Chapter 22
Genetic Engineering of Plants for Heavy Metal Removal from Soil

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22.1 Introduction

Heavy metals are elements with metallic properties and have atomic mass >20 and specific gravity above 5 g cm^{-3} (Rascio and Navari-Izzo 2011). The most common heavy metal contaminants are arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), and zinc (Zn) (Ha et al. 2014). Metals are natural components present in the soil (Tangahu et al. 2011). A large amount of hazardous materials including heavy metals were released into the environment from natural (e.g., geological erosion and saline seeps) and extensive anthropogenic (e.g., mining, agriculture, industry, wastewater treatment, construction) activities, which cause soil, air, and water pollution (Arthur et al. 2005). Some metals are micronutrients (requires at low concentrations) necessary for plant growth, such as Zn, Cu, manganese (Mn), nickel (Ni), and cobalt (Co), while others have unknown biological functions, such as Cd, Pb, and Hg (Appenroth 2010). At higher concentration these metals exert toxic effects on plant and animal health including human (Table 22.1). Unlike organic contaminants heavy metal does not undergo biodegradation and persist in the soil for a long time; therefore, its removal from the soil is receiving great attention.

The conventional (e.g., soil excavation, land filling, soil washing) and physicochemical (e.g., thermal treatment, chemical extraction, encapsulation) methods were employed to remediate contaminated soil. These methods are expensive, inefficient especially for large-scale cleanup, and non-eco-friendly as they destroy natural habitat and leave unsightly scars on the landscape. Consequently, alternative biological methods such as bioremediation (use of living organisms), phytoremediation (use of plants), and zoo remediation (use of animals) (Gifford et al. 2006) are developed for environmental remediation. Of these,

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Table 22.1 General properties, sources, and toxic effects of some heavy metals on plant and human health

Heavy metal	General properties	Sources	Effects on plants	Effects on human
Arsenic (As)	Z: 33 at. wt.: 74.92 SD: 5.73 MP: 817 °C BP: 613 °C	Mining, ore dressing, and smelting of nonferrous metals, production of As and As compounds, petroleum and chemical industry, pesticides, beer, table salt, tap water, paints, pigments, cosmetics, glass and mirror manufacture, fungicides, insecticides, treated wood and contaminated food, dyestuff, and tanning industry	Inhibits photosynthesis, inhibits growth, biomass, and yield; death	As intake: leads to gastrointestinal symptoms; severe disturbances of the cardiovascular and central nervous systems; bone marrow depression; hemolysis; hepatomegaly; melanosis; polyneuropathy; encephalopathy; lung, bladder, kidney, and skin cancer; and other skin lesions
Cadmium (Cd)	Z: 48 at. wt.: 112.4 D: 8.65 g cm^{-3} MP: 321 °C BP: 765 °C A soft, silvery white, ductile metal with a faint bluish tinge	Industrial processes, farming practices, volcanic eruption, mining, ore dressing, smelting of nonferrous metals, battery manufacturing, cigarettes, processed and refined foods, large fish, shellfish, tap water, auto exhaust, plated containers, galvanized pipes, air pollution from incineration, occupational exposure	Chlorosis, growth inhibition, reduction in photosynthesis, water and nutrient uptake, browning of root tips, death	Cd inhalation can cause acute pulmonary and sporadic effects. Cd exposure may cause kidney damage. Carcinogenic
Chromium (Cr)	Z: 24 at. wt.: 51.996 D: 7.14 g cm^{-3} MP: 1,900 °C BP: 2,642 °C A lustrous, brittle, hard metal	Cr compound production, leather-working industry, metal and plastic electroplate, dyestuff and dying by acidic medium,	Alteration in germination, inhibition of plant growth, chlorosis, nutrient imbalance, wilting of tops, root injury,	Cr breathing: irritation on the lining of the nose, nose ulcers, runny nose, asthma, cough, etc. Skin contact:

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Table 22.1 (continued)

Heavy metal	General properties	Sources	Effects on plants	Effects on human
		production and application of dyestuff, metal Cr smelting	inhibition of chlorophyll biosynthesis, photosynthesis, yield	skin ulcers, skin redness, skin swelling. Cr exposure: liver and kidney damage, skin irritation
Copper (Cu)	Z: 29 at. wt.: 63.546 D: 8.94 g cm^{-3} MP: 1,356 °C BP: 2,868 °C A rosy-pink transition metal	Mining; milling; smelting; agriculture; waste disposal; local sources, such as foundries and smelters; application of fungicides and sewage sludge. Cu added to tap water; pesticides; intrauterine devices; dental amalgams; nutritional supplements, especially prenatal vitamins; birth control pills; weak adrenal glands; and occupational exposure	Chlorosis, necrosis, stunting, and inhibition of root and shoot growth. Inhibition of enzyme activity or protein function, impaired cell transport processes, and oxidative damage and metabolic disturbances	Cu toxicity: excessive oxidative stress and tissue damage, abdominal pain, nausea, vomiting, headache, lethargy, diarrhea, respiratory difficulties, hemolytic anemia, gastrointestinal bleeding, liver and kidney failure, and death
Lead (Pb)	Z: 82 at. wt.: 207 SD: 11.35 MP: 327.5 °C BP: 1,740 °C A bluish-white metal of bright luster and is soft, very malleable, ductile, and a poor conductor of electricity	Construction and application of pipes, used for batteries, cable coverings, plumbing, ammunition, fuel, additives, paint pigments, PVC plastics, X-ray shielding, crystal glass production, pesticides, tap water, cigarette smoke, hair dyes, paints, inks, glazes, etc.	Morphology, growth, photosynthesis, inhibition of enzyme activities, water imbalance, alteration in membrane permeability, oxidative stress	Pb exposure: headaches, irritability, abdominal pain, affects the nervous system, Pb encephalopathy, carcinogenic
Mercury (Hg)	Z: 80 at. wt.: 200.59 SD: 13.5 MP: −39 °C	Production and application of Hg catalyst in chemical industry, Hg	Obstruction of water flow, interference of mitochondrial activity,	Hg exposure: lung and kidney damage; other chronic

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Table 22.1 (continued)

Heavy metal	General properties	Sources	Effects on plants	Effects on human
	BP: −357 °C Metallic form, it volatilizes readily at room temperature	battery manufacturing, smelting and restoring of Hg, Hg compound production, pesticide and medicine making, production and application of fluorescent light and Hg lamps, Hg slime from caustic soda production, dental amalgams, large fish, shellfish, medications, manufacture of paper, chlorine, adhesives, fabric softeners, and waxes	oxidative stress, disruption of biomembrane lipids and cellular metabolism, affects photosynthesis	poisoning-like neurological and psychological symptoms, such as tremor; changes in personality, restlessness, anxiety, sleep disturbance, and depression
Nickel (Ni)	Z: 28 at. wt.: 58.71 D: 8.9 g cm^{-3} MP: 1,455 °C BP: 2,732 °C	Residue from the production of nickeliferous compounds; abandoned nickeliferous catalysts; nickeliferous residue and waste from electroplate technology; nickeliferous waste from analysis, assay, and testing activity; hydrogenated oils (margarine, commercial peanut butter, and shortening); shellfish; air pollution; cigarette smoke; plating; occupational exposure	Inhibits chlorophyll biosynthesis, chlorosis, necrosis, water and nutrient imbalance, disorder of cell membrane functions, wilting, browning of root tips	Ni exposure: skin allergies, lung fibrosis, kidney and cardiovascular system poisoning, stimulation of neoplastic transformation, carcinogenic
Zinc (Zn)	Z: 30 at. wt.: 65.39 D: 7.133 g cm^{-3} MP: 419.6 °C BP: 907 °C A bluish-white,	Mining, ore dressing, smelting of nonferrous metals, metal and plastic electroplate, pigment, beaded	Inhibition of root growth, senescence, chlorosis, oxidative stress	High Zn intake relative to Cu and induce Cu deficiency. In humans multiple adverse effects

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Table 22.1 (continued)

Heavy metal	General properties	Sources	Effects on plants	Effects on human
	relatively soft metal	paint and rubber working, Zn compound production, Zinoky battery product industry		include decrease in Cu-dependent enzymes such as superoxide dismutase, ceruloplasmin, and cytochrome C oxidase and changes in immunological parameters, cholesterol, and its lipoprotein distribution

Z atomic number, *at. wt.* atomic weight, *SD* specific density, *D* density, *MP* melting point, *BP* boiling point

References: Denkhaus and Salnikow (2002), Hasanuzzaman and Fujita (2013), Jarup (2003), Martin and Griswold (2009), Sunitha et al. (2013), Tangahu et al. (2011), Uriu-Adams and Keen (2005)

phytoremediation has emerged as inexpensive, eco-friendly, and publicly acceptable remediation technology that utilizes plants and associated microorganisms not only to remove, transform, or stabilize contaminants in the water and soil but also help in CO_2 sequestration, soil stabilization, watershed management, biodiversity improvement, providing diverse sources of energy, and aesthetics (Dickinson et al. 2009).

However, the slow rate of metal removal and incomplete metabolism, as an autotroph plant, lack catabolic enzyme machinery necessary to achieve degradation/full mineralization of xenobiotic substances which stymied the progress of phytoremediation. This results into the accumulation of toxic metabolites as it is into the plant tissues that could be released into the environment (Aken 2008). The possibility of the release of toxic substances into the food chain limits the widespread utilization of phytoremediation. Genetic engineering of plants has the potentiality to overcome these challenges. This chapter summarizes our current knowledge of transgenic plants for heavy metal removal from contaminated area.

22.2 Basic Strategies of Phytoremediation for Metals

The use of plants and their associated microbes to bioremediate contaminated soil, sediments, and water is known as phytoremediation (Arthur et al. 2005; Cherian and Oliveira 2005). The plants used several different phytoremediation strategies such as rhizofiltration, phytostabilization, phytodegradation, phytoextraction, and

phytovolatilization to decontaminate soil and water contaminated with several organic pollutants (Arthur et al. 2005). Out of these, rhizofiltration-, stabilization-, extraction-/accumulation-, and volatilization-based phytoremediation strategies were utilized by the plants to remove metals from contaminant soils (EPA 1998).

In rhizofiltration, plant root system is employed for the elimination of metals from contaminated wastewater, where they absorb and accumulate metals in the roots (Dushenkov et al. 1995). Similarly, the hairy roots induced in some plants by the *Agrobacterium* infection were also utilized for rhizofiltration of radionuclides and heavy metals (Eapen et al. 2003; Straczek et al. 2009).

Phytostabilization simply prevents/reduces the mobility and bioavailability of metals in the environment through immobilization of soil by plant roots (Salt et al. 1995). Therefore, in phytostabilization, even if metal concentration is not reduced, the migration of metals in the surrounding environment is prevented (Li et al. 2000).

In phytoextraction, plants absorb metals from contaminated soils and concentrate/accumulate them into harvestable plant parts (shoot, leaves, etc.). After harvesting, the plant parts may be ashed or utilized for metal recovery followed by disposal of the ashes in a landfill (Kumar et al. 1995). It is the most effective among several phytoremediation methods, although technical difficulties exist in their applications (Krämer 2005).

Phytovolatilization involves the use of plants to take up the metals from the soil, transforming them into volatile form, and release them through transpiration into the atmosphere (Bizily et al. 2003; Rugh et al. 1998). The details of all of these technologies are summarized by Arthur et al. (2005).

22.3 Improving Metal Phytoremediation with Genetic Engineering of Plants

Widespread utilization of phytoremediation can be limited by the small habitat range or size of plants expressing remediation potential and insufficient abilities of native plants to tolerate and accumulate contaminants (Arthur et al. 2005). Several approaches such as agronomic practices (planting density, fertilization) (Chaney et al. 2000), use of soil amendments (organic acids, synthetic chelators) (Joner 2013), and conventional breeding are utilized to increase biomass and metal uptake capacity of the suitable plants used for metal phytoremediation (Pilon-Smits and Pilon 2002).

However, in order to achieve a better phytoremediation efficiency, plants should have the ability to grow outside their area of collection, extensive root system, and fast growth rate; accumulate high amounts of heavy metals in their easily harvestable parts; tolerate soil pollution; and also produce a great quantity of biomass in contamination condition (Pilon-Smits 2005). The development of plants having all these traits is not possible through conventional breeding methods as these are

time-consuming and laborious and have several other ecological, physiological, and biological constraints. On the other hand, the precision of biotechnological approaches, mainly genetic engineering, contributed rapid and significant changes in the crop improvement by offering a wide array of novel genes and traits which can be effectively inserted into candidate plants to improve their phytoremediation potential for metal removal.

22.4 Transgenic Plants for Heavy Metal Removal

Transgenic plants expressing desirable genes from different organisms are developed to increase the heavy metal remediation efficiency of plants. Two principle strategies have been pursued in the phytoremediation techniques, i.e., improved *in planta* and *ex planta* metabolism that may lead to enhanced removal of xenobiotics/heavy metal (Fig. 22.1). *In planta* process includes uptake and diffusion through the roots, trunk, or leaves, sorption and transformation, and/or sequestration via tree metabolic activity manipulation. Alternatively, *ex planta* process includes genetic engineering of plants for extracellular synthesis of reactive enzymes, metal-selective ligands (phytosiderophores or chelating agents), or plant-associated microorganisms (bacteria and entophytes) in the rhizosphere (James and Strand 2009; Ma and Nomoto 1996; Raskin 1996).

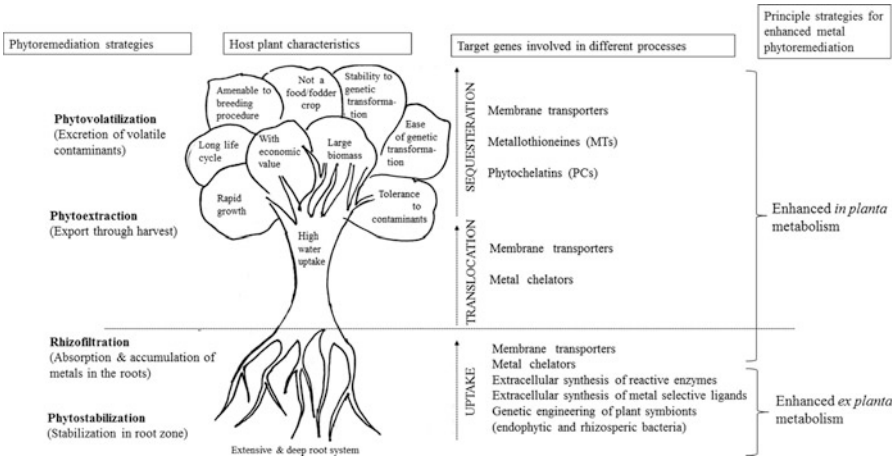

Fig. 22.1 Schematic representation of phytoremediation strategies and important characteristics of host plants along with targeted genes involved in various processes and suitable for genetic modification of plants to enhance phytoremediation of heavy metals

22.4.1 Host Systems

The plant system which is transformed for enhanced phytoremediation ability should possess certain characteristics such as extensive deep rooting system, fast growth rate with high biomass, tolerance to contaminants, ease of genetic transformation, stability of gene expression, and amenable to breeding procedures, and it should not be a food or fodder crop, and it has an advantage if it has an economic value (Kotrba et al. 2009). No single plant species can have all these characteristics. However, depending on phytoremediation strategies, types of heavy metal, and its intended final use, the plant species have to be selected.

22.4.2 Model Systems for Genetic Engineering Study

There is a great diversity in biological species present on the earth, and it is practically impossible to analyze each of them in detail. Since, phylogenetically related species display strong similarities in their genetic makeup, physiology, and behavior, at the molecular level, principle cellular signaling pathways are highly conserved. Biologists recognized the merits of employing model organisms as representatives of their species/subspecies to investigate the phenomena and mechanisms of development in great depth. Moreover, for most model organisms, protocols for transgenesis, full genome sequence information, and numerous bioinformatic resources are available, which offer significant informative data and tools that helps not only to improve plant phytoremediation properties in a highly targeted manner but also to disentangle biological complexity, to unravel networks of molecular interactions, and finally to predict mechanisms in distantly related species (Pitzschke 2013).

22.4.2.1 Whole Plant Systems

Arabidopsis and tobacco are considered as two of the best model species for phytoremediation-related transformation studies on metal tolerance and accumulation and for testing *in planta* expression of potential target gene constructs prior to transformation of other candidate species. The development of optimized tissue culture protocols and well-established genetic transformation methods makes *Arabidopsis* and tobacco ideal candidates for transformation studies. Additionally, this small nonfood plant with short generation time and prolific seed production provides an advantage to test and clarify the roles/functions of transgenes in a very short time.

22.4.2.2 In Vitro Cultures

In vitro culture involves growing plant cells and tissues aseptically on defined medium in environmentally controlled conditions (temperature, photoperiod, and darkness) (George et al. 2008). The in vitro dedifferentiated cells (callus or cell suspension), differentiated organs (roots and shoots), and genetically transformed (hairy roots and shooty teratomas) culture are convenient laboratory tools for phytoremediation studies related to metal removal (Doran 2009).

The in vitro culture systems offer a number of advantages over a whole plant system as follows. (1) Once established, these in vitro culture systems can be propagated indefinitely and are available on demand. (2) The in vitro (callus, cell suspensions) culture systems used as a suitable material to carry out nuclear transformation studies and also play an important role in the selection and confirmation of successful transformants having desired trait prior to time-consuming process of plant regeneration are carried out. (3) Use of in vitro cultures allows experiments to be carried out using material derived from the same parent plant avoiding the effects of variability between individual specimens. (4) An in vitro screening reduces not only the growth period and the treatment time length of the plants but also the space required for the experiments. (5) The in vitro systems allow the independent study of the complex interaction among plant/soil/microbiota to evaluate the participation of specific enzymes, organic compounds, transporters, or peptides involved in the plant response to the pollutants (Boominathan and Doran 2002; Doran 2009; Flocco and Giulietti 2007). (6) Cell cultures are also a useful system for metabolic engineering and for obtaining rapid evidence of the ecotoxicological behavior of chemicals and heavy metals in plants with less analytical expense (Golan-Goldhirsh et al. 2004). (7) Moreover, the environmental factor variability is also reduced, physiological activities can be increased by modifying the culture conditions (e.g., employing biotic and abiotic stress), and it is easier to isolate and analyze metabolites (Hu and Du 2006; Shanks and Morgan 1999). (8) When non-differentiated tissues are employed, genetic and epigenetic changes can be observed due to somaclonal variations (Lee and Phillips 1988). However, this variation and in vitro selection seem to be an appropriate technology for the development of new plant variants with enhanced metal accumulation and extraction properties (Herzig et al. 2003; Jan et al. 1997; Nehnevajova et al. 2007). (9) This approach also allows the analysis of metal accumulation properties of each organ (Kartosentono et al. 2001; Nedelkoska and Doran 2000a) and the possibility to develop industrial bioreactor models (Giri and Narasu 2000; Kim et al. 2002).

In spite of the number of advantages offered by in vitro culture systems in phytoremediation research, it is neither practical nor a feasible technology for direct large-scale phytoremediation applications. However, in vitro cell suspensions and hairy root culture are frequently used as model systems for understanding the metabolic and tolerance mechanisms that function in whole plants (Doran 2009).

22.4.3 Methods of Gene Transfer to the Plants

Significant achievements in vector-mediated/indirect and vectorless/direct gene transfer strategies have tremendously been assisting scientists to incorporate genes from any organisms to plants in a specific and targeted approach. Among the various vector-mediated transformation strategies, *Agrobacterium tumefaciens* plant-pathogenic soil bacterium has been widely used as a vector to create transgenic plants. This bacterium is known as "natural genetic engineer" of plants because these bacteria have natural ability to transfer a portion of its Ti (tumor inducing) plasmid and T-DNA (transfer DNA) to the genome of the host plant upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti plasmid in place of unwanted sequences (Block 1993; Gelvin 2003). The T-DNA transfer and its integration into the plant genome are governed by various *Agrobacterium* and plant tissue-specific factors which include genotype of the plant, type of explant, plasmid vector, bacterial strain, composition and pH of culture medium, temperature and time of cocultivation, tissue damage, and suppression or elimination of *Agrobacterium* infection after cocultivation (Ziemienowicz 2014). *A. tumefaciens*-mediated transformation method has been extensively utilized for engineering dicotyledonous plants including tobacco (Gisbert et al. 2003; Pomponi et al. 2006), *A. thaliana* (Kim et al. 2005; Shukla et al. 2013), and Indian mustard (Gasic and Korban 2007; LeDuc et al. 2004) for heavy metal phytoremediation. However, monocotyledon plants and forest trees are considered as recalcitrant to *Agrobacterium*-mediated transformation. Therefore, considering the limitations of vector-mediated (*Agrobacterium*) gene transfer methods, the vectorless/direct gene transfer methods have been developed. The microprojectiles or biolistics or particle gun for gene transfer is widely employed in plant genetic engineering studies (Chen et al. 1998; Rugh et al. 1998). In this method, tungsten or gold microparticles coated with DNA of interest are accelerated to high velocity by a particle gun apparatus into living plant tissue. These particles with high kinetic energy penetrate the cells and membranes and carry foreign DNA inside of the bombarded cells (Block 1993).

Over the years, gene transfer techniques have been refined, and many underlying mechanisms have been decoded. However, there are several experimental prerequisites to be followed for effective gene delivery. Each plant has its own requirements, and it is necessary to establish a separate protocol of transformation individually. The choice of gene to be incorporated, type of vector, genotype, promoter, and reproducible regeneration system are some of the parameters that determine success of plant genetic transformation.

22.4.4 Target Genes

The plants were able to grow and colonize in heavy metal-contaminated sites by the development of several metal exclusion and tolerance mechanisms (Viehweger 2014). An integrated physiological, anatomical, biochemical, and molecular studies reveal the metal tolerance mechanism of plants as well as lead to the identification of target genes involved in various processes including metal uptake, translocation, and sequestration in plant. Therefore, the classical approach to engineer plants for enhanced heavy metal remediation consists in the strengthening of endogenous systems by upregulation of the expression or activity of target genes. Some of the target genes useful for genetic engineering of plants for enhanced heavy metal phytoremediation are enlisted below.

22.4.4.1 Membrane Transporters

Membrane transporters are likely to play a central role in translocation of heavy metals from source to root and to shoot and its further sequestration into specialized compartments (viz., vacuoles, chloroplast, mitochondria) to ensure sufficient levels to the necessary compartments while safely storing metals under times of excess (Cherian and Oliveira 2005; Palmer and Guerinot 2009). Recent developments in molecular biology, bioinformatics, omics, and sequencing technologies lead not only to the identification of novel vacuolar/plasma membrane-localized transporters but also understanding their role in heavy metal uptake, translocation, and sequestration. Many membrane transporters belonging to heavy metal ATPases, natural resistant-associated macrophage proteins (NRAMPs), cation diffusion facilitators (CDF), and ZIP (zinc-regulated transporter, iron-regulated transporter-related protein) gene families were identified in plant genome (Hall and Williams 2003; Krämer 2010). The manipulation of membrane transporter genes and its transfer to the non-accumulator plant potentially offer an enhanced phytoremediation process with increased heavy metal uptake and tolerance as shown in Table 22.2. Our knowledge of the molecular nature and regulation of transporters has expanded vastly over the past years. Fundamental research into transport mechanisms in plants is leading to the development of genetic-engineered plants provided with specialized membrane transporters can be geared up for cleanup of heavy metal-contaminated soils (phytoremediation), mining of rare metals (phytomining), and enhanced human nutrition through accumulation of nutritionally important metals in plant tissues (biofortification) which in turn could expand available arable land (Schroeder et al. 2013).

Table 22.2 Summary of transgenic plants developed for heavy metal phytoremediation

Gene family	Gene	Product	Origin	Target plant	Effect	Reference
Membrane transporters	AtMHX1	Vacuolar transporter	*A. thaliana*	Tobacco	Reduced tolerance to Mg and Zn	Shaul et al. (1999)
	AtCAX2	Vacuolar transporter	*A. thaliana*	Tobacco	15–20 % more metal ions in the shoots and higher root tonoplast transport in transgenic plants than in controls	Hirschi et al. (2000)
	ZAT1	Zn transporter	*A. thaliana*	*Arabidopsis*	Enhanced Zn tolerance and 2× higher Zn accumulation in roots	van der Zaal et al. (1999)
	ZntA	Heavy metal transporter	*E. coli*	*Arabidopsis*	Transgenic plant grew better than WT in medium with 0.7 mM Pb (II) or 70 μM Cd (II) and showed higher fresh weight	Lee et al. (2003)
	YCF1	Transport protein	Yeast	*Arabidopsis*, poplar	Tolerance to 1 mM Pb (II) and increased biomass and Cd tolerance on agar media	Song et al. (2003)
	AtNramp1	Fe transporter		*Arabidopsis*	At 600 μM iron concentrations, only the transgenic plants survived for longer periods	Curie et al. (2000)
	AtNramp3	Fe transporter		*Arabidopsis*	Increased accumulation of Fe, on Cd^{2+} treatment, and Cd hypersensitivity	Thomine et al. (2000)
	NtCBP4	Tobacco calmodulin-binding channel protein	*N. tabacum*	*N. tabacum*	Confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity in transgenic plants	Arazi et al. (1999)

	CAX2	Low affinity Ca^{2+}, heavy metal cation/H^+ antiporter 2	*A. thaliana*	*N. tabacum*	Confers high Cd^{2+} transport and selectivity in tonoplast vesicles and increased Cd accumulation in roots of transgenic plants	Korenkov et al. (2007a)
	CAX4	Low affinity Ca^{2+}, heavy metal cation/H^+ antiporter 4	*A. thaliana*	*N. tabacum*	Confers tolerance to high toxic levels of Cd, Zn, and Mn in transgenic tobacco plants	Korenkov et al. (2007b)
	merC	Bacterial Hg^{2+} importer of unknown mode of transport and energy-coupling mechanism	*A. ferrooxidans*	*A. thaliana*	Hg^{2+} hypersensitivity biomass reduced by 6.4 times when grown on medium with 3 μM Hg^{2+}. Leaves submersed into test solution with 100 μM Hg^{2+} accumulated over a 3-h period 3.2 more Hg	Sasaki et al. (2006)
	PgIREG1	Vacuolar transporter	*Psychotria gabriellae*	Yeast and *Arabidopsis*	Confers Ni tolerance when expressed in yeast and in transgenic plants	Merlot et al. (2014)
	MerE	Mercury transporter	*E. coli*	*A. thaliana*	The transgenic *Arabidopsis* expressing MerE accumulated significantly more methylmercury and mercuric ions into plants than the WT *Arabidopsis*	Sone et al. (2013)
Phytochelatins (PCs)	TaPCS1	Phytochelatin synthase	Wheat	Tobacco	High Pb (1 mM) and Cd (50 mM) tolerance, longer roots, higher and greener leaves at seedling stage than WT	Gisbert et al. (2003)
	AtPCS1	Phytochelatin synthase	*A. thaliana*	*Arabidopsis*	Hypersensitivity to $CdCl_2$ and $ZnCl_2$	Lee et al. (2003)

(continued)

Table 22.2 (continued)

Gene family	Gene	Product	Origin	Target plant	Effect	Reference
	AtPCS1	Phytochelatin synthase	*A. thaliana*	*Arabidopsis*	20–100× more biomass on 250–300 mM arsenate and hypersensitivity to Cd	Li et al. (2004)
	OASTL	Cysteine synthase	*A. thaliana*	*Arabidopsis*	9× increase in Cd tolerance on medium with 200 mM $CdCl_2$	Domínguez-Solís et al. (2004)
	OASTL	Cysteine synthase	*A. thaliana*	*Arabidopsis*	Tolerance up to 400 mM $CdCl_2$ with exogenous cysteine supply and 72 % more Cd accumulation (mature plants grew on 250 mM $CdCl_2$ for 14 days)	Domínguez-Solís et al. (2004)
	OASTL	Cysteine synthase		Tobacco	Tolerance up to 300 mM Cd, 250 mM Se, and 500 mM Ni and produced higher biomass when grown on agar medium	Kawashima et al. (2004)
	AtPCS1	Phytochelatin synthase	*A. thaliana*	*N. tabacum*	2.2 times longer roots and 1.6 times higher Cd^{2+} accumulation in roots and shoots from hydroponic solution with 30 μM Cd^{2+} and 250 μM glutathione	Pomponi et al. (2006)
	AtPCS1	Phytochelatin synthase	*A. thaliana*	*B. juncea*	1.9 and 1.4 times longer roots on media with 100 μM Cd^{2+} and 500 μM AsO_4^{3-}, respectively	Gasic and Korban (2007)
	AtPCS1 and MTL4	Phytochelatin synthase and genetic fusion of four human metallothioneins	*A. thaliana* and *H. sapiens*	*M. huakuii*/*A. sinicus*	Roots of *A. sinicus* colonized with rhizobia *M. huakuii* producing AtPCS1 and	Ike et al. (2007)

					AtPCS1 + MT4 accumulated, respectively, 2.5 and 3 times more Cd from soil containing 1 ppm Cd. Colonized nodules increased Cd concentration [only] by 30 %	
	TaPCS1	Phytochelatin synthase	*Triticum aestivum*	*N. glauca*	1.6 times longer roots on media with 800 μM Pb^{2+} or 50 μM Cd^{2+}. Shoots of transformed line NgTP1 accumulated from polluted soil, respectively, 6.0, 3.3, 4.8, 18.2, and 2.6 times more Pb, Cd, Zn, Cu, and Ni	Martínez et al. (2006)
	CdPCS1	Phytochelatin synthase	*Ceratophyllum demersum*	*Arabidopsis*	*Arabidopsis* showed a significant enhanced accumulation of heavy metal(loid)s in aerial parts without significant difference in growth parameters in comparison to WT *Arabidopsis* plants	Shukla et al. (2013)
Metallothioneins	MT2	Metallothionein	Human	Tobacco, rapeseed	Enhanced Cd tolerance at the seedling stage	Misra and Gedamu (1989)
	MT1	Metallothionein	Mouse	Tobacco	Tolerated 200 mM $CdCl_2$ at the seedling level	Pan et al. (1994)
	CUP1	Metallothionein	Yeast	Cauliflower	Tolerated 400 mM $CdCl_2$ in hydroponic medium	Hasegawa et al. (1997)
	CUP1	Metallothionein	Yeast	Tobacco	2–3× higher Cu content than the control but no Cd tolerance	Thomas et al. (2003)

(continued)

Table 22.2 (continued)

Gene family	Gene	Product	Origin	Target plant	Effect	Reference
	PsMTA	Metallothionein	Pea	*Arabidopsis*	8× higher Cu accumulation	Evans et al. (1992)
	HisCUP1	CUP1 with hexahistidine extension	Recombinant fusion	*N. tabacum*	By 75–90 % higher Cd^{2+} accumulation from sandy soil with 0.2 ppm of Cd and humus soil with 0.4 ppm Cd (2.5 ppb exchangeable with Ca^{2+})	Macek et al. (2002)
					Transgenic plant tolerated up to 16.2 ppm Cd in sandy soil	Pavlíková et al. (2004)
	merP	Hg^{2+}-binding protein encoded within TnMERI1	*Bacillus megaterium*	*A. thaliana*	Capable of germination and growth on media with 12.5 μM Hg^{2+} accumulating 5.35 μg Hg^{2+} g^{-1} of fresh seedling weight	Hsieh et al. (2009)
	ScMTII	Metallothionein	*Saccharomyces cerevisiae*	*N. tabacum*	The transgenic tobacco plant accumulated 3.5–4.5-fold more Cd above the threshold level of 100 mg Cd kg^{-1}	Daghan et al. (2013)
Metal chelators	HvNAS1	Nicotianamine synthase gene	*Hordeum vulgare*	*Arabidopsis*	Confers enhanced tolerance of high levels of metals, in particular Ni	Kim et al. (2005)
				Tobacco	Transgenic tobacco plants with a high level of nicotianamine grew well in a Ni-enriched serpentine soil without developing any symptoms of Ni toxicity	Kim et al. (2005)

	Citrate synthase	Citrate synthase	–	*N. tabacum*	Overproduction of citrate was shown to result in aluminum (Al) tolerance in transgenic plants	de la Fuente et al. (1997)
				Carica papaya	Overproduction of citrate was shown to result in Al tolerance in transgenic plants	de la Fuente et al. (1997)
	NAAT	Nicotianamine aminotransferase	Barley	Rice	The transgenic plants released more phytosiderophores and grew better on iron-deficient soils	Takahashi et al. (2001)
Manipulation of enzymes involved in heavy metal phytoremediation	gshI	Bacterial γ-glutamylcysteine synthetase (γ-ECS)	*E. coli*	*B. juncea*	2.1 times longer roots in media with 200 μM Cd^{2+}. By 90 % higher shoot Cd levels when grown in media with 50 μM Cd^{2+}	Zhu et al. (1999a)
					When grown on polluted soil, shoots showed 1.5, 2.0, 2.0, and 3.1 times higher Cd, Zn, Cu, and Pb levels, respectively	Bennett et al. (2003)
				Populus	Cd accumulation from soil containing 225 ppm Cd higher by 2.5 and 3 times with plants producing γ-ECS to cytosol and plastids, respectively	Koprivova et al. (2002)

(continued)

Table 22.2 (continued)

Gene family	Gene	Product	Origin	Target plant	Effect	Reference
	gshI and arsC	Bacterial γ-glutamylcysteine synthetase and bacterial arsenate reductase	*E. coli*	*A. thaliana*	6 times higher biomass yield from medium with 200 μM AsO_4^{3-}. 3 times higher As accumulation from medium with 125 μM AsO_4^{3-}	Dhankher et al. (2002)
	gshII	Bacterial and yeast glutathione synthetase	*E. coli*	*B. juncea*	1.5 times longer roots on medium with 200 μM Cd^{2+}. By 20 % enhanced Cd^{2+} accumulation from media with 50 μM Cd^{2+}	Zhu et al. (1999b)
	GSH1	Bacterial and yeast glutathione synthetase	*S. cerevisiae*	*A. thaliana*	No effect on Cd^{2+}, AsO_4^{3-}, and AsO_2^{-} tolerance. Increased accumulation of Cd (4 times from media with 30 ppm Cd^{2+}) and As (2.5 and 4.4 times from media with 28 ppm AsO_4^{3-} and AsO_2^{-}, respectively)	Guo et al. (2008)
	GSH1 and AsPCS1	Bacterial and yeast glutathione synthetase and phytochelatin synthase of garlic	*S. cerevisiae* and *A. sativum*	*A. thaliana*	2 times longer roots on media with 50 μM Cd^{2+}, 150 μM AsO_4^{3-}, or 50 μM AsO_2^{-}. Increased accumulation of Cd (10 times from media with 30 ppm Cd^{2+}) and As (3 and 10 times from media with 28 ppm AsO_4^{3-} and AsO_2^{-}, respectively)	Guo et al. (2008)
	APS1	ATP sulfhydrylase	*A. thaliana*	*B. juncea*	1.5 times longer roots and 1.4 times higher biomass with plantlets grown on medium with 400 μM SeO_4^{2-}. Improved accumulation of Se and S: 3 times higher Se	Pilon-Smits et al. (1999)

					levels in shoots when plantlets grown on medium with 40 μM SeO_4^{2-}. Doubled levels of glutathione, both in shoots and roots	
	SMT	Selenocysteine methyltransferase	*A. bisulcatus*	*B. juncea*	No phytotoxicity of 25 μM SeO_3^{2-} in medium (97 % growth inhibition with WT). By 40 % reduced growth on medium with 25 μM SeO_4^{2-} (60 % inhibition with WT). Se accumulation from media with 200 μM SeO_4^{2-} and 100 μM SeO_3^{2-} increased 4 and 2 times, respectively	LeDuc et al. (2004)
	SMT and APS1	Selenocysteine methyltransferase and ATP sulfhydrylase (*Arabidopsis*)	*A. bisulcatus* and *A. thaliana*	*B. juncea*	Se accumulation from media with 200 μM SeO_4^{2-} increased 9 times (6 times compared to single-transformed APS1 plant)	LeDuc et al. (2006)
	merA and merB	Mercuric reductase and organomercurial reductase	*E. coli* Tn21	*N. tabacum* chloroplasts	Doubled biomass yield with seedlings grown on medium with 400 μM phenyl–Hg^+	Ruiz et al. (2003)
	merApe9	Encoding the same polypeptides as bacterial merA but with codons optimized for plants		*A. thaliana*	Germinating on media with 50–100 μM Hg^{2+} (25 μM Hg^{2+} is lethal to germination of WT seeds). 2.5 times higher rate of Hg volatilization from hydroponic medium with 5 μM Hg^{2+} (rate of 5 μg Hg^0 $[g\ FW]^{-1}\ min^{-1}$)	Rugh et al. (1996)

(continued)

Table 22.2 (continued)

Gene family	Gene	Product	Origin	Target plant	Effect	Reference
				N. tabacum	Germinating on media with 100–350 μM Hg^{2+} (50 μM Hg^{2+} is lethal to germination of WT seeds). Increased rate of Hg volatilization from solution with 25 μM Hg^{2+}: 9 times by roots (23.8 μg Hg^0 [g FW]$^{-1}$ min^{-1}), 8 times by leaves (6.9 μg Hg^0 g^{-1} min^{-1}), and 5 times by stem (4.1 μg Hg^0 g^{-1} min^{-1})	He et al. (2001)
	merApe9 and merB	Encoding the same polypeptides as bacterial merA and merB, respectively, but with codons optimized for plants		*A. thaliana*	Seedlings with MerB localized in ER volatized Hg from solution with 25 μM phenyl–Hg^+ at rate of 760 ng Hg^0 [g FW]$^{-1}$ min^{-1}.	Bizily et al. (2003)
	merBpe			*A. thaliana*	Germinating on media with 5 μM CH_3–Hg^+ or phenyl–Hg^+	Bizily et al. (1999)
	merApe9 and merBpe			*A. thaliana*	Germinating on media with 10 μM CH_3–Hg^+ (1 μM CH_3–Hg^+ is lethal to germination of WT seeds). Seedlings volatize Hg from solution with 25 μM phenyl–Hg^+ at a rate of 60 ng Hg^0 [g FW]$^{-1}$ min^{-1}	Bizily et al. (2000)

				S. alterniflora	Callus culture can tolerate 500 μM Hg^{2+} and 100 μM phenyl–Hg^{+} (225 μM Hg^{2+} or 50 μM phenyl–Hg^{+} is lethal to WT callus)	Czakó et al. (2006)
	merA18			*L. tulipifera*	Germinating on media with 50 μM Hg^{2+} (25 μM Hg^{2+} is lethal to germination of WT seeds). Hg volatilization from hydroponic media with 10 μM Hg^{2+} improved 10 times (rate of 1.2 μg Hg^{0} $[g\ FW]^{-1}\ day^{-1}$)	Rugh et al. (1998)
	CGS1	Cystathionine-γ-synthase	*A. thaliana*	*B. juncea*	Doubled root length on medium with 200 μM SeO_3^{2-} but no effect on tolerance to SeO_4^{2-}. Doubled rate of Se volatilization from media with 40 μM SeO_3^{2-} (up to 0.23 g Se $[g\ FW]^{-1}\ day^{-1}$) or 40 μM SeO_4^{2-} (up to 0.3 g Se $[g\ FW]^{-1}\ day^{-1}$)	Van Huysen et al. (2003)
	Human CYP2E1 and glutathione *S*-transferase (GST)	Human CYP2E1 and glutathione *S*-transferase (GST)		Alfalfa	Alfalfa plants exhibited strong resistance toward the mixtures of Cd and trichloroethylene that were metabolized by the introduced GST and CYP2E1 in combination	Zhang and Liu (2011)

WT wild-type plants

22.4.4.2 Phytochelatins (PCs)

Phytochelatins (PCs) are low molecular weight cysteine-rich metal-binding peptides whose synthesis is induced by heavy metals. PCs are synthesized nontranslationally from glutathione by the enzyme phytochelatin synthase to form molecules of (γ-EC) nG (where $n \sim 2–11$) (Cobbett 2000; Krämer 2010). They chelate heavy metals with their thiol (-SH) group of cysteine, and the resulting metal–phytochelatin complexes are sequestered into vacuoles. This results into development of heavy metal tolerance in plants by decreasing free heavy metal ion concentration in plant fluids. A number of structural variants are identified in a wide variety of plant species, and different metals, including Cd, Hg, Ag, Cu, Ni, Au, Pb, and Zn, are found to induce PC production; however, Cd is by far the strongest inducer (Mejáre and Bülow 2001; Pal and Rai 2010). Transgenic plants with increased phytochelatin level through overexpression of phytochelatin synthase resulted in enhanced heavy metal tolerance (Table 22.2).

22.4.4.3 Metallothioneins (MTs)

Metallothioneins (MTs) are ubiquitous, low molecular weight (5–10 kDa), cysteine-rich proteins present in plants, animals, fungi, and cyanobacteria. In plants, MTs are suggested to be involved in metal tolerance or homeostasis, detoxification, and distribution as they are able to bind metal ions by the formation of mercaptide bonds with the numerous cysteine residues. Recent reports show that MTs are also involved in the scavenging of reactive oxygen species (Hassinen et al. 2011; Freisinger 2011; Leszczyszyn et al. 2013). Each MT exhibits preferences for a special metal ion due to coordination residues other than cysteine and differences in folding and stability in dependence on the bound metal (Leszczyszyn et al. 2007). However, various MT genes are cloned and transferred to several plants resulting in constitutive enhancement of heavy metal tolerance in transgenic plants (Eapen and D'Souza 2005) (Table 22.2).

22.4.4.4 Metal Chelators (MCs)

Metal chelators are mostly low molecular weight organic compounds, viz., organic acids (malate, citrate) and amino acids (nicotianamine, histidine), which can act as metal-binding ligands. MCs are involved in the uptake, transport, and sequestration of possibly toxic-free metal ions present in cytosol or plant fluids to the vacuolar compartment, providing metal tolerance to the plants (Haydon and Cobbett 2007). Overexpression of metal chelator genes in plants showed enhanced heavy metal tolerance (Table 22.2).

22.4.4.5 Manipulation of Enzymes Involved in Heavy Metal Phytoremediation

Genetic-engineered plants to express the enzymes responsible for detoxification, tolerance, and chelation of heavy metals, oxidative stress response/mechanisms, and plant architecture are developed and showed enhanced phytoremediation capacity (Table 22.2). Transgenic plants overexpressing the genes encoding the enzymes for histidine biosynthesis, ACC deaminase, Hg^2 reductase, glutathione synthetase, arsenate reductase, and aldolase/aldehyde reductase were shown to become more tolerant to the toxic levels of metals and carried out phytoextraction with increasing potential (Chatterjee et al. 2013).

22.4.5 Optimization of Transgene Expressions in Transgenic Plants

The expression levels of transgene in transgenic plants are generally low in the case of heterologous gene expression system. Since the additional transcriptional and translational enhancers and constitutive and tissue-specific promoters are needed to optimize expression of transgenes that results into increased phytoremediation capacity of transgenic plants.

22.4.5.1 Use of Constitutive Promoters

The promoter is used to manage gene expressions. The selection of a promoter in a particular expression system depends on the entry and integration of align gene into the host genome. Phytoremediation-related plant transformation studies have largely utilized the cauliflower mosaic virus 35S (CaMV 35S) (Thomine et al. 2000), and actin promoters (Dhankher et al. 2002) have been employed to drive constitutive high level of expression of integrated genes in most of the plant tissues.

22.4.5.2 Inducible Promoters

The performance of inducible promoters is not conditioned to endogenous factors but to environmental conditions and external stimuli that can artificially be controlled. The expression pattern of the gene may also be programmed to be only under certain environmental conditions (e.g., stress, light) by using different promoters. The double transgenic plant with strong tolerance to the As and enhanced As accumulation was developed by co-expressing two bacterial genes. The *E. coli* arsenate reductase, *arsC*, gene was expressed in leaves as driven by a light-induced

soybean RuBisCO small subunit 1 (*SRS1*) promoter and results into arsenate reduction in leaves. In addition, the *E. coli* γ-glutamylcysteine synthetase, γ-*ECS*, was expressed in both roots and shoots, driven by strong constitutive *Actin2* promoter, and leads to enhanced biosynthesis of thiol-rich peptides for AsIII complexation. Thus, they provide increased AsV tolerance (Dhankher et al. 2002).

22.4.5.3 Tissue-Specific Expression of Transgenes

Tissue-specific promoters direct the expression of a gene in specific tissues (e.g., roots, leaves, tubers, fruits and seeds, etc.). The expression of metal hyper accumulator genes in easily harvestable plant tissues like leaves has an advantage in phytoextraction and phytomining. However, fruit-/seed-specific expression of transgene for heavy metal phytoremediation face objections due to fruits/seeds is eaten by birds and other organisms. This leads to the biomagnification of heavy metal in the food chain.

Addressing the problem of high Cd accumulation in rice plants including seeds, Li et al. (2007) focused their efforts on reducing Cd accumulation in rice seeds by silencing the expression of phytochelatin synthase (PCS) gene, *OsPCS1*. If *OsPCS1 gene* was constitutively silenced, the transgenic rice plant may become sensitive to Cd exposure that would affect the growth of plants on Cd-contaminated soil. Hence, the knockdown of *OsPCS1* gene by RNAi under the control of the seed-specific promoter ZMM1 (from maize) in rice showed reduced Cd accumulation in rice seeds as compared to the wild plants. Furthermore, this transgene-induced RNA interference approach can be used to control heavy metal accumulation in the edible part of the food crops that would be grown on metal-contaminated soils or irrigated with metal-contaminated water. Thus, it is possible to enhance tolerance to heavy metal and restrict its entry into the food chain.

22.4.5.4 Organelle Targeting

Transgene integration in the various targeted organelle compartments like chloroplast, mitochondria, and vacuoles facilitates sequestration/detoxification of toxic heavy metals in the organelle. This prevents adverse interaction of heavy metals with cytoplasmic environment. Plant chloroplast genetic engineering offers several advantages over nuclear transformation, i.e., very high levels of transgene expression to 46 % of total leaf protein, transgene containment by the maternal inheritance/cytoplasmic inheritance of the plastid genome, the absence of gene silencing and positioning effect, the ability to express multiple genes in a single transformation event, and the ability to express bacterial genes without codon optimization. However, chloroplast transformation technology success was limited by several challenges, viz., it requires a species-specific vectors and achieving homoplasmy (integration of transgene into each chloroplast genome and elimination of untransformed genome).

Phytoremediation of organomercurial compounds via chloroplast genetic engineering was achieved by integrating the native *mer* operon containing the *merA* (mercuric ion reductase) and *merB* (organomercurial lyase) genes into the tobacco chloroplast genome. The transgenic plants showed better growth and resistance to very high concentrations of PMA (phenylmercuric acetate) up to 400 μM as compared to control untreated plants (Ruiz et al. 2003). Therefore, chloroplast engineering could be a beneficial approach for Hg phytoremediation as well.

22.4.6 Genetic Engineering of Plant Symbionts

The plant-associated bacteria including endophytic, phyllospheric, and rhizospheric bacteria can affect plant growth and development by fixing atmospheric nitrogen, increasing bioavailability of essential mineral nutrients, and synthesizing phytohormones and enzymes involved in plant growth hormone metabolism (Weyens et al. 2009a, b). The potential of plants and their associated microorganisms in degradation and removal of environmental pollutants/heavy metals has been recently recognized. However, not every plant-associated bacterium possesses the ability to degrade every toxic compound, and not every bacterium that has a degrading capacity toward a contaminant limits their widespread application in phytoremediation (Newman and Reynolds 2005). However, the success rate of phytoextraction of heavy metals using endophytic bacteria remains slow because of the lack of proper strains with heavy metal resistance and detoxification capacities (Luo et al. 2011). To overcome these constraints, plant-associated bacteria are engineered with the appropriate characteristics to achieve enhanced phytoremediation (Menn et al. 2000).

Several plant-associated bacteria are reported to accelerate the phytoremediation in metal-contaminated soils by promoting plant growth and health (Miransari 2011; Rajkumar et al. 2012). On the other hand, the rate of phytoremediation is limited by metal availability, uptake, translocation, and phytotoxicity (Weyens et al. 2009a, b). Therefore, to improve the efficiency of phytoremediation of toxic metal-contaminated soils, plant-associated bacteria can be engineered with pathways for the synthesis of natural metal chelators such as citric acid to increase metal availability for plant uptake or alternatively with metal sequestration systems to reduce phytotoxicity and increase metal translocation to aerial plant parts (Sessitsch and Puschenreiter 2008). *Lupinus luteus* grown on a nickel-enriched substrate and inoculated with an engineered nickel-resistant bacterium *Burkholderia cepacia* L. S.2.4 *ncc-nre* showed significantly increased nickel concentration in roots (Lodewyckx et al. 2001).

A. thaliana PCS1 gene along with a genetic fusion of four mammalian MT-coding sequence was expressed in *Mesorhizobium huakuii* subsp. rengei (strain B3) and resulted in approximately 25-fold increase in Cd accumulation as compared to its natural capability (Rajkumar et al. 2012). The colonization of Chinese milk vetch (*Astragalus sinicus*) with the B3 strain in rice paddy soil containing

1 mg kg^{-1} Cd promoted uptake of the metal in roots but not in nodules, by three times. This strategy would be useful in the rhizofiltration or transient phytostabilization of heavy metals in soil.

The possible advantages of using genetic-engineered endophytic microorganisms to improve xenobiotic remediation were summarized by Newman and Reynolds (2005), a major advantage being where genetic engineering of a xenobiotic degradation pathway is required, bacteria are easier to manipulate than plants. In addition, quantitative gene expression of pollutant catabolic genes within the endophytic populations could be a useful monitoring tool for assessing the efficiency of the remediation process. The unique niche of the interior plant environment provides the xenobiotic degrader strain with an ability to reach larger population sizes due to the reduced competition. Another important advantage of using endophytic pollutant degraders is that any toxic xenobiotics taken up by the plant may be degraded *in planta*, thereby reducing phytotoxic effects and eliminating any toxic effects on herbivorous fauna residing on or near contaminated sites (Ryan et al. 2008).

22.5 Hairy Root Cultures for Heavy Metal Removal

Agrobacterium rhizogenes is a plant-pathogenic gram-negative soil bacterium, initiating hairy root disease in most of dicotyledonous plants upon infection. These hairy roots no longer require the continuous presence of the inciting bacterium for proliferation, demonstrating that the plant cells have been transformed. The molecular basis of this transformation revolves around the activities of a large (~200 kb) root-inducing (Ri) plasmid resident in *A. rhizogenes* virulent strains. Specifically, a portion of the Ri plasmid, the transferred DNA (T-DNA), delineated by 25 base pair border repeats, is transferred into the plant cells, integrated into the nuclear DNA, and expressed. This transformation process results into induction of hairy roots from infected plant tissue. A number of excellent review articles have been published describing *Agrobacterium* and plant genes involved in T-DNA transfer and integration (Gelvin 2000, 2003) and *Agrobacterium* and plant cell interaction (McCullen and Binns 2006); hence, these aspects are not described in this chapter.

The hairy root disease is characterized by plagiotropic root growth, a high degree of lateral branching, and the profusion of root hairs, although the tissue maintains a highly differentiated and functional root organ (Santos-Díaz 2013). "Hairy root" cultures have several properties—fast growth; greater genetic, phenotypic, and biochemical stability; growth in hormone-free media; and easily established—and maintenance and propagation in the laboratory offers a more reliable and reproducible in vitro model experimental system (cf. Sect. 22. 4.2.2), which have promoted their use in phytoremediation (Doran 2009; Georgiev et al. 2012; Shanks and Morgan 1999; Zhou et al. 2013). Table 22.3 highlights examples of hairy root cultures used for metal removal.

Table 22.3 Plant hairy root cultures used to remove metal

Plant species	*A. rhizogenes* strain used	Explant	Heavy metal	Remediation strategy/focus of study	Reference
Alyssum bertolonii	–	–	Nickel	Phytoextraction	Nedelkoska and Doran (2001)
Alyssum tenium	15834	–	Nickel	Phytoextraction	Boominathan and Doran (2002, 2003b)
Armoracia rusticana	A4	Callus	Uranium	Rhizofiltration	Soudek et al. (2011)
Brassica juncea	–	–	Uranium	Rhizofiltration	Eapen et al. (2003)
Brassica napus	LBA 9402	Leaf	Chromium	Rhizoremediation	Ontañon et al. (2014)
Calystegia sepium	–	Various plant organs	Cadmium	Phytoextraction	Metzger et al. (1992)
Chenopodium amaranticolor	–	–	Uranium	Rhizofiltration	Eapen et al. (2003)
Daucus carota	–	–	Uranium	Rhizofiltration	Straczek et al. (2009)
Euphorbia hirta	15834	–	Copper	Phytoextraction	Nedelkoska and Doran (2000b)
Hyptis capitata	A4	–	Copper	Phytoextraction	Nedelkoska and Doran (2000b)
Nicotiana tabacum	15834	Seedling	Cadmium	Phytoextraction	Boominathan and Doran (2003a, b)
Nicotiana tabacum	15834	–	Copper	Phytoextraction	Nedelkoska and Doran (2000b)
Nicotiana tabacum	15834	Seedling	Nickel	Phytoextraction	Boominathan and Doran (2002, 2003b)
Nicotiana tabacum cv. Wisconsin	LBA 9402	Leaf	Arsenic	Phytoextraction	Talano et al. 2014
Polycarpaea longiflora	15834	–	Copper	Phytoextraction	Nedelkoska and Doran (2000b)
Solanum nigrum	–	–	Zinc	Phytoextraction	Subroto et al. (2007)
Solanum nigrum	Bearing (Ri plasmid C58ci)	–	Cadmium	Rhizofiltration	Macek et al. (1994)

(continued)

Table 22.3 (continued)

Plant species	*A. rhizogenes* strain used	Explant	Heavy metal	Remediation strategy/focus of study	Reference
Thlaspi caerulescens	15834	Seedling	Cadmium	Phytoextraction	Nedelkoska and Doran (2000a)
Thlaspi caerulescens	15834	Seedling	Cadmium	Organic acid complexation, heavy metal distribution, and antioxidative defenses in hairy root culture	Boominathan and Doran (2003a, b)

22.6 Transgenic Plants: Lab to Land Transfer/Testing

The myriad of reports on transgenic plants with enhanced heavy metal accumulation, tolerance, and volatilization published in recent years could be used for phytoremediation of heavy metal-contaminated sites. However, the majority of phytoremediation studies are restricted to the model plants and have been carried out under controlled laboratory conditions using metal-spiked hydroponic system or agar medium. Up to now, these transgenic plants never have been tested/used in real metal-contaminated sites for remediation. On the other hand, phytoextraction capacity of transgenic phytochelatin-overproducing mustard plant was tested in a greenhouse study using metal-contaminated soil from Leadville, Colorado (Bennett et al. 2003). Both types of transgenics accumulated significantly higher levels of Cd and Zn in their shoots than untransformed plants.

In another greenhouse pot experiment using naturally seleniferous soil, the ATP sulfurylase (APS) transgenic *Brassica* accumulated threefold higher Se levels than wild-type (WT) *Brassica juncea*, while cystathionine-γ-synthase (CgS) transgenics contained 40 % lower Se levels than WT plant (Huysen et al. 2004). In a field experiment on Se-contaminated sediment in the San Joaquin Valley (CA, USA), the APS transgenics accumulated fourfold higher Se levels than wild-type *B. juncea* (Bañuelos et al. 2005), and on the same site, the selenocysteine lyase (cpSL) and selenocysteine methyltransferase (SMT) transgenic showed twofold higher Se accumulation compared to WT *B. juncea*, in agreement with earlier laboratory experiments (Bañuelos et al. 2007). The results obtained from the different transgenics under controlled laboratory conditions were essentially the same as those obtained with greenhouse and field experiment (Pilon-Smits and LeDuc 2009).

Additionally, the number of plants with enhanced heavy metal phytoremediation capability was developed through the genetic engineering tools and tested under the laboratory conditions; however, this is often where the process of evaluating phytoremediation ends. Ideally, these transgenic plants need to be tested in in situ field trials to assess its phytoremediation capability. Such established field trials are,

therefore, immediately required to make it a commercially viable and acceptable technology (Cherian and Oliveira 2005).

22.7 Transgenic Plants for Phytoremediation: Key Considerations for Risk Assessment and Mitigation Strategies

Genetic-engineered plants are rapidly being developed which deal with heavy metal accumulation, tolerance, and resistance and have been used not only for phytoextraction of various metals (Pilon-Smits and Pilon 2002) but also to enhance crop productivity in areas with suboptimal soil metal levels or heavy metal-contaminated soil which could expand available arable land (Guerinot and Salt 2001). Furthermore, transgenic plants are also used for biofortification of crop plants (Guerinot and Salt 2001; Schroeder et al. 2013). However, each GE plants/product undergoes an environmental risk assessment (ERA) prior to commercialization to assess potential harmful effects on the environment. The ERA directs the assessment based on the product concept, crop, trait(s), intended use (e.g., import versus cultivation), receiving environment, and potential interaction among these factors (Prado et al. 2014). The safety assessment strategy ensures that the safety of transgenic crops is reviewed by multiple regulatory agencies in accordance with different risk assessment strategies and with national and international safety assessment guidelines (Paoletti et al. 2008). This is essential to educate the community about the risks and benefits of transgenic plants for phytoremediation so that this technology can gain full regulatory and public acceptance and realize its full commercial potential (Linacre et al. 2003). The risk assessment scenario for transgenic plants is given in detail by Häggman et al. (2013) and Prado et al. (2014).

Some of the possible risks associated with transgenic plants for metal phytoremediation are accumulation of toxic metals in edible parts leading to metal entry into the food chain, which in turn affects animal and human health, and uncontrolled spread of transgene to the wild relatives leading to super weeds due to higher fitness offered by the transgene. The risk of biomagnification can be minimized by using nonedible plants and growing transgenic plants in restricted area. The various physical (placement of barriers to pollen spread, restriction of location or timing of crop planting, containment of engineered crops) and physiological (harvest engineered plants before flowering, engineer traits into plants that self-pollinate, engineer traits into plants that are sterile, perform genetic modifications on plastids) barriers are used to limit gene flow (through pollen) between related plant species (Daniell 2002; Häggman et al. 2013; Pilon-Smits and Pilon 2002; Ruiz and Daniell 2009). Additionally, transgenic for phytoremediation is likely to involve genetic use restriction technologies designed to impede transgene movement in the environment (Hills et al. 2007).

Further, considerations for the use of transgenics for the phytoremediation are the same as those involved with growing transgenics for other purposes and should also be evaluated and weighed against the risk of alternative remediation methods (Pilon-Smits and LeDuc 2009).

22.8 Future Perspectives and Conclusion

Phytoremediation is considered as an effective, low cost, environmental friendly, preferred remediation technology, and potentially applicable cleanup option to remove contaminants from soil- or water-contaminated areas (Dickinson et al. 2009). Transgenic plants expressing various genes from different sources have been developed as a means to increase heavy metal tolerance, accumulation, and volatilization that facilitates more effective heavy metal phytoremediation (Table 22.2). Most of the transgenic research was carried out on the model plant species, viz., *Arabidopsis*, tobacco, and *Brassica*. However, *B. juncea* (L.), an edible oil-producing crop, is being consumed by humans or animals in one form or another. Ecologically, use of edible crops for phytoremediation is not viable because the heavy metals enter into the food chain by consumption of either humans or animals. Therefore, further research is needed to explore more efficient nonedible candidate plant which is suitable for metal phytoremediation and genetic transformation. In this scenario, Gupta et al. (2013) suggested that aromatic plants (*Vetiveria zizanioides*, *Cymbopogon martinii*, *C. flexuosus*, *C. winterianus*, *Mentha sp.*, *Ocimum basilicum*) producing essential oils could be a better choice for the heavy metal phytoremediation as these plants are nonedible and are not being consumed directly by humans or animals. In addition, the essential oil obtained from aromatic plants is free from the risk of heavy metal accumulation from plant biomass which offers economic benefits. Furthermore, the use of halophytes is suggested as the optimal candidate for phytostabilization or phytoextraction of heavy metal-contaminated saline soils as many halophytes can accumulate or excrete heavy metals (Wang et al. 2014).

The advent of the genomic era, next-generation sequencing technologies, and rapid progress in molecular biology research have led to the fast screening and identification of novel genes and proteins responsible for metal tolerance, accumulation, and volatilization. This will open up further avenues for the creation of new transgenic plants having one or several genes (gene stacking) with desirable properties for heavy metal phytoremediation. Additionally, it might be possible to control transgene expressions in specific tissues/compartments under specific conditions (Pilon-Smits and Pilon 2002; Ruiz and Daniell 2009).

The recent novel omics approaches (genomics, transcriptomics, proteomics, metabolomics, secretomics, high-throughput and next-generation technologies) combined with new bioinformatics techniques will allow us to understand how integrated biological communities (plants and microbes) interact to adapt to contaminant stress and enhance soil remediation. Furthermore, the plant–microbe

metaorganism approach can be modified to maximize growth, appropriate assembly of microbial communities, and, ultimately, phytoremediation activity (Bell et al. 2014; Schenk et al. 2012). To achieve further improvements in phytoremediation, it will need coordinated efforts from plant physiologists, agronomists, soil scientists, molecular biologists, microbiologists, chemists, environmental engineers, and government regulators (Lee 2013; Pilon-Smits 2005).

The enormous amount of knowledge regarding transgenic plants for phytoremediation is generated through emerging scientific tools and methodologies combined with established practices and techniques. However, translation of this knowledge into usable technologies is the need of the hour to accelerate phytoremediation as an eco-friendly and cost-effective technology.

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