Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox*

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Summary

• Efficient root-to-shoot translocation is a key trait of the zinc/cadmium hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox*, but the extent of variation among different accessions and the underlying mechanisms remain unclear.

• Root-to-shoot translocation of Cd and Zn and apoplastic bypass flow were determined in 10 accessions of *T. caerulescens* and one of *T. praecox*, using radiolabels ^{109}Cd and ^{65}Zn. Two contrasting accessions (Pr and Ga) of *T. caerulescens* were further characterized for *TcHMA4* expression and metal compartmentation in roots.

• Root-to-shoot translocation of ^{109}Cd and ^{65}Zn after 1 d exposure varied 4.4 to 5-fold among the 11 accessions, with a significant correlation between the two metals, but no significant correlation with uptake or the apoplastic bypass flow. The F_2 progeny from a cross between accessions from Prayon, Belgium (Pr) and Ganges, France (Ga) showed a continuous phenotype pattern and transgression. There was no significant difference in the *TcHMA4* expression in roots between Pr and Ga. Compartmentation analysis showed a higher percentage of ^{109}Cd sequestered in the root vacuoles of Ga than Pr, the former being less efficient in translocation than the latter.

• Substantial natural variation exists in the root-to-shoot translocation of Cd and Zn, and root vacuolar sequestration may be an important factor related to this variation.

Key words: cadmium, compartmentation, hyperaccumulation, *Thlaspi caerulescens*, *Thlaspi praecox*, translocation, zinc.

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Introduction

Hyperaccumulation of metals or metalloids by plants involves at least three key processes: efficient absorption by roots, efficient root-to-shoot translocation and hypertolerance through internal detoxification (Baker *et al.*, 2000; Pollard *et al.*, 2002; Assunção *et al.*, 2003b; McGrath & Zhao, 2003). *Thlaspi caerulescens* is one of the best known examples of hyperaccumulators, being able to hyperaccumulate zinc, as well as cadmium or nickel in some accessions. The mechanisms of uptake and tolerance in this plant species have been studied intensively in recent years. Tolerance is achieved mainly through vacuolar sequestration and complexation with ligands (Küpper *et al.*, 1999, 2004; Salt *et al.*, 1999; Ma *et al.*, 2005; Ueno *et al.*, 2005). Enhanced uptake is possibly attributed to hyperexpression of metal transporter genes in *T. caerulescens* compared with

nonhyperaccumulating species (Pence *et al.*, 2000; Assunção *et al.*, 2001; van de Mortel *et al.*, 2006).

Although Zn hyperaccumulation is a species-wide constitutive property in *T. caerulescens*, there is substantial natural variation among different accessions in the uptake of and tolerance to Zn, which is even more pronounced in the case of Cd and Ni (Meerts & van Isacker, 1997; Escarré *et al.*, 2000; Lombi *et al.*, 2000; Assunção *et al.*, 2003a; Roosens *et al.*, 2003). Physiological evidence suggests that there are multiple transporters mediating the influx of Zn and Cd with different affinities for the two metals in different accessions with contrasting accumulation patterns (Lombi *et al.*, 2001; Zhao *et al.*, 2002). Furthermore, quantitative trait locus (QTL) analyses of segregating populations from intraspecific crosses between different *T. caerulescens* accessions have identified a number of QTLs for Zn and Cd accumulation, some of which are colocated, while the others are metal-specific (Assunção *et al.*, 2006; Deniau *et al.*, 2006).

The process of root-to-shoot translocation is less well understood. When compared with nonhyperaccumulating species, *T. caerulescens* and another Zn hyperaccumulator, *Arabidopsis halleri*, have a much higher level of expression of the P_{1B}-type ATPase genes, especially *TcHMA4* or *AhHMA4* (Bernard *et al.*, 2004; Hammond *et al.*, 2006; van de Mortel *et al.*, 2006; Talke *et al.*, 2006). There is strong evidence that AtHMA4 mediates efflux of Zn and Cd from the xylem parenchyma cells to the xylem vessels, and therefore plays a key role in their transport from root to shoot in *Arabidopsis thaliana* (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2004). TcHMA4 is possibly also involved in the xylem loading of Zn and Cd in *T. caerulescens* (Papoyan & Kochian, 2004), and its high expression in roots of this plant species may explain the efficient root-to-shoot translocation. It has been reported that *AhHMA4* colocalizes with a major QTL for Cd and Zn tolerance in *A. halleri* measured by root elongation (Courbot *et al.*, 2007; Willems *et al.*, 2007). The elevated expression of *AhHMA4* may serve as an efficient mechanism for improving Cd and Zn tolerance in plants under the conditions of Cd/Zn excess by maintaining low cellular Cd^{2+} and Zn^{2+} through efflux of these ions.

Another possible reason for the efficient translocation is that hyperaccumulating plants may sequester smaller amounts of metals in roots than nonhyperaccumulating species. Lasat *et al.* (1998) tested this hypothesis by analysing the compartmentation of ^{65}Zn in the root tissues of *T. caerulescens* and of the nonhyperaccumulator *Thlaspi arvense*. They found that *T. arvense* roots retained a larger proportion of ^{65}Zn in the vacuole compartment than *T. caerulescens*, which may explain the lower translocation efficiency in the former than the latter. There is also a possibility that some Zn and Cd may enter the xylem vessels via the apoplastic pathway, although the extent of contribution by this pathway has not been determined experimentally. White *et al.* (2002) argued for a significant role for the apoplastic pathway in Zn hyperaccumulation in *T. caerulescens*. Their argument is based mainly on a comparison of literature data on Zn influx in roots and accumulation in shoots. However, as admitted by White *et al.* (2002), their calculations excluded the possible contribution by low-affinity transporters, which would be more prominent in the high concentration range of external Zn. Metal-specific accumulation patterns observed in different accessions of *T. caerulescens* also cannot be explained by a significant apoplastic pathway because of its lack of selectivity for different metals (Ernst *et al.*, 2002).

Previous studies have alluded to the variations in the shoot-to-root ratios of Zn and Cd concentrations among different accessions of *T. caerulescens* (Schat *et al.*, 2000; Assunção *et al.*, 2003a; Roosens *et al.*, 2003), suggesting different efficiencies of root-to-shoot translocation. However, the reasons underlying these variations are not clear. The main aim of the present study was to characterize the natural variations in the efficiencies of root-to-shoot translocation of Zn and Cd among different accessions of *T. caerulescens* and *Thlaspi praecox*; the latter was recently identified as a hyperaccumulator of Zn and Cd (Vogel-Mikus *et al.*, 2005). Specifically, we investigated the relationship between the translocation efficiency of Zn and Cd, and their relationship with the apoplastic bypass flow. Furthermore, the expression level of *TcHMA4* and compartmentation of Cd in roots were compared in two accessions of *T. caerulescens* with contrasting translocation efficiency.

Materials and Methods

Plant culture

Ten accessions of *Thlaspi caerulescens* J. & C. Presl. and one accession of *Thlaspi praecox* Wulfen were used. Table 1 shows the abbreviation for each accession, location and the metal status of the soil at the accession sites. Seeds were sown in plastic trays filled with fine vermiculite. After germination, seedlings were watered with a basal nutrient solution containing (in μM) 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 250 K_2HPO_4, 50 KCl, 10 H_3BO_3, 1.8 $MnSO_4$, 0.2 Na_2MoO_4, 0.31 $CuSO_4$, 50 Fe(III)-EDDHA (ethylenediamine-di(*o*-hydroxyphenylacetic acid)), 1 $ZnSO_4$. The nutrient solution was buffered at *c*. pH 6.0 with 2 mM MES (2-morpholinoethanesulphonic acid, pH adjusted with KOH). All experiments were carried out in a controlled environment room with the following conditions: 12 h day length with a light intensity of 350 μmol photons m^{-2} s^{-1} supplied by sodium vapour lamps, 20 : 16°C day : night temperature, and 70 : 80% day : night relative humidity.

Uptake and root-to-shoot translocation of ^{109}Cd and ^{65}Zn

Seedlings (5 wk old) were transferred to 55-ml plastic pots (one seedling per pot) and grown for 3 wk in the basal

Table 1 Location and site characteristics of different *Thlaspi* accessions used in the present study

Species	Accession	Abbreviation	Site characteristics
T. caerulescens	Bizkaia, Spain	Bi	Zn/Pb mine spoil
	Black Rock, UK	Br	Zn/Pb mine spoil
	Clough Wood, UK	Cw	Zn/Pb mine spoil
	Ganges, France	Ga	Zn/Pb mine spoil
	La Calamine, Belgium	Lc	Zn/Pb mine spoil
	Lellingen, Luxembourg	Le	Uncontaminated soil
	Monte Prinzera, Italy	Mp	Ultramafic soil (serpentine)
	Prayon, Belgium	Pr	Zn/Pb mine spoil
	Viviez, France	Vi	Soil impacted by Zn smelter
	Whitesike, UK	Wh	Zn/Pb mine spoil
T. praecox	Žerjav, Northern Slovenia	Tp	Soil impacted by Pb mining and smelting

nutrition solution, which was renewed once every 3 d. Each of the 11 accessions was replicated in six pots. The nutrient solution was then replaced with an uptake solution containing 5 μM $CdSO_4$ labelled with 1 KBq ^{109}Cd and 5 μM $ZnSO_4$ labelled with 2 KBq ^{65}Zn in the basal nutrition solution (pH 6.0). Nutrient solution was aerated during the uptake period. After 24 h uptake, seedlings were rinsed with deionized water and separated into shoots and roots, blotted dry and weighed. The radioactivities of ^{109}Cd and ^{65}Zn were determined by gamma spectroscopy (Wallac Wizard 1470, PerkinElmer, Boston, MA, USA).

An additional experiment was conducted to determine the distribution of ^{109}Cd and ^{65}Zn between roots and shoots in two accessions (Pr and Ga) of *T. caerulescens* following the initial radiolabel feeding period. Seedlings were fed with ^{109}Cd and ^{65}Zn for 24 h as described above. Roots were then rinsed briefly with deionized water and transferred to the same nutrient solution without ^{109}Cd and ^{65}Zn labels. Seedlings from five replicates were harvested at 1, 2, 3 and 5 d. Radioactivities of ^{109}Cd and ^{65}Zn in roots and shoots were determined.

To investigate the genetic basis controlling the variation in Cd and Zn translocation from roots to shoots, an F_2 progeny from the cross of Pr × Ga of *T. caerulescens* (Zha *et al.*, 2004) was used. One hundred and twenty F_2 seedlings and 20 each of the parental controls were precultured to 8 wk old. Uptake and distribution of ^{109}Cd and ^{65}Zn after 24 h feeding were determined as described above.

Apoplastic bypass flow

The apoplastic bypass flow was quantified using the apoplastic tracer dye trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS), following the method of Yeo *et al.* (1999). PTS is a water-soluble, membrane-impermeable fluorescent dye, which is transported to plant shoots via apoplastic bypass flow (Yeo *et al.*, 1999). Seedlings of the 11 accessions were precultured as described above. Seedlings (8 wk old) were placed in 55-ml pots with the uptake solution containing 30 mg PTS l^{-1} and the basal nutrition solution including 5 μM $ZnSO_4$ and 5 μM $CdSO_4$ (pH 6.0). The control solution was identical to the uptake solution, but without PTS. Each treatment was replicated in four pots for each *Thlaspi* accession. After 24 h, seedlings were separated into shoots and roots, rinsed thoroughly with deionized water, blotted dry and weighed. The shoots were cut into fine pieces and extracted with 10 ml deionized water in a water bath at 90°C for 2 h. The fluorescence of the extracts was measured with a luminescence spectrometer (PerkinElmer Luminescence Spectrometer LS 50B) at $\lambda_{excitation}$ 403 nm and $\lambda_{emission}$ 510 nm. The mean fluorescence value of the control for each accession was subtracted from the treatment with PTS. The data were expressed as the fluorescence intensity at $\lambda_{emission}$ 510 nm g^{-1} shoot fresh weight.

Expression of *TcHMA4*

The expression levels of *TcHMA4* in roots and shoots of the accessions Ga and Pr were quantified using real-time RT–PCR. Seedlings (20 d old) were transferred to 1.2-l pots (four plants per pot) and treated with 10 μM or 100 μM Zn, or 10 μM Zn + 5 μM Cd, for 8 d. Each treatment was replicated four times. Nutrient solutions were aerated continuously and renewed every 3 d. Total RNA was extracted from the shoot and root tissues using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), then converted to cDNA using the SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA). For quantitative determination of mRNA by real-time RT–PCR, the cDNAs for *TcHMA4* were amplified by PCR (ABI PRISM 7500 real-time PCR system; Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) with the primers 5′-tcgcttctataagaagacttagg-3′ and 5′-catcgaccacaattccatcaatag-3′ (GenBank no. AJ567384) (45 s at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 62°C and 45 s at 72°C). Actin was used as an internal control. The primers for actin were 5′-gagactttcaatgcccctgc-3′ and

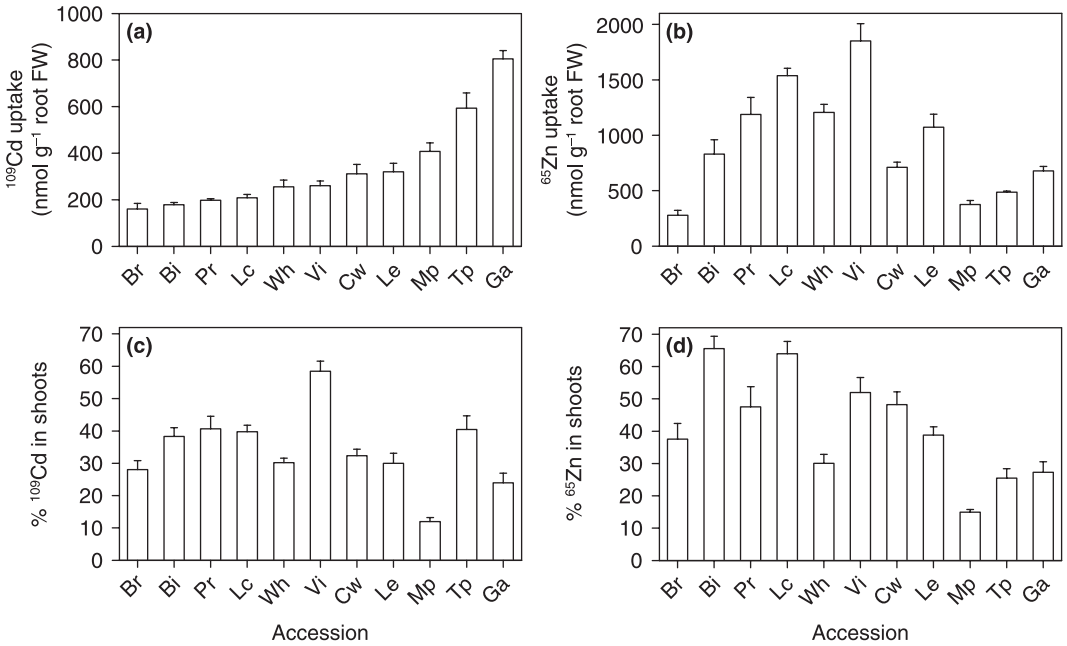

Fig. 1 Variation in the uptake of (a) ^{109}Cd; (b) ^{65}Zn and the percentage of (c) ^{109}Cd; (d) ^{65}Zn translocated to shoots among 11 accessions of *Thlaspi caerulescens* and *Thlaspi praecox*. Data are mean + SE ($n = 6$).

5′-ccatctccagagtcgagcaca-3′. The quantitative PCR data for *TcHMA4* were normalized with the expression level of actin.

^{109}Cd compartmentation in roots

A short-term ^{109}Cd efflux experiment was conducted to estimate Cd compartmentation in roots of the Pr and Ga accessions of *T. caerulescens*. The method was similar to that used for ^{65}Zn by Lasat *et al.* (1998). Seedlings (8 wk old) were incubated in separate 55-ml pots with an uptake solution containing 0.5 mM $CaCl_2$, 2 mM MES (pH 6.0) and 5 μM $CdSO_4$ labelled with 5 KBq ^{109}Cd. After uptake for 24 h, roots of intact seedlings were rinsed briefly with deionized water, wiped gently with tissue, and placed in an aerated efflux solution, which was identical with the uptake solution except without ^{109}Cd. At 14 different intervals from 2 to 360 min, a 2.5-ml aliquot of the efflux solution was taken for the determination of ^{109}Cd radioactivity, and the efflux solution was renewed. During the efflux experiment, plants were placed under shade to reduce transpiration. At the end of the experiment, seedlings were rinsed with deionized water, separated into shoots and roots, blotted dry, weighed, and counted for ^{109}Cd radioactivity. The ^{109}Cd efflux data were fitted successively to three linear equations to estimate the pool size and efflux half-life corresponding to the cell wall, cytoplasm and vacuole compartments, as described by Lasat *et al.* (1998).

Statistical analysis

ANOVA was used to test the significance of difference among accessions or treatments.

Results

Uptake and root-to-shoot translocation of ^{109}Cd and ^{65}Zn in 11 *Thlaspi* accessions

After seedlings were exposed to radiolabelled Cd and Zn (5 μM each) for 24 h, uptake of ^{109}Cd and ^{65}Zn, expressed on the basis of root FW, varied 5.0- and 6.7-fold, respectively, among the 11 *Thlaspi* accessions ($P < 0.001$; Fig. 1a,b). Consistent with previous reports (Lombi *et al.*, 2000; Roosens *et al.*, 2003), the Ga accession of *T. caerulescens* had the highest Cd uptake, while the Vi accession had the highest Zn uptake. The *T. praecox* accession from northern Slovenia also had a high Cd uptake, confirming its Cd hyperaccumulating ability observed in the field specimens (Vogel-Mikus *et al.*, 2005). Interestingly, there was no significant correlation ($r = 0.35$, $n = 11$, $P = 0.29$) between Cd and Zn uptake (Fig. 2a).

Within 24 h, 12–59 and 15–66% of ^{109}Cd and ^{65}Zn, respectively, had been transported to shoots (Fig. 1c,d). The variation in translocation efficiency was 4.9- and 4.4-fold for Cd and Zn, respectively, among the 11 accessions ($P < 0.001$). Unlike uptake, the translocation efficiency of Cd and Zn correlated significantly ($r = 0.64$, $n = 11$, $P < 0.05$; Fig. 2b).

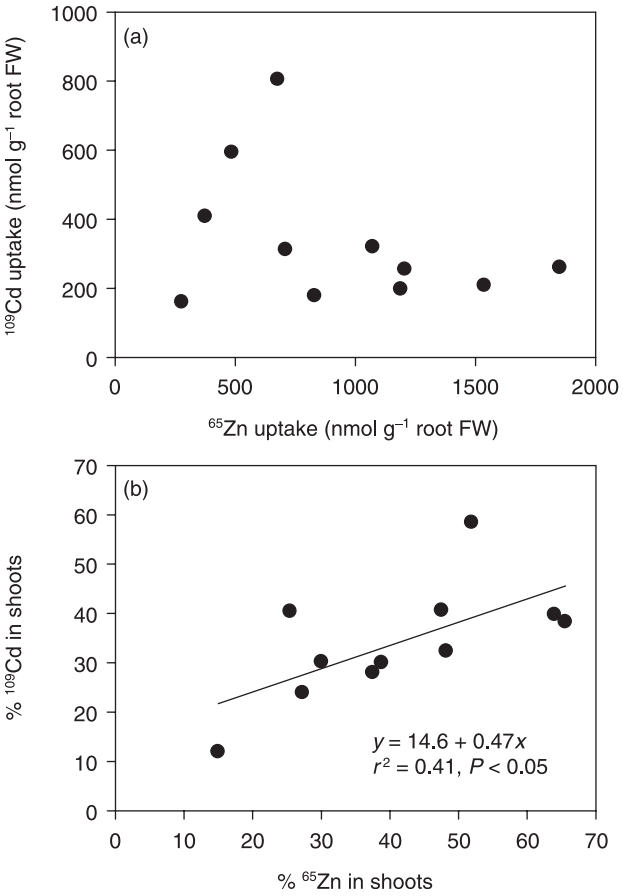

Fig. 2 Relationship (a) between ^{109}Cd and ^{65}Zn uptake; (b) between percentage of ^{109}Cd and ^{65}Zn translocated to shoots among 11 accessions of *Thlaspi caerulescens* and *Thlaspi praecox*.

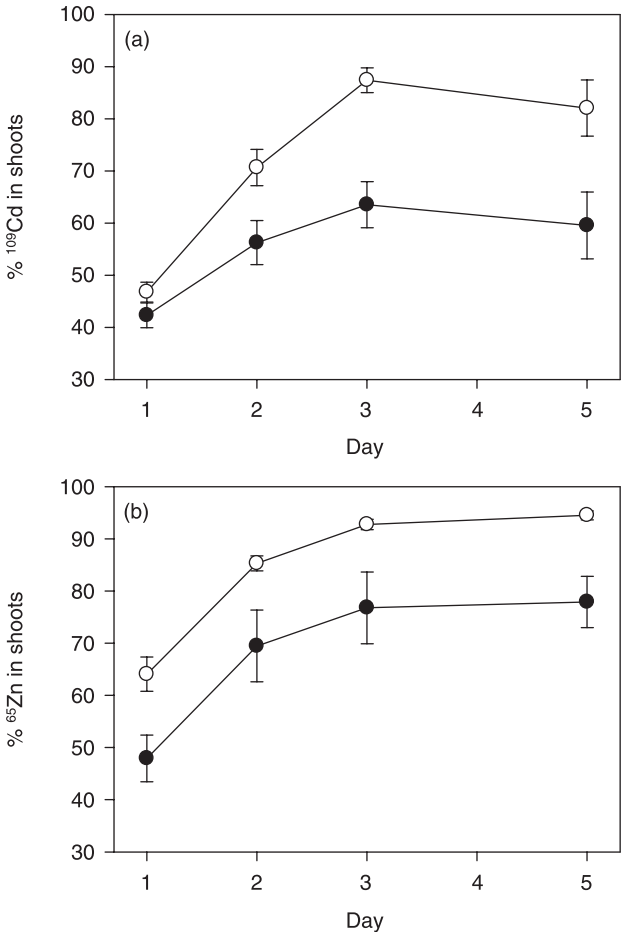

Fig. 3 Percentage of (a) ^{109}Cd; (b) ^{65}Zn translocated to shoots in the Ga (closed circles) and Pr (open circles) accessions of *Thlaspi caerulescens* following ^{109}Cd and ^{65}Zn exposure for 1 d. Data are mean ± SE ($n = 5$).

Neither the Cd nor the Zn translocation efficiency correlated significantly with the root : shoot biomass ratio (for Cd: $r = 0.15$, $n = 11$, $P = 0.66$; for Zn: $r = 0.50$, $n = 11$, $P = 0.11$; data not shown). It is conceivable that low translocation efficiency may be a result of rapid root uptake owing to saturation of the translocation machinery; in this case there should be a negative correlation between uptake and translocation efficiency. However, there was no significant correlation ($r = 0.31$, $n = 11$, $P = 0.35$) between Cd translocation efficiency and Cd uptake by roots (data not shown). In the case of Zn, its translocation efficiency appeared to increase with uptake, although the correlation also did not reach a significant level ($r = 0.57$, $n = 11$, $P = 0.06$; data not shown).

To investigate the translocation efficiency of Cd and Zn over a longer period, the Ga and Pr accessions were exposed to radiolabelled Cd and Zn (5 μM each) for 1 d, followed by 4 d in the normal nutrient solution without ^{109}Cd and ^{65}Zn. Figure 3 shows that the percentage of both ^{109}Cd and ^{65}Zn in shoots increased with time up to 3 d after feeding. Similarly to the data presented in Fig. 1, the Pr accession had a higher translocation efficiency for both metals than the Ga accession ($P < 0.001$). For both Cd and Zn translocation efficiency, there were no significant interactions between accession and time.

Apoplastic bypass flow

The PTS concentration in shoots varied 4.9-fold among the 11 accessions ($P = 0.011$; Fig. 4a), showing a strong correlation with the root : shoot biomass ratio ($r = 0.91$, $n = 11$, $P < 0.001$; Fig. 4b). However, there was no significant correlation between PTS concentration and Cd or Zn translocation efficiency (for Cd: $r = 0.02$, $n = 11$, $P = 0.95$; for Zn: $r = 0.27$, $n = 11$, $P = 0.42$; Fig. 4c,d).

Root-to-shoot translocation of ^{109}Cd and ^{65}Zn in F_2 progeny from the Pr × Ga cross

A previous study (Zha *et al.*, 2004) reported a cosegregation analysis of Cd and Zn accumulation in the F_2 progeny from

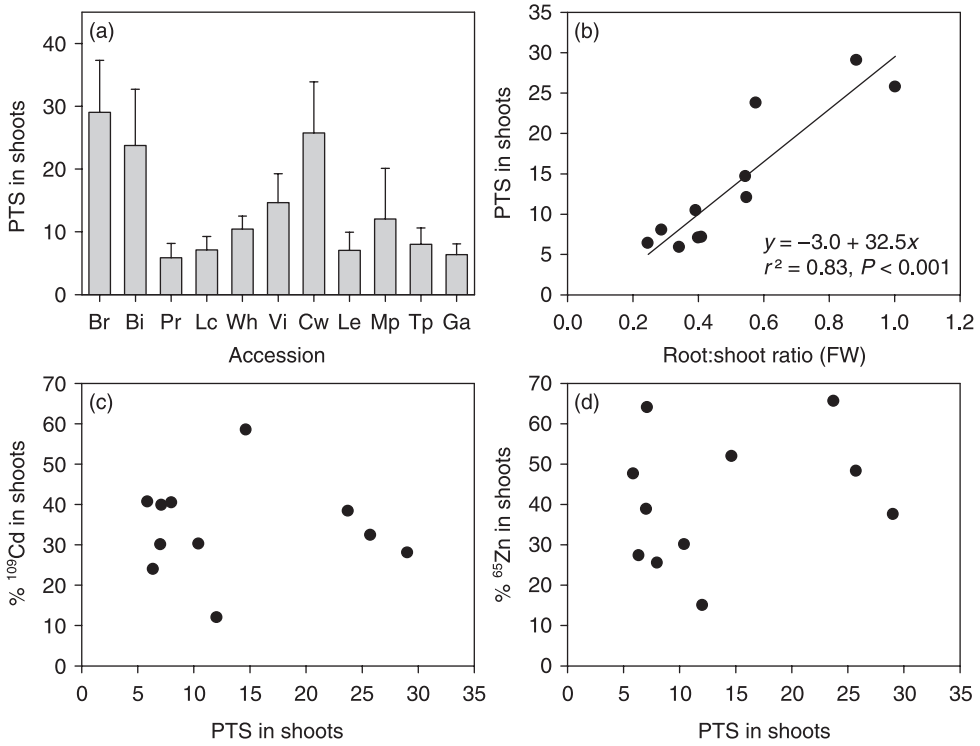

Fig. 4 Variation in the apoplastic bypass flow as measured by the fluorescence of trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS) in shoots among 11 accessions of *Thlaspi caerulescens* and *Thlaspi praecox* (a); correlation between PTS in shoots and root : shoot biomass ratio (b); correlation between PTS in shoots and percentage of ^{109}Cd (c) or ^{65}Zn (d) translocated to shoots. PTS is expressed as fluorescence intensity g^{-1} shoot FW. Data in (a) are mean + SE ($n = 4$).

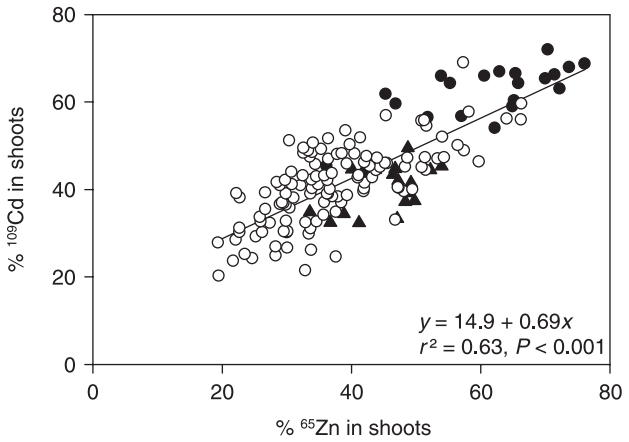

Fig. 5 Relationship between percentage of ^{109}Cd and ^{65}Zn translocated to shoots in parental controls and F_2 progeny of the Pr × Ga cross of *Thlaspi caerulescens*. Triangles, Ga; closed circles, Pr; open circles, F_2.

the Pr × Ga and Ga × Pr crosses, using both soil and hydroponic experiments with a relatively long (40–45 d) exposure to Cd and Zn. In the present study, we analysed the segregation patterns of Cd and Zn translocation in the F_2 progeny from the Pr × Ga cross and the parental controls, after plants were given radiolabelled Cd and Zn (5 μM each) for 24 h. Similar to the results with 11 accessions described above (Fig. 2b), there was a strong correlation ($r = 0.79$, $n = 160$, $P < 0.001$) between the percentage of ^{109}Cd and ^{65}Zn transported to shoots in the parental and F_2 plants. A single linear regression described the data well for both parental controls and F_2 plants (Fig. 5). Figure 6 shows the phenotypic distributions in the parental controls and F_2 plants. The Pr accession had a higher root-to-shoot translocation efficiency for both Cd and Zn than the Ga accession, and the phenotypes of the two overlapped slightly. The distribution patterns in the F_2 were continuous, but biased toward the phenotype range of Ga. There was evidence of transgression for both Cd and Zn translocation efficiency beyond the lowest parental range, but not above the highest parental range.

Expression of *TcHMA4*

Real-time RT–PCR was used to quantify the transcript abundance of *TcHMA4*, a prime candidate gene implicated in the root-to-shoot transport of Zn and Cd in *T. caerulescens* (Bernard *et al.*, 2004; Papoyan & Kochian, 2004). Figure 7 shows the relative levels of transcript abundance in shoot and root tissues of the Ga and Pr accessions grown with different concentrations of Zn and Cd. The transcript level in roots was

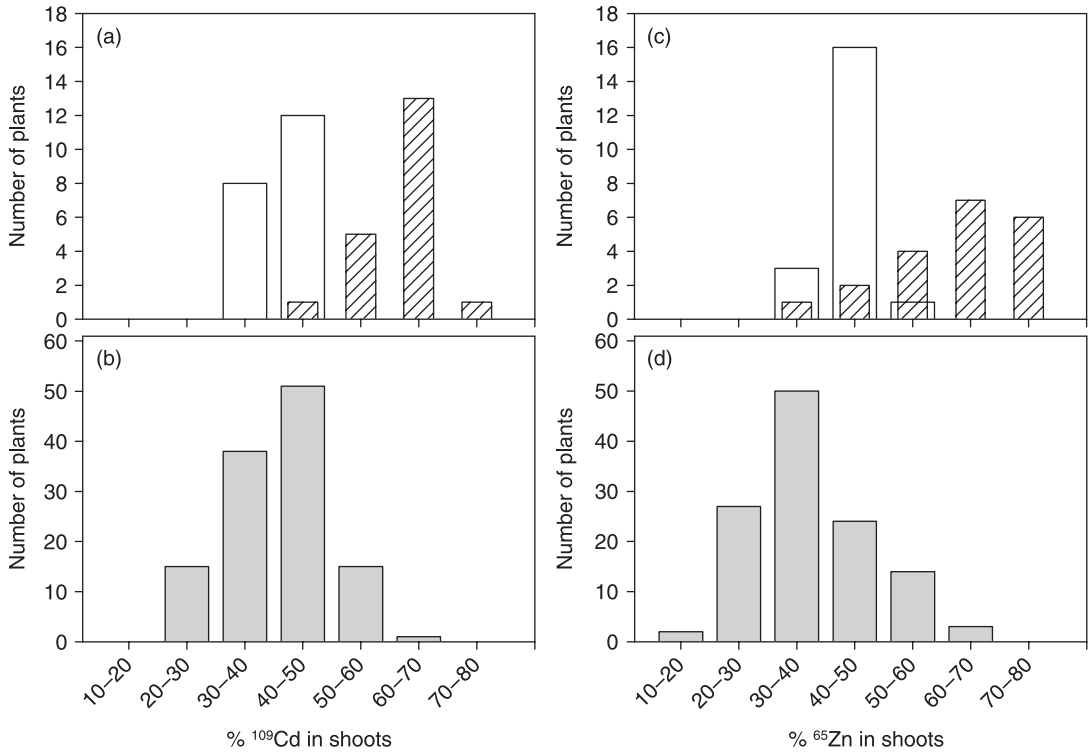

Fig. 6 Phenotype distribution of percentage of ^{109}Cd and ^{65}Zn translocated to shoots in parental controls (a,c) and F_2 progeny (b,d) of the Pr × Ga cross of *Thlaspi caerulescens*. ^{109}Cd (a,b) and ^{65}Zn (c,d). Hatched bars, Pr; open bars, Ga; grey bars, F_2.

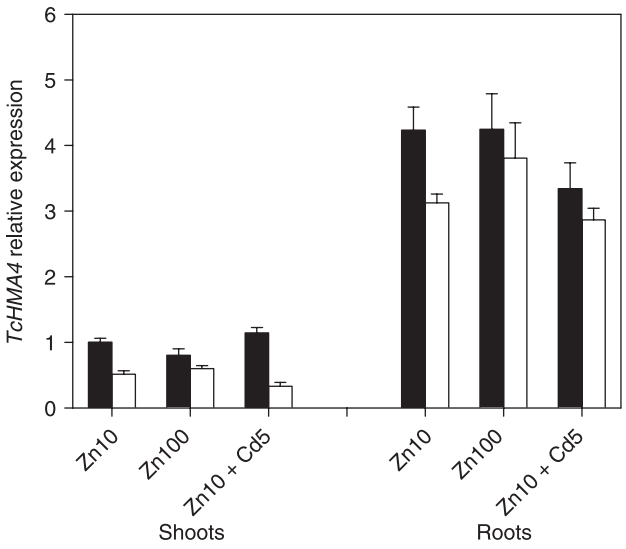

Fig. 7 Relative expression level of *TcHMA4* in roots and shoots of the Ga (closed bars) and Pr (open bars) accessions of *Thlaspi caerulescens* as influenced by Zn and Cd treatments. *TcHMA4* transcript abundance of root and shoot tissues from different treatments were compared with that of the Ga shoot tissue from the 10 μM Zn treatment. Data are mean + SE (n = 4).

three to nine times higher than that in shoots of either accession. Two-way ANOVA involving accession and Zn/Cd treatments was performed on the root or shoot *TcHMA4* data. There was a near-significant (P = 0.051) difference in the transcript level in roots between the Ga and Pr accessions, with Ga having approx. 20% higher transcript abundance than Pr. The transcript level in shoots was *c*. twofold higher in Ga than in Pr (P < 0.001). The different Zn and Cd treatments had no significant effect on the expression of *TcHMA4*.

^{109}Cd compartmentation in roots

A possible explanation for the difference in the Cd and Zn translocation efficiency among different accessions may lie in the compartmentation of the metals in the root cells. Cadmium compartmentation in the root cells of the Ga and Pr accessions was investigated using the method of short-term ^{109}Cd efflux. Similarly to previous studies on ^{65}Zn efflux from *T. caerulescens* roots (Lasat *et al*., 1998), the time-dependent kinetics of ^{109}Cd efflux from Ga and Pr roots could be resolved into three linear phases (Fig. 8), representing Cd efflux from cell walls (0–15 min), cytoplasm (30–60 min) and vacuoles (180–360 min). The amounts of ^{109}Cd in the three compartments and the rate of efflux (efflux half-life $t_{1/2}$), estimated from the three linear regression lines, are shown in Table 2. After exposure to ^{109}Cd for 1 d, Ga roots accumulated more

Table 2 ^{109}Cd compartmentation and half-life ($t_{1/2}$) for ^{109}Cd efflux from different root cell compartments of the Ga and Pr accessions of *Thlaspi caerulescens*

Accession		Cell wall	Cytoplasm	Vacuole
Ga	Amount of ^{109}Cd (nmol g^{-1} FW)	145.5 ± 31.0	87.1 ± 14.6	659.8 ± 34.1
	Percentage distribution of ^{109}Cd	16.2 ± 3.2	9.6 ± 1.2	74.2 ± 3.1
	Efflux half-life $t_{1/2}$ (min)	2.4 ± 0.4	25.9 ± 3.8	2306 ± 231.0
Pr	Amount of ^{109}Cd (nmol g^{-1} FW)	83.8 ± 2.2	26.6 ± 2.8	149.0 ± 10.8
	Percentage distribution of ^{109}Cd	32.6 ± 1.1	10.3 ± 1.0	57.1 ± 1.6
	Efflux half-life $t_{1/2}$ (min)	3.3 ± 0.6	27.5 ± 2.1	2332 ± 360.9

Values are mean ± SE.

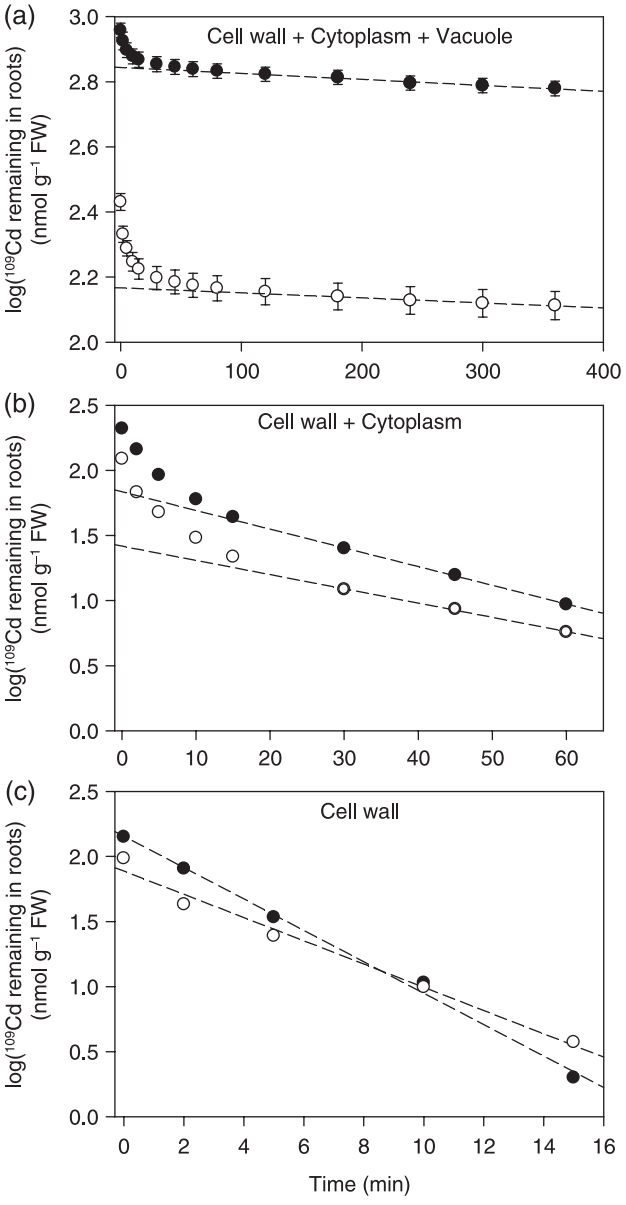

Fig. 8 Short-term efflux of ^{109}Cd from roots of the Ga (closed circles) and Pr (open circles) accessions of *Thlaspi caerulescens*. The ^{109}Cd efflux data were resolved into three linear curves representing cell wall, cytoplasm and vacuoles according to Lasat *et al*. (1998). Data points in (a) are mean ± SE (n = 5).

^{109}Cd than Pr roots, with 1.7-, 3.3- and 4.4-fold larger amounts of ^{109}Cd in the root cell walls, cytoplasm and vacuoles, respectively, than those of Pr roots. Proportionally more ^{109}Cd was in the vacuoles of Ga roots (74%) than in those of Pr roots (57%). Similar proportions (approx. 10%) of root ^{109}Cd were found in the cytoplasm in both Ga and Pr roots, whereas the latter had a higher percentage in the cell walls than the former. The half-time for ^{109}Cd efflux from the cell walls, cytoplasm and vacuoles was similar between Ga and Pr.

Discussion

The results from the present study, using radiolabelling and short-term (1 d) exposure to relatively low levels of Cd and Zn, show large variations in the uptake of Cd and Zn among 10 accessions of *T. caerulescens* and one accession of *T. praecox* (Fig. 1). There was no significant correlation between uptake of the two metals (Fig. 2), supporting the hypothesis that multiple transport systems are involved in the uptake of Cd and Zn by *T. caerulescens* (Lombi *et al*., 2001; Zhao *et al*., 2002; Zha *et al*., 2004). These results corroborate and extend previous investigations using fewer accessions (two to four) and longer exposure time (weeks) (Lombi *et al*., 2000; Assunção *et al*., 2003a; Zha *et al*., 2004). By contrast, Roosens *et al*. (2003) reported a significant correlation between Zn and Cd concentrations in shoots of seven accessions of *T. caerulescens* after exposure to the two metals for 14 d. Metal concentration in shoots after a long exposure period is a measure of accumulation resulting from both uptake and root-to-shoot translocation, both of which are likely to be under regulation by the internal metal status (Papoyan *et al*., 2007), whereas the method used in our study is a better measure of root uptake *per se*.

The focus of the present study was on the root-to-shoot translocation of Cd and Zn, which is an important process of metal hyperaccumulation, yet still poorly understood. The percentages of Cd and Zn transported to shoot within 24 h exposure varied widely among the 11 accessions examined (Fig. 1). Several interesting observations emerged from the present work. First, the translocation efficiency did not correlate with uptake for either metal, suggesting independent variation in uptake and translocation among different accessions

of *Thlaspi*. This is consistent with the observation made by Schat *et al*. (2000) with three accessions of *T. caerulescens*. Second, there was a positive correlation between the translocation efficiency of Cd and Zn (Figs 2,5), suggesting the possibility of a common transport mechanism from roots to shoots for the two metals. This correlation was significant not only in the 11 accessions tested, but also in the F_2 progeny from the Pr × Ga cross, indicating a genetic correlation between the translocation efficiency of the two metals. Third, the translocation efficiency did not correlate with the apoplastic bypass flow measured with a membrane-impermeable fluorescence dye (Fig. 4). Previously, White *et al*. (2002) argued for a significant role of apoplastic pathway in the xylem loading of Zn in *T. caerulescens*. This hypothesis is not supported by the data shown in Fig. 4, which suggest that the variation among accessions in Cd and Zn translocation is not controlled by the apoplastic pathway. Recently, van der Mortel *et al*. (2006) reported the presence of two layers of endodermis in the root tissues of *T. caerulescens*, as well as enhanced expression of lignin/suberin biosynthesis genes compared with that in roots of *A. thaliana*. Their results strengthen the argument for a dominant role of the symplastic pathway in the transport of Cd and Zn to xylem.

To investigate possible reasons accounting for the variation in translocation efficiency among accessions, we chose two accessions (Ga and Pr) for further characterization. These two accessions differed significantly not only in uptake, but also in the efficiency of root-to-shoot transport of Cd and Zn. Accession Ga had the highest uptake of Cd (Fig. 1); its extraordinary ability to accumulate Cd has been reported before (Lombi *et al*., 2000; Assunção *et al*., 2003a; Roosens *et al*., 2003). However, the translocation efficiency for both Cd and Zn was rather low in Ga. In comparison, accession Pr had a consistently higher translocation efficiency for both metals (Figs 1, 3). In the F_2 progeny from the Pr × Ga cross, the phenotype of Cd and Zn translocation to shoots exhibited a continuous pattern (Fig. 6). There was also evidence of transgression at the low end of the phenotype range. These results suggest a multilocus model controlling the variation in efficiency of metal translocation, with the trait-enhancing alleles originating from both Pr and Ga. The lack of transgression toward the high end of the phenotype range (Fig. 6) may be because of saturation of the translocation capacity, or other unknown causes.

One of the key steps in transport from roots to shoots is loading of metals into the xylem vessels in the stelar region of roots. HMA4, a metal-transporting P_{1B}-type ATPase, has been shown to play a key role in the root-to-shoot transport of Zn and Cd in *A. thaliana*, probably acting as an efflux pump located on the plasma membranes of xylem parenchyma cells, and delivering Zn^{2+} and Cd^{2+} to the xylem vessels (Mills *et al*., 2003; Hussain *et al*., 2004; Verret *et al*., 2004; Mills *et al*., 2005). A role in loading Zn^{2+} and Cd^{2+} into the xylem has also been implicated for TcHMA4 in *T. caerulescens* (Papoyan & Kochian, 2004). We therefore investigated whether expression of *TcHMA4* could account for the difference between Ga and Pr in the translocation efficiency of Zn and Cd. Similarly to other studies on *T. caerulescens* (Bernard *et al*., 2004; Papoyan & Kochian, 2004), *TcHMA4* was expressed more in roots than in shoots (Fig. 7). The less efficient Ga accession had a somewhat higher expression level in roots than Pr, although the difference did not quite reach the significance level. Similarly, Bernard *et al*. (2004) found no significant difference in *TcHMA4* transcript abundance among three accessions of *T. caerulescens*, including the accessions from Pr and St Félix-de-Pallières, which is near Ga. Therefore it is unlikely that the difference between Pr and Ga in the root-to-shoot translocation of Cd and Zn is attributable to different expression levels of *TcHMA4*. We also found that exposure to different Zn (10 or 100 μM) or Cd (0 or 5 μM) treatments for 8 d did not significantly affect *TcHMA4* expression (Fig. 7). Bernard *et al*. (2004) showed a weak but statistically insignificant induction of *TcHMA4* expression by Cd. In roots of *A. halleri*, the transcript level of *AhHMA4* showed little response to different Zn or Cd treatments (Talke *et al*., 2006). By contrast, Papoyan & Kochian (2004) reported that *TcHMA4* expression in the root of the Pr accession was significantly induced by both Zn deficiency and exposure to high Zn or Cd levels.

Sequestration of metals in root vacuoles could decrease their availability for translocation to shoots, as has been shown by Lasat *et al*. (1998), who compared the compartmentation of ^{65}Zn in the root tissues of *T. caerulescens* and of the nonhyperaccumulator *T. arvense*. Similarly, Yang *et al*. (2006) found that the nonhyperaccumulating ecotype of *Sedum alfredii* retained 2.7-fold more Zn in root vacuoles than the hyperaccumulating ecotype of the same species. We compared ^{109}Cd compartmentation in the root tissues of Pr and Ga. Although Ga had higher concentrations of ^{109}Cd in all three root compartments because of a higher rate of uptake, proportionally more ^{109}Cd was stored in the vacuoles than in the Pr accession (Table 2). The difference in vacuolar sequestration may explain the difference between the two accessions in Cd translocation efficiency. It remains to be investigated whether the activity of tonoplast transporters for Cd differs between the two accessions.

In conclusion, this study has revealed a large variation in the root-to-shoot translocation of Cd and Zn among different accessions of *Thlaspi*, which was independent of the variation in root uptake. These accessions are valuable materials for dissecting the traits responsible for Zn and Cd hyperaccumulation, especially translocation. The efficiency of root-to-shoot translocation was not related to apoplastic bypass flow among the 11 *Thlaspi* accessions examined. In the two accessions of *T. caerulescens* chosen for further characterization, difference in Zn and Cd translocation was not correlated with the expression level of *TcHMA4* in roots. The accession with a higher translocation efficiency stored proportionally less Cd in root vacuoles, suggesting that vacuolar sequestration may play a role in controlling the root-to-shoot translocation of metals.

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