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Chapter 9
Biochemical and Functional Responses of *Arabidopsis thaliana* Exposed to Cadmium, Copper and Zinc

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Abstract Phytoremediation has been accepted advantageous over commonly used civil engineering remediation methods in costs, practice and the scale at which the processes operate. Understanding the metabolic answer and the adaptation of plants towards toxic metal exposure opens the way to future phytoremediation of contaminated sites. The majority of these metals get accumulated in plants and may either directly or indirectly find their way into the food chain causing severe secondary consequences. In particular, excess cadmium (Cd), copper (Cu) and zinc (Zn) are known to induce stress effects in all plant species. However, while Cu and Zn are normally present in different soils, and are part of or act as cofactors of many cell macromolecules, plants have no metabolic requirement for Cd. *Arabidopsis thaliana* L. is considered a model plant for many studies as its genomic sequence was completely identified and its mechanisms in genomic, transcriptomic and proteomic regulation are often similar to other plant species. The molecular, biochemical, physiological and morphological characteristics of this species are strongly affected by the exposure to Cd, Cu and Zn. The aim of this work is to give an up-to-date overview on the recent breakthroughs in the area of responses and adaptation of *A. thaliana* to Cd, Cu and Zn, three of the most common metals found in polluted soils, both alone and in combination. This chapter aims to contribute to a better understanding of the fundamental aspects of detoxification of metals and general responses in phytoremediation. The numerous and easily available genetic resources developed in *A. thaliana* should be extended to fast growing plant species of high biomass having significant tolerance to metals and suitable for phytoremediation purposes.

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• Phytoremediation • Zinc

Abbreviations

Cd   Cadmium
CKs   Cytokinins
Cu   Copper
GSH   Reduced glutathione
IAA   Indole-3-acetic acid
MTs   Metallothioneins
PCS   Phytochelatin synthase
PCs   Phytochelatins
Zn   Zinc

9.1   Introduction

Excess cadmium (Cd), copper (Cu) and zinc (Zn) are known to induce stress
effects in all plant species. However, while Cu and Zn are normally present in
different soils, and are part of or act as cofactors of many cell macromolecules
(e.g., Cu/Zn-proteins and cytochromes), plants have no metabolic requirement for
Cd. Cadmium levels in soils are generally low (an average of 0.3 μM) and, for this
reason, plants usually do not experience this metal under normal environmental
conditions. Nevertheless, Cd is efficiently absorbed by plant roots, translocated *via*
xylem, and compartmentalized in vacuoles, and it influences the transcription of
several genes (Vangronsveld et al. 2009; Cuypers et al. 2010).

Elements, such as Cu, Zn and Cd belong to the group of so-called 'heavy
metals'. In small amounts, most of these elements are indispensable for many
organisms, but their increased doses induce acute or chronic poisoning. Some
environments, such as the serpentine soils, have naturally high concentrations of
metals. Moreover, mining and industrialization have also led to soils with increased
metal contents. In recent years, phytoremediation techniques have been applied on
Cd and other metals as well as on organics (Vangronsveld et al. 2009). Many
studies have been focusing on plant metal uptake and transport, their entry into
regular metabolic channels of the plant, and plant metal chelation and sequestration.
Furthermore, important responses induced by Cu, Zn and Cd, such as the synthesis
of phytochelatins (PCs) and metallothioneins (MTs), were observed in many plant
species (Kvesitadze et al. 2006).

Metals enter the plant cells from air, soil and water, but plants absorb
contaminants primarily through their roots and leaves, whose absorption differ
essentially from each other. Due to their positive charge, Cd, Zn and Cu are present
mainly as soil-bound cations or complexed in organic molecules (e.g., humic/fulvic

acids, extracellular enzymes, aliphatic and aromatic hydrocarbons, alcohols, phenols, amines, etc.), and so they enter the roots dissolved in water, like nutrients, and they move towards the transport tissue (xylem) mainly along the apoplast. To a lesser extent, Cd, Zn and Cu are bound to negatively-charged dust particles, so plants interact with these metals during their precipitation from the atmosphere when they penetrate into leaves via the cuticle (Kvesitadze et al. 2006). Once absorbed by roots and leaves, environmental contaminants are translocated to different plant organs by the same physiological processes transporting nutrients.

The plant's abilities to absorb, conjugate, compartmentalize and accumulate metals within its cells determines the ecological detoxification potential of a species. High biomass production, well-developed roots, and a strong defense system are the most important overall criteria for plants to be successfully applied in phytoremediation processes of metal contaminated soils (Kabata-Pendias and Mukherjee 2007). Some plant species are able to accumulate high amounts of metals in their roots and subsequently translocate them to the above-ground organs (the so-called "phytoextraction"), eliminating in this way contaminants from the soils, sediments and sludges. There are several plant species that not only tolerate large quantities of metals but hyperaccumulate them. Hyperaccumulators are defined as plants that can accumulate 10,000 μg g^{-1} dry weight Zn or Mn, 1,000 μg g^{-1} dry weight Ni, Co, As, Se, or Cu, or 100 μg g^{-1} dry weight Cd, and they have gained great interest as potential sources of genes for developing plants for phytoremediation (Hassinen et al. 2007). As hyperaccumulating plants have extraordinary capabilities of uptake and metal tolerance, they are the best sources of genes to be used for phytoremediation purposes. Despite the recent exploitation of high-throughput methodologies, such as cDNA analysis and microarrays, the overall picture of plant metal tolerance, accumulation, and translocation is far from being complete (Hassinen et al. 2007). It is also noteworthy that other techniques are used to remediate metal-contaminated soils, such as phytostabilization.

Arabidopsis thaliana L. ("*Arabidopsis*" throughout the text) is considered a model plant for many studies as its genomic sequence was completely identified and its mechanisms in genomic, transcriptomic and proteomic regulation are often similar to other plant species. The molecular, biochemical, physiological and morphological characteristics of *Arabidopsis* are strongly affected by the exposure to Cd, Cu and Zn (Tan-Kristanto et al. 2003; Van Belleghem et al. 2007; Remans et al. 2008; Smeets et al. 2009; Semane et al. 2010; Watanabe et al. 2010; Cuypers et al. 2010) (Fig. 9.1). Furthermore, a cumulative role in toxic metal accumulation and tolerance in this species was observed (Verbruggen et al. 2009). *Arabidopsis* offers many advantages in the search for novel genes and for this reason, many studies have been carried out in this species regarding the tolerance and accumulation of metals other than Cd/Cu/Zn, such as iron (Duy et al. 2007; Stacey et al. 2008), lead (Kim et al. 2006; Liu et al. 2009), caesium and strontium (Kanter et al. 2010), aluminum (Goodwin and Sutter 2009), selenium (Zhang et al. 2007; Dutilleul et al. 2008), uranium (Vanhoudt et al. 2008), and arsenic (Li et al. 2006; Singh and Ma 2007), and mercurial derivatives (Bizily et al. 1999; Li et al. 2006).
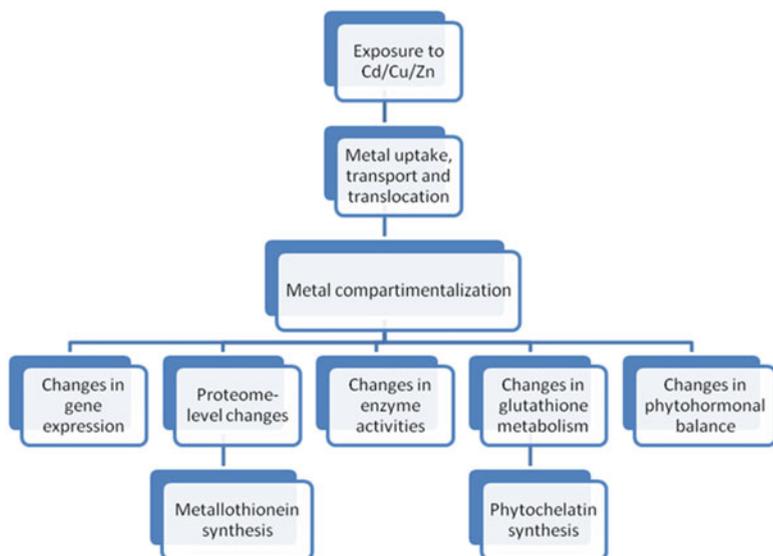
Fig. 9.1 Effects of cadmium, copper and zinc on *Arabidopsis thaliana* (Data from Sanità di Toppi et al. 2003; Tan-Kristanto et al. 2003; Van Belleghem et al. 2007; Remans et al. 2008; Smeets et al. 2009; Semane et al. 2010; Watanabe et al. 2010; Cuypers et al. 2010)

The great importance of the studies on *Arabidopsis* responses to metals is because of the fact that it belongs to the family of Brassicaceae (Cruciferae), whose cultivated species are known to be good accumulators of toxic metals, allocating large amounts of most toxic metals to above-ground organs. Nowadays, about 400 plant species are known to hyperaccumulate metals, and an important part of them belong to the family of Brassicaceae (Pence et al. 2000; Wójcik et al. 2005a, b; van de Mortel et al. 2006; Courbot et al. 2007; Gasic and Korban 2007; Hassinen et al. 2007; Mijovilovich et al. 2009; Vangronsveld et al. 2009). Furthermore, many genera of Brassicaceae (e.g., *Brassica*, *Alyssum*, *Arabis*, *Arabidopsis*, *Berkheya, Bornmuellera, Cardamine*, *Cochlearia, Peltaria*, *Pseudosempervivum, Stanleya*, *Streptanthus*, and *Thlaspi*) are well known in terms of their utility in toxic metals-remediation strategies. In particular, the oleiferous genus *Brassica* includes good Cd/Cu/Zn-accumulating species and it also has a great agronomic importance, being the third most important source of vegetable oil in the world after palm and soybean oil. Finally, the basic biology, ecology, population genetics and molecular evolution are often similar among different plant species. Therefore, the wealth of functional and genomic tools of *Arabidopsis* could be applied to gain insights into adaptive evolution of ecologically important traits and genome-wide processes at the basis of metal uptake and compartmentalization among other plant species (Clauss and Koch 2006; Przedpełska and Wierzbicka 2007).

On this basis, the aim of this work is to give an up-to-date overview on the recent breakthroughs in the area of responses and adaptation of *Arabidopsis* to Cd,

Cu and Zn, three of the most common metals found in increased concentrations in contaminated soils, both alone and in combination. As *Arabidopsis* is a model plant for many genomic, transcriptomic and proteomic studies, this chapter could be important for a better understanding of the fundamental aspects of detoxification of metals (in terms of tolerance and accumulation potential) and for their use in phytoremediation approaches.

9.2 The Environmental Impact of Cadmium, Copper and Zinc

9.2.1 Cadmium

Cadmium is a major environmental contaminant that enters human food via accumulation in crop plants (Kabata-Pendias and Mukherjee 2007), and is considered as being one of the most ecotoxic metals that exhibits adverse effects on all biological processes in humans, animals, and plants (Cuypers et al. 2010). It is produced mainly as a by-product in mining and refining of Zn, with uses in the production of batteries, pigments, coatings and stabilizers. In uncontaminated soils, its content is highly governed by soil texture and ranges from 0.22 to 0.51 mg kg^{-1} dry soil. In contaminated soils (>1 mg kg^{-1}), the major sources of pollution are atmospheric deposition and P-fertilizers. Contents of Cd in plants vary in the range of 5–400 µg kg^{-1} dry matter, and are higher in roots than in shoots. The behavior of Cd in plants is closely related to Zn as both metals are bivalent cations with a high affinity for sulfur, particularly sulphydryl groups. Some researchers have reported on the association of Cd with other elements in plants (e.g., Zn, Cu, Se, P, Cl) and have highlighted the key role of pH, carbonates, and organic carbon in Cd soil bioavailability (Kabata-Pendias and Mukherjee 2007). The accumulation or exclusion of Cd by plants differ at the family levels, but *Brassicaceae* and *Fabaceae* species show the highest and the lowest tolerance to Cd, respectively (Kabata-Pendias and Mukherjee 2007).

9.2.2 Copper

Copper is used for the production of conductor materials and in the manufacturing of motors and electrical equipment, and for the fabrication of household articles, coins, art objects and ammunition (Kabata-Pendias and Mukherjee 2007). Copper is also widely used in agriculture (fertilizers, pesticides etc.) and, due to its bacteriostatic properties, it is also used as a feed additive in livestock and poultry nutrition. The general values for the average total Cu contents in soils of different types are reported to range between 20 and 30 mg kg^{-1}. The phytoavailability of Cu is influenced by its chemical form, and is not a function of its total concentration

but of several soil variables, such as pH, oxidation and reduction potential, organic matter, soil texture, mineral composition, temperature, and water regime. Contaminated soils contain up to 5,000 mg kg^{-1} of Cu, whereas Cu content in plants usually ranges from 3 to 15 mg kg^{-1}. Plants growing on Cu-polluted sites tend to accumulate increased amounts of this metal (up to 1,000–10,000 mg kg^{-1} in some plants of the Labiatae family), especially near industrial areas, and in soils treated with Cu-bearing herbicides. Copper is only slightly mobile in plants, as it is strongly bound by nitrogen and proteins.

9.2.3 Zinc

Zinc is an important component of various alloys and is a catalyst in different chemical productions (e.g., rubber, pigments, plastic, lubricants, and pesticides). It is used in the industry of batteries, automotive equipment, medical applications, and pipes and household devices (Kabata-Pendias and Mukherjee 2007). This metal, compared to Cd, has a relatively low toxicity for humans, but the ingestion or inhalation of larger doses of Zn, especially in forms of inorganic compounds, can be harmful to individuals. The micronutrient Zn has an essential role in physiological and metabolic processes in plants as a cofactor or as a structural element in 300 catalytic and noncatalytic proteins, but it is very toxic when available in elevated amounts (van de Mortel et al. 2006). In uncontaminated soils, Zn contents averages from 10 to 300 mg kg^{-1}, and silicates, carbonates, phosphates, oxides and organic matter may contribute to Zn retention. In strongly contaminated soils, where Zn can reach levels from 1,000 to 10,000 mg kg^{-1}, the metal derives from atmospheric deposition, fertilizers, pesticides, sewage sludge, leaching from galvanized materials, manure, waste, slag and ashes. Contents of Zn in food plants varies in the range of 18–47 mg kg^{-1}, and it is higher in roots than in foliage. Sensitive terrestrial plants die when soil Zn concentration exceeds 100–200 mg kg^{-1} soil, but some species are known to hyperaccumulate Zn, as for example *Thlaspi* species that can contain above 10,000 mg Zn kg^{-1} and were applied for the phytoremediation of contaminated soil (Sarret et al. 2002; McGrath et al. 2006). The mobility of Zn within plants highly varies depending on species and plants' nutritional status.

9.3 Metal Uptake, Transport and Translocation

9.3.1 Occurrence of Metal Ions Throughout the Plant

As leaf Cd concentrations in excess of 5–10 μg g^{-1} dry matter are toxic to most plants, they have evolved mechanisms to limit Cd translocation to the shoot (Sanità di Toppi et al. 2003). Generally, the presence of Cd in the rhizosphere

inhibits root elongation and influences root anatomy, but apoplastic movement of Cd to the xylem can be restricted by the development of the exodermis, endodermis, and other extracellular barriers (Lux et al. 2011).

Making use of nuclear microscopy techniques (NMP), Ager et al. (2002, 2003) studied leaves of wild type and transformed lines of *Arabidopsis* overexpressing the *Atcys-3A* gene (Domínguez-Solís et al. 2001) grown in a Cd-enriched Hoagland medium. They proved that Cd is preferentially sequestered in the central region of epidermal trichomes, where it is likely complexed to phosphate and sulfur. The fact that *Arabidopsis* trichomes participate in metal detoxification has also been suggested by Wienkoop et al. (2004), who identified proteins involved in sulfur metabolism and detoxification in these cells by specific cell sampling and shotgun peptide sequencing (nano LC/MS/MS). The preference for Cd localization in the peripheral parts of leaf blades was confirmed by Wójcik and Tukiendorf (2004).

Van Belleghem et al. (2007) examined the subcellular Cd localization in roots and leaves of *Arabidopsis* exposed to different Cd levels (from 0 to 50 μM) by means of energy-dispersive X-ray microanalysis (EDXMA). They found that in the root cortex Cd is associated with phosphorus (Cd/P) in the apoplast, and sulfur (Cd/S) in the symplast, and that the transport route of Cd through the cortex is mainly apoplastic. In the endodermis, where Cd transport is forced through symplast, sequestration of Cd/S was present in cells as granular deposits. In the central cylinder, Cd transport occurred mainly in the apoplast. Furthermore, large amounts of precipitated Cd in the phloem suggest that Cd re-translocation from the shoot occurs. In leaves, Cd was detected in tracheids but not in the mesophyll tissue. Extensive symplastic and apoplastic sequestration in the root parenchyma combined with re-translocation back to the roots via the phloem confirms the Cd-excluder strategy of *Arabidopsis*. In *Arabidopsis* plants grown on Cd-containing hydroponics (5–100 μM), examined by Wójcik and Tukiendorf (2004) using EDXMA, Cd was not detectable in the cytoplasm, vacuoles and organelles within roots or cell walls of tissues other than the pericycle, so confirming the preference of the apoplastic route for Cd transport (Van Belleghem et al. 2007).

The situation in the hyperaccumulator species *Thlaspi caerulescens*, related to *Arabidopsis*, is quite different, as Cd in roots is mainly located in cortex parenchyma cells, endodermis, parenchyma cells of the central cylinder and xylem vessels, whereas in leaves it accumulates in the vacuoles of cells lying on the way of water migration from the vascular cylinder to epidermal cells (Wójcik et al. 2005a, b). In this species, Cd is passively transported by the transpiration stream and the mechanisms of Cd detoxification in roots seem to be both apoplastic and symplastic.

Sarret et al. (2002) determined the chemical forms of Zn in the Zn-tolerant and hyperaccumulator *Arabidopsis halleri* and in the non-tolerant and non-accumulator *Arabidopsis lyrata* by combining chemical analyses and X-ray analyses. Plants were grown with various Zn concentrations (100 and 250 μM $ZnSO_4$). In aerial parts of *A. halleri*, Zn was predominantly octahedrally coordinated and complexed to malate. A secondary organic species was identified in the bases of the trichomes,

which contained elevated Zn concentrations, and in which Zn was tetrahedrally coordinated and complexed to carboxyl and/or hydroxyl functional groups.

Kashem et al. (2010) used *A. halleri* grown for 3 weeks in $ZnSO_4$ levels ranging from 0.2 to 2,000 μM. Plants proportionally absorbed Zn excess by roots on the basis of the Zn concentration applied, but they did not show reductions in shoot and root dry weight. The percentage of Zn translocation in shoot varied from 69% to 90% of the total Zn, suggesting that the shoot was the major sink of Zn accumulation in this species. The concentration of Zn found in shoots indicated that *A. halleri* has an extraordinary ability to tolerate and accumulate Zn. In the roots of *A. halleri,* Zn phosphate, Zn malate and Zn citrate were the three Zn species detected. Zinc phosphate was mainly found in both the roots and aerial part of *A. lyrata* – a non-accumulator species.

9.3.2 Metal Transporters in Uptake and Translocation

To absorb and translocate metal ions, plants utilize a large number of membrane transporters (Wintz et al. 2003). P-type H^+-pumps, the organellar proton pumps and many ATP-binding cassette (ABC) proteins are primary active transporters (Ludewig and Frommer 2002; Cobbett et al. 2003a, b) involved in metal transport.

P_{1B}-type ATPases transport metal ions (Cu^+, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, etc.) across biological membranes in plants (Cobbett et al. 2003a, b). *Arabidopsis* is remarkable for its large number of type 1B metal transporting ATPases if compared to other organisms. It is likely that these transporters are involved in the metabolism of Cu and Zn and possibly a third metal (Cobbett et al. 2003a, b). Among *Arabidopsis* P-type pumps, HMA2 (Heavy Metal Associated 2 protein) is responsible for Zn^{2+} efflux from the cells and therefore is required for maintaining low cytoplasmic Zn levels and normal Zn homeostasis (Eren and Argüello 2004). Observations indicate a primary role for HMA2 and HMA4, whose expression is predominantly in the vascular tissues of roots, stems, and leaves, in essential Zn translocation (Hussain et al. 2004; Talke et al. 2006; Courbot et al. 2007). HMA2, has N- and C-terminal domains that can bind Zn ions with high affinity (metal-binding domains, MBDs), but only the N-terminal domain seems to be essential for functioning *in planta* while the C-terminal domain may contain a signal important for the subcellular localization of the protein (Wong et al. 2009; Zimmermann et al. 2009). Besides being essential in Zn translocation, a nearly complete abolition of root-to-shoot Cd translocation resulting from the loss of function of HMA2 and HMA4 in the phytochelatin (PC) -deficient *cad1-3 Arabidopsis* mutant indicates their importance in Cd translocation in *Arabidopsis* (Wong and Cobbett 2009). Both *hma2* and *hma4* mutations also confer increased sensitivity to Cd in PC-deficient mutants of *Arabidopsis*, suggesting that they may also influence Cd detoxification (Hussain et al. 2004). Another protein of this family, HMA7, is involved in Cu^+ transport. While the MBD of HMA7 features a CxxC sequence motif characteristic for

Cu^+ binding sites, those of HMA2 and HMA4 contain a CCxxE motif, unique for plant Zn^{2+}-ATPases (Zimmermann et al. 2009). HMA3, belonging to the P_{1B-2} subgroup, likely plays a role in the detoxification of biological (Zn) and non-biological (Cd, Co and Pb) metals by participating in their vacuolar sequestration (Morel et al. 2009). Abdel-Ghany et al. (2005) identified two previously uncharacterized genes (*PAA1* and *PAA2*) coding for P-type ATPase in *Arabidopsis*, that are located in the chloroplast and are required for sequential Cu transport over the envelope and thylakoid membrane, respectively in order to foresee an efficient photosynthetic electron transport. Another P-type ATPase gene of *Arabidopsis* is *RAN1*, involved in Cu homeostasis (Hirayama et al. 1999). The *ran1* mutants have a non-functional ethylene response phenotype because the ethylene receptors are Cu-dependent proteins.

ABC proteins mediate the transport of substances by coupling the release of chemical energy stored in ATP to substrate translocation. An important ABC protein involved in Cd transport, accumulation and tolerance is *Arabidopsis* MRP7, localized both in the tonoplast and in the plasma membrane. The over-expression of this transporter increases Cd-tolerance and results in enhanced Cd root-to-shoot transport and accumulation in leaf vacuoles, indicating more efficient detoxification (Wojas et al. 2009).

NRAMPs (natural resistance-associated macrophage proteins) have been characterized in animals and plants as divalent transition metal transporters involved in metal metabolism and host resistance to certain pathogens. In *Arabidopsis*, over-expression of *AtNramp3* and *AtNramp4* results in Cd hypersensitivity and in Cd and Fe accumulation. These genes are normally expressed in both roots and aerial parts, and disruption of the *AtNramp3* gene leads to slightly enhanced Cd resistance of root growth (Thomine et al. 2000). For this reason, *AtNramp* genes likely encode metal transporters of both the metal nutrient Fe and Cd. Furthermore, members of the ZIP family, in particular, *ZIP2* and *ZIP4*, are involved in Cu transport, whereas *AtOPT3* participates in the transport of various cations (Cu, Mn, Fe) (Wintz et al. 2003). Recently, Verbruggen et al. (2009) described and summarized exhaustively the strategy that non-hyperaccumulator species like *Arabidopsis* use for Cd uptake and accumulation. In *Arabidopsis*, the ZIP transporter IRT1 seems to be a main entry for Cd.

Two members of the *Arabidopsis* Yellow Stripe-Like (YSL) family, *AtYSL1* and *AtYSL3* codify for two oligopeptide transporter families, predicted to be integral membrane proteins involved in delivery of metal micronutrients to and from vascular tissues (Waters et al. 2006). Indeed, leaf Fe concentrations are decreased in the double mutant, whereas Mn, Zn, and especially Cu concentrations are elevated, whereas in seeds of double-mutant plants, the concentrations of Fe, Zn, and Cu are low. Haydon and Cobbett (2007) have identified Zn-sensitive *Arabidopsis* mutants for the gene *ZINC-INDUCED FACILITATOR 1* (*ZIF1*) that encodes a member of the major facilitator superfamily (MFS) of membrane proteins, one of the two largest families of membrane transporters found on earth. Shoots of *zif1* mutants showed increased accumulation of Zn but not of other metal ions, and overexpression of *ZIF1* confers increased Zn tolerance

and interveinal leaf chlorosis, suggesting that ZIF1 is involved in a novel mechanism of Zn sequestration, possibly by transport of Zn-ligand complexes into vacuoles. van der Zaal et al. (1999) isolated a Zn transporter gene, *ZAT1*, and observed that transgenic *Arabidopsis* plants in which *ZAT1* was over-expressed exhibited enhanced Zn accumulation in roots and increased Zn-tolerance. However, transgenic plants expressing an anti-sense construct showed no altered phenotype. Finally, among further implicated proteins are IRT3 and ZIP10, which have been proposed to contribute to cytoplasmic Zn influx in *Arabidopsis* (Talke et al. 2006).

9.4 Metal Compartmentalization

9.4.1 Proteins and Transporters Involved in Metal Homeostasis

Different proteins and transporters are involved in Cd/Cu/Zn detoxification/ homeostasis in *Arabidopsis*. A complete inventory of *Arabidopsis* metallochaperone-like proteins containing a predicted HMA domain revealed a large family of 67 proteins. In a recent study, Tehseen et al. (2010) found that 45 proteins, the HIPPs, have a predicted isoprenylation site while 22 proteins, the HPPs, do not. Sequence comparisons divided the proteins into seven major clusters (I–VII). Cluster IV is notable for the presence of a conserved Asp residue before the CysXXCys, metal binding motif. Promoter-GUS reporter expression analysis indicated variable spatial expression of these HIPPs, but it is probable that HIPPs have a role in Cd-detoxification, possibly by binding Cd.

Regarding Zn, AtMTP1 has been demonstrated to be a Zn transporter localized in the vacuolar membrane and mediates Zn detoxification and storage by vacuolar sequestration of Zn (Desbrosses-Fonrouge et al. 2005). AtMTP1 is not produced throughout the plant, but primarily in the subpopulation of dividing, differentiating and expanding cells, and contributes to cellular metal accumulation and to basal metal tolerance in cells of growing tissues.

The main detoxification pathway of Cd in roots relies on phytochelatin (PC) complexation (cfr. next section) and vacuolar transport of Cd–PCs complexes of low molecular weight (LMW). In the vacuole, high molecular-weight complexes (HMW) that contain sulphides (S^{2-}) may be formed, but the stability of those complexes is still not well understood. Cadmium can also be transported to the vacuole by the activity of different transporters (cation exchangers, HMA3) or as Cd-GS$_2$ complexes by an unidentified ABC transporter, and part of the vacuolar Cd(II) pool could be refluxed back into the cytosol by NRAMP activity. Metallothioneins (MTs), a particular class of proteins later discussed in detail, can also act as potential Cd ligands in the cytosol.

9.4.2   *Phytochelatins and Metallothioneins*

Phytochelatins (PCs; general formula (γ-Glu-Cys)nGly)), are polymerized chains of glutamic acid and cysteine residues synthesised from reduced glutathione (GSH) (γ-Glu-Cys-Gly) in a reaction mediated by phytochelatin synthase (PCS) (Cobbett 2000; Sanità di Toppi et al. 2003). They are a class of metal-binding peptides playing an important role in metal homeostasis and detoxification, and they represent a major detoxifying pathway for metals in plants and many other organisms (Sanità di Toppi and Gabbrielli 1999; Wójcik and Tukiendorf 2004). Moreover, Cd application induced the biosynthesis of PCs in root and shoot tissues of *Arabidopsis* (Wójcik and Tukiendorf 2004; Połeć-Pawlak et al. 2005). On the other hand, no PC accumulation was detected in Cd-treated *Thlaspi caerulescens* plants (hyperaccumulator strategy), suggesting that naturally selected tolerance in this species is not associated with enhanced PC synthesis (Wójcik et al. 2005a, b; Verbruggen et al. 2009).

In *Arabidopsis*, Cd movement through the root symplast is restricted by the production of PCs and the sequestration of Cd-chelates in vacuoles, and PC deficiency resulted in an increase in shoot Cd concentrations (Wong and Cobbett 2009). Whether long-distance transport of PCs occurs during metal detoxification remains unknown. The findings of Gong et al. (2003) and Chen et al. (2006) showed that transgenic expression of *TaPCS1* (a phytochelatin synthase gene from wheat) in *Arabidopsis* suppresses the metal sensitivity of PC-deficient Arabidopsis mutants *cad1-3*. The same authors demonstrated that PCs can be translocated from roots to shoots, and that the transgenic expression of the *TaPCS1* gene increases long-distance root-to-shoot Cd transport and reduces Cd accumulation in roots.

The *Arabidopsis CAD1* (= *AtPCS1*) gene encodes a PCS, and *cad1* mutants are phytochelatin deficient and Cd hypersensitive (Vatamaniuk et al. 1999; Peterson and Olivier 2006). In *Arabidopsis*, the PCS1 activity is positively related to GSH levels of up to 60 mM (depending on Cd levels), and it is enhanced if the concentration of Cd(II) increases (with maximum activity at approximately 60 μM Cd, depending on GSH levels). *Arabidopsis* PCS1 possesses a Cd(II) binding site where Cd (II) binds to activate the enzyme, but it likely has also a second Cd(II) binding site where Cd(II) binds to induce an inhibitory effect, as demonstrated by mathematical models (Ogawa et al. 2011). Noteworthy, the *Arabidopsis* genome also contains a highly homologous gene *AtPCS2* that encodes a functional PCS apparently non-redundant with *AtPCS1* (Cazalé and Clemens 2001). Localization studies of both *Arabidopsis* PCS forms, whose action is likely cytosolic, revealed a ubiquitous presence of AtPCS1 in *Arabidopsis* seedlings, while AtPCS2 was only detected in the root tip (Blum et al. 2010).

With an over-expression of *Arabidopsis* PC synthase gene (*AtPCS1*), a hypersensitivity reaction due to the very high levels of PCs occurred in plants subjected to Cd (50 or 85 μM $CdCl_2$, and this hypersensitivity was also observed for Zn but not for Cu (Lee et al. 2003a, b). The toxicity could be due to the depletion of GSH to produce PCs or to the increasing energy request (ATP) due to the high PC chelation

and storage in the vacuole. This indicates that PC homeostasis is of key importance for metal chelation in *Arabidopsis*. The overexpression of the same *Arabidopsis* gene in tobacco plants enhances Cd^{2+} tolerance and accumulation but only when GSH is added to the culture medium (Pomponi et al. 2006), highlighting the importance of GSH/PCs balance in the cells. In another study (Lee et al. 2003a, b), transgenic *Arabidopsis* lines were generated following transformation with a construct containing the *AtPCS1* cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35::AtPCS1*). These plants exhibited ~14-fold increase in the level of the *AtPCS1* transcript. After being exposed to 85 μM $CdCl_2$ for a 3-day period, ~30% increase in the level of PC production occurred in transgenic lines if compared to wild-type, but transgenic lines showed higher sensitivity to Cd than wild-type seedlings. In the same work, *Arabidopsis* plants of other transgenic lines exhibited approximately two-fold increase in Cd tolerance compared to wild-type plants. Surprisingly, these six Cd-tolerant lines, showing only ~15% increase in PC content, presented increased Cd accumulation in their shoots. In Cd-tolerant lines, the positive effect of PC may be higher than its negative effect; while, in Cd-hypersensitive transgenic lines the reverse may be true. Lee et al. (2003a, b) concluded that PC has a positive effect in chelating non-essential metals, while it has an unknown negative effect (e.g. chelating essential metals or disrupting disulfide bond). The authors presume that the unknown toxic effect of PCs may be similar to the toxic effects of both Cys and GSH, both components of PCs, as these inhibit plant growth at supra-optimal concentrations.

Tennstedt et al. (2009) indicated a contribution of PCS expression to Zn^{2+} sequestration, using a known PC-deficient *Arabidopsis* mutants (*cad1-3* and *cad1-6*) with respect to Zn homeostasis. They found that PC-deficient mutants show pronounced Zn^{2+} hypersensitivity and significant reduction in root Zn accumulation. Plants grown under control conditions consistently showed PC2 accumulation. Moreover, in wild type plants, Zn^{2+}-elicited (20 μM for 5 days) PC2 accumulation in roots reached about 30% of the level of Cd^{2+}-elicited (0.5 μM for 5 days) PC2 accumulation, suggesting that PC formation is essential for Zn^{2+} tolerance and provides a driving force for the accumulation of Zn. This function might also help explain the occurrence of PCS genes throughout the plant kingdom and in a wide range of other organisms.

Wójcik et al. (2009) found that Cu (ranging from 5 to 50 μM) did not induce phytochelatin accumulation nor significantly affected the GSH level in *Arabidopsis* plants, but it caused changes in the root structure and leaf chloroplasts ultrastructure, suggesting that GSH is not directly involved in Cu detoxification and tolerance in this species.

Metallothioneins (MTs) are proteins found in various eukaryotes having a low molecular weight (less than 10 kD), a large fraction of cysteine residues, and a high metal content with coordination of metal ions in metal-thiolate clusters (Cobbett and Meagher 2002). In the *Arabidopsis* genome, seven functional MT genes have been reported. However, exhaustive analysis of the genome sequence suggests the presence of at least four additional MT genes (Cobbett and Meagher 2002). It was also observed that MT gene expression in *Arabidopsis* could be induced

by Cu and, to a lesser degree, by Zn and Cd. In a comparative study of different *Arabidopsis* genotypes (Murphy and Taiz 1995), variation in Cu tolerance among genotypes, measured by root growth inhibition, was highly correlated with the expression of MT2a in Cu-treated plants. It seems that MTs in *Arabidopsis* are able to play a role in metal tolerance, and their primary role may be in metal homeostasis (Cobbett and Meagher 2002). In the study of Guo et al. (2008), six Arabidopsis MTs (MT1a, MT2a, MT2b, MT3, MT4a, and MT4b) were expressed in Cu- and Zn-sensitive yeast mutants. All four types of MTs provided similar levels of Cu tolerance and accumulation to the yeast mutants, and the type-4 MTs (MT4a and MT4b) conferred greater Zn tolerance and higher accumulation of Zn than other MTs to the mutants. To examine the functions of MTs in plants, the authors studied *Arabidopsis* plants that lack MT1a and MT2b, demonstrating that the lack of MT1a, but not MT2b, led to a 30% decrease in Cu accumulation in roots of plants exposed to 30 μM CuSO$_4$. Furthermore, when MT deficiency was combined with PC deficiency, growth of the *mt1a-2 mt2b-1 cad1-3* triple mutant was more sensitive to Cu and Cd if compared to the *cad1-3* mutant, suggesting that MTs (and MT1a in particular) are important for plant metal homeostasis, and that they function cooperatively with PCs to protect plants from Cu and Cd toxicity. Zhigang et al. (2006) analysed the protective function of a plant type-2 MT after its expression in *Arabidopsis* seedlings. When BjMT2 cDNA was expressed in *Arabidopsis* under the regulation of the 35S promoter, seedlings exhibited an increased tolerance against Cu^{2+} and Cd^{2+} based on shoot growth and chlorophyll content. Analysis of transiently transformed cells of *Arabidopsis* leaves by confocal laser scanning microscopy (CLSM) revealed exclusive cytosolic localization of a BjMT2::EGFP (enhanced green fluorescent protein) fusion protein in control and metal-exposed plant cells. Remarkably, ectopic expression of BjMT2 reduced root growth in the absence of metal exposure, whereas in the presence of 50 or 100 μM Cu^{2+} root growth in control and transgenic lines was identical.

9.5 Effects of the Cd, Cu and Zn at Different Biological Organization Levels

9.5.1 Gene Expression

Different genomic and transcriptomic approaches have been used to investigate the effects of metals on genomic and transcriptomic level (Hassinen et al. 2007). Furthermore, data on genetic markers, analysis of quantitative trait loci and microarray data are publicly available via TAIR (http://www.arabidopsis.org/), where some of the datasets are related to metals. From a combination of these data, it appears that a higher rate of cysteine biosynthesis is required in *Arabidopsis* under Cd stress for a better plant protection or adaptation mechanism. In fact, the transcription of some key genes for cysteine biosynthesis, such as *Atcys-3A*

(cytosolic *O*-acetylserine(thiol)lyase) and *SAT* (serine acetyltransferase) gene family, is significantly induced by exposure of *Arabidopsis* plants to Cd stress, and this is accompanied by increases in cysteine and glutathione (GSH) levels (Barroso et al. 1999; Dominguez-Solís et al. 2001; Howarth et al. 2003). As the employment of cysteine-rich chelating compounds such as PCs is one of the defense systems against toxic metals in plants, Harada et al. (2002) studied Cd stressed ($CdCl_2$ up to 200 μM for 4 h) *Arabidopsis* plants to investigate the response of the genes involved in GSH biosynthesis. They found significant increases in transcripts for ATP sulfurylase, APS reductase and sulfite reductase, all of which are involved in cysteine synthesis, and in total thiols (mainly GSH, PCs and cysteine), suggesting that under Cd stress, *Arabidopsis* activates the sulfur assimilation pathway to provide an enhanced supply of GSH for PC biosynthesis. AtATM3, an ATP-binding cassette transporter of *Arabidopsis*, is a mitochondrial protein involved in the biogenesis of iron-sulfur clusters and iron homeostasis, and its gene is upregulated in roots of plants treated with Cd^{2+} or Pb^{2+} (100 μM $CdCl_2$ or 1 mM $Pb(NO_3)_2$ for 24 h) (Kim et al. 2006). In addition, the authors found that that *AtATM3*-overexpressing or *AtATM3*-constitutively-expressing plants are more tolerant to Cd, whereas *AtATM3* mutant plants were more sensitive to Cd than their wild-type controls. Since non-protein thiols, such as GSH and PCs, are positively correlated with metal resistance and the closest homolog of AtATM3 in fission yeast (*Schizosaccharomyces pombe*), HMT1, is a vacuolar membrane-localized phytochelatin-Cd transporter, Kim et al. (2006) hypothesized that GSH-Cd(II) complexes formed in the mitochondria are exported by AtATM3, hence contributing to Cd resistance in *Arabidopsis*. Transcriptional regulation in response to Cd treatment was also investigated in both roots and leaves of *Arabidopsis* treated with low (5 μM) or high (50 μM) Cd concentrations for 2, 6, and 30 h, using a genome microarray (Herbette et al. 2006). One of the main responses observed in roots was the induction of genes involved in sulfur assimilation-reduction and GSH metabolism. In addition, HPLC analysis of GSH and PC content showed a transient decrease of GSH after 2 and 6 h of metal exposure in roots correlated with an increase of PC contents. Altogether, the results suggested that plants subjected to Cd activate the sulfur assimilation pathway by increasing transcription of related genes to provide an enhanced supply of GSH for PC biosynthesis.

To understand the mechanisms of Cd-induced NO synthesis in roots and leaves of *Arabidopsis*, a microarray analysis was performed by Besson-Bard et al. (2009) in Cd-exposed plants (30 μM $CdCl_2$ for 24 h). The authors found that NO contributes to Cd toxicity by favoring Cd^{2+} versus Ca^{2+} root uptake and by initiating a cellular pathway resembling those activated upon iron deprivation. They identified 43 genes encoding proteins related to iron homeostasis, proteolysis, nitrogen assimilation/metabolism, root growth, and transporters such as *IRT1*, that encodes for a Cd, Mn, Zn and Co cation transporter (Korshunova et al. 1999; Rogers et al. 2000).

To investigate the cellular responses of *Arabidopsis* to Zn, van de Mortel et al. (2006) examined in detail the transcription profiles of roots of *Arabidopsis* plants

grown with 0, 2 and 25 µM of Zn. A total of 608 Zn-responsive genes with at least a three-fold difference in expression level were detected in response to changes in Zn supply. A large fraction of these genes are of yet unknown function, but many of them appear to be involved in metal homeostasis, abiotic stress response, and lignin biosynthesis. In Cu-exposed *Arabidopsis* plants (1 mM $CuSO_4$ for 30 min), Mira et al. (2002) described two Cu-induced messengers encoding a vegetative storage protein (VSP2) that could act as a temporary storage of amino acids during processes in which these are mobilized, as it happens when plants are subjected to severe oxidative stress.

To confirm transcript data obtained from e.g. microarray analysis or to study the transcript levels of specific genes under metal stress, it is essential to perform quantitative RT-PCR measurements. In this regard, Remans et al. (2008) used a strategy for accurate normalisation of the measured gene expression using a minimum of three reference genes [*AT5G15710* (F-box protein), *AT2G28390* (SAND family protein) and *AT5G08290* (mitosis protein YLS8)] in roots and leaves of *Arabidopsis* exposed to Cd (2–10 µM) and Cu (0.5–2 µM) during 24 h. That metals induce oxidative stress in plants was illustrated by the influence of Cd and/or Cu on ROS (reactive oxygen species) producing and antioxidative defence mechanisms in *Arabidopsis*, addressed by either induction or reduction of specific pro- and antioxidant gene transcripts (Smeets et al. 2008, 2009; Remans et al. 2010; Cuypers et al. 2010).

9.5.2 Proteins

The effects of metals at the proteome level are less investigated, and extensive proteomic studies to unravel the mechanisms of metal uptake and tolerance in plants are yet to be completed. A significant proportion of the *Arabidopsis* genome encodes membrane proteins, especially transport proteins and putative sensors that cope with these conditions (Ludewig and Frommer 2002). Thus, the necessity to regulate uptake of nutrient metals, many of which can be cytotoxic at high concentrations, is particularly important.

Roth et al. (2006) found alterations in the root proteome of hydroponically grown *Arabidopsis* plants exposed to 10 µM Cd^{2+} for 24 h, a condition that triggers PC synthesis. Two dimensional gel electrophoresis and western analysis indicated significant changes in protein abundance upon Cd^{2+} treatment. Most of the identified proteins belong to four different classes: (1) metabolic enzymes such as ATP sulfurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; (2) glutathione *S*-transferases; (3) latex allergen-like proteins; and (4) unknown proteins. Their results showed a selective enrichment of the protein family glutathione *S*-transferases, suggesting the generation of internal sinks for reduced sulfur after exposure of plants to Cd. On the other hand, Semane et al. (2010) studied the leaf proteome of 3-week-old *Arabidopsis* seedlings exposed for 1 week to 1 µM Cd. Their data indicated that plants adapted their metabolism to cope with the Cd exposure and only moderate protein changes were observed,

whereas at higher levels (10 μM Cd) growth reduction, chlorosis of rosette leaves, lipid peroxidation and enhanced peroxidase activity occurred. In particular, 21 proteins were up-regulated in response to Cd, functionally grouped into five classes: (1) proteins involved in oxidative stress response and GSH/PC metabolism, (2) photosynthesis and energy production, (3) protein metabolism, (4) gene expression, and (5) proteins with various or unknown function.

Kung et al. (2006) screened for copper-interacting proteins in *Arabidopsis* roots *via* copper-immobilized metal affinity chromatography (Cu-IMAC). They identified 35 proteins involved in redox/hydrolytic reactions, amino acid metabolism, glutathione metabolism, phosphorylation, translation machinery, membrane-associated proteins, and vegetative storage proteins. Finally, they predicted and scored six potential Cu- interacting motifs present in Cu-IMAC-isolated proteins with higher frequency than in the whole *Arabidopsis* proteome. To understand better Zn-responsive proteins, Fukao et al. (2009) investigated Zn-treated (300 μM $ZnSO_4$), *Arabidopsis* roots. Ten up-regulated and 17 down-regulated proteins were identified, 15 of which showed a significant correlation with previously reported transcriptomic data.

Besides an open screening for changes in protein abundance under metal stress, also studies on specific proteins are conducted. Li et al. (2010) suggested that NRT1.8 functions in the removal of nitrate from xylem vessels. Indeed, long-distance transport of nitrate requires xylem loading and unloading, a successive process that determines nitrate distribution and subsequent assimilation efficiency. Interestingly, *NRT1.8* appeared to be the only nitrate assimilatory pathway gene that was observed to be strongly up-regulated by Cd^{2+} in roots, and the *nrt1.8-1* mutant showed a nitrate-dependent Cd^{2+}-sensitive phenotype. Further analyses showed that Cd^{2+} stress increases the proportion of nitrate allocated to wild-type roots compared with the *nrt1.8-1* mutant. The NRT1.8 transporter likely plays an important role in protecting the plant against Cd toxicity, and possibly against a wide range of biotic and abiotic stresses (Gojon and Gaymard 2010).

9.5.3 Phytohormones

Arabidopsis plants exposed to stress caused by metals often resemble, in terms of the redistribution of growth, plants altered in phytohormone metabolism (Pasternak et al. 2005; Kai et al. 2007).

Indole-3-acetic acid (IAA) and its metabolites are the most widely auxins in plants (Hansen and Halkier 2007; Kai et al. 2007; Ludwig-Müller 2007). Key components of the cell cycle and signal-transduction pathways that promote and attenuate auxin-dependent lateral roots initiation have been identified in *Arabidopsis* (Casimiro et al. 2003). Pasternak et al. (2005) observed that in *Arabidopsis* Cu-exposed plants (30 up to 100 μM $CuSO_4$) root hair density was significantly increased and an acceleration of the emergence of lateral roots occurred. These authors observed that phenotypes of *Arabidopsis* plants exposed to $CuSO_4$ resemble plants altered in auxin metabolism.

Fig. 9.2 Seedlings of *Arabidopsis thaliana* not exposed to metals (control (**a**), and exposed for 12 days to 10 μM Cd (**b**), 5 μM Cu (**c**) and 150 μM Zn (**d**)) (Source: A. Sofo)

Significant morphological changes (degree of root branching and number of root hairs) together with increased IAA root levels were found by Sofo et al. (unpublished data) in roots of *Arabidopsis* plants exposed to Cd, Cu and Zn, applied separately or in different combinations (Fig. 9.2). Map-based cloning was used to find the *ILR2* gene that affects IAA-leucine resistance in the *Arabidopsis ilr2-1* mutant (Magidin et al. 2003). This gene encodes a protein that is polymorphic among *Arabidopsis* accessions and it was found to modulate a metal transporter, thus providing a link between auxin-conjugate metabolism and metal homeostasis. Considerable effort has also been directed at clarifying the processes and factors contributing to IAA homeostasis during metal exposure, but the entire picture remains to be elucidated,

as IAA synthesis is regulated in response to different and complex signaling pathways (Hansen and Halkier 2007).

Another class of hormones, cytokinins (CKs) are implied in meristem activity, *de novo* bud formation, release from apical dominance, leaf expansion, reproductive development, and senescence. In many plant species, moderately high Cd levels (5–50 μM) and longer exposures to Cd (3 weeks) significantly decreases cytokinin export from the root tips to the shoot (Prasad 1995). In *Arabidopsis* plants grown in the presence of Cd, Cu and Zn, applied separately or in different combinations, reduced CKs levels in shoots, associated to CKs increases in roots, were observed by Sofo et al. (unpublished data) (Fig. 9.2). Since in *Arabidopsis* the genes encoding ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases (*ipt*) and/or the activities of the corresponding enzymes (IPTs) are of basic importance in CK biosynthesis (Miyawaki et al. 2006), the application of metals likely could up-regulate the transcription of these genes.

In *Arabidopsis*, Cd-induced inhibitory effects were reported to be concomitant with an increase in endogenous abscisic acid (ABA) levels in plant tissues indicating the possibility of this phytohormone mediating a part of the metal-imposed phytotoxicity (Sharma and Kumar 2002). Furthermore, it was observed that Cu or Cd applied on *Arabidopsis* seedlings, causes a rapid increase of jasmonic acid, followed by a rapid decrease observed during 7 successive hours (Maksymiec et al. 2005), so indicating that jasmonic acid is connected with the mechanism of toxic action of both metals in plants. Interestingly, jasmonic acid applied on *Arabidopsis* seedlings exposed to Cu or Cd (100 μM up to 144 h) enhances their sensitivity to these metals, in terms of reductions of membrane peroxidation and quantum yield of PSII, so demonstrating an important role of jasmonic acid in metal stress signaling (Maksymiec et al. 2007). Indeed, salicylhydroxamate and propyl gallate, two inhibitors of jasmonic acid synthesis, are able to prevent some deleterious inhibitory effect of Cu and Cd in *Arabidopsis* (Maksymiec and Krupa 2002).

Finally, Cu and Cd, but not Zn, were found to elicit the greatest amount of ethylene produced by *Arabidopsis* plants (Arteca and Arteca 2007). These authors found that inflorescence stalks and root tips produced the greatest amount of ethylene in response to $CuSO_4$ or $CdSO_4$ over a range of concentrations from 0 to 800 μM, whereas all other plant parts tested released significantly lower levels. It was found that increasing leaf age, light and high temperatures caused a dramatic decrease in Cu/Cd-induced ethylene production in both inflorescence stalks and leaves of *Arabidopsis*.

9.6 The Multi-pollution Context

Monometallic exposure is very unusual in real-world situations, so it is very important to study both the metal-specific effects and the mechanisms induced when the plants are exposed to more metals simultaneously (Smeets et al. 2009). In fact, the combined exposure to more metals could enhance some of the effects

that are induced with only one metal. Moreover, it can happen that plants able to hyperaccumulate some metals without showing any physiological damage, can be strongly susceptible to other ones (Mijovilovich et al. 2009). For instance, seed is a developmental stage that is highly protected against external stresses in the plant life cycle. However the toxicity of Cu, Zn and particularly Cd, alone or in combination, on seed germination in *Arabidopsis* was proven (Li et al. 2005). The same authors highlighted that Cu and Zn are significantly less effective on reducing seedling growth when compared to Cd.

The studies on *Arabidopsis* plants subjected simultaneously to different metals are quite scarce. In one of these studies from Maksymiec and Krupa (2006), a considerable increase of hydrogen peroxide accumulation and superoxide radicals was observed during the first hours of exposure of plants to excess Cu and Cd (100 μM $CuSO_4$ and $CdSO_4$). Furthermore, excess Cd, in contrast to Cu, increased the SOD activity. The metal-induced oxidative stress was also confirmed by other studies. Smeets et al. (2009) observed a specific Cd-related induction of NADPH oxidases and metal-specific patterns of superoxide dismutases in 3-week-old *Arabidopsis* seedlings exposed to Cu and/or Cd (10 μM $CdSO_4$ and $CuSO_4$) for 24 h. Both metals induced gene expression of several H_2O_2-quenching enzymes and lipid peroxidation possibly by the activation of lipoxygenases. Skórzyńska-Polit et al. (2006) studied the activity and cellular localization of lipoxygenases (LOX) in *Arabidopsis* plants grown under excess Cd and Cu (both at 0, 5, and 50 μM) in solution cultures for 7 days. LOX was localized mainly in the cytoplasm as well as inside the chloroplasts and its activity was significantly higher in metal-exposed plants than in control plants. Moreover, the authors observed that the changes in ultra-structure of the leaf parenchyma cells were more evident in plants treated with Cd than those exposed to Cu. LOXs are particularly important during oxidative-stress responses, as they catalyse the dioxygenation of polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene backbone, producing hydroperoxy fatty acids, which are highly reactive compounds that are toxic to cells, but are also precursors of oxylipin signalling molecules like jasmonates.

9.7 Conclusions and Perspectives

Understanding the metabolic responses and the adaptation of plants towards metal exposure opens the way to future phytoremediation of contaminated soils. The majority of these metals accumulate in plants and may either directly or indirectly find their way into the food chain causing severe secondary consequences. For this reason, phytoremediation has been accepted advantageous over commonly used physical remediation methods in costs, practice and the scale at which the processes operate. Usually, fast growing plants of high biomass are considered potential candidates for toxic metal accumulation but may be hampered with significant tolerance capacity, limiting their use for phytoremediation purposes. Identifying the particular gene(s) underlying a specific adaptation to metals is

a major challenge in modern biology (Roosens et al. 2008). Therefore, the study of naturally occurring variation in *Arabidopsis* provides a bridge between functional genetics and evolutionary analyses. Nevertheless, the use of this species to study adaptation is limited to those traits in common with other plant species used for phytoremediation. Therefore, in order to fully understand the genetics of adaptation of plants to metals, the numerous and easily available genetic resources developed in *Arabidopsis thaliana* should be extended to other plant species.

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