14 Plant Metallothionein Genes and Genetic Engineering for the Cleanup of Toxic Trace Elements

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CONTENTS

14.1 INTRODUCTION

The use of plants beyond the necessity of food and fiber is the beginning of environmental biogeotechnology. Toxic metal pollution of the biosphere has accelerated rapidly since the onset of the Industrial Revolution and heavy metal toxicity poses major environmental and health problems. In this regard, plants that accumulate and/or exclude metals are increasingly considered for phytoremediation and phytostabilization. Lead is one of the most frequently encountered heavy metals in polluted environments. The primary sources of this metal include mining and smelting of metalliferous ores; burning of leaded gasoline; disposal of municipal sewage and industrial wastes enriched in Pb; and use of Pb-based paint [1].

The threat that heavy metals pose to human and animal health is aggravated by their long-term persistence in the environment. For instance, Pb is one of the more persistent metals and has been estimated to have a soil retention time of 150 to 5000 years. Also, the average biological half-life of cadmium has been estimated to be about 18 years. The use of biological materials to clean up heavy metal-contaminated soils has been targeted as an efficient and affordable form of bioremediation [2]. One affordable solution might be expressing metal-accumulating genes in nonaccumulating plants showing interesting skills for bioremediation in order to turn them into hyperaccumulators.

14.2 MOLECULAR/ADAPTIVE PHYSIOLOGY AND GENETICS OF METAL HYPERACCUMULATION IN PLANTS

Cloning and characterization of metallothionein (MT) gene families in plants has progressed considerably in the last decade (Table 14.1). MTs and phytochelatins in plants contain a high percentage of cysteine sulfhydryl groups, which bind and sequester heavy metal ions in very stable complexes. Phytochelatins bind Cu and Cd with high affinity and are induced by various metals [62,63]. Phytochelatins may play a role in plant Cd tolerance. Howden and Cobbett [64] have isolated *Arabidopsis* mutants with increased sensitivity to Cd while Cu tolerance was almost unchanged [65,66]. These *cad1* mutants were deficient in PC synthesis and showed greatly reduced levels of PC synthase activity.

MTs not only bind to metals but also regulate intracellular concentrations and detoxify lethal concentrations of metals [59,67]. Various MT genes, such as mouse MTI; human MTIA (alpha domain); human MTII; Chinese hamster MTII; yeast *CUP1*; and pea *PsMTA*, have been transferred to *Nicotiana* sp., *Brassica* sp., or *A. thaliana* [20–23,68–77]. As a result, varying degrees of constitutively enhanced Cd tolerance have been achieved compared with the control. Metal uptake was not markedly altered; in some cases, no differences were present and, in others, maximally 70% less or 60% more Cd was taken up by the shoots or leaves.

Only one study has been reported on a transgenic plant generated with MT of plant origin. When pea (*Pisum sativum*) MT-like gene *PsMTA* was expressed in *A. thaliana*, more Cu (several-fold in some plants) accumulated in the roots of transformed plants than in those of controls [22]. S. Karenlampi (Finland) and her associates have isolated an MT gene from metal-tolerant *Silene vulgaris* and transferred it into several metal-sensitive yeasts. Increases in Cd and Cu tolerance were observed in the modified yeasts. These studies suggest that the MT gene may be useful in improving metal tolerance of plants.

The hyperaccumulator *Thlaspi caerulescens* and the related nonaccumulator *T. arvense* differ in their transcriptional regulation of *ZNT1* (zinc transporter 1) capable of conferring uptake of Cd^{2+} and Zn^{2+} [78]. Expression of *ZNT1* and root zinc uptake rates is elevated in *T. caerulescens* when compared to *T. arvense*. Zinc-mediated down-regulation of *ZNT1* transcript levels in the hyperaccumulator occurs at about 50-fold higher external metal concentrations compared to the nonhyperaccumulator. In several nickel hyperaccumulators, metal exposure elicits a large and dose-dependent increase in the concentrations of free histidine, which can act as a specific chelator able to detoxify Ni^{2+} and which enhances the rate of nickel translocation from the rooting medium into the xylem for transport into the shoot via the transpiration stream.

In the shoots of hyperaccumulating plants, metal detoxification is achieved by metal chelation and subcellular compartmentalization into the vacuole and the apoplast [79–83] and by sequestration within specific tissues, e.g., in the epidermis or in trichomes. The plant detoxification systems remain to be characterized at the molecular level. The generation and analysis of crosses between hyperaccumulators and related nonhyperaccumulators will be one key tool in identifying the genes responsible for the metal hyperaccumulator phenotype.

Based on a preliminary genetic analysis of a number of F2 progeny from crosses between the cadmium- and zinc-tolerant zinc hyperaccumulator *Arabidopsis halleri* ssp. *halleri* (L.) and the closely related, nontolerant nonaccumulator *A. lyrata* ssp. *petraea* (L.), it was postulated that only a small number of major genes were involved in zinc hyperaccumulation and zinc tolerance in *A. halleri* [34,84].

14.3 PLANT METALLOTHIONEIN GENES AND GENETIC ENGINEERING FOR PHYTOREMEDIATION OF TOXIC METALS

The development of a phytoremediation technology for some trace elements requires the transfer of genes into plants across species borders. The molecular basis of trace element detoxification and hyperaccumulation in plants has been increasingly investigated [20,85–87].

TABLE 14.1
Cloning and Characterization of Metallothionein (MT) Gene Families in Plants Including cDNA Encoding for Metallothinoein-like proteins

Plant Name	Protein/Gene/Gene Family	Reference
	Type 1	
Mimulus		3
Pea	*PsMTA*	4
Barley	*ids-1*	5
		6
Maize		7
		8
Wheat	*wali1*	9
White clover		10
Arabidopsis	*MT1/MT1a*	11–13
	MT1c	12
Brassica napus		14
Rice	*OsMT-1*	15,16
Cotton	*MT1-A*	17
Vicia faba (fava bean)	*MT1a*	18
	MT1b	
Red fescue		19
Brassica napus, Nicotiana tabacum	*MT2*	20
Nicotiana tabacum	*MTl*	21
Pisum sativum	*PsMT A*	22
Yeast	*CUP1*	23
Arabidopsis	*Glutathione-S-transferase (parB)*	24
Glycine max (soybean)	*Ferritin*	25
	ZAT (AtMTP1)	26
	Arabidopsis thaliana (CAX2)	27
	Nicotiana tabacum (NtCBP4)	28
	FRE1 and *FRE2*	29
	Arabidopsis thaliana (AtNramp1)	30
	Arabidopsis thaliana (AtNramp3)	31
	Arabidopsis thaliana (merA)	32
	Arabidopsis thaliana (merB)	33
	AHA2	34
	Type 2	
Soyabean		35
Arabidopsis		36
	MT2/MT2a	11,12
	MT2b	12
Ricinus communis (castor bean)		37
Vicia faba (fava bean)		38
Kiwi fruit	*PLIWI504*	39
Coffee		40
Chinese cabbage		41
Sambucus		37
Nicitiana Tabacum (tobacco)		42
		43
Trifolium repens (white clover)		44

TABLE 14.1
Cloning and Characterization of Metallothionein (MT) Gene Families in Plants Including cDNA Encoding for Metallothionein-like Proteins (continued)

Plant Name	Protein/Gene/Gene Family	Reference
Brassica campestris		45
Rice	*OsMT-2*	46
Tomato (three sequences)	$LeMT_A$	47
	$LeMT_B$	48
		48
Brassica napus		49
Brassica juncea (five sequences)		50
Apricot		51
Common rice plant		52
	Type 3	
Rice		53
		54
		16
	Type 4	
Kiwi fruit	*Clone 503*	39
Apple		55
Papaya		56
Banana	*Clone3-6*	57
Rice(two sequences)		16
Sweet cherry		58
	Others	
Arabidopsis	*MTIb*	12
Arabidopsis	*MT3*	59
Tomato		47
Brassica campestris		45
Douglas fir	*PM 2.1*	60
Strawberry	*FMET1*	61
Banana	*Clone 3-23*	57

Despite the difficulty in predicting the effects of microbial genes in a complex multicellular organism like a plant, the successful introduction of a modified bacterial mercuric ion-reductase gene into yellow poplar (*Liriodendron tulipifera*) and *Nicotiana tabacum* demonstrates that bacterial genes may be extremely valuable in phytoremediation. Two *Arabidopsis* mutants resistant to high levels of aluminum have been characterized [34]. The genes have yet to be cloned, but one of the mutants, on chromosome 1, secretes organic acids to bind Al in the soil before it enters the plant. The second mutant, mapped to chromosome 4, increases the flux of hydrogen outside the root, changing the pH, which transforms the Al^{3+} ions into aluminum hydroxides and aluminum precipitates. These forms are incapable of entering the plant via the roots.

The *Arabidopsis* transgenic plants with mer (mercury) operon have conferred tolerance to gold [86ñ88]. Implications for the glutathione, phytochelatin synthetase pathways in transgenics for remediations is increasingly gaining the attention of scientists [88,89]. Metallothioneins (low molecular weight proteins with high cysteine content and a high affinity for binding metal cations such as those of cadmium, copper, and zinc) from animal sources were introduced into plants in

a transgenic approach, mainly to reduce metal accumulation in shoots by trapping the metal in the roots. Expression of a mammalian MT in *N. tabacum* L. under the control of a constitutive promoter was able to reduce the translocation of cadmium into the shoots. Following exposure to a low cadmium concentration (0.02 μ*M*) in the rooting medium, leaf cadmium concentrations were 20% lower in the transgenics than in wild-type plants. However, under field conditions, a consistent difference between transgenic and control plants could not be observed in leaf cadmium content or plant growth.

These results demonstrate that trace element uptake observed on nonsoil substrates under glasshouse or growth chamber conditions may not be extrapolated to predict the performance of transgenic plants on soil substrates or under field conditions. Plants overexpressing mammalian MTs were reported to be unaffected by concentrations of 100 to 200 μ*M* cadmium, whereas growth of *N. tabacum* control plants was severely inhibited at external cadmium concentrations of 10 μ*M* [20].

After thorough examination of a number of wild plant species growing on soils highly contaminated by heavy metals in eastern Spain, *Nicotiana glauca* R. Graham (shrub tobacco) was selected for biotechnological modification because it showed the most appropriate properties for phytoremediation [90]. This plant has a wide geographic distribution, grows fast with a high biomass, and is repulsive to herbivores. Following *Agrobacterium*-mediated transformation, the induction and overexpression of a wheat gene encoding phytochelatin synthase (*TaPCS1*) in this particular plant greatly increased its tolerance to metals such as Pb and Cd; it developed seedling roots 160% longer than those of wild-type plants. In addition, seedlings of transformed plants grown in mining soils containing high levels of Pb (1572 ppm) accumulated double the concentration of this heavy metal than that in wild-type plants. These results indicate that the transformed *N. glauca* represents a highly promising new tool for use in phytoremediation efforts.

It has been suggested that phytoremediation would rapidly become commercially available if metal-removal properties of hyperaccumulator plants, such as *T. caerulescens*, could be transferred to high biomass-producing species, such as Indian mustard (*Brassica juncea*) or maize (*Zea mays*) [91]. Biotechnology has already been successfully employed to manipulate metal uptake and tolerance properties in several species. For example, in tobacco (*Nicotiana tabacum*), increased metal tolerance has been obtained by expressing the mammalian metallothionein, metal-binding proteins, and genes [68,71].

Possibly, the most spectacular application of biotechnology for environmental restoration has been the bioengineering of plants capable of volatilizing mercury from soil contaminated with methylmercury. Methylmercury is a strong neurotoxic agent that is biosynthesized in Hg-contaminated soils. To detoxify this toxin, transgenic plants (*Arabidopsis* and tobacco) were engineered to express bacterial genes *merB* and *merA*. In these modified plants, *merB* catalyzes the protonolysis of the carbon–mercury bond with the generation of Hg^{2+}, a less mobile mercury species. Subsequently, *merA* converts Hg(II) to Hg(0), a less toxic, volatile element, which is released into the atmosphere [87,92].

Although regulatory concerns restrict the use of plants modified with *merA* and *merB*, this research illustrates the tremendous potential of biotechnology for environmental restoration. In an effort to address regulatory concerns related to phytovolatilization of mercury, Bizili et al. [33] demonstrated that plants engineered to express *MerB* (an organomercurial lyase under the control of a plant promoter) may be used to degrade methylmercury and subsequently remove ionic mercury via extraction. Despite recent advances in biotechnology, little is known about the genetics of metal hyperaccumulation in plants. In particular, the heredity of relevant plant mechanisms, such as metal transport and storage [93] and metal tolerance [94], must be better understood. Recently, Chaney et al. [95] proposed the use of traditional breeding approaches for improving metal hyperaccumulator species and possibly incorporating significant traits, such as metal tolerance and uptake characteristics, into high biomass-producing plants. Experiments have been conducted with genetically engineered plants for enhanced uptake of metals.

14.3.1 Mercuric Ion Reduction and Resistance

Bacteria have the ability to reduce a number of heavy metals to less toxic forms. Mercury resistance in Gram-negative bacteria is encoded by an operon, which includes mercuric ion reductase gene (*mer*A) among them. *Mer*A is a soluble NADPH-dependent, FAD-containing disulfide oxidoreductase. This enzyme converts toxic Hg^{2+} to the less toxic metallic mercury (Hg^0). *Escherichia coli* cells expressing the *mer*A gene were shown to possess a weak reduction activity toward Au^{3+} and Ag^+ in addition to reduction of Hg^{2+} [96]. The *mer*A gene also weakly increased Hg^{2+} tolerance of *Saccharomyces cerevisiae* [97]. These studies suggested that the *mer*A gene might affect metal tolerance when expressed in plant.

Initial attempts to express the bacterial *mer*A gene from Tn21 in plants to produce Hg^{2+} resistance were unsuccessful in spite of the use of very efficient plant expression systems. No full-length *mer*A RNA or *mer*A-encoded protein was detected. The original bacterial *mer*A sequence is rich in CpG dinucleotide having a highly skewed codon usage, both of which are particularly unfavorable to efficient expression in plants because they are exposed to methylation and subsequent gene silencing [87,98]. Therefore, a mutagenized *mer*A sequence (*mer*Ape9) was constructed, modifying 9% of the coding region, and was transformed to *Arabidopsis thaliana*. The seeds germinated and the seedlings grew on medium containing up to 100 m*M* Hg, although the transgenic plants expressed only low levels of *mer*A mRNA. Transgenic seedlings evolved two to three times the amount of Hg^0 compared to control plants. Plants were also resistant to toxic levels of Au^{3+}.

Rugh et al. [97] give a good example of a successful modification of a bacterial metal tolerance gene for expression in plants. Recently, Rugh and coworkers [98] reported on the development of transgenic yellow poplar (*Liriodendron tulipifera*) for mercury phytoremediation using *mer*A gene (*mer*A18) modified even further to optimize the codon usage in the plant. Transgenic plants evolved ten times the amount of Hg^0 compared to control plants. Thus far, this system has not been tested in field conditions. This is a convincing indication that genetic engineering may improve a plant's capacity to phytoremediate metal-polluted soils.

Partial success has been reported in the literature. For example, in an effort to correct for the small size of hyperaccumulator plants, Brewer [99] generated somatic hybrids between *T. caerulescens* (a Zn hyperaccumulator) and *Brassica napus* (canola) followed by hybrid selection for Zn tolerance. High biomass hybrids with superior Zn tolerance were recovered. These authors have also advocated a coordinated effort to collect and preserve germ plasm of accumulator species. Initially, phytoremediation trials were performed using plants known to accumulate metals and/or to possess metal tolerance — *Silene vulgaris* (Moench) Garcke L.; Brassicaceae plants *Brassica oleracea* and *Raphanus sativus*; and metal hyperaccumulators like *Thlaspi caerulescens* and *Alyssum* L. spp. [100]. Metal hyperaccumulators were most efficient at metal removal in these field trials.

In order to clean up a moderately contaminated soil, 6 and 130 croppings would be needed for zinc and cadmium, respectively. In pot trials, a low rate of biomass production, common to most hyperaccumulators, was shown to limit zinc removal from a contaminated soil by *T. caerulescens*. High biomass nonaccumulator Brassica crops were more effective [101]. For phytoextraction to become a viable technology, dramatic improvements would be required in hyperaccumulator biomass yield or nonaccumulator metal accumulation [102]. Plants able to tolerate and accumulate several metals are required; polluted soils often contain high levels of several contaminant trace elements. Soils polluted with arsenic, cadmium, lead, or mercury provide major targets for remediation. To date, no plants that reproducibly hyperaccumulate lead or mercury have been identified. In most naturally occurring tolerant plants studied to date, tolerance to arsenic or lead appears to be based on exclusion from the plant [103–108].

The development of a phytoremediation technology for some trace elements is thus likely to require the transfer of genes into plants across species borders. Although little is known about the molecular basis of trace element detoxification and hyperaccumulation in plants, a number of trace element detoxification systems from bacteria and yeast have been characterized genetically and

functionally at the molecular level for the detoxification of metals [108]. Despite the difficulty in predicting the effects of microbial genes in a complex multicellular organism like a plant, the successful introduction of a modified bacterial mercuric ion-reductase gene into yellow poplar (*Liriodendron tulipifera*) and *Nicotiana tabacum* demonstrates that bacterial genes may be extremely valuable in phytoremediation [92,98].

Furthermore, *ZIP* genes that confer Zn uptake activities in yeast have also recently been described. Moffat [34] reported the characterization of two *Arabidopsis* mutants that were resistant to high levels of aluminum. The genes have yet to be cloned, but one of the mutants, on chromosome 1, secretes organic acids to bind Al in the soil before it enters the plant. The second mutant, mapped to chromosome 4, increased the flux of hydrogen outside the root, changing the pH, which transformed the Al^{3+} ions into aluminum hydroxides and aluminum precipitates. These forms are incapable of entering the plant via the roots.

Rate-limiting steps in selenium assimilation and volatilization have been deduced in Indian mustard. ATP sulfurylase was determined to be involved in selenate reduction and, when overexpressed in Indian mustard, conferred Se accumulation, tolerance, and volatilization. The *Arabidopsis* transgenic plants with *mer* operon have conferred tolerance to gold. Schmöger et al. (2000) [87] demonstrated that phytochelatins are involved in detoxification of arsenic. This has important implications for the glutathione, phytochelatin synthetase metabolic pathway transgenics.

Vatamaniuk et al. (1999) [108] 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. 1999 [83] identified a wheat cDNA, *TaPCS1,* that increased Cd resistance in wild-type yeast. Just like *AtPCS1,* tl resistance mediated by *TaPCS1* was associated with an increase in Cd accumulation and was dependent on GSH. Both *AtPCS1* and *TaPCS1* metal tolerance is GSH dependent. For further evidence of the role of PCs, refer to chapter 16 in this book. Overexpression of glutathione synthetase and g-glutamyl cysteine synthetase enhances cadmium accumulation and tolerance in Indian mustard plants (Zhu et al., 1999 a, b) [109, 110]. These studies show that the manipulation of GSHa concentrations has significant potential for increasing the plant accumulation of metals (Meagher 2000) [111].

Because most metal hyperaccumulators are slow growing and have low biomass, bioengineering of nonaccumulators is essential for effective phytoremediation. Conventional breeding approaches have also been proposed to improve plants for metal extraction. However, the success of this approach is doubtful due to sexual incompatibility between the parent lines [93]. Biotechnology has the potential of overcoming this limitation. However, comprehensive knowledge of the genetic basis for hyperaccumulation is essential for effective use of biotechnology to design transgenic plants capable of efficient phytoremediation.

14.4 ZINC-TRANSPORTING GENES IN PLANTS

Zinc is a constituent of several enzymes: carbonic anhydrase dehydrogenases; aldolases; Cu/Zn superoxide dismutase; isomerases; transphosphory-lases; and RNA and DNA polymerases. Therefore, Zn deficiency results in malfunction or no function of these enzymes. Zn-metalloproteins (Zn-finger motif) are regulators of gene expression (DNA-binding transcription factors). In the absence of these, RNA polymerase cannot complete its function of transcribing genetic information from DNA into RNA. Zinc is a cofactor of more than 200 enzymes, such as oxidoreductases, hydrolases, transferases, lyases, isomerases, and ligases. Many of the metalloenzymes are involved in the synthesis of DNA and RNA and protein synthesis and metabolism.

Metal-transporting genes have been identified in *Arabidopsis* (Brassicaceae). Overexpression of an *Arabidopsis* zinc transporter cation diffusion facilitator (CDF) gene enhanced resistance to Zn accumulation. Transgenic plants showed increased Zn uptake and tolerance and antisense of this gene led to wild-type Zn tolerance in transgenic plants. Zinc transporters can be manipulated to increase selectivity and accumulation of metal ions. In Brassicaceae, about 21 species belonging

to three genera (*Cochlearia, Arabidopsis*, and *Thlaspi)* are reported to be zinc hyperaccumulators. Zhao et al. [112] isolated and identified the gene *ZNT1* as one of the micronutrient transport genes with high sequence homology with other Zn transport genes isolated from yeast.

A family of zinc transporter genes that responds to zinc deficiency has also been identified in *Arabidopsis*. Zn hyperaccumulation in *Thlaspi caerulescens* is because of the *ZNT1* gene, which encodes a high-affinity Zn transporter. This gene is constitutively expressed at a much higher level in *T. caerulescens* than in *T. arvense*, where its expression is stimulated by Zn deficiency. In fact, plant Zn status is shown to alter the normal regulation of Zn transporter genes in *T. caerulescens*. An important aspect of Zn hyperaccumulation and tolerance in *T. caerulescens* is also the production of low molecular weight compounds involved in Zn detoxification in the cell (cytoplasm and vacuole) and in the long-distance transport of Zn in the xylem vessels.

Citrate was not shown to play an important role in Zn chelation and malate had constitutively high concentrations in the shoots of the accumulator *T. caerulescens* and the nonaccumulator *T. ochroleucum*. More recently, direct measurements of the *in vivo* speciation of Zn in *T. caerulescens* using the noninvasive technique of x-ray absorption have revealed that histidine is responsible for the transport of Zn within the cell, whereas organic acids (citrate and oxalate) chelate Zn during long-distance transport and storage. Another constitutive aspect of *T. caerulescens* is the high Zn requirement for maximum growth, compared to other species. This probably depends on the strong expression of the metal sequestration mechanism, which would subtract a large amount of intracellular Zn to normal physiological processes even when the Zn supply is low.

Several transporters implicated in the uptake of divalent nutrient cations like Ca^{2+}, Fe^{2+}, or Zn^{2+} appear to be able to transport other divalent cations. For example, heterologous expression in yeast of IRT1 from *A. thaliana*, an iron-repressed transporter in the *ZIP* family of metal transporters, suggests a broad-range specificity of transport for Cd^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, and possibly other divalent cations (TC 2.A.5.1).

The expression of IRT1 is strongly induced in plants under conditions of iron deficiency and is repressed in iron-replete plants. This correlates well with the finding that cadmium uptake is enhanced in iron-deficient pea seedlings. However, these transporters are tightly regulated at the transcriptional and post-transcriptional levels, and, to date, no reports on plants engineered to overexpress transporters of the *ZIP* family have been issued. Tobacco plants engineered to contain increased amounts of NtCBP4 protein (TC 1.A.1.5.1), a putative cyclic-nucleotide and calmodulin-regulated cation channel in the plasma membrane, displayed an increased sensitivity to lead, a 1.5- to 2.0-fold shoot accumulation of lead, and an increased nickel tolerance. Yeast cells expressing the wheat *LCT1* cDNA (TC 9.A.20.1.1), encoding a low-affinity cation transporter, were hypersensitive to Cd^{2+} and accumulated increased amounts of cadmium [84]. Plants overexpressing *AtNramp3* (TC 2.A.55), a member of the Nramp family of metal transporters, were hypersensitive to Cd^{2+}, but enhanced cadmium accumulation was not observed.

In phytoremediation, it must be considered that a transporter capable of transporting a specific contaminant metal cation is capable of transporting other competing cations, like Ca^{2+} or Zn^{2+}, under natural soil conditions if the latter ions are present in large excess. Therefore, it is desirable to better understand what governs the specificity of membrane transporters, in order to generate mutated transporters with altered specificities [84]. Understanding the regulation of *ZIP* family members in *T. caerulescens* and analyzing *Arabidopsis* mutants with altered metal responses will also help to identify novel target genes and strategies for the generation of plants with enhanced metal uptake [93].

To date, numerous examples have been demonstrated to have the potential for phytoremediation — for example, Pb, Ni, Zn, Al, Se, Au, and As. Arazi et al. [28] have described a tobacco plasma membrane calmodulin-binding transporter that confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity. Zinc-transporter To investigate the *in vivo* role of this gene, transgenic plants with the ZAT coding sequence exhibited increased Zn resistance and accumulation in the roots at high Zn concentrations [114].

In maize, zinc accumulation was found to be genetically controlled and affected by additive genes [115]. Four genes were found to be the minimum segregation factors in the (high × low) crosses for Zn accumulation. Zinc deficiency also increases root exudation of amino acids, sugars, and phenolic substances at different degrees in different species [116].

Three wheat genotypes (*Triticum aestivum* and *T. turgidum*) differed in their root-growth response to low zinc levels [117]. The zinc-efficient genotype increased root and shoot dry matter and developed longer and thinner roots (a greater proportion of fine roots with diameter of 0.2 mm) compared with the less efficient genotype. Due to a larger root surface area, the efficiency of zinc uptake increased. In wheat, Zn can be remobilized from leaves under Zn deficiency. Also, spinach, potato, navy bean, tomato, sorghum, and maize show great variations in Zn efficiency [118,119].

Arabidopsis thaliana has multiple Zn transporters designated ZIP1, *ZIP2*, *ZIP3*, and *ZIP4*. Grotz et al. [119] demonstrated the specificities of each of the *ZIP* genes with their experiments. They tested other metal ions for their ability to inhibit Zn uptake mediated by these proteins. Zn uptake by *ZIP1* was not inhibited by a tenfold excess of Mn, Ni, Fe, and Co. Zn was the most potent competitor, demonstrating that *ZIP1* prefers Zn as its substrate over theses metal ions. Cd and Cu also inhibited Zn uptake, but to a lesser extent. This suggests that Cd and Cu may also be substrates for *ZIP1*.

The *ZIP* family members have 309 to 476 aa; this range is largely due to variation in the number of residues between transmembrane domains III and IV, a domain designated as "variable." The amino acid sequences of all the known *ZIP* family members were aligned, and a dendrogram describing their sequence similarities was generated [120]. *ZNT1* is 379 aa in length and shares the same structural features exhibited by the other members of the *ZIP* family, including eight putative transmembrane domains and a highly hydrophilic cytoplasmic region predicted to reside between transmembrane domains three and four. The putative cytoplasmic domain contains a series of histidine repeats, which may define a metal-binding region for the transporter. Zinc transporters can be manipulated to increase selectivity and accumulation of metal ions.

Pence et al. [113] reported on the cloning and characterization of a high-affinity Zn^{2+} transporter cDNA, *ZNT1*, from the Zn/Cd-hyperaccumulating plant, *Thlaspi caerulescens*. Through comparisons to a closely related, nonaccumulator species, *Thlaspi arvense*, the researchers determined that the elevated ability of *T. caerulescens* to take up Zn and Cd was due, in part, to an enhanced level of expression of Zn transporters. Previous physiological studies by the group indicated that the hyperaccumulating ability of *T. caerulescens* was linked to Zn transport at a number of sites in the plant. The researchers transformed a Zn transport-deficient strain of yeast, ZHY3, with a cDNA library from *T. caerulescens*. By screening for growth on low-Zn medium, they were able to isolate seven clones, five of which represented a 1.2-kb cDNA designated *ZNT1* (for Zn transporter) that restored the yeast's ability to grow under low-Zn conditions. The *ZNT1* gene displayed considerable identity to two *Arabidopsis* thaliana metal transporter genes, *ZIP4* (for transporting Zn) and *IRT1* (for transporting Fe).

For purposes of comparison, they then cloned the homolog of *ZNT1* (designated *ZNT1*-arvense) from the nonhyperaccumulator species *T. arvense*. Expression studies using northern blots of RNA isolated from the roots and shoots of both *Thlaspi* species revealed that *ZNT1* is expressed in *T. caerulescens* at extremely high levels. In contrast, expression of *ZNT1* in *T. arvense* could only be detected at a very low level in shoots and roots, and then only when the plants had been exposed to conditions of Zn deficiency. To further explore the role of Zn status on *ZNT1* expression, Pence et al. [113] exposed both species to a range of Zn concentrations. They found that when *T. caerulescens* was grown in a nutrient solution containing an excess of Zn (50 μM), the transcript level of *ZNT1* decreased, indicating that *ZNT1* is not expressed constitutively in *T. caerulescens*. *ZNT1* transcript levels in *T. arvense* appeared to be unaffected by exposure to excess Zn.

Transport studies in *T. caerulescens* show that *ZNT1* mediates high-affinity Zn transport. In many plant species, the induction of a high-affinity transporter is characteristic of a nutrient-deficiency response and would correlate with the expression pattern observed for *ZNT1* in *T. arvense*.

The authors speculate that the hyperaccumulation phenotype in *T. caerulescens* may then be due to a mutation in the plant's ability to sense or respond to Zn levels — that is, these plants may be functioning as if they are constantly experiencing Zn deficiency. They propose that this is likely the result of a change in global regulation linked to the plant's overall Zn status; this supports the concept that the Zn hyperaccumulation phenotype, at least in this species, is due to a change in the regulation and not the constitutive expression of a single gene.

Several mutants with altered response to heavy metals have been isolated from *A. thaliana*. Cadmium-hypersensitive mutants with defects in phytochelatin synthetase and possibly in g-glutamylcysteine synthetase and glutathione synthetase have been isolated by Howden et al. [65,66]. Chen and Goldsbrough [120] found an increased activity of g-glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. Some of these genes may prove useful in modifying suitable target plants for phytoremediation, although there are doubts about the usefulness of genes involved in phytochelatin synthesis [122].

14.5 FERRITIN EXPRESSION IN RICE

Ferritin is an iron storage protein found in animals, plants, and bacteria. It comprises 24 subunits, which may surround in a micellar up to 4500 ferric atoms [123]. It provides iron for the synthesis of iron proteins such as ferredoxin and cytochromes. It also prevents damage from free radicals produced by iron/dioxygen interactions. Ferritin has been found to provide an iron source for treatment of anemia. It was thus proposed that increase of the ferritin content of cereals by genetic modification may help to solve the problem of dietary iron deficiency. To increase the Fe content of rice, Goto et al. [25] transferred soybean ferritin gene into the plant. Using the rice seed storage protein glutelin promoter (GluB-1), they could target the expression of ferritin in developing seeds. The Fe content in transformed seeds was threefold compared to that in control seeds.

14.6 GENETIC MANIPULATION OF ORGANIC ACID BIOSYNTHESIS

It has been proposed that metal tolerance could be based on the organic acid-formed complexes. Ernst [123] observed high malate concentrations in Zn- and Cu-tolerant plants; also the content of citrate was increased. Hyperaccumulators are heavily loaded with these acids and acid anions might have some function in metal storage or plant internal metal transport. Free histidine has been found as a metal chelator in xylem exudates in plants that accumulate Ni and the amount of free histidine increases in Ni exposure [80]. By modifying histidine metabolism, it might be possible to increase the Ni-accumulating capacity of plants.

During the past few years, several metal transporters have been isolated from *Arabidopsis*: Zn transporters *ZIP1*, *3, 4* [120]; Fe transporter IRT1 [125]; and Cu transporter *COPT1* [126, 127]. Several transporters, like *ZIP1*, *ZIP3* and *IRT1*, are expressed in response to metal deficiency. *IRT1* may also play a role in the uptake of other metals because Cd, Zn, Co, and Mn inhibited Fe uptake of *IRT1* [125]. Changing the regulation of the expression of these transporters may modify the uptake of metals to the cells or organelles in a useful way.

Most of the studies aimed at determining the role of organic acid excretion have been carried out by comparing different plant species or nonisogenic lines of the same plant species. Plant transformation allows the production of genetically identical plants that differ only in one or a few genes. Taking advantage of this technology, the author's research group produced transgenic plants with an enhanced capacity to synthesize and excrete citrate. It was reasoned that, by overproducing citrate (one of the most powerful cation chelators in the organic acid group), the actual relevance of organic acids in several aspects of the plant–soil relationship could be elucidated.

To produce citrate-overproducing (CSb) plants, the coding sequence of the bacterial citrate synthase gene was placed under control of the 35S CaMV promoter and the nopaline synthase 3% end sequence (35S–CSb). This construct was used to transform tobacco and papaya plants. To determine whether the expression of a citrate synthase in plant cells leads to an increase in their citrate content, total and root extracts of transgenic lines were examined by HPLC and compared to control plants. It was found that the tobacco lines expressing the 35S–CSb construct had up to tenfold higher levels of citrate in their root tissue. The amount of citrate exuded by the roots of these transgenic lines was also increased up to fourfold as compared to control plants [81].

De la Fuente et al. [81] characterized the novel transgenic CSb lines to determine whether these plants were tolerant to aluminum. Experimental evidence suggests that the citrate-overproducing plants could tolerate a tenfold higher Al concentration than control plants. A mitochondrial citrate synthase of *Arabidopsis thaliana* was introduced into carrot (*Daucus carota*) cells by *Agrobacterium tumefaciens*. Several transgenic carrot cell lines that produced the *Arabidopsis* CS polypeptides and had high CS activity were identified. The increase in CS expression resulted in an enhanced capacity of phosphate uptake from insoluble sources of P in these transgenic cells [81,88].

More recently, this research group has shown that transgenic *Arabidopsis* plants that express high levels of the carrot citrate synthase have an enhanced aluminum tolerance. It has been reported that organic acid excretion by lupin plants constitutes a drain of 5 to 25% of the total fixed C; however, this does not appear to affect dry matter production significantly. This fact has also been confirmed in transgenic tobacco plants that overproduce citrate, which grow efficiently even at high levels of P-fertilization [83]. The insights obtained from transgenic models highlight the potential of organic acid manipulation to generate novel crops more efficient in the use of soil P and well adapted for growth in marginal soils [89].

14.7 MOLECULAR GENETIC AND TRANSGENIC STRATEGIES FOR PHYTOREMEDIATION HYPERACUMULATION

Transgenic plants capable of tolerating high levels of accumulated cadmium and lead have been developed recently [128]. These plants take up heavy metals more rapidly than traditional bioremediation plants do, thus making them potential hyperaccumulators with application for phytoextraction and rhizofiltration in the field [129-131].

Observing that certain *Saccharomyces cerevisiae*, which possess the *YCF1*, or yeast cadmium factor 1 protein, are known to pump cadmium [Cd(II)] into vacuoles, Li et al. [129] tested whether *YCF1* would also confer resistance to lead [Pb(II)]. Also known as vacuolar glutathione S-conjugate transporter, *YCF1* belongs to the ATP-binding cassette superfamily [2,3]. Li's team confirmed that *YCF1* gene expression permitted *S. cerevisiae* to withstand the toxic effects of 3 m*M* lead (Pb II) and 0.1 m*M* cadmium (Cd II) concentrations in growth media. This protection against lead and cadmium toxicity was due to the uptake and storage of the heavy metals in yeast vacuoles.

Next, Li's group attempted to determine whether *YCF1* expression in plants produced the same results. *Arabidopsis thaliana* was investigated as a model for *YCF1* expression. First, the *YCF1* gene was created using RT-PCR from *YCF1* expressing yeast. For expression in *A. thaliana*, Li and colleagues subcloned the *YCF1* gene into two vectors: PBI121 and pCambia1302. To enhance expression in plants, the pCambia1302 vector was cloned with four copies of the CaMV 35S promoter. *Agrobacterium tumefaciens* was used for transformation of *A. thaliana*. Green fluorescent protein tagged to *YCF1*, used as an expression reporter, indicated the presence of *YCF1* protein in the vacuolar as well as in the plasma membrane of the transformed *Arabidopsis* cells.

Li and coworkers investigated the uptake and sequestering of lead and cadmium in the plants. Transformed *A. thaliana* was grown on gravel supplemented with half-concentration Murashige–Skoog agar medium containing 0.75 m*M* lead or 70 μ*M* cadmium. After 3 weeks, the plant

tissues were analyzed for metal uptake using atomic absorption spectroscopy. Li's findings showed that the transgenic plants were as effective as naturally occurring hyperaccumulators. Although the transgenic plants accumulated less than twofold higher concentrations of Cd and Pb compared to wild-type, this is likely much less than the hyperaccumulator plants (mentioned earlier). Plants that exclude heavy metals have been demonstrated by expressing bacterial heavy metal transporter in *Arabidopsis* that enhances resistance to and decreases uptake of heavy metals [131].

Genetic strategies and transgenic plant and microbe production and field trials will fetch phytoremediation field applications [20,104,114,132–134]. Mercury is a worldwide problem as a result of its many diverse uses in industry. Mercury has been used in bleaching operations (chlorine production, paper, textiles, etc.); as a catalyst; as a pigment for paints; for gold mining; and as a fungicide and antibacterial agent in seeds and bulbs. Elemental mercury, Hg(0), can be a problem because it is oxidized to Hg^{2+} by biological systems and subsequently is leached into wetlands, waterways, and estuaries. Additionally, mercury can accumulate in animals as methylmercury (CH_3–Hg^+), dimethylmercury ($CH_3)_2$–Hg) or other organomercury salts. Organic mercury produced by some anaerobic bacteria is one to two orders of magnitude more toxic in some eukaryotes; it is more likely to biomagnify than ionic mercury and efficiently permeates biological membranes. Monomethyl–Hg is responsible for severe neurological degeneration in birds, cats, and humans.

Certain bacteria are capable of pumping metals out of their cells and/or oxidizing, reducing, or modifying the metal ions to less toxic species. One example is the *mer* operon, which contains genes that sense mercury (*mer*B); transport mercury (*mer*T); sequester mercury to the periplasmic space (*mer*P); and reduce mercury (*mer*A). *Mer*B is a subset of the *mer* operon and is capable of catalyzing the breakdown of various forms of organic mercury to Hg^{2+}. *Mer*B encodes an enzyme, organomercurial lyase, that catalyses the protonolysis of the carbon–mercury bond. One of the products of this reaction is ionic mercury [92,135]:

$$Hg^{2+}.\ R\text{–}CH_2\text{–}Hg^+ \text{ ----}mer\text{B----}>R\text{–}CH_3 + Hg(II)$$

$$Hg(II) + NADPH \text{ ----}mer\text{A----}>Hg(0) + NADP^+ + H^+$$

Hg(0) (elemental mercury) can be volatilized by the cell.

14.8 CONCLUSIONS

Tree crop improvement through biotechnology is important for the remediation of contaminated environments because trees have extensive root systems to ensure an efficient uptake of pollutants (cadmium, mercury, pesticides) from soil and provide the possibility of several cycles of decontamination with the same plants. Transgenic trees with increased tolerance to heavy metals might be a solution towards sustainable development. Therefore, if overexpressed in annual and perennial crops in combination with bacterial enzymes for GSH synthesis, specific glutathione-S-transferases and phytochelatin synthase might go a long way. The genetic manipulation of organic acid metabolism could be used to develop transgenic varieties more adapted to marginal soils and more efficient in the assimilation of nutrients.

Alkaline soils of semiarid climates, which have traditionally sustained traditional rain-fed cultivation — however low P or Fe availability may be (even when fertilizers have been used) — have strongly limited more productive crop management [85,87]. The knowledge of organic acid biosynthesis, its universal occurrence in plants, and the effectiveness of organic acid exudation in conferring Al tolerance and enhanced P uptake from sparingly soluble P compounds makes the organic acid pathway a promising target to develop transgenic varieties better adapted to grow in marginal soils. A major opportunity is to modify the quality and quantity of these organic compounds to target the rhizosphere; in this way, genetic manipulation can contribute to a better understanding of the specific

carbon substrates preferred by beneficial microorganisms, such as N2-fixing soil bacteria and mycorrhizal fungi.

Currently, many different genes involved in organic acid biosynthesis have been cloned from several organisms. Through genetic engineering, it could be possible to overexpress these genes under the regulation of strong and tissue-specific plant promoters. The incorporation of transgenic crops into integrated plant management and land use strategies could represent a promissory option for agricultural expansion with a lower environmental cost. It is certainly a research priority to achieve a more sustainable agriculture for future generations.

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