Physiology and Molecular Biology of Trace Element Hyperaccumulation

Sylvain Merlot, Vanesa Sanchez Garcia de la Torre,
and Marc Hanikenne

Abstract

Some trace elements are essential for plants but become toxic at high concentration. Remarkably, about 500 plant species worldwide are able to accumulate tremendous amounts of metals in their leaves and are therefore called metal hyperaccumulators. In the context of sustainable development, there is a regain of interest for metal hyperaccumulation mechanisms that may become instrumental for improving metal phytoextraction from contaminated soils to produce metals with a lower net impact on the environment. In addition, studying the molecular mechanisms of hyperaccumulation in diverse plant species is necessary in order to understand the evolution of this extreme and complex adaptation trait. Our current knowledge of metal hyperaccumulation is mostly based on the analysis of a few species from the Brassicaceae family, and suggests that the underlying mechanisms result from an exaggeration of basic mechanisms involved in metal homeostasis. However, the development of Next Generation Sequencing technologies opens today the possibility for studying new hyperaccumulator species that therefore may reveal more diversity in these mechanisms. The goal of this chapter is to provide background information on metal hyperaccumulation and give a clear picture of what we know currently about the molecular mechanisms involved in this trait. We also attempt to outline for the reader the future scientific challenges that this field of research is facing.

S. Merlot (✉) • V. Sanchez Garcia de la Torre
Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette cedex, France
e-mail: sylvain.merlot@i2bc.paris-saclay.fr

M. Hanikenne
InBioS - Functional Genomics and Plant Molecular Imaging, and PhytoSYSTEMS, University of Liège, Liège, Belgium

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1 Introduction

Investigating the mechanisms involved in metal hyperaccumulation allows us to observe extreme adaptation of metal homeostasis networks in plants and identify key players in metal distribution and tolerance in plant tissues. The study of metal hyperaccumulator species also permits examination of mechanisms underlying the evolution of this extreme adaptive trait (Shahzad et al. 2010; Hanikenne and Nouet 2011; Hanikenne et al. 2013). Several comprehensive reviews have been published recently on this topic (Verbruggen et al. 2009b; Krämer 2010; Hanikenne and Nouet 2011) and we refer readers to those reviews. Whereas hyperaccumulator species typically accumulate specifically one metal when growing in their natural environment, some species have the ability to tolerate and accumulate several metals when grown *ex situ*. This is well documented for the hyperaccumulator species of the Brassicaceae family *Noccaea caerulescens*, in which ultramafic-adapted accessions such as Puy de Wolf (France), Monte Prinzera (Italy), or Puente Basadre (Spain) are able to accumulate Ni but also Zn and Cd (Assunção et al. 2003; Peer et al. 2003; Escarré et al. 2013; Gonneau et al. 2014; Callahan et al. 2016). This ability to tolerate and accumulate several metals likely reflects the relatively low specificity of some mechanisms involved in metal transport and chelation.

Several metals that are accumulated in hyperaccumulator species (e.g. Zn, Ni, Mn) are essential nutrients but become toxic at high concentrations for most plants (i.e. non-accumulating species). Therefore, all plant species have developed mechanisms to regulate essential metal homeostasis according to their needs and metal availability in soils (Burkhead et al. 2009; Palmer and Guerinot 2009; Thomine and Vert 2013). Our current knowledge suggests that the molecular mechanisms involved in metal hyperaccumulation are derived essentially from the mechanisms involved in metal homeostasis. In several examples, genes involved in metal homeostasis are differentially expressed in hyperaccumulators compared to related non-accumulator species, as a result of gene duplication and/or changes in promoter activity (Talke et al. 2006; van de Mortel et al. 2006; Krämer et al. 2007; Hanikenne et al. 2008; Shahzad et al. 2010). However, specific genes linked to hyperaccumulation may be discovered as molecular analysis of hyperaccumulation will extend in the future to additional non-model species from various plant families owing to the development of high-throughput sequencing technologies (Verbruggen et al. 2013; Halimaa et al. 2014b; Merlot et al. 2014). For most metals, the hyperaccumulation trait appeared independently in distant plant families. Therefore, some of the mechanisms involved in metal hyperaccumulation may be specific to a plant family or a species, whereas others may be convergent among distant hyperaccumulators. For instance, several examples of convergent evolution have been identified between the Brassicaceae *Arabidopsis halleri* and *N. caerulescens* (see below and Krämer et al. 2007; Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012), suggesting important functional constraints in the metal homeostasis network.

Because of its singularity, metal hyperaccumulation may appear as an exception having minimal relevance. However, from a scientific point of view, metal hyperaccumulation in plants is fascinating, and understanding the mechanisms involved in this trait may provide tools for producing metals with lower impact on the environment in the near future. The goal in this chapter is to outline our current knowledge about the molecular mechanisms of metal hyperaccumulation in plants and to highlight possible future developments in this important field of research.

2 Molecular Physiology of Metal Hyperaccumulation

2.1 Main Steps of Metal Hyperaccumulation

When exposed to excess metals, most plant species adopt a so-called excluder strategy to prevent metal accumulation in photosynthetically active shoot tissues (Krämer 2010). This goal can be achieved by limiting metal absorption by roots, increasing metal efflux from root tissues, and/or increasing metal storage in root cell walls and vacuoles. In contrast, achieving metal hyperaccumulation and hypertolerance requires modifications at specific nodes of the metal homeostasis network to ensure that the metal flux in the plant is directed towards shoot tissues (Fig. 1; Clemens et al. 2002). At the physiological level, these alterations include some or all of the following steps:

1. An enhanced metal mobilization and uptake in roots;
2. An efficient radial metal transport towards the root vascular tissues; this includes a reduction of metal storage in root vacuoles;
3. An increased transport of metal from the root to the shoot, with efficient xylem loading; this

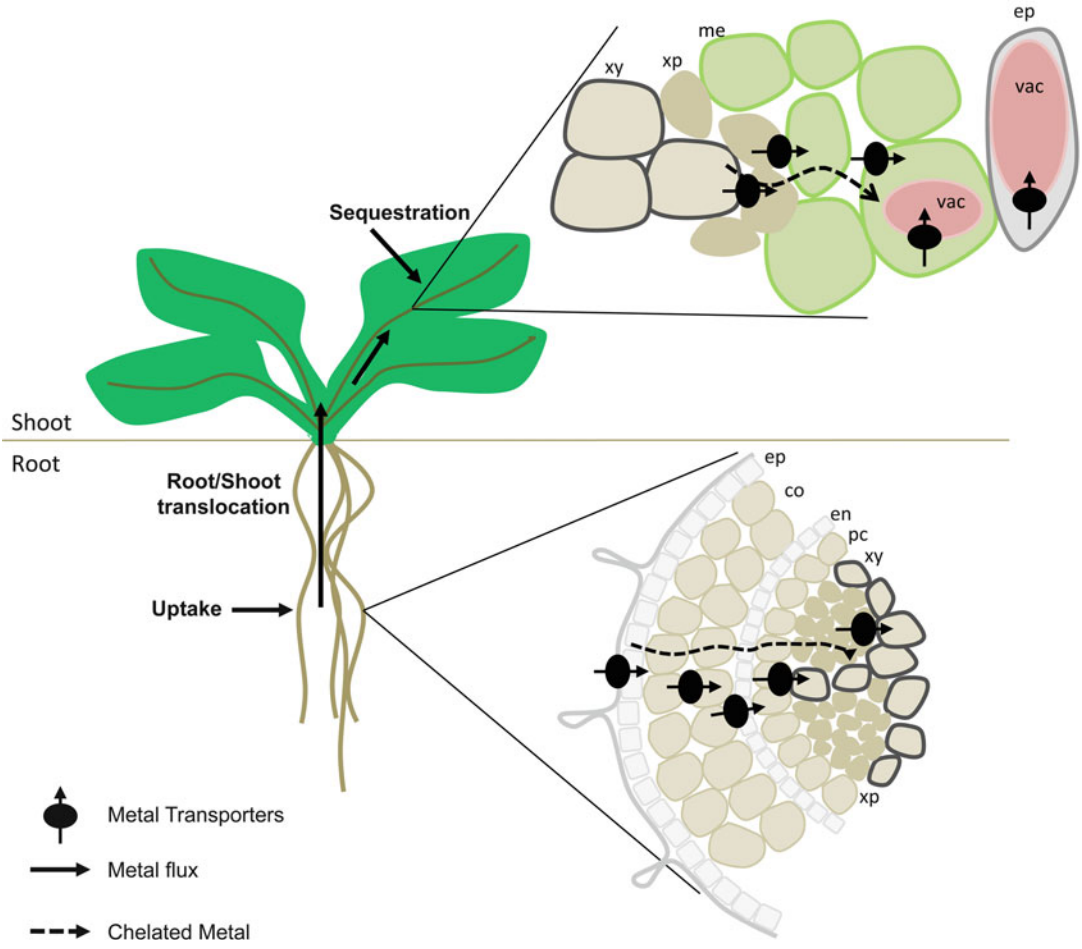

Fig. 1 Model of the physiology of metal hyperaccumulation and hypertolerance. Enhanced metal uptake and radial transport in roots, xylem loading/unloading and vacuolar storage in shoots all make major contributions to the traits. In roots, it is possible that the metal can travel in the apoplasm up to the endodermis cell layer before cellular uptake (not represented). Note that the tissue (epidermis or mesophyll) involved in metal storage varies depending on the species and the metal (see text). *co* cortex, *en* endodermis, *ep* epidermis, *me* mesophyll, *Zinc-NA* Zinc-Nicotianamine chelates, *pc* pericycle, *vac* vacuole, *xp* xylem parenchyma, *xy* xylem. Figure modified from Hanikenne and Nouet (2011)

step contributes to metal tolerance by enabling metal storage (and thus detoxification) in shoot tissues;

4. An efficient mechanism for xylem unloading and metal distribution in shoots together with a high vacuolar storage capacity.

Processes occurring in both roots and shoots thus contribute to metal tolerance and hyperaccumulation. Root-based processes play an essential role in hyperaccumulation whereas shoot-based processes are required for hypertolerance, as suggested from grafting experiments between *N. caerulescens* and the non-Zn-accumulating *Microthlaspi perfoliatum* (Guimarães et al. 2009).

2.2 Metal Distribution in Shoots

Metal distribution in shoot tissues is specific to both the species and the metal considered. This topic has been extensively reviewed recently (Fernando et al. 2013; Leitenmaier and Küpper 2013). Briefly, in most cases, metals (Zn, Cd, Ni or Se) accumulate at the base of the trichomes and in the vacuoles of epidermal cells. In contrast, mesophyll cells that are the main site of photosynthesis accumulate lower amounts of metals (Küpper et al. 1999, 2001; Lombi et al. 2002; Cosio et al. 2005). In the vacuoles of epidermal cells, metals can reach very high concentrations (e.g. several hundred mM; Küpper et al. 1999; Fernando et al. 2006b). There are, however, exceptions. For instance, Zn and Cd are stored in the vacuoles of mesophyll cells in both Zn- and Cd-hyperaccumulators *A. halleri* and *Sedum alfredii*, in which Zn is mostly bound to malate (Küpper et al. 2000; Sarret et al. 2002, 2009; Tian et al. 2011; Lu et al. 2014; Isaure et al. 2015). In the hyperaccumulator *Sedum plumbizincola*, Zn accumulates mostly in leaf epidermal cells, but also in large amounts in mesophyll cells of young leaves (Cao et al. 2014). In this species, Cd is mostly bound to cell walls in leaves (Peng et al. 2017). Accumulation of Mn in mesophyll cells is also observed in several Mn-hyperaccumulators (Fernando et al. 2006a, b, 2013).

3 Identification of Molecular Processes Involved in Metal Hyperaccumulation

In the last 15 years, a number of complementary approaches have been used to identify the molecular actors underlying hyperaccumulation and hypertolerance. These approaches included:

I. Screens of cDNA libraries in yeast in order to isolate genes contributing to metal transport and tolerance (e.g. Lasat et al. 2000; Pence et al. 2000; Bernard et al. 2004; Papoyan and Kochian 2004);
II. Quantitative genetics analyses aiming to identify Quantitative Traits Loci (QTLs) co-segregating with the traits in progenies of crosses between an hyperaccumulator and a related non-accumulator species (e.g. Dräger et al. 2004; Deniau et al. 2006; Filatov et al. 2007; Courbot et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Baliardini et al. 2015).
III. Transcriptomic studies comparing gene expression levels in hyperaccumulator and related non-accumulator species (e.g. Becher et al. 2004; Weber et al. 2004, 2006; Chiang et al. 2006; Craciun et al. 2006; Filatov et al. 2006; Hammond et al. 2006; Talke et al. 2006; van de Mortel et al. 2006; van de Mortel et al. 2008; Gao et al. 2013; Han et al. 2015).

These candidate genes are mostly involved in metal transport, metal chelator synthesis, or metal-induced oxidative stress response. Note that the last is not discussed in this chapter. Several candidate genes were further characterized functionally (e.g. Pence et al. 2000; Persans et al. 2001; Dräger et al. 2004; Kim et al. 2004; Hanikenne et al. 2008; Gustin et al. 2009; Lin et al. 2009; Shahzad et al. 2010; Ueno et al. 2011; Milner et al. 2012; Deinlein et al. 2012; Merlot et al. 2014; Baliardini et al. 2015; Nouet et al. 2015; Charlier et al. 2015). However, only a few candidates (e.g. *HMA4*, see below) were confirmed by reverse genetics in hyperaccumulator species as major players in metal hyperaccumulation and tolerance; their functions are described in detail in the following sections.

A large part of our knowledge on metal hyperaccumulation comes from the study of two model Zn and Cd hyperaccumulating species of the Brassicaceae family, *A. halleri* and *N. caerulescens*, which are related to the sensitive and non-accumulating species *A. thaliana* (Yogeeswaran et al. 2005; Clauss and Koch 2006). These two species have been instrumental in successfully improving our understanding of the physiological, molecular, and genetic bases of metal hyperaccumulation and associated hypertolerance (see Krämer et al. 2007; Milner and Kochian 2008; Pauwels et al. 2008; Roosens et al. 2008; Verbruggen et al. 2009b; Krämer 2010; Hanikenne and Nouet 2011). Those successes relied on availability of the *A. thaliana* genome sequence (The Arabidopsis Genome Initiative 2000), and on dedicated tools and resources combined with relatively high gene sequence conservation among Brassicaceae species (94% and 88% identity with *A. thaliana* for *A. halleri* and *N. caerulescens*, respectively) (Talke et al. 2006; van de Mortel et al. 2006). It is expected that our knowledge will rapidly broaden with new species becoming accessible to molecular and genomic analyses (Gao et al. 2013; Verbruggen et al. 2013; Merlot et al. 2014).

4 Mechanisms of Zn and Cd Hyperaccumulation

As mentioned above, most of our knowledge about Zn and Cd hyperaccumulation was acquired using *A. halleri* and *N. caerulescens* (Fig. 2). More recent models include (i) the Crassulaceae *S. alfredii* and *S. plumbizincola* from Asia, which are the only species reported to hyperaccumulate Cd other than the Brassicaceae (Yang et al. 2004, 2006; Deng et al. 2007; Krämer 2010; Wu et al. 2013; Cao et al. 2014; Ma et al. 2015; Peng et al. 2017); and (ii) the Amaranthaceae *Gomphrena claussenii* from South America, which is highly tolerant to Zn and Cd and presents indicator levels of Zn and Cd accumulations (Villafort Carvalho et al. 2013, 2015).

Arabidopsis halleri and *N. caerulescens* display constitutive Zn hyperaccumulation and hypertolerance, although intraspecific variation for those traits has been reported (Bert et al. 2000, 2002; Reeves et al. 2001; Assunção et al. 2003; Molitor et al. 2005; Besnard et al. 2009). Similarly, hyperaccumulation of Cd shows substantial intraspecific variation (Escarré et al. 2000; Bert et al. 2002; Roosens et al. 2003; Verbruggen et al. 2013; Meyer et al. 2015). Metal hyperaccumulation evolved independently in the two species (Krämer 2010). However, both share a set of alterations of their metal homeostasis networks in comparison to the non-accumulator *A. thaliana*, which is described in detail below.

4.1 Uptake of Zn and Cd

Prior to uptake, it is suggested that metals are actively mobilized from the soil, by acidification and/or chelate secretion (Clemens et al. 2002). A recent report, however, suggested that the roots of *A. halleri* secrete elevated levels of nicotianamine (NA), a metal chelator able to form NA-Zn complexes (Curie et al. 2009; Clemens et al. 2013), which may reduce root Zn uptake and increase tolerance (Tsednee et al. 2014). Another report indicated higher organic acid levels and Zn mobilization in the dissolved organic matter within the rhizosphere of hyperaccumulator compared to non-accumulator accessions of *S. alfredii* (Li et al. 2012).

Several divalent metal transporters of the ZIP (Zrt-Irt-like Protein) family are highly expressed in roots and/or shoots of both *A. halleri* and *N. caerulescens* (Talke et al. 2006; Krämer et al. 2007; Lin et al. 2009, 2016). It presumably results in enhanced rates of root metal uptake or mobilization from root storage sites. By contributing to Zn radial transport towards the xylem in roots, it also may contribute to metal partitioning between root and shoot tissues. Several

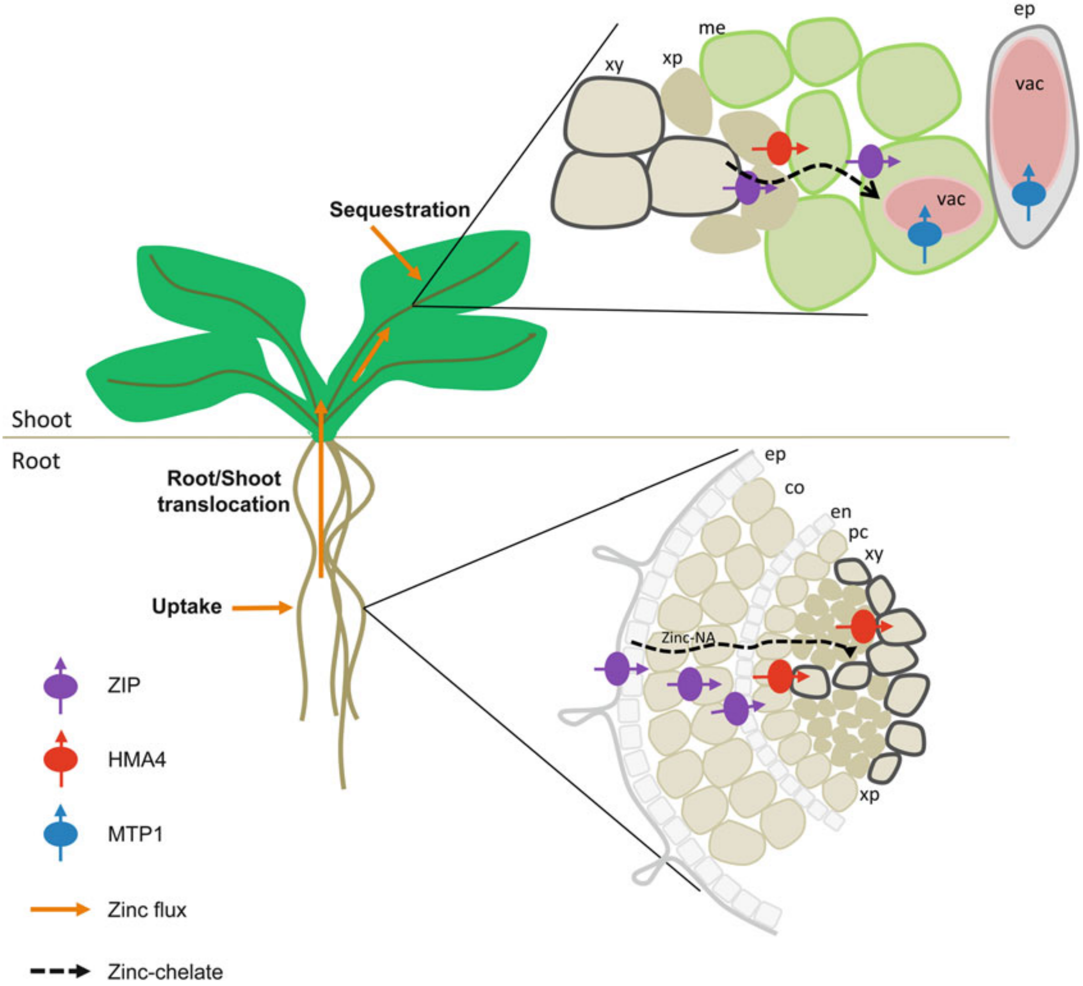

Fig. 2 Model for Zn hyperaccumulation and hypertolerance in the Brassicaceae *A. halleri* and *N. caerulescens*. Enhanced functions of ZIP transporters in cellular uptake, of the P-type ATPase HMA4 in xylem loading/unloading and of MTP1 in vacuolar storage all make major contributions to the traits. The exact functions and localizations of individual ZIPs are unknown. In roots, the metal chelator nicotianamine (NA) possibly favours Zn radial transport towards the xylem by symplastic inter-cellular mobility of Zn by either allowing Zn movement through plasmodesmata and/or preventing vacuolar storage. Vacuolar storage in shoots occurs in the epidermis in *N. caerulescens* and in the mesophyll in *A. halleri*. Additional metal homeostasis genes that are highly expressed in both hyperaccumulators are discussed in the text. Note that similar mechanisms have been identified as more active in a metal hyperaccumulating population of the Crassulaceae *S. alfredii* compared to a non-accumulating population. *co* cortex, *en* endodermis, *ep* epidermis, *me* mesophyll, *Zinc-NA* Zinc-Nicotianamine chelates, *pc* pericycle, *vac* vacuole, *xp* xylem parenchyma, *xy* xylem. Figure modified from Hanikenne and Nouet (2011)

ZIP genes are induced by Zn deficiency under the control of the bZIP19 and bZIP23 transcription factors in *A. thaliana* (Assunção et al. 2010). Their high expression in *A. halleri* and *N. caerulescens* roots could be the direct consequence of the high activity of HMA4 (Heavy Metal ATPase 4; see below), which depletes Zn in roots (Talke et al. 2006; Hanikenne et al. 2008; Gustin et al. 2009). Note that several ZIP genes are also highly expressed in *S. plumbizincola* (Peng et al. 2017). Further work will be required to determine their individual function in Zn hyperaccumulation and to assess whether Cd uptake is determined by specific mechanisms or via Zn- and/or Fe-transport mechanisms (Meyer and Verbruggen 2012).

4.2 Root-to-Shoot Transfer of Zn and Cd

Increased rate of root-to-shoot metal transfer is key to achieving metal hyperaccumulation in shoots. It requires enhanced radial transport to xylem, decreased vacuolar storage in root cells, and efficient xylem loading. Several *NAS* (nicotianamine synthase) genes are highly expressed in *A. halleri* and *N. caerulescens* (Weber et al. 2004; van de Mortel et al. 2006; Deinlein et al. 2012). *NAS* transcript levels are also higher in roots of a hyperaccumulator accession compared to those of a non-hyperaccumulator accession in *S. alfredii* (Liang et al. 2014). Elevated levels of NA have been measured in roots of *A. halleri* compared to *A. thaliana* (Weber et al. 2004; Deinlein et al. 2012). It was further shown, using *A. halleri* RNAi lines, that high expression of the *NAS2* gene provides increased NA levels for Zn symplastic mobility towards the xylem and for controlling the rate of Zn xylem loading in roots (Deinlein et al. 2012; Cornu et al. 2015). The amino-acid histidine (His), whose concentration weakly correlates with Zn content in *N. caerulescens*, was shown to enhance Zn xylem loading and thus contribute to reduce Zn storage in roots (Callahan et al. 2007; Kozhevnikova et al. 2014).

In *A. halleri*, Zn and Cd loading into the xylem is driven by the HMA4 protein (Talke et al. 2006; Courbot et al. 2007; Hanikenne et al. 2008), which is a plasma membrane P-Type ATPase pump that uses the energy released from the hydrolysis of Adenosine triphosphate (ATP) to transport metal against the electro-chemical gradient (Hussain et al. 2004; Wong and Cobbett 2009; Pedersen et al. 2012; Hanikenne and Baurain 2014). The *HMA4* gene co-segregates with QTLs for Zn and Cd tolerance and accumulation (Courbot et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Meyer et al. 2016). High expression of *HMA4* is required for both hyperaccumulation and hypertolerance in *A. halleri* (Talke et al. 2006; Hanikenne et al. 2008). Increased gene dosage of *HMA4* was selected during the evolutionary history of *A. halleri* and evolved through tandem triplication and activation in *cis* of the promoters of all three copies (Hanikenne et al. 2008, 2013). The *A. halleri HMA4* locus was shaped by positive selection, resulting in a selective sweep and in ectopic gene conversion (Hanikenne et al. 2013). The three *HMA4* copies are active mainly in vascular tissues of *A. halleri*, which allows acting in xylem metal loading in roots and possibly in metal distribution in leaves. It also may ensure metal exclusion from metal-sensitive tissues (e.g. root tip, cambium). By controlling highly active Zn xylem loading, HMA4 also acts as a physiological regulator: it depletes the root Zn pool, which triggers a Zn-deficiency response resulting in high expression of several *ZIP* genes (Hanikenne et al. 2008). In agreement, modeling of the Zn supply-dependent spatio-temporal evolution of Zn concentration in root symplast and apoplast of *A. thaliana* predicted that slight changes in *HMA4* transcript levels have a major impact on the radial distribution of Zn in roots and the root-to-shoot Zn gradient (Claus et al. 2013). It was further shown that a certain extent of functional differentiation exists among the three *AhHMA4* copies when expressed in *A. thaliana*, stemming from differences in expression levels rather than in expression profile. Interestingly, *AhHMA4* copy 3 was subjected to the strongest, possibly most recent, positive selection during the evolutionary history of *A. halleri* (Hanikenne et al. 2013), thus linking sequence diversity patterns and function *in vivo* (Hanikenne et al. 2013; Nouet et al. 2015).

HMA4 is also highly expressed in *N. caerulescens* as well as in Zn- and Cd-hyperaccumulator accessions of *S. alfredii* and *S. plumbizincola*, where it very likely plays similar roles to the *A. halleri HMA4* (Bernard et al. 2004; Papoyan and Kochian 2004; van de Mortel et al. 2006; O'Lochlainn et al. 2011; Craciun et al. 2012; Zhang et al. 2016; Peng et al. 2017). Moreover, the gene coding for the ZIP transporter *ZNT1* of *N. caerulescens* is highly expressed in cortex, endodermis, and pericycle root cells. When expressed in *A. thaliana*, it contributes to Zn and Cd tolerance and accumulation. The *NcZNT1* gene may therefore be involved in Zn and Cd influx into cells responsible for xylem loading, providing metals for transport by HMA4 (Milner et al. 2012; Lin et al. 2016). The ortholog of *ZNT1* in *A. halleri*, *ZIP4*, is also highly expressed and may contribute to a similar function (Talke et al. 2006). Note that NRAMP1 (Natural Resistance-Associated Macrophage Protein 1) may also play a similar role for Cd in *N. caerulescens* (Milner et al. 2014). Moreover, the vacuolar metal efflux transporters NRAMP3 and NRAMP4 are highly expressed in *N. caerulescens* and *A. halleri* roots and were proposed to limit vacuolar storage and increase metal mobility (Weber et al. 2004; Oomen et al. 2009). NRAMP3 is also highly expressed in *S. plumbizincola* (Peng et al. 2017).

Once in the xylem sap, metals are transported to the shoot owing to the evapo-transpiration stream. In this compartment, Zn is mainly bound to organic acids such as malate and citrate (Monsant et al. 2011; Lu et al. 2013; Cornu et al. 2015).

4.3 Storage of Zn and Cd in Leaves

It is suggested that HMA4 and ZIP transporters play an important role in Zn unloading and distribution in shoot tissues (Krämer et al. 2007; Hanikenne and Nouet 2011). However, their exact contribution, as well as that of metal ligands or other transporters, to these processes remains to be detailed. Zn storage in vacuoles is most likely ensured by the MTP1 (Metal Tolerance Protein 1) protein in *A. halleri* (Dräger et al. 2004; Talke et al. 2006; Shahzad et al. 2010), although its role remains to be formally established through the analysis of knock-down plants. MTP1 is a vacuolar transporter implicated in Zn tolerance (Krämer 2005). The MTP1 gene is constitutively highly expressed in both root and shoot of *A. halleri*, and is present in four to five copies that are located on three distinct linkage groups in the genome (Dräger et al. 2004; Talke et al. 2006; Willems et al. 2007; Shahzad et al. 2010). The two most highly expressed copies each co-segregate with QTLs for Zn tolerance (Dräger et al. 2004; Talke et al. 2006; Willems et al. 2007; Shahzad et al. 2010).

MTP1 is also highly expressed in *Noccaea* (formerly *Thlaspi*) *goesingense*, another Zn and Ni hyperaccumulator, in *N. caerulescens*, and in Zn-hyperaccumulating populations of *S. alfredii* (Milner and Kochian 2008; Gustin et al. 2009; Zhang et al. 2011). It likely plays in these species a similar role as in *A. halleri*.

Finally, no detailed information is currently available on the molecular mechanisms of Cd storage in *A. halleri* shoot vacuoles (Meyer and Verbruggen 2012). Indeed, MTP1 is not associated with high Cd tolerance or accumulation in *A. halleri* (Courbot et al. 2007; Willems et al. 2010), although MTP1-related proteins were shown to transport Cd in several species, including the Ni hyperaccumulator *N. goesingense* (Persans et al. 2001; Migocka et al. 2015). In *N. caerulescens*, HMA3 (Heavy Metal ATPase 3) may contribute to this process (Ueno et al. 2011). In addition, differential regulation of *MTP1* by Cd in related *Noccaea* species suggests that it may contribute to Cd handling (Martos et al. 2016).

4.4 Additional Candidate Genes for Zn and Cd Accumulation and Tolerance

A few additional candidate genes for a role in Zn or Cd tolerance have been functionally characterized. In *A. halleri*, the PDF1.1 (Plant Defensin 1.1) protein was identified through a cDNA screen in yeast as a contributor to Zn tolerance. It also confers Zn tolerance when ectopically overexpressed in *A. thaliana* and is more highly expressed in shoots of *A. halleri* compared to *A. thaliana* (Mirouze et al. 2006). It localizes in intracellular compartments (Oomen et al. 2011). PDFs were initially known as secreted antifungal proteins and are characterized by a cysteine-stabilized, α-helix β-sheet, three-dimensional structure (De Coninck et al. 2013; van der

Weerden and Anderson 2013). Family wide comparison of *A. halleri* and *A. thaliana PDF1* genes revealed that the molecular function of the *A. thaliana* and *A. halleri* proteins in Zn tolerance and antifungal activity is conserved, and that functional differences in the two species may result from differential expression levels and regulation (Shahzad et al. 2013; Nguyen et al. 2014).

The fine mapping of a QTL in *A. halleri* allowed the identification of *CAX1* (cation/hydrogen exchanger 1) as a candidate gene for Cd tolerance (Courbot et al. 2007; Baliardini et al. 2015). CAX1 is localized in the vacuolar membrane and plays a key role in Ca homeostasis (Conn et al. 2011). *CAX1* is more expressed in the roots of *A. halleri* compared to those of *A. thaliana*, and high expression of *CAX1* co-segregated with Cd tolerance in a back-cross 1 population of an *A. halleri*/*A. lyrata* cross. The CAX1 QTL is conditional on Ca supply in the medium and is detected at low Ca supply only. The *A. thaliana cax1* mutant analysis suggests that, at low Ca supply, CAX1 may be required to tolerate Cd-induced oxidative stress (Baliardini et al. 2015, 2016).

5 Mechanisms of Ni Hyperaccumulation

Today, more than 400 nickel (Ni) hyperaccumulator species have been identified worldwide. These species are scattered in more than 40 plant families, mostly dicotyledons (Krämer 2010; van der Ent et al. 2013; Cappa and Pilon-Smits 2014). Despite this large diversity and the interest in understanding the underlying mechanisms, as they may be instrumental to improve Ni phytoextraction, only a limited number of studies have focused on the molecular mechanisms of Ni hyperaccumulation. Ni is an essential microelement for plants because it is required for urease activity (Polacco et al. 2013). Plants have, therefore, evolved mechanisms for the regulation of Ni homeostasis and Ni hyperaccumulation that likely derive from these mechanisms (Fig. 3). In *A. thaliana*, the mechanisms involved in Ni homeostasis are strongly linked to Fe homeostasis (Schaaf et al. 2006; Morrissey et al. 2009; Nishida et al. 2011). Interestingly, in Ni hyperaccumulators of the *Alyssum* genus, it was shown that Mn treatment reduces Ni accumulation, thus suggesting that in some species Ni hyperaccumulation may also use mechanisms primary involved in Mn homeostasis (Leigh Broadhurst et al. 2009; Ghaderian et al. 2015).

5.1 Uptake of Ni

Efficient uptake of Ni by the roots of hyperaccumulators requires divalent metal importers (e.g. ZIP, NRAMP) or transporters able to carry conjugated forms of Ni (e.g. Yellow Stripe-Like (YSL) family). However, the identity of the transporters involved in Ni uptake in hyperaccumulators is still not clearly established.

In *A. thaliana*, the metal transporter IRT1 that is required for the uptake of Fe from soil was shown to also be involved in Ni uptake (Vert et al. 2002; Nishida et al. 2011, 2012). Interestingly, the high expression of the *IRT1* ortholog in the roots of *N. caerulescens* (*NcIRT1*) is correlated with Ni hyperaccumulation in the Monte Prinzera accession (Halimaa et al. 2014b). In addition, *de novo* sequencing of *NcIRT1* in Monte Prinzera revealed sequence polymorphism in the large cytoplasmic loop of IRT1 that may have a role in transport specificity and/or regulation (Halimaa et al. 2014a). However, in other Ni hyperaccumulator accessions of *N. caerulescens* (i.e. Puy de Wolf and Bergenbach), we were unable to detect correlation between *NcIRT1* expression and Ni hyperaccumulation (V. S. Garcia de la Torre, S. Merlot, unpublished data). These data suggest that diverse transporters may be involved in the uptake of Ni in hyperaccumulators. Indeed, several members of the ZIP and NRAMP families from *Noccaea* species have been linked to Ni transport or accumulation, but further studies will be required to support their implication in an efficient uptake of Ni (Mizuno et al. 2005, 2007; Wei et al. 2009; Halimaa et al. 2014b).

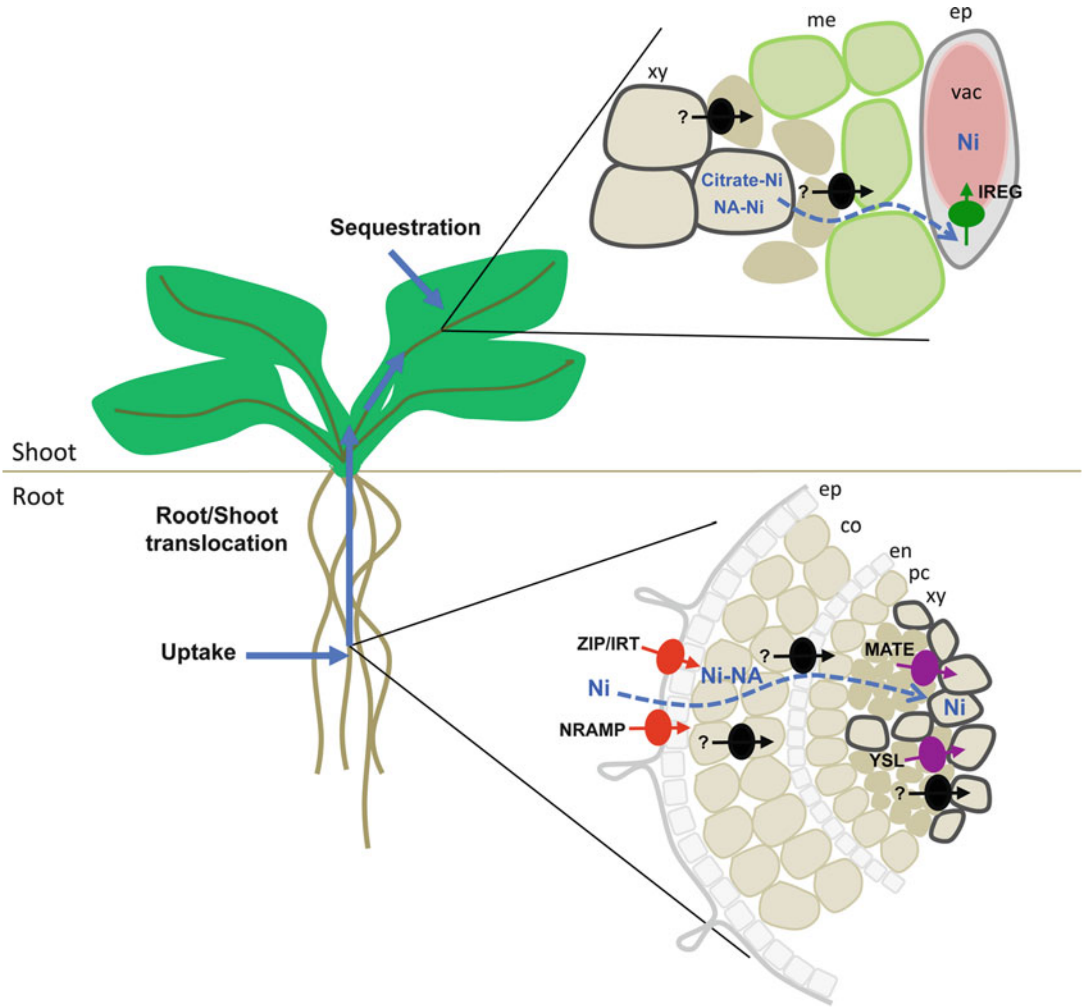

Fig. 3 Proposed mechanisms of Ni transport in Hyperaccumulators. Efficient Ni uptake is mediated by metal transporters (ZIP/IRT, NRAMP. . .) located at the plasma membrane of root epidermal cells (ep). Ni is then transported through the cortex (co) and the endodermis (en) by a combination of Ni export and import transporter activities. During this step, Ni is chelated [e.g. by nicotianamine (Ni-NA)] to reduce its reactivity in the cytoplasm. Binding to His might prevent vacuolar sequestration to favor radial transport. In the pericycle (pc), Ni is loaded in the xylem (xy) together with chelator molecules (e.g. NA, Citrate. . .) by YSL and MATE transporters and transported to the shoot. The mechanisms involved in xylem unloading and transport to the epidermal cell are not well known but may be similar as the one involved in Ni transport in roots. In epidermal cell, Ni is transported and stored in the vacuole (vac) by IREG transporters. Figure modified from Hanikenne and Nouet (2011)

5.2 Root-to-Shoot Transfer of Ni

The long-distance transport of Ni from roots to shoots requires several steps that involve metal transporters and chelators that are able to bind Ni in different pH environments. In hyperaccumulators, a large proportion of Ni is found as complexes with carboxylic acids including citrate and malate (for reviews see Callahan et al. 2006; Sarret et al. 2013). These organic acid complexes are stable in acidic compartments such as vacuoles and xylem. In particular, citrate-Ni was identified in the xylem sap of the Ni hyperaccumulator *Alyssum serpyllifolium*

(Alves et al. 2011). Interestingly, an ortholog of the *A. thaliana* citrate transporter FRD3 of the Multidrug And Toxic compound Extrusion family (MATE) is more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (van de Mortel et al. 2006). AtFRD3 and its orthologue in rice, OsFRDL1, are involved in the translocation of Fe from roots to shoots (Rogers and Guerinot 2002; Yokosho et al. 2009). Therefore, high expression of MATE transporters in the root pericycle of hyperaccumulators would increase the loading of xylem with citrate and therefore favor the translocation of Ni-citrate complex from root to shoot. However, to date no direct correlation has been established between MATE transporters and Ni hyperaccumulation. It is interesting to note that *FRD3* is also highly expressed in *A. halleri*, which hyperaccumulates Zn. FRD3 transporters may, therefore, have a general function in metal hyperaccumulation, favouring long-distance transport of metal from root to shoot (Talke et al. 2006; Charlier et al. 2015). NA also has a strong affinity for Ni over a wide pH range and is proposed to bind Ni in more neutral compartments such as cytoplasm or phloem (Callahan et al. 2006; Rellan-Alvarez et al. 2008; Alvarez-Fernandez et al. 2014). Accordingly, over-expression of NA synthase in transgenic *A. thaliana* increases Ni tolerance but is not sufficient to improve Ni accumulation (Pianelli et al. 2005). The NA-Ni complex was identified in the xylem sap of *N. caerulescens* (Mari et al. 2006), in the latex of the Ni hyperaccumulator *Pycnandra acuminata* (Schaumölffel et al. 2003), and in extracts of several hyperaccumulator species (Callahan et al. 2012). Transporters of the YSL family have been shown to transport NA-metal complexes (Curie et al. 2009; Conte and Walker 2012). Several genes coding for YSL transporters are more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (Gendre et al. 2007). Among these transporters, NcYSL3 that is able to transport the NA-Ni complex is expressed in the vasculature of roots and leaves, suggesting a role in long-distance Ni transport.

Finally, the amino acid histidine (His), whose concentration in some hyperaccumulators of the *Alyssum* and *Noccaea* genera correlates with Ni accumulation, is proposed to play a role in the radial transport of Ni (Krämer et al. 1996; Richau et al. 2009). His has a strong affinity for Ni and the Ni-His complex, and has been identified in samples from *Alyssum* and *Noccaea* hyperaccumulators (Krämer et al. 1996; Persans et al. 1999; Callahan et al. 2006; McNear et al. 2010). In the Ni hyperaccumulator *A. lesbiacum*, genes involved in His biosynthesis are constitutively and highly expressed compared to the non-accumulator *A. montanum*. The overexpression of the first enzyme of the His biosynthetic pathway, ATP-phosphoribosyltransferase, in *Arabidopsis thaliana* increases Ni tolerance but not Ni content, thus suggesting that other mechanisms are necessary for accumulation of this metal (Wycisk et al. 2004; Ingle et al. 2005). Treatment of plants with Ni-His increases xylem loading and inhibits Ni uptake from root vacuoles (Richau et al. 2009). It was therefore proposed that the high concentration of His in roots of Ni hyperaccumulators prevents vacuolar storage, favoring radial transport and xylem loading (Kerkeb and Krämer 2003; Richau et al. 2009). However, the mechanisms responsible for the inhibition of Ni vacuolar sequestration by His are still unknown. Moreover, it is currently not known if this strategy is widely conserved in Ni hyperaccumulators and if this is relevant in natural environments (e.g. ultramafic-derived soil), where N is limiting (Alves et al. 2011; Centofanti et al. 2013).

5.3 Storage of Ni in Leaves

In most hyperaccumulators that have been studied, Ni is stored in the vacuole of leaf epidermal cells (for review see Sarret et al. 2013). Several lines of evidence indicate that Ferroportin (FPN)/Iron Regulated (IREG) transporters play an essential role in the sequestration of Ni in vacuoles.

In *A. thaliana*, *AtIREG2* is expressed in roots in response to Fe-starvation and the AtIREG2 protein localization on the vacuole. The analysis of the *ireg2* mutant indicated that AtIREG2 is involved in the storage of Ni excess in the vacuole of root cells (Schaaf et al. 2006; Morrissey et al. 2009). On the contrary, a second IREG transporter in *A. thaliana*, named FPN1/IREG1, is localized on the plasma membrane and is proposed to play a role in xylem loading of metals in roots (Morrissey et al. 2009). Interestingly, the ortholog of *AtIREG2* in *A. lyrata* is genetically linked to ultramafic adaptation (Turner et al. 2010). Recent comparative transcriptomic analysis using RNA-Seq technology revealed that a high expression of the ortholog of *AtIREG2* in roots of *N. caerulescens* Monte Prinzera is linked to Ni accumulation (Halimaa et al. 2014b). Independent RNA-Seq analyses also indicate that *NcIREG2* is strongly and constitutively expressed in leaves of the *N. caerulescens* accessions Puy de Wolf and Bergenbach (V. S. Garcia de la Torre, S. Merlot, unpublished results).

The PgIREG1 transporter from the Ni hyperaccumulator *Psychotria gabriellae* (Rubiaceae) localizes in the vacuolar membrane and is able to transport Ni when expressed in yeast. PgIREG1 therefore seems to be the functional homolog of AtIREG2. Interestingly, *PgIREG1* is highly expressed in leaves of *P. gabriellae* in environmental conditions and is more expressed than in the closely related, non-accumulator *P. semperflorens* (Merlot et al. 2014). These data further suggest that the high expression of IREG transporters in leaves is a convergent mechanism for Ni hyperaccumulation. However, whereas over-expression of *AtIREG2* and *PgIREG1* in transgenic *Arabidopsis* plants significantly increases Ni tolerance, it does not increase Ni accumulation, further supporting the hypothesis that other mechanisms are required for hyperaccumulation (Schaaf et al. 2006; Merlot et al. 2014). Other families of divalent metal exporters such as MTP transporters could mediate the transport of Ni in vacuoles, but their role in hyperaccumulation needs to be further supported (Persans et al. 2001).

6 Hyperaccumulation of Other Trace Elements

Species that are able to hyperaccumulate Mn have been identified in more than 10 genera mostly in the Myrtaceae (e.g. *Gossia*) and Proteaceae (e.g. *Virotia*) families (Fernando et al. 2013; Losfeld et al. 2015). As for other metals, Mn hyperaccumulation likely evolved from basic mechanisms involved in Mn homeostasis; however, these mechanisms are poorly investigated to date in Mn hyperaccumulators (Pittman 2005; Fernando et al. 2013; Socha and Guerinot 2014). In hyperaccumulators, Mn was found to accumulate in the vacuole of non-photosynthetic epidermal cells, but also more surprisingly in photosynthetic palisade mesophyll cells in the hyperaccumulator *Virotia neurophylla* (Fernando et al. 2012). These differences in Mn localization suggest that some mechanisms involved in Mn accumulation and detoxification might be divergent among hyperaccumulators. The high concentration of Mn found in leaves of several species is linked to their strategy to acquire P. For example, Proteaceae species excrete carboxylates in their rhizosphere that solubilize not only P but also micronutrients including Mn (Lambers et al. 2015). Several families of metal transporters, such as NRAMP, ZIP, YSL, and MTP have been shown to be able to transport Mn in plants, but their role in hyperaccumulation is not clearly established (Fernando et al. 2013; Socha and Guerinot 2014). Most NRAMP transporters are able to transport Mn in the cytoplasm, either from the external medium or the vacuole, and therefore could participate in several steps of Mn hyperaccumulation. In particular, the NRAMP1 transporter from *A. thaliana* was shown to be the main transporter involved in Mn uptake in roots (Cailliatte et al. 2010). The ShMTP8 transporter (previously known as ShMTP1) was isolated from the Mn-tolerant species *Stylosanthes hamata* (Fabaceae) as a transporter that confers Mn resistance when expressed in yeast, and was proposed to mediate accumulation of Mn in the vacuoles of plant cells (Delhaize et al. 2003). Further molecular studies on Mn hyperaccumulators will be required to identify those mechanisms that are key to Mn hyperaccumulation.

In addition to the above-mentioned metals, mechanisms involved in the hyperaccumulation of the metalloid element Se in plants are extensively studied because of their relevance for improving Se phytoremediation and biofortification. We only briefly address Se hyperaccumulation here and refer interested readers to thorough reviews on Se homeostasis and hyperaccumulation (Zhu et al. 2009; Barillas et al. 2011; Schiavon and Pilon-Smits 2016; White 2016). Selenium hyperaccumulation has been described for 45 taxa scattered among six families. More than half of Se hyperaccumulators have been described in the genus *Astragalus* (Fabaceae), but other well-described Se hyperaccumulators have been found in the genera *Stanleya* (Brassicaceae), *Oonopsis*, and *Xylorhiza* (Asteraceae). The distribution of Se hyperaccumulators among plant families suggests that Se hyperaccumulation likely evolved independently at least six times (Cappa and Pilon-Smits 2014). Selenium is available to plants mostly as selenate (SeO_4^{2-}), a structural homologue of sulphate, or selenite (SeO_3^{2-}), depending on the nature of the soil (Elrashidi et al. 1987). In cultivated soils, selenate uptake by root cells is catalyzed by high-affinity sulphate transporters of the SULTR family (Shibagaki et al. 2002; El Kassis et al. 2007; Barberon et al. 2008). Transporters of this family are highly and constitutively expressed in Se hyperaccumulators of the *Astragalus* and *Stanleya* genera (Freeman et al. 2010; Cabannes et al. 2011; Schiavon et al. 2015). The Se hyperaccumulation trait is also linked to the capacity of Se hyperaccumulators to preferentially take up selenate over sulphate. Sequence analysis of SULTR1 transporters from *Astragalus* identified a Gly to Ala polymorphism linked to the hyperaccumulation trait; however, it is not demonstrated yet if this difference explains the preferential uptake of selenate over sulphate (Cabannes et al. 2011). In rice growing in anaerobic soils (e.g. paddy fields), selenite forms are transported in root cells by the phosphate transporters OsPT2 (Zhang et al. 2014) and by aquaporins (Zhao et al. 2010). Long-distance transport of selenate to the shoot is also proposed to be mediated by SULTR transporters (Takahashi et al. 2000). In shoots, the main fraction of selenate is metabolized into organoselenium compounds (SeCys and SeMet) in chloroplast (Zhu et al. 2009; Barillas et al. 2011; White 2016). In hyperaccumulator species, genes involved in the synthesis of organoselenium compounds and in the methylation of SeCys to produce the non-toxic form MeSeCys were shown to be constitutively more expressed than in non-accumulator species (Pickering et al. 2003; Freeman et al. 2010; Schiavon et al. 2015), indicating that the metabolism of Se is key for its hyperaccumulation.

7 Perspectives and Conclusions

7.1 Interaction of Hyperaccumulators with Biotic Environment

The so-called ‘elemental defense’ hypothesis proposes that metal hyperaccumulation provides a defense against pathogens and/or herbivores by direct toxicity (Boyd and Martens 1992; Hörger et al. 2013). Indeed, it has been shown that Ni and Zn accumulation can protect different Brassicaceae species from bacterial and fungal infection (Boyd et al. 1994; Ghaderian et al. 2000; Fones et al. 2010). However, it is also well documented that in environmental conditions, specific populations of bacteria are associated with the root system of metal hyperaccumulators (Aboudrar et al. 2012; Cabello-Conejo et al. 2014; Lucisine et al. 2014; Muehe et al. 2015). Endophytic bacteria have also been identified in the shoots and roots of metal hyperaccumulators (Idris et al. 2004; Mengoni et al. 2009a; Nonnoi et al. 2012). However, since the majority of these bacteria are not cultivable, current studies mostly describe endophytic populations using metagenomics approaches (Chen et al. 2014; Luo et al. 2011; Sessitsch et al. 2012; Visioli et al. 2014). Interestingly, it was shown that the inoculation of

N. caerulescens and *A. serpyllifolium* with cultivable endophytic bacteria increases Ni translocation to shoots (Ma et al. 2011b; Visioli et al. 2015). Little is known about the interactions between metal hyperaccumulators and associated bacteria. Metal hyperaccumulators represent an extreme niche for metal-tolerant bacteria (Mengoni et al. 2009b). On the other hand, metal-tolerant bacteria can improve plant growth and confer protection against abiotic stress by the production of hormones (auxins, cytokinins, etc.), or protect the host plant against other pathogens by the production of antagonistic substances or by competition for space and nutrients (Ma et al. 2011b; Reinhold-Hurek and Hurek 2011). Metal-tolerant bacteria can also produce organic acids and metal chelators that can favor metal solubility, transport, and tolerance (Idris et al. 2006; Ma et al. 2011a; Visioli et al. 2015). These observations suggest intimate interactions between hyperaccumulators and associated bacteria; however, mechanisms and genes involved in these interactions are mostly unknown. The development of next-generation sequencing technologies and associated meta-transcriptomic (or dual-transcriptomics) analyses will uncover those genes expressed by plants and associated bacteria involved in their interaction (Camilios-Neto et al. 2014; Pankievicz et al. 2016). Understanding these mechanisms will be instrumental to improving metal phytoextraction or to producing secondary metabolites such as metal chelators of bacterial origin that can be used in metal-based therapies (Franz 2013).

7.2 Evolution of Hyperaccumulation Mechanisms

After this brief review of our current knowledge of the molecular mechanisms of Zn, Cd, or Ni hyperaccumulation, it is apparent that several candidate genes involved in these processes are involved in the control of metal homeostasis in non-accumulator plants. These genes display an enhanced function in hyperaccumulators, through gene-copy number amplification and/or altered regulation, which profoundly modifies the metal flux in the plants towards shoot accumulation. Many examples have also been presented that highlight the high level of convergent evolution between *A. halleri*, *N. caerulescens*, and *S. alfredii*. This convergent evolution likely reflects—and sheds light on—functional constraints of the metal homeostasis network (Krämer et al. 2007; Krämer 2010; Verbruggen et al. 2009a; Hanikenne and Nouet 2011). The key function of HMA4 in several hyperaccumulator species represents a potent example of this convergent evolution (Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012).

Metal hyperaccumulation and associated tolerance in plants are complex traits that have required the fine-tuning of multiple mechanisms during the course of evolution. If several key players have now been identified, how tolerance and hyperaccumulation traits evolved remains an open ‘chicken or egg’ question. Hence, Bayesian inference suggested that speciation between *A. halleri* and *A. lyrata* closely coincided with *HMA4* duplication (Roux et al. 2011). The complex signature of selection detected at the *HMA4* locus of *A. halleri* further supports the key role of this gene in the evolution of the hyperaccumulation trait (Hanikenne et al. 2013). Recent adaptations to anthropogenic metal-polluted sites possibly occurred independently within distinct phylogeographic units of the *A. halleri* European distribution (Pauwels et al. 2012). Hypertolerance of metallicolous populations thus potentially evolved using a variety of genetic mechanisms (Meyer et al. 2009, 2010; Pauwels et al. 2012). Moreover, the recent study by Meyer et al. (2016) suggests that, if HMA4 contributes to Zn tolerance in both metallicolous and non-metallicolous populations of *A. halleri* (Hanikenne et al. 2013), the function of MTP1 in Zn tolerance may have evolved later in metallicolous populations that colonized recently polluted soils. Indeed, the co-segregation of *MTP1* with Zn tolerance is only observed in a backcross 1 population of a cross between a French metallicolous individual (i.e. living on metal-polluted soil) of *A. halleri* and *A. lyrata*, and is lost when a Slovakian non-metallicolous (i.e. living on non-polluted soil) *A. halleri* individual is used as parent (Meyer et al. 2016). In contrast, the co-segregation of *HMA4* with Zn tolerance is independent of the edaphic origin of the *A. halleri* populations used in the analysis (Willems et al. 2007; Meyer et al. 2016). This interpretation partially contradicts the hypothesis that MTP1 is required for metal detoxification accommodating the high HMA4-dependent metal flux into *A. halleri* shoots, which was proposed based on the observation that expression of *AhHMA4* in non-accumulator plants resulted in increased sensitivity to excess Zn (Hanikenne et al. 2008; Barabasz et al. 2010).

The study of metal hyperaccumulation in distant plant families combined with the comparison of distinct accessions with contrasting accumulation capabilities within a species will likely shed light on the evolution of the hyperaccumulation and hypertolerance traits. Such study may indeed reveal the commonalities and differences in the mechanisms underlying these traits, highlighting evolutionary divergence and convergence. It also may reveal evolutionary 'intermediates' (i.e. genotypes that do not display the full extent of hyperaccumulation or tolerance), which may allow ordering the evolutionary events that took place during the adaptation of the metal homeostasis network.

7.3 How Can Phytoextraction Technologies Benefit from Molecular Knowledge?

To date, most of our knowledge on metal hyperaccumulation arises from studies on a few model hyperaccumulator species (i.e. *A. halleri* and *N. caerulescens*) of the Brassicaceae family. In the future, it will be necessary to pursue molecular studies and improve genetic manipulation of these species, in order to identify and demonstrate the role of key mechanisms involved in metal hyperaccumulation. However, these species have a low biomass and a relatively restricted distribution worldwide. Therefore, one of the coming challenges for the development of agromining/phytoextraction will be to transfer knowledge of the mechanisms involved in metal hyperaccumulation to species having a high potential for phytoextraction. As mentioned above, the development of Next Generation Sequencing technologies opens the possibility for study of 'non-model' species at the genomic and transcriptomic levels.

As for other crop plants, this molecular knowledge will be instrumental for the selection of genotypes having the best potential for metal phytoextraction. The level of expression of key genes involved in metal hyperaccumulation can be used as markers to predict metal accumulation capacities. These marker genes can also be used to study the interaction between metal accumulation and agricultural practices (e.g. fertilization), for improving biomass production while maintaining efficient metal accumulation.

Finally, genome-editing technologies such as CRISPR-CAS9 are currently implemented in plants to specifically modify the sequence of target genes (Doudna and Charpentier 2014). This technology offers several advantages compared to traditional transformation technologies used to produce Genetically Modified Organisms (GMO), and therefore could be better accepted by the civil society and political stakeholders to engineer crop plants for metal phytoextraction. Using this recent technology, we can, for example, imagine specifically introducing point mutations in the sequence of genes involved in metal accumulation in order to increase their activity, improve specificity, or modify selectivity towards metals of interest (Rogers et al. 2000; Menguer et al. 2013; Pottier et al. 2015).

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