Transgenic Plants in Phytoremediation: Recent Advances and New Possibilities

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Phytoremediation, the use of plants and their associated microbes to remedy contaminated soils, sediments, and groundwater, is emerging as a cost-effective and environmentally friendly technology. Due in large part to its aesthetic appeal, this technology has gained increasing attention over the past 10 years. Phytoremediation uses different plant processes and mechanisms normally involved in the accumulation, complexation, volatilization, and degradation of organic and inorganic pollutants. Certain plants, called hyperaccumulators, are good candidates in phytoremediation, particularly for the removal of heavy metals. Phytoremediation efficiency of plants can be substantially improved using genetic engineering technologies. Recent research results, including overexpression of genes whose protein products are involved in metal uptake, transport, and sequestration, or act as enzymes involved in the degradation of hazardous organics, have opened up new possibilities in phytoremediation. This paper provides a critical review of the recent progress made toward the development of transgenic plants with improved phytoremediation capabilities and their potential use in environmental cleanup.

Introduction

Extensive mining, agriculture, industry, and military operations have released enormous amounts of toxic compounds into the environment. Of these, heavy metals or metalloids and organic pollutants pose a serious threat to plants and animals, including humans. It is, therefore, urgent to adequately remove these pollutants from the contaminated sites. The conventional methods of remediation of these sites, including soil excavation and land filling, soil washing, and immobilization or extraction by physicochemical techniques, are rather ineffective and expensive. Moreover, they often destroy natural habitats and leave unsightly scars on the landscape (1). As a result, cost-effective alternative methodologies have arisen, such as bioremediation. In bioremediation, microorganisms are used to breakdown contaminants into harmless products. This process requires high microbial density and a continuous nutrient input in cases such as the use of bioreactors (2).

As an alternative, the use of plants as cleaners of metal or organic pollutants has gained increasing importance in recent years, giving rise to the concept of phytoremediation (3, 4). Phytoremediation is an emerging low-cost technology that utilizes plants to remove, transform, or stabilize contaminants including organic pollutants located in water, sediments, or soils. The advantages of phytoremediation over usual bioremediation by microorganisms are that plants, as autotrophic systems with large biomass, require only modest nutrient input and they prevent the spreading of contaminants through water and wind erosion (5). Plants also supply nutrients for rhizosphere bacteria, allowing the growth and maintenance of a microbial community for further contaminant detoxification.

Numerous plant species have been identified for the purpose of phytoremediation (Table 1). Certain plant species, known as hyperaccumulators, are attractive candidates as they are able to accumulate potentially phytotoxic elements to concentrations 50–500 times higher than average plants (6). The high bioconcentration factor and the efficient root-to-shoot transport system endowed with enhanced metal tolerance provide hyperaccumulators with a high potential de-toxification capacity (7). However, many of the hyperaccumulators are slow growing and have reduced biomass production, thus requiring several years for decontamination of the polluted sites. Trees, on the other hand, appear as an attractive alternative due to their extensive root system, high water uptake, rapid growth, and large biomass production (8, 9).

The remedial capacity of plants can be significantly improved by genetic manipulation and plant transformation technologies (3, 9, 10, 11). The identification of unique genes from hyperaccumulators, and their subsequent transfer to fast-growing species, have proven to be highly beneficial as demonstrated by the recent success of transgenic plants with improved phytoremediation capacity (12, 13).

This review aims to present an overview of recent research designed to improve the phytoremediation capacity of plants by transgenic approaches, including genes and traits being manipulated for organic and inorganic pollutant decontamination. The use of transgenic tree species in phytoremediation programs is also highlighted.

Factors Affecting Phytoremediation

There are several options for the remediation of contaminated sites using plants. Different phytotechnologies have already been put into practice and each one uses different plants or plant properties. Faster growth rate, high biomass, hardiness, and tolerance to pollutants are some of the favorable plant properties being exploited for remediation. In addition, various biological processes such as plant–microbe interactions can affect the remediation efficiency. However, the
underlying mechanisms of the biological processes involved are largely unknown. Therefore, a more thorough understanding of these processes is urgently needed. Among these, plant uptake of water and contaminants, plant–microbe interactions, enhanced microbial activity in the rhizosphere, fate and transport of contaminants in plant root zone, further translocation, and tolerance mechanisms are of paramount importance in developing improved phytoremediation technologies.

Types of Pollutants

There are two major classes of contaminants: organic and inorganic. Organic contaminants include different compounds such as petroleum hydrocarbons, chlorinated solvents, halogenated hydrocarbons such as trichloroethylene (TCE), and explosives such as trinitrotoluene (TNT). When compared to inorganics, the organic pollutants are relatively less toxic to plants because they are less reactive and do not accumulate readily.

Inorganic compounds include heavy metals such as mercury, lead, and cadmium, and nonmetallic compounds such as arsenic and radionuclides like uranium. Some metals are essential for the normal growth and development of life forms. However, at high concentrations, metals become toxic and lead to oxidative stress with production of reactive oxygen species and free radicals, which are highly damaging to cells (14, 15). Some metal ions are particularly reactive and can interfere with the structure and function of proteins in living systems. High concentration of inorganic pollutants may also lead to replacement of other essential nutrients (16).

Fate and Transport of Organic Contaminants in Plants

Uptake and Transport. The use of plants to remove organic pollutants was derived from the observation that organic pollutants disappear more quickly from vegetated soil than from barren soil (17) and this was later confirmed in studies of plant-mediated degradation of petroleum contaminants (18).

As organic compounds are usually man-made and xenobiotic in plants, there are no transporters for their uptake and the usual mechanism of uptake is by simple diffusion (passive uptake). When organic contaminants come into contact with roots, they may be sorbed to the root structure. The hydrophobic or hydrophilic nature of the organic compounds also determines their possible uptake. Hemicellulose in the cell wall and the lipid bilayer of plant membranes can bind hydrophobic organic pollutants effectively. Hydrophobicity was related to the octanol–water partition coefficient (log K_{ow}) of the organics. The greater the hydrophobicity of the chemical (as measured by the log K_{ow}), the greater its tendency to partition out of the aqueous phase and onto roots. Organics with a log K_{ow} between 0.5 and 3 are hydrophobic enough to move through the lipid bilayer of membranes, and still water soluble enough to travel into the cell fluids. On the other hand, if organics are too hydrophilic (log K_{ow} < 0.5) they cannot pass through membranes and will never get into the plant (19). Briggs et al. (20) defined the root concentration factor (RCF) as the ratio of organic chemical sorbed on the root to that in the hydroponic solution (RCF = equilibrium concentration in roots:equilibrium concentration in external solution). Burken and Schnoor (21) also reported a similar relationship for organic contaminants from waste sites using hydroponically grown hybrid poplars. Also, the root uptake of chemicals depends on the plant’s uptake efficiency, the transpiration rate, and the chemical concentration in soil water. The transpiration stream concentration factor (TSCF) is the ratio of the compound concentration in the xylem fluid relative to the external solution, and is a measure of uptake into the plant shoot. The correlation found for organics with respect to TSCF was similar to that in the case of hydrophobicity and RCF. Therefore, compounds with a log K_{ow} between 0.5 and 3 are easily transported to the xylem and translocated to the shoot.

Further, organic pollutants can be degraded or mineralized by plants, either independently or in association with microorganisms. For example, organics like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, and petroleum hydrocarbons are sufficiently degraded by rhizospheric microbial activity (22). Plants have significant metabolic activity in both roots and shoots, and some of the enzymes involved in these metabolic processes, namely nitroreductases, dehalogenases, laccases, peroxidases, etc., are useful in the remediation process (23, 24).

Enzymatic Transformation. Phytotransformation is a process by which plants uptake organic pollutants and, subsequently, metabolize or transform them into less toxic metabolites. Once taken up and translocated the organic chemicals generally undergo three transformation stages: (a) chemical modification (oxidations, reductions, hydrolysis); (b) conjugation (with glutathione, sugars, amino acids); and (c) sequestration or compartmentation (conjugants are converted to other conjugates and deposited in plant vacuoles or bound to the cell wall and lignin) (25).

Plant enzymes that typically catalyze the first phase of the reactions are P450 monooxygenases and carboxylesterases (26, 27). The second phase involves conjugation to glutathione (GSH), glucose, or amino acids, resulting in soluble, polar compounds (28). For instance, detoxification of herbicides in plants is attributed to conjugation with glutathione catalyzed by glutathione S-transferase (GST) (29). It was also reported that a group of GSTs mediate conjugation of organics to GSH in the cytosol (30, 31). Sometimes organic pollutants, such as atrazine and TNT, are partially degraded and stored in vacuoles as bound residues (32). The third phase of plant metabolism is compartmentation and storage of soluble metabolites either in vacuoles or in the cell wall matrix. The glutathione S-conjugates are actively transported to the vacuole or apoplastic by ATP-dependent membrane pumps (33). Also, an alternate conjugation–sequestration mechanism for organics exists in plants and involves coupling of a glucose or malonyl group to the organic compound, followed by the transport of the conjugate to the vacuole or the apoplast (26).
Fate and Transport of Inorganic Contaminants in Plants

Uptake of Inorganics. Plant tolerance to heavy metals depends largely on plant efficiency in the uptake, translocation, and further sequestration of heavy metals in specialized tissues or in trichomes and organelles such as vacuoles. The uptake of metals depends on their bioavailability, and plants have evolved mechanisms to make micronutrients bioavailable. Chelators such as siderophores, organic acids, and phenolics can help release metal cations from soil particles, increasing their bioavailability. For example, organic acids (malate, citrate) excreted by plants act as metal chelators. By lowering the pH around the root, organic acids increase the bioavailability of metal cations (34). However, organic acids may also inhibit metal uptake by forming a complex with the metal outside the root. Citrate inhibition of Al uptake and resulting aluminum tolerance in several plant species is an example of this mechanism (35–37). Copper tolerance in Arabidopsis is also the result of a similar mechanism (38). The presence of rhizosphere microbes may also affect plant uptake of inorganics. For example, rhizosphere bacteria can enhance plant uptake of mercury and selenium (39). However, the exact mechanisms of these plant–microbe interactions are largely unknown. It is possible that the microbe-mediated enhanced uptake may be due either to a stimulatory effect on root growth or to microbial production of metabolites that could affect plant gene expression of transporter proteins, or to a microbial effect on the bioavailability of the element (40).

Translocation and Sequestration. Once taken up by root cells, metal ions find their way to the shoot and then to their final intracellular destination, such as the vacuoles, by a process called translocation. It is believed that the increased tolerance of hyperaccumulators is associated with the presence of high-affinity chelating molecules in the cytoplasm. For example, phytochelatins (cysteine- and glutathione-rich compounds) help to sequester metals such as Ag, Cd, Cu, and Ni and thus protect cells from their harmful effects on surrounding proteins (41). In Thlaspi caerulescens, Zn is believed to be complexed with histidine in root cells and organic acids in the shoot and finally the complexed metals are transported and sequestered in the vacuoles, which account for the plant hypotolerance to metals (7). After chelation by GSH or phytochelatins (PCs), a transporter (usually an ABC-type) actively transports the metal–chelate complex to vacuoles where it may undergo further complexation with sulfide (42).

Membrane transport systems are likely to play a central role in the translocation process. Many gene families that are involved in metal transport were identified. Some of them are heavy-metal ATPases, natural resistance-associated macrophage proteins (NRAMPs), cation diffusion facilitators, the Zrt- and Irt-like proteins family, and cation antiporters (43). Transporters are also important in the regulation and storage of metals in the cell wall or in organelles, such as vacuoles, and in epidermal cells or trichomes (44–46). The specificity of membrane transporters is important but it is still poorly understood, thus needing further exploration (47); therefore, better knowledge of the transporters, chelators, sequestration, and tolerance mechanisms would be helpful in engineering plants for improved phytoremediation capabilities toward inorganic pollutants. Some of the promising results in this direction will be discussed in later sections.

Phytoremediation Strategies

Phytoremediation is a broad expression comprising different strategies used by plants to decontaminate soil and water, namely, rhizofiltration, phytostabilization, phytodegradation, phytotransformation, and phytovolatilization (48).

Rhizofiltration involves the elimination of aquatic waste material by the plant root system (49, 50). In this process, plants are grown in hydroponics and later transplanted into metal-polluted wastewater, where they absorb and accumulate metals in roots and shoots (51). Hairy roots induced in some of the hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclides and heavy metals (52, 53). Phytostabilization is an important strategy, in which plants reduce the mobility and bioavailability of pollutants in the environment either by immobilization or by preventing their migration (54, 55).

In phytoextraction, plants remove metals from the soil and concentrate them in the harvestable parts (60). Phytotransformation involves the uptake of contaminants from the soil, followed by translocation and accumulation, mainly in the shoot tissue. Plant biomass is then harvested and subsequently incinerated to remove the contaminant permanently from the site.

Phytovolatilization is used to extract volatile elements such as selenium and mercury (in some cases) from sludge and soils and release them through transpiration to the atmosphere as a detoxified vapor (13, 61–63). In general, phytoextraction is considered as the main option to remove heavy metals and metalloids, whereas phytodegradation is applied mostly to organic contaminants. The use of conventional plants in phytoremediation and the details of all of these phytotechnologies were previously elaborated (19, 64).

Transgenic Plants and Phytoremediation

Phytoextraction. Phytoextraction is the best solution for the removal of contaminants that cannot be degraded. More than 400 species of natural hyperaccumulators were identified (7). One of the most studied hyperaccumulators is Thlaspi caerulescens (pennywort) (65). Two important factors that make a plant an efficient phytoextractor are its biomass production and its bioconcentration efficiency (bioconcentration is defined as the ratio between the concentration of the pollutant in the harvestable parts of the plant and its concentration in the soil). Although hyperaccumulators are good candidates for phytoremediation, many of them are low biomass plants. Using genetic engineering methodologies, it is now possible to transfer the appropriate genes or hyperaccumulation traits into high biomass plants. Also, transfer and overexpression of genes taken from bacteria, yeast, or animals into plant systems have been attempted for improved remediation potential. A number of trace-element detoxification systems have been characterized genetically and functionally at the molecular level in yeast and bacteria. The introduction of such genes into plants has already yielded promising results (61, 66). Some of the genes or traits manipulated in transgenic plants for improved tolerance and phytoextraction potential are outlined below (Table 2).

Three classes of peptides have been implicated in heavy-metal homeostasis in plants: metallothioneins (MTs), phytochelatins (PCs), and glutathione (GSH). The thiol peptide GSH (γ-Glu-Cys-Gly) and its variant homoglutathione (H-GSH, γ-Glu-Cys-β-Ala) are considered to influence the form and toxicity of heavy metals such as As, Cd, Cu, Hg, and Zn, in several ways (67).

Metallothioneins (MTs). These are cysteine-rich proteins that have high affinity to cations such as Cd, Cu, and Zn (57),

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<table>
<thead>
<tr>
<th>gene</th>
<th>product</th>
<th>source</th>
<th>target plant</th>
<th>performance</th>
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<tr>
<td>MT2</td>
<td>metallothionein</td>
<td>human</td>
<td>tobacco, rapeseed</td>
<td>enhanced Cd tolerance at the seedling stage</td>
<td>Misra and Gedamu 1989</td>
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<td>MT1</td>
<td>metallothionein</td>
<td>mouse</td>
<td>tobacco</td>
<td>tolerated 200 mM CdCl₂ at the seedling level</td>
<td>Pan et al. 1994</td>
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<td>CUP1</td>
<td>metallothionein</td>
<td>yeast</td>
<td>cauliflower</td>
<td>tolerated 400 mM CdCl₂ in hydroponic medium</td>
<td>Hasegawa et al. 1997</td>
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<td>CUP1</td>
<td>metallothionein</td>
<td>yeast</td>
<td>tobacco</td>
<td>2–3× higher Cu content than the control, but no Cd tolerance</td>
<td>Thomas et al. 2003</td>
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<td>PsMTA</td>
<td>metallothionein</td>
<td>yeast</td>
<td>tobacco</td>
<td>8× higher Cu accumulation</td>
<td>Evans et al. 1992</td>
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<td>gshII</td>
<td>glutathione synthetase</td>
<td>E. coli</td>
<td>Indian mustard</td>
<td>longer root length at 0.15 mM Cd, 25% higher shoot Cd concentrations, and 3× higher Cd accumulation per shoot than WT plants</td>
<td>Zhu et al. 1999</td>
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<td>gshI</td>
<td>γ-glutamyl-cystein</td>
<td>E. coli</td>
<td>Indian mustard</td>
<td>3–5× higher γ-ECS and GSH levels and 90% higher shoot Cd concentrations when grown at 0.05 mM external Cd than in WT plants</td>
<td>Bennett et al. 2003</td>
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<td>gshI, gshII, and APS1</td>
<td>γ-Glu-Cys synthetase, glutathione synthetase, and ATP sulfurylase</td>
<td>E. coli, A. thaliana</td>
<td>Indian mustard</td>
<td>1.5–2× more Cd and Zn than WT, while APS did not accumulate 4.3, 2.8, and 2.3× more Se, with gshI, gshII, and APS1, respectively, than WT</td>
<td>Banuelos et al. 2005</td>
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<td>gshI</td>
<td>γ-Glu-Cys synthetase</td>
<td>E. coli</td>
<td>Poplar</td>
<td>Cd tolerance and increase of total sulfur in shoot</td>
<td>Arisi et al. 1997, 2000</td>
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<td>gshI</td>
<td>γ-Glu-Cys synthetase</td>
<td>E. coli</td>
<td>Poplar</td>
<td>increased phytochelatin synthesis and higher Cd accumulation in roots</td>
<td>Rennenberg and Will 2000</td>
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<tr>
<td>gshI</td>
<td>γ-Glu-Cys synthetase</td>
<td>E. coli</td>
<td>Poplar</td>
<td>higher accumulation of Cd, Cr, and Cu, and increased GST activity following Zn exposure</td>
<td>Bittsanszky et al. 2005</td>
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<td>SAT</td>
<td>serine acetyl transferase</td>
<td>T. goesingense</td>
<td>Arabidopsis</td>
<td>5× increase in shoot Ni tolerance and 1.5× increase in root resistance when grown on 100 mM Ni medium</td>
<td>Freeman et al. 2004</td>
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<td>TaPCS1</td>
<td>phytochelatin synthase</td>
<td>Wheat</td>
<td>Tobacco</td>
<td>high Pb (1 mM) and Cd (50 mM) tolerance, longer roots, higher and greener leaves at seedling stage than WT</td>
<td>Gisbert et al. 2003</td>
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<td>AtPCS1</td>
<td>phytochelatin synthase</td>
<td>A. thaliana</td>
<td>Arabidopsis</td>
<td>hypersensitivity to CdCl₂ (50 and 85 mM) and to ZnCl₂ (0.5 and 1.0 mM)</td>
<td>Lee et al. 2003</td>
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<td>AtPCS1</td>
<td>phytochelatin synthase</td>
<td>A. thaliana</td>
<td>Arabidopsis</td>
<td>20–100× more biomass on 250–300 mM arsenate and hypersensitivity to Cd</td>
<td>Li et al. 2004</td>
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<td>GR</td>
<td>glutathione reductase</td>
<td>E. coli</td>
<td>Indian mustard</td>
<td>plasticid transformants with enhanced tolerance to cadmium (100 mM CdSO₄) with 200 mM CdCl₂</td>
<td>Pilon-Smits et al. 2000</td>
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<td>OASTL</td>
<td>cysteine synthase</td>
<td>A. thaliana</td>
<td>Arabidopsis</td>
<td>9× increase in Cd tolerance on medium tolerance up to 400 mM CdCl₂ with exogenous cysteine supply and 72% more Cd accumulation (mature plants grew on 250 mM CdCl₂ for 14 days)</td>
<td>Dominguez-Solis et al. 2001, 2004</td>
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<td>source</td>
<td>target plant</td>
<td>performance</td>
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<td>OASTL</td>
<td>cysteine synthase</td>
<td>tobacco</td>
<td>tobacco</td>
<td>tolerance up to 300 mM Cd, 250 mM Se, and 500 mM Ni and produced higher biomass when grown on agar medium</td>
<td>Kawashima et al. 2004</td>
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<td>SMT</td>
<td>selenocysteine methyltransferase</td>
<td>A. bisulcatus</td>
<td>arabidopsis</td>
<td>increased accumulation of MeSeCys to an average of 0.5 mmol g⁻¹ dry weight</td>
<td>Ellis et al. 2004</td>
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<td>APS</td>
<td>ATP sulfurylase</td>
<td>A. thaliana</td>
<td>indian mustard</td>
<td>2-3x higher Se accumulation in shoot and 1.5-fold higher in roots than WT</td>
<td>Pilon-Smits et al. 1999</td>
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<tr>
<td>APS</td>
<td>ATP sulfurylase</td>
<td>A. thaliana</td>
<td>indian mustard</td>
<td>2.5x higher shoot concentration of different metals both at seedling and mature stages</td>
<td>Wangelin et al. 2004</td>
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<td>APS and CGS</td>
<td>ATP sulfurylase, cystathionine-γ-synthase</td>
<td>A. thaliana</td>
<td>indian mustard</td>
<td>2.5x higher shoot Se in APS and 40% lower shoot Se levels in CGS plants than in WT</td>
<td>Van Huysen et al. 2004</td>
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<td>ArsC and gshl</td>
<td>arsenate reductase and γ-Glu-Cys synthetase</td>
<td>E. coli</td>
<td>arabidopsis</td>
<td>2-3x more As per gram of tissue than WT or plants expressing ArsC or γ-ECS alone</td>
<td>Dhankher et al. 2002</td>
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<td>NiCBP4</td>
<td>cation channel</td>
<td>tobacco</td>
<td>tobacco</td>
<td>30-50% higher Cd concentrations than WT controls tolerance to 200 mM NiCl₂ and hypersensitivity to Pb enhanced Pb tolerance (below 0.1 mM Pb²⁺ concentrations) and attenuated accumulation reduced tolerance to Mg and Zn</td>
<td>Arai et al. 1999</td>
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<td>AtMHX1</td>
<td>vacuolar transporter</td>
<td>A. thaliana</td>
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<td>15-20% more metal ions in the shoots and higher root tonoplast transport in transgenic plants than in controls</td>
<td>Sunkar et al. 2000</td>
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<td>AtCAX2</td>
<td>vacuolar transporter</td>
<td>A. thaliana</td>
<td>tobacco</td>
<td>enhanced Zn tolerance and 2x higher Zn accumulation in roots</td>
<td>Hirschi et al. 2000</td>
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<td>ZAT1</td>
<td>Zn transporter</td>
<td>A. thaliana</td>
<td>arabidopsis</td>
<td>enhanced Zn tolerance and 2x higher Zn accumulation in roots</td>
<td>Van der Zaal et al. 2004</td>
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<td>ZntA</td>
<td>heavy-metal transporter</td>
<td>E. coli</td>
<td>arabidopsis</td>
<td>transgenic plants grew better than WT in medium with 0.7 mM Pb(II) or 70 µM Cd(II), and showed higher fresh weight</td>
<td>Lee et al. 2003</td>
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<td>YCF1</td>
<td>transport protein</td>
<td>yeast</td>
<td>Arabidopsis, poplar</td>
<td>tolerance to 1 mM Pb(II) and increased biomass and Cd tolerance on agar media</td>
<td>Song et al. 2003</td>
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<td>FRE1 and FRE2</td>
<td>ferric reductase</td>
<td>yeast</td>
<td>tobacco</td>
<td>1.5x higher iron content in transgenic shoots compared to WT plants</td>
<td>Samuelsen et al. 1998</td>
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<td>AtNramp1</td>
<td>Fe transporter</td>
<td>arabidopsis</td>
<td>arabidopsis</td>
<td>at 600 µM iron concentrations only the transgenic plants survived for long periods increased accumulation of Fe, on Cd²⁺ treatment and Cd hypersensitivity</td>
<td>Curie et al. 2000</td>
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<td>AtNramp3</td>
<td>Fe transporter</td>
<td>arabidopsis</td>
<td>arabidopsis</td>
<td>increased accumulation of Fe, on Cd²⁺ treatment and Cd hypersensitivity</td>
<td>Thomine et al. 2000</td>
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They confer heavy-metal tolerance and accumulation in yeast. Overexpression of genes involved in the synthesis of metallothioneins may lead to enhanced metal uptake and enhanced metal translocation or sequestration, depending on the type of chelator and on its role and location (19, 35, 68). For instance, the overexpression of MT genes in tobacco and oil seed rape resulted in enhanced Cd tolerance (69). An MT yeast gene (CUP1), when overexpressed in cauliflower, resulted in a 16-fold higher Cd tolerance (70). In a related study, the yeast metallothionein (CUP1) promoted copper uptake in tobacco. The CUP1 transformants accumulated up to 7 times more copper in older versus younger leaves during copper stress. Compared to the control, the pooled leaves of transgenic plants contained 2–3 times more copper, when grown in copper-contaminated soil (71). Similarly, higher Cu accumulation is reported in Arabidopsis thaliana by the overexpression of a pea MT gene (72). Another MT of plant origin (PsMTA- from Pisum sativum), when overexpressed in A. thaliana, accumulated 8-fold more Cu in roots than control plants (73).

**Phytochelatins (PCs).** Phytochelatins are a class of post-translationally synthesized (cysteine-rich metal-chelating) peptides that play a pivotal role in heavy-metal tolerance in plants and fungi by chelating these substances and decreasing their free concentrations (67). Following Agrobacterium-mediated transformation, the induction and overexpression of a wheat gene encoding phytochelatin synthase (TaPCS1) in Nicotiana glauca (shrub tobacco) greatly increased its tolerance to metals such as Pb and Cd. When grown for 6 weeks in a metal-contaminated soil, the transgenic plants accumulated higher Pb concentrations (50% increase in the aerial parts and 85% in roots). Transgenic seedlings also had longer roots and higher and greener leaves than unmodified plants. This wild plant has a wide geographic distribution, longer roots and higher and greener leaves than unmodified plants. This wild plant has a wide geographic distribution, longer roots and higher and greener leaves than unmodified plants. The increased cysteine availability in these transgenic lines allowed them to grow under severe metal stress (up to 400 μM cadmium in the growth medium). Further investigation on these transgenic lines showed that most of the cadmium had accumulated in the trichomes. Similarly, overexpression of Atcys-3A in A. thaliana resulted in a 9-fold increase in Cd tolerance (79). Thus, molecular engineering of the cysteine biosynthesis pathway, together with the modification of the number of leaf trichomes, may have a considerable potential in increasing heavy-metal accumulation for phytoremediation purposes.

**GSH Enzymes Involved in the Sulfate Assimilation Pathway and Others.** GSH in plants, generally described as γ-glutamylcysteine synthetase, plays an essential role in heavy-metal detoxification and is the direct precursor of PCs, which are metal-binding peptides. Overexpression of two glutathione synthesizing enzymes, γ-glutamylcysteine synthetase (γ-ECS) or glutathione synthetase (GS), in Brassica juncea (indian mustard) showed enhanced Cd tolerance and accumulation (80, 51). In addition to conferring tolerance to Cd, the overexpression of γ-ECS led to an increase in total shoot sulfur (S) suggesting an added advantage of enhanced S assimilation. Similar results were also obtained in poplar overexpressing γ-ECS (81, 82). To determine the importance of glutathione reductase (GR) for heavy-metal accumulation and tolerance, a bacterial GR was overexpressed in Indian mustard, targeted to the cytosol or the plastids. Overexpression of GR in plastids resulted in 20–50 times higher GR activity, whereas in cytosolic transformants, GR activity was only 2–4 times higher than in the WT. When grown in the presence of 100 μM CdSO₄, the plastidic transformants showed enhanced Cd tolerance and decreased Cd accumulation in the shoot. When compared to WT plants, these transgenics showed no chlorosis and their chlorophyll fluorescence and photochemical quenching were higher (83).

In another study, transgenic Indian mustard plants, overexpressing the γ-ECS, GS, and adenosine triphosphate sulfurylase (APS), were compared to assess the phytoremediation potential regarding mixtures of metals (84). When compared to WT plants, the ECS and GS transgenics showed higher shoot concentrations of Cd (+50%) and Zn (+43% for GS and +93% for ECS). The ECS transgenic plants also had higher levels of Cr (+170%), Cu (+140%), and Pb (+200%), relative to WT plants. However, the APS transgenics showed no significant difference in shoot metal concentration relative to WT plants. These results demonstrate the enhanced capability of transgenic plants to phytoextract soil contaminated with different metal pollutants. Similarly, overexpression of APS led to increased selenate uptake and tolerance in Indian mustard (85). In these transgenic plants Se accumulation was 2–3-fold higher in shoots and 1.5-fold higher in roots compared to WT plants. A recent study of transgenic APS plants showed 2.5-fold higher shoot concentrations of different metals (such as Cd, Cu, Hg, and Zn) at the seedling stage and other metals at the mature stage (86). These studies indicate that overexpression of ATP sulfurylase may be a promising approach to create plants with enhanced phytoextraction capacity for mixtures of metals. One of the recent studies showed the performance of APS and cystathionine-γ-synthase (CGS) transgenic plants and their capacity to accumulate selenium (Se) from soils naturally rich in this element (11). After 10 weeks on Se soil, the APS transgenics contained 2.5-fold higher Se concentration in shoots, whereas the CGS transgenics contained 40% lower levels of Se in shoots than WT plants. Another significant achievement in using transgenic plants for selenium decontamination was the successful field trial of transgenic Indian mustard plants to remove selenium from Se- and boron-contaminated saline sediments (87). The APS, ECS, and GS transgenic plants accumulated 4.3, 2.8, and 2.3-fold more Se in their leaves than WT plants (87). Similarly, in other studies, transgenic plants overexpressing selenocysteine methyltransferase (SMT) showed enhanced tolerance and accumulation of Se (12, 13). Overproduction of SMT in Arabidopsis increased the accumulation of Se-methylenecysteine (MeSeCys) from essentially zero in
control plants to an average of 0.5 μmol g⁻¹ dry weight in the highest SMT accumulating line with MeSeCys concentrations ranging up from 0.99 to 1.3 μmol g⁻¹ dry weight in transgenic lines (12).

A transgenic system for removing arsenate (As) from soil was tested in *A. thaliana* by inserting two genes, *arsC* (arsenate reductase) and ECS (γ-glutamylcysteine synthetase) from *Escherichia coli* (88). When grown on As, the transgenic plants accumulated 4- to 17-fold higher fresh shoot weight and accumulated 2- to 3-fold more arsenate per gram of tissue than WT plants or those expressing γ-ECs or *arsC* alone (88). Similarly, when *arsC* was overexpressed in tobacco and *Arabidopsis*, both transgenic plants showed significantly higher Cd tolerance (with 30–50% higher Cd concentrations) than WT controls (89). Recent studies on the hyperaccumulator species *Thlaspi* showed that plant tolerance toward heavy metals, such as Cd and Ni, was due to antioxidative defense, and antioxidants such as glutathione were shown to play an important role (90, 91).

**Metal Transporters**. Transport of metals and alkali cations across plant plasma membrane and organelar membranes is essential for plant growth, development, signal transduction, and toxic metal phytoremediation. Transporters play an important role in plants by shutting potentially toxic cations across membranes. Improved metal tolerance and accumulation was achieved in several plant species by modifying metal transporters such as *CAX2*, *ZAT*, *NtCBP4*, FRE1, or FRE2. Increased Ca, Cd, and Mn tolerance in tobacco plants was reported by the overexpression of a calcium vacuolar transporter *CAX2* from *Arabidopsis* (92). *CAX2*-expressing plants had higher root tunoplast transport of all three ions (Ca, Cd, and Mn) than the control. However, no altered accumulation of Mg and Zn was observed when another vacuolar transporter, AMXH, was overexpressed in tobacco (93). Van der Zaal and collaborators (94) studied the effect of overexpression of a Zn transporter (ZAT) in *A. thaliana*. The transgenic plants had enhanced Zn resistance and 2-fold higher Zn accumulation in roots. *NtCBP4* is a metal transporter gene from tobacco encoding a calmodulin binding protein. Its overexpression showed enhanced tolerance and reduced accumulation of Ni, and enhanced accumulation and reduced tolerance of Pb (95). This was the first report of a plant protein involved in metal uptake across the plasma membrane modulating plant tolerance and accumulation of Pb.

To further investigate the possible modulation of Pb tolerance in plants, Sunkar et al. (96) produced transgenic plants overexpressing a truncated version of this protein (designated as *NtCBP4ΔC* and lacking the calmodulin binding domain). In contrast to the phenotype of transgenic plants expressing the full-length gene, transgenic plants expressing the truncated gene showed improved tolerance to Pb in addition to attenuated accumulation of Pb. Similarly, when *Arabidopsis* plants were transformed with a bacterial transporter gene *ZntA*, which encodes a Pb(II)/Zn(II) pump, the plants developed improved resistance through reduced uptake of these metals (97). The shoots of the transgenic plants had decreased Pb and Cd content. Moreover, the transgenic protoplasts showed lower accumulation of Cd and faster release of preloaded Cd than WT protoplasts. These results show that *ZntA* can be functionally expressed in plant cells and that it may be useful for the development of crop plants that are safe from environmental contamination.

Recently, Song et al. (98) studied the utility of a yeast transport protein *YCF1*, which (conjugated with glutathione) detoxifies cadmium by transporting it to vacuoles, for the remediation of lead and cadmium contamination. When overexpressed in *A. thaliana*, the transgenic plants showed improved Pb(II) and Cd(II) resistance, enhanced cadmium accumulation in vacuoles, and higher lead and cadmium contents. The overexpression of the Fe transporter *AtNramp1* showed increased Fe tolerance (99), whereas the overexpression of *AtNramp3* resulted in reduced Cd tolerance without any difference in Cd accumulation (100). The overexpression in tobacco of two yeast genes encoding ferric reductase (FRE1, FRE2) led to an increase of 1.5-fold in the iron content of shoots of transgenic plants (101).

**Phytovolatilization**

Contaminants such as mercury (Hg), arsenate (As), and selenium (Se) are a serious problem in many parts of the world and plant genetic engineering has been attempted as a strategy to remove these metals from soil (102–104). Volatilization of As and Hg has been reported for microorganisms, but these elements do not appear to be volatilized to significant levels by nontransgenic plants.

**Mercury Tolerance and Volatilization.** Mercury is a global pollutant cycling between air, water, and soil as a result of natural processes and anthropogenic activities. Although not all Hg-contaminated sites are vulnerable to methylmercury formation (105), Hg in soil poses a serious threat and must be removed. Two different approaches have been employed in plant-based systems to remove Hg from the soil (106). The first approach involves the use of transgenic plants encoding genes from Hg-detoxifying bacteria, which have increased Hg resistance and enhanced volatilization capacity. *Arabidopsis* and tobacco plants genetically modified with bacterial organomercurial lyase (merB) and mercuric reductase (merA) genes (61, 102, 107) absorbed elemental Hg(II) and methyl mercury (MeHg) from the soil, releasing volatile Hg(0) from the leaves into the atmosphere (108). Transgenic *A. thaliana* plants expressing *merB* genes were significantly more tolerant to methylmercury and were shown to convert methyl mercury to inorganic mercury, a form that is 100-fold less toxic to plants (103, 109). Bizly et al. (109) were also successful in producing *merA–merB* double transgenics which are capable of converting organic mercury all the way to elemental mercury, which could be released in the volatile form. Transgenic *Arabidopsis* plants expressing both *merA* and *merB* were able to grow on up to 10 μM methylmercury concentrations, which is 40-fold higher than the maximum concentration tolerated by WT seedlings (109). Furthermore, when roots of the *merA* and *merB* transgenics were supplied with 25 μM organomercurials in solution, elemental mercury was volatilized at an estimated rate between 14.4 and 85.0 μg Hg(g⁻¹ fresh biomass) day⁻¹. In another study, *merA* tobacco plants were able to remove 3–4-fold more mercury from hydroponic medium than control plants (110). Organic mercury detoxification in plants was also achieved by subcellular targeting (particularly to endoplasmic reticulum) of methylmercury lyase (63). The results of this study clearly indicate that *Arabidopsis* plant lines with ER-targeted or cell wall secreted versions of *merB* more efficiently resist and detoxify organic mercury than plants expressing *merB* in the cytoplasm. Another possibility may be the sequestration and root specific expression of *merA* and *merB* genes to detoxify charged mercurials prior to transport to shoots. Plant expression of modified mercury transport genes, *merP* and *merT*, may provide a means of improving mercury uptake with organelle and tissue specific targeting (111).

The second approach used in plants to remove Hg from soils is based on the use of nontoxic thio-containing solutions to induce Hg accumulation in above-ground tissues of high-biomass (nontransgenic) plant species. In one such study, experiments were carried out in plant growth chambers and in the field to investigate plant-mercury accumulation and volatilization in the presence of thiosulfate (S₂O₃⁻) containing solutions (1 g S₂O₃₂⁻/kg substrate) (106). Application of (NH₄)₂S₂O₃ to substrates increased, up to 6 times, the Hg concentration in the shoots and roots of Indian mustard,
relative to controls. However, the volatilization rates were significantly higher in control plants (irrigated with water) as compared to plants treated with \((\text{NH}_4)_2\text{S}_2\text{O}_3\). Similarly, in a previous study, ammonium thiosulfate has been used to induce Indian mustard to accumulate 40 mg Hg/kg of shoot tissue from a lead—copper—zinc metal mine contaminated with Hg \((112)\). The development of strategies to improve plant Hg uptake and sequestration in harvestable parts is an alternative option \((106)\).

**Selenium Tolerance and Volatilization.** Selenium is a major environmental pollutant and doses above the Se requirements can result in toxic effects \((104)\). The oxidized forms of Se (selenate or selenite) are highly soluble and are easily removed by plants, whereas inorganic forms, such as selenide or elemental Se, are less bioavailable. Selenium and sulfur are nutrients with very similar chemical properties. Their uptake and assimilation occurs through common pathways from selenate and sulfate, respectively, activated by ATP sulfurylase. Phytoremediation has a great potential for selenium decontamination. Two plant species, *B. juncea* (Indian mustard) and *Chara canescens* (muskgrass), were identified as good candidates for this process, as they could extract and accumulate relatively high concentrations of Se \((10)\). Enhanced phytoremediation of Se is achieved by overexpression of enzymes catalyzing rate-limiting steps such as ATP sulfurylase and CGS \((85, 113)\) and has already been discussed in earlier sections in the context of phytoextraction. Here we limit our discussion to phytovolatilization of Se.

Volatilization of Se involves assimilation of inorganic Se into the organic selenoamino acids selenocysteine (SeCys) and selenomethionine (SeMet). Selenomethionine can be methylated to dimethylselenide (DMSe) and is volatile \((114)\). Overexpression of CGS in *Brassica* promoted selenium volatilization and the CGS seedlings were more tolerant to selenite than the WT. The CGS plants contained Se levels that were 20–40% lower in shoots and 50–70% lower in roots than in the WT when supplied with selenite \((113)\). These results suggest that selenate-to-selenite reduction is rate limiting for selenite tolerance and accumulation. Therefore, overexpression of CGS offers a promising approach for the production of plants with enhanced capacity to remove Se from contaminated sites in the form of the low-toxicity volatile dimethylselenide. A gene encoding the enzyme SMT has been cloned from the Se-hyperaccumulator, *A. bisulcatus* \((115)\), and when overexpressed in *Arabidopsis* and Indian mustard it increased selenium tolerance, accumulation, and volatilization. SMT transgenic seedlings tolerated Se, particularly selenite, significantly better than the WT, producing 3–7-fold higher biomass and 3-fold longer roots \((13)\).

The main advantage of phytovolatilization is that it can completely remove the pollutant from the site, without the need for plant harvesting and disposal, as in other cases. However, there is some skepticism regarding the safety of the volatilization of these elements into the atmosphere. Recent risk-assessment studies on Se and Hg volatilization indicated that, in phytoremediation, these elements are dispersed and diluted to such an extent that they do not pose a serious threat \((106, 110, 116)\). The plants engineered for improved phytovolatilization capabilities and their performance are summarized in Table 3.

**Phytodegradation**

The use of plants capable of removing organic pollutants (such as chloroacetanilide, TNT, TCE, atrazine, etc.) by phytodegradation is an important option for phytoremediation \((1, 32, 59, 117)\). In this process, plant enzymes act on organic pollutants and mineralize them either completely into inorganic compounds, such as CO₂, water, and Cl₂, or partially into stable intermediates that are stored in the plant \((118)\). Some of the important classes of enzymes involved in

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<th>TABLE 3. Performance of Transgenic Plants Overexpressing Genes for Improved Phytovolatilization Efficiency</th>
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<td><strong>gene</strong></td>
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<td>merA and merB</td>
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<td>SMT</td>
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phytodegradation of organic pollutants are peroxidases, peroxigenases, laccases, phosphatases, nitroreductases, and dehalogenases (119). Endophytic microorganisms are also frequently involved in phytodegradation (120).

Transgenic plants with increased capacity for degradation of organic pollutants such as chloroaacetanilide and TNT were already obtained (8, 58). To engineer plant tolerance to TNT, two bacterial enzymes (PETN reductase and nitroreductase), able to reduce TNT into less harmful compounds, were overexpressed in tobacco plants. The two genes, *ons* and *nfs*, under the control of a constitutive promoter provided the transgenic plants with increased tolerance to TNT at concentrations that severely affected the development of WT plants (2, 58).

Another good candidate for enhancing the phytoremediation potential of plants is the mammalian cytochrome P450 2E1. This cytochrome oxidizes a wide range of compounds including TCE and ethylene dibromide (EDB). Transgenic tobacco plants overexpressing the human P450 2E1 resulted in the enhanced metabolism of TCE and EDB (121). In another study, two species of human P450 cDNA were introduced in tobacco cells by Agrobacterium-mediated transformation and tested against atrazine metabolism. Transgenic cultures were able to produce larger amounts of nonphytotoxic (primary oxidized) metabolites than non-transformed cultures (122).

It is also interesting to note that the overexpression of enzymes involved in glutathione synthesis (ECS and GS) offers a promising approach to obtaining plants with enhanced tolerance not only to heavy metals, but also to certain organic pollutants such as atrazine, metolachlor, and phenanthrene (122). *B. juncea* plants, overexpressing ECS and GS genes, showed enhanced tolerance to atrazine (50 and 100 mg L⁻¹). While root growth of WT seedlings was 50% inhibited by 100 mg L⁻¹ atrazine, in ECS and GS transgenic plants only a 20–30% inhibition of root growth was noticed. Moreover, as compared to WT plants, the transgenic ones showed a slightly higher tolerance to CDNB (1-chloro-2,4-dinitrobenzene) (tested at 5 and 10 mg L⁻¹), metolachlor (50 and 100 mg L⁻¹), and phenanthrene (100 and 200 mg L⁻¹) (123).

In recent years, some crop plants were also genetically engineered with mammalian P450 cytochrome genes to confer herbicide resistance (25, 124, 125). Such transgenic plants metabolize exogenous chemicals and show cross-tolerance toward different classes of herbicides. Plants with cross-tolerance can be used in herbicide rotation systems, to avoid or delay the evolution of herbicide-resistant weeds. These engineered plants are also expected to reduce the environmental load of agricultural chemicals on farmland (126–128).

All these studies indicate that engineering plants with higher enzyme activities involved in rate-limiting steps may be important to improve the efficiency of phytodegradation of organic pollutants. Although the metabolic fate of organic pollutants in plants is well studied, much work still remains to be done to enhance our knowledge about the potential risks and benefits of transgenic plants in phytoremediation. Transgenic plants engineered for improved phytodegradation efficiency and their performances under different organic pollutants are summarized in Table 4.

**Transgenic Trees and Phytoremediation**

Nontransgenic trees have been used for phytoremediation of heavy-metal contaminated sites (5). Although transgenic approaches for tree improvement have been mainly focused on traits such as biomass and wood quality (129), there are already several examples of transgenic trees engineered for phytoremediation purposes (Tables 2–4).

An important characteristic of tree species that makes them suitable candidates for remediation is their large biomass (above and below ground) and the long life cycle. Among the various tree species, poplar and willow trees have gained increasing importance due to their extensive root system, high water uptake, rapid growth, and large biomass production. Poplar plants have already been used to remove atrazine, trichloroethylene, and selenium from polluted sites (32, 59, 130). A promising development in transgenic research using trees for phytoremediation is the overexpression of the bacterial mercuric reductase in yellow poplar (61). Three modified *merA* constructs were used for the transformation of *Liriodendron tulipifera* L. (yellow poplar) proembryogenic masses and mercury volatilization were found to be 10-fold higher in transgenic plantlets as compared to the wild type. The transgenic plants volatilized Hg(0) at an average rate of approximately 1 μg g⁻¹ tissue day⁻¹ when grown on an agar media containing 10 μM HgCl₂ (61). Che et al. (131) used an identical approach on *Populus deltoides* (eastern cottonwood) trees. Transgenic plants expressing modified *merA9* and *merA18* genes accumulated significantly higher biomass than control plants on a Georgia piedmont soil contaminated with 40 ppm Hg(II). These results indicate the high potential of trees engineered with *merA* genes for in situ remediation of mercury-contaminated soil or wastewater.

Similarly, transgenic poplars, overexpressing γ-glutamylcysteine synthetase (γ-ECS), could be used for phytoremediation of heavy metals and herbicides due to the higher uptake capacity for Cd and increased GSH levels (8, 132). The suitability of a WT poplar hybrid and two transgenic poplar lines overexpressing γ-ECS was investigated for phytoremediation of soils artificially contaminated with the chloroaacetanilide herbicides, acetochlor or metolachlor. The transgenic plants showed increased herbicide tolerance, due to elevated endogenous γ-ECS and GSH levels, resulting in rapid herbicide degradation (8). Although the overexpression of γ-ECS allowed a higher cadmium accumulation in tissues, it had only a marginal effect on cadmium tolerance (132, 133).

Bittsanszky and co-workers (134) investigated the phytoremediation potential of four poplar lines, *Populus nigra* (N–SL clone), *Populus canescens*, and two transgenic *P. canescens* clones, regarding zinc stress by overexpressing the bacterial γ-ECS in chloroplast and cytosol using in vitro leaf disk cultures. However, in this work, the Zn uptake did not differ in transgenic and untransformed clones, although accumulation of other metals, such as Cd, Cr, and Cu content, was significantly higher in some of the transgenic lines. One of the preliminary studies on the overexpression of a yeast transport protein YCF1 in poplar plants has shown enhanced biomass in the transgenic plants versus the WT, when grown in the presence of Pb(II) (98).

**Perspectives**

Phytoremediation, as a new and promising technology, has gained wide acceptance and is currently an area of active research in plant biology. A good number of plants have already been identified as potential candidates for phytoremediation applications. Efforts are being made to understand the underlying genetic and biochemical processes involved in metal uptake, transport, and storage by hyperaccumulating plants (45, 52, 54, 64, 91). The knowledge gained from such studies in conjunction with biotechnology has helped to improve, substantially, the phytoremediation capability of plants. For example, new transgenic plants have been developed with improved capacity for metal uptake, transport, and accumulation as well as for detoxification of organic pollutants. However, many gaps still persist in our understanding of the processes of plant–microbe interactions, metal accumulation, and ion homeostasis.

To obtain further gains, research in the following areas appears to be worth pursuing in the future. (1) Manipulation
<table>
<thead>
<tr>
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<th>organic pollutant</th>
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<tr>
<td>Onr</td>
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of metal transporters and their cellular targeting to specific cell types, such as vacuoles, to allow for safe compartmentalization of heavy metals in locations that do not disturb other cellular functions. (2) Genetic manipulation of the chloroplast genome may be, for some plants, an alternative approach to achieve high gene expression while avoiding the risk of transgene escape via pollen (135). (3) Identification of candidate plants with substances that may deter the herbivores from feeding and the subsequent transformation of such plants with altered or improved metal tolerance capabilities. Such a system will help avoid the transfer of metals to the food chain (76). (4) Development of transgenic plants with enhanced plant–microbe interaction or rhizosphere microbial activity. It may be possible either to develop transgenic plants that have the ability to secrete metal-selective ligands capable of solubilizing elements for phytoremediation, or to find simple molecules with selective chelation ability which plants can make and secrete into the rhizosphere (111). (5) Transgenic research in phytoremediation should also address the problem of mixed contamination occurring in many of the polluted sites. A multigene approach involving the simultaneous transfer of several genes into suitable candidate plants may help to remove contaminants of mixed or complex nature. (6) Not much data are yet available on the field performance of transgenic plants in phytoremediation. Established field trials are, therefore, urgently needed to make it a commercially viable and acceptable technology.

To further advance our knowledge, phytoremediation research requires more collaborative studies involving expertise from different fields such as botany, plant physiology, biochemistry, geochemistry, agricultural engineering, microbiology, and genetic engineering among others. In the years to come, as in other areas, plant genetic engineering for improved phytoremediation could also benefit from the data of genomic and postgenomic projects including proteomics.

Acknowledgments
We thank Dr. Danika L. LeDuc (University of California at Berkeley) for critical reading of the manuscript and for her valuable comments and suggestions that helped us to improve it. Dr. Margarida O. Krause is gratefully acknowledged for revising the English. The postdoctoral support from FCT (SFRH/BPD/9053/2002) to Sam Cherian is also acknowledged.

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Received for review June 16, 2005. Revised manuscript received September 16, 2005. Accepted October 6, 2005.

ES051134L