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Accumulation and Detoxification of Metals by Plants and Microbes

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1. Introduction

Excessive toxic metal levels in soils pose potential hazards to human and animal health as well as the ecosystem in general. Anthropogenic sources of heavy metal deposition have increased as the result of the Industrial Revolution. Agriculture, mining, smelting, electroplating, and other industrial activities have resulted in the deposition of undesirable concentration of metals, such as As, Cd, Cr, Cu, Ni, Pb and Zn, in the soil.

Although trace metals are important part of the soil ecosystem, the accumulation of these metals may be harmful to people, animals, plants and other organisms contacting the soil or groundwater. Unlike many other pollutants, heavy metals are difficult to be removed from the environment as they cannot be chemically or biologically degraded, and are ultimately indestructible. Now-a-days, various heavy metals constitute a global environmental hazard.

Use of microorganisms and plants for the decontamination of heavy metals has attracted growing attention because of their low cost and high efficiency. Microorganisms could be used to clean up metal contamination by removing metals from contaminated water, sequestering metals from soils and sediments or solubilizing metals to facilitate their extraction.

In this article, we describe how bacteria and plants accumulate and detoxify metal ions, engineering approaches to enhance the metal tolerance, accumulation and detoxification in microorganisms and plants. We also describe bioremediation using symbiosis between plants and microorganisms.

2. Phytoremediation

Phytoremediation is the use of plants to remove pollutants from the environment or to render them harmless. Phytoremediation of toxic metals may be of high significance because of the lack of alternative technologies that are affordable and effective. While organic molecules can be degraded in microbial bioremediation, toxic metals can be remediated only by gathering trace amount of dispersed metals in soil or water and removing them from the environment. It may provide an economically viable solution for the remediation of metal-polluted sites. Thus, several sub-sets of metal phytoremediation have been developed and targeted for commercialization.

a) *Phytoextraction*: in which high-biomass, metal-accumulating plants and appropriate soil amendments are used to transport and concentrate metals from the soil into the above-ground shoots, which are harvested with conventional agricultural methods.
b) *Rhizofiltration*: in which plant roots grown in aerated water, precipitate and concentrate toxic metals from the polluted effluents.
c) *Phytostabilization*: in which plants stabilize the pollutants in soil, thus rendering them harmless.
d) *Phytovolatilization*: in which plants extract volatile metals (e.g., mercury and selenium) from soil and volatilize them from foliage.

Here, we focus only on phytoextraction and phytovolatilization strategies. These strategies might become viable alternatives to mechanical and chemical approaches in remediation of metals from the contaminated soils.

2.1 Phytoextraction of Metals

Phytoextraction is based on the genetic and physiological capacity of specialized plants to accumulate, translocate, and resist high amounts of metal. The idea of using plants to remove metals from soils came from the discovery of different wild plants that accumulate high concentrations of metals in their foliage. Naturally occurring plants called “metal hyperaccumulators” can accumulate 10-500 times higher levels of metal elements than crops (Chaney et al. 1997). The degree of accumulation of metals such as Ni, Zn, and possibly Cu, observed in hyperaccumulators often reaches 1-5% of their dry weight (Raskin et al. 1997). There is a report that *Brassica* (mustard) species or varieties of *Brassica juncea* (Indian mustard) have an enhanced ability to accumulate metals from hydroponics solution into their above ground (harvestable) parts. These plants concentrate toxic heavy metals (Pb, Cu and Ni) to a level up to several percent of their dried shoot biomass (Kumar et al. 1995).

2.1.1 Uptake and Accumulation of Toxic Heavy Metals by Plants

There are many processes that influence metal accumulation in plants e.g. metal mobilization and uptake from soils, compartmentation and sequestration within the root, efficiency of xylem to load and transport metal, distribution of metal in the aerial parts, sequestration, and storage in leaf cells (Clemens et al. 2002).

Uptake and bioavailability of heavy metals. Phytoextraction occurs when heavy metals are ready to be absorbed by roots (bioavailability). Bioavailability depends on metal solubility in soil solution. Some metals, such as Zn and Cd, occur primarily in exchangeable, and readily bioavailable form. Others, such as Pb, occur as soil precipitate, a significantly less bioavailable form. Plants roots increase metal bioavailability by extruding protons to acidify the soil and mobilize the metals. This mechanism has been observed for Fe mobilization in some Fe-deficient dicotyledonous plants (Crowley et al. 1991). Moreover lowering the soil pH affects both metal bioavailability and metal uptake into roots. In *T. caerulescens*, uptake of Mn and Cd was dependent on the soil acidity (Brown et al. 1995).

The formation of metal-chelate complexes prevents precipitation and sorption of the metals thereby maintaining their availability for the plant uptake (Salt and Rauser 1995). Addition of synthetic chelates such as EDTA is very effective in facilitating the plant uptake of Cd, Cu, Ni and Zn (Raskin et al. 1997).

Transport of heavy metals. Plants have evolved highly specific mechanisms to take up, translocate, and store macro-nutrients (N, P, K, S, Ca, and Mg) and essential micro-nutrients (Fe, Zn, Mn, Ni, Cu, and Mo). Molecular physiology of the plant transport systems for elemental nutrients and pollutant is still in its infancy. Plant genes encoding metal transporters have been identified and characterized. The IRT1 (iron-regulated transporter) is the first member of the ZIP gene family to be identified. The IRT1 is an Fe(II) transporter that takes up iron from the soil. The *IRT1* was cloned for functional expression in a yeast mutant (*fet3 fet4*) defective for iron uptake (Eide et al. 1996). IRT1 is able to complement the metal uptake defects of the *Saccharomyces cerevisiae zrt1 zrt2* zinc uptake mutants and the *S. cerevisiae smf1* manganese uptake mutant (Korshunova et al. 1999). Although IRT1 was originally identified as the Fe transporter, the studies of complementation and uptake in yeast provided information that IRT1 was able to transport both Mn and Zn in addition to Fe. There are several evidences that point to a role for IRT1 in mediating the accumulation of Cd in iron deficient plants: (1) Cd was shown to compete with Fe uptake in yeast expressing IRT1 (Eide et al. 1996), (2) yeast-expressing IRT1 was more sensitive to Cd (Rogers et al. 2000) than wild type, and (3) plants engineered to over express IRT1 accumulated Cd in greater amounts than wild-type plant (Guerinot 2000). Another member of ZIP protein is zinc transporter (ZIP), which contains *ZIP1*, *ZIP2*, and *ZIP3* genes of *Arabidopsis*. Expression of these genes restored zinc-limited growth of *zrt1 zrt2* yeast mutant

(Grotz et al. 1998). In the plant, *ZIP1* and *ZIP3* are expressed in roots in response to zinc deficiency, thus these genes play a direct role in zinc uptake from the soil. The Zn(II) transport activity of these three proteins is inhibited by Mn(II), Co(II), Cd(II), and Cu(II), indicating that ZIP proteins may transport potentially toxic metals as well as nutrients. From cross-species, microarray transcript profiling reveals high constitutive expression of metal homeostasis gene, such as ZIP6 in shoots and ZIP9 in roots of the zinc hyper accumulator *Arabidopsis halleri* (Weber et al. 2004; Becher et al. 2004). These transporter genes offer a good starting point for the understanding how metals cross membranes.

Translocation of an element from roots to shoots. Accumulator plant must have the ability to translocate an element from roots to shoots at high rate. The transport processes are stimulated by metal influx into root and leaf cells, and metal loading into the xylem. Many other factors are also involved in the metalic elements.

Transporter proteins. Because of their charge, metal ions cannot move freely across the cellular membrane, which are lipophilic structures. Therefore, membrane proteins must mediate ion transport into cells with transport functions known as transporters. Transporter proteins play an important role in the translocation of an elements, since they contain the binding domains, which bind to specific ions and transfer bound ions from extracellular space through the hydrophobic environment of the membrane into the cell. There is an evidence for higher Zn^{2+} uptake capacity in hyper accumulator, *Thlaspi caerulescens* as compared to the non-hyper accumulating relative *T. arvense* (Lasat et al. 1996). This might be attributable to higher expression levels of Zn^{2+} transporters such as the ZIP member ZNT1 (Pence et al. 2000).

Chelators. Cations of heavy metals are often bound to soil particles because of soil cation exchange capacity. The binding affinity of cations reduces cation movement in vascular plants, particularly in the negatively charged cells of the xylem. A solution to this problem is chelation, which means as the process of a cation binding to a compound, resulting in a uncharged complex that can move more freely through a variety of substrates. Several chelators, both natural and synthetic are known to perform this function in soil and plants.

Natural	Synthetic
Phytochelatin (PC)	EDTA (ethylene diamine tetra acetic acid)
Metallothionein (MT)	EGTA (ethylene glycol tetra acetic acid)
Organic acids	

The use of specific chemicals, synthetic chelates, has been shown to dramatically stimulate Pb accumulation in plants. These compounds prevent Pb

precipitation and keep the metal as soluble chelate-Pb complexes available for uptake into roots and transport within plants. For example, addition of EDTA at a rate of 10 mmol/kg soil, increased Pb accumulation in shoots of maize up to 1.6 wt% of dry biomass (Blaylock et al. 1997). In a subsequent study, Indian mustard exposed to Pb and EDTA in hydroponics solution was able to accumulate more than 1% Pb in dry shoots (Vassil et al. 1998).

Chelation with certain ligands, for example histidine and citrate, appears to route metals primarily to the xylem. Histidine is very important for Ni tolerance and transport in hyper accumulators, since large increases in histidine levels and coordination of Ni with histidine have been reported in the xylem sap of Ni hyper accumulator, *Alyssum lesbiacum* (Kramer et al. 1996). Organic acid, citrate had been also shown to complex with some toxic metals during transport of metals to the shoot of hyper accumulating and non-hyper accumulating plant species (Senden et al. 1992).

Phytochelatins (PCs) are known to play an essential role in the heavy-metal detoxification by chelating heavy metals in the cytosol and sequestering PC-Cd^{2+} complexes in the vacuoles via transport across the tonoplast (Ortiz et al. 1995; Salt and Rauser 1995). In addition, there is an evidence to demonstrate that PCs provide a major mechanism for regulating long distance Cd^{2+} transport in *Arabidopsis*. Transgenic expression of wheat phytochelatin synthase (TaPCS1) cDNA in the *Arabidopsis* PC-deficient mutant, *cad1-3*, revealed the suppression of the heavy metal sensitivity of *cad1-3*. PCs can be transported from roots to shoots and transgenic expression of the *TaPCS1* gene increases long-distance root-to-shoot Cd^{2+} transport and reduces Cd^{2+} accumulation in roots (Gong et al. 2003).

2.1.2 Detoxification of Metal Ions by Plants

Chelation. Chelation of metals in the cytosol by high affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance. Two major classes of heavy metal chelating peptides are presented in plants, metallothioneins (MTs) and phytochelatins (PCs).

Metallothioneins make up a super family of cysteine-rich metal-chelating proteins. The chelation of divalent or monovalent cations is mediated through the cysteine residues, which are often highly conserved between species. The biological role of MT is focused on the sequestration of toxic heavy metal ions, such as Cd^{2+}, in order to prevent them from interacting with other cellular components, and on the homeostatic regulation of essential metal ions, such as Zn^{2+}.

MTs are widely distributed among living organisms, and they are fairly well conserved in mammals, plants, and fungi (Butt and Ecker 1987; Huckle et al. 1993). Based on structure, MTs can be subdivided into three classes. Class I includes those polypeptides related to mammalian species (Kagi 1991). These proteins are encoded in structural genes and synthesized by transcription and

translation. Mammalian MTs are usually composed of 61 amino acids (molecular mass, 6 to 7 kDa) and lack aromatic amino acids or histidines. Two distinct domains of these proteins coordinate 7 divalent or 12 monovalent metal ions with 20 Cys residues. These metal ions present along the sequence in the form of Cys-X-Cys or Cys-Cys motifs (X is another amino acid residue). Class II MTs originate from yeasts (e.g., *Saccharomyces cerevisiae*, *Candida glabrata*, and *Candida albicans* (Mehra and Winge 1991)), or cyanobacteria [e.g., *Synechococcus sp.* (Olafson et al. 1988)]. A well-known member of class II is the *S. cerevisiae* MT responsible for copper tolerance, called CUP1. This protein contains 12 cysteine residues organized in Cys-X-Cys, Cys-Cys, and Cys-X-X-Cys motifs, which originate eight binding sites for monovalent and four binding sites for divalent metal ions (Weige et al. 1985). In animal and plant species, MTs synthesis is induced by the metal ions, such as Cd, Zn, Hg, Ag and Pb (Kagi 1991). In plant-species, metal-induced expression of MT genes has also been reported in both maize and rice (Chevalier et al. 1995; Hsieh et al. 1995). RNA expression of MT genes in *Arabidopsis* could be induced by copper, and to a lesser degree by Zn and Cd (Zhou and Goldsbrough 1994). Thus, plant MTs may function as metal-binding proteins that can mediate metal tolerance. However, direct evidence that MTs are required for a specific function in metal metabolism, tolerance or another process is currently lacking.

These MTs bind Cd, Zn, Hg, Cu, and Ag. Toyama et al. (2002) demonstrated that As^{3+} bound to MT-2 by ICP-AES and MALDI-TOF-MS. The maximum molar ratio of As^{3+} to human MT-2 is more than 6:1. Hong et al. (2001) developed high yield expression and single step purification of human thionein and metallothionein. hMT was expressed in *E. coli* as an intein (protein splicing element) fusion protein in the absence of added metals and purified by intein-mediated purification with an affinity chitin-binding tag. This procedure constitutes a novel and simple strategy to prepare thionein (T), the metal-free form, or MT when reconstituting T with metals *in vitro*. The yield was 8 mg of T or 6 mg of pure Cd7- or Zn7-MT from 1-liter culture.

Class III metallothioneins are known as phytochelatins (PCs). Phytochelatins are the naturally occurring metal-binding peptides. They are short peptides composed of only three amino acids, namely, Glu, Cys and Gly, with Glu and Cys residues linked through a γ-carboxymide bond. The structure of such peptides can be represented by $(\gamma\text{-Glu-Cys})_n$-Gly, where n ranges from 2 to 11. PCs have been identified in a wide variety of plant species and in some microorganisms (Cobbett 2000). They are structurally related to glutathione [GSH; (γ-Glu-Cys)-Gly] and presumed to be the products of biosynthetic pathway. Numerous physiological, biochemical, and genetic studies have confirmed that GSH is the substrate for PCs biosynthesis. The PCs pathway is involved in the synthesis of γ-Glu-Cys from Glu and Cys by γ-glutamylcysteine synthetase (GCS), then glutathione synthetase (GS) catalyzes the synthesis of GSH. PCs synthesis was presumed to be involved in the transpeptidation of the

γ-Glu-Cys moiety of GSH onto initially a second GSH molecule to form PC_2 or onto a PC molecule to produce a PC (n+1) oligomer. This γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15) has been termed PC synthase (PCS). *In vitro*, the activity of the partially purified enzyme was active only in the presence of metal ions. The best activator was Cd followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cation. The PC biosynthesis continued until the activated metal ions were chelated either by the formed PCs or by the addition of a metal chelator such as EDTA (Loeffler et al. 1989).

Vacuolar compartmentalization. Vacuolar compartmentalization appears to be the reason for hypertolerance of natural hyper accumulator plant. The vacuole is generally considered to be the main storage site for metals in yeast and plant cells. The role of Cd detoxification and tolerance is played by the vacuolar compartmentalization, which prevents the free circulation of Cd ions in the cytosol and forces them into a limited area. Cd stimulates synthesis of PCs, which rapidly form a low molecular weight Cd-PC. The Cd-PC complex will be transported into the vacuole by a Cd/H antiport and an ATP-dependent PC-transporter (Salt and Wagner 1993; Salt and Rauser 1995). A gene, which codes for a PC-transporter in yeast was isolated namely *Hmt1* gene. The *Hmt1* gene encodes a member of a family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane (Ortiz et al. 1992, 1995). The gene product is responsible for transporting Cd-PC complex into the vacuole. Inside the vacuole the Cd-PC complexes acquire acid-labile sulphur (S^{2-}) and form a high molecular weight Cd-PC-sulfide complex, that may be essential for Cd resistance in the yeast (Speiser et al. 1992).

Compartmentalization of metals in the vacuole is a part of the tolerance mechanism of some metal hyper accumulators. The Ni hyper accumulator *T. goesingense* enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole (Kramer et al. 2000). High-level of metal ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles (Persans et al. 2001). Intact vacuoles isolated from tobacco and barley exposed to Zn have been shown to accumulate this metal (Krotz et al. 1989; Burken and Schnoor 1996).

The strategies for uptake, accumulation and detoxification of heavy metals by higher plants are summarized in Figure 1.

2.1.3 Ideal Plant for Phytoremediation

Populations of metal-tolerant hyperaccumulating plants can be found in naturally occurring metal-rich sites (Baker and Brooks 1989). However, these plants are not ideal for phytoremediation since they are usually small and have a low biomass production. In contrast, plants with good growth usually show low metal accumulation capability as well as low tolerance to heavy metals.

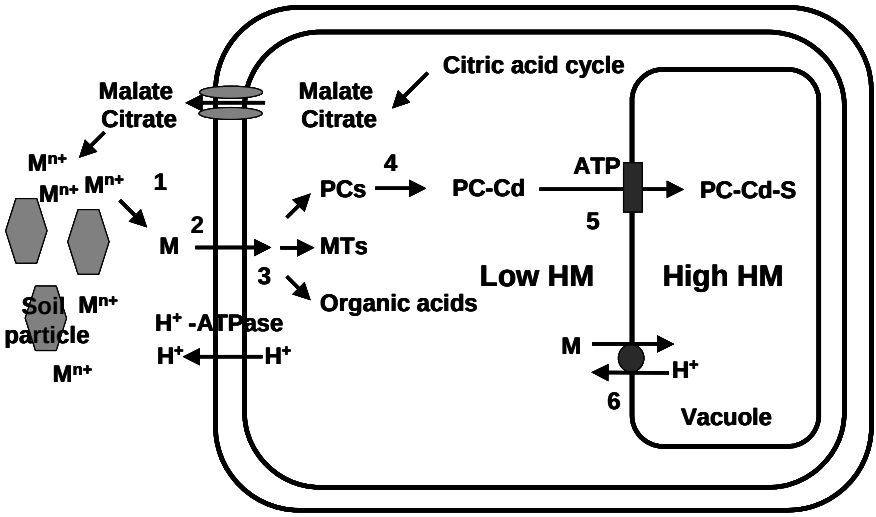

Fig. 1. Summary of potential cellular mechanisms for metal uptake, accumulation and detoxification in higher plants. 1. Metal ions are mobilized by secretion of chelators and by acidification of the rhizosphere. 2. Uptake of hydrated metal ions or metal-chelate complexes is mediated by various uptake systems residing in the plasma membrane. 3. Metals are chelated in cytosol by various ligands. 4. PCs form complex with Cd. 5. PC-Cd complex is transported into the vacuole. 6. Metals are transported and accumulated in the vacuole (Modified after Hall 2002; Clemens et al. 2002)

A plant suitable for phytoremediation should be fast growing, develop a large biomass, be tolerant to and accumulate high concentrations of toxic metals in the shoot, and be easily cultivated and harvested (Karenlampi et al. 2000). There are fast-growing hyper accumulators that can produce a large biomass. Examples are the Ni hyperaccumulators *Alyssum bertolonii* and *Berkheya coddii*, which produced 9 and 22 t/ha of shoot dry matter, respectively, in small-scale field experiments (Robinson et al. 1997a, b). The arsenic hyperaccumulator, *Pteris vittata* can also produce a relatively large biomass under favorable climate (Ma et al. 2001). However, fast-growing species that can hyper accumulate Zn, Cd, Cu, Pb and Cr have been not yet reported. Approaches to find metal-tolerant hyperaccumulating plants for phytoremediation involve searching for, and studying natural hyperaccumulators, or developing genetically engineered plants that possess above traits to achieve some of the properties of hyper accumulators. Although most of the cultivated plants are not hyper accumulator for metals, some of them are good candidate of breeding to accumulate toxic metals since their transformation systems have been developed and cultivation conditions in the fields are well known. A winter-growing legume Chinese milk vetch (*Astragalus sinicus*) is widely used as a green manure in rice fields in China

and Japan (Murooka et al. 1993). This plant is suitable for use in bioremediation in the rice paddy.

2.1.4. Genetic Engineering in the Improvement of Plants for Phytoremediation

Several criteria must be considered for engineering plant for phytoremediation. First, the plant must be able to solubilize and uptake heavy metals that are tightly bound to soil particles. Second, a mechanism must exist to transfer the heavy metal from the root to the shoot. Third, the heavy metal must be deposited in a compartment where it does not interfere with cellular metabolism.

Genetic engineered plants for metal uptake and translocation. In phytoremediation, heavy metal uptake and translocation are essential components of heavy metal hyperaccumulation. Citric and Malic acid are two compounds, which have been shown to complex heavy metals in the plant roots. After loss of one H^+, each acid contains a COO^- group which binds to the cation positive charge. Plants secrete acids, which aid in the uptake of non-bioavailable metals. These acids protect cellular function when the acid-Cd complex is brought into the root. Citric acid-metal complexes have been reported to be translocated via the xylem (Senden et al. 1992). If a plant could be genetically altered to produce higher levels of endogenous citric or malic acid, then perhaps phytoextraction could be enhanced.

Free histidine has been found as a metal chelator in xylem exudates in plants that accumulate Ni and the amount of free histidine increases with Ni exposure (Kramer et al. 1996). By modifying histidine metabolism, it might be possible to increase the Ni- accumulating capacity of plants.

The expression of the metal transporter genes, such as the *IRT1* (iron-regulated-transporter) gene, and the wheat Ca^{2+} transporter LCT1 gene mediate the uptake of Na^+ and Cd^{2+} in yeast (Schachtman et al. 1997; Clemen et al. 1998). Therefore, the introduction of such genes to plants may enhance the metal ions uptake by the plant roots.

Transporter proteins, isolated from hyperaccumulating species, such as Zn transporter protein (ZNT1) can only uptake Zn, but not the toxic ions (i.e., Cd). Molecular study for alteration of gene for transport of other metals may be useful for phytoextraction. Moreover, several Zn transporters like ZIP1, ZIP3 (Grotz et al. 1998) and IRT1 are expressed in response to metal deficiency. Changing the regulation of the expression of these transporters may modify the uptake of metals to the cells or organelles. By substituting various conserved residues in ZIP family transporters with alanine produces mutant versions of IRT1 that apparently no longer transport Fe^{2+} and Mn^{2+} but retain Zn^{2+} and Cd^{2+} transport activity (Roger et al. 2000). Expression of these genes might enhance metal accumulation in transgenic plants.

Genetic engineered plants with altered metal tolerance and accumulation. Increased resistance to metal is another important trait that can improve the efficiency of phytoextraction. As mentioned above that hyper tolerance is essential for the hyper accumulation phenotype to occur in natural hyper accumulators. Hyper tolerance is achieved by internal detoxification and probably involves compartmentation and complexation. With the aim of creating plants that can tolerate and accumulate high levels of toxic metals, various *MT* genes (mouse *MTI*, human *MTI* (alpha domain), human *MTII*, yeast *CUP1*, pea *PsMTA*) were introduced into plants, such as *Nicotiana sp*., *Brassica sp*. or *A. thalina*. Transgenic plants, that express MTs, have been scored for enhanced Cd tolerance, but metal uptake was not markedly altered (Maiti et al. 1988 1989; Misra and Gedamu 1989; Evans et al. 1992; Pan et al. 1994a, b; Hasegawa et al. 1997).

Modification or over-expression of the enzymes that are involved in the synthesis of glutathione and PCs might be a good approach to enhance heavy metal tolerance and accumulation in plants. Over-expression of γ-glutamylcysteine synthetase enhanced Cd^{2+} tolerance and accumulation in Indian mustard (Zhu et al. 1999).

Co-expressed with both *arsC* gene, which encodes arsenate reductase (ArsC) and γ-glutamylcysteine synthetase gene, *Escherichia coli* showed substantially greater tolerance to arsenic than wild type. The transformant accumulated two-to threefold higher concentrations of arsenic in the shoots (Dhankher et al. 2002).

Over-expression of vacuolar transporters and channels involved in metal tolerance from *Saccharomyces cerevisiae* named YCF1 in *A thaliana* significantly increased tolerance towards high concentration of Pb and Cd and led to a more than two fold higher accumulation of these metals in shoots of transgenic plants when compared to control plant (Song et al. 2003). In addition, over expressing of protein that localized to vacuole membrane of poplar named metal-tolerance proteins (MTPs) (cation diffusion facilitator (CDF) family) in *Arabidopsis* confers Zn tolerance (Blaudez et al. 2003). Expression of *Arabidopsis* vacuolar low-affinity Ca^{2+}/H^{+} antiporter, CAX2, in Tobacco (*Nicotiana tabacum*) altered the Ca^{2+}, Cd^{2+} and Mn^{2+} content of plants and made transgenic plants more tolerant to Mn^{2+} stress (Hirschi et al. 2000). Thus, introduction of the vacuolar metal transporters into plants may have an important impact on improving phytoremediation.

Introduction of metal binding peptides or proteins involved in intracellular metal sequestration of proteins (MTs, PCs) may increase metal tolerance in plants by prevention of cellular proteins from metal ions. Enhanced accumulation may be achieved by over-expression of plasma membrane transporters under the control of non-metal-responsive promoters. In addition, expression of modified transporters, which altered the metals uptake to the cells or organelles, might enhance metal uptake by plants. Moreover, expression of transporter protein in roots and/ or shoots with an efficient chelator may increase metal ions translocation from roots to shoots.

2.2 Phytovolatilization

Phytovolatilization is the transformation of toxic elements into relatively harmless forms. Many elements (e.g. arsenic, mercury, selenium) can exist in a variety of states, including different cationic and oxyanionic species and thio- and organo-metallics. These forms vary widely in their transport and accumulation in plants and in their toxicity to humans and other life forms.

2.2.1 Mercury Phytoremediation

Mercury pollution is a worldwide problem in aquatic environments, resulting from its industrial use in bleaching operations as a catalyst, as a pigment in paints, and in the mining of gold. The Hg(0), becomes problematic, since biological systems can reoxidize it to Hg(II). Microbes present in the sediment capable of converting Hg(II) to methylmercury ($CH3Hg^+$) tend to accumulate in vertebrates and fish. Mercury-resistant bacteria eliminate organomercurals by producing an enzyme, organomercurial lyase (MerB), which catalyzes the protonolysis of the carbon-mercury bond (Begley et al. 1986). The products of this reactions are a less toxic inorganic species, Hg(II), and a reduced carbon compound.

$$R\text{-}CH_2\text{-}Hg^+ + H^+ \xrightarrow{\text{MerB}} R\text{-}CH_3 + Hg(II)$$

These bacteria also synthesize a second enzyme, mercuric ion reductase (MerA), that catalyzes the reduction of the inorganic product, Hg(II), to a volatile and much less reactive elemental form, Hg(0) (Fox and Walsh 1982).

$$RSHg^+ + NADPH \xrightarrow{\text{MerA}} Hg(0) + RSH + NADP^+$$

Hg phytoremediation has been already developed. Yellow poplar expressing a modified *merA*, released ten times more elementary Hg than untransformed plantlets (Rugh et al. 1998).

Transgenic plants expressing MerB were significantly more tolerant to methylmercury and other organomercurials compared with untransformed plants. The MerB plants effectively converted the highly toxic methylmercury to $Hg^{2+,}$ which is about 100 times less toxic in plants (Bizily et al. 1999).

The MerA MerB double-transgenics showed the highest tolerance to organic mercury (up to 10 μM) compared to MerB transgenic (5 μM) and MerA and wild type plants (0.25 μM). The MerA MerB double transgenic plants were 50-fold more tolerant to organic mercury compared to wild type and were shown to volatilize elemental mercury when supplied with organic, whereas the single transgenics and the wild type plant did not. Thus, the MerA MerB double transgenic plants converted organic mercury to elemental mercury, which was released from the plant through volatilization (Bizily et al. 2000).

So far, this system has not been tested in the field conditions. This is, however, the first clear indication that genetic engineering may improve the plant's capacity to phytoremediate metal-polluted soils.

Phytoremediation is recognized as a fast-growing and cost-effective technology to remediate hazardous toxic metals from the contaminated sites. Summary of the processes of phytoaccumulation and phytovolatilization are shown in Figure 2. Accumulation of metal ions is dependent on uptake and bioavailability of heavy metals, transport and translocation of heavy metals from roots to shoot. Detoxification of heavy metals involved chelation, compartmentalization and volatilization. Novel proteins involved transport and translocation of metal ions have been identified and characterized from a variety of organisms. However, a clear role of these proteins yet remains to be elucidated.

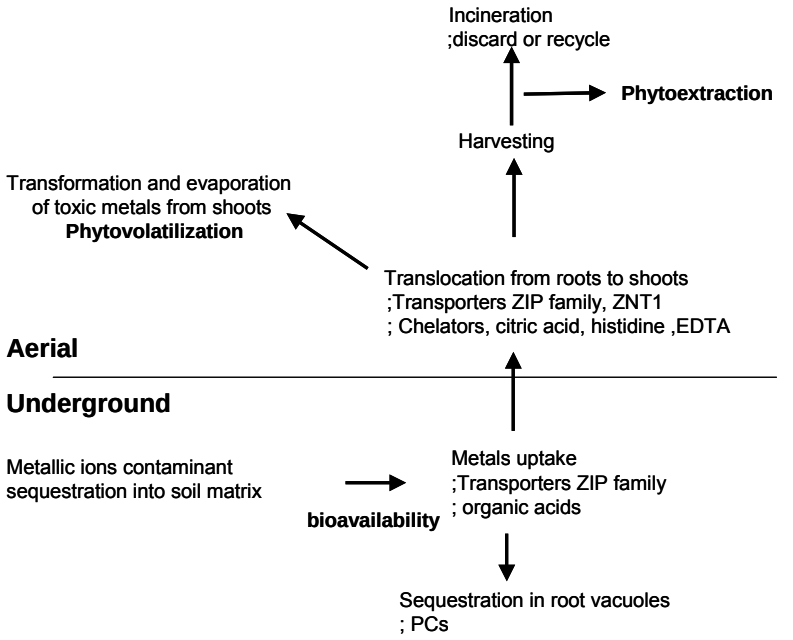

Fig. 2. Scheme of metallic ions decontamination in natural phytoremediation processes (modified after Singh et al. 2003)

3. Microbial Remediation of Metal-polluted Soils

3.1 Expression of Metal-binding Proteins or Peptides in Bacteria

Due to the difficulty in the removal of heavy metals from environment, many researchers attempt to get rid of heavy metals by microbial remediation. A

promising way of improving bioremediation processes is to genetically engineer bacterial strains to confer increased abilities to accumulate toxic heavy metals. Attempts to enhance the metal content of bacterial cells have been made by over expressing metal-binding peptides or proteins.

3.1.1 Expression of Heterologous Metallothioneins (MTs)

With the aim of enhancing the tolerance, sequestration or accumulation of heavy metals, bacteria with the high metal-binding capacity of MTs have been widely exploited. MTs from various sources have been expressed intracellularly in *Escherichia coli* monkey (Murooka and Nagaoka 1987), yeast (Berka et al. 1988; Sayers et al. 1993), human (Yamashita et al. 1994; Odawara et al. 1995), and plant (Kille et al. 1990). In many instances, however, problems of the stability and short half-life of the expressed heterologous proteins were encountered. This problem was linked to the high cysteine content of MTs, which might interfere with cellular redox pathways in the cytosol (Raina and Missiakas 1997). The small molecule of MT can be stabilized by fusion to large molecules, such as β–galactosidase. The human MT (hMT) fused to β–galactosidase enhanced uptake of Cd by the recombinant *E. coli.* In the same manner, the increased molecule size of hMT resulted in improved stability and productivity in *E. coli* (Hong et al. 2001). hMT was synthesized with prokaryotic codons and linked by a gly-gly-gly tripeptide linker to form a tetrameric hMT. The tetrameric MTL4 bound 28 gram atom of Cd or Zn (Hong et al. 2000, Murooka et al. 2001). The problem of stability and short half life of intracellular heterologus expression of MTs has been circumvented by the surface display of proteins. The metal-binding proteins have been anchored to the LamB, protein that spans the outer membrane. Yeast and mammalian MTs expressed on the surface of *E. coli* as fusions to LamB, enhanced the metal binding capacity of the cells between 10 - 20-fold (Sousa et al. 1998). Fusion of metallothionein to the autotransporter β-domain of the IgA protease of *Neisseria gonorrhoeae*, which targeted the hybrid protein towards the bacterial outer membrane, was performed on a natural inhabitant of soil bacterium, *Ralstonia eutropha*. The resulting bacterial strain was found to have an enhanced ability for immobilizing Cd^{2+} from external media (Valls et al. 2000).

Expression of both metal transporter proteins and metal binding peptides may enhance strain's ability to accumulate metal ions. There is a report that expression of both Hg^{2+} transport systems (MerT and MerP) and glutathione S-transferase fusion protein of *Saccharomyces cerevisiae* or pea MT in *E. coli* significantly increased the bioaccumulation of Hg^{2+} (Chen and Wilson 1997).

3.1.2 Expression of Phytochelatins and Synthetic Phytochelatins

Phytochelatins are short peptides composed of only three amino acids, namely, Glu, Cys and Gly, with Glu and Cys residues linked through a γ-carboxylamide

bond. The structure of such peptides can be represented by $(\gamma\text{-Glu-Cys})_n$-Gly, where n range from 2 to 11. PCs are enzymatically synthesized from glutathione by PC synthase (EC 2.3.2.15) (Cobbett 2000).

Over-expression of PC synthase in bacterial strains appears to be a promising way to improve the heavy metal (such as Cd) or metalloid (such as As) content of organisms for use in bioremediation. There are reports of increasing in Cd accumulation in *Mesorhizobium huakuii* subsp. *rengei* B3 and *E. coli* cells expressing the *Arabidopsis thalina* gene encoding PC synthase (Sriprang et al. 2003; Sauge-Merle et al. 2003). Recently, the synthetic peptide $(\text{Glu-Cys})_n$-Gly, in which Glu and Cys are linked by an α-carboxyamide bond was successfully expressed onto the cell surface using Lpp-OmpA fusion system in *E. coli*, resulting in 15- or 20-fold increases in Cadmium and mercury accumulation (Bae et al. 2000; Bae et al. 2001). However, *E. coli* strains are not suitable for *in situ* soil remediation, since they are not adapted to these environments. A more realistic approach is to engineer soil bacteria that can survive in contaminated environments for an extended period. The surface expression of synthetic PC with 20 cysteines (EC20) using the truncated ice nucleation protein (INPNC) anchor in the robust bacterium, *Moraxella* sp. increases a 10-fold mercury (Hg^{2+}) accumulation. The expression of surface protein is more efficient in *Moraxella* sp. than *E. coli* (Bae et al. 2002).

3.1.3 Expression of Synthetic Metal-binding Peptides

Novel metal binding peptides might offer a higher affinity, higher metal-binding capacity and/or specificity and selectivity for a target metal ion than known metal-binding proteins. Peptides with unique binding properties can either be designed *de novo* or selected by screening peptide libraries. Various peptides comprising different sequences of cysteines or histidines have been tested for binding Cd. Recently, metal-binding peptides that contain either histidines $(\text{GHHPHG})_2$ or cysteines (GCGCPCGCG) were engineered to LamB and expressed on the surface of *E. coli*. Surface display of these peptides increased the bioaccumulation of Cd by 4-fold and 2-fold, respectively. Moreover, a His_6 peptide has been expressed on the surface of *E. coli* as a fusion to the OMP LamB. This construct resulted in a 5-fold increase in Cd accumulation, when the peptide was expressed as a single copy and 11-fold increase when expressed in tandem (Sousa et al. 1996; Mejare and Bulow 2001).

3.2 Metal and Metalloid Remediation as the Result of Changes in Redox State

Microorganisms can detoxify metals by valence transformation, extracellular chemical precipitation, or volatilization.

Microbial reduction of the highly soluble oxidized form of selenium, Se^{6+}, to insoluble elemental selenium, Se^0, by microorganisms that conserve energy to

support growth from Se^{6+} reduction is a natural mechanism for the removal of selenium from contaminated surface and groundwater. The *Bacillus sp*. SF-1 has been isolated as a selenate-reducing bacterium that can tolerate and efficiently reduce very high concentration of selenate (Se^{6+}) (up to about 150 mg-Se/L) into selenite and, subsequently, into elemental Se (Kashiwa et al. 2001).

Enzymatic reduction of Cr(VI) to less mobile and less toxic Cr(III) has been one of the most widely studied forms of metal bioremediation (Lovley 1995; Wang and Shen 1995). The NAD(P)H-dependent chromium reductase, which has ability to reduce Cr(VI), was found in some bacteria such as *Pseudomonas ambigua* (Suzuki et al. 1992), *P. putida* (Ishibashi et al. 1990), *Enterobacter cloacae* (Wang et al. 1989) and *Pseudomonad* (CRB5) (McLean and Beveridge 2001). The Cr (VI) reduction occurs under aerobic and/or anaerobic conditions.

In bioremediation of heavy metals, microorganisms have been mostly used to treat industrial waste streams, with the organisms either immobilized onto different support matrixes or in a free-living state, enclosed in treatment tanks or other kinds of reactor vessels. Subsequently, the metal-loaded biomass can be either disposed appropriately or treated to recover the metals.

4. Heavy Metal Bioremediation using “Symbiotic Engineering”

Rhizobia grow slowly for long periods in soil, but if they infect a compatible legume they can grow rapidly; successful infection by a single bacterium can lead to the formation of a nitrogen-fixing nodule on the root of legume, containing over 10^8 bacterial progeny (Downie 1997). This special character is useful for biotechnological application for the expression of genes, such as metallothionein that sequester heavy metals from contaminated soil. Once symbiosis is established, the heavy metals will be accumulated in nodules. This would be an alternative and less expensive method to remove heavy metals from the soil.

Mesorhizobium huakuii subsp. *rengei* strain B3 (Murooka et al. 1993; Nuswantara et al. 1999) is a bacterium that establishes symbiosis with *Astragalus sinicus* (Chinese milk vetch, or renge-soh in Japanese), a legume used as a green manure in rice field in Japan and Southern China, by eliciting the formation of nitrogen-fixing root nodules (Chen et al. 1991). Symbiosis between leguminous plants and rhizobia is initiated when flavonoids and related plant compounds induce the bacteria to produce molecular signals, which stimulate nodule organogenesis (Fisher and Long 1992). Bacteria enter the developing nodule via infection threads and are taken up by plant host cells in an endocytosis-like process. The rhizobia undergo differentiation into a distinct cell type called as bacteroid, which is capable of fixing atmospheric nitrogen into ammonia to be available to the host plants (Mylona et al. 1995).

Likewise, *A. sinicus* is widely used as a natural fertilizer in rice fields during the idle period. It would be more interesting, if one can use this legume plant to increase fertility and at the same time remove heavy metals from the soil. Sriprang et al. (2002) developed a novel plant-bacterial remediation system for heavy metals by the introduction of the chimeric *MTL4* gene to *M. huakuii* subsp. *rengei* B3. This is also the first report that a foreign gene was expressed in bacteroids in the nodules. Murooka proposed this new technology to be called as "Symbiotic Engineering".

4.1 Heavy Metal Bioremediation with Oligomeric MTs

Sriplang et al. (2002) developed a plant-bacterial remediation system for heavy metals by the expression of tetrameric hMT (MTL4) in *M. huakuii* subsp. *rengei* B3. The *MTL4* gene (Hong et al. 2000) was fused to the *nifH* and *nolB* promoters, which generated nodule- specific expression of the *MTL4* gene. The expression analysis of the *MTL4* gene was demonstrated in the free-living cells in the presence of Cd^{2+} and Cu^{2+} under the low oxygen condition. The *MTL4* under the *nifH* and *nolB* promoters was expressed and increased the accumulation of Cd^{2+}, but not Cu^{2+} in free-living cells. The expression of the integrated *nifH-MTL4* gene in the chromosome of strain B3 was also expressed stably and accumulated Cd^{2+}in the bacterial cells. By inoculation of the recombinant B3, *A. sinicus* established symbiosis with the recombinant B3 that was grown in Cd^{2+} and Cu^{2+}-polluted soils. The symbionts with recombinant plasmids pNHMT4 and pNBMT4 increased Cd^{2+} accumulation in nodules 2.3 and 6.6- fold, respectively, whereas no significantly increase in Cu^{2+} accumulation was noted. Accumulation of Cd^{2+} in nodules was at the same level in different external Cd concentrations in soils. This might be due to the limitation of the production of the MTL4 protein. The basal level of Cd^{2+} accumulation in nodules by tri-peptide glutathione (GSH) in legume root nodules (Moran et al. 2000) has a crucial role in protecting the plants against xenobiotics, heavy metals and oxidative stress (Noctor and Foyer 1998). By our calculation, one nodule can adsorb as much as 1.4 nmol Cd^{2+}. Based on the average nodulation per plant in the rice field (100 nodules), it is estimated that 140 nmol of Cd^{2+} can be removed from soil by one plant containing the recombinant B3.

4.2 Heavy Metal Bioremediation with Phytochelatin

The *Arabidopsis* gene for phytochelatin synthase (*AtPCS*) in *M. huakuii* subsp. *rengei* B3 was also expressed (Sriprang et al. 2003). The *AtPCS* gene was expressed under the control of the *nifH* promoter, which regulates the nodule-specific expression of the *nifH* gene. The expression of the *AtPCS* gene was demonstrated in free-living cells under low-oxygen conditions. The

PCS was expressed and catalyzed the synthesis of PCs in strain B3. Cells that expressed the AtPCS gene, whereas no PCs were found in control cells that harbored the empty plasmid, synthesized a range of PCs, with values of n from 2 to 7. The presence of $CdCl_2$ activated PCS and induced the synthesis of substantial amounts of PCs. Cells that contained PCs accumulated 36 nmoles of Cd^{2+}/ mg dry weight of cells. The expression of the *AtPCS* gene in *M. huakuii* subsp. *rengei* B3 increased the ability of cells to bind Cd^{2+} by 9- to 19-fold approximately. The PCS protein was detected by immunostaining in bacteroids of mature nodules of *A. sinicus* containing the *AtPCS* gene. When recombinant *M. huakuii* subsp. *rengei* B3 established the symbiotic relationship with *A. sinicus*, the symbionts increased Cd^{2+} accumulation in nodules by 1.5-fold.

4.3 Advantages of Bioremediation using Symbiotic Engineering

A limitation of the using microbes for bioremediation is that although the metal was bound microbe, but after decomposition of microbes, the metals are still present in the soils. This consideration suggests that for the majority of metal contaminants, the most effective *in situ* remediation strategies may need to combine microbial methods for binding of metals from soil with methods that can effectively uptake metals from soil and prevent the recycle of metals to soil. Plants uptake such released metals from roots and nodules. Bacteroids in nodules can be easily engineered with metal binding peptides. Expression of both *MTL4* and *AtPCS* genes in B3 strain resulted in the additive accumulation of cadmium in the free-living cells. However, accumulation of cadmium in the nodules, in which the two genes were expressed, was not much increased as compared with each single gene expression. This result suggests that uptake of cadmium into the nodule is very limited. Thus, Murooka et al. (unpublished results) expressed the *Arabidopsis* gene for *AtPCS* and iron-regulated transporter (IRT1) in *M. huakuii* subsp. *rengei* B3. The *AtPCS* gene was integrated in the chromosome under the control of the *nifH* promoter, which regulates the nodule-specific expression of the *nifH* gene. The IRT1 gene was expressed under the control of the *nolB* promoter, which regulates the nodule-specific expression of the *nolB* gene. The presence of single copy of *AtPCS* in the chromosome showed slightly increased in Cd^{2+} accumulation, 2.9 Cd^{2+}/ mg dry weight of cells. The presence of multicopy of *AtPCS* in the chromosome showed increased in Cd^{2+} accumulation 20 Cd^{2+}/ mg dry weight of cells. The expression of both the *AtPCS* and *IRT1*gene in recombinant *M. huakuii* subsp. *rengei* B3 increased the ability of cells to bind Cd^{2+} 1.7 to 2.5-fold approximately compared to cells expressed only *AtPCS*.

Thus, genetically engineered symbiotic system, “symbiotic engineering” has a great potential for bioremediation of heavy metals-polluted soil. This bioremediation technique can be applicable to use in symbiosis between mycorrhiza and plants.

5. Conclusion

Bioremediation is the use of plants and microorganisms to extract sequester or detoxify pollutants. Phytoremediation is the use of plants to clean up chemical-contaminated soils. Bioremediation offers a low-cost method for soil or water remediation and some extracted metals may be recycled for value. This review describes traits of metal- hyper accumulating plants for phytoextraction of metals. The hyper accumulators must have high ability to mobilize and uptake of trace elements/metal ions, into the root, shoot and other viable parts of the plant with the aids of chelators and transporter proteins. Chelation of metal ions by various ligands and vacuole compartmentalization play important role in detoxification in hyper accumulators. Alternatively, phytovolatilization of Hg by plants offer great promise for decontamination of metal ions from soil. Potential transgenic approaches for the development of effective phytoremediation technology have been achieved.

Using of microorganisms to remedy heavy metals has been developed. A promising way of improving bioremediation processes is to genetically engineer bacterial strains to confer increased abilities to accumulate toxic heavy metals. Attempts to enhance the metal content of bacterial cells have been made by over expressing metal-binding peptides or proteins, synthetic metal binding peptides. A novel phytoremediation system using symbiosis between leguminous plants and rhizobia was also developed. This system uses both advantages of plants and microorganisms, particularly engineered genes can be transformed to plants through infection with recombinant microorganisms.

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