Chapter 3
Functional Significance of Metal Ligands in Hyperaccumulating Plants: What Do We Know?

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3.1 Introduction

Due to their physical and chemical properties, the transition metals (e.g., Mn, Fe, Ni, Cu, Zn) can act as specific cofactors for numerous metalloproteins that are involved in different metabolic processes in the cell. Such processes include respiration, nitrogen fixation, and photosynthesis, and thus these transition metals are involved in the maintenance of the functional and structural integrity of plant cells. On the other hand, when they are present in excess, the transition metals can interfere with many metabolic processes, mainly through inhibition of correct protein/enzyme functioning and by causing oxidative damage. At metal-polluted sites, plants also have to deal with the presence of nonessential metals and metalloids (e.g., Cd, Pb, Hg, As, Se), therefore the regulation of their metal homeostasis can be even more challenging.

Metal-hyperaccumulating plants represent a relatively minor group of plant species, and they can accumulate and tolerate metals in their shoots to levels that are several orders of magnitude higher than in other plants. The phenomenon of metal hyperaccumulation has puzzled scientists since its first description by Baumann in 1885 (Brooks 2000). The challenge of holding metal levels within vital limits in the cell requires metal homeostasis mechanisms that can balance the activities of the transporters that mediate import of these metals into the cell, their distribution to the organelles, and their export from the cell. This homeostasis needs metal sensors that must correctly distinguish between the particular inorganic elements (Waldron et al. 2009).

Transcriptional control is especially important in the regulation of this essential transition-metal homeostasis in plants (Pilon et al. 2009). Recent studies have

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indicated that the copy number and expression of specific metal-transporter genes can impact metal hyperaccumulation by triggering root metal deficiency, and consequentially enhancing metal uptake (Hanikenne et al. 2008). In addition, under such circumstances, preventing the association of the inappropriate metals with proteins might be even more challenging than the acquisition of the correct metal by the proteins (Waldron et al. 2009). Consequently, there is still considerable ambiguity around the basic questions of why and how metal hyperaccumulation actually evolved (Rascio and Navari-Izzo 2010).

3.2 Problems in Maintaining Metal Homeostasis

When in contact with organic material, metals tend to bind to specific functional groups, or ligands, of the organic molecules. The four classes of functional polypeptide-derived or *endogenous* ligands recognized in plants are: (a) amino groups at the N terminus; (b) carboxylate groups at the C terminus; (c) carbonyl and amide groups of the main amino acid chain; and (d) side groups of the main amino acid chain (e.g., amide, amino, carboxyl, hydroxyl, imidazole, phenol, selenol, sulfide, thiol groups). Ligands that do not derive from polypeptides are known as *exogenous*, and these range from simple inorganic entities (e.g., oxide, hydroxide, sulfide, water, and other solvent-derived molecules, and physiological ligands, such as dioxygen and nitric oxide) to polydentate organic compounds (e.g., porphyrins and corrins) (Degtyarenko 2000).

The natural order of stability of complexes with transient divalent metals is known as the Irving–Williams series. For high-spin complexes of divalent ions of the first-row transition metals, the stability constants for the formation of complexes follows the order: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II), and this order has been shown to hold for a wide variety of ligands. When compared with other divalent cations, such as Ca and Mg, the stabilities of these transition-metal complexes are much greater (Irving and Williams 1948). Biology must therefore overcome this trend for some proteins to bind uncompetitive metals and for others to bind competitive ones.

To achieve metal homeostasis, the amount of each metal within a cell has to be sensed, to allow adjustments of the actions of the transporters at the plasma membrane or on internal compartments. Metal importers, metal exporters, and metal stores maintain a limited supply of competitive metals. At low metal concentrations in plants, proteins compete with other molecules for these metals, rather than the metals competing with other metals for the proteins. Numerous proteins have therefore developed dependencies on metal species that cannot be readily replaced. In extreme cases only, metals with similar properties can be replaced with metals that might have once been plentiful, but are now scarce (Waldron et al. 2009). On metal-polluted sites, however, the metals that are in abundance can compete with the original metals for the active binding sites on proteins causing metal imbalances and misfunctioning. Obviously, metal-hyperaccumulating plants have found solutions to overcome these situations.

One way to ensure that the correct metal is acquired by a metalloenzyme is to exploit the delivery proteins, i.e., the metallochaperones. Metals are passed from metallochaperones to cognate apoproteins by means of ligand-exchange reactions that determine which proteins gain access to the metals supplied (Waldron et al. 2009). A complete inventory of *Arabidopsis thaliana* metallochaperone-like proteins reveals a large family of 67 proteins. These include proteins containing heavy-metal-associated (HMA) domains and heavy metal ATPases (Tehseen et al. 2010). Metallochaperones in plants provide the plant needs during the life cycle and contribute to metal redistribution to storage organs during plant senescence (Mira et al. 2001). The presumed roles of these metallochaperones at the cellular level is illustrated in Fig. 3.1.

When metals are present at toxic concentrations, the metallochaperones can bind the excess metals, and hence they contribute to metal detoxification. Homologs of all three groups of metallochaperones described in yeast have also been described in *Arabidopsis*. By analogy to their functions in other organisms, the ATX1-like Cu chaperones interact with the P-type ATPase HMA5, with a presumable role in Cu compartmentalization and detoxification (Andrés-Colás et al. 2006). This might

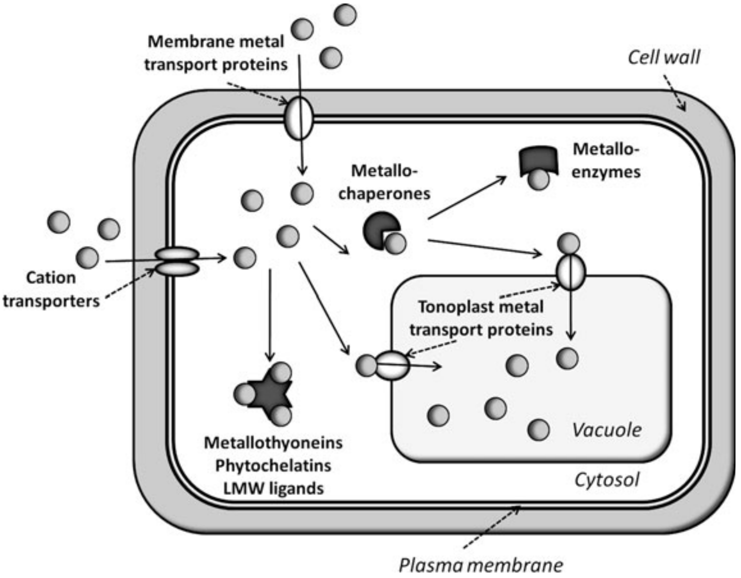

Fig. 3.1 A proposed role of metalchaperones in plant cell is trafficking metals to metalloenzymes. When metals are present in access metallochaperones are proposed to interact with tonoplast metal transporters to facilitate metal compartmentation and detoxification. In addition, low cytosolic free metal concentrations can also be maintained by scavenging systems including phytochelatins, metallothioneins, and low molecular weight (LMW) ligands such as amino acids, glutathione, and LMW thiols

also apply to metal-hyperaccumulating plant species. In the metal-hyperaccumulating *Thlaspi caerulescens*, the overexpression of the P-type ATPase HMA4 confers increased Cd tolerance through its functioning as a Cd-efflux transporter. This contribution to enhanced xylem loading makes HMA4 a key player in the metal-hyperaccumulation character of *T. caerulescens* (Papoyan and Kochian 2004), probably with the engagement of one of the metallochaperones. The involvement of HMA4 in metal hyperaccumulation has also been indicated by new insights into the metal hyperaccumulation and hypertolerance of *Arabidopsis halleri*. After a reduction in the expression of *AhHMA4* using RNA interference, Hanikenne et al. (2008) demonstrated that both, an increase in the copy number, and an elevated expression of the individual *AhHMA4* gene copies contribute to hyperaccumulation and hypertolerance of Zn by enhancing the xylem loading and transport of Zn to shoots, and acting as a physiological master switch in the up-regulation of Zn-deficiency-response gene expression in roots. Clearly, more studies are needed before we can determine whether metal hyperaccumulation per se developed as a metal homeostasis response to metal imbalances that are triggered by metal deficiency in plants, and decipher the role(s) of the metallochaperones in the metal homeostasis of metal-hyperaccumulating plants.

3.3 Soil-Metal Partitioning and Toxicity

Metal speciation and the related solid–liquid partitioning greatly affect the mobility and bioavailability of metals in the soil. The major determinants that affect soil metal speciation are: (a) adsorption of metals to organic matter, oxyhydroxides, and clay minerals; and (b) precipitation of metals as pure or mixed solids, and their complexation with inorganic or organic ligands or mineral colloids. Among the properties of the soil, pH is the most important factor affecting the retention of free metal ions (Degryse et al. 2009). Indeed, total metal concentrations are known to be poor predictors of metal toxicity (Smolders et al. 2009).

The leaching of metals from the soil into the groundwater is also related to the dissolved metal concentrations and the amounts of metal in the solid phase that buffers the metal in solution. The free metal is considered to be the major determinant of metal bioavailability. The part of the solid-phase metal that is rapidly exchangeable with the solution phase is known as the “labile” phase, and this should also be a better predictor of metal-ion buffering in solute transport models than the total metal concentrations (Degryse et al. 2009). Therefore, the uptake of trace metals from solution by plants is commonly believed to depend on the free metal ion activity, rather than on the total metal concentration, and this has been explained by the free-ion activity model (FIAM) of the toxicities of trace metals towards higher plants. Many strong synthetic chelators provide further evidence for this view, whereas the presence of weak soluble ligands (e.g., organic acids and amino acids) alters the aqueous speciation of the trace metals and their availability to higher plants (Parker et al. 2001). Consequently, the uptake of metals such as Cd and Zn from such solutions might actually be greater than the uptake from ligand-free solutions with the same Cd and Zn ion activities. This can be partially explained by intact ligand–metal uptake complexes, and partially by breaks in the endodermal barrier of root apices and of the lateral root initiation sites, where metal–ligand complexes are transported with the water flow via the apoplastic pathway. Unfortunately, reported exceptions to the FIAM do not appear to occur in a predictable and systematic way, with regard to metal or organic ligand strengths, or plant species (McLaughlin et al. 1997; Parker et al. 2001).

In bulk soils, the situation is even more complex because of limitations in metal transport to the root surface, complexation of metals with organic ligands in the soil, and the tendency of roots to alter the chemistry of the rhizosphere (Degryse et al. 2006). Metal-hyperaccumulating plants are known to form “hot spots,” meaning that in the rhizosphere of metal-hyperaccumulating plants, the metal concentrations are greatly increased, compared to those of non-metal-accumulating plants. As metals are known to stabilize the soil organic metal content (Lützow et al. 2006), which is partially due to decreased activities of the soil microorganisms that are involved in organic-matter decomposition, it is of no particular surprise that more organic matter is found in the *Thlaspi goesingense* rhizosphere than in that of non-metal-accumulating plants from the same sampling site (Wenzel et al. 2003). An increase in the organic matter content might therefore enhance metal uptake further through the uptake of intact metal–organic complexes. Indeed, field-collected data reinforce the hypothesis relating to ligand-induced Ni solubility in the rhizosphere of *T. goesingense*, which was attributed to the formation of Ni-organic acid complexes (Wenzel et al. 2003). In addition, increased mobilization of Cd and Zn in the rhizosphere of the metal-hyperaccumulating *Sedum alfredii* can at least partially be attributed to the synthesis of short-chain organic acids (e.g., oxalic, tartaric, formic, acetic acids) by the rhizosphere bacteria associated with the roots, although the presence of metals in the substrate might have a large effect on this property (Li et al. 2010). Despite their importance, metal-tolerance mechanisms of arbuscular mycorrhizal fungi have been poorly documented, which is primarily due to the difficulties in their cultivation. Indeed, there are some discrepancies in the current literature regarding the ability of arbuscular mycorrhizal fungi to exude organic acids. It appears likely, however, that there are substantial differences between fungal isolates in this regard, which might also be related to metal-toxicity alleviation, as demonstrated in the case of aluminum (Klugh and Cumming 2007). Although there are now few hundreds of known metal-hyperaccumulating plants, it is estimated that to date only 10% of these have had their rhizospheres examined (Alford et al. 2010). Therefore, further research is needed to resolve the diversity of rhizosphere microorganisms of metal-hyperaccumulating plants and their interactions that are involved in metal uptake.

3.4 Metal Immobilization and Transport in the Root

From the large body of evidence that has accumulated over the last decade, it has become obvious that not one, but rather several mechanisms provide metal immobilization and promote tolerance to elevated metal concentrations in metal-hyperaccumulating plants. This system begins immediately in the poorly explored apoplasts, where metal ions are first adsorbed by the carbohydrate network of the root/cell wall. The dependence of metal uptake on pH indicates that acidic carboxyl (R–COOH) and hydroxyl (R–OH) groups of cell wall constituents, together with amines, phosphates, thiols, and others, are the most likely sites for ion exchange (Kratochvil and Volesky 1998). In addition to physical and chemical properties of a metal, metal–cell wall interactions also largely depend on the number of particular functional groups with which the metals can interact. When comparing monocotyledonous and dicotyledonous plant species, the later have higher pectin content and higher numbers of available free carboxyl and hydroxyl groups in their cell walls, and so these can adsorb more metals on the root surface because of this greater cation-exchange capacity (Mari and Lebrun 2005). The capacity of the root cell walls probably governs further uptake of metals by symplastic pathways up to the shoots, and it is well established that dicotyledonous plants are more efficient metal accumulators than monocotyledonous plants.

In the root cortex, metals and metal complexes have to enter the symplast before they can pass through the endodermis (Wang et al. 2010). In the cytoplasm, metals can either bind to free amino acids, the nonproteinogenic nicotianamine, protein ligands that are rich in Cys residues, such as metallothioneins, metallochaperones, phytochelatins, and low molecular weight thiols (Callahan et al. 2006; Krämer and Clemens 2005; Hernández-Allica et al. 2006; Trampczynska et al. 2010).

The chelation of metals with specific ligands can influence metal partitioning and transport. Root Zn in *T. caerulescens* was shown to be coordinated to histidine, which is believed to have an important role in Zn homeostasis in the root (Salt et al. 1999). Histidine also has a relatively high association constant for Ni. In addition, exposure of the hyperaccumulating *Allysum lesbiacum* to Ni elicitates high levels of free histidine synthesis and enhanced xylem loading. Histidine has therefore been proposed to be an important determinant of Ni accumulation in this species. However, the same property was not confirmed for *T. goesingense* (Kerkeb and Krämer 2003; Persans et al. 1999).

Root phytochelatin synthesis is accompanied by higher levels of Cd retention in the roots of the nonhyperaccumulating *Thlaspi arvense* compared to the metal-hyperaccumulating *T. caerulescens*, which indicates the importance of phytochelatin synthesis in accelerated metal sequestration in roots, in the vacuoles, of nonaccumulating plant species, when compared to metal hyperaccumulators (Ebbs et al. 2002; Wong and Cobbett 2008). It is generally believed that extensive phytochelatin synthesis that would significantly contribute to hypertolerance in metal-hyperaccumulating plants would be too costly. Instead, the elevated levels of glutathione and low molecular weight thiols that are found in the root tips of *T. goesingense* and *T. caerulescens* (Hernández-Allica et al. 2006) are believed to contribute to reduced production of reactive oxygen species, and the withstanding of relatively high cytosolic metal concentrations (Freeman et al. 2004). Thus, these elevated levels of glutathione probably also have an important role in the enhancement of xylem loading.

The metallothioneins are metal-binding proteins that show differential expression patterns in differing plant organs. It was proposed that Cys arrangements in the metallothioneins confer differential metal-binding properties to the different molecules. Although their exact functions remain a matter of debate, they appear to function in metal tolerance and/or homeostasis. They can bind six to eight Cu, Zn, Cd, Hg, and Pb ions as M_3 or M_4 clusters. In addition to their functions as safe depositories of metal ions, they might also allow metal delivery to transport proteins (Callahan et al. 2006). Expression of metallothionein genes in metal-hyperaccumulating plants can also exceed the expression found in nonaccumulating plant species, which supports their role in metal hyperaccumulation. In addition, the expression patterns of the metallothionein genes can differ across hyperaccumulators and nonaccumulators. However, it has been suggested that these are not directly involved in metal accumulation, but rather contribute to metal tolerance, presumably by contributing to the elemental homeostasis under excess metal conditions (Rosens et al. 2004).

In the xylem sap of *T. caerulescens*, Zn can be found as its free ion or it can be chelated to organic acids that are responsible for root-to-shoot transport. Apparently, at low pH values of the xylem sap (pH ~5.5), the imidazole nitrogen of histidine becomes protonated, which results in a decrease in the stability of the Zn–histidine complex, which favors complexation of Zn with citrate (Salt et al. 1999). About half of the cellular Zn has also been shown to be bound to nicotianamine, which has been shown to be a predominant chelator for Zn shoot transport rather than for Zn storage (Mari et al. 2006; Trampczynska et al. 2010). In addition to forming stable complexes with Zn, nicotianamine can also bind Fe, Ni, Cu, Mn, and Co. Transporters from the yellow stripe-like (YSL) transporter family are believed to be involved in long-distance transport of metal–nicotianamine complexes in plants, and in roots they are believed to mediate metal uptake into cells that are directly involved in xylem loading (Curie et al. 2009). On the other hand, root xylem loading of Cd by HMA2 and HMA4 was recently described as the decisive mechanism for Cd translocation to the shoot in *A. thaliana* (Wong and Cobbett 2008). Although small differences can be seen even between different populations, Cd in the *Thlaspi* species is mostly transported coordinated to thiols and organic acids (Küpper et al. 2004; Ebbs et al. 2008; Vogel-Mikuš et al. 2010).

3.5 Metal Immobilization and Compartmentalization in the Shoot

Metal-localization studies have shown that, in general, mechanisms of metal accumulation at the plant organ, tissue, and cellular levels lead to metal immobilization within tissues that are less metabolically active, thus maintaining plant functions across a wide range of metal concentrations (Küpper et al. 1999; Küpper et al. 2001; Vogel-Mikuš et al. 2008). Epidermal cells of leaves are recognized as efficient sinks for Ni, Zn, and Cd, and they represent important tolerance mechanism in the *Thlaspi* species (Küpper et al. 1999; Küpper et al. 2001; Krämer et al. 2000; Vogel-Mikuš et al. 2008). However, as the mesophyll is composed of considerably larger volume percentage of the leaf, the total mesophyll Cd and Zn concentrations might be even more important as metal sinks, despite the lower mesophyll concentrations when compared to epidermal tissues, with the vacuoles of mesophyll cells as the predominant stores (Ma et al. 2005; Vogel-Mikuš et al. 2008). In plants with trichomes, trichome cells might accommodate the majority of Cd and Zn, as demonstrated for *A. halleri* (Küpper et al. 2000). At the cellular level, the most pronounced mechanism of metal accumulation is sequestration into the vacuole, although in aluminum-accumulating tea plants, for example, the predominant mechanism of metal immobilization is through binding of the aluminum to the cell walls of epidermal cells (Tolrà et al. 2011).

Immobilization and compartmentalization of metals in metabolically less active parts of tissues and cells indicate that the processes of metal homeostasis are highly regulated and involve several metal-chelating molecules that can be found in the cytoplasm, the vacuole, and even in the cell wall. Ligands that bind metals more tightly (strong ligands) are considered to be mainly in the cytosol, especially in young plant tissues, where the vacuoles are not yet fully developed (Küpper et al. 2004; Vogel-Mikuš et al. 2010). In the vacuoles, however, organic acids that are considered to be weak metal ligands usually provide the majority of the binding sites for metal ions.

Early studies of the coordination of metals in metal-hyperaccumulating plants showed that histidine coordinates Ni, but it was not recognized as the primary determinant of Ni hyperaccumulation in *T. goesingense* (Persans et al. 1999). Rather, the Ni–histidine complexes appeared to be present in the cytoplasm, whereas most of the intracellular Ni found in the vacuoles was coordinated to citrate (Krämer et al. 2000), thus supporting the above-mentioned hypothesis. In addition, complexation of Ni with nicotianamine can facilitate vacuolar Ni transport (Trampczynska et al. 2010). The ability of *T. goesingense* to hyperaccumulate Ni seems to be governed by enhanced accumulation of Ni within the leaf vacuoles. The transporter responsible for this vacuolar accumulation, TgMTP1, has also been recognized to confer tolerance to Ni, Cd, Co, and Zn (Persans et al. 2001). Enhanced cellular protection of *T. goesingense* from lipid peroxidation is additionally provided by elevated glutathione levels (Freeman et al. 2004). Similarly, most of the Zn in *T. caerulescens* is coordinated to citrate, whereas chelation with histidine, the cell wall, and oxalate contribute little to Zn shoot immobilization (Salt et al. 1999). High organic acid concentrations are a constitutive property of *T. caerulescens*, and these might be responsible for the high Zn requirement in this species due to extensive vacuolar sequestration (Tolrà et al. 1996). Instead, shoot Cd appears to be coordinated mainly with oxygen or sulfur ligands depending on the developmental stage of the plant (Küpper et al. 2004; Vogel-Mikuš et al. 2010). As the storage of metals coordinated with oxygen ligands in vacuoles of leaves is presumably less costly than the synthesis of strong metal-specific ligands, vacuolar Cd storage is the predominant tolerance mechanism of mature and senescent leaves. In young tissues, however, sulfur ligands predominate, which might also reflect smaller proportions of metals being sequestered in vacuoles. For phloem transport, Cd is bound to thiol (–SH) ligands, most likely glutathione, although it might also be bound to nicotianamine, since in *T. praecox*, around one third of the ligands present in the phloem belong to O/N groups. In seeds, as well as the sulfur ligands that represented two-thirds of all of the Cd ligands, phytate has been suggested as an additional Cd-coordination molecule that is not found in vegetative tissues. Before vacuoles become fully functional, coordination of Cd to strong ligands in embryonic tissues seems to be a reasonable solution related to ontogenesis. In addition, larger portions of Zn and Cd coordination to histidine in old and senescent leaves reinforce the hypothesis on ontogenesis as an important determinant for metal coordination, which is presumably related to metal remobilization during senescence and the related deterioration of vacuoles (Küpper et al. 2004; Vogel-Mikuš et al. 2010).

Metal coordination has also been reported to change with metal concentration. By increasing the shoot Cd concentrations in *T. caerulescens*, the proportion of oxygen ligands in mature leaves decreases relative to the proportions of histidine and sulfur ligands (Küpper et al. 2004). This indicates that increasing Cd concentrations in tissues lead to increases in the proportion of cytosolic Cd. However, more studies will be needed to reveal the primary ligands of the different metals over the wider range of metal concentrations.

3.6 In Situ Analyses of Metal Ligands in Plants

Due to the reactivities of these metals towards biological components, metal immobilization is considered to be the key step in metal hypertolerance. The mechanisms of metal immobilization have, however, received significantly less attention than the mechanisms of metal uptake and transport, primarily because of the need for the development and adoption of noninvasive and nondestructive techniques that can be aimed at precisely identifying the localization of metal ions within plant tissues and the ligands involved in their chelation (Mari and Lebrun 2005).

Even today, with all of the advantages of the modern analytical equipment that is available to us, the determination of metal ligands in plant tissues still represents a challenge. This is due to the relatively low metal concentrations in the tissues and the ease with which metal–ligand complexes can be lost or modified during sample preparation. The speciation and chemical form of metals in plants can be determined by specialized techniques, such as HPLC coupled to element-specific detection by inductively coupled plasma mass spectrometry (ICP-MS) or electron-spray ionization mass spectrometry (ESI-MS). The most popular technique, HPLC-ICP-MS, uses conventional HPLC with either reverse-phase or ion-exchange columns to separate the metal–ligand compounds, which are then detected by an ICP-MS tuned to specific metal isotopes. However, to be able to propose a reliable structure of the metal–ligand complexes detected, in addition to this mass spectrometry data, chemical and physiochemical data are often required.

From the plant physiology point of view, the main disadvantage of these techniques is that the metal–ligand compounds have to be removed from the matrix (extracted). During the extraction procedure, the metal–ligand environment can be altered by chemical pretreatment of the samples, as the release of the contents from different cell compartments can result in the formation of complexes that are not present in vivo (Callahan et al. 2006). To avoid the studying of artifacts due to sample preparation procedures, the use of complementary techniques, such as nuclear magnetic resonance (NMR), and direct speciation methods using either X-ray absorption or diffraction techniques (e.g., extended X-ray absorption fine structure [EXAFS], X-ray absorption near-edge structure [XANES], X-ray diffraction [XRD], and microprobe analysis) that give access to structural information of solid samples is of great importance in these types of studies. These techniques mentioned have become increasingly popular because of the minimal sample preparation needed and the resulting decreased risk of species transformation (Caruso et al. 2003). In metal-hyperaccumulating plants, the majority of studies that have dealt with identification of metal ligands have used the EXAFS and XANES X-ray absorption techniques, e.g., Persans et al. (1999) for determination of Ni coordination in *T. goesingense*; Küpper et al. (2004) for determination of Cd and Zn ligands in leaves of *T. caerulescens*; and Vogel-Mikuš et al. (2010) for determination of Cd ligands in vegetative tissues and seeds of *T. praecox*. With the development of synchrotron radiation stations with beam lines that are dedicated to (sub)micro-X-ray fluorescence mapping, it now even becomes possible to localize the metals in tissues at the cellular and subcellular levels. XANES and/or EXAFS spectra can also be recorded to provide information concerning the chemical oxidation stages and coordination geometries of elements in complexes (XANES), and information on the local environment of the atoms investigated in the sample (i.e., the number and species of neighboring atoms, their distance from the selected atom, and the thermal or structural disorder of their positions) (Gardea-Torresday et al. 2005 and ref. therein, Fakuda et al. 2008). Typical examples of these types of studies can be found in Isaure et al. (2006) and Fakuda et al. (2008), where micro-XRF was used for the localization of Cd, and micro-XANES for the determination of the Cd ligands in *A. thaliana* and *A. halleri*. In addition, Kachenko et al. (2010) used the same techniques for localization and arsenic speciation in the leaves of the fern *Pityrogramma calomelanos* var. *austroamericana*.

3.7 Conclusions

There are thus several levels of metal immobilization in plants. Some appear to have developed from the need to deliver metals to metalloproteins, whereas others appear to result from more specific responses of metal-hyperaccumulating plants to high metal concentrations. There are clear differences in the regulation of metal homeostasis between non-metal-accumulating and metal-hyperaccumulating plants. To date, little is known about the plant metal perception and metal sensing, although the greater metal requirements of metal-hyperaccumulating plants might arise from reduced metal sequestration in the root and more intensive root-to-shoot metal transport. Recent discoveries lead us to believe that metal hyperaccumulation is connected to the self-imposed metal deficiency imbalance responses that are regulated by mechanisms of metal homeostasis and that are just beginning to emerge. Intense metal sequestration in the shoot, on the other hand, provides mechanisms for intense metal tolerance of hyperaccumulating plants. Taken together, it appears likely that metal-hyperaccumulating plants have what it takes for successful remediation of metal-enriched soils. The question remains, do we know how to successfully explore these plant traits to achieve our goals?

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