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Cellular mechanisms for heavy metal detoxification and tolerance

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Abstract

Heavy metals such as Cu and Zn are essential for normal plant growth, although elevated concentrations of both essential and non-essential metals can result in growth inhibition and toxicity symptoms. Plants possess a range of potential cellular mechanisms that may be involved in the detoxification of heavy metals and thus tolerance to metal stress. These include roles for the following: for mycorrhiza and for binding to cell wall and extracellular exudates; for reduced uptake or efflux pumping of metals at the plasma membrane; for chelation of metals in the cytosol by peptides such as phytochelatins; for the repair of stress-damaged proteins; and for the compartmentation of metals in the vacuole by tonoplast-located transporters. This review provides a broad overview of the evidence for an involvement of each mechanism in heavy metal detoxification and tolerance.

Key words: Detoxification, heat shock proteins, heavy metal tolerance, metallothioneins, mycorrhiza, phytochelatins, plasma membrane, vacuolar compartmentation.

Introduction

Heavy metals such as Cu and Zn are essential for normal plant growth and development since they are constituents of many enzymes and other proteins. However, elevated concentrations of both essential and non-essential heavy metals in the soil can lead to toxicity symptoms and the inhibition of growth of most plants. The toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular/molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects (Van Assche and Clijsters, 1990). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Dietz *et al*., 1999). Some plant species, however, have evolved tolerant races that can survive and thrive on such metalliferous soils, presumably by adapting mechanisms that may also be involved in the general homeostasis of, and constitutive tolerance to, essential metal ions as found in all plants. Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress. These all appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell and thus preventing the damaging effects described above, rather than developing proteins that can resist the heavy metal effects. Thus, for example, there is little evidence that tolerant species or ecotypes show an enhanced oxidative defence; rather tolerant plants show enhanced avoidance and homeostatic mechanisms to prevent the onset of stress (de Vos *et al*., 1991; Dietz *et al*., 1999). The strategies for avoiding heavy metal build-up are diverse. Extracellularly they include roles for mycorrhizas and for cell wall and extracellular exudates. Tolerance could also involve the plasma membrane, either by reducing the uptake of heavy metals or by stimulating the efflux pumping of metals that have entered the cytosol. Within the protoplast a variety of potential mechanisms exist, for example, for the repair of

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Abbreviations: HSP, heat shock protein; MT, metallothionein; PC, phytochelatin.

stress-damaged proteins involving heat shock proteins or metallothioneins, and for the chelation of metals by organic acids, amino acids or peptides, or their compartmentation away from metabolic processes by transport into the vacuole. This range of mechanisms is summarized in Fig. 1.

Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plants, and is manifested by an interaction between a genotype and its environment (Macnair *et al*., 2000), although the term is frequently used more widely in the literature to include changes that may occur experimentally in the sensitive response to heavy metals. In a number of thorough genetic studies, such adaptive metal tolerance has been shown to be governed by a small number of major genes with perhaps contributions from some more minor modifier genes (Macnair, 1993; Macnair *et al*., 2000; Schat *et al*., 2000). The question of whether this means that only a single biochemical or molecular change is required to produce tolerance to a specific metal remains to be resolved. Related to this question is the occurrence of multiple tolerance and co-tolerance where plants can grow on soils enriched in combinations of several heavy metals. This tolerance could result from a less specific mechanism that confers a broad resistance to several different metals (co-tolerance) or may involve a series of independent metal-specific mechanisms (multiple tolerance) (Schat *et al*., 2000). However, the evidence for co-tolerance is not strong, suggesting that specific mechanisms are involved for each metal present at a toxic concentration (Macnair *et al*., 2000; Schat *et al*., 2000).

This paper provides an overview of the variety of potential mechanisms that may be involved in the detoxification and tolerance to heavy metals at the cellular level, mainly in relation to Cu, Cd, Ni, and Zn since these have been the most widely studied. It does not cover mechanisms that may operate at the whole plant level, such as root-to-shoot transport or the role of trichomes, nor the special case of hyperaccumulators unless in relation to particularly relevant examples.

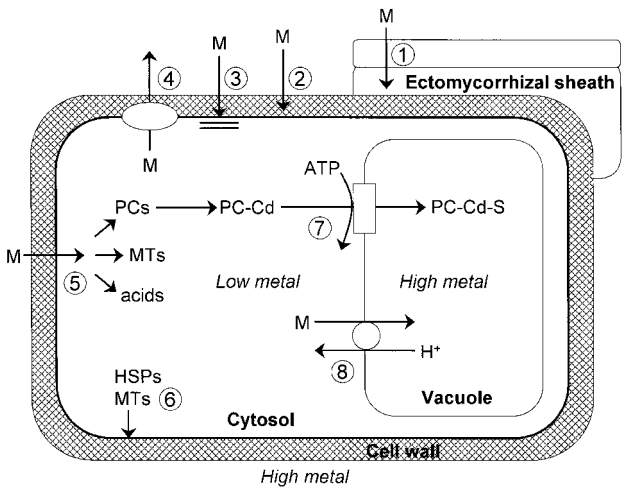

Fig. 1. Summary of potential cellular mechanisms available for metal detoxification and tolerance in higher plants. 1. Restriction of metal movement to roots by mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reduced influx across plasma membrane. 4. Active efflux into apoplast. 5. Chelation in cytosol by various ligands. 6. Repair and protection of plasma membrane under stress conditions. 7. Transport of PC-Cd complex into the vacuole. 8. Transport and accumulation of metals in vacuole. (Modified after Marschner, 1995.)

Mycorrhizas

Although not always considered in general reviews of plant metal tolerance mechanisms, mycorrhizas, and particularly ectomycorrhizas that are characteristic of trees and shrubs, can be effective in ameliorating the effects of metal toxicity on the host plant (Marschner, 1995; Hüttermann *et al*., 1999; Jentschke and Godbold, 2000). However, the mechanisms involved in conferring this increase in tolerance have proved difficult to resolve; they may be quite diverse and show considerable species and metal specificity since large differences in response to metals have been observed, both between fungal species and to different metals within a species (Hartley *et al*., 1997; Hüttermann *et al*., 1999). For example, Colpaert and Van Assche showed that the ectomycorrhizal fungus *Paxillus involutus* retained Zn and that this reduced the Zn content of *Pinus sylvestris*, whereas another species *Thelephora terrestris* retained little Zn and even increased the Zn content of the host (Colpaert and Van Assche, 1992). Similarly, although the mycorrhizal species *Suillus bovinus* and *T. terrestris* both protected *P. sylvestris* against Cu toxicity, the amount of Cu retained by the two fungi varied considerably (Van Tichelen *et al*., 2001). Again, considerable variation was observed between the ability of five ectomycorrizal fungi to grow in culture with a range of nine different heavy metals (Tam, 1995). The mechanisms employed by the fungi at the cellular level to tolerate heavy metals are probably similar to some of the strategies employed by higher plants, namely binding to extracellular materials or sequestration in the vacuolar compartment. Thus in the fungus *Pisolithus tinctorius*, tolerance to Cu and Zn was achieved by binding to extrahyphal slime (Tam, 1995), whereas detoxification of Cd in *Paxillus involutus* involved binding of Cd to the cell walls and accumulation of Cd in the vacuole (Blaudez *et al*., 2000).

In relation to the role of ectomycorrhizas in metal tolerance by the host plant, most mechanisms that have been proposed involve various exclusion processes that restrict metal movement to the host roots. These have been extensively reviewed and assessed (Jentschke and

Godbold, 2000) and include absorption of metals by the hyphal sheath, reduced access to the apoplast due to the hydrophobicity of the fungal sheath, chelation by fungal exudates, and adsorption onto the external mycelium. Clearly, from the variation between species described above, these different exclusion mechanisms are likely to vary in significance between different plant/fungal interactions.

There are fewer reports on the role played by arbuscular mycorrhizas in metal tolerance. Weissenhorn *et al*. showed that the effects of maize root colonization by arbuscular mycorrhiza could either reduce the heavy metal content of the plants or increase metal absorption from polluted soils, depending on growth conditions, the fungus and the metal (Weissenhorn *et al*., 1995). However, a *Glomus* isolate (Br1) obtained from zinc violets (*Viola calaminaria*) growing on a heavy metal soil was shown to support the growth of maize and alfalfa on heavy metal soils more effectively than a commonly used *Glomus* isolate (Hildebrandt *et al*., 1999). In a related study, it was shown that the maize grown in the presence of the heavy metal *Glomus* isolate Br1 contained considerably lower heavy metal concentrations than plants grown without mycorrhiza or with the common *Glomus* strain (Kaldorf *et al*., 1999). Furthermore, elemental microbeam analysis indicated that the growth of maize in heavy metal soils was, at least in part, due to the selective immobilization of metals within the root tissues that contain the fungal cells.

The cell wall and root exudates

The binding properties of the cell wall and its role as a mechanism of metal tolerance has been a controversial one. Earlier reports have been reviewed (Ernst *et al*., 1992) and there have only been a few more papers on this topic. Although the root cell wall is directly in contact with metals in the soil solution, adsorption onto the cell wall must be of limited capacity and thus have a limited effect on metal activity at the surface of the plasma membrane. It is also difficult to explain metal-specific tolerance by such a mechanism (Ernst *et al*., 1992). However, Bringezu *et al*. reported that the heavy metal-tolerant *Silene vulgaris* ssp. *humilis* accumulated a range of metals in the epidermal cell walls, either bound to a protein or as silicates (Bringezu *et al*., 1999).

One related process concerns the role of root exudates in metal tolerance. Root exudates have a variety of roles (Marschner, 1995) including that of metal chelators that may enhance the uptake of certain metals. In an investigation into the role of Ni-chelating exudates in Ni hyperaccumulating plants, it was observed that the Ni-chelating histidine and citrate accumulated in the root exudates of non-hyperaccumulating plants, and thus could help to reduce Ni uptake and so play a role in a Ni-detoxification strategy (Salt *et al*., 2000). Since the range of compounds exuded is wide, other exudates could play a role in tolerance to other metals. The clearest example of a role for root secretions in tolerance is in relation to organic acids and the detoxification of the light metal Al (Ma *et al*., 2001). Buckwheat, for example, secretes oxalic acid from the roots in response to Al stress, and accumulates non-toxic Al-oxalate in the leaves (Ma *et al*., 1997); thus detoxification occurs both externally and internally. In wheat and maize there is evidence that such secretion from the roots is mediated by Al-activated anion channels in the plasma membrane (Ma *et al*., 2001).

Plasma membrane

The plant plasma membrane may be regarded as the first 'living' structure that is a target for heavy metal toxicity. Plasma membrane function may be rapidly affected by heavy metals as seen by an increased leakage from cells in the presence of high concentrations of metals, particularly of Cu. For example, it was shown that Cu, but not Zn, caused increased K^+ efflux from excised roots of *Agrostis capillaris* (Wainwright and Woolhouse, 1977). Similarly, others concluded that damage to the cell membrane, monitored by ion leakage, was the primary cause of Cu toxicity in roots of *Silene vulgaris*, *Mimulus guttatus*, and wheat, respectively (De Vos *et al*., 1991; Strange and Macnair, 1991; Quartacci *et al*., 2001). Such damage could result from various mechanisms including the oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as the H^+-ATPase, or changes to the composition and fluidity of membrane lipids (Meharg, 1993). Certainly direct effects of Cu and Cd treatments on the lipid composition of membranes have been reported (Ros *et al*., 1990; Fodor *et al*., 1995; Hernandez and Cooke, 1997; Quartacci *et al*., 2001) which may have a direct effect on membrane permeability. In addition, Cd treatments have been shown to reduce the ATPase activity of the plasma membrane fraction of wheat and sunflower roots (Fodor *et al*., 1995) while, in *Nitella*, Cu-induced changes in cell permeability were attributed to non-selective conductance increases and inhibition of the light-stimulated H^+-ATPase pump (Demidchik *et al*., 1997).

Thus tolerance may involve the protection of plasma membrane integrity against heavy metal damage that would produce increased leakage of solutes from cells (De Vos *et al*., 1991; Strange and Macnair, 1991; Meharg, 1993). However, there is little evidence to show how this might be achieved. For example, metal-tolerant plants do not appear to possess enhanced tolerance to free radicals or reactive oxygen species, but rather rely on

improved mechanisms for metal homeostasis (Dietz *et al*., 1999). Again these effects on membranes are metal-specific since, in contrast to Cu, Zn protects membranes against oxidation and generally does not cause membrane leakage (Ernst *et al*., 1992; Cakmak, 2000). Another factor that may be involved in the maintenance of plasma membrane integrity in the presence of heavy metals could be enhanced membrane repair after damage (Salt *et al*., 1998). This could involve heat shock proteins or metallothioneins, and evidence for this is discussed in following sections.

Apart from tolerance involving a more resistant plasma membrane or improved repair mechanisms, the cell membrane may play an important role in metal homeostasis, either by preventing or reducing entry into the cell or through efflux mechanisms. Many of these cations, of course, are essential and so complete exclusion is not possible; selective efflux may be more realistic. In bacteria, most resistance systems are based on the energy-dependent efflux of toxic ions (Silver, 1996). It appears that the metabolic penalty for having more specific uptake mechanisms, and thus restricting the entry of toxic ions, is greater than that of having inducible efflux systems (Silver, 1996).

The number of examples of exclusion or reduced uptake mechanisms in higher plants is quite limited. The clearest example of reduced uptake as an adapted tolerance mechanism is in relation to arsenic toxicity (Meharg and Macnair, 1990, 1992). In *Holcus lanatus* roots, phosphate and arsenate appear to be taken up by the same systems. However, an arsenate-tolerant genotype showed a much lower rate of uptake for both anions than the non-tolerant genotype, and also showed an absence of the high-affinity uptake system. The altered phosphate and arsenate uptake system was genetically correlated to arsenate tolerance (Meharg and Macnair, 1992). Further work has suggested that arsenate tolerance in *H. lanatus* requires both this adaptive suppression of the high affinity transport system, together with constitutive phytochelatin (PC) production since arsenate can still accumulate to high levels in tolerant plants (Hartley-Whitaker *et al*., 2001*a*). PCs are discussed in detail in a later section. More recently, a plasma membrane transporter in tobacco that confers Ni tolerance and Pb hypersensitivity has been described (Arazi *et al*., 1999). The transporter, designated Nt CBP4, is a calmodulin-binding protein that is structurally similar to certain K^+ and non-selective cation channels. Transgenic plants that over-expressed this transporter showed improved Ni tolerance and hypersensitivity to Pb which were associated with reduced Ni accumulation and enhanced Pb accumulation. Although the normal physiological function of this transporter remains to be established, it could provide a possible mechanism for Ni tolerance.

An alternative strategy for controlling intracellular metal levels at the plasma membrane involves the active efflux of metal ions, although there is very little direct evidence for such a process in plants. However, in bacteria, efflux pumping is the basis of most toxic ion resistance systems, involving transporters such as P-type ATPases (see below) or cation/H^+ antiporters (Silver and Ji, 1994; Silver, 1996); efflux pumping systems have been identified for Cu, Cd, Zn, Co, and Ni (Silver, 1996). Efflux transporters may also play a role in metal ion homeostasis in animal cells. For example, a plasma membrane Zn transporter (ZnT-1) was isolated from rat kidney (Palmiter and Findley, 1995). Cells transformed with a mutant ZnT-1 lacking the first membrane-spanning domain showed Zn sensitivity; it was proposed that normally ZnT-1 transports Zn out of cells and that its absence produces increased sensitivity of the mutant cells to Zn toxicity. It was thought that Zn efflux involves some form of secondary active transport. Another group of transporters that appear to be involved in Cu homeostasis by a copper export system are the heavy metal CPx-ATPases, a branch of the P-type ATPases (Solioz and Vulpe, 1996; Williams *et al*., 2000). Defects in these ATPases have been linked to two human disorders, Menkes disease and Wilson disease, that result from defective Cu export and thus the accumulation of Cu in some tissues (Solioz and Vulpe, 1996). In Chinese hamster ovary cells there is evidence that the Menkes P-type ATPase continuously recycles from Golgi to plasma membrane; elevated concentrations of Cu shift the distribution of the ATPase from Golgi to the plasma membrane leading to the efflux of a potentially toxic ligand (Petris *et al*., 1996).

Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general, and thus could play a key role in tolerance. These include the heavy metal CPx-ATPases, the Nramps, and the CDF (cation diffusion facilitator) family (Williams *et al*., 2000), and the ZIP family (Guerinot, 2000). Recently, a role for the Nramps in Fe and Cd uptake has been reported (Thomine *et al*., 2000); interestingly, disruption of an *AtNramp 3* gene slightly increased Cd resistance, whereas overexpression resulted in Cd hypersensitivity in *Arabidopsis*. In the Zn/Cd hyperaccumulator *Thlaspi caerulescens*, Pence *et al*. cloned a transporter, *ZNT1*, that mediates high-affinity Zn uptake as well as low affinity Cd uptake, and is expressed at high levels in the roots and shoots (Pence *et al*., 2000). Increased expression, resulting from changes in the plant Zn status, led to increased Zn influx in the roots. However, the transport function, specificity and cellular

location of most of these proteins in plants is as yet unknown. From the evidence presented above for bacterial and mammalian systems, the CPx-ATPases and the CDF family (that includes the ZnT Zn efflux transporters of humans and rodents) would seem the most likely candidates for a metal efflux tolerance mechanism.

Heat shock proteins

Heat shock proteins (HSPs) characteristically show increased expression in response to the growth of a variety of organisms at temperatures above their optimal growth temperature. They are found in all groups of living organisms, can be classified according to molecular size and are now known to be expressed in response to a variety of stress conditions including heavy metals (Vierling, 1991; Lewis *et al*., 1999); they act as molecular chaperones in normal protein folding and assembly, but may also function in the protection and repair of proteins under stress conditions.

There have been several reports of an increase in HSP expression in plants in response to heavy metal stress. Tseng *et al*. showed that, in rice, both heat stress and heavy-metal stress increased the levels of mRNAs for low molecular mass HSPs (16–20 kDa) (Tseng *et al*., 1993), while Neumann *et al*. indicated that HSP17 is expressed in roots of *Armeria maritima* plants grown on Cu-rich soils (Neumann *et al*., 1995). Small heat shock proteins (e.g. HSP17) were also shown to increase in cell cultures of *Silene vulgaris* and *Lycopersicon peruvianum* in response to a range of heavy metal treatments (Wollgiehn and Neumann, 1999); however, no or very low amounts of HSPs were found in plants growing on metalliferous soils, suggesting that HSPs are not responsible for the heritable metal tolerance of *Silene*.

Working with cell cultures of *L. peruvianum*, it was shown that a larger HSP (HSP70) also responds to Cd stress (Neumann *et al*., 1994). It is of interest that antibody localization showed that HSP70 was present in the nucleus and cytoplasm, but also at the plasma membrane. This suggests that HSP70 could be involved in the protection of membranes against Cd damage. Expression of HSP70 also increased in the seaweed *Enteromorpha intestinalis* after exposure to a variety of stressors including Cu (Lewis *et al*., 2001). Thus, in relation to earlier discussions of tolerance mechanisms involving a more resistant plasma membrane or improved repair mechanisms, HSPs could have an important role in this respect. Interestingly, it was reported that a short heat stress given prior to heavy-metal stress induces a tolerance effect by preventing membrane damage, as judged by ultrastructural studies (Neumann *et al*., 1994). Clearly more molecular evidence is required to support such an important repair or protective role.

Phytochelatins

Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance. Potential ligands include amino acids and organic acids, and two classes of peptides, the phytochelatins and the metallothioneins (Rauser, 1999; Clemens, 2001). The phytochelatins have been the most widely studied in plants, particularly in relation to Cd tolerance (Cobbett, 2000; Goldsbrough, 2000).

The phytochelatins (PCs) are a family of metal-complexing peptides that have a general structure $(\gamma\text{-Glu Cys})_n\text{-Gly}$ where $n = 2–11$, and are rapidly induced in plants by heavy metal treatments (Rauser, 1995; Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). PCs are synthesized non-translationally using glutathione as a substrate by PC synthase (Grill *et al*., 1989; Rauser, 1995), an enzyme that is activated in the presence of metal ions (Cobbett, 2000). The genes for PC synthase have now been identified in *Arabidopsis* and yeast (Clemens *et al*., 1999; Ha *et al*., 1999; Vatamaniuk *et al*., 1999).

Evidence has been presented both for and against a role for PCs in heavy metal tolerance (for reviews see Ernst *et al*., 1992; Meharg, 1994; Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). However, a clear role in Cd detoxification has been supported by a range of biochemical and genetic evidence. Howden *et al*. isolated a series of Cd-sensitive mutants of *Arabidopsis* that varied in their ability to accumulate PCs; the amount of PCs accumulated by the mutants correlated with the degree of sensitivity to Cd (Howden *et al*., 1995*a*, *b*). Using *Brassica juncea*, it has been shown that Cd accumulation is accompanied by a rapid induction of PC biosynthesis and that the PC content was theoretically sufficient to chelate all Cd taken up; this protects photosynthesis but did not prevent a decline in transpiration rate (Haag-Kerwer *et al*., 1999). Again, Inouhe *et al*. showed that cultured cells of azuki beans that were Cd hypersensitive also lacked PC synthase activity (Inouhe *et al*., 2000). Using *Arabidopsis*, Xiang and Oliver showed that treatment with Cd and Cu resulted in increased transcription of the genes for glutathione synthesis, and the response was specific for those metals thought to be detoxified by PCs (Xiang and Oliver, 1998): interestingly jasmonic acid treatment activated the same set of genes, although jasmonic acid production was not stimulated by heavy metals in plant cell cultures (Blechert *et al*., 1995). Zhu *et al*. overexpressed the γ-glutamylcysteine synthetase gene from *E. coli* in *Brassica juncea* resulting in increased biosynthesis of glutathione and PCs and an

increased tolerance to Cd (Zhu *et al*., 1999). A similar approach was taken with *Arabidopsis*; γ-glutamylcysteine synthetase was expressed in both sense and antisense orientations resulting in plants with a wide range of glutathione levels (Xiang *et al*., 2001). Plants with low glutathione levels were hypersensitive to Cd, although elevating the levels above wild-type did not increase metal resistance.

Recently, genes encoding for PC synthases in higher plants and yeast have now been identified, and it has been shown that the *Arabidopsis* gene could confer substantial increases in metal tolerance in yeast (Clemens *et al*., 1999; Vatamaniuk *et al*., 1999). The gene for PC synthase (*CAD1*) has been identified in *Arabidopsis* as well as an homologous gene in *Schizosaccharomyces pombe* (Ha *et al*., 1999); a mutant of the latter with a targeted deletion of this gene was PC-deficient and Cd-sensitive. To compare the involvement of PCs in metal detoxification, the sensitivity of the *cad 1–3* mutant was tested for sensitivity to a range of heavy metals in both *Arabidopsis* and *S. pombe* (Ha *et al*., 1999). PCs appeared to be important in the detoxification of Cd and arsenate, but played no role in the detoxification of Zn, Ni and selenite ions. In contrast to the *S. pombe* mutant, *cad 1–3* showed slight sensitivity to Cu and Hg. A possible role for PCs in Cu tolerance had also been suggested (Salt *et al*., 1989) from studies on copper-tolerant *Mimulus guttatus*; exposure to Cu in the presence of buthionine sulphoximine (BSO), a potent inhibitor of γ-glutamyl-cysteinyl synthetase, caused a considerable reduction in root growth that was not seen in the presence of inhibitor alone. However, in contrast, when Cu-sensitive and Cu-tolerant ecotypes of *Silene vulgaris* were exposed to concentrations of Cu giving either no or 50% inhibition of growth for each ecotype, they showed equal PC synthesis in the root tips (Schat and Kalff, 1992); it was concluded that differential Cu tolerance in *S. vulgaris* does not rely on differential PC production. Thus the role of PCs in Cu tolerance remains to be resolved. An involvement of PCs in arsenate tolerance has also been proposed (Hartley-Whitaker *et al*., 2001*a*, *b*; see section on Plasma membrane).

Not all studies have supported a role for PCs in metal tolerance (Steffens, 1990; Ernst *et al*., 1992). De Knecht *et al*. concluded that differential Cd tolerance in *Silene vulgaris* was not due to a differential production of PCs (De Knecht *et al*., 1992, 1994). Although PCs may play some role in Cd detoxification in *S. vulgaris*, PC production in greater amounts is not the mechanism that results in increased Cd tolerance. Again, treatment with the inhibitor BSO was found to increase the Zn-tolerance of *Festuca rubra* roots, arguing against a key role for PCs in the Zn-tolerance mechanism in these tissues (Davies *et al*., 1991*a*). Thus, although evidence for the role for PCs in detoxification is strong, especially for Cd, these peptides may play other important roles in the cell, including essential heavy-metal homeostasis, sulphur metabolism or, perhaps, as anti-oxidants (Rauser, 1995; Dietz *et al*., 1999; Cobbett, 2000). Their participation in the detoxification of excess concentrations of some heavy metals may be a consequence of these other functions (Steffens, 1990). Certainly the role of PCs in adaptive tolerance has been questioned (Meharg, 1994; Schat *et al*., 2000). It was suggested that the general lack of examples of co-tolerance (see Introduction) indicates that adaptive tolerance is unlikely to be produced by changes in relatively non-specific binding compounds such as PCs (or metallothioneins or organic acids) (Macnair *et al*., 2000).

The final step in Cd detoxification, certainly in the fission yeast and probably in higher plants, involves the accumulation of Cd and PCs in the vacuole (see Salt *et al*., 1998; Schat *et al*., 2000; for reviews). This accumulation appears to be mediated by both a Cd/H^+ antiporter and an ATP-dependent ABC transporter, located at the tonoplast (Salt and Wagner, 1993; Salt and Rauser, 1995; Rea *et al*., 1998); the stabilization of the Cd-PC complex in the vacuole involves the incorporation of acid-labile sulphide. In the fission yeast, a Cd-sensitive mutant has been isolated that is able to synthesize PCs, but is unable to accumulate the Cd-PC-sulphide complex (Ortiz *et al*., 1992); the mutant has a defect in a gene (*hmt 1*) that encodes an ABC-type transporter. Similar transporters may well be involved in Cd compartmentalization in higher plants (Salt and Rauser, 1995; Rea *et al*., 1998).

Metallothioneins

Higher plants contain two major types of cysteine-rich, metal-binding peptides, the metallothioneins (MTs) and the phytochelatins (discussed above). MTs are gene-encoded polypeptides that are usually classified into two groups. Class 1 MTs possess cysteine residues that align with a mammalian (equine) renal MT; Class 2 MTs also possess similar cysteine clusters but these cannot be easily aligned with Class 1 MTs (de Miranda *et al*., 1990; Robinson *et al*., 1993; Prasad, 1999). MT genes have now been identified in a range of higher plants (Prasad, 1999) including *Arabidopsis* where, in addition to Class 1 and Class 2 MT genes, MT3 and MT4 types have been recognized (Goldsbrough, 2000). Other species are also thought to contain an extensive MT gene family and more than one class of MT gene (Giritch *et al*., 1998), while expression studies have revealed tissue-specific patterns (Garcia-Hernandez *et al*., 1998; Charbonnel-Campaa *et al*., 2000; Goldsbrough, 2000). In plants, there is a lack of information concerning the metals likely to be bound by MTs, although Cu, Zn and Cd have been

the most widely studied (Tomsett and Thurman, 1988; Robinson *et al*., 1993; Goldsborough, 2000).

Although MTs can be induced by Cu treatments and there is evidence for a role in heavy metal tolerance in fungi and animals (Hamer, 1986), the role of MTs in heavy metal detoxification in plants remains to be established (Zhou and Goldsbrough, 1994; Zenk, 1996; Giritch *et al*., 1998; Schat *et al*., 2000). However, it has been reported that MT2 mRNA was strongly induced in *Arabidopsis* seedlings by Cu, but only slightly by Cd and Zn (Zhou and Goldsbrough, 1994); when genes for MT1 and MT2 from *Arabidopsis* were expressed in an MT-deficient yeast mutant, both genes complemented the mutation and provided a high level of resistance to Cu. van Vliet *et al*. showed that MT genes can be induced by Cu, and that the expression of MT2 RNA is increased in a Cu-sensitive mutant of *Arabidopsis* that accumulates high concentrations of Cu (van Vliet *et al*., 1995). 10 ecotypes of *Arabidopsis* were surveyed and a clear correlation between the Cu sensitivity of seedlings and the expression of MT2 RNA was shown (Murphy and Taiz, 1995*a*, *b*). Clearly more evidence is needed to establish a relationship between Cu sensitivity and MT production. By contrast, in a study of the effects of Cd exposure on *Brassica juncea*, it was reported that MT2 expression was delayed relative to PC synthesis (Haag-Kerwar *et al*., 1999) and they argued against a role for MT2 in Cd detoxification. Thus the role of MTs remains to be established. They could clearly play a role in metal metabolism, but their precise function is not clear; they may have distinct functions for different metals (Hamer, 1986). Alternatively, they could function as antioxidants, although evidence is lacking (Dietz *et al*., 1999), while a role in plasma membrane repair is another possibility (Salt *et al*., 1998).

Organic acids and amino acids

Carboxylic acids and amino acids such as citric, malic and histidine are potential ligands for heavy metals and so could play a role in tolerance and detoxification (for reviews see Rauser, 1999; Clemens, 2001); however strong evidence for a function in tolerance, such as a clear correlation between amounts of acid produced and exposure to a metal, has not been produced to support a widespread role. For example, a 36-fold increase was reported in the histidine content of the xylem sap on exposure to Ni in the Ni-hyperaccumulating plant *Alyssum lesbiacum* (Kramer *et al*., 1996). In addition, supplying histidine to a non-accumulating species greatly increased both its Ni tolerance and the capacity for Ni transport to the shoot. However, the histidine response may not be a widespread mechanism of Ni tolerance since it was not observed in another Ni-hyperaccumulator, *Thlaspi goesingense* (Persans *et al*., 1999). A possible role for the histidine found in root exudates as a Ni-detoxifying agent has been discussed earlier, as has the role of organic acids in Al tolerance (see section on Cell wall and root exudates).

Vacuolar compartmentalization

Efflux of ions at the plasma membrane (discussed above) or transport into the vacuole are two ways of reducing the levels of toxic metals in the cytosol and so are potentially important mechanisms for heavy metal tolerance. One well-documented example, the accumulation of Cd and PCs in the vacuole involving an ABC transporter, has already been described (see section on PCs), but there is evidence that the vacuole may be important in the accumulation of other metals involving other tonoplast transport systems.

Earlier studies showed that the vacuole is the site for the accumulation of a number of heavy metals including Zn and Cd (for reviews see Ernst *et al*., 1992; De, 2000). Apart from the Cd–PC accumulation process, the best evidence for a role of vacuolar accumulation in relation to metal tolerance is for Zn. For example, meristematic cells of *Festuca rubra* roots show increased vacuolation on treatment with Zn (Davies *et al*., 1991*b*), while uptake analysis using Zn^{65} with barley leaves suggested that rapid compartmentation of Zn into the vacuole was an important mechanism for dealing with high levels of Zn (Brune *et al*., 1994). Further studies on barley leaves showed that, although Cd, Zn and Mo were found mainly in the vacuole, Ni was primarily found in the cytosol and this appeared to be related to the development of leaf damage (Brune *et al*., 1995); however, compartmentalization in the roots was not examined. Analysis of transport systems at the tonoplast has added support to a vacuolar mechanism of tolerance. Verkleij *et al*. isolated tonoplast vesicles from roots of Zn-tolerant and -sensitive ecotypes of *Silene vulgaris* (Verkleij *et al*., 1998). They showed that at high Zn concentrations, Zn transport was 2.5 times higher into vesicles from the tolerant lines than from the sensitive ones, suggesting that the tonoplast plays an important role in naturally selected Zn tolerance. Using plant crosses, this increased tonoplast uptake system was shown to correlate genetically with Zn tolerance (Chardonnens *et al*., 1999). More recently, an *Arabidopsis* gene (*ZAT*) was isolated that is closely related to the animal *ZnT* (Zn transporter) genes (see section on Plasma membrane) (Van der Zaal *et al*., 1999). ZAT mRNA seemed to be expressed constitutively throughout the plant and was not induced by higher Zn concentrations. However, overexpression of *ZAT* in transgenic plants led to a significant increase in Zn resistance and an enhanced accumulation in the root under high Zn treatments. Thus the Zn transporter could be involved in sequestration of Zn in the vacuole and thus in Zn tolerance in plants.

Detailed information on other heavy metal transport systems at the tonoplast is limited. Two genes *CAX1* and *CAX2* have been isolated from *Arabidopsis* and shown to be vacuolar-located high and low efficiency H^+/Ca^{2+} exchangers; while *CAX1* is thought to be involved in vacuolar Ca^{2+} accumulation, it was suggested that *CAX2* could be a high capacity H^+/heavy metal cation transporter (Hirschi *et al.*, 1996). Although there is evidence for H^+ antiport systems for Ca and Cd in oat root tonoplasts, no evidence was found for a Ni/H^+ antiporter or a nucleotide-dependent Ni pump and suggested that the vacuole is not a major site for Ni accumulation in this tissue (Gries and Wagner, 1998). Brune *et al.* came to a similar conclusion concerning Ni after a study of heavy metal compartmentation in barley leaves (Brune *et al.*, 1995).

Conclusions

This review has focused on recent evidence that identifies potential cellular/molecular mechanisms that may be involved in the resistance and tolerance of plants to excess concentrations of heavy metals in the environment. Generally, the strategy adopted by plants aims to avoid the build-up of excess metal levels in the cytosol, and thus to prevent the onset of toxicity symptoms. This is achieved by the use of various mechanisms that are present and likely to be employed in general metal homeostasis in all plants. It appears likely that specific mechanisms are employed for specific metals in particular species. These include mechanisms that reduce uptake into the cytosol by entrapment in the apoplastic space, chelation of metals in the cytosol by a range of ligands, or efflux from the cytosol, either into the apoplast or into the vacuole (Fig. 1). It is also possible that more than one mechanism may be involved in reducing the toxicity of a particular metal (Table 1). For example, a recent report has shown that arsenate tolerance in *Holcus lanatus* involves both adaptive suppression of the plasma membrane uptake system and a role for PC production (Hartley-Whitaker *et al.*, 2001*a*).

These processes involved in reducing toxicity are of considerable current interest because an understanding of the means of manipulating metal tolerance could be important in the development of crops for phytoremediation purposes, particularly for highly contaminated soils (Salt *et al.*, 1998). However, the evidence reviewed in this paper strongly suggests that there is no single mechanism that can account for tolerance to a wide range of metals. Although adaptive tolerance appears to be under relatively simple genetic control, tolerance to individual metals involves distinct metal-specific mechanisms; co-tolerance is not a widespread phenomenon. This means that breeding plants for broad phytoremediation purposes will involve a large number of genetic changes (Macnair *et al.*, 2000). Beyond these cellular mechanisms, of course, is the problem of understanding tolerance at the whole plant level and this introduces a further layer of complexity that is beyond the scope of this review.

It is clear that evidence for a role in tolerance, particularly for adaptive tolerance, for most of the mechanisms discussed is quite limited. For example, although a whole new range of metal transporter families have been identified in plants in the last five years or so that could play a key role in tolerance, much remains to be established concerning their metal ion specificity, their cellular and tissue location, and their role in metal homeostasis (Williams *et al.*, 2000). Another important area for future research is the relationship between the role of chaperones in the sequestration and intracellular trafficking of Cu and other metals and metal homeostasis and detoxification, since there is evidence that Cu chaperones deliver Cu to Cu pumps for transport into various cellular compartments (Himelblau and Amasino, 2000; Clemens, 2001). Again, although jasmonic acid has been implicated, there is almost no information available on the signal transduction pathway(s) involved in the response to heavy metals (Xiang and Oliver, 1998). However, the increased application of molecular genetics techniques will have a huge impact on our understanding of metal tolerance. With the completion of the

Table 1. *Summary of potential mechanisms involved in the detoxification of and tolerance to specific metals*

Mechanism	Metal	Key reference
Mycorrhizas	Zn, Cu, Cd	Jentschke and Godbold (2000)
Cell wall, exudates	Various, including Ni, Al	Salt *et al.* (2000); Ma *et al.* (1997)
Plasma membrane		
Reduced uptake	Arsenate	Meharg and Macnair (1992)
	Ni	Arazi *et al.* (1999)
Active efflux	Various, including Zn	Silver (1996)
	(evidence not for plants)	Palmiter and Findley (1995)
Phytochelatins	Cd	Cobbett (2000)
Metallothioneins	Cu	Murphy and Taiz (1995)
Organic acids, amino acids	Various	Rauser (1999)
Heat shock proteins	Various, including Cd	Neumann *et al.* (1994)
Vacuolar compartmentation	Zn	Van der Zaal *et al.* (1999)

Arabidopsis genome project, followed eventually by genome sequences for other plants, the full range of genes that are potentially involved in heavy metal homeostasis and tolerance will be revealed. One approach will be the use of gene arrays to study the co-ordinated expression of transporters and chelators in response to heavy metal treatments. The increased availability of gene deletion mutants, or of plants over- or underexpressing certain key genes, will again provide valuable evidence in relation to tolerance mechanisms. Such information will allow detailed models to be constructed of the various responses that occur when plants, both sensitive and tolerant, are subjected to heavy metal stress.

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