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Metal Toxicity in Plants: Perception, Signaling and Remediation

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Preface

The extensive increase of world population and industrial management has produced numerous environmental problems such as pollution (e.g. water, air, soil, noise and radiation), accumulation of heavy metals in soil and reduction in water quality. These facts can produce severe deterioration of natural resources, disturbance of ecosystems and affect human health. The term "heavy metal" refers to metallic elements with a high specific gravity (more than 5) or density which are very toxic even at very low concentrations. Some of these elements are referred as the trace elements, including iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo), cobalt (Co) and zinc (Zn), which are essential for biological systems in small quantities by participating in redox reactions and acting as enzyme cofactors (Sanitá di Toppi and Gabbrielli 1999). However, these metals can be toxic at high concentrations. Other heavy metals, such as cadmium (Cd), mercury (Hg), lead (Pb), aluminum (Al) or arsenic (As), have no function as nutrients and are very toxic to plants, animals and humans. The toxicity of these metals is based on their chemical properties which allow them to promote the production of reactive oxygen species (ROS), inactivation of enzymes, basically by reaction with SH-groups, and displacement of other cations or metals from proteins (Sanitá di Toppi and Gabbrielli 1999).

Heavy metals appear in the environment through natural sources or by anthropogenic activities such as mining, fossil fuel combustion, phosphate fertilizers used in agriculture and metal-working industries (Clemens 2006). These human activities have produced a severe environmental concern in some parts of the world because of the contamination by metals in day-to-day life, which can even compromise the health of future generations, due to the persistence of the metals in the environment by their bioaccumulation through the food chain (Clemens 2006). Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plantss and is manifested by an interaction between the genotype and its environment (McNair et al. 2000). Some plants have developed resistance to high metal concentrations, basically by two mechanisms, avoidance and tolerance. The first mechanism involved exclusion of metals outside the roots, and the second mechanism consists basically in complexing the metals to avoid

protein and enzyme inactivation. Some plants can also accumulate metals in their tissues at concentrations higher than those found in the soil, and these plants as referred as hyperaccumulator. Most hyperaccumulator plant species belongs to Brassicaceae family. Heavy metal hyperaccumulation in plants is due to a combination of metal transporters and chelator molecules. Chelation of metals in cytosols by high affinity ligands is potentially a very important mechanism of heavy metal detoxification and tolerance. Potential ligands include amino acids, nicotianamine, phytochelatins and metallothioneins (Clemens 2001). Phytochelatins have been the most widely studied in plants with a general structure (γ-Glu Cys)$_n$-Gly where $n = 2$–11, and are rapidly induced in plants by heavy metal treatments (Rauser 1995). Hyperaccumulation can be exploited as a very useful tool to clean contaminated soils, water and sediments by the process called phytoremediation which essentially uses green plants to clean-up contaminants.

During the last two decades, ROS has gain importance in different aspects of heavy metal stress. Under physiological conditions, there is a balance between production and scavenging of ROS in all cell compartments. However, this balance could be perturbed by a number of adverse environmental factors. One of the major consequences of heavy metal action is enhanced production of ROS giving rise to damage to membranes, nucleic acids, and proteins (Halliwell and Gutteridge 2000). However, ROS are double-faced molecules acting as signal molecules regulating a large gene network in response against biotic and abiotic stress. On the other hand, nitric oxide (NO) also gained much importance in the last decade, as basically NO is a gaseous reactive molecule with a pivotal signaling role in many developmental and cell response processes (Besson-Bard et al. 2008). Recently, an increasing number of studies have been reported on the effects of NO alleviating toxicity of heavy metal including Cd and As (Xiong et al. 2010). Changes in the levels of both molecules are associated in the perception of stress and can trigger the defence cellular responses against adverse environmental conditions. In plants, hormones also play a critical role in the regulation of growth/development and modulation in plant responses against stresses. ROS and plant hormones interplay in the regulation of those processes, although the mechanisms involved are not well known in most cases.

The number of publications focused on heavy metal toxicity in plants has been growing exponentially in the last decade. The purpose of this book is to present the most recent advances in this field, mainly on the uptake and transport of heavy metals in plants, mechanisms of toxicity, perception of metals and the regulation of cell responses under metal stress. Another key feature of this book is related to the studies in recent years on signaling and remediation processes taking advantage of recent technological advances including "omic" approaches. Transcriptomic, proteomic and metabolomic studies have become very important tools to analyze the dynamics of changes in gene expression, and the profiles of protein and metabolites under heavy metal stress. This information is also very useful to draw the complex signaling and metabolic network induced by heavy metals in which hormones and reactive oxygen species also have an important role. Understanding the mechanism involved in sequestration and hyperaccumulation is very important

in order to develop new strategies of phytoremediation are reviewed in several chapters of this book. The information included in this book will bring very stimulating insights into the mechanism involved in the regulation of plant response to heavy metals, which in turn will contribute to improving our knowledge of cell regulation under metal stress and the use of plants for phytoremediation.

The editors are grateful to the authors for contributing their time, knowledge and enthusiasm to bring this book into being.

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Contents

Heavy Metal Bindings and Their Interactions with Thiol Peptides and Other Biological Ligands in Plant Cells

Mashiro Inouhe, Huagang Huang, Sanjay Kumar Chaudhary, and Dharmendra Kumar Gupta

Abstract Plants have developed their potentials for uptake, transport and accumulation of terrestrial elements in order to coordinate their developmental and life-cycle performance. The utilization and toxicity of the metallic elements in plants are principally based on their own chemical properties in water and the interaction with their counterpart anions and cooperative molecules. Biochemical partners of the metals are various organic ligands composed of C, H, O, N, P, or S. Their roles are shared by two cell sites – the outside *apoplast* and the inside *symplast*. The apoplast equips the polymeric ligands of polysaccharides, phenolics, and proteins with carboxylic and some other functional groups capable of conjugating metals in the cell surfaces, but excess heavy metals in the primary cell wall are toxic to plants. Mobile organics in the apoplast have another function in xylem transport or biological interactions in the rhizosphere underground. The symplast (and vacuole) contains a variety of organic ligands such as organic acids, amino acids, polyamines, nicotianamine, phytates, soluble phenolics, and thiol-peptides called *cadystins* or *phytochelatins* (PCs). These can bind most heavy metals to make the

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lesser toxic binding forms and hence affecting their movements, transports, accumulations and their final fates in vivo in plants. PCs have the general structure of (γ-glutamyl-cysteinyl)$_n$-glycine ($n = 2–11$) and they are synthesized from glutathione ($n = 1$). The PC−metal conjugates are formed in the cytoplasm and transported to vacuole to make more stable complex mixtures with inorganic sulfur (S^{2-}). By contrast, little evidence supports the idea that PCs have a central role in xylem transport or the immobilization in shoots of heavy metals. Hyperaccumulators of Cd, Zn, Ni or As have a feature to carry out massive transport of them from root to shoot using other prevailing O- or N-bond ligands, besides the ability to form PCs. These suggest that the distinctive mechanisms for metal transports through the xylem sap system may be established independently of the PC-detoxification mechanism in the roots. Intentional and practical readjustment of the PC-dependent versus PC-independent systems in situ can improve the relative efficiency of the heavy metal mobility to shoot sites and the total accumulation capacity in the vascular plants.

1 Introduction

As a consequence of the industrial revolution there is an enormous and increasing demand for heavy metals that leads to highly anthropogenic emission into the biosphere (Ayres 1992). Apart from some emissions into the atmosphere in the form of dust particles or gases, these heavy metals stay largely in the aquatic and soil phases of this planet. Contamination also occurs extensively or locally even under natural environmental conditions where there are no directly connected human activities. Heavy metal pollution of environment is one of major ecological concern because of its impact on human health through the food chain and its high persistence in the environment (Piechalak et al. 2002). Meanwhile, various species of plants are very useful for cleaning up the metal-contaminated soil or water as a very eco-friendly technique called phytoremediation. This technology based on the potential and capacity of plants capable of accumulating heavy metals to shoot sites via root with no remarkable metabolic impediment or growth retardation of the organs. Here, what is required is an understanding of the plant mechanisms: how the plant neutralizes the toxic metals in roots (detoxification mechanism), how it transports them from roots to shoot (transport mechanism), and how it stores or fixes them stably in a special shoot sites (accumulation/immobilization mechanism), otherwise discharge or elimination will occur. All these mechanisms are closely connected to the problem of which biological ligands are bio-synthesized, co-transported, and further utilized for the respective metals in plants (Fig. 1).

   The tolerance characteristics of plants to heavy metal ions are diverse among the metal ions involved (Foy et al. 1978; Woolhouse 1983; Verkleij and Schat 1990). Especially a group of metals called "Borderline class" metals including Mn, Zn, Fe, Ni, Cd, Pb and Cu etc. are capable of binding to multiple types of naturally occurring chemicals or components in plants, although "Class A" metals, such as K, Ca, Na, Mg, Al, and Cs prefer the O-donor ligands, all of which bind through
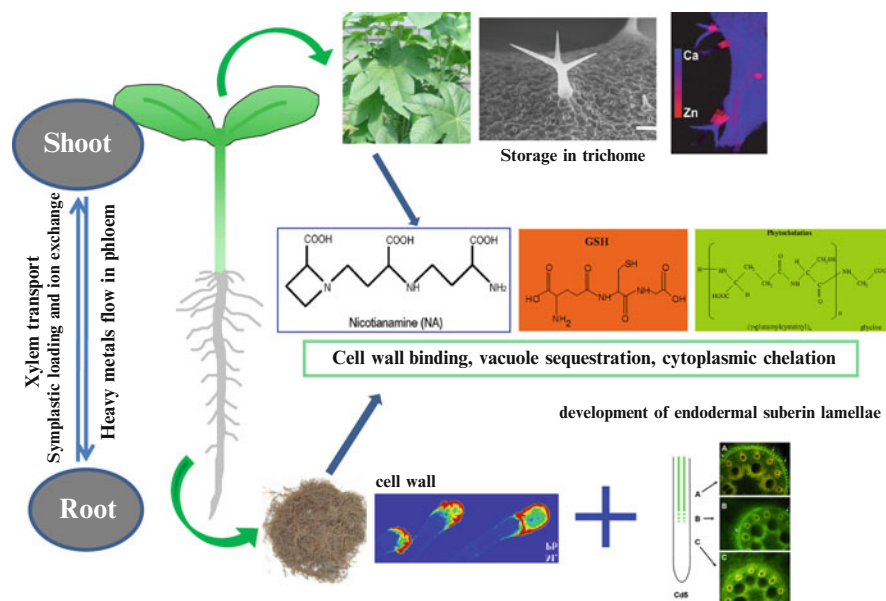
Fig. 1 Simplified scheme involved in heavy metal accumulation and homeostasis in plants

oxygen (−COOH, –H$_2$PO$_4$, –OH, –CHO etc.), rather than the S- or N-bond ligands (−SH, –SS–, –NH$_2$, =NH etc.) preferred by "Class B" metals (Woolhouse 1983). Nevertheless, the tolerance against those toxic ions can be expressed in a highly specific manner for each metal in general in plants, and co-tolerance appears relatively rare (Hall 2002; Inouhe 2005). One of the fundamental bases of the mechanisms can be addressed to either the alteration of the metal-sensitive metabolism and structure or the development of new metal-sequestering principles within some cellular compartments (Mehra and Winge 1991). As for the latter detoxification mechanism, various types of metal-binding complexes have been identified from plants. Among them the best characterized are phytochelatins (PCs) and the related thiol-peptides. Details of the structures, biosynthesis, analytical methods, genetics and the other many aspects of them are available in many publications (Rauser 1995, 1999; Zenk 1996; Cobbett and Goldsbrough 2002; Inouhe 2005). Furthermore, a variety of other organic ligands capable of conjugating to various metals in vivo have been reported with their possible roles similar to or distinct from those of PCs in plants (Callahan et al. 2006; Sharma and Dietz 2006; Haydon and Cobbett 2007). Based on recent information, we here survey their biochemical characteristics and the possible functions in bindings, detoxification, transport and accumulation of representative heavy metals such as Cd, Zn, Cu, and Ni in plant cells. Next, their localization and distribution in different sites of the plant body including their consolidate bindings to polymeric ligands in the structures are compared to facilitate our understanding on the possible roles of PCs and non-PC ligands contained in them.

2 Biological Ligands for Heavy Metal Conjugation and Detoxification in Plant Cells

2.1 Phytochelatins

To protect themselves from the toxicity of metal ions, plant cells have developed a mechanism to inactivate metal ions thus preventing enzymatic and structural proteins (Kneer and Zenk 1992). This mechanism consists of the biosynthesis of a set of iso-peptides PCs with varying chain lengths such as $(\gamma\text{-Glu-Cys})_n\text{-Gly}$; where $n = 2–11$ (Fig. 1). PCs (or *cadystins*) were first discovered in fission yeast *Schizosaccharomyces pombe* exposed to Cd (Murasugi et al. 1981) and then in many plants (Grill et al. 1989; Rauser 1995). PCs are formed directly from glutathione (GSH, a reduced form) by the activity of PC synthase (γ-Glu-Cys dipeptidyl transpeptidase: EC 2.3.2.15), in the last step of the following metabolic sequence: $Glu + Cys \rightarrow \gamma\text{-Glu-Cys}$ (γEC peptide) $\rightarrow \gamma\text{-Glu-Cys-Gly}$ (GSH) $\rightarrow$ PCs. The first and second steps of this sequence are mediated by γEC synthetase (EC 6.3.2.2) and GSH synthetase (EC 6.3.2.3), respectively. PC synthase (PCS) consists of 95,000 Mr tetramers of protein subunits and has a Km of 6.7 mM for GSH, and its activities to produce PCs are post-translationally regulated by a range of heavy metals and metalloids (Grill et al. 1989). This enzyme continues the reaction until the activating metal ions are chelated by the PCs formed, providing an auto-regulated mechanism of the PC biosynthesis in which the reaction products chelate the activating metals thereby terminating the reaction (Loeffler et al. 1989).

Since the first isolation of PC synthase gene (*PCS1*, *CAD2*) in 1999 (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999), various PCS genes have been isolated from different species of plants and other organisms such as yeast, nematode, slime molds and cyanobacteria (Vatamaniuk et al. 2002; Tsuji et al. 2004; Pal and Rai 2010). The PCS activities have been detected in plants such as *Silene cucubalis* (Grill et al. 1989), *Arabidopsis* (Howden et al. 1995), *Pisum sativum* (Klapheck et al. 1995), *Cicer arietinum* (Gupta et al. 2002), and tomato (Chen et al. 1997), but not in azuki bean (Inouhe et al. 2000). In tomato, PCS activity was detected mainly in the roots and stems and not leaves or fruit (Chen et al. 1997), but the tissue-specific PCS expression or PC biosynthesis are not well understood in the other plants.

PCs play an important role in detoxification of various heavy metal ions in plants (Rauser 1995; Zenk 1996; Cobbett 2000). Chelation of heavy metals with PCs produced in cytoplasm and compartmentalization of the PC-metal complexes in vacuoles are generally considered as the "first line" of defence mechanisms by plants (Clemens 2006). PC synthesis can be stimulated in cells exposed to Cd and various other metals such as Cu, Zn, Pb and Ag, or metalloid As, and thePCs formed are capable of binding to all these ions via the sulfhydryl (−SH) and carboxyl (−COOH) residues (Grill et al. 1987). *Arabidopsis* mutants lacking enzymes involved in GSH synthesis (Howden and Cobbett 1992) or deficient in PCS activity

(Howden et al. 1995) were hypersensitive to Cd. Inhibition studies of PC biosynthesis via GSH using either mutants or inhibitor further demonstrated fundamental roles of PCs in the metal detoxification in yeast, fungi, green algae, aquatic plants, and many higher plants and their cell cultures (Inouhe 2005). In addition, overexpression of PCS genes efficiently increases the Cd-tolerance in plants as well as in yeast and bacteria. For example, transgenic plants of *Brassica juncea*, overexpressing GSH synthetase, γ-glutamylcysteine synthetase or PCS, are more tolerant to Cd stress (Zhu et al. 1999a, b; Wawrzyn'ski et al. 2006; Gasic and Korban 2007). However, there are exceptions to such a relationship. Firstly, some transgenic *Arabidopsis* lines overexpressing PCS are hypersensitive to Cd since these are probably depleted in GSH pools and thus more susceptible to Cd-induced oxidative stress (Li et al. 2004). The discrepancy suggests that the tolerance levels of plants to heavy metal toxicity may be correlated to the total levels or balance of "thiol" compounds in the cells (Cobbett and Goldsbrough 2002; Gupta et al. 2002). In yeast *Saccharomyces cerevisiae*, exposure of cells to Cd led to a global drop in sulfur-containing protein synthesis and in a redirection of sulfur metabolite fluxes towards the GSH pathway (Lafaye et al. 2005). More recently, simultaneous overexpression of GSH synthetase and PCS in *Arabidopsis* was found to increase the tolerance and accumulation of Cd and As (Guo et al. 2008), which also supports the need to maintain a proper balance of thiol metabolism under stress conditions. Secondly, besides the metabolic balance, transports of PC-metal conjugates from cytoplasm to vacuole are required for metal tolerance and accumulation in plant cells (Clemens 2006). In *B. juncea*, a change of expression of a GSH transporter BjGT1 in response to Cd exposure has been reported (Bogs et al. 2003) also indicating that GSH plays a prominent role in Cd accumulation and detoxification. ABC transporters have been identified in yeast and fission yeast that directly mediate the vacuolar transport of Cd complexes and thus are involved in the final step of Cd detoxification (Ortiz et al. 1995; Li et al. 1997). Recent analyses of AtMRPs, a subfamily of *Arabidopsis* ABC transporters, showed that AtMRP3 was induced by Cd and not by oxidative stress (Bovet et al. 2003), suggesting that ABC transporters in plants, as in yeast, are involved in heavy metal fluxes.

Massive PC production is accompanied by a coordinated transcriptional induction of biosynthesis of enzymes involved in sulfate uptake (Nocito et al. 2002; Herbette et al. 2006) and assimilation into Cys (Harada et al. 2001; Gupta et al. 2002; Weber et al. 2006) and GSH (Xiang and Oliver 1998; Wawrzyn'ski et al. 2006). This suggests the requirement for the reduced sulfur in the PC biosynthesis and heavy-metal responses of plants. Sulfur is taken up by roots and translocated to different organs through specific transporters on membranes and mainly in the apoplastic route. Sulfate transporters of Group 1 (e.g. SULTR1;1 and SULTR1;2) are the high-affinity transporters expressed primarily in roots of sulfur-starved plants and they function to overcome sulfur limiting conditions (Leustek 2002). Expression of Group 1 sulfate transporters is negatively regulated by cytokinins through their receptor gene *CRE1* (Maruyama-Nakashita et al. 2004). Thus, a decline in the cytokinin content (Veselov et al. 2003) may indirectly indicate increased expression of Group 1 sulfate transporters. Sulfate transporters from

Group 2 (e.g. SULTR2;1) are involved in xylem loading, while those of Group 4 (SULTR4;1 and SULTR4;2) are localized in vacuoles and chloroplasts (Leustek 2002) and thus may play an important role in transport of sulfate from roots to shoots and finally to chloroplasts, an organelle where major fraction of sulfate is assimilated to Cys after a series of reactions: sulfate + ATP → APS (adenosine 5′–phosphosulfate) → sulfite → sulfide → Cys. These four steps are mediated by ATP sulfurylase, APS reductase, ferredoxin-dependent sulfite reductase, and O-acetylserine (thiol) lyase, respectively. Then the synthesised Cys and GSH in the source organs are transported to roots and other sink organs by translocation and further used for PC formation.

The long-distance transports between source and sink organs are essential for the nutritional correlations in vascular plants. As a typical example, PCs might play a role in Cd transport from root to shoot demonstrating that a PC-dependent "overflow protection mechanism" would contribute to keeping Cd accumulation low in the root, causing extra Cd transport to the shoot (Gong et al. 2003). However, overexpression of *Arabidopsis* PCS in tobacco plants enhances Cd tolerance and accumulation but not its translocation to the shoot (Pomponi et al. 2006). Some levels of PCs are detected in phloem sap in rice (Kato et al. 2010) but not in xylem sap in *Arabidopsis halleri* (Ueno et al. 2008). Thus the special role of PCs in long-distance transport of heavy metals has not been fully substantiated in plants, especially hyper-accumulating species.

Chickpea roots are capable of forming a substantial level of thiol compounds that are apparently different from GSH and PCs, the major compounds identified are homo-phytochelatins (hPCs), consisting mainly of hPC_2 and hPC_3. These peptides are synthesized from homo-glutathione (hGSH) in response to Cd and As almost to the equivalent levels of PCs, but not to Cu, Zn, Ni and Co, suggesting that hPCs may have an important role in Cd and As-sequestering and signaling in chickpea roots (Gupta et al. 2002, 2004). Some other PC-related peptides were reported in different plant sources (Table 1). Although their physiological roles in the absence or presence of heavy metals are not well understood at present, PCs and PC-related peptides can be thought to have a role in the homeostasis and metabolism of essential metal ions in plants (Rauser 1999; Zenk 1996; Cobbett 2000). In vitro experiments have shown that PC-Cu and PC-Zn complexes could reactivate the apoforms of the copper-dependent enzyme diamino-oxidase and the Zn-dependent enzyme carbonic anhydrase, respectively (Thumann et al. 1991). In addition, roles for PCs in Fe or sulfur metabolism have also been proposed (Zenk 1996;

Table 1 Various PC-like peptides produced by plants and yeast

PC-related γ-EC peptides	Structure	Plant sources
Homophytochelatin	(γ -Glu-Cys)n-Ala	Leguminosae
Hydroxymethyl-PC	(γ -Glu-Cys)n-Ser	Gramineae
iso-Phytochelatin (Glu)	(γ -Glu-Cys)n-Glu	Maize
iso-Phytochelatin (Gln)	(γ -Glu-Cys)n-Gln	Horse radish
Desglycine phytochelatin	(γ -Glu-Cys)n	Maize, yeast

Adapted from Rauser (1995); Zenk (1996); Klapheck et al. (1995); Inouhe (2005)

Toppi and Gabbrielli 1999). PCs and PC-related peptides are thiol compounds functionally equivalent or superior to Cys and GSH. These are therefore biologically active compounds that function to prevent oxidative stress in plant cells (Gupta et al. 2010).

2.2 Organic Acids, Nicotianamine, Amino Acids, and Phytates

Organic acids (OAs) have been associated with metal hyperaccumulation and tolerance in a range of plant species and have been proposed as important cellular ligands for Zn, Cd and Ni (Salt et al. 1999; Kupper et al. 2004). The carboxylic acids known to be present in high concentrations in the cell vacuoles of photosynthetic tissues include citric, isocitric, oxalic, tartaric, malic, malonic and aconitic (Callahan et al. 2007). Many studies have implied that these acids play a role in hyperaccumulation (Rauser 1999; Salt et al. 1999; Romheld and Awad 2000; Chiang et al. 2006). Analysis of tissues from metal hyperaccumulator species using X-ray absorption techniques has identified OAs as the predominant ligands. By X-ray absorption spectrometry (XAS) and extended X-ray absorption fine structure (EXAFS) analysis, citrate was identified as the predominant ligand for Zn in leaves of *Thlaspi caerulescens* (Salt et al. 1999). Similarly, Ni-citrate accounted for one-quarter of the Ni species in leaves of the Ni hyperaccumulator *T. goesingense* and in the related nonaccumulator *T. arvense* (Kramer et al. 2000). The identification of the vacuole as the major subcellular compartment for Zn, Cd and Ni and the favoring of the formation of metal-OA complexes in the acidic environment of the vacuolar lumen suggest that citrate and malate are probably relevant only as ligands for these metals within vacuoles (Kramer et al. 2000; Ma et al. 2005).

Studies have demonstrated that the primary constituents of root exudates are low-molecular weight organic acids (LMWOAs) that play essential roles in making sparingly soluble soil Fe, P, and other metals available to growing plants (Romheld and Awad 2000). Acetic, lactic, glycolic, malic, maleic, and succinic acids were found in rhizosphere soils of tobacco and sunflower (Chiang et al. 2006). Concentrations of these LMWOAs exudates increased with increasing amendment of Cd concentrations in the rhizosphere soils. After the loss of H^+, each acid contains a COO^- group, which binds to the cations. Correlation coefficients between concentrations of Cd amendment versus LMWOAs exudates of tobacco and sunflower were 0.85 and 0.98, respectively (Chiang et al. 2006). Positive correlations have been found between external Zn and organic acid concentrations in the roots of hyperaccumulator plants *A. halleri* (Zhao et al. 2000). These results suggest that the different levels of LMWOAs present in the rhizosphere soil may play an important role in the solubilization of heavy metals that bind with soil particles into soil solution and followed by uptake by plants. However, this mechanism does not draw a sharp line between toxic and essential metals for uptake and

further utilization. This role may be covered by other specific biological ligands or transporters in the root and shoot tissues.

Nicotianamine (NA), a non-proteinaceous amino acid synthesized in all plants by the condensation of three S-adenosyl-methionine molecules through the activity of the enzyme nicotianamine synthase (NAS), is ubiquitously present in higher plants (Fig. 1). It is known to be involved in chelation of metals such as Fe, Cu, Zn for their enhanced extraction by roots and/or transport to shoost, especially under mineral-deficient conditions (Takahasi et al. 2003; Mari et al. 2006). However, recent evidence supports their possible functions in heavy metal-tolerance and hyperaccumulation in plants. The hyperaccumulation of Zn and Cd is a constitutive property of the metallophyte A. halerii. Recently, Weber et al. (2004) have used *Arabidopsis* gene chips to identify those genes that are more active in roots of *A. halleri* than *A. thaliana* under controlled conditions. Two genes showing highest levels of expression in *A. halleri* roots code for a NAS and a putative Zn^{2+} uptake system. In addition, roots of *A. halleri* also show higher levels of both NA and NAS. *A. halleri* presents a 2-fold increase of its NA root content probably linked to the constitutive expression of the *AhNAS2* gene. Expression of NAS in *S. pombe* cells has demonstrated that formation of NA can confer Zn^{2+} tolerance. Taken together, these observations suggest active roles of NA in plant Zn homeostasis and NAS in hyperaccumulation of Zn in *A. halleri* (Weber et al. 2004). Recently, it was reported that the overexpression of *TcNAS* in *A. thaliana* transgenic plants also confers Ni resistance (Pianelli et al. 2005), strengthening the idea that NA could play a role in metal tolerance and hyperaccumulation.

Plant cells contain many other small organic ligands with variable functional groups, including amino acids, polyamines, nucleotides, phytates and other phosphate sugars. Of these, polyamines appear to act as a messenger or a molecule to stabilize or protect the cell membranes rather than as direct binding ligands to toxic heavy metals (Sharma and Dietz 2006). Nucleotides, phytates and sugar phosphates can conjugate to Ca, Mn, Mg, Al and other metals through their O-bonds. Especially, the importance of phytates in coordination and storage of phosphate and metals such as Zn, Mg, and K in vacuole and cytoplasm and also in the detoxification of Cd has been widely suggested (Van Steveninck et al. 1992; Hayden and Cobbett 2006). Amino acids are the most abundant amphoteric ions with variable forms and residues, existing in 10–100 mM orders of concentrations and serving multiple functions in plant cells. Cysteine (Cys) is a thiol compound that has a S-donor residue equivalent to a GSH molecule. However, its internal level does not usually exceed that of GSH or PCs, probably because of the restricted supply of total S available for it and its quick turnover and utilization for the other thiol ligands and proteins. Acidic amino acids, glutamic acid (Glu) and aspartic acid (Asp), provide an extra carboxyl group ($-COOH$), and their amides, glutamine (Gln) and asparagine (Asn), provide an acid amide group consisting of both O- and N-donors ($-CO-NH_2$). All these are generally rich in phloem sap, for example, at near 300 mM in cereals and 50 mM in some dicotyledonous plants (Oshima et al. 1990; Winter et al. 1992), and can be potential ligands for translocational metal cations. Histidine (His) is the most characterized imidazole ($=NH$)-containing

amino acid that plays a central role in binding to and transport of Ni, especially in Ni-hyper-accumulating plants (Kramer et al. 2000; Callahan et al. 2006). Two His molecules can make a stable complex chelating to one Ni (Callahan et al. 2006). Furthermore, proline (Pro) has been most extensively studied for its unique and important function as a compatible solute in many plants affected by water-deficit and salinity stress, but interestingly, heavy metals such as Cu, Cd, Zn or Pb also significantly stimulate the accumulation and/or biosynthesis of Pro in many plants (Sharma and Dietz 2006). Possible roles of Pro as a direct N-donor ligand conjugating to heavy metals are not established as yet, but will be more attractive in combination with its role as osmotic protectant or antioxidant under complex conditions including salinity and drought stress.

As mentioned above, there are possible interactions between different soluble organic ligands and different metals in cytoplasm, vacuole and other apoplastic solutions in shoots and roots. These solutions also contain inorganic anions such as sulfate, phosphate, nitrate, borate, carbonate, chloride and silicate. These inorganic anions and counterpart cations affect the organic ligand's interactions with metals in each site at different but almost constant pH conditions (Callahan et al. 2006). Some bindings between metals and ligands are not specific and not stable, especially under varied pH and ion-strength conditions. Conversely the regulated conditions can promise a unique and established mechanism for metal transport and binding systems in land plants.

2.3 Soluble Phenolics

At the end of this section on the soluble form of metal-binding ligands, we introduce a unique but increasingly well-recognized example of phenolics. Phenolic compounds are derived mainly from trans-cinnamic acid, which is formed from L-phenylalanine in a reaction catalyzed by L-phenylalanine ammonia-lyase (PAL). These compounds are constitutively expressed in higher plants and can effectively prevent oxidative stress caused by unfavorable environmental factors. Since the levels of phenolics are affected sensitively by heavy metal accumulations, they are suitable candidates to act as biomarkers (Santiago et al. 2000). Such compounds can be used as early indicators of environmental stress on a target organism before morphological or ultrastructural damage occurs. They are also useful as cytological and biochemical indicators because they are compartmented as secondary metabolites at the different tissue- and sub-cellular levels in response to the environment, and the specified localization reflects their biochemical properties or roles in plants. In general, glycosides of phenolics are localized in hydrophilic regions of the cell such as vacuoles and apoplasts, while aglycones are localized in lipophilic regions (Sakihama et al. 2002). All these are also known as potent bio-ligands capable of binding or precipitating heavy metal ions in different cell sites. Furthermore, known insoluble phenolics such as lignin are localized in the cell wall especially more differentiated secondary cell walls in many plants and can

perform as metal-accumulating polymeric ligands. Tong et al. (2004) have reported that compartmentation and the formation of complexes with phenol derivatives in the vacuole may be another example of the mechanisms of resistance to heavy metals. Precipitation of phenolics generally revealed a significant higher electron-opacity over all protoplasm in bilberry leaves collected in a polluted forest in comparison to leaves from an unpolluted locality (Bialonska et al. 2007). These results indicate that the distribution and properties of phenolics depend on the level of heavy metals accumulated in the cell and the phenolics accumulated in vacuoles and apoplasts may play a significant role in scavenging of free radicals produced in plant cells (Bialonska et al. 2007). In a herbaceous plant chamomile (*Matricaria chamomilla*), soluble phenolics in the root and leaf rosettes were elevated by high doses of Cu and Cd, whereby Cu had a more expressive effect in roots and Cd in leaf rosettes, respectively (Kovacik et al. 2008). Low doses of Cd and Cu did not affect soluble phenolics in either the leaf rosettes or the roots. Recently, Janas et al. (2010) suggested that higher phenolics accumulation in vacuoles and cell walls of lentil (*Lens culinaris* Medic.) seedlings treated with Cu ions might be involved in scavenging ROS produced in the Cu-treated plant cells. They also confirmed that the induction of phenolics in Cu-treated seedlings had an important role in the lentil root protection against this metal. The concentration of polyphenolic compounds (particularly isoflavonoids like genistein and genistein-(malonyl)-glucoside) was significantly higher for lupin (*Lupinus albus* L.) roots when grown in a 20-µM Cu solution as compared to the control, and these phenolic compounds can bind Cu ions (Jung et al. 2003). In addition, plants exposed to 20 and 62 µM Cu accumulated high Cu amounts in root cell walls whereas only low amounts reached the symplasm. Therefore, it is proposed that the complexation of Cu^{2+} in the rhizosphere and in the roots apoplasm by phenolic compounds could have restricted Cu toxicity to the plant (Jung et al. 2003). Going back further, Suresh and Subramanyam (1998) had already studied the role of polyphenolic compounds involved in Cu binding onto the cell walls of fungus *Neurospora crassa*. Their ESR (electron spin resonance) and FTIR (Fourier transformation infrared) studies of the Cu-polyphenol complexes indicated Cu to be bound as Cu(I) present in a distorted octahedral geometry and bound through oxygens belonging to phenolic hydroxyls and/or nitrite groups. The authors proposed that both groups might participate in a binding mechanism and supposed that nitrophenols are the responsible ligands located in the cell wall. Similar bindings are likely in plant cells.

3 Heavy Metal Localization and Distribution

3.1 Localization of Heavy Metals in Cells and Tissues of Different Plant Organs

As shown in Fig. 2, general mechanisms for detoxification and accumulation of heavy metals in plants are the distribution of the metals to apoplastic compartments
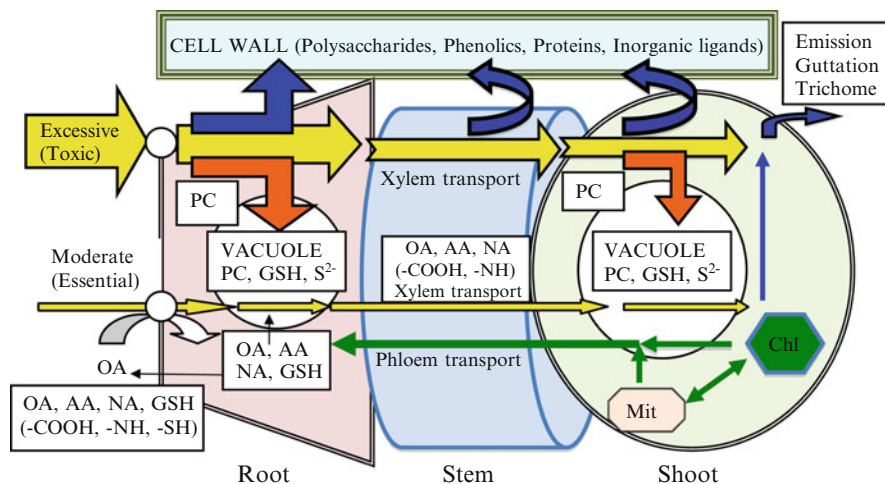
Fig. 2 Possible metal localization and presence of major metal-binding ligands in a model plant with a standard root, stem and shoot system. In each organ, tissues and cells are conventionally divided into apoplasic and symplastic sites. The former including xylem (sap) in the conductive tissues of each organ, and rizosphere connected to or surrounding the root system underground, and also in some cases vacuoles (apoplast but inside the protoplasm). The latter includes phloem (sap) and cytoplasm in each organ. The xylem and phloem systems support large parts of the stem and other tissues, and they play considerable roles in mineral/water transport from root to shoot and vice versa, with assimilatives as long-distance transports. Trichomes in shoot (leaf) also consist of apoplastic and symplastic sites but develop their special structure and functions for metal binding and accumulation. *Mit* mitochondria, *Chl* chloroplast, *PC,* phytochelatin, *GSH* glutathione, *OA* organic acid, *NA* nicotianamine, *AA* amino acid, *-COOH* carboxyl group, *-NH* amino- or imino- group, *-SH* sulfhydryl group

like cell walls or trichome, and the chelation of the metals by a ligand in cytoplasm, followed by the sequestration of the metal−ligand complex into the vacuole, in the different organs such as roots, stems and leaves (Yang et al. 2005). Generally, the heavy metal contents in plant organs decrease in the following sequence; root > leaves > stems > inflorescence > seeds. However, this order sometimes varies with plant species, especially in hyperaccumulators, of which the shoots have the highest heavy metal content. Roots usually manifest the maximum content of heavy metals. Leaves vary with age in their ability to accumulate heavy metals, some heavy metals accumulate preferentially in the youngest leaves of plants, whereas in others, the maximum content is found in senescing leaves. Preventing Cd ions from entering the cytosol by the plant cell walls theoretically represents the best detoxification mechanism (Ma et al. 2005). Cd stress may be alleviated by sequestration of Cd in the cell wall or the vacuole in Cd-tolerant genotypes of barley, especially in short-term Cd-exposed experiments. Cell walls of the root can act as a first barrier against Cd stress in immobilizing excesses of Cd (Wu et al. 2005). Available evidence suggests that Cd binds to the secondary wall and middle lamellae in maize roots (Khan et al. 1984). On the other hand, in bush bean, Cd was mainly bound to pectic sites and hystidyl groups of the cell wall in roots and leaves (Leita et al. 1996). In white lupin, the cell wall was found to retain up to 47% of the

absorbed Cd in leaves, 51% in stems, and 42% in the roots, although 20–40% of total Cd was associated with PCs (Vazquez et al. 2006), implying that this plant may use cell wall binding as a more effective mechanism of Cd detoxification than PCs. However, excess and non-specific metal binding to primary cell walls did not appear to be the tolerance mechanism in tomato suspension-cultured cells and roots of some dicotyledonous plants (Inouhe et al. 1991, 1994). In these cases, where the cells are actively growing, the cytoplasmic formation of PCs followed by metal binding and transport to vacuoles can be more effective mechanisms of Cd detoxification than wall bindings.

3.2   Distribution of Heavy Metals and Conjugating Ligands in Root

Besides bioavailability, uptake and translocation efficiencies determine metal accumulation and distribution in plants (Clemens 2006). Roots are the plant organs in closest contact with metal-contaminated soils; therefore, they are the most affected by metals. Resistance to excess metals can be achieved by avoidance when the plant is able to restrict metal uptake into the cells, or tolerance when the plant is able to survive in the presence of excess metals inside. Having been taken up by the root and transported to various cells and tissues within the plant, heavy metals concentrate there to cause injury in a sensitive plant, or as an inactivated form in a tolerant plant.

Cd-tolerant tobacco species (*Nicotiana rustica*) indicated greater labeled cadmium (^{109}Cd) content in the roots than the leaves, the major part of which was stored in the distal part as a tolerance strategy (Bovet et al. 2006). In hyperaccumulator *A. halleri* roots exposed to 100 μM Cd and 500 μM Zn hydroponically, Zn and Cd accumulated in the cell walls of the rhizodermis (root epidermis), mainly due to precipitation of Zn/Cd phosphates (Kupper et al. 2000). In roots, scanning electron microscope combined with energy dispersive spectrometry (SEM-EDS) confirmed that the highest Zn concentration was found in xylem parenchyma cells and epidermal cells, while for Cd, a gradient was observed with the highest Cd concentration in rhizodermal and cortex cells, followed by central cylinder. Light microscope results showed that Zn and Cd distributed mainly along the walls of epidermis, cortex, endodermis and some xylem parenchyma (Hu et al. 2009). Energy-dispersed X-ray (EDX) microanalysis revealed details about the subcellular localization of Cd in *A. thaliana*, ecotype Columbia (Van Belleghem et al. 2007). The results indicated that the localizations of Cd in the root cortex were associated with phosphorus (Cd/P) in the apoplast and sulfur (Cd/S) in the symplast, suggesting phosphate and PC sequestration, respectively. In the endodermis, sequestration of Cd/S was present as fine granular deposits in the vacuole and as large granular deposits in the cytoplasm. In the central cylinder, symplastic accumulation followed a distinct pattern illustrating the importance of passage cells for

the uptake of Cd. Furthermore, in the apoplast, a shift of Cd/S granular deposits from the middle lamella towards the plasmalemma was observed. Large amounts of precipitated Cd in the phloem suggest retranslocation from the shoot (Van Belleghem et al. 2007). On the other hand, subcellular localization of Pb and Cd in *Iris pseudacorus* showed that numerous Pb deposits were found on the inner surface of dead cell walls in the cortex treated with 2,070 mg L^{-1} Pb, there were no Pb deposits in the cell walls and cytoplasm of the neighbor cells (Zhou et al. 2010). Cd deposits were found in the cell wall and on the outer surface of the cells in a triangular intercellular space bordering with three cortical cells treated with 1,000 mg L^{-1} Cd for 16 days sand culture. The ultrastructure showed that Cd deposits in some cell walls were not well distributed and not found in the cytoplasma and vacuoles, showing that Cd was mainly transported by the way of apoplasts (Zhou et al. 2010); Han et al. (2007) found similar results that some Cd deposits were located not only in the cell walls but also in the vicinity of the plasma membranes and membrane-bound organelles in the root cells of *Iris lactea* var. *chinensis*. This observation also supports the apoplastic transport of Cd in the plan but cannot exclude the possibility that Cd deposits accumulated in the cell walls might negatively affect the enzymes and other protein functions in this compartment.

The increase of the cell walls (CWs) capacity to bind Pb by formation of cell wall thickenings (CWTs) rich in JIM5 pectins, callose and lipids in *Funaria hygrometrica* plant cells treated with Pb might be regarded as the next step in the development of the plant resistance strategy against this metal based on immobilizing toxic ions within apoplast (Krzeslowska et al. 2009). Binding metal ions within CWs is the important resistance strategy of plant cells in response to Cd (Fig. 1). This has been shown recently for *T. caerulescens* (Wojcik et al. 2005); *Salix viminalis* (Vollenweider et al. 2006) and *Linum usitatissimum* (Douchiche et al. 2007, 2010). In the last named, it was found moreover that exposing plants to Cd resulted in significant increases of both the cell wall thickness and JIM5 pectins formation level in CWs (Douchiche et al. 2007). In *S. viminalis,* the main Cd sink was pectin-rich collenchyma CWs of the veins. Moreover, also in this case, the amount of pectins slightly increased in collenchyma cells in response to Cd. Active storage of Cd in this plant was indicated by homogeneous CWTs containing cellulose and proanthocyanidins (Vollenweider et al. 2006). Thus, similarly to *Funaria* protonemata treated with Pb, both *L. usitatissimum* seedlings tissues and *S. viminalis* collenchyma increased the capacity of cell walls for Cd detoxification by formation of thicker cell wall and increasing the level of polysaccharides, especially that of pectin (Krzeslowska et al. 2009).

3.3 Distribution of Heavy Metals and Conjugating Ligands in Shoots

As already noted, there are well-documented differences across plant species in the partitioning of Cd between organs. Compared to other toxic metals or metalloids

(e.g., Pb and As), Cd has a higher propensity to accumulate in shoots other than the roots. Still, there is normally more Cd in roots than in leaves, and even less in fruits and seeds (Wagner 1993). The tendency of tobacco plants to translocate Cd quite efficiently to the leaves contributes to the fact that tobacco smoke is an important Cd source for smokers (Lugon Moulin et al. 2004). But recently, some research showed that tobacco develops an original mechanism of metal detoxification by the exudation of metal/Ca-containing particles through leaf trichomes (Choi et al. 2001; Choi and Harada 2005; Sarret et al. 2006).

An energy-dispersive X-ray (EDX) analysis system equipped to variable pressure scanning electron microscopy (VP-SEM) revealed that the tobacco trichomes exudates contain amounts of heavy metals. Overexpression of cysteine synthase confers Cd tolerance to tobacco, and the endogenous concentration of Cd was 20% less in transgenic plants than in wild-type plants. The numbers of both long and short trichomes in the transgenic plants were 25% higher than in that of wild-type plants, indicating the active excretion of Cd from trichomes in transgenic plants (Harada and Choi 2008). Upon Cd or Zn treatment, the number of trichomes was increased more than 2-fold (Choi et al. 2001; Sarret et al. 2006). Confocal laser scanning electron microscopy showed metal accumulation in the tip cells in trichomes. The chemical forms of the exuded grains were identified as metal-substituted calcite (calcium carbonate) by using synchrotron-based X-ray microanalyses (Sarret et al. 2006, 2007). Observation by VP-SEM indicated that large crystals of 150 μm in size were formed on head cells of both short and long trichomes. An EDX analysis system fitted with VP-SEM revealed the crystals to contain amounts of Cd and Ca at much higher concentrations than in the head cells themselves.

TEM demonstrated crystal formation in amorphous osmiophilic deposits in vacuoles in tobacco (Choi et al. 2001). The majority of Ni is stored either in *Alyssum* leaf epidermal cell vacuoles or in the basal portions only of the numerous stellate trichomes. Broadhurst et al. (2004) reported simultaneous and region-specific localization of high levels of Ni, Mn, and Ca within *Alyssum* trichomes as determined by SEM/EDX. The metal concentration in the trichome basal compartment was about 15–20% dry weight, the highest ever reported for healthy vascular plant tissue (Broadhurst et al. 2004). In aerial parts, Zn was predominantly octahedral coordinated and complexed to malate.

In *A. halleri*, secondary organic species were identified in the bases of the trichomes, which contained elevated Zn concentrations, and in which Zn was tetrahedrally coordinated and complexed to carboxyl and/or hydroxyl functional groups (Sarret et al. 2002). In *A. halleri* leaves, the trichomes had by far the largest concentration of Zn and Cd. Inside the trichomes, there was a striking subcellular compartmentation, with almost all the Zn and Cd being accumulated in a narrow ring in the trichome base. Another phenomenon is that the epidermal cells other than trichomes were very small and contained lower concentrations of Zn and Cd than mesophyll cells. In particular, the concentrations of Cd and Zn in the mesophyll cells increased markedly in response to increasing Zn and Cd concentrations in the nutrient solution. This indicates that the mesophyll cells in the leaves of

A. halleri are the major storage site for Zn and Cd, and play an important role in their hyperaccumulation (Kupper et al. 2000). In contrast, Cd was detected in tracheids of *A. thaliana* but not in the mesophyll tissue (Van Belleghem et al. 2007). In *Potentilla griffithii* leaves, Zn and Cd shared a similar distribution pattern, and both were mostly accumulated in epidermis and bundle sheath. However, in leaves of 40 mg L^{-1} Cd treatment, which caused the phytotoxicity, Cd was also found in the mesophyll cells. The major storage site for Zn and Cd in leaves of *P. griffithii* was vacuoles, and to a lesser extent cell walls or cytosol. The present study demonstrates that the predominant sequestration of Zn and Cd in vacuoles of epidermis and bundle sheath of leaves may play a major role in strong tolerance and hyperaccumulation of Zn and Cd in *P. griffithii* (Hu et al. 2009).

4 Conclusion

It is obvious that plants utilize various types of biological ligands to conjugate, co-transport and partition heavy metal elements (Fig. 2). The biochemical and genetic bases of the Cd-tolerance phenotypes of plants may involve both the PC-dependent and -independent processes. The former involves several different processes: the activation of PC synthase, GSH biosynthesis, and accumulation of acid-labile sulfide, sulfur assimilation and transport of the Cd-PC complexes into vacuoles. All these would be required for the formation of the stable and nontoxic Cd–complexes in the vacuole or other sites in the cells of most plants, where the PC synthase is a key factor for the tolerance phenotypes to Cd and other heavy metals. The PC-independent mechanisms are apparently present in more differentiated higher plants that habituate on terrestrial system. Their hyperaccumulation phenotype of metal/metalloids from soil and water can be attributed to the highly developed apoplastic transport systems. The low constant pH condition and changeable solute components in the xylem sap and other apoplastic sites may allow more variable and more complicated interactions between the metal and biological ligands in plants. This might be a potential for the differentiation and specification of a unique hyperaccumulator to be evolved on ground. Readjustment of both the symplastic and apoplastic activities including the formations of PC-dependent and -independent metal-binding ligands and their transport systems can be beneficial for more effective and intentional approaches to conduct the remediation technique under contaminated soil and water environments.

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