Chapter 3
Transgenic Approaches for Phytoextraction of Heavy Metals

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1 Introduction

A heavy metal is a member of an ill-defined subset of elements that include the transition metals, some metalloids, lanthanides, and actinides, all of which are known to exhibit metallic properties (Babula et al. 2008). The heavy metals have a specific gravity of more than 5 g/cm^3 in their standard state. Of the 90 naturally occurring elements, 53 can be considered as heavy metals, of which 17 are of importance for organisms and are bioavailable (Weast 1984). Iron (Fe), manganese (Mn), and molybdenum (Mo) are important as micronutrients; zinc (Zn), nickel (Ni), copper (Cu), vanadium (V), cobalt (Co), tungsten (W), and chromium (Cr) are toxic and required in traces; arsenic (As), mercury (Hg), silver (Ag), antimony (Sb), cadmium (Cd), lead (Pb), and uranium (U) are not nutritionally important and show toxicity towards various living forms (Godbold and Hüttermann 1985; Breckle 1991; Nies 1999; Schützendübel and Polle 2002). According to Wood (1974), most of the heavy metals can be categorized as toxic and their concentration in the soil varies between 1 and 100,000 mg/kg (Blaylock and Huang 2000). Arsenic (As), though a nonmetal, is often studied in heavy metal contamination since the reaction of most living forms to this element is similar to their reaction to the metal ions.

Large areas of developed as well as developing countries have been contaminated with high concentration of heavy metals that are the result of air emissions from combustion plants, oil, mining and other industrial processes, incinerators, and military and waste practices (Bhargava et al. 2012a, b). Heavy

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metal-contaminated soils pose a health hazard to all forms of life primarily humans, plants, and animals (Bhargava et al. 2012a) and cause serious danger to public health by entering the food chain or by leaching into drinking water. Besides this, they may have negative impact on ecosystems and other natural resources. The situation is compounded by the fact that heavy metals cannot be easily degraded and are difficult to remove from the environment (Sriprang and Murooka 2007). Since heavy metals can only be transformed from one oxidation state or organic complex to another, they need to be removed from the contaminated sites (Garbisu and Alkorta 2001).

2 Toxicity of Heavy Metals to Plants and Animals

Several metals are considered hazardous wastes that can accumulate in living forms and have a relatively large half-life. Heavy metals are detrimental to the growth of plants and survival of animal life (Sandalio et al. 2001; John et al. 2009; Bhargava et al. 2012b).

2.1 Toxicity of Heavy Metals to Plants

Plants are sensitive to heavy metals in a variety of ways that are enumerated below:

1. Uptake and accumulation of metals by binding to extracellular exudates and constituents of the cell wall
2. Extrusion of metals from cytoplasm to the extranuclear compartments
3. Complexation of the metal ions inside the cells by complex molecules
4. Concentration of osmolytes and osmoprotectants and induction of enzyme systems
5. Alteration of plant metabolism (Cho et al. 2003)

Heavy metals are known to induce changes at morphological, physiological, and molecular levels in the plants (Hall 2002; DalCorso et al. 2013). Heavy metal toxicity in plants varies according to the plant species, type of metal, their concentration, chemical structure, and edaphic factors (Schützendübel and Polle 2002; Nagajyoti et al. 2010). These are absorbed through the root systems, and once inside the plant, they induce destruction of chlorophyll, deficiency of essential minerals, and inhibition of root penetration (Kim et al. 2003; Manousaki et al. 2008; Shakya et al. 2008; Lamb et al. 2010; Singh et al. 2013). The uptake and accumulation of nutrients is influenced by alteration in the water absorption and solute permeability caused by the heavy metals (Hernández et al. 1997). The accumulation of heavy metals in plants and their subsequent release during decomposition facilitates their recycling in the ecosystem (Kim et al. 2003). This pathway regulates the level of toxic metals in the biosphere.

2.2 Toxicity of Heavy Metals to Humans

Some of the heavy metals are of importance to man, but their dietary intake has to be maintained at regulatory limits since excesses may lead to toxicity resulting in clinically diagnosable symptoms (Nolan 2003; Young 2005). However, metals like Cd, Pb, As, and methylated forms of Hg do not have much importance in human physiological processes and are toxic even at low concentrations (Holum 1983; McCluggage 1991; EU 2002; Young 2005). The effects of the heavy metals could range from toxic (acute, chronic, or subchronic), mutagenic, teratogenic to even carcinogenic (Duruibe et al. 2007). The toxicity, persistence, and nonbiodegradable nature of the heavy metals are potent threats to the living organisms. The presence of metals has been correlated with birth defects, liver damage, renal damage, cancer, and a range of disorders in various parts of the human body (ATSDR 2001). This toxicity is compounded by the fact that several heavy metals accumulating in the human body have a very large half-life (Salt et al. 1995).

Cadmium is one of the most important pollutants in terms of food chain contamination (Liu et al. 2005). Cd intake below the FAO/WHO tolerable weekly intake of 70 μg/day does not constitute a health risk. The metal poses considerable risk to human and animal health at concentrations that are not generally toxic for plants. Cadmium accumulates in the human kidney for a considerably long period (20–30 years) and produces deleterious effects on the respiratory and skeletal systems (Jin et al. 2004; Johri et al. 2010). Cd accumulates in the kidney cortex and causes renal tubular dysfunction (Strehlow and Barltrop 1988; McKenna and Chaney 1991). More severe exposure leads to bone defects like osteomalacia, osteoporosis, and spontaneous fractures (Alfvén et al. 2000). Subchronic airborne particle exposure to Cd leads to pulmonary effects like emphysema, bronchiolitis, and alveolitis, while high exposure leads to cadmium pneumonitis, an obstructive lung disease characterized by chest pain, bloody sputum, and death of lung tissues (McCluggage 1991; EU 2002; Young 2005).

One of the greatest concerns for human health is caused by lead contamination. Lead poisoning is an environmental and public health hazard that has acquired global proportions. Exposure to Pb occurs through multiple pathways such as air, food, water, dust, and soil (Lasat 2002; Gupta et al. 2013). The danger of Pb is compounded by low mobility even under high precipitation. The toxic effects of Pb include apoptosis, excitotoxicity, alteration in the lipid metabolism, inhibition of superoxide dismutase, suppression of the activity-associated Ca^{2+}-dependent release of acetylcholine, dopamine and amino acid neurotransmitters, disruptive effects on dopamine systems, and toxic effects on both astroglia and oligodendroglia (Lidsky and Schneider 2003). Lead poisoning initially causes damage to the central and peripheral nervous systems and inhibition of hemoglobin synthesis and finally leads to dysfunction of the kidneys and cardiovascular and reproductive systems (Ferner 2001; LWTAP 2004; Ogwuegbu and Muhanga 2005; Gupta et al. 2013).

Zinc is relatively harmless as compared to several other metal ions having similar chemical properties (Plum et al. 2010). Only exposure to high doses has toxic effects.

Zinc causes similar symptoms as lead poisoning that often leads to confusion (McCluggage 1991). Common signs of Zn toxicosis are diarrhea, vomiting, anemia, icterus, and liver and kidney failure (Fosmire 1990). Excess amount of Zn causes system dysfunctions resulting in growth impairment and reduced reproductive capacity (INECAR 2000; Nolan 2003).

The functional significance of Hg in human physiology is not known. Hg poisoning causes neurological disorders and extensive damage to the brain and central nervous system, abortion, corrosive esophagitis, hematochezia, congenital malformation, erethism, acrodynia, gingivitis, stomatitis, and kidney injury (Ferner 2001; LWTAP 2004; Charles et al. 2013; Miller et al. 2013).

Arsenic is known to coagulate proteins and forms complexes with coenzymes leading to inhibition of production of adenosine triphosphate, the main energy-yielding molecule in the body (INECAR 2000). Arsenic is carcinogenic with high exposure often causing death (Ogwuegbu and Ijioma 2003; USDOL 2004; Charles et al. 2013). Arsenic toxicity causes an immune disorder in which the body's immune system attacks its own peripheral nervous system which results in muscle weakness (Kantor 2006; Richards 2007).

Apart from plants, heavy metals have a detrimental effect on other forms of life. Livestock and wildlife have long been reported to be suffering from heavy metal poisoning (Rosenfeld and Beath 1964; Ohlendorf et al. 1986; Maracek et al. 1998).

3 Phytoremediation Technologies

The term phytoremediation refers to the remediation methods that efficiently utilize plant systems to remove pollutants (inorganic and organic) or render them harmless (Salt et al. 1998; Ali et al. 2013). This technique can be applied to reduce the pollutants present in soil, water, or air. The origin of the term phytoremediation can be traced to two words, the Greek word "phyto" meaning plant and the Latin suffix "remedium" meaning curing or restoring. This technique uses various approaches as depicted in Fig. 3.1.

Technologies for phytoremediation can be categorized into the types as shown below:

1. Phytoextraction. Phytoextraction refers to the removal of contaminants from soils by plants and their transportation and concentration in the harvestable parts. This technique utilizes plants that concentrate contaminants in their aerial parts so that the contaminant-enriched biomass can be properly disposed of (Kramer 2005). Several plant species are known that flourish in the presence of high concentration of contaminants in the soil and even hyperaccumulate them in their shoots (Baker and Brooks 1989).
2. Phytostabilization. Phytostabilization is the utilization of plants in mechanical stabilization of polluted soil for preventing bulk erosion, leaching, and reducing

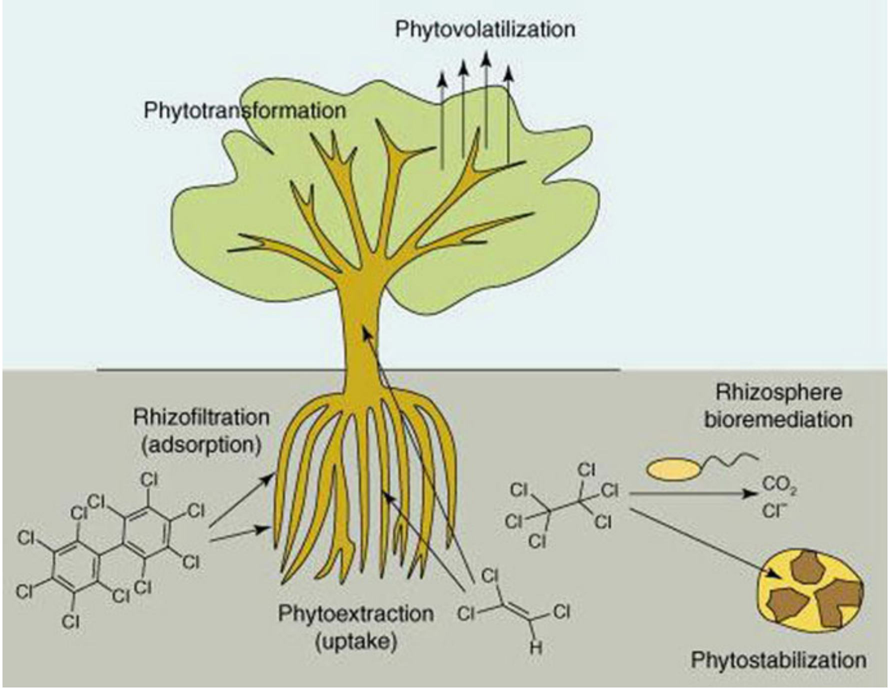

Fig. 3.1 Different phytoremediation strategies employed to clean up the environment (Reprinted from Aken (2008), with permission from Elsevier)

airborne transport of pollutants (Gonzaga et al. 2006). Such plants develop extensive root system, possess tolerance to the contaminant, and immobilize the contaminant in the rhizosphere. These plants provide a cover of vegetation for the contaminated site, thus preventing water and wind erosion (Kramer 2005).

3. Phytovolatilization. This involves biologically converting soil metals into volatile forms and releasing them into the environment (Chaney et al. 1997; Garbisu and Alkorta 2001; Lasat 2002; McGrath et al. 2002; Ernst 2005; Sakakibara et al. 2007). Contaminants like mercury or selenium, once taken up by the plant roots, can be converted into nontoxic forms and volatilized through various plant parts (Prasad 2003).
4. Phytoimmobilization. This involves decreasing the mobility and bioavailability of pollutants by the plants primarily through alteration of soil factors which lowers mobility of the pollutant and sorption on roots.
5. Phytotransformation. Phytotransformation, also referred to as phytodegradation, is the sequestering and breakdown of pollutant by plants into simple compounds that are integrated with plant tissue and foster plant growth (EPA 1998).
6. Rhizofiltration. Rhizofiltration is the form of bioremediation that makes use of plant roots to absorb, concentrate, and/or precipitate pollutants from contaminated

water bodies (Salt et al. 1998). This process removes contaminants by trapping them into harvestable plant parts, but is restricted for treatment of aquatic bodies.

Of the abovementioned techniques, phytoextraction seems to be quite attractive (Chaney 1983) and has been receiving increasing attention for decontaminating polluted soils. The advantages of this technique are low costs, generation of metal-rich plant residue, minimal environmental disturbance, and public acceptance (Kumar et al. 1995; Bhargava et al. 2008). The time required for remediation of the metal-contaminated site may range from 1 to 20 years depending on the type of contaminant, extent of contamination, growing season, and on how efficiently a metal is removed by the plant (Kumar et al. 1995; Blaylock and Huang 2000). Phytoextraction is most promising for the remediation of vast stretches of land contaminated with heavy metals at shallow depths (Kumar et al. 1995; Blaylock and Huang 2000).

4 Plants and Heavy Metals

Plants have been divided into three categories with respect to their response to excess amount of metals in their growing substrate (Baker 1981, 1987):

1. Excluders: These survive by avoidance mechanisms and are sensitive over a wide range of metal concentrations in the soil. Excluders prevent uptake of toxic metals into root cells (de Vos et al. 1991). These species avoid spread of contamination due to erosion and can be used for stabilization of soil (Lasat 2002).
2. Indicators: Indicator plants accumulate metals in their aboveground biomass which mirrors external concentration of metal in the soils (McGrath et al. 2002).
3. Accumulators: These can survive by physiological tolerance mechanisms and accumulate metals in the aboveground biomass at varied soil concentrations (Bhargava et al. 2012a). Accumulators do not prevent metals from entering the roots, thus allowing bioaccumulation of metals.

5 Hyperaccumulators

The term “hyperaccumulator,” introduced by Brooks et al. (1977), refers to those plant species that can uptake metals, transport them to aerial parts of the plant, and accumulate up to 100-fold greater amount of the metal as compared to the non-accumulator plants (Baker and Brooks 1989; Baker et al. 2000; McGrath and Zhao 2003). Metal hyperaccumulation refers to the uptake and sequestration of extremely high concentrations of metal in the aboveground biomass of the plant growing in normal field conditions (Pollard 2000). Metal hyperaccumulators have the natural capacity to accumulate heavy metals in their aboveground tissues without exhibiting any toxicity symptoms (Bhargava et al. 2012a). A metal hyperaccumulator can concentrate the metals to a level of 0.1 % (of the leaf dry weight) for Ni, Co, Cr, Cu, Al, and Pb; 1 % for Zn and Mn; and 0.01 % for Cd and Se (Baker and Brooks 1989; Baker et al. 2000). Metal hyperaccumulators have been reported to occur in over 450 species of vascular plants from 45 angiosperm families that comprise Brassicaceae, Asteraceae, Caryophyllaceae, Cyperaceae, Cunoniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphorbiaceae (Padmavathiamma and Li 2007; Bhargava et al. 2012a). The highest number taxa that are established for hyperaccumulation of metals are found in Brassicaceae (approximately 11 genera and 87 species), especially in the genera *Thlaspi* and *Alyssum*, wherein accumulation of more than one metal has been reported (Reeves and Baker 2000; Prasad and Freitas 2003; Verbruggen et al. 2009; Vamerali et al. 2010).

6 Transgenics for Improved Phytoextraction

The goal of remediating soils contaminated with heavy metals is extraction of the contaminant from the large soil volume and its concentration into various plant parts for harvest and easy disposal (Bhargava et al. 2012a). Biomass production and bioconcentration efficiency are the two factors that categorize a plant as an efficient phytoextractor (Cherian and Oliveira 2005). Bioconcentration is the ratio between the concentration of the pollutant in the aboveground parts of the plant and the concentration of the pollutant in the soil. Plants ideal for phytoextraction should have high biomass and deep roots, should be easily harvestable, and should accumulate considerable amount of the contaminant in their aboveground parts. However, no plant fulfils all these criteria. Most of the plants that accumulate metals exhibit metal selectivity, show sluggish growth, produce little biomass, and cannot be utilized for remediation other than in their natural habitats (Kamnev and van der Lelie 2000). Thus, in spite of the fact that the amount of metal concentration per unit of plant biomass is high, little amount of metal is removed from the contaminated site during a certain period of time (Bhargava et al. 2012a). Apart from this, the use of hyperaccumulator plants can be limited because of less information about their agronomic characters, breeding potential, pest management, and physiological processes (Cunningham et al. 1995).

An interesting alternative can be modification of a rapidly growing non-accumulator plant to modify it to achieve some of the features of the hyperaccumulators. Plant breeders and environmental researchers have long strived to develop such improved plant varieties which can be used for effective phytoextraction. Conventional breeding approaches coupled with suitable agronomic practices like soil fertilization and conditioning, proper plant density, crop rotation, weed control, and irrigation practices can go a long way in enhancing the phytoextraction capacity vis-á-vis metals (Lasat 2000). Traditional plant breeding uses the available genetic variation within taxa to bring together the traits that can aid in successful phytoextraction (Li et al. 1995; Liu et al. 2005; Bhargava et al. 2008). However, several anatomical constraints severely restrict sexual compatibility between taxa and pose serious limitations in developing hybrids with increased phytoextraction capability. Biotechnology, especially recombinant DNA technology, has opened new gateways in phytoremediation technology by offering the opportunity for direct gene transfer that would overcome sexual incompatibility, if present (Bhargava et al. 2012a; Mani and Kumar 2013). This approach of the development of recombinant plants having increased uptake, accumulation, and tolerance can be considered as a good alternative. Recent progress in determining the molecular basis for metal accumulation and tolerance by hyperaccumulators has been significant and has provided us a path to tread for achieving this goal (Clements et al. 2002). Genetic engineering has enabled us to transfer specific genes conferring metal tolerance into plants having high biomass. Development of transgenic plants containing alien gene from various life forms and overexpressing these genes have been carried out for improving the phytoextraction potential, and considerable success has been achieved in this direction (Cherian and Oliveira 2005). The transgenic plants exhibiting greater phytoextraction capacity are generally produced by the manipulation of genes from microbes and mammals both of which have the metabolic machinery (degradative enzymes) required to achieve complete mineralization of organic molecules (by virtue of being heterotrophs) (Aken 2008). Apart from this, the introduction of trace-element detoxification systems from yeast and bacteria into plants also holds immense potential. A number of strategies have been followed to achieve this goal which are provided below (Pilon-Smits and Pilon 2002).

6.1 Metal-Binding Molecules

Plants respond to metal stress by mechanisms like chelation and sequestration of metals by use of particular ligands. Plant metal tolerance and accumulation are significantly influenced by overproduction of metal chelator molecules. The two metal-binding ligands best characterized and found across most taxonomic groups are the metallothioneins (MTs) and phytochelatins (PCs) (Cobbett 2000; Cobbett and Goldsbrough 2002; Kim et al. 2013). PCs, a family of thiol-rich peptides, were first reported in fission yeast (*Schizosaccharomyces pombe*) (1981) and later in plants (1985). PCs consist of three amino acids (glutamine, cysteine, and glycine) of which the Glu and Cys residues are linked through a γ-carboxylamide bond. PCs consist of repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly with the basic structure (γ-Glu-Cys)*n*-Gly [(PC)*n*] where n has been reported as high as 11, but usually ranges from 2 to 5 (Memon and Schroder 2009). MTs are ubiquitous, low molecular weight (500–14,000 Da), cysteine-rich proteins that give rise to metal-thiolate clusters and were originally isolated in 1957 as Cu-, Zn-, and Cd-binding proteins in equine kidney (Gratao et al. 2005; Yang et al. 2009). Both PCs and MTs have been widely reported in the plant kingdom ranging from algae, gymnosperms, monocots, and dicots and are known to play a crucial role in the uptake, transportation, tolerance, and accumulation of heavy metals in plants (Hartley-Whitaker et al. 2001; van Hoof et al. 2001; Cobbett and Goldsbrough 2002; Kim et al. 2013; Zagorchev et al. 2013).

Table 3.1 Expression of metallothionein genes in transgenic plants (Reprinted from Bhargava et al. 2012a, with permission from Elsevier)

MT gene	Source	Plant species genetically modified	Reference
mt-IA	Mouse (*Mus musculus)*	*Nicotiana tabacum*	Pan et al. (1994)
mt-ß-glucuronidase fusion	Chinese hamster (*Cricetulus griseus)*	*Nicotiana tabacum*	Hattori et al. (1994)
mt-II	Humans (*Homo sapiens*)	*Nicotiana tabacum*	de Borne et al. (1998)
cup1	Yeast (*Saccharomyces cerevisiae*)	*Nicotiana tabacum*	Thomas et al. (2003)
tymt	Cattail (*Typha latifolia*)	*Arabidopsis thaliana*	Zhang et al. (2004)
hmt	Humans (*Homo sapiens*)	*Medicago varia*	Watrud et al. (2006)
mt-1	Mouse (*Mus musculus)*	*Lycopersicon esculentum*	Sheng et al. (2007)
smtA	Cyanobacteria (*Synechococcus* sp.)	*Arabidopsis thaliana*	Xu et al. (2010)
femt3	Buckwheat (*Fagopyrum* sp.)	*Nicotiana debneyii*	Nikolic et al. (2010)
ccmt1	Pigeon pea (*Cajanus cajan*)	*Arabidopsis thaliana*	Sekhar et al. (2011)
psmtA	Pea (*Pisum sativum*)	*Populus alba*	Turchi et al. (2012)

Overexpression of MTs has opened up new avenues for enhancing heavy metal tolerance and accumulation, especially of Cd and Cu. A number of MT genes from various organisms like mouse, hamster, humans, yeast, cyanobacteria, and plants have been overexpressed in plants for enhancing heavy metal uptake and accumulation (Table 3.1) (Bhargava et al. 2012a). Overexpressed MT genes in *Brassica napus* and *Nicotiana tabacum* have led to increased Cd tolerance in the transgenic plants (Misra and Gedamu 1989). Overexpression of MT genes of plant origin has also resulted in increased metal accumulation in many plants. Enhanced Cu accumulation was reported in *Arabidopsis thaliana* when the pea MT genes were overexpressed in the plants (Evans et al. 1992). Overexpression of the MT yeast gene (*CUP1*) in cauliflower led to a 16-fold increase in tolerance towards Cd (Hesegawa et al. 1997). In tobacco, the overexpression of yeast metallothionein (*CUP1*) promoted 7-fold increase in Cu uptake during copper stress (Thomas et al. 2003). Compared to the control, the genetically engineered plants accumulated 2–3 times more copper when grown in copper-rich soils.

Modification or overexpression of the enzymes involved in the synthesis of PCs has been successfully employed to genetically transform plants having high biomass into efficient phytoremediators (Zhu et al. 1999; Bhargava et al. 2012a). Several reports are available wherein overexpression of the genes encoding enzymes that stimulate the synthesis of cysteine and glutathione has resulted in an increase in the formation of PCs. The tolerance of transgenic plants to metals such as Pb and Cd was reportedly enhanced by the induction and overexpression of a wheat gene encoding phytochelatin synthase (TaPCS1) in shrub tobacco using *Agrobacterium-mediated* transformation (Gisbert et al. 2003). The recombinant plants had longer roots and greener leaves as compared to the normal plants. When grown in metal-contaminated soil, the transgenic plants showed significant increase in Pb concentration in the aboveground parts as well as in roots. An *Arabidopsis* PC synthase (AtPCS1) when overexpressed showed increased production of PCs (1.3- to 2.1-fold) in the transgenics as compared to the wild-type plants (Lee et al. 2003). The lines showed hypersensitivity to Cd and Zn stress but not for Cu. The enzyme cysteine synthase [O-acetyl-L-serine (thiol) lyase] belongs to the family of transferases and is responsible for catalyzing the last step for L-cysteine biosynthesis in plants producing L-cysteine and acetate (Cherian and Oliveira 2005). The tolerance of recombinant plants overexpressing the cysteine synthase cDNA towards a range of heavy metals (Cd, Ni, Pb, Cu, and Se) was analyzed by Kawashima et al. (2004). When grown on an agar medium containing Ni, Cd, and Se, the recombinant tobacco plants exhibited more tolerance than the wild types and showed comparatively higher fresh weight and root length. Two transgenic *Arabidopsis* lines were obtained by employing genetic transformation using the *Atcys-3A* cDNA construct expressing the cytosolic *O*-acetylserine-(thiol) lyase (Dominguez-Solis et al. 2004). There was enhancement in the cysteine availability in the recombinants which enabled the plants to survive under conditions of heavy Cd stress with most of the metal accumulating in the trichomes. Similar results were obtained when *Atcys-3A* was overexpressed in *A. thaliana* which resulted in increased Cd tolerance (Dominguez-Solis et al. 2001). Thus, modification of the cysteine biosynthesis pathway along with an alteration in the number of leaf trichomes may be highly beneficial in increasing heavy metal accumulation. The induction of PCs by Cd suggests that metal tolerance and accumulation can be improved by biosynthesis of phytochelatins, but supporting evidence that this approach would yield a Cd phytoextraction plant is lacking (Bhargava et al. 2012a). Although Cd tolerance has been increased 3–7-fold by overexpression of the enzyme PC synthase, this increase is quite small as compared to the 200-fold higher tolerance exhibited by *T. caerulescens* for Zn and Cd (Heiss et al. 2003; Chaney et al. 2005; Wang et al. 2006). The overexpression of PCs or MTs for Cd accumulation in tobacco plants has yielded similar results (Lugon-Moulin et al. 2004).

Ferritins are a broad superfamily of ubiquitous, intracellular iron storage proteins found in animals, fungi, bacteria, algae, and higher plants, but have not been reported in yeast (Briat et al. 2010a, b). These were first discovered and isolated from the spleen of horses. These proteins play a crucial role in the iron homeostasis and help alleviate oxidative stress through detoxification of excess iron (Briat et al. 2010b). The 450 kDa globular ferritin protein has the shape of an inorganic microcrystalline hollow sphere that surrounds about 24 subunits and can accommodate between 2,000 and 4,000 ferric iron atoms (Arosio and Levi 2002; Li et al. 2012). The overexpression of ferritin in transgenic tobacco and rice showed manifold increase in the iron content in different plant parts (Goto et al. 1998, 1999). Overexpression of ferritin in transgenic tobacco plants was achieved by Vansuyt et al. (2000) that led to an increase in the iron content in leaves and seed, but the results were soil dependent. However, due to paucity of work and lack of conclusive results, more detailed studies are needed to recommend use of ferritins in phytoextraction.

Siderophores are low molecular mass, iron-chelating compounds produced by microorganisms and members of family Poaceae that increase the availability and uptake of iron into roots (Neubauer et al. 2000; Devez et al. 2009). Apart from sequestering iron, siderophores can chelate various other metals like Cr, Cd, Cu, Ni, Pb, and Zn (Nair et al. 2007). This specific chelation of metals can be of great relevance to decontamination of soil having high metal content.

6.2 Membrane Transporters

The acquisition, transport, distribution, and compartmentalization of metals within different tissues and cells are extremely essential for healthy plant growth and development and are also extremely important for remediation of toxic metals (Hall and Williams 2003; Cherian and Oliveira 2005). Transporters play a crucial role by shuttling potentially toxic cations across the membranes. The genetic manipulation of metal transporters can be of immense importance in enhancing metal accumulation and tolerance in plants. Although a number of membrane transporter gene families have been identified using powerful genetic and molecular techniques (Table 3.2) (Bhargava et al. 2012a), the molecular physiology of the plant transport systems is still in its infancy. The prominent cation transporters are mostly in the ZIP (ZRT, IRT-like protein), NAS (nicotianamine synthase), NRAMP (natural resistance-associated macrophage protein), YSL (yellow stripe-like transporter), CDF (cation diffusion facilitator), SAM (S-adenosylmethionine synthetase), FER (Ferritin Fe(III) binding), HMA (heavy metal ATPase), CTR (copper transporters), and IREG (iron-regulated transporter) family (Guerinot 2000; Williams et al. 2000; Talke et al. 2006; van de Mortel et al. 2006; Kramer et al. 2007; Memon and Schroder 2009; Maestri et al. 2010).

Two yeast genes FRE1 and FRE2 that encoded ferric reductase were introduced in tobacco (*Nicotiana tabacum*) under the cauliflower mosaic virus 35S promoter for enhancing uptake of iron (Samuelsen et al. 1998). The transgenic plants accumulated higher iron content in the shoots than the wild-type plants. Overexpression of NtCBP4, the metal transporter gene from tobacco encoding a calmodulin-binding protein, was carried out and the recombinants showed enhanced tolerance to Ni^{2+} and hypersensitivity to Pb^{2+} (Arazi et al. 1999). Thus, selective ion tolerance in crops can be introduced using NtCBP4 which was involved in metal uptake across the plasma membrane. Sunkar et al. (2000) prepared a truncated version of NtCBP4 designated as NtCBP4ΔC that was devoid of the C-terminal, the calmodulin-binding domain and part of the cyclic nucleotide-binding domain. Overexpression of this truncated protein resulted in the transgenic plants showing enhanced Pb tolerance and attenuated accumulation of Pb. The transgenic plants obtained by overexpression of antiporter calcium exchanger 2 (*CAX2*) from *A. thaliana* in *N. tabacum* accumulated more Cd^{2+} and Mn^{2+} and exhibited elevated tolerance to high Mn^{2+}

Table 3.2 Important metal transporter genes in different plant species involved in heavy metal tolerance and accumulation (Reprinted from Bhargava et al. 2012a, with permission from Elsevier)

Family	Gene	Plant	Metal transported	Reference
Zn-regulated transporter (ZRT)	*zip1-12*	*Arabidopsis thaliana*	Zn	Weber et al. (2004), Roosens et al. (2008)
	zip4	*Oryza sativa*	Zn	Ishimaru et al. (2005)
	zip	*Medicago truncatula*	Zn	Lopez-Millan et al. (2004)
	znt1-2	*T. caerulescens*	Zn	van de Mortel et al. (2006)
Fe-regulated transporter (IRT)	*irt1*	*Arabidopsis thaliana*	Fe	Kerkeb et al. (2008)
	irt1-2	*Lycopersicon esculentum*	Fe	Bereczky et al. (2003)
	irt1-2	*T. caerulescens*	Fe	Schikora et al. (2006), Plaza et al. (2007)
Natural resistance-associated macrophage proteins (NRAMP)	*nramp1-3*	*Lycopersicon esculentum*	Fe	Bereczky et al. (2003)
	nramp4	*Thlaspi japonicum*	Fe	Mizuno et al. (2005)
	nramp1	*Malus baccata*	Fe	Xiao et al. (2008)
Cation diffusion facilitator (CDF)	*mtp1*	*Arabidopsis thaliana*	Zn	Kawachi et al. (2008)
	mtp1	*Arabidopsis halleri*	Zn	Willems et al. (2007)
	mtp1	*Thlaspi goesingense*	Zn, Ni	Kim et al. (2004)
	mtp1	*Nicotiana tabacum*	Zn, Co	Shingu et al. (2005)
Al-activated malate transporter (ALMT)	*almt1*	*Triticum* spp.	Al	Sasaki et al. (2004)
	almt1	*Secale cereale*	Al	Collins et al. (2008)
P-type, ATPase (heavy metal associated)	*hma8*	*Glycine max*	Cu	Bernal et al. (2007)
	hma9	*Oryza sativa*	Cu, Zn, Cd	Lee et al. (2007)
	hma4	*Arabidopsis halleri*	Cd	Courbot et al. (2007)
	hma3	*Arabidopsis thaliana*	Co, Zn, Cd, Pb	Morel et al. (2008)
Nicotianamine synthase (NAS)	*nas2, nas3*	*Arabidopsis halleri*	Zn	Talke et al. (2006)
Copper transporter	*copt1*	*Arabidopsis thaliana*	Cu	Sancenon et al. (2004) Andres-Colas et al. (2010)
Yellow stripe-like (YSL)	*ysl2*	*Arabidopsis thaliana*	Fe, Cu	DiDonato et al. (2004)
	ysl3	*T. caerulescens*	Fe, Ni	Gendre et al. (2006)

levels mainly due to increased transport of metal ions in root tonoplast vesicles (Hirschi et al. 2000). It was suggested that modulation of CAX2 could be of immense importance in enhancing plant ion tolerance.

Another approach of developing efficient hyperaccumulator plant by using metal transporters is to alter their metal specificity. The *Arabidopsis* root membrane protein (IRT1) has an important role in the uptake of Fe, Cd, Zn, and Mn (Rogers et al. 2000). However, the substitution of glutamic acid residue with alanine at position 103 resulted in the loss of Zn transport capacity. Two other mutations, at position 100 or 136, eliminated the transport of both Mn and Fe. The experiments clearly demonstrate that the transport profile of a ZIP family member can be altered to raise plants with an altered specificity for selective metals.

6.3 Metal Metabolism

The introduction of an entirely new pathway from a new organism can be a good strategy to remove toxic metals from the environment. This approach was followed to convert toxic methylmercury to volatile elemental mercury by introduction of two bacterial genes (*merA* and *merB*). This strategy has been successfully applied in the engineering of plants capable of removing methyl-Hg from contaminated soils (Rugh et al. 1996; Pilon-Smits and Pilon 2002). *MerA* gene encodes mercuric reductase, an enzyme that reduces ionic mercury to elemental mercury, while *MerB* gene encodes organomercurial lyase, which converts methylmercury to ionic mercury (Summers 1986). *Arabidopsis* was the first to be engineered with the gene *merA* from an Hg-resistant bacterium (Rugh et al. 1996). Researchers later developed transgenic *Arabidopsis* plants wherein *merB* enzyme was targeted to the endoplasmic reticulum (Bizily et al. 2003). The recombinants showed 10 to 70 times higher specific activity to degrade organic Hg than the recombinants with cytoplasmic *merB*. Bizily et al. (2000) accomplished the complete pathway from methyl Hg to metallic Hg by developing double transgenics in *Arabidopsis* in which both *merA* and *merB* were expressed. These double transgenics were 50 times more tolerant to concentrations of methyl Hg in comparison to the wild-type plants and 10 times more tolerant than plants transformed with *merB* alone. In contrast to the *merA-merB* double transgenics, the wild-type plants and single transgenics did not volatilize elemental mercury on supply of organic mercury. The double transgenics efficiently converted organic mercury to elemental mercury and released it in volatile form (Pilon-Smits and Pilon 2002).

The same strategy discussed above has been followed to raise plants having the capacity to volatize mercury from other plant species. Transformation of yellow poplar (*Liriodendron tulipifera*) and eastern cottonwood (*Populus deltoides*) with *merA* led to increased tolerance of the transformants to ionic Hg (Rugh et al. 1998; Che et al. 2003). The recombinant plants cottonwood plants showed normal growth in 25 μM Hg(II), a concentration sufficient to kill the wild-type plants. The recombinants produced up to 4 times more elemental Hg as compared to the wild types, proving the ability of the transgenic plants for efficient uptake and transformation of

Hg to less toxic form. Heaton et al. (2003) successfully transformed rice (*Oryza sativa*) by incorporating the *merA* gene. The recombinants slowly converted Hg^{2+} to the less toxic volatile form and were tolerant to Hg^{2+} concentrations that were detrimental to the wild-type plants (Heaton et al. 2003). The transgenics obtained by double transformation of *Spartina alterniflora* (Smooth Cordgrass or Saltmarsh Cordgrass), the common wetland deciduous grass, with *merA* and *merB* showed increased tolerance to phenylmercuric acetate and mercuric chloride (Czako et al. 2006). Eastern cottonwood when transformed with both *merA* and *merB* exhibited high resistance to phenylmercuric acetate and had a greater rate of detoxification as compared to the control plants (Lyyra et al. 2007). In the transgenics, *merB* released Hg^{2+} by catalyzing the protonolysis of the C-Hg bond, which was subsequently converted to the less toxic, volatile elemental Hg^0 by *merA*. The transgenics released 10 times of elemental Hg as compared to the wild-type plants. However, public acceptance to this approach is low because Hg^0 volatilizes and can redeposit on soil or water bodies (Chaney et al. 2007). In this case, the contaminant instead of being destroyed simply transformed from soil-bound to the airborne form.

6.4 Oxidative Stress Resistance Mechanisms

Another interesting alternative to improve metal tolerance is the overexpression of enzymes involved in general stress resistance mechanisms (Pilon-Smits and Pilon 2002). Nine genes from *Arabidopsis, N. tabacum*, wheat, and yeast involved in oxidative stress response were expressed in an *Arabidopsis* ecotype by Ezaki et al. (2000). The transgenic plants were highly resistant to Al due to the resistance conferred by *Arabidopsis* blue-copper-binding protein gene (*AtBCB*) and three genes from tobacco, viz., glutathione S-transferase gene (*parB*), peroxidase gene (*NtPox*), and GDP-dissociation inhibitor gene (*NtGDI1*). Overexpression of an alfalfa gene encoding a NADPH-dependent aldose/aldehyde reductase in tobacco provided the transgenic plants greater tolerance against oxidative damage induced by heavy metals (Oberschall et al. 2000). Reduced accumulation of Cd and enhanced tolerance to the metal has been reported by the overexpression of glutathione reductase (Pilon-Smits et al. 2000). In another study, the overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in *Lycopersicon esculentum* (family: Solanaceae) led to more accumulation and less deleterious effects of heavy metals in the transgenic plants in comparison to the non-transgenic ones (Grichko et al. 2000).

7 Transgenics in the Field

Although transgenics show promise for phytoextraction of heavy metals during laboratory studies using hydroponic systems or when grown on agar media, their field testing has been rarely carried out. This necessitates thorough testing of the transgenic plants for ascertaining the actual risks involved with the use of such plants in the field for phytoextraction. Field trials with transgenic poplars carried out in former mining areas in Russia and Germany to assess the risk of transgenic poplars developed for the remediation of contaminated soils have shown that the transgenic plants were genotypically stable with no adverse impact on the environment (Peuke and Rennenberg 2005). Theoretical risk assessment on the use of metal-volatilizating plants has shown that the use of transgenic plants having phytoextraction capacity is relatively safe (Lin et al. 2000; Meagher et al. 2000; Rugh et al. 2000). Still, safety issues on the use of phytoextraction technology remain that range from entry of metals in the food chain, accumulation of metals in the topsoil, and spread of the contaminant in the plant material to new environments (Perronnet et al. 2000; Linacre et al. 2003; Mertens et al. 2005, 2007). Although gene escape by the use of transgenics is not a significant problem, there should be proper analysis of the gene frequency for numerous generations over contaminated and noncontaminated soils by greenhouse or pilot experiments performed in the fields (Pilon-Smits and Pilon 2002). Also, the use of transgenics should be undertaken in consonance with the classical breeding approaches which would lower the risk of outcrossing to wild taxa (Bhargava et al. 2012a). The transgenic plant species should be carefully chosen in such a way that they do not have any compatible wild relatives. This may also require use of male-sterile transgenics and harvesting of the plants before blooming (Pilon-Smits and Pilon 2002).

8 Conclusions and Future Perspective

The role of transgenic plants as metal hyperaccumulators is emerging as a cutting-edge area of research and is likely to gain commercial significance in the broader realm of bioremediation. However, most of the studies have been carried in controlled conditions for short durations (Sheoran et al. 2011). Intense research efforts are needed to explore and optimize this underutilized technique for greater field use. Phytoextraction is an environmental friendly, cost-effective, aesthetically pleasing approach which is most suitable for not only the developing countries but also the developed world. An amalgamation of modern biotechnology with conventional breeding is likely to be fruitful in the cleaning of heavy metal-contaminated sites in the coming years.

Developing transgenic plants using biotechnological approaches for efficient phytoextraction of heavy metals requires comprehensive knowledge of the genetic and biological processes in metal hyperaccumulators (Sheoran et al. 2011). This knowledge can help us to develop an ideal hyperaccumulator plant having greater capacity for metal uptake, tolerance, and accumulation (Sheoran et al. 2011). The sequencing of complete genome of hyperaccumulators could go a long way in identifying promising functional noncoding regions which would reduce the burden of comprehensive laboratory and field testing (Wray and Babbitt 2008). The identification of genes associated with metal tolerance using genome sequencing can open up new avenues for the creation of transgenics having desired properties that would help in establishing phytoextraction as a potent technology for environmental cleanup. The understanding of genome evolution in the hyperaccumulator species should be improved by amalgamation of ecological and molecular genomics (Verbruggen et al. 2009). Detailed study of the adaptive evolution across candidate genes associated with metal tolerance and accumulation could yield promising results (Bhargava et al. 2012a). Another possible approach could be the simultaneous expression of several genes in specific cellular components instead of a single gene.

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