
Engineered Plants for Heavy Metals and Metalloids Tolerance

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Abstract

Agricultural soils around the world are slightly to moderately polluted with heavy metals such as As, Cd, Hg, Cr, Cu, Pb, Ni, and Zn as a result of industrialization, widespread application of pesticides, fertilizers, and anthropogenic activities. Plants experience toxic effects of heavy metals in the form of oxidative stress, reduction in overall growth, and productivity. To minimize these toxic effects, plants have evolved an arsenal of mechanisms such as preventing uptake via roots or blocking transport to above-ground parts. If everything fails, the toxic metal inside the cell is dealt with using a range of detoxification and storage strategies including chelation with thiols and amino acids and subsequent sequestration into subcellular compartments. In this chapter, we have reviewed general strategies for heavy metal tolerance and detoxification by plants. Also plants engineered for heavy metal transport, oxidative stress tolerance pathways, and other mechanisms such as stress-associated protein have substantially advanced our understanding of heavy metal tolerance by plants. In future, as a result of ongoing climate change, frequent floods, storms, and more use of underground and recycled water from industrial and municipal wastes for crop irrigation can further increase the heavy metals in the agricultural soils. Therefore, to minimize the impact of heavy metals on global agricultural production, it will be of utmost importance to further our knowledge of heavy metal tolerance and detoxification by plants.

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7.1 Introduction

Heavy metals and metalloids are of significant environmental concerns. Exposure to heavy metals and metalloids not only adversely affects human and environmental health but also inhibits growth and productivity of crop plants and thus poses serious threats to global agricultural production and food security (Bray et al. 2000). Toxicity to plants and their growth inhibition can occur due to elevated concentrations of both essential and nonessential heavy metals present in the soil. Heavy metals cause cellular damage by either inducing oxidative stress such as lipid peroxidation through production of reactive oxygen species (ROS) or free radicals or by binding to the sulphhydryl groups of various enzymes and proteins such as the transcription factors (Assche and Clijsters 1990; Dietz et al. 1999). Because of the global climate change, the burden of environmental stresses including heavy metals on crop plants is likely to increase in future. With decrease in groundwater use for irrigation, metals that naturally occur in soil and water are increasing in concentration and thus enter the food chain affecting human and environmental health. Water deficiency is also induced by other abiotic stresses such as salinity, heat, and cold; thus, all these stresses go hand in hand, making it absolutely essential to understand the responses of plant to water deficiency (Bohnert et al. 1995). Apart from drought and salinity, heavy metals and metalloids such as mercury (Hg), cadmium (Cd), lead (Pb), chromium (Cr), nickel (Ni), copper (Cu), arsenic (As), and selenium (Se) constitute of inorganic pollutants and are present as positive or negatively charged ions in the soil (Paulose et al. 2008).

7.1.1 Heavy Metal Toxicity in Plants and Effects on Crop Productivity

7.1.1.1 Arsenic

Arsenic (As) contamination is widespread in the environment. Natural processes such as volcanic eruptions and hot water springs along with human intervention with activities such as mining, smelting, use of pesticides, herbicides etc. contribute to arsenic pollution in the environment. It is a carcinogen and has been shown to cause major health problems around the world including but not limiting to cancer of the liver, kidney, and lungs (Kaiser 2001). Arsenic-contaminated soils, sediments, and water supplies are major sources of food chain contamination. High levels of As have been reported in the underground drinking water and food crops such as rice (*Oryza sativa*) grown in many parts of Southeast Asia especially India and Bangladesh where this staple food is widely irrigated using arsenic-contaminated groundwater subjecting millions of people to arsenic poisoning risk (Clark et al. 2000). There have been several reports of unacceptable levels of arsenic being present in edible crops, which were grown on contaminated lands (Larsen et al. 1992; Das et al. 2004; Williams et al. 2005). Arsenic is present in soil and water in organic forms – monomethylarsenate (MMA) and dimethylarsenate (DMA) as well as inorganic forms – arsenate (AsO_4^{-3} , referred to as AsV) and arsenite (AsO_3^{-3} , referred to as AsIII), but it is the latter that is a more toxic form and needs remediation (Bentley and Chasteen 2002; Chen et al. 2005). The metalloid arsenic (As) and other heavy metals are phytotoxic and the elevated concentrations of As in soil causes a significant loss of crop yield (Xiong et al. 1987;

Marin et al. 1993; Zhu et al. 2008). In addition, arsenate (AsV), being a phosphate analogue, competes with phosphate uptake, causes the inhibition of phosphate and other nutrient uptake, and thus further decreases crop production (Meharg and Macnair 1992; Abedin et al. 2002; Dhankher et al. 2006; LeBlanc et al. 2013). It has been reported that in 2003, 1/5th of the total cultivable land of China had already been accounted toward heavy metal-contaminated land. These widespread heavy metal contamination has led to a loss of about 1000 million tons of grain production directly impacting the economy (Gu et al. 2003). Arsenic gets accumulated in the soil due to the use of sewage sludge and recycled water on the agricultural land due to shortage of surface water. Therefore, it is essential to develop strategies to resist crops from uptaking arsenic or if absorbed then need to be converted to a nontoxic form.

7.1.1.2 Mercury

Mercury is among the most hazardous of the heavy metals (Keating et al. 1997), primarily because its charged species have great affinity for the thiol group on cysteine residues of proteins and other important biological molecules (Liu et al. 1992). Early studies demonstrated that mercury species inactivate metabolic enzymes and structural proteins (Boyer 1954; Falchuk et al. 1977). The strong interaction of mercury species with cellular ligands may also account for its tendency to accumulate in organisms. Organomercurial species are more toxic in some eukaryotes and are more likely to biomagnify across trophic levels than ionic mercury [Hg (II)] (Bizily et al. 1999). Fish contaminated with monomethylmercury ($\text{CH}_3\text{-Hg}^+$) at Minamata bay in Japan was a widespread ecological disaster (Minamata Disease Research Group 1968). Consumption of the fish contaminated with $\text{CH}_3\text{-Hg}^+$ leads to severe neurodegeneration in birds, cats, and humans. Methylmercury has also been found in lakes and estuaries into which only inorganic forms of mercury have been released (Balogh et al. 2006; Hammerschmidt et al. 2006). For plants, Hg is highly toxic, affecting major cellular metabolic pathways, and thus causes severe reduction in plant growth and crop yields

(Boeing 2000; Patra and Sharma 2000; Patra et al. 2004).

7.1.1.3 Cadmium

The regulatory limit of Cd in agricultural soil is 100 mg/kg (Salt et al. 1995). Cadmium pollution has increased in the environment due to mining, industrial usage, and anthropogenic activities. Cd is abundantly used in surface coatings, pigment formulation, manufacture of batteries, stabilization of polyvinylchloride (plastics), manufacture of automobiles, and aerospace and military applications. Cd has application where high stability and resistance to heat, cold, and light are required. Cd released to the environment tends to concentrate in soils and sediments, where it is potentially available to rooted plants (Prasad 1995). In bean plants (*Phaseolus vulgaris*), leaf cell expansion growth and relative water content of primary leaves decreased by about 10 % compared to control after 48-h exposure to 3 μM Cd (Barceló and Poschenreider 1990). Cd is an effective inhibitor of photosynthesis (Krupa 1988; Greger and Ogren 1991; Krupa et al. 1993). The linear relationship between net photosynthesis and inhibition of transpiration observed in clover, lucerne, and soybean suggests the closure of stomata by Cd (Huang et al. 1987). In experiments with bean plants (*P. vulgaris*), Cd inhibited net photosynthesis by increasing stomatal and mesophyll resistance to CO_2 uptake (Krupa et al. 1993). Therefore, exposure to Cd causes reduction in photosynthesis, water, and nutrient uptake.

7.1.1.4 Zinc

Zinc is an essential nutrient for plants as well as human beings. It plays important roles as component of enzymes for protein synthesis and energy production and maintains structural integrity of biomolecules (Hänsch and Mendel 2009). Zn^{2+} is an integral part of a large number of zinc finger containing proteins, transcription factors, oxidoreductases, and hydrolytic enzymes such as metalloproteases (Krämer and Clemens 2006). Concentrations of Zn in contaminated soils frequently exceed 150–300 mg/kg of soil (de Vries et al. 2007). Similar to Cd, exposure to excess levels of Zn results in chlorosis, reduced growth

of both roots and shoots, and early senescence in plants (Choi et al. 1996; Ebbs and Kochian 1997; Fontes and Cox 1998). Excess Zn can also cause Mn, Cu, and phosphate deficiencies in plants (Lee et al. 1996).

7.1.1.5 Copper

Copper (Cu) is also an essential micronutrient for plant growth and plays important roles in processes such as CO₂ assimilation, ATP synthesis, mitochondrial respiration, oxidative stress protection, and cell wall synthesis. Under physiological conditions, copper exists in the two-oxidation states Cu¹⁺ and Cu²⁺ and can interchange between these forms (monovalent copper is unstable). This allows copper to function as a reducing or oxidizing agent in biochemical reactions. But at the same time, this property also makes copper potentially toxic as copper ions can catalyze the production of free radicals, in particular through Fenton chemistry, thus leading to the damage of proteins, DNA, and other biomolecules (Hänsch and Mendel 2009). Exposure of plants to excess Cu generates oxidative stress and ROS (Stadtman and Oliver 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules and affects crop yields (Hegedüs et al. 2001).

7.1.1.6 Lead

Lead naturally occurs in the earth's crust in small amounts as a bluish-gray metalloid (Gupta et al. 2010). Contamination of Pb occurs from its use in pesticides and fertilizers, combustion of Pb containing fossil fuels, and Pb additive containing municipal sewage dumping, from its use in industrial processes such as mining and smelting (Gupta et al. 2010). Many commercially available products such as batteries, medical equipment (i.e., x-ray shields, fetal monitors), paints, ceramic glazes, television glass, ammunition, etc. also contain small concentrations of Pb (Gupta et al. 2010). Pb has the tendency to accumulate in the body organs (i.e., brain), which may lead to poisoning or even death and has also shown to affect the gastrointestinal tract, kidneys, and central nervous system. Pb exposure has detrimental effect on small children and they can have impaired

development, lower IQ, shortened attention span, hyperactivity, and mental deterioration.

Basic forms of Pb released into the soil and water including ground and surface are ionic lead (Pb²⁺), Pb oxides and hydroxides, and Pb-metal oxyanion complexes with ionic Pb and Pb hydroxyl complexes being the most stable forms (Evanko and Dzombak 1997). Pb is very difficult to remove once it is introduced in the soil matrix as it is a very sticky metal because of its ability to form a precipitate within the soil matrix along with anions such as phosphate ions which decreases its solubility and, in many cases, is not readily bioavailable (Gupta et al. 2010). The top few inches of soil are where Pb is mainly found, bound to the organic matter through adsorption, ion exchange, precipitation, and/or complexation (Hart et al. 1999; Gill and Tuteja 2011). Many plants transport a very small concentration of Pb²⁺ in the aboveground plant tissues and retain maximum concentration in their roots via sorption and precipitation. Pb exposure causes stunted root growth and accelerated cell death in rice (Huang and Huang 2008). As with any other heavy metals, one of the major consequences of Pb toxicity is the enhanced production of reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, H₂O₂ (Shu et al. 2012).

7.1.1.7 Chromium

Chromium (Cr) is a heavy metal that causes serious environmental contamination in soil, sediments, and groundwater (Shanker et al. 2005). The tanning industry is one of the major consumers of water and most of it is discharged as wastewater, which contains high amount of Cr (1.07–7.80 mg/l). Inorganic Cr exists in two forms, trivalent Cr (III) and hexavalent Cr (VI), the latter being more toxic, powerful epithelial irritant and a proven human carcinogen established by the International Agency for Research on Cancer (IARC), the Environmental Protection Agency (EPA), and the World Health Organization (WHO). Toxicity of Cr has been studied in many plants. Excess of Cr causes inhibition of plant growth, chlorosis in young leaves, nutrient imbalance, wilting of tops, and root injury (Chatterjee

2000; Dixit et al. 2002; Sharma et al. 2003; Scoccianti et al. 2006).

7.1.1.8 Selenium

Selenium (Se) is a metalloid that naturally occurs in the shale rocks and is also produced by some anthropogenic sources such as oil refineries, power plants, etc. and thus is present in excess, potentially in toxic levels in the environment (Wilber 1983; Fordyce 2005). At low levels, Se serves as an essential nutrient with normal dietary intake in the range of 50–400 µg Se/day and has several health benefits like prevention of heart diseases, muscle disorders, and cancer and is also involved in viral suppression and functioning of the immune system. Higher levels are highly toxic and cause stomach cancer, defects in the development, and failure in the reproductive system (Terry et al. 2000; Ellis and Salt 2003). Excessive levels of Se can also accumulate in food chain, which can cause serious health defects in human and wildlife population. In soil and water, Se is present in several forms such as selenide (Se^{2-}), elemental selenium (Se^0), selenite (Se^{4+}), and selenate (Se^{6+}) and many organic forms like dimethyl selenide (DMSe) and dimethylselenenylsulfide (DMSeS), which have different bioavailabilities. Selenate (Se^{6+}) form of Se is formed in alkaline, well-aerated soils, making it highly soluble, and does not form stable adsorption complexes with other components present in the soil. As a consequence, they are more bioavailable for plant uptake, whereas acidic soils favor the formation of elemental selenium (Se^0) and selenides (Se^{2-}) that are quite insoluble and therefore are less bioavailable for plant uptake (Zayed et al. 1998; Terry et al. 2000).

near food crops for heavy metal tolerance is that the genes encoding metal-binding peptides such as metallothioneins (MTs), glutathione (GSH), and phytochelatins (PCs) caused increased metal uptake along with metal tolerance. The phytotoxic effects suffered by crops grown on soil with heavy metal and metalloid residues could be overcome by developing crops resistant to these metals. However, progress toward developing such genetics-based strategies has been hindered by the lack of understanding of the basic molecular and biochemical mechanisms of heavy metal uptake and detoxification in plants. Despite these limitations, a significant progress has been made to engineer plants with genes, either individually or in combination, for increasing tolerance and detoxification of heavy metals such as As, Hg, Cd, Pb, Se, Ni, etc. Further plants have been engineered for remediation of several metals and metalloids (Pilon-Smits et al. 1999; Rugh 2001; Dhankher et al. 2002, 2006; van Huysen et al. 2003; Li et al. 2005; Dixit and Dhankher 2011; Paulose et al. 2013).

Plants utilize several strategies/mechanisms to achieve tolerance toward heavy metals and metalloids. These include thiol-mediated chelation followed by sequestration (Schmoger 2000; Song et al. 2010), uptake or exclusion through transporters, and complexation with phytochelatins followed by vacuolar storage (Dhankher et al. 2002) and chelation by metallothioneins and anthocyanins or by binding with carboxylic acids such as citrate, malate, or amino acids such as histidine and nicotianamine. A list of genes used for engineering plants for enhanced tolerance to heavy metals and metalloids is presented in Table 7.1.

7.2 Strategies for Heavy Metal Detoxification and Enhanced Tolerance in Plants

Some plants have the natural ability to detoxify, accumulate, and tolerate high levels of heavy metal stresses using processes at the cellular levels, and other plants could be engineered to carry out such processes. The major challenge to engi-

7.2.1 Chelation with Metal-Binding Peptides

In non-hypertolerant plants, binding of metals by strong ligands is the main detoxification strategy. The best-known types of ligands for this purpose are thiols, including GSH and its precursor gamma-glutamylcysteine (γ EC dipeptide), phytochelatins (PCs), and metallothioneins (MTs)

Table 7.1 Heavy metal and metalloids tolerant genes used for engineering plants for enhanced tolerance to various toxic metals and metalloids

Plant species	Gene source	Gene	Metal/Metalloid tolerance	Phenotypes	Reference
<i>Arabidopsis thaliana</i>	<i>Escherichia coli</i>	<i>merB</i>	Hg	Tolerance to Hg	Bizily et al. (1999)
<i>A. thaliana</i>	<i>Nicotiana tabacum</i>	<i>CBP4</i>	Pb, Ni	Higher Pb accumulation and reduced uptake of Ni in shoots	Bizily et al. (1999)
<i>A. thaliana</i>	<i>Escherichia coli</i>	<i>ArsC, γ-ECS</i>	As	Enhanced tolerance and accumulation of As	Dhankher et al. (2002)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>ACR2</i>	As	Enhanced accumulation of As in shoots	Dhankher et al. (2006)
<i>Nicotiana glauca</i>	<i>Triticum aestivum</i>	<i>PCS1</i>	Pb, Cd	Enhanced tolerance to Pb and Cd	Gisbert et al. (2003)
<i>Nicotiana tabacum</i>	<i>Arabidopsis thaliana</i>	<i>MT2b</i>	As	Enhanced As root-to-shoot transport	Grispen et al. (2009)
<i>A. thaliana</i>	<i>Allium sativum</i> , <i>Saccharomyces cerevisiae</i>	<i>PCS1, GSH1</i>	Cd, As	Enhanced tolerance and accumulation of As and Cd	Guo et al. (2008)
<i>A. thaliana</i>	<i>Allium sativum</i> , <i>Saccharomyces cerevisiae</i>	<i>PCS1, YCF1</i>	As, Cd	Enhanced tolerance to and accumulation of As and Cd	Guo et al. (2012)
<i>Brassica oleracea</i> ,	<i>Saccharomyces cerevisiae</i>	<i>CUP1</i>	Cd	Enhanced tolerance to Cd	Hasegawa et al. (1997)
<i>A. thaliana</i>	<i>Saccharomyces cerevisiae</i>	<i>ZIF</i>	Zn	Enhanced tolerance and accumulation of Zn	Haydon and Cobbett (2007a, b)
<i>A. thaliana</i>	<i>Schizosaccharomyces pombe</i>	<i>HMT1</i>	Cd	Enhanced tolerance to and accumulation of Cd, Cu, As and Zn	Huang et al. (2012)
<i>Pteris vittata</i>	<i>Pteris vittata</i>	<i>ACR3</i>	As	Tolerance and hyperaccumulation of As	Indriolo et al. (2010)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>ATM3</i>	Cd	Enhanced resistance to Cd	Kim et al. (2006)
<i>Brassica juncea</i>	<i>Astragalus bisulcatus</i>	<i>SMT</i>	Se	Enhanced tolerance to Se	LeDuc et al. (2004)
<i>Brassica juncea</i>	<i>Arabidopsis thaliana</i> , <i>Astragalus bisulcatus</i>	<i>SMT, APS1</i>	Se	Enhanced accumulation of Se	LeDuc et al. (2006)
<i>Vicia faba</i>	<i>Arabidopsis thaliana</i>	<i>MT2a, MT3</i>	Cd	Tolerance to Cd by reducing ROS production	Lee et al. (2004)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>HMA3</i>	Cd, Co, Pb, Zn	Increased tolerance to and accumulation of Cd, Co, Pb and Zn	Morel et al. (2009)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>ABCC1</i>	Cd	Increased resistance to Cd	Park et al. (2012)
<i>Brassica juncea</i>	<i>Arabidopsis thaliana</i>	<i>APS1</i>	Se	Increased uptake, reduction and tolerance of Se	Pilon-Smits et al. (1999)
<i>A. thaliana</i>	<i>Escherichia coli</i>	<i>merA</i>	Hg	Resistance to toxic levels of Hg	Rugh et al. (1996)
<i>Nicotiana tabacum</i> , <i>A. thaliana</i>	<i>Escherichia coli</i>	<i>merA, merB</i>	Hg	Increased tolerance and accumulation of Hg and phenyl-Hg	Ruiz et al. (2003)
<i>Oryza sativa</i>	<i>Triticum aestivum</i>	<i>HsfA4a</i>	Cd	Enhanced tolerance to Cd	Shim et al. (2009)
<i>A. thaliana</i>	<i>Saccharomyces cerevisiae</i>	<i>YCF1</i>	Cd, Pb	Increased tolerance to and accumulation of Cd and Pb	Song et al. (2003)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>ABCC1/ABCC2</i>	As	Increased tolerance to As	Song et al. (2010)
<i>Nicotiana tabacum</i>	<i>Saccharomyces cerevisiae</i>	<i>CUP1</i>	Cu	Increased accumulation of Cu	Thomas et al. (2003)
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	<i>ZAT</i>	Zn	Increased tolerance to and accumulation of Zn	van der Zaal et al. (1999)
<i>Brassica juncea</i>	<i>Escherichia coli</i>	<i>GSH1</i>	Cd	Enhanced tolerance and accumulation of Cd	Zhu et al. (1999)

Note: This table does not represent all the genes in the published literature but includes only a subset of genes used for engineering plants for heavy metal tolerance

(Leitenmaier and Kupper 2013). Most of the divalent cations (Cd, Hg, Pb, Cu, Zn) and oxyanions (AsV and AsIII) are highly reactive and has a strong affinity toward thiol groups such as those in γ EC, GSH, PCs, and MTs (Schmoger 2000; Dhankher et al. 2002; Zimeri et al. 2005).

7.2.1.1 Chelation with Glutathione

Glutathione (GSH) is the key redox molecule in plant cells. It plays important roles in protecting cells from oxidative stress caused by exposure to environmental stresses including toxic metals, ozone exposure, and biotic stresses such as insect and pathogen infestation. Plants detoxify toxic metals and metalloids through a GSH-dependent pathway. GSH homeostasis in plants is maintained by the γ -glutamyl cycle, which involves GSH synthesis and degradation and the recycling of component amino acids (Paulose et al. 2013). GSH synthesis is catalyzed by two enzymes – γ -glutamylcysteine synthetase (γ -ECS) and glutathione synthetase (GS). Overexpression of these enzymes enhanced the tolerance to toxic metals and metalloids such as Hg, Cd, Pb, Cr, and As (Dhankher et al. 2002; Li et al. 2005). Once toxic metal is bound to thiol peptides, it is relatively less toxic to plants, and the metal-thiol complexes are then sequestered into vacuoles by glutathione-conjugating pumps, GCPs (Wang et al. 2002; Dhankher et al. 2002; Indriolo et al. 2010; Song et al. 2010). Therefore, enhancing the levels of GSH and its derivatives favors the trapping of toxic metals in thiol complexes resulting in enhanced tolerance and accumulation. This strategy is employed in most of the metal hyper-accumulators (Freeman et al. 2004, 2005).

The role of GSH and PCs in Cd tolerance has been studied in great details. These peptides chelate Cd cations with varying affinities, leading to vacuolar sequestration for metal-peptide complexes (Kneer and Zenk 1992; Ortiz et al. 1992; Howden 1995; Li et al. 1996). Their involvement in detoxifying heavy metals was discovered almost 30 years ago (Grill et al. 1985, 1989). Cadmium stress affects sulfur uptake and assimilation pathway in plants (Zhu et al. 1999), which directly affects the synthesis of amino acids such as cysteine that requires sulfur for its synthesis.

Cysteine is a precursor in synthesis of GSH. Zhu et al. (1999) demonstrated the role of γ -ECS in increasing cadmium tolerance and accumulation by overexpressing γ -ECS encoding *E. coli* gene *gshI* in *Brassica juncea*. Along with transgenics growing better in presence of toxic concentrations of Cd, increased concentrations of PCs, γ -GluCys, and GSH were also seen in the transgenic seedlings, leading to believe that increased production of these compounds lead to increased Cd tolerance and accumulation (Zhu et al. 1999). Metalloid toxicity in non-hypertolerant plants has been observed in *cadI* mutant plants that are either biochemically (Schat 2002) or genetically mutant for PC synthase leading to Cd hypersensitivity (Howden 1995). *Arabidopsis* engineered to express bacterial GSH1 showed strong tolerance to As and weak tolerance to Hg, whereas it failed to provide any tolerance to Cd stress (Li et al. 2005). Transgenic tobacco (*N. tabacum*) plants expressing serine acetyltransferase (SAT, involved in the production of a cysteine precursor O-acetylserine), GSH1, and PCS, either separately or in combination, have shown increased Cd concentration in roots (Wawrzynski et al. 2006).

Arsenic is present in soil and water in organic forms such as MMA and DMA as well as inorganic forms such as AsV and AsIII, but it is the latter that is a more toxic form and needs remediation (Bentley and Chasteen 2002; Chen et al. 2005). In 2002, Dhankher and coworkers engineered *Arabidopsis* to detoxify As and created a genetics-based strategy for accumulation of As in the aboveground tissue for phytoremediation by co-expressing arsenate reductase, ArsC, and γ -glutamylcysteine synthetase, γ -ECS. The bacterial ArsC reduces AsV to AsIII (Rosen 1999), and γ -ECS is the first step for the synthesis of GSH and PCs and enhances the thiol peptide levels in plants. Transgenic lines expressing light-regulated ArsC were hypersensitive to AsV, whereas the γ -ECS transgenic lines were moderately resistant as compared to wild-type plants. Double transgenic plants made by genetic crossing of As-hypersensitive ArsC plants and moderately resistant γ -ECS overexpressing plants were super-resistant to AsV as compared to the plants

expressing γ -ECS alone. The double transgenic *ArsC*+ γ -ECS plants accumulated threefold more As in the aboveground tissues (Dhankher et al. 2002).

Thlaspi species hyperaccumulate Ni up to 3 % of their shoot dry weight (Yadav 2010). The concentrations of GSH, Cys, and O-acetylserine (OAS) in shoot tissue are strongly correlated with the ability to hyperaccumulate Ni in various *Thlaspi* hyperaccumulators collected from serpentine soils. Examples of such hyperaccumulators reported are *T. goesingense*, *T. oxyceras*, and *T. rosulare*, and non-accumulator relatives are *T. perfoliatum*, *T. arvense*, and *A. thaliana* (Kramer et al. 1997; Wenzel and Jockwer 1999; Guerinet and Salt 2001; Peer et al. 2003; Freeman et al. 2004). High concentrations of OAS, Cys, and GSH in Austrian Ni hyperaccumulator *T. goesingense* coincide with constitutively high activity of both SAT and glutathione reductase (GR) enzymes. *Arabidopsis* overproducing SAT from *T. goesingense* has been found to cause accumulation of OAS, Cys, and GSH, mimicking the biochemical changes observed in the Ni hyperaccumulators. In these transgenic *Arabidopsis*, GSH concentration was strongly correlated with increased resistance to Ni-induced growth inhibition and oxidative stress. This observation concluded that high levels of GSH conferred tolerance to Ni-induced oxidative stress in *Thlaspi* Ni hyperaccumulators (Freeman et al. 2004). *Sedum alfredii*, a perennial herb which is also a known Zn/Cd hyperaccumulator, was used as an indicator species by Gupta and coworkers to demonstrate that enzymatic and nonenzymatic antioxidants such as cysteine, nonprotein thiols (NPSH), glutathione (GSH), and PCs have important roles in detoxification of toxicity induced by Pb (Gupta et al. 2010). The above-mentioned antioxidants were found in higher concentration in the Pb accumulating *S. alfredii* species when compared with its non-accumulating counterpart suggesting that the capacity of the ecotypes to accumulate different levels of Pb depends upon their ability to detoxify Pb through production of these antioxidants.

In case of mercury, hyperaccumulation can be achieved by overexpression of each of the three enzymes – γ -ECS, GS, and PS – involved in the GSH and PC biosynthesis pathway that will increase thiol sinks for Hg (II). Plants transformed with *merB* and γ -ECS will trap Hg (II) in the form of thiol-Hg complexes and result in enhanced organic and ionic Hg resistance. Constitutive expression of PCS expression has previously been achieved, which further increases PC sink for Hg (II). However, the second approach of phytoextraction has not been fully achieved yet and more research needs to be done in understanding Hg phytoextraction as such Hg accumulation in plant tissues can be toxic to wildlife. Also, plant tolerance to Hg is generally low, and therefore phytoremediation can be limited by plant tolerance.

7.2.1.2 Chelation with Phytochelatin

Phytochelatin form a family of structures with increasing repetitions of the γ -GluCys dipeptide followed by a terminal Gly, (γ -GluCys)_n-Gly, where n is generally in the range of 2–8. PCs are structurally related to glutathione (GSH, γ -GluCysGly), and numerous physiological, biochemical, and genetics studies have confirmed that GSH is the substrate for PC biosynthesis (Cobbett 2000; Cobbett and Goldsbrough 2002). PCs are synthesized from GSH by the enzyme phytochelatin synthase, PCS (Grill et al. 1989). *Arabidopsis cad1* mutant was the first PC synthase mutated or inactivated gene. The *cad1* mutants were PC deficient but had wild-type levels of GSH, suggesting a defect in the PCS gene (Howden 1995). The *Arabidopsis cad1/AtPCS1* gene (Howden 1995; Ha 1999; Vatamaniuk et al. 1999) and a similar gene in wheat, *TaPCS1*, have been shown to confer resistance to Cd and Pb when expressed in the yeasts *S. cerevisiae* and *N. glauca*, respectively (Clemens et al. 1999; Cobbett and Meagher 2002; Gisbert et al. 2003). *Arabidopsis cad1-3* mutant was found to be more sensitive to As(V) and Cd and slightly sensitive to Cu, Ag, and Hg, while there was no difference observed when exposed to Zn, selenite, and Ni

ions (Ha 1999). Using *B. juncea*, it has been shown that Cd accumulation is accompanied by a rapid induction of PC biosynthesis, and the levels of PC present were theoretically sufficient to chelate all the Cd that is taken up by plants (Haag-Kerwer et al. 1999). A possible role for PCs in Cu tolerance had also been suggested from studies on copper-tolerant *Mimulus guttatus* (Salt et al. 1989). The constitutive overexpression of TaPCS1 in shrub tobacco (*N. glaucum*) substantially increased its tolerance to Pb²⁺ and Cd²⁺ and greatly improved accumulation of Cu²⁺, Zn²⁺, Pb²⁺, and Cd²⁺ in shoots (Martínez et al. 2006).

In the published literature, it has also been observed that excessive levels of PC in transgenic plants lead to increase in heavy metal accumulation without enhancing tolerance and causing hypersensitivity to heavy metals (Pomponi et al. 2006). A similar effect was observed when AtPCS was overexpressed in *Arabidopsis* leading to Cd hypersensitivity (Lee et al. 2003). As far as the role of PCs in copper tolerance/sensitivity, cadmium-tolerant transgenic plants that overexpressed AtPCS1 were not tolerant of copper stress, thereby supporting the hypothesis that PC is not primarily involved in Cu tolerance mechanism. Investigation into Cu tolerance in *cad2-1*, a Cd-sensitive and glutathione (GSH)-deficient *Arabidopsis* mutant, leads to *cad2-1* mutant being more resistant to Cu stress than wild-type plants. This was likely due to the high level of cysteine present in *cad2-1* mutants. However, when the growth medium was supplemented with cysteine, the wild-type plants also exhibited Cu tolerance. Moreover, *S. cerevisiae* that expressed AtPCS1 showed tolerance to Cd but hypersensitivity to Cu. All these results indicate that PCs are not a major factor in determining Cu tolerance in plants (Lee and Kang 2005). Historically, there has been some disagreement about the role of PCs in metal tolerance and not all studies have supported this role (Steffens 1990; Hall 2002). Although evidence for the role for PCs in Cd detoxification is strong, these peptides may play other important roles in the cell, including metal homeostasis and sulfur metabolism or as antioxidants (Rauser 1995; Dietz et al. 1999; Cobbett 2000; Hall 2002). The participation of PCs in

heavy metal detoxification may be a consequence of these other functions (Steffens 1990).

7.2.1.3 Chelation with Metallothioneins

Metallothioneins (MTs) are low-molecular-weight and highly cysteine-rich metal-binding peptides, which play important roles in toxic metal detoxification and metal ion homeostasis. MTs in plants differ considerably from those found in mammals and fungi as they contain mercaptide groups they are able to bind metal ions. Based on arrangement of cysteine residues, metallothioneins from plants can be classified into four subfamilies or classes – MT1 to MT4. Class 1 MTs are characterized by the presence of Cys–X–Cys motifs, whereas in Class 2 MTs both Cys–Cys and Cys–XX–Cys pairs are located toward the N-terminal domain (Robinson et al. 1993). They are able to detoxify metals, achieve homeostasis, and allow metal transport due to their ability to reversibly bind both toxic and essential metal ions.

Human MT2 and mouse MT2 were among the first MT genes expressed in transgenic plants (Lefebvre and Laliberte 1987; Misra and Gedamu 1989). In case of wheat and rice, MTs are not only induced by metal ions, such as Cu and Cd, but also by abiotic stresses such as extreme temperature and nutrient deficiency (Cobbett and Goldsbrough 2002). Plant MTs sequester excess of metals by coordinating metal ions with the multiple cysteine thiol groups (Robinson et al. 1993) and have particular affinity for Zn²⁺, Cu⁺, and Cu²⁺ as shown by the expression of the pea gene PsMTa in *E. coli* (Tommey et al. 1991). Overexpression of yeast metallothionein gene (*CUP1*) created Cd-tolerant transgenic cauliflower, which grew well in the presence of 400 μM Cd and accumulated more Cd, especially in the upper leaves (Hasegawa et al. 1997). Also CUP1 overexpression in *N. tabacum* resulted in increased copper accumulation (Thomas et al. 2003). Gene silencing demonstrated that the MT1 class isoforms are required to protect *Arabidopsis* plants from toxic effects of the heavy metal Cd(II) and possibly As. The study (Zimeri et al. 2005) used RNA interference to knock

down expression of the *Arabidopsis* class I MT genes: MT1a, MT1b, and MT1c. The MT1 knockdown lines showed greatest sensitivity to Cd(II) and accumulated less Cd, Zn, and As than wild-type plants (Zimeri et al. 2005). When expressed in *N. tabacum*, AtMT2b enhanced root-to-shoot transport of arsenic resulting into decreased As tolerance but increased accumulation in shoots (Grispen et al. 2009). The role of AtMT2a and AtMT3 in Cd detoxification and resistance was studied recently in which the two genes fused to GFP and RFP protected the guard cell chloroplasts of *Vicia faba* from degradation when exposed to high concentrations of cadmium (Lee et al. 2004). It was also identified that the two genes might not be playing a role in cadmium detoxification through vacuole sequestration but are most likely involved in reducing the levels of ROS generated upon cadmium exposure (Lee et al. 2004). Overexpression of CcMT2 from legume *Cajanus cajan* in *Arabidopsis* induced both Cd and Cu tolerance and allowed both metals to accumulate without affecting the expression of endogenous transporters (Sekhar et al. 2011). A heat shock transcription factor A4a (*HsfA4a*) from *Triticum aestivum* when overexpressed in rice enhanced Cd tolerance in rice plants and decreased Cd accumulation in rice plants with knocked-down expression of OsHsfA4a. Under cadmium stress, upregulation of *HsfA4a* along with increased expression of MT genes in wheat and rice such as *OsMT-I-1a* was seen in roots of these plants suggesting that MT has a role to play in cadmium tolerance through *HsfA4a* (Shim et al. 2009). One of the MT genes from sugarcane, *ScMT2-1-3*, not only showed increased tolerance and detoxification to Cd²⁺ and Cu²⁺ when expressed in transgenic *E. coli* but was also upregulated under Cu²⁺ stress and downregulated under Cd²⁺ stress (Guo et al. 2013). A decrease in the levels of peroxidase (POD) activity and malondialdehyde (MDA) accumulation was observed in tobacco plants overexpressing *TaMT3* gene under 35S promoter, leading to believe that *TaMT3* has a role to play in providing increased tolerance to cadmium stress (Zhou et al. 2014). Upon Pb exposure, increased expression of two genes *HiHMA4* and *HiMT2a* coding

for a P1B-type ATPase and an MT was observed in roots and leaves of Pb hyperaccumulator plant species *Hirschfeldia incana*, a member of *Brassicaceae* (Auguy et al. 2013). When characterized further, these were seen to play a role in greater lead tolerance and were involved in greater lead accumulation. *OsMT1a* expression was induced specifically by Zn²⁺ treatment. Both transgenic plants and yeasts harboring *OsMT1a* accumulated more Zn²⁺ than wild-type controls, suggesting *OsMT1a* is most likely to be involved in zinc homeostasis. Transgenic rice plants overexpressing *OsMT1a* demonstrated enhanced tolerance to drought. The examination of antioxidant enzyme activities demonstrated that catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) were significantly elevated in transgenic plants. Furthermore, the transcripts of several Zn²⁺-induced CCCH zinc finger transcription factors accumulated in *OsMT1a* transgenic plants, suggesting that *OsMT1a* not only participates directly in ROS scavenging pathway but also regulates expression of the zinc finger transcription factors via the alteration of Zn²⁺ homeostasis, which leads to improved plant stress tolerance (Yang et al. 2009). Therefore, according to Grennan AK (2011), “although many recent studies have started to reveal the roles of MTs in plants, there is still much more information needed. The large diversity in the metal-binding regions of plant MTs suggests that they have the ability to bind a greater range of metals than their animal counterparts and, consequently, a greater range of function.”

7.2.1.4 Other Metal-Binding Peptides

Carboxylic acids such as citrate, malate, oxalate, and amino acids such as histidine and nicotianamine are potential non-thiol ligands for heavy metal detoxification in plants (Rausser 1999). Histidine was first shown to bind a major proportion of Ni in the Ni hyperaccumulator, *Alyssum lesbiacum* (Kramer et al. 1996), and later it was shown to bind Zn in hyperaccumulators as well (Salt et al. 1999; Küpper et al. 2004). Free histidine is an important metal-binding ligand, and in its monodentate form it may not bind to metals very strongly, but multi-histidine residue can

make a strong interaction with metals. Nicotianamine (NA), as a polydentate ligand with three carboxyl groups and three nitrogen, makes a strong ligand for Fe^{2+} , Zn^{2+} , Ni^{2+} , and Cu^{2+} (Beneš et al. 1983). They are structurally very similar to the iron-phytosiderophore mugineic acid. NA provides six alternating carboxylate and amine functions; their relative positions favor the formation of six-coordinate metal complexes (Callahan et al. 2006; Leitenmaier and Kupper 2013). They bind to both iron (II) and iron (III) (Pich et al. 1994; Von Wiren 1999). NA has been shown to be important for Cu metabolism (Liao et al. 2000); at the same time they were also found to bind a substantial proportion of total Cu at toxic concentrations in *N. caerulea* (Mijovilovich et al. 2009). NA has also been shown to bind Zn (Tramczynska et al. 2010), in Cd/Zn hyperaccumulators *Arabidopsis halleri* and *N. caerulea* (Becher et al. 2004; Weber et al. 2004). An RNAi-mediated knockdown of nicotianamine synthase in *A. halleri* revealed a severe diminishing of Zn and Cd accumulation, which proves importance of NA for hyperaccumulation of these metals (Deinlein et al. 2012). In summary, when present in low concentrations, NA likely is important as a ligand mostly in compartments with low abundance of stronger ligands (e.g., the vacuole, xylem, and phloem). At the very high NA levels in hyperaccumulators, however, it might also take part in binding of these metals in the cytoplasm (Leitenmaier and Kupper 2013).

Anthocyanins have also emerged as important non-thiol ligands in metal binding, which have been shown to be associated with molybdenum (MO) accumulation (Hale et al. 2001). Oxalates were shown to bind Cu in Cu-tolerant lichens and fungi (Fomina et al. 2005), and they have been shown to bind Cu in Cu-sensitive Cd/Zn-hyperaccumulator *N. caerulea* (Mijovilovich et al. 2009). Organic acids have been shown to facilitate enhanced uptake of metals into the roots of hyperaccumulators (Li et al. 2012). The strategy of binding to oxalate in hyperaccumulators makes sense as Cu and manganese (Mn) oxalates are hardly soluble and much more stable which

diminishes their bioavailability as compared to binding to smaller organic acids like malate or citrate (Leitenmaier and Kupper 2013).

7.3 Compartmentation and Sequestration of Heavy Metals

Chelation of metal ions is an important aspect of metal ion detoxification, but a potential mechanism for increased metal tolerance is the sequestration of metal ions and metal-chelate complexes into subcellular compartments. There is considerable evidence that sequestration to the vacuole plays a significant role in detoxifying metal ions in a number of organisms. Manipulation of these sequestration mechanisms may be a necessary to increase plant's tolerance to toxic concentrations of heavy metals (Cobbett and Meagher 2002).

7.3.1 Transport and Storage of GSH- and PC-Bound Metals to Vacuoles

The HMT1 gene encodes a member of the family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane and is required for the transport of PCs or PC-Cd complexes into vacuolar membrane vesicles (Huang et al. 2012). In fission yeast (*Schizosaccharomyces pombe*) the Cd-sensitive, *hmt1* mutant is unable to accumulate vacuolar PC-Cd (Ortiz et al. 1992). YCF1, a member of ABC family of transporters, is a classical example of transport of both GSH conjugates and $(\text{GSH})_2\text{Cd}$ complexes into vacuolar compartment (Li et al. 1996). YCF1 has also been shown to sequester GSH conjugates of Hg (Gueldry et al. 2003) and As(III) (Ghosh et al. 1999) into vacuoles. In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of both the Cd and PCs accumulated were confined to the vacuole (Vögeli-Lange and Wagner 1990). AtMRP3 can also transport GS conjugates of Cd (Tommasini et al. 1998). Recently, Guo et al. (2012) confirmed increased

tolerance to and accumulation of Cd and As by transgenic *Arabidopsis* overexpressing PCS1 in combination with either YCF1 (Guo et al. 2012) or GSH1 (Guo et al. 2008). It was recently discovered that when *SpHMT1*, which is a PC-Cd transporter, from fission yeast (*S. pombe*) was expressed in *Arabidopsis*, greater tolerance and accumulation was achieved for Cd, Cu, As, and Zn ions as well as enhanced vacuolar sequestration could also be achieved. Chardonens et al. (1999) have shown that Zn-tolerant lines of *Silene vulgaris* have increased tonoplast transport of Zn compared with non-tolerant lines. *Arabidopsis* Zn-induced facilitator1 (AtZIF1) is a kind of MFS (major facilitator superfamily) transporter, still part of ABC transporter group, and mRNA of ZIF1 was induced by Zn, and its mutant showed to accumulate more Zn in the shoot and also be sensitive to Cd. Its localization studies indicated it being present in the tonoplast suggesting that it might be playing a role in transport of Zn and its complexes to the vacuoles (Haydon and Cobbett 2007a,b). The CPX or type 1B subclass of P-type ATPase transporters is important in heavy metal detoxification and homeostasis in many organisms, including prokaryotes, fungi, plants, and animals (Cobbett and Meagher 2002). HMA3, a P_{1B}-type heavy metal ATPase, was found to mediate leaf vacuolar storage of Cd, Co, Pb, and Zn in *Arabidopsis* (Morel et al. 2009).

The homologue of yeast efflux transporter *ACR3* was identified in an As hyperaccumulator, *Pteris vittata* (*PvACR3*), and was shown to rescue arsenic-sensitive phenotypes of yeast deficient for *ACR3*. Upregulation of *ACR3* was seen in plant tissues in contact with As, and its knock-down leads to sensitive phenotypes in the presence of As confirming the role of *PvACR3* in As tolerance through vacuolar sequestration of As-bound compounds (Indriolo et al. 2010). The role of ABC transporter family members such as that of ABCC1 in transporting and detoxifying As has also been shown in various species such as yeast, protozoa, and *C. elegans* (Papadopoulou et al. 1994; Broeks et al. 1996; Song et al. 2003; Schwartz et al. 2010; Guo et al. 2012). Two vacuolar PC transporters AtABCC1 and AtABCC2 in *Arabidopsis* were shown to play a role in the tol-

erance and transport of PC-bound metalloids such as As(III)-PC into the vacuole (Song et al. 2010). They also showed that in the absence of these transporters, *Arabidopsis* plants were extremely sensitive to As. When heterologously expressed, in the presence of PC background, greater tolerance and accumulation of As could be seen. Greater As tolerance in *Arabidopsis* was also achieved when AtABCC1 was overexpressed along with AtPCS1 (Song et al. 2010). Overexpression of AtABCC1 in *Arabidopsis* also resulted in enhanced Cd(II) tolerance and accumulation (Park et al. 2012).

7.4 Heavy Metal Transporters

Metal transporters play a significant role in the uptake and transport of essential and nonessential metals and metalloids across plasma membrane of plant species which are needed for processes such as plant growth and development, signal transduction, and toxic metal detoxification (Krämer et al. 2007; Paulose et al. 2008). With the advances in “omics” in the last few years, a lot of metal transporters have been identified in various plant families. These metal transporters have been broadly categorized into two groups – metal uptake transporters and efflux transporters as described below.

7.4.1 Metal Uptake Transporters

Out of several different types and families of metal uptake transporters, certain metal ions such as Cd, Co, Mn, and Zn get transported through some ZIP (zinc-regulated transporter) transporters because of their nonmetal specific nature. ZAT overexpression in *Arabidopsis* provided strong tolerance to and accumulation of zinc upon exposure to 400 μ M ZnSo₄ (van der Zaal et al. 1999). Under Zn deficiency, transcripts of ZIP1, ZIP2, and ZIP3 from *A. thaliana* were shown to be induced only in the roots and ZIP4 was found in both roots and shoots (Grotz et al. 1998). When expressed in yeast, another ZIP member from *Arabidopsis*-AtIRT1, which nor-

mally uptakes iron, was seen to uptake Zn (Korshunova et al. 1999) and transport Cd leading to Cd-sensitive yeast cells (Rogers et al. 2000). An important role of two ZIP genes, ZNT1 and ZNT2, from Ni hyperaccumulator *Thlaspi japonicum* was also identified in providing tolerance to yeast cells when subjected to greater concentrations of Ni (Mizuno et al. 2005). ZIP proteins from *Medicago trunculata* (MtZIP) were shown to rescue *zrt1/zrt2* mutant in yeast in the presence of Zn and other metals. Under Zn deficiency, ZIP4 from rice was highly expressed in both root and shoots and also complemented a Zn-uptake-deficient mutant strain (Ishimura et al. 2005).

Another class of transporter involved in uptake of toxic metals is the family of natural resistance-associated macrophage protein (NRAMP). Six genes in *Arabidopsis* genome code for NRAMP proteins and have been divided into two groups – group one consisting of *AtNRAMP1* and 6 and group two containing *AtNRAMP2* through 5 (Maser 2001). Cadmium sensitivity and accumulation were increased in yeast cells in which *AtNRAMP 1, 3, and 4* had been expressed (Thomine et al. 2003). Overexpression and characterization of *AtNRAMP3* and *AtNRAMP4* T-DNA lines further confirmed their role in Cd transport in plants (Thomine et al. 2003). *TjNRAMP4* from *T. japonicum*, which is a homologue of *AtNRAMP4*, also showed increased Ni accumulation and sensitivity when expressed in wild-type yeast cells (Mizuno et al. 2005).

Yellow stripe-like protein family transporters (YSL) allow the uptake of metals that are complexed with secondary amino acids such as phyto-siderophores or nicotianamines and got their name from maize mutant, yellow stripe 1, which is unable to uptake iron-phytosiderophore complex (Curie et al. 2001). First YS1 was identified in maize and was termed ZmYS1, which complemented Zn-uptake mutant *zap1* in yeast (Schaaf et al. 2004). Eight genes (*AtYSL1* through 8) from *Arabidopsis* were found homologous to ZmYS (Paulose et al. 2008). The role of the transporters from this family in uptake of metals such as Zn, Cd, and other divalent cations is still unclear. OPT3, a member of *Arabidopsis* oligopeptide transporter (OPT) family, was shown to be involved in move-

ment of iron into developing seeds (Stacey et al. 2008). *opt3-2* mutants, which have reduced levels of OPT3, exhibited constitutive expression of iron deficiency responses in roots regardless of iron supplementation, resulting in overaccumulation of iron in leaves, but decreased levels of iron in seeds (Chu 2010, dissertation thesis). Recently, OPT3 has been shown to be a phloem-specific transporter that mediates Fe loading into the phloem. By loading of Fe into the phloem in leaves, OPT3 has been shown to regulate both signaling of Fe demand from shoots to roots and Fe transport to developing tissues (Zhai et al. 2014) and plays a key role regulating Fe, Zn, and Cd distribution within the plant (Mendoza-Cózatl et al. 2014).

7.4.2 Metal Efflux Transporters

Another group of transporters is broadly termed as metal efflux transporters under which the CDF family members (cation diffusion facilitator) have been shown to transport metal cations such as Zn and Cd (Nies 2003). Plant CDFs have been given the name metal tolerance proteins (MTPs) (Delhaize 2003; Kim et al. 2004; Paulose et al. 2008). Twelve CDF members have been identified in *A. thaliana* and the first CDF characterized was termed as AtMTP1. This gene, when overexpressed and knocked down in plants, showed tolerance to Zn with less tissue accumulation of Zn. MTP1 has also been identified in *A. halleri*, a metal accumulator species, and was shown to complement the mutant phenotype of a yeast strain that lacks vacuolar zinc resistance gene (ZRC1) and cobalt transporter gene, COT1 (Dräger et al. 2004). All three MTP1s in *A. halleri* have shown Zn tolerance. Several allelic variants of MTP1 from Ni/Zn-hyperaccumulator plant *T. goesingense*, also a homologue of AtMTP1, have conferred resistance to yeast strains mutant for ZRC1 and COT1. Overexpression of *TgMTP1* also decreases the concentration of Zn in the cells by effluxing it out (Kim et al. 2004).

Arabidopsis HMA2, HMA3, and HMA4 are the members of IB-2 subgroup of P_{1B}-type ATPase transporter family, and it was shown that *AtHMA2* was strongly induced by Zn and Cd

exposure and seen to transport Zn outside the cytoplasm. Knocked-down *hma2* mutants, compared to wild-type *Arabidopsis* plants, accumulated higher levels of Zn and Cd (Eren and Argüello 2004). In the presence of higher Cd concentrations, HMA4 from *T. caeruleus* enabled the yeast strain grew better and also effluxed Cd out of the cells through the plasma membrane. High and low concentrations of Zn were also able to induce the expression of *TcHMA4* (Bernard et al. 2004; Papoyan and Kochian 2004). HMA4 gene from *Arabidopsis* is located and expressed at the plasma membrane of the root vascular tissues but its role in metal loading in the xylem was confirmed when its overexpression leads to an increase in the amount of Zn and Cd in the shoots. However, a mutant of this gene showed less translocation of the Zn and Cd from roots to shoots (Verret et al. 2004). The role of HMA4 in root-to-shoot Zn translocation has also been shown earlier by Mills et al. (2005). They also explained the role of *AtHMA4* in metal detoxification by showing that plants' sensitivity to higher concentrations of Cd and Zn increases when *AtHMA4* is disrupted. The role of HMA2 and HMA4 transporters was also studied in *hma2 hma4* double mutants. Decreased amount of Zn in the shoots and nutrient-deficiency phenotype of this mutant, when subjected to zinc-free growth medium, pointed toward the role of these two genes in Zn homeostasis. Promoter analysis of HMA2 and HMA4 revealed that they are located in the vascular tissues of roots, leaves, and stems, and HMA2 was also found to be present at the plasma membrane confirming their role in Zn translocation. These were also thought to be involved in Cd detoxification as in a phytochelatin-deficient mutant background, their sensitivity to Cd increased (Hussain et al. 2004; Wong et al. 2009). *AtHMA3* transporter, a member of the P_{1B-2} subgroup of the P-type ATPase family located in the vacuolar membrane of *Arabidopsis*, has been shown to play a role in transport of heavy metal in plants. When overexpressed in *Arabidopsis*, transgenic plants were more tolerant to Cd, Co, Pb, and Zn. Greater accumulation of Cd was also observed. Sensitive phenotypes of T-DNA knockout lines were also

seen in the presence of Cd and Zn. Confocal microscopy revealed participation of *AtHMA3* in vacuolar sequestration of Cd (Morel et al. 2009).

AtATM3, an ATP-binding cassette transporter from *Arabidopsis*, is a mitochondrial protein involved in the biogenesis of iron-sulfur clusters and iron homeostasis in plants. AtATM3-overexpressing plants were shown to exhibit enhanced resistance to Cd, whereas *atatm3* mutant plants were more sensitive to Cd than wild-type controls. The role of AtATM3 in regulating cellular levels of nonprotein thiols (NPSH) was also observed. Increased expression of AtATM3 was seen when GSH biosynthesis was inhibited with increased expression of GSH1 under Cd stress and in the *atatm3* mutant suggesting that it may be involved in the transport of GSH-Cd conjugates across mitochondrial membrane (Kim et al. 2006). Recently, it has been shown that protein with strong similarity to AtATM3 transport Cd conjugates (Hanikenne et al. 2005) and CeHMT1, a close homologue of AtATM3, has been shown to be required for Cd tolerance (Vatamaniuk et al. 2005).

The multidrug and toxin extrusion (MATE) family is the most recently categorized one among five multidrug efflux transporter families (Kuroda and Tsuchiya 2009). Magalhaes et al. (2007) used positional cloning to identify the gene encoding a member of the MATE family, an aluminum-activated citrate transporter, responsible for the major sorghum (*Sorghum bicolor*) aluminum (Al) tolerance. Similarly another study in 2009 showed that expression of an expressed sequence tag, belonging to MATE gene family, correlates with the citrate efflux phenotype. This study provided genetic and physiological evidence that citrate efflux is a second mechanism for Al resistance in wheat (Ryan et al. 2009). Iron homeostasis-related FRD3 (ferric reductase defective 3) gene, which encodes a multidrug and toxin efflux (MATE) transporter, is responsible for reduced Zn tolerance in *A. thaliana*. FRD3 works as a multimer and is involved in loading Zn into xylem. Cross-homeostasis between Fe and Zn, therefore, appears to be important for Zn tolerance in *A. thaliana* with FRD3 acting as an essential regulator (Pineau et al. 2012).

MRPs (multidrug resistance-associated proteins) constitute another efflux transporter family belonging to ATP-binding cassette transporter superfamily (ABC) and transport metals that have formed conjugates with GSH. Plant orthologs of yeast cadmium factor 1 (*ScYCF1*) which provides Cd resistance by pumping GSH-Cd conjugates into vacuoles were identified in *Arabidopsis*. The first MRP gene termed as *AtMRP1* showed a similar function as *ScYCF1* (Szczyepka et al. 1994; Li et al. 1997). *AtMRP3*, 6, 7, and 14 were subsequently identified and shown to be upregulated by Cd (Bovet et al. 2003), with *AtMRP3* also seen to be involved in transport of Cd. *AtMRP3* promoter was also induced upon metals such as Cd, Ni, As, Co, and Pb exposure in *A. thaliana* and *N. tabacum* (Zientara et al. 2009).

AtPDR12, a member of the pleiotropic drug resistance protein (PDR) family of ABC transporters was found in *Arabidopsis*. The mRNA level of only *AtPDR12* increased in both shoots and roots of Pb(II)-treated *Arabidopsis*, suggesting that it may be involved in the detoxification of Pb(II). The GFP: *AtPDR12* fusion protein at the plasma membrane suggested that *AtPDR12* functions as a pump to exclude Pb(II) and/or Pb(II)-containing toxic compounds from the cytoplasm (Lee et al. 2005). Cd tolerance in plants has also been achieved through ABC transporter PDR8 (pleiotropic drug resistance 8) located at the plasma membrane that extrudes Cd out of the cell (Kim et al. 2007).

Apart from these transporter families described above, there are some other transporters that do not fall under these families such as the IREG and PCR1 family from *Arabidopsis*, and have been shown to be involved in Ni tolerance and transport and cadmium tolerance, respectively (Schaaf et al. 2004; Song et al. 2004). Tobacco *NtCBP4* (a calmodulin-binding protein) was isolated from tobacco cDNA library, located at the plasma membrane, and was shown to provide tolerance against heavy metal toxicity. The *NtCBP4* transgenic lines showed improved tolerance to Ni by limiting Ni accumulation and hypersensitivity to Pb due to increased accumula-

tion of Pb. These results lead to believe that *NtCBP4* is involved in the uptake of metals across the plasma membrane (Arazi et al. 1999).

Metalloid such as arsenic is naturally present in soil in the form of arsenate (AsO_4^{-3}). Due to chemical similarity between AsO_4^{-3} and inorganic phosphate, $\text{Pi}(\text{PO}_4^{-3})$, As(V) is usually taken up into the plants via phosphate transporters. Out of 9 high-affinity phosphate transporters (PHT1-9) in *Arabidopsis*, PHT1 and PHT4 have been involved in As(V) transport. Overexpression of PHT1 resulted into plants being sensitive to As(V) (Catarcha et al. 2007), while their single and double mutants have been shown to be As(V) tolerant indicating that As(V) uptake is compromised by mutations in these Pi transporters (Shin et al. 2004). Recently, LeBlanc et al. created transgenic *Arabidopsis* overexpressing PHT1 or PHT7 from *Arabidopsis* in combination with or without YCF1, leading to increased As accumulation and tolerance in *Arabidopsis* (LeBlanc et al. 2013).

7.5 Other Genes for Metal Tolerance

Apart from strategies discussed above, plants can be engineered with genes that have other means of dealing with heavy metal toxicity either by volatilization of metals and metalloids or via interacting with proteins in other metabolic pathways as described below.

7.5.1 Phytovolatilization

Phytovolatilization of a metal or metalloid ion involves the accumulation of metal (loid) species in plant cells and their subsequent conversion to an evaporable, usually less toxic, form such that it can be liberated to atmosphere. The main advantage of phytovolatilization is the removal of metal (loid) from a site without the need for plant harvesting and disposal (Kotrba et al. 2009).

Bacterial resistance to Hg^{2+} and organomercurial compounds has been attributed to organomercurial lyase (*MerB*), which converts methylmercury and other organomercurials to Hg^{2+} and mercuric reductase (*MerA*) that reduces elemental Hg^{2+} to nontoxic volatile Hg^0 (Summers 1986). These bacterial mercury resistance genes were cloned and used to genetically engineer plants for methylmercury phytoremediation. Overexpression of *merA*, *merB*, or a combination of both, in *A. thaliana* (Rugh et al. 1996; Bizily et al. 1999, 2003), *N. tabacum* (Ruiz et al. 2003), and rice (*Oryza sativa*, Heaton et al. 2003), resulted in Hg^{2+} and organomercurial tolerant phenotypes. More than tenfold higher volatilization rate was achieved by the targeting of *MerB* in the endoplasmic reticulum of *merA/merB*, where *MerB* exhibited more than 20 times higher specific activity than in plants with cytoplasmically distributed *MerB* (Bizily et al. 2003).

Selenium (Se) occurs naturally in two forms – selenate and selenite. Due to a similar nature of selenate and sulfate, they are normally taken up and assimilated by the same transporters and pathways (Ng and Anderson 1979; Zayed and Terry 1992; Anderson 1993). One such transporter involved in sulfate transport into plant cells is sulfate permease, and in certain plant species such as *Brassica napus* and *Stylosanthes hamata*, selenate was seen inhibiting the sulfate uptake by this transporter (Hawkesford 2003; Smith et al. 1995). ATP sulfurylase is an enzyme involved in sulfate reduction in plants (Setya et al. 1996). It converts selenate to selenite leading to a decrease in selenate concentration, and recent studies have shown that ATP sulfurylase is also involved in selenate reduction in plants (Pilon-Smits et al. 1999). Overexpression of APS1 in Indian mustard (*B. juncea*) showed that under supplied selenate, activity of ATP sulfurylase in these overexpressed plants was much higher in shoots but not roots compared to wild-type plants. These plants were able to reduce selenate better. Greater selenium accumulation and tolerance was also seen in APS transgenic plants (Pilon-Smits et al. 1999). The biosynthesis of MetSeCys, catalyzed by selenocysteine methyltransferase (SMT) in hyperaccumulating plant species inactivates SeCys for synthesis of SeMet and proteosynthesis.

MetSeCys could then be converted to volatile dimethyldiselenide (DMDS_e; Terry et al. 2000). Most plants that do not produce DMDS_e to convert SeMet from SeCys to volatile dimethylselenide (DMSe), which has been reported to be 500–700 times less toxic than selenate and selenite in soil (Wilber 1980). In addition to an improved Se accumulation in selenocysteine methyltransferase, *B. juncea* overexpressing SMT and ATP sulfurylase showed a higher DMSe production than wild-type control plants and an acquired ability to produce DMDS_e (LeDuc et al. 2004). Volatile forms were then efficiently evaporated from the leaves of transgenics grown in culture media and polluted soil (LeDuc et al. 2004; Bañuelos et al. 2007).

7.5.2 Genes Involved in Oxidative Stress Response and Misfolded Protein Repair

As the concentration of heavy metals inside the cell reaches to a point of saturation, the plant as a system begins to experience oxidative stress caused by the production of ROS and the inhibition of metal-dependent antioxidant enzymes (Schützendübel and Polle 2002). In general, heavy metal-induced ROS production causes oxidative damage to photosynthetic pigments; biomolecules such as lipids, proteins, and nucleic acids; and leakage of electrolytes via lipid peroxidation causing dramatic reductions in plant growth and productivity. Plants respond to oxidative stress by production of antioxidative enzymes such as SOD, APX, and GR and nonenzymatic free radical scavengers (Aust et al. 1985). Heavy metal toxicity is reported to increase activity of glucose-6-phosphate dehydrogenase and peroxidase in the leaves of plants grown in polluted soils (Van Assche and Clijsters 1987). In *Nicotiana plumbaginifolia*, leaves exposed to excess Fe have been shown to induce expression of ascorbate peroxidase (APX) and catalases (CAT) (Kampfenkel et al. 1995). Similarly the expression of CAT3 from *B. juncea* was induced upon Cd treatment (Minglin et al. 2005). A brief treatment with low concentration of Al to chickpea seedlings (*Cicer arietinum*) resulted in higher

SOD, APX, and guaiacol peroxidase (GPX) activity (Singh et al. 2012). Peroxiredoxins (PRXes) are peroxide-degrading enzymes with mitochondrial and chloroplastic variants. A T-DNA insertion mutant of *A. thaliana* lacking the expression of mitochondrial PrxIIF (AtPrxIIF knockout) was more sensitive to Cd in terms of root growth than the controls, signifying the involvement of PrxIIF in cellular detoxification of Cd such that root growth is maintained under Cd stress up to a certain threshold (Finkemeier et al. 2005).

Superoxide dismutases (SODs) are enzymes that play a pivotal role in metabolizing singlet O₂, preventing formation of deleterious reactive oxygen species (ROS) including hydrogen peroxide (H₂O₂), hypochlorite (OCl⁻), peroxyxynitrate (ONO₂), and hydroxyl radical (HO⁻) (Miller 2012). SOD activity is also induced in tomato seedlings after prolonged Cd treatment (Dong et al. 2006). SOD activity increases significantly in wheat leaves, following exposure to high levels of Cd, probably reflecting the accumulation of superoxide (Lin et al. 2007). On the other hand, Rodríguez-Serrano and coworkers showed a reduced SOD activity in pea plants exposed to Cd. Cd in particular has been shown to reduce the GSH/GSSG ratio and activate antioxidant enzymes such as SOD and GR (Rodríguez-Serrano et al. 2009). Heavy metals also induce the synthesis of stress-related proteins and signaling molecules, such as HSPs, SAPs, salicylic and abscisic acids, and ethylene (Manara 2012). Heat shock proteins (HSPs) are expressed not only in response to elevated temperatures but also in response to other abiotic stresses such as drought and heavy metal stress. Under heavy metal stress, protein-folding mechanism can go haywire resulting in misfolded proteins. Heat shock proteins can act as molecular chaperon ensuring correct folding and repair of misfolded protein (Vierling 1991). Heavy metals induce the expression of low-molecular-weight HSPs in rice (Tseng et al. 1993). *Zea mays* plants exposed to varying soil concentrations of Cu, Ni, Pb, and Zn showed increased chloroplast small HSP contents, with increased time of exposure. These HSPs in turn were shown to protect photosynthesis from heavy metal toxicity (Heckathorn et al. 2004).

Members of stress-associated protein (SAP) family (Vij and Tyagi 2006) were recently shown to provide tolerance to multiple abiotic stress including toxic metals (Mukhopadhyay et al. 2004). OsiSAP8 was shown to be induced in response to heavy metals such as Zn, Cu, Hg, and Cd (Kanneganti and Gupta 2008). Similarly overexpression of AtSAP10 in *Arabidopsis* provided strong tolerance to Ni and Mn. Due to their unique ability to provide tolerance to multiple abiotic stresses, members of SAP family are also the ideal candidates to engineer plants for heavy metal tolerance to tackle the challenges of global climate changes and the effects associated with it (Dixit and Dhankher 2011).

7.6 Heavy Metal Tolerance and Climate Change Adaptations

As global climate change is happening and not in distant future, the burden of environmental stresses including heavy metals and metalloids on crop plants is likely to continue to increase. Flood events can transport heavy metals, cyanide, and hydrocarbons from a contaminated area to a non-contaminated one (Harmon and Wyatt 2008; Hilscherova et al. 2007; Boxall et al. 2009). Climate change is likely to increase frequency of heavy precipitation events worldwide, which would result in transport of historical contaminants from previously undisturbed sediments. This could have implications for residue of toxic metal levels in food crops (Casteel et al. 2006). As irrigation demands may increase because of warmer and drier summers, water of poorer quality, including partially treated wastewater from industries and municipalities, will likely be applied to crops which may result in additional contaminant loadings to crops (Rose et al. 2001). Changes in temperature and precipitation could also increase aerial inputs of volatile and dust-associated contaminants. Finally, changes in bioavailability may occur with less bioavailable forms of contaminant being converted to more bioavailable forms. For example, Booth and Zeller (2005) suggested that increases in temper-

ature could enhance the methylation rate of Hg. The use of composting for treatment of municipal waste is increasing, with a portion of the resulting compost being used in agriculture. This is likely to increase loadings of microbes, heavy metals, and persistent organic pollutants in agricultural land (Déportes et al. 1995; Boxall et al. 2009). Furthermore, with the expansion of crop cultivation on soils that are not optimal for the growth of crop plants such as contaminated lands, development of abiotic stresses including heavy metals stress tolerant plants is becoming increasingly important (Kathuria et al. 2007). Improving the tolerance of major crop plants to heavy metals and other abiotic stresses has been a main goal in agricultural research for a long time. Transgenic approaches offer attractive alternatives to conventional techniques for the genetic improvement of abiotic stress tolerance. The development of new cultivars with enhanced heavy metal tolerance will undoubtedly have an important effect on global food production and food safety.

7.7 Conclusions

Crop production is declining around the world due to several biotic and abiotic stresses, which include heat, cold, drought, heavy metals, etc. (Abedin et al. 2002; Van Nguyen and Ferrero 2006). In the past years, a lot of focus has been on improving plant species and their tolerance toward these stresses but not much has been achieved because of the limited knowledge of the gene/network of genes that might be involved in providing such tolerance to multiple stresses. The research in this direction could have a significant impact on global food security, human health enhancement, and the environment, if more knowledge and information is gained on the hyperaccumulating species and phytoremediation strategy through the use of transgenics. Once this system is fully understood in the model plant species, the knowledge and information gained can be applied on other agricultural crops to engineer crops that will be better able to withstand such abiotic stresses and still produce sustainable

yield. This will also help to grow crops for food and biomass production on not so cultivable lands, thus making them more cultivable over time. Developing crops more resilient to heavy metals and other abiotic stresses will enable them to grow on marginal to moderately contaminated soils without losing crop yields.

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