Planta (2001) 212: 475–486

Planta

Review

Molecular mechanisms of plant metal tolerance and homeostasis

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Received: 14 July 2000 / Accepted: 22 September 2000

Abstract. Transition metals such as copper are essential for many physiological processes yet can be toxic at elevated levels. Other metals (e.g. lead) are nonessential and potentially highly toxic. Plants – like all other organisms – possess homeostatic mechanisms to maintain the correct concentrations of essential metal ions in different cellular compartments and to minimize the damage from exposure to nonessential metal ions. A regulated network of metal transport, chelation, trafficking and sequestration activities functions to provide the uptake, distribution and detoxification of metal ions. Some of the components of this network have now been identified: a number of uptake transporters have been cloned as well as candidate transporters for the vacuolar sequestration of metals. Chelators and chaperones are known, and evidence for intracellular metal trafficking is emerging. This recent progress in the molecular understanding of plant metal homeostasis and tolerance is reviewed.

Key words: Chelation – Hyperaccumulation – Metal homeostasis – Metal tolerance – Metal trafficking – Metal transport

Introduction

A number of heavy metals, including copper and zinc, are essential micronutrients required for a wide variety of physiological processes. Zn^{2+}, for example, serves as cofactor for many enzymes, and a vast number of protein sequences contain Zn^{2+}-binding structural domains (Clarke and Berg 1998). Copper is a vital component of electron-transfer reactions mediated by proteins such as superoxide dismutase, cytochrome *c* oxidase and plastocyanin. However, these same metals can be toxic at supraoptimal concentrations. The reactivity of copper, for instance, can lead to the generation of harmful reactive oxygen species. Furthermore, plants encounter metals such as cadmium, lead or mercury, which are generally considered nonessential and are potentially highly toxic due to their reactivity with S and N atoms in amino acid side chains. In order to maintain the concentration of essential metals within physiological limits and to minimize the detrimental effects of nonessential metals, plants, like all other organisms, have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals.

The main components of metal homeostasis are transport, chelation and sequestration processes (Fig. 1). Their regulated activities ensure the proper delivery and distribution of metal ions at the cellular and at the organismal level, resulting in a basic level of metal tolerance. Loss of one of these critical processes leads to hypersensitivity. For example, the phytochelatin-synthesis-deficient *Arabidopsis* mutant *cad1* is more sensitive to Cd^{2+} than wild-type plants (Howden et al. 1995; see below).

Basic metal tolerance is ubiquitous. Current evidence suggests that plants share several common metal-tolerance mechanisms. Some plant species and genotypes, however, can grow on soil that naturally, or due to human activities, contains growth-prohibiting concentrations of metals. These plants belong to a specialized flora that has colonized Ni-rich serpentine soils, or areas polluted by, for instance, Zn smeltering or mining activities (Ernst 1974). They possess naturally selected higher levels of tolerance ("hypertolerance", Chaney et al., 1997). Mostly, this hypertolerance is specific for certain metals (Schat and Vooijs 1997).

Some plants not only tolerate higher levels of metals but hyperaccumulate them. About 400 different species belonging to a wide range of taxa have been described as hyperaccumulators (Baker and Brooks 1989). Hyperaccumulators generally refer to plants able to accumulate

Abbreviations: GSH = reduced glutathione; MT = metallothionein; PC = phytochelatin

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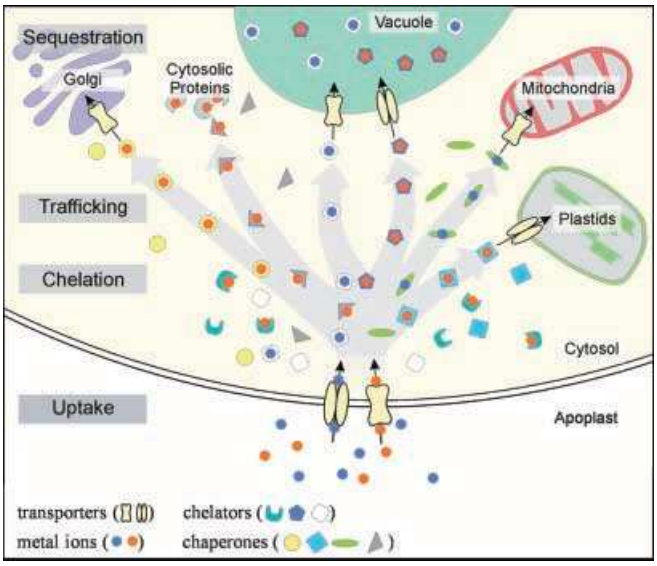

Fig. 1. Simplified hypothetical scheme of the cellular plant metal homeostasis network. Following uptake through transporters, metal ions are bound by chelators and chaperones. Chelators buffer cytosolic metal concentrations; chaperones are involved in metal trafficking. Essential metal ions are delivered specifically to metal-requiring cytosolic proteins and to organelles. Uptake into the organelles is catalyzed by metal-ion pumps that directly interact with specific chaperones. Free chaperones can then bind a metal ion again. Detoxification and storage of excess metal is achieved by sequestration in the vacuole. Transporters residing in the tonoplast mediate the passage of metal ions and metal-chelator complexes

>0.1% of dry weight of elements such as Ni, Co or Pb. For Zn the limit is >1%, for Cd >0.01% of dry weight. The hyperaccumulation phenotype is the basis for the concept of phytoremediation, i.e. the use of metal-hyperaccumulating plants for the clean-up of metal-contaminated soil (Chaney et al. 1997). Hyperaccumulation is mainly observed with Ni, Zn, Co and Se. Also, four hyperaccumulators of Pb and one hyperaccumulator each for Cd and As have been identified. It is interesting to note that approximately 75% of the hyperaccumulators characterized to date have been Ni hyperaccumulators (Baker and Brooks 1989).

The focus of this review is the recent advances in our molecular understanding of plant metal homeostasis and tolerance mechanisms. This will include a discussion of the transport of metal ions into the cell, since metal-uptake rates directly influence metal tolerance and sensitivity. The metals mainly considered are those that have been studied most intensively with regard to tolerance: Zn, Cu, Ni and Cd. The metal ions are relevant for organisms. However, redox-active metals such as Cu occur in different oxidation states in biological systems. Thus, copper will be mentioned in the elemental form. For biologically redox-inert metals (e.g. Zn, Cd) the ionic forms will be used in the context of experiments where the oxidation state is clear.

Much of the fundamental insight into eukaryotic metal homeostasis has come from experiments involving *Saccharomyces cerevisiae*, and to some degree *Schizosaccharomyces pombe*. Mammalian and plant homologs exist for many of the molecular components identified in yeast to date. Thus, studies on yeast metal homeostasis will be covered in some detail.

A number of important aspects such as the mobilization of metals, the role of mycorrhizal colonization for tolerance, etc. will not be included due to space constraints. I apologize to all colleagues whose work cannot be cited within the scope of this review.

Uptake of metal ions

In recent years we have seen tremendous advances in our molecular understanding of the entry of both essential and nonessential metal ions into plant cells. By complementation of *S. cerevisiae* uptake mutants, cDNAs encoding micronutrient transporters have been cloned (Fig. 2). *Arabidopsis* COPT1 rescues the high-affinity copper uptake deficiency of *S. cerevisiae* mutant *ctr1-3* and is hypothesized to represent a copper transporter (Kampfenkel et al. 1995). However, since the *COPT1* mRNA is not detectable in root tissue, plant transporters involved in Cu uptake from the soil remain to be identified.

Uptake Fe^{2+} and Zn^{2+} is mediated by a group of transporters belonging to the ZIP family (for *Z*RT, *I*RT related *P*roteins, Fox and Guerinot 1998; TC 2.A.5 according to the classification system for transmembrane solute transporters, Saier 2000), members of which can be found in a diverse range of eukaryotes. First isolated was IRT1 from *Arabidopsis* (Eide et al. 1996). IRT1 suppresses the growth defects of the iron-transport-deficient *S. cerevisiae fet3 fet4* mutant under iron-limiting conditions, and IRT1-expressing yeast cells

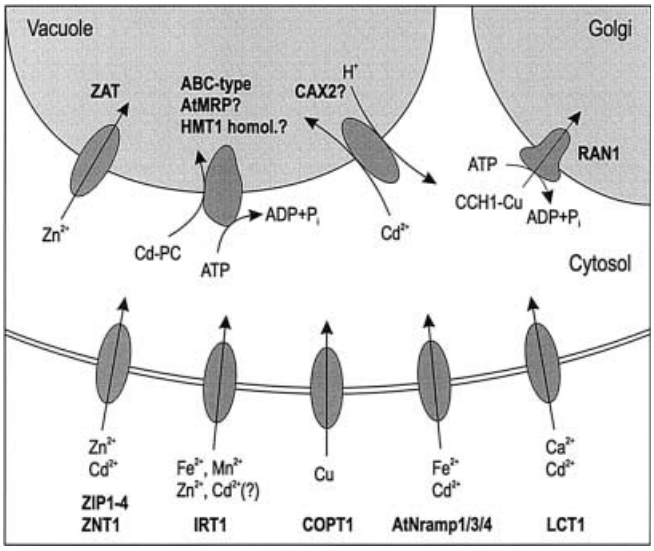

Fig. 2 Plant metal transporters identified to date. Plant proteins that have been shown in heterologous systems to be involved in the uptake, trafficking or sequestration of metal ions are shown with their respective substrates. The localization is depicted as hypothesized for these transporters. HMT1 is an ABC-type transporter from *Schizosaccharomyces pombe*, of which no plant homolog has been identified to date

exhibit a novel Fe(II)-uptake activity. *IRT1* transcription is induced in *Arabidopsis* roots by iron starvation, which makes this transporter a likely candidate for mediating Fe^{2+} uptake from the soil. Additional studies in yeast showed that IRT1 has a broad substrate range and also transports Mn^{2+}, Zn^{2+} and possibly Cd^{2+} (Korshunova et al. 1999).

The cloning of IRT1 has led to the discovery of the related ZIP family. *Saccharomyces cerevisae* ZRT1 and ZRT2 were identified based on sequence similarity to IRT1 and shown to represent high-affinity and low-affinity Zn^{2+} transporters, respectively (Zhao and Eide 1996a,b). A *zrt1 zrt2* yeast mutant, which requires elevated zinc levels in the medium for growth, was then used to clone plant Zn^{2+} transporters. *Arabidopsis* ZIP transporters 1–3 confer Zn^{2+} uptake activity with an apparent K_m in the nanomolar range to yeast cells (Grotz et al. 1998; Guerinot and Eide 1999). *ZIP1* and *ZIP3* are expressed mainly in roots and are induced under zinc-deplete conditions, suggesting a role in Zn^{2+} uptake from the soil. A fourth gene, *ZIP4*, was identified as a genomic sequence. The *ZIP4* transcript level is elevated in both root and shoot tissues of zinc-starved plants. The assumed role of ZIP transporters in zinc nutrition is supported by the recently published findings on ZIP homologs in different *Thlaspi* species. Physiological studies had demonstrated that Zn^{2+} uptake into roots of the Zn^{2+} hyperaccumulator *T. caerulescens* shows a similar K_m when compared with uptake by the non-hyperaccumulating relative *T. arvense*, but a 4.5-fold higher V_{max} (Lasat et al. 1996). A Zn^{2+} transporter (ZNT1) was then cloned from *T. caerulescens* by complementation of *zrt1 zrt2* and found to be homologous to ZIP4 from *Arabidopsis* (Pence et al. 2000). The K_m of ZNT1-mediated Zn^{2+} uptake in yeast cells lies in the range of the K_m of Zn^{2+} uptake into *T. caerulescens*. Interestingly, the higher V_{max} of Zn^{2+} uptake in *T. caerulescens* correlates with a significantly higher expression of ZNT1 in these plants, indicating that increased transporter expression could be one factor determining metal accumulation rates.

Most recent data on a third ZIP homolog from *S. cerevisae*, ZRT3, demonstrated that members of this family are not only involved in the uptake of metal ions. ZRT3 is proposed to function in the mobilization of accessibly stored zinc from the vacuole (MacDiarmid et al. 2000). The protein localizes to the vacuolar membrane and *ZRT3* expression is induced under zinc-deplete conditions. Also, using a bioassay for the cytosolic labile zinc pool it was found that even when it had excess zinc a *zrt3* mutant was unable to mobilize that store.

Another family of proteins is emerging as a major factor involved in plant metal uptake, Nramp (= *N*atural *r*esistance *a*ssociated *m*acrophage *p*roteins, TC 2.A.55) metal transporters. Nramp1 was originally identified in mouse as a gene conferring bacterial resistance (Vidal et al. 1993). Homologous sequences have since been identified in bacteria, fungi, plants and animals. Mutations in these Nramp-related genes are implicated in a diverse range of phenotypes such as ethylene insensitivity in *Arabidopsis* (EIN2, Alonso et al. 1999) and abnormal taste behaviour in *Drosophila* (MVL, Rodrigues et al. 1995). Metal-transport activity has been demonstrated for the rat DCT1 (= Nramp2) as well as for SMF1 from *S. cerevisiae* (Gunshin et al. 1997; Supek et al. 1996). Recently, Nramp metal transporters from *Arabidopsis* and rice have been characterized. Based on sequence comparison, this family of proteins can be divided into two classes, AtNramp1 and OsNramp1 belonging to one, AtNramp2–5 and OsNramp2 to the other (Curie et al. 2000). AtNramp1 complements the *fet3 fet4* mutant and is up-regulated under iron-limiting conditions. Therefore, it is likely to play a role in iron homeostasis (Curie et al. 2000). Similar results were obtained for AtNramp3 and AtNramp4 (Thomine et al. 2000). Furthermore, analysis of an AtNramp3 insertion mutant and AtNramp3-overexpressing plants indicated that AtNramp3 is also involved in Cd^{2+} uptake. Disruption of the gene slightly enhances Cd^{2+} tolerance, whereas overexpression causes Cd^{2+} hypersensitivity.

With the exception of the recently described Cd-carbonic anhydrase of marine diatoms (Lane and Morel 2000), no biological function is known to date for the potentially highly toxic heavy metals Pb and Cd. Thus, it is unlikely that transporters with specificities for the respective metal cations exist. Instead, these nonessential metals are likely to enter cells through cation transporters with a broad substrate specificity. It is well documented that iron-deficiency leads to enhanced uptake of other metal ions (Cohen et al. 1998). This is attributable to the induction of such cation transporters under low-iron conditions. As mentioned above, direct evidence has now been obtained for Cd^{2+}-transport activity of IRT1, ZNT1 and AtNramp3 (Korshunova et al. 1999; Pence et al. 2000; Thomine et al. 2000). A search among putative cation transporter cDNAs for effects on growth in the presence of Cd^{2+} upon expression in yeast led to the observation, that wheat LCT1 (TC 9.A.20) renders yeast cells more Cd^{2+} sensitive (Clemens et al. 1998). LCT1 had originally been cloned by complementation of the K^+ high-affinity uptake-deficient yeast mutant CY162 and had been shown to also mediate Na^+ influx (Schachtman et al. 1997). Additional studies revealed that LCT1 expression leads to increased Cd^{2+} and Ca^{2+} uptake activity in *S. cerevisiae* (Clemens et al. 1998).

The first example of a plant transporter possibly mediating Pb^{2+} uptake has been described by Arazi et al. (1999). They overexpressed a calmodulin-binding cyclic nucleotide-gated channel (NtCBP4), which was found to localize to the plasma membrane, in tobacco. This resulted in an increased sensitivity toward Pb^{2+} and correlated with enhanced Pb^{2+} accumulation. Interestingly, NtCBP4 overexpressors at the same time are more Ni^{2+} tolerant. Possible explanations for this observation are, as suggested by the authors, interaction of NtCBP4 with Ni^{2+}, which attenuates uptake, or the suppression of other, more Ni^{2+}-selective channels, by NtCBP4 overexpression. Uptake studies with NtCBP4 will help our understanding of the reported phenotypes.

From the data gathered to date about plant metal transporters it is obvious that multiple pathways exist for most metal ions. Genetic studies will be needed to test which transporters contribute most to the uptake of which metal.

Chelation

The reactivity and limited solubility of most metal ions require constant chelation once they are taken up into the cell. This has most clearly been demonstrated for copper in yeast cells (Rae et al. 1999). It was shown that yeast cells contain less than one "free" copper atom per cell (see below). The metal ions are bound by chelators and chaperones. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations. Chaperones specifically deliver metal ions to organelles and metal-requiring proteins. In plants the principal classes of known metal chelators include phytochelatins, metallothioneins, organic acids and amino acids.

Phytochelatins

Phytochelatins (PCs) (=cadystins in *S. pombe*) are small, metal-binding peptides of the general structure $(\gamma\text{-Glu-Cys})_n$-Gly ($n=2$–11) (Kondo et al. 1984; Grill et al. 1985; Jackson et al. 1987). They have been found in virtually all plants tested and certain fungi (Gekeler et al. 1989). PCs are synthesized non-translationally from reduced glutathione (GSH) in a transpeptidation reaction (Grill et al. 1989). The extensive literature on structure and synthesis of PCs and related molecules such as $(\gamma\text{-Glu-Cys})_n$-Ser, $(\gamma\text{-Glu-Cys})_n$-$\beta$-Ala, $(\gamma\text{-Glu-Cys})_n$-Glu and $(\gamma\text{-Glu-Cys})_n$ has been reviewed recently (Rauser 1995, 1999) and will not be discussed here.

Phytochelatin synthesis, catalyzed by the enzyme PC synthase (Grill et al. 1989), is induced within minutes following exposure to a variety of metals or metalloids. Interestingly, some inducers do not seem to serve as substrates for chelation. Low-molecular-weight cytosolic PC-Cd complexes, which are transported into the vacuole where high-molecular-weight complexes are formed under incorporation of S^{2-} (see below), are well characterized. Other metals that could be detected in complexes with PCs include Ag, Cu (Maitani et al. 1996) and As (Schmöger et al. 2000). The significance of PC-Cd complex formation for the detoxification of Cd^{2+} in plants was supported by the isolation of the *Arabidopsis cad1* mutant, which contains wild-type levels of GSH, yet is PC-deficient and Cd^{2+} hypersensititve (Howden et al. 1995). The *CAD1* gene has been cloned recently by positional cloning and was found to encode a PC synthase (the *CAD1* gene is therefore now also called *AtPCS1*) as shown by detection of GSH- and metal-dependent PC synthesis in *Escherichia coli* cells expressing AtPCS1 (Ha et al. 1999). Independently, *AtPCS1* and *TaPCS1* from wheat were isolated in screens for plant cDNAs conferring Cd^{2+} tolerance (Vatamaniuk et al. 1999; Clemens et al. 1999). *S. cerevisiae* cells expressing these genes display a Cd^{2+}-tolerance phenotype that is GSH-dependent and correlates with the synthesis of PCs. Purified recombinant PCS proteins from *Arabidopsis* and *S. pombe* catalyze the formation of PCs from GSH (Vatamaniuk et al. 1999; Clemens et al. 1999). The cloning of *PCS* genes and their regulation has also been reviewed recently (Cobbett 2000a,b).

The *Arabidopsis cad1-3* mutant is highly sensitive to Cd^{2+} and AsO_4^{2-} compared to the wild type and also displays slightly elevated sensitivities towards Cu, Hg and Ag (Ha et al. 1999). An *S. pombe SpPCS*-knock-out is Cd^{2+}, AsO_4^{2-} and to a lesser extent Cu hypersensitive (Ha et al. 1999; Clemens et al. 1999). The overexpression of AtPCS1 in *S. cerevisiae* leads to an elevation of Cd^{2+}, Hg and arsenate tolerance (Vatamaniuk et al. 1999). However, sensitivity towards Zn^{2+} and Ni^{2+} is not altered, and effects on Cu sensitivity are small. These data underscore the importance of PCs for the detoxification of a range of metals and metalloids. They also demonstrate that PC formation cannot represent the only mechanism of metal tolerance in plants. Furthermore, PCs appear not to be involved in the generation of naturally selected metal tolerance (see below).

Metallothioneins

Metallothioneins (MTs) are ubiquitous low-molecular-weight, cysteine-rich proteins, which bind metal ions in metal-thiolate clusters (Hamer 1986). The archetypical class-I MTs from mammalian cells are known to confer Cd^{2+} tolerance (Masters et al. 1994). The metal predominantly bound is Zn^{2+}, which is why mammalian MTs are hypothesized to be involved in Zn^{2+} buffering. Yeast MTs, which belong to class II (i.e. the alignment of Cys residues is divergent from that of class-I MTs), appear to bind mainly Cu. They constitute one of the cytosolic Cu stores. The copper-inducible *S. cerevisiae* MT CUP1 contributes to copper detoxification (Hamer et al. 1985). A disruption of *cup1* causes Cu hypersensitivity; the overexpression of *cup1* enhances Cu tolerance. As with class-I MTs, Cd^{2+} binding could also be detected. MT-Cd complexes are formed in *S. pombe* cells expressing a *S. cerevisiae* MT (Yu et al. 1994).

The first MT identified in plants was the wheat E_c (for early Cys-labelled) protein. It was isolated from mature embryos and shown to bind Zn^{2+} (Lane et al. 1987). To date, more than 50 MT-like sequences have been found in various plants (Rauser 1999). They differ considerably from the mammalian and fungal MTs. Most of the genes are also diverse from the MT-II-like wheat E_c sequence and have been categorized into different types based predominantly on the Cys arrangements (Robinson et al. 1993). It has been hypothesized that the differences in Cys motifs (e.g. type 1: exclusively C-X-C; type 2: combination of C-X-C, C-C and C-X-X-C) could account for differences in metal specificities (Robinson et al. 1993). In *Arabidopsis*, at least three type-1 sequences, two type-2 sequences, 2 E_c-type sequences and at least two sequences of a different type, designated MT3, have been found (Zhou and Goldsbrough 1995; Murphy et al. 1997).

Numerous studies have been published on the expression of plant MT-like genes. Evidence for developmental regulation was found as well as responsiveness to a variety of stimuli including metal exposure, hormone treatments, cold, osmotic stress or heat stress (reviewed and listed by Rauser 1999). A detailed investigation of *Arabidopsis* MT1a and MT2a expression using reverse transcription-polymerase chain reaction (RT-PCR) and in-situ hybridization revealed distinct patterns for the two genes (Garcia-Hernandez et al. 1998). While both mRNA species were detected in root maturation zones and leaf trichomes, only MT1a appeared to be expressed also in vascular tissue and mesophyll cells. The induction of MT2a by Cu treatment, which was described earlier and was found to correlate with Cu tolerance of ten *Arabidopsis* ecotypes (Murphy and Taiz 1995), was restricted to the cotyledons.

Understanding the role of MT-like genes in plants has been hampered by the lack of protein data. Attempts to purify MTs from plant tissues have been unsuccessful with the exception of wheat E_c and, more recently, *Arabidopsis* MT1 and MT2 (Murphy et al. 1997). Only for wheat E_c, has association of the protein with metals been demonstrated. The difficulty in obtaining purified MTs has been attributed to the vulnerability of the MTs to proteolysis, particularly within the long linker region typical of plant MT-like sequences, that is found between the Cys domains (Kille et al. 1991).

Functional data have been obtained from heterologous systems. The copper-sensitivity of the *S. cerevisiae cup1*Δ strain can be suppressed by expression of *Arabidopsis* MT1 and MT2 (Zhou and Goldsbrough 1994). Also, these genes confer an increase in Cd^{2+} tolerance. *Arabidopsis* MT2 partially rescues the Zn^{2+} hypersensitivity of a *Synechococcus* mutant deficient in the Zn^{2+} MT *smtA* (Robinson et al. 1996). *Escherichia coli* cells expressing a *Pisum sativum* type-1 MT as a glutathione *S*-transferase (GST) fusion accumulated more Cu; a similar experiment using fava bean type-1 and type-2 MTs yielded binding of Cu and Cd^{2+}. In both cases, binding of Zn^{2+} was less pronounced (Evans et al. 1992; Foley et al. 1997).

In summary, these data suggest a role of MT-like genes and their respective products in plant metal homeostasis. Proposed functions include detoxification of metals (copper in particular), cytosolic zinc buffering, scavenging of metals during leaf senescence or involvement in metal secretion via leaf trichomes (Robinson et al. 1996; Garcia-Hernandez et al. 1998; Rauser 1999). Genetic and biochemical data will help to find unequivocal evidence for MT function – which even for the more extensively studied class I-MTs of mammals is still considered "enigmatic" (Palmiter 1998).

Organic acids, amino acids

Due to the reactivity of metal ions with S, N and O, carboxylic and amino acids represent potential ligands. Citrate, malate and oxalate have been implicated in a range of processes, including differential metal tolerance, metal transport through the xylem and vacuolar metal sequestration (reviewed in Rauser 1999). Citric acid, for instance, has been hypothesized to be a major Cd^{2+} ligand at low Cd^{2+} concentrations (Wagner 1993), has been shown to form complexes with Ni^{2+} in Ni-hyperaccumulating plants (Sagner et al. 1998) and has been suggested to contribute to Zn^{2+} accumulation and tolerance (Godbold et al. 1984). Similarly, malate was proposed as a cytosolic zinc chelator in zinc-tolerant plants (Mathys 1977). However, with the exception of Al tolerance (Delhaize and Ryan 1995), unequivocal evidence for a function of organic acids in plant metal tolerance has been difficult to obtain. Clear correlations between the concentration of a particular organic acid and the degree of exposure to a particular metal – which have to be postulated in the light of the metal specificity of most homeostatic and tolerance mechanisms – have not been observed. Genetic data are missing and powerful non-invasive analytical tools such as X-ray absorption spectroscopy are restricted to information on the ligand environment of a metal ion. Modelling of metal speciation (Rauser 1999) is limited by the lack of knowledge about additional potential metal ligands and their respective association constants. Moreover, ligands for a metal ion change depending on, for instance, compartmentalization, availability of other ions and pH. When Cd^{2+} binding was analyzed in *Brassica juncea* by X-ray absorption spectroscopy, interaction with O or N ligands was shown for the xylem, while in root tissue an S ligand (PCs) was detected (Salt et al. 1995).

The one example known to date for a significant and proportional change in amino acid or organic acid concentration, elicited by a change in metal exposure, is the so-called histidine response (Krämer et al. 1996). A comparative analysis of two *Alyssum* (Brassicaceae) species, the Ni-hyperaccumulating *A. lesbiacum* and the non-hyperaccumulating *A. montanum*, revealed significantly higher Ni tolerance and shoot Ni content, but not root Ni content, in *A. lesbiacum*. This finding indicated differences in root-to-shoot translocation as a potential factor determining tolerance and accumulation rate. Indeed, exposure of *A. lesbiacum* to Ni caused an increase in amino acid content of xylem sap, which could not be observed in *A. montanum*. This was almost exclusively attributable to an enormous increase in histidine levels. A linear relationship between xylem Ni and histidine concentrations was found over a wide range of Ni exposures. The assumed role of histidine for the chelation of Ni was confirmed by extended X-ray absorption fine-structure analysis of *A. lesbiacum* xylem sap, which produced evidence for coordination of Ni with histidine.

Other Ni-hyperaccumulating *Alyssum* species, *A. murale* and *A. bertolonii*, showed a rise in histidine levels proportional to Ni exposure similar to *A. lesbiacum* (Krämer et al. 1996). However, recent evidence suggests that the His response might not be the general Ni-tolerance mechanism of plants. When the Ni-hyperaccumulator *Thlaspi goesingense* was analyzed, no such histidine response was found (Persans et al. 1999).

Histidine concentrations in xylem sap and shoots remained basically unchanged after transfer of plants to high Ni medium while root histidine concentrations actually declined. When compared to the non-hyperaccumulator *T. arvense*, no significant differences in histidine concentrations in xylem sap, roots and shoots were found during Ni exposure. Also, three cDNAs encoding enzymes involved in histidine biosynthesis were cloned from *T. goesingense*. No changes in expression were observed upon Ni treatment.

Nicotianamine is a non-proteinaceous amino acid, synthesized from three molecules of *S*-adenosyl methionine. It is a precursor of the phytosiderophore mugeneic acid, but unlike mugeneic acid it is not extruded by roots and it has been found in all plants investigated (Stephan and Scholz 1993). Nicotianamine is a chelator of Fe^{2+} and other divalent metal ions as well as Fe^{3+} (von Wirén et al. 1999). Its physiological role has been mainly studied in the nicotianamine-deficient tomato mutant *chloronerva*, which exhibits severe growth impairment and intercostal chlorosis. This phenotype is attributed to disturbances of the internal transport of Fe and other metals, Cu in particular, through the phloem (Stephan and Scholz 1993; Pich and Scholz 1996). Nicotianamine synthase genes have now been identified. The *chloronerva* gene has been isolated by map-based cloning (Ling et al. 1999), and nicotianamine synthases from barley have been purified and the corresponding genes cloned (Herbik et al. 1999; Higuchi et al. 1999).

Chaperones and metal trafficking

With the recent identification of Cu chaperones and Cu transporters from *S. cerevisiae* and humans, intracellular metal trafficking pathways are now emerging (reviewed by O'Halloran and Culotta 2000). The yeast copper chaperone, yCCS, encoded by *LYS7*, is essential for expression of the active, Cu-containing form of yeast superoxide dismutase (ySOD1) (Culotta et al. 1997). When the function of yCCS was studied in more detail, it was found that the number of ySOD1 molecules and the number of Cu atoms per cell are not affected by the absence of yCCS in a knock-out strain, indicating that yCCS is not involved in Cu uptake or in stabilizing ySOD1. However, the ratio of active copper-bound form of the enzyme to the inactive apo form was drastically lowered. Hence, yCCS appeared more likely to directly activate ySOD1, and Cu delivery to purified denatured apo-SOD1 was tested in vitro. It was found that yCCS, Cu(I)-GSH and copper salts reactivated ySOD1. In the presence of Cu-scavenging molecules such as bathocuproine sulfonate, however, reactivation was seen only with yCCS. From this observation it was concluded, that yCCS directly activates ySOD by metal insertion and that yCCS is essential only at negligibly low cytosolic concentrations of Cu. Accordingly, the requirement for yCCS could be overcome by raising the intracellular Cu concentration. SOD activity increased when medium Cu concentrations were elevated and in the absence of the MTs CUP1 and CRS5. Taken together, these data demonstrate that copper chaperones are essential in providing the copper atoms for several Cu-requiring proteins, because they can compete for Cu with other chelators and metal-sequestrating processes at the extremely low cytosolic concentrations of free Cu ions. Based on the number of Cu atoms and SOD molecules, the ratio of active to apo-SOD1 and the association constant for Cu binding to apo-SOD, Rae et al. (1999) estimated the concentration of "free" Cu in a yeast cell to be less than 10^{-18} M, which corresponds to less than one "free" copper atom per cell.

In addition to yCCS two other cytosolic copper chaperones are known from *S. cerevisiae*. COX17 delivers Cu to the cytochrome *c* oxidase complex (Glerum et al. 1996). ATX1 is involved in copper transfer to post-Golgi vesicles via interaction with CCC2, a Cu-pumping P-type ATPase residing in the membrane of post-Golgi vesicles (Fu et al. 1995; Pufahl et al. 1997). Homologs of ATX1 have been found in humans (HAH1, Klomp et al. 1997) and in *Arabidopsis* (CCH, Himelblau et al. 1998). HAH1 directly interacts with closely related P-type ATPases, the Wilson and Menkes' disease proteins (WDP, MNK)(Hamza et al. 1999). Wilson and Menkes' disease are genetic Cu transport disorders caused by mutations in the respective ATPases (Bull and Cox 1994). Mutations in the transporters associated with the genetic defects impair Cu delivery to the ATPase. Interestingly, MNK was shown to re-localize from the Golgi to the plasma membrane in medium with elevated Cu concentrations. Under these conditions MNK is thought to remove excess Cu by pumping it out of the cell (Petris et al. 1996). This observation illustrates the close relationship of metal trafficking and detoxification.

Recently, an *Arabidopsis* gene (*RAN1*) with significant similarity to the Menkes' and Wilson disease genes was identified, that is required for the ethylene signaling pathway (Hirayama et al. 1999). *ran1* mutants show ethylene phenotypes in response to an ethylene antagonist. RAN1 protein functions as a Cu-ATPase. Expression in *S. cerevisiae* rescues *ccc2*Δ cells. In analogy to CCC2, RAN1 is hypothesized to reside in post-Golgi vesicles and to be involved in delivery of copper into proteins of the secretory pathway. Among those are the members of the ETR1 family of ethylene receptors, which are known to require Cu for ethylene binding (Rodriguez et al. 1999). RAN1 appears to play a role in several Cu-dependent processes. This is indicated by the rosette-lethal phenotype of the strong *ran1-3* loss-of-function mutant (Woeste and Kieber 2000), which is not suppressed by ethylene-insensitive mutants.

The identification of RAN1 and the *Arabidopsis* ATX1 homolog CCH1 suggests that in plant cells a Cu trafficking network exists which is analogous to the one described in yeast and human cells (Himelblau and Amasino 2000; Woeste and Kieber 2000). Following uptake through high-affinity transporters such as COPT1, Cu ions are chelated by specific Cu chaperones and delivered to Cu pumps for transport into organelles or directly to cytosolic Cu-dependent proteins. Both Cu chelation and Cu pumping activity are not only required

for Cu acquisition but also for other processes. For instance, during leaf senescence Cu and other metal ions are mobilized to growing parts of the plant in order to minimize loss of valuable nutrients. CCH1 and RAN1 are up-regulated in senescent leaves and therefore might be contributing to the scavenging of Cu ions which is required for this mobilization (Himelblau and Amasino 2000).

Other specific chelator proteins involved in trafficking of essential metal ions await identification in plants. From the emerging similarity of the Cu-trafficking networks in eukaryotes it appears likely that homologs for the other known Cu chaperones will be found in plants. Furthermore, it remains to be seen in plants, as well as in other organisms, which analogous mechanisms exist for less reactive essential metal ions such as Zn^{2+}.

Intracellular sequestration

Excess metal ions have to be removed from the cytosol. Principally this can be achieved by efflux or by compartmentalization. The main storage compartment for toxic compounds in plant cells is the vacuole and there is evidence for vacuolar sequestration of metal ions in plants (Vögeli-Lange and Wagner 1990). Transporters potentially mediating this process have been identified in *S. cerevisae*, *S. pombe* and plants (Fig. 2).

ABC-type transporters

Phytochelatins are found in low-molecular-weight (LMW) and high-molecular-weight (HMW) Cd-binding complexes in plant and *S. pombe* cells (reviewed in Rauser 1995). It is generally assumed that LMW complexes are formed in the cytosol and subsequently transported into the vacuole where more Cd^{2+} and sulfide are incorporated to produce the HMW complex, which represents the main storage form of Cd. The first molecular insight into the vacuolar sequestration of Cd came from the cloning of *HMT1*, which complemented a *S. pombe* mutant deficient in the formation of the HMW complex (Ortiz et al. 1992). HMT1 is a protein with similarity to ABC-type transporters (TC 3.A.1). It localizes to the vacuolar membrane and mediates the MgATP-energized, vanadate-inhibitable transport of PC-Cd complexes and apo-PCs (Ortiz et al. 1995). A similar transport activity was detected in the tonoplast of oat root cells, indicating the operation of an HMT1-like mechanism in plant cells (Salt and Rauser 1995). No HMT1 homolog has been identified in plants, however.

In *S. cerevisae*, the ABC-type transporter YCF1 mediates Cd^{2+} tolerance. YCF1 shares sequence homology with the human multi-drug resistance-associated protein (MRP) and was demonstrated to function as a glutathione *S*-conjugate pump (Szczypka et al. 1994; Li et al. 1996). Substrate for YCF1 is bis(glutathionato)cadmium as determined by transport studies using vacuolar membrane vesicles isolated from a *ycf1*Δ strain and an isogenic wild-type strain (Li et al. 1997). MRP-related sequences have been found in *Arabidopsis* (Lu et al. 1997, 1998; Tommasini et al. 1998). Because of the apparent lack of an HMT1 homolog in plants, the AtMRPs are considered likely candidates as mediators of PC-Cd transport across the tonoplast (Rea et al. 1998). Although AtMRP3 partially complements a *ycf1*Δ strain (Tommasini et al. 1998) no data have been published showing PC-Cd transport activity in vacuoles of *S. cerevisiae* cells expressing either of the known AtMRPs. This has led to the speculation that a chaperone-like co-reactant, absent from the in vitro assays, might be required for AtMRP3-dependent transport of bis(glutathionato)cadmium or PC-Cd complexes (Rea et al. 1998).

Cation diffusion facilitators

The first members of the *C*ation *D*iffusion *F*acilitator (CDF) family of metal transporters (TC 2.A.4) were found in bacteria (Nies 1992) and have now been identified in yeast, animals and plants (Paulsen and Saier 1997; van der Zaal et al. 1999). The main substrates for these mechanistically poorly understood transporters apparently are Zn^{2+} and Cd^{2+} . In *Ralstonia eutropha*, CzcD mediates Zn^{2+} efflux (Anton et al. 1999), and the *S. cerevisiae* proteins COT1 and ZRC1 confer cobalt and zinc/cadmium tolerance, respectively, when overexpressed (Kamizono et al. 1989; Conklin et al. 1992). Both proteins localize to the vacuolar membrane, indicating an involvement in metal sequestration (Li and Kaplan 1998). *S. pombe* cells constitutively express a *ZRC1* homolog that contributes to Zn^{2+} tolerance, as disruption of this gene renders *S. pombe* cells Zn^{2+} hypersensitive (Clemens, unpublished). From mammals, four Zn^{2+} transporters that belong to the CDF family are known. Rat ZnT1 complemented the Zn^{2+} sensitivity of a hamster cell line. Since ZnT1 protein was detected in the plasma membrane, this phenotype was attributed to a Zn^{2+} efflux activity (Palmiter and Findley 1995). For two other ZnTs, a function in Zn^{2+} transport out of the cytosol into different compartments was suggested (Palmiter et al. 1996, 1998).

One CDF has been studied in *Arabidopsis* to date, at least two additional sequences can be found in the genome (accession numbers AL353032 and AC004561). An *Arabidopsis* cDNA, named ZAT, with homology to the mammalian ZnTs was introduced into *Arabidopsis* in sense and antisense orientation under 35S control (van der Zaal et al. 1999). Transgenic plants obtained with the sense construct showed higher growth rates than control plants on medium supplemented with 250 μM $ZnCl_2$. Cadmium sensitivity was not altered. The Zn^{2+}-tolerance phenotype was accompanied by an increase in root Zn^{2+} accumulation when plants were analyzed in hydroponic culture. Shoot zinc content was not different from that of control plants. These findings were interpreted by the authors as an indication of ZAT involvement in Zn^{2+} sequestration. It remains to be determined whether the ZAT protein resides in the tonoplast as suggested.

Other vacuolar transporters

As mentioned earlier, more Cd^{2+} is incorporated into PC-Cd complexes in the course of HMW-complex formation in the vacuole. This additional Cd^{2+} has been hypothesized to reach the vacuole via a direct transport of Cd^{2+} ions (Rauser 1995). A candidate pathway is the Cd^{2+}/H^+ antiport activity detected in tonoplast-enriched vesicles derived from oat root cells (Salt and Wagner 1993). The pH-dependent saturable Cd^{2+} uptake displayed an apparent K_m of 5.5 μM, which the authors estimated to lie in the range of cytosolic Cd^{2+} concentrations in plants growing on Cd-polluted soil. It was suggested that molecularly the same transporter might account for this Cd^{2+}/H^+ antiport and the vacuolar Ca^{2+}/H^+ antiport (Salt and Wagner 1993). Plant genes encoding transporters with the latter activity have been cloned. *Arabidopsis* CAX1 and CAX2 (belonging to transporter family 2.A.19) rescue the *S. cerevisiae* double mutant *vcx1 pmc1*, defective in vacuolar Ca^{2+} uptake (Hirschi et al. 1996). Both proteins resemble microbial Ca^{2+}/H^+ antiporters. In vacuolar membrane-enriched vesicles from *S. cerevisiae* mutant cells expressing either CAX1 or CAX2, a ΔpH-dependent Ca^{2+} uptake was detectable, which was absent from control vesicles. CAX1 and CAX2 were found to differ significantly in affinities for Ca^{2+}. CAX1 was characterized as high-affinity (K_m of 13.1 μM), CAX2 as low-affinity (K_m of >100 μM). The K_m of CAX2 was considered too high to be of physiological relevance for maintaining the Ca^{2+} resting state. Instead, based on preliminary selectivity experiments the authors proposed for CAX2 a function as a high-affinity, high-capacity H^+/heavy metal cation antiporter (Hirschi et al. 1996). This would render CAX2 a candidate for the Cd^{2+}/H^+ antiport. However, no additional data adding support to this hypothesis have been published.

Naturally selected tolerance (hypertolerance)

"Metallophytes", i.e. plants that are able to grow on soils enriched in heavy metals such as zinc, copper, nickel, cadmium or lead, have evolved increased levels of tolerance, which are usually metal-specific (Verkleij and Schat 1990). Genetically this hypertolerance is believed to be controlled by a small number of genes (Macnair 1993). The molecular mechanisms accounting for hypertolerance are little understood. Numerous studies have been conducted with *Silene vulgaris*, which has many ecotypes differing in tolerance of Cu, Cd or Zn (Ernst 1974). All evidence available from comparative studies on sensitive and tolerant ecotypes of *S. vulgaris* suggests that the typical plant response to metal exposure, the formation of phytochelatins – which clearly is involved in the detoxification of a range of metals (see above) – is not responsible for differential metal tolerance to Cd, Cu and Zn. The analysis of Cd-sensitive and Cd-tolerant *S. vulgaris* revealed that the sensitive plants produced PCs more rapidly and in higher amounts at the same external Cd^{2+} concentrations (de Knecht et al. 1994). PC composition and degree of sulfide incorporation into HMW complexes were the same (de Knecht et al. 1994). When exposed to their respective no-effect or IC_{50} Cu concentrations, Cu-sensititve and Cu-tolerant ecotypes showed equal PC synthesis (Schat and Kalff 1992). Similarly, Zn tolerant *S. vulgaris* plants produced less PCs than Zn-sensitive plants both following exposure to the same Zn concentration and to equal-effect Zn concentrations (Harmens et al. 1993).

As described earlier, Ni hypertolerance is correlated with the histidine response in at least some species. It was demonstrated that spraying of plants with histidine can result in an increase in Ni tolerance (Krämer et al. 1996). For Ni hypertolerant *Thlaspi goesingense*, enhanced Ni tolerance of leaf protoplasts was observed in comparison with *T. arvense* (Krämer et al. 1997), indicating the operation of a detoxification mechanism at the cellular level. Sequestration of Ni in the vacuole appears to contribute to this (Krämer et al. 2000).

Enhanced vacuolar sequestration has also been implicated in differential Zn and Cd tolerance (Chardonnens et al. 1998; Verkleij et al. 1998). The most direct evidence was obtained from *S. vulgaris*. A Zn-tolerant ecotype displayed a 2.5- to 3-fold enhancement of MgATP-energized transport of Zn^{2+} across the tonoplast when compared to a Zn-sensitive ecotype. In crosses of those ecotypes the enhanced Zn uptake into the vacuole co-segregated with higher Zn tolerance (Chardonnens et al. 1999). Future work will show whether this is a correlation found generally in Zn-hypertolerant plants – and possibly Ni- or Cd-hypertolerant plants – and which transporters, e.g. ZAT homologs (van der Zaal et al. 1999), are involved.

Conclusions

From the work on Cu homeostasis in human and yeast cells, in particular, it has become apparent that a complex regulated network controls the trafficking of essential metal ions. Transport, chelation and sequestration processes balance the intracellular amounts of Cu, Zn and other essential metals. Several genetic disorders and mutant phenotypes have been linked to disturbances of metal homeostasis, highlighting the physiological importance of this network.

In recent years we have seen the identification of a number of molecular components involved in metal homeostasis from various eukaryotic systems. In plants, uptake transporters for Fe^{2+}, Zn^{2+}, Cu, and Mn ions have been cloned and possible entry pathways for Cd^{2+} and Pb^{2+} have been determined. Phytochelatin synthase genes have been isolated, as well as Cu chaperone genes. RAN1 was characterized as a potential target protein for chaperones, and candidate vacuolar transporters for metal ions or metal complexes have been identified. Histidine response, enhanced vacuolar metal uptake and increased uptake transporter expression have been found as determinants of plant metal hypertolerance and hyperaccumulation.

Still, we are only beginning to understand the mechanisms of metal tolerance and homeostasis in plants and other organisms. Many components have yet to be identified, several fundamental questions remain to be answered. A key process is the sensing of the metal status of a cell and subsequent regulatory steps. Clearly there is transcriptional regulation of genes following metal exposure and in response to metal deficiency. For instance, cDNA-AFLP expression profiling (Bachem et al. 1996) in the metallophyte *Arabidopsis halleri* showed, that a large number of genes is either up- or down-regulated upon metal treatment (Vess and Clemens, unpublished). However, the respective signal transduction pathway is not known from plants (Xiang and Oliver 1998). In *S. cerevisiae*, metal-responsive transcription factors (ACE1, MAC1, ZAP1) have been described, which appear to directly and specifically bind the respective metal and might function as metal sensors (Winge 1998). The microarray-based analysis of *S. cerevisiae* gene expression under different Zn regimes revealed that about 15% of the genes are altered in their expression under zinc deficiency; 46 genes appeared to be directly activated by ZAP1 (Lyons et al. 2000).

Most of the molecular insight obtained so far concerns cellular processes. Very little is known about the mechanisms of homeostasis and tolerance at the organismal level. Factors determining the compartmentalization, e.g. the metal accumulation in trichomes (Salt et al. 1995), are unidentified. With the exception of the histidine response and nicotianamine, understanding of the control of metal translocation from roots to shoots is also lacking. Tremendous differences can be found among plant species and even genotypes in the root/shoot ratio of metal accumulation. Many different factors including uptake into root cells, sequestration in root cells, efflux, long-distance transport, uptake into leaf cells, and storage determine this ratio.

The growing interest in problems related to metal transport, trafficking and tolerance, the use of model systems such as *S. cerevisiae* and *S. pombe*, and the beginning of molecular analysis of model hyperaccumulators like *Arabidopsis halleri* and some of the *Thlaspi* species, will help to further elucidate the molecular mechanisms of plant metal tolerance and homeostasis.

I am grateful to Drs. Ute Krämer and Eugene Kim for critically reading the manuscript and to Christoph Vess for preparing the figures. Research in my laboratory is supported by the Deutsche Forschungsgemeinschaft.

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