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·**Review**·

Molecular Mechanisms and Genetic Basis of Heavy Metal Tolerance/ Hyperaccumulation in Plants

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Abstract: Phytoremediation has gained increased attention as a cost-effective method for the remediation of heavy metal-contaminated sites. Because some plants possess a range of potential mechanisms that may be involved in the detoxification of heavy metals, they manage to survive under metal stresses. High tolerance to heavy metal toxicity could rely either on reduced uptake or increased plant internal sequestration, which is manifested by an interaction between a genotype and its environment. The growing application of molecular genetic technologies has led to increased understanding of mechanisms of heavy metal tolerance/accumulation in plants and, subsequently, many transgenic plants with increased heavy metal resistance, as well as increased uptake of heavy metals, have been developed for the purpose of phytoremediation. In the present review, our major objective is to concisely evaluate the progress made so far in understanding the molecular/cellular mechanisms and genetic basis that control the uptake and detoxification of metals by plants.

Key words: amino acids; heat shock proteins; metal-binding proteins; metal transporters; metallothioneins; organic acids phytochelatins; phytoremediation.

Many metal ions are essential as trace elements, but at higher concentrations they become toxic. Because it is really difficult to remove heavy metals from the environment and the low levels observed pose a high risk of heavy metal accumulation in the food chain, many heavy metals constitute a global environmental hazard. At high concentrations, these essential heavy metals, as well as the non-essential metals, not only can become extremely toxic, causing abnormal symptoms, but the entry of heavy metals into the food chain is also of concern because it can cause chronic health problems. So, the possibility of transfer of these contaminants to humans through the food chain has led researchers to pay considerable attention to this issue.

Phytoremediation, as a cost-effective and environmentally friendly method, is an emerging technology based on the use of plants to clean up polluted sites (Cunningham *et al.* 1997) that has attracted growing attention because of its distinctive potential and advantages compared with conventional technologies, such as soil replacement, solidification, and washing strategies. Plants ideal for phytoremediation should grow fast, have high biomass, and tolerate and accumulate a range of heavy metals in their harvestable parts. To date, there are more than 400 plant species known to hyperaccumulate heavy metals (Brooks 1998), most of which fall short of biomass. Only recently, some ideal plants have been reported. For example, Chinese brake fern (*Pteris vittata* L.) has been reported to be an arsenic hyperaccumulator. This plant has a considerable biomass, is fast growing, easy to propagate, and perennial (Ma *et al.* 2001; Chen *et al.* 2002). Approaches

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to find metal-tolerant hyperaccumulating plants for phytoremediation involves searching for and studying natural hyperaccumulators or developing genetically engineered plants that possess these traits.

In general, a high level of tolerance to heavy metals could rely on either reduced uptake or increased plant internal sequestration, which is manifested by an interaction between a genotype and its environment (Macnair *et al.* 2000; Hall 2002). Because some plants possess a range of potential mechanisms that may be involved in the detoxification of heavy metals, they are tolerant to metal stresses. These mechanisms involve: (i) binding to the cell wall; (ii) reduced uptake or efflux pumping of metals at the plasma membrane; (iii) chelation of the metal in the cytosol by various ligands, such as phytochelatins, metallothioneins, and metal-binding proteins; (iv) repair of stress-damaged proteins; and (v) the compartmentation of metals in the vacuole by tonoplast-located transporters (Hall 2002).

Therefore, an understanding of the molecular mechanisms and genetic basis is an important aspect of developing plants as agents for the phytoremediation of contaminated sites (Salt *et al.*1998; Cobbett 2000). Using molecular techniques, some high-biomass non-accumulators that are fast growing can be engineered to achieve some of the properties of the hyperaccumulators. Determining the molecular mechanism of metal accumulation will be a key point in achieving this goal.

1 Molecular/Cellular Mechanisms of Heavy Metal Hyperaccumulation in Plants

1.1 Metal transporters

Metal cation homeostasis is essential for plant nutrition and resistance to toxic heavy metals. Therefore, heavy metal transport is a very exciting and developing field in plant biology**.** Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance. These include 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, cation diffusion facilitator (CDF) family proteins (Williams 2000), and the zinc-iron permease (ZIP) family (Guerinot 2000). Of course, many plant metal transporters remain to be identified at the molecular level.

The 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, and Pb across cell membranes (Williams 2000). Responsive-to-antagonist 1 (RNA1), a functional CPx-ATPase, plays a key role in the operation of the ethylene signaling pathway in plants. Hirayama *et al*. (1999) identified an *Arabidopsis* mutant RNA1 that shows ethylene phenotypes in response to treatment with *trans*-cyclooctene, a potent receptor antagonist. Genetic epistasis studies revealed an early requirement for RAN1 in the ethylene pathway. Functional evidence from yeast complementation studies suggested that RAN1 transports copper and it was proposed that this CPx-ATPase may have a role in delivering copper to the secretory system, which is required in the production of functional hormone receptors. The CPx-ATPases are thought to be important not only in obtaining sufficient amounts of heavy metal ions for essential cell functions, but also in preventing the accumulation of these ions to toxic levels.

The Nramp family defines a novel family of related proteins that have been implicated in the transport of divalent metal ions. Thomine *et al*. (2000) reported that Nramp proteins play a role in Fe and Cd uptake; interestingly, disruption of an *AtNramps3* gene slightly increased Cd resistance, whereas overexpression resulted in Cd hypersensitivity in *Arabidopsis.*

The CDF proteins are a family of heavy metal transporters implicated in the transport of Zn, Cd, and Co that have been identified in some plants. Certain members of the CDF family are thought to function in heavy metal uptake, whereas others catalyse efflux, and some

are found in plasma membranes whereas others are located in intracellular membranes. The recent study by van der Zaal *et al.* (1999) suggests that the protein zinc transporter of *Arabidopsis thaliana* (*ZAT1*) may have a role in zinc sequestration. Enhanced zinc resistance was observed in transgenic plants overexpressing *ZAT1* and these plants showed an increase in the zinc content of the root under conditions of exposure to high concentrations of zinc. However, this transporter is not confined to root tissue; northern blotting analysis indicated that *ZAT1* was constitutively expressed throughout the plant and was not induced by exposure to increasing concentrations of zinc.

Until now, 15 members of the ZIP gene family have been identified in the *A. thaliana* genome. Various members of the ZIP family are known to be able to transport iron, zinc, manganese, and cadmium. Pence *et al*. (2000) cloned the transporter *ZNT1*, a ZIP gene homolog, in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. They found that *ZNT1* mediates high-affinity Zn uptake as well as low-affinity Cd uptake. Northern blot analysis indicated that enhanced Zn transported in *T. caerulescens* results from a constitutively high expression of *ZNT1* in the roots and shoots. Sequence analysis of *ZNT1* indicated that it is member of a recently discovered micronutrient transport gene family, which includes the *Arabidopsis* Fe transporter IRT1 and the ZIP Zn transporters (Pence *et al*. 2000). Working with *T. caerulescens* from a different source population, Assuncao *et al*. (2001) have also cloned two ZIP cDNA (*ZNT1* and *ZNT2*) and, similarly, have found them to be highly expressed in root tissue. The fact that downregulation of transcript levels was not observed in response to high concentrations of zinc suggests that a constitutively high level of expression of these transporters may be a distinctive feature of hyperaccumulator plants. Lombi *et al*. (2002) have also cloned an ortholog of the *A. thaliana* iron transporter IRT1 from *T. caerulescens* that also belongs to the ZIP gene family.

Of course, many plant metal transporters remain to be identified at the molecular level and the transport function, specificity, and cellular location of most of these proteins in plants remains unknown.

1.2 Amino acids and organic acids

Plants produce a range of ligands for Cd, Cu, Ni, and Zn. Carboxylic acids and amino acids, such as citric, malic, and histidine (His), are potential ligands for heavy metals and, so, could play a role in tolerance and detoxification (Rauser 1999; Clemens 2001; Hall 2002).

The Cd-and Zn-citrate complexes are prevalent in leaves, even though malate is more abundant. In the xylem sap moving from roots to leaves, citrate, and His are the principal ligands for Cu, Ni, and Zn. Recently, Salt *at al.* (1999) identified putative Zn-His complexes in the roots of the closely related Zn hyperaccumulator *T. caerulescens*. Kramer *et al.* (1996) observed a 36-fold increase in the concentration of free His in the xylem exudate of the Ni-hyperaccumulator *Alyssum lesbiacum* after exposure to Ni. However, no significant change was observed in the non-accumulator *Alyssum montanum* and a significant linear correlation in the xylem exudate concentrations of free His and Ni in several Ni-hyperaccumulators in the genus *Alyssum* was also observed (Kramer *et al.* 1996). Furthermore, the addition of equimolar concentrations of exogenous *L*-His to an Ni-amended hydroponic rooting medium enhanced Ni flux into the xylem in the non-accumulator *A. montanum*, as well as in the non-accumulator *Brassica juncea* L. cv. *vitasso*. In *B. juncea*, reducing the entry of *L*-His into the root by supplying *D*-His instead of *L*-His or *L*-His in the presence of a 10-fold excess of *L*-alanine did not affect root Ni uptake, but reduced Ni release into the xylem. Compared with *B. juncea*, root His concentrations were constitutively approximately 4.4-fold higher in the hyperaccumulator *A. lesbiacum* and did not increase within 9 h of exposure to Ni (Kerkeb and Kramer 2003). However, no increase was observed in the concentration of free His in root, shoot, or xylem sap in the other Ni-hyperaccumulator *Thlaspi goesingense* in response to Ni exposure (Persans *et al.* 1999).

1.3 Phytochelatins

Many plants cope with the higher levels of heavy

metals by binding them in complexes with a class of peptides called phytochelatins (PCs) and sequestering the complexes inside their cells.

The structure of PCs is $(\gamma\text{-Glu-Cys})_nX$, in which X is Gly, γ-Ala, Ser, or Glu and n=2–11 depending on the organism, although the most common forms have two to four peptides. The biosynthesis of PCs is induced by many metals, including Cd, Hg, Ag, Cu, Ni, Au, Pb, As, and Zn; however, Cd is by far the strongest inducer (Grill *et al.* 1987, 1989). The metal binds to the constitutively expressed enzyme γ-glutamylcysteinyl dipeptidyl transpeptidase (PC synthase), thereby activating it to catalyse the conversion of glutathione to phytochelatin (Zenk 1996). Glutathione, the substrate for PC synthase, is synthesized from its constituent amino acids in two steps. The first step is catalysed by γ-glutamyl-Cys synthetase (γ-ECS) and the second step is catalysed by glutathione synthetase (GS). γ-Glutamyl-Cys synthetase is feedback regulated by glutathione and is dependent on the availability of cysteine (Mejáre and Bülow 2001). Structural analyses of PC-metal complexes by EXAFS studies (Strasdeit 1991; Pickering *et al.* 1999) and optical spectroscopy have documented a ligandation of Cd^{2+}, Ag^{+}, Hg^{2+}, and Pb^{2+} by thiolate coordination (Mehra *et al.* 1995, 1996a, 1996b), as is known for corresponding metallothionein-metal complexes (Howden and Cobbett 1992). In general, PC biosynthesis is triggered by heavy metal cations such as Cd^{2+} and Zn^{2+}. The analysis of a PC-deficient *Arabidopsis* mutant showed a detoxifying role for PCs, at least for Cd^{2+} and Hg^{2+} (Howden and Cobbett 1992).

Phytochelatins are also reported to be involved in the homeostasis of Zn^{2+} and Cu^{+}/Cu^{2+} by providing a transient storage form for the ions (Grill *et al.*1988; Thumann *et al.* 1991). The induction of PCs by the anion arsenate has been observed in a survey for peptide-inducing metal ions (Grill *et al.* 1987) and suggests a unique mode of PC synthase activation. However, Maitani *et al.* (1996) failed to demonstrate an As-PC complex. This result indicates that PCs do not fulfill a detoxifying function during As poisoning. Raab *et al.* (2004) have developed a method to ascertain the nature of As-PC complexes in extracts of the As-tolerant grass *Holcus lanatus* and the As-hyperaccumulator *Pteris cretica* using parallel metal (loid)-specific (inductively coupled plasma-mass spectrometry) and organic-specific (electrospray ionization-mass spectrometry) detection systems. In an *in vitro* experiment using a mixture of reduced glutathione (GSH), PC_2, and PC_3, As preferred the formation of the arsenite ($As^{(III)}$)-PC_3 complex over GSH-$As^{(III)}$-PC_2, $As^{(III)}$-$(GSH)_3$, $As^{(III)}$-PC_2, or $As^{(III)}$-$(PC_2)_2$ (GS is glutathione bound to As via the sulfur of cysteine). In *H. lanatus*, the $As^{(III)}$-PC_3 complex was the dominant complex, although GSH, PC_2, and PC_3 were found in the extract. *P. cretica* only synthesizes PC_2 and forms dominantly the GSH-$As^{(III)}$-PC_2 complex. In both plant species, As is dominantly in non-bound inorganic forms, with 13% being present in PC complexes for *H. lanatus* and 1% in *P. cretica* (Raab *et al.* 2004).

1.4 Metallothioneins

Detoxification of metals by the formation of complexes is used by most of the eukaryotes. Metallothioneins (MTs) are low molecular weight (6–7 kDa), cysteine-rich proteins found in animals, higher plants, eukaryotic microorganisms, and some prokaryotes (Kägi 1991). They are divided into three different classes on the basis of their cysteine content and structure. The Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs (in which X denotes any amino acid) are characteristic and invariant for metallothioneins. No aromatic amino acids or histidines are found in MTs. MTs found in a few higher plants are also low molecular weight proteins with a high cysteine content, but the cysteines distribute differently than they do in mammalian MTs; therefore, these proteins are designated class II (mammalian MTs comprise class I). The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi (Kägi 1991).

Although it is believed that MTs could play a role in metal metabolism, the role of MTs in plants remains to be determined owing to a lack of information and their

precise function is not clear (Hall 2002).

1.5 Heat shock proteins

Heat shock proteins (HSPs) characteristically show increased expression in response to the growth of a variety of organisms at temperatures above their optimal growth temperature. They are found in all groups of living organisms, can be classified according to molecular size, and are now known to be expressed in response to a variety of stress conditions, including heavy metal stresses (Vierling 1991; Lewis 1999). HSPs act as molecular chaperones in normal protein folding and assembly, but may also function in the protection and repair of proteins under stress conditions.

Today, there have been a couple of reports of increased HSP expression in plants in response to heavy metal stress. Neumann *et al.* (1995) observed that HSP17 is expressed in the roots of *Armeria maritime* plants grown on Cu-rich soils. It was also reported that a short heat stress given prior to heavy metal stress induces a tolerance effect by preventing membrane damage. Clearly, more molecular evidence is required to support such an important repair or protective role.

1.6 Other metal-binding proteins

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 the toxic metals (e.g. Cd, Hg, and Pb) are sequestered rather than essential trace metals, such as Zn and Cu.

Ryu *et al.* (2003) isolated and characterized a novel Cu-binding protein (BP) in the Asian periwinkle *Littorina brevicula*, which is highly resistant to a wide range of heavy metal concentrations and has its metal-binding protein(s) induced in the presence of Cd and An. In their study, following purification by Sephacryl S-100 chromatography, Ryu *et al.* (2003) found that Cu-BP contained an equal amount of Zn in non-exposed physiological conditions. However, Zn is replaced by Cu at the binding site upon the addition of excess Cu (100 μmol/L $CuCl_2$) to the cytosol or after a long period (60 d) of exposure of the periwinkles to the metal ion (150 μg/L $CuCl_2$). Ryu *et al.* (2003) also determined the molecular weight of the purified protein as 11.38 kDa using MALDI-TOF MS analyses. This Cu-BP is distinct from common mollusc MT in that it contains a significantly lower number of Cys (eight residues) and high levels of the aromatic amino acids Tyr and Phe. In addition, the protein contains His and Met, which are absent in the MT-like Cd-BP of *L. brevicula*. The Cu-BP of *L. brevicula* functions in the regulation of Zn as well as Cu, which is an essential component of hemocyanin under physiological conditions. This protein is possibly involved in the detoxification mechanism against a heavy burden of Cu (Table 1).

2 Genetic Engineering of Plants for Phytoremediation

The application of powerful genetic and molecular

Table 1 Peptides and proteins contributing to heavy metal tolerance or accumulation

Peptides and proteins	Related heavy metals	References
Phytochelatins	Cd, Zn, Hg, Cu, Ag, Ni, Au, Pb, As	Grill *et al*. 1987, 1989; Grill *et al*. 1988; Thumann *et al*. 1991; Howden and Cobbett 1992; Mehra *et al*. 1995, 1996a, 1996b; Maitani *et al*. 1996; Raab *et al.* 2004
Metallothioneins	Cd, Zn, Hg, Cu, Au, Ag, Co, Bi	Kägi 1991; Hall 2002
Heat shock proteins	Cu	Neumann *et al*. 1995
Cpx-type heavy metal ATPases	Cu, Zn, Cd, Pb	Hirayama *et al*. 1999
Nramp	Cd	Thomine *et al*. 2000
CDF family proteins	Zn, Co, Cd	van der Zaal *et al*. 1999
ZIP family	Cd, Zn, Mn	Pence *et al*. 2000; Assuncao *et al*. 2001; Lombi *et al*. 2002
Metal-binding protein	Zn, Cu, Cd	Ryu *et al*. 2003

CDF, cation diffusion facilitator; Nramp, natural resistance-associated macrophage protein; ZIP, zinc-iron permease.

techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters and their homologs in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants.

2.1 Isolation of the genes that contribute to heavy metal resistance in plants

Today, the two primary strategies used to isolate and identify genes that contribute to heavy metal resistance in plants have been functional complementation of yeast mutants defective in metal ion transport with plant cDNA expression libraries and the identification of putative transporters by virtue of sequence similarities with databases of plant cDNA and genomic sequences that have determined.

Until now, a few genes that contribute to Cd resistance in plants have been identified. Thomine *et al*. (2000) isolated *AtNramp* cDNAs from *Arabidopsis* and observed that these genes complement the phenotype of the metal uptake-deficient yeast strain *smf1*. The *AtNramp*s show homology to the *Nramp* gene family in bacteria, yeast, plants, and animals. Expression of *AtNramp* cDNAs increases Cd^{2+} sensitivity and Cd^{2+} accumulation in yeast. In *Arabidopsis*, *AtNramps* are expressed in both roots and aerial parts under metal-replete conditions. The results of Thomine *et al*. (2000) show that *Nramp* genes in plants encode metal transporters and that *AtNramps* transport both the nutrient metal Fe and the toxic metal Cd. Louie *et al*. (2003) created a library enriched in Cd-induced cDNAs from Cd-tolerant *Datura innoxia* using suppressive subtractive hybridization. Two differential screening steps were used to screen the Cd-induced library, resulting in eight putative Cd-specific cDNAs of a pool of 94 clones. Reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm that four of these eight clones were Cd specific. One of the four Cd-specific cDNAs had homology to a sulfur transferase family protein in *A. thaliana*. Song *et al*. (2004) screened an *Arabidopsis* cDNA library using a yeast (*Saccharomyces cerevisiae*) expression system using the Cd(II)-sensitive yeast mutant *ycf1* and then yielded a small Cys-rich membrane protein (*Arabidopsis* plant cadmium resistance; *AtPcrs*). Database searches revealed that there are nine close homologs in *Arabidopsis*. Homologs were also found in other plants. Four of the five homologs that were tested also increased resistance to Cd(II) when expressed in *ycf1*. It was found that *AtPcr1* localizes at the plasma membrane in both yeast and *Arabidopsis*. *Arabidopsis* plants overexpressing *AtPcr1* exhibited increased Cd(II) resistance, whereas antisense plants that showed reduced *AtPcr1* expression were more sensitive to Cd(II). The overexpression of *AtPcr1* reduced Cd uptake by yeast cells and also reduced the Cd content of both yeast and *Arabidopsis* protoplasts treated with Cd. Thus, it appears that the *Pcr* family members may play an important role in the Cd resistance of plants (Moffat 1999).

2.2 Isolation of genes for PC synthase

To date, several groups have isolated genes for the PC synthases, which make the metal-binding peptides when the cell is exposed to toxic metals (Moffat 1999). Ha *et al.* (1999) isolated the *CAD1* gene, using a positional cloning strategy, which was proposed to encode PC synthase in *Arabidopsis* and their experiments showed that expression of the *CAD1* mRNA is not influenced by the presence of Cd. The position of the gene was mapped using molecular markers and a candidate gene identified from the *Arabidopsis* genome initiative genomic sequence. Zhu *et al.* (1999) overexpressed the *Escherichia coli* counterparts of ECS (*gshI*) and glutathione synthetase (*gshII*) in Indian mustard (*Brassica juncea*), resulting in transgenic plants that accumulate more Cd than wild-type plants. Overexpression of *E. coli gshII* in the cytosol increased Cd concentrations in the shoot up to 25% and total Cd accumulation per shoot up to threefold compared with the wild type. Moreover, Cd accumulation and tolerance was correlated with the level of *gshII* expression and Cd-treated GS plants had higher concentrations of glutathione, PC, thiolsulfur, and Ca than wild-type

plants. Overexpression of *E. coli gshI* in the plastids resulted in transgenic plants that, in a hydroponic system, grew better than the wild-type plants even though shoot Cd concentrations were 40%–90% higher than in the wild-type plants. The overexpression of *E. coli gshI* increased the biosynthesis of glutathione (1.5–2.5-fold) and PCs in transgenic plants. Oven *et al*. (2002) isolated and functionally expressed a cDNA *GmhPCS1* encoding homophytochelatin synthase from *Glycine max*, a plant known to accumulate homophytochelatins rather than PCs upon exposure to heavy metals. The catalytic properties of *GmhPCS1* were compared with the PC synthase AtPCS1 from *A. thaliana*. When assayed only in the presence of glutathione, both enzymes catalysed PC formation; *GmhPCS1* accepted homoglutathione as the sole substrate for the synthesis of homophytochelatins, whereas *AtPCS1* did not. Heiss *et al*. (2003) isolated a PCS cDNA clone from *B. juncea* L. cv. *vitasso*, a candidate species for phytoremediation, and revealed a close relationship of BjPCS1 with PCS proteins from *A. thaliana* and *T. caerulescens*.

2.3 Introduction of heterologous MTs into transgenic plants

Plant MT-like genes have been isolated from several plant species, including maize, soybean, rice, wheat, tobacco, and *Brassica napus*, but their role in metal detoxification has not yet been established. Type I MT-like genes are expressed predominantly in the roots, whereas type II MT-like genes are expressed primarily in the leaves (Mejáre and Bülow 2001).

Transgenic plants that express MTs have been scored for enhanced Cd tolerance and Cd accumulation or modified Cd distribution. A human *MT-II* gene was introduced into tobacco and oilseed rape and it was found that the growth of these transgenic seedlings was unaffected up to Cd concentrations of 100 μmol/L (Misra and Gedamu 1989). The human *MT-II* gene and MT-II fused to the β-glucuronidase gene were expressed in tobacco under the control of the CaMV 35S promoter with a double enhancer (35S2). *In vitro*-grown transgenic seedlings expressing the fusion protein accumulated 60%–70% less Cd in their shoots than did control plants (Elmayan and Tepfer 1994) (Table 2).

3 Genetic Basis of Metal Hyperaccumulation

Most research on hyperaccumulators has focused on the physiological mechanisms of metal uptake, transport, and sequestration, but relatively little is known about the genetic basis of hyperaccumulation. Persistent exposure of natural populations to inadequate or toxic micronutrient availability would be expected to provoke evolutionary adaptation, providing that the appropriate genetic variation is available in the populations in question. The plant species occurring on 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* (Schat 1999). All these species have been shown to exhibit a

Table 2 Genes isolated and introduced into plants with increased heavy metal resistance and uptake

Genes	Plants	Related heavy metal	References
AtNramps	*Arabidopsis*	Cd	Thomine *et al.* 2000
A library enriched in Cd-induced cDNAs	*Datura innoxia*	Cd	Louie *et al*. 2003
AtPcrs	*Arabidopsis*	Cd	Song *et al.* 2004
CAD1	*Arabidopsis*	Cd	Ha *et al*. 1999
gshI and *gshII*	*Brassica juncea*	Cd	Zhu *et al*. 1999
PCS cDNA clone	*B. juncea*	Cd	Heiss *et al*. 2003

very pronounced inter-population variation in the degree of heavy metal tolerance. Plants from metalliferous sites are often five- to 50-fold more tolerant to particular heavy metals than plants from non-metalliferous sites (Schat and Ten Bookum 1992).

Genetic variation among plants in their 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. 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.

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.* (2002) 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*. (1999, 2000) has proved it 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 population was highly variable, including individuals that accumulated as little Zn as *A. petraea*, individuals that accumulated as much as *A. halleri*, and a range of intermediates. The segregation of tolerance to Cu, Zn, and Cd in these crosses appeared to be governed largely by either one major gene or two additive genes, depending on the level of tolerance of the tolerant parent (Schat 1999). 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. The F_2 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 *S. vulgaris* mine populations (Schat *et al.*1996; Schat 1999).

As molecular physiology provides greater insights into the specific genes that control metal accumulation, we may learn more about the genetic and regulatory factors that influence variable expression of the hyperaccumulating phenotype.

4 Future Prospects

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

In the past years, most studies focusing on the physiological mechanisms of hyperaccumulation have made great progress; however, an 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 tolerance will be identified.

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