New Phytologist

Research

Co-segregation analysis of cadmium and zinc accumulation in *Thlaspi caerulescens* interecotypic crosses

H. G. Zha[1,3,*], R. F. Jiang[2,3,*], F. J. Zhao[3], R. Vooijs[4], H. Schat[4], J. H. A. Barker[5] and S. P. McGrath[3]

[1]Soil and Environment Bioremediation Research Centre, State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China; [2]College of Resources and Environmental Sciences, China Agricultural University, Beijing 100094, China; [3]Agriculture and Environment Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK; [4]Department of Ecology and Physiology of Plants, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands; [5]Crop Performance and Improvement Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

Author for correspondence:
F. J. Zhao
Tel: +44 1582763133
Fax: +44 1582760981
Email: Fangjie.Zhao@bbsrc.ac.uk

Received: *2 April 2004*
Accepted: *7 April 2004*

doi: 10.1011/j.1469-8137.2004.01113.x

Summary

• Cadmium (Cd) hyperaccumulation in *Thlaspi caerulescens* varies among ecotypes. Here we investigated segregation of Cd and zinc (Zn) accumulation in F_2 crosses between high (Ganges) and low (Prayon) Cd-accumulating ecotypes.

• Accumulation was measured in plants grown in compost treated with 5 and 100 mg kg^{-1} Cd and Zn, respectively, and in hydroponics with 50 μM Zn and 10 or 50 μM Cd. Another hydroponic experiment examined the relationship between Cd tolerance and accumulation.

• Parental phenotype distributions for shoot metal concentrations were distinct for Cd, but not consistent for Zn. Shoot Cd and Zn in F_2s varied continuously, with significant transgression for Zn in all treatments. Shoot Cd correlated strongly with shoot manganese (Mn), and to a lesser degree with shoot Zn. Shoot Cd concentrations in the Cd nontolerant F_2s were lower than, or similar to, those in the Cd-tolerant F_2s.

• We conclude that Cd and Zn accumulation is governed by multiple genes, and that Cd tolerance and accumulation are independent traits in *T. caerulescens*. Two uptake systems with distinctive affinities for Cd, Zn and Mn are proposed.

Key words: *Thlaspi caerulescens*, cadmium (Cd), zinc (Zn), hyperaccumulation, tolerance, genetic analysis.

Introduction

Metal hyperaccumulation by higher plants is a rare and scientifically interesting phenomenon. Metal hyperaccumulators are able to accumulate metal(s) in the aboveground parts to concentrations two to three orders higher than normal plant species. Generalizing across species, there are three common traits that characterize metal hyperaccumulating plants: efficient root uptake, efficient root to shoot transport and a greatly elevated tolerance that is achieved through internal detoxification (McGrath *et al.*, 2002; Pollard *et al.*, 2002).

Thlaspi caerulescens (Brassicaceae) is probably the best-known example of a metal hyperaccumulator, which occurs widely in central and western Europe, particularly on metalliferous soils (Reeves & Brooks, 1983; Baker & Brooks, 1989). Zinc hyperaccumulation has been shown to be a constitutive and species-wide trait in *T. caerulescens*, although there is still considerable variation between different populations when grown under controlled conditions (Baker *et al.*, 1994; Meerts & van Isacker, 1997; Escarré *et al.*, 2000; Lombi *et al.*, 2000; Assunção *et al.*, 2003a; Roosens *et al.*, 2003). In general, populations from calamine sites (rich in Zn) tend to accumulate less Zn than those from nonmetalliferous sites, but the tolerance to Zn is higher in the former than in the latter (Meerts & van Isacker, 1997; Escarré *et al.*, 2000; Assunção *et al.*, 2003a). Recent studies have begun to shed light on the genetic background of Zn accumulation and tolerance and the relationship between these traits. Genetic analysis of the F_2 and F_3 progeny from crosses between a calamine and a nonmetallicolous population of *T. caerulescens* showed that Zn accumulation

*The first two authors contributed equally to this work

and tolerance segregated largely independently, although there was a significant degree of association between low accumulation and high tolerance (Assunção *et al.*, 2003b). In that study, the phenotype distribution of shoot Zn concentration in the F_3 families was more or less continuous, suggesting that the difference in Zn accumulation between calamine and nonmetallicolous populations is under the control of more than one gene. By contrast, in a recent study of the F_1 offspring from crosses between a number of calamine and nonmetallicolous populations, Frerot *et al.* (2003) suggested that the differences in Zn hyperaccumulation between crosses could be explained by a monogenic system with two alleles, with the dominant allele restricting Zn hyperaccumulation to a lower level observed in the calamine populations. However, such interpretation remains only speculative until the phenotype distribution patterns in the F_2 progeny are obtained.

Macnair *et al.* (1999) made interspecific crosses between the Zn hyperaccumulating and tolerant *Arabidopsis halleri* and the nonhyperaccumulating, nontolerant species *Arabidopsis petraea*. They found that the F_1 of this cross was Zn tolerant, and had a Zn accumulation phenotype similar to *A. halleri*, indicating that the genes governing tolerance and accumulation in *A. halleri* are dominant. Furthermore, Zn accumulation and tolerance appeared to segregate in the F_2 independently, suggesting that the traits for Zn accumulation and tolerance are genetically independent. Macnair *et al.* (1999) showed that the character of Zn hyperaccumulation in the F_2 was both highly heritable and highly variable, with no distinct classes in the phenotype distribution. They speculated that the number of genes governing this character was probably not many, but equally probably not just one (Macnair, 2003).

T. caerulescens is also able to accumulate Cd, a metal that is nonessential and highly toxic to higher plants. However, Cd accumulation appears to vary much more widely between populations than Zn accumulation. Lombi *et al.* (2000) showed that two *T. caerulescens* populations from southern France were far superior to several British and Belgian populations in Cd accumulation when grown under comparable conditions, whereas Zn accumulation was broadly similar among these populations. In fact, Assunção *et al.* (2003a) suggested that Cd hyperaccumulation is a population-specific rather than a species-wide property of *T. caerulescens*. Studies of metal influx kinetics provided strong physiological evidence for a high affinity Cd uptake system that is highly expressed in the Ganges ecotype (from southern France) but is largely lacking in the Prayon ecotype (from Belgium) (Lombi *et al.*, 2001b; Zhao *et al.*, 2002). The superior Cd accumulation ability of the southern French populations of *T. caerulescens* was also observed in the field specimens collected from the region, with shoot Cd concentrations reaching up to 3600 mg kg^{-1} d. wt (Robinson *et al.*, 1998; Reeves *et al.*, 2001). Results from pot and field experiments showed that the *T. caerulescens* populations from southern France have a promising potential for the phytoremediation of Cd contaminated soils (Lombi *et al.*, 2001a; Schwartz *et al.*, 2003; Zhao *et al.*, 2003). Furthermore, the Ganges ecotype appears to be more tolerant to Cd than Prayon (Lombi *et al.*, 2000; Roosens *et al.*, 2003); the former was able to tolerate up to 10 000 mg Cd kg^{-1} d. wt in shoots without showing phytotoxicity in a hydroponic experiment (Lombi *et al.*, 2000).

The contrasting Cd accumulation between the Ganges and Prayon ecotypes makes them particularly valuable materials for studies of the mechanisms of Cd hyperaccumulation. Comparative physiological studies have been reported previously (Lombi *et al.*, 2001b, 2002; Zhao *et al.*, 2002; Assunção *et al.*, 2003a; Roosens *et al.*, 2003). However, the genetic basis for the differences between the two contrasting ecotypes with regard to Cd hyperaccumulation has not been analysed. This paper reports a study of the segregation patterns of Cd and Zn accumulation in F_2 progeny derived from reciprocal crosses between the Ganges and Prayon ecotypes of *T. caerulescens*.

Materials and Methods

Plant origins and crossing scheme

Seeds of *T. caerulescens* J. & C. Presl were collected in Belgium (Prayon ecotype) and in southern France (Ganges ecotype). Both sites are metalliferous, containing highly elevated concentrations of Zn, Cd and Pb (Roosens *et al.*, 2003). Plants were grown exactly as described in Assunção *et al.* (2003b). After 6 wk in hydroponics, the plants were vernalized for 5 wk in a controlled-environment room (4°C; 90% RH; 150 μmol m^{-2} s^{-1} at plant level; 12 h d^{-1}) and subsequently returned to the original growth conditions. Interecotypic reciprocal pair crosses were made as in Assunção *et al.* (2003b), and the parent plants were allowed to produce additional seeds by self-fertilization, which were used as the parental controls in the experiments described below. F_2 was generated through self-fertilization in two F_1 full siblings with reciprocal parental ancestry.

Cd and Zn accumulation in the F_2 progeny of the crosses between Ganges and Prayon

Pot experiment The F_2 seeds of the Ganges × Prayon (G × P) and Prayon × Ganges (P × G) crosses as well as the seeds of the parental controls were sown in trays of fine vermiculite. After germination, seedlings were watered with a full nutrient solution containing (in μM) 1000 $Ca(NO_3)_2$, 500 $MgSO_4$, 500 K_2HPO_4, 100 KCl, 10 H_3BO_3, 1.8 $MnSO_4$, 0.2 Na_2MoO_4, 0.31 $CuSO_4$, 0.5 $NiSO_4$, 100 Fe(III)-EDDHA (ethylenediamine-di(*o*-hydroxyphenylacetic acid)), and 5 $ZnSO_4$. The nutrient solution was buffered at pH 6.0 with 2 mM MES (2-morpholinoethanesulphonic acid, pH adjusted with KOH). Three weeks later, 30 seedlings of each parent, 117 seedlings of the G × P F_2s and 117 seedlings of the P × G F_2s were transferred to plastic pots, each filled with 350 g general-purpose

compost. The compost had been spiked with 5 mg Cd kg^{-1} and 100 mg Zn kg^{-1}. Cd and Zn were added in a solution of $CdCl_2$ and $ZnCl_2$ and mixed thoroughly with the compost before transplanting. Pots were placed randomly on a bench inside a glasshouse with the following conditions: 16 h day length with natural sunlight supplemented with SONT sodium-vapour lamps to maintain a minimum intensity of 350 μmol photons m^{-2} s^{-1}, and 20°C/16°C day/night temperature. Plants were watered with deionized water throughout the growth period. A number of seedlings died shortly after transplanting, leaving 29 Ganges, 28 Prayon, 98 G × P F_2s and 108 P × G F_2s. On day 45 after transplanting, all leaves and petioles were harvested, leaving only the shoot apexes for re-growth. Leaves were rinsed with deionized water, blotted dry and weighed. The samples were then dried at 60°C for 48 h, and the d. wts were recorded. Plant samples were ground and digested with a mixture of HNO_3 and $HClO_4$, and the concentrations of Cd, Zn and other elements were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES; Fisons ARL Accuris, Ecublens, Switzerland). Before harvest, leaf morphology and colour of all plants were assessed and recorded.

Hydroponic experiment Efficient translocation of metals from roots to shoots is an important characteristic of metal hyperaccumulators. However, this aspect could not be examined in the pot experiment described above, because roots were not harvested. A hydroponic experiment was therefore set up to further characterize the phenotypes of Cd and Zn accumulation. Three-week-old seedlings of the parents Ganges and Prayon, and of the F_2s of the P × G cross were transferred to hydroponic culture. Forty-five seedlings in total, comprising 5 seedlings each of Ganges and Prayon and 35 seedlings of F_2s, were transferred to a black plastic tank containing 31 l of nutrient solution. Seedlings were arranged randomly in each tank. Nutrient composition was the same as above; except that Zn was supplied at 50 μM. Cadmium was supplied at two concentrations: 10 and 50 μM (as $CdCl_2$). The first concentration was chosen to reflect the Cd exposure level at the Ganges site because soil solutions extracted from the Ganges soils contained 5–10 μM Cd (unpublished data), whereas the second concentration provided a test of Cd tolerance in the parental controls and the F_2s. Each Cd concentration was repeated in 4 tanks. Nutrient solution was aerated continuously and renewed once every week. The experiment was conducted inside a controlled environment growth chamber with the following conditions: 16 h day length with a light intensity of 350 μmol photons m^{-2} s^{-1} supplied by Osram HQI lamps, 20°C/16°C day/night temperature, and 60% RH. Symptoms of individual plants were recorded on 10 and 37 d after the initiation of treatments (DAT), and plants harvested on 40 DAT. Shoots were separated from roots, rinsed thoroughly with deionized water, blotted dry and weighed. Plant samples were dried at 60°C for 48 h and the d. wts determined. The concentrations of Cd, Zn and other elements in shoots and roots were determined as described above.

Cd accumulation and tolerance in F_2 plants

A hydroponic experiment was carried out to examine the relationship between Cd tolerance and accumulation in F_2 plants. The experimental design was similar to that used by Assunção *et al.* (2003b) for Zn tolerance. Ninety-eight 3-week-old seedlings of the F_2 plants of the G × P cross were transferred to 350 ml black plastic pots, with one seedling per pot. Plants were grown with normal nutrient solution as described above, with 5 μM Zn and no Cd, for 5 wk in a controlled environment growth chamber with conditions the same as in the phenotyping hydroponic experiment. Nutrient solution was aerated for 4 h per day and renewed once every week. Starting from the 6th week, all plants were sequentially exposed to 100, 200 and 500 μM Cd (as $CdCl_2$) for 1 wk per level of exposure. After each level of exposure, plants showing chlorotic and stunted growth symptoms, together with 8 randomly selected healthy plants, were harvested for Cd analysis (see above). Only 4 plants remained healthy after exposure to 500 μM Cd for 1 wk.

Statistical analysis

ANOVA was used to test the statistical significance of the difference between the two parental controls, between the F_2 plants from the two reciprocal crosses in the pot experiment, or between the Cd treatments in the hydroponic experiment. Correlation coefficients were calculated between elemental concentrations in F_2 plants. Where appropriate, data were transformed logarithmically before statistical analysis to obtain homogeneity of variances. Transgression, i.e. segregation beyond the limits of the phenotype distributions of the parental controls, was tested for by comparing the observed and expected frequencies of F_2s in the lowest and the highest 2.5%-area sections of the parental control distributions. Metal concentrations were log-transformed and the 2.5% area limits were calculated as the antilog of the means ± t*SD. Expected frequencies were calculated assuming that 75% of the F_2s were distributed like the parental distribution to be tested against. The probabilities of observed frequencies were considered to equal the relative frequencies of the Poisson distributions with the expected frequencies as the means.

Results

Cd and Zn accumulation phenotypes: pot experiment

In the pot experiment, Ganges produced 20% more shoot biomass than Prayon ($P < 0.05$). The range of shoot Cd concentration for Ganges (260–610 mg kg^{-1} d. wt, $n = 29$)

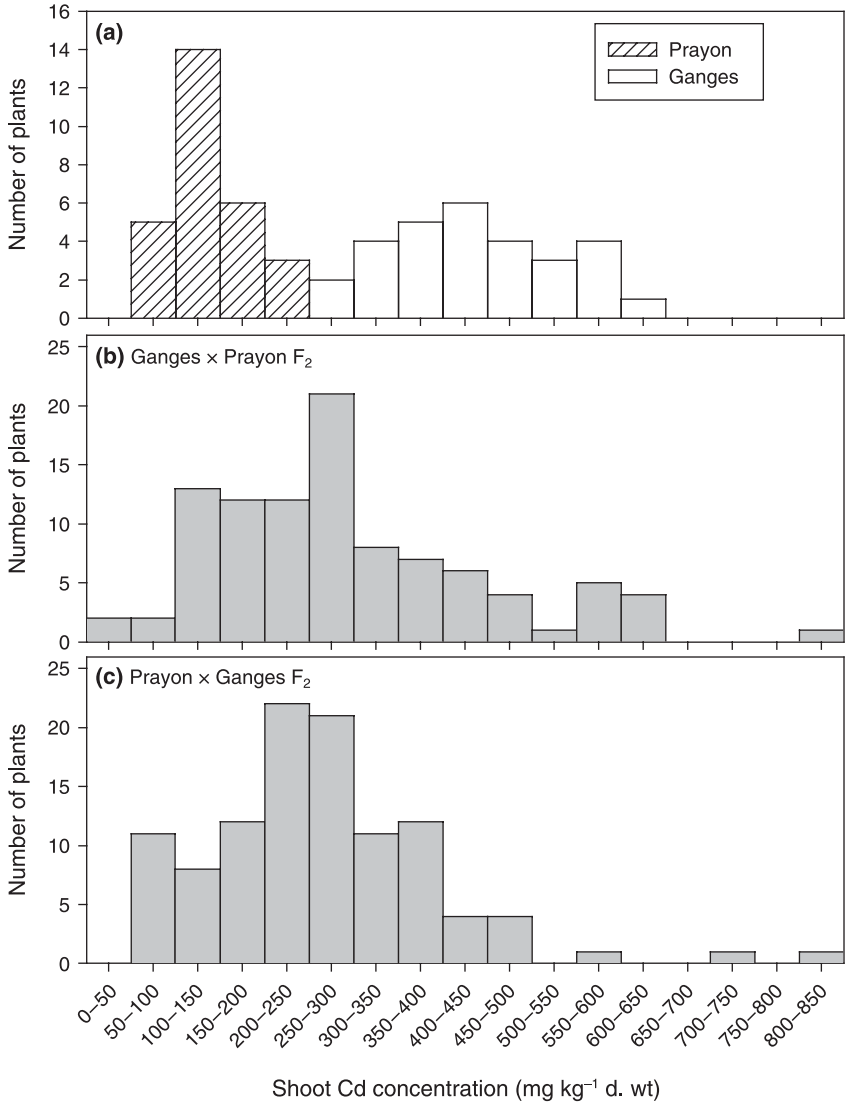

Fig. 1 Frequency distributions of shoot Cd concentration in the Ganges and Prayon parental controls (a), and in the F_2 from the crosses of G × P (b) and P × G (c). *Thlaspi caerulescens* plants were grown on compost spiked with 5 mg Cd kg^{-1} and 100 mg Zn kg^{-1}.

did not overlap with that for Prayon (69–249 mg kg^{-1} d. wt, $n = 28$) (Fig. 1a), indicating distinct phenotypes of Cd accumulation. On average, shoot Cd concentration of Ganges was 3.1-fold higher than that of Prayon ($P < 0.001$).

Among the F_2 plants of the G × P and P × G crosses, morphological characteristics, such as leaf shape and leaf colour, segregated as similar to either parents or displaying intermediate phenotypes. The morphological segregation indicates that the crosses were indeed successful. The two sets of F_2 plants clearly segregated in their distribution of shoot Cd concentration (Fig. 1b,c). All but 2–3 F_2 plants had a shoot Cd concentration within the lower and upper limits of the Prayon and Ganges phenotype range, respectively. The patterns of phenotype distribution in the F_2 plants from the reciprocal crosses of G × P and P × G were similar, and the difference between the means of shoot Cd concentration of the two sets of F_2 plants was not significant ($P = 0.16$). In the G × P set, 39% and 49% of the F_2 plants fell within the ranges of shoot Cd concentration of Prayon and Ganges, respectively. In the P × G set, the corresponding percentages were 48% and 48%. The segregation ratios were not consistent with a 1 : 3 ratio that is indicative of a monogenic model ($\chi^2 = 16.3$ and 33.5 for the G × P and P × G crosses, respectively, $P < 0.001$). There was no significant transgression beyond the phenotype distributions of the parental controls.

By contrast to the Cd accumulation pattern, the distribution patterns of shoot Zn concentration of Ganges and Prayon clearly overlapped (Fig. 2a). On average, Ganges accumulated approximately 50% higher concentration of Zn in the shoots than Prayon ($P < 0.001$). In the F_2 plants, there was significant transgression beyond the lower limit of the Prayon parental distribution ($P < 10^{-5}$). About 13% of the F_2s accumulated less Zn than the Prayon parental control. All these plants, except one that was stunted, showed normal biomass and

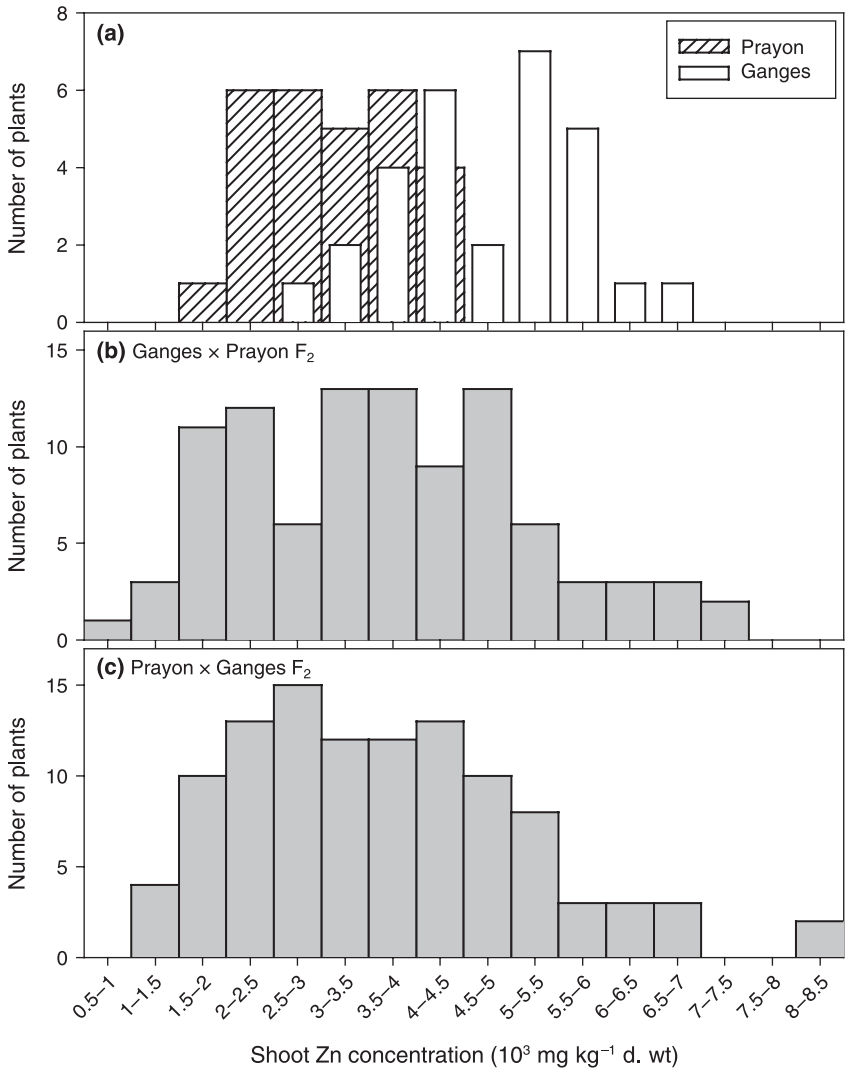

Fig. 2 Frequency distributions of shoot Zn concentration in the Ganges and Prayon parental controls (a), and in the F_2 from the crosses of G × P (b) and P × G (c). *Thlaspi caerulescens* plants were grown on compost spiked with 5 mg Cd kg^{-1} and 100 mg Zn kg^{-1}.

morphology. However, there was no significant transgression beyond the upper limit of the Ganges parental distribution (Fig. 2b,c).

In both sets of the crosses, approximately 5% of the F_2 plants showed interveinal chlorotic symptoms in leaves. Chlorotic plants had a significantly ($P < 0.001$) lower shoot biomass (mean = 0.39 g d. wt per plant, $n = 11$) than nonchlorotic plants (mean = 1.21 g d. wt per plant, $n = 195$). There were no significant differences between chlorotic and nonchlorotic plants in the concentrations of Zn or Fe in shoots. However, chlorotic plants had a significantly ($P < 0.01$, ANOVA on log-transformed data) lower Cd concentration in shoots (mean = 198 mg kg^{-1}) than nonchlorotic plants (mean = 283 mg kg^{-1}), indicating that chlorotic plants accumulated less Cd in the shoots.

In addition, the shoot Cd concentration in the F_2 plants from both crosses correlated most strongly with shoot Mn concentration, followed by the correlation with shoot Zn or Fe concentrations (Table 1). However, the partial correlation coefficient between Cd and Fe (i.e. at constant concentrations of other elements) was not significant. There was little correlation between Cd and either Ca or Mg in the shoots. Figure 3 shows the relationships between the concentrations of Cd and Zn or Mn in shoots, in both F_2 plants and in their parental controls.

Cd and Zn accumulation phenotypes: hydroponic experiment

Prayon produced larger shoot and root biomass than Ganges at 10 μM Cd, but at 50 μM Cd the two ecotypes produced similar biomass (Table 2). Ganges was markedly superior to Prayon in Cd accumulation, with approximately 4-fold larger concentrations of Cd in roots and shoots at 10 μM Cd, and about 2-fold larger concentrations at 50 μM Cd (Table 2). By contrast, the differences in Zn accumulation between the two ecotypes were much smaller and opposite to the ecotypic difference in Cd accumulation; shoot Zn concentrations

New Phytologist

Table 1 Correlation and partial correlation coefficients between shoot Cd, Zn, Mn, Fe, Ca and Mg concentrations of the F_2 plants in the pot experiment (n = 206, including both G × P and P × G)

	Correlation coefficients				
	Cd	Zn	Mn	Fe	Ca
Zn	0.49***				
Mn	0.74***	0.32***			
Fe	0.32***	0.07 ns	0.43***		
Ca	0.03 ns	−0.10 ns	0.27***	0.25***	
Mg	−0.10 ns	−0.23**	0.17*	0.25***	0.82***
	Partial correlation coefficients				
	Cd	Zn	Mn	Fe	Ca
Zn	0.33***				
Mn	0.68***	0.00 ns			
Fe	0.09 ns	−0.04 ns	0.22**		
Ca	0.02 ns	0.07 ns	0.12 ns	−0.01 ns	
Mg	−0.18 *	−0.16 ns	0.09 ns	0.13 ns	0.80***

ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in Ganges were 20% and 60% lower than those in Prayon in the 10 and 50 μM Cd treatments, respectively. The effect of increasing solution Cd supply on shoot Zn concentration was also opposite in the two ecotypes; increasing solution Cd decreased shoot Zn concentration in Ganges by 30% but increased shoot Zn in Prayon by 33%. The positive effect of Cd on shoot Zn concentration in Prayon was probably an indirect effect of a decreased shoot biomass at the higher Cd concentration (Table 2), because the total amount of Zn accumulated in the shoots was decreased significantly ($P < 0.01$) by Cd. The concentrations of Mn in both shoots and roots of Ganges were significantly larger than those of Prayon, and were decreased by the increasing Cd in solution (Table 2). There were no significant differences in shoot Fe, Ca and Mg concentrations between the two ecotypes.

The contrasting affinities for Cd and Zn in the two ecotypes are best illustrated by the molar ratio of Cd to Zn in plants (Table 2). In the 10 μM Cd treatment, which had a Cd : Zn molar ratio of 0.2 in the nutrient solution, shoot Cd : Zn ratio in Ganges was 3-fold higher than the solution

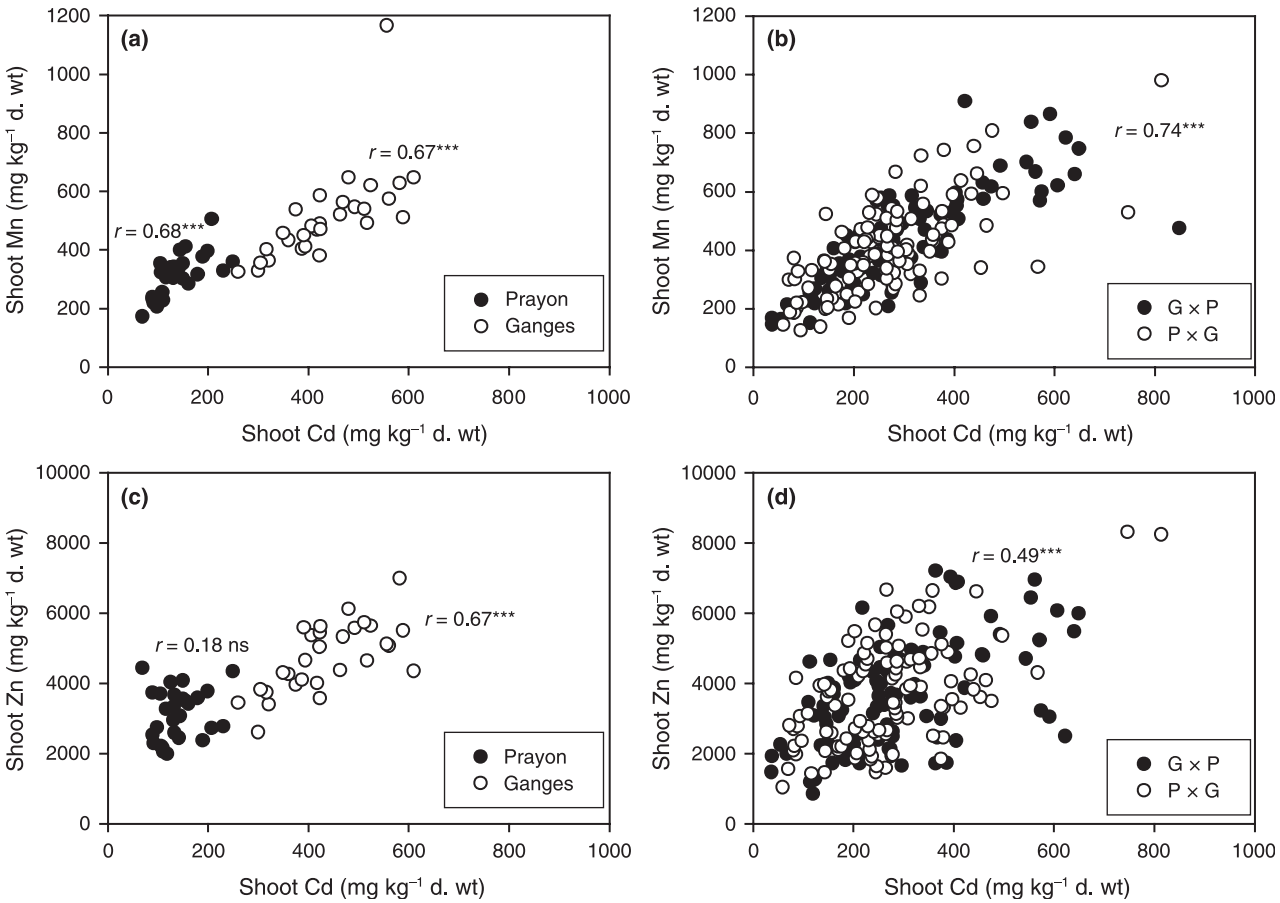

Fig. 3 Relationships between shoot Cd, Mn and Zn in the Ganges and Prayon parental controls (a, c), and in the F_2 from the crosses of G × P and P × G (b, d). *Thlaspi caerulescens* plants were grown on compost spiked with 5 mg Cd kg^{-1} and 100 mg Zn kg^{-1}. Significance of the correlation: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 2 Differences between the Prayon and Ganges ecotypes of *Thlaspi caerulescens* in plant growth and metal concentrations as influenced by Cd treatment in the hydroponic experiment (n = 20)

		Biomass (g d. wt per plant)			Shoot concentration (mg kg^{-1} d. wt)					Root concentration (mg kg^{-1} d. wt)				Molar ratio of Cd/Zn		Shoot/Root concentration ratio	
Cd (μM)	Ecotype	Shoot	Root	R/S ratio	Cd	Zn	Mn	Fe	Ca	Mg	Cd	Zn	Mn	Shoot	Root	Cd	Zn
10	Prayon	0.80	0.25	0.33	1180	5760	105	76	31 677	5696	646	994	27	0.12	0.40	1.9	6.2
10	Ganges	0.40	0.17	0.45	4649	4537	154	90	29 653	6053	2456	1064	37	0.60	1.34	1.9	4.3
50	Prayon	0.46	0.16	0.35	5641	7635	88	89	27 128	6496	2677	1097	20	0.45	1.42	2.2	7.1
50	Ganges	0.38	0.15	0.39	10 397	3168	106	89	27 532	6371	6763	1202	24	1.92	3.32	1.6	2.7
ANOVA	Ecotype	***	*	***	***	***	***	ns	ns	ns	***	ns	***	***	***	**	***
F prob.	Cd conc	**	**	ns	***	ns	***	ns	***	***	***	*	***	***	***	ns	ns
	Ecotype × Cd	**	*	ns	***	***	***	ns	*	ns	***	ns	ns	***	***	***	***

ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ratio, but the molar ratio was about half of the solution ratio in Prayon. In the 50 μM Cd treatment with a Cd : Zn molar ratio of 1.0 in the nutrient solution, shoot Cd : Zn molar ratio in Ganges was about 2-fold higher than the solution ratio, but was less than half of the solution ratio in Prayon. A similar pattern was seen with the root Cd : Zn molar ratios, although the root ratios were higher than the solution ratios for both ecotypes. This may be because apoplastically bound Cd and Zn in roots were not removed in our experiment, and the metal binding sites on cell walls may have a overall higher affinity for Cd than for Zn in both ecotypes. Alternatively, the root to shoot translocation process, including xylem loading, may impose further selectivity for metals, for Cd in Ganges and for Zn in Prayon, in addition to the root uptake process. Both ecotypes transferred the majority of Cd or Zn taken up to the shoots: approximately 85% and 95% of Cd and Zn in Prayon, respectively, and approximately 80% and 90% of Cd and Zn in Ganges, respectively. The shoot : root concentration ratio was larger for Zn than for Cd in both ecotypes (Table 2). Whilst the ecotypic difference in the shoot/root ratio for Cd was small and only significant at 50 μM Cd, the ratio for Zn was substantially higher in Prayon than in Ganges, suggesting a more efficient translocation from roots to shoots in the former.

Because F_2 plants were grown in four replicate tanks in each Cd treatment, it is important to know whether the tanks were comparable in terms of the conditions for plant growth and metal uptake, before all F_2 plants can be pooled for analysis of segregation patterns. One way of checking this is to compare the growth and metal uptake by the parental controls in different tanks. ANOVA showed that for both Ganges and Prayon with either 10 or 50 μM Cd, there were no significant differences (P in the range of 0.09 and 0.71) between the four replicate tanks in shoot or root biomass, or the concentrations of Cd and Zn in shoots or roots, indicating that the conditions were indeed comparable in the four replicate tanks. Figure 4 shows the frequency distributions of shoot Cd concentration for the parental controls and F_2 plants at the two concentrations of solution Cd. Again, Ganges and Prayon exhibited distinct and nonoverlapping phenotypes of shoot Cd concentration, whereas a considerable fraction of the F_2 plants were intermediate between Prayon and Ganges. At 10 μM Cd, 6% and 53% of the F_2 plants were within the phenotype ranges of the Prayon and Ganges, respectively, whilst at 50 μM Cd the corresponding percentages were 18% and 40%, respectively. There was no significant transgression at 10 μM Cd. At 50 μM Cd there was significant segregation beyond the upper limit of the Ganges distribution ($P < 0.0001$). However, about 35% of these plants were stunted, suggesting that their extreme Cd accumulation phenotypes might have been produced by toxicity (see below). The patterns of phenotype distribution for root Cd concentration in the parents and F_2 plants were similar to those for shoot Cd (data not shown).

By contrast to shoot Cd concentration, the distributions of shoot Zn concentration in Ganges and Prayon overlapped in the 10 μM Cd treatment (Fig. 5a). In the 50 μM Cd treatment, shoot Zn concentration of Prayon varied widely (4998–10210 mg kg^{-1} d. wt) and was larger than in the Ganges plants (Fig. 5c). Shoot Zn concentration of the F_2 plants varied mostly within the combined range of the parental controls (Fig. 5b,d). In the 50 μM Cd treatment, in which the phenotype distributions of the two parents did not overlap, more F_2s (42% of the total) were within the range of the Ganges phenotype than in the Prayon's range (20% of the total). Similar to the pot experiment, there was significant transgression beyond the lower limits of the parental control distributions ($P < 0.0001$), both at 10 and at 50 μM Cd, with about 12% and 8% of the F_2s exhibiting lower shoot Zn concentrations than the least accumulating parent, respectively. These plants showed normal biomass and morphology. There was no significant transgression beyond the upper limit of the most accumulating parental control distribution.

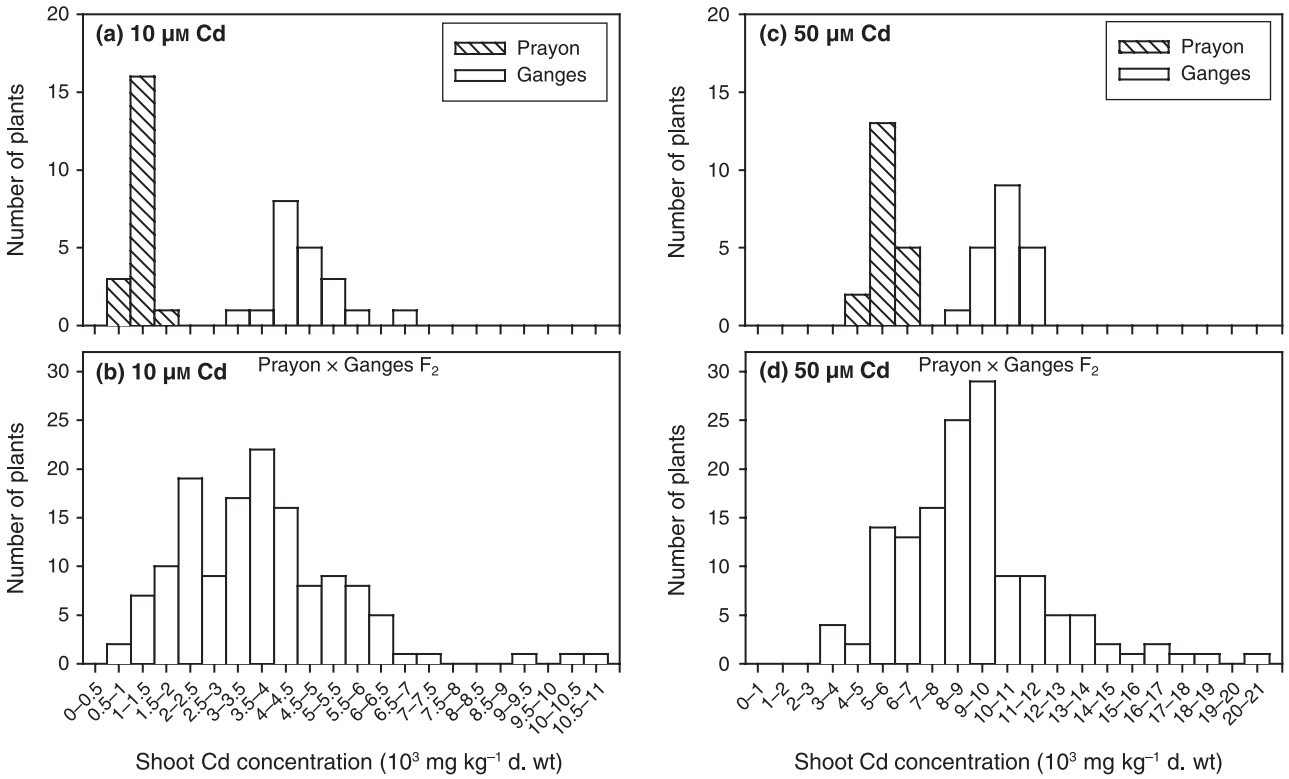

Fig. 4 Frequency distributions of shoot Cd concentration in the Ganges and Prayon parental controls (a, c), and in the F_2 from the cross of P × G (b, d). *Thlaspi caerulescens* plants were grown hydroponically with 50 μM Zn and either 10 μM Cd (a, b) or 50 μM Cd.

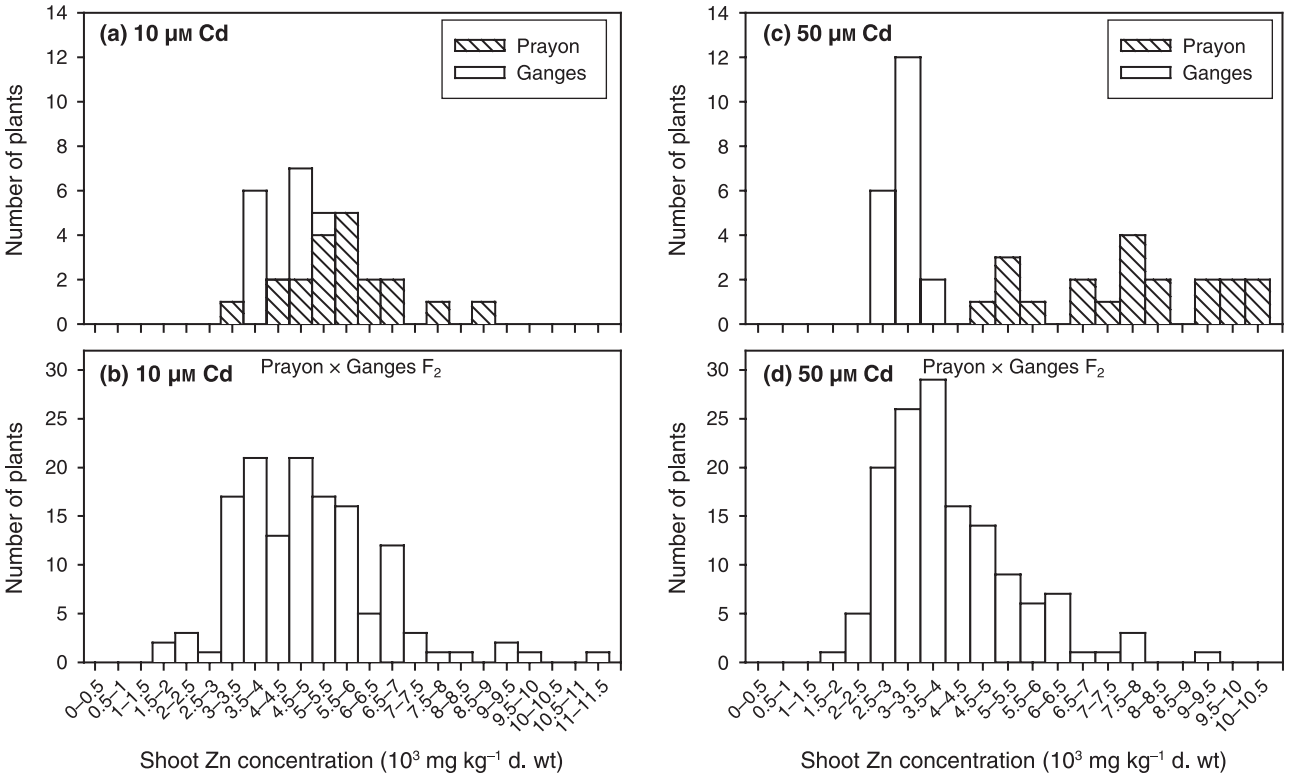

Fig. 5 Frequency distributions of shoot Zn concentration in the Ganges and Prayon parental controls (a, c), and in the F_2 from the cross of P × G (b, d). *Thlaspi caerulescens* plants were grown hydroponically with 50 μM Zn and either 10 μM Cd (a, b) or 50 μM Cd.

Table 3 Percentages of the parental control and F_2 plants of *Thlaspi caerulescens* showing the symptoms of chlorosis or stunted growth in the hydroponic experiment

Cd treatment (μM)	Plant	Chlorotic at 10 DAT*	Chlorotic at 37 DAT	Stunted growth observed on 37 DAT
10	Ganges	35	0	0
	Prayon	0	0	10
	F_2	49	1.5	12
50	Ganges	75	45	0
	Prayon	0	0	95
	F_2	43	18	32

*DAT, days after the initiation of treatment.

Two types of visual symptoms were observed in the parent and F_2 plants, i.e. interveinal leaf chlorosis and stunted growth due to an inhibition of the growth point on the shoot apex. None of the Prayon plants showed any chlorotic symptoms on either 10 or 37 d after the initiation of the Cd treatments (DAT) at both Cd concentrations, whereas 35% and 75% of the Ganges plants were chlorotic on 10 DAT at 10 and 50 μM Cd, respectively (Table 3). Chlorotic symptoms in the Ganges plants disappeared after prolonged Cd exposure (37 DAT) in the 10 μM Cd treatment, but not entirely in the 50 μM Cd treatment. By contrast, no Ganges plants showed stunted growth, whereas growth in 10% and 95% of the Prayon plants was stunted at 10 and 50 μM Cd, respectively (Table 3). It is likely that the transient leaf chlorotic symptoms were due to Cd-induced Fe deficiency and the stunted growth due to Cd toxicity *per se*. Using stunted growth as the criterion for tolerance to Cd, Ganges was tolerant and Prayon not tolerant to the prolonged exposure of 50 μM Cd. Both symptoms were also observed in some F_2 plants (Table 3). In the 50 μM Cd treatment, Cd tolerant and nontolerant (stunted growth) plants segregated by 94 : 45 in the F_2, which was not consistent with a ratio of 3 : 1 ($\chi^2 = 4.03$, $0.01 < P < 0.05$). However, it should be noted that this experiment was not designed to test the segregation pattern of Cd tolerance.

Plants with or without symptoms were grouped and analysed for the significance of the difference in shoot metal concentrations. There were no significant differences between the chlorotic and nonchlorotic plants in the concentrations of Fe, Cd and Zn in shoots in either parents or F_2 plants. On average, shoot biomass of the F_2 plants showing stunted growth was about half of the normal plants. There were significant differences ($P < 0.01$) between normal and stunted F_2 plants in the concentrations of shoot Cd and Zn. Stunted plants had 20–40% higher concentrations of Cd and Zn than normal plants ($P < 0.01$), but the amounts of Cd and Zn accumulated in the shoots were significantly ($P < 0.001$) smaller in the stunted plants than in normal plants.

Similar to the pot experiment, there were strong correlations between the concentrations of Cd and Mn in the shoots of the F_2 plants (Table 4), although the relationship was clearly influenced by the concentration of Cd in the nutrient solution (Fig. 6b). Increasing solution Cd from 10 to 50 μM

Table 4 Correlation and partial correlation coefficients between shoot Cd, Zn, Mn, Fe, Ca and Mg concentrations of the F_2 plants of *Thlaspi caerulescens* in the hydroponic experiment

Correlation coefficients

	10 μM Cd (n = 137)					50 μM Cd (n = 139)				
	Cd	Zn	Mn	Fe	Ca	Cd	Zn	Mn	Fe	Ca
Zn	0.63***					0.71***				
Mn	0.79***	0.37***				0.83***	0.55***			
Fe	0.31***	0.35***	0.45***			0.33***	0.42***	0.34***		
Ca	0.29***	0.44***	0.48***	0.16 ns		0.60***	0.59***	0.59***	0.06 ns	
Mg	0.45***	0.56***	0.52***	0.42***	0.55***	0.63***	0.45***	0.64***	0.34***	0.46***

Partial correlation coefficients

	10 μM Cd (n = 137)					50 μM Cd (n = 139)				
	Cd	Zn	Mn	Fe	Ca	Cd	Zn	Mn	Fe	Ca
Zn	0.72***					0.48***				
Mn	0.84***	−0.60***				0.63***	−0.22**			
Fe	−0.36***	0.36***	0.47***			−0.11 ns	0.40***	0.21*		
Ca	−0.49***	0.48***	0.56***	−0.35***		0.00 ns	0.41***	0.28***	−0.36***	
Mg	−0.06 ns	0.27**	0.16 ns	0.19*	0.26**	0.20*	−0.07 ns	0.20*	0.18*	0.13 ns

ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

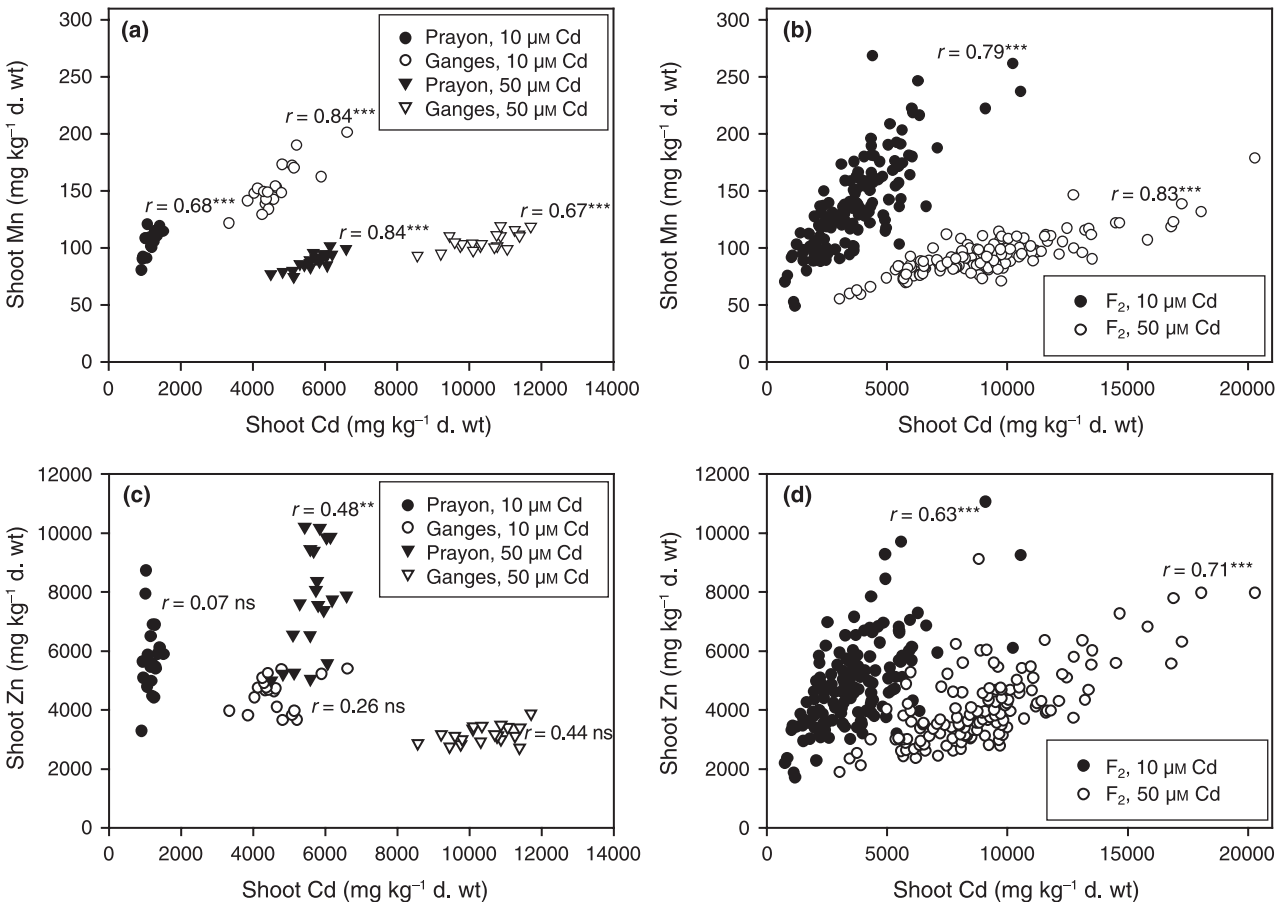

Fig. 6 Relationships between shoot Cd, Mn and Zn in the Ganges and Prayon parental controls (a, c), and in the F_2 from the cross of P × G (b, d). *Thlaspi caerulescens* plants were grown hydroponically with 50 μM Zn and either 10 μM Cd or 50 μM Cd. Significance of the correlation: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

suppressed Mn accumulation in the shoots of the F_2 plants. There were also significant correlations between shoot Cd and Mn in the two parental controls separately (Fig. 6a). Furthermore, Mn accumulation in shoots was more suppressed in Ganges by increasing the Cd concentration in nutrient solution than in Prayon. The F_2 plants also showed a significant correlation between the concentrations of Cd and Zn in the shoots (Table 4, Fig. 6d). Although there were significant differences between the two ecotypes in shoot Zn concentration (Table 2), the correlations between shoot Zn and Cd were not strong within each ecotype at each solution Cd concentration (Fig. 6c). The correlations between shoot Cd and Fe, Ca and Mg in the F_2 plants were also significant (Table 4). However, the corresponding partial correlation coefficients were either not significant or negative, suggesting weak links between the concentrations of these elements.

Cd accumulation and tolerance in F_2 plants

In this experiment, F_2 plants from the P × G cross were exposed sequentially to increasing concentrations of Cd in the nutrient solution. Nontolerant plants developed leaf chlorosis and stunted growth symptoms. At the first concentration step (100 μM Cd), eight F_2 plants showed clear chlorotic and stunted growth symptoms and varied widely in the concentration of Cd in shoots, with five plants containing < 600 mg Cd kg^{-1} d. wt and the remaining three plants containing 4000–5500 mg Cd kg^{-1} d. wt (Fig. 7). By contrast, eight randomly selected healthy plants contained 1900–6400 mg Cd kg^{-1} d. wt. The mean Cd concentration of the healthy (tolerant) plants was significantly higher than that of the chlorotic (nontolerant) plants ($P < 0.05$, ANOVA performed on log-transformed data). At the 200 and 500 μM Cd steps, more plants became chlorotic and stunted than the number of plants remaining healthy. But the differences in shoot Cd concentration between chlorotic and healthy plants were not significant.

Discussion

Ecotypic differences in Cd and Zn accumulation

In both pot and hydroponic experiments, the Ganges and Prayon ecotypes of *T. caerulescens* showed distinct Cd

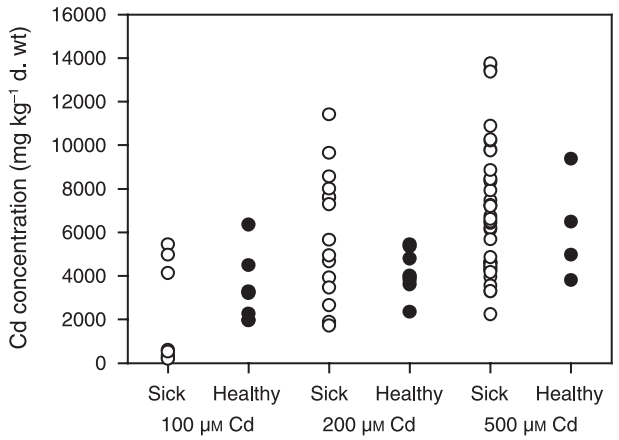

Fig. 7 Shoot Cd concentrations in individual F_2 plants from the G × P cross showing healthy or toxicity (sick) symptoms in the hydroponic experiment. *Thlaspi caerulescens* plants were grown hydroponically with 5 μM Zn, and exposed sequentially with increasing concentrations of Cd.

accumulation phenotypes that were not overlapping. Ganges was far superior to Prayon in Cd accumulation, although the scale of the difference between the two ecotypes was dependent on Cd concentration in the growth medium. For example, the difference in shoot Cd concentration was 4-fold when the plants were exposed to 10 μM Cd in the hydroponic experiment, but decreased to 2-fold when solution Cd was increased to 50 μM. Much larger differences (40- to 120-fold) were obtained when the two ecotypes were grown in field plots with a gradient of total soil Cd from 2 to 12 mg kg^{-1} (Lombi *et al.*, 2000). Note that the bioavailability of Cd in that field experiment would be far lower than in the pot experiment of this study in which soluble Cd was added to compost at 5 mg kg^{-1}. In fact, Cd accumulation by Prayon in the field experiment (< 10 mg Cd kg^{-1} shoot d. wt) (Lombi *et al.*, 2000) was no greater than the food crop sugar beet (*Beta vulgaris*) which was grown in the same experimental plots but in a different year (McGrath *et al.*, 2000). Similarly, a calamine population of *T. caerulescens* from Belgium (similar to Prayon) was found to accumulate less Cd than the non-hyperaccumulator *Thlaspi arvense* when the plants were exposed to 0.5 or 5 μM Cd (Assunção *et al.*, 2003a). Yet, when Prayon was exposed to 50 μM Cd in the hydroponic experiment, shoot Cd concentration reached 5600 mg kg^{-1} d. wt (Table 2). These results indicate that the difference between Ganges and Prayon lies mainly in the root uptake systems and/or root to shoot transport processes, which are operating at the low environmental Cd concentrations, i.e. systems with a high affinity for Cd. However, the fact that the shoot : root Cd ratio did not differ much between the two ecotypes (Table 2) suggests that it is root uptake rather than root to shoot transport that determines the large ecotypic difference. Indeed, kinetic studies showed the existence of a high-affinity Cd uptake system (K_m in the sub μM range) that was very active in the Ganges roots but was largely lacking in the Prayon roots (Lombi *et al.*, 2001b; Zhao *et al.*, 2002).

Previous studies showed either little difference between Ganges and Prayon in Zn accumulation, or that the difference was much smaller than that observed for Cd accumulation (Lombi *et al.*, 2000, 2001b; Zhao *et al.*, 2002; Assunção *et al.*, 2003a; Roosens *et al.*, 2003). The present study provided further insight into the ecotypic difference in Zn accumulation, showing that the difference was inconsistent and dependent on the experimental conditions. In the pot experiment, shoot Zn phenotypes of Ganges and Prayon were overlapping, with the former having a larger mean concentration than the latter (Fig. 2). However, in the hydroponic experiment, Prayon had a larger mean concentration of shoot Zn than Ganges, with either overlapping (in the 10 μM Cd treatment) or nonoverlapping (in the 50 μM Cd treatment) phenotype distributions (Fig. 5). Furthermore, Zn accumulation was affected by the Cd treatments differently in the two ecotypes: increasing solution Cd decreased Zn accumulation in Ganges but increased shoot Zn concentration in Prayon. Similarly, Roosens *et al.* (2003) also showed that Cd inhibited Zn accumulation in two populations of *T. caerulescens* from the Ganges region, but not in populations from other regions including Prayon. By contrast, Zn was found to inhibit Cd uptake in Prayon but not in Ganges (Lombi *et al.*, 2001b; Zhao *et al.*, 2002). Clearly, the two ecotypes showed markedly different selectivity for Zn and Cd, with Ganges discriminating for Cd against Zn, and Prayon the opposite (Table 2). Taken together, the physiological evidence available so far suggests that there is an uptake system with a high affinity for Zn but low affinity for Cd in Prayon, and that in addition to such an uptake system in Ganges, there is a system with a high affinity for Cd but low affinity for Zn. This latter system is likely to be largely responsible for Cd uptake in the Ganges ecotype, but also contributes to its Zn uptake, although the contribution to Zn uptake would depend on the ratio of Cd : Zn in the substrate. Thus, when Cd : Zn molar ratio in the substrate is low (e.g. in the pot experiment, the molar ratio of Cd to Zn added to the compost was about 0.03), Ganges accumulates not only more Cd but also more Zn than Prayon. However, when Cd : Zn molar ratio in the substrate is high (e.g. in the 50 μM Cd treatment in the hydroponic experiment, the ratio was 1.0), Ganges accumulates more Cd but less Zn than Prayon. Possible candidates for the uptake system with a high affinity for Zn and a low affinity for Cd are transporters of the ZIP-family members, ZNT1 and ZNT2, both of which are highly expressed in the roots of different populations of *T. caerulescens* (Pence *et al.*, 2000; Assunção *et al.*, 2001; Lombi *et al.*, 2002). When expressed in yeast, *ZNT1* was found to mediate high affinity uptake of Zn and low affinity uptake of Cd (Pence *et al.*, 2000). Candidates for the high affinity Cd and low affinity Zn uptake system that is prevalent in the Ganges ecotype are still elusive, although there is some

evidence that the ZIP-family member IRT1 may be involved (Lombi *et al.*, 2002; Roosens *et al.*, 2003).

Genetic background for the ecotypic differences in Cd and Zn accumulation

The occurrence of transgressive segregation for shoot Zn concentrations in the F_2, both in the pot and in the hydroponic experiment, clearly suggests that there are at least two Zn accumulation genes with differential expression in Prayon and Ganges, one of them with highest expression in Ganges and the other one in Prayon. This supports the hypothesis that the predominant accumulation mechanisms operating in Ganges and Prayon must be of a different kind. However, significant transgression beyond the upper limits of the most accumulating parental controls, to be expected from the combination of the two systems, is not apparent, possibly due to down-regulation of Zn uptake by excessive Zn accumulation in the shoot (Pence *et al.*, 2000). The absence of a comparable transgression in the segregation pattern of the shoot Cd concentration seems to argue against the existence of two systems for Cd hyperaccumulation. On the other hand, in the pot experiment and at 10 μM Cd in hydroponics, a putative Zn-preferring system with low affinity for Cd might not have contributed detectably to Cd accumulation, which would explain the absence of significant transgression. Indeed, at 50 μM Cd there was significant transgression but, as discussed in the Results, these results could have been affected by toxicity.

The reciprocal crosses between Ganges and Prayon produced F_2 progeny that showed similar phenotypic distribution patterns of Cd accumulation and insignificant difference in the means of shoot Cd concentrations (Fig. 1), suggesting that there was little or no maternal inheritance with regard to Cd accumulation in *T. caerulescens*. In both pot and hydroponic experiments, the F_2 plants showed a more or less continuous distribution pattern in shoot Cd concentration (Figs 1 and 4), which confirms the hypothesis that the difference in Cd accumulation between the two ecotypes is governed by more than one gene. Similarly, other genetic studies have shown that multiple genes are probably involved in Zn accumulation in *T. caerulescens* (Assunção *et al.*, 2003b) and *A. halleri* (Macnair *et al.*, 1999), and in Cd accumulation in *A. halleri* (Bert *et al.*, 2003). The studies on *A. halleri* involved interspecific crosses between a hyperaccumulator and a nonhyperaccumulator, whereas our study and that of Assunção *et al.* (2003b) used interecotypic crosses of the same hyperaccumulator species. Therefore, results from interspecific crosses should not be considered to be predictive with regard to those from interecotypic hyperaccumulator crosses. Our data show that higher percentages of F_2 plants fell within the Ganges phenotype range for Cd accumulation than in the Prayon phenotype range, and this was also the case for Zn accumulation in the 50 μM Cd treatment in which the Ganges parent clearly accumulated less Zn than Prayon (Fig. 5). The results suggest that the alleles from Ganges are at least partially dominant over those from Prayon.

Relationship with other metals

In the F_2 from the crosses between Ganges and Prayon, there was a strong and highly significant correlation between shoot Mn and Cd concentrations. Between 55% and 70% of the variability of shoot Mn concentration in the F_2 could be explained by the variability of shoot Cd concentration. In addition, in both experiments, Ganges accumulated significantly more Mn than Prayon. Mn accumulation was clearly suppressible by Cd in both the F_2 and the parents, particularly Ganges. There was also a significant correlation between shoot Zn and Cd concentrations in the F_2, with 24–50% of the variability of Zn accumulation being explained by the variability of Cd accumulation. These correlations indicate that component(s) of the metal transport system in *T. caerulescens* are capable of transporting multiple metals, including Cd, Mn and Zn. It is likely that such component(s) were derived primarily from Ganges. It is tempting to speculate that one of the components is IRT1, which has been shown to mediate transport of multiple metals including Fe(II), Cd, Mn and Zn (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Vert *et al.*, 2002). Furthermore, expression of the *IRT1* gene was found to be much greater in the roots of Ganges than in Prayon, particularly in response to the limitation of Fe supply (Lombi *et al.*, 2002). However, the correlation between shoot Fe and Cd concentrations in the F_2 was not strong. This is perhaps not surprising because Fe acquisition involves both reduction and *trans*-membrane transport processes in dicotyledonous species and the concentration of Fe in shoots does not necessarily reflect the physiological availability of Fe to plants (Marschner, 1995). Even severely chlorotic plants were found to have broadly similar Fe concentrations to nonchlorotic plants in our experiments. However, we observed that the Ganges seedlings were more likely than Prayon to show leaf chlorosis, which was probably caused by Fe deficiency induced by exposure to Cd (Table 3), even when there was an ample supply of Fe in the nutrient solution. Similarly, Roosens *et al.* (2003) suggested that Ganges may be bordering on incipient Fe deficiency when exposed to Cd.

Ecotypic difference in Cd tolerance

Previous studies showed that Ganges was more tolerant to Cd than Prayon (Lombi *et al.*, 2000; Roosens *et al.*, 2003). This was confirmed in the present study. Clearly, the enhanced Cd tolerance in Ganges compared to Prayon is not due to a decreased uptake of Cd, but must be due to an enhanced internal detoxification mechanism, e.g. vacuolar sequestration. To test the relationship between Cd tolerance and accumulation, F_2 plants were exposed to Cd sequentially increasing Cd

concentrations in a hydroponic experiment. In the first step of Cd exposure, nontolerant F_2 plants appeared to fall into two groups with regard to Cd accumulation in shoots: a high Cd accumulation group similar to the tolerant F_2s and a low accumulation group (Fig. 7). In the second and third steps of Cd exposure, tolerant and nontolerant F_2 plants did not differ significantly in Cd accumulation. In the phenotyping hydroponic experiment, about one-third of the F_2 plants were nontolerant under prolonged exposure to 50 μM Cd, with the other two-thirds being tolerant. At the end of Cd exposure, the nontolerant F_2 plants had significantly higher concentrations of Cd and Zn than the tolerant F_2s. However, this difference may be a result, rather than the cause, of a much reduced shoot biomass in the nontolerant F_2 plants. Overall, the results suggest that Cd tolerance and accumulation are independent traits in *T. caerulescens*, as has been shown for the tolerance and accumulation of Cd (Bert *et al.*, 2003) and Zn (Macnair *et al.*, 1999) in *A. halleri*. Macnair *et al.* (1999) suggested that a single major gene was responsible for the Zn tolerance in *A. halleri*, whereas the Cd tolerance in *A. halleri* appears to be controlled by more than one gene (Bert *et al.*, 2003). It was not possible to determine the number of genes governing the elevated Cd tolerance in Ganges compared to Prayon in the present study. Further co-segregation studies of the F_3 plants from the same crosses should provide more definitive answers regarding the relationship between Cd tolerance and accumulation.

In conclusion, our results suggest that the pronounced ecotypic difference between Ganges and Prayon in Cd and Zn accumulation is governed by multiple genes, and that Cd accumulation and tolerance are genetically independent traits in *T. caerulescens*. The ecotypic difference in Cd accumulation was attributed mainly to the root uptake process rather than root to shoot translocation. The study provides further evidence for a root uptake system that has a high affinity for Cd and a low affinity for Zn and is highly active in the Ganges ecotype. This system probably also contributes to Mn uptake, as well as Zn uptake when the ratio of Cd to Zn in the substrate is low. By contrast, the low Cd accumulating ecotype Prayon is dominated by uptake systems with a low affinity for Cd.

Acknowledgements

We thank Rothamsted International for a fellowship to R. F. Jiang, and the Royal Society of London for a Joint-Project Fellowship (Q-827) to H. G. Zha. We are grateful to Miss Sarah Dunham and Mr Adrian Crosland for their assistance in the experimental and analytical work. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. H. G. Zha received further funding from Natural Science foundation of China (Grant no. 40125005).

References

Assunção AGL, Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003a. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Assunção AGL, Martins PD, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **24**: 217–226.

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003b. A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159**: 383–390.

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. *Biorecovery* **1**: 81–126.

Baker AJM, Reeves RD, Hajar ASM. 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C Presl (Brassicaceae). *New Phytologist* **127**: 61–68.

Bert V, Meerts P, Saumitou-Laprade P, Salis P, Gruber W, Verbruggen N. 2003. Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249**: 9–18.

Eide D, Broderius M, Fett J, Guerinot ML. 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences, USA* **93**: 5624–5628.

Escarré J, Lefebvre C, Gruber W, Leblanc M, Lepart J, Riviere Y, Delay B. 2000. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**: 429–437.

Frerot H, Petit C, Lefebvre C, Gruber W, Collin C, Escarré J. 2003. Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **157**: 643–648.

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB. 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Molecular Biology* **40**: 37–44.

Lombi E, Tearall KL, Howarth JR, Zhao FJ, Hawkesford MJ, McGrath SP. 2002. Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **128**: 1359–1367.

Lombi E, Zhao FJ, Dunham SJ, McGrath SP. 2000. Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytologist* **145**: 11–20.

Lombi E, Zhao FJ, Dunham SJ, McGrath SP. 2001a. Phytoremediation of heavy metal-contaminated soils: Natural hyperaccumulation versus chemically enhanced phytoextraction. *Journal of Environmental Quality* **30**: 1919–1926.

Lombi E, Zhao FJ, McGrath SP, Young SD, Sacchi GA. 2001b. Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. *New Phytologist* **149**: 53–60.

Macnair MR. 2003. The hyperaccumulation of metals by plants. *Advances in Botanical Research* **40**: 63–105.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London Series B – Biological Sciences* **266**: 2175–2179.

Marschner H. 1995. *Mineral nutrition of higher plants, 2nd edn*. London, UK: Academic Press.

McGrath SP, Zhao FJ, Dunham SJ, Crosland AR, Coleman K. 2000. Long-term changes in the extractability and bioavailability of zinc and cadmium after sludge application. *Journal of Environmental Quality* **29**: 875–883.

McGrath SP, Zhao FJ, Lombi E. 2002. Phytoremediation of metals, metalloids, and radionuclides. *Advances in Agronomy* **75**: 1–56.

Meerts P, van Isacker N. 1997. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, Kochian LV. 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens. Proceedings of the National Academy of Sciences, USA* **97**: 4956–4960.

Pollard AJ, Powell KD, Harper FA, Smith JAC. 2002. The genetic basis of metal hyperaccumulation in plants. *Critical Reviews in Plant Sciences* **21**: 539–566.

Reeves RD, Brooks RR. 1983. European species of *Thlaspi* L. (Cruciferae) as indicators of nickel and zinc. *Journal of Geochemical Exploration* **18**: 275–283.

Reeves RD, Schwartz C, Morel JL, Edmondson J. 2001. Distribution and metal-accumulating behaviour of *Thlaspi caerulescens* and associated metallophytes in France. *International Journal of Phytoremediation* **3**: 145–172.

Robinson BH, Leblanc M, Petit D, Brooks RR, Kirkman JH, Gregg PEH. 1998. The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. *Plant and Soil* **203**: 47–56.

Roosens N, Verbruggen N, Meerts P, Ximenez-Embun P, Smith JAC. 2003. Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. *Plant, Cell & Environment* **26**: 1657–1672.

Schwartz C, Echevarria G, Morel JL. 2003. Phytoextraction of cadmium with *Thlaspi caerulescens. Plant and Soil* **249**: 27–35.

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briata JF, Curie C. 2002. IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* **14**: 1223–1233.

Zhao FJ, Hamon RE, Lombi E, McLaughlin MJ, McGrath SP. 2002. Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens. Journal of Experimental Botany* **53**: 535–543.

Zhao FJ, Lombi E, McGrath SP. 2003. Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens. Plant and Soil* **249**: 37–43.