Compartmentation and complexation of metals in hyperaccumulator plants

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INTRODUCTION

Many heavy metals such as copper, manganese, nickel, and zinc are well-known as essential trace elements for all living organisms, mainly as active centers of enzymes. And surprisingly even cadmium, which never has been thought to be essential for any organism, has been found to be a micronutrient for the marine alga Thalassiosira weissflogii (Bacilariophyceae) where it builds up the active center of a carbonic anhydrase (Lane and Morel, 2000). For land plants no essential role for cadmium is known to date, although it has been shown to be beneficial for Cd hyperaccumulators as a defense against pathogens and herbivores (Jiang et al., 2005), and in the southern French populations of the Cd/Zn hyperaccumulator Noccaea caerulescens (Brassicaceae) even a growth reduction in absence of Cd was observed without influence of pathogens or herbivores (Liu et al., 2008).

Hyperaccumulators are being intensely investigated. They are not only interesting in scientific context due to their “strange” behavior in terms of dealing with high concentrations of metals, but also because of their use in phytoremediation and phytomining, for which understanding the mechanisms of hyperaccumulation is crucial. Hyperaccumulators naturally use metal accumulation as a defense against herbivores and pathogens, and therefore deal with accumulated metals in very specific ways of compartmentation and complexation, different from non-hyperaccumulator plants and also non-hyperaccumulated metals. For example, in contrast to non-hyperaccumulators, in hyperaccumulators even the classical phytochelatin-inducing metal, cadmium, is predominantly not bound by such sulfur ligands, but only by weak oxygen ligands. This applies to all hyperaccumulated metals investigated so far, as well as hyperaccumulation of the metalloid arsenic. Stronger ligands, as they have been shown to complex metals in non-hyperaccumulators, are in hyperaccumulators used for transient binding during transport to the storage sites (e.g., nicotianamine) and possibly for export of Cu in Cd/Zn hyperaccumulators (metallothioneins (MTs)). This confirmed that enhanced active metal transport, and not metal complexation, is the key mechanism of hyperaccumulation. Hyperaccumulators tolerate the high amount of accumulated metals by sequestering them into vacuoles, usually in large storage cells of the epidermis. This is mediated by strongly elevated expression of specific transport proteins in various tissues from metal uptake in the shoots up to the storage sites in the leaf epidermis. However, this mechanism seems to be very metal specific. Non-hyperaccumulated metals in hyperaccumulators seem to be dealt with like in non-hyperaccumulator plants, i.e., detoxified by binding to strong ligands such as MTs.

Keywords: hyperaccumulator/hypertolerance, excluder, metal sequestration, metal transport, metal storage cells, organic acids, phytochelatin

In many places worldwide, heavy metals can occur in very high concentrations that are detrimental or even lethal to most plant species. These high concentrations are sometimes due to natural factors, leading to natural metallophyte communities, for example in special regions in Africa like the “copper belt” in the Republic of Congo where copper ore naturally comes to the surface (e.g., Duvigneaud, 1958; Malaisse et al., 1999). In many other cases, however, they are caused by human activities (e.g., Romic and Romic, 2003). As prominent examples, they are known to be results of mining (and smelters), where cadmium is often found together with zinc (Buchauer, 1973; Van Geen et al., 1997; Liu et al., 2005), and also high amounts of copper in the soil are found in mining areas (Ke et al., 2007). The same applies for manganese in parts of Southern China (Li et al., 2007). But also application of sewage sludge (McBride et al., 1997) and dust from car tires (Cd is a softener for rubber) along road sides contributed to Cd pollution (Fergusson et al., 1980). Metal pollution is also found in rivers all over Europe (Audry et al., 2004), especially in regions rich in vineyards, as copper is still used as a classical reagent against fungal attacks toward vine plants (Küpper et al., 1996; Ribolzi et al., 2002; He et al., 2009; Mackie et al., 2012; Ruyters et al., 2013).
Based on their ability to cope with metals in the medium they grow on/in, plants can be divided into three types:

(1) Indicator plants are usually sensitive to heavy metals. They can be used as an indicator for metal in the soil because the internal heavy metal concentration is a linear function of the bioavailable heavy metal concentration in the soil (or water for aqueous plants/nutrient solution for hydroponics).

(2) Excluders are a type of plants being able to tolerate heavy metals in the soil up to a threshold concentration by preventing the accumulation of metal in the cells. This exclusion is achieved either by blocking the uptake in the roots (see, e.g., Lux et al., 2011 for a detailed review) or by active (energy dependent) efflux pumps (Baker, 1983; van Hooft et al., 2001). Most metal (hyper)tolerant plants belong to this group (Baker, 1981).

(3) Most important for this topic are plants that can not only tolerate high concentrations of specific elements, but take them up actively and accumulate them to several percent of the dry mass of their above-ground parts. Originally described by Sachs (1865), since 1977 those plants are called hyperaccumulators (Brooks et al., 1977). Many studies on many metals, plant and pathogen species have shown that the hyperaccumulation phenotype naturally serves as a defense against pathogens and herbivores (Boyd and Martens, 1994; Martens and Boyd, 1994; Boyd et al., 2002; Hanson et al., 2003; Ihee et al., 2005 and many further studies since then, discussed in detail, e.g., in the reviews by Küpper and Kronenck, 2005, 2007). In this context, it should be mentioned that hyperaccumulation and hypertolerance are, according to studies on several hyperaccumulator species, genetically independent characters (Macnair et al., 1999; Bert et al., 2003). However, this “independence” is limited because a hyperaccumulator would poison itself if the accumulated metal would not be tolerated via specific mechanisms. More recent works also genetically question this independence, showing partially overlapping quantitative trait loci for accumulation and tolerance (Frerot et al., 2010; Willems et al., 2010). Hyperaccumulators have been found for many heavy elements and within many groups of plants and algae, including at least the following: Al, As, Cd, Cu, I, Mn, Ni, Se, Zn. More hyperaccumulators and hyperaccumulated elements are likely to be found as screening for this phenotype is ongoing and there is still a lot of debate about the definition of hyperaccumulation thresholds. However, nickel hyperaccumulators are by far the most numerous group (several hundred species are known) and biotechnology-oriented research focused on those metals where phytomining and/or phytoremediation are attractive uses, i.e., As, Cd, Cu, Ni, and Zn (see recent reviews, e.g., by Whiting et al., 2004; Chaney et al., 2005, 2007; Küpper and Kronenck, 2005, 2007). In this review, also we put most emphasis on the latter group of metals, but mention other hyperaccumulated elements as well when their complexation and/or compartmentation is different from this “core group” of hyperaccumulated elements. Furthermore, it should be noted that the mechanisms leading to accumulation of metals in hyperaccumulators are specific for one element or at maximum a few elements (see Figure 1 for a short comparison between accumulated and non-accumulated metals). For example, while N. caerulescens accumulates up to several percent of its dry mass of both zinc and (only in case of the southern French populations) >2% of cadmium as well, its accumulation of copper is not elevated compared to non-accumulator species (Mijovilovich et al., 2009), the same applies to As, and it accumulates even less Mn than excluder species (Martínez-Alcántara et al., 2013). Thus it is important to mention that the hyperaccumulation ability always depends strongly on the population and the metal – it is not possible to say that a specific species as such is a hyperaccumulator.

 Depending on the type of plant, a number of strategies to resist the toxicity of heavy metals are known (for a quick summary, see Figure 1). In general, metal tolerance mechanisms include sequestration in specific cell types and intracellular compartments where metals can do the least harm, changing the speciation to less toxic forms by redox mechanisms, precipitation, or binding of metals to ligands, as well as export of metals out of the cells and plants. Since in principle this has been reviewed earlier (Prasad and Hagemeyer, 1996; Cobett and Goldbrough, 2002; Küpper and Kronenck, 2005; Verbruggen et al., 2009; Krämer, 2010 for general reviews; for cadmium accumulation Küpper and Leitenmaier, 2013), the current review will focus on recent new developments in this field that are relevant for a range of different metals.

As the most important mechanisms of metal tolerance in hyperaccumulating plants, compartmentation in specific storage cells as well as various ligands for detoxification and storage of metals will be discussed in the following. Furthermore, differences in metal compartmentation/complexation between hyperaccumulation and non-hyperaccumulating metal metabolism will be highlighted (for an overview, see also Figure 1).

COMPLEXATION

In non-hyper tolerant indicator plants, binding of metals by strong ligands is the main detoxification strategy. The best known type of ligands for this purpose are thiol, including glutathione, phytochelatins (PCs), and metallothioneins (MTs). In addition, some non-thiol ligands are involved, and the non-thiol ligands have a decisive role in hyperaccumulators. Already in 1996 histidine had been shown to be a main ligand of Ni in the Ni hyperaccumulator Alysum lesbiacum (Krämer et al., 1996). In more recent times, the non-protogenic amino acid nicotianamine has been shown to be involved in metal accumulation. Nicotianamine, glutathione, and MTs also have other functions for non-hyperaccumulated metals, such as transient binding during transport to the storage sites, storage and metal re-mobilization at times when metals are needed for active centers of enzymes, etc. PCs, in contrast, seem to be synthesized specifically for metal(loid) detoxification, particularly in the case of As and Cd (reviewed by Cobett and Goldbrough, 2002).

CYSTEINE

Cysteine is well-known to be the most important metal-binding amino acid residue in proteins. Furthermore, as discussed below, it is a building block of PCs, the main As and Cd detoxifying compounds in non-hyperaccumulating plants. As a free amino acid, however, it has not been identified to be involved in binding metals for detoxification.

HISTIDINE

Histidine is, besides the third ligand cysteine, the second most important amino acid residue forming metal-binding sites in metalloproteins, and both residues often are combined in the same site.
Less known, however, is the role of the free amino acid histidine as a metal ligand. It was first shown to bind a major proportion of Ni in the Ni hyperaccumulator Alyssum lesbiacum (Krämer et al., 1996), but later it was found to be involved in binding zinc in hyperaccumulators as well (Salt et al., 1999; Küpper et al., 2004). In Alyssum lesbiacum, histidine was suggested to enhance loading of Ni into the xylem by a co-release of Ni and His into the xylem (Kerkeb and Krämer, 2003). In this context it is important to say that free histidine as a simple monodentate ligand is not binding metals very strongly, in contrast to the multi-histidine residue metal-binding sites in proteins. In this way, free histidine is important as a ligand most likely only in compartments where stronger ligands are scarce, e.g., the vacuole, xylem, or transport vesicles inside the cytoplasm, although low pH might limit complexation of metals by histidine in the vacuole and xylem. In the phloem, also stronger metal ligands (MTs, see below) were found, but histidine might bind metals there if the higher affinity sites are occupied. In the cytoplasm itself, in contrast, histidine most likely is an important ligand only as an amino acid residue of metal-binding proteins unless these high-affinity sites are already saturated with an excess of metal and all other detoxification ligands would be occupied (i.e., conditions of severe toxicity). Because of this, an alternative explanation seems more likely for the results of Richau et al. (2009). These authors demonstrated that addition of histidine to roots of N. caerulescens enhances loading of the xylem with Ni, but can block loading of Ni into isolated vacuoles. From the latter observation they concluded that Ni would bind to free His in the cytoplasm. In view of the facts discussed above, it seems more likely that His did not bind Ni directly in the cytoplasm, but in transport vesicles. Metal-rich vesicles have been observed in the cytoplasm of cells of hyperaccumulators (Neumann and De Figueiro, 2002; Leitenmaier and Küpper, 2011), but so far it was not possible to analyze metal speciation in such small compartments.

NICOTIAMINE

As a polydentate ligand with three carboxyl groups and three nitrogen atoms, this molecule is a rather strong ligand with pKs of 12.1 for Fe^{2+}, 14.7 for Zn^{2+}, 16.1 for Ni^{2+}, and 18.6 for Cu^{2+} (Beneš et al., 2002).
While histidine and nicotianamine are becoming more known as tel et al., 2006, 2008) led to the question whether it may play a role in long-distance zinc transport in hyperaccumulators and hyperaccumulation of these metals (Deinlein et al., 2012). This hypothesis was further supported by the finding that binding of zinc to nicotianamine was shown in vivo (Trampczynska et al., 2010). Soon, it was shown to be important for copper metabolism (Pitcher et al., 1994; Pitch and Scholz, 1996; Liao et al., 2000; Irtelli et al., 2009), and it was found to bind a significant proportion of total copper at physiologically relevant concentrations in N. caerulescens (that does not hyperaccumulate copper; Mijovic et al., 2009). Also binding of zinc to nicotianamine was shown in vivo (Trampczynska et al., 2010).

Taking the information above together, when present in low concentrations nicotianamine likely is important as a ligand mostly in compartments with low abundance of stronger ligands (e.g., the vacuole, xylem, and phloem). At the very high nicotianamine levels in hyperaccumulators, however, it might also take part in binding of these metals in the cytoplasm, although direct evidence for that (e.g., extended X-ray absorption fine structure (EXAFS) spectroscopy with subcellular resolution on intact frozen-hydrated tissues) is still missing because it is currently not yet possible due to technical reasons.

OTHER NON-THIOL LIGANDS/DETOXIFYING ANIONS

While histidine and nicotianamine are becoming more known as relevant metal ligands in plants, other non-thiol ligands have been shown to make a major contribution in particular cases, and it is possible that their actual importance is grossly underestimated. One such example are anthocyanins, which have been shown to be associated with molybdenum accumulation (Hale et al., 2001). Another such case is oxalate, which was first shown to bind copper in copper-tolerant lichens and fungi (Chudzholm et al., 1987; Fonnasa et al., 2005; Purvis et al., 2008) and also has been reported (by X-ray absorption spectroscopy (XAS) studies) to be the major ligand for manganese in Phytolacca acinosa Rohn. and Phytolacca americana (Phytolaccaeae; Du et al., 2009; Xu et al., 2009). Furthermore, oxalate turned out to contribute a major part to copper binding in the copper sensitive Cd/Zn hyperaccumulator N. caerulescens, particularly in the more Cu-tolerant individuals of the population (Mijovic et al., 2009). While binding to oxalate will really detoxify copper and manganese, since copper and manganese oxalates are hardly soluble and a rather stable complex, binding to smaller organic acids like malic or citric acid will not significantly diminish bioavailability of toxic metals. For this reason, at first glance it is surprising that in various hyperaccumulator plants most of the hyperaccumulated metal(loid)s are associated with such organic acids (As: Wang et al., 2002; Cd: Küpper et al., 2006; Cu: Küpper et al., 2009; Mn, in various species outside the family of Phytolaccaeae: Fernando et al., 2010; Ni: Sagner et al., 1998; Zn: Salt et al., 1999). It is logical, however, in context with the compartmentation of metals in vacuoles of such plants, see next chapter of this review for details. In these compartments, in all plants high levels of malate and citrate are stored, so that storage of the metal(loid)s in the same compartment will inevitably lead to a weak association of both.

Organic acid ligands may also play a role in enhanced uptake of metals into the roots of hyperaccumulators. A recent study (Li et al., 2012) has shown that dissolved organic matter (DOM) in the rhizosphere of a hyperaccumulator ectype of Sedum alfredii contained more organic acids and mobilized zinc better than DOM from a non-accumulator ectype of S. alfredii.

METALLOTHIONEINS

Metallothioneins are the best known metal detoxifying ligands, because they have been known from animal physiology for a very long time (reviewed by Cobbett and Goldsbrough, 2002). Soon, they were found in plants as well, although in less variants than in animals (for a review, see Freisinger, 2011). Their role in plants, however, seems to be significantly different from animals. In plants (non-hyperaccumulator plants as well as hyperaccumulators), their main role seems to be metal homeostasis under normal, non-toxic physiological conditions. For example, upregulation of MTs has been shown to occur during senescence of plant tissues for re-mobilization of copper (original research: Buchanan-Wollaston, 1994; Guo et al., 2003; Hense et al., 2007), and MT expression has been shown in the phloem (Vlaisavlje, 2003; Barnes et al., 2004). Furthermore, Zn-MTs seem to be involved in physiological Zn disposition in developing seeds. Here, the best studied case is the MT Ec1 from wheat (Kawashima et al., 1992; Penuza and Freisinger, 2007; Loebus et al., 2011). In terms of detoxification, they seem not to be very important for Cd, but do play a role for Cu (reviewed by Cobbett and Goldsbrough, 2002). The study of Garcia-Hernandez et al. (1998) suggested that MT1 is important for scavenging copper mainly in leaf veins; as copper-induced MT1 expression was observed mainly in leaf veins and to a lesser extent in mesophyll cells, while hardly any induction of MT1 in response to copper stress was observed in roots.

In hyperaccumulator plants, MTs are not important for storage of the hyperaccumulated metals, as these are generally not bound by thiol ligands (see other Non-Thiol Ligands/Detoxifying Anions). Nevertheless, they were found to be overexpressed in the Cd/Zn hyperaccumulator N. caerulescens compared to related non-accumulators (Pepys and Kochian, 2004). In our opinion, the most likely reason for this seemingly contradictory facts is detoxification of copper by MTs in hyperaccumulators of other metals (e.g., Cd, Zn, Ni) that are sensitive toward copper (Walker and Bernal, 2008; Mijovic et al., 2009). This is likely important for Cd/Zn hyperaccumulation, because due to
Andresen et al., 2013), as they frequently occur in the environment. Phytochelatins were discovered as heavy metal detoxifying ligands almost undetectable levels if better than ACS grade chemicals (e.g., Suprapur/T raceSelect), ultraclean water and acid washed labware (Andresen et al., 2013). In the latter study, PC levels dropped to insignificant (Schat et al., 2002). Comparing different ecotypes of N. caerulescens, these authors furthermore showed that in the non-Cd-hyperaccumulating, Cd-sensitive populations blockage of PC synthesis made the already Cd-sensitive populations (and only those) even more sensitive to Cd toxicity, i.e., they behaved like all non-hypertolerant/non-accumulator other species would do. Additionally, metal hyperaccumulator plants were found to have lower PC levels than related non-accumulator plants (Ebbs et al., 2002). Furthermore, in contrast to MTs (see above), PCs do not seem to be involved in binding of the non-hyperaccumulated but toxic copper in the Cd/Zn hyperaccumulator N. caerulescens (Mijović et al., 2009). This could again be shown by EXAFS spectroscopy. Copper PCs have a completely different EXAFS spectrum than Cu-MTs, with only one strong Cu–Cu interaction peaking at 2.56 Å and a weak contribution at 4.94 Å (Poletti et al., 2000), but no contribution at distances where peaks in the Fourier transform were observed (Mijović et al., 2009).

**Glutathione**

Glutathione is the main building block which PC synthesis uses for making PCs. Besides this indirect involvement in metal detoxification, there are also some indications that glutathione itself acts as a low-affinity (monodentate) ligand for Cd. Pietrini et al. (2003) have found, using 50–100 μM cadmium to induce toxicity in Phragmites australis, a 30-fold increase in the concentration of reduced glutathione in leaves. In contrast, cadmium and copper at more realistic concentrations (0.03–3 μM total dissolved Cd) that already caused a strong induction of PCs in phytoplankton did not lead to upregulation of glutathione synthesis under metal stress conditions (Ahner et al., 2002). In hyperaccumulators, no upregulation of glutathione levels in response to metal uptake has been reported so far, and (as mentioned before) thiol ligands are generally not much involved in metal storage in hyperaccumulators (As: Wang et al., 2002; Cd: Küpper et al., 2004; Cu: Küpper et al., 2009; Ni: Sagner et al., 1998; Zn: Salt et al., 1999). However, a constitutive high level of glutathione biosynthesis may contribute...
While metal uptake through the root is the first important step in hyperaccumulation in land plants, most of the metal is stored in the shoots. Storage in the above-ground tissues is part of the definition of hyperaccumulators, and since the original discovery almost 150 years ago (Sachs, 1865) many studies throughout many decades have shown this phenotype (reviews, e.g., by Baker, 1981; Brooks, 1998; Kupper and Krońek, 2005). As a particularly detailed recent long-term study dealing with cadmium uptake, Lovy et al. (2013) show that in the Cd/Zn hyperaccumulator *N. caerulescens*, exposed to constant Cd concentrations throughout the complete growth cycle, 86% of the Cd taken up was allocated to the shoots.

As a prerequisite for efficient transport from the roots to the shoots, the first important step in hyperaccumulation is a reduced sequestration into the root vacuoles of the heavy metal accumulating plant. As just discussed, during the last decade numerous studies have been undertaken successfully to identify genes that are involved in metal transport in hyperaccumulators. Attempts to isolate and characterize the corresponding proteins have been successful, and the significance of the role of these proteins in metal hyperaccumulation, as revealed already by their first discovery almost 150 years ago (Sachs, 1865), has now been established.

**COMPARTMENTATION**

**ENHANCED TRANSLLOCATION OF METALS FROM ROOTS TO THE SHOOT**

As just discussed, during the last decade numerous studies have been successful in demonstrating metals in plant roots. Moreover, the significance of the role of the heavy metal accumulating plants in the transport of metals, especially Cd and Zn, has now been established.

**OVEREXPRESSED TRANSPORT PROTEINS**

**TRANSPORT TOWARD THE FINAL STORAGE CELLS VIA OVEREXPRESSED TRANSPORT PROTEINS**

Xylem loading and xylem transport and thus distribution of metals away from the roots and toward the final storage sites are key steps in metal hyperaccumulation, as was commented by White et al. (2002).

It has been found that metal hyperaccumulation is mediated, at least in part, by an up to 200 times higher expression of various metal transporter genes in hyperaccumulators compared to non-accumulating plants (Pence et al., 2000; Assunção et al., 2001; Becher et al., 2004; Papoyan and Kochian, 2004; Hanikenne et al., 2008, reviewed by Verbruggen et al., 2009). As the name suggests, hyperaccumulators are characterized by strong accumulation, compared to the substrate, of a certain element. This is usually expressed as a bioaccumulation factor, which is commonly defined as the ratio of the concentration of an element inside the organism compared to the metal concentration in the substrate. To achieve a bioaccumulation coefficient far greater than one [characterizing hyperaccumulators as revealed already by their first discovery almost 150 years ago (Sachs, 1865)], the metal to be pumped into their storage sites against the concentration gradient. Altogether, most likely many steps of the metal transport from root uptake until passage over the vacuolar membrane of the storage cells are against the concentration gradient. Therefore, all these transport steps require an active, i.e., energy consuming transport system (Salt and Wagner, 1993). Furthermore, specificity of the transport is tightly controlled. This is obvious in *N. caerulescens*, which hyperaccumulates Cd and Zn but does not accumulate copper when it is supplied to the nutrient solution at the same concentration (Mijuvlovich et al., 2009). Furthermore, this species is, apart from some more resistant individuals, highly sensitive toward copper (Mijuvlovich et al., 2009). This copper sensitivity is a severe problem for phytoremediation of sites that are contaminated with a combination of several metals (Walker and Bernal, 2004).

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future. Investigations of the biochemical and biophysical properties of the N. caerulescens version of HMA4, NcHMA4, have shown that the ATPase function of this transporter is activated most strongly by Cd and Zn. Gels and western blots (using an antibody specific for NcHMA4) of crude root extract and of the purified protein revealed a size of NcHMA4 of about 50–60 kDa, while the mRNA for the NcHMA4 gene predicts a single protein with a size of 128 kDa. This indicates the occurrence of post-translational processing (Parameswaran et al., 2007). Whether this shortened but functional protein is the product of the C-terminally truncated copies of the gene (Craiciu et al., 2012) still remains unknown. In recent work by Leitenmaier et al. (2011), NcHMA4 showed activity of NcHMA4 with Ca$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ under various concentrations (0.03–10 μM tested), and all three metal ions activated the ATPase at a concentration of 0.3 μM, while there were clear differences in activation energy (E_a) observed, depending on the metal applied. According to EXAFS, NcHMA4 is bound Cd primarily by thiolate sulfur from cysteine, and not by imidazole nitrogen from histidine. These properties correspond well with what is known from a study of a human copper ATPase, ATP7A (Hung et al., 2007). Maximal activation of NcHMA4 and HsATP7b occurred at about the same metal concentration, and also the measured turnover rates were similar (Leitenmaier et al., 2011 vs. Hung et al., 2007). Therefore, also the other properties, investigated in only one of these two existing biochemical studies of CPx-type ATPase holoproteins, should be at least somewhat similar in the other. In this way, research on plant metal transport proteins has also medical relevance as mutations of the human copper ATPases cause well-known deadly diseases (Menkes and Wilson disease).

The expression of HMA4 not only in the roots but also in shoots clearly shows that it must have more functions than xylem loading. Expression is similarly high also in shoots, again with a maximum in the vascular bundle, which could mean that this protein is involved in xylem unloading as well (Craiciu et al., 2012). Such a role dual role both in loading and unloading of the xylem by the same protein could be achieved simply by insertion of the protein in either the xylem-facing side of these cells (~loading) or the opposite side (~unloading). How the passage through the mesophyll to the epidermal storage cells is accomplished is still an open and important question, as shown also by the fact that HMA4 overexpression in a non-accumulator causes this plant to poison itself due to lack of mechanisms detoxifying the Cd and Zn arriving in the shoots (Hanikenne et al., 2008). Uptake over the cytoplasmic membrane of the epidermal storage cells is not well-characterized either, but a recent study on the cellular distribution and regulation of gene expression levels (Küpper and Kochian, 2010) indicates genes that may be candidates for causing this difference in metal accumulation between epidermal cells: NcZNT3 was highly expressed in epidermal storage cells, much higher than in other cell types. The ZIP-family of transporters, to which ZNT3 belongs, is generally localized in the plasma membrane (review by Guerinot, 2000).

**FINAL STORAGE SITE: VACUOLES OF LARGE EPIDERMAL CELLS**

Hyperaccumulators have to store the enormous amounts of taken up metal in a way that it does not harm important enzymes and especially not photosynthesis, therefore it is crucial to keep the metal concentration in the cytoplasm of mesophyll cells as low as possible. It makes sense for hyperaccumulating plants to store metal in the vacuoles because in this organelle only enzymes like phosphatases, lipases, and proteases (Wink, 1993; Carter et al., 2004) are present, which have not been found to be a target of heavy metal toxicity. Additionally it has been shown that high amounts of metals are stored specifically in the vacuoles of large epidermal cells (Küpper et al., 1999, 2001; Frey et al., 2000), where no chloroplasts are located and therefore also during transport through the cytoplasm harboring the chloroplasts, photosynthesis cannot be inhibited. Preferential storage of hyperaccumulated metals in the epidermis has been shown for the majority of hyperaccumulator species and for elements as chemically diverse as Al, As, Cd, Ni, Se, and Zn (e.g., Küpper et al., 1999, 2001; Frey et al., 2000; Lombi et al., 2002; Carr et al., 2003; Bhatia et al., 2004; Bidwell et al., 2004; Broadhurst et al., 2004; Cousio et al., 2005; Freeman et al., 2006). The approximate volume of this storage site multiplied by the metal concentration in it (data, e.g., for Zn from Küpper et al., 2009) indicates that about 70% of the total accumulated zinc in mature leaves is stored in the epidermis of N. caerulescens, and it is likely similar for most other species investigated by the other authors listed above. If the storage capacity of the epidermis is exceeded, enhanced storage seems to occur in the mesophyll (Küpper et al., 2001). This leads to inhibition of photosynthesis especially in those cells that accumulate most of the toxic metal (Küpper et al., 2007).

In the vacuoles of the epidermal metal storage cells, heavy metal concentrations of several hundred millimoles per liter can be reached (Küpper et al., 2001, Küpper et al., 2009), which already indicated that hyperaccumulation must involve active pumping of the metals into specific storage sites. The alternative hypothesis that the metal enrichment in the epidermis is caused by the transpiration stream (i.e., influx of metal-containing water into the epidermis, and evaporation of the water) could be excluded by a study on epidermal protoplasts. The drastically different cadmium uptake rates into epidermal storage cells compared to mesophyll cells in this study clearly showed that the epidermal accumulation is due to differences in active metal transport and not differences in passive mechanisms like transpiration stream transport or cell wall adhesion (Leitenmaier and Küpper, 2011).

Furthermore, it turned out that the transport over the cytoplasmic membrane is faster than the sequestration into the vacuole, making the latter the rate-limiting step in epidermal accumulation. Altogether, it seems likely that the transport steps over the plasma and tonoplast membranes of leaf epidermal storage cells are driving forces behind the hyperaccumulation phenotype (Leitenmaier and Küpper, 2011). Protein families involved in vacuolar sequestration may be the Nramp's (natural resistance-associated macrophage proteins), CDF's (cation diffusion facilitator), and CAX's (cation exchanger; reviewed by Hall and Williams, 2005) as well as CPx-type ATPases. Until now, already several transporters for vacuolar sequestration of zinc (and possibly cadmium) and nickel have been investigated and could be partially characterized (Yamka et al., 1999; Desbrosses-Fontouque et al., 2005; Elbaz et al., 2006; Haydon and Cobbett, 2007; Morel et al., 2009). Several CDF transporters for vacuolar sequestration of Zn (and possibly
Cd and Co) have been characterized, all are homologous, almost identical in sequence. These are MTP1, ZAT, and ZTP (Van der Zaal et al., 1999; Assunção et al., 2001; Becher et al., 2004; Dräger et al., 2004). The Arabidopsis thaliana homolog of this protein, AtMTP1, has been shown to mediate Zn detoxification and leaf Zn accumulation and is known to be localized in the vacuolar (tonoplast) membrane (Dubreucq-Jonrouge et al., 2003). Due to the chemical similarity of cadmium and zinc, transporters designed for Zn inevitably will transport Cd as well for purely chemical reasons. Mhx, a homolog of an Arabidopsis thaliana vascular metal (Fe, Mg, Zn) versus proton exchanger and member of the CDF protein family, was found to be highly expressed in the leaf vacuolar membrane of Arabidopsis halleri (Elbaz et al., 2006).

HMA3, a CAX- (Pyr/Al) type heavy metal ATPase, was found to mediate leaf vacuolar storage of Cd, Co, Pb, and Zn in Arabidopsis thaliana (Morel et al., 2009). The strongly elevated expression of HMA3 was shown to play a decisive role in Cd accumulation not only in N. caerulescens (Ueno et al., 2011b), but also in rice (Ueno et al., 2010, 2011a), reconfirming also the importance of the sequestration into vacuoles for the hyperaccumulation phenotype. However, the reported effects of the vacuolar sequestration on shoot metal concentrations as mediated by HMA3 are opposite in rice compared to N. caerulescens, because the localization of this expression is different. In rice, HMA3 sequesters Cd into root vacuoles, diminishing the Cd available for transport into the shoots (Ueno et al., 2010). In N. caerulescens, in contrast, it was shown to be expressed highly expressed also in the shoots, thus sequestering Cd there (Ueno et al., 2011b). For purely biophysical reasons (transport equilibria), this will cause enhanced xylem unloading in the shoots, and thus enhanced root-shoot translocation (i.e., a sink-driven transport).

The natural overexpression of NRAMPs was identified both in rice and in N. caerulescens to play an important role in Cd tolerance and possibly Cd accumulation (Oomen et al., 2009; Wei et al., 2008 and Takahashi et al., 2011).

At this final step, Al represents an exception: in the known Al hyperaccumulator, Camellia sinensis (tea), Al is accumulated mostly in the cell walls, with very low concentrations inside the cells even when the samples are properly prepared to prevent artefactual cell wall localization (Carr et al., 2003).

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