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REVIEW

Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation

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Abstract

A relatively small group of hyperaccumulator plants is capable of sequestering heavy metals in their shoot tissues at high concentrations. In recent years, major scientific progress has been made in understanding the physiological mechanisms of metal uptake and transport in these plants. However, relatively little is known about the molecular bases of hyperaccumulation. In this paper, current progresses on understanding cellular/molecular mechanisms of metal tolerance/hyperaccumulation by plants are reviewed. The major processes involved in hyperaccumulation of trace metals from the soil to the shoots by hyperaccumulators include: (a) bioactivation of metals in the rhizosphere through root–microbe interaction; (b) enhanced uptake by metal transporters in the plasma membranes; (c) detoxification of metals by distributing to the apoplasts like binding to cell walls and chelation of metals in the cytoplasm with various ligands, such as phytochelatins, metallothioneins, metal-binding proteins; (d) sequestration of metals into the vacuole by tonoplast-located transporters. The growing application of molecular-genetic technologies led to the well understanding of mechanisms of heavy metal tolerance/accumulation in plants, and subsequently many transgenic plants with increased resistance and uptake of heavy metals were developed for the purpose of phytoremediation. Once the rate-limiting steps for uptake, translocation, and detoxification of metals in hyperaccumulating plants are identified, more informed construction of transgenic plants would result in improved applicability of the phytoremediation technology.
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Keywords: Phytoremediation; Heavy metals; Hyperaccumulation; Molecular bases; Biotechnology

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Introduction

Heavy metals are the main group of inorganic contaminants and a considerable large area of land is contaminated with them due to use of sludge or municipal compost, pesticides, fertilizers, and emissions from municipal waste incinerators, car exhausts, residues from metalliferous mines, and smelting industries [1,2]. Although metals are present naturally in the Earth's crust at various levels and many metals are essential for cells (e.g. copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), zinc (Zn)), all metals are toxic at higher concentrations. Specifically, any metal (or metalloid) species may be considered a "contaminant" if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect [3]. Metal concentrations in soil typically range from less than one to as high as 100 000 mg kg^{-1}. Irrespective of the origin of the metals in the soil, excessive levels of many metals can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products [4], pose significant hazards to human, animal, and ecosystem health [5]. This includes the metals/metalloids, such as arsenic (As), cadmium (Cd), chromium (Cr), Cu, lead (Pb), mercury (Hg), Ni, selenium (Se), silver (Ag), and Zn. Other less common metallic species that can be considered contaminants include aluminum (Al), cesium (Cs), cobalt (Co), Mn, molybdenum (Mo), strontium (Sr), and uranium (U) [3].

The threat of heavy metals to human and animal health is aggravated by their long-term persistence in the environment [6]. For instance, Pb, one of the more persistent metals, was estimated to have a soil retention time of 150–5000 years and was reported to maintain high concentration for as long as 150 years after sludge application to soil [7]. Also, the average biological half-life of Cd has been estimated to be about 18 years [8] and 10 years once in the human body [9]. Another reason for toxic heavy metals causing concern is that the metals may be transferred and accumulated in the bodies of animals or human beings through food chain, which will probably cause DNA damage and carcinogenic effects by their mutagenic ability [10], e.g. some species of Cd, Cr, and Cu have been associated with health effects ranging from dermatitis to various types of cancer [11,12]. In addition, some metals occur in the environment as radioactive isotopes (e.g. ^{238}U, ^{137}Cs, ^{239}Pt, ^{90}Sr), which can greatly increase the health risk [13].

Plants that take up heavy metals from the soil offer an alternative and less expensive method to strip heavy metals directly from the soil. Plants have constitutive and adaptive mechanisms for accumulating or tolerating high contaminant concentrations in their rhizospheres. The use of such plants to cleanup soils and water contaminated with pollutants, a technique known as phytoremediation, is emerging as a new tool for in situ remediation. Phytoremediation takes advantage of the fact that a living plant acts as a solar-driven pump, which can extract and concentrate certain heavy metals from the environment [14]. This remediation method maintains the biological properties and physical structure of the soil. The technique is environmentally friendly, potentially cheap, visually unobtrusive, and offers the possibility of bio-recovery of the heavy metals [21]. Phytoremediation strategies can offer suitable approaches for decontaminating polluted soil, water, and air by trace metals as well as organic substances (Table 1).

As early as the 19th century, Baumann [15] identified plants capable of accumulating uncommonly high Zn levels. Minguzzi and Vergnano [16] identified plants capable of hyperaccumulating up to 1% Ni in shoots. Following the identification of these and other hyperaccumulating species, a great deal of research has been conducted to elucidate the physiology and biochemistry of metal hyperaccumulation in plants [21]. However, for this technology to become efficient and cost-effective on a commercial scale, there are some limitations that need to be overcome [17]. Plants ideal for phytoremediation should be: (a) fast-growing, (b) have high biomass, (c) extensive root system, (d) be easy to harvest, and (e) tolerate and accumulate a range of heavy metals in their harvestable parts. While no such plant has been described so far, high biomass non-accumulators that are fast-growing can be engineered to achieve some of the properties of the hyperaccumulators [18]. Determining the molecular mechanism of metal accumulation will be key point to achieving this goal.

Most heavy metal accumulating plants identified so far have root penetration to only shallow depths and a small biomass. To allow remediation within a reasonable period, metal uptake and plant yield have to be enhanced dramatically [14]. This can be done by continuing the search for metal hyperaccumulators, as well as by engineering common plants with hyperaccumulating genes. However, this approach can be only

Table 1. List of phytoremediation strategies

Phytoremediation techniques	Action mechanism	Medium treated
Phytoextraction	Direct accumulation of contaminants into plant shoots with subsequent removal of the plant shoots	Soil
Rhizofiltration (phytofiltration)	Absorb and adsorb pollutants in plant roots	Surface water and water pumped through roots
Phytostabilization	Root exudates cause metals to precipitate and biomass becomes less bioavailable	Groundwater, soil, mine tailings
Phytovolatilization	Plants evaporate certain metal ions and volatile organics	Soil, groundwater
Phytodegradation (plant-assisted bioremediation)	Microbial degradation in the rhizosphere region	Groundwater within the rhizosphere and soil
Phytotransformation	Plant uptake of organic contaminants and degradation	Surface- and groundwater
Removal of aerial contaminants	Uptake of various volatile organics by leaves	Air

used when the molecular mechanisms of metal uptake, tolerance, accumulation, and translocation are better understood.

The primary objective of this review is to concisely evaluate the progress made so far in understanding the molecular mechanisms that control uptake and detoxification of heavy metals in plants, and the complex interactions between the metal, soil, and plant systems that are instrumental in uptake, translocation, and storage of metals in plants. A detailed bibliography is provided for interested readers to delve further into this complex topic.

Major processes involved in trace metal hyperaccumulation by plants

Hyperaccumulation of heavy metals by higher plants is a complex phenomenon. It involves several steps, such as: (a) transport of metals across the plasma membrane of root cells; (b) xylem loading and translocation; and (c) detoxification and sequestration of metals at the whole plant and cellular levels [19]. The first hyperaccumulators characterized were members of the Brassicaceae and Fabaceae families. More than 400 plant species have been reported so far that hyperaccumulate metals [3,20] and a considerable number of species show the capacity to accumulate two or more elements [3,21,22,23]. While most of these plant species have been reported to accumulate Ni, some of them also accumulate Co, Cu, and Zn. A few species accumulate Mn and Cd. The mechanisms of metal hyperaccumulation in these plants are so far not fully understood. Generally speaking, the accumulation ability of a given metal is determined by the uptake capacity and intracellular transportation of plant. The major processes that are assumed to be influencing metal accumulation rates in plant [18,24,26] are illustrated in Fig. 1.

Bioactivation of trace metals in the rhizosphere

The hyperaccumulating plants show extraordinary ability to absorb metals from the soil and accumulate them in the shoots under low and high metal levels [24,48]. Most of heavy metals have low mobility in soils, and are not easily absorbed by plant roots. For instance, there was no significant correlation between Zn accumulation and total Zn in soil for *Thlaspi caerulescens* [25], but a close relationship was noted between shoot Zn accumulation and soil extractable Zn level [38]. The bioavailability and plant uptake of heavy metals in the soils are affected by metal content, pH, Eh, water content, organic substances, and other elements in the rhizosphere. Plant roots and soil microbes and their interaction can improve metal bioavailability in rhizosphere through secretion of proton, organic acids, phytochelatins (PCs), amino acids, and enzymes (Fig. 2).

Secretion of protons by roots could acidify the rhizosphere and increase the metal dissolution. Bernal et al. [27] found that pH affected proton release and plant growth of the Ni hyperaccumulator (*Alyssum murale*) under solution culture condition. However, the difference seemed not large enough for totally explaining the acidification of rhizosphere and improved metal dissolution. It was observed that the pH in the

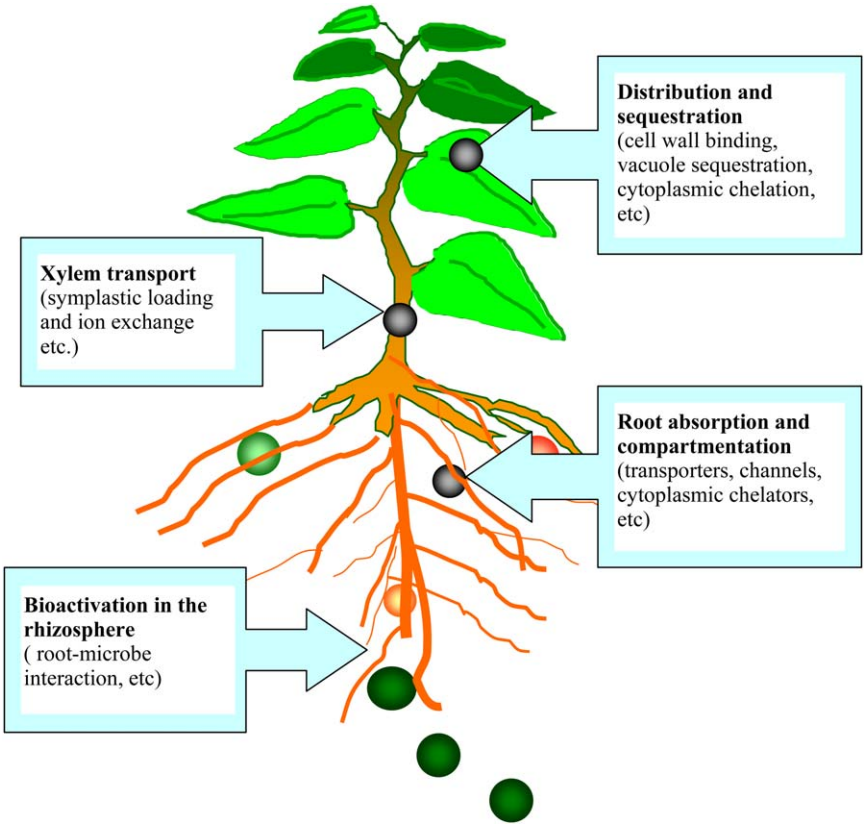

Fig. 1. Major processes proposed to be involved in heavy metal hyperaccumulation by plants.

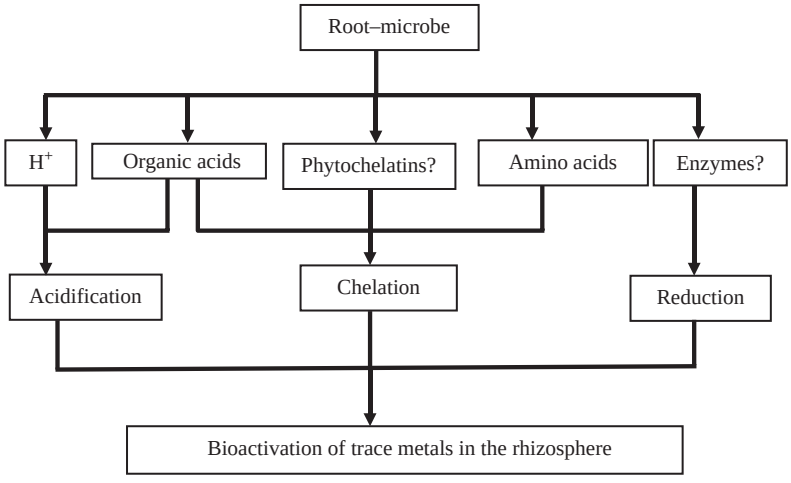

Fig. 2. Processes possibly involved in heavy metal mobilization in the rhizosphere by root–microbe interaction.

rhizosphere soil of the Cu accumulating plant species (*Elsholtzia splendens*) was significantly lower than in the bulk soil when plants were grown in Cu and other metal contaminated soil under field experiment conditions [28]. The proton extrusion of the roots is operated by plasma membrane H^+-ATPase (E.C. 3.6.3.6) and H^+-pumps. The molecular bases and effects on these membrane proteins by other factors have been researched. In Lupin, P deficiency induces citrate exudation by enhancing the activity of plasma membrane H^+-ATPase and H^+ export [29]. AtHMA4 is an *Arabidopsis thaliana* P-1B-ATPase which transports Zn and Cd. Verret et al. [30] demonstrated that AtHMA4 is localized at the plasma membrane and expressed in tissues surrounding the root vascular vessels. The ectopic overexpression of AtHMA4 improved the root growth in the presence of toxic concentrations of Zn, Cd, and Co. A null mutant exhibited a lower translocation

of Zn and Cd from the roots to shoot. In contrast, the AtHMA4 overexpressing lines displayed an increase in Zn and Cd accumulation by shoots.

The secretion of organic acids can mobilize heavy metals and enhance root absorption. Krishnamurti et al. [31] reported that Cd-organic complexed Cd took about 40% of the total in the soil and was positively related to phytoavailability of Cd. Many low molecular organic acids could influence Cd release from absorbed Cd in the soil and increase Cd solubility through forming of Cd-LMWOA complexes. Cieslinski et al. [32] found many low molecular weight organic acids, such as acetic acid and succinate in the rhizosphere of the Cd-accumulating genotype of wheat (Kyle), but not the non-accumulating genotype (Arcola). Al-induced exudation of maleate, as a basis for the mechanism of Al tolerance in wheat, was found to be accompanied by changes in PM surface potential and the activation of H^+-ATPase [33]. However, contrasting results were reported on the role of root exudation for metal hyperaccumulation in *Thlaspi caerulescens*. Some researchers concluded that there was no relationship between root exudation and metal hyperaccumulation in *T. caerulescens* [34–36], whereas other groups reported that root–microbe interaction changed soil conditions in the rhizosphere and increased the solubility of the retained Zn in the rhizosphere soil of the Zn hyperaccumulator *T. caerulenscens*. We found that root exudates of the Zn/Cd hyperaccumulating plant species *Sedum alfredii* Hance could extract more Zn and Pb from the contaminated soil [37]. The transport and hyperaccumulation of Ni were enhanced by amino acid histidine in *Allysum* [50]; the biochemical characterization of root exudates and molecular bases of root exudation in increasing heavy metal mobilization in the rhizosphere need to be further studied.

It has been proven that phytosiderophores can be released under Fe deficiency from cereals to increase the mobilization of Fe, Zn, Cu, and Mn in the soil [38]. The molecular bases involved in phytosiderophore synthesis and secretion have been extensively and intensively studied [26,39,40]. However, no evidences are available yet whether hyperaccumulators can release PCs to the rhizosphere to increase metal uptake.

Root reductases in some dicots can reduce Fe^{3+} or Cu^{2+} under low Fe and Cu supply to increase plant uptake of Fe, Cu, Mn, and Mg [41]. Genes of ferric reductases have been isolated from Arabidopsis and maize [26], NAOH-dependent Fe^{3+}-chelate reductase (NFR) belongs to the cytochrome b5 reductases (b5R) class. NRF gene of *A. thaliana* closely resembles mammalian b5Rs and maize NFR has 66% and 65% similarity to that of mammal and yeast, respectively [42]. However, the characterization of root reductase in hyperaccumulators with relation to metal transformation in the rhizosphere is not understood.

Compared to the bulk soil, the rhizosphere is populated by large concentrations of microorganisms which mainly consist of bacteria and mycorrhizal fungi. Those root-colonizing bacteria and mycorrhizae may significantly increase the bioavailability of various heavy metal ions for uptake. Firstly, they have been shown to catalyze redox transformations leading to changes in soil metal bioavailability. For example, a strain of *Xanthomonas maltophyla* was shown to catalyze the reduction and precipitation of highly mobile Cr^{6+}–Cr^{3+}, a significantly less mobile and environmentally less hazardous compound [43]. The same strain was also found to induce the transformation of other toxic metal ions including Pb^{2+}, Hg^{2+}, Au^{3+}, Te^{4+}, Ag^+, and oxyanions, such as SeO_4^- [44]. Using a different mechanism, As mobility can be enhanced by the activity of the dissimilatory non-reducing bacterium *Shewanella alga* [45]. In addition, certain soil microorganisms have been shown to significantly enhance Zn accumulation in the shoot of the hyperaccumulator *T. caerulescens* by facilitating an increase in the solubility of non-labile Zn in the soil and thus enhancing its bioavailability to this plant [46]. Secondly, soil microorganisms have been shown to exude organic compounds which stimulate bioavailability and facilitate root absorption of a variety of metal ions including Mn^{2+} and possibly Cd^{2+} [9]. Moreover, fungal symbiotic associations have the potential to enhance root absorption area, and stimulate the acquisition of plant nutrients including metal ions [17]. The effect of mycorrhizal associations on metal root uptake is not clear and appears to be metal and plant specific. The bulk of evidence tends to indicate an inhibition of metal uptake by mycorrhizae. However, it is possible that adapted mycorrhizal fungi may play an important role in hyperaccumulation of some metals and metalloids. In the field phytoremediation trial, we found that soluble Pb and Zn in the rhizosphere of the hyperaccumulating species (*Sedum alfredii* H.) were higher than in the bulk soil [47], and the amount of Zn extracted by the plant *Sedum alfredii* was much higher in the second year than in the first year. However, the root–microbe interaction mode for increasing metal bioavailability in the rhizosphere needs to be further characterized.

Metal absorption and transporters

Transport proteins and intracellular high-affinity binding sites mediate the uptake of metals across the plasma membrane. A comprehensive understanding of the metal transport processes in plants is essential for formulating effective strategies to develop genetically engineered plants that can accumulate specific metals. Several classes of proteins have been implicated in heavy

metal transport in plants. These include the heavy metal (or CPx-type) ATPases that are involved in the overall metal-ion homeostasis and tolerance in plants, the natural resistance-associated macrophage protein (Nramp) family of proteins, and the cation diffusion facilitator (CDF) family proteins [51], zinc–iron permease (ZIP) family proteins, etc. (Table 2).

CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential as well as potentially toxic metals like Cu, Zn, Cd, Pb across cell membranes [51]. These transporters use ATP to pump a variety of charged substrates across cell membranes and are distinguished by the formation of a charged intermediate during the reaction cycle. Heavy metal transporters have been classified as type IB and are called the CPx-ATPases because they share the common feature of a conserved intra-membranous cystein–proline–cystein, cystein–proline–histidine or cystein–proline–serine (CPx) motif, which is thought to function in heavy metal transduction. *Arabidopsis* P-type ATPase (PAA1) was the first CPx-ATPase reported in higher plants [52]. Most CPx-type ATPases identified so far have been implicated in Cu or Cd transport. The physiological role of the heavy metal ATPases in higher plants is not known. Since the *Arabidopsis* CPx-ATPases show fairly low similarities to each other, it is possible that they transport different substrates. They may be present in the plasma membrane and function as efflux pumps removing potentially toxic metals from the cytoplasm, or may also be present at various intracellular membranes and be responsible for the compartmentalization of heavy metals, e.g. sequestration in the vacuoles, golgi, or endoplasmic reticulum.

Since intracellular levels of heavy metals must be carefully controlled, transporters represent good candidates for regulation. How they may be regulated in higher plants is not known yet. Potentially, this could occur at the transcriptional level (control on initiation rates, mRNA stability, differential mRNA splicing) or at the post-translational level (targeting, stability), which has been observed in bacteria and yeast [51].

As mentioned earlier, Nramp is a novel family of related proteins that have been implicated in the transport of divalent metal ions. The corresponding genes that code for these proteins are called the *Nramp* genes (the genes are italicized to distinguish them from the proteins). In higher plants, three *Nramp* homologs have been identified in rice [52]. Subsequently, two *Arabidopsis* genes showing similarity to *Nramp*s have also been identified [53]. Initial results suggest that *Arabidopsis Nramp* homologs encode functional metal transporters [54]. Northern analysis indicates that the rice *Nramp* genes *OsNramp1* is primarily expressed in the roots and *OsNramp2* in the leaves and *OsNramp3* in both tissues of rice [52]. This distinct pattern of expression could mean that they are regulated differently and have distinct functions in different tissues, or that they transport distinct but related ions in different parts of the plant.

CDF proteins have been primarily implicated in the transport of Zn, Co, and Cd in bacteria and some eukaryotes. In the *A. thaliana* genome, 12 nucleotide sequences are predicted to encode members of this family of metal-ion transporters [55]. They might more appropriately be designated as ''cation efflux'' transporters, because the proteins appear to function generally in cation efflux out of the cytoplasmic compartment, whether across the plasma membrane to the cell exterior or across endomembranes into intracellular compartments, such as the vacuole [55].

A related Zn transporter (*ZAT1*) from *Arabidopsis* was reported by Van der Zaal [56], which belongs to another important superfamily of metal-ion transporters known as ZIP gene family [51,57]. And 15 members of this family have been identified in the *A. thaliana* genome [55]. Various members of ZIP are known to be able to transport Fe, Zn, Mn, and Cd, and a *ZIP* gene homolog, *ZNT1*, has been identified in the hyperaccumulator *T. caerulescens* [58]. Working with *T. caerulescens* from a different source population, Assuncao et al. [59] have also cloned two *ZIP* cDNAs (*ZNT1* and *ZNT2*) and similarly found them to be highly expressed in root tissue. Lombi et al. [19] have also cloned from *T. caerulescens* an ortholog of the *A. thaliana* iron

Table 2. Genes of transporters isolated from plants involved in heavy metal uptake

Genes	Plant	Elements	References
OsNramp1 OsNramp2	Rice	Mn	[52]
Cpx-type heavy metal ATPases	*Arabidopsis*	Cu, Zn, Cd, Pb	[49,51,52,100]
Nramp	*Arabidopsis* rice	Cd, divalent metals	[52–54]
CDF family proteins	*Arabidopsis*	Zn, Co, Cd	[55]
ZIP family (ZAT1, ZAT2, ZAT3)	*Arabidopsis* *T. caerulescens*	Cd, Zn, Mn	[56] [19,58,59]

transporter IRT1, which also belongs to the ZIP gene family.

In order to enhance metal uptake, (a) the number of uptake sites could be increased, (b) the specificity of the uptake proteins could be altered, and (c) sequestration capacity could be enhanced by increasing the number of intracellular high-affinity binding sites or the rates of transport across organelles.

Distribution and detoxification of metals in plants

A general mechanism for detoxification of heavy metals in plants is the distribution of metals to apoplast tissues like trichone and cell walls, and chelation of the metals by a ligand, followed by the sequestration of the metal–ligand complex into the vacuole. Table 3 lists some reports on metal distribution in hyperaccumulating or accumulating plant species. Cell walls may play an important role in detoxifying metals in plant cells of the Ni and Zn/Cd hyperaccumulating plant species. About 60–70% of Ni and/or Zn accumulated is distributed in the apoplast cell walls [60,61]. However, molecular bases of metal detoxification by cell walls are not well understood.

Complexation with ligands can be extracellular, e.g. the mechanism of Al tolerance by efflux of organic acids like maleate and citrate from roots. Intracellular complexation involves peptide ligands, such as metallothioneins (MTs) and PCs. MTs are cystein-rich polypeptides, first identified in mammalian tissues as Cd-binding peptides [9]. Several MT genes, and also proteins have been identified in plants. While MTs are gene-encoded, PCs are enzymatically synthesized. PCs have been identified in a wide variety of plant species including monocots, dicots, gymnosperms, algae [62].

PCs are a family of peptides that were first identified in yeast. Most of the current knowledge of these peptides has resulted from molecular-genetic studies on yeast and *Arabidopsis* during the last few years. PCs are rapidly induced in cells and tissues exposed to a range of heavy metal ions, such as Cd, Ni, Cu, Zn, Ag, Hg, and Pb, and anions, such as arsenate and selenite [63,85]. PCs consist of only three amino acids, glutamine (Glu), cystine (Cys), and glycine (Gly). They are structurally related to the tripeptide glutathione (GSH), and are enzymatically synthesized from GSH. PCs form a family of structures with increasing repetitions of the -Glu-Cys dipeptide followed by a terminal Gly, (-Glu-Cys)*n*-Gly, where *n* is generally in the range of 2–5, but can be as high as 11 [64]. It was shown that GSH-deficient mutants of *Arabidopsis* are also PC deficient and Cd sensitive [65]. GSH-dependent PC synthase activity was identified in cultured cells of *Silene cucubalis* [66]. The enzyme is active only in the presence of metal ions, such as Cd, Cu, Zn, Ag, Hg, and Pb. Similar activities have been identified in *Arabidopsis* [67], tomato [68], and pea [69]. Several studies have suggested that PC synthase activity is regulated at the level of enzyme activation by metal ions. The fact that PC synthase activity has an important role in Cd tolerance was shown in *Vigna angularis* (Azuki beans) that are hypersensitive to Cd. Cell-suspension cultures of Azuki bean did not synthesize PCs when challenged

Table 3. Heavy metal distribution in hyperaccumulators at tissue/cellular level

Tissue/organ	Element	Plant species	References
Trichone	Zn, Cd	*Arabidopsis halleri*	[101]
	Cd	*Brassica juncea*	[9]
	Ni	*Alyssum lesbiacum*	[102]
Epidermal	Zn	*T. caerulescens*	[101]
	Zn	*T. caerulescens*	[104]
	Ni	*Alyssum*	[102]
Mesophyll	Zn	*Arabidopsis halleri*	[101]
	Cd	*Sedum alfredii* H.	[106]
Cell wall	Ni	*T. goesingense*	[60]
	Cu	*Elsholtzia splendens*	[103]
	Zn	*Sedum alfredii* H.	[60]
	Pb	*Sedum alfredii* H.	[105]
Vacuole	Zn	*T. caerulescens*	[101]
	Zn	*T. caerulescens*	[104]
	Cd	*Sedum alfredii* H.	[106]
	Zn	*Sedum alfredii* H.	[60]

with Cd. These cells also lacked PC synthase activity. Externally applied GSH failed to stimulate PC synthesis or confer Cd tolerance to the cells, which demonstrated the importance of PC synthesis in Cd tolerance in plants [70].

In spite of the identification of PC synthase activity a decade ago, the identification of a corresponding gene remained elusive until recently. PC synthase genes were first isolated almost simultaneously by two research groups. Vatamaniuk et al. [71] identified an *Arabidopsis* cDNA, named *AtPCS1*. Expression of AtPCS1 protein mediated an increase in Cd accumulation, pointing to a possible role in Cd chelation or sequestration. Clemens et al. [72] identified a wheat cDNA, *TaPCS1*, that increased Cd resistance in wild-type yeast. Just like *AtPCS1*, the Cd resistance mediated by *TaPCS1* was associated with an increase in Cd accumulation and was GSH dependent. Both *AtPCS1* and *TaPCS1* mediated Cd tolerance is GSH dependent and functions in vacuole-deficient mutants, suggesting a cytosolic localization, and mediates in vivo PC biosynthesis in yeast. Further evidence of the role of PCs in metal hyperaccumulation comes from the overexpression of a bacterial glutathione synthetase, an enzyme that synthesizes GSH, by Indian mustard plants [73]. These transgenic plants have increased GSH and PC concentrations and increased Cd tolerance and accumulation relative to controls. Also, overexpression of plant PC synthetase in transgenic yeast increased the tolerance to and the accumulation of Cd [71]. These studies show that the manipulation of GSH and PC concentrations has significant potential for increasing the accumulation of heavy metals by plants.

Regulation of PC synthase activity is likely to be the most important regulatory mechanism of the PC-biosynthetic pathway. Evidence pointing to the role of post-translational activation was obtained using plant cell cultures. PC biosynthesis was reported to occur within minutes of exposure to Cd and is independent of de novo protein synthesis. In *Silene cucubalis* cell cultures [66], tomato [67], and *Arabidopsis* [68], PC synthase expression is independent of heavy metal exposure. These studies suggest that PC synthase is regulated by enzyme activation by heavy metals. However, there are conflicting reports regarding the induction of transcript levels in response to heavy metal exposure. PC biosynthesis probably varies between different plant species, being regulated at transcriptional or post-transcriptional levels, or both. This suggests that PC synthase activity is regulated differently in different plant species.

PC biosynthesis may also be regulated by the biosynthesis of glutathione. In transgenic plants of Indian mustard (*Brassica juncea*), an increase in the expression of enzymes of the GSH biosynthetic pathways led to an increase in PC biosynthesis and Cd tolerance [73]. Wild-type Indian mustard plants responded to increased Cd exposure with increased levels of γ-glutamylcysteine synthetase (γ-*ECS*) transcript, which codes for the first enzyme in the PC-biosynthetic pathway [74]. Similarly, there is also evidence supporting post-transcriptional regulation of γ-ECS expression. [75].

Metal-binding proteins and peptides in plants can enhance metal tolerance/accumulation. These metal-binding peptides or proteins should be preferentially metal specific such that only metals with toxic effect (e.g. Cd, Hg, and Pb) are sequestered and not essential metals, such as Zn and Cu. Ryu et al. [76] isolated and characterized a novel copper-binding protein (Cu-BP) in the Asian periwinkle, *Littorina brevicula*, which is highly resistant to a wide range of heavy metal concentrations and its metal-binding protein(s) are induced in the presence of Cd and Zn. The molecular weight of the purified protein has been determined as 11.38 kDa. This Cu-BP is distinct from common mollusk MT in that it contains significantly lower number of Cys (eight residues) and high levels of aromatic amino acids, Tyr and Phe. The protein additionally contains His and Met, which are absent in the MT-like Cd-BP of *L. brevicula*. Cu-BP of *L. brevicula* functions in the regulation of Zn as well as Cu, which is an essential component of hemocyanin in physiological conditions. This protein possibly is involved in the detoxification mechanism against a heavy burden of Cu.

Sequestration of metals in vacuoles

The vacuole is generally considered to be the main storage site for metals in yeast and plant cells and there is evidence that PC-metal complexes are pumped into the vacuole in fission yeast (*Schizosaccharomyces pombe*) [77] and in plants [9]. Vacuolar accumulation of Ni is essential for Ni resistance in the yeast *Saccharomyces cerevisiae* [78,79]. This vacuolar accumulation of Ni is driven by the pH gradient that exists across the vacuolar membrane of yeast [79]. Surprisingly, this type of pH gradient dependent Ni transport could not be observed in roots of Ni sensitive oat seedlings [80], and only a minor accumulation of Ni could be detected in vacuoles isolated from leaves of Ni sensitive barley.

Compartmentalization of metals in the vacuole is also part of the tolerance mechanism of some metal hyperaccumulators [81]. The Ni hyperaccumulator *Thlaspi goesingense* enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole [60]. High-level expression of a vacuolar metal-ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles [82].

Within plant cells, PC-metal complexes bound by GSH or PCs are shuttled to the vacuole by an ABC-type transporter protein in the tonoplast [83]. The same type of transporter is involved in shuttling GSH-conjugated anthocyanins to the vacuole [84]. In fact, anthocyanins can also bind metals [13], and have been suggested to play a role in metal sequestration. Other metal-binding molecules that are involved in metal complexation in the vacuole are organic acids [60]. To date, the best characterized of the known vacuolar transporters and channel involved in metal tolerance is YCF1 from *Saccharomyces cerevisiae*. YCF1 is an MgATP energized glutathione S-conjugate transporter responsible for vacuolar sequestration of organic compounds after their S-conjugation with glutathione, as well as GSH-metal complexes. It catalyzes the transport of bis(glutathionato)cadmium (Cd-GS_2) into vacuoles [86], as well as As-GS_3 and Hg-GS_2 [87].

Genetic basis of metal hyperaccumulation

Most research on hyperaccumulators has focused on the physiological mechanisms of metal uptake, transport, and Sequestration [3,4], but relatively little is known regarding the genetic basis of Hyperaccumulation [81,89]. Persistent exposure of natural populations to inadequate or toxic micronutrient availability would be expected to provoke evolutionary adaptation, provided that the appropriate genetic variation is available in the populations in question [24]. The plant species occurring on strongly metal-enriched soils provide striking examples of microevolutionary adaptation to toxic heavy metal availability. Most of these species are 'facultative' metallophytes: they occur on both normal and metalliferous soil types. Well-known examples are *Festuca ovina*, *F. rubra*, *Agrostis capillaries*, *A. gigantean*, *A. stolonifera*, *A. canina*, *Deschampsia cespitosa*, *D. flexuosa*, *Minuartia verna*, *T. caerulescens*, and *Silene vulgaris* [24,26]. All these species have been shown to exhibit a very pronounced inter-population variation in the degree of heavy metal tolerance. Plants from metalliferous sites are often 5–50-fold more tolerant to particular metals than plants from non-metalliferous sites [22,85].

Genetic variation between plants in the ability to accumulate metals is of great theoretical importance, because it is the raw material on which natural selection acts to influence the evolution of hyperaccumulation. Although some degree of hyperaccumulation occurs in all members of the species that can hyperaccumulate, there is evidence of quantitative genetic variation in the ability to hyperaccumulate, both between and within populations [13,59]. Such variation does not appear to correlate positively with either the metal concentration in the soil or the degree of metal tolerance in the plants [18,57].

The genotypic differences between populations described above are of great interest to researchers trying to understand and manipulate the genetics of hyperaccumulation. Relatively few studies have been designed to test the magnitude and genetics of within-population variability. Pollard et al. [57] have conducted a similar study on *T. caerulescens* from five populations representing a variety of soil types in Britain and Spain, including Zn/Pb mine soil, serpentine soils high in Ni/Co/Cr, and non-metalliferous soils. Plants grown from seeds, collected as sib families, were cultivated hydroponically on solutions of uniform metal concentration (either Zn or Ni). Populations varied in their metal hyperaccumulation when grown in the uniform hydroponic solution. An analysis of variance revealed these differences between populations to be statistically significant.

Studies using controlled crosses, inter-specific hybrids, and molecular markers are beginning to shed light on the genetic control of this variation. Macnair et al. [88,89] have proved possible to generate F_1 hybrids between *A. halleri* and the non-accumulator *A. petraea* (L.)Lam., which can then be back-crossed with the parental species to make an F_2 array. The F_2 were highly variable, including individuals that accumulated as little Zn as *A. petraea* and individuals that accumulated as much as *A. halleri*, as well as a range of intermediates. The segregation of tolerance to Cu, Zn, and Cd in these crosses appeared to be largely governed by either one major gene, or two additive genes, dependent on the level of tolerance of the tolerant parent [90]. In general, the inheritance of adaptive high-level metal tolerance appears to be governed by a single major gene in other metallophyte species as well. F2 crosses between equally tolerant plants from different geographically isolated mines do not segregate. No more than two loci for Cu tolerance, two for Zn tolerance, and one or two for Cd tolerance have been found among plants from a total of four Cu tolerant, five Zn tolerant, and three Cd tolerant isolated *Silene vulgaris* mine populations [90,91]. The genetic bases and regulatory factors that influence variable expression of specific genes that control metal accumulation in the hyperaccumulating phenotype need to be clarified.

Genetic engineering for phytoremediation

Metal hyperaccumulators are notorious for small size and slow growth. These properties have an adverse impact on the potential for metal phytoextraction and severely restrict the employment of effective agronomic practices, such as mechanical harvest [81]. To overcome

these disadvantages, conventional breeding approaches have been proposed to improve plants for metal extraction [47]. Unfortunately, the success of this approach may be precluded due to sexual incompatibility caused by anatomical differences between parents. Biotechnology has the potential to overcome this limitation by allowing direct gene transfer [99]. Research data indicate that manipulation of relevant plant features, including metal tolerance, is a realistic possibility.

Cellular mechanisms for metal tolerance can be classified into two basic strategies. One strategy is to keep the concentration of toxic metal ions in the cytoplasm low by preventing the metal from being transported across the plasma membrane, either by increased binding of metal ions to the cell wall or by reduced uptake through modified ion channels, or by pumping the metal out of the cell with active efflux pumps, a mechanism that is widespread in metal-tolerant bacteria [81]. The other strategy is to detoxify heavy metal ions entering the cytoplasm through inactivation via chelation or conversion of a toxic ion into a less toxic or easier to handle form and/or compartmentalization.

Modification or overexpression of the enzymes that are involved in the synthesis of GSH and PCs might be a good approach to enhance heavy metal tolerance and accumulation in plants. Zhu et al. [73] overexpressed the *Escherichia coli* counterparts of γ-ECS and GSH synthetase in Indian mustard plants that accumulate more Cd than wild-type plants. Rugh et al. [92] modified yellow poplar trees with two bacterial genes, *merA* and *merB*, to detoxify methyl-Hg from contaminated soil. In transformed plants, *merB* catalyzes the release of Hg^{2+} from methyl-Hg, which is then converted to Hg^0 by *merA*. Elemental Hg is less toxic and more volatile than the mercuric ion, and is released into the atmosphere. Pilon-Smits et al. [93] overexpressed the ATP-sulfurylase (APS) gene in Indian mustard. The transgenic plants had four-fold higher APS activity and accumulated three times more Se than wild-type plants. Recently, Dhankher et al. [94] reported a genetics-based strategy to remediate As from contaminated soils. They overexpressed two bacterial genes in *Arabidopsis*. One was the *E. coli* AsrC gene encoding arsenate reductase that reduces arsenate to arsenite coupled to a light-induced soybean *rubisco* promoter. The second gene was the *E. coli* γ-ECS coupled to a strong constitutive actin promoter. The AsrC protein, expressed strongly in stem and leaves, catalyzes the reduction of arsenate to arsenite, whereas γ-ECS, which is the first enzyme in the PC-biosynthetic pathway, increases the pool of PCs in the plant. The transgenic plants expressing both AsrC and γ-ECS proteins showed substantially higher As tolerance; when grown on As, these plants accumulated a 4–17-fold greater fresh shoot weight and accumulated 2–3-fold more As than wild-type plants.

Even though there is a variety of different metal tolerance mechanisms, and there are many reports of transgenic plants with increased metal tolerance and accumulation, most, if not all, transgenic plants created to date rely on overexpressing genes involved in the biosynthesis pathways of metal-binding proteins and peptides [6,73,95–97], genes that can convert a toxic ion into a less toxic or easier to handle form [92,98], or a combination of both [94]. A summary of the most effective transgenes and the effects of their expression on tolerance, accumulation, and volatilization of metals in plants is given in Table 4. For effective use of biotechnology to design transgenic plants capable of efficient phytoremediation, a comprehensive knowledge of the genetic basis for hyperaccumulation is essential, especially those biological processes in natural metal hyperaccumulators that have enhanced metal acquisition, translocation, tolerance, and accumulation abilities and thus are the most promising source of potential phytoremediation genes.

Future research perspectives

Heavy metal hyperaccumulators have received increased attention in recent years, due to the potential of using these plants for phytoremediation of metal contaminated soils [2,3,5,7,44]. However, there are some limitations for this technology to become efficient and cost-effective on a commercial scale, as most of the metal hyperaccumulating plants identified have small biomass, and are not very adaptable to harsh environment. These limitations need to be overcome by achieving a good understanding of the mechanisms of metal hyperaccumulation in plants.

In the past years, most researches focusing on the physiological mechanisms of hyperaccumulation have made great progress; however, the understanding of a range of molecular/cellular mechanisms will undoubtedly change our concept of metal acquisition and homeostasis in higher plants. With the completion of the *Arabidopsis* genome project, eventually followed by genome sequences for other plants, the full range of genes that are potentially involved in heavy metal homeostasis and accumulation will be identified [94]. The problem of low biomass phytoremediators can be overcome by increasing plant yield and metal uptake by engineering common plants with hyperaccumulating genes. If non-native transgenic plants are used for phytoremediation, proper control of their dissemination has to be adopted to avoid the introduction of new weed species. Major targets of phytoremediation technology are likely to be larger contaminated areas, such as agricultural lands polluted by industrial or mining activities. Expanded research programs on the basic

Table 4. Genes introduced into plants and the effects of their expression on heavy metal tolerance, accumulation, or volatilization

Gene	Product	Source	Target	Maximum observed effect[a]
merA	Hg(II) reductase	Gram-negative bacteria	*Liriodendron tulipifera*	50 μmol l^{-1} $HgCl_2$; 500 mg $HgCl_2$ kg^{-1}
			Nicotiana tabacum	*V*: Hg-volatilization rate increase 10-fold
merA	Hg(II) reductase	Gram-negative bacteria	*Arabidopsis thaliana*	*T*: 10 μmol l^{-1} CH_3HgCl (>40-fold)
merB	Organomercurial lyase	Gram-negative bacteria	*A. thaliana*	*V*: Up to 59 pg Hg(0) mg^{-1} fresh biomass min^{-1}
APS1	ATP sulfurylase	*A. thaliana*	*B. juncea*	*A*: Two-fold increase in Se concentration
MT-I	MT	Mouse	*N. tabacum*	*T*: 200 μmol l^{-1} $CdCl_2$ (20-fold)
CUP1	MT	*Saccharomyces cerevisiae*	*B. oleracea*	*T*: 400 μmol l^{-1} $CdCl_2$ (approximately 16-fold)
gsh2	GSH synthase	*E. coli*	*B. juncea*	*A*: Cd concentrations 125%
gsh1	γ-Glu-Cys synthase	*E. coli*	*B. juncea*	*A*: Cd concentrations 190%
NtCBP4	Cation channel	*N. tabacum*	*N. tabacum*	*T*: 250 μmol l^{-1} $NiCl_2$ (2.5-fold), Pb-sensitive *A*: Pb concentrations 200%
ZAT1	Zn transporter	*A. thaliana*	*A. thaliana*	*T*: Slight increase
TaPCS1	PC	Wheat	*Nicotiana glauca* R. Graham	*A*: Pb concentrations 200%

[a]Relative values refer to control plants not expressing the transgene. References are given in the text. *A*: accumulation in the shoot; GSH: glutathione, MT: metallothionein; *T*: tolerance; *V*: volatilization.

and applied processes and problems affecting each individual class of pollutants are needed before significant progress is to be made. Deployment of phytoremediation under diverse conditions and contaminants require evaluation of field performance, which is complicated by the difficulty to characterize the mass balance of metal contaminants as well as the complexity of interactions that take place between soil, metal, and plants. A multidisciplinary research effort that integrates the work of plant biologists, soil chemists, microbiologists, and environmental engineers is essential for greater success of phytoremediation as a viable soil cleanup technique.

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