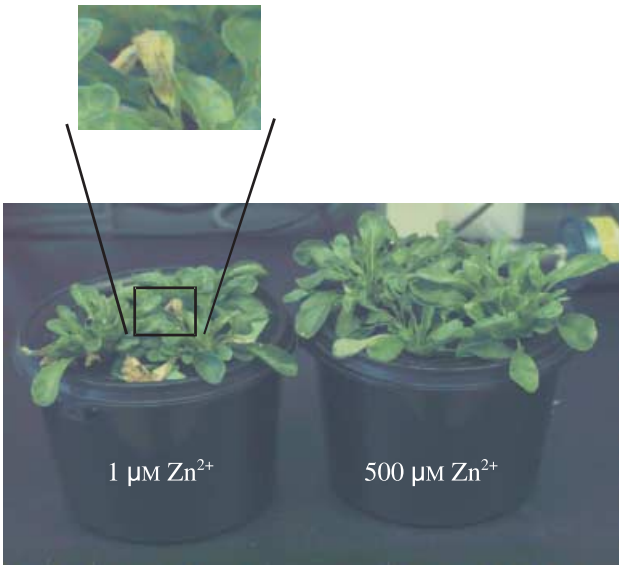

Fig. 1 *Thlaspi caerulescens* plants grown on hydroponic nutrient solution containing 1 μM or 500 μM zinc (Zn), and 200 μM cadmium (Cd); Zn and Cd were added simultaneously. The magnified section highlights the necrotic symptoms caused by high Cd on the leaves of low-Zn-grown plants.

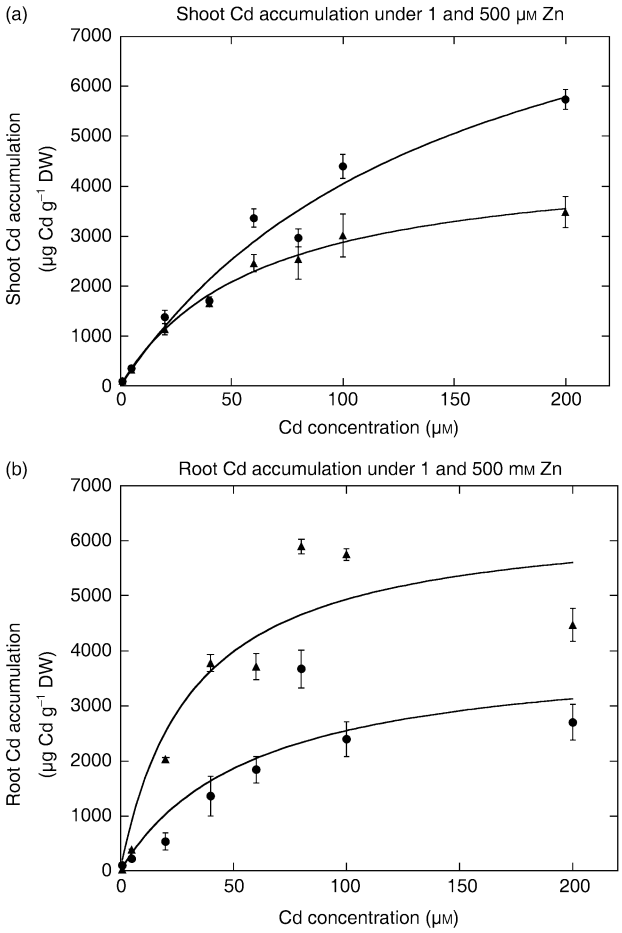

Fig. 2 (a) Zinc (Zn)-dependent accumulation of cadmium (Cd) in shoots of *Thlaspi caerulescens* plants grown on nutrient solution containing 1 μM (triangles) or 500 μM (circles) Zn. (b) Zn-dependent accumulation of Cd in the roots of *T. caerulescens* plants grown on nutrient solution containing 1 μM (triangles) or 500 μM (circles) Zn. ANOVA indicated that shoot Cd accumulation was significantly different between low- and high-Zn-grown plants at Cd concentrations of 60–200 μM Cd ($P = 0.004$ and $F = 9.9$). For root Cd accumulation, ANOVA analysis indicated that accumulation values for low- vs high-Zn-grown plants were significantly different over the entire Cd concentration range ($P = 0.003$ and $F = 9.6$).

desorption period. We had previously found that this desorption regime was optimal for desorbing radioactive Zn or Cd from *T. caerulescens* root cell walls while minimizing efflux of radiotracer that had been transported into the root symplasm during the uptake period. Following desorption, roots were excised, blotted dry, weighed, and the radioactivity was counted using a Perkin Elmer 'WIZARD 3' 1480 Automatic Gamma Counter.

Results

Effect of plant Zn status on Cd tolerance in *T. caerulescens*

It has been well established that *T. caerulescens* is a hyper-accumulator plant species capable of tolerating extremely high concentrations of toxic heavy metals such as Cd and Zn. In the present study, we addressed the influence of changing the plant Zn status from our standard conditions for Zn sufficiency (plants grown on 1 μM Zn) to a high-Zn status (plants grown on 500 μM Zn) and the ability to tolerate a high concentration of Cd (200 μM) in the nutrient solution. *T. caerulescens* plants grown under the high-Zn regime were able to tolerate high concentrations of Cd more effectively and outperformed plants grown under Zn-sufficient conditions (Fig. 1). The Zn-sufficient-grown plants exhibited symptoms of Cd toxicity, including reduced shoot biomass and leaf necrosis (data not shown), while the high-Zn-grown plants were asymptomatic. The simplest hypothesis was to attribute this to high Zn concentrations in the nutrient solution, leading to a reduction in Cd accumulation, which resulted in reduced Cd toxicity. To test this hypothesis, we measured root and shoot Cd accumulation in low- and high-Zn-grown *T. caerulescens* plants. Contrary to our expected results, shoot cadmium accumulation was significantly higher in plants grown under the high-Zn regime than in plants grown in low-Zn conditions, and this difference was more significant as the Cd concentrations in the nutrient solution increased (> 40 μM) (Fig. 2a). In roots, the pattern was reversed as the higher-Zn regime resulted in significantly lower concentrations of root Cd accumulation (Fig. 2b). It should be noted that because the roots were in direct contact with the nutrient

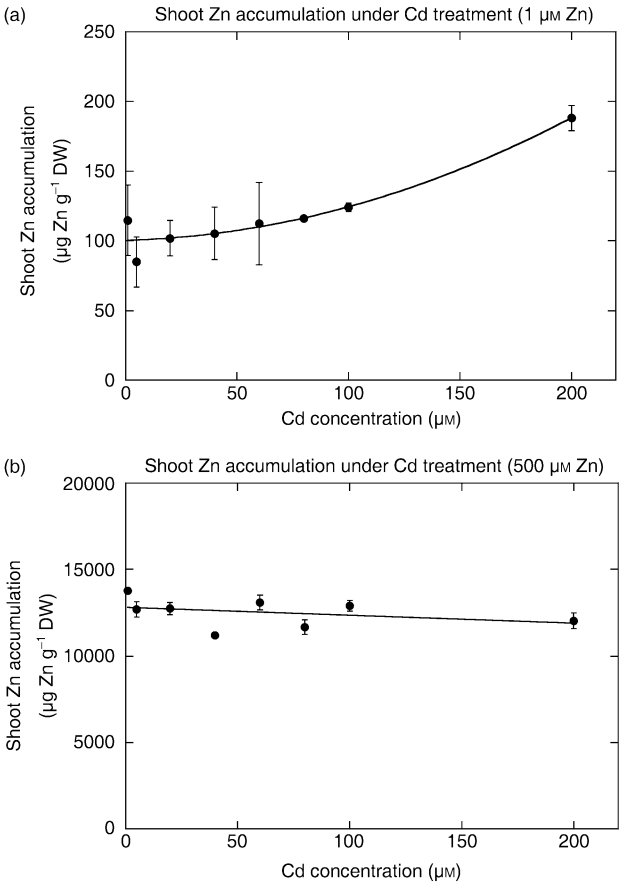

Fig. 3 Cadmium (Cd)-dependent accumulation of zinc (Zn) in the shoots of *Thlaspi caerulescens* plants grown on nutrient solution containing 1 μM Zn (a) or 500 μM Zn (b) and Cd concentrations ranging from 0 to 200 μM. Statistical analysis using the one-tailed *t*-test indicated that for the data in (a), shoot Zn accumulation for plants grown on the highest Cd concentration (200 μM) was significantly greater than shoot Zn accumulation for plants grown on the lower Cd concentrations (*P*-values between 0.01 and 0.1).

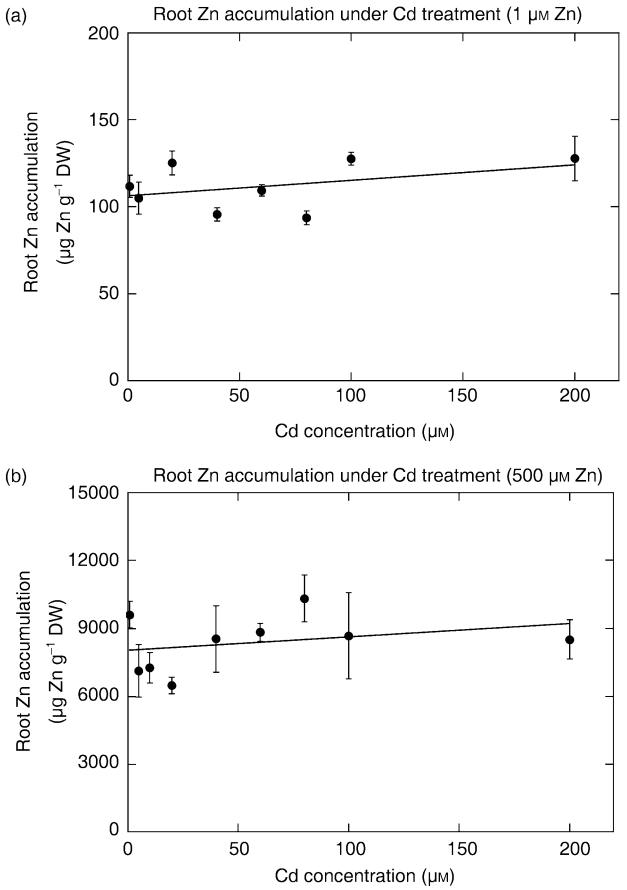

Fig. 4 Cadmium (Cd)-dependent accumulation of zinc (Zn) in roots of *Thlaspi caerulescens* plants grown on nutrient solution containing 1 μM Zn (a) and 500 μM Zn (b) over a range of Cd concentrations.

solution, and a significant fraction of the root-associated Cd and Zn is in the cell wall, this effect could primarily be on root cell wall binding of Cd and not root Cd uptake.

Given that high plant Zn status stimulated shoot Cd accumulation, we also examined the effect of increasing plant Cd status on plant Zn accumulation. As seen in Fig. 3(a) for normal Zn-grown (1 μM) plants, increasing the plant Cd status (from 1 to 200 μM) resulted in a significant, twofold increase in shoot Zn accumulation. However, in shoots of high-Zn-grown plants, where shoot Zn concentrations were increased 100-fold compared with normal-Zn-grown plants, changes in plant Cd status had no effect on shoot Zn accumulation, which remained relatively constant at shoot Zn concentrations of approx. 13 000 μg g^{-1} Zn (Fig. 3b). Examination of root Zn concentrations indicated that Zn accumulation in roots from plants grown in high Zn were also 100-fold greater that those found in roots from plants grown under normal Zn conditions. However, in contrast to the shoot accumulation data, the Zn content in roots from both normal and high-Zn treatments remained unchanged regardless of the Cd concentration present in the growth media (Fig. 4a,b).

We also examined the effect of plant Zn and Cd status on the accumulation of the heavy metal micronutrients Cu, Ni, and Mn. The findings for plant Cu and Ni accumulation were very similar, and hence only the findings for Cu accumulation are presented in Fig. 5. As was seen for the effect of high-Zn status on shoot Cd accumulation, the high-Zn regimen led to a significant increase in shoot Cu accumulation (Fig. 5a). Additionally, as was seen for the effect of high-Zn status on root Cd accumulation, inclusion of 500 μM Zn in the growth solution resulted in a reduction of root Cu concentrations by as much as threefold (Fig. 5b). The interactions between varying plant Cd status and Cu (and Ni) accumulation were somewhat different from those seen for Zn accumulation. Increasing Cd concentrations in the growth solution resulted in moderate increases in root Cu accumulation, while the same treatment inhibited shoot Cu accumulation, in both

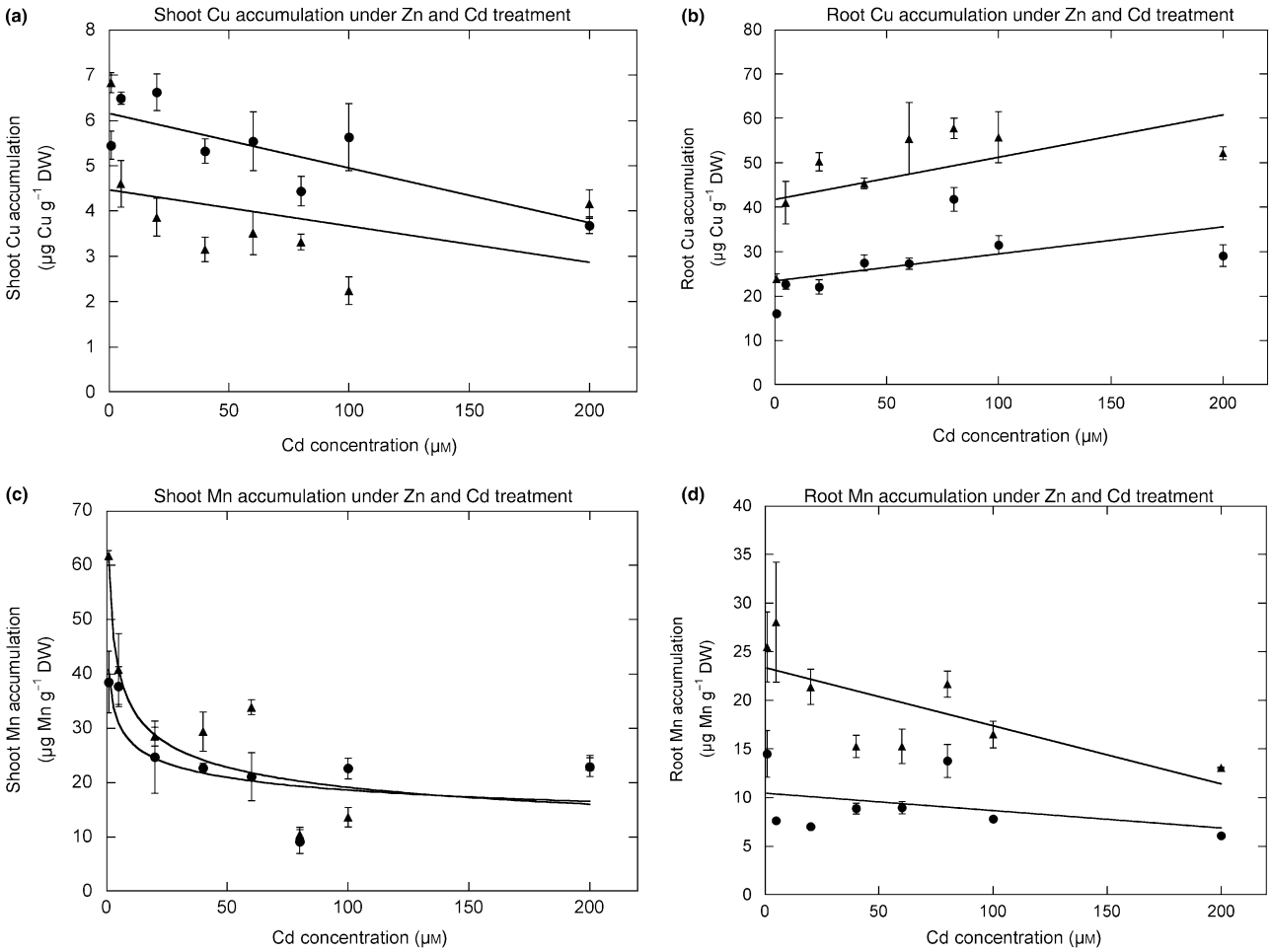

Fig. 5 Cadmium (Cd)- and zinc (Zn)-dependent uptake of the micronutrients copper (Cu), and manganese (Mn) in *Thlaspi caerulescens* plants. Cu accumulation in the shoots (a) and roots (b), and Mn accumulation in the shoots (c) and roots (d) of plants grown in 1 μM Zn (triangles) or 500 μM Zn (circles). ANOVA indicated that shoot and root Cu accumulation values were significantly different in response to growth on low- vs high-Zn (shoots, $P = 2.2 \times 10^{-5}$; $F = 21.6$) (roots, $P = 3.1 \times 10^{-9}$; $F = 50.8$). ANOVA indicated that the inhibition of both shoot and root Mn accumulation by Cd was significant (shoots, $P = 9.7 \times 10^{-8}$ and $F = 21.0$ for low-Zn-grown plants, $P = 0.001$ and $F = 5.6$ for high-Zn-grown plants; roots, $P = 0.05$ and $F = 2.6$ for low-Zn-grown plants, $P = 7.7 \times 10^{-5}$ and $F = 8.8$ for high-Zn-grown plants).

low- and high-Zn-grown plants (Fig. 5a,b). Unlike plant accumulation of Cu and Ni, Mn accumulation in roots and shoots was insensitive to changes in plant Zn status, and was significantly inhibited by increasing concentrations of Cd (Fig. 5c,d).

Short-term measurement of root unidirectional Cd^{2+} influx using ^{109}Cd radiotracer techniques

Short-term ^{109}Cd radiotracer root uptake techniques, which our laboratory has previously shown to measure unidirectional metal influx into the root symplasm effectively (Lasat *et al.*, 1996), were used to investigate the effect of high vs low plant Zn status on root Cd uptake. As seen in Fig. 6, *T. caerulescens* plants grown on the high-Zn regime maintained significantly higher root Cd^{2+} influx rates, particularly at lower Cd concentrations, than plants grown on the sufficient Zn concentration (1 μM Zn). The difference in root Cd^{2+} influx was much greater for uptake at the lower concentrations of Cd (1, 5, and 10 μM; Fig. 6a) than for root Cd influx at higher Cd concentrations (60–100 μM Cd; Fig. 6b). These findings clearly indicate that the increase in plant heavy metal accumulation with increasing plant Zn status is the result, at least in part, of enhanced root metal uptake.

Discussion

Plant species such as the Zn/Cd/Ni hyperaccumulator *T. caerulescens* have the unique ability to hyperaccumulate multiple heavy metals at the same time from a complex nutrient or soil solution. In the present study, we investigated the interactions between Zn and Cd transport and accumulation,

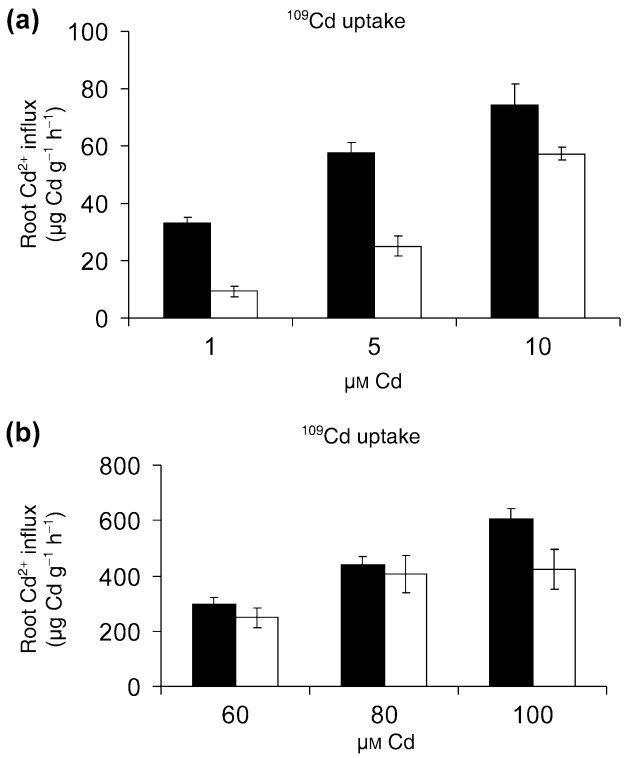

Fig. 6 Root Cd (^{109}Cd) influx for *Thlaspi caerulescens* plants grown on 1 μM Zn (open bars) and 500 μM Zn (closed bars) at low (1, 5, or 10 μM) (a), and high (60, 80, and 100 μM) Cd concentrations (b) in the uptake solution. ANOVA indicated that the difference in root ^{109}Cd influx at the low Cd concentrations for low- vs high-Zn-grown plants was significant ($P = 0.008$ and $F = 8.6$), while the difference in Cd influx was not significant at the high Cd concentrations ($P = 0.14$ and $F = 2.3$).

in order to gain a better understanding of the overall hyperaccumulation phenotype. We compared *T. caerulescens* plants grown on a low, but sufficient Zn concentration with high-Zn- and -Cd-grown plants in terms of their ability to tolerate and accumulate these two heavy metals, as well as other micronutrients that also are heavy metals, namely Cu, Ni, and Mn. We had previously determined when we first began working with *T. caerulescens* that growth on our modified Johnson's nutrient solution containing 1 μM Zn yielded fully Zn-sufficient *T. caerulescens* seedlings. This observation was based on no visible symptoms of Zn deficiency and no decrease in shoot or root biomass when compared with *T. caerulescens* plants grown on Johnson's solution containing between 5 and 500 μM Zn (Pence, 2002). We found that a high-Zn regimen leads to a significantly higher degree of Cd tolerance, when compared with that experienced by plants grown on a 'normal' or sufficient Zn concentrations (Fig. 1). At first glance, this phenomenon could have been interpreted as simple competition between Zn^{2+} and Cd^{2+} ions at the entry point for metals into the plant (i.e. the active site for metal transporters in the root-cell plasma membrane). In a nonhyperaccumulator plant, ionic competition between Zn and Cd results in a decreased accumulation of one metal upon exposure to a higher concentration of the other metal. For example, high-Zn treatment has been shown to decrease the ability to accumulate Cd in wheat seedlings (Hart *et al.*, 2002). In the present study, *T. caerulescens* roots showed similar interactions, such that high-Zn-grown plants maintained significantly lower root Cd concentrations compared with roots from plants grown at lower Zn concentrations. Given some similarities in physicochemical characteristics (e.g. similar ionic radius), Cd can mimic and replace other divalent metals such as Zn at critical binding sites in proteins and other macromolecules. Thus, it is plausible that nonaccumulating and hyperaccumulator plant species share similar patterns of heavy metal interactions at the root, which do not involve active processes associated with heavy metal transport and tolerance. Such interactions would rely on the high affinity of divalent cations for the fixed negative charges in the cell wall, with the majority of the divalent metal cations residing in the apoplast of roots exposed to the metals via nutrient or soil solution (Lasat *et al.*, 1996; Hart *et al.*, 1998).

However, upon examination of heavy metal accumulation in the shoots, the present study revealed that, in contrast to nonhyperaccumulator species, *T. caerulescens* exhibits more complex interactions between Zn and Cd than can be explained by simple competitive interactions. It was found that shoot Zn and Cd accumulation in *T. caerulescens* was positively correlated; plants grown on high Zn are able not only to tolerate high concentrations of Cd in the root-bathing media, but also to accumulate significantly more Cd in the shoots compared with the plants grown on a lower concentration of Zn (Fig. 2a). For example, plants grown on 1 μM Zn and 100 μM Cd accumulated 2800 μg g^{-1} Cd in the shoots, while plants grown on 500 μM Zn and the same concentration of Cd were found to accumulate Cd in the shoots to a concentration of 4200 μg g^{-1}.

Ionic interactions, such as the stimulation of shoot Cd and Zn accumulation in response to elevated plant Zn or Cd status, appear to involve processes associated with heavy metal translocation from the soil to the shoot. It is generally believed that since Cd has no known biological function, its entry into the plant takes place via transport processes that are normally functioning for Zn (Marschner, 1995). Therefore, an increase in the Zn concentration in the soil environment will increase simple ion competition for transporter proteins between Zn and Cd, which should normally lead to a decrease in plant Cd accumulation and not the stimulated shoot Cd accumulation seen here in *T. caerulescens*. Interestingly, this response is broader than just an effect of plant Zn status on Cd accumulation. This study shows that elevated plant Cd status also increases shoot Zn accumulation. These findings clearly indicate that Zn and Cd transport and accumulation in *T. caerulescens* are interconnected, with the accumulation of one metal positively affecting the accumulation of the other metal in the shoot.

Given the current lack on information concerning the mechanisms underlying this response, we can only hypothesize about its basis. One possibility is that upon long-term exposure to high concentrations of Zn or Cd, an additional mechanism/degree of metal tolerance is induced, allowing the plants to accumulate even higher concentrations of heavy metals in the shoot. However, we have circumstantial evidence that argues against such a scenario of enhanced metal tolerance. As seen in Fig. 6, the high-Zn-grown plants maintain a much higher root Cd^{2+} influx than plants with low Zn status, even at Cd concentrations in the uptake solution that are not toxic to *T. caerulescens* plants. This observation suggests that there is a response to the high plant metal status that is more global than simply induction of increased metal tolerance.

In contrast to nonaccumulating plant species that tend to sequester the absorbed heavy metal in the root, previous physiological analyses of Zn/Cd hyperaccumulation in *T. caerulescens* have suggested that, in hyperaccumulator species, translocation of heavy metals from the soil solution to the shoot is a key step in hyperaccumulation (Lasat *et al.*, 1996, 1998; Papoyan & Kochian, 2004). Three major transport components are involved in this process: (i) metal transport from the soil solution into the root; (ii) radial transport across the root and subsequent loading into the xylem; and (iii) transport of the metal via the xylem to the leaf tissue, followed by the storage in leaf epidermal and mesophyll cells. Several transporters, such as ZNT1 (Pence *et al.*, 2000) and HMA4 (Hussain *et al.*, 2004; Papoyan & Kochian, 2004), have been identified and have been suggested to regulate the first and second steps of this process, respectively. Although our results indicate a significant stimulation of root Cd influx (i.e. changes in the first step) in response to increasing plant Zn status, we also recognize that this response may be an indirect consequence of an alteration in xylem loading and transport (i.e. the second step). Since nonaccumulator plants do not have strong metal detoxification mechanisms in the shoots, most of the metals are excluded from the shoot via root sequestration, most likely to protect the photosynthetic apparatus in leaf cells which is extremely sensitive to heavy metals (Kupper *et al.*, 1999, 2002). On the other hand, hyperaccumulator plants very actively translocate heavy metals into the shoots, presumably because of the existence of heavy metal tolerance mechanisms operating in the shoot. HMA4 is a heavy metal ATPase localized in the xylem parenchyma of *T. caerulescens* (as well as in *Arabidopsis*) and it has been suggested that it serves as a micronutrient/heavy metal transporter mediating the loading of these metals into the xylem (Bernard *et al.*, 2004; Hussain *et al.*, 2004; Papoyan & Kochian, 2004; Verret *et al.*, 2004) in both nonaccumulating and hyperaccumulating plant species. HMA4 expression was found to be significantly higher in *T. caerulescens* than in *Arabidopsis* (Bernard *et al.*, 2004; Papoyan & Kochian, 2004). Interestingly, while the expression of HMA4 in *Arabidopsis thaliana* is down-regulated upon exposure to heavy metals (Mills *et al.*, 2003), its expression in *T. caerulescens* is up-regulated upon exposure to high concentrations of Cd and Zn (Papoyan & Kochian, 2004). These qualitative and quantitative differences in the expression profile of this heavy metal transporter (apparently involved in xylem loading) could underlie the explanation for the Zn and Cd interactions observed in this study. If, in fact, Cd and Zn transport share a common transport pathway such as HMA4 in *T. caerulescens*, and this transporter cannot discriminate effectively with respect to the transport of these two metals, increasing the concentration of either ion in the soil would up-regulate the expression of this xylem loading transporter, which would in turn increase the accumulation of both metals in the shoot. Analysis of the effect of Cd and Zn status on the accumulation of other divalent heavy metal micronutrients (i.e. Ni, Cu, Mn) suggest that the response of *T. caerulescens* to elevated metal status is not restricted to just Zn and Cd, supporting our hypothesis that *T. caerulescens* plants grown on high Zn exhibit an elevated concentration of heavy metal and micronutrient xylem loading.

In summary, the present study shows that *T. caerulescens* plants grown on elevated concentrations of Zn and/or Cd for extended periods of time exhibit a significantly enhanced ability to accumulate metals in the shoot, including Cd, Zn, Cu, and Ni. This stimulated shoot metal accumulation is associated with enhanced root metal influx and, presumably, enhanced xylem transport of these metals from the root to shoot. Based on previous physiological and molecular findings indicating that xylem loading of metals plays a key role in the hyperaccumulation phenotype, we hypothesize that this transport step also plays a crucial role in the processes identified here. We also suggest that this enhanced xylem loading, triggered by exposure to high heavy metal concentrations for extended periods, may translate into improved heavy metal tolerance, as the metals are more efficiently translocated to the shoots where highly effective metal tolerance mechanisms operate.

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