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MULTI-AUTHOR REVIEW

The molecular mechanism of zinc and cadmium stress response in plants

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Abstract When plants are subjected to high metal exposure, different plant species take different strategies in response to metal-induced stress. Largely, plants can be distinguished in four groups: metal-sensitive species, metal-resistant excluder species, metal-tolerant non-hyperaccumulator species, and metal-hypertolerant hyperaccumulator species, each having different molecular mechanisms to accomplish their resistance/tolerance to metal stress or reduce the negative consequences of metal toxicity. Plant responses to heavy metals are molecularly regulated in a process called metal homeostasis, which also includes regulation of the metal-induced reactive oxygen species (ROS) signaling pathway. ROS generation and signaling plays an important duel role in heavy metal detoxification and tolerance. In this review, we will compare the different molecular mechanisms of nutritional (Zn) and non-nutritional (Cd) metal homeostasis between metal-sensitive and metal-adapted species. We will also include the role of metal-induced ROS signal transduction in this comparison, with the aim to provide a comprehensive overview on how plants cope with Zn/Cd stress at the molecular level.

Keywords Plant stress adaptation · Molecular regulation · Zn · Cd · ROS

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Introduction

Heavy metal contamination in soil caused by human activities, such as mining and industrial activities, is a serious problem all over the world [1–3]. Heavy metals are a poorly defined set of chemical elements, mostly belonging to the so-called transition metals, but often also including elements like Pb and Tl. Quite a few of the lower molecular weight heavy metals are essential minerals, like Zn, Co, Cu, Ni, Mn, Mo and Fe, but most are non-essential for biology. All of them are potentially toxic, depending on their bioavailable concentrations and sensitivity of the organism that is exposed [4–6]. Most toxic to humans are the elements that resemble essential minerals, such as Hg, Pb, and Cd, which are more likely to enter the cell through the existing mineral uptake machinery. Cd exposure, for example, can cause emphysema and osteoporosis, leading to irreversible damage to lungs, kidneys, and bones in humans [7]. Toxicity of these metals is not limited to humans or animals, but affects many organisms, including plants. Under excess heavy metal exposure, plants will display reduced biomass, leaf chlorosis, inhibited root growth, and morphological alterations, often leading to plant death at excessive exposures [8]. While humans and animals can move and thus avoid heavy metal-contaminated areas, plants can not, and need to evolve ways to deal with the heavy metals they encounter in their direct environment.

Among plant species, there is a wide variation in sensitivity to heavy metal exposure. In general, plants have developed two major strategies to resist high heavy metal exposure [9, 10]. The first one is the *excluder strategy*, in which the plants try to avoid heavy metals entering the roots, for instance by restricting soil metal bioavailability or by reducing expression of metal uptake transport

proteins. The solubility of metals in the rhizosphere is affected by pH, cation exchange capacity, concentrations of organic compounds or metal chelating compounds, properties of the minerals, and the activity of microorganisms [11]. Secretion of protons and exudation of carboxylates from roots can acidify the rhizosphere and increase metal solubility, bioavailability, and toxicity [12]. Organic acids that are excreted by roots can form a complex with heavy metals in the rhizosphere and thus inhibit the uptake of metals [13]. Microorganisms can decrease metal solubility in soil by ways of biosorption, extracellular sequestration, transportation, bioprecipitation, and chelation of metals by siderophores [14], but bacterial siderophore excretion can also enhance bioavailability. With the excluder strategy, plants are trying to keep the metal concentrations in roots low, despite the elevated metal concentration in the soil. However, in areas highly contaminated with heavy metals, such as close to a metal smelter, it is often too demanding to exclude toxic metals from plant roots due to the high metal concentrations in the soil. To cope with such a situation, plants have developed a different mode of action, which involves taking up metals and reducing the damage.

This second strategy is the *tolerance strategy*, which relies on confinement and detoxification of metals in a controlled way. This not only permits plants to withstand high metal exposure but also to accumulate metals to sometimes extremely high concentrations. Again a division can be made, this time between plants that tolerate high uptake of metals, but restrict their accumulation to roots, and plants that accumulate metals and preferentially transport metals to the above-ground parts [15]. The latter type of plants is often referred to as heavy metal hyperaccumulators, a term coined by Jaffré et al. [16]. Both types combine a high tissue tolerance to toxic metals with a high ability to accumulate them. In either type, metals are detoxified by chelation in the cytosol, sequestration in vacuoles, or confinement in the apoplast. If metals are translocated from roots to above-ground tissues, via xylem, and distributed over aerial tissues, they are often compartmentalized or sequestrated in photosynthetically inactive tissues, like epidermis, or in storage tissues, such as trichomes and old leaves [17]. Plant heavy meal hypertolerance and/or hyperaccumulation are clear ecophysiological adaptations to metalliferous soils [18].

Whichever strategy plants use to limit the negative effects of metal toxicity, the metal response needs to be tightly regulated to reduce damage by toxic metals but ensure proper homeostasis of essential minerals. Plant responses to heavy metal stress are the combined results of cellular transport mechanisms and activation of signal transduction pathways. These processes are metal-dependent and plant species-dependent. In this review, we will discuss how plants respond at the cellular and molecular level to high exposures of zinc (Zn) and cadmium (Cd), two metals commonly found in the environment with toxic effects on plants and humans. In addition, we will discuss how plants regulate the response to metal exposure through signal transduction. Furthermore, we will compare the different molecular mechanisms and signal transductions between the majority of plant species, which are heavy metal sensitive, and the few species that have evolved extreme adaptation to heavy metal exposure.

Plant response to heavy metals

When discussing the toxic effects of heavy metals, one should be aware that the toxicity of metals to plants depends on the external bioavailable metal concentration, the exposure time, the plant genotype, and the general condition of the plant. In addition, the dose–response curves to essential elements and non-essential elements are different (Fig. 1) [19]. Therefore, it is convenient to categorize heavy metals into two groups, the essential micronutrients and the non-essential elements. Heavy metal micronutrients, such as Co, Cu, Fe, Mn, Mo, Ni, and Zn, play an essential role in plant cell growth and development [20]. For example, Zn is a cofactor of many enzymes through which it is involved in protein binding, regulation of enzyme activity, transcriptional regulation, translational regulation, and signal transduction [21]. Because of the importance of essential micronutrients in plant physiology, it is cardinal to maintain homeostasis of these heavy metals in plant cells. This means that metal concentrations should be maintained within a relatively narrow range to avoid deficiency and toxicity effects. Plants fortunately possess tight regulatory mechanisms to maintain heavy metal micronutrient homeostasis. Only when this mechanism has reached its capacity, and heavy metal micronutrient concentrations rise above a certain threshold level, will their toxicity will be imminent [22]. Non-essential heavy metals, such as Cd, Pb, Tl, and Hg, which are not known to have a biological function, are generally toxic to plants [5]. Cd for instance is a powerful enzyme inhibitor. Cd exposure also results in the degeneration of mitochondria, and in aberrations of mitosis, leading to inhibition of cell proliferation and cell division in meristems [23]. Cd also damages the photosynthetic apparatus, causing production of reactive oxygen species (ROS) in photosynthetically active tissues [24]. These non-essential heavy metals can evoke a strong response of plant cells even when only applied at low concentrations [22]. The molecular mechanism to exclude, detoxify, or compartmentalize non-essential heavy metals plays a crucial role in plant survival under heavy metal exposure.

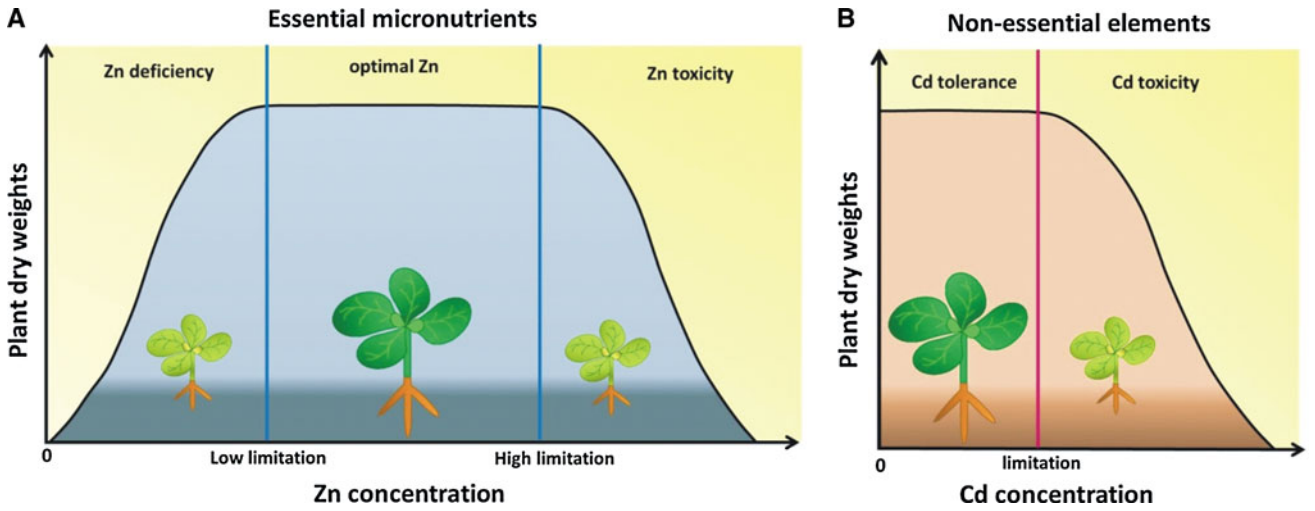
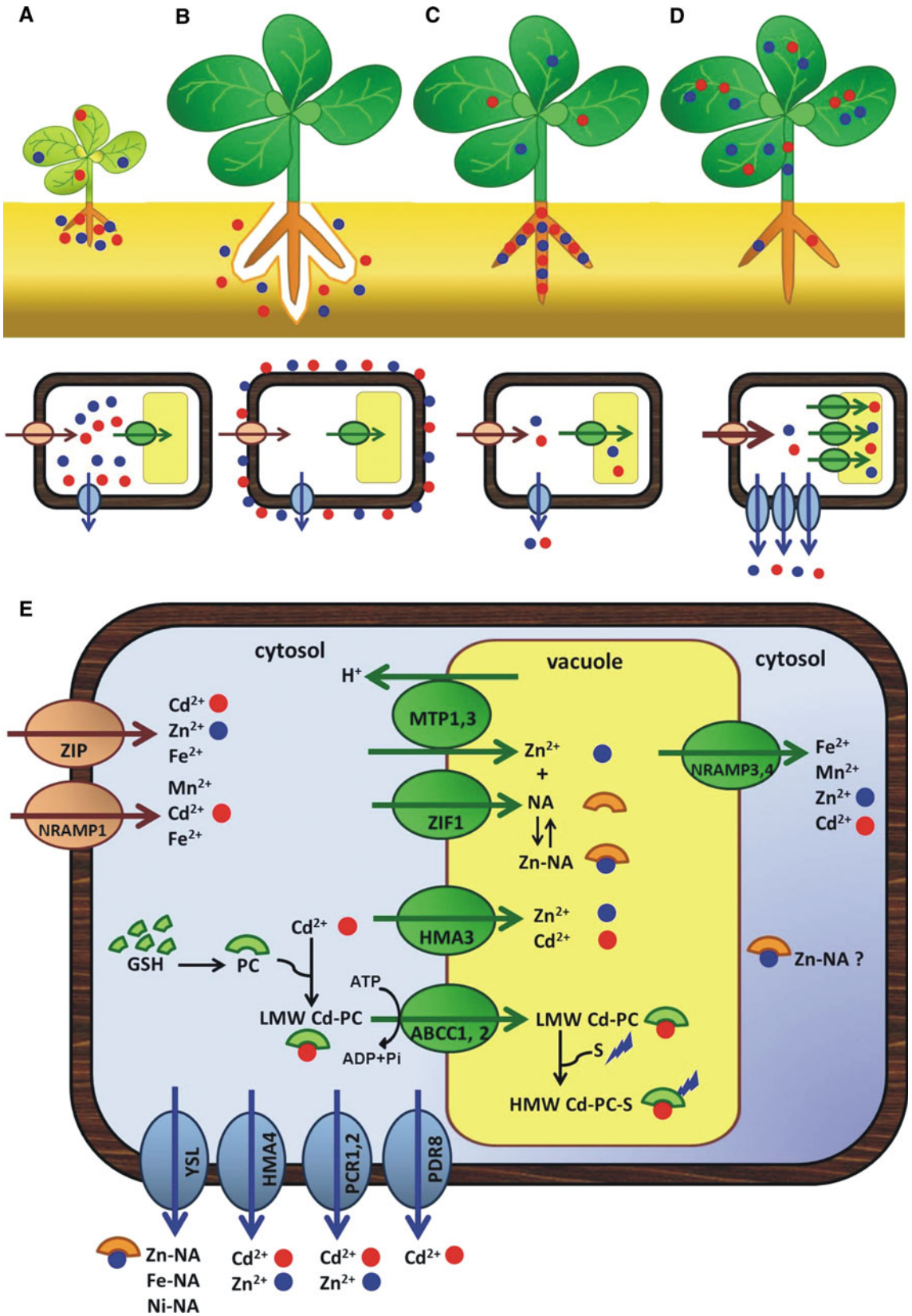


Fig. 1 Dose–response curves of plants to essential micronutrients and non-essential elements (modified from Alloway [19]). The dose–response curve is shown as plant dry weight against metal concentration. **a** For essential micronutrients, there are growth-limiting low and high Zn concentrations. At Zn concentrations below the lower limit, plants will show a Zn-deficient phenotype; however, when encountering concentrations over the highest limit, plants will show a Zn-toxicity phenotype. To obtain normal growth and development, plants must keep the concentration of essential micronutrients like Zn within the optimal range (i.e., metal homeostasis). **b** Non-essential elements are not necessary for plant growth and there is no lower limitation for such elements. Taking Cd as an example, when the applied Cd concentration is below the limit, plants will be tolerant and survive, however, when the encountered concentration exceeds this limit, plants will become sensitive and display a Cd-toxicity phenotype

The consequences of prolonged metal exposure for plant cells are membrane disintegration, ion leakage, lipid peroxidation, DNA/RNA degradation, and eventually cell death. At the cellular level, higher plants potentially use one or more of the following six ways to avoid or endure heavy metal exposure (Fig. 2) [25]. (1) Reduce metal bioavailability; (2) control metal influx; (3) chelate metals; (4) promote metal efflux; (5) compartmentalize and sequester metals; (6) detoxify metal-induced ROS. In the subsequent sessions, we will introduce the differential molecular mechanisms of plant responses to Zn and Cd. The differences in the respective regulatory mechanisms will be compared, including comparison between metal-sensitive and metal-tolerant plant species.

Reducing metal bioavailability

The bioavailability of metals in soil determines if metals are accessible for plants to be taken up or not. Bioavailability is largely determined by the rhizosphere. Rhizosphere microbes can change metal solubility, mobility, availability, specificity, precipitation by alteration of the soil pH, and secretion of metal chelators [26]. The presence of mycorrhiza, a symbiotic interaction between specific soil fungi and roots of most vascular plant species, can enhance heavy metal detoxification and tolerance of plants by selective metal exclusion and metal chelator secretion of the fungi [27]. For instance, the ectomycorrhizal fungus *Paxillus involutus* possesses specific Zn retaining capacity in its mycelium, which can immobilize Zn and prevent its transport to host plants, thus increasing the Zn tolerance of its host, Scots pine (*Pinus sylvestris*). There is also genetic variation among different mycorrhizal fungi genotypes, since a Zn-sensitive strain of the ectomycorrhizal fungus, *Suillus bovinus*, inoculated on Scots pine seedlings, provided a stronger Zn stress response (reduced chlorophyll concentration and inhibited N assimilation) compared to pine seedlings inoculated with a Zn-tolerant fungal strain [28]. The same holds for Cd tolerance. *Pinus pinaster* seedlings inoculated with the same fungus showed higher shoot biomass, less Cd-sensitivity symptoms, and lower shoot Cd concentrations than seedlings inoculated with *Rhizopogon roseolus* [29]. Next to mycorrhizal fungi, bacterial microbes can also affect plant metal exposure. Application of *Pseudomonas aeruginosa* to black gram (*Vigna mungo*) seeds or of *Methylobacterium oryzae* or *Burkholderia* sp. to tomato, can reduce Cd accumulation in plant roots and enhance plant growth [30]. *P. aeruginosa* can also reduce the uptake of Cd in pumpkin and mustard by decreasing Cd bioavailability in the soil [31, 32]. To prevent the entrance of Zn into their plant host, ectomycorrhizal fungi tend to sequester Zn into their vacuoles, while Cd is bound to thiol-containing compounds such as glutathione and γ-glutamylcysteine [33]. It is clear that the effects of microbes and mycorrhiza to heavy metal stress response usually depends on the plant species, the microbial varieties or mycorrhiza types, and the metal. Several studies have recently been published on the selection of


A
B
C
D
E
cytosol
vacuole
cytosol
H+
MTP1,3
Zn2+
+
NA
Zn-NA
ZIP
Cd2+
Zn2+
Fe2+
NRAMP1
Mn2+
Cd2+
Fe2+
ZIF1
NRAMP3,4
Fe2+
Mn2+
Zn2+
Cd2+
HMA3
Zn2+
Cd2+
Zn-NA ?
GSH
PC
Cd2+
ATP
LMW Cd-PC
ABCC1, 2
ADP+Pi
LMW Cd-PC
S
HMW Cd-PC-S
YSL
HMA4
PCR1,2
PDR8
Zn-NA
Fe-NA
Ni-NA
Cd2+
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◀ **Fig. 2** Molecular mechanism in response to heavy metal stress in plants. When plants are exposed to high metal concentrations (in this case Zn, *blue dots*, and Cd, *red dots*), they can be discerned in four types depending on their response (**a**–**d**). **a** Heavy metal-sensitive plants, which cannot keep metals out of their roots or prevent transport to the shoot, and which will succumb due to the toxic effects of metals on root and shoot cells. **b** Heavy metal-resistant excluder plants, which are able to keep metals outside the roots or take care of rapid efflux in case toxic metals have entered root cells. **c** Heavy metal-tolerant non-hyperaccumulator plants, in which metals can enter root cells where they are sequestrated into root vacuoles, preventing translocation to shoots. **d** Heavy metal-hypertolerant hyperaccumulator plants, in which metals are actively taken up through the root, and largely loaded into xylem for root to shoot transport. In the shoot, the metals are safely sequestered in vacuoles. The molecular mechanism to keep metal homeostasis in plant cells is shown in **e**. It involves metal influx transporters (*dark purple*), such as ZIP and NRAMP1 proteins, which are responsible for the uptake of metals into cytosol; metal tonoplast located transporters (*green*), such as MTP1, 3, ZIF1, HMA3, and ABCC1 and 2, which are responsible for metal (Zn or Cd), chelator (NA), or metal–chelator complex (Cd–PC) sequestration into the vacuole, or remobilization from the vacuole (NRAMP3/4); and metal efflux transporters (*blue*), such as HMA4, which acts to exclude excess metals out of the cytosol and is involved in the metal translocation towards the shoot. The cell wall is shown in *brown*, the vacuole is shown in *yellow*, and the cytosol is show in *grey*. *ZIP* ZRT-IRT-like protein family, *NRAMP* natural resistance-associated macrophage protein, *MTP* metal tolerance protein, *ZIF1* zinc-induced facilitator 1, *HMA* heavy metal ATPase; *YSL*, yellow stripe 1-like, *PCR* plant cadmium resistance, *PDR8* pleiotropic drug resistance 8, *NA* nicotianamine, *GSH* glutathione, *PC* phytochelatin, *S* sulfide, *LMW* low molecular weight, *HMW* high molecular weight

metal-hypertolerant microbes and mycorrhiza for bioremediation purposes, either in using microorganisms to remove metal pollutants [26, 34], or indirectly by microorganisms that promote soil metal bioavailability thus enhance metal uptake by metal hyperaccumulator plants [35–37].

Another way of preventing entry of metals into the plant is by binding metals to specific root exudates or to the plant cell walls. Root exudates, including low molecular weight compounds (amino acids, organic acids, sugars, phenolics) and high molecular weight compounds (polysaccharides and proteins), can be actively secreted or passively leaked from root cells to the soil [38]. The presence of root exudates affects the metal availability either by direct chelation of metals or indirectly by acidification of the rhizosphere and reduction of elements [39]. Thus, root exudates can enhance or inhibit metal uptake by plant roots and further regulate metal tolerance and accumulation of plants. For instance, barley cultivar "Sahara" accumulates more Zn than "Clipper" because of the higher root exudation of organic acids (malate, maleate, fumarate, and *cis*-aconitate) and amino acids (alanine, valine, proline, aspartic acid, and glutamic acid) [40]. Exudation can also account for differences between species, such as in the comparison between *Oenothera picensis*, with higher organic acid root exudation than *Imperata condensate*, accounting for its higher tolerance to elevated soil Cu concentrations [41].

The cell wall, the first plant structure getting in touch with metals, contains suberin and low-methylesterified pectin. Suberin is often found in root endodermis and exodermis cell walls, where it acts as a barrier to control the uptake of water and mineral ions to provide control over nutritional mineral accumulation and transportation [42]. Low-methylesterified pectin comprises polysaccharides for the binding of divalent and trivalent metal ions [43]. Most essential and non-essential metals are commonly entering plants as divalent cations (Zn^{2+}, Cd^{2+}, Fe^{2+}, Mn^{2+} or Cu^{2+}). Thus, altering the composition of the cell wall to enhance binding of metal cations to the cell wall can contribute to reducing the inadvertent uptake of toxic metals by plants. Zn-treated tomato suspension cells for instance produced a higher cell wall biomass, with higher Zn-retaining ability by cell wall polymers and higher Zn-binding capacity compared to non-treated suspension cells [44]. When comparing hyperaccumulating (HE) and non-hyperaccumulating ecotypes (NHE) of *Sedum alfredii*, both showed similar Zn absorption abilities, however, the Zn affinity to root cell walls was different [45]: Zn bound to root cell walls of HE plants was more available for xylem loading than NHE. This implies that metal binding capacity and affinity of the cell wall can be modified by the plant, and thus contribute to preventing metals from entering the plant. Modification of cell wall lignins can be studied at the transcriptomic level using microarray analysis, focusing on expression of lignin biosynthesis related genes, such as 4-coumarate-CoA ligases *4CCL1*, *4CCL2*, *4CCL3*, *4CCL8*, *4CCL9*, and *4CCL14*; cinnamoyl-CoA reductase *CCR2*; and hydroxycinnamyl alcohol dehydrogenase *CAD1* [46–48]. When comparing root gene expression under deficient, sufficient, and excess Zn conditions between the Zn-hypertolerant and hyperaccumulating species *Noccaea* (formerly *Thlaspi*) *caerulescens* and the non-tolerant and non-hyperaccumulating-related species *Arabidopsis thaliana*, a large group of lignin biosynthesis-related genes are expressed at higher levels in *N. caerulescens*. This may relate to the formation of the extra endodermis reinforcements that are found in roots of *N. caerulescens* [21, 49]. A similar phenomenon was found in response to Cd stress. When comparing differential gene expression of roots under Cd exposure from *N. caerulescens* (Cd tolerant), and *A. thaliana* (Cd sensitive), lignin biosynthesis genes and cell expansion-related genes were found to be up-regulated in *A. thaliana* under excess Cd conditions, while *N. caerulescens* maintained its constitutively high expression of these genes [50]. This means that also Cd-sensitive species like *A. thaliana* make use of lignin depositions as a

physical barrier to prevent Cd from entering the plant. Metal precipitation is another way to restrict metal entry, as was found in the heavy metal hyperaccumulator *Arabidopsis halleri*, in which precipitates of Zn and Cd phosphates accumulate in the cell walls of the root epidermis [17]. These examples demonstrate the potential role of structural modifications of root cell walls to reduce the level of bioavailable metals and thus induce heavy metal tolerance.

Control of metal influx

Metals can enter the plant symplast in several ways: by simple diffusion, by passive transport through channel proteins, or by active transport through carrier proteins, the latter of which is most important, at least offers most control. These carrier proteins are generally referred to as metal transporters. They often have different affinities for different heavy metals, which is why they can confer some level of discrimination at the plasma membrane between desired essential elements and unwanted non-essential elements. When plants face an excess of Zn or Fe, they will rapidly try to inhibit excessive metal influx through down-regulation of the relevant transporters at the transcriptional and often also post-transcriptional or post-translational level. Fe status, for instance, alters the function of the IRT1 transporter through transcriptional and post-translational regulation [51–53]. Zn and Cd are most likely crossing the plasma membrane via members of the ZIP transporter family (ZRT-IRT like protein; **Z**inc-regulated transporter, **I**ron-regulated transporter **P**rotein) [54]. In *A. thaliana* this family comprises 15 genes, *ZIP1* to *ZIP12* and *IRT1* to *IRT3*). The *A. thaliana ZIP4* gene appears to be an important factor to control Zn import, as it encodes a plasma membrane-localized Zn specific transporter, which expression is upregulated under Zn deficiency, and repressed by excess Zn in *A. thaliana* [49, 55, 56]. Interestingly, the *ZNT1* gene, a *ZIP4* orthologue from *N. caerulescens*, is much higher expressed, almost irrespective of the Zn exposure levels, when compared to its orthologues in *A. thaliana* or *Thlaspi arvense*, another related non-hyperaccumulator [49, 57], which is well in accordance with the constitutive Zn hyperaccumulation in *N. caerulescens*. In addition to *ZIP4*, also the *ZIP1*, *ZIP3*, *ZIP5*, *ZIP9*, *ZIP10*, *ZIP12* and *IRT3* genes of *A. thaliana* appear to be involved in Zn-uptake, as their expression in roots is induced in response to Zn deficiency [49, 55, 58]. Expression of all of these *ZIP* genes is under control of two basic-region leucine zipper (bZIP) transcription factors, bZIP19 and bZIP23, which act redundantly to control the initial Zn deficiency response of *A. thaliana* [55]. Whether the control of *ZIP* Zn transporters in metal hyperaccumulators is also controlled by the two bZIP transcription factors is not yet known. Although these transcription factors in *A. thaliana* hardly respond at the transcriptional level to alterations in Zn exposure levels, the expression of their target *ZIP* genes is tightly controlled, with strongly reduced expression at high Zn exposure.

Still, also at elevated levels, Zn is able to enter the root cells, through other metal uptake transporters or in a more passive way. Zn excess often evokes an Fe-deficiency response, with induced expression of Fe uptake transporters like *IRT1*. Since these also have affinity for Zn, inadvertent uptake of Zn cannot be completely avoided. This means that the essential mineral homeostasis mechanisms are better at controlling metal uptake under deficiency conditions than at high exposure levels.

Cd chemically resembles Zn and Fe. To date, there has been no Cd-specific influx transporter found for plant cells and the uptake of Cd is likely to occur through available metal uptake ZIP transporters (or alike), which have high specific transport affinity for Zn or Fe, but also low affinity for Cd [59, 60]. The uptake of Cd in root cells thus appears to be an opportunistic event. Still, the Zn/Cd specialized hyperaccumulating Ganges accession of *N. caerulescens* shows a much higher maximum Cd influx than the Cd-tolerant, and Cd-excluding, accession Prayon, which results in a fivefold higher Cd concentration in xylem sap of Ganges [61]. This accession also shows strong induction of *IRT1* under Fe-deficiency conditions, which may account for the high Cd uptake, although this has not been proven conclusively [62, 63].

Metal chelation

Metals that get past the plasma membrane need to be chelated by various ligands in order to reduce their undesired interaction with cellular compounds. Such ligands can be oligopeptides, organic acids, amino acids, or proteins. Metal–ligand complexation is an important part of the molecular mechanism of metal homeostasis. Therefore, the production of metal chelators is of cardinal importance in the plant metal exposure response. Heavy metal hyperaccumulating species usually show higher chelator accumulation than non-hyperaccumulating species, which supports the importance of chelators in metal detoxification, metal sequestration, and metal efflux. The next section will particularly address the role of Zn and Cd chelators.

Nicotianamine (NA) is a nonproteinogenic amino acid that shows high binding affinity in vitro to a range of transition metals, such as Cu, Ni, Co, Zn, Fe, and Mn. The formation of Zn-NA complexes has also been detected in vivo, in *Schizosaccharomyces pombe* [64, 65]. Metal-NA complexes can be transported over cellular membranes by YSL proteins, which are thus important components in the

regulation of metal homeostasis in plants [66]. NA is synthesized from three molecules of *S*-adenosyl-methionine by the enzyme nicotianamine synthase (NAS) [67]. The expression of *NAS* genes determines the NA concentrations and thus contributes to NA-mediated metal homeostasis. *NAS* genes are found in varying copy numbers in different plant species. *Solanaceous* species appear to have only one *NAS* gene, Graminae species mostly have three *NAS* genes, and in *A. thaliana* (and other Brassicaceae), there are four NAS genes: *NAS1*, *2*, *3*, *4*, which act functionally redundant in metal homeostasis [68]. Differences in *NAS1*, *NAS2*, and *NAS3* gene expressions are seen when comparing the Zn/Cd hyperaccumulator species *A. halleri* and *N. caerulescens* to related non-accumulators [49, 69, 70]. In correspondence with the *NAS* transcript levels, *A. halleri* has a higher NA concentration in roots than *A. thaliana* [71]. Knocking down *NAS2* gene expression in *A. halleri* by RNA interference (RNAi) results in reduced NA contents in roots, which inhibits the root-to-shoot translocation of Zn and renders these plants virtually non-hyperaccumulators [71]. In addition to YSL transporters, which transport NA-metal complexes over the plasma membrane [66, 72], recently a tonoplast localized transporter, zinc-induced facilitator 1 (ZIF1), was shown to be involved in transporting NA from the cytosol into the vacuole, thus facilitating NA–Zn complex formation in the vacuoles [73]. In *A. thaliana*, the *ZIF1* gene is up-regulated when exposed to excess Zn [74] and plants tend to retain the Zn in the vacuoles of root cells. As a result, less Zn is available for root-to-shoot translocation of Zn, preventing Zn to accumulate in the shoots where it is potentially more harmful than in roots due to interference with the photosynthetic machinery. The formation of NA–Zn complexes contributes to Zn loading into xylem in Zn hyperaccumulators, and turns out to be a useful storage form of chelated Zn in roots of non-accumulators under excess Zn exposure. *NAS*, *YSL*, and *ZIF1* genes, controlling NA synthesis and (metal-chelated) membrane transport, form an important regulatory mechanism of controlling metal distribution of the plant.

Glutathione (GSH), a γ-Glu-Cys-Gly tripeptide, is important because of its tripartite role in metal detoxification as a metal chelator, a cellular antioxidant, and as an ROS signaling molecule [75, 76]. GSH can act as a metal chelator through its thiol groups, which have high metal binding affinity. The expression of GSH synthesis genes, *GSH1* and *GSH2*, is induced by Cd treatments in *A. thaliana*, contributing to Cd tolerance, while a decrease in GSH levels reduces Cd tolerance [77, 78]. Also in rice, Cd-tolerant plants have higher levels of GSH than Cd-sensitive plants [79]. In addition, GSH plays an essential role in Fe-mediated Zn tolerance in *A. thaliana* [80]. GSH acts as a moderator of cellular oxidation status, as it can exist in a reduced (GSH) and an oxidized form (GSSG). The reduced form can donate a reducing equivalent ($H^+ + e^-$) to unstable molecules like ROS. The resulting reactive GSH will soon react with another reactive GSH to become GSSG. It thus also acts as a ROS signaling molecule, mainly determined by the GSH:GSSG ratio, which reflects the oxidative state of the plant cell [75]. The transition between GSH and GSSG also affects activity of several antioxidant enzymes, such as glutathione reductase (GR), ascorbate peroxidase (APX), and catalases [76]. Under Cd treatment, a decreased GSH/GSSG ratio was observed in *A. thaliana*, which was accompanied by enhanced GR and APX activities [77]. The hyperaccumulating ecotype of *Sedum alfredii* shows a higher GSH/GCCG ratio and less ROS production than the non-hyperaccumulating ecotype under excess Cd [81]. In summary, GSH plays an important role in the detoxification of heavy metals and metal-induced oxidative stress response.

Phytochelatins (PCs) are oligomers of GSH $(Glu\text{-}Cys)_n$-Gly ($n = 2$–10), synthesized by the enzyme phytochelatin synthase (PCS). The synthesis of PCs was initially found to be essential for tolerance to Cd, as concluded from analysis of *pcs* (*cad*) mutants defective in PCS [82–84], but later it was also shown to be relevant for tolerance to excess Zn [85]. When Cd enters plant cells, low molecular weight (LMW) PCs will first form a PC–Cd complex. This LMW PC–Cd complex is subsequently sequestered into vacuoles by ATP-binding cassette (ABC) transporters; for example, two ABCC-type transporters from *A. thaliana*, *ABCC1* and *ABCC2*, can enhance Cd tolerance and accumulation through vacuolar sequestration of PC–Cd [86, 87]. LMW PC–Cd complexes bind sulfides to form a stable, high molecular weight (HMW) PC–Cd complex, which is stored in the vacuole [88, 89]. While Cd detoxification by PC–Cd complexation and vacuolar storage is important for most plant species [90], it does not appear to play a role in Cd detoxification in Cd hyperaccumulator species [91–93]. When characterizing and comparing PCS between the Cd-hyperaccumulators *A. halleri* and *N. caerulescens*, and the non-hyperaccumulator *A. thaliana*, it turned out that the *PCS1* gene from *A. halleri* and *N. caerulescens* showed lower expression than its orthologue from *A. thaliana*, in accordance with PC accumulation data [94]. This demonstrates that PCs do not constitute the major Cd detoxification pathway in Cd hyperaccumulators. Instead of PCs, the induction of antioxidative mechanisms appears to play a more important role in conferring Cd tolerance. For example, the root superoxide dismutase (SOD) activity, leaf peroxidase (POD) activity, catalase (CAT) activity, and free proline concentrations are higher in the Cd-tolerant species *Solanum nigrum*, compared to the non-hyperaccumulator *S. melongena*, while there is no indication of elevated PC levels in the tolerant species [95]. Similarly, investigations of PC synthesis and Cd accumulation in the

Cd-hyperaccumulating ecotype of *Sedum alfredii* showed that Cd is hardly retained in roots, but transported and accumulated in leaf cell walls, in which PCs take no part [96].

For metal non-hyperaccumulating species, the balance between GSH and PCs is important for Cd tolerance. For example, overexpressing the *AtPCS1* gene can result in both Cd-sensitive and Cd-tolerant transgenic plants [97, 98]. The explanation is that elevated synthesis of PCs results in the depletion of GSH, which contributes to enhanced oxidative stress and the Cd-sensitive phenotype. However, if synthesis of PCs is not tremendously increased, though sufficient for increased PC–Cd complexation, the transgenic plants can maintain their GSH levels for sufficient contribution to Cd-induced antioxidative stress response and consequently they become Cd-tolerant [76]. In conclusion, PC accumulation plays an important role in Cd detoxification in non-tolerant, non-hyperaccumulating plant species, but is of little importance in Cd hyperaccumulators, which appear to rely more on efficient Cd sequestration and enhanced ROS detoxification.

Metallothioneins (MTs) are small cysteine-rich proteins that are found in most eukaryotes and contain metal-binding motifs that provide sulfhydryl for interacting with bivalent metal ions [88]. Based on the type of cysteine residues, plant MTs are classified into four types. In *A. thaliana* there are six MTs, belonging to four types: *MT1a*, *MT2a*, *MT2b*, *MT3*, *MT4a*, and *MT4b*. Yeast complementation experiments with these six *MTs* showed that most can enhance tolerance to and accumulation of Cu. Only *MT4* types confer Zn tolerance and accumulation and *MT1*, *2*, and *3* types enhance tolerance to Cd, but often not Cd accumulation [99]. In *A. thaliana*, *MT1a* is responsible for Cu homeostasis under elevated Cu, and is required for Cd tolerance, Cd accumulation, and Zn accumulation [99, 100]. Phytohormones abscisic acid (ABA) and gibberellic acid (GA) regulate the contribution of *MT4a* and *MT4b* to Zn accumulation in seeds and Zn nutrient supplementation of young seedlings [101]. MTs and PCs can work cooperatively to protect *A. thaliana* from Cu and Cd toxicity [99]. *MT1* and *MT2* are expressed at much higher levels in *N. caerulescens* compared to *A. thaliana*, implying that they are important for metal tolerance [102]. Indeed, *MT1*, *2*, and *3* from *N. caerulescens* can confer Cd tolerance to yeast or increase the intracellular Cd concentrations [102, 103]. The higher *NcMT2* expression in metallicolous *N. caerulescens* accession La Calamine, compared to the non-metallicolous accession Lellingen, is thought to contribute to the metal-adapted phenotype through improved Cu homeostasis at high Zn and Cd exposure [103, 104]. MTs not only play a role as metal chelators but also as ROS scavengers to reduce oxidative stress. The redox-sensing residue on MT, cysteine, acts as a ROS scavenger [105, 106]. Zn-induced *MT1a* can improve rice stress tolerance through the regulation of zinc-finger transcription factors via alternating Zn homeostasis, and also participate in the ROS scavenging pathway by altering the levels of the antioxidant enzymes catalase, peroxidase, and ascorbate peroxidase [107]. Also, rice MT2b is an reactive oxygen scavenger involved in the H_2O_2 signaling pathway [108]. Similar functions are found for *A. thaliana* MT2a and cotton MT3a [109, 110]. MT3 from *Tamarix hispida* is a ROS scavenger that contributes to increased Cd, Zn, and Cu tolerance; also, the enhanced activity of superoxide dismutase, catalase, and glutathione peroxidase under metal treatments participates in scavenging of ROS [111]. The exact role of MTs as ROS scavenger in metal hyperaccumulator species is still not clear.

Promotion of metal efflux

Another solution to overcome excessive entering of toxic metals into plant cells is to release these metals again from the cells. They can either be returned back to the soil solution or remain in the apoplast. The direction of metal efflux corresponds to the metal accumulation phenotype of the plant. In non-tolerant, non-hyperaccumulating plants, root efflux transporters direct metals to the soil solution, while in heavy metal hyperaccumulators, the efflux system is directed towards loading of metals into the xylem, on their way to the shoot.

Zn efflux is an important factor in plant Zn homeostasis, and thus Zn efflux transporters have been found. However, so far, no Cd-specific efflux transporter has been found in plants, which means that Cd efflux always accompanies the transport of other metals by action of efflux transporters with higher affinities for other metals than Cd. An important cellular metal efflux transporter family is the P_{1B}-type ATPase family [112]. One prominent Zn efflux transporter is HMA4 (Heavy Metal ATPase 4). This plasma membrane-localized transporter is normally responsible for loading Zn into the xylem. It is involved in Zn and Cd uptake, as found in *A. thaliana* where it is upregulated in roots under elevated Zn exposure but repressed by Cd exposure [113]. A *hma4* null mutant results in low Zn and Cd translocation ability, while overexpressing *HMA4* enhances root tolerance to Zn, Cd, and Co, and increases Zn and Cd accumulation [114]. Another P_{1B}-type ATPase, HMA2, is equally important for Zn homeostasis in plants [114]. In *Arabidopsis*, *HMA2* and *HMA4* act redundantly in Zn and Cd root-to-shoot translocation [116, 117]. HMA4 also appears to be the major determinant in explaining root-to-shoot transport of Zn and Cd in the heavy metal hyperaccumulators *A. halleri* and *N. caerulescens*. The gene is much higher expressed in these species compared to related non-accumulators [49, 70, 118, 119]. This appears to be caused by local gene multiplication

events in both species [120]. RNAi-mediated knock-down of *HMA4* gene expression in *A. halleri* resulted in loss of the Zn/Cd hypertolerance and hyperaccumulation ability in *A. halleri*, including increased sensitivity to high Zn and Cd exposure and reduced root to shoot translocation efficiency [119].

In *A. thaliana*, there are other efflux transporters involved in Zn or Cd redistribution, translocation, and detoxification, such as the ones encoded by the *Plant Cadmium Resistance 1* and *2* (*PCR1* and *PCR2*) genes. Activity of the plasma membrane-localized Cd efflux transporter, PCR1, enhances Cd tolerance by exporting Cd out of the cell and thus reducing Cd contents [121]. Also, PCR2 functions as a Zn efflux transporter, which contributes to Zn distribution and detoxification in *A. thaliana* [123]. Another plasma membrane-localized transporter, pleiotropic drug resistance 8 (PDR8), confers Cd tolerance in *A. thaliana* by pumping Cd^{2+} across the plasma membrane, out of root epidermal cells [123]. It is not clear if these transporters are also relevant in heavy metal hyperaccumulating species.

Metal sequestration and remobilization

If a plant is not able to prevent entry or enhance efflux, it will have to face the symplastic entry of metals and deal with it. This means plants need to sequester the metals at "safe" sites. This can either be in the cell, in specific organelles, in storage tissues, or even outside the cells. To give an example of the latter, in *N. caerulescens*, the Cd-hyperaccumulating ecotype Ganges can store much more Cd in the cell walls of epidermal cells than the poor Cd-accumulating ecotype Prayon [124]. Metal sequestration is an important strategy to reduce the cytoplasmic metal concentrations. Preferred organelle is the vacuole, which provides a well-controlled internal storage reservoir, normally already acting as storage buffer for nutrient minerals to account for temporary deficiencies [125]. Tonoplast transporters, which not only transport metals but also chelators (e.g., ZIF1 for NA) [73], are needed for vacuolar import. Non-tolerant species mostly promote enhanced vacuolar sequestration in the roots, thus preventing transport to photosynthetic leaves where metals can be potentially more harmful than in roots. In contrast, the heavy metal-adapted species have strong metal sequestration abilities in shoot cells and reduce root vacuolar sequestration. This difference in compartmentalization promotes metal loading into the xylem and creates a safe metal storage sink in leaves. Several proteins are involved, of which we will discuss the most prominent ones.

The metal tolerance protein 1 (MTP1), belongs to the cation diffusion facilitator (CDF) protein family and is probably the most important Zn vacuolar sequestration transporter in plants. It acts as a tonoplast located Zn^{2+}/H^{+} antiporter [126–128]. In heavy metal hyperaccumulators like *Thlaspi* (currently *Noccaea*) *goesingense*, *N. caerulescens*, *A. halleri* and also the hyperaccumulating ecotype of *Sedum alfredii*, this gene is higher expressed in shoots than in comparable non-hyperaccumulators [57, 129–132]. The higher *MTP1* expression in *A. halleri* compared to its close non-hyperaccumulator relatives *A. lyrata* or *A. thaliana*, is provided by several additional copies of the gene, distributed over four loci, compared to only one locus in the other two species [133]. Two of these loci are co-segregating with zinc tolerance QTLs in a back cross population between both species [134]. Next to MTP1, tonoplast localized MTP3 also contributes to Zn tolerance and Zn sequestration in response to excess Zn in *A. thaliana* [135], but the role of *MTP3* in hyperaccumulators is still not clear. Other *MTPs*, such as *MTP8* and *MTP11,* show increased gene expression in *N. caerulescens* and *A. halleri*, compared to *A. thaliana*, which suggests they may be important for heavy metal homeostasis and tolerance in these species [49, 58, 70, 136, 137].

Heavy metal ATPase 3 (HMA3) is another member of the P_{1B}-type ATPase superfamily to which HMA2 and HMA4 also belong [138]. It was found to be a prominent candidate for Cd sequestration. The HMA3 transporter from rice, isolated from the low Cd-accumulating rice cultivar Nipponbare, is located at the tonoplast of root cells to limit Cd root-to-shoot translocation through selective sequestration of Cd into the root vacuoles [139, 140]. HMA3 also confers Cd vacuolar storage in *A. thaliana* and overexpression in this species enhances Cd, Co, Pb, and Zn tolerance, probably through regulation of the vacuolar sequestration of these metals, even though actual Zn transport of this protein has not been confirmed in yeast [141, 142]. The gene is much higher expressed in shoots of *A. halleri* than in *A. thaliana*, independent of Zn exposure levels. Expression of the *A. halleri HMA3* in yeast increased Zn tolerance, suggesting it to be also involved in regulation of cellular Zn status in *A. halleri* [143]. Similarly, the HMA3 protein of *N. caerulescens* is much higher expressed in the Cd-hyperaccumulating accession Ganges, than in the poor Cd accumulator Prayon. Like for *HMA4* and *MTP1*, copy number variation appears to largely account for the difference in expression level between both accessions. Unlike HMA3 from *A. thaliana*, HMA3 from *N. caerulescens* is not found to transport Zn, only Cd [143]. The *HMA3* expression pattern in *N. caerulescens* also deviates from that in *A. thaliana*, with higher expression in leaf epidermis and mesophyll cells, which is in line with the shoot Cd hyperaccumulation ability of *N. caerulescens*.

Next to HMA3, the chloroplast envelope-located HMA1 transporter is found to be involved as a metal sequestration

transporter. It acts as a Cu-ATPase to import Cu into chloroplasts and also contributes to Zn detoxification by reducing Zn content in the plastid [145, 146]. In yeast, AtHMA1 activity can be activated by Zn, Cu, Cd, and Co [147]. Knocking down *HMA1* gene expression in *A. thaliana* results in reduced Cu/ZnSOD enzyme activity, which means it is involved in the antioxidant defense [145]. Although the function of *HMA1* in hyperaccumulator species has not been studied in detail, enhanced *HMA1* expression is observed in *A. halleri* where it may contribute to Zn hypertolerance [143].

When examining the gene expression of transporters involved in metal efflux (*HMA4*) and metal sequestration (*HMA3* and *MTP1*), these genes show higher expression in hypertolerant and/or hyperaccumulating species than in non-tolerant and/or non-accumulating species. The enhanced expression is often due to multiple copies of the genes, which is likely to be caused by gene duplication during evolution of metal exposure adaptation. A study of speciation between hyperaccumulator *A. halleri* and non-hyperaccumulator *A. lyrata* indicates that the historical split between both species coincides with the initial duplication of the *HMA4* gene, which suggests that this may have contributed to, if not caused, the separation of both species [148].

The natural resistance-associated macrophage protein (NRAMP) family contains six members in *A. thaliana*. Although these proteins are involved in the regulation of heavy metal homeostasis, they have quite different roles to play. The metal influx AtNRAMP1 transporter confers Fe, Mn, and Cd uptake in yeast [149]. In *A. thaliana*, this plasma membrane-localized transporter works as a major high-affinity Mn transporter, needed for acquisition of Mn from the soil [150]. AtNRAMP 3 and AtNRAMP 4 act as tonoplast localized metal efflux transporters, needed for vacuolar remobilization of Fe and Mn, but also contributing to Cd tolerance. They are essential for remobilization of Fe during seed germination at low Fe supply and to maintain optimal photosynthesis and plant growth at low Mn supply [151, 152]. AtNRAMP3 can transport Cd and AtNRAMP4 can transport both Zn and Cd. Overexpressing *AtNRAMP4* results in a Zn-and Cd-hypersensitive phenotype under Fe deficiency in *A. thaliana* [153]. The *N. caerulescens* NRAMP3 and NRAMP4 show the same metal transport abilities as their *A. thaliana* orthologues; however, *NcNRAMP3* and *NcNRAMP4* are expressed at higher levels than the *A. thaliana NRAMPs*, and thus contribute to enhanced root-to-shoot metal transport [154, 155]. AtNRAMP6 is localized to a vesicular-shaped endomembrane compartment and functions as an intracellular metal transporter that regulates the distribution and availability of Cd within *A. thaliana* [156]. Its role in heavy metal hyperaccumulation is still not clear.

Detoxification of metal-induced reactive oxygen species

Heavy metal toxicity induces the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), which are highly reactive molecules that interact with various cellular components leading to oxidative damage to macromolecules like nucleic acids, proteins, sugars, and lipids, causing oxidative stress in plant cells and (intra)cellular membrane damage that could result in cell death in severe cases [157]. To resist such oxidative stress, plant cells possess a comprehensive antioxidant system to scavenge ROS and detoxify them. This is mainly conferred by the action of several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) [158]. SOD is the only enzyme able to scavenge $O_2^{\bullet -}$. CAT, APX, and GR play a crucial role in the scavenging and detoxification of H_2O_2. Thus, the presence and concentration of these antioxidant enzymes indicates the status of the antioxidative defense in plant cells. In heavy metal-sensitive plant species, ROS levels will rise substantially if there are not sufficient antioxidant enzymes available. Consequently, ROS-induced cellular damage will induce local programmed cell death and will generally affect plant growth and development. Heavy metal-tolerant species normally produce high levels of ROS scavenging antioxidant enzymes, providing a sufficiently efficient antioxidant defense mechanism against heavy metal-induced oxidative stress [159].

Zn plays a dual role in the ROS-induced oxidative stress in plant cells. Zn excess leads to ROS production, while Zn is also an important cofactor of SOD, which catalyses the removal of $O^{\bullet}$ and thus protects cells from ROS-induced damage [160]. Zn is not a redox-active metal, which means it is not directly involved in the Haber–Weiss and Fenton reactions, as are known for Fe. Nevertheless, excess Zn leads to the production of $OH^{\bullet}$ and $CH3^{\bullet}$ radicals, as observed in cell walls of *Verbascum thapsus*, and the accumulation of H_2O_2 in the leaf apoplast [161]. Increased activity of the antioxidant enzymes SOD and APX and also monodehydroascorbate reductase (MDHAR) accounts for the accumulation of ROS-scavenging ascorbate and phenolics, thus enhancing the survival of *V. thapsus* under excess Zn exposure-induced oxidative stress [161]. Excess Zn-induced toxicity in *Brassica napus* was found to be caused by intervention in its nutrient balance and induction of oxidative stress due to decreased SOD and APX activities [162]. Higher activities of the antioxidant enzymes SOD, CAT, APX, GR, and DHAR also accounted for the higher Zn tolerance of Zn hyperaccumulating ecotypes of *S. alfredii,*

compared to non-hyperaccumulating ecotypes, under high Zn exposure [163].

The Zn- and Cd-induced antioxidative mechanism in plant cells mainly acts through enhancing activity or presence of antioxidant enzymes or increasing accumulation of antioxidants. For example, the enhanced presence of antioxidative enzymes (such as CAT and SOD) under Cd stress in Cd hyperaccumulating species/ecotypes like *N. caerulescens*, *Brassica juncea*, *S. alfredii*, and *S. nigrum* results in less ROS accumulation and increased Cd detoxification ability than in the related non-hyperaccumulating species/ecotypes, such as *N. tabacum*, *S. alfredii*, and *S. melongena* [81, 164, 165].

The ROS response mechanism induced by Zn or by Cd exposure can act through different defense pathways. For example, the Zn/Cd hyperaccumulator *Arabis paniculata* adapts to Zn excess stress by enhancing expression of proteins involved in energy metabolism and protein metabolism to accelerate plant growth and correct misfolded proteins; but it resists Cd exposure stress by promoting the antioxidative defense and cellular metabolism to maintain cellular redox homeostasis [166]. Similarly, *N. caerulescens* under high Cd exposure shows higher APX activity but lower SOD activity than when under high Zn exposure [167]. Although Cd and Zn may induce the antioxidant mechanisms by different pathways, Zn plays a synergistic role in Cd-induced antioxidant defense because of its role as an enzyme cofactor. Zn supplementation enhances the activities of antioxidant enzymes SOD, CAT, APX, and GR, and increases the accumulation of antioxidants like ascorbic acid and GSH, as found in Cd-treated *Solanum lycopersicum*, *S. alfredii*, and *Triticum aestivum* [168–170]. Next to Zn, also low Mg can have a synergistic effect on Cd toxicity in plants, probably by maintaining Fe status, increasing the antioxidative capacity and protecting the photosynthetic apparatus [171].

Signal transduction of heavy metal stress

Next to controlling cellular metal contents, by the activity of various transporters, and reducing the toxic effects due to ROS generation, plants need to sense the presence of metals. More specifically, they need to sense alterations in the cytoplasmic and organellar concentrations of heavy metals to anticipate overaccumulation and prepare for detoxification. In this section, we report on heavy metal-induced signal transduction in response to excess metal exposure. When talking about the signaling transduction, a basic scheme should be kept in mind (Fig. 3), meaning that plant stressors (such as Cd) can induce signaling perception through an external signal (such as calcium or miRNA), upon which the signals are rapidly transmitted (e.g., through MAP kinases) to the responsible transcription factors. These will interact with gene promoter elements to induce the required gene expression response [172].

Dual role of ROS

Not only Zn but also ROS play a dual role in metal stress response. ROS act both as oxidative molecules, aggressively reacting with cellular macromolecules, and as signal transduction molecules [173]. For example, overproduction of H_2O_2 leads to serious oxidative damage and is thus a danger to cellular function. However, H_2O_2 is also an important signaling molecule, which regulates plant development, hormone signaling, programmed cell death, and biotic and abiotic stress response and tolerance [158]. Thus, keeping control over ROS generation in plant cells during metal exposure is important to keep control of developmental processes and general stress response. In this section, we will further outline the role of ROS in heavy metal stress signal transduction and response.

Different metals induce different pathways to regulate the induction of ROS signals [159]. Although the mode of Zn-regulating ROS signal transduction is still not clear, the action of another heavy metal micronutrient, Cu, has been well studied. Excess Cu induces the accumulation of ROS either directly, by acting as a redox-active molecule, or indirectly, by inhibiting microRNA398 (miR398) expression, which was found to be regulated by SQUAMOSA promoter-binding (like) protein binding to the GTAC(T) motif of the miR398 promoter [174, 175]. Cd-induced ROS accumulation is mediated and indirectly regulated by NADPH oxidase and Ca, but also includes involvement of miR398. NADPH oxidase is localized on the plasma membrane and produces superoxide ($O_2^{\bullet -}$) from oxygen (O_2) when challenged with excess metals [176–178]. Excess Cd also inhibits the expression of miR398, which normally targets copper/zinc superoxide dismutase genes *CSD1* and *CSD2* for degradation. These encode the most important SODs in plant cells [179, 180]. Reduced miR398 results in increased *CSD 1* and *CSD2* expression, which enhances CSD accumulation, and subsequent detoxification of superoxide into less toxic H_2O_2 in mitochondria [179, 181–183]. Thus, the reduction of miRNA398 induces ROS detoxification and antioxidant accumulation, which further reduces ROS accumulation in plant cells [184]. Cd-induced production of ROS can also be mediated by a different process, involving protein phosphatases, Ca^{2+} channels, and cGMP, as found in pea [185]. Excess Cd can lead to Ca^{2+} deficiency, which results in the downregulation of antioxidant enzymes CAT and CSD [186, 187]; as a result, less ROS are scavenged.

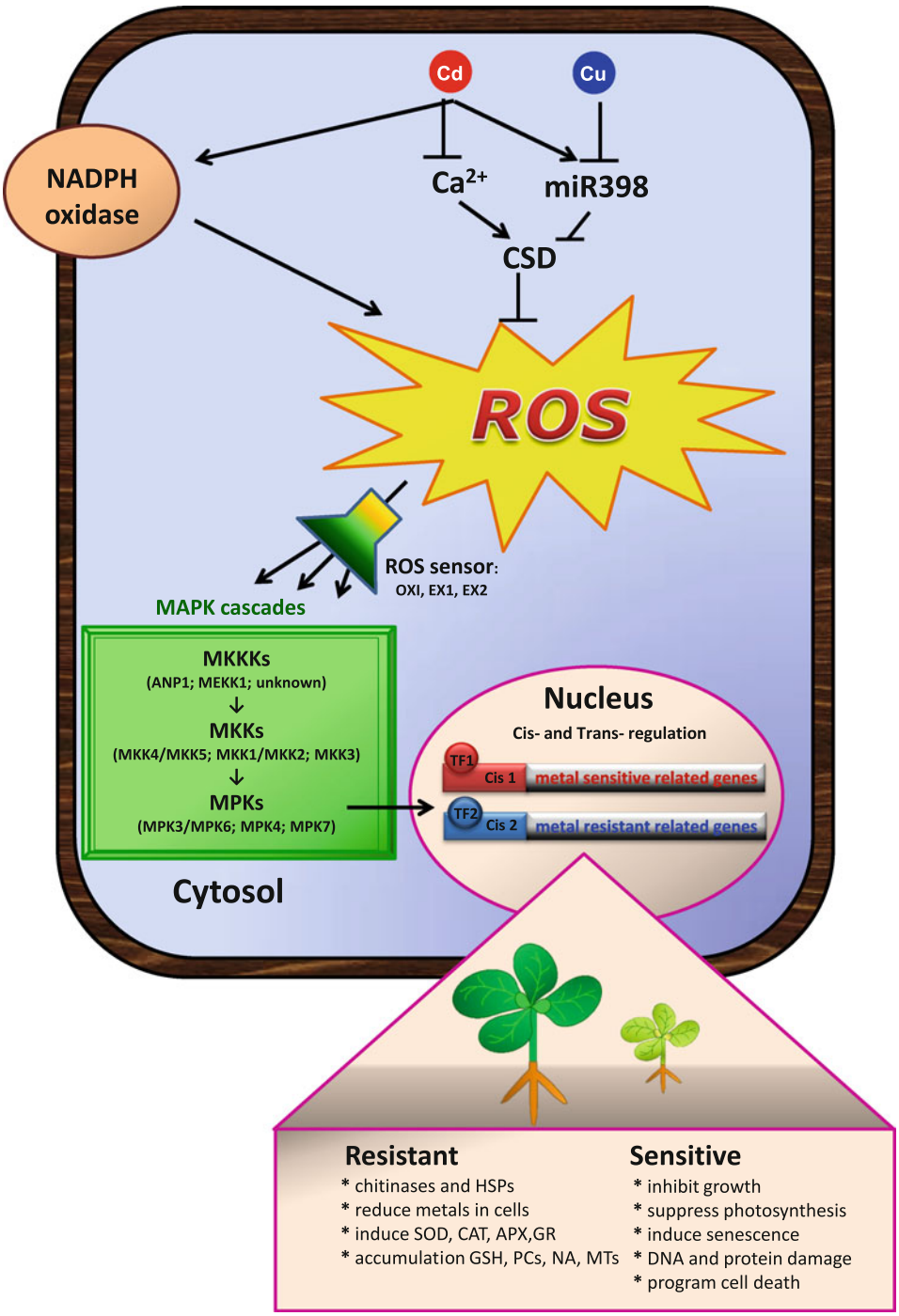

Fig. 3 Signal transduction in response to heavy metal stress in plants. Heavy metals, such Cd (*red*), but also Cu (*blue*), can affect ROS accumulation through three ways, (1) excess Cd^{2+} induces miR398 expression, which inhibits the function of Cu/Zn/SOD (CSD) and further induces ROS accumulation; (2) excess Cd^{2+} inhibits the regulatory role of Ca^{2+}, which also stimulates ROS accumulation through inhibited CSD activity; (3) excess Cd^{2+} enhances NADPH oxidases, which leads to additional H_2O_2 production. The accumulated ROS are detected by ROS sensors, the OXI1, EX1, and EX2 proteins, and used as a signal to induce MAPK (mitogen-activated protein kinase; MPK) cascades (*green rectangle*), which are a series of phosphorylations from MKKKs (MAPK kinase kinases) to MKKs (MAPK kinases), and then to MPKs (MAP kinases). The MAPK cascades can activate functions of transcription factors (*trans*-regulatory elements) in the nucleus, which regulates gene expression through the binding to *cis*-regulatory elements, and further controls the plant response to heavy metals, to be sensitive or tolerant. MAPK cascade-mediated gene expressions in ROS signaling pathways have been well studied by Pitzschke et al. [212]. *ROS* reactive oxygen species, *OXI1* oxidative signal-inducible 1, *EX1* or *2* Executer 1 or *EX2*, *SOD* superoxide dismutase, *CAT* catalase, *APX* ascorbate peroxidase, *GR* glutathione reductase, *GSH* glutathione, *PCs* phytochelatins, *NA* nicotianamine, *MTs* metallothioneins, *ANP1 Arabidopsis* nicotiana protein kinase 1-related kinase 1

ROS and stress response

Heavy metal stress-induced ROS production and accumulation in plant cells is detected by the OXI1 (Oxidative Signal-Inducible 1) protein for H_2O_2, and the EX1 (Executer 1) or EX2 proteins for superoxide [188, 189]. These proteins can stimulate the rapid signal transduction of a downstream mitogen-activated protein kinase (MAPK) cascade. A MAPK cascade is a series of phosphorylation steps from MKKKs (MAPK kinase kinase), via MKKs (MAPK kinase), to MPKs (MAPK) [190]. MAPKs are serine/threonine kinases, which can phosphorylate a large number of transcriptional factor (*trans*-regulatory elements), such as DREB (Dehydration-Responding Element Binding proteins), bZIP (basic region leucine ZIPper), NAC (NAM, ATAF1,2, CUC2), AP2 (Activator Protein 2), RAV (Related to ABA-insensitive3/Viviparous1), WRKY (containing a conserved WRKYGQK domain and a zinc-finger-like motif), MYB (MYeloBlastosis), bHLH (basic Helix-Loop-Helix), Zat (Zinc finger, C2H2-EAR-motif-containing repressor), and GRAS (GAI, RGA, SCR), and further change the expression of *cis*-regulatory elements, such as IDRS (Iron-Dependent Regulatory Sequences), and ABA (abscisic acid), SA (salicylic acid) or IAA (auxin) responsive elements [191, 192]. For example, activation of OXI1, a serine/threonine kinase, is required for full activation of MAP kinases MPK3 and MPK6 [188]. Colcombet and Hirt [193] proposed that ROS act upstream of several MAPK cascades, one of which is the H_2O_2-activated ANP1 (*Arabidopsis Nicotiana* protein kinase 1-related kinase 1) phosphorylation of MKK4/MKK5 and subsequently MPK3/MPK6; a second one is the H_2O_2-activated MEKK1 phosphorylation, via MKK1/MKK2, to MPK4 [194]; and finally, the H_2O_2-activated phosphorylation of an as yet unknown component via MKK3, to MPK7. The activation MAPK cascade is plant-species and metal variety-dependent. In alfalfa (*Medicago sativa*) seedlings, excess Cu can activate four MAPKs (SIMK, SAMK, MMK2, MMK3) responses, and does this faster than Cd. It can also activate SIMKK, which Cd cannot [195]. In *A. thaliana*, Cd can trigger activation of MPK3/MPK6 in a ROS dosage-dependent way [196]. In rice, increasing myelin basic protein kinase (MBP) activities by excess Zn exposure can be inhibited by ROS treatments [197].

The signal coming from a MAPK cascade will be further transduced to transcription factors, which can bind to specific *cis*-regulatory elements and induce metal responsive gene expression. In metal-sensitive plants, the MAPK cascades activate *trans*- and *cis*-regulatory mechanisms that are insufficient to provide metal tolerance, while in metal-tolerant plants, MAPK cascades successfully activate an adequate metal tolerance mechanism. In metal-sensitive plants, the Cd-ROS-MAPK signal causes damage through different processes: (1) interruption of the hormonal signaling pathway; (2) suppression of photosynthesis; (3) damaging macromolecules; (4) induction of senescence; and (5) induction of programmed cell death. Interfering with hormone signaling will inhibit plant growth and development. For example, Cd-induced ROS can activate auxin oxidase, which degrades auxin, and changes the auxin-regulated morphogenetic response in *A. thaliana* rosette leaves [198, 199]. The effect on photosynthesis includes restraining photosystem II (PSII) activity, inhibiting the PSII photoreaction, lowering photophosphorylation, reducing the activity of chloroplast enzymes RuBPC and phosphoribulokinase, decreasing photosynthetic pigments (such as total chlorophyll content and chlorophyll *a*/*b* ratio), diminishing net photosynthesis in leaves, and reducing chloroplast metabolism [200]. Macromolecules like proteins and nucleic acids are damaged by oxidation, impaired DNA repair, and poor protein folding. Enhanced lipid peroxidation causes membrane damage that may lead to cell death [201, 202]. Senescence is induced through H_2O_2-induced MEKK1 expression, which enhances the expression of transcription factor WRKY53, a positive regulator of leaf senescence in *A. thaliana* [203, 204]. Finally, the metal-induced production of H_2O_2 acts as signaling molecule to trigger the expression of the WRKY75, Zat11 and NAM transcription factors that stimulate programmed cell death in plants [205].

In heavy metal-adapted plants, the Cd-induced ROS-MAPK response is different and does not lead to the same damage as observed in metal-sensitive plants. This appears to be due to the heavy metal-induced accumulation of repair proteins, such as chitinases, and molecular chaperones, such as heat shock proteins (HSPs). Different isoforms of chitinases are expressed in response to metal treatments, implying a specific role in the metal response mechanism [206]. Overexpressing fungal chitinases in tobacco or *A. thaliana* can confer Cd tolerance in transgenic plants [207, 208]. Induction of HSPs in response to oxidative heavy metal stress is thought to involve the molecular chaperone activity of HSPs, to prevent misfolding of proteins, protein aggregation, and the degradation of (denatured) proteins under stress [209]. Cd stress induces the expression of several HSP proteins, such as HSP70s, chaperonin, and mitochondrial HSP60s [210]. The interaction between *OsHSP70* and *MAPKs* protects rice roots from Cu toxicity [211].

Conclusions

When facing heavy metal stress in nature, plants have evolved one of four different responses to deal with the stress (Fig. 2a). These responses are guided by the molecular mechanism in plant cells (Fig. 2b) and by a

series of signal transductions (Fig. 3). ROS play a dual role in these mechanisms and affect the plant response to heavy metal stress. Most plant species are not sufficiently armed against heavy metal stress, which means their response leads to metal sensitivity, exhibited by inhibited root growth and leaf chlorosis. Large amounts of toxic metals will enter plant cells, which have no sufficient mechanisms to detoxify them. Thus, excess metals induce accumulation of ROS, which results in a further negative impact on plant growth and development, suppressed photosynthesis, damage to nucleic acids and proteins, enhanced programmed cell death, and induction of senescence. The other response to metal stress is metal tolerance, in which plant growth and development are not much affected by metal stress, at least plants do not succumb. To resist heavy metal exposure, the entrance of metals into cells needs to be avoided or otherwise regulated; toxic metals ending up in the cytosol should be efficiently detoxified; metal-induced accumulation of ROS should be immediately scavenged; the cell membrane system should be protected; and the injured cells should be quickly repaired. Plant cells can detoxify metal-induced damage through the accumulation of repair proteins (chitinases and HSPs), antioxidants (GSH), antioxidant enzymes (SOD, CAT, APX, and GR) and accumulation of metal chelators (PCs, NA, and MTs).

Metal-tolerant plants usually take one of three strategies to maintain metal homeostasis. One involves heavy metal exclusion, in which metals are excluded outside the cells or bound to cell walls, to reduce the metal concentration in the cell. Another involves sequestration of heavy metals, as performed by heavy metal-tolerant non-hyperaccumulators, in which metals are taken up by root influx transporters and subsequently sequestered into vacuoles or rapidly exported outside the cell. The third is practiced by heavy metal-hypertolerant hyperaccumulator species, in which metals are very efficiently detoxified in vacuoles by several tonoplast transporters, while large amounts of metals are transported from roots to shoots via xylem. Interestingly, the required enhanced expression of involved transporters in heavy metal hyperaccumulators is often caused by gene copy number multiplication, which appears to act prior to subsequent differentiations in gene expression regulation.

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