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# Intraspecific variation of metal preference patterns for hyperaccumulation in *Thlaspi caerulescens*: evidence from binary metal exposures

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**Abstract** Metal preferences with regard to accumulation were compared between populations of the heavy metal hyperaccumulator *Thlaspi caerulescens*, originating from calamine, serpentine and non-metaliferous soils. Plants were exposed for 3 weeks to factorial combinations of concentrations of different metals in binary mixture in hydroponics. The nature and degree of the interactions varied significantly between populations. In the calamine, non-Cd/Ni-hyperaccumulating population, La Calamine, there were no one-sided or mutual antagonistic interactions among the metals with regard to their accumulation in the plant. In three other populations capable of Cd and Ni hyperaccumulation, from calamine, serpentine and non-metalliferous soil respectively, there were

one-sided or mutual antagonistic interactions between Cd and Zn, Cd and Ni, and Ni and Zn, possibly resulting from competition for transporters involved in uptake or plant-internal transport. Significant synergistic interactions, probably resulting from regulation of transporter expression, were also found, particularly in the La Calamine population. All the populations seemed to express a more or less Zn-specific high-affinity system. The serpentine and the non-metallicolous populations seemed to possess low-affinity systems with a preference for Cd and Zn over Ni, one of which may be responsible for the Ni hyperaccumulation of the serpentine population in its natural environment. The calamine population from Ganges also seemed to express a strongly Cd-specific high-affinity system which is in part responsible for the Cd-hyperaccumulation phenotype exhibited by this population in its natural environment.

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## Introduction

*Thlaspi caerulescens* is a hyperaccumulator of Zn and, occasionally, Cd and Ni (Baker and Brooks 1989; Lombi et al. 2000; Assunção et al. 2003). Among ecotypes and local populations, there is a high level of uncorrelated variation in the degrees and metal-specificity patterns for accumulation, root-to-

shoot transport and tolerance (Meerts and Van Isacker 1997; Escarré et al. 2000; Schat et al. 2000; Lombi et al. 2000; Pollard et al. 2002; Assunção et al. 2003). This variation provides good opportunities for a further dissection of the genetics of these traits. Quantitative trait locus (QTL) analyses of segregating *T. caerulescens* intraspecific crosses between plants from populations with contrasting metal accumulation and tolerance phenotypes have identified a number of QTLs for Zn and Cd accumulation (Assunção et al. 2006; Deniau et al. 2006). In both of the crosses analyzed, both parents contributed trait-enhancing alleles at the Zn accumulation loci, suggesting that population-specific mechanisms may significantly contribute to the Zn hyperaccumulation trait, indeed (Assunção et al. 2006; Deniau et al. 2006). In conformity with this viewpoint, transgressive segregation for Zn accumulation has been observed in two different intraspecific crosses (Zha et al. 2004; Deniau et al. 2006). Moreover, some of the QTLs for Zn accumulation co-located with a QTL for Cd accumulation, whereas others were specific for Zn. On the other hand, there were also QTLs for Cd accumulation that did not co-locate with one for Zn, suggesting that Zn and Cd hyperaccumulation may be in part accomplished by different mechanisms with distinct patterns of metal-preference and differential expression among populations (Deniau et al. 2006).

Metal hyperaccumulation most probably relies on enhanced uptake, enhanced translocation to the shoot and enhanced vacuolar sequestration in leaf cells (Lasat et al. 1996; Pence et al. 2000; Assunção et al. 2001; Persans et al. 2001; Dräger et al. 2004). These processes are not well understood yet. However, transmembrane metal transporters seem to play a decisive role in these processes (Pence et al. 2000; Assunção et al. 2001; Persans et al. 2001; Dräger et al. 2004).

Enhanced Zn uptake in *T. caerulescens* has been putatively attributed to members of the ZIP family of metal transporters, ZNT1 and ZNT2. These transporters were found to be much higher expressed in *T. caerulescens*, especially in roots, than in the non-hyperaccumulator congener, *T. arvense* (Pence et al. 2000; Assunção et al. 2001). ZNT1 has been shown to mediate high-affinity Zn transport and low-affinity Cd transport in yeast (Pence et al. 2000). However, Assunção et al. (2001) did not find appreciable differences in *ZNT1/ZNT2* mRNA expression among

three *T. caerulescens* populations with strongly different degrees of Zn, Cd and Ni accumulation. Two of these populations hyperaccumulated Cd and Ni under controlled conditions. The other one, though showing the high shoot to root concentration ratios typical of a hyperaccumulator, did not, because the uptake rates for these metals were even lower than in *T. arvense* (Assunção et al. 2003). Therefore, it seems that ZNT1 and ZNT2 are not responsible for the hyperaccumulation of Cd or Ni, at least. Also, the strongly different degrees of Zn hyperaccumulation in these populations suggested that transporters other than ZNT1 or ZNT2 might in fact account for the bigger part of the Zn uptake capacity in the high-accumulation populations, although there is also the possibility that ZNT1 and ZNT2 would be differentially regulated at the post-transcriptional level. In addition, Lombi et al. (2000, 2001) and Zhao et al. (2002), comparing two *T. caerulescens* calamine populations with strongly different Cd accumulation capacities, obtained physiological evidence of a transporter with high preference for Cd over Zn and Mn, the activity of which was found to be at least five-fold higher in the high-accumulation population than in the low-accumulation one. Recent microarray transcript profiling in *T. caerulescens* and another Zn/Cd hyperaccumulator, *Arabidopsis halleri* (Becher et al. 2004; Weber et al. 2004; Hammond et al. 2006; Talke et al. 2006; Van de Mortel et al. 2006) have shown that these species express a large number of metal transporters, including members of the CDF, HMA and NRAMP families, at much higher levels than non-hyperaccumulators do. Their precise roles in the hyperaccumulation phenomenon are elusive yet.

In a previous study (Assunção et al. 2003), *T. caerulescens* populations from contrasting soil types, i.e. serpentine, calamine and non-metalliferous, were characterized with regard to the accumulation of Zn, Cd and Ni in hydroponic culture. Results suggested that all these populations exhibit unique accumulation features with regard to the patterns of metal-specificity and concentration-dependency, suggesting that they possess different mechanisms of metal accumulation with different degrees and patterns of metal-specificity. However, in the latter study plants were grown under single metal exposure. It may be expected that apparent metal-preference patterns can be different under combined exposure, such as often found in nature, due to interactions between metals. In

this study we aimed at a further characterization of the degree and nature of the variation in metal preference among these populations, in particular with regard to the question of whether the strongly different foliar metal compositions in the natural environment can be explained by differential metal preference patterns or by differential soil metal compositions. For this purpose, *T. caerulea* populations from serpentine, calamine and non-metalliferous soil were exposed to factorial combinations of concentrations of different metals in binary mixture.

## Materials and methods

### Plant culture and root and shoot metal accumulation assay

Seeds collected from *T. caerulea* J. & C. Presl populations at La Calamine, Belgium (LC), Lellingen, Luxemburg (LE), Saint Laurent le Minier–Avinières (5 km from Ganges), Southern France (GA), and Monte Prinzerà, Italy (MP), as well as from a *T. arvensis* road side population at Amsterdam, the Netherlands, were sown on moist peat. Three-week-old seedlings were transferred to 1-l polyethylene pots (one seedling per pot), filled with modified half-strength Hoagland's solution: 3 mM KNO<sub>3</sub>, 2 mM Ca

(NO<sub>3</sub>)<sub>2</sub>, 1 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 1 μM KCl, 25 μM H<sub>3</sub>BO<sub>3</sub>, 2 μM ZnSO<sub>4</sub>, 2 μM MnSO<sub>4</sub>, 0.1 μM CuSO<sub>4</sub>, 0.1 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 20 μM Fe (Na)EDTA. The pH buffer MES was added at a 2-mM concentration, and the pH was set at 5.5, using KOH. The solutions were replaced twice a week. Germination and plant culture were performed in a climate chamber (20/15°C day/night; 250 μmol m<sup>-2</sup> s<sup>-1</sup> at plant level; 14 h day<sup>-1</sup>; 75% RH).

After 1 week, the plants were exposed to nutrient solution supplemented with ZnSO<sub>4</sub>, CdSO<sub>4</sub> or NiSO<sub>4</sub>. In each assay two metals were supplied together, in factorial combinations of the following concentrations: 1, 10 and 100 μM in the Zn/Ni assay and 0.5, 5 and 50 μM in the Cd/Zn and Cd/Ni assays. The nutrient solution was the same as during preculture, but buffered at a slightly lower pH, i.e. 5.25. At the given pH and FeEDTA concentrations part of the added metals particularly of Ni, are expected to displace iron from the EDTA complex, and thus to become complexed themselves (Table 1), for equilibrium complexation rates, according to the GEO-CHEM-PC Version 2.0 program (Parker et al. 1995). However, based on measurements of the loss of dissolved complexed iron, the kinetics of these displacement reactions are slow, i.e. less than 2.5 μM after 1 day, even at 50 μM Ni (H. Schat, unpublished data), indicating that the free ionic metal

**Table 1** Percentages of each metal (Zn, Cd and Ni) as a free metal and complexed with EDTA (italicized data), for equilibrium complexation rates, according to the GEOCHEM-PC Version 2.0 program (Parker et al. 1995)

Percentages of each metal as a free metal and complexed with EDTA

Zn/Cd	0.5/0.5	0.5/5	0.5/50	5/0.5	5/5	5/50	50/0.5	50/5	50/50
Zn	46.91%	49.52%	68.27%	49.52%	43.32%	69.41%	68.19%	69.35%	76.99%
	<i>48.93%</i>	<i>46.07%</i>	<i>25.57%</i>	<i>46.08%</i>	<i>52.06%</i>	<i>24.30%</i>	<i>25.66%</i>	<i>24.37%</i>	<i>15.88%</i>
Cd	47.30%	49.94%	68.87%	49.94%	52.51%	70.03%	68.79%	69.96%	77.37%
	<i>49.21%</i>	<i>46.34%</i>	<i>25.73%</i>	<i>46.36%</i>	<i>43.58%</i>	<i>24.45%</i>	<i>25.82%</i>	<i>24.52%</i>	<i>15.92%</i>
Zn/Ni	1/1	1/10	1/100	10/1	10/10	10/100	100/1	100/10	100/100
Zn	48.11%	32.44%	91.25%	53.32%	67.15%	91.20%	77.64%	83.31%	90.75%
	<i>47.62%</i>	<i>62.04%</i>	<i>0.31%</i>	<i>41.94%</i>	<i>26.86%</i>	<i>0.31%</i>	<i>15.17%</i>	<i>8.94%</i>	<i>0.30%</i>
Ni	1.25%	2.34%	74.37%	1.57%	3.04%	74.36%	6.01%	10.39%	74.22%
	<i>98.66%</i>	<i>97.49%</i>	<i>19.89%</i>	<i>98.32%</i>	<i>96.75%</i>	<i>19.87%</i>	<i>93.53%</i>	<i>88.80%</i>	<i>19.60%</i>
Ni/Cd	0.5/0.5	0.5/5	0.5/50	5/0.5	5/5	5/50	50/0.5	50/5	50/50
Ni	1.20%	1.35%	3.30%	1.56%	1.77%	4.38%	56.27%	56.32%	56.76%
	<i>98.71%</i>	<i>98.55%</i>	<i>96.44%</i>	<i>98.32%</i>	<i>98.10%</i>	<i>95.29%</i>	<i>39.54%</i>	<i>39.46%</i>	<i>38.87%</i>
Cd	47.60%	50.28%	69.26%	53.71%	56.55%	73.99%	91.99%	91.94%	77.79%
	<i>48.88%</i>	<i>45.98%</i>	<i>25.32%</i>	<i>42.31%</i>	<i>39.23%</i>	<i>20.17%</i>	<i>0.81%</i>	<i>0.81%</i>	<i>15.15%</i>

The nine treatments corresponding to the factorial combinations of concentrations from each binary metal exposure (Zn/Cd, Zn/Ni and Ni/Cd) are shown (see the “Materials and methods” section).

activities must have remained relatively close to the initial values. Precipitation of metal salts did not occur throughout the experiment. Five plants were used per treatment permutation. The nutrient solutions were replaced twice a week. It was calculated that this replacement rate was sufficient to prevent a more than 30% depletion of the metals from the nutrient solution prior to renewal. After 3 weeks, the plants were harvested, after desorbing the roots systems with ice-cold 5-mM  $\text{PbNO}_3$  for 1 h. Roots and shoots were dried overnight at 70°C, wet-ashed in a 4:1 mixture of  $\text{HNO}_3$  (65%) and  $\text{HCl}$  (37%) in Teflon bombs at 140°C for 7 h and analyzed for Zn, Cd and Ni using flame atomic absorption spectrometry (Perkin Elmer 1100B). Total plant metal accumulation was calculated as the total amount of plant metal ( $\mu\text{mol}$ ) per g total plant weight.

### Statistics

Statistic analysis was performed with log-transformed values using three-way ANOVA, followed by post hoc comparison using Tukey's HSD test.

## Results

Zn, Cd and Ni accumulation were determined in *T. caerulescens* populations grown hydroponically under binary metal exposure for 3 weeks. Data for exposure levels that exceeded the threshold for leaf chlorosis and growth inhibition in the populations in question (Assunção et al. 2003) have not been presented and the 100- $\mu\text{M}$  Ni exposure level is lacking in case of GA.

### Zinc and cadmium

In agreement with previous studies (Assunção et al. 2003), there were highly significant differences between populations in Zn and Cd accumulation on a total plant dry weight basis. Also the nature of the Zn/Cd interaction varied significantly between populations (Table 2). In the non-Cd-hyperaccumulating population, LC, there was neither significant inhibition of Zn accumulation by Cd, nor significant inhibition of Cd accumulation by Zn (Fig. 1). Instead, there was an apparent stimulation of Cd accumulation in the presence of 50  $\mu\text{M}$  Zn (Fig. 1b). Conversely, at the 0.5- $\mu\text{M}$  Zn supply level, Zn uptake was signifi-

cantly enhanced by 50  $\mu\text{M}$  Cd in LC and GA (Fig. 1a). In the Cd hyperaccumulating populations, LE and GA, Zn accumulation was inhibited by Cd at high external Zn concentrations, particularly in the GA population (Fig. 1a). Zn inhibited Cd accumulation in LE, GA and MP, but the patterns of concentration-dependency varied strongly between populations. At low external Cd (0.5  $\mu\text{M}$ ), Zn-imposed inhibition of Cd accumulation was apparent in LE and MP, but not in GA. At higher external Cd, Zn had not much effect on Cd accumulation in LE, but it clearly inhibited Cd accumulation in GA (Fig. 1b).

### Zinc and nickel

Ni accumulation and the nature of the Zn/Ni interaction varied significantly between populations (Table 3). There was no significant overall effect of the Ni concentration in the nutrient solution on Zn accumulation and the third order population  $\times$  Zn  $\times$  Ni interaction was not significant (Table 3, Fig. 2a). Ni accumulation, however, was strongly suppressed by Zn in the Ni hyperaccumulating populations LE, MP and GA, but not in the non-Ni-hyperaccumulating population, LC (Fig. 2b).

### Nickel and cadmium

The nature of the Ni/Cd interference varied significantly between populations (Table 4). Again, in the non-Cd/Ni-hyperaccumulating population, LC, there was neither an inhibitory effect of Ni on Cd accumulation, nor of Cd on Ni accumulation (Fig. 3). In all of the Cd/Ni-hyperaccumulating populations, there was a consistent pattern, that is a strong inhibition of Ni accumulation by Cd, but no inhibition of Cd accumulation by Ni (Table 4). Instead, at the 5- $\mu\text{M}$  Cd supply, 50  $\mu\text{M}$  Ni significantly enhanced Cd uptake in LC and LE, though not in GA (Fig. 3a).

## Discussion

Our results clearly show interactions between Zn, Cd and Ni with regard to their accumulation in the plant. Antagonistic interactions have often been assumed to result from competition for common metal trans-

**Table 2** Three-way ANOVA of Zn accumulation and Cd accumulation in *T. caeruleus* populations, LE, LC and GA, grown for 3 weeks in nutrient solution supplemented with factorial combinations of concentrations of Zn and Cd

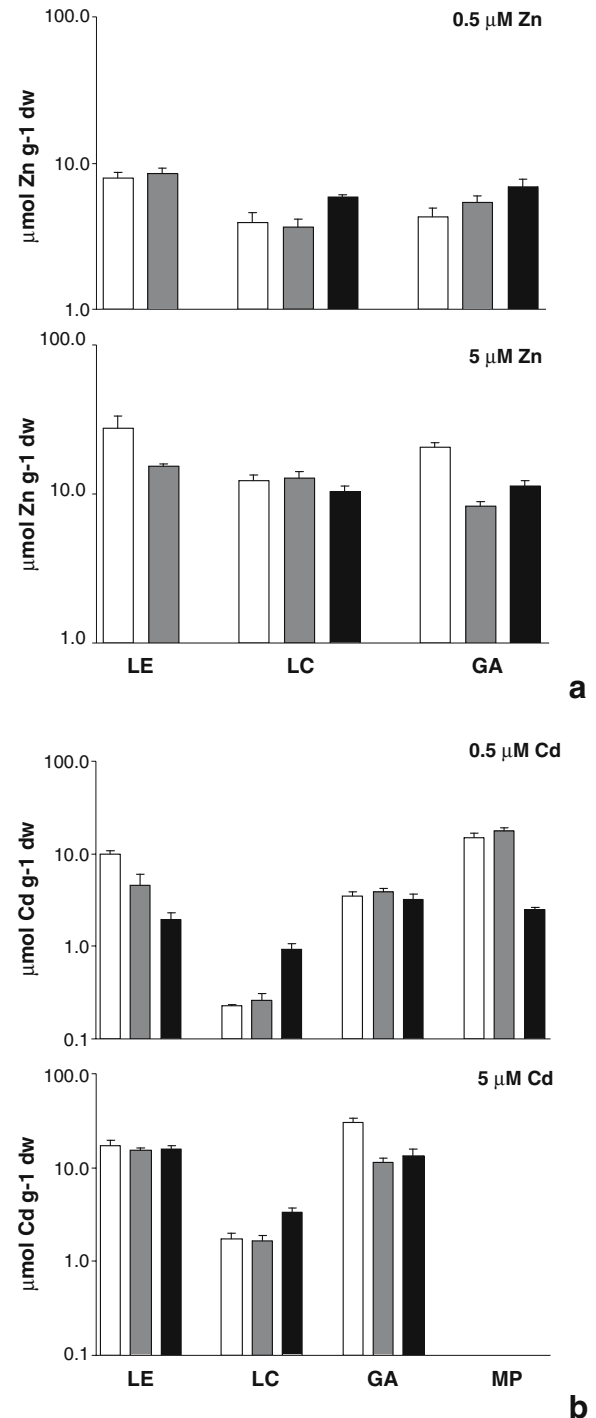
Source	df	MS	F	P
<b>Zn accumulation</b>				
Population	2	0.366	34.348	0.000
Zn	1	2.689	252.061	0.000
Cd	1	9.597E-02	8.995	0.005
Population×Zn	2	1.630E-02	1.528	0.229
Population×Cd	2	2.156E-02	2.021	0.145
Zn×Cd	1	0.184	17.181	0.000
Population×Zn×Cd	2	7.373E-02	6.910	0.003
Within groups	42	1.067E-02		
<b>Cd accumulation</b>				
Population	2	7.584	446.930	0.000
Zn	1	0.217	12.812	0.001
Cd	1	5.433	320.177	0.000
Population×Zn	2	5.812E-02	3.425	0.042
Population×Cd	2	0.217	12.797	0.000
Zn×Cd	1	1.529E-02	0.901	0.348
Population×Zn×Cd	2	0.187	11.046	0.000
Within groups	42	1.697E-02		

The analysis was confined to two concentrations of each metal (0.5 and 5  $\mu\text{M}$ ). Four to five plants were analysed per treatment level and accumulation data were log-transformed prior to statistical analysis.

porters (e.g., Zhao et al. 2002). Many trans-membrane metal transporters, involved in a variety of processes, such as uptake into the root, efflux from root or shoot cells, xylem loading or deloading, and vacuolar sequestration or remobilization, are capable to transport different metals (Persans et al. 2001; Vert et al. 2002; Thomine et al. 2003; Verret et al. 2004; Mills et al. 2005), albeit with different affinities (Pence et al. 2000). Therefore, competitive interactions between metals might occur in all the phases of the (hyper) accumulation process, that is, from their uptake into

**Fig. 1** Zn (a) and Cd (b) accumulation (mean $\pm$ SE) in *T. caeruleus* populations LE, LC, GA and MP. Plants were exposed for 3 weeks to nutrient solution supplemented with 0.5, 5, 50  $\mu\text{M}$  Zn and 0.5, 5, 50  $\mu\text{M}$  Cd, supplied together in factorial combinations of concentrations. Each graph represents the accumulation of the metal supplied in the concentration stated on the right/top in combination with the different concentrations of the other metal from the binary mixture. White bars (0.5  $\mu\text{M}$ ); grey bars (5  $\mu\text{M}$ ); black bars (50  $\mu\text{M}$ ). Four to five plants were analysed per treatment level. The data of the highest concentrations are used (when they exist) to show their effect on the accumulation of the other metal (shown in a and b)

the root up to their final compartmentalisation in the cell walls, vacuoles or trichomes of the leaves. Competition taking place after the uptake phase might eventually slow down the overall accumulation rate,

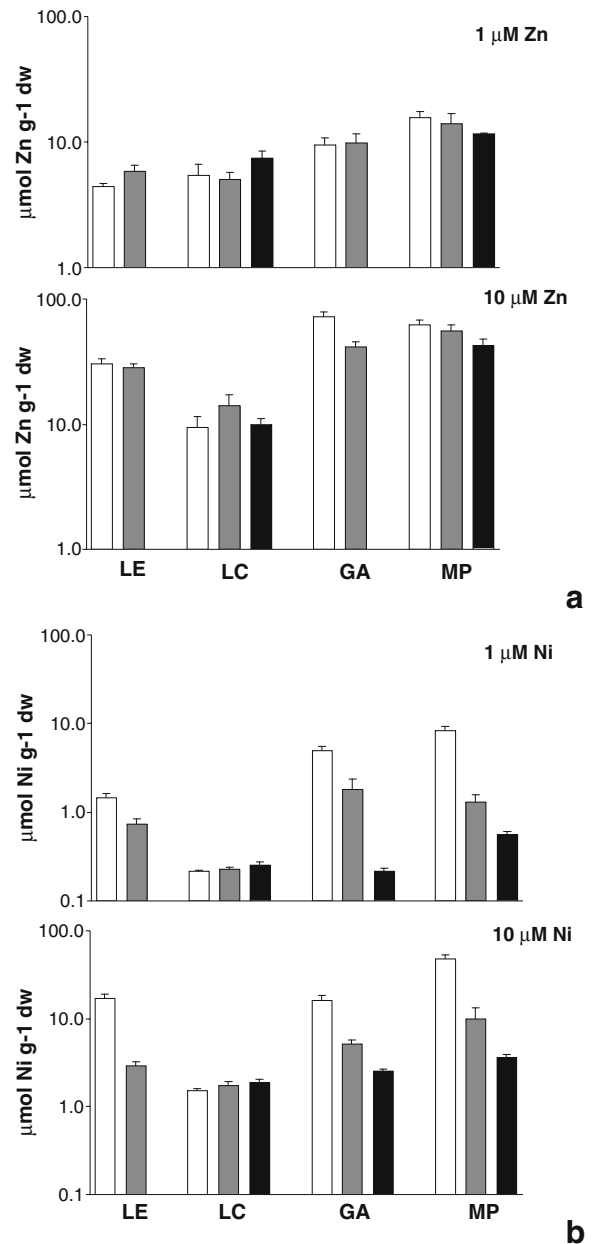


**Table 3** Three-way ANOVA of Zn accumulation and Ni accumulation in *T. caeruleus* populations, LE, LC, GA and MP, grown for 3 weeks in nutrient solution supplemented with factorial combinations of concentrations of Zn and Ni

Source	df	MS	F	P
<b>Zn accumulation</b>				
Population	3	1.457	61.325	0.000
Zn	1	7.845	330.221	0.000
Ni	1	3.759E-03	0.158	0.692
Population×Zn	3	0.227	9.554	0.000
Population×Ni	3	5.236E-02	2.204	0.095
Zn×Ni	1	7.300E-03	0.307	0.581
Population×Zn×Ni	3	5.294E-02	2.228	0.093
Within groups	69	2.376E-02		
<b>Ni accumulation</b>				
Population	3	4.848	198.458	0.000
Zn	1	4.271	174.819	0.000
Ni	1	11.827	484.131	0.000
Population×Zn	3	0.608	24.902	0.000
Population×Ni	3	0.136	5.551	0.002
Zn×Ni	1	6.581E-02	2.694	0.105
Population×Zn×Ni	3	7.403E-02	3.030	0.035
Within groups	69	2.443E-02		

The analysis was confined to two concentrations of each metal (1 and 10  $\mu\text{M}$ ). Four to five plants were analysed per treatment level and accumulation data were log-transformed prior to statistical analysis.

due to feed-back down-regulation of the uptake. Alternatively, it is conceivable, though less likely, that antagonistic interactions could also directly result from regulation, without any competitive interaction during transport, e.g. if enhanced uptake of one metal would less strongly down-regulate its own accumulation than that of the other. On the other hand, synergistic interactions must result from up-regulation of the uptake or transport mechanism. In case of essential metals, such as iron and zinc, the transporters responsible for uptake and transport are strongly up-regulated under conditions of deficiency (Grotz et al. 1998; Assunção et al. 2001; Connolly et al. 2002; Vert et al. 2002). It is conceivable that interactions between metals could induce deficiency responses, e.g. if a metal would interfere with the sensing machinery of another metal, or create a state of “physiological deficiency” of another metal, e.g. by displacing it from its essential functional binding sites. In fact, such explanations might apply to the Cd-induced enhancement of Zn accumulation in LC and GA (Fig. 1a), particularly because the response is



**Fig. 2** Zn (a) and Ni (b) accumulation (mean±SE) in *T. caeruleus* populations LE, LC, GA and MP. Plants were exposed for 3 weeks to nutrient solution supplemented with 1, 10, 100  $\mu\text{M}$  Zn and 1, 10, 100  $\mu\text{M}$  Ni, supplied together in factorial combinations of concentrations. Each graph represents the accumulation of the metal supplied in the concentration stated on the right/top in combination with the different concentrations of the other metal from the binary mixture. White bars (1  $\mu\text{M}$ ); grey bars (10  $\mu\text{M}$ ); black bars (100  $\mu\text{M}$ ). Four to five plants were analysed per treatment level. The data of the highest concentrations are used (when they exist) to show their effect on the accumulation of the other metal (shown in a and b)

**Table 4** Three-way ANOVA of Cd accumulation and Ni accumulation in *T. caeruleus* populations, LE, LC and GA, grown for 3 weeks in nutrient solution supplemented with factorial combinations of concentrations of Cd and Ni

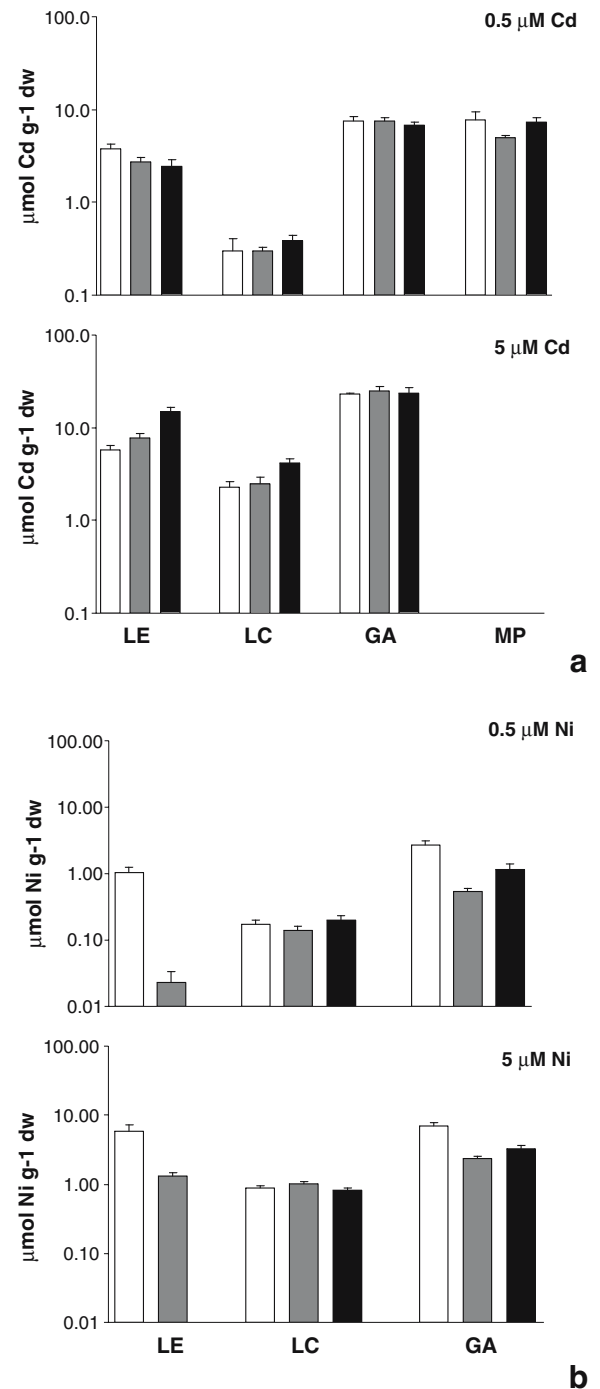
Source	df	MS	F	P
<b>Cd accumulation</b>				
Population	2	7.763	357.806	0.000
Cd	1	5.018	231.288	0.000
Ni	1	1.225E-02	0.564	0.456
Population×Cd	2	0.524	24.137	0.000
Population×Ni	2	4.779E-03	0.220	0.803
Cd×Ni	1	2.781E-02	1.282	0.263
Population×Cd×Ni	2	3.876E-02	1.787	0.179
Within groups	47	2.170E-02		
<b>Ni accumulation</b>				
Population	2	3.038	113.495	0.000
Cd	1	4.681	174.864	0.000
Ni	1	10.707	399.996	0.000
Population×Cd	2	1.463	54.671	0.000
Population×Ni	2	0.556	20.758	0.000
Cd×Ni	1	0.745	27.837	0.000
Population×Cd×Ni	2	0.248	9.263	0.000
Within groups	47	2.677E-02		

The analysis was confined to two concentrations of each metal (0.5 and 5  $\mu\text{M}$ ). Four to five plants were analysed per treatment level and accumulation data were log-transformed prior to statistical analysis.

confined to the lowest Zn supply level, and to the populations adapted to Zn-toxic soils, which have been shown to exhibit enhanced requirements for Zn (Tolra et al. 1996; Shen et al. 1997). It is more difficult to reconcile Zn-induced Cd accumulation, such as found in LC (Fig. 1b), or Ni-induced Cd accumulation, such as in LE and LC (Fig. 3b), with a deficiency response, since Cd is not known to have any essential function in plant metabolism. In these cases it is conceivable that Cd is inadvertently taken up and transported via an induced essential metal transport system. Recently,

**Fig. 3** Cd (a) and Ni (b) accumulation (mean $\pm$ SE) in *T. caeruleus* populations LE, LC, GA and MP. Plants were exposed for 3 weeks to nutrient solution supplemented with 0.5, 5, 50  $\mu\text{M}$  Cd and 0.5, 5, 50  $\mu\text{M}$  Ni, supplied together in factorial combinations of concentrations. Each graph represents the accumulation of the metal supplied in the concentration stated on the right/top in combination with the different concentrations of the other metal from the binary mixture. White bars (0.5  $\mu\text{M}$ ); grey bars (5  $\mu\text{M}$ ); black bars (50  $\mu\text{M}$ ). Four to five plants were analysed per treatment level. The data of the highest concentrations are used (when they exist) to show their effect on the accumulation of the other metal (shown in a and b)

Papoyan et al. (2007) also described mutual stimulation of Cd and Zn accumulation in the Prayon ecotype of *T. caeruleus*, which is a calamine ecotype with accumulation characteristics similar to those of LC. In the present study synergisms between metals at the





level of their accumulation in the plant is particularly evident in LC. However, in the other calamine population, GA, the phenomenon is also apparent from the fact that a higher concentration of a particular metal often less effectively inhibits another metal's accumulation than a lower one does (e.g. Figs. 1a and 3b). However, such synergisms are not confined to the calamine populations, since the non-metallicolous LE population shows enhanced Cd accumulation under Ni exposure (Fig. 3a).

In general, a detailed analysis of interactions between metals requires short-term kinetics analysis, preferably with radio-labelled metals. Since we measured metal accumulation after 3 weeks of exposure, it is not possible to establish the mechanisms of interaction with certainty, nor to identify the phase of the accumulation process in which the interactions take place. However, it is possible to provide plausible hypotheses, based on circumstantial evidence.

From the concentration-dependency patterns of the Cd/Zn interaction, it can be inferred that Zn hyperaccumulation can be mediated by different accumulation mechanisms with distinct metal-specificities. At the lowest external Zn concentration tested, that is 0.5  $\mu\text{M}$ , there was relatively little variation in the degrees of Zn accumulation among the populations, and there was no significant suppression of Zn accumulation by Cd in either of them (Fig. 1a). At the external Zn concentration of 5  $\mu\text{M}$  there was much more variation in Zn accumulation between the populations. Under this condition, Zn accumulation was still unsuppressed by Cd in the population with the lowest Zn accumulation rate, LC, but strongly Cd-suppressed in LE and GA, down to a level comparable to that in LC (Fig. 1a). These results suggest that each of the populations might possess a highly Zn-preferent high-affinity system accounting for a major part of the Zn accumulation at low external Zn concentrations. The Cd-suppressible component of Zn accumulation, which is particularly manifest in LE and GA at high external Zn and Cd concentrations (5  $\mu\text{M}$  Zn/5 and 50  $\mu\text{M}$  Cd), might represent an additional, low-affinity accumulation system. Cd-suppressible low-affinity Zn accumulation is apparent in LE and GA, but not in LC, which would largely explain the relatively low rates of accumulation of Zn, Cd and Ni in the latter population.

Likewise, from the Cd/Zn interactions it can be inferred that there must be different systems for Cd

hyperaccumulation. At the lowest external Cd concentration, that is 0.5  $\mu\text{M}$ , Cd accumulation was non-Zn-suppressible in GA, as well as in the non-Cd-hyperaccumulating population, LC, but appeared to be almost completely Zn-suppressible in LE and MP (Fig. 1b). Thus, in agreement with earlier studies (Lombi et al. 2001; Zhao et al. 2002), Cd hyperaccumulation in GA appears to depend on a highly Cd-specific high-affinity uptake system. On the other hand, Cd hyperaccumulation in LE and MP is Zn-suppressible, albeit only at low external Cd concentrations, suggesting that another, less Cd-preferent uptake system should be involved. It is tempting to assume that the system responsible for Cd hyperaccumulation in LE and MP would be identical with the one that mediates low-affinity Zn accumulation in these populations. Also in GA, there seems to be an additional system for Cd hyperaccumulation, though only at high external Cd concentrations. This system might be less Cd-preferent than that operating in LE and MP, in view of the more or less symmetrical Cd/Zn interference in the higher concentration range (Fig. 1b).

The Ni/Zn and Ni/Cd interactions show that Ni accumulation was unaffected by Zn or Cd in the non-Ni-hyperaccumulating population, LC, but strongly suppressed by both Cd and Zn in LE, MP and GA, down to levels comparable to those obtained in LC (Figs. 2b and 3b). Conversely, there was no significant Ni-imposed inhibition of either Zn or Cd accumulation in either of the populations (Figs. 2a and 3a). These results clearly suggest that Ni hyperaccumulation is mediated by the less metal-specific systems that mediate low-affinity Zn and Cd accumulation in LE, MP and GA (see above). Apparently, these low-affinity systems seem to prefer Cd over Zn and Ni (LE, MP), or Cd and Zn over Ni, at least (GA). A comparably strong preference for Zn over Ni under binary exposure has been observed in *Thlaspi pindicum* and *T. alpinum* ssp. *sylvium*, which are both Ni-hyperaccumulating serpentine endemics (Taylor and Macnair 2006). In the latter study, iron was supplied as FeEDDHA which is expected to resist displacement by Ni, showing the apparent preference for Zn over Ni in our study is most probably not due to Ni complexation by EDTA.

The present state of knowledge considering the precise functions and metal-specificity patterns of the transporters presumed to be involved in metal hyperaccumulation is insufficient to provide plausible

mechanistic explanations for the variation in metal accumulation patterns observed in this study. It is conceivable that transcriptome comparisons between *T. caerulescens* populations will provide clues. Further QTL analysis will be necessary too.

In summary, different metal accumulation systems seem to be involved in heavy metal hyperaccumulation among the total of *T. caerulescens* populations studied. First, all the populations seem to express, to a comparable degree, a highly Zn-specific high-affinity accumulation system. This system would be expected to explain, at least in part, the apparent hyperaccumulation of Zn in either of the populations at their sites of origin (Assunção et al. 2003). Second, apart from LC, all the populations seem to express low-affinity systems for Cd, Zn and Ni accumulation, which apparently prefers Cd over Zn and Zn over Ni (LE and MP), or Cd and Zn over Ni (GA), at least. The ecological significance of such non-specific systems is not entirely clear. In nature, they would be expected to mediate Ni hyperaccumulation, though only in situations where the plant-available soil Ni concentrations are strongly in excess of those of Zn and Cd, such as in serpentine soils. In agreement with this viewpoint, in the natural environment Ni hyperaccumulation is only apparent in MP, where the soil at the population site contains about 40-fold more Ni than Zn, and hardly any Cd at all (Assunção et al. 2003). Since the plant-available soil Zn concentrations usually exceed those of Cd by orders of magnitude, it is not likely that these less specific systems could ever account for Cd hyperaccumulation under natural conditions. However, the mechanism operating in LE and MP, which is relatively Cd-selective, might account for the relatively high foliar Cd concentrations, in comparison with non-hyperaccumulator plants, in these populations under natural conditions (Assunção et al. 2003). Also, the less specific systems might as well contribute to Zn hyperaccumulation in non-metallicolous and metallicolous natural populations. The importance of these systems is also illustrated by their absence or low expression in LC. In their natural environments, LC accumulates less than two times more Cd than LE, although the soil Cd concentration is two to three orders of magnitude higher at the LC population site. Finally, in agreement with previous studies (Lombi et al. 2001; Zhao et al. 2002), there is a highly Cd-specific high-

affinity system, which is exclusively (over)expressed in the GA population and explains this population's Cd hyperaccumulation phenotype in its natural environment (800–1,500  $\mu\text{g g}^{-1}$  DW in leaves), at least in part. The difference with LC (60–100  $\mu\text{g g}^{-1}$  DW in leaves) may be due to the combination of the presence of the high-affinity system in GA and the absence of a low-affinity system in LC, since the soil metal compositions at these localities are very similar (Assunção et al. 2003).

The question remains whether the clearly population-specific accumulation patterns reflect general adaptations to particular soil metal compositions. As suggested by the striking differences between the two calamine populations, LC and GA, this may not be the case, suggesting that selective factors other than soil metal compositions may play a role here. The variation among serpentine and non-metallicolous populations is unknown yet, but seems to be considerable too (H. Schat, unpublished results).

In conclusion, when exposed to binary combinations of Zn, Cd and Ni, all the *T. caerulescens* populations under study exhibited unique concentration-dependent metal preference patterns with regard to accumulation, most likely because of differential expression of a number of different accumulation systems with distinct metal-affinity patterns. Differential metal preference, next to variation in soil metal composition, appears to explain part of the variation in foliar metal compositions among the populations in their natural environment.

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