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Heavy Metal Uptake and Detoxification Mechanisms in Plants*

Shruti Mishra and R.S. Dubey
Department of Biochemistry, Faculty of Science,
Banaras Hindu University, Varanasi-221005, India

Abstract: Plants take up heavy metals from their surroundings via a number of different plasma membrane localized transporters. Inside the plant system toxicity of heavy metals is manifested by an array of physiological and metabolic disturbances if metal detoxification processes are delayed or not efficient. Immobilization of heavy metals by root exudates and in some cases mycorrhizal association constitute the extracellular mode of detoxification whereas chelation of heavy metals by phytochelatins, metallothioneins, organic acids and amino acids, induced synthesis of proline and synthesis of stress proteins are implicated in preventing metal accumulation in fully reactive form in the cytosol. Plant cells have developed sophisticated toxic metal sensing proteins that transmit signals to activate expression of metal detoxification and homeostasis genes in order to cope with the elevated heavy metal concentrations. In transducing signals of heavy metal origin involvement of second messengers viz., Ca^{2+} , jasmonic acid and H_2O_2 as well as participation of ethylene and salicylic acid are likely. This review aims to give succinct overview of plant metal uptake and detoxification mechanisms.

Key words: Detoxification, heavy metals, metallothioneins, mycorrhiza, phytochelatins, sequestration, signal transduction, stress proteins

Introduction

Soil contamination with heavy metals has become a world-wide problem particularly in areas with high anthropogenic pressures leading to losses in agricultural yield as well as hazardous health effects when these metals enter the food chain (Frankenberger, 2002; Jonak *et al.*, 2004).

Plants require relatively small number of elements for their growth and survival. Natural soils however contain many other elements that are non-essential and in spite of the selectivity of root cell membranes, may be detected in plant tissues in trace amounts. Many of these elements, especially metals like Cd, Pb, As, Hg can be highly toxic. In addition, required micronutrients such as Cu, Ni and Zn can also be toxic to the plants when present in higher concentrations in the soil environment. Accumulation of heavy metals in the soil influences soil properties and adversely affects the establishment and growth of plants whose roots initially develop in the most contaminated layer (Bhattacharjee and Mukherjee, 2004; Jha and Dubey, 2004). Uptake of metals by plants occurs via the root and the extent of absorption mostly depends on the ionic potential of the element concerned. Sometimes metals can be absorbed actively or passively or undergo accumulation along with a macronutrient cation. There occurs a competition among the metals for chelation, movement to the root

Corresponding Author: Dr. R.S. Dubey, Professor, Department of Biochemistry, Faculty of Science,
Banaras Hindu University, Varanasi-221005, India
Tel: 91 542 2317190 Fax: 91 542 2368174

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and uptake at the root-tip. Available data suggest that heavy metals if not detoxified rapidly enough, may trigger multiple deteriorative events in plants by altering major physiological and biochemical processes, ultimately leading to visible plant injuries and yield losses (Carrier *et al.*, 2003; Jha and Dubey, 2004; Kukier and Chaney, 2004).

During the past two decades concerted research efforts have addressed the biology of heavy metal toxicity at the whole plant, cellular and subcellular levels. A detail understanding of the uptake of heavy metals by plants, components associated with metal tolerance and signal transduction pathway in presence of metal is essential in order to produce metal accumulating and metal tolerant plant species by using genetic engineering techniques. This article reviews our current status of knowledge related to the uptake of metals, the strategies adopted by plants in their detoxification as well as the possible involvement of signal molecules in metal detoxification processes mainly in relation to Cd, Pb, Hg, As, Ni and the metal Al. Although aluminium is not a heavy metal, but its mode of action and detoxification processes involved to reduce its toxicity are of particular concern due to potential phytotoxic nature of aluminium especially in acid soils.

Uptake of Heavy Metals

Root cell walls initially bind metal ions from the soil and thereafter *via* high affinity binding sites and plasma membrane localized transport systems; metal ions are taken up across plasma membrane. Generally uptake of metal ions occurs through secondary transporters such as channel proteins and/or H⁺-coupled carrier proteins. Uptake of cations through secondary transporters is facilitated by the membrane potential of the plasma membrane, which is negative on the inner side of the membrane (Hirsch *et al.*, 1998). A number of different types of metal transporters are implicated in plants and often more than one transport system exists for one metal (Williams *et al.*, 2000). Summary of putative Cd, As, Ni and Zn transporters identified in plants to date has been presented in Fig. 1.

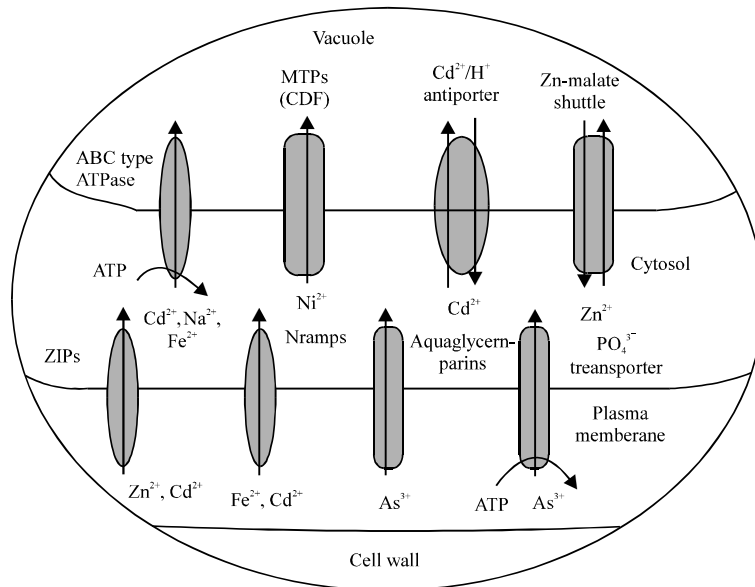


Fig. 1: Transporters of heavy metals Cd, As(V), As(III), Ni and Zn

In uptake and transport of Zn^{2+} and Cd^{2+} , ZNT (zinc-iron permease [ZIP] gene homolog) proteins have been implicated. The histidyl area of the variable region of these transport proteins is involved in metal binding and their subsequent transport (Pence *et al.*, 2000). The metal ion Cd^{2+} can also enter the plant cell by transporters for essential cations such as Fe^{2+} (Thomine *et al.*, 2000). In *Arabidopsis*, AtNramps (Natural resistance associated macrophage proteins) genes encode the metal transporters, which transport both mineral nutrient Fe and toxic metal Cd (Thomine *et al.*, 2000). Lead is taken up by plant roots primarily through passive absorption (Tung and Temple, 1996), although certain proteins have been identified that mediate cross membrane movement of Pb. In tobacco a protein NtCBP4 has been identified which is involved in metal uptake across plasma membrane. Expression of NtCBP4 in transgenic tobacco confers Pb-hypersensitivity (Arazi *et al.*, 2000). Plants take up arsenate (AsV) via phosphate transporter. A competition occurs between phosphate and arsenate at the uptake level for the same transport system as evidenced from several studies in plant species like *Oryza sativa* (Abedin *et al.*, 2002), *Brassica juncea* (Pickering *et al.*, 2000). Arsenate accumulation follows Michaelis-Menten kinetics at a concentration range that coincides with the level of activity of the high affinity phosphate uptake system (Abedin *et al.*, 2002). A class of multifunctional channels called aquaglyceroporins has been identified in plants, which transport neutral organic solutes such as urea and glycerol. Transport of arsenite (AsIII) via these aquaglyceroporins has been reported in plants (Meharg and Jardine, 2003).

No specific Ni-uptake system has been characterized in higher plants. However, two distinct high affinity systems have been described in prokaryotes- the single component Ni^{2+} permeases, which belong to the nickel/cobalt transporter (NiCoT) family and the Nik systems, which belong to ABC transporter family (Eitinger and Mandrand-Berthelot, 2000). The absorption of organic and inorganic Hg from soil by plants is low (Lodeniuss, 1990) and after uptake by plants, restricted transport of Hg is observed through phloem (Panda Kamal *et al.*, 1992). However, air-borne mercury, in certain cases, is absorbed by plant leaves (Wojciechowska-Mazurek *et al.*, 1995).

Extracellular Avoidance of Metal Build-up

At extracellular level plants respond to metals either by immobilizing them with their root exudates or some mycorrhizal association helps them in performing this task. In many plant species primary barrier against entry of heavy metals into plant roots operates at the root level. Cd can be immobilized by cell wall (Leita *et al.*, 1996) and extracellular carbohydrates (Wagner, 1993). In roots and leaves of bush bean, Cd ions mostly seem to bind pectic sites and histidyl groups of the cell wall (Leita *et al.*, 1996). Possible involvement of histidine and citrate has been suggested in root exudates that reduces Ni-uptake and helps in Ni-detoxification (Salt *et al.*, 2000).

In several bean species differences in Al tolerance have been attributed to the capacity of roots to exude citric acid, a strong Al chelator (Ma, 2000). In cereals also better tolerance to Al toxicity is associated with root exudation of citric acid, malic acid, succinic acid, oxalic acid and other organic acids (Bertrand and Guary, 2002). Activation of organic acid efflux occurs rapidly without any measurable delay after exposure to Al in several plant species (Ryan *et al.*, 2001). Efflux of citrate and malate from plant roots in response to Al occurs by activation of anion-channels located in the plasma membrane (Ryan *et al.*, 2001). In lower plants, like algae, it has been shown that metals can be trapped by polysaccharides, alginates and other slimes secreted by the algae (Lee and Volesky, 1997).

There are accumulating evidences in support of mycorrhizal involvement in host plant metal detoxification more specially the ectomycorrhizal association (Blaudez *et al.*, 2000; Jentscheke and Godbold, 2000). The fungi employ the same strategy for metal detoxification as adopted by higher

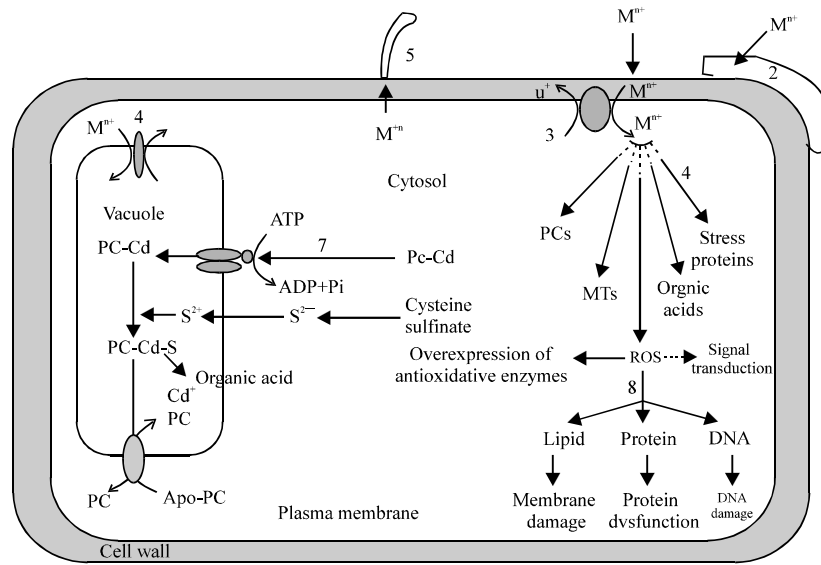


Fig. 2: Cellular mechanisms proposed to be involved in metal uptake, sequestration and detoxification in plants. 1. Extracellular avoidance of metal buildup through immobilization by root exudates. 2. Ectomycorrhizal association restricts metal movement to roots. 3. Metal ions are taken up by plant roots through channel proteins and/or H^+ -coupled carrier proteins. 4. From cytosol metals are transported and accumulated in vacuoles, the events are aided by vacuolar electrogenic proton fluxes. 5. Glandular trichomes and epidermal structures (hydropotes) sequester metals in leaves. 6. Metal ions in cytosol can be detoxified *via* these routes. 7. Mechanisms involved in Cd chelation and compartmentalization in the vacuole. 8. Metal ions escaped from the complexation damage cellular macromolecules *via* the production of ROS

plants i.e., binding to extracellular materials or sequestration in the vacuolar compartment (Blaudez *et al.*, 2000). Ectomycorrhizas restrict metal movement to host roots by exclusion processes (Jentschke and Godbold, 2000). Some mycorrhization stimulates the phenolic defense system in mycorrhizal symbiosis (Schützendübel and Polle, 2002). Cellular mechanisms proposed to be involved in metal uptake, sequestration and its detoxification in plants have been outlined in Fig. 2.

Intracellular Detoxification Pathways *Phytochelatin*

Higher plants, algae and fungi can respond to exposure of several metals by the synthesis of thiol containing metal binding peptides called phytochelatin (PCS) (Cobbett, 2000). The immobilized metals in form of metal phytochelatin complexes are very stable and less toxic than free metal ions. PCS are considered to be an important component of the metal detoxifying mechanisms of higher plants (Zenk, 1996; Hartley-Whitaker *et al.*, 2001; Küpper *et al.*, 2004). PCS are derived from glutathione (GSH) and possess the general structure (γ -Glu-Cys) $_n$ -Gly where $n = 2-11$ (Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). Their biosynthesis occurs due to the trans-peptidation of γ -glutamyl-cysteinyl dipeptides from GSH by the action of constitutively present enzyme PC synthase that is activated due to heavy metal ions. When PCS are synthesized in sufficient amount to chelate

metal ions, the enzyme is turned off and the reaction is terminated (Chen *et al.*, 1997). The unusual bonding between glutamate and cysteine i.e., carboxyamide linkage in phytochelatin suggests that they are not synthesized on ribosomes and are thus not a direct gene product but are enzymically synthesized polypeptides (Harada *et al.*, 2004). The cysteine residues of PCS ensure metal coordination via their thiol group and the high percentage of glutamic acid residues makes PCS extremely water soluble (Bertrand and Guary, 2002). The N-terminal and downstream γ -peptidyl bonds of PCS probably serve to protect thiol peptides from general protease action but not from the specific action of γ -glutamyl transpeptidases during the salvage process (Satofuka *et al.*, 2001).

PCS have been classified as class III metallothioneins and are induced in all autotrophic plants analyzed so far. The biosynthesis of PCS is induced by many metals like Cd, Hg, Ag, Cu, Ni, Au, Pb and Zn; however, Cd is the strongest inducer (Zenk, 1996). Despite the fact that many metals are able to trigger PC synthesis, only some of them (i.e., Cd, Hg, Pb, Cu, Ag) are able to form complexes with PCS (Bertrand and Guary, 2002). Following Cd exposure, a rapid induction in PC level has been observed in *Brassica napus* (Carrier *et al.*, 2003). Lead exhibits several coordination numbers when it binds with PCS; the number depends on the length of the PC chain; PCS with longer chain bind Pb more strongly than those with shorter chain length (Piechalak *et al.*, 2002). Arsenate and arsenite both induce the synthesis of PCS (Pickering *et al.*, 2000; Hartley-Whitaker *et al.*, 2001). In *Brassica juncea*, an As-non-accumulator, As III-thiolate complex has been reported and the thiolate has probably its origin from glutathione or PCS (Pickering *et al.*, 2000). Maitani *et al.* (1996) reported rapid induction of Hg-binding PCS in root cultures of *Rubia tinctorum* following metal exposure. In addition, a number of structural variants of PCS, such as $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$, $(\gamma\text{-Glu-Cys})_n\text{-Ser}$ and $(\gamma\text{-Glu-Cys})_n\text{-Glu}$ have been identified in certain plant species (Cobbett, 2000).

Considerable efforts have been made to study the genes encoding PC synthases as well as catalytic mechanism of PC synthases in higher plants. PC synthase like polypeptides are of widespread occurrence in plants and a catalytic homologue of this enzyme has been identified in prokaryotes (Tsuji *et al.*, 2004). The enzyme PC synthase mediates a bisubstrate reaction in which the thiol group(s) of at least one of the substrate is blocked, usually, but not necessarily through the formation of metal thiolate. The chain extension proceeds along C π N with cleavage of the Cys-Gly peptide bond of the donor (Rea *et al.*, 2004). It is proposed that the strongly conserved N-terminal half of the enzyme is responsible for catalysis and that activation arises from the binding of the metal ions to cysteine residues, within this domain (Ruotolo *et al.*, 2004). This finding is in consistence with the observation that three most extreme *Arabidopsis cad1* alleles have amino acid substitutions in this region (Ha *et al.*, 1999). The C-terminal domain of PC synthase contributes stability to the full-length polypeptide and participates in defining the range of metals to which the enzyme is responsive (Ruotolo *et al.*, 2004).

A lot of research efforts are underway to characterize the gene encoding PC synthase in higher plants. Clemens *et al.* (1999) and Ha *et al.* (1999) have successfully characterized structural genes of this enzyme in *Arabidopsis*, wheat and yeast. Two Cd-responsive novel genes coding for the proteins ATMEKK1 and a putative farnesylated protein, with two metal binding motifs have been reported in *Arabidopsis* that endow marked tolerance to the plants towards Cd (Suzuki *et al.*, 2001).

Plant age dependent differences in the complexation and speciation of heavy metals has also been observed. In *Thlaspi caerulescens* plants the nature of Cd binding changed depending on the tissue age (Küpper *et al.*, 2004). In young leaves predominant Cd ligands appeared to be sulphur-ligands (e.g., phytochelatin, metallothioneins and other cysteine rich peptides) while in senescing leaves oxygen-ligands were predominantly involved.

Metals when get complexed with PCS are transported to vacuoles. In this way PC-metal complexes isolate the toxic metals from various metal sensitive enzymes in the plant cell cytoplasm and possibly form a more complex aggregation in the vacuole (Cobbett, 2000). The metal-PC complex is transported from the cytosol to the vacuole by either metal/H⁺ antiporters or ATP-dependent ABC transporters of tonoplast (Salt and Rauser, 1995; Rea *et al.*, 1998). Involvement of a ABC-transporter YCF1 (yeast cadmium factor 1) has been reported in yeast which catalyzes Mg ATP-energized transport of bis(glutathionato)cadmium [Cd.GS₂] into vacuole (Li *et al.*, 1997). In *S. pombe* heavy metal tolerance factor 1 (HMT1) catalyzes the Mg ATP-energized uptake of Cd.PCs and apoPCs into the vacuoles (Ortiz *et al.*, 1995). Similar Mg-ATP dependent transport of PC and Cd-PC complexes have been shown in vacuolar membrane vesicles of oat roots and this transport pathway appears to be an analog of HMT1 of *S. pombe* (Ortiz *et al.*, 1995). In Cd-hyperaccumulator *Thlaspi caerulescens* plants about 90% of the total Cd present in mesophyll cells remains localized in vacuoles (Ma *et al.*, 2005). Therefore in these plants vacuolar sequestration has been suggested as an important mechanism of internal detoxification of Cd in leaves (Ma *et al.*, 2005). Vacuolar transportation of As-PC complexes has also been reported in several plant species (Meharg and Hartley Whitaker, 2002; Raab *et al.*, 2004), however, thiol conjugated forms of As(III) have not been observed (Raab *et al.*, 2004). In *S. cerevisiae* MRP (multidrug resistance-associated protein) homolog, Ycf1p has vacuolar localization and catalyzes sequestration of bis(glutathionato)arsenic [As(GS)₂] in the vacuole (Ghosh *et al.*, 1999).

PC-metal complexes not only serve as an important component of intracellular heavy metal detoxification mechanisms in plants by translocating metals across the tonoplast and sequestering in vacuoles but these complexes can also undergo long-distance transport from roots to shoots. A gene, TaPCS1 from wheat, when transgenically expressed in *Arabidopsis* roots, enhances long distance root-to-shoot Cd²⁺ transport and reduces Cd-accumulation in roots (Gong *et al.*, 2003). In vacuoles inorganic sulfide and sulfite ions are incorporated in the PC-metal complexes making them more stable (Bertrand and Guary, 2002). Sulfide ions in the phytochelatin complex stabilize the pH at which dissociation of metal occurs (Steffens, 1990). Phytochelatin complexes containing labile sulfur ions are more stable and resistant to proteolytic degradation than those lacking these constituents (Steffens, 1990). In vacuoles, from PC-metal-sulfide complexes metals are liberated and the PC moiety is degraded (Grill *et al.*, 1988). The metals are complexed with vacuolar organic acids while the individual amino acids can again enter into the cytosol (Zenk, 1996).

Besides detoxification of metals, PCS also appear to have certain other important roles in the cell like in maintaining homeostasis of heavy metals (Thomine *et al.*, 2000), in sulfur metabolism (Rauser, 1995) as well as in antioxidative defense of the plant. PCS may have higher antioxidant activity than other well-known antioxidants (Giles *et al.*, 2003). Certain studies have indicated that anthocyanins also have a role in metal sequestration. They can bind metal ions and with the help of ABC-type transporter proteins glutathione-conjugated anthocyanin-metal complexes are then transported to the vacuoles (Marrs, 1996).

Metallothioneins

Metallothioneins (MTs) are cysteine rich gene encoded proteins capable of sequestering metals by forming metal-thiolate clusters. They have molecular weight in the range 5-20 kDa and can be divided into two different classes on the basis of their cysteine content and structure. MTs are proteins typical of vertebrates but MT genes have also been identified in a wide range of plant species (Goldsbrough, 2000). The expression of MTs in plants growing in absence of metal excess has also been observed (Bertrand and Guary, 2002).

Introduction of mammalian MT gene into *B. compestris*, *B. napus* and *N. tabacum* confers Cd tolerance by increasing complexation of metal with MT and minimizing translocation of Cd to the shoots (Maiti *et al.*, 1989). A number of genes in plants have been found to encode MT-like proteins having sequence similarity to animal MT proteins. The history of plant MTs dates back to 1987, when the first plant metallothionein protein termed Ec protein (early cysteine-labeled) was detected in wheat (Lane *et al.*, 1987). Later, a number of MT-encoding genes were identified in *Arabidopsis*. A total of eight genes constitute MT-gene family in *Arabidopsis* (Zhou and Goldsbrough, 1994). MTs have highest complexing affinity for Cu and are induced by exposure to it (Murphy *et al.*, 1997). On Cu treatment, strong induction in MT2 mRNA level has been observed in *Arabidopsis*. When genes for MT1 and MT2 from *Arabidopsis* were expressed in an MT-deficient yeast mutant, both genes complemented the mutation and provided a high level of resistance to Cu (Zhou and Goldsbrough, 1994). Similarly, in Cu-sensitive mutants of *Arabidopsis* it was shown that MT genes could be induced by Cu. A PsMT_A gene identified in the roots of *P. sativum* regulates the synthesis of MT-like proteins (Robinson *et al.*, 1992).

There appears to be similarity between MTs and PCS for complexation with metals as observed in plants such as datura and maize (Rivai *et al.*, 1990). The role of MTs as antioxidants has also been advocated (Wong *et al.*, 2004). A decrease in MT levels could result in increased level of free transition metal ions (Briat, 2002). According to Wong *et al.* (2004) a rice metallothionein protein OsMT2b appears to function as an ROS scavenger, which depicts the antioxidative role of plant metallothioneins. Similarly, NO-scavenging capacity and involvement in plasma membrane repair appear to be additional roles of MTs (Salt *et al.*, 1998).

Stress Proteins

When subjected to heavy metal treatment, most of the plants trigger the synthesis of certain sets of novel proteins. Induction of protein synthesis under metal toxicity plays an important role in the maintenance of heavy metal homeostasis and/or detoxification by endowing plasma membranes with the capacity to serve as an initial barrier against the inflow of free heavy metal ions into the cells (Suzuki *et al.*, 2002).

Some of these proteins belong to the family of heat shock proteins (HSPs). These proteins are referred to more commonly as 'stress proteins'. HSPs act as molecular chaperones and help in normal protein folding and assembly, but may also function in the protection and repair of proteins under stressful conditions. It has been demonstrated that the DNA of Cd-stressed cells produces specific mRNA transcripts which regulate the synthesis of stress proteins (Edelman *et al.*, 1988). In several plant species Cd-exposure induces the synthesis of a considerable number of stress proteins with molecular mass ranging from 10-70 kDa. In *Oryza sativa*, Cd supply induced the synthesis of new proteins with mol. wts. 70, 42, 26, 23, 15 and 11 kDa (Reddy and Prasad, 1995; Prasad, 1997). A 18 kDa Cd inducible protein with 4-SH groups per molecule was isolated by Shah and Dubey (1998a) from rice seedlings. *Armeria maritima* plants grown on Cu-rich soils showed increased expression of HSP17 (Neumann *et al.*, 1995). Enhanced level of HSP17 was also observed in cell cultures of *Silene vulgaris* and *Lycopersicon esculentum* in response to a range of heavy metals (Wollgiehn and Neumann, 1999). Whereas, increased expression of HSP70 was observed in seaweed *Entormorpha intestinalis* after Cu-exposure (Lewis *et al.*, 2001).

A Cys-rich membrane protein with mol. wt. 16 kDa isolated from *Arabidopsis*, termed as AtPcrs plays an important role in Cd resistance of *Arabidopsis* plants and similar homologs have also been reported in *Lycopersicon esculentum* and *Oryza sativa* (Song *et al.*, 2004). In *Arabidopsis* again,

increased expression of another metal binding protein gene *cdl19* has been reported after exposure of plants to Cd, Hg, Fe and Cu (Suzuki *et al.*, 2002). In *Hordeum vulgare* seedlings increased synthesis in the amount of apoplastic polypeptides was observed in response to Zn treatment (Brune *et al.*, 1994). Ni also induces synthesis of proteins in many plant species. It has been observed that Ni induces synthesis of proteins at the transcriptional or translational level (Blinda *et al.*, 1997).

Expression of pathogenesis related proteins (PR proteins) has also been reported in certain plants under metal toxicity, but this response solely appears to be metal specific. Synthesis of a 16-kDa polypeptide related to PR-10 family pathogenesis related proteins is induced in *Lupinus luteus* plants under Cd, Zn and Cu treatment (Przymusiński *et al.*, 2004). The precise role of PR proteins in heavy metal tolerance is not known, however, it appears that these proteins have ribonuclease like function and are associated with the defense mechanism of plants during pathogen attack (Bantignies *et al.*, 2000). It is believed that the defense reaction of cells might have much more general character, regardless of the kind of stress (Przymusiński *et al.*, 2004).

Germin and germin-like proteins (GLPs) constitute a ubiquitous family of plant proteins that seem to be involved in many developmental and stress-related processes. Increased expression of one of the wheat germin genes, namely *gf-2.8* has been observed in certain plants exposed to the heavy metal ions Cd^{2+} , Cu^{2+} and Co^{2+} (Berna and Bernier, 1999).

Chelation of Metals by Organic Acids and Amino Acids

In many plant species an alternative approach for detoxification of metals involves formation of metal-organic acid or metal-amino acid complexes. A wide range of metals, viz., Cd, Pb, Ni, Mn and Al are either partially or in certain cases completely detoxified by this way. Plants are protected from the toxic effects of Ni in their tissues by its complexation with the anions of hydrocarboxylic acids such as citrate, malate and malonate (Reeves 1992; Salt and Krämer, 2000) and the amino acid histidine (Krämer *et al.*, 1996). Generally, the coordination complexes formed by heavy metals with exclusively oxygen-donor centers do not have especially high stability constants (Pollard *et al.*, 2002) whereas histidine acts as a tridentate ligand, at typical cytoplasmic pH values, with its two nitrogen and one oxygen-donor centers and is therefore more efficient and selective chelator of heavy metal Ni than the hydroxycarboxylic acids (Pollard *et al.*, 2002). Recently, complexation of Ni with another high affinity ligand, nicotinamine, has also been reported (Vacchina *et al.*, 2003). In non-polluted soils ($[Cd] < 0.3 \mu M$), Cd usually gets complexed with vacuolar citrate and when Cd concentration rises PC plays a crucial role (Wagner, 1993). Cd and Zn resistance of *Atriplex halimus* L. is due to precipitation of metals in oxalate crystals (Lutts *et al.*, 2004). In *Oryza sativa* synthesis of oxalate represents a significant response mechanism that enhances tolerance to Pb (Yang *et al.*, 2000).

Organic acid mediated detoxification is an important strategy adopted by plants to reduce Al toxicity. Organic acids chelate with Al and reduce its toxic effects at the cellular level (Ma, 2000). The complexes of Al-citrate (1:1) in hydrangea and Al-oxalate (1:3) in buckwheat have been well characterized (Ma, 2000). Little is known about the way by which Al:organic acid complexes are transported across membranes but it is likely that specific transporters are involved. Chelation of metals with ligands such as histidine, citrate, etc. helps in routing them primarily to the xylem (Krämer *et al.*, 1996; Salt and Krämer, 2000).

Proline

The amino acid proline is the most widespread metabolite that accumulates in many plant species under toxicity due to metals (Shah and Dubey, 1998b). In higher plants accumulating proline appears to be involved in the chelation of excess cytoplasmic metal ions, which show a preference for nitrogen or oxygen coordination (Farago and Mullen, 1979). Cd induced accumulation of proline has been observed in rice and lettuce plants (Shah and Dubey, 1998b; Bertrand and Guary, 2002). Rice plants grown for 15 days in sand cultures containing 160 μM Al^{3+} showed about 1.8 to 2.7 times increased level of proline compared to the plants growing without Al^{3+} (Sharma and Dubey, 2005). In several metal tolerant plant species constitutive proline levels are higher than the sensitive plants (Bertrand and Guary, 2002). There is no conclusive evidence for a direct role of proline in the cellular detoxification of heavy metals and there are different opinions regarding the mechanisms by which proline can alleviate metal toxicity. It has been shown that under heavy metal toxicity proline maintains a favourable water balance in plant tissues by serving as osmoprotectant (Paleg *et al.*, 1984). It also acts as protein stabilizer (Sharma and Dubey, 2004), metal chelator (Farago and Mullen, 1979), inhibitor of lipid peroxidation (Mehat and Gaur, 1999), free radical scavenger (Alia *et al.*, 2001), etc. Due to its zwitterionic and high hydrophilic character, proline appears to provide protection to the enzymes and biomolecules (Siripornadulsil *et al.*, 2002). Under *in vitro* conditions addition of proline in the enzyme assay medium provides considerable protection to the enzymes, the activities of which are inhibited otherwise due to the metals Cd^{2+} , Pb^{2+} , Ni^{2+} and Al^{3+} (Sharma and Dubey, 2005). Proline accumulation in metal stressed plants possibly does not appear to be a direct effect of heavy metal stress but as a consequence of the water stress induced by metals (Bertrand and Guary, 2002).

Antioxidative Defense Mechanism

Build up of toxic concentrations of heavy metals within the plant tissues result, at some stage of stress exposure, in an increased formation of reactive oxygen species (ROS) (Shah *et al.*, 2001; Verma and Dubey, 2003). In general these ROS (O_2^- , OH and H_2O_2) are products of normal cellular metabolism, their production is under tight control due to cellular antioxidative defense system. Presence of ROS causes oxidative damage to biomolecules such as lipids, proteins, nucleic acids etc. (Shah *et al.*, 2001; Blokhina *et al.*, 2003). Induction in the activities of antioxidative enzymes as well as increase in the level of non-enzymic antioxidants are the strategies adopted by plants to scavenge ROS and to reduce oxidative damage caused due to ROS under heavy metal stress (Shah *et al.*, 2001; Fecht-Christoffers *et al.*, 2003; Verma and Dubey, 2003).

Recent reports suggest that though antioxidative defense system is not directly involved in heavy metal detoxification yet ROS play important role as intermediate signaling molecules to regulate the expression of genes for plant's defense system (Orzco-Cárdenas *et al.*, 2001; Vranova *et al.*, 2002).

Heavy Metal Sequestration

In Vacuoles

Various metabolites and ions are stored inside the vacuoles. Vacuolar sequestration of a number of heavy metals like Cd, Ni, As, Zn is known which diverts metal ions from metabolically active compartments (cytosol, chloroplasts, mitochondria) and minimizes the harmful effects of metal ions to vital cellular processes. Transporters are present in internal membranes to allow regulation of stored metals in organelles. Active accumulation of most of the metal ions is driven by the electrochemical potential by electrogenic proton influxes *via* the vacuolar H^+ -ATPase (Kakinuma *et al.*, 1993). Cd is transported across the tonoplast by a $\text{Cd}^{2+}/\text{H}^+$ antiport mechanism (Carrier *et al.*, 2003). Both vacuolar

(Bidwell *et al.*, 2004) and extravaacuolar localization of Ni ion occurs *via* a pH-gradient dependent manner in yeast (Nishimura *et al.*, 1998) whereas in Ni-hyperaccumulator plant *Thalpi goesingense* vacuolar metal transport proteins termed as metal-tolerance proteins (TgMTPs) are involved in compartmentalization of Ni in vacuoles in shoots (Persans *et al.*, 2001). Arsenic is also primarily stored in the vacuoles (Lombi *et al.*, 2002). Transport of Zn to the vacuole is mediated by 'Zn-malate shuttle'; malate being liberated in exchange for oxalate or citrate and is shuttled back to cytoplasm (Ernst *et al.*, 1992) but the storage form of Zn in the vacuoles is not much studied. A gene (*ZAT*) closely related to animal ZnT (Zn transporter) gene has been isolated from *Arabidopsis* (Van der Zaal *et al.*, 1999). Overexpression of *ZAT* gene in transgenic plants led to enhanced accumulation of Zn in the roots under high Zn treatment (Van der Zaal *et al.*, 1999).

In Trichomes and Hydropotes

Apart from vacuolar sequestration, plants possess additional morphological features that are also involved in heavy metal sequestration and detoxification. Several reports have confirmed the involvement of glandular trichomes and epidermal structures (hydropotes) in the chelation, sequestration and detoxification of the metals.

Trichomes are epidermal hairs present at the surface of plant leaves and have diversified roles in exudation of various molecules, protection against wind and sunlight, storage of metals, etc. Retardation in growth and about two-fold increase in the number of trichomes was observed in Cd-exposed tobacco seedlings (Choi *et al.*, 2001). A significant proportion of Ni has been found in trichomes of *Alyssum lesbiacum* plants (Krämer *et al.*, 1997). At the bases of *Arabidopsis halleri* trichomes, elevated concentrations of Zn have been found. In these trichomes Zn remains tetrahedrally coordinated and complexed to carboxyl and/or hydroxyl functional groups of certain secondary organic species (Sarret *et al.*, 2002). Further studies using variable-pressure scanning electron microscopy and energy dispersive X-ray analysis indicated that head cells of tobacco trichomes actively exclude toxic Cd by forming and excreting Cd/Ca-containing crystals (Choi *et al.*, 2001). Specific overexpression of a gene coding for a metallothionein (MT2) has been reported in trichomes (Garcia-Hernandez *et al.*, 1998), which suggests that trichomes constitute important sites for accumulation as well as detoxification of toxic metal ions.

In the semiaquatic and aquatic plants of the families Menyanthaceae and Nymphaeaceae; hydropotes located on the abaxial epidermis of the leaf laminae accumulate Cd (Lavid *et al.*, 2001a). It is suggested that usual polymerization of polyphenols by peroxidases in hydropotes gets enhanced after uptake of heavy metals and thereby detoxification of metals occurs by their binding with polyphenols in these glands (Lavid *et al.*, 2001b; 2001c).

Heavy Metal Detoxification and Signal Transduction Pathway

Though no unified signal transduction pathway exists in higher plants for detoxification of heavy metals, signal transduction cascades have been hypothesized which trigger the detoxification process. Likely involvement of the second messengers- calcium or fatty acid derivative jasmonic acid (JA) has been suggested (Maksymiec and Baszynski, 1999). In addition, activation of distinct mitogen activated protein kinase pathways has been observed in response to heavy metal stress (Jonak *et al.*, 2004). It was observed by Xiang and Oliver (1998) that *Arabidopsis* plants treated with Cd or Cu responded by increasing transcription of the genes of glutathione (precursor of PC) synthesis and that jasmonic acid also activated same set of genes. However, it is not clear whether JA is involved in heavy metal induced expression of genes in *Arabidopsis*. Similarly, Cu²⁺ ions induced a marked increase in the

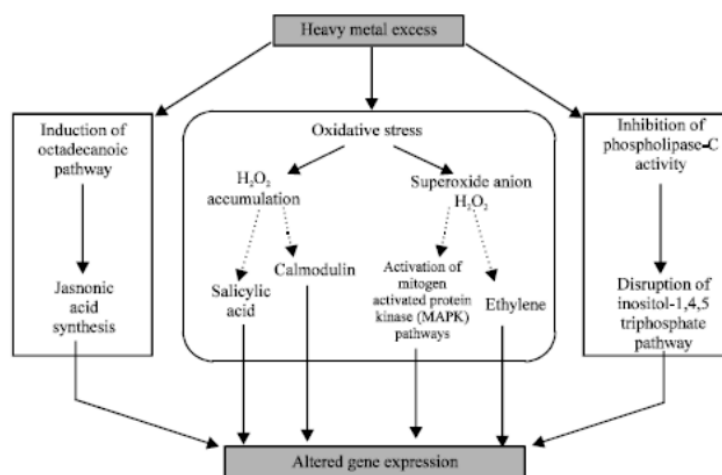


Fig. 3: Potential routes by which heavy metals can influence gene expression.

content of jasmonic acid in rice (Rakwal *et al.*, 1996). Both excess metal ions and JA or its derivative methyl jasmonate exert similar effects in plants (Maksymiec and Krupa, 2002). These observations suggest that possible pathways for the action of heavy metals and jasmonic acid appear to be similar. Maksymiec *et al.* (2002), suggested that heavy metals might induce octadecanoic pathways leading to synthesis of JA which would consequently act as elicitor and would generate metal toxicity symptoms. A generalized view of the potential routes through which heavy metals influence gene expression, has been presented in Fig. 3.

Most of the heavy metals enhance production of H_2O_2 and it is quite possible that accumulating H_2O_2 may somehow switch-on the signal transduction pathway meant for heavy metal detoxification. Role of H_2O_2 as a signaling molecule is increasingly being realized that activates expression of several genes (Orozco-Cárdenas *et al.*, 2001).

Gene expression patterns change when plants encounter excessive amounts of heavy metals. Some of the heavy metal responsive genes code for signal transduction components, such as the *Arabidopsis* mitogen-activated protein kinase kinase kinase (MAPKKK), MEKK1, transcription factors, stress induced proteins etc. (Xiang and Oliver, 1998; Suzuki *et al.*, 2001; Louie *et al.*, 2003). In several plant species, mitogen activated protein kinase (MAPK) pathways are activated in response to heavy metal stress (Xiang and Oliver, 1998; Suzuki *et al.*, 2001; Louie *et al.*, 2003). Jonak *et al.* (2004) observed that exposure of alfalfa (*Medicago sativa*) seedlings to excess Cu or Cd ions activated four distinct MAPKs: SIMK, MMK2, MMK3 and SAMK. There exists possibility that MAPK activation by excess heavy metals is mediated by ROS, since heavy metals are known to disturb redox control of the cell. Several studies in different plant species support this view of MAPK activation by ROS (Nakagami *et al.*, 2004; Rentel *et al.*, 2004). A number of signal transduction components are involved in the detection of H_2O_2 and the activation of defense mechanisms in plants. These include: A two-component histidine kinase, a receptor like protein kinase, the MAPKK, AtANP1 (also the NtNPK1) and the MAPKs, AtMPK316 and Ntp46MAPK (Desikan *et al.*, 2001; Vranova *et al.*, 2002). In addition, calmodulin has been implicated in H_2O_2 signaling (Desikan *et al.*, 2001). So far, the

evidence for an involvement of MAPK pathway in heavy metal stress is scarce in plants and significance of MAPK signaling in response to heavy metal excess has yet to be fully understood.

Ethylene is a natural plant growth regulator involved in the control of a wide range of developmental responses (Kunkel and Brooks, 2002; Milone *et al.*, 2003). Heavy metals induce ethylene biosynthesis (Milone *et al.*, 2003) and it is well known that production of ethylene influences the plant defense response (Kunkel and Brooks, 2002).

Salicylic acid (SA) has been identified as an important signaling element involved in establishing the response of plants after abiotic stress (Alvarez, 2000). The significance of SA in signaling under heavy metal stress is supported by the fact that heavy metals induce an increase in SA content (Metwally *et al.*, 2003). SA activates defense related genes either by H₂O₂ mediated signal transduction pathway or by directly affecting mechanisms of metal detoxification (Chen *et al.*, 1993; Metwally *et al.*, 2003). SA inhibits two major H₂O₂ scavenging enzymes catalase and ascorbate peroxidase which cause cellular H₂O₂ concentration to rise and this accumulating H₂O₂ subsequently acts as second messenger and activates defense related genes (Chen *et al.*, 1993). Inositol-1,4,5,-triphosphate (IP₃) also participates in transducing signals under metal stress conditions. Inhibition of phospholipase-C activity and subsequent disruption of the IP₃ signal transduction pathway has been reported under aluminium excess (Jones and Kochian, 1995).

Conclusions

The increasing release of heavy metals Cd, Pb, As, Hg, Ni into the environment has caused concern for human and plant health. These heavy metals serve as major pollutants of the environment and drastically affect plant growth and its metabolism ultimately leading to severe losses in yield. Metal ions reach within the plant tissues through a number of membrane transporters localized in the root. A number of metal transporters and their gene families have now been identified but still their metal ion specificities need to be established and there are still certain heavy metals for whom no specific membrane transporters are known in higher plants. Once inside the cell these metal ions interact with biomolecules and cellular organelles, various metabolic processes are adversely affected. Plants have developed defense mechanisms to combat heavy metal induced injury. Extracellularly plants immobilize metals near root region either with the help of root exudates or with mycorrhizal association. Among the intracellular detoxification mechanisms adopted by plants the most promising one involves sequestration of metal ions by the formation of complexes between metal cation and the peptides phytochelatins synthesized within the plant tissues. Formation of metal complexes with organic acids or amino acids appears to be an alternate approach to detoxify metals within the plant tissues. Synthesis of stress specific proteins, amino acid proline and increased production of reactive oxygen species are observed under toxicity with metals Cd, Pb, Cu, etc. More than one mechanism may be involved in mitigating the toxicity of particular metal. Much work is needed to fully unveil the mechanisms associated with toxicity of heavy metals in plants as well as the mechanisms associated with plant metal-tolerance. Tolerance of plants to certain metals is associated with increased synthesis of phytochelatins, proline and elevated levels of antioxidant enzymes. Overexpression of animal metallothionein gene in higher plants and cyanobacteria as well as overproduction of phytochelatins in transgenic plants confer heavy metal tolerance in the transformants. On encountering with excessive amounts of heavy metals, gene expression patterns in plants change. In *Arabidopsis* it has been shown that heavy metal responsive genes code for signal transduction components however, in higher plants pathways through which metal stress stimuli are transduced await further investigation.

The concept of phytoremediation of contaminated soils has been increasingly supported by research in recent years. The understanding of heavy metal detoxification processes afforded by genetic and molecular investigations, would allow us to explore the mechanisms by which some species are capable of higher-accumulation of heavy metals and how they may be best used for phytoremediation. The understanding of cellular signaling induced by heavy metal stress will be a vital tool in further improving this technology. With these few examples it appears that once the salient components associated with metal tolerance are well-established, future trials of genetic engineering may provide successful transformants with improved tolerance to heavy metals.

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