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Review

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Molecular mechanisms of metal hyperaccumulation in plants

Author for correspondence:
Nathalie Verbruggen
Tel: +32 2 650 21 28
Fax: +32 2 650 54 21
Email: nverbru@ulb.ac.be

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Nathalie Verbruggen[1], Christian Hermans[1] and Henk Schat[2]

[1]Laboratoire de Physiologie et de Génétique Moléculaire des Plantes, Université Libre de Bruxelles, Campus Plaine – CP242 – Bd du Triomphe, B-1050 Brussels, Belgium; [2]Institute of Molecular and Cellular Biology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV, Amsterdam, the Netherlands

Contents

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Summary

Metal hyperaccumulator plants accumulate and detoxify extraordinarily high concentrations of metal ions in their shoots. Metal hyperaccumulation is a fascinating phenomenon, which has interested scientists for over a century. Hyperaccumulators constitute an exceptional biological material for understanding mechanisms regulating plant metal homeostasis as well as plant adaptation to extreme metallic environments. Our understanding of metal hyperaccumulation physiology has recently increased as a result of the development of molecular tools. This review presents key aspects of our current understanding of plant metal – in particular cadmium (Cd), nickel (Ni) and zinc (Zn) – hyperaccumulation.

I. Hyperaccumulation: the phenomenon

High tolerance to trace elements (including metals or metalloids) has evolved in a number of plant species. Tolerant plants are often excluders, limiting the entry and root-to-shoot translocation of trace metals. However, a class of rare plants called hyperaccumulators combines extremely high tolerance to, and foliar accumulation of, trace elements. Hyperaccumulators have recently gained considerable interest because of their potential use in phytoremediation (Chaney *et al.*, 1997, 2005; reviewed by Pilon-Smits, 2005), phytomining (Li *et al.*, 2003) and food crop biofortification (Broadley *et al.*, 2007; Palmgren *et al.*, 2008).

Hyperaccumulators can accumulate exceptional concentrations of trace elements in their aerial parts without visible toxicity symptoms. Foliar concentration thresholds for hyperaccumulation are summarized in Table 1. The most extreme example reported to date is probably the latex of *Sebertia acuminata*, which can contain up to 26% (w/w) Ni (Jaffré *et al.*, 1976).

Table 1 Hyperaccumulators of trace metals (adapted from Reeves & Baker, 2000; A. Baker pers. comm.; except for cadmium: Bert *et al*., 2002; Yang *et al*., 2004; Vogel-Mikuš *et al*., 2005)

Trace element	Concentration criterion (% in leaf dry matter)	Number of taxa	Number of families represented
Antimony	≥ 0.1	2	2
Cadmium	≥ 0.01	4	2
Cobalt	≥ 0.1	(26)	11
Copper	≥ 0.1	(35)	15
Lead	≥ 0.1	14	7
Manganese	≥ 1.0	10	6
Nickel	≥ 0.1	390	42
Thallium	≥ 0.1	1	1
Zinc	≥ 1.0	14	6

Numbers in brackets refer to unconfirmed data (A. Baker, pers. comm.).

Table 2 Foliar metal concentrations in *Thlaspi caerulescens* populations

	La Calamine (μmol g^{-1} DW)	Lellingen	Ganges	Monte Prinzera
Zn	6.5	28.0	60.5	53.9
Cd	1.8	17.3	29.9	4.5
Ni	1.5	17.3	16.9	48.3

Plants were grown for 4 wk in modified Hoagland's solution, of which the last 3 wk with 10 μM Zn, 5 μM Cd or 10 μM Ni (data from Assunção *et al*., 2003a).

Over 450 plant species (0.2% of angiosperms) have been identified as hyperaccumulators of trace metals (Zn, Ni, Mn, Cu, Co and Cd), metalloids (As) and nonmetals (Se), the majority of them being Ni hyperaccumulators (75%) (Brooks *et al*., 1974, 1977; Baker & Brooks, 1989; Reeves & Baker, 2000; Ellis & Salt, 2003; Reeves, 2003, 2006; Sors *et al*., 2005; Milner & Kochian, 2008) (Table 1). As previously noted by Macnair (2003), the phenomenon of hyperaccumulation has been overreported. For example, Cu and Co hyperaccumulation in a number of species from the copper belt in Congo appeared to be the result of airborne contamination of the leaf surface, rather than root uptake and translocation (Faucon *et al*., 2007).

II. Macroevolution of hyperaccumulation

Hyperaccumulators occur in over 34 different families. The Brassicaceae family is relatively rich in them, in particular the genera *Alyssum* and *Thlaspi*. Since Ni hyperaccumulation occurs in a broad range of unrelated families, it is certainly of polyphyletic origin (Macnair, 2003). A relatively large fraction of the Ni hyperaccumulators described so far belongs to the Odontarrhena section in the genus *Alyssum* (Brassicaceae). Within that section, Mengoni *et al*. (2003) established that the nonhyperaccumulators are distributed over different clades and that the trait might have been lost or, less likely, acquired more than once. With few exceptions, among them *Sedum alfredii* (Crassulaceae), Zn and Cd hyperaccumulation is confined to the *Brassicaceae*, in particular to the genus *Thlaspi* and the species *Arabidopsis halleri*. Within the *Thlaspi* genus, hyperaccumulation is confined to the *Noccaea* section, and all the members of this section are hyperaccumulators, suggesting that hyperaccumulation is monophyletic within this genus (Macnair, 2003). Some species or populations of *Thlaspi* hyperaccumulate Ni in their natural (serpentine) environment, but a number of these are capable of Zn hyperaccumulation, whereas *Alyssum* hyperaccumulators do not hyperaccumulate Zn (Assunção *et al*., 2001, 2008; Taylor & Macnair, 2006).

How did hyperaccumulation evolve? The selective factors causing the evolution of hyperaccumulation are unknown and difficult to identify retrospectively. The different nonmutually exclusive hypotheses in the literature are: increased metal tolerance, protection against herbivores or pathogens, inadvertent uptake, drought tolerance, and allelopathy (Boyd & Martens, 1992). The hypothesis of protection against herbivores and pathogens is certainly the most popular one (Boyd & Martens, 1994; Pollard & Baker, 1997; Jhee *et al*., 1999; Huitson & Macnair, 2003; Noret *et al*., 2007, reviewed in Boyd, 2007; Galeas *et al*., 2008).

III. Microevolution of hyperaccumulation: variation within hyperaccumulator species

There is a huge variability between populations of hyperaccumulator species in their capacity to tolerate and accumulate metals, which provides opportunities to analyze the underlying genetic determinants. Such variation has been extensively reported for *Thlaspi caerulescens* (Table 2) and *Arabidopsis halleri*, which are the two most studied hyperaccumulator species to date. In *T. caerulescens*, all populations hyperaccumulate

Zn, but when grown on the same substrate, populations from metallicolous soils generally accumulate less Zn than populations from nonmetallicolous soils (Meerts & Van Isacker, 1997; Escarré *et al.*, 2000; Dubois *et al.*, 2003; Frérot *et al.*, 2003). Also in *A. halleri*, Zn hyperaccumulation is constitutive, but there is heritable variation in degree between local populations. However, on average, there is no difference between metallicolous and nonmetallicolous populations under controlled conditions (Bert *et al.*, 2002; Macnair, 2002). High Zn tolerance seems to be constitutive in both species, but again there is considerable variation in degree, with the highest tolerances found in metallicolous populations (Bert *et al.*, 2000; Assunção *et al.*, 2003a,c; Pauwels *et al.*, 2006). When grown in Zn-enriched soil, nonmetallicolous plants show reduced performance, compared with metallicolous plants, demonstrating that the basic degrees of Zn tolerance in nonmetallicolous plants are insufficient to sustain maximum performance in metalliferous soil (Meerts & Van Isacker, 1997; Escarré *et al.*, 2000; Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008). As evidenced by molecular markers, the enhanced Zn tolerance in metallicolous populations are of polyphyletic origin, resulting from independent local microevolutionary adaptation (Pauwels *et al.*, 2006; Jiménez-Ambriz *et al.*, 2007).

For Cd accumulation, intraspecies variation seems considerably higher than for Zn (Bert *et al.*, 2002; Assunção *et al.*, 2003a,b,c). Cd hyperaccumulation has been reported in only four species, which are also Zn hyperaccumulators, suggesting that Cd and Zn hyperaccumulation relies on common genetic determinants, at least partially (Table 1). In *S. alfredii*, both Zn and Cd hyperaccumulation are not constitutive at the species level, but confined to metallicolous populations (Yang *et al.*, 2006; Sun *et al.*, 2007). In *T. caerulescens* the variation in Cd accumulation among local populations is correlated with the variation in Zn accumulation, at least in some studies (Assunção *et al.*, 2003c; Roosens *et al.*, 2003). Xing *et al.* (2008) observed a strong correlation between the root-to-shoot translocation rates of Cd and Zn among a large number of *T. caerulescens* and *T. praecox* populations. These studies confirm the hypothesis of common determinants for Cd and Zn hyperaccumulation. However, Cd and Zn accumulation are uncorrelated among *T. caerulescens* populations from southern France, where the metallicolous ones accumulate more Cd but less Zn than the nonmetallicolous populations, when grown in the same soil (Escarré *et al.*, 2000). In *T. caerulescens*, some populations accumulate less Cd than the nonhyperaccumulator congeneric species, *T. arvense*, irrespective of the Cd supply. However, all the *T. caerulescens* populations exhibit higher Cd concentrations in leaves than in roots (Assunção *et al.*, 2003c). By contrast, *A. halleri*, in which Cd hyperaccumulation is not constitutive at the species level, accumulates more Cd in roots than in shoots (Bert *et al.*, 2003). In *T. caerulescens*, Cd hypertolerance is not constitutive at the species level, but confined to populations from calamine soils. Some exceptional populations from southern France (the region around the village of Ganges) even require Cd for optimal growth (Roosens *et al.*, 2003; Liu *et al.*, 2008), suggesting a possible biological role for Cd in those populations as demonstrated in the marine diatom *Thalassiosira weissflogii* (Lee *et al.*, 1995; Lane & Morel, 2000; Lane *et al.*, 2005). Thus, in *T. caerulescens*, enhanced root-to-shoot Cd transport seems to be constitutive, but Cd hypertolerance and high-level Cd hyperaccumulation seem to result from recent microevolutionary change at a local scale.

Like Cd hyperaccumulation, Ni hyperaccumulation does not seem to be constitutive in *T. caerulescens* but is confined to serpentine populations (Assunção *et al.*, 2003c). Enhanced Ni tolerance may be constitutive at the species level although variation exists among populations. (Assunção *et al.*, 2003c). Under equimolar supply of Zn and Ni, serpentine populations of *T. caerulescens*, *T. pindicum* and *T. alpinum* prefer Zn over Ni (Assunção *et al.*, 2001, 2008; Taylor & Macnair, 2006), suggesting that Ni hyperaccumulation results from local microevolutionary adaptation, and that hyperaccumulation of Zn, rather than of Ni, may be the basic condition in these species. The Turkish serpentine endemics *T. oxyceras*, *T. rosulare* and *T. violascens*, on the other hand, don't seem to prefer Zn over Ni (Peer *et al.*, 2003), suggesting that different strategies for Ni hyperaccumulation may have been evolved within the Noccaea clade.

IV. Genetic analysis of metal accumulation and tolerance

In this section we aim to review our current knowledge of the genetic architecture of metal (Zn, Ni and Cd) accumulation and tolerance.

Zn hyperaccumulation and hypertolerance are to a large extent constitutive at the species level in *A. halleri* and *T. caerulescens*, which is a major handicap in the genetic analysis of these traits. Macnair *et al.* (1999) were the first to circumvent this handicap by analyzing an interspecific cross between *A. halleri* and *A. l. petraea*. It may be expected that major hyperaccumulation or tolerance loci that control the segregation in interspecific *A. halleri* × *A. l. petraea* crosses may not do so in intraspecific *T. caerulescens* crosses. This may not be true for nonconstitutive traits, such as Cd or Ni hyperaccumulation and Cd tolerance (see earlier).

From an analysis of a F_2 generation of the *A. halleri* × *A. lyrata* subsp. *petraea* cross, Macnair *et al.* (1999) concluded that Zn tolerance and Zn hyperaccumulation were under independent genetic control. Another progeny from an *A. halleri* × *A. l. petraea* F_1 plant backcrossed to *A. l. petraea* (called BC_1) was used by Bert *et al.* (2003) to analyze the co-segregation of Cd tolerance and Cd accumulation. They found independent inheritance of these characters, but a degree of co-segregation of Cd tolerance with Zn tolerance, and Cd accumulation with Zn accumulation, suggesting partial pleiotropic control of these characters. The same BC_1 has been used to map quantitative

Table 3 Summary of quantitative trait loci (QTL) analysis

Cross	Trait	QTL	LG	LOD	%PVE	Trait-enhancing allele
(a)						
LE × LC	ZnR	ZnR1	3	4.6	21.7	LE
LE × LC	ZnR	ZnR2	5	3.6	16.6	LC
LC × GA	ZnS	ZnS1[b]	1	6.0	14.7	LC
LC × GA	ZnS	ZnS2	4	4.0	9.6	LC
LC × GA	ZnS	ZnS3	7	7.4	18.1	GA
LC × GA	ZnR	ZnR3[b]	3	4.0	14.9	LC
LC × GA	ZnR	ZnR4[c]	6	8.5	54.4	GA
LC × GA	CdS	CdS1[a]	3	5.5	23.8	GA
LC × GA	CdR	CdR1[a]	3	7.3	9.6	GA
LC × GA	CdR	CdR2[c]	6	8.7	33.1	GA
(b)						
Ah × Al	ZnT	ZnT1a	3	6.5	12.2	Ah
Ah × Al	ZnT	ZnT2	4	7.3	11.2	Ah
Ah × Al	ZnT	ZnT3	6	4.5	5.6	Ah
Ah × Al	ZnS	CdT1a	3	9.9	42.9	Ah
Ah × Al	ZnS	CdT2	4	4.5	23.7	Ah
Ah × Al	ZnR	CdT3	6	4.4	15.9	Ah

(a) QTLs for zinc (Zn) or cadmium (Cd) accumulation in roots and shoots in crosses between plants from different *Thlaspi caerulescens* populations. Plants were grown in modified Hoagland's with 2 μM Zn and 5 μM Cd (LC × GA) or 10 μM Zn (LE × LC) (data from Assunção *et al*., 2006; Deniau *et al*., 2006). Co-locating QTLs have been marked with the same superscripted letters (LE, Lellingen; LC, La Calamine; GA, Ganges; S, shoot; R, root; PVE, percentage of phenotypic variance explained; LG, linkage group).
(b) QTLs for Zn (Willems *et al*., 2007) and Cd (Courbot *et al*., 2007) tolerance in a BC1 backcross progeny of an *Arabidopsis halleri* × *A. lyrata* subsp. *petraea* cross (*Ah* × *Al*). ZnT/CdT, Zn/Cd tolerance; further as above.

trait loci (QTLs) for Zn and Cd tolerance (Courbot *et al*., 2007; Willems *et al*., 2007). Three additive QTLs were found for Zn tolerance and for Cd tolerance, all of them with the trait-enhancing alleles originating from the *A. halleri* parent (Table 3). One QTL for Cd tolerance co-located with a Zn tolerance locus (Table 3), confirming the hypothesis of partial pleiotropic control of Zn and Cd tolerance (Bert *et al*., 2003). Filatov *et al*. (2007), using an F_2 generation of the *A. halleri* × *A. l. petraea* cross made by Macnair *et al*. (1999), mapped two QTLs for Zn accumulation to different linkage groups.

Zha *et al*. (2004), using an F_2 generation of an intraspecific *T. caerulescens* cross between two calamine plants, from Prayon (PR) and southern France (GA), the latter with much higher Cd accumulation capacity, analyzed the segregation and co-segregation of Cd and Zn accumulation and Cd tolerance. Zn, Cd and Mn accumulation in the F_2 were phenotypically correlated. They found transgressive segregation of Zn accumulation, but not of Cd accumulation. QTLs for Zn and Cd accumulation have been mapped in two *T. caerulescens* intraspecific crosses (Table 3). Assunção *et al*. (2006) found two QTLs for root Zn accumulation in an F_3 cross between plants from a Belgian calamine site (LC) and from a nonmetalliferous site in Luxemburg (LE), the latter with higher Zn accumulation capacity. Deniau *et al*. (2006) mapped QTLs for Cd and Zn accumulation in an F2 cross between two plants from GA and LC, the latter with lower Cd accumulation capacity. There was one common locus for Cd and Zn in root, one for Zn in root and Zn in shoot, and one for Cd in root and Cd in shoot. The trait-enhancing alleles at the three Cd accumulation loci were all derived from the GA parent. In the two interspecific crosses, both of the parents contributed trait-enhancing alleles to the Zn accumulation loci. In agreement with this, Deniau *et al*. (2006) found transgressive segregation for Zn accumulation, but not for Cd accumulation. The Cd and Zn accumulation rates in the F_2 plants were moderately, but significantly phenotypically correlated, in agreement with the hypothesis that Cd and Zn accumulation, in so far as they segregated, are partly governed by common genetic determinants, but additionally by more metal-specific determinants.

Richau & Schat (2008) analyzed the segregation of Zn and Ni accumulation in F_3 and F_4 families derived from a cross between *T. caerulescens* from a calamine site (LC) and from a serpentine site at Monte Prinzera, Italy (MP), the latter showing much higher Zn and Ni accumulation capacity. The segregation of both Ni and Zn accumulation was not transgressive in this cross and Zn and Ni accumulation in the F_3 was phenotypically correlated. Partitioning of the phenotypic correlation showed that the correlation was entirely genetic, implying that Zn and Ni accumulation, in so far as they segregated in this cross, are pleiotropically controlled by the same genes. This is in agreement with the Zn-suppressible Ni hyperaccumulation in MP (Assunção *et al*., 2001, 2008).

The genetics of metal hypertolerance have been poorly studied in *T. caerulescens*. Zn tolerance segregated in LC × LE F_2 crosses, but was uncorrelated with Zn accumulation (Assunção *et al*., 2003b). Similarly, Zha *et al*. (2004) and Richau & Schat (2008) did not find any phenotypic correlation between Cd accumulation and Cd tolerance, or between Ni accumulation and Ni tolerance, respectively. Thus, metal tolerance and metal accumulation, in so far they segregate in intraspecific *T. caerulescens* crosses, seem to be under nonpleiotropic genetic control.

The high synteny between the genetic maps of *A. halleri* and *A. thaliana* facilitated the identification of candidate genes at QTLs for Zn and Cd tolerance. Those genes will be discussed in the following section.

V. Mechanisms of trace metal hyperaccumulation

Physiological studies have paved the way for a basic understanding of metal hyperaccumulation mechanisms, including enhanced metal uptake, increased xylem loading and increased detoxification in the shoot (Lasat *et al*., 1996, 1998; Krämer *et al*., 1996, 1997; Lombi *et al*., 2001; Zhao *et al*., 2002, 2006; Assunção *et al*., 2003a; Xing *et al*., 2008).

Analyzing trace metal tolerance and accumulation has been greatly enhanced by the use of molecular resources developed for *A. thaliana*. High-throughput technologies, in particular microarray, have allowed the complexity of the hyperaccumulation phenomenon to be tackled. These studies support the idea that genes that are thought to be involved in hyperaccumulation and hypertolerance are not species-specific or novel, but rather differently expressed and regulated, compared with nonhyperaccumulator species. However as no complete genome sequences of hyperaccumulators are yet available, this assumption cannot be fully verified. Comparative transcriptomics studies on hyperaccumulators and related nonaccumulating nontolerant species have identified a large array of genes that are constitutively (in the absence of excess of metallic ions) highly expressed (Weber *et al.*, 2004, 2006; Becher *et al.*, 2004; Craciun *et al.*, 2006; Filatov *et al.*, 2006; van de Mortel *et al.*, 2006; Talke *et al.*, 2006). These molecular insights are for the moment restricted to species related to *A. thaliana.* But the ever-growing availability of full-genome sequences will allow the development of microarrays in other plant families.

Hyperaccumulation of Zn is probably the best-understood example of metal hyperaccumulation at the molecular level. The existence of species closely related to *A. thaliana* displaying Zn hyperaccumulation has allowed rapid progress. The most closely related hyperaccumulator species is *A. halleri*. These species diverged *c.* 5 million yr ago and they show *c.* 94% nucleotide identity within coding regions. Genes differentially expressed in *A. halleri* compared with *A. thaliana* (Becher *et al.*, 2004; Weber *et al.*, 2004, 2006; Talke *et al.*, 2006) or in progenies from crosses between *A. halleri* and *A. l. petraea* segregating for Zn hyperaccumulation (Filatov *et al.*, 2006) have recently been reviewed by Roosens *et al.* (2008). Similar studies have taken place in *T. caerulescens* (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006, 2008). *T. caerulescens* and *A. thaliana* diverged *c.* 20 million yr ago and share *c.* 88% nucleotide identity in coding regions (van de Mortel *et al.*, 2006). In total, > 2000 genes were found to be at least five times significantly more expressed in *T. caerulescens* compared with Arabidopsis, of which 1147 had an unknown function. To pinpoint genes directly involved in Zn homeostasis, only those regulated by Zn supply (deficiency or excess) in Arabidopsis and/or in hyperaccumulators were used to compose a shortlist of candidate genes (van de Mortel *et al.*, 2006; Talke *et al.*, 2006). Striking convergence was observed between the genes identified in the two Brassicaceae. Many of them can be categorized in functions associated with metal homeostasis, while others take part in basic metabolism, without direct relation to metal tolerance or accumulation (Table 4).

Comparative root transcriptome analyses between the same hyperaccumulators and *A. thaliana* or *A. l. petraea* were also performed in response to Cd^{2+} exposure with the aim to identify genes that are important for adaptation to this toxic heavy metal (Craciun *et al.*, 2006; Weber *et al.*, 2006; van de Mortel *et al.*, 2008). Cd is considered as a major environmental pollutant, which enters the food chain mainly by plant uptake. Therefore, it is important to identify the genes responsible for the accumulation of Cd in the different organs of plants to be able to control this trait. There was a constitutive overexpression of genes involved in stress response in *A. halleri* and *T. caerulescens*, as well as of genes involved in metal homeostasis. The study in *T. caerulescens* emphasized the potential role of genes involved in lignin, glutathione and sulfate metabolism.

In short, transcriptomics studies have confirmed the importance of metal transport and detoxification processes in hyperaccumulation but have also shed light on other putative modifications of cellular processes (which will be addressed in the last paragraph of this section).

We will now review our current understanding of key steps involved in hyperaccumulation, illustrated in Fig. 1.

1. Uptake of metal from the soil to the root

Metal avaflability and mobility in the rhizosphere can be influenced by root exudates, such as siderophores, organic acids and protons, as well as by rhizosphere microorganisms (Zhao *et al.*, 2001; Whiting *et al.*, 2001; Wenzel *et al.*, 2003). However, there is no definite answer to the question of whether, and how, hyperaccumulators and nonhyperaccumulators, or their root-associated microbial communities, have different effects on the metal availability in their rhizospheres. In *T. caerulescens*, high metal uptake has been associated with enhanced root proliferation in artificially metal-enriched soil patches under experimental conditions (Whiting *et al.*, 2000). However, not all *T. caerulescens* populations exhibit this metal root foraging (Haines, 2002), and its relevance under natural conditions is unknown.

Enhanced Zn root uptake seems to be driven by overexpression of members of the ZIP family (**z**inc-regulated transporter, **i**ron-regulated transporter **p**rotein; reviewed by Krämer *et al.*, 2007) (Fig. 1). Early work by Pence *et al.* (2000) has highlighted the constitutive overexpression in *T. caerulescens* roots of *ZNT1* resulting in root Zn uptake with higher V_{max} (but similar K_m) than in the related nonaccumulator species, *T. arvense*, as observed in a previous radiotracer flux study (Lasat *et al.*, 1998). *ZNT1* mediates high-affinity Zn transport as well as low-affinity Cd uptake (Pence *et al.*, 2000) and is the homolog of *AtZIP4*. High expression of *ZNT1* was later confirmed in microarray analyses (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006). Many Zn-transporting ZIP members, including *ZNT1*, are Zn-regulated in nonaccumulators, that is, they are only detectably expressed under conditions of Zn deficiency, whereas they are expressed more or less independently of the Zn supply in hyperaccumulators (Pence *et al.*, 2000; Assunção *et al.*, 2001).

Physiological studies on *T. caerulescens* have provided strong evidence that multiple uptake systems are involved in the root uptake of Cd and Zn (Lombi *et al.*, 2002, Zhao *et al.*, 2002;

Table 4 List of genes more expressed in *Arabidopsis halleri* (references 1, 2, 3, 5) and/or *Thlaspi caerulescens* (references 4, 6, 8, 9) compared with nontolerant nonaccumulator relatives (*A. thaliana* or *T. arvense*)

Related function	Name	Annotation	Organ	References
Metal uptake into cells	*ZIP4*	ZIP family of metal transporters	R + S	1, 2, 4, 6
	ZIP6	ZIP family of metal transporters	R + S	1, 3, 4, 5
	ZIP9	ZIP family of metal transporters	R + S (*)	1, 2, 4, 5, 6
	ZIP10	ZIP family of metal transporters	R (lower in S)	4, 5, 6
	IRT1	ZIP family of metal transporters	R	1, 6
	IRT3	ZIP family of metal transporters	R + S	1, 4, 5
	ZIP7	ZIP family of metal transporters	S	4
Metal vacuolar sequestration	*MTP1*	Cation diffusion facilitator	R + S	1, 2, 5, 6
	MTP8	Cation diffusion facilitator	R + S	4, 5, 6
	MPT11	Cation diffusion facilitator	S	5
	CAX2	Ca^{2+}: cation antiporter	R + S	1, 2, 4
	AtHMA3	P-type metal ATPase	S	1, 3, 4, 6
Metal remobilization from the vacuole	*NRAMP3*	Natural resistance associated macrophage	R + S (Ah)	2, 3, 5
	NRAMP1	Natural resistance associated macrophage	S	4, 6
	NRAMP5	Natural resistance associated macrophage	S	4, 6
Xylem loading/unloading of metal/ligands/metal–ligand complexes	*HMA4*	P-type metal ATPase	S	1, 2, 3, 4, 5, 6
	FRD3	Multidrug and toxin efflux family transporter	R + S (Ah)	4, 5, 6, 8
	YSL3	Yellow-stripe-like transporter	R + S	7
	YSL6	Yellow-stripe-like transporter	S	5
	YSL7	Yellow-stripe-like transporter	S	7
Synthesis of metal ligands	*NAS1*	Nicotinamine synthase		4, 6
	NAS2	Nicotinamine synthase	R	2, 5, 6
	NAS3	Nicotinamine synthase	S (Ah) + R(Tc)	1, 4, 5, 6
	NAS4	Nicotinamine synthase	R + S (Tc)	2, 4, 6, 8
	SAMS1	S-adenosyl-methionine synthetase	S	5
	SAMS2	S-adenosyl-methionine synthetase	S	5
	SAMS3	S-adenosyl-methionine synthetase	R + S	5
	AOSA2	Cysteine synthase	R + S	2, 5
Other roles in iron homeostasis	*FER1*	Ferritin Fe(III) binding	S	5, 6
	FER2	Ferritin Fe(III) binding	S	5
	IREG2	Iron regulated transporter 2	R	5, 6
	At4g35830	Cytoplasmic aconitase	S	3
Stress protection/response	*PDI1*	Protein disulfide isomerase 1	S	5
	PDI2	Protein disulfide isomerase 2	S	5
	At1g45145	H-type thioredoxin	S	3
Homeostasis of macronutrient	*PHT1-4*	Phosphate:H1 symporter family	R + S	1, 4, 5

Gene names refer to *A. thaliana.* 1, Becher *et al*. (2004); 2, Weber *et al*. (2004); 3, Filatov *et al*. (2006); 4, Hammond *et al*. (2006); 5, Talke *et al*. (2006); 6, van de Mortel *et al*. (2006); 7, Gendre *et al*. (2007); 8, van de Mortel *et al*. (2008). When high gene expression was observed in only one of the two species, the name of the species is added (Ah, *A. halleri*; Tc, *T. caerulescens*).
*, no transcript in *A. halleri* shoot except under Zn-deficiency conditions.

Cosio *et al*., 2004, Roosens *et al*., 2004). Those include a system with a strong preference for Zn over Cd, and another one with a preference for Cd over Zn. Based on physiological evidence, the high rate of Cd hyperaccumulation in the populations from southern France (Lombi *et al*., 2002; Cosio *et al*., 2004; Roosens *et al*., 2004) is attributable to the expression of a Cd-preferent accumulation system, which also accumulates Zn when there is a lot more Zn than Cd in the nutrient solution (Zhao *et al*., 2002). In the populations from northern Europe, Zn accumulation is predominantly due to a Zn-preferent accumulation system (Zhao *et al*., 2002). The transgression of Zn accumulation found in the interspecies crosses between calamine populations of Prayon or La Calamine (B) and Ganges (southern France) (see Section IV) would then be explained by independent recombination of the genes controlling both systems.

Studies of metal influx showed that V_{max} for Cd uptake was *c*. five times higher in the Ganges population than in Prayon; K_m was similar, and Cd uptake was enhanced by Fe deficiency in Ganges but not in Prayon (Lombi *et al*., 2001). Cd was able to compete with Fe uptake in populations from the Ganges region but not in Prayon (Roosens *et al*., 2003). In the latter population, Ca uptake was inhibited by Cd addition, suggesting that, in addition to two Zn uptake systems, at least two other

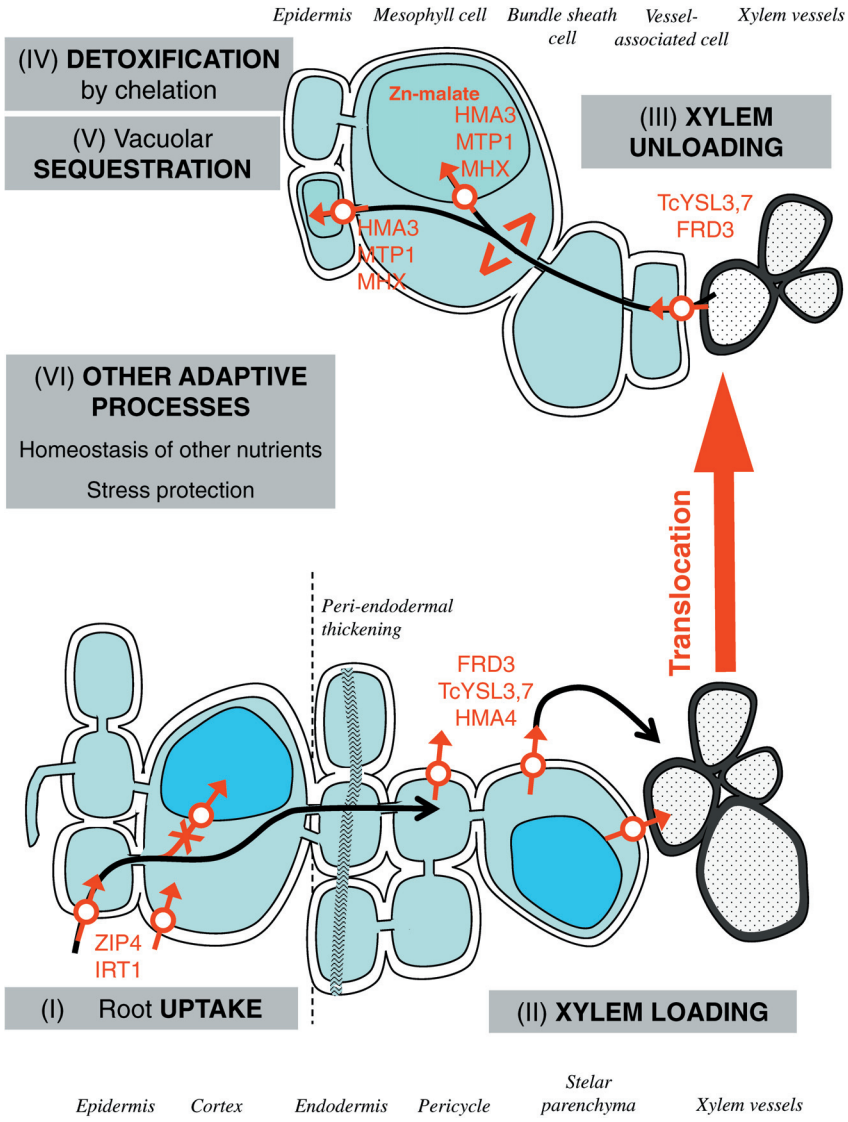

Fig. 1 Overview of our current understanding of adaptations to hyperaccumulate metals, in particular zinc (Zn). Those adaptations are highlighted in red. (I) Enhanced Zn uptake into root cells is thought to be driven by ZIP4 and to some extent by IRT1. ZIP3, ZIP9, and ZIP10 may also play a role, yet to be established. In *T. caerulescens* a peri-endodermal thickening (apoplastic barrier along the root axis) was observed. (II) Active xylem loading depends on reduced vacuolar root sequestration (main Zn storage is thought to be in the cortex) and enhanced activity of HMA4 in stelar parenchyma, leading to efficient efflux of Zn from symplasm. Efflux of citrate driven by FRD3 seems to play a role in Zn translocation. Loading of Zn in the xylem can also occur as Zn-nicotianamine complex by YSL proteins. (III) In the leaves, Zn is unloaded in vessel-associated cells by YSL proteins, and possibly by FRD3 in *Arabidopsis halleri*. Enhanced uptake of Zn in shoot symplastic pathway may be favored by ZIP6 in *A. halleri*. (IV) Detoxification is assumed to be operated by chelation of metals. < > refers to chelation. Possible ligands of Zn in the cytoplasm are histidine and nicotianamine. (V) Vacuolar sequestration in the leaves is thought to be the main pathway of detoxification of metals. Zn is mainly stored in vacuoles of mesophyll (*A. halleri*) and/or epidermal cells (*Thlaspi caerulescens*), through the activity of MTP1, and possibly HMA3 and MHX. In the vacuoles, a large pool of malate favors the formation of Zn-malate complexes. (VI) Other adaptive processes include homeostasis of other nutrients, in particular Fe (role of FER1, FER2, IREG2, NRAMP3 in *A. halleri* and *T. caerulescens*; NRAMP1,5 in *T. caerulescens*); P (PHT1-4), enhanced stress responses/protection (higher GSH level, higher level of defensins, etc.).

uptake systems are involved in Cd uptake (Roosens *et al.*, 2003). IRT1 has been suggested to be responsible for Cd hyperaccumulation in the Ganges population (Lombi *et al.*, 2001). However, in contrast to AtIRT1, TcIRT1 seems to be incapable of Cd transport (Plaza *et al.*, 2007).

Microarray analyses have highlighted the overexpression of more ZIP members in *A. halleri* and *T. caerulescens* (the homologs of *AtZIP3*, *AtZIP6*, *AtZIP9*, *AtZIP10* and *AtIRT3*), but their roles in plants and in Zn hyperaccumulation remain to be established (Becher *et al.*, 2004; Weber *et al.*, 2004, 2006; Filatov *et al.*, 2006; Hammond *et al.*, 2006; van de Mortel *et al.*, 2006, 2008; Talke *et al.*, 2006; reviewed in Krämer *et al.*, 2007). Candidate transporters responsible for Ni hyperaccumulation in serpentine *T. caerulescens* have not been identified. Several Ni-hyperaccumulating populations prefer Zn over Ni in experimental conditions (Assunção *et al.*, 2001), suggesting that Ni is taken up by a Zn transporter in these populations. However, in other populations, there may be Ni-preferent transporters (Peer *et al.*, 2003).

2. Translocation from the root to the shoot

Efficient translocation of metal ions to the shoot requires radial symplastic passage and active loading into the xylem (Clemens, 2006; Xing *et al.*, 2008). The need of ligands for all trace metals in the xylem is controversial. Salt *et al.* (1999) showed that most Zn in the xylem sap of *T. caerulescens* was present as the free hydrated Zn^{2+} ion. Similarly, Ueno *et al.* (2008) showed that Cd occurred mainly in the free ionic form in the xylem sap in *A. halleri*.

Availability of trace metals for translocation to the shoot implies limited sequestration in vacuoles of root cells (Fig. 1).

Lasat *et al.* (1998) observed a much lower accumulation of Zn in root vacuoles, as well as a faster release of Zn from the root vacuoles in *T. caerulescens*, compared with *T. arvense.* Yang *et al.* (2006) reported that in the nonaccumulating ecotype of *S. alfredii*, 2.7-times more Zn was retained in the root vacuoles than in the hyperaccumulating ecotype. Recently, Xing *et al.* (2008) reported variation in root vacuolar Cd fractions, associated with different translocation efficiencies, between two *T. caerulescens* populations.

Physiological studies of hyperaccumulators also demonstrated higher metal concentrations in the xylem sap due to enhanced xylem loading (Lasat *et al.*, 1998). Several types of transporters are involved in this process.

P-type ATPase-HMA Molecular studies and mutant analysis have identified particular P-type ATPases as being responsible for the Cd and Zn loading of the xylem from the surrounding vascular tissues. The P_{1B}-type ATPases, also known as the heavy metal transporting ATPases (HMAs), play an important role in transporting transition metal ions against their electrochemical gradient using the energy provided by ATP hydrolysis. In bacteria, P_{1B} ATPases are the main players in metal tolerance (Monchy *et al.*, 2007). HMAs cluster into two classes: those transporting monovalent cations (Cu/Ag group) and those transporting divalent cations (Zn/Co/Cd/Pb).

HMA4 was the first gene encoding a plant P_{1B}-type ATPase of the divalent transport group to be cloned and characterized in *A. thaliana* (Mills *et al.*, 2003). HMA4 is localized at the plasma membrane. A role for HMA4 in Zn homeostasis, Cd detoxification, and in the translocation of these metals from the root to the shoot has been demonstrated in *A. thaliana*, where *HMA4* expression seems to be confined to the stele (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2004, 2005). In both *A. halleri* and *T. caerulescens*, *HMA4* is more expressed in both roots and shoots compared with Cd/Zn-sensitive close relatives (Bernard *et al.*, 2004; Papoyan & Kochian, 2004; Hammond *et al.*, 2006; van de Mortel *et al.*, 2006; Talke *et al.*, 2006; Courbot *et al.*, 2007), strongly supporting the idea that HMA4 plays an important role in tolerance and/or accumulation of both metals. HMA4 would be involved in cytosolic metal efflux and particularly in root detoxification by translocating Cd and Zn to the shoot. In *A. halleri*, this hypothesis was reinforced by QTL analysis showing co-localization of a major QTL for Zn and Cd tolerance with the *HMA4* gene (Courbot *et al.*, 2007; Willems *et al.*, 2007). *HMA4* is currently the only gene for which there is genetic evidence for a role in both Zn and Cd tolerance. High *HMA4* expression in the BC_1 between *A. halleri* and *A. l. petraea* co-segregated with the *HMA4* allele originating from the *A. halleri* parent and with Cd tolerance (Courbot *et al.*, 2007). Recently, Hanikenne *et al.* (2008) showed that the enhanced *HMA4* expression in *A. halleri* results from triplication of a genomic region containing *HMA4*, as well as from altered cis-regulation. These authors demonstrated the importance of *HMA4* in the hyperaccumulation of Zn and hypertolerance to Cd using RNAi-mediated silencing. *A. halleri* plants (from a Cd-hypertolerant accession) with a lowered expression of *HMA4* translocated less Zn from the root to the shoot (Zn accumulation was shown in the pericycle cells) and were more sensitive to Cd and Zn treatments. Cd translocation was not measured. Interestingly, when expressed in *A. thaliana* under the activity of its own promoter, *AhHMA4* increased Zn and Cd sensitivity because of the absence of a detoxification mechanism with sufficient capacity to cope with the strongly enhanced metal accumulation in the leaves (Hanikenne *et al.*, 2008).

Transcriptomic studies in hyperaccumulators have also revealed a higher expression of genes encoding metal ligands or metal-ligand complex transporters (ligands are reviewed in the following subsection). Some of these transporters seem to play a role in trace metal translocation.

MATE MATE is a large family of multi-drug and toxic compound extrusion (or efflux) membrane proteins. Some members of the family were shown to function as drug/cation antiporters that remove toxic compounds and secondary metabolites from the cytosol by exporting them out of the cell or sequestering them to the vacuole (Delhaize *et al.*, 2007). FRD3 is a member of the MATE subfamily, which is thought to efflux citrate into the root vascular tissue. Citrate is necessary for the transport of Fe and possibly also Zn (Durrett *et al.*, 2007). *FRD3* is constitutively overexpressed in *A. halleri* and *T. caerulescens* compared with *A. thaliana* and may play a role in Zn translocation (van de Mortel *et al.*, 2006; Talke *et al.*, 2006).

OPT OPT is a superfamily of oligopeptide transporters including the yellow-stripe 1-like (YSL) subfamily. Some YSL transporters are involved in the loading and unloading of nicotianamine-metal chelates from the vascular tissues. There is evidence for a role of YSL transporters in the Zn and Ni hyperaccumulation of *T. caerulescens*, especially for *TcYSL3* and *TcYSL7*, which are expressed in xylem parenchyma and phloem (Gendre *et al.*, 2007, reviewed in Haydon & Cobbett, 2007). Furthermore, TcYSL3 was shown to transport Ni-NA chelates (Gendre *et al.*, 2007).

3. Sequestration in the shoot vacuoles

The ability to hyperaccumulate Zn, Ni and Cd seems to be governed, at least in part, by an enhanced capacity of metal storage in leaf vacuoles. Several families of transporters are involved in this process.

CDF The family of cation diffusion facilitators (CDF) in plants, also called metal transporter proteins (MTPs), contains members involved in the transport of Zn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+} not only from cytoplasm to organelles or apoplasm,

but also from the cytoplasm to the endoplasmic reticulum (Peiter *et al.*, 2007). *ZAT* (*ZINC TRANSPORTER OF ARABIDOPSIS THALIANA*), recently renamed *AtMTP1*, encodes a Zn transporter involved in vacuolar sequestration in *A. thaliana*. Overproduction of the Zn transporter *ZAT* in *A. thaliana* resulted in higher Zn tolerance and a twofold higher Zn accumulation in roots (Van der Zaal *et al.*, 1999). MTP1 homologs seem to be involved in the Zn hypertolerance trait. In *A. halleri*, *MTP1* was highly expressed, in both roots and shoots, due to expanded copy number (Dräger *et al.*, 2004). Two *MTP1* copies co-segregated with Zn tolerance in the BC_1 generation of a cross between *A. halleri* and *A. l. petraea* (Dräger *et al.*, 2004) and co-localized with two QTLs for Zn tolerance (Willems *et al.*, 2007). In *T. caerulescens* the *AtMTP1* homolog, *ZTP1*, was highly expressed in leaves and may play a role in vacuolar sequestration too (Assunção *et al.*, 2001).

In the Ni/Zn hyperaccumulator *T. goesingense*, other CDF members, TgMTP1t1 and TgMTP1t2 (derived from one single copy genomic sequence), were proposed to be involved in Ni vacuolar detoxification (Persans *et al.*, 2001). However a more recent study revealed that TgMTP1 seems to be localized at the plasma membrane, where it could mediate both Ni and Zn efflux from the cytoplasm (Kim *et al.*, 2004).

Other CDF members may play a role in the hypertolerance of other trace metals. ShMTP is involved in the vacuolar storage of Mn in the Mn-hypertolerant tropical legume *Stylosanthes hamata*. *ShMTP* conferred higher tolerance and accumulation of Mn when overexpressed in *A. thaliana* (Delhaize *et al.*, 2003). The role of CDF in Mn hyperaccumulators has not yet been investigated.

HMA A role of the AtHMA3 homolog in Zn hyperaccumulation was suggested by comparative transcriptome analysis between *A. halleri* (shoot) or *T. caerulescens* (root) and *A. thaliana* or *T. arvense* (Becher *et al.*, 2004; Hammond *et al.*, 2006; van de Mortel *et al.*, 2006). Yeast expression studies supported a role for AhHMA3 in Zn vacuolar transport (Becher *et al.*, 2004) but not for AtHMA3 (Gravot *et al.*, 2004). The difference in phenotypes can be due to the different strengths of the mutants defective in Zn transport that were used in the two studies. By contrast, the same authors showed evidence for Cd transport by AtHMA3 but not by AhHMA3.

CaCA In the Ca^{2+}/cation antiporter (CaCA) superfamily, MHX is a vacuolar Mg^{2+} and Zn^{2+}/H^+ exchanger (Shaul *et al.*, 1999): MHX protein was present in the leaves of *A. halleri* at much higher concentrations than in *A. thaliana* and was therefore proposed to play a role in Zn vacuolar storage. Since transcript levels were similar in both species, a difference in post-transcriptional regulation was postulated (Elbaz *et al.*, 2006).

Members of other CaCA subfamilies may also play a role in metal detoxification. CAX is the acronym for **ca**tion **ex**changer. It is a large family of membrane proteins, which was recently subdivided into 'true' CAX (CAX1–CAX6) and CCX (calcium cation exchanger) (CCX 1–5, previously named CAX 7–11) (Shigaki *et al.*, 2006). Apart from a recent report on CCX3 (Morris *et al.*, 2008), only CAX members of *A. thaliana* belonging to the true CAX clade have been characterized until now, and all seem to be involved in metal vacuolar sequestration, in particular of Cd. *In vitro* studies have shown a Cd^{2+}/H^+ antiport activity in tonoplast-enriched vesicles from oat roots with a K_m of 5.5 μM (Salt & Wagner, 1993). Clemens (2006) questioned the availability of Cd^{2+} ions in the micromolar range *in planta*. Nevertheless there is experimental evidence that AtCAX2 and AtCAX4 can transport Cd^{2+} into the vacuoles. Under the activity of the constitutive 35S CaMV promoter, overexpression of *AtCAX2* and *AtCAX4* resulted in higher accumulation of Cd into the root vacuoles (Korenkov *et al.*, 2007). Some of the *CAX* Arabidopsis homologs are highly expressed or differentially expressed in response to Zn in Zn/Cd hyperaccumulators: *CAX2* (Becher *et al.*, 2004, Weber *et al.*, 2006) and *CAX8*/*CCX2* in *A. halleri* (Craciun *et al.*, 2006); *CAX3* and *CAX7*/*CCX1* in *T. caerulescens* (van de Mortel *et al.*, 2006, 2008). However, their metal specificities have not been investigated, and yeast expression experiments suggest that CAX2 does not transport Zn (Becher *et al.*, 2004).

ABC The superfamily of ABC (ATP-binding cassette) transporters is involved in many physiological processes. Some of the ABC transporters are involved in vacuolar sequestration of various metals or xenobiotics. In two subfamilies, MRP and PRD, members are involved in the transport of chelated heavy metals or the organic acids necessary for the transport of heavy metals. There is strong evidence for a role in trace metal homeostasis (Song *et al.*, 2003; Hanikenne *et al.*, 2005; Kim *et al.*, 2006) and they may be expected to contribute to trace metal hyperaccumulation, in particular to vacuolar sequestration. Two ABC genes were identified in *T. caerulescens*: the *AtMRP10* homolog was shown to be differentially expressed in the shoots of two *T. caerulescens* populations displaying contrasting Zn tolerance and accumulation (Hassinen *et al.*, 2007) and *ATH13* was more expressed in the shoot compared with *A. thaliana* (van de Mortel *et al.*, 2008). However, direct evidence for a role of these genes in vacuolar sequestration is lacking.

4. Detoxification by chelation of trace metals

It is assumed that most of the hyperaccumulated metals are bound to ligands, such as organic acids, amino acids, peptides and proteins. While some aspects of metal detoxification by ligands have been discovered, there is no complete picture of the different chelators involved in the different stages of the plant-internal transport and storage of metals in hyperaccumulators. Furthermore, the nature of the chelators differs, depending on

the location within the plant and the age of the plant (Salt *et al.*, 1999). In *T. caerulescens* plants, a significant fraction of the Zn within the roots seemed to be associated with histidine, while in the shoot, most Zn was associated with organic acids (Salt *et al.*, 1999). Küpper *et al.* (2004) found that a large fraction of the foliar Cd was bound to sulfur ligands in *T. caerulescens*, whereas Ueno *et al.* (2005) deduced that Cd in the shoot was rather bound to organic acids, mainly malate.

For detailed analysis on ligands, we refer to the reviews of Callahan *et al.* (2006) and Haydon & Cobbett (2007). We summarize here some of the key ligands that seem to play a role in hyperaccumulation.

Histidine Histidine (His) is considered to be the most important free amino acid involved in hyperaccumulation (Callahan *et al.*, 2006). It forms stable complexes with Ni, Zn and Cd and it is present at high concentrations in hyperaccumulator roots (Persans *et al.*, 1999). In the Ni hyperaccumulator *Alyssum lesbiacum*, Ni exposure induced a dose-dependent increase in His in the xylem sap, which was not found in the nonhyperaccumulator congeneric species, *A. montanum*. Moreover, exogenously supplied His greatly enhanced Ni tolerance and both Ni and His loading into the xylem in *A. montanum*, but not in *A. lesbiacum*, probably due to the much higher constitutive root His pool in the latter species (Krämer *et al.*, 1996; Kerkeb & Krämer, 2003). In line with this, Ingle *et al.* (2005) observed an enhanced expression of the first enzyme of the His biosynthetic pathway, ATP-phosphoribosyltransferase, in *A. lesbiacum*, as compared with *A. montanum*. However, His overproducing transgenic *A. thaliana* lines displayed elevated Ni tolerance, but did not exhibit increased Ni concentrations in xylem sap or in leaves (Wycisk *et al.*, 2004; Ingle *et al.*, 2005). This suggests that His-dependent Ni xylem loading may not be universal in Brassicaceae, and that additional factors are required in at least *A. thaliana*. The mechanism of His-coupled Ni xylem loading has not been identified yet. Recent results obtained with *T. caerulescens* suggest that Ni-His complex formation strongly inhibits the retention of Ni in root cell vacuoles (K. H. Richau & H. Schat, pers. comm.).

Nicotianamine Synthesis of nicotianamine (NA) from 3 S-adenosyl-methionine (SAM) by NA synthase (NAS) is present in all plants. NA forms strong complexes with most transition metal ions. The role of NA seems to be in the movement of micronutrients throughout the plant (Stephan & Scholz, 1993). The study of the semi-lethal tomato *chloronerva* mutant, deficient in NAS, showed impairment in Fe and Cu distribution (Herbik *et al.*, 1996).

Nicotianamine seems to be involved in metal hyperaccumulation, both in *A. halleri* and in *T. caerulescens*, in which several *NAS* genes showed higher transcript levels. Higher expression of SAM synthetase genes may also be involved in enhanced nicotianamine synthesis (Talke *et al.*, 2006). Higher NA content was observed in *A. halleri* together with higher *AhNAS2* transcript and protein levels in roots (Weber *et al.*, 2004) and higher transcript levels of *AhNAS3* in the shoot compared with *A. thaliana* (Becher *et al.*, 2004). NA was proposed to act as a Zn cytosolic buffer, keeping Zn ions in a detoxified form, available for translocation to the shoot. Microarray analysis in *T. caerulescens* indicated that *NAS1*, *3* and *4* are more expressed than in *Arabidopsis* (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006). In this species a role for NA in Ni hyperaccumulation was proposed and Ni–NA complexes were identified in Ni-exposed roots (Vacchina *et al.*, 2003; Mari *et al.*, 2006). *TcNAS1* was constitutively highly expressed in the shoot of *T. caerulescens* plants (Mari *et al.*, 2006). In response to Ni, *NAS* was also induced in roots, where it chelated absorbed Ni and facilitated its transport to the shoot. Increase in Ni tolerance could be gained upon *NAS* overexpression in nontolerant species (Douchkov *et al.*, 2005; Kim *et al.*, 2005; Pianelli *et al.*, 2005). More recent results in *Thlaspi* hyperaccumulators support the assertion that NA is involved in hyperaccumulation of Ni but not of Zn (Callahan *et al.*, 2007).

Organic acids (citrate, malate) Because of the low association constants of organic acids with metals, Callahan *et al.* (2006) argued against a role for organic acids in the hyperaccumulation mechanism (such as long-distance transport), in spite of their constitutively elevated concentrations in hyperaccumulators (Lee *et al.*, 1978; Ueno *et al.*, 2005; Montargès-Pelletier *et al.*, 2008). So their role may be limited to vacuolar sequestration. The formation of metal–organic acid complexes is favored in the acidic environment of the vacuole (Haydon & Cobbett, 2007). A large proportion of Zn in the shoot was associated with malate in *A. halleri* (Sarret *et al.*, 2002). Ni seems to be mainly associated with citrate in the shoots of *Alyssum* (Lee *et al.*, 1978) and *T. goesingense* (Krämer *et al.*, 2000).

Glutathione Glutathione (Glu-Cys-Gly; GSH) is a major cellular antioxidant. It can form complexes with several metals and is the precursor of phytochelatins. Increased production of glutathione in *T. goesingense* and other *Thlaspi* Ni hyperaccumulators is thought to provide protection against oxidative damage due to high Ni concentrations (Freeman *et al.*, 2004). Enhanced glutathione synthesis is driven by constitutive activation of the sulfur assimilation pathway, in particular through enhanced activity of mitochondrial serine acetyltransferase (SATm) (Freeman *et al.*, 2004). The metal tolerance profile of *T. goesingense* was mimicked in *A. thaliana* expressing the *T.g SATm* gene (Freeman & Salt, 2007). Cd exposure also enhanced sulfate and GSH metabolism in *T. caerulescens* (van de Mortel *et al.*, 2008), and appeared to increase the foliar and root GSH concentrations in a hyperaccumulating *S. alfredii* population, but not in a nonaccumulating one, where GSH decreased owing to PC

synthesis (Sun *et al.*, 2007). These results suggest that there is a role for GSH in hyperaccumulation, which is probably associated with its antioxidant activity.

Phytochelatins Phytochelatins (PCs) (general formula $(GluCys)_nGly$, where $n = 2–11$) are synthesized enzymatically from glutathione in the presence of certain metals and metalloids and are ubiquitous in plants (reviewed in Clemens, 2006). Although PCs do have a role in basal metal detoxification, they do not seem to be involved in Cu, Cd, Zn, Co and Ni hypertolerance (Ebbs *et al.*, 2002; Schat *et al.*, 2002; Hernandez-Allica *et al.*, 2006). In hyperaccumulators, just as in nonhyperaccumulators, PCs are mainly induced in the roots, in particular by Cd, but not (or barely) by Zn or Ni, and considerable rates of Cd-induced PC accumulation have only been found in Cd-sensitive, nonmetallicolous or serpentine populations of *T. caerulescens* and in a nonaccumulating *S. alfredii* (Schat *et al.*, 2002; Sun *et al.*, 2007). Arsenic, which is normally a very effective inducer of PC synthesis in other species, induces only inconsiderable PC concentrations in the roots of the As hyperaccumulator, *Pteris vittata* (Zhao *et al.*, 2003). These results suggest that PCs are generally not essential for the hyperaccumulation phenotype.

Metallothioneins Variation in expression levels of MT family members between plant populations has been associated with variation in Cu tolerance (Murphy & Taiz, 1995; Van Hoof *et al.*, 2001; Jack *et al.*, 2007). MTs of the types 1, 2 and 3 are predominantly regulated by Cu, and seem to function in Cu accumulation and phloem Cu transport (Guo *et al.*, 2008).

Overexpression of several members of the MT family (type 1, 2 and 3) compared with Arabidopsis, and variations in expression levels between populations have been reported for *T. caerulescens* (Roosens *et al.*, 2004, 2005; Rigola *et al.*, 2006; Hassinen *et al.*, 2007). Type 3 MT was particularly strongly expressed in Cd-hyperaccumulating populations from southern France and was further induced by Cu addition. Several lines of evidence suggested that TcMT3 may be involved in Cu homeostasis (Roosens *et al.*, 2004). Recently, V. I. Hassinen *et al.* (pers. comm.) studied the co-segregation of various MTs with Zn accumulation in different crosses between *T. caerulescens* populations with different *MT* expression levels and found no significant co-segregation.

5. Other adaptive processes in hyperaccumulators

In hyperaccumulators there is evidence for modification of the homeostasis of metals other than the hyperaccumulated ones, such as Cu (Roosens *et al.*, 2004; Talke *et al.*, 2006), Mn (Talke *et al.*, 2006; Krämer *et al.*, 2007) and, in particular, Fe homeostasis (Filatov *et al.*, 2006; Hammond *et al.*, 2006; Talke *et al.*, 2006; van de Mortel *et al.*, 2006). Genes previously associated with iron homeostasis, such as a cytosolic aconitase gene, *IRT1*, *FERRITIN genes* (*FER1*, *FER2*), *NRAMP3*, *IRON REGULATED TRANSPORTER 2* (*IREG2*) and *FERRIC CHELATE REDUCTASE* genes (*FRO2*), are overexpressed in *A. halleri* and/or *T. caerulescens* (Becher *et al.*, 2004; Weber *et al.*, 2004; Filatov *et al.*, 2006; van de Mortel *et al.*, 2006, 2008; Talke *et al.*, 2006). A compelling example is NRAMP3, whose expression co-segregated with the Zn-hyperaccumulation phenotype in the F_3 progeny from a cross between *A. halleri* and *A. l. petraea* (Filatov *et al.*, 2006). In *A. thaliana*, *NRAMP3* is expressed in the vascular bundles of roots, stem and leaves. AtNRAMP3 is localized on the tonoplast and is proposed to remobilize vacuolar pools of Fe, Cd and Mn (Thomine *et al.*, 2003). The roles of AtNRAMP3 homologs in metal hyperaccumulation are not known. There is no evidence for a Zn transport activity for At or TcNRAMP3 (Thomine *et al.*, 2003; Oomen *et al.*, 2009). In support of a putative role in tolerance, the *nramp3nramp4* double mutant of *A. thaliana* displayed strong hypersensitivity to high Zn and Cd exposures. However, metal tolerance conferred by *TcNRAMP* expression in the *nramp3nramp4* mutant did not exceed that of wild-type *A. thaliana* (Oomen *et al.*, 2009).

Impact of hyperaccumulation on macronutrient homeostasis is suggested by overexpression of genes predicted to encode K^+ transporters and high-affinity phosphate transporters (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006).

Evolution of hyperaccumulation seems to have been accompanied by modification of signals and proteins usually involved in pathogen response. There is a constitutive overaccumulation of salicylic acid (SA) in nickel hyperaccumulators in the *Thlaspi* genus (Freeman *et al.*, 2005). SA is a key signal involved in plant pathogen response and may thus contribute to the elevated expression of pathogen-responsive genes. Overexpression of defensins/*PDF* genes was observed in *A. halleri* and *T. caerulescens* compared with *A. thaliana* (Becher *et al.*, 2004; van de Mortel *et al.*, 2006; Talke *et al.*, 2006). *A. halleri* defensin cDNAs (*AhPDF*) specifically induced higher Zn tolerance in yeast (Mirouze *et al.*, 2006). Defensins accumulated to a higher degree in *A. halleri* than in *A. thaliana*, and transgenic *A. thaliana* plants overexpressing *AhPDF* also showed slightly increased tolerance to Zn. The function of defensins is unclear. Plant defensins have usually been associated with an antifungal activity. The current hypothesis is that defensins interfere with divalent metal cation (perhaps Zn^{2+}) channels (Mirouze *et al.*, 2006).

VI. General discussion and research perspectives

1. Hyperaccumulation dissected with physiological and molecular tools

Hyperaccumulators constitute an exceptional biological material and gene reservoir that can be exploited to understand adaptation to extreme metallic environments. Physiological and classical genetic studies have been complemented by molecular studies, in particular transcriptome analysis.

Presently it is not always easy to reconcile the results of the different research approaches applied.

Physiological studies Physiological studies in *T. caerulescens* have revealed considerable diversity among populations with regard to the capacities and the specific metal-affinity patterns of the accumulation systems, although there may also be a basic system which is common to all the populations (Assunção *et al.*, 2001, 2003a,c, 2008; Zhao *et al.*, 2002). This system is possibly driven by *ZNT1* and *ZNT2* (the *AtZIP4* and *AtIRT3* homologs, respectively), which are highly expressed in all *T. caerulescens* populations investigated so far. As such, this variation is also reflected by genetic studies, yielding a broad segregation of accumulation traits, often with significant transgression in case of Zn. Moreover, QTL analyses of Cd and Zn accumulation yielded QTLs for Cd or Zn exclusively, but also a common one, and, in the case of Zn accumulation, with trait-enhancing alleles originating from different parents. Although intraspecific, these variations are up to one order of magnitude (Table 2). Unfortunately, QTL candidate genes have not been identified yet, primarily because the maps were AFLP-based, which makes it impossible to reconcile results with molecular work. Physiological and genetic analyses of *A. halleri* × *A. l. petraea* crosses also yielded consistent results, that is, the co-segregation of Cd and Zn tolerance was explained by a common QTL (Courbot *et al.*, 2007; Willems *et al.*, 2007). QTL analysis in this cross yielded candidate genes underlying the QTLs for Zn and Cd tolerance, in particular *HMA4* for Cd and Zn tolerance, and *MTP1A* and *MTP1B* for Zn tolerance. In agreement with the QTL analysis, Cd and Zn tolerance (and Zn accumulation) were partly lost in *A. halleri* upon silencing *HMA4* (Hanikenne *et al.*, 2008). To date, this is the only case of consistency among physiological, genetic and molecular results.

Microarray analyses Microarray analyses have undoubtedly advanced our knowledge of hyperaccumulation by providing promising candidate genes. However, differential expression of genes does not imply that they contribute to metal tolerance or accumulation (Roosens *et al.*, 2008). Differential expression can be at the origin or a consequence of the hyperaccumulation trait; or merely a result of divergence between species. Differential expression can also be the result of adaptation to environmental factors other than excess metals or, generally speaking, to linkage disequilibrium through selection at multiple loci. To distinguish genes directly involved in metal tolerance and/or accumulation from all the other genetic changes, regulation by the hyperaccumulated metal was taken as an additional selection criterion (van de Mortel *et al.*, 2006, 2008; Talke *et al.*, 2006). It is, however, possible that important players are not differentially regulated by Zn and have been lost in that selection. Another filter strategy was to combine genomics and classical genetics approaches and to do comparative transcriptomic analysis in contrasting phenotypes derived from crosses between a hyperaccumulator and a nonhyperaccumulator (Craciun *et al.*, 2006; Filatov *et al.*, 2006). However, the *HMA4* gene was not identified in these two studies, which were performed on progenies of crosses between *A. halleri* and *A. l. petraea*. In the study by Craciun *et al.* (2006), a comparative cDNA-AFLP analysis was applied to a cross segregating for Cd tolerance. Because this technique is based on a double digestion, it can generate short fragments, which are lost in the analysis. This was, for example, the case for *HMA4*. The reason why the gene did not pop up in the microarray analysis by Filatov *et al.* (2006) is elusive. An additional limitation of interspecies transcriptional studies is an underappreciation of genes with lower expression in hyperaccumulators. For example, twofold lower signal intensity was recorded for *T. caerulescens* compared with the *A. thaliana* signal intensities in the study of van de Mortel *et al.* (2006). Down-regulated genes can be important for hyperaccumulation; for example, down-regulation of genes encoding root vacuolar metal transporters is expected to result in decreased vacuolar metal sequestration in the root, allowing for a higher availability of metals for xylem loading. Finally, some genes can be expressed similarly at the transcript level, while being differentially regulated at the post-transcriptional level, resulting in different protein levels. This has been illustrated by the MHX case (Elbaz *et al.*, 2006).

Despite these drawbacks, a picture emerged for molecular mechanisms underlying hyperaccumulation, in particular for Zn (Fig. 1). The study of hyperaccumulators has further unraveled the role of genes involved in metal homeostasis previously identified in *A. thaliana*. There is a remarkably convergent core set of genes encoding members of the *ZIP*, *CDF*, *HMA* and *NRAMP* transporter families, as well as *FRD3* and *NAS* genes overexpressed in Zn hyperaccumulators studied so far. The identification of those genes enables transgenic strategies to engineer plants with higher tolerance capacities or modified accumulation of trace metals. Those transgenic plants could be designed for phytoremediation, biofortification or enhanced food safety (crops with lower accumulation of toxic metals in edible organs). The expectations from plant genetic modifications (PGM) are limited by ever-increasing regulation over PGM, as already mentioned by Baker & Whiting (2002), and green dogmatism.

The study of hyperaccumulators has also provided valuable insights into fundamental mechanisms of metal homeostasis that all plants possess in order to regulate the cellular concentrations of essential and nonessential metal ions. Yet many genes up-regulated in hyperaccumulators are still of unknown function. Nevertheless, at least some light has been shed on the molecular mechanisms of metal-ion uptake processes, chelation, translocation, detoxification and sequestration of trace metals. However, comparative studies with the model plant *A. thaliana* should be carefully considered because they are based on the assumption that conservation implies function. This assumption does not seem to be always true. For example TcZNT1 proteins

are the AtZIP4 homologs but they seem to transport different metals: AtIRT1 can transport Cd in addition to Fe, but the two homologs in *T. caerulescens* cannot (Plaza *et al.*, 2007).

2. Prospects for further research on hyperaccumulators

Little is known about the evolution of the mutations leading to the observed regulatory changes in the transcriptome. Trait evolution seems to be associated primarily with regulatory changes in gene expression. Bioinformatics methods offer a way to identify promising functional noncoding regions and to narrow the focus for experimental tests (Wray & Babbitt, 2008). Such tools are of limited help without the availability of complete hyperaccumulator genome sequences. Our understanding of hyperaccumulator genome evolution could be greatly improved by merging molecular and ecological genomics. One of the exciting research avenues is the search for signatures of recent adaptive evolution across candidate genes for metal tolerance or accumulation. Strong reductions in silent polymorphism across the genomic neighborhood of candidate genes would indicate whether the target gene has recently experienced fixation of an allele through directional positive selection.

The phenomenon of constitutive overexpression of a large array of genes seems to be a common process in the adaptation of plants to extreme environments. *Thellungilla halophila* (salt cress) is a close relative of *A. thaliana*, with which it displays 95% identity at the cDNA level (Inan *et al.*, 2004; Taji *et al.*, 2004; Gong *et al.*, 2005). Salt cress plants are salt-tolerant and can grow in 500 mM NaCl medium, without special morphological alterations. It appeared that a large number of genes that are stress-inducible in *A. thaliana* were constitutively overexpressed in *T. halophila*. The possible importance of epigenetics in plant adaptation to extreme environments has not yet been investigated.

Four possible mechanisms to account for the large transcriptional modifications seen in Zn hyperaccumulators have been summarized by Talke *et al.* (2006):

- specific cis-elements or trans-factors controlling transcript levels
- deregulation of the Zn sensing machinery
- higher Zn requirement
- indirect consequences of an alteration in the metal homeostasis network of another element.

Gene duplication (e.g. *HMA4*, *MTP1*, *ZIP3*, *ZIP9* in *A. halleri* and *ZIP4/ZNT1-2*, and *IRT1* in *T. caerulescens*) may contribute to the ability of hyperaccumulators to express genes at a very high level. In the case of *HMA4*, modification of cis-regulatory elements contributes to the high expression level of each copy (Hanikenne *et al.*, 2008). The possible importance of trans-acting factors has been little studied. A total of 131 genes encoding putative transcription factors were up-regulated (more than fivefold higher expression) in *T. caerulescens* compared with *A. thaliana* (van de Mortel *et al.*, 2006) but no clear candidates have popped up (van de Mortel *et al.*, 2008).

A deregulation of the root Zn deficiency response (Becher *et al.*, 2004; Talke *et al.*, 2006; Weber *et al.*, 2004, 2006) may be related to the higher Zn requirement and root-to-shoot translocation rates in hyperaccumulators. Overexpression of the *AhHMA4* gene in Arabidopsis induced typical markers of Zn deficiency. However, the degree of overexpression measured in hyperaccumulators is in general far beyond that induced by Zn deficiency in nonhyperaccumulator species. We currently have very little knowledge about the way plants gauge their metal status. Metal-sensor proteins have been demonstrated in bacteria (Busenlehner *et al.*, 2003) and yeast (Rutherford & Bird, 2004) but have not been established in plants. The existence of metal sensors in plants does suggest an interesting line of future study.

Another major issue of gene regulation is the tissue specificity of gene expression. Although microarray studies have provided new insights, the tissue specificity of genes has not been addressed yet. Metals in hyperaccumulators are unevenly distributed over foliar tissues, and there may be differences not only between hyperaccumulator species but even between populations in the tissue-specific accumulation of the same metal. For example, in *T. caerulescens*, the highest concentrations of Cd and Zn are found in the epidermal vacuoles (Ma *et al.*, 2005), but in *A. halleri* the highest concentrations are found in mesophyll cells (Küpper *et al.*, 1999, 2000). Thus, global transcriptomic analysis at the organ level might obscure useful information. For most of the genes, neither the tissue-specific expression patterns nor the cellular localization of their products are known. This greatly limits the prediction of their role in hyperaccumulation. Future advances in the molecular study of hyperaccumulation will require tissue-specific information at both the single gene and global levels. The development of a cell sorting system is presently hampered by technical bottlenecks such as low transformation efficiency. The possibility of transforming specific cell types of hyperaccumulators may help to decipher tissue specific adaptations. In the future, combination of cell sorting with microarray analysis will undoubtedly identify new players in hyperaccumulation mechanisms and provide novel genes for bioengineering of phytoextraction or biofortification.

The complexity of hyperaccumulation is far from being understood not only at the tissue level, but also at the subcellular level. Metal transport has been studied mainly at the plasma membrane or at the tonoplast. It is expected, however, that general metal homeostasis, including processes at all other endomembranes, will be modified in hyperaccumulators. Furthermore, transport mechanisms are still poorly understood for most of the metal transporters highlighted in this review. The form of the metal that is handled by transporters is also a matter of debate and the possible role of metal-chaperones has so far only been demonstrated for Cu.

In general, there is a lack of studies at the protein level (in particular of membrane proteins) in hyperaccumulators. In the absence of protein data or functional study, the biological

significance of changes at transcript levels remains to be established.

Despite important recent progress enabled by the application of new techniques, only a tiny piece of the whole hyperaccumulation puzzle has been uncovered. Plant scientists' skills are urgently required to unlock the remaining secrets of hyperaccumulators.

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References

Assunção AGL, Bleeker P, ten Bookum WM, Vooijs R, Schat H. 2008. Intraspecific variation of metal preference patterns for hyperaccumulation in *Thlaspi caerulescens*: evidence from binary metal exposures. *Plant and Soil* **303**: 289–299.

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.

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

Assunção AGL, Da Costa Martins P, 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, Pieper B, Vromans J, Lindhout P, Aarts MGM, Schat H. 2006. Construction of a genetic linkage map of *Thlaspi caerulescens* and quantitative trait loci analysis of zinc accumulation. *New Phytologist* **170**: 21–32.

Assunção AGL, Schat H, Aarts GM. 2003a. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**: 351–360.

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, Whiting SN. 2002. In search of the Holy Grail – a further step in understanding metal hyperaccumulation? *New Phytologist* **155**: 1–4.

Becher M, Talke IN, Krall L, Krämer U. 2004. Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37**: 251–268.

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N. 2004. A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569**: 140–148.

Bert V, Bonnin I, Saumitou-Laprade P, de Laguérie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155**: 47–57.

Bert V, Macnair MR, De Laguerie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae) *New Phytologist* **146**: 225–233.

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.

Boyd RS. 2007. The defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. *Plant and Soil* **293**: 153–176.

Boyd RS, Martens SN. 1992. The raison d'être for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD, eds. *The vegetation of ultramafic (serpentine) soils*. Andover, UK: Intercept, 279–289.

Boyd RS, Martens SN. 1994. Nickel hyperaccumulated by *Thlaspi montanum* var. *montanum* is acutely toxic to an insect herbivore. *Oikos* **70**: 21–25.

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. *New Phytologist* **173**: 677–702.

Brooks RR, Lee J, Jaffré T. 1974. Some New Zealand and New Caledonian plant accumulators of nickel. *Journal of Ecology* **62**: 493–499.

Brooks RR, Lee J, Reeves RD, Jaffré T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration* **7**: 49–57.

Busenlehner LS, Pennella MA, Giedroc DP. 2003. The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. *FEMS Microbiology Reviews* **2–3**: 131–43.

Callahan DL, Baker AJM, Kolev SD, Wedd AG. 2006. Metal ion ligands in hyperaccumulating plants. *Journal of Biological Inorganic Chemistry* **11**: 2–12.

Callahan DL, Kolev SD, O'Hair RA, Salt DE, Baker AJ. 2007. Relationships of nicotianamine and other amino acids with nickel, zinc and iron in *Thlaspi* hyperaccumulators. *New Phytologist* **176**: 836–48.

Chaney RL, Angle JS, McIntosh MS, Reeves RD, Li YM, Brewer EP, Chen KY, Roseberg RJ, Perner H, Synkowski EC *et al.* 2005. Using hyperaccumulator plants to phytoextract soil Ni and Cd. *Zeitschrift Naturforschung* **60**: 190–198.

Chaney R, Malik M, Li Y M, Brown SL, Brewer EP, Angle JS, Baker AJM. 1997. Phytoremediation of soil metals. *Current Opinion in Biotechnology* **8**: 279–284.

Clemens S. 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88**: 1707–1719.

Cosio C, Martinoia E, Keller C. 2004. Hyperaccumulation of Cadmium and Zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. *Plant Physiology* **134**: 716–725.

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N. 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase. *Plant Physiology* **144**: 1052–1065.

Craciun A, Courbot M, Bourgis F, Salis P, Saumitou Laprade P, Verbruggen N. 2006. Comparative cDNA-AFLP analysis of Cd-tolerant and -sensitive genotypes derived from crosses between the Cd hyperaccumulator *Arabidopsis halleri* and *Arabidopsis lyrata* ssp. *petraea*. *Journal of Experimental Botany* **57**: 2967–2983.

Dechamps C, Noret N, Mozek R, Escarré J, Lefèbvre C, Gruber W, Meerts P. 2008. Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. *New Phytologist* **177**: 167–177.

Delhaize E, Gruber BD, Ryan PR. 2007. The roles of organic anion permeases in aluminium resistance and mineral nutrition. *FEBS Letters* **581**: 2255–2262.

Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR. 2003. Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. *The Plant Cell* **15**: 1131–1142.

Deniau A, Pieper B, Ten Bookum WM, Lindhout P, Aarts MGM, Schat H. 2006. QTL analysis of cadmium and zinc accumulation in the heavy metal hyperaccumulator *Thlaspi caerulescens. Theoretical and Applied Genetics* **113**: 907–920.

Douchkov D, Gryczka C, Stephan UW, Hell R, Baümlein H. 2005. Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. *Plant, Cell & Environment* **28**: 365–374.

Dräger DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Krämer U. 2004. Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39**: 425–439.

Dubois S, Cheptou P-O, Petit C, Meerts P, Poncelet M, Vekemans X, Lefèbvre C, Escarré J. 2003. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Durrett TP, Gassmann W, Rogers EE. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology* **144**: 197–205.

Ebbs S, Lau I, Ahner B, Kochian L. 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens. Planta* **214**: 635–640.

Elbaz B, Shoshani-Knaani N, David-Assael O, Mizrachy-Dagri T, Mizrahi K, Saul H, Brook E, Berezin I, Shaul O. 2006. High expression in leaves of the zinc hyperaccumulator *Arabidopsis halleri* of AhMHX, a homolog of an *Arabidopsis thaliana* vacuolar metal/proton exchanger. *Plant, Cell & Environment* **29**: 1179–1190.

Ellis DR, Salt DE. 2003. Plants, selenium and human health. *Current Opinion in Plant Biology* **6**: 273–279.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière 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.

Faucon MP, Shutcha N, Meerts P. 2007. Revisiting copper and cobalt concentrations in supposed hyperaccumulators from SC Africa: influence of washing and metal concentrations in soil. *Plant Soil* **301**: 29–36.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MM. 2006. Comparison of gene expression in segregating families identifies genes and genomic regions involved in a novel adaptation, zinc hyperaccumulation. *Molecular Ecology* **15**: 3045–3059.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MR. 2007. A quantitative trait loci analysis of zinc hyperaccumulation in *Arabidopsis halleri*. *New Phytologist* **174**: 580–590.

Freeman JL, Garcia D, Kim D, Hopf A, Salt DE. 2005. Constitutively elevated salicylic acid signals glutathione-mediated nickel tolerance in *Thlaspi* nickel hyperaccumulators. *Plant Physiology* **137**: 1082–1091.

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer W, Pickering IJ, Salt DE. 2004. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *The Plant Cell* **16**: 2176–2191.

Freeman JL, Salt DE. 2007. The metal tolerance profile of *Thlaspi goesingense* is mimicked in *Arabidopsis thaliana* heterologously expressing serine acetyltransferase. *BMC Plant Biology* **7**: 63.

Frérot H, Petit 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.

Galeas ML, Klamper EM, Bennett LE, Freeman JL, Kondratieff BC, Quinn CF, Pilon-Smits EA. 2008. Selenium hyperaccumulation reduces plant arthropod loads in the field. *New Phytologist* **177**: 715–724.

Gendre D, Czernic P, Conéjéro G, Pianelli K, Briat J-F, Lebrun M, Mari S. 2007. TcYSL3, a member of the YSL gene family from the hyperaccumulator *Thlaspi caerulescens*, encodes a nicotianamine-Ni/Fe transporter. *Plant Journal* **49**: 1–15.

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ. 2005. Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana. Plant Journal* **44**: 826–39.

Gravot A, Lieutaud A, Verret F, Auroy P, Vavasseur A, Richaud P. 2004. AtHMA3, a plant P1B-ATPase, functions as a Cd/Pb transporter in yeast. *FEBS Letters* **561**: 22–28.

Guo WJ, Meetam M, Goldsbrough PB. 2008. Examining the specific contributions of individual Arabidopsis metallothioneins to copper distribution and metal tolerance. *Plant Physiology* **146**: 1697–706.

Haines BJ. 2002. Zincophilic root foraging in *Thlaspi caerulescens*. *New Phytologist* **155**: 363–372.

Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJ, Whiting SN, May ST, Broadley MR. 2006. A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytologist* **170**: 239–260.

Hanikenne M, Krämer U, Demoulin V, Baurain D. 2005. A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschizon merolae*. *Plant Physiology* **137**: 428–446.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* **453**: 391–395.

Hassinen VH, Tervahauta AI, Halimaa P, Plessl S, Peräniemi H, Aarts MGM, Servomaa K, Kärenlampi SO. 2007. Isolation of Zn-responsive genes from two accessions of the hyperaccumulator plant *Thlaspi caerulescens*. *Planta* **225**: 977–989.

Haydon MJ, Cobbett CS. 2007. Transporters of ligands for essential metal ions in plants. *New Phytologist* **174**: 499–506.

Herbik A, Giritch A, Horstmann C, Becker R, Balzer HJ, Bäumlein H, Stephan UW. 1996. Iron and copper nutrition-dependent changes in protein expression in a tomato wild type and the nicotianamine-free mutant chloronerva. *Plant Physiology* **111**: 533–540.

Hernandez-Allica J, Garbisu C, Becerril JM, Barrutia O, Garcia-Plazaola JI, Zhao FJ, McGrath SP. 2006. Synthesis of low molecular weight thiols in response to Cd exposure in *Thaspi caerulescens*. *Plant, Cell & Environment* **29**: 1422–1429.

Huitson SB, Macnair MR. 2003. Does zinc protect the zinc hyperaccumulator *Arabidopsis halleri* from herbivory by snails? *New Phytologist* **159**: 453–459.

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbetta CS. 2004. P-Type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *The Plant Cell* **16**: 1327–1339.

Inan G, Zhang Q, Li P, Wang Z, Cao Z, Zhang H, Zhang C, Quist TM, Goodwin SM, Zhu J *et al.* 2004. Salt cress. A halophyte and cryophyte *Arabidopsis* relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. *Plant Physiology* **135**: 1718–1737.

Ingle RA, Mugford ST, Rees JD, Campbell MM, Smith JAC. 2005. Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. *The Plant Cell* **17**: 2089–2106.

Jack E, Hakvoort HWJ, Reumer A, Verkleij JAC, Schat H, Ernst WHO. 2007. Real-time PCR analysis of metallothionein-2b expression in metallicolous and nonmetallicolous populations of *Silene vulgaris* (Moench) Garcke. *Environmental and Experimental Botany* **59**: 84–91.

Jaffré T, Brooks RR, Lee J, Reeves RD. 1976. *Sebertia acuminata*: a hyperaccumulator of nickel from New Caledonia. *Science* **193**: 579–580.

Jhee EM, Dandridge KL, Christy AM, Pollard AJ. 1999. Selective herbivory on low-zinc phenotypes of the hyperaccumulator *Thlaspi caerulescens* (Brassicaceae). *Chemoecology* **9**: 93–95.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Kerkeb L, Krämer U. 2003. The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiology* **131**: 716–24.

Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE. 2004. The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. *Plant Journal* **39**: 237–251.

Kim D, Takahashi M, Higuchi K, Tsunoda K, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK. 2005. Increased nicotianamine biosynthesis confers enhanced tolerance to high levels of metals, in particular nickel, to plants. *Plant Cell Physiology* **46**: 1809–1818.

Kim DY, Bovet L, Kushnir S, Noh EW, Martinoia E, Lee Y. 2006. AtATM3 Is Involved in Heavy Metal Resistance in Arabidopsis. *Plant Physiology* **140**: 922–932.

Korenkov V, Park SH, Cheng NH, Sreevidya C, Lachmansingh J, Morris J, Hirschi K, Wagner GJ. 2007. Enhanced Cd^{2+} selective root-tonoplast-transport in tobaccos expressing *Arabidopsis* cation exchangers. *Planta* **225**: 403–411.

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC. 1996. Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**: 635–638.

Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE. 2000. Subcellular localization and speciation of nickel in hyperaccumulator and nonaccumulator *Thlaspi* species. *Plant Physiology* **122**: 1343–1353.

Krämer U, Smith RD, Wenzel WW, Raskin I, Salt DE. 1997. The role of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Hálácsy. *Plant Physiology*. **115**: 1641–1650.

Krämer U, Talke I, Hanikenne M. 2007. Transition metal transport. *FEBS Letters* **581**: 2263–2272.

Küpper H, Lombi E, Zhao FJ, McGrath SP. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212**: 75–84.

Küpper H, Mijovilovich A, Meyer-Klaucke W, Kroneck PM. 2004. Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by x-ray absorption spectroscopy. *Plant Physiology* **134**: 748–757.

Küpper H, Zhao FJ, McGrath SP. 1999. Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **119**: 305–311.

Lane TW, Morel FM. 2000. A biological function for cadmium in marine diatoms. *Proceedings of the National Academy of Sciences, USA* **97**: 4627–4631.

Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FM. 2005. A cadmium enzyme from a marine diatom. *Nature* **435**: 42.

Lasat MM, Baker AJM, Kochian LV. 1996. Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of Thlaspi. *Plant Physiology* **112**: 1715–1722.

Lasat MM, Baker AJM, Kochian LV. 1998. Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiology* **118**: 875–883.

Lee J, Reeves RD, Brooks RR, Jaffré T. 1978. The relation between nickel and citric acid in some nickel-accumulating plants. *Phytochemistry* **17**: 1033–1035.

Lee JG, Roberts SB, Morel FMM. 1995. Cadmium: a nutrient for the marine diatom *Thalassiosira weissflogii*. *Limnology and Oceanography* **40**: 1056–1063.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R, Nelkin J. 2003. Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant and Soil* **249**: 107–115.

Liu MQ, Yanai J, Jiang RF, Zhang F, McGrath SP, Zhao FJ. 2008. Does cadmium play a physiological role in the hyperaccumulator *Thlaspi caerulescens*? *Chemosphere* **71**: 1276–1283.

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

Ma JF, Ueno D, Zhao FJ, McGrath SP. 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Planta* **220**: 731–736.

Macnair MR. 2002. Within and between population genetic variance for zinc accumulation in *Arabidopsis halleri*. *New Phytologist* **155**: 59–66.

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 Biological Sciences* **266**: 2175–2179.

Mari S, Gendre D, Pianelli K, Ouerdane L, Lobinski R, Briat J-F, Lebrun M, Czernic P. 2006. Root-to-shoot long-distance circulation of nicotianamine and nicotianamine–nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* **57**: 4111–4122.

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

Mengoni A, Baker AJM, Bazzicalupo M, Reeves RD, Adigüzel N, Chianni E, Galardi F, Gabbrielli R, Gonnelli C. 2003. Evolutionary dynamics of nickel hyperaccumulation in *Alyssum* revealed by ITS nrDNA analysis. *New Phytologist* **159**: 691–699.

Mills RF, Francini A, da Rocha PSCF, Baccarini PJ, Aylett M, Krijger GC, Williams LE. 2005. The plant P-1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS Letters* **579**: 783–791.

Mills RF, Krijger GC, Baccarini PJ, Hall JL, Williams LE. 2003. Functional expression of AtHMA4, a P-1B-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal* **35**: 164–176.

Milner MJ, Kochian LV. 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany* **102**: 3–13.

Mirouze M, Sels J, Richard O, Czernic P, Loubet S, Jacquier A, François IE, Cammue BP, Lebrun M, Berthomieu P *et al.* 2006. A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. *Plant Journal* **47**: 329–342.

Monchy S, Benotmane MA, Janssen P, Vallaeys T, Taghavi S, van der Lelie D, Mergeay M. 2007. Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialized in the maximal viable response to heavy metals. *Journal of Bacteriology* **189**: 7417–7425.

Montargès-Pelletier E, Chardot V, Echevarria G, Michot LJ, Bauer A, Morel JL. 2008. Identification of nickel chelators in three hyperaccumulating plants: an X-ray spectroscopic study. *Phytochemistry* **69**: 1695–1709.

Morris J, Tian H, Park S, Sreevidya CS, Ward JM, Hirschi KD. 2008. AtCCX3 is an Arabidopsis endomembrane H^+-dependent K^+ transporter. *Plant Physiology* **148**: 1474–1486.

van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H, Ghandilyan A, Tsiatsiani S, Aarts MG. 2008. Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in Arabidopsis thaliana and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **31**: 301–324.

van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM. 2006. Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **142**: 1127–1147.

Murphy A, Taiz L. 1995. Comparison of metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. Correlation with copper tolerance. *Plant Physiology* **109**: 945–954.

Noret N, Meerts P, Vanhaelen M, Dos Santos A, Escarré J. 2007. Do metal-rich plants deter herbivores? A field test of the defence hypothesis. *Oecologia* **152**: 92–100.

Oomen RJFJ, Wu J, Lelievre F, Blanchet S, Richaud P, Barbier-Brygoo H, Aarts MG, Thomine S. 2009. Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **181**: 637–650.

Palmgren MG, Clemens S, Williams LE, Krämer U, Borg S, Schjørring JK, Sanders D. 2008. Zinc biofortification of cereals: problems and solutions. *Trends Plant Science*. **13**: 464–73.

Papoyan A, Kochian LV. 2004. Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136**: 3814–3823.

Pauwels M, Frerot H, Bonnin I, Saumitou-Laprade P. 2006. A broad-scale analysis of population differentiation for Zn tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *European Society for Evolutionary Biology* **19**: 1838–1850.

Peer WA, Mamoudian M, Lahner B, Reeves RD, Murphy AS, Salt DE. 2003. Identifying model metal hyperaccumulating plants: germplasm analysis of 20 Brassicaceae accessions from a wide geographical area. *New Phytologist*, **159**: 421–430.

Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D. 2007. A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. *Proceedings of the National Academy of Sciences, USA* **104**: 8532–8537.

Pence NS, Larsen PB, Ebbs SD, Letham DBL, 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.

Persans MW, Nieman K, Salt DE. 2001. Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. *Plant Biology* **98**: 9995–10000.

Persans MW, Yan X, Patnoe JM, Krämer U, Salt DE. 1999. Molecular dissection of the role of histidine in nickel hyperaccumulation in *Thlaspi goesingense* (Hálácsy). *Plant Physiology* **121**: 1117–1126.

Pianelli K, Mari S, Marquez L, Lebrun M, Czernic P. 2005. Nicotianamine over-accumulation confers resistance to nickel in *Arabidopsis thaliana*. *Transgenic Research* **14**: 739–748.

Pilon-Smits E. 2005. Phytoremediation. *Annual Review of Plant Biology*. **56**: 15–39.

Plaza S, Tearall KL, Zhao FJ, Buchner P, McGrath SP, Hawkesford MJ. 2007. Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* **58**: 1717–28.

Pollard AJ, Baker AJM. 1997. Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **135**: 655–665.

Reeves RD. 2003. Tropical hyperaccumulators of metals and their potential for phytoextraction. *Plant and Soil* **249**: 57–65.

Reeves RD. 2006. Hyperaccumulation of trace elements byplants. In: Morel JL, Echevarria G, Goncharova N, eds. *Phytoremediation of metal-contaminated soils. NATO science series: IV: earth and environmental sciences*, Vol. 68. New York, NY, USA: Springer, 1–25.

Reeves RD, Baker AJM. 2000. Metal-accumulating plants. In: Raskin I, Ensley BD, eds. *Phytoremediation of toxic metals: using plants to clean up the environment*. New York, NY, USA: John Wiley, 193–229.

Richau KH, Schat H. 2008. Intraspecific variation of nickel and zinc accumulation and tolerance in the hyperaccumulator *Thlaspi caerulescens*. *Plant and Soil* **314**: 253–262.

Rigola D, Fiers M, Vurro E, Aarts MG. 2006. The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New Phytologist* **170**: 753–765.

Roosens NH, Bernard C, Leplae R, Verbruggen N. 2004. Evidence for copper homeostasis function of metallothionein (MT3) in the hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **577**: 9–16.

Roosens NH, Leplae R, Bernard C, Verbruggen N. 2005. Variations in plant metallothioneins: the heavy metal hyperaccumulator *Thlaspi caerulescens* as a study case. *Planta* **222**: 716–729.

Roosens NH, Willems G, Saumitou-Laprade P. 2008. Using *Arabidopsis* to explore zinc tolerance and hyperaccumulation. *Trends in Plant Science* **13**: 208–215.

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.

Rutherford JC, Bird AJ. 2004. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. *Eukaryotic Cell* **3**: 1–13.

Salt DE, Prince RC, Baker AJM, Raskin I, Pickering IJ. 1999. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environmental Science and Technology* **33**: 713–717.

Salt DE, Wagner GJ. 1993. Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd^{2+}/H^{+} antiport activity. *Journal of Biological Chemistry* **268**: 12297–12302.

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MA, Manceau A. 2002. Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **130**: 1815–1826.

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM. 2002. The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and nonhyperaccumulator metallophytes. *Journal of Experimental Botany* **53**: 2381–2392.

Shaul O, Hilgemann DW, de-Almeida-Engler J, Van Montagu M, Inzé D, Galili G. 1999. Cloning and characterization of a novel Mg(2+)/H(+) exchanger. *EMBO Journal* **18**: 3973–3980.

Shigaki T, Rees I, Nakhleh L, Hirschi KD. 2006. Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. *Journal of Molecular Evolution* **63**: 815–25.

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y. 2003. Engineering tolerance and accumulation of lead and cadmium in transgenic plants. *Nature Biotechnology* **21**: 914–919.

Sors TG, Ellis DR, Na GN, Lahner B, Lee S, Leustek T, Pickering IJ, Salt DE. 2005. Analysis of sulfur and selenium assimilation in *Astragalus* plants with varying capacities to accumulate selenium. *Plant Journal* **42**: 785–797.

Stephan UW, Scholz G. 1993. Nicotianamine: mediator of transport of iron and heavy metals in the phloem? *Physiologia Plantarum* **88**: 522–529.

Sun Q, Ye ZH, Wang XR, Wong MH. 2007. Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*. *Journal of Plant Physiology* **164**: 1489–98.

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K. 2004. Comparative genomics in salt tolerance between Arabidopsis and Arabidopsis-related halophyte salt cress using Arabidopsis microarray. *Plant Physiology* **135**: 1697–1709.

Talke I, Hanikenne M, Krämer U. 2006. Zinc dependent global

transcriptional control, transcriptional de-regulation and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142**: 148–167.

Taylor SI, Macnair MR. 2006. Within and between population variation for zinc and nickel accumulation in two species of *Thlaspi* (Brassicaceae). *New Phytologist* **169**: 505–514.

Thomine S, Lelièvre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H. 2003. AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant Journal* **34**: 685–695.

Ueno D, Iwashita T, Zhao FJ, Ma JF, 2008. Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd-hyperaccumulator *Arabidopsis halleri*. *Plant Cell Physiology* **49**: 540–548.

Ueno D, Ma JF, Iwashita T, Zhao FJ, McGrath SP. 2005. Identification of the form of Cd in the leaves of a superior Cd accumulating ecotype of *Thlaspi caerulescens* using Cd-NMR. *Planta* **221**: 928–936.

Vacchina V, Mari S, Czernic P, Marquès L, Pianelli K, Schaumlöffel D, Lebrun M. 2003. Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation. *Analytical Chemistry* **75**: 2740–2745.

Van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JAC, Hooykaas PJJ. 1999. Overexpression of a novel *Arabidopsis* gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology* **119**: 1047–1055.

Van Hoof NALM, Hassinen VH, Hakvoort HWJ, Ballintijn KF, Schat H, Verkleij JAC, Ernst WHO, Karenlampi SO, Tervahauta AI. 2001. Enhanced copper tolerance in *Silene vulgaris* (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. *Plant Physiology* **126**: 1519–1526.

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P. 2004. Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576**: 306–312.

Verret F, Gravot A, Auroy P, Preveral S, Forestier C, Vavasseur A, Richaud P. 2005. Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His(11) stretch. *FEBS Letters* **579**: 1515–1522.

Vogel-Mikuš K, Regvar M, Mesjasz-Przybyłowicz J, Przybyłowicz WJ, Simčič J, Pelicon P, Budnar. 2005. Spatial distribution of cadmium in leaves of metal hyperaccumulating *Thlaspi praecox* using micro-PIXE. *New Phytologist* **179**: 712–721.

Weber M, Harada E, Vess C, von Roepenack-Lahaye E, Clemens S. 2004. Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37**: 269–281.

Weber M, Trampczynska A, Clemens S. 2006. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell & Environment* **29**: 950–963.

Wenzel WW, Bunkowski M, Puschenreiter M, Horak O. 2003. Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and excluder plants on serpentine soil. *Environmental Pollution* **123**: 131–138.

Whiting SN, Leake JR, McGrath SP, Baker AJM. 2000. Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **145**: 199–210.

Whiting SN, de Souza MP, Terry N. 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. *Environmental Science Technology* **35**: 3144–50.

Willems G, Drager DB, Courbot M, Gode C, Verbruggen N, Saumitou-Laprade P. 2007. The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri* (*Brassicaceae*): an analysis of quantitative trait loci. *Genetics* **176**: 659–674.

Wray G, Babbitt CC. 2008. Enhancing gene regulation. *Science* **321**: 1300–1301.

Wycisk K, Kimb EJ, Schroeder JI, Krämer U. 2004. Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*. *FEBS Letters* **578**: 128–134.

Xing JP, Jiang RF, Ueno D, Ma JF, Schat H, McGrath SP, Zhao FJ. 2008. Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi* caerulescens and *Thlaspi praecox*. *New Phytologist* **178**: 315–325.

Yang XE, Li TQ, Long XX, Xiong XH, He ZH, Stoffella PJ. 2006. Dynamics of zinc uptake and accumulation in the hyperaccumulating and nonhyperaccumulating ecotypes of *Sedum alfredii* Hance. *Plant and Soil* **284**: 109–119.

Yang XE, Long XX, Ye HB, He ZL, Calvert DV, Stoffella PJ. 2004. Cadmium tolerance and hyperaccumulation in a new Zn hyperaccumulating plant species (*Sedum alfredii* Hance). *Plant and Soil* **259**: 181–189.

Zha HG, Jiang RF, Zhao FJ, Vooijs R, Schat H, Barker JHA, McGrath SP. 2004. Co-segregation analysis of cadmium and zinc accumulation in *Thlaspi caerulescens* interecotypic crosses. *New Phytologist* **163**: 299–312.

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, Hamon RE, McLaughlin MJ. 2001. Root exudates of the hyperaccumulator *Thlaspi caerulescens* do not enhance metal mobilization. *New Phytologist* **151**: 613–620.

Zhao FJ, Jiang RF, Dunham SJ, McGrath SP. 2006. Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist* **172**: 646–654.

Zhao FJ, Wang JR, Barker JHA, Schat H, Bleeker PM, McGrath SP. 2003. The role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris vittata*. *New Phytologist* **159**: 403–410.