16 Detoxification/Defense Mechanisms in Metal-Exposed Plants

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CONTENTS

16.1 INTRODUCTION

Trace element "biogeogenic cycling" in the environment is an integral function of the ecosystem (aquatic, terrestrial, and atmospheric systems). Metal enrichments in these compartments may result from natural sources or from human activities, such as smelting, mining, processing, agricultural, and waste disposal technologies. Metals are present in the Earth's crust in various quantities [1]. Their relative abundance, however, differs greatly in regions over the globe, and the region at which a metal is found in high concentration serves as the source of the metal. Although a metal may be present in high concentration in a region, it does not pose any threat to the environment until the landmass of the region is used for agroindustry. This is because the metals present remain tightly bound to their Lewis components as sulfides, oxides, or carbonates, as the case may be [1], and the ore particles also remain tightly packed along with the particles of the soil, which makes them highly immobilized. It is only the mining of the ore, and subsequent uses of the extracted metals that lead to far and wide contamination of the environment. From the figures of the crustal abundance

of various metals and their production per annum (Table 16.1), the magnitude of contamination or pollution by these metals as a result of anthropogenic activities may be imagined.

The concentration of a metal that existed in a region before the advent of industrial activity is termed its natural or background level. This is a result of release of the metal due to natural weathering of the metal-bearing formations in the area. The knowledge of natural contamination of a metal provides a true reference point for estimating the extent of pollution from the element and allows the contemporary situation to be seen in perspective — i.e., whether it is in excess from the point of view of its toxicity to organisms (Figure 16.1). However, the natural, or background levels of metals for some areas may be difficult to obtain because they may not exist due to human intervention; this is particularly true for lead, mercury, cadmium, and arsenic. In fact, although naturally occurring geochemical materials are the primary source of metals in the environment, not many examples are known for which the interaction between natural weathering processes and mineralized zones is completely devoid of a human contribution.

Anthropogenic activities lead to pollution of the three nonliving components of the environment — air, water, and soil — and the biosphere by metals [4]. The magnitude of pollution depends largely upon the nature and intensity of the activities; the most important among them are mining; industrial processing of ores and metals; and the use of metals and metal components, which affect the environment in a wide variety of ways [2,4,8–10]. However, this discussion will be restricted to terrestrial contamination and the adaptive process that plants undergo to face the challenge of the presence of high levels of natural or man-induced metals around them.

Excluders prevent metal uptake into roots and avoid translocation and accumulation in shoots [11]. They have a low potential for metal extraction, but they can be used to stabilize the soil to avoid further contamination spread due to erosion. Resistance of plants to heavy metal ions can be achieved by an avoidance mechanism, which includes mainly the mobilization of metal in root and in cell walls. Tolerance to heavy metals is based on the sequestration of heavy metal ions in vacuoles; on binding them by appropriate ligands like organic acids, proteins, and peptides; and on the presence of enzymes that can function at high levels of metallic ions (Figure 16.2 and Figure 16.3) [12].

The effective xylem loading of hyperaccumulators may be due to smaller sequestration of metals in the root vacuoles of hyperaccumulators [13]. Translocation of Ni from roots to shoots may involve specific ligands in some hyperaccumulator species. Kramer et al. [14] showed that spraying histidine on the leaves of the nonaccumulating *Alyssum montanum* greatly increased Ni tolerance and capacity for Ni transport to the shoots. The detoxification of heavy metals commences only when they enter the cells and occurs in the cell by the process of chelation, compartmentalization, or precipitation [15].

Metallothionein (MT)-II genes have been identified in plants [16,17]. Although detection of plant metallothioneins has been problematic, evidence suggests that they have the ability to bind heavy metals. Also, accumulation of heavy metals in plants has been shown to induce the production of phytochelatins (PCs), a family of thiol-rich peptides [18]. The synthesis of PCs has been documented to be induced by a variety of metals. However, PCs have been shown to be primarily involved in Cd and Cu tolerance [19]. A recent study suggested that PCs may also be involved in As detoxification [20].

The processes of heavy metal uptake, accumulation, distribution, and detoxification have been studied in a wide range of crop and herbaceous species [21]. The mechanisms involved in perennials have been partially investigated and reported to be considerably tolerant [22]. Several sequestration and detoxification strategies are reported to take place in plants exposed to elevated doses of toxic trace elements (Figure 16.4 and Figure 16.5) [23].

Complexation with phytochelatin peptides synthesized from glutathione has been identified as an important mechanism for detoxifying metals such as Cd, Pb, and Zn. Yet, phytochelatins do not appear to be the primary mechanism. Large increases in histidine levels and coordination of Ni with histidine have been reported in the xylem sap of *Alyssum lesbiacum*, suggesting that histidine is important for Ni tolerance and transport in hyperaccumulators [24].

TABLE 16.1
Worldwide Metal Production and Uses

Metal	Crustal abundance (mg kg^{-1})	Yearly production (× 1000 tonnes)	Major uses	Principal ores
Aluminum	83,000	16,200	Cable and wire for high-voltage electric transmission and various parts of autos, aircraft, and electrical equipment	Bauxite, Al_2O_3
Arsenic	1.80[a]	50	Making alloys for bullets and shot, storage batteries, herbicides, insecticides, and wood preservatives	Arsenide
Bismuth	0.20	4	Finds uses in phamaceuticals, electronics, cosmetics, and pigments, and as catalyst	Principally in flue dust as bismuthinite, Bi_2S_3, during smelting of Pb, Zn, or Cu
Chromium	110	10,800	Used in metal plating, making stainless-steel, wear-resistant and cutting-tool alloys, and as an anticorrosive	Chromite, $FeOCr_2O_3$
Cadmium	0.2	19	Used in electroplating, making Ni/Cd batteries, alloys, control rods in nuclear reactors, and pigments, and as stabilizer of polyvinyl chloride (PVC) plastic	Greenockite, CdS
Copper	63	8,700	Mainly used in making alloys and electrical products, the only wire used in windings in generators, motors, and transformers	As metal sulfides and oxides
Gold	0.0035	1.61	Used in jewelry and as the basis of currency	Calavarite ($AuTe_2$), Petzite [$(Ag,Au)_2Te$]
Iron	58,000	508,000	Most widely produced metal, usually as steel; also used in many alloys for special purposes	Hematite, Fe_2O_3, goethite, $Fe_2O_4.H_2O$, magnetite, Fe_3O_4
Lead	12	3,400	Making storage batteries, petrol additive, pigments, ammunition, cable sheathing	Galena, PbS
Manganese	1,300	22,000	Used as oxygen and sulfur scavenger in steel; manufacture of alloys, dry cells, chemicals	Found mainly as oxides
Mercury	0.089	6	Used as cathode in chlor-alkali cells, and also used in making paints, electrical apparatuses, fungicides	Cinnabar, HgS
Molybdenum	1.30	89	Used in making alloys, pigments, chemicals, lubricants, and as catalyst	Molybdenite, MoS_2, wulfenite, $PbMoO_4$
Nickel	89	800	Used in making coins, storage batteries, alloys, and as catalyst	Pentlandite [$(Fe,Ni)_9S_8$], Nicolite (NiAs)
Selenium	0.075	1.6[a]	Used in electronics, glass, pigments, photocopying	Mainly as clausthalite, PbSe, crokesite $(Cu,Tl,Ag)_2Se$
Silver	0.075	14	Finds uses mainly in making photographic materials and jewelry	Found with sulfide minerals

TABLE 16.1
Worldwide Metal Production and Uses (continued)

Metal	Crustal abundance (mg kg^{-1})	Yearly production (× 1000 tonnes)	Major uses	Principal ores
Tin	1.70	190	Used in coatings, solders; in making bearing alloys, bronze	Cassiterite, stannite
Titanium	6,400	4,200	Mainly used in making aircraft engines and their parts, also in making valves, pumps, paint pigments	As oxide, TiO_2
Vanadium	140	32	Used in making strong steel alloy	Primarily occurs as V(III) in igneous rocks
Zinc	94	7,200	Widely used in making brass (alloy), paint pigments; in galvanization	Found as sulfides, oxides, and silicates

Represents production during 1985.
Note: All production figures are of 1987.

Sources: Adapted from Manahan, S.E., *Environmental Chemistry*, Lewis Publishers, Boston, 1990, chap. 17; Ochiai, E.-I., *Bioinorganic Chemistry, an Introduction*, Allyn and Bacon, Inc., Boston, 1977, chap. 1; Fergusson, J.E., *The Heavy Elements: Chemistry, Environmental Impact and Health Effects*, Pergamon Press, New York, 1990, chap. 2; Evans, A.M., in *Introduction to Mineral Exploration*, Evans, A.M., Ed., Blackwell Science, Oxford, 1995, chap. 1; Chaterjee, K.K., *An Introduction to Mineral Economics*, Wiley Eastern Limited, Bombay, 1993, chap. 6; and Wedepohl, K.H., in *Metals and Their Compounds in the Environment: Occurrence, Analysis and Biological Relevance*, Merian, E., Ed., John Wiley & Sons, Inc., New York, 2000, chap. 1.

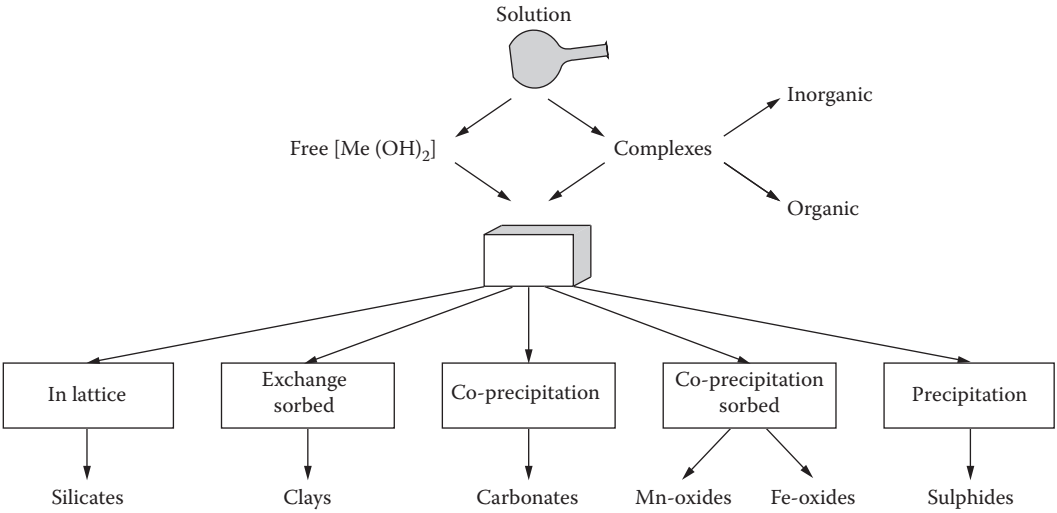

FIGURE 16.1 Fate of trace elements in the environment.

There are many indications that organic acids are involved in heavy metal tolerance, transport, and storage in plants, including for Al, Cd, Fe, Ni, and Zn. In plants that hyperaccumulate the metals as stated previously, the levels of citric, malic, malonic, and oxalic acids have been correlated with elevated concentrations of these metals in the biomass. Plant vacuoles are a major repository for organic acids, so an association between metals and organic acids suggests that metal detoxification occurs by vacuolar sequestration. However, other strategies for metal tolerance and

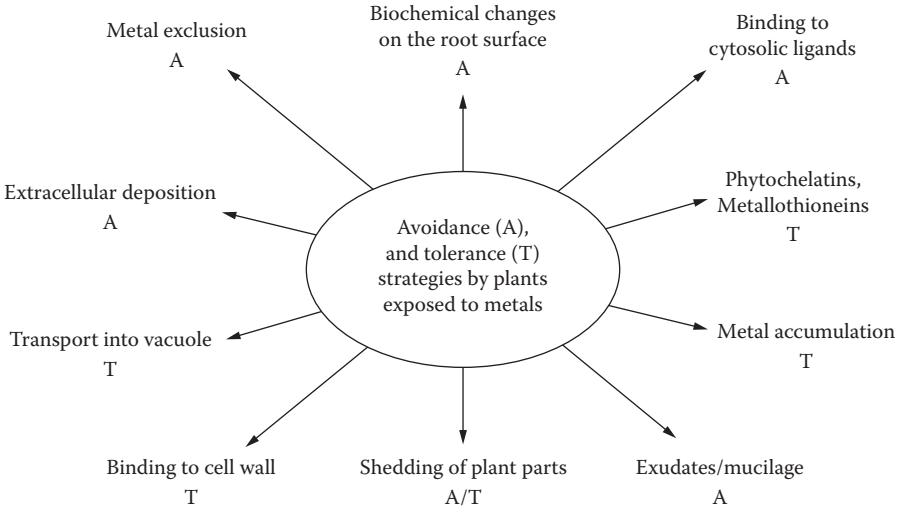

FIGURE 16.2 Avoidance and tolerance strategies adapted by plants exposed to elevated doses of trace elements.

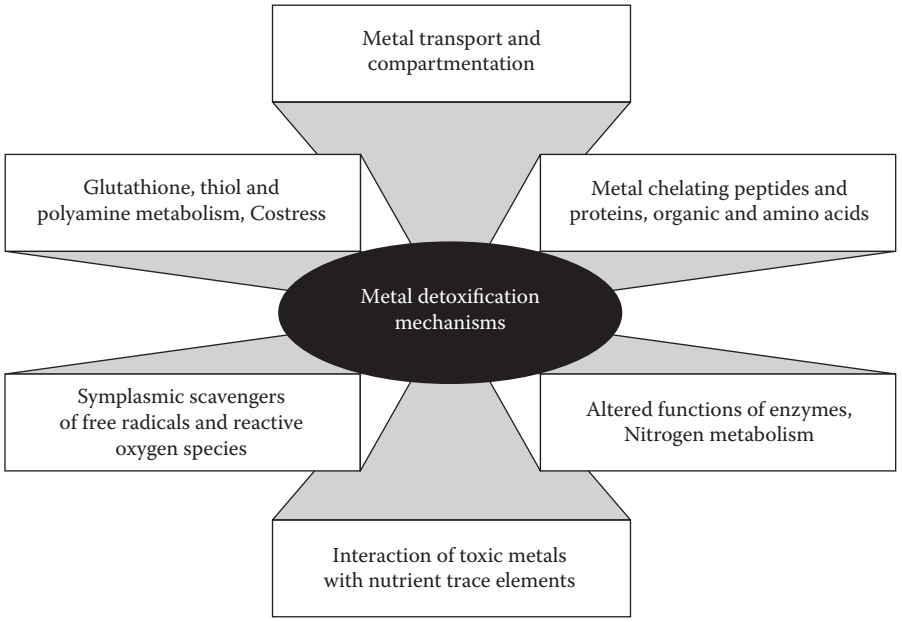

FIGURE 16.3 Metal detoxification mechanisms exhibited by vascular plants.

accumulation, such as binding to the cell wall or localization in the apoplast, may also be involved. The distribution of metals within plant tissues is therefore an important property that can act as an indirect indicator of detoxification mechanism. The distribution of metals between the apoplasm and symplasm of tissues, and between the cytosol and vacuole in cells, requires transmembrane transport; thus, the energizing and functioning of membrane processes may be of key significance in hyperaccumulation. The first, and still most common, parameter to characterize metal tolerance is the tolerance index, TI, which is calculated as:

$$TI = \text{response at elevated test metal concentration/response at control conditions}$$

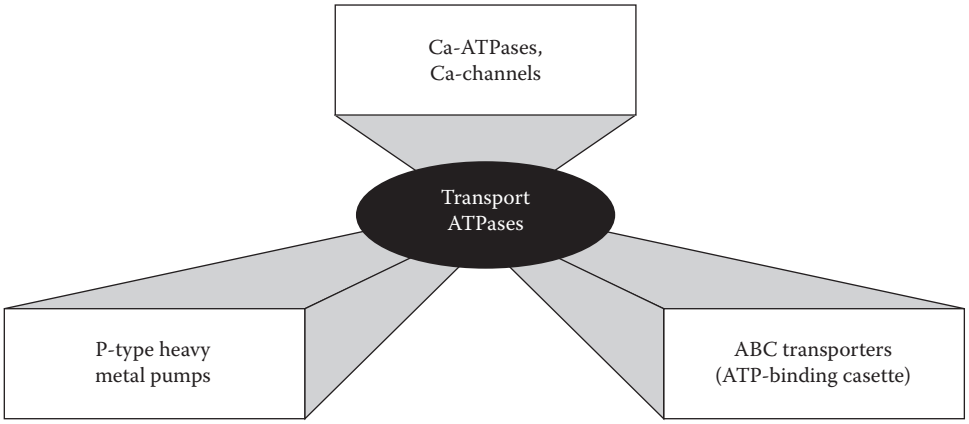

FIGURE 16.4 Role of plasmalemma in heavy metal tolerance involving transport ATPases.

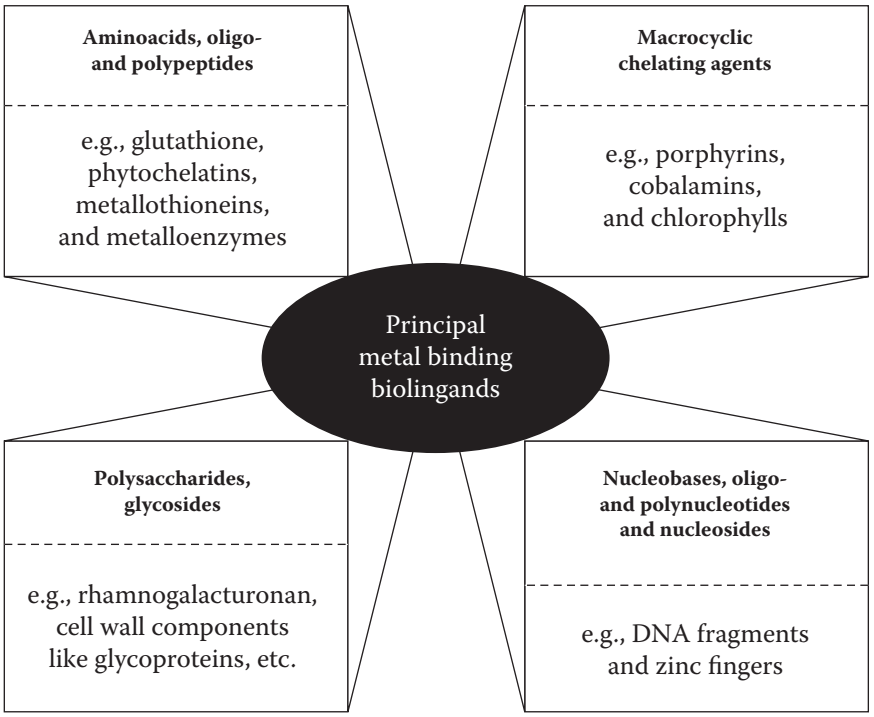

FIGURE 16.5 Principal metal-binding bioligands in vascular plants.

where response is a measurable character, e.g., increase in root length in the classical root elongation test. Alternatively, the effect index (EI) can be calculated as EI = 1 – TI. The response to control conditions and to the elevated metal concentration can be measured sequentially or in parallel [25].

16.2 HEAVY METAL CONTAMINATION OF SOIL AND ASSOCIATED AGRICULTURAL AND ENVIRONMENTAL PROBLEMS

Table 16.1 shows the yearly figures of production of heavy metals. Although the figures are of much environmental concern, these are of little importance as far as contamination of soil is concerned. This is because the use of heavy metals as industrial produce by mankind remains

confined to cities and suburban areas, which may constitute less than 10 to 15% of the total inhabitable land mass. More importantly, the heavy metals used as industrial produce mostly find their way into aquatic environments through the drainage system and run-off water during the rainy season; from there, their return to the atmosphere and landmass through biogeochemical cycling is very slow [4].

Furthermore, it may also be noted that the use of heavy metals like Hg and As as components of pesticides in agriculture has been nearly discontinued, and the contamination of the land mass by these through agricultural practices is now only history. Also, although the use of fertilizers may result in contamination of the environment by various heavy metals present in them [26,27], this is unlikely to be of much significance because these are continuously removed from the soil along with each harvest.

Considering the network of the roads connecting one city to another, a significant source of heavy metals in the terrestrial environment, particularly of Pb, could be automobile exhaust [28–30]. Chamberlain [31] estimated that, since 1946, automobile-generated aerosol lead added about 3 μg g^{-1} lead to the topsoil in rural areas and <10 μg g^{-1} in urban areas in the U.K. However, this is said to be small compared to natural levels and the lead added from industrial discharges [4]. Furthermore, with increasing use of unleaded gasoline, the threat of contamination of the land mass may not remain of much importance. In fact, the threat of heavy metal pollution of the land mass, which may require some remedial measures such as phytoremediation, mainly results from two sources: (1) mining activities in the region for ore rich in one or more heavy metals; and (2) atmospheric emission by industries.

Mining of the earth for ore is the first step towards increasing contamination of the landmass by various heavy metals depending upon the type of the ore. The mining operation lets the ore particles loose; otherwise, they are bound tightly among each other, remaining virtually immobile. They become prone to be blown away by wind, thus contaminating a vast area around the mine, particularly in the windward direction. Additionally, the mining operation leaves stretches of mined lands devoid of vegetation because of their high metal contents. This problem of contamination of uncontaminated agricultural lands will further increase with increase in the area of mining and the mining operation. It is generally in practice to use only ores rich in metal for cost-effective extraction, but when the currently available stock of the metal-rich ores ends, the ores less rich in metal content may eventually be processed, leading to spatial increase in heavy metal-contaminated/polluted agricultural and other lands.

Processing of the ores for the extraction of metals is the second major step during which metals find their way into land mass; the metals escaping out of the chimneys of smelters are ultimately deposited in agricultural fields or other land, which may be far from the smelting unit. Atmospheric metal enrichment, leading subsequently to pollution of soil, is also associated with other higher temperature anthropogenic activities, like burning of fossil fuels, production of cements, etc. For illustration, the emission of a few heavy metals due to burning of coal is given in Table 16.2. Despite modern technological advances, smelting operations and fossil fuel burning in industries continue to be important sources of heavy metals in the terrestrial environment.

The environmental problem associated with Al needs special mention, although it is not a heavy metal when its specific gravity is taken into consideration. The two sources of metals to the terrestrial environment described earlier hold true for this metal also. In addition, Al as such occurs in high levels in soil, which may be appreciated from its high crustal abundance [1]. Wherever the soil pH is acidic, this causes serious agricultural losses. It has been estimated that approximately 40% of the world's cultivated lands and up to 70% of potentially arable lands are acidic [34], which speaks of the gravity of environmental problems and economical losses associated with Al contamination of soil. Al is most often found as oxide or silicate precipitates that are not toxic to plants. However, in acidic soil (pH < 5.0), Al speciates to soluble octahedral hexahydrate form, $Al(H_2O)_6^{3+}$ (commonly called Al^{3+} [35]) — the phytotoxic species responsible for agricultural losses.

TABLE 16.2
Emission[a] of a Few Heavy Metals from the Burning of Fossil Fuels

Heavy metals	Emission from coal burning	Emission from oil burning
Cobalt	700	30
Chromium	1400	50
Copper	2100	23
Nickel	2100	1600
Vanadium	3500	8200
Mercury	400	1600
Cadmium	140	2
Selenium	420	30
Arsenic	5000	10
Zinc	7000	40
Lead	3500	50

[a]Tons per year.

Sources: Adapted from Forstner, U. and Wittmann, G., *Metal Pollution in the Aquatic Environment*, Springer–Verlag, New York, 1979, chap. B; Bertine, K.K. and Goldber, E.D., *Environ. Sci. Technol.*, 11, 297, 1977; and Ruch, R.R. et al., *Environ. Geol. Notes*, 61, 1, 1973.

16.3 HEAVY METAL DETOXIFICATION MECHANISMS IN PLANTS

By virtue of their stationary status, plants, unlike animals, cannot migrate to avoid unfavorable fluctuation or changes in their environment; thus, they must change their metabolic activities suitably to allow them to cope with the changing environment or otherwise perish. The resulting changes in their metabolism are called "stress response," which may enable the plant to survive under the condition of stress. This may occur for a short time only, known as acclimation, or the changes induced may be good enough to support continuous growth of the plant, known as adaptation. The latter quality is being or may be exploited to find the solution to increasing heavy metal contamination of landmasses and forms the basis of "phytoremediation."

Cunninghum and Ow [36] envisioned the working of phytoremediation as follows: "By growing plants over a number of years the aim is to either remove the pollutants from the contaminated matrix or to alter the chemical and physical nature of the contaminants within the soil so that it no longer presents a risk to human health and the environment." Thus, plants resistant to heavy metals can be used under the concept of phytoremediation in one or more of the following ways:

- To remove the metals from the soil
- To chelate the metals in the soil and bind the soil particles tightly so that their erosion by wind, as well as further contamination of the land in the windward direction, is prevented
- To make possible the use of the metal-contaminated land for agriculture

It is explicit that the plants to be used under the first category, i.e., for the removal of metals from soil, should be hyperaccumulators of the heavy metals contaminating the land; to be used under the second category, plants may or may not be hyperaccumulators, but should be resistant to the metals present in the soil and able to grow well, with good rooting systems. For plants to be used under the third category, in addition to being resistant to the metals contaminating the soil, it is necessary that they do not take up and accumulate them in their tissues; otherwise the agricultural products would be highly contaminated with the metals.

Heavy metal-resistant plants are available in nature in hyperaccumulator and excluder categories. However, their use is limited because the character is not present in the desired species or the character requires additional desirable traits for effective phytoremediation. For example, a metal hyperaccumulator plant may not have the desired root structure, such as root depth and root density, and/or may not be fast growing with high biomass turnover, which could improve the metal accumulation or soil decontamination potential of the plant. Similarly, the metal-excluding character may not be present in the required crop species. Although breeding programs could be envisioned to put the desired traits together, knowledge of the biochemical and molecular bases of tolerance of plants to metals under both categories may allow scientists to take a biotechnological approach and introduce the required trait into the species of interest in a more comfortable and cost-effective manner.

Research on understanding the mechanism of metal tolerance dates back to as early as the 1950s, when only ecological and physiological differences between plants from metal-enriched and noncontaminated habitats were studied [37,38]. The investigation gained momentum only in the late 1960s when time- and cost-effective techniques for the analysis of metals — atomic absorption spectrophotometry — was developed [39]. However, during that period, the research was mainly concentrated upon the uptake of metals and their cellular compartmentalization [40,41].

From the 1970s, the physiological and genetic aspects of metal tolerance began to be studied using the rewarding approach of comparison of metal-tolerant and nontolerant cultivars of a species, or even isogenic line of a species, which differed as far as possible only in resistance to one or more metals [42,43]. Currently, understanding of tolerance of heavy metals in plants narrows basically to two categories: (1) resistance by exclusion of the metals; and (2) resistance by uptake, but subsequent sequestration of the metals to inactive form inside the cells. In addition, another concept is emerging in this field: heavy metal tolerance involving antioxidative machinery, which is worth discussing.

16.4 EXTRACELLULAR DETOXIFICATION OF METALS OTHER THAN ALUMINUM

The indication of possible involvement of plant exudates in ameliorating the toxic effect of metals other than Al mainly comes from work on algal systems, and that only on cyanobacteria. The indication mainly stems from the fact that many organic compounds, like amino acids [44,45]; mercaptans [46]; organic acids [47]; peptides [48]; and spent medium [46], supplied along with the heavy metals (Cu, Cd, Ag) alleviated their toxicity, and the fact that cyanobacteria produce weak acids, strong metal-complexing agents like hydroxamate siderophore [47]; amino acid-containing compounds [49]; catechol siderophore [50]; metallothioneins [51]; and unknown chelators [52,53].

However, the relationship between the excretion of organic compounds and their detoxification role has never been experimentally demonstrated [54]. Protective effects of plant exudates against heavy metal toxicity have not been reported. Recently, however, Arduini et al. [55] worked on Cd and Cu distribution in various Mediterranean tree seedlings and suggested that the well-developed root cap in plants has a protective role against metal uptake.

16.5 HEAVY METAL DETOXIFICATION THROUGH INTRACELLULAR SEQUESTRATION

Currently, it is accepted in principle that plants' resistance to heavy metals, unlike that to Al^{3+}, is achieved through their uptake and proper sequestration inside the cell, rather than by their exclusion that works for Al^{3+}. Plants' adaptation of a totally different mechanism for tolerance to heavy metals than for tolerance to Al^{3+} is probably because many essential metals in the heavy metal category

must be taken up from the environment for various metabolic functions to continue [1], but their chemical properties match greatly with many of the nonessential heavy metals, thus making it difficult for plants to go for their selective uptake. Thus, the nonessential heavy metals are also taken up along with the essential ones when present in the environment.

Additionally, the essential heavy metals are also more or less as toxic to the organisms as the nonessential ones at a similar concentration. Thus, probably the only way for plants to counter the presence of elevated levels of essential or nonessential heavy metals in the environment is to sequester them properly out of the cytoplasm in inactive form. The problems associated with the presence of elevated levels of heavy metals in the environment rather may be viewed under broad perspectives: it is essential for plants to have mechanisms that (1) maintain the internal concentrations of essential metals between deficient and toxic limits; and (2) keep the nonessential metals below their toxicity threshold [18].

At present, approximately 400 plant species belonging to at least 45 families are hyperaccumulators of heavy metals to various degrees [56,57]. The field-collected samples of plants from metal-rich soils have been found to contain metals like Cu, Co, Cd, Mg, Ni, Se, or Zn up to levels that are 100 to 1000 times those normally accumulated by plants [56,57]. The concentration of some metals may reach as high as 1000 ppm or more on dry mass basis [58,59]. The exact mechanism involved in such hyperaccumulation is, however, still debatable. A few reports of involvement of organic acids in the hyperaccumulation process have been issued; however, general agreement in the scientific community is that the hyperaccumulation, and thus the resistance to the heavy metals accumulated, is facilitated by thiol-rich polypeptide, similar in function to that of metallothionein rich in amino acid cysteine, which was first discovered in horse kidney [60].

16.6 COMPLEX FORMATION WITH PHYTOCHELATINS

Polypeptides are designated "metallothionein" when they show several of the feature characteristics of equine renal metal-binding protein, like high metal content; high cysteine content with absence of aromatic amino acid and histidine; an abundance of Cys-*x*-Cys sequence, where *x* is an amino acid other than Cys; spectroscopic feature characteristics of metal thiolates; and metal thiolate clusters [61,62]. These have been subdivided into three classes:

- Class I: polypeptides with locations of cysteine closely related to those in equine renal metallothionein (MT)
- Class II: polypetides with location of cysteine only distantly related to those in equine renal metallothionein
- Class III: atypical nontranslationally synthesized metal thiolate polypeptides

The class III metallothioneins (MTs) are only known from the plant kingdom. They occur in the organisms as metal-binding complexes of various sizes, *Mr* (molecular mass) 3000 to 10,000, depending upon the ionic strength of the eluants; this suggests that these are accretions of multiple peptides of various lengths with the metal atom(s). The incorporation of varying amounts of sulfide or sulfite ions also contributes to the size heterogeneity of the class III MTs. Nevertheless, two broad categories of the complexes are generally recognized: low molecular weight (LMW) and high molecular weight (HMW). The grouping is based on the good resolution of the metal-binding complexes obtained in the extracts from fission yeast exposed to Cd in some of the earliest known experiments in the line [63,64].

Currently, several situations are known to exist in plants, ranging from good resolution of LMW and HMW complexes [65], partial resolution of the LMW complexes on the trailing shoulder of abundant HMW complexes [66], to no evidence for LMW complex [67]. Such variations are attributed to differences between organisms; type of nutrient medium for growth; concentration of the gel-filtration matrix; and column bed dimensions [18].

The indication of the existence of class III MTs in plants was first provided by Rauser and Curvetto [68] in roots of *Argostis* tolerant to Cu, and by Weigel and Jager [69] in bean exposed to Cd. The metal-binding complex was characterized to some extent only by Murasugi et al. [63,70] in extract from fission yeast, *Schizosaccharomyces pombe*, grown on Cd solution. Subsequently, these have been reported to be produced in cultured plant cells [71], algae [72], and virtually all higher plants tested [71,73]. These have also been reported to be induced by a variety of metals, including Cd, Cu, Zn, Pb, Hg, Bi, Ag, and Au [74–76]; some of these are soft metals on the Pearson's scale of softness. In addition, the complex is also induced by multiatomic anions like SeO_4^{2-}, SeO_3^{2-}, and AsO_4^{3-} [74]. Their induction depends not only on the type of the metal, but also on the plant species [77].

The information on composition and structure of phytochelatins (PCs) comes from the pioneering work of two groups: Kondo et al. [78,79] on fission yeast, *Schizosacchromyces pombe*, and Grill et al. [71] on cultured cells of *Rauvolfia serpentina*. The complexes produced by *S. pombe* have a structure identical to those of plants, consisting of heterogeneous population of polypeptides; each represents repeating units of γ-glutamyl-cysteine (γ-Glu-Cys) followed by a single C terminal glycine [γ-Glu-Cys)$_n$-Gly] with the number of repeating units (n) ranging from 2 to 11 [71,78,79]. Before their classification as class III MTs, various trivial names were given, depending upon one or more features associated with them — for example, as cadystin — as were found to be induced by cadmium in fission yeast [63,64,70,78,79]; phytochelatin (PC), representing the metal binding peptides of kingdom phyta [71]; γ-glutamyl peptides, after the presence of γ-glutamyl [80]; and poly(γ-glutamylcysteinyl)glycine, after their basic constituents [81].

However, none of these trivial names is appropriate because metals other than Cd also induce these polypeptides. Moreover, fungi are not considered to belong to kingdom phyta and diverse γ-glutamyl di- and tripeptides are known [61]. Nevertheless, the term "phytochelatin" is popularly used because fungi are always considered as plants. Besides, the term is meaningful — suggesting the chelating function of the molecule — and hence this will continue to be used in this chapter.

16.7 PHYTOCHELATINS: PRIMARY STRUCTURE AND CLASSES

The primary structure of phytochelatins (PCs) was basically derived from the Cd-binding chemical analysis of the Cd-binding complexes from various sources in the early phase of work in this field [71,72,78,79,81,82]. The polypeptides were found to be composed of three amino acids: Glu, Cys, and Gly, which occurred in the molar ratio: 2:2:1 and 3:3:1 in *S. pombe* [79] and *Datura innoxia* [81]; 3:3:1 and 4:4:1 in tomato [82]; 4:4:1 in *R. serpentine* [71]; and up to 5:5:1 in *Chlorella fusca* [72]. The primary structure in general is of nature (γ-Glu-Cys)$_n$-Gly, with n = 2 to 5. The value of n up to 11 has also been observed depending upon the plant species [73,74].

The primary structure of the polypeptides described previously is related to glutathione (GSH), γ-Glu-Cys-Gly. However, in some plants such as soybean (*Glycine max*), glutathione is replaced by homoglutathione with a nonprotein amino acid β-alanine (γ-Glu-Cys-β-Ala). In such plants, glycine at the carboxy terminal in the polypeptide is replaced by β-Ala and thus has been named homophytochelatin (h-PC) [83]. This has the general structure (γ-Glu-Cys)$_n$-β-Ala and has been observed in 36 species of legume; 13 species produce only h-PC and 23 species produce h-PC and PC, depending upon whether the plant produces only h-GSH or GSH and h-GSH [83].

The third family of the PC polypeptide was observed by Klapheck et al. [84] in certain species of Poaceae (rice, wheat, and oats) in which the terminal Gly is replaced by serine (Ser) showing the primary structure (γ-Glu-Cys)$_n$-Ser. These peptides are related to the tripeptide hydroxymethyl-glutathione (γ-Glu-Cys-Ser), so the polymer is termed hydroxymethyl-phytochelatin (hm-phytochelatin). In addition, the species studied also produced (γ-Glu-Cys)$_n$-Gly and (γ-Glu-Cys)$_n$ (desglycyl or desGly peptide).

The most recent addition in the family of phytochelatin polypeptides is that related to novel tripeptide γ-Glu-Cys-Glu, with the structure (γ-Glu-Cys)$_n$-Glu. This was identified in maize exposed

to Cd [85]. Maize also produces in abundance another family of the polypeptides, the desGly-peptides [(γ-Glu-Cys)$_n$], which was first noticed as a minor constituent of Cd-binding complexes in the fission yeast [85]. The production of (γ-Glu-Cys)$_n$-Gly also occurred.

Thus, there are five families of polypeptides in class III MTs. They have the common features such as (1) Glu occupies the amino-terminal position; (2) Cys forms the next residue-forming peptide bond with the γ-carboxyl group of Glu; and (3) γ-Glu-Cys pairs are repeated two or more times with the subscript (*n*) specifying the exact number of repeats. The division of the polypeptides into five classes is only on the basis of the variation in the carboxy terminal amino acid. All the five classes of phytochelatins thus belong to one specific family of dipeptide γ-Glu-Cys, and the complement of (γ-Glu-Cys)$_n$ peptide from the five families largely varies according to the species.

16.8 PHYTOCHELATIN: SYNTHESIS

The γ-glutamyl linkages present in PCs suggest that these polypeptides are not primary gene products, i.e., they are not the translational products of mRNA and must be formed by ribosome-independent enzyme reactions. The similarity of PCs to GSH in containing γ-Glu-Cys moiety suggested that this could be involved in the synthesis of the polypeptides. A number of other observations also support the function of GSH as a precursor of PCs.

- The metal-induced synthesis of PCs is accompanied by a depletion of the GSH pool in cell cultures [74,86,87] and in plant tissue root [88–90].
- GSH is synthesized by the action of γ-glutamylcysteine (γ-Glu-Cys) synthetase (EC 6.3.2.2), which joins Glu with Cys followed by addition of Gly by GSH synthetase (EC 6.3.2.3). The activity of one or both of the enzymes increases upon exposure of the plants to Cd [88,91]. Furthermore, the plant cells incubated with buthione sulfoximie (BSO), a potent inhibitor of γ-Glu-Cys synthetase, are unable to synthesize PCs, and addition of GSH reestablishes PC synthesis [86–88].
- The mutants of *S. pombe* that lack γ-Glu-Cys synthetase or GSH synthetase do not synthesize PCs in response to Cd [92]. It has been demonstrated that, in cells of *Datura innoxia*, [^{35}S]GSH is rapidly incorporated into PCs after exposure to cadmium [93]. In the presence of BSO and GSH, PCs produced upon exposure of tomato cells to cadmium incorporate little [^{35}S]cysteine, indicating that these peptides are not synthesized by sequential addition of cysteine and glutamate to GSH [94].

At least three possible pathways of biosynthesis of PCs can be visualized:

- Transpeptidation, with GSH or the oligomeric PC peptide acting as an acceptor for the successive addition of γ-Glu-Cys moieties from GSH by transpeptidation reaction
- Dipeptide addition in which γ-Glu-Cys units, synthesized by the action of γ-Glu-Cys synthetase, are transferred to GSH and/or the oligomeric PC peptides
- Polymerization of the γ-Glu-Cys units to (γ-Glu-Cys)$_n$ oligomeric molecules that are transferred subsequently to Gly in a similar fashion known for GSH synthetase

Grill et al. [95] identified one activity in the extract from *Silene cucubalus* (= *vulgaris*) that conformed to the first possible pathway. *In vitro* experiments with 15-fold purified enzyme activity fraction with GSH as substrate showed induction of PC synthesis immediately after the addition of 0.1 m*M* Cd^{2+}. (γ-Glu-Cys)$_2$-Gly appeared without a noticeable lag phase. Heptapeptide (n = 3) was detected 15 min after the addition of Cd^{2+} and, after a further 20 min, nanopeptide (n = 4) was detected. (The reaction came to a halt after 100 min and resumed after a second addition of Cd^{2+}.)

When (γ-Glu-Cys)$_2$-Gly was present along with GSH, heptapeptide formation occurred immediately after the addition of Cd^{2+}. In the presence of only (γ-Glu-Cys)$_2$-Gly as substrate, the

formation of first heptapeptides and then nanopeptides occurred. Simultaneously, the concentration of the pentapeptide (the substrate) decreased with concomitant release of GSH. These coworkers concluded that, in addition to adding γ-Glu-Cys unit to GSH, the enzyme, which was named γ-glutamylcysteine dipetidyl transpeptidase (trivial name phytochelatin synthetase or PC synthetase) could also add this to PC molecules. Also, the source of γ-Glu-Cys moiety can be GSH as well as the PC molecules.

Chen et al. [96] in addition to confirming the conclusion of Grill et al. [95] also showed that PCs could not be synthesized by the enzyme (PC synthetase) extract from tomato cells in the presence of γ-Glu-Cys alone or γ-Glu-Cys and Gly. They concluded that the enzyme identifies only two substrates, GSH and PCs, and probably has two binding sites, one specific for GSH and the other, less specific, for GSH and PCs. This conclusion was also in context of observations of Klapheck et al. [97]; they worked on pea (*Pisum sativum* L.), which produced GSH and h-GSH, and showed that the crude enzyme preparation from root produced $(\gamma\text{-Glu-Cys})_2$-Gly in the presence of GSH. Given only γ-Glu-Cys-β-Ala (h-GSH) or γ-Glu-Cys-Ser (hm-GSH), however, the rate of production of their n_2 oligomers was much less.

However, in the presence of GSH and h-GSH or hm-GSH the synthesis of the respective β-alanyl or seryl n_2 oligomers was greatly increased. This led them to conclude that the enzyme has a γ-Glu-Cys donor-binding site specific for GSH and a less specific γ-Glu-Cys acceptor-binding site that is able to bind several tripeptides — namely, GSH, h-GSH, hm-GSH, and, of course, the PCs. Thus, the synthesis of PCs as well as of h-PCs and hm-PCs is possible only by a single enzyme. Whether the enzyme preparation of Chen et al. [96] and Klapheck et al. [97] could carry out PCs synthesis — even in the absence of GSH — with only added $(\gamma\text{-Glu-Cys})_2$-Gly has not been checked. If so, then the possibility of existence of GSH specific binding site does not exist. Of course, it may be possible that the specificity of γ-Glu-Cys donor site of the enzyme lies in the recognition of Gly residue at the carboxy terminal end of the tripeptide, or the PCs.

The other two pathways of $(\gamma\text{-Glu-Cys})_n$-Gly synthesis are indicated in the observation of Hayashi et al. [98]. The crude preparation of the enzyme differs from that described previously in two respects: (1) Cd is not necessary for its catalysis; and (2) some $(\gamma\text{-Glu-Cys})_2$ appears in the reaction mixture along with PCs with GSH as substrate. Incubation of GSH with γ-Glu-Cys, $(\gamma\text{-Glu-Cys})_2$, or $(\gamma\text{-Glu-Cys})_3$ in the presence of the enzyme produces $n + 1$ oligomers of the $(\gamma\text{-Glu-Cys})_n$ provided — i.e., PCs are produced by dipeptide addition, the second pathway. The preparation also polymerized γ-Glu-Cys into $(\gamma\text{-Glu-Cys})_{2,3}$, suggesting a dipeptide transfer function of the enzyme. This is the only work that suggests a biosynthetic origin for $(\gamma\text{-Glu-Cys})_n$. Furthermore, GSH synthetase added Gly to $(\gamma\text{-Glu-Cys})_{2,3}$, giving $n = 2$ and $n = 3$ oligomers of the PCs, respectively. This allowed Hayashi et al. [98] to propose that polymerization of γ-Glu-Cys to $(\gamma\text{-Glu-Cys})_n$, followed by GSH synthetase adding Gly, could be a third pathway for PC biosynthesis.

Klapheck et al. [97], however, believe the production of $(\gamma\text{-Glu-Cys})_n$ to be a result of catabolic processes by the action of carboxypeptidase on $(\gamma\text{-Glu-Cys})_n$-Gly removing the Gly moiety. By analogy to the reactions catalyzed by carboxypeptidase C, a proteolytic enzyme that also acts as dipetidyl transpeptidase [99], desGly-PCs may also arise from a hydrolytic activity of PC synthetase — i.e., cleavage of the Gly after binding of a PC molecule at the donor-binding site and transfer to water instead of to a γ-Glu-Cys acceptor [97].

The assumption of catabolic process in the formation of $(\gamma\text{-Glu-Cys})_n$ is strengthened from the observation of production of $(\gamma\text{-Glu-Cys})_n$-Glu in maize [85]. The tripeptide γ-Glu-Cys-Glu is found in maize only after the Cd-induced appearance of $(\gamma\text{-Glu-Cys})_n$-Gly and $(\gamma\text{-Glu-Cys})_n$, offering the possibility that the family of γ-Glu-Cys peptides with amino-terminal Glu are degradation products of other thiol peptides. Only action of γ-glutamyl transpeptidase for cleaving intramolecular γ-Glu linkages would be required [18]. Study on the Cd-sensitive mutant of *Arabidopsis thaliana* also supports only transpeptidation of γ-Glu-Cys moiety from GSH to GSH or $(\gamma\text{-Glu-Cys})_n$-Gly as the probable pathway for PC synthesis; the mutant is deficient in GSH synthesis, producing significantly less PCs despite having PC synthetase activity similar to the wild type [66,100]. Nevertheless, the

presence of desGly-PCs at the beginning of Cd incubation and at low Cd concentrations does indicate that *de novo* synthesis of these peptides is possible [84].

16.9 PHYTOCHELATINS: INDUCTION BY HEAVY METALS

Voluminous literature exists on the induction of PCs by heavy metals and their possible involvement in metal tolerance and has also been reviewed by many [18,39,61,101,102]. In fact, the phytochelatin response, or the synthesis of heavy metal-binding polypeptides, is one of the few examples in plant stress biology in which it can be readily demonstrated that the stress response (PC synthesis) is truly an adaptive stress response. Nevertheless, there are several exceptions. During the course of the stress response studies, attention has been focused not only on the rate of synthesis of the polypeptides, but also on the role of the precursors and the enzymes involved in their synthesis. Most of the information, however, comes from the work involving the heavy metal, cadmium, in response to which the induction of PC synthesis was first detected.

The argument in favor of the possible involvement of PCs in heavy metal tolerance mainly comes from its induction and accumulation by a wide range of plant species, including algae, in response to Cd, and also in response to a range of heavy metals [39,61,66,75,77,84,100–104]. In cell suspension cultures (of *Rauvolfia serpentina*), it has been observed that the tendency of metals to induce PCs decreases in the order [74]:

$$\text{Hg} >> \text{Cd, As, Fe} > \text{Cu, Ni} > \text{Sb, Au} > \text{Sn, Se, B} > \text{Pb, Zn}$$

In the root culture of *Rubia tinctorum*, the PC induction by various heavy metals was in the order [76]:

$$\text{Hg} >> \text{Ag} > \text{Cd} >> \text{As} > \text{Cu} > \text{Pd} > \text{Se} > \text{Ni} > \text{Pb} > \text{Zn} > \text{In} > \text{Ga}$$

For the metals common to both cases, the order of induction is more or less similar, except for Ni and Se. The order, however, is based on the total metal concentration in the culture medium. For free ionic metals, the order may be different. This is evident from the work of Huang et al. [105], who applied the metal concentrations to the cell suspension culture depending upon their toxicity. Furthermore, Grill et al. [95] showed that the activation of the purified enzyme from cell suspension cultures of *Silene cucubalus* by Hg was only 27% of activation produced by equimolar concentration of Cd.

Further support in favor of the possible role of PCs in providing plants resistance to heavy metals comes from the study on metal (mostly Cd)-tolerant culture cell lines and strains of algae and mutants. The uptake of Cd by Cd-tolerant plant cell lines is somewhat greater than by the nontolerant ones prior to Cd becoming toxic [105–107].The Cd-tolerant cells bind more than 80% of the cellular Cd as Cd–PC complex, but little binding of Cd occurs in the nontolerant cells, which grow poorly and die prematurely [87,105–107]. Furthermore, Gupta and Goldsbrough [108] observed that the tomato cell lines selected for resistance to various concentrations of Cd showed increased Cd and PC accumulation concomitant with increase in their tolerance level. At least 90% of the Cd in the most tolerant cell line was associated with Cd–PC complexes.

The evidence of PCs' protective function against heavy metal toxicity also comes from studies of the influence of the precursors of PCs, and the enzyme(s) involved in their synthesis, on the resistance of cell cultures or intact plants to the metals. Upon exposure of Cu-sensitive and Cu-tolerant *Silene cucubalus* (L.) to Cu, the loss of GSH pool was only observed in the former [109], suggesting that the maintenance of GSH pool for continued synthesis of phytochelatin is necessary for survival under metal stress. In a similar study on tomato cells, it was observed that the tolerance of CdR6-0 cells (cells selected for Cd tolerance) was associated with their enhanced capacity to synthesize GSH, nearly twofold higher than the unselected CdS cells, to maintain the production

of PC [110]. It has also been demonstrated that the transgenic Indian mustard (*Brassica juncea*) overexpressing GSH synthetase contains greater amounts of GSH and phytochelatin, accumulates more Cd, and shows greater tolerance to Cd than the wild type [111]. Furthermore, the growth of the Cd-tolerant cells [87,105] or that of the nontolerant cells [94] remains unaffected in the presence of BSO alone, but is greatly inhibited in the presence of BSO together with Cd. The cells' growth is, however, restored in the presence of exogenous GSH and is accompanied by PC synthesis [86,94].

Howden et al. [66,100] used a genetic approach to establish the relationship between Cd-tolerance and phytochelatin synthesis and its accumulation. They could isolate an allelic series of Cd-sensitive mutants, *cad1* (*cad1-1*, *cad1-2*…), and a second Cd-hypersensitive mutant, *cad2*, affected at a different locus. They observed that the hypersensitivity of *cad1* mutants to Cd was associated with deficiency in their ability to accumulate PCs due to deficient PC synthetase activity, and that of *cad2* was associated with deficient GSH level resulting in deficient PC synthesis. Genetic studies using *S. pombe* have also shown that GSH-deficient mutants are also PC deficient and Cd hypersensitive [92,112].

In contrast to the preceding, many studies on naturally evolved heavy metal-tolerant varieties of plants, as well as on laboratory-selected tolerant cell lines, do not demonstrate a clear relationship between heavy metal resistance and PC production, thus creating doubts on the involvement of PCs in metal tolerance:

- Tolerant plants often do not produce more PCs than nontolerant ones [103,109,113].
- Although the level of PC in a Cu-sensitive ecotype of *Silene cucubalus* increases significantly at 0.5 μ*M* concentration of Cu, which is a nontoxic concentration for those plants, significant increase in the level of PC in the Cu-tolerant plants occurs only at 40 μ*M* or high Cu-concentrations, which are toxic for the ecotype [109].
- Distinctly Cu-tolerant (Marsberg) and nontolerant (Amsterdam) ecotypes of *Silene vulgaris* produce equal amount of PCs if they are grown at Cu-concentrations that cause equal degree of root growth inhibition, but such concentrations of the metal for tolerant plants are always greater than those for the nontolerant plants [113].
- The roots of Cd-tolerant plants of *S. vulgaris* exposed to a range of Cd concentrations accumulated greater amounts of the metal than the roots of Cd-sensitive plants, but contained significantly less PCs than the latter, particularly at the higher exposure concentrations [103].

16.10 HMW PC AND METAL TOLERANCE

Although the accumulation of PCs could be a major component of the heavy metal detoxification process, the increased tolerance to metals may involve other aspects of PC function. The first argument in favor of this came from Delhaize et al. [87], who observed that, although Cd-sensitive and Cd-tolerant cells of *Datura innoxia* synthesized the same amount of PCs during the initial 24-h exposure to 250 μ*M* Cd, the concentration was toxic to the Cd-sensitive cells only, as revealed by a cell viability study. However, they differed in their ability to form PC–Cd complexes: the sensitive cells formed complexes later than the tolerant cells. In addition, the complexes formed by the sensitive cells were of lower molecular weight than those of tolerant cells and did not bind all the Cd, unlike in the tolerant cells. Thus, the rapid formation of PC–Cd complexes sequestering most of the Cd within a short period could be a necessity for plants or cells showing tolerance to heavy metals. Evidence in support of this also comes from work on Cd-sensitive mutants of *Arabidopsis thaliana*, *CAD1*, which is deficient in its ability to sequester Cd [114].

Furthermore, Gupta and Goldsbrough [108] observed that the cell lines of tomato selected for their tolerance to various concentrations of Cd showed a trend towards accumulation of HMW PCs in addition to showing their enhanced synthesis. At least 90% of the Cd in the most tolerant cells was associated with PC complexes containing large amounts of SH. Thus, the size of PCs may be

a determining factor in tolerance to heavy metals. It has been shown that PC_7 is more efficient than PC_2 in complexing Cd per mole of γ-Glu-Cys [115]. Moreover, exposure of maize seedlings to increasing concentrations of Cd results in the accumulation of longer PCs, with PC_4 the largest peptide accumulating PC [89].

Yet another way by which the metal-binding capacity of PCs (per mole of PC-SH) is increased is upon their association with acid labile sulfur (S^{2-}), which has been reported to increase the stability of Cd–PC complexes as well in *S. pombe* [80]. The relevance of the presence of S^{2-} in PC–metal complexes to metal detoxification is substantiated by the observation that the mutants of *S. pombe* that produce PC–Cd complexes without sulfide are hypersensitive to Cd [92]. In addition, Cd-tolerant *Silene vulgaris* plants exhibit a higher S:Cd ratio in the PC complexes than the Cd-sensitive plants [116]. It has also been observed that the HMW PC–metal complexes contain greater amount of S^{2-} than the LMW PC–metal complexes.

Two distinct peaks for HMW and LMW PC–metal complexes have not generally been observed in plants and have only been described for tomato [117] and a Se-tolerant variety of *Brassica juncea* [118]. Nevertheless, the two forms do exist even if they may not be distinctly separated. The acid labile sulfur associated with the two forms varies from species to species. HMW Cd-binding complexes in maize seedlings exposed to 3 μ*M* Cd show a S^{2-}:Cd molar ratio of 0.18; no acid labile sulfur occurred in the LMW complexes [119]. *Brassica juncea* grown in synthetic medium with 100 μ*M* Cd for 7 days produced HMW complexes with S^{2-}:Cd molar ratio of 1.0 and LMW complexes with ratio 0.42 [118). Incompletely resolved complexes from roots of tomato exposed to 100 μ*M* Cd for 4 weeks had continuous S^{2-}:Cd molar ratios ranging from 0.15 to 0.41 for the HMW complexes and from 0.04 to 0.13 for the LMW complexes [117].

The yeasts *S. pombe* and *C. glabrata* grown in different media for 16 to 48 h and exposed to 500 or 1000 μ*M* Cd showed complexes with S^{2-}:Cd molar ratios of 0.11 to 0.55 [80,120]. How sulfide, Cd, and PC peptides interact within the complex is unclear for the cases in which the ratio is low. Cd–PC complexes with S^{2-}:Cd ratio greater than 0.4 appear as dense aggregates of 2-nm diameter particles called CdS crystallites. In yeast, each crystallite contains about 80 CdS units stabilized by a coating of about 30 peptides of glycyl [$(\gamma\text{-Glu-Cys})_n$Gly] and desGly [$(\gamma\text{-Glu-Cys})_n$] forms [120]. Reese et al. [117] showed the presence of such crystallites in plant (tomato) in PC–Cd formations with S^{2-}:Cd ratio of 0.41, but the crystallites were of less than 2-nm diameter coated with only $(\gamma\text{-Glu-Cys})_n$Gly peptides.

The number of γ-Glu-Cys dipeptide repeats influences the stability of the complexes. Complexes formed with shorter peptides ($n = 1$ and $n = 2$) are more labile, and accretion of the crystallite to larger particles is more facile [117]. In yeasts *S. pombe* and *Candida glabrata,* although $(\gamma\text{-Glu-Cys})_{2,3}$ peptides are present in C-binding complexes, $(\gamma\text{-Glu-Cys})_{2-4}$-Gly peptides are usually more concentrated [120,121]. In tomato, the number of γ-Glu-Cys units varies from 3 to 6 with $n = 4$ the predominant peptides [117]. The Cd-binding complexes from several other sources are composed of $(\gamma\text{-Glu-Cys})_n$-Gly, with $n = 3$ and $n = 4$ oligomers the most abundant [108,122]. In soybean (*Glycine max*), $n = 1,2,3,4$ oligomers of $(\gamma\text{-Glu-Cys})_n$-β-Ala form the Cd-binding complexes [83]. The HMW complexes in maize are formed by the peptides from three families: $(\gamma\text{-Glu-Cys})_n$-Gly; $(\gamma\text{-Glu-Cys})_n$-Glu; and $(\gamma\text{-Glu-Cys})_n$, of which $(\gamma\text{-Glu-Cys})_n$ peptides remain present in highest concentrations, followed by $(\gamma\text{-Glu-Cys})_n$-Gly and $(\gamma\text{-Glu-Cys})_n$-Glu. The $n = 3$ oligomers of the three families form the highest constituent followed by an equally dominating concentration of $n = 4$ oligomers [119].

The preponderance of $n = 3$ and $n = 4$ oligomers in Cd-binding complexes from maize corroborates the increasing affinity of Cd for longer peptides [123], and their presence in HMW complex together with the acid labile sulfur speaks to the importance of HMW Cd complexes in metal (Cd) detoxification. Rauser and Meuwly [119] in their study showed that the concentration of $n = 3$ and $n = 4$ oligomers increased in maize with increase in the number of days of its exposure to Cd, and the HMW complexes sequestered 59% of the Cd after day 1, which increased from 88 to 92% by days 4 to 7.

16.11 CELLULAR COMPARTMENTALIZATION OF PC–METAL COMPLEXES AND METAL TOLERANCE

Another important aspect of PC-mediated tolerance of plants to heavy metals is probably the effective transportation of the metal to vacuoles for storage in which they could be playing an important role. Arguments in favor of this come from several observations. Vogeli–Lange and Wagner [124] isolated mesophyll protoplast from tobacco exposed to Cd and showed that the vacuoles contained 110 ± 8% of the protoplast Cd and 104 ± 8% of the protoplast PCs. These workers envisioned the synthesis of PCs in cytosol and transfer of Cd and the peptides, perhaps as complex, across the tonoplast into the vacuole, where the metal is chelated by the peptides and organic acids.

Working on tomato cells, Gupta and Goldsbrough [125] observed the highest level of PCs after 4 days of their exposure to Cd, which coincided with the peak of cellular Cd concentration (0.6 m*M*). At this time, there was an eightfold molar excess of PC over Cd. However, the PCs could not be detected after 12 days and the cellular concentration of Cd was still 0.2 m*M* (the intracellular concentration of Cd decreased as a result of increase in the cell mass). This led them to suggest that PCs possibly function as transport carriers for Cd into the vacuole, where the acidic pH favors dissociation of the Cd–PC complexes, followed by breakdown of the PCs and possible sequestration of the metal in some other form, in agreement with the model proposed by Vogeli–Lange and Wagner [124].

Later, while working on Cd-tolerant and Cd-sensitive plants of *Silene vulgaris*, De Knecht et al. [103] observed that, in response to a range of Cd concentrations, the root tips of Cd-tolerant plants exhibited a lower rate of PCs production accompanied by a lower rate of larger chain PC synthesis than those of Cd-sensitive plants, although both the plants (root tips) accumulated nearly similar levels of Cd at a particular metal-exposure concentration. Second, the tolerant plants reached the same PC concentration as the sensitive plants only after exposure to high Cd concentrations, and at an equal PC concentration the composition of PC and the amount of sulfide incorporated per unit PC-thiol were the same in both the populations.

The authors concluded that the lower concentration of PCs in the Cd-tolerant plants than in the Cd-sensitive plants could be because of greater transport of Cd–PC complexes in vacuoles in the former and, as suggested by Vogeli–Lange and Wagner [124] and Gupta and Goldsbrough [125], the PC–Cd complexes might be getting dissociated in the vacuole because of its acidic pH, followed by breakdown of the PCs or their reshuttling into the cytoplasm. Thus, the observed lower PC concentration in Cd-tolerant plants might be a result of a lower Cd concentration in the cytoplasm caused by (1) a faster transport of the metal into the vacuole when compared to that in the Cd-sensitive plants; and (2) return of the dissociated PCs (in the vacuole) into the cytoplasm, obviating the need of their fresh synthesis for the additional Cd uptake.

Because the enzymes involved in PC synthesis are present in cytoplasm but PCs are also found in the vacuole, a transport mechanism must be involved, and an insight into this comes from the work on *S. pombe*. A Cd-hypersensitive mutant, deficient in producing HMW complex, was observed [92]. This was found to be as a result of mutation within the *hmt1* (heavy metal tolerance 1) gene encoding an ATP-binding cassette (ABC)-type protein associated with vacuolar membrane [126]. ABC-type proteins represent one of the largest known families of membrane transporters. They can mediate tolerance to a wide diversity of cytosolic agents. The presence of HMT1 protein in the vacuolar membrane suggests the possibility of an ABC-type transporter-mediated resistance to Cd, by its sequestration in the vacuole [127].

The yeast *hmt1*$^-$ mutant harboring *hmt1*-expressing multicopy plasmid (pDH35) exhibited enhanced resistance to Cd compared to the wild-strain (*hmt1*$^-$ mutant) and accumulated more Cd with HMW complex formation [127]. The vacuolar vesicle derived from the *hmt1*$^-$ mutant complemented with *hmt1* cDNA (*hmt1*$^-$/pDH35), i.e., HMT1 hyperproducer exhibited ATP-dependent uptake of LMW apophytochelatin and LMW–Cd complexes, but that from the *hmt1*$^-$ mutant did

not show any such activity. HMW-Cd complex was not an effective substrate for the transporter proteins. The vacuolar uptake of Cd^{2+}, which was ATP dependent, was also observed, but was not attributable to HMT1. The electrochemical potential generated by vacuolar ATPase did not drive transport of peptides or complexes.

The observation of Ortiz et al. [127] is also supported by work on oat tonoplast vesicles [128,129]. Tonoplast vesicles from oat roots have a Cd^{2+}/H^{+} antiporter [129]. The vesicles also show MgATP-dependent transport of PCs and Cd–PC complex [128], and the peptide transport is not driven by electrochemical potential generated by the vacuolar ATPase. Based on the information available, Rauser [18] proposed a model, somewhat similar to that proposed by Ortiz et al. [127], for the transport of Cd and Cd-binding complexes across the tonoplast. PCs synthesized in the cytosol combine with Cd to form LMW complex that is moved across the tonoplast by ABC-type transporters. Apo–PCs are also transported by them. The energy required for the transport is derived from ATP.

Once inside the vacuole, more Cd, transported by Cd^{2+}/H^{+} antiporter, is added to the LMW, along with Apo–PCs and sulfide complexes, to produce HMW complexes. Genetic and biochemical analyses suggest that the formation of sulfide moiety in the HMW PC–Cd-S^{2-} complex involves purine metabolism, which serves as the source of sulfide [130,131]. The sulfide-rich HMW complex is more stable in the acidic environment of the vacuole and has a higher Cd-binding capacity than the LMW complex. The LMW complex functions as a cytosolic carrier and the vacuolar HMW complex is the major storage form of cellular Cd. Whether LMW and HMW complexes in plants are compartmentalized as depicted in the model and are of the same peptide composition, however, awaits direct evaluation. Nevertheless, the studies [127,128] do indicate a central role of vacuole in sequestration and detoxification of Cd, and maybe heavy metals in general, and that tolerance to metals could also be due to increased ability of plants to transport them into the vacuole (see Figure 15.2 in Chapter 15).

16.12 ROLE OF PCS IN DETOXIFICATION OF HEAVY METALS OTHER THAN CADMIUM

As stated earlier, synthesis of PCs is induced by most heavy metals, including the multiatomic anions [74,76], in most of the higher plants [73,83]. It has also been observed that the enzyme involved in its synthesis, PC synthetase, needs the presence of heavy metals for its activation; a crude preparation of the enzyme from *S. vulgaris* was activated best by Cd^{2+}, and by Ag^{+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, and Au^{+} in decreasing order [95]. No activation of the enzyme was detected by the metals of the hard-acceptor category including Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Na^{+}, and K^{+}. The trend of activation observed by Grill et al. [95], however, was not observed for PC synthetase from tobacco cells, except that Cd was the most effective activator, followed by Ag^{+}. The activation by Cu^{2+} was next to Ag^{+}, and Pb^{2+}, Zn^{2+}, and Hg^{2+} produced only weak stimulation of the enzyme activity [96]. Thus, although the enzyme has a rather nonselective domain for binding with metals, it is mostly activated by heavy metals.

This strongly suggests that intracellular metabolism of heavy metals, other than Cd as well, might be largely mediated through PCs. The view also stems from the fact that the heavy metal ions that activate PC synthetase *in vitro* are also able to induce PC synthesis *in vivo* [96], with one exception: Ni^{2+} induced PC synthesis *in vivo*, but did not activate PC synthetase activity *in vitro*. Furthermore, the indication of possible involvement of PC in heavy metal tolerance also comes from genetic evidence; in addition to being sensitive to Cd^{2+}, phytochelatin-deficient *cad1* mutants of *Arabidopsis* are also sensitive to Hg^{2+} [114], and GSH-deficient strains of *S. pombe* show reduced tolerance to Pb^{2+} as well as showing no tolerance to Cd^{2+} [112].

PC synthetase activity is detected mostly in roots, but not in leaves or fruits [61,101]. The constitutive presence of PC synthetase in roots suggests an important role of PCs in metal

detoxification. Because plants assimilate various metal ions from soil, the first organ exposed to these ions is the root. Localization of PC synthetase to roots and stems probably provides an effective means of restricting the heavy metals to these organs by chelation in the form of Cd–PC complexes. It has been demonstrated that PCs are able to protect enzymes from heavy metal poisoning *in vitro* [65,132]; many metal-sensitive plant enzymes (rubisco, nitrate reductase, alcohol dehydrogenase, glycerol-3-phosphate dehydrogenase, and urease) were more tolerant to Cd in the form of a Cd–PC complex compared with the free metal ion. Free PCs could reactivate the metal-poisoned enzymes (nitrate reductase poisoned by Cd–acetate) *in vitro* more effectively than other chelators such as GSH or citrate [65].

Recognition of PCs as the chelators of heavy metals in general and protectors of plants against their toxic effect, however, requires careful consideration. For instance, in tobacco cells not selected for metal tolerance, BSO increased the toxicity of Cd but not of Zn or Cu, as if the control of sequestration differed between the metals [133]. This may, of course, be true, but has not been properly demonstrated. Second, although most of the heavy metals are able to induce synthesis of PCs in plants, only a few of them (Cd, Cu, and Ag) form complexes with the peptides [76]. Recently, As has been reported to form complexes with PCs in arsenate-tolerant *Holcus lanatus* [134]. In fact, PC–metal complex formation has been reported mostly for Cd. A few reports of PC forming complexes with Cu are also available [74,75,135], and formation of PC–Zn complexes has been observed in cells (of *Rauvolfia*) grown in micronutrient concentration of Zn [65]. PCs have also been reported to form complexes with Hg and Pb *in vitro* [136,137].

Nevertheless, genetic evidence is that PCs are involved in tolerance to these metals; PC-deficient *cad1* mutants of *Arabidopsis* are also sensitive to Hg^{2+} [114], and GSH-deficient strains of *S. pombe* show reduced tolerance to Pb^{2+} [112]. Thus, although the involvement of PCs in making plants resistant to heavy metals other than Cd cannot be overlooked, more information is required on their induction by individual heavy metals in different plant species. Also, information is required on the formation of PC–metal complexes and cellular localization of the metals (individual) and Apo–PC and metal–PC complexes before the functional significance of PCs known for Cd can be generalized for all heavy metals.

16.13 COMPLEX FORMATION WITH ORGANIC ACIDS

Organic acids are the other group of biomolecules that can function as chelators of heavy metals inside the cell, converting the metals to almost inactive and nontoxic forms. With regard to Al, at least two organic acids are known to function as chelators. One is citric acid [138]; nearly two-thirds of Al in hydrangea leaves remain present in the cell sap in soluble form as Al–citrate complex at a 1:1 molar ratio of Al to citrate, a nontoxic form of Al.

Another acid that has been reported to form intracellular complex with Al is oxalic acid [139]. About 90% of Al in buckwheat remains present as soluble oxalate Al complex in the symplasm, and the intracellular concentration of Al detected is as high as 2 m*M*. The complex occurs in molar ratio of 1:3, Al:oxalate. Oxalic acid can form three species of complexes with Al at an Al:oxalic acid molar ratio of 1:1, 1:2, and 1:3. The 1:3 Al–oxalate complex is the most stable, with a stability constant of 12.4 [140]. This stability constant is much higher than that of Al–citrate (8.1) or Al:ATP (10.9), meaning that formation of 1:3 Al–oxalate complex can prevent binding of Al to cellular components, thereby detoxifying Al very effectively. The report is in contrast to the order of stability constant for Al–organic acid complexes: Al–citrate > Al–oxalate > Al–malate [141]. It is not known, however, whether the Al complexes of citrate or oxalate remain located in cytoplasm or in the vacuole.

Among the heavy metals reported to be chelated by organic acids inside the cells are Zn and Ni. After exposure to high concentrations of various heavy metals, vacuoles of the Zn- and Ni-tolerant plants, as well as those of the nontolerant plants, often contain high concentrations of zinc and nickel [142,143], as well as some Cu and Pb [144] and Cd [145–147]. The results of the studies

on Zn- and Ni-tolerant plants suggested that organic acids could be involved in their sequestration in the vacuole; the Zn-tolerant plants, including *Silene vulgaris*, exhibited enhanced accumulation of malate [148,149] and the Ni-tolerant plants showed accumulation of malate, malonate, or citrate [148,150] upon their exposure to Zn and Ni, respectively. The details of their transportation and sequestration inside the vacuole and the roles of the organic acids in the process are not available.

In one of the models for the transport of Zn into vacuole, it has been postulated that malic acid would bind Zn in the cytosol, thereby detoxifying it, and the Zn–malate complex would be transported over the tonoplast into the vacuole where it would dissociate [39]. After this, malate would be retransported into the cytosol. Vacuolar Zn would remain bound to stronger chelators, such as citrate, oxalate, etc., when present. Brune et al. [151] reported that barley mesophyll cell vacuoles contain appreciable concentration of phosphate (30 to >100 mol m^{-3}); malate (>10 mol m^{-3}); sulphate (>4 mol m^{-3}); citrate (~1 mol m^{-3}); and amino acids (>10 mol m^{-3}) when grown in hydroponic culture. They hypothesized that these organic and inorganic salts interact with the divalent cations, thereby buffering the vacuolar free Zn concentration to low values even in the presence of high Zn levels (292 mmol m^{-3}) in the vacuolar space.

According to Wang et al. [152], citrate is the most efficient ligand for metal complexation in the vacuole at vacuolar pH values of 6 to 6.5. The results of Brune et al. [151] demonstrate the importance of compartmentalization and transport as homeostatic mechanisms within leaves to handle possibly toxic zinc levels in shoots. The dependence of plants on organic acids for detoxification of Zn could be the reason for poor induction of PCs by the metal [74,76].

The mechanism of detoxification adapted by plant probably varies from metal to metal and, for a metal, from species to species, and it is difficult to reconcile the idea of tolerance by means of any single mechanism. For example, Zn-tolerant *Agrostis capillaries* and *Silene vulgaris*, which exhibit increases in malate levels [153], are only slightly Ni tolerant [43]; Ni-tolerant *Alyssum bertolonii*, which is very rich in malate [150], is nontolerant to Zn [39]. Similarly, as stated earlier, BSO increases the toxicity of Cd to the tobacco cells not selected for Cd tolerance, but not of Zn or Cu. Again, for Al detoxification, plants follow several strategies.

16.14 ANTIOXIDATIVE SYSTEM IN METAL TOLERANCE

It is generally considered that virtually all the biochemical effects of heavy metals may ultimately lead to damage of cells and tissues [1]. Thus, arguments are made that heavy metal tolerance could also be linked, to some extent, with reactive oxygen scavenging capability of a plant species [154]. However, little direct evidence supports this hypothesis, although indirect evidence does suggest such a relationship.

De Vos et al. [109] observed that 20 μ*M* Cu reduces the GSH pool in the roots of Cu-sensitive *Silene cucubalus*, and this is accompanied by enhanced MDA accumulation, indicating oxidative damage of membrane. However, even at 30-μ*M* concentration, Cd caused less decrease in the GSH pool than copper and no lipid peroxidation despite inducing nearly 13-fold increase in PC levels, compared to only 6-fold increase induced by Cu. In the Cu-tolerant plants, the decrease of GSH level was significantly less than in the Cu-sensitive plants. Because GSH is an important antioxidant, it may be concluded that tolerance to copper in Cu-tolerant *S. cucubalus* could be because of its more efficient antioxidative system than that of Cu-sensitive plants. Gallego et al. [155] also correlated oxidative damage in sunflower cotyledons induced by Cd with decrease in the GSH pool.

Further indirect evidences come from the study of responses of the antioxidative enzymes to metal treatment. Cakmak and Horst [156] observed significant increase in peroxidase activity in soybean root in response to Al treatment with concomitant increase in MDA content, indicating the induction of oxidative stress with the plant responding by increasing the level of one of its antioxidative enzymes. Subsequently, Ezaki et al. [157] reported Al stress induced appearance of two cationic peroxidases and two moderately anionic peroxidases in tobacco cells. They also produced evidence that at least one of the isoenzymes is produced by enhanced expression of

pAL201 gene and opined the possibility of the isoenzyme having some function in Al resistance. Significant enhancement in the activity of peroxidase in response to heavy metals has also been reported [158–162]. Hendry et al. [158] observed enhancement in the activity of peroxidase in the Cd-sensitive, but not in the Cd-tolerant, plants of *Holcus lanatus* in response to Cd.

The activity of ascorbate peroxidase has also been reported to increase in plants in response to heavy metals [161–163]. Working on *Phaseolus aureus*, Shaw and Rout [161] observed metal-specific (Hg and Cd) differences in the response of the enzyme by the older seedlings when compared to the younger ones, with Hg inducing the activity of the enzyme while Cd had not. This was accompanied by death of the Cd-treated older seedlings after exposure for more than 36 h, suggesting that the enhanced synthesis of ascorbate peroxidase in response to Hg could be protecting the plant against the oxidative stress induced by the metal; this was not possible in the case of Cd treatment. Like peroxidase, ascorbate peroxidase is also induced more in the metal-sensitive plant (*Alssum maritinum*) than in the tolerant plant (*A. argentums*, a nickel hyperaccumulator) [163].

Reports are also available showing enhancement in the activity of catalase, probably the main H_2O_2 scavenging enzyme, in response to heavy metal treatment [1], suggesting possible involvement of the enzyme in heavy metal tolerance. Furthermore, the perimedullar tuber tissue of potato cultivar resistant to Cd showed a higher constitutive level of catalase and also significantly greater increase in the activity of the enzyme in response to exposure to Cd when compared to the Cd-sensitive cultivar [164]. The observation is in contrast to that observed for peroxidase and ascorbate peroxidase and suggests that the enzyme could be of greater importance than peroxidase and ascorbate peroxidase in reducing the metal-induced increase in H_2O_2 level, leading to tolerance of the plant (Cd-tolerant cultivar) to the metal (Cd).

Superoxide dismutase (SOD) has been considered to play the most significant role in active oxygen species scavenging because its action prevents the accumulation of $O_2^{\bullet -}$ radical, which could lead to generation of toxic $HO^{\bullet}$ [1]. It is also the most widely studied enzyme in the context of environmental stresses. In fact, its involvement in tolerance to environmental stresses — particularly drought and frost, which lead to oxidative stress — is well established. This is on the basis of observation of its enhanced synthesis in response to environmental stresses or observation of increase in threshold of tolerance to environmental stresses in the organisms manipulated for enhanced expression of the enzyme [165–168]. The enzyme is also considered to be involved actively in heavy metal tolerance in plants. This is because of observation of significant increase in the activity of enzyme in plants exposed to various heavy metals [156,169,170]. More importantly, like catalase, the activity of SOD in response to heavy metals is increased more in the metal-tolerant plants than in the sensitive ones [158]; this speaks further in favor of an important role of the enzyme in heavy metal tolerance.

Although the observations of various workers presented here suggest an active involvement of the antioxidative components in heavy metal tolerance in addition to the involvement of other processes, it must be kept in mind that contradictory observations have also been reported [1]. Furthermore, the database in support of the involvement of antioxidative machinery in metal tolerance is very limited, particularly the observation from the studies involving metal-tolerant and metal-sensitive varieties of a species. Therefore, at this stage, it would be premature to draw a definite conclusion in favor of the involvement of the antioxidative system in metal tolerance in plants; it would be wise at present to treat the idea as a supposition. Nevertheless, it is worth mentioning that *Arabidopsis* transgenic line, AtPox(4-1), showing enhanced expression of peroxidase shows significantly less lipid peroxidation upon exposure to Al and greater tolerance to the metal than the nontransgenic plant [171].

16.15 METAL–METAL INTERACTIONS

Toxicological evidence of plant and nonessential metal interactions comes from a variety of experiments with variable doses and durations of exposure; this leaves a dearth of information on

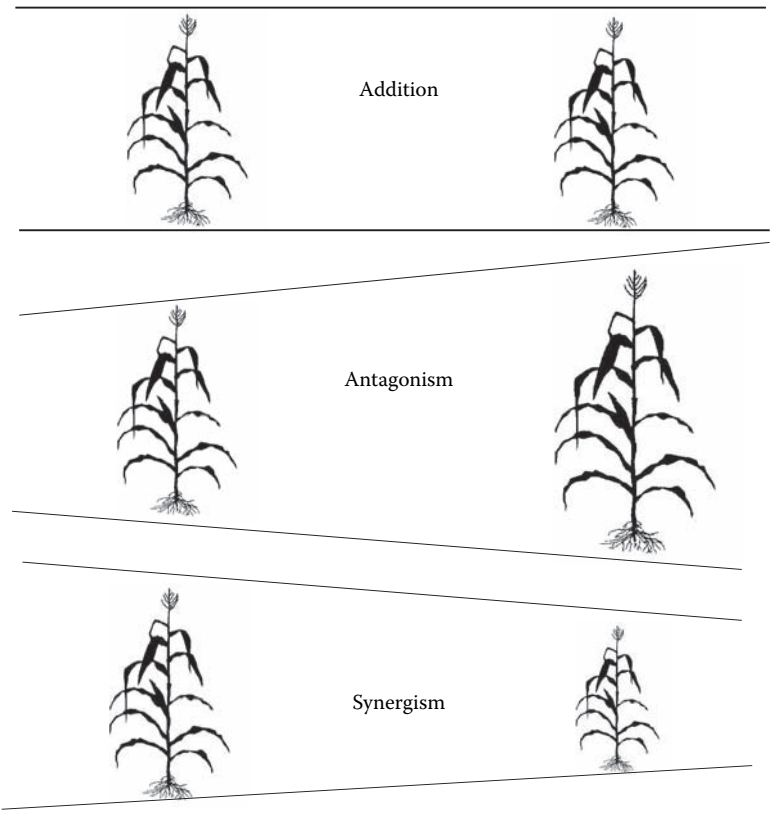

FIGURE 16.6 Metal–metal interactions on model plant systems.

the outcome of interaction of essential (Cu, Zn) and nonessential (Pb, Hg) metals. Therefore, Rauser [172] felt that it might be prudent to begin checking crucial laboratory responses using plants in various levels and durations of exposure of pollution using essential and nonessential metals. Heavily polluted soils and waters often contain mixtures of metals that may have antagonistic, synergistic, or no effects (additive) on plants. Therefore, metal–metal interactions on model plant systems bear exploring. Symeonidis and Karataglis [173] divided plant responses to combinations of metals in the growth medium into three groups (Figure 16.6):

- *Additive:* relative growth under conditions of multiple metal stress is equal to the product of the relative growth produced by the individual metals in isolation (e.g., Cu–Co).
- *Antagonistic:* relative growth under conditions of multiple metal stress is greater than that of the product of the relative growth produced by the individual metals in isolation (e.g., Cu–Cd, Ca–Cd).
- *Synergistic:* relative growth under growth conditions of multiple metal stress is less than that of the product of the relative growth produced by individual metals in isolation (e.g., Cu–Zn).

Cd and Zn belong to group IIB transition elements with similar electronic configuration and valence state; both have affinity to sulfur, nitrogen, and oxygen ligands. Thus, both these elements have similar geochemical and environmental properties [174]. Most of the ores are mixtures of metals in which potentially toxic metals (As, Cd, and Hg) other than the sought-after elements

may also be present. Following extraction, which varies in efficiency but is never complete, the contaminant metals are also released into the environment freely.

Ore extraction of Zn from mines and nonferrous metal production processes in smelters with subsequent release of zinc effluents to the environment is normally accompanied by cadmium environmental pollution because zinc ore (ZnS) generally contains 0.1 to 5% and sometimes even higher cadmium [175]. Similarly tyres containing ZnO and sewage sludges applied to agricultural soils as fertilizers also contain Cd as a major contaminant. Thus, this association of Cd and Zn in the environment, their chemical similarity, and the interactive functions are of considerable importance [176]. Factors regulating essential and nonessential metal accumulation at the organismal and cellular level is vital for understanding phytotoxicity [177].

Cadmium has even been described as an antimetabolite of Zn by scientists because of observed Zn deficiency in most of the Cd-treated systems. It has been hypothesized that elements whose physical and chemical properties are similar will act antagonistically to each other biologically [176]. In recent years, a number of workers have documented responses of plants to combinations of Zn and Cd in soil, as well as in solution culture in soil-crop systems under actual field conditions [174].

Aravind and Prasad [178–180] conclusively demonstrated that Zn showed an antagonistic interaction with Cd and alleviated Cd toxicity in *Ceratophyllum demersum*, a freshwater macrophyte. The possible mechanisms identified include:

- Zn inhibited Cd uptake directly by competition and indirectly by controlling the H^+ATPase, leading to a reduced intracellular concentration of Cd.
- Zn reduced Cd-increased peroxidation, membrane leakage, and lipolysis.
- Zn inhibited the formation of toxic reactive oxygen species triggered by Cd-influenced NADPH oxidase.
- Zn substantially increased activities of the antioxidant enzymes like SOD, CAT, POD, and APX, significantly quenching the formation of ROS.
- Zn restored and enhanced the functioning of carbonic anhydrase in Cd-exposed *C. demersum* by competitive substitution.

16.16 CONCLUSION

Thus, it can be seen that metals, including heavy metals, are nature's gift to mankind, and modern civilization would not have developed without bringing them into use. However, at the same time, metals — particularly the heavy metals — are very toxic to the living organism; thus, suitable measures must be taken to prevent excessive exposure to them and to immobilize them in the areas of their "hot spots." It is increasingly realized that this can be achieved by the use of plants. Plants can remediate metal pollutants in mainly two ways:

- Phytostabilization: plants convert pollutants to less bioavailable forms and/or prevent pollutants' dispersal by wind erosion or leaching.
- Phytoextraction: plants accumulate pollutants in their harvestable tissues, thus decreasing the concentration of the pollutants in the soil.

With regard to mercury, another concept under phytoremediation is phytovolatilization. Metal-contaminated soil can also be remediated by the use of proper soil amendment practices, which can decrease or increase the metal uptake by plants. When crop plants are grown, the amendment should be such as to decrease the metal availability to the plants, which should also preferably be metal excluders.

Metal resistance in plants could be a result of its exclusion or uptake and proper sequestration inside the cells. This varies depending upon the metals as well as on the plant species. For Al, resistance is mostly due to (1) its exclusion mediated by secretion of organic acids by the roots, which form complexes with the metal, making it unavailable to the plants; and/or (2) efflux of H^+, which increases the rhizosphere pH due to which Al^{3+} species in proximity with the root gets converted to less toxic and less available forms. Resistance to Al due to intracellular complex formation with oxalic acid has also been reported.

For the heavy metals in general, the resistance is achieved by intracellular sequestration inside the vacuoles. This is believed to be mostly mediated through the formation of complexes with phytochelatins, the nontranslationally synthesized low molecular weight polypeptides. However, the evidence for this is mostly available from work on Cd. Although most of the heavy metals are known to induce synthesis of phytochelatins, only a few, like Cd, Cu, and Ag, are reported to form stable complexes with them. In fact, more studies are needed involving different plant species and their ecotypes tolerant and sensitive to various heavy metals before phytochelatins can be recognized as a detoxifier of heavy metals in general.

Moreover, some reports indicate that the accumulation of the heavy metals like Zn and Ni is accompanied by enhanced accumulation of organic acids, although the details of their chelation and sequestration are not available. Also, a totally different mechanism is used for detoxification of Hg by certain bacteria in which the organic and inorganic forms of the element are converted to volatile nontoxic form. Furthermore, the involvement of antioxidative machinery in heavy metal tolerance is increasingly advocated, although not sufficiently substantiated. The information available thus far gives an indication that, in plants, heavy metal tolerance, or metal tolerance in general, could be a result of integrated functioning of more than one mechanism rather than a result of a singular process (Table 16.3). It is hoped that future research will lead to further understanding of the various processes involved in heavy metal tolerance, or metal tolerance in general, and throw light on the nature of the interaction between them.

TABLE 16.3
Adaptive Plant Ecophysiological, Molecular Basis of Metal Tolerance and Detoxification Processes

Mechanism	Key ref.
Plasma membrane (passive uptake and active efflux)	177, 182
Ferritins, metallothioneins, glutathione derived peptides (phytochelatins)	18, 20, 102, 134, 172, 183–190
Over expression of glutathione (precursor for metal sequestration)	111, 191, 192
Low molecular weight organic acids and amino acids	14, 24, 181, 193–203
Heat shock proteins	204–206
Vacuolar compartmentation	177, 182, 183, 207
Metal transporters = cation efflux family (formerly cation diffusion family)	12, 208–214
Hairy root cultures, rhizofiltration and metal complexation	215–218
Rhizosphere biotechnology and physiology and biochemistry of metal tolerance	23, 219–221
Genetic and transgenic strategies for metal hyperaccumulation	177, 222–231
Mycorrhizae	232–240
Naturally occurring metal accumulators	241, 242
Metal–metal interactions (antagonism type) e.g., zinc prevents cadmium toxicity; ferritin prevents HM toxicity	178–180, 186

Source: Modified from Prasad, M.N.V. (Ed.) *Heavy Metal Stress in Plants: from Biomolecules to Ecosystems*, 2nd ed. Springer–Verlag, Heidelberg, 2004, 462; xiv.

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