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REVIEW ARTICLE

Transition metal transporters in plants

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Abstract

Transition metals such as Fe, Cu, Mn, and Zn are essential minerals for normal plant growth and development, although they can be toxic when present in excess. Thus, for healthy plant growth, a range of transition metals must be acquired from the soil, distributed around the plant, and their concentrations carefully regulated within different cells and organelles. Membrane transport systems are likely to play a central role in these processes. The application of powerful genetic and molecular techniques has now identified a range of gene families that are likely to be involved in transition metal transport. These include the heavy metal ATPases (HMAs), the Nramps, the cation diffusion facilitator (CDF) family, the ZIP family, and the cation antiporters. This review provides a broad overview of the range of potential transport systems now thought to be involved in the uptake, distribution and homeostasis of transition metals in plants.

Key words: CDF family, heavy metal ATPases, membrane transport, Nramp, transition metals, ZIP family.

Introduction

Plants require a range of transition metals as essential micronutrients for normal growth and development. These metals are essential for most redox reactions which, in turn, are fundamental to cellular function. Fe is a key component of haem proteins (e.g. cytochromes, catalase, and Fe-S proteins such as ferredoxin) and a range of other enzymes. Cu is an integral component of certain electron transfer proteins in photosynthesis (e.g. plastocyanin) and respiration (e.g. cytochrome *c* oxidase) and is involved in lignification (laccase), while Mn is less redox active but is also involved in photosynthesis (e.g. O_2 evolution). Zn is non-redox-active but has a key structural and/or catalytic role in many proteins and enzymes. Other transition metals such as Ni and Mo are also essential micronutrients for plant function. When any of these metals are present in short supply, a range of deficiency symptoms can appear and growth is reduced (Marschner, 1995). However, although essential, these metals can also be toxic when present in excess with the production of reactive oxygen species and oxidative injury being particularly important (Schützendübel and Polle, 2002). Thus their concentrations within cells must be carefully controlled, and so plants and other organisms possess a range of potential mechanisms for metal ion homeostasis and tolerance, including membrane transport processes (Clemens, 2001; Hall, 2002). Thus for healthy plant growth and development, a range of transition metals must be acquired from the soil, transported around the plant, and distributed and compartmentalized within different tissues and cells. Clearly membrane transport systems are likely to play a key role in these events.

Until quite recently, very little was known about the molecular mechanisms of metal transport across cell membranes. However, within the last 10 years or so rapid progress has been made, particularly with the budding yeast *Saccharomyces cerevisiae*, and with the application of this knowledge of transport processes in yeast to other eukaryotes (Eide, 1998; Nelson, 1999; Van Ho *et al*., 2002). With the use of genetic and molecular techniques such as sequence comparison to identify transporters, functional complementation of yeast mutants and plant transformation to regulate gene activities, a wide range of gene families have now been identified in plants that are likely to be involved in transition (or heavy) metal transport (Table 1; Fig. 1). These include the heavy metal (or CPx-type) ATPases, the natural resistance-associated macrophage proteins (Nramps) and the cation diffusion facilitators (CDFs) (Williams *et al*., 2000), the ZIP family (Guerinot, 2000), and the cation antiporters (Gaxiola *et al*.,

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Table 1. *Proposed specificity and location of the main transition metal transporters*

Name	Family members in *A. thaliana*	Proposed specificity	Cellular location	Main tissue expression
Heavy metal ATPases (P_{1B})	8			
RANI		Cu	Post-Golgi	Whole plant
PAA1		Cu	Chloroplast	
Ca^{2+}-ATPases (P_{2A})	4			
ECA1		Mn^{2+}	ER	Roots?
Nramp	6 (+EIN2)			
AtNramp 1		Fe, Mn		Roots
AtNramp 2		–		Roots
AtNramp 3		Fe, Cd, Mn	Vacuole	Roots/shoots
AtNramp 4		Fe, Mn		Roots/shoots
OsNramp 1		Mn, Fe		Roots
OsNramp 2		Mn		Leaves
OsNramp 3		Mn		Roots/leaves
LeNramp1		Fe		Root vascular parenchyma
CDF	8			
ZAT (AtMTP1)		Zn	Vesicular/vacuolar	All tissues
TcZTP1		Zn	Intracellular membranes	Leaves, roots
TgMIP1		Cd, Co, Zn, Ni	Vacuole	Leaves
ShMTP1		Mn	Intracellular membranes	Roots, leaves
ZIP	15			
IRT1		Fe, Zn, Mn, Cd	PM	Roots
IRT2		Fe, Zn		Roots
OsIRT1		Fe		Roots
LeIRT1		Fe (broad?)		Roots
LeIRT2		broad?		Roots
TcZNT1		Zn, Cd		Roots, shoots
TcZNT2		–		Roots
ZIPs1-3		Zn		Roots
ZIP4		Zn	Plastids	Roots, shoots
GmZIP1		Zn	Peribacteroid membrane	Root nodules
Cation/H^+ antiporters	12			
CAX 2		Ca, Mn, Cd	Vacuole	Roots
ABC transporters	128	PC-Cd, GS-Cd		
ID17		Fe		
COPT1-5	5	Cu		Leaves, stems, flowers
GNGC channels	20	Ni, Pb	PM	

2002). However, in many cases, clear evidence of their location and function in plants, and even their substrate specificity, is lacking. Furthermore, although it is sometimes assumed that metal cation movement across membranes by some of these transport systems is driven by the proton motive force generated by ATP-powered H^+ pumps, direct evidence for such co-transport in plants is generally lacking. For most of these transporters the gene families are quite large. For example, in *Arabidopsis*, there are eight heavy metal ATPases (Mills *et al*., 2003), six Nramps (Williams *et al*., 2000) and 15 ZIPs (Mäser *et al*., 2001). This diversity may be required for a variety of reasons: to provide the high and low affinity systems needed to cope with varying metal availability in the soil; to provide the specific requirements for transport at the different cellular and organellar membranes within the plant; and to respond to a variety of stress conditions. Of course, some of these gene family members may be functionally redundant. Techniques such as functional analysis in yeast, expression studies in plants under different metal availabilities and analysis of mutant phenotypes will eventually provide the answers to many of these questions. Wider interest in these transporter families include their potential for the genetic modification of certain species, either to improve the metal quality of crops for human and animal nutrition or for the purposes of phytoremediation, a technology using plants to remove toxic elements from soil.

This paper aims to provide an overview of the range of potential transport systems now thought to be involved in the acquisition, distribution and homeostasis of transition metals in plants. It will focus on the general properties of these transporter families, rather than their detailed molecular structure. It will review what is known about their substrate specificity, location and function in relation to the general picture of transition metal nutrition of plants.

Heavy metal ATPases: CPx-type ATPases

The P-type ATPases form a diverse superfamily of transporters that are found in all three kingdoms and function to pump a range of cations across cellular

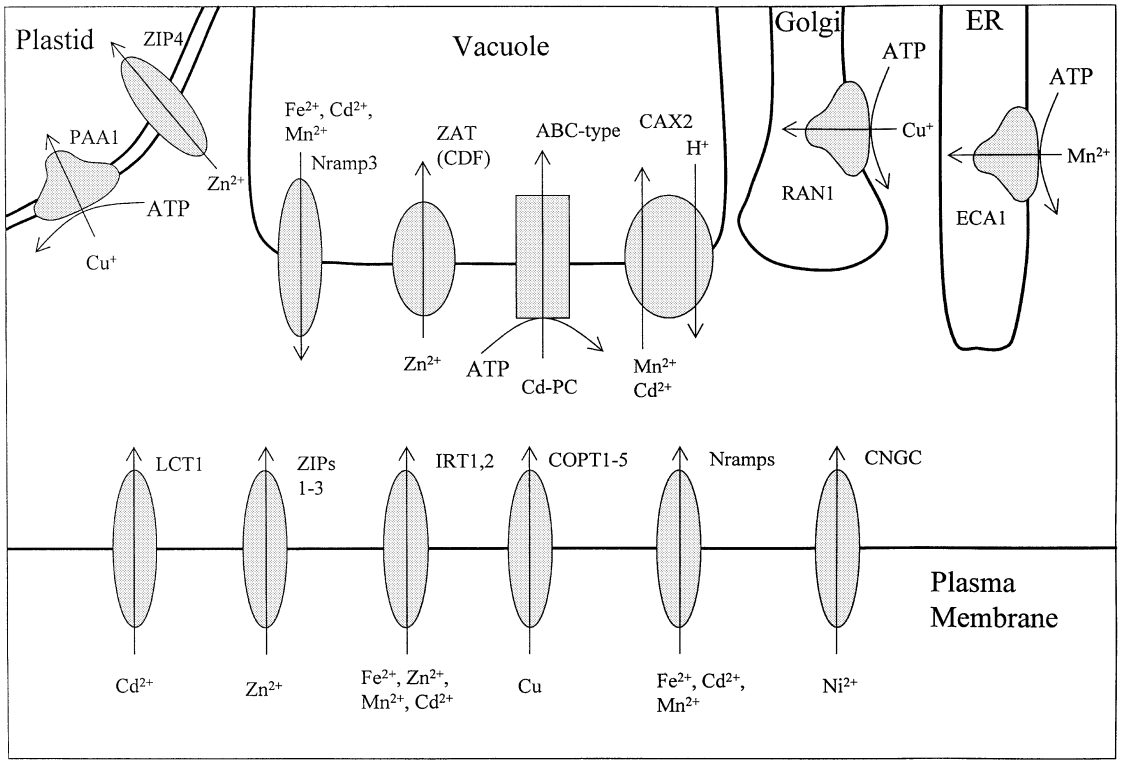

Fig. 1. Summary of putative transition metal transporters identified in plants to date. The membrane localization of some of these transporters, particularly those at the plasma membrane, remains to be confirmed. Modified after Clemens (2001).

membranes. They include the H^+-ATPases of plants and fungi, the Na^+/K^+-ATPases of animals and the Ca^{2+}-ATPases found in many organisms (Axelsen and Palmgren, 2001). They share a common enzymic mechanism that involves the formation of a phosphorylated intermediate in the reaction cycle, hence the name P-type (Serrano, 1989; Palmgren and Harper, 1999; Axelsen and Palmgren, 2001). Typically P-type ATPases contain 8–12 transmembrane domains with a large cytoplasmic loop, usually between TM-4 and TM-5, that contains several highly conserved motifs including the phosphorylation site (Palmgren and Axelsen, 1998; Palmgren and Harper, 1999). However, the heavy metal ATPases (HMAs) are predicted to have eight transmembrane domains with the large cytoplasmic loop between TM-6 and TM-7 (for reviews, see Williams *et al*., 2000; Mills *et al*., 2003).

The P-type ATPase superfamily has been classified into five major families (types I–V), each of which may be divided into two or more sub-families that group according to their transport specificity (Palmgren and Axelsen, 1998; Axelsen and Palmgren, 2001). The P_{1B} sub-family is thought to be involved in heavy metal transport. This group has also been described as the CPx-ATPases since they contain a conserved intramembranous cysteine-proline-cysteine/histidine/serine sequence (Solioz and Vulpe, 1996). Phylogenetic analysis of the P_{1B} sub-family suggested the existence of two main groups which seem to correlate with transport specificity: either for the monovalent Cu^+/Ag^+ cations or the divalent $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ cations (Solioz and Odermatt, 1995; Beard *et al*., 1997; Axelsen and Palmgren, 2001; Arnesano *et al*., 2002; Mills *et al*., 2003). *Arabidopsis* contains eight P_{1B}-type ATPases (Mills *et al*., 2003; http://mips.gsf.de), an unusually high number compared with non-plant species analysed to date; for example, yeast and humans each contain two HMAs (Palmgren and Axelsen, 1998). Only two of the *Arabidopsis* HMAs have been assigned transport specificity to date. RAN1 (AtHMA7) falls into the Cu^+/Ag^+ cluster and is thought to act by delivering Cu^+ across post-Golgi membranes to create functional ethylene receptors (Hirayama *et al*., 1999; Woeste and Kieber, 2000). It is interesting in relation to the transport specificity discussed above that the Ag^+ ion is known to be an effective inhibitor of ethylene responses; thus Ag^+ could be incorporated into the ethylene receptors causing a loss of function and thus a loss of ethylene effects (Hirayama *et al*., 1999). PAA1 (AtHMA6) also falls into the Cu^+/Ag^+ cluster and studies with *paa1* mutants suggest a role in Cu transfer across the chloroplast envelope (Shikanai *et al*., 2003). Phylogenetic analysis indicates that, of the other HMAs in *Arabidopsis*, HMAs 5 and 8 fall into the Cu^+/Ag^+ cluster, while HMAs 1–4 are candidates for the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ sub-class (Axelsen and Palmgren, 2001; Mills *et al*., 2003). *AtHMA4* is expressed in a range of tissues

with the highest expression in roots, and is up-regulated in roots exposed to elevated levels of Zn and Mn (Mills *et al*., 2003). *AtHMA4* was also able to complement functionally, at high Zn concentrations, the *E. coli* mutant *zntA* that is defective in the endogenous Zn efflux ATPase (Mills *et al*., 2003).

Apart from the proposed functions of RAN1 (AtHMA7) and PAA1 (AtHMA6) described above, little else is known about the role of this sub-family in plants. However, studies on bacterial and animal systems strongly support a role for HMAs in metal ion homeostasis that involves ATP-powered efflux from the cytoplasm. For example, the basis of most heavy metal resistance systems in bacteria involve the efflux of toxic ions to the outside, and includes the involvement of P-type ATPases (Solioz and Odermatt, 1995; Silver, 1996; Beard *et al*., 1997). In animals, defects in P_{1B}-type ATPases have been linked to two human disorders, Menkes disease and Wilson disease, that result from impaired Cu export and thus the accumulation of toxic concentrations in some tissues (Solioz and Vulpe, 1996). In Chinese hamster ovary cells, there is evidence that the Menkes ATPase is redistributed from Golgi to plasma membrane in response to elevated levels of Cu, allowing efflux of the potentially toxic metal (Petris *et al*., 1996). However, to date, there is no evidence for a role in efflux by P-type ATPases in heavy metal tolerance in plant cells (Hall, 2002).

An interesting aspect of P_{1B}-type ATPase function involves their potential interaction with metallochaperones. Metallochaperones function to guide and insert a Cu cofactor into the active site of a target protein; they are needed because of the vast capacity of the cytoplasm for Cu sequestration (Harrison *et al*., 2000; Huffman and O'Halloran, 2001; Arnesano *et al*., 2002). A comparative structural genome analysis of metallochaperones and their partner ATPases suggest that the chaperones protect Cu from unwanted binding in the cytoplasm, but are able to recognize their partner proteins and allow rapid metal transfer between the two (Arnesano *et al*., 2002). The best characterized metallochaperone is the ATX1protein from yeast (Eide, 1998). This works in conjunction with CTR1, a plasma membrane Cu transporter, and with CCC2, a Cu-transporting CPx-ATPase that pumps Cu into the post-Golgi secretory compartment. Here Cu is loaded into a multicopper oxidase that is essential for high affinity iron uptake (Eide, 1998; Williams *et al*., 2000; Huffman and O'Halloran, 2001). Homologues of the yeast ATX1 have now been found in other eukaryotes including *Arabidopsis* (copper chaperone, CCH), while *Arabidopsis* also possesses COPT1, a putative Cu transporter that shows some sequence similarity to CTR1 (Kampfenkel *et al*., 1995; Williams *et al*., 2000). Thus a similar system to the one described for the maintenance of the iron uptake pathway in yeast could operate in *Arabidopsis*, with one of the HMAs playing a similar role to CCC2. Indeed, as described above, the HMA, RAN1 (AtHMA7), could act with CCH and COPT1 to deliver Cu to form a functional ethylene receptor at the plasma membrane (Hirayama *et al*., 1999; Williams *et al*., 2000). Thus the HMAs could play a general role in the intracellular trafficking of a range of essential metals in plants. In addition to the ATX1 homologue, *Arabidopsis* also contains homologues to the two other Cu chaperones characterized in yeast, CCS and COX17 (Wintz and Vulpe, 2002).

In addition to the HMAs, at least one other sub-family of the P-type ATPase superfamily is probably involved in transition metal transport, specifically of Mn^{2+}. The *Arabidopsis* ECA1 is a member of the P_{2A} sub-family of Ca^{2+}-ATPases of the endo- and sarcoplasmic reticulum that also transport Mn^{2+} (Axelsen and Palmgren, 2001). ECA1 is able to restore growth of a yeast mutant defective in a Ca^{2+} pump and growing on a medium containing Mn, while the ECA1 polypeptide is able to form a Mn^{2+}-dependent phosphoprotein (Liang *et al*., 1997). More recently, mutants of ECA1 were shown to grow poorly on low Ca^{2+} or high Mn^{2+} (Wu *et al*., 2002). When expressed in yeast, ECA1 provides increased tolerance of a yeast mutant to toxic levels of Mn^{2+} and Zn^{2+}. It was proposed that ECA1 functions to pump Ca^{2+} and Mn^{2+} into the endoplasmic reticulum to support plant growth under low Ca^{2+} or excess Mn^{2+} (Wu *et al*., 2002). Yeast also contains a P-type ATPase, PMR1, related to Ca^{2+}-ATPases that is localized to the Golgi and transports both Ca^{2+} and Mn^{2+} into the secretory system (Eide, 1998).

ABC transporters

The ATP-binding cassette (ABC) superfamily is a very large and diverse family of membrane proteins found in all three kingdoms and involved in a wide range of transport functions (Rea *et al*., 1998; Rea, 1999; Davies and Coleman, 2000; Theodoulou, 2000; Martinoia *et al*., 2002). All ABC transporters are characterized by the possession of one or two copies of two basic structural features: a highly hydrophobic transmembrane domain, each containing 4 or 6 transmembrane spans, and a peripheral (cytosolic) ATP-binding domain or nucleoside-binding fold (Rea, 1999; Theodoulou, 2000). Most ABC proteins are primary pumps driven by ATP hydrolysis and transport a wide range of substrates including ions, sugars, lipids, peptides, pigments, xenobiotics, and antibiotics. To date, two major sub-classes of this superfamily have been identified in plants: these are the multidrug resistance-associated proteins (MRPs) and the multidrug resistance proteins (MDRs). Although the first ABC transporter to be cloned was an MDR-like gene from *Arabidopsis*, only certain MRPs in plants have been characterized functionally to date (Rea *et al*., 1998; Theodoulou, 2000). In particular, a role in the transport of glutathione *S*-conjugates (GS-conjugates) into the vacuole has been the

most fully described. Using isolated vacuoles and vacuolar membrane vesicles, the uptake of GS-conjugates was shown to be energized by MgATP, sensitive to vanadate and independent of the H^+ gradient (Martinoia *et al*., 1993; Li *et al*., 1995). It is now well established that this GS-conjugate pump is an ABC transporter.

In relation to transition metal transport, there is now evidence for a role for ABC transporters in the uptake into the vacuole of Cd, in the form of a heavy metal chelate. In the fission yeast, a Cd-sensitive mutant was isolated that was unable to accumulate a sulphide-containing phytochelatin-Cd complex. A yeast gene, *HMT1*, was isolated that complements this mutant and shares sequence identity with the ABC transporter family (Ortiz *et al*., 1992). Evidence that a similar mechanism might operate in plants was presented by Salt and Rauser (1995) who showed that the transport of a phytochelatin-Cd across the oat root tonoplast was energized by MgATP and highly sensitive to orthovanadate. Thus an hmt1-like mechanism may operate at the tonoplast, although no homologue has yet been identified in plants (Clemens, 2001). However, the MRPs are considered likely candidates to transport phytochelatin–Cd or GS–Cd complexes across the tonoplast (Rea *et al*., 1998). Recently, Bovet *et al*. (2003) found that transcript levels in the roots of four MRPs were up-regulated in *Arabidopsis* after Cd treatment with the increase for *AtMRP3* being particularly pronounced. Interestingly, *AtMRP3* was also induced by Cu treatment, but only slightly by Zn. The induction of *AtMRP3* appeared to be due to the Cd itself rather than via oxidative stress, although there is no evidence as yet that *AtMRP3* functions directly in Cd transport (Bovet *et al*., 2003).

A possible role for ABC transporters in Mn^{2+} transport is suggested by work on the cyanobacterium *Synechocystis*. Oxygen evolution activity and growth rates could be restored in photosynthesis-deficient mutants by the addition of Mn, while complementation and gene inactivation studies showed the involvement of an ABC transporter system (Bartsevich and Pakrasi, 1995, 1996). Recently, a new iron-regulated ABC transporter, *IDI7*, has been identified in barley roots and localized to the tonoplast (Yamaguchi *et al*., 2002). *IDI7* has features typical of an ABC transporter and is induced by Fe-deficiency, although the nature of the transported substrate is not known.

Nramps

The Nramps (Natural resistance associated macrophage proteins) describe a highly conserved family of integral membrane proteins that are involved in metal ion transport in a wide range of organisms, including bacteria, fungi, plants, and animals. The Nramp gene was first identified in the mouse where it is found in the phagosomes of infected macrophages. It is thought to determine sensitivity to bacterial infection by regulating the concentrations of essential divalent cations such as Fe and Mn in the macrophage compartment; mutations in *Nramp 1* result in mice susceptible to bacterial infection (Supek *et al*., 1997; Nelson, 1999). In yeasts, three Nramps have been identified (SMF 1–3) that mediate the uptake of Mn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and Fe^{2+} (Supek *et al*., 1997; Liu *et al*., 1997; Chen *et al*., 1999). Further evidence for a role in metal ion transport came from a study of the DCT1/Nramp 2 gene in mammals. When expressed in oocytes, DCT1 from rats was shown to be a H^+-coupled transporter of Fe^{2+}, but with a broad substrate specificity that includes Zn^{2+}, Mn^{2+}, Cu^{2+}, and Ni^{2+} (Gunshin *et al*., 1997). Further evidence for mammalian Nramps suggests that Nramp 2 is a bivalent cation/H^+ symporter whereas Nramp 1 is an antiporter that can transport metals in either direction against a H^+ gradient (Goswami *et al*., 2001).

In plants, this family was first identified in rice where three Nramps (OsNramp 1–3) were reported (Belouchi *et al*., 1995, 1997). Genes from this family have now been identified in a range of higher plants, including six Nramps in *Arabidopsis* (Williams *et al*., 2000; Mäser *et al*., 2001). The plant Nramps appear to cluster into two sub-families: one includes AtNramps 1 and 6 and the other Nramps 2–5 (Thomine *et al*., 2000; Mäser *et al*., 2001). The rice OsNramps 1 and 3 group with the first sub-family and OsNramp 2 with the second. In addition, in *Arabidopsis*, mutations in the *EIN2* locus give rise to an ethylene-insensitive phenotype (Alonso *et al*., 1999); *EIN2* shows some homology, but much lower, with members of the Nramp family.

Like other Nramps, the plant proteins are highly conserved throughout evolution and contain 12 predicted transmembrane domains with a characteristic 'consensus transport motif' between TM-8 and TM-9 (Gunshin *et al*., 1997; Curie *et al*., 2000; Williams *et al*., 2000). This motif bears similarities to a signature previously recorded in a number of bacterial transport proteins, and particularly with a highly conserved portion of the permeation pore of the animal *shaker*-type K^+ channel (Belouchi *et al*., 1997).

Yeast mutants defective in metal uptake systems have been used to investigate the transport specificities of plant Nramps. AtNramp 1 and OsNramp 1 are able to complement yeast mutants defective in Fe transport, but AtNramp 2 and OsNramp 2 are not (Curie *et al*., 2000). Similarly, AtNramps 3 and 4 are able to support Fe and Mn uptake in transport-deficient yeast, and also increased their Cd accumulation and sensitivity (Thomine *et al*., 2000). However, no transport activity could be demonstrated for EIN2 when expressed in yeast and other systems (Alonso *et al*., 1999). In *Arabidopsis* roots, AtNramps 1, 3 and 4 are up-regulated under conditions of Fe deficiency (Curie *et al*., 2000; Thomine *et al*., 2000), while AtNramp3 is also clearly linked to Fe and Cd uptake and sensitivity (Thomine *et al*., 2000). AtNramp 3 is expressed in the

vascular bundles of roots, stems and leaves and thus may function in long-distance metal transport (Thomine *et al*., 2003). It is up-regulated under conditions of Fe-starvation and localizes to the vacuolar membrane, and thus may function in mobilizing metal pools from the vacuole (Thomine *et al*., 2003). In tomato, *LeNramp1* is specifically expressed in roots, is also up-regulated by Fe-deficiency, and localizes to the root vascular panenchyma (Bereczky *et al*., 2003); it is proposed that *LeNramp1* functions in mobilizing Fe in the vascular tissue under conditions of Fe deficiency. It is also interesting to note that an Nramp transcript from barley is down-regulated in the presence of Cd at adequate nitrogen supply, but strongly up-regulated by Cd under N-deficiency (Finkemeier *et al*., 2003). Thus, overall, these results indicate that the different members of the Nramp family may perform different physiological functions, and that at least some of the Nramps are involved in Fe and Cd uptake and homeostasis. Evidence from yeast and mammalian systems described above, however, suggests that the plant Nramps could have a broader substrate range than this. Further evidence will come from studies of yeast complementation, expression under different metal treatments, and the analysis of transgenic and mutant plants.

Cation diffusion facilitator (CDF) family

First identified in bacteria, this family is now known to occur in yeast, animals and plants where they are involved in heavy metal transport, particularly of Zn, Cd and Co (Paulsen and Saier, 1997; Eide, 1998; van der Zaal *et al*., 1999). These proteins are predicted to have six TM domains and to possess an N-terminal signature sequence and a C-terminal cation binding domain (Paulsen and Saier, 1997; Mäser *et al*., 2001). Eukaryote members also have a histidine-rich domain between TM-4 and TM-5 that may function as a Zn-binding region (Huang and Gitschier, 1997; Williams *et al*., 2000). Members of this family exhibit an unusual degree of size variability, ranging from about 280 to 740 residues (Paulsen and Saier, 1997); the one plant member characterized from *Arabidopsis*, ZAT, contains 398 amino acid residues (van der Zaal *et al*., 1999).

At least seven Zn transporters that belong to the ZnT family have been identified in mammals (Palmiter and Findley, 1995; Huang and Gitschier, 1997; Williams *et al*., 2000; Kirschke and Huang, 2003). ZnT1 is localized in the plasma membrane of rat kidney and is thought to efflux Zn out of the cells; mutants lacking the first membrane-spanning domain show increased Zn sensitivity (Palmiter and Findley, 1995). The other members of this group appear to be localized on intracellular membranes and are involved in the transport of Zn into intracellular compartments such as the Golgi apparatus (Huang and Gitschier, 1997; Mäser *et al*., 2001; Kirschke and Huang, 2003). These mammalian genes are known to be related to two proteins from yeast (*S. cerevisiae*), ZRC1 and COT1, that are thought to be intracellular Zn transporters, perhaps localized at the vacuole (Eide, 1998; Li and Kaplan, 1998); ZRC1 also confers resistance to Cd as well as Zn, while COT1 confers resistance to Co. In the yeast, *Schizosaccharomyces pombe*, another CDF member, Zhf, appears to be localized in the endoplasmic reticulum, and again is involved in cellular Zn homeostasis (Clemens *et al*., 2002). MacDiarmid *et al*. (2002) showed that the major Zn^{2+} transport activities of yeast vacuolar vesicles required a H^+ gradient generated by the V-ATPase; this could be a common energization mechanism for this family of transporters.

A plant CDF transporter gene was first characterized in *Arabidopsis* and designated *ZAT* (van der Zaal *et al*., 1999). It is most closely related to the mammalian ZnTs and shows from 35–40% identity with ZnT 2-4. *ZAT* is expressed constitutively throughout the plant and was induced by increased Zn concentrations. However, overexpression of *ZAT* in transgenic plants led to a significant increase in Zn resistance and a strongly increased Zn content under high Zn exposure (van der Zaal *et al*., 1999). It was proposed that ZAT is involved in the vesicular/vacuolar sequestration of Zn and thus is involved in Zn homeostasis and tolerance. Recently, the ZAT1p transporter from *Arabidopsis* has been heterologously expressed in *E. coli*, purified and reconstituted into proteoliposomes where it was shown to support the uptake of Zn^{2+} (Bloß *et al*., 2002).

A ZAT gene, *ZTP1*, has also been identified in the Zn hyperaccumulator, *Thlaspi caerulescens* (Assunção *et al*., 2001). It is mainly expressed in the leaves, but also in roots, and shows highest expression in plants from a calamine (enriched in Zn, Cd and Pb) soil compared to a serpentine (Ni-enriched) or non-metalliferous soil. These plants are also the most Zn-tolerant, suggesting a role for ZTP1/ZAT-like transporters in Zn intracellular compartmentation and tolerance (van der Zaal *et al*., 1999; Assunção *et al*., 2001). In another hyperaccumulator, *Thlaspi goesingense*, Persans *et al*. (2001) have characterized a CDF transporter (*TgMTP1*) that is thought to account for the accumulation of metal ions within the shoot vacuoles. A single genomic sequence (*TgMTP1*) gives rise to an unspliced (*TgMTP1t1*) and a spliced (*TgMTP1t2*) transcript. Expression studies in yeast suggest that *TgMTP1t1* confers tolerance to Cd, Co and Zn, while *TgMTP1t2* confers tolerance to Ni. Both are highly expressed in *T. goesingense* compared with non-accumulator species (Persans *et al*., 2001). A CDF transporter, *ShMTP1*, has also been identified in the tropical legume *Stylosanthes hamata* that can grow in acid, high Mn^{2+} soils (Delhaize *et al*., 2003). *ShMTP1* confers Mn^{2+} tolerance when expressed in yeast and *Arabidopsis* through seques-

tration into internal organelles and probably functions as a proton/Mn^{2+} antiporter (Delhaize *et al*., 2003).

A search of the *Arabidopsis* genome shows the existence of eight genes for proteins with homology to the CDF family, while ESTs for family members have been identified in a range of other higher plants (Mäser *et al*., 2001). Mäser *et al*. (2001) have proposed that this family be renamed the cation efflux family (CE), and that the plant members be named the metal tolerance proteins (MTPs) rather than ZATs to allow a wider range of transport specificities to be established.

The ZIP family

The ZIPs (ZRT, IRT-like proteins) are involved in the transport of Fe, Zn, Mn, and Cd with family members differing in their substrate range and specificity (Guerinot, 2000; Mäser *et al*., 2001). About 85 ZIP family members have now been identified from bacteria, archaea and all types of eukaryotes, including 15 genes in *Arabidopsis* (Mäser *et al*., 2001). Alignment of the predicted amino acid sequences suggests that ZIPs can be grouped into four sub-families, although all of the higher plant genes appear to fall into a single group (Mäser *et al*., 2001). The ZIP proteins are predicted to have eight transmembrane domains with the amino- and carboxyl- terminal ends situated on the outer surface of the plasma membrane (Guerinot, 2000). They range quite widely in overall length, this being due to a variable region between TM-3 and TM-4. This region is predicted to be on the cytoplasmic side and is a potential metal-binding domain rich in histidine residues. A similar histidine-rich domain has been described for the CDF family (see above). The most conserved region of these proteins lies in TM-4 and is predicted to form an amphipathic helix containing a fully conserved histidine that may form part of an intramembranous metal binding site involved in transport (Guerinot, 2000; Mäser *et al*., 2001); the transport function after heterologous expression in yeast is eliminated when conserved histidines or certain adjacent residues are replaced by mutation (Rogers *et al*., 2000).

Iron is an essential element but is often of limited availability in the soil due to its low solubility. Plants have evolved two broad strategies to overcome this problem (Marschner, 1995; Fox and Guerinot, 1998). Grasses secrete phytosiderophores that chelate Fe^{3+} and the complex is then absorbed by the roots (known as Strategy II). The first identification of a putative phytosiderophore transporter is described in a later section. All other plants rely on a reductive mechanism to mobilize Fe^{3+} (Strategy I). They acidify the soil and reduce ferric ions by a plasma membrane-located Fe(III) chelate reductase. Ferrous ions are then taken up by the roots by means of a Fe^{2+} transporter. AtIRT1 (iron-regulated transporter 1), the first member of the ZIP family to be identified (Eide *et al*., 1996), was cloned from *Arabidopsis* by functional complementation of an iron-uptake-deficient yeast mutant (*fet3 fet4*). IRT1 is now thought to be the major transporter for high affinity Fe uptake by roots (Connolly *et al*., 2002; Vert *et al*., 2002). *IRT1* mRNA is detectable by 24 h on transfer to Fe-deficient conditions and both mRNA and protein levels peak after 72 h; *IRT1* mRNA and protein are undetectable 12 h after transfer back to Fe-sufficient conditions (Connolly *et al*., 2002). Plants overexpressing *AtIRT1* also accumulate higher concentrations of Cd and Zn than wild types under Fe-deficient conditions, indicating an additional role in the transport of these metals (Connolly *et al*., 2002) which is also supported by transport studies in yeast (Eide *et al*., 1996; Korshunova *et al*., 1999). In the *T. caerulescens* ecotype Ganges, the uptake of Cd but not Zn was significantly enhanced by Fe deficiency, and this may be related to an increase in the abundance of *TcIRT1-G* mRNA in root tissues (Lombi *et al*., 2002). *irt1-1*, an *Arabidopsis* knockout mutant, was chlorotic and showed severe growth defects, although this condition could be rescued by the exogenous application of Fe (Vert *et al*., 2002); the protein localized to the plasma membrane and, under Fe-deficient conditions, *IRT1* was expressed predominantly in the external layers of the root. Mutants of *IRT1* also showed significant changes in photosynthetic efficiency and developmental defects that were consistent with a deficiency in Fe transport and homeostasis (Henriques *et al*., 2002; Varotto *et al*., 2002).

Interestingly, AtIRT2 (another ZIP) is also expressed in root epidermal cells under Fe deficiency. However, it cannot substitute for loss of IRT1 (Grotz and Guerinot, 2002) and it appears to have a greater specificity as regards substrates; although it can complement Fe and Zn uptake mutants it does not seem to transport Cd or Mn in yeast (Vert *et al*., 2001). This suggests that these transporters have different functions in *Arabidopsis*.

LeIRT1 and *LeIRT2* have now been reported in tomato and are both predominantly expressed in roots (Eckhardt *et al*., 2001). *LeIRT1*, but not *LeIRT2*, is strongly enhanced by iron limitation and, together with particular P and K transporter genes, it is also up-regulated by P and K deficiencies in the root medium, suggesting a possible co-regulation of the transporter genes for certain essential minerals (Wang *et al*., 2002). Studies in yeast suggest that LeIRT1 and 2 also have a broad range (Eckhardt *et al*., 2001). OsIRT1 from rice, which has high homology to the *Arabidopsis AtIRT1* gene, is also predominantly expressed in roots and is induced by Fe-and Cu-deficiency (Bughio *et al*., 2002).

Based largely on yeast complementation studies, further information is available on the functional properties of plant ZIP transporters. The ZIP transporters 1–3 from *Arabidopsis* restore Zn uptake to yeast Zn uptake mutants *zrt1 zrt2* and are proposed to play a role in Zn transport

(Grotz *et al*., 1998; Guerinot, 2000). ZIPs 1, 3 and 4 are expressed in the roots of Zn-deficient plants, while ZIP4 is also found in the shoots and is predicted to have a chloroplast targeting sequence (Grotz *et al*., 1998; Guerinot, 2000). The proposed role of ZIP transporters in Zn nutrition is supported by the characterization of homologues from other species. The yeast ZIPs, ZRT1 and ZRT2, were shown to be high and low affinity Zn transporters, respectively (Eide, 1998; Guerinot, 2000); ZRT1 is glycosylated and present at the plasma membrane. A third ZIP homologue in yeast, ZRT3, is proposed to function in the mobilization of stored Zn from the vacuole (MacDiarmid *et al*., 2000). A *ZIP* gene homologue, *TcZNT1*, from the Zn/Cd-hyperaccumulating plant, *Thlaspi caerulescens* was shown to mediate high-affinity Zn^{2+} uptake and low-affinity Cd^{2+} uptake following expression in yeast (Pence *et al*., 2000). The transporter was expressed at high levels in both root and shoot of *T. caerulescens*, while overexpression of the transporter caused by changing Zn status resulted in increased Zn influx in the roots. Assunção *et al*. (2001) reported that *TcZNT1* and *TcZNT2* were predominantly expressed in roots although their expression was barely Zn responsive. By contrast, in the non-hyperaccumulator *T. arvense*, these genes were exclusively expressed under conditions of Zn deficiency (Assunção *et al*., 2001). A member of the ZIP family, *GmZIP1*, has now been identified in soybean (Moreau *et al*., 2002). By functional complementation of the *zrt1 zrt2* yeast cells, *GmZIP1* was found to be highly selective for Zn, while yeast Zn uptake was inhibited by Cd. *GmZIP1* was specifically expressed in the nodules and not in roots, stems or leaves, and the protein was localized to the peribacteroid membrane, indicating a possible role in the symbiosis (Moreau *et al*., 2002).

Cation/H^+ antiporters

The plant tonoplast contains a number of cation/H^+-antiporters, and those involved in the regulation of cytosolic Ca^{2+} and Na^+ concentrations by transport into the vacuole are particularly well characterized (Hirschi, 2001; Maeshima, 2001; Gaxiola *et al*., 2002). Two genes, *CAX1* and *CAX2*, have been cloned from *Arabidopsis* using yeast complementation and shown to be high and low efficiency Ca^{2+}/H^+ exchangers, respectively (Hirschi *et al*., 1996). It is predicted that these proteins have 11 transmembrane domains and a central hydrophilic region rich in acidic amino acid residues between TM-6 and TM-7 (Hirschi, 2001; Maeshima, 2001).

Earlier transport studies using tonoplast-enriched vesicles suggested that a Cd^{2+}/H^+ antiporter might be involved in the accumulation of Cd^{2+} into the vacuole (Salt and Wagner, 1993), and there is evidence that this could be a role for CAX2 (Hirschi *et al*., 2000). Expression of *CAX2* in yeast suppresses growth defects due to Ca^{2+} and Mn^{2+}. Furthermore, tobacco plants expressing *CAX2* are able to accumulate more Ca^{2+}, Cd^{2+} and Mn^{2+}, and this also results in increased Cd^{2+} and Mn^{2+} transport in root tonoplast vesicles (Hirschi *et al*., 2000). Further *CAX* genes have been identified (Cheng *et al*., 2002), while completion of the *Arabidopsis* genome indicates that a large number of homologues to these transporters exist (Mäser *et al*., 2001). However, to date, CAX2 is the only CAX transporter shown to be capable of vacuolar Mn^{2+} transport, although transport appears to be of low affinity (Shigaki *et al*., 2003). Further characterization of these transporters may reveal a wider role in metal ion homeostasis, including transport into organelles such as chloroplast and mitochondria since some homologues contain organellar-targeting sequences (Mäser *et al*., 2001).

Copper transporter family

A putative Cu influx transporter (COPT1) has been isolated from *Arabidopsis* by complementation of a yeast mutant defective in Cu uptake (Kampfenkel *et al*., 1995). This cDNA codes for a highly hydrophobic protein of 169 amino acids that has three potential transmembrane domains. Expression of COPT1 in yeast is associated with an increased sensitivity to Cu toxicity (Kampfenkel *et al*., 1995). Furthermore COPT1 shows a high sequence similarity to *CTR2*, a potential low affinity Cu transporter in yeast, and both are members of a broad family of eukaryotic copper transporters (CTR) (Eide, 1998; Van Ho *et al*., 2002). Yeast also possesses two high affinity plasma membrane-located Cu transporters, *CTR1* and *CTR3* (Van Ho *et al*., 2002). COPT1 transcripts showed highest expression in leaves and were also found in stems and flowers, but were absent from roots (Kampfenkel *et al*., 1995). Recently Sancenón *et al*. (2003) have identified a five-member family (COPT1–5) of potential Cu transporters from *Arabidopsis*, including the previously identified COPT1 described above. COP1, 2, 3, and 5 were able functionally to complement and show Cu transport activity when expressed in a yeast high affinity Cu transport mutant. Tissue-specific expression was investigated by RT-PCR; except for COPT4, expression was generally higher in leaves and stems than in roots (Sancenón *et al*., 2003).

Other putative transition metal transporters

A family of putative cation transporters that are homologous to animal cyclic nucleotide-gated channels have been identified in *Arabidopsis* and other plants (Schuurink *et al*., 1998; Kohler *et al*., 1999; Arazi *et al*., 1999). Twenty genomic GNGC sequences have been identified in *Arabidopsis*: the proteins contain six putative transmembrane domains and a calmodulin-binding site that overlaps that for cyclic nucleotide binding (Mäser *et al*., 2001;

White *et al*., 2002). These channels appear to be plasma membrane-located and non-selective, being permeable to both monovalent and divalent cations (Schuurink *et al*., 1998; Arazi *et al*., 1999; White *et al*., 2002). In relation to this review, transgenic plants overexpressing the tobacco NtCBP4 protein showed improved tolerance to Ni^{2+}, an essential transition element, and hypersensitivity to Pb^{2+} that was associated with reduced Ni^{2+} accumulation and enhanced Pb^{2+} accumulation (Arazi *et al*., 1999). A link to Pb^{2+} transport was strengthened by the observation that plants expressing a truncated version of NtCBP4 showed improved tolerance to Pb^{2+} and reduced accumulation of the metal (Sunkar *et al*., 2000). Further work is clearly needed to establish the full substrate range of these channels.

Magnesium transporters may also be involved in transition metal transport. Shaul *et al*. (1999) cloned a transporter from *A. thaliana*, called AtMHX, that is localized to the vacuolar membrane and functions as a H^+ exchanger with Mg^{2+} or Zn^{2+}, but not Ca^{2+}. AtMHX is expressed mainly in the vascular cylinder and may have a role in Mg^{2+} and Zn^{2+} partitioning between different organs of the plant. A novel family of Mg^{2+} transport genes has also been identified in *Arabidopsis* (Li *et al*., 2001). The gene family, *AtMGT*, has 10 members encoding putative Mg^{2+} transporters, and one protein, AtMGT1, is localized to the plasma membrane using green fluorescent protein as a reporter. AtMGT1 shows highest affinity for Mg^{2+}, but there is some evidence that Ni^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, and Cu^{2+} might also be transported although high concentrations are required (Li *et al*., 2001).

Another transporter with a potential role in both Ca^{2+} and Cd^{2+} uptake is the *LCT1* (low-affinity cation transporter) gene cloned from wheat (Schachtman *et al*., 1997; Clemens *et al*., 1998). When expressed in yeast, *LCT1* renders the growth of yeast more sensitive to Cd^{2+} and this is associated with increased uptake of Cd^{2+} by a high affinity system. No *LCT1* homologues have been found in *Arabidopsis* or in genomes from other non-plant species (Mäser *et al*., 2001).

In relation to Strategy II iron uptake discussed earlier (see section on ZIPS), a putative transporter for the Fe(III–phytosiderophore complex has now been identified (Curie *et al*., 2001). The maize Yellow Stripe mutant (ys1) shows Fe inefficiency due to a defect in the uptake system for Fe-phytosiderophores (von Wiren *et al*., 1994). YS1 was shown to be a membrane protein that restores growth in a yeast iron uptake mutant when grown on a Fe(III) – phytosiderophore medium (Curie *et al*., 2001). Under iron-deficiency, expression of *ys1* increased in both roots and shoots. There are eight predicted YELLOW STRIPE 1-LIKE (YSL) proteins in *Arabidopsis* although their function is unknown (Curie *et al*., 2001).

Recently, a new family of multidrug resistance efflux transporters has been identified in bacteria and termed the MATE (multidrug and toxic compound extrusion) family (Brown *et al*., 1999; Morita *et al*., 2000). A clone bearing limited homology to this family has been identified in *Arabidopsis* and the protein termed AtDTX1 (for *Arabidopsis thaliana* Detoxification 1) (Li *et al*., 2002). The MATE family appears to be a large gene family of at least 56 members in *Arabidopsis*, many more than in other sequenced organisms, with the proteins possessing 12 putative transmembrane domains (Li *et al*., 2002; Rogers and Guerinot, 2002). Functional analysis of AtDTX1 using a bacterial mutant showed that it serves as a carrier for a range of toxic compounds and is also capable of detoxifying Cd^{2+}; it appears to be plasma membrane located (Li *et al*., 2002). Its potential role in heavy metal tolerance clearly needs further study. Another member of this family, *FRD3* (ferric reductase defective 3), has also been cloned and is thought to have an important role in iron homeostasis in *Arabidopsis*, although it probably does not transport Fe directly (Rogers and Guerinot, 2002).

Molybdenum is an essential element although plants require very low amounts (Marschner, 1995). As yet no gene responsible for a Mo transporter has been identified, although there is some evidence that molybdate may be taken up by the phosphate and/or sulphate transporter (Mendel and Hänsch, 2002).

Conclusions

Transition metals are essential for normal plant growth and development. They must be taken up from the soil, distributed around the plant, and their concentrations regulated in different tissues, cells and organelles. Membrane transporters must play a central role in these diverse activities. Within the last ten years, our understanding of such transporters has developed rapidly, and a number of gene families have now been identified that could carry out these diverse functions (Table1). At least one putative transporter has been identified for all the essential transition metals except Mo, although in most cases clear evidence of how and where they operate within the plant is still required.

A number of features stand out. Many of the gene families in plants are large relative to those in other organisms. For example, *Arabidopsis* has eight heavy metal ATPases whereas most other eukaryotes studied to date have only one or two per genome. There are six Nramps in *Arabidopsis*, but only three in yeast and two each in mice and humans. Even more strikingly, there are 15 ZIP genes in *Arabidopsis* while yeast has only three. In addition, most transition metals appear to be potential substrates for at least two gene families. Thus Fe and Mn are transported by both the Nramps and the ZIPs, while Mn may also be a substrate for both a Ca^{2+}-ATPase and the cation/H^+ antiporters. Again, Zn is a substrate for both the CDFs and the ZIPs. Evidence from yeast suggests that

some of these gene families such as the Nramps may also have a much broader substrate range than indicated so far for plants, and this will widen the range of transporters potentially available for any specific metal. Their tissue and cellular localization within the plant will also influence their *in planta* substrates. Some of these transporters will be involved in active efflux and others in influx, and they are also likely to show important differences in their affinities for particular metals.

The wide range and size of the gene families identified as putative transition metal transporters is perhaps not difficult to explain. As outlined previously, there are several stages involved in the sequence from initial acquisition from the soil to the final destination and functioning of the metals. As with yeast (Eide, 1998; Van Ho *et al*., 2002), both high and low affinity mechanisms will be required to cope with differing metal availabilities. A range of cellular and organellar membranes may be involved, and both uptake and efflux mechanisms may be needed (Fig. 1). A range of genes may allow different regulatory mechanisms to operate, and allow responses to a variety of environmental stress conditions. Another interesting possibility is the co-ordination and co-regulation of the uptake of certain essential minerals indicated by the observed up-regulation of the expression of transporter genes for Fe, K and P_i under different mineral deficiencies (Wang *et al*., 2002). With the range of biochemical, molecular and genetic techniques that can now be applied to these problems, including the rapidly expanding use of microarrays and gene knockouts, the next few years will see rapid progress in defining more clearly the specificity, location, regulation, and physiological role of these transition metal transporters.

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