

Chapter 2

Plant Responses to Heavy Metal Toxicity

Anna Manara

Abstract Plants, like all other organisms, have evolved different mechanisms to maintain physiological concentrations of essential metal ions and to minimize exposure to non-essential heavy metals. Some mechanisms are ubiquitous because they are also required for general metal homeostasis, and they minimize the damage caused by high concentrations of heavy metals in plants by detoxification, thereby conferring tolerance to heavy metal stress. Other mechanisms target individual metal ions (indeed some plants have more than one mechanism to prevent the accumulation of specific metals) and these processes may involve the exclusion of particular metals from the intracellular environment or the sequestration of toxic ions within compartments to isolate them from sensitive cellular components. As a first line of defense, many plants exposed to toxic concentrations of metal ions attempt to prevent or reduce uptake into root cells by restricting metal ions to the apoplast, binding them to the cell wall or to cellular exudates, or by inhibiting long distance transport. If this fails, metals already in the cell are addressed using a range of storage and detoxification strategies, including metal transport, chelation, trafficking, and sequestration into the vacuole. When these options are exhausted, plants activate oxidative stress defense mechanisms and the synthesis of stress-related proteins and signaling molecules, such as heat shock proteins, hormones, and reactive oxygen species.

Keywords Metal homeostasis • Metal uptake • Metal transport • Chelation • Oxidative stress

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2.1 Signal Transduction in Response to Heavy Metals

The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metal, and is characterized by the synthesis of stress-related proteins and signaling molecules, and finally the transcriptional activation of specific metal-responsive genes to counteract the stress (Maksymiec 2007). The relevant signal transduction pathways include the Ca-calmodulin system, hormones, ROS signaling, and the mitogen-activated protein kinase (MAPK) phosphorylation cascade, which converge by activating the above-mentioned stress-related genes. Different signaling pathways may be used to respond to different heavy metals (DalCorso et al. 2010).

2.1.1 The Ca-Calmodulin System

Ca^{2+} signaling features in responses to a number of abiotic stress factors, including temperature extremes, osmotic stress, oxidative stress, anoxia, and mechanical perturbation (Knight 1999). Excess heavy metals modify the stability of Ca channels, thus increasing calcium flux into the cell. Intracellular Ca is a secondary messenger, which interacts with calmodulin to propagate the signal and ultimately to regulate downstream genes involved in heavy metal transport, metabolism, and tolerance (Yang and Poovaiah 2003). Higher intracellular Ca levels are observed in plants exposed to Cd, inducing adaptive mechanisms that alleviate the toxic effects of the heavy metal (Skórzyńska-Polit et al. 1998). The Ca-calmodulin system is also involved in the response to other heavy metal toxicity, such as Ni and Pb. Transgenic tobacco plants expressing NtCBP4 (*Nicotiana tabacum* calmodulin-binding protein) tolerate higher levels of Ni^{2+} but are hypersensitive to Pb^{2+} , reflecting the exclusion of Ni^{2+} but the accumulation of more Pb^{2+} than wild-type plants (Arazi et al. 1999).

2.1.2 Hormones in the Heavy Metal Response

Plant hormones are involved in many physiological and developmental processes, and play a crucial role in the adaptation to abiotic stress as shown by the regulation of hormone synthesis in the presence of heavy metals (Peleg and Blumwald 2011). For example, plants exposed to toxic levels of Cd, Cu, Fe, and Zn produce higher levels of ethylene, but Co does not have the same effect (Maksymiec 2007; Wise and Naylor 1988). Cd and Cu stimulate ethylene synthesis by upregulating ACC synthase expression and activity (Pell et al. 1997). Cu and Cd also induce the rapid accumulation of jasmonic acid (JA) in *Phaseolus coccineus* (Maksymiec et al. 2005), and Cu has also been shown to have this effect in rice (Rakwall et al. 1996).

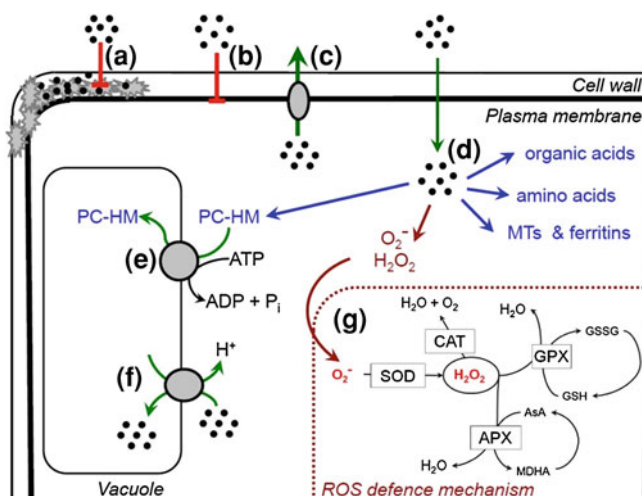


Fig. 2.1 The response to heavy metal toxicity in higher plants. As discussed in the text, plant responses to heavy metals include: **a** metal ion binding to the cell wall and root exudates; **b** reduction of metal influx across the plasma membrane; **c** membrane efflux pumping into the apoplast; **d** metal chelation in the cytosol by ligands such as phytochelatins, metallothioneins, organic acids, and amino acids; **e** transport of metal–ligand complexes through the tonoplast and accumulation in the vacuole; **f** sequestration in the vacuole by tonoplast transporters; **g** induction of ROS and oxidative stress defense mechanisms as described in Fig. 1.2. Metal ions are shown as black dots

and *Arabidopsis thaliana* (Maksymiec et al. 2005). Salicylic acid (SA) is involved in heavy metal stress responses, as shown by the increase in SA levels in barley roots in the presence of Cd and the ability of exogenous SA to protect roots from lipid peroxidation caused by Cd toxicity (Metwally et al. 2003).

2.1.3 The Role of Reactive Oxygen Species

As stated above, one of the major consequences of heavy metal accumulation is the production of ROS, which as well as causing widespread damage may also function as signaling molecules. Heavy metals such as Cd can produce ROS directly via the Fenton and Haber–Weiss reactions, and indirectly by inhibiting antioxidant enzymes (Romero-Puertas et al. 2007). In particular, H_2O_2 acts as a signaling molecule in response to heavy metals and other stresses (Dat et al. 2000). H_2O_2 levels increase in response to Cu and Cd treatment in *A. thaliana* (Maksymiec and Krupa 2006), upon Hg exposure in tomato (Cho and Park 2000) and in response to Mn toxicity in barley (Cho and Park 2000). This increase in H_2O_2 accumulation changes the redox status of the cell and induces the production of antioxidants and the activation of antioxidant mechanisms (Fig. 2.1).

2.1.4 The MAPK Cascade

The MAPK cascade in plants is a response to both biotic and abiotic stresses, including pathogens, temperature extremes, heavy metal stress, drought, and wounding (Jonak et al. 1996; He et al. 1999). The pathway is also used in hormone signal transduction and in response to developmental stimuli (Jonak et al. 2002). The MAPK cascade involved three kinases sequentially activated by phosphorylation: the MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK), and the MAPK. At the end of this cascade of phosphorylation, MAPKs phosphorylate different substrates in different cellular compartments, including transcription factors in the nucleus. In this way, the MAPK cascade allows the transduction of the information to downstream targets. Four isoforms of MAPK were shown to be activated in alfalfa (*Medicago sativa*) seedlings exposed to Cu or Cd (Jonak et al. 2004) and a MAPK gene is also activated by Cd treatment in rice (Yeh et al. 2004). All these signaling pathways finally converge in the regulation of transcription factors that activate genes required for stress adaptation, particularly in the context of heavy metals this means genes for the activation of metal transporters and the biosynthesis of chelating compounds.

2.2 Metal Ion Uptake from Soil

Metal availability and motility in the rhizosphere is influenced by root exudates and microorganisms (Wenzel et al. 2003). Higher plants possess highly effective systems for the acquisition of metal ions and other inorganic nutrients from the soil. These are based on a small number of transport mechanisms, suggesting that different heavy metal cations are co-transported across the plasma membrane in the roots. Because toxic heavy metals such as Cd and Pb have no known biological function, it is likely that specific transporters do not exist. Instead, these toxic metals enter into the cells through cation transporters with a wide range of substrate specificity.

2.2.1 Metal Ion Binding to Extracellular Exudates and to the Cell Wall

As a first line of defense against heavy metals, plant roots secrete exudates into the soil matrix. One of the major roles of root exudates is to chelate metals and to prevent their uptake inside the cells (Marschner 1995). For example, Ni-chelating histidine and citrate are present in root exudates and these reduce the uptake of Ni from soil (Salt et al. 2000). The binding of metal ions such as Cu and Zn in the apoplast also helps to control the metal content of root cells (Dietz 1996). Cation

binding sites are also present on the root cell wall, and this allows metal exchange thus influencing the availability of ions for uptake and diffusion into the apoplast (Allan and Jarrel 1989). The cell wall can play a key role in the immobilization of toxic heavy metal ions by providing pectic sites and hystidyl groups, and extra-cellular carbohydrates such as callose and mucilage, and thus prevents heavy metals uptake into the cytosol (Fig. 2.1). Thus, different tobacco genotypes with chemically distinct root cell wall surfaces have different sensitivities to Mn toxicity (Wang et al. 1992). These data suggest that the chemical properties of the cell wall might modulate plant metal uptake and consequently metal tolerance. However, the role of the cell wall in metal tolerance is not completely understood. The cell wall is in direct contact with metal ions in the soil but only a limited number of absorption sites are available, suggesting the cell wall has only a minor impact on metal tolerance (Ernst et al. 1992). However, *Silene vulgaris* ssp. *humilis* is a heavy metal-tolerant plant that accumulates different heavy metals by binding them to proteins or silicates in the epidermal cell walls (Bringezu et al. 1999).

2.2.2 Metal Ion Transport through the Plasma Membrane in Root

Plants possess various families of plasma membrane transporters involved in metal uptake and homeostasis. At the cellular level, metal transporters on the plasma membrane and tonoplast are required to maintain physiological concentrations of heavy metals, but they may also contribute to heavy metal stress responses (Fig. 2.1). These transporters belong to the heavy metal P_{1B}-ATPase, the NRAMP, the CDF (Williams et al. 2000), and the ZIP families (Guerinot 2000). The biological function, cellular location, and metal specificity of most of these transporters in plants are still unknown. In plants most of these metal ions transporters were identified by complementation in *Saccharomyces cerevisiae* mutants defective in metal uptake.

2.2.2.1 The ZIP Family

One of the principal metal transporter family involved in metal uptake is the ZIP family. ZIP family of transporters have been identified in many plant species (as well as bacteria, fungi and animals) and are involved in the translocation of divalent cations across membranes. Certain ZIP proteins are induced in *A. thaliana* roots and shoots in response to Fe or Zn loading, and thus appear to be part of a stress response. Most ZIP proteins are predicted to comprise eight transmembrane domains and have a similar topology, with the N- and C-termini exposed to the apoplast, and a variable cytoplasmic loop between transmembrane domains III and IV that contains a histidine-rich domain putatively involved in metal binding (Guerinot 2000) and specificity (Nishida et al. 2008).

The first ZIP transporter to be characterized was the *A. thaliana* IRT1. This was identified by functional complementation of the *S. cerevisiae* *fet3fet4* double mutant, which is impaired in iron transport (Eide et al. 1996). In *A. thaliana*, IRT1 is expressed in root cells and accumulates in response to iron deficiency, suggesting a role in Fe^{2+} uptake from the soil (Vert et al. 2002). Many metal transporters present low ion selectivity, and additional studies in yeast showed that AtIRT1 can also transport Mn^{2+} , Zn^{2+} , and Cd^{2+} (Korshunova et al. 1999). IRT1, in plant, is also involved in the uptake of heavy metal divalent cations such as Cd^{2+} and Zn^{2+} (Cohen et al. 1998). Furthermore, when expressed in yeast, *AtIRT1* enhanced the Ni^{2+} -uptake activity (Nishida et al. 2011). In *A. thaliana*, *AtIRT1* is induced in response to excess Ni and is involved in Ni^{2+} transport and accumulation. In *S. cerevisiae* the ZRT1 and ZRT2 transporters were identified on the basis of sequence similarity to IRT1 and they are respectively high- and low-affinity Zn^{2+} transporters (Zhao and Eide 1996a, b). The *zrt1zrt2* double mutant yeast was then used to clone the *A. thaliana* Zn^{2+} transporters, AtZIP1, AtZIP2, and AtZIP3 by functional complementation (Grotz et al. 1998). ZIP1 and ZIP3 are expressed principally in the roots and are induced under Zn limiting conditions. In *A. thaliana*, the analysis of the genomic sequence revealed the presence of a fourth member of the family, *AtZIP4*, which is expressed in roots and shoots, and it is also induced by Zn restriction, supporting their proposed role in Zn nutrition. ZIP transporters in plants are also involved in Cd uptake from soil into the root cells and transport Cd from root to shoot (Krämer et al. 2007). In hyperaccumulator species, ZIP transporters are necessary (but not sufficient) for the enhanced accumulation of metal ions and metal accumulating capacity correlates with ZIP expression (Krämer et al. 2007). In *S. cerevisiae*, ZRT3 is a further transporter identified by functional complementation but this appears to be involved in the mobilization of Zn from vacuole and not only in the uptake from the environment (MacDiarmid et al. 2000). There is some evidence that Ni is taken up by Zn transporters (Assunção et al. 2001) although candidate Ni-specific transporters have also been identified (Peer et al. 2003).

2.2.2.2 The NRAMP Family

NRAMP metal transporters have been shown to transport a wide range of metals, such as Mn^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} , Cd^{2+} , Ni^{2+} , and Co^{2+} , across membranes, and have been identified in bacteria, fungi, plants, and animals (Nevo and Nelson 2006). In plants, NRAMP transporters are expressed in roots and shoots and are involved in transport of metal ions through the plasma membrane and the tonoplast (Krämer et al. 2007). NRAMPs in *A. thaliana* are thought to transport Fe and Cd, with NRAMP1 playing a specific role in Fe transport and homeostasis (Thomine et al. 2000). The *AtNramp1* gene complements the yeast *fet3fet4* double mutant, and is induced under limiting Fe conditions. (Curie et al. 2000). The overexpression of *AtNramp1* in transgenic *A. thaliana* plants leads to an increase in plant resistance to toxic iron concentration (Curie et al. 2000).

2.2.2.3 The Copper Transporters Family

The Copper Transporters (CTR) family of transporter has firstly been identified in yeast and mammalian and subsequently also in plants. CTR proteins comprise a putative metal-binding motif in the extracellular domain, three predicted transmembrane domains, and a conserved and essential MXXXM motif within the putative second transmembrane domain (Puig and Thiele 2002). The *A. thaliana* copper transporter COPT1 was identified by functional complementation of the *S. cerevisiae* mutant *ctr1-3*, which is defective in copper uptake (Kampfenkel et al. 1995a). In *A. thaliana*, COPT1 has been shown to transport copper, and it also has a role in growth and pollen development (Sancenón et al. 2004).

2.2.3 Reduced Metal Uptake and Efflux Pumping at the Plasma Membrane

The plasma membrane plays an important role in plant response to heavy metals by preventing or reducing the uptake of metals into the cell or by active efflux pumping outside the cell. There are few examples of ion exclusion or reduced uptake as a sole protective mechanism in plants. Although an arsenate-tolerant genotype of *Holcus lanatus* absorbs less arsenate than an equivalent non-tolerant genotype (Meharg and Macnair 1992) due to the suppression of the high-affinity arsenate transport system combined with the constitutive synthesis of PCs (Hartley-Whitaker et al. 2001). Active efflux systems are more common and are used to control heavy metals accumulation inside the cell. This mechanism is well documented in bacteria (Silver 1996) and in animal cells (Palmiter and Findley 1995). Differently, there are only few evident indications of plasma membrane efflux transporters involved in heavy metal response in plants. Comparing data obtained for bacteria and mammals, the most likely candidate heavy metal efflux pumps in plants (based on sequence similarity to microbial and animal proteins) are the P_{1B}-ATPases and the CDF families of transporters. P_{1B}-type ATPases belong to P-type ATPase superfamily and use energy from ATP hydrolysis to translocate diverse metal cations across biological membranes (Axelsen and Palmgren 2001). P_{1B}-type ATPases share common structural characteristics, such as eight predicted transmembrane domains, a CPx (Cys-Pro-Cys/His/Ser) intra-membrane motif that is hypothetically involved in metal translocation (Ashrafi et al. 2011), and a putative N- or C-terminal metal binding domain (Colangelo and Gueriot 2006). P_{1B}-ATPases pump metal ions out of the cytoplasm against their electrochemical gradient, into either the apoplast or into the vacuole. The eight P_{1B}-type ATPases in *A. thaliana* and rice were renamed heavy metal ATPases (HMAs) (Baxter et al. 2003). HMAs are divided into two classes, one involved in transport of monovalent cations (Cu/Ag) and the second in the transport of divalent cations (Zn/Co/Cd/Pb) (Baxter et al. 2003). HMAs are more selective than the

transporters involved in metal uptake, e.g., HMA2, HMA3, and HMA4 export Zn and Cd exclusively (Krämer et al. 2007). Therefore, *hma2 hma4* double mutants and, to a lesser extent, the *hma4* single mutant contain low levels of Zn in the shoots, display Zn deficiency symptoms, but other micronutrients are unaffected (Hussain et al. 2004). In addition, they show increased Cd sensitivity and decrease in Cd root-to-shoot translocation (Hussain et al. 2004; Wong and Cobbett 2009). Both AtHMA2 (Hussain et al. 2004) and AtHMA4 (Verret et al. 2005) are located on the plasma membrane and heterologous expression of AtHMA4 in yeast induces tolerance to Zn and Cd toxicity, thus suggesting that this transporter can act as efflux pump (Mills et al. 2005).

ABC transporters are also involved in metal ion efflux from the plasma membrane. For example, AtPDR8 is localized in the plasma membrane of *A. thaliana* root hairs and epidermal cells, conferring both metal tolerance (Kim et al. 2007) and pathogen resistance (Kobae et al. 2006). AtPDR8 is induced in the presence of Cd and Pb, transgenic plants overexpressing the protein do not accumulate Cd in the roots or shoots and are tolerant to normally toxic levels of Cd and Pb. In contrast, mutants accumulate higher levels of Cd and are sensitive to both metals. Probably, AtPDR8 acts as an efflux pump of these metals at the plasma membrane (Kim et al. 2007).

2.3 Root-to-Shoot Metal Translocation

Once taken up by the roots, metal ions are loaded into the xylem and transported to the shoots as complexes with various chelators. Organic acids, especially citrate, are the major chelators for Fe and Ni in the xylem (Tiffin 1970; Leea et al. 1977). In addition, amino acids are potential metal ligands, for instance Ni may also be chelated by histidine and translocated (Kramer et al. 1996), and the methionine derivative NA is involved in the transport of Cu (Pich and Scholz 1996). Several types of transporter proteins are involved in the root-to-shoot transport of metals. Metal ions are also translocated from source to sink tissue via phloem. Therefore, phloem sap contains metals arising from source tissue, like Fe, Cu, Zn, and Mn (Stephan et al. 1994). Into the phloem, only NA was identified as potential metal chelator of Fe, Cu, Zn, and Mn (Stephan and Scholz 1993). NA is involved in the long distance transport of metals inside the xylem and phloem, but other chelators are required for loading. High molecular weight compounds that chelate Ni, Co, and Fe are found in the phloem of *Ricinus communis* plants (Wiersma and Van Goor 1979; Maas et al. 1988) and Zn-chelating peptides are found in *Citrus* spp. (Taylor et al. 1988) but they have yet to be characterized in detail.

2.3.1 The HMA Family of Transporters

The P-type ATPases reclassified as HMAs (see above) function not only as efflux pumps to remove metal ions from the cell, but also as internal transporters to load Cd and Zn metals into the xylem from the surrounding tissues. HMA4 is the first gene encoding for P-type ATPase cloned and characterized in *A. thaliana* (Mills et al. 2003). AtHMA4 is a plasma membrane transporter of divalent ions required for Zn homeostasis and Cd detoxification as it participates in the cytosolic efflux and in the root-to-shoot translocation of these metals (Mills et al. 2003; Verret et al. 2004). Overexpression of the AtHMA4 protein not only increases Zn and Cd tolerance, but also enhances the root-to-shoot translocation of both metals suggesting a role also in metal root-to-shoot transport (Verret et al. 2004). AtHMA5 is expressed constitutively in roots and induced by Cu in other plant organs, but *hma5* mutants are hypersensitive to Cu and accumulate this metal in roots to a greater extent than wild-type plants, suggesting a role in root-to-shoot translocation and Cu detoxification (Andrés-Colás et al. 2006).

2.3.2 The MATE Family of Efflux Proteins

MATE is a family of membrane-localized efflux proteins involved in extrusion of multidrug and toxic compound from the cell. FRD3 is a MATE protein that participates in iron-citrate efflux, i.e., the loading of Fe^{2+} and citrate into the vascular tissue in the roots. Xylem exudates from *frd3* mutant plants contain less citrate and Fe than wild-type plants, whereas those from transgenic plants overexpressing FRD3 produce more citrate in root exudates. Ferric-citrate complexes are required for the translocation of Fe to the leaves because Fe moves through the xylem in its chelated form (Durrett et al. 2007).

2.3.3 The Oligopeptide Transporters Family

Oligopeptide Transporters (OPT) is a superfamily of oligopeptide transporters including the YSL subfamily. The YSL family, specific for plants, takes its name from the maize Yellow stripe 1 protein (ZmYS1) that mediates Fe uptake by transporting Fe(III)-phytosiderophore complexes (Curie et al. 2001). The ZmYS1 transporter is able to translocate Fe, Zn, Cu, Ni, and, to a lesser extent, Mn and Cd (Schaaf et al. 2004). These ions can be chelated by either phytosiderophores or NA (Roberts et al. 2004). Eight putative YSL transporters have been identified in *A. thaliana* based on similarity to the maize gene (Colangelo and Guerinot 2006). AtYSL1 is expressed in the leaf xylem parenchyma, in pollen, and young siliques; mutants have a low Fe-NA complex content and cannot germinate normally,

indicating a role of this protein in the transport of chelated Fe (Le Jean et al. 2005). AtYSL2 is expressed in shoot and root vascular tissues and is localized mainly in the lateral plasma membrane, consistent with a role in the lateral movement of metals into the veins (DiDonato et al. 2004; Schaaf et al. 2005). AtYSL2 is modulated by Fe, Cu (DiDonato et al. 2004), and Zn (Schaaf et al. 2005) and can transport both Fe and Cu as NA complexes (DiDonato et al. 2004).

2.4 Heavy Metal Chelation in the Cytosol

Inside the cell, heavy metal ions that are not immediately required metabolically may reach toxic concentrations, and plant cells have evolved various mechanisms to store excess metals to prevent their participation in unwanted toxic reactions. If the toxic metal concentration exceeds a certain threshold inside the cells, an active metabolic process contributes to the production of chelating compounds. Specific peptides such as PCs and MTs are used to chelate metals in the cytosol and to sequester them in specific subcellular compartments. A large number of small molecules are also involved in metal chelation inside the cells, including organic acids, amino acids, and phosphate derivatives (Rausser 1999) (Fig. 2.1).

2.4.1 Phytochelatins

PCs are the best-characterized heavy metal chelators in plants, especially in the context of Cd tolerance (Cobbett 2000). PCs are a family of metal-binding peptides with the general structure (γ -Glu-Cys) $_n$ Gly ($n = 2-11$) (Cobbett and Goldsbrough 2002). They are present in plants and fungi. The cysteine thiol groups allow PCs to chelate metals and form complexes with a molecular weight of 2.5–3.6 kDa (Cobbett 2000). PCs are synthesized in the cytosol and then transported as complexes to the vacuole. Their synthesis is rapidly activated in the presence of heavy metals such as Cd, Cu, Zn, Ag, Au, Hg, and Pb (Rausser 1995; Cobbett 2000). Synthesis involves the chain extension of GS by PCS a constitutively expressed cytosolic enzyme whose activity is controlled post-translationally because the metal ions chelated by PCs are required for enzyme activity (Grill et al. 1989; Cobbett 2000). Due to their metal affinity, PCs are thought to be involved in the homeostasis and trafficking of essential metals such as Cu and Zn (Thumann et al. 1991) and in the detoxification of heavy metals, but they do not seem to be involved in hyperaccumulation (Ebbs et al. 2002). Contrasting evidence has been reported for the role of PCs in heavy metal tolerance. They have a clear role in the response of plants and yeast to Cd, e.g., they are induced rapidly in *Brassica juncea* following the intracellular accumulation of Cd, thus protecting the photosynthetic apparatus despite a decline in transpiration and leaf expansion (Haag-Kerwer et al. 1999). Furthermore, the Cd sensitivity of various *A. thaliana*

mutants correlated with their ability to accumulate PCs (Howden et al. 1995). Cd and Cu treatment also induces the transcription of genes involved in the synthesis of GS, the precursor of PCs (Xiang and Oliver 1998). Transgenic *A. thaliana* plants with low GS levels are more sensitive to Cd, whereas those with elevated GS levels have similar Cd tolerance to wild-type plants (Xiang et al. 2001). Similarly, overexpression of the *Escherichia coli* γ -glutamylcysteine synthetase gene in *B. juncea* increased the synthesis of GS and PCs, resulting in greater Cd tolerance (Zhu et al. 1999).

The *A. thaliana* gene for PC synthase (*CAD1*) was identified by using the Cd-sensitive, PC-deficient *cad1* mutant (Ha et al. 1999). *cad1* mutant produces normal levels of GSH but is deficient in PCs and hypersensitive to Cd (Howden et al. 1995). A *Schizosaccharomyces pombe* mutant with the same characteristics has also been identified (Ha et al. 1999). The expression of *AtPCS1* from *A. thaliana* and *TaPCS1* from wheat in *S. cerevisiae* increases PC synthesis and induces Cd tolerance (Vatamaniuk et al. 1999; Clemens et al. 1999). Furthermore, purified recombinant *A. thaliana* and *S. pombe* PC synthases catalyze the production of PCs from GSH in vitro (Vatamaniuk et al. 1999; Clemens et al. 1999).

To evaluate the role of PCs in the heavy metal stress response, the sensitivity of *cad1* mutants to different metals was analyzed. The *A. thaliana cad1-3* mutant is more sensitive to arsenate and Cd than wild-type plants, while there is no difference considering Zn, selenite, and Ni ions (Ha et al. 1999). In contrast to the *S. pombe pcs* mutant, *cad1-3* was also slightly sensitive to Cu and Hg and showed intermediate sensitivity to Ag (Ha et al. 1999). The role of PCs in Cu tolerance remains to be determined. Studies of the copper-tolerant plant *Mimulus guttatus* confirmed a role for PCs in Cu tolerance (Salt et al. 1989). In contrast, Cu-sensitive and Cu-tolerant ecotypes of *S. vulgaris* produced similar amounts of PC when the root tips were exposed to Cu, suggesting differential tolerance arises from other mechanism (Schat and Kalff 1992; De Knecht et al. 1994). Therefore, although many evidences for the role of PCs in plant response and detoxification are reported, not all studies have supported an effective role of PCs in metal tolerance.

It is also notable that excessive PC levels in transgenic plants increase the accumulation of heavy metals without enhancing tolerance (Pomponi et al. 2006) and can even confer hypersensitivity to heavy metals. Indeed, an excessive expression of *AtPCS* genes confers a hypersensitivity to Cd stress (Lee et al. 2003). This probably reflects additional important roles for PCs in the cell, such as essential metal homeostasis, antioxidant mechanisms, and sulfur metabolism (Rausser 1995; Dietz et al. 1999; Cobbett 2000). Their role in heavy metal stress response probably may be a side effect of these functions (Steffens 1990).

The final stage of PC activity, particularly in the Cd response, involves their accumulation as complexes in the vacuole (Salt et al. 1998), where they eventually form HMW complexes after incorporation of S^{2-} . PC-Cd complexes are transported into the vacuole by Cd/ H^+ antiporters and ATP-dependent ABC transporters in the tonoplast (Salt and Wagner 1993; Salt and Rausser 1995). In *S. pombe*, a Cd-sensitive mutant has been isolated; this strain can synthesize PCs but not accumulate the Cd-PC-sulfide complexes (Ortiz et al. 1992).

The mutant results to have a mutation in the gene *hmt1* that encodes for an ABC-type transporter, suggesting that this gene mediated transport and compartmentalization of heavy metals. Similar ABC-type transporters may also be involved in the compartmentalization of Cd in higher plants (Salt and Rauser 1995).

PCs also mediate the root-to-shoot transport of Cd. Transgenic *A. thaliana* plants expressing wheat *TaPCS1* accumulate small amounts of Cd in the roots but have an increased Cd transport to the shoot, reflecting the increased transport efficiency (Gong et al. 2003).

2.4.2 Metallothioneins and Ferritins

Like PCs, MTs are a major family of LMW cysteine-rich metal-binding peptides. MTs have been found in many organisms, although the MTs in plants differ considerably from those found in mammals and fungi. As they contain mercaptide groups they are able to bind metal ions. *Class 1* MTs contain cysteine motifs that align with mammalian MTs, whereas *Class 2* MTs contain similar cysteine clusters but they do not align with *Class 1* MTs (Robinson et al. 1993). *Class 1* MTs are characterized by the exclusive presence of Cys–X–Cys motifs, whereas in *Class 2* MTs both Cys–Cys and Cys–XX–Cys pairs are located in the N-terminal domain (Robinson et al. 1993). In vertebrates, MTs contain a stretch of 20 highly conserved cysteine residues, whereas plant and fungal MTs do not contain this motif (Cherian and Chan 1993).

In *S. cerevisiae* the MT-encoding gene *CUP1* is synthesized and activated by metal ions, such as Cu (Füerst et al. 1988). In plants, MTs are induced by various abiotic stresses but are also expressed during development (Rauser 1999). In wheat and in rice, MTs are induced by metal ions, such as Cu and Cd, and by abiotic stresses such as temperature extremes and nutrient deficiency (Cobbett and Goldsbrough 2002). Plant MTs sequester excess of metals by coordinating metal ions with the multiple cysteine thiol groups (Robinson et al. 1993), and have particular affinity for Zn^{2+} , Cu^+ , and Cu^{2+} as shown by the expression of the pea gene *PsMTa* in *E. coli* (Tommey et al. 1991). *A. thaliana* MT gene expression is activated in response to Cu and Cd treatment, but not by Zn, e.g., MT2 is expressed in response to Cu, but only marginally in the presence of Cd and Zn (Zhou and Goldsbrough 1994). In *A. thaliana*, MT1a and MT2a are expressed in the trichomes and the phloem, indicating they take part in both heavy metal sequestration and in metal ion transport (García-Hernández et al. 1998). *A. thaliana mt1a* mutants are hypersensitive to Cd and accumulate much lower levels of As, Cd, and Zn than wild-type plants, showing that MTs play a role in both metal tolerance and metal accumulation (Zimeri et al. 2005). This is supported by overexpression experiments, e.g., *Vicia faba* guard cells overexpressing *A. thaliana AtMT2a* and *AtMT3* can tolerate higher levels of Cd than untransformed cells (Lee et al. 2004). Similarly, the overexpression of *CcMT2* from legume *Cajanus cajan* in *A. thaliana* induces both Cd and Cu tolerance and

allows both metals to accumulate without affecting the expression of endogenous transporters (Sekhar et al. 2011).

Although animal and fungal MTs have a clear role in heavy metal detoxification (Hamer 1986) the precise relationship between plant MTs and heavy metals is unknown (Zhou and Goldsbrough 1994; Zenk 1996; Giritch et al. 1998). MTs are known to participate in Cu homeostasis (Cobbett and Goldsbrough 2002) and *A. thaliana* MT1 and MT2 complement the *S. cerevisiae* MT-defective *cup1* mutant and confer Cd tolerance (Zhou and Goldsbrough 1994). *A. thaliana* MT2 can also partially rescue the Zn hypersensitivity of a *Synechococcus amtA* mutant, which is deficient for an endogenous Zn^{2+} -MT gene (Robinson et al. 1996). The expression of pea MT type I as a GSH transferase fusion in *E. coli* increases Cu accumulation (Evans et al. 1992), and the expression of *B. juncea* MT2 in *A. thaliana* enhances Cd and Cu tolerance (Zhigang et al. 2006). In contrast, MT2 expression is delayed in *B. juncea* plants exposed to Cd although there is a rapid induction of PC biosynthesis (Haag-Kerwer et al. 1999). These results indicate that there is no correlation between MT2 expression and Cd accumulation in leaves and the precise role of MTs in plants under heavy metal stress remains to be established. MTs probably have different functions in response to different heavy metals and could also participate in additional antioxidant protection mechanism and plasma membrane repair (Hamer 1986).

Ferritins are ubiquitous multimeric proteins that can store up to 4500 iron atoms in a central cavity (Harrison and Arosio 1996). Animal ferritins can also store other metals, including Cu, Zn, Cd, Be, and Al (Price and Joshi 1982; Dedman et al. 1992) whereas plant ferritins have only been shown to store Fe. Plant ferritins are synthesized in responses to various environmental stresses, including photoinhibition and iron overloading (Murgia et al. 2001, 2002). Ferritin gene expression in plants is dually regulated by ABA and by antioxidants and serine/threonine phosphatase inhibitors (Savino et al. 1997). Ferritins are therefore a front-line defense mechanism against free iron-induced oxidative stress (Ravet et al. 2009). The major function of plant ferritins is not to store and release iron, as previously reported, but to scavenge free reactive iron and prevent oxidative damage (Ravet et al. 2009).

2.4.3 Organic Acids, Amino Acids, and Phosphate Derivatives

Organic acids and amino acids can bind heavy metals and may therefore be deployed in response to metal toxicity (Rausser 1999). However, a clear correlation between heavy metal accumulation and the production of these compounds has not been established. Organic acids such as malate, citrate, and oxalate confer metal tolerance by transporting metals through the xylem and sequestering ions in the vacuole, but they have multiple additional roles in the cell (Rausser 1999).

Citrate, which is synthesized in plants by the enzyme citrate synthase, has a higher capacity for metal ions than malate and oxalate, and although its principal

role is to chelate Fe^{2+} it also has a strong affinity also for Ni^{2+} and Cd^{2+} (Cataldo et al. 1988). Malate is a cytosolic Zn-chelator in zinc-tolerant plants (Mathys 1977).

Also amino acids and derivatives are able to chelate metals conferring to plants resistance to toxic levels of metal ions. Histidine is considered the most important free amino acid in heavy metal metabolism. Thanks to the presence of carboxyl, amino, and imidazole groups, it is a versatile metal chelator, which can confer Ni tolerance and enhance Ni transport in plants when supplied in the growth medium, perhaps reflecting its normal role as a chelator in root exudates (Callahan et al. 2006). Histidine levels also increase in the xylem of *Alyssum lesbiacum*, a Ni hyperaccumulator, when the plant is exposed to Ni (Kramer et al. 1996).

The amino acid derivative NA is an aminocarboxylate synthesized by the condensation of three S-adenosyl-L-methionine molecules in a reaction catalyzed by NA synthase (Shojima et al. 1990). NA chelates Fe, Cu, and Zn in complexes (Stephan et al. 1996) and then accumulates within vacuoles (Pich et al. 1997); it is not secreted from the roots (Stephan and Scholz 1993). NA is also involved in the movement of micronutrients in plants (Stephan and Scholz 1993). The physiological role of NA has been studied extensively in the tomato mutant *chloronerva*, which lacks a functional NA synthase gene and is characterized by the abnormal distribution and accumulation of Fe (Scholz et al. 1985) and Cu (Herbik et al. 1996). NA is also the precursor of the phytosiderophore mugineic acid, which binds Zn^{2+} , Cu^{2+} , and Fe^{3+} (Treeby et al. 1989). This derivative is synthesized in grasses by the deamination, reduction, and hydroxylation of NA (Shojima et al. 1990).

Phytate (myo-inositol hexakisphosphate) is the principal form of reserve phosphorous in plants (Hocking and Pate 1977) and is often localized in the roots and seeds (van Steveninck et al. 1993; Hubel and Beck 1996). The molecule comprises six phosphate groups which allow the chelation of multiple cations, including Ca^{2+} , Mg^{2+} , and K^{+} , but also Fe^{2+} , Zn^{2+} and Mn^{2+} (Mikus et al. 1992). The distribution of phytate and its ability to chelate multiple metal species suggest it could be mobilized as a detoxification strategy. In support of this, the addition of Zn to the culture medium leads to the production of Zn^{2+} -containing phytate globoids in the root endoderm and pericycle cells of certain crops (van Steveninck et al. 1993). Therefore, a controlled synthesis or mobilization of phytate in these cell layers plays a key role in metal ion loading to the aerial parts of plants.

2.5 Metal Sequestration in the Vacuole by Tonoplast Transporters

When metal ions are accumulated in excess inside the cytosol, plants have to remove them in order to minimize their toxic effects. Plants respond to high intracellular concentrations of metal ions by using efflux pumps either to export the ions to the apoplast (as discussed above) or to compartmentalize them within the

cell. The main storage compartment for metal ions is the vacuole, which in plants accounts for up to 90% of the cell volume (Vögeli-Lange and Wagner 1990). Several families of intracellular transporters involved in this process have been identified in plants and yeast and they appear to be highly selective.

2.5.1 The ABC Transporters

ABC transporters can transport xenobiotics and heavy metals into the vacuole, and two subfamilies (MRP and PDR) are particularly active in the sequestration of chelated heavy metals. Plant cell vacuoles are, in fact, the major site for accumulation and storage of PC-Cd complexes. PC-Cd complexes are generated in the cytosol and are then translocated by ABC transporters (Vögeli-Lange and Wagner 1990). In the vacuoles more Cd and sulfide are incorporated to form HMW complexes, the main Cd storage form. The first vacuolar ABC transporter HMT1 was identified by its ability to complement a *S. pombe* mutant that cannot produce HMW complexes (Ortiz et al. 1992). HMT1 is localized in the tonoplast and transports PC-Cd complexes into the vacuole in a Mg-ATP-dependent manner (Ortiz et al. 1995). A similar protein has been identified in oat roots, but HMT1 homologs are yet to be found in other plants (Salt and Rauser 1995). In *S. cerevisiae*, the tonoplast ABC pump YCF1 transports Cd into the vacuole as a bis(glutathionato)Cd complex, and confers Cd tolerance (Li et al. 1997). MRP-related sequences like YCF1 have been found in *A. thaliana* (Lu et al. 1997, 1998) and are the most likely candidates for PC-Cd transport across the tonoplast because HMT1 homologs are scarce in plants. In *A. thaliana*, two transporters belonging to the ABC family, AtMRP1 and AtMRP2 have been shown to transport PC-Cd complexes into the vacuole but the role of AtMRP3 in Cd transport remains unclear (Lu et al. 1997, 1998). AtMRP3 partially complements yeast $\Delta ycf1$ mutants but there is no evidence of PC-Cd transport into the vacuole (Tommasini et al. 1998). Moreover, many plants produce Mg-ATP-dependent transporters of GS-S-conjugates (Martinoia et al. 1993).

2.5.2 The CDF Transporters

Members of the CDF transporter family (also called MTPs in plants) are involved in the transport of metal ions from the cytoplasm to the vacuole (Krämer et al. 2007), and to the apoplast and endoplasmic reticulum (Peiter et al. 2007). CDF transporters have been characterized primarily in prokaryotes (Nies 1992) but are also found in many eukaryotes, where they transport divalent metal cations such as Zn, Cd, Co, Fe, Ni, and Mn (Montanini et al. 2007). Eukaryotic CDF transporters are characterized by six transmembrane domains, a C-terminal cation efflux domain, and a histidine-rich region between transmembrane domains IV and V (Mäser et al. 2001) that may act as a sensor of metal concentration (Kawachi et al.

2008). The CDF family can be divided into four phylogenetic groups (Mäser et al. 2001), but groups I and III are the most interesting in plants since these are the ones involved in metal tolerance and accumulation (Krämer et al. 2007). The *A. thaliana* ZAT1 transporter (later renamed AtMTP1) is closely related to the animal ZnT Zn transporter and its function is the vacuolar sequestration of Zn (van der Zaal et al. 1999). The gene is constitutively expressed and is not induced by Zn, but its overexpression in transgenic plants exposed to high levels of Zn confers resistance to Zn toxicity and leads to Zn accumulation in the roots without altering Cd sensitivity (van der Zaal et al. 1999). These data suggest that AtMTP1 transports Zn into the vacuole and may represent a Zn tolerance mechanism. Another tonoplast transporter, AtMTP3, is also involved in the transport of Zn into the vacuole (Kramer et al. 2007).

2.5.3 The HMA Transporters

As stated above, P_{1B}-ATPases (HMAs) are involved in the efflux of metal ions from the cytoplasm, and those on the tonoplast (such as AtHMA3) are thought to contribute to Cd and Zn homeostasis by sequestration into the vacuole (Krämer et al. 2007). However, AtHMA3 may play a role in the detoxification of a wider range of heavy metals through storage in the vacuoles, because overexpression induces tolerance to Cd, Pb, Co, and Zn (Morel et al. 2009).

2.5.4 CaCA Transporters

The CaCA superfamily is ubiquitous in both prokaryotes and eukaryotes and is an integral component of Ca²⁺ cycling systems that involve the efflux of Ca²⁺ across membranes against a concentration gradient. This is achieved by using a counter-electrochemical gradient of other ions, such as H⁺, Na⁺, or K⁺ (Emery et al. 2012). Examples of members of the CaCA families that may be involved in metal homeostasis are MHX and CAX. MHX is a vacuolar Mg²⁺ and Zn²⁺/H⁺ antiport expressed predominantly in xylem-associated cells; overexpression in tobacco increases sensitivity to Mg and Zn although the concentration of these metals in shoots is unchanged (Shaul et al. 1999). The CAX family are Ca²⁺/H⁺ antiports that also recognize Cd²⁺, suggesting this is an important route for Cd sequestration in the vacuole (Salt and Wagner 1993). In *A. thaliana*, only CAX proteins such as AtCAX2 and AtCAX4 seem to be involved in the vacuolar accumulation of Cd. The overexpression of AtCAX2 and AtCAX4 results in the accumulation of more Cd in the vacuoles (Korenkov et al. 2007). AtCAX4 is expressed mainly in root tips and primordia and is induced by Ni and Mn. Root growth in response to Cd and Mn is altered in *cax* mutants whereas overexpression induces symptoms that are compatible with Cd accumulation (Mei et al. 2009).

2.5.5 NRAMP Transporters

NRAMP transporters such as AtNRAMP3 and AtNRAMP4 are localized in the tonoplast and are probably functionally redundant. The *nramp3 nramp4* double mutant has a Fe-deficient phenotype in seedlings that can be rescued by providing excess Fe, although the Fe content is the same as in wild-type plants suggesting that AtNRAMP3 and AtNRAMP4 are required to mobilize Fe from the vacuole (Thomine et al. 2003; Lanquar et al. 2005). The overexpression of AtNRAMP3 increases Cd sensitivity (Thomine et al. 2000) and reduces the accumulation of Mn (Thomine et al. 2003), indicating a possible role in the homeostasis of metals other than Fe.

2.6 Oxidative Stress Defence Mechanisms and the Repair of Stress-Damaged Proteins

If the intracellular concentration of metal ions saturates the defense mechanisms discussed above then the plant will begin to suffer oxidative stress caused by the production of ROS and the inhibition of metal-dependent antioxidant enzymes (Schützendübel and Polle 2002). Under these circumstances, plants activate their antioxidant responses, including the induction of enzymes such as CAT and SOD and the production of non-enzymatic free radical scavengers. There are many examples of this process, such as the induction of APX and CAT in *Nicotiana plumbaginifolia* leaves exposed to excess Fe (Kampfenkel et al. 1995b), and the induction of CAT3 in *B. juncea* plants exposed to Cd (Minglin et al. 2005). In pea plants, Cd causes the oxidation of CAT thus reducing its activity, so the plant responds by upregulating the transcription of the corresponding gene (Romero-Puertas et al. 2007). The tomato *chloronerva* mutant is NA-deficient and contains abnormally high levels of Fe and Cu in leaves, resulting in the activation of antioxidant enzymes such as cytosolic APX and Cu/Zn SOD (Pich and Scholz 1993; Herbick et al. 1996). SOD activity is also induced in tomato seedlings after prolonged Cd treatment (Dong et al. 2006). SOD activity also increases significantly in wheat leaves, but only following exposure to high levels of Cd, probably reflecting the accumulation of superoxide (Lin et al. 2007). Nevertheless, previous studies have shown that SOD activity is reduced in pea plants exposed to Cd toxicity (Romero-Puertas et al. 2007). An increase in APX mRNA is also observed in *Brassica napus* cotyledons subjected to toxic levels of Fe (Vansuyt et al. 1997). Several metals are able to induce Fe and Mn-SOD in plants (del Río et al. 1991).

The production of ROS is also countered by the activation of the ascorbic acid-GS scavenging system. In *Phaseolus vulgaris* and *Pisum sativum*, Cd treatment induces APX (Romero-Puertas et al. 2007). In addition, GR activity, another enzyme taking part in the cycle, is upregulated in roots and acts as a defence mechanism against Cd-generated oxidative stress (Yannarelli et al. 2007).

GS plays a key role in metal tolerance, because it can act as a ROS scavenger, metal chelator and as a substrate for PC biosynthesis (Krämer 2010). Indeed, the expression of the *E. coli* GSS gene *gshII* in *B. juncea* increased Cd tolerance in the seedlings and increased the capacity for Cd accumulation in adult plants (Zhu et al. 1999).

Heavy metals, in particular Cd, reduce the GSH/GSSG ratio and activate antioxidant enzymes such as SOD and GR (Romero-Puertas et al. 2007). GSH (Glu-Cys-Gly) is the major intracellular antioxidant inside the cell and is the precursor of PCs (Cobbett 2000). It can also form complexes with metal ions, particularly Cd (Wójcik and Tukiendorf 2011). GS synthesis is activated in response to heavy metal stress.

In common with other forms of abiotic stress, heavy metals induce the synthesis of stress-related proteins and signaling molecules, such as HSPs, SAPs, salicylic and abscisic acids, and ethylene. HSPs are found in all cells and are expressed not only in response to elevated temperatures but also to other stresses, including heavy metals, where they protect and repair proteins, and act as molecular chaperones to ensure correct folding and assembly (Vierling 1991). For example, heavy metals induce the expression of low molecular weight HSPs in rice (Tseng et al. 1993), and in cell culture of *S. vulgaris* and *Lycopersicon peruvianum* (Wollgiehn and Neumann 1999), the latter also producing the larger protein HSP70 (Neumann et al. 1994). HSP70 is localized in the nucleus and the cytoplasm, and is also found at the plasma membrane suggesting a protective role for membranes. These observations suggest that HSPs could have an important role in heavy metal response mechanism involving plasma membrane protection and in the repair of stress-damaged proteins.

The SAPs contain A20 or AN1 zinc finger domains (sometimes both) and, like HSPs, also respond to multiple abiotic stresses in plants, including cold, drought, salt, heavy metals, hypoxia, and wounding; they may function as transcriptional regulators or by direct protein–protein interactions (Dixit and Dhankher 2011). AtSAP10 is expressed in *A. thaliana* roots and is induced within 30 min by exposure to As, Cd, and Zn (Dixit and Dhankher 2011). Plants overexpressing AtSAP10 are metal tolerant, they accumulate Ni and Mn in the shoots and roots but there is no change in Zn content (Dixit and Dhankher 2011).

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