Chapter 3
Plants that Hyperaccumulate Heavy Metals

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Abstract Heavy metal hyperaccumulators are plants that can tolerate and accumulate extremely high concentrations of metals in their shoots. This reflects the enhancement of physiological processes such as metal uptake, mobilization, translocation, and detoxification by chelation and vacuolar sequestration. Hyperaccumulation occurs in approximately 500 taxa of angiosperms and is particularly common among the Brassicaceae. Several candidate genes have been proposed as determinants of heavy metal hyperaccumulation. They predominantly encode transporters involved in metal translocation and storage, and also chelators and genes involved in stress responses.

Keywords Hyperaccumulator · Elemental defense · Metal transporter · Metal ligand

3.1 Defining Hyperaccumulator Plants

The first plant species reported to accumulate extremely high levels of metals was *Alyssum bertolonii*, whose Ni content was greater than 1 mg g^{-1} dry weight (Minguzzi and Vergnano 1948). However, the term "hyperaccumulator" was coined only in 1976 to describe plants whose shoot metal concentration is some orders of magnitude higher than adjacent plants (Jaffrè et al. 1976). This definition implies high rates of metal uptake in roots, translocation, and accumulation in

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Table 3.1 Hyperaccumulation thresholds for the most relevant heavy metals, in comparison with the average content in plant tissues and toxicity levels

Element	Average range in plant tissues (mg/kg dw)[a]	Critical toxicity level (mg/kg dw)[b]	Threshold for hyperaccumulators (mg/kg dw)[a,b]
As	<0.01–4[c]	<2–80	>1,000
Cd	0.03–0.5	6–10	>100
Co	0.01–3[d]	0.4-several	>1,000
Cu	2–20	20–30	>1,000
Cr	0.2[e]	0.2–1	>1,000
Pb	0.1–5	0.6–28	>1,000
Mn	1–700	200–3,500	>10,000
Hg	0.005–0.2	0.001–5[f]	>1,000
Ni	0.4–4	10–50	>1,000
Se[h]	0.01–0.2	3–100	>1,000
Tl	0.1–1.5[g]	20	>1,000
Zn	15–150	100–300	>10,000

[a] *from* Maestri et al. (2010)

[b] *from* Krämer (2010)

[c] *from* National Research Council, Committee on Medical and Biological Effects of Environmental Pollutants (1977)

[d] *from* Sillanpää and Jansson (1992)

[e] *from* National Research Council, Committee on Biologic Effects of Atmospheric Pollutants (1974)

[f] *from* Patra et al. (2004)

[g] *from* Kazantzis (2000)

[h] Although Se is not a heavy metal, it is included because a number of Se hyperaccumulators have also been discovered

shoots, thus excluding plants which accumulate metals in the roots alone (Maestri et al. 2010). Inevitably, hyperaccumulation implies hypertolerance, i.e., the ability of the plants to detoxify heavy metals stored in aerial tissues (Krämer 2010). Thresholds have been set for different metals and metalloids to define plants as hyperaccumulators (Table 3.1).

Hyperaccumulation is an extreme trait that has evolved many times but is relatively uncommon in terrestrial higher plants. The metal hyperaccumulators identified thus far belong to approximately 500 taxa, accounting for 0.2% of all angiosperms (Baker et al. 2000; Krämer 2010). The number of hyperaccumulator taxa discovered for the main heavy metals is shown in Table 3.2. Most of the known hyperaccumulators are biennial or short-lived perennial herbs, shrubs or small trees. They are mainly endemic to metal-rich soils and are often unable to compete with other species in non-selective soils, possibly due to the higher metabolic costs of metal accumulation and detoxification (Baker et al. 2000). The hyperaccumulation trait is particularly well represented among the Brassicaceae. A phylogenetic tree of the Brassicaceae showing the positions of the main hyperaccumulator species is shown in Fig. 3.1.

Ni hyperaccumulation is the most common trait, reflecting the large number of Ni-enriched serpentine soils worldwide, in particular in the Mediterranean area

Table 3.2 Number of hyperaccumulator plants discovered to date for relevant heavy metals, as reported by Krämer (2010), with modification regarding Cd hyperaccumulator species cited by Liu et al. (2009)

Element	Taxa (no.)	Families (no.)
As	15	2
Cd	8	5
Co	(26)[a]	(11)
Cu	(35)	(15)
Pb	(14)	(7)
Mn	10	6
Ni	390	42
Se[b]	20	7
Tl	1	1
Zn	15	6

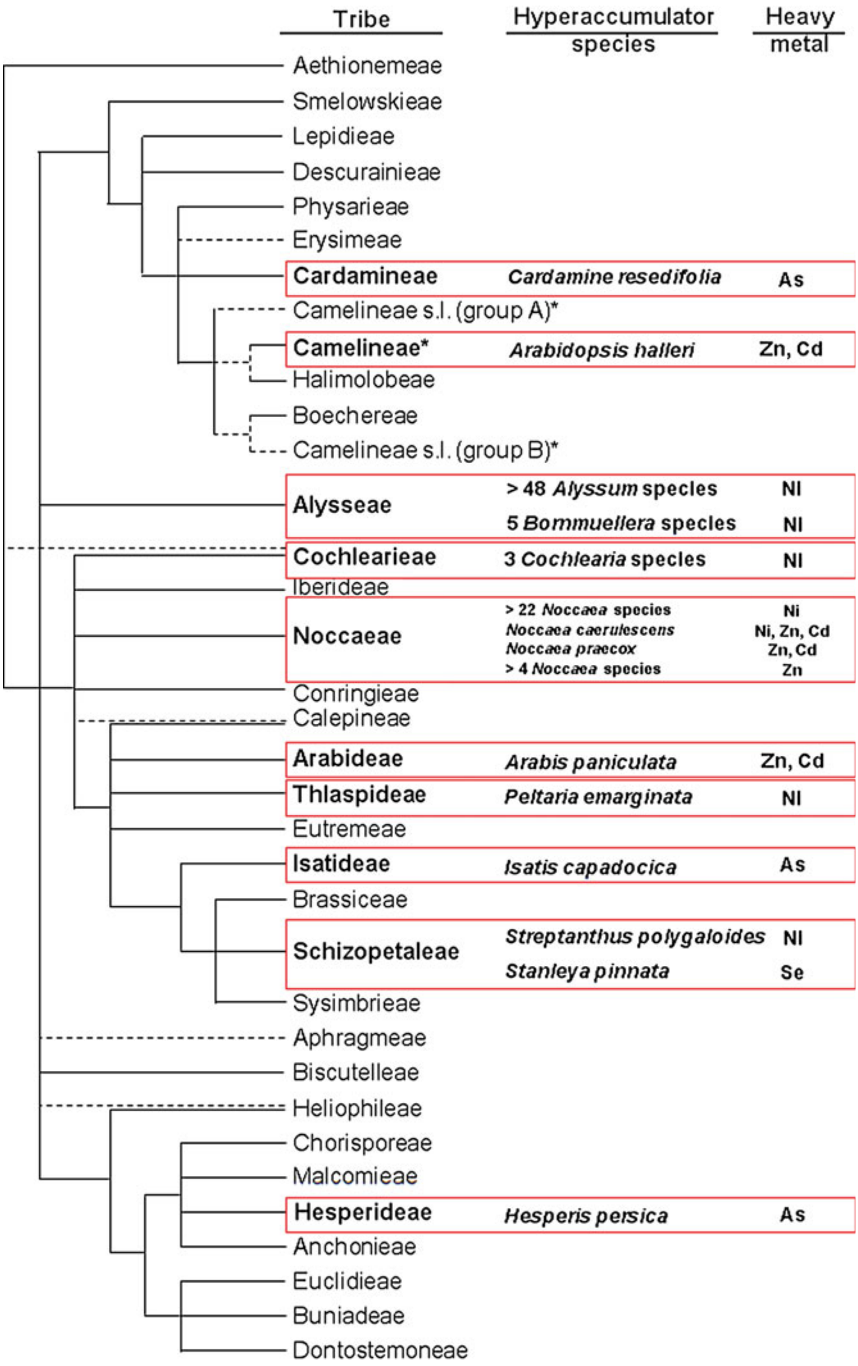
[a] Parentheses indicate that overreporting may have occurred due to contamination with soil particles or minerals

[b] Although Se is not a heavy metal, it is included because a number of Se hyperaccumulators have also been discovered

and in New Caledonia. Among the nearly 400 known metal hyperaccumulator species, approximately 25% are from the families Brassicaceae and Euphorbiaceae (Krämer 2010). Evident from Fig. 3.1, Ni hyperaccumulation has evolved independently six times in the Brassicaceae (Krämer 2010) and occurs most frequently in the genus *Alyssum* (Baker et al. 2000), almost exclusively in the section Odontarrhena (Krämer 2010). As far as Zn is concerned, the majority of hyperaccumulators belong to the Brassicaceae, with probably three independent evolutionary events. Zn hyperaccumulation tends to correlate with Cd and Pb accumulation because these metals share similar chemical properties (Krämer 2010). The only known Cd hyperaccumulator species outside the Brassicaceae are *Viola baoshanensis* (Violaceae; Liu et al. 2004), *Salsola kali* (Chenopodiaceae; de la Rosa et al. 2004), *Sedum alfredii* (Crassulaceae; Deng et al. 2007) and *Phytolacca americana* (Phytolaccaceae; Liu et al. 2009). Finally, As hyperaccumulation has been reported in only two angiosperm species, both belonging to the Brassicaceae (Karimi et al. 2009). Interestingly, the only other known As hyperaccumulators are some fern species from the genus *Pteris* (Zhao et al. 2002), among which the most studied is *P. vittata* (Wang et al. 2002). Species that hyperaccumulate other metals, such as Se (Reeves and Baker 2000) and Pb (Baker et al. 2000), have also been identified.

Two model species for hyperaccumulation, *Arabidopsis* (formerly *Cardaminopsis*) *halleri* and *Noccaea* (formerly *Thlaspi*) *caerulescens*, are particularly suitable for genetic analysis thanks to their strong similarity and extensive synteny with *A. thaliana*.

Arabidopsis halleri is a self-incompatible perennial diploid species that can tolerate and hyperaccumulate Zn and Cd. It shares 94% sequence identity with *A. thaliana* within coding regions (Clauss and Koch 2006) and appears to have


Tribe
Hyperaccumulator species
Heavy metal
Aethionemeae
Smelowskieae
Lepidieae
Descurainieae
Physarieae
Erysimeae
Cardamineae
Cardamine resedifolia
As
Camelineae s.l. (group A)*
Camelineae*
Arabidopsis halleri
Zn, Cd
Halimolobeae
Boechereae
Camelineae s.l. (group B)*
Alysseae
> 48 Alyssum species
Ni
5 Bornmuellera species
Ni
Cochlearieae
3 Cochlearia species
Ni
Iberideae
Noccaeae
> 22 Noccaea species
Ni
Noccaea caerulescens
Ni, Zn, Cd
Noccaea praecox
Zn, Cd
> 4 Noccaea species
Zn
Conringieae
Calepineae
Arabideae
Arabis paniculata
Zn, Cd
Thlaspideae
Peltaria emarginata
Ni
Eutremeae
Isatideae
Isatis capadocica
As
Brassiceae
Schizopetaleae
Streptanthus polygaloides
Ni
Stanleya pinnata
Se
Sysimbrieae
Aphragmeae
Biscutelleae
Heliophileae
Chorisporeae
Malcomieae
Hesperideae
Hesperis persica
As
Anchonieae
Euclidieae
Buniadeae
Dontostemoneae

◀ **Fig. 3.1** Phylogenetic tree of the Brassicaceae family, as reported by Lysak and Koch (2011). Currently accepted tribes are listed in the *first column*. Main hyperaccumulator species among the Brassicaceae are indicated in the *second column* together with the accumulated heavy metals, and are highlighted with *red squares*. *Dashed lines* indicate uncertain phylogenetic relationships. Branches are not drawn to scale. *Recent hypothesis for the subdivision of the Camelineae tribe, as suggested by Bailey et al. (2006)

diverged from its non-tolerant sister species *A. lyrata* around 337,000 years ago, with a speciation event coinciding with major adaptive changes that conferred hypertolerance (Roux et al. 2011). *A. halleri* is found mainly in Central and Eastern Europe, although the subspecies *gemmifera* occurs in Japan and Taiwan (Al-Shehbaz and O'Kane 2002). All *A. halleri* populations, from both non-contaminated and metalliferous soils, are constitutively able to hyperaccumulate Zn and Cd, although the degree of hyperaccumulation is variable and heritable (Macnair 2002; Meyer et al. 2010).

Noccaea caerulescens is a self-compatible diploid species, biannual or perennial, which shares an average sequence identity of 88% with *A. thaliana* within the coding regions (Assunção et al. 2003a; Rigola et al. 2006). Zn hypertolerance and accumulation is constitutive in this species, although the trait shows more variability than in *A. halleri* (Verbruggen et al. 2009; Plessl et al. 2010; Krämer 2010). Some *N. caerulescens* ecotypes can also accumulate Cd and Ni. Furthermore, variations in Cd hyperaccumulation among different ecotypes seem to correlate with different degrees of Zn accumulation (Assunção et al. 2003b; Roosens et al. 2003). In some populations from Southern France, Cd may even be necessary for optimal growth (Roosens et al. 2003). Like Cd, Ni hyperaccumulation in *N. caerulescens* appears to be non-constitutive and confined to some populations from serpentine soils (Assunção et al. 2003b). The most studied ecotypes are: Prayon (Belgium) and Ganges (France), both of which hyperaccumulate Zn and, in different degrees, Cd; Monte Prinzera (Italy) that accumulates Zn and Ni; La Calamine (Belgium), a Zn/Cd-hypertolerant population with low accumulation rates; and Lellingen (Luxembourg), a non-metalliferous population (Assunção et al. 2003a, b; Verbruggen et al. 2009).

3.2 Ecological Role of Metal Hyperaccumulation in Plants

Metal hyperaccumulation is an adaptive solution that may be disadvantageous for plants because it is associated with high energy costs and therefore slows metabolism and growth. However, the trait has evolved independently several times in different taxa, indicating that it must provide some evolutionary advantages. Several different explanations for metal hyperaccumulation have been proposed although in most cases there is no supporting experimental data. Six hypotheses were reviewed by Boyd and Martens (1992): metal tolerance/disposal, induction of drought resistance, interference, inadvertent uptake, and defense against herbivores and pathogens. Of these, the inadvertent uptake hypothesis gives no selective value to metal hyperaccumulators, regarding the trait as a by-product of other physiological processes.

According to the metal tolerance/disposal hypothesis, the accumulation of heavy metals in the aerial parts of the plant may contribute to tolerance by removing metals from sensitive tissues and eliminating them through the loss of leaves (Rascio and Navari-Izzo 2011). The drought resistance theory suggests that heavy metals could work as osmolytes in the cells. Neither hypothesis is supported by any experimental evidence.

The interference hypothesis takes allelopathy into consideration. Hyperaccumulators would be able to inhibit neighboring plants by creating a high metal-content zone, allowing them to compete with faster growing plants for space and light. However, most studies of elemental allelopathy have been inconclusive and do not take into consideration important criteria, such as the role hyperaccumulators play in increasing the metal content in the surrounding area (Morris et al. 2009). Exhaustive work comparing the Se hyperaccumulators *Atragalus bisulcatus* and *Stanleya pinnata* with the non-accumulators *Astragalus drummondii* and *Stanleya elata* growing in seleniferous and non-seleniferous soils demonstrated that plants can affect Se accumulation in their neighbors, and that Se in the soil influences competition and facilitation between plants. Therefore, Se hyperaccumulators may affect the composition of plant communities by allowing growth of Se-tolerant species (El Mehdawi et al. 2012).

Finally, the elemental defense hypothesis considers the role of heavy metals in defense against herbivores and pathogens, and is the most supported theory. The role of Ni (Jhee et al. 2006b), Cd (Jiang et al. 2005), Zn (Behmer et al. 2005), As (Rathinasabapathi et al. 2007), and Se (Galeas et al. 2008; Quinn et al. 2010) in protecting plants from biotic stresses has been confirmed. Biotic stress resistance is a direct effect of metal accumulation, since metal-tolerant pathogens show a greater ability to colonize hyperaccumulator plants (Fones et al. 2010). Defense is mediated both by the toxicity of heavy metals and their deterrent action, since herbivores seem to prefer plants that accumulate low levels of metals (Pollard and Baker 1997; Boyd et al. 2002). For example, many experiments conducted on *Brassica juncea* plants grown with or without Se, and exposed to caterpillars (*Pieris rapae*) and a fungal pathogen of the root system (*Fusarium* sp.), showed that caterpillars strongly preferred leaves without Se, and Se-containing plants were less susceptible to fungal infection (Hanson et al. 2003).

Elemental defense provides advantages over chemical defense because heavy metal uptake requires less metabolic effort than the biosynthesis of chemical toxins and deterrents, and inorganic elements cannot be degraded by herbivores, although some of them are able to chelate metals and therefore develop tolerance (Rascio and Navari-Izzo 2011). This is supported by the low levels of glucosinolate found in the metal hyperaccumulators *Streptanthus polygaloides* (Davis and Boyd 2000) and *N. caerulescens* (Tolrà et al. 2001). However, joint effects between different metals and between metals and chemical compounds have been demonstrated

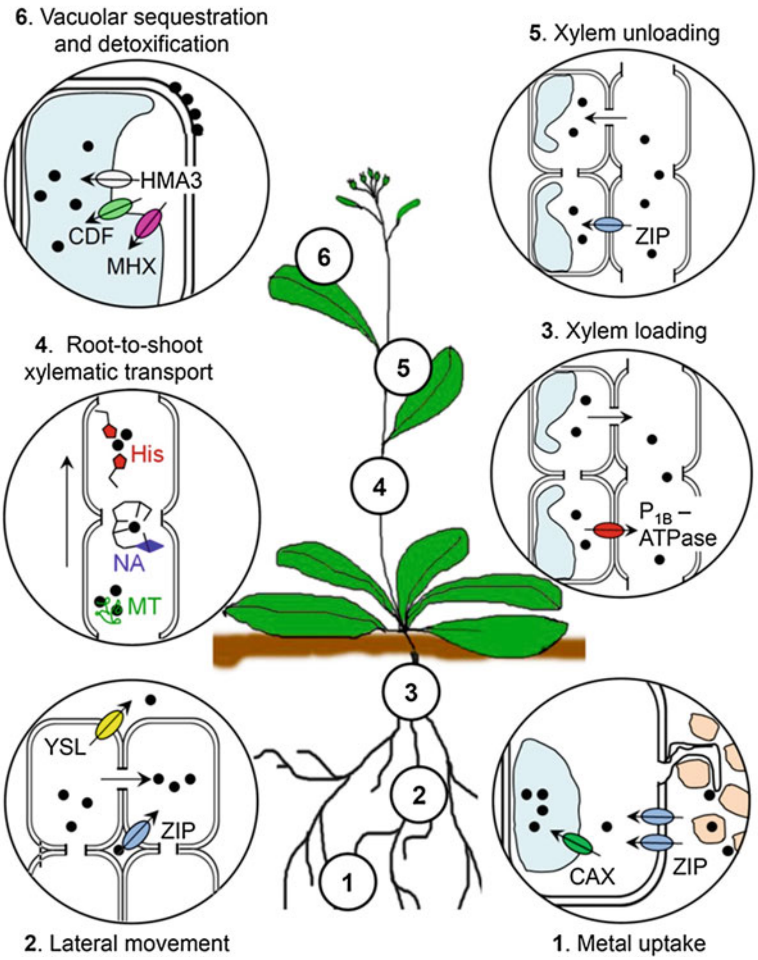


Fig. 3.2 Main mechanisms that are involved in metal accumulation by hyperaccumulating plants. The most relevant metal transporters and chelators described in this chapter are reported. The *black dots* represent metal ions and the *black arrows* indicate the direction of their transport. Areas inside the cells indicate the vacuole

(Jhee et al. 2006a). Recently, a proteomic approach aiming to unravel differences in the *A. halleri* proteome following treatment with Cd and Zn highlighted that proteins involved in plant defense mechanisms against biotic stress are down-regulated by heavy metals. In other words, if a high metal concentration in the shoot provides protection, then other defense mechanisms can be temporarily saved. These data also suggest there is cross-talk between heavy metal signaling and defense signaling (Farinati et al. 2009).

3.3 Determinants for Metal Hyperaccumulation

As stated above, the distinctive characteristic of hyperaccumulator plants is the partitioning of heavy metals in the aerial tissues, whereas most plant species confine metals to the roots, thus preventing damage to the photosynthetic machinery. Hyperaccumulation is achieved by enhancing certain physiological processes, such as uptake into the roots, symplastic mobility, xylem loading and unloading, and metal detoxification by chelation or vacuolar sequestration in the shoots (for a review see Verbruggen et al. 2009; Krämer 2010).

Hypertolerance and hyperaccumulation are quantitative characters (Bert et al. 2003; Assunção et al. 2006; Deniau et al. 2006; Filatov et al. 2006, 2007; Courbot et al. 2007; Willems et al. 2007). Although they coexist in hyperaccumulator species, segregation experiments using *inter*specific and *intra*specific crosses demonstrate that they are genetically independent (Macnair et al. 1999; Assunção et al. 2003c; Bert et al. 2003). At least three QTLs for Zn hyperaccumulation have been identified in *A. halleri* (Filatov et al. 2007) as well as one major QTL for Cd accumulation (Willems et al. 2010). In *N. caerulescens*, two QTLs have been identified for Zn and Cd accumulation in the roots, three for Zn accumulation in shoots, and one for Cd accumulation in shoots (Deniau et al. 2006). Different comparative approaches, including transcriptomic (Becher et al. 2004; Weber et al. 2004; Hammond et al. 2006; Rigola et al. 2006; Talke et al. 2006; van de Mortel et al. 2006, 2008) and proteomic analysis (Ingle et al. 2005b; Tuomainen et al. 2010), have been used to isolate candidate determinants of hyperaccumulation. Several genes are overexpressed in hyperaccumulators in comparison to non-accumulator species, including genes encoding metal transporters and chelators, and genes involved in generic stress responses. The main mechanisms involved in metal hyperaccumulation are summarized in Fig. 3.2.

3.3.1 Metal Transporters

3.3.1.1 ZIP Family

The ZIP family has been shown to promote cation (particularly Zn) uptake and accumulation in *A. thaliana* (Lin et al. 2009), suggesting that ZIP genes may play an important role in metal hyperaccumulation. Some ZIP genes have been isolated from hyperaccumulator species, including the *N. caerulescens* genes encoding NcZNT1 and NcZNT2, which are homologous to AtZIP4 (Assunção et al. 2001), as well as NcZNT5 and NcZNT6, which are homologous to AtZIP5 and AtZIP6, respectively (Wu et al. 2009). Mizuno et al. (2005) cloned the genes for TjZNT1, which transports Zn, Cd, and Mn, and TjZNT2, which is more specific for Zn and Mn, from the Ni hyperaccumulator *Thlaspi japonicum*. AhIRT3, which is involved in Fe and Zn transport, was identified in *A. halleri* (Lin et al. 2009), as well as CsZIP1 from the Mn hyperaccumulator *Chengiopanax sciadophylloides* (Mizuno et al. 2008).

Several ZIP genes do appear to be overexpressed in hyperaccumulator species, including *A. halleri* (*ZIP4* and *ZIP6*: Becher et al. 2004; *ZIP9*: Weber et al. 2004; *ZIP6*: Filatov et al. 2007; *IRT3*, *ZIP3*, *ZIP4*, *ZIP6*, *ZIP9*, and *ZIP10*: Talke et al. 2006) and *N. caerulescens* (*IRT3*, *ZIP6* and *ZIP7*: Hammond et al. 2006; *ZIP3*, *ZIP4* and *ZIP9*: van de Mortel et al. 2006; *ZIP1* and *ZIP8*: van de Mortel et al. 2008) in comparison to non-accumulators. The overexpression of ZIP genes may in some cases reflect gene duplication events, e.g., *AhZIP3*, *AhZIP6* and *AhZIP9* in *A. halleri* (Talke et al. 2006).

3.3.1.2 CDF Family

Members of the CDF family are important for the maintenance of metal homeostasis by mediating the efflux of metal ions from the cytosol into the apoplast or vacuole (Gustin et al. 2011). In particular, the *A. thaliana* CDF protein AtMTP1 induces Zn tolerance and accumulation when overexpressed in transgenic *A. thaliana* plants, suggesting a potential role in hyperaccumulation (van der Zaal et al. 1999).

Accordingly, AtMTP1 homologs in hyperaccumulators appear to have an important role. *MTP1* is strongly expressed in *A. halleri* (Becher et al. 2004; Dräger et al. 2004; Talke et al. 2006) and *N. caerulescens* (Assunção et al. 2001) in comparison to non-accumulator species, and cosegregates with the QTL for Zn tolerance in *A. halleri* (Willems et al. 2007; Shahzad et al. 2010). *MTP1* is also induced in the presence of Cd in *N. caerulescens*, suggesting a role in the response to Cd toxicity (Küpper and Kochian 2010).

A. halleri MTP1 genes have been studied in detail and five paralogs have been detected, named *AhMTP1-A1*, *-A2*, *-B*, *-C*, and *-D* (Dräger et al. 2004; Shahzad et al. 2010). *AhMTP1-D* is not fixed in at least one metalliculous population. The *AhMTP1* copies share on average 97.5% sequence identity, and respectively 91 and 93% identity with their orthologs in *A. thaliana* and *A. lyrata*. Stronger divergences are present in the promoter regions and are correlated with different expression levels in the different species (Shahzad et al. 2010). The *A. halleri* paralogs are differentially expressed and are modulated by Zn (Dräger et al. 2004; Shahzad et al. 2010). Exhaustive analysis of MTP1 has also been carried out in the hyperaccumulators *N. caerulescens* (Assunção et al. 2001) and *Thlaspi goesingense* (Kim et al. 2004; Gustin et al. 2009). In particular, the overexpression of TgMTP1 in *A. thaliana* induces a systematic response that includes the increased expression of Zn transporters (*ZIP3*, *ZIP4*, *ZIP5*, and *ZIP9*), suggesting that TgMTP1 may induce Zn accumulation by initiating a Zn deficiency response (Gustin et al. 2009).

AhMTP8 and *AhMTP11* are also overexpressed in *A. halleri* in comparison to *A. thaliana* (Talke et al. 2006). Similarly, *NcMTP8* is expressed more strongly in the presence of excess Zn in *N. caerulescens* in comparison to *A. thaliana* (van de Mortel et al. 2006). *NcMTP11* and *NcMTP12* also show higher expression levels in *N. caerulescens* in comparison to *T. arvense* (Hammond et al. 2006). These metal transporters belong to group I of the CDF family (Krämer et al. 2007) and are homologous to ShMTP8 (formerly ShMTP1) from the Mn-tolerant legume *Stylosanthes hamata*, which transports Mn into the vacuoles (Delhaize et al. 2003). NcMTP11 and NcMTP12 may therefore contribute to the homeostasis of metals other than Zn and Cd.

3.3.1.3 P_{1B}-Type ATPases

P_{1B}-type ATPases (HMAs) play a prominent role in the homeostasis of different metals. In *A. thaliana* they are involved both in Zn and Cd root-to-shoot translocation (AtHMA2 and AtHMA4: Wong and Cobbett 2009) and metal detoxification by vacuolar storage (AtHMA3: Morel et al. 2009), confirming their important role in heavy metal tolerance and accumulation.

HMA1 (Becher et al. 2004), *HMA3* (Becher et al. 2004; Filatov et al. 2006) and *HMA4* (Talke et al. 2006) are overexpressed in *A. halleri* in comparison to *A. thaliana*. *AhHMA4* was found to co-localize with the QTLs for Zn (Willems et al. 2007; Roosens et al. 2008) and Cd tolerance (Courbot et al. 2007) and for Zn and Cd accumulation (Willems et al. 2010). The downregulation of *AhHMA4* by RNA interference demonstrates that Zn and Cd tolerance and Zn hyperaccumulation are mainly due to AhHMA4, which seems to be responsible for loading metals into the xylem and their redistribution in the leaf blade (Hanikenne et al. 2008). Three almost identical gene copies are present in the *A. halleri* genome, suggesting a recent duplication event. In addition to the higher copy number, promoter modifications have enhanced the expression of *AhHMA4* (Hanikenne et al. 2008). The speciation event that separated *A. halleri* from its sister species *A. lyrata* may have coincided with the duplication of *HMA4* (Roux et al. 2011).

The P_{1B}-type ATPases *NcHMA3* and *NcHMA4* are overexpressed in *N. caerulescens* in comparison to *T. arvense* (Hammond et al. 2006) and *A. thaliana* (van de Mortel et al. 2006). *NcHMA4* is expressed more strongly in *N. caerulescens* roots than shoots and confers Cd resistance in yeast (Bernard et al. 2004). Four tandem copies of *NcHMA4* are present in the *N. caerulescens* genome. The paralogs share 88–99% sequence identity as well as 76–78% and 62–66% identity, respectively, with *A. thaliana* and *A. halleri*, indicating that the gene amplification is a relatively recent event within the *N. caerulescens* lineage (Ó Lochlainn et al. 2011). Each gene copy is constitutively expressed at high levels as in *A. halleri* (Ó Lochlainn et al. 2011). NcHMA3 is localized in the tonoplast and is highly specific for Cd. It is expressed at a higher level in the Ganges ecotype, which accumulates more Cd than Prayon. Overexpression of *NcHMA3* in *A. thaliana* induces Cd tolerance and low levels of Zn tolerance (Ueno et al. 2011).

3.3.1.4 NRAMP Family

NRAMP transporters are involved in the remobilization of metals (especially Fe) from the vacuole, which suggests a putative role in hyperaccumulation (Lanquar et al. 2005).

Accordingly, it was shown that *NRAMP3* is overexpressed in *A. halleri* (Weber et al. 2004; Filatov et al. 2006; Talke et al. 2006) and *N. caerulescens* (van de Mortel et al. 2006) in comparison to *A. thaliana*. NcNRAMP3 and NcNRAMP4 from *N. caerulescens* have been characterized and are similar in terms of localization and biological activity to their orthologs in *A. thaliana* (Oomen et al. 2009). NcNRAMP3 can transport Fe, Mn, and Cd in yeast, whereas NcNRAMP4 transports Zn in addition (Oomen et al. 2009). The expression of NcNRAMP3 in yeast also induces the accumulation of Cd but reduces Ni accumulation, underlining its role in heavy metal homeostasis (Wei et al. 2009). These data indicate that the differing roles of NRAMP proteins in *A. thaliana* and the hyperaccumulator *N. caerulescens* may reflect different levels or patterns of gene expression (Oomen et al. 2009). An NRAMP4 ortholog has also been cloned in the Ni-hyperaccumulator species *T. japonicum*, and the expression of TjNRAMP4 in yeast induces Ni accumulation and sensitivity, inferring a role in Ni homeostasis (Mizuno et al. 2005).

3.3.1.5 YSL Family

YSL transporters interact with a variety of heavy metals as phytosiderophore and NA chelates (Schaaf et al. 2004). Their involvement in the lateral translocation of metals into the veins (DiDonato et al. 2004) suggests a role in root-to-shoot translocation and therefore in metal hyperaccumulation. Nevertheless, few YSL genes appear to be overexpressed in hyperaccumulator species by transcriptomic approach, only *YSL6* in *A. halleri* (Talke et al. 2006) and *YSL7* in *N. caerulescens* (van de Mortel et al. 2006), suggesting that YSL proteins contribute minimally to the regulation of metals other than Fe. However, three *N. caerulescens* YSL genes (*NcYSL3*, *NcYSL5*, and *NcYSL7*) are expressed at higher levels than their orthologs in *A. thaliana* (Gendre et al. 2007). These genes are constitutively expressed at high levels around the vasculature, and they are not inducible by heavy metals. NcYSL3 can transport both Fe- and Ni–NA complexes in yeast assays (Gendre et al. 2007).

3.3.1.6 CaCA Superfamily

As discussed above, the MHX and CAX transporters are the only members of the CaCA family that appear to be involved in heavy metal accumulation in plants. MHX is a vacuolar Mg^{2+} and Zn^{2+}/H^{+} antiport (Shaul et al. 1999). In *A. halleri*, the *AhMHX* gene is present as a single copy; the transcript is present mainly in the shoots and at similar levels to its ortholog in *A. thaliana*. However, the MHX protein is constitutive and much more abundant in *A. halleri* than in *A. thaliana*, indicating some form of post-transcriptional regulation (Elbaz et al. 2006).

Some CAX genes are overexpressed in metal hyperaccumulators, including *CAX2* in *A. halleri* (Becher et al. 2004; Weber et al. 2004), and *CAX2* (Hammod et al. 2006), *CAX3* (van de Mortel et al. 2008), and *CAX7* (van de Mortel et al. 2006) in *N. caerulescens*. Moreover, *NcCAX3* is induced by Cd (van de Mortel et al. 2008). These data suggest CAX genes are involved in metal hyperaccumulation. However, the metal specificity of CAX transporters has not been investigated thus far, although CAX2 does not transport Zn (Becher et al. 2004).

3.3.2 Metal Ligands

3.3.2.1 Histidine

Histidine is the most versatile free amino acid in terms of metal hyperaccumulation, and has a particularly high affinity for Ni (Callahan et al. 2006). The Ni-hyperaccumulator *Alyssum lesbiacum* accumulates high levels of histidine in the xylem sap when exposed to excess Ni (Krämer et al. 1996). Interestingly, Ni tolerance and Ni transport to shoots can also be induced in the non-accumulator species *Alyssum montanum* by feeding with histidine, underlining its important role in hyperaccumulation. Similarly, feeding the non-accumulator *Brassica juncea* with histidine increases Ni translocation by the xylem, although it has no impact on Ni uptake (Kerkeb and Krämer 2003).

The role of histidine biosynthesis in Ni accumulation was tested in *A. thaliana* by introducing the bacterial ATP phosphoribosyl transferase enzyme *StHisG*, which catalyzes the first step of histidine biosynthesis and is insensitive to feedback inhibition by histidine. The transgenic plants were much more Ni tolerant than wild type plants, confirming the important role of the free histidine pool (Wycisk et al. 2004). The relationship between histidine biosynthesis and Ni hyperaccumulation was also studied in the Ni hyperaccumulator *Alyssum lesbiacum*, by monitoring transcript and protein levels. This showed that there was no transcriptional regulation in response to excess Ni, but the levels of all enzymes (especially ATP phosphoribosyl transferase, ATP-PRT) were constitutively higher than those in the weak accumulator *Alyssum serpyllifolium* and the non-accumulator *Alyssum montanum* (Ingle et al. 2005a). The overexpression of AlATP-PRT from *Alyssum lesbiacum* in *A. thaliana* conferred Ni tolerance but had no impact on Ni accumulation (Ingle et al. 2005a) and there was no modulation of histidine biosynthesis by Ni in the Ni-hyperaccumulator *Thlaspi goesingense* (Persans et al. 1999). These data suggest that additional factors are necessary to develop a complete hyperaccumulation phenotype.

3.3.2.2 Nicotianamine

NA is the principal metal ligand in plants and it can form complexes with most transition metal ions (Verbruggen et al. 2009). A role for NA in Zn and Cd hyperaccumulation has been proposed because the genes in the NA biosynthesis pathway are upregulated in hyperaccumulators. *NAS2*, which is responsible for the last step in the pathway, is constitutively expressed in *A. halleri* roots at a higher level than its ortholog in *A. thaliana* (Becher et al. 2004; Weber et al. 2004). *NAS3* is overexpressed in shoots (Becher et al. 2004). *SAMS2*, which generates the NA precursor SAM, is also expressed at higher levels in *A. halleri* than *A. thaliana* (Talke et al. 2006). *N. caerulescens NAS3* and *NAS4* are also expressed at higher levels than their *A. thaliana* orthologs, and *NAS4* is constitutively expressed (van de Mortel et al. 2006, 2008). NA is also involved in Ni chelation, because Ni–NA complexes are found in *N. caerulescens* roots exposed to Ni (Vacchina et al. 2003). *NcNAS1* is constitutively expressed in shoots, whereas NA accumulation in roots appears to be Ni dependent (Mari et al. 2006). Furthermore, NcNAS1 overexpression in *A. thaliana* induces Ni tolerance and accumulation (Pianelli et al. 2005). Finally, NA is probably involved in the accumulation and mobilization of other metals, such as Fe, because *AhNAS4* cosegregates with a QTL for Fe accumulation in *A. halleri* (Willems et al. 2010).

3.3.2.3 Metallothioneins

Although no correlation with hyperaccumulation has been demonstrated, MTs are induced by several heavy metals e.g. in *A. thaliana* (Murphy and Taiz 1995) and are involved in metal tolerance and accumulation (Zimeri et al. 2005).

The *N. caerulescens MT2a* and *MT2b* genes are expressed at higher levels than their orthologs in *A. thaliana* (van de Mortel et al. 2006) and the *N. caerulescens MT2a* and *MT3* genes are expressed at higher levels than their orthologs in *T. arvense* (Hammond et al. 2006). The MT proteins were also expressed at higher levels in a metal-adapted *N. caerulescens* population in comparison to non-metalliculous populations (Hassinen et al. 2009). However, the different expression levels of *NcMT2a*, *NcMT2b*, and *NcMT3* do not correlate with the Cu, Cd, and Zn accumulation capacity and tolerance profiles in transgenic *A. thaliana* (Hassinen et al. 2009). There is no evidence of a direct connection between MTs as metal ligands and hyperaccumulation, thus it is likely that the increased tolerance induced by MT expression in some experiments is due to alternative roles, such as for ROS scavenging (Hassinen et al. 2011).

3.3.3 Response to Stress

3.3.3.1 Glutathione

Antioxidants are important for hyperaccumulators to address the potential oxidative stress caused by heavy metal ions. GS plays a key role for metal tolerance because it can act as a ROS scavenger, a metal chelator, and as a substrate for PC biosynthesis (Krämer 2010). The overexpression of genes involved in cysteine and GS biosynthesis has been reported in hyperaccumulators, e.g., the cysteine synthetase gene *OASA2* is expressed at higher levels in *A. halleri* than *A. thaliana* (Becher et al. 2004; Weber et al. 2004), and the glutathione-S-transferase protein GSTF10 is induced by heavy metals (Farinati et al. 2009). Similarly, *GSTF16* is overexpressed in *N. carulescens* shoots in comparison to *T. arvense* (Hammond et al. 2006), and other GST genes are expressed at higher levels than their *A. thaliana* orthologs (van de Mortel et al. 2008). Enhanced GS biosynthesis correlates with Ni tolerance, and the concentrations of GS, cysteine, and O-acetyl-L-serine (OAS) appear to correlate with Ni accumulation in different *Thlaspi* species, both hyperaccumulator and non-accumulator (Freeman et al. 2004). *A. thaliana* plants transformed with the enzyme serine acetyltransferase (SAT) from *T. goesingense* produce GS, cysteine, and OAS at similar levels to hyperaccumulator species and tolerate higher levels of Ni (Freeman et al. 2004), Zn, and Co, but not Cd (Freeman and Salt 2007). This probably reflects the fact that TgSAT is less sensitive to feedback inhibition induced by cysteine than AtSAT and can therefore accumulate higher levels of GS (Na and Salt 2011).

3.3.3.2 Lignin

The cell wall is an important site for metal storage in plants because it provides a large number of metal-binding sites (Maestri et al. 2010). Genes involved in phenylpropanoid (*PAL2*), lignin (CytP450 family), and suberin biosynthesis (*CER3*, *CER6* and some LTP genes) are overexpressed in *N. caerulescens* in comparison to *A. thaliana* (van de Mortel et al. 2006, 2008). Lignin and suberin deposition in *N. caerulescens* results in the lignification of endodermal cells, followed by the formation of a second layer of endodermis and the development of the Casparian strip, which does not occur in *A. thaliana* or *T. arvense*. Lignification and suberification may therefore help to prevent metal efflux from the vascular cylinder (van de Mortel et al. 2006, 2008).

3.3.3.3 Defensins

Metal hyperaccumulation also induces the expression of genes involved in stress-response signaling (biotic and abiotic) including defensins, which are strongly overexpressed in both *A. halleri* (Becher et al. 2004; Talke et al. 2006) and *N. caerulescens* (van de Mortel et al. 2006) in comparison to *A. thaliana*. Some defensins in *A. halleri* are modulated by Zn (Mirouze et al. 2006). The overexpression of AhPDF1.1 induces Zn tolerance in both yeast and *A. thaliana*. This can be explained by a Zn-chelation hypothesis, in which defensins (like MTs) use their cysteine-rich domains to bind metal ions. Alternatively, defensins may interfere with divalent cation transporters, reflecting their structural similarity to some channel-blocking peptides (Mirouze et al. 2006).

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