Review

The ZIP family of metal transporters

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Abstract

Members of the ZIP gene family, a novel metal transporter family first identified in plants, are capable of transporting a variety of cations, including cadmium, iron, manganese and zinc. Information on where in the plant each of the ZIP transporters functions and how each is controlled in response to nutrient availability may allow the manipulation of plant mineral status with an eye to (1) creating food crops with enhanced mineral content, and (2) developing crops that bioaccumulate or exclude toxic metals. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Metal ions need to be transported from the soil solution into the root and then distributed throughout the plant, crossing both cellular and organellar membranes. Because trace elements are often present in the soil solution in exceedingly low amounts, plants must use high-affinity transport systems to accumulate these ions. A number of genes involved in metal transport in plants have already been identified. Some were identified by functional complementation of yeast mutants and others on the basis of sequence similarity, using a variety of approaches including database mining, degenerate polymerase chain reaction (PCR), and heterologous hybridization techniques. Many of these genes belong to previously described transporter families such as the P-type ATPases [1] and the Nramp proteins [2]; both of these families are reviewed elsewhere in this issue. The classification of a transporter to a family of

known function immediately provides a great deal of information about what role particular transporters may play. Recent studies on metal transport in *Arabidopsis* have identified the founding members of a new family of metal transporters, the ZIP family [3], that now has representatives in all the eukaryotic kingdoms - animals, plants, protists and fungi. Here I will briefly review what we have learned about this family of transporters, including the various roles attributed to ZIP family members, and I will point out some areas in need of further study.

2. Overview of the ZIP family

The ZIP family takes its name from the first members to be identified 'ZRT, IRT-like Protein'. IRT1 (iron-regulated transporter) is an *Arabidopsis* cation transporter that is expressed in the roots of iron deficient plants [4] and ZRT1 and ZRT2 (zinc-regulated transporter) are, respectively, the high- and low-affinity zinc transporters of yeast [5,6]. At this time, over 25 ZIP family members have been identi-

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fied. These genes fall into roughly two subfamilies based on amino acid similarities (Fig. 1). Subfamily I includes 15 genes in plants (11 from *Arabidopsis*, two from tomato, one from pea and one from rice), two yeast genes (*ZRT1* and *ZRT2*), and a gene from the protozoan *Trypanosoma brucei*. Subfamily II includes 8 genes in the nematode *Caenorhabditis elegans*, one gene in *Drosophila* and two genes in humans. Although genomics is allowing the rapid expansion of the ZIP family, functional data is lacking for many of the family members.

Fig. 2. Predicted topology of a ZIP family member. ZIP proteins are predicted to cross the membrane eight times (TOPPRED II). There is a variable region between transmembrane domains 3 and 4 that is histidine-rich and predicted to reside in the cytoplasm.

Most ZIP proteins are predicted to have eight potential transmembrane domains and a similar membrane topology in which the amino- and carboxy-terminal ends of the protein are located on the outside surface of the plasma membrane (Fig. 2). ZIP proteins range from 309 to 476 amino acids in length; this difference is largely due to the length between transmembrane domains III and IV, designated the 'variable region'. In most cases, the variable region contains a potential metal-binding domain rich in histidine residues that is predicted to be cytoplasmic. For example, in IRT1, this motif is HGHGHGH. Similar potential metal-binding domains have also been found in efflux proteins belonging to the cation diffusion facilitator (CDF) family [7]. A number of the *C. elegans* ZIP proteins have this potential metal-binding site located in positions other than the variable region. The most conserved portion of the ZIP family proteins occurs in transmembrane domain IV, which is predicted to form an amphipathic helix with a fully conserved histidine residue. This histidine residue, along with an adjacent (semi) polar residue, may comprise part of an intramembranous heavy metal binding site that is part of the transport pathway [3].
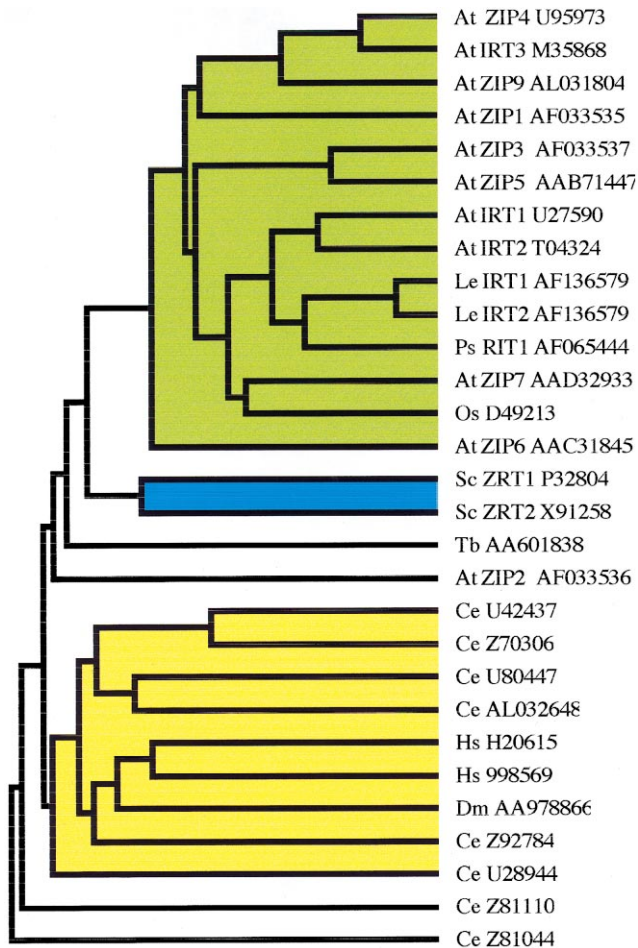

Fig. 1. Dendrogram showing amino acid sequence similarity relationships among the ZIP family members. Computer database comparisons were performed using BLAST and the multiple sequence alignment was performed using PILEUP and PRETTY (GCG). Three distinct clades are evident and are highlighted in color: green, plants; blue, fungi; yellow, animals. At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Le, *Lycopersicon esculentum*; Ps, *Pisum sativa*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; Tb, *Trypanosoma brucei*.
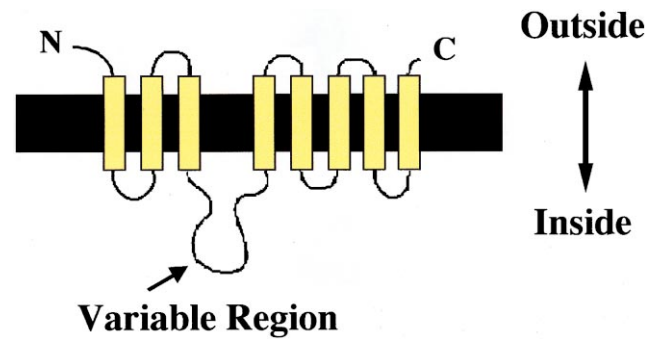
3. A role in iron transport

Iron, an essential nutrient, is not readily available to plants growing in soil. Although iron is the fourth most abundant element in the earth's crust, it is found mainly as stable, insoluble oxyhydroxide poly-

mers that effectively limit free Fe(III) to an equilibrium concentration of 10^{-17} M at neutral pH, a value far below that required for the optimal growth of plants [8]. In addition to the solubility problem, the chemical properties of iron require cells to place limitations on its accumulation. Fe(II) and Fe(III) can act catalytically to generate hydroxyl radicals that can damage cellular constituents such as DNA and lipids [9]. Thus, uptake and storage of iron is a highly regulated process.

All plants except the grasses are thought to rely on a reductive mechanism to mobilize rhizosphere Fe(III) [8,10]. The initial reduction of Fe(III) is carried out by a plasma membrane-bound Fe(III) chelate reductase, encoded by the *FRO2* gene [11]. The resulting Fe(II) is then transported across the root epidermal cell membrane by an Fe(II)-specific transporter [4]. Our current hypothesis is that IRT1, the first member of the *ZIP* gene family to be identified, is an Fe(II) transporter that takes up iron from the soil. *IRT1* was cloned by functional expression in a yeast mutant (*fet3 fet4*) defective for iron uptake [4]. Interestingly, IRT1 does not resemble either Fet4, the low-affinity Fe(II) transporter in yeast [12] or the high-affinity yeast transporter, FTR1 [13]. Yeast expressing *IRT1* possess Fe(II) uptake activity. Moreover, in *Arabidopsis*, *IRT1* mRNA is only expressed in roots under iron-limiting growth conditions.

Because iron deficiency is the leading human nutritional disorder in the world today, there is great interest in using our knowledge of iron metabolism to enhance the iron content of food [14]. It is not yet clear how many steps in iron acquisition and storage will have to be manipulated to achieve this goal. Fe(III) reduction is thought to be the rate-limiting step for iron acquisition from soil [15]; with the genes for the Fe(III) reductase and for the presumptive Fe(II) transporter now cloned, we are in a position to test this assumption. In addition to increasing iron uptake from the soil, we must also consider that plant sources of Fe include both leafy vegetables and seeds, which derive their iron from xylem and phloem, respectively. Sending more Fe to leaves versus seeds will thus entail modifying iron distribution within the plant accordingly. There is also the issue of how much iron can be safely stored in plant tissues. Several laboratories have already engineered

plants to overexpress the iron storage protein ferritin [16–18]. Plants that overaccumulate ferritin have higher iron content but also behave as if they were iron-deficient [16]. This points out the need to increase iron uptake at the same time as increasing iron storage capability.

Although IRT1 was originally identified as an Fe transporter, we now know from complementation and uptake studies in yeast that IRT1 is able to transport both Mn and Zn in addition to Fe [19]. Thus, in plants, the *IRT1* gene is transcriptionally responsive to Fe deficiency but once expressed, it may be capable of transporting other divalent metals in addition to Fe. Such IRT1-mediated transport may explain the increase in Mn and Zn that has been reported in iron deficient plants (e.g., [20]).

Iron-deficient plants have also been reported to accumulate Cd [21]. There are several pieces of evidence that point to a role for IRT1 in mediating the accumulation of Cd in iron deficient plants: (1) Cd was shown to compete with Fe for uptake in yeast expressing *IRT1* [4], (2) yeast-expressing IRT1 are more sensitive to Cd (unpublished observations), and (3) plants engineered to overexpress IRT1 can accumulate Cd in greater amounts than wild-type plants (unpublished observations). Because Cd contamination poses a serious threat to human health and uptake into plants is the primary avenue by which Cd enters the food chain, Cd uptake via IRT1 may be undesirable. However, if the goal is to have plants remove Cd from soils contaminated with heavy metals, the ability of IRT1 to transport Cd might prove to be useful. Of course, various processes besides transport from the soil are involved in the accumulation of potentially toxic metals by plants, including chelation and cellular compartmentalization. For example, Cd is known to accumulate primarily in roots because the majority of the Cd that enters the root is compartmentalized into the vacuole as the free cation or complexed with thiol-rich peptides known as phytochelatins [22,23]. Only limited amounts of Cd are actually translocated to the shoot, and the little that is transported moves as a complex with organic acids [24]. So, one would predict that simply overexpressing the transporter would not result in Cd accumulation in shoots.

A pea isolog of IRT1, RIT1, is 79% similar and 63% identical to IRT1 (GenBank accession number
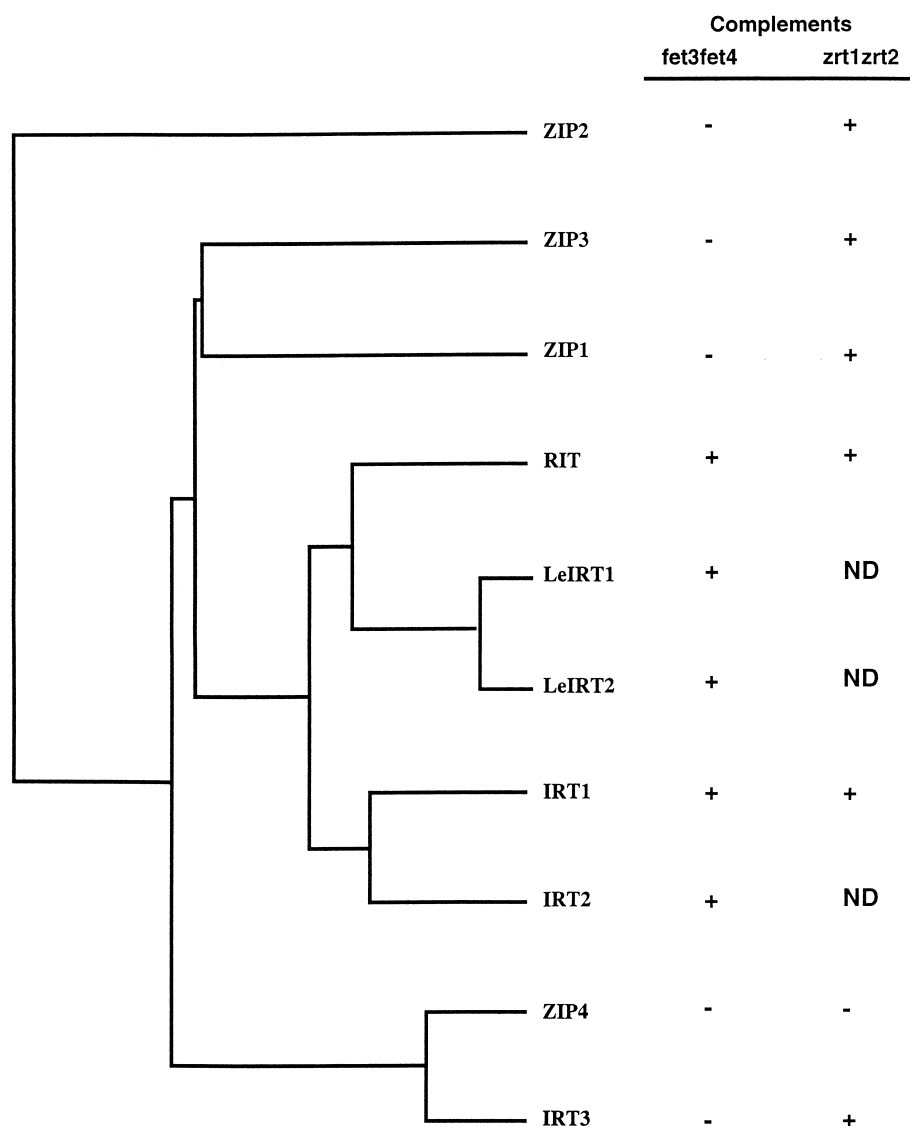
Fig. 3. Dendrogram showing amino acid similarity relationships among the plant ZIP family members for which there is functional data on transport capability. The ability to complement the *fet3fet4* mutant of yeast indicates that these transporters can use iron as a substrate; the ability to complement *zrt1zrt2* indicates that zinc can be transported.

AF065444). When expressed in yeast, RIT1 can complement the iron-transport-deficient yeast strain *fet3-fet4* as well as the zinc-transport-deficient yeast strain *zrt1zrt2* [25]. Using radiotracer techniques, RIT has been shown to mediate high-affinity Fe and Zn uptake and low-affinity Cd uptake [25]. Like IRT1, RIT1 is expressed in roots in response to iron deficiency. Two IRT1 isologs have also been identified in tomato, LeIRT1 and LeIRT2 (GenBank accession numbers AF136579 and AF136580). LeIRT1 and LeIRT2 are 96.6% similar and 91% identical to each other and approximately 81% similar and 67–

68% identical to IRT1. Both tomato genes can complement the *fet3fet4* strain of yeast (Eckhardt et al., documentation for accession numbers. AF136579 and AF136580). At this point, all of the family members that can mediate iron transport cluster together (Fig. 3).

4. A role in manganese transport

Manganese is an essential element that is required in trace amounts by virtually all organisms. As a

redox-active cofactor in Mn-superoxide dismutase, Mn plays an important role in detoxification of free radical forms of oxygen. Mn is also required for light-induced oxygen evolution during photosynthesis. Do the ZIP proteins play a role in manganese transport in plants? IRT1 can transport manganese when expressed in yeast as measured by (1) complementation of a manganese transport defective mutant, smf1, and (2) radiolabeled Mn uptake assays using yeast transformed with *IRT1* [19]. In yeast, *SMF1* encodes a Mn transporter belonging to the Nramp family of transporters [26]. We are in the process of testing all the plant ZIP family members to see which ones are capable of complementing the *smf1* mutant. We obviously need to determine if expression of any of the ZIP family members responds to Mn deficiency. Most importantly, we will need to test *Arabidopsis* mutants that no longer express each of the various transporters to see if any are deficient in cation transport. However, redundancy could prevent us from seeing a phenotype if one ZIP protein is able to functionally substitute for another.

5. A role in zinc transport

Zinc is an essential component of more than 300 enzymes including RNA polymerase, alkaline phosphatase, alcohol dehydrogenase, Cu/Zn superoxide dismutase, and carbonic anhydrase. Moreover, greater than 3% of the protein sequences inferred from the completely sequenced genomes of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* contain sequence motifs characteristic of zinc binding structural domains such as the zinger finger domain [27]. Zn is taken up as a divalent cation [28]. Once taken up, zinc is neither oxidized nor reduced; thus, the role of zinc in cells is based on its behavior as a divalent cation that has a strong tendency to form tetrahedral complexes [29].

5.1. Yeast

After IRT1, the next two members of the ZIP family to be assigned functions were the zinc transporters ZRT1 and ZRT2 of yeast. These proteins were originally identified on the basis of their similarity to IRT1 [4]. ZRT1 and ZRT2 are 44% identical

and 67% similar to each other, and approximately 30–35% identical and 54–65% similar to IRT1.

Kinetic studies of zinc uptake by yeast cells grown with different amounts of zinc in the medium had suggested the presence of at least two uptake systems. One system has a high affinity for zinc with an estimated apparent K_m of 10 nM Zn(II) and is only active in zinc-limited cells [5]. The second system has a lower affinity for zinc (apparent K_m of 100 nM Zn(II)) and is detectable in zinc-replete cells [6]. The *ZRT1* gene encodes the transporter protein of the high-affinity system [5]. The level of *ZRT1* mRNA correlates with activity of the high-affinity system; overexpressing *ZRT1* increases high-affinity uptake whereas disrupting the *ZRT1* gene eliminates high-affinity activity and results in poor growth of the mutant on zinc-limiting medium. In similar studies, it was determined that the *ZRT2* gene encodes the transporter of the low-affinity-uptake system [6]. More recently, the ZRT1 protein was shown to be glycosylated and localized to the plasma membrane of the cell [30]. Additional, but as yet uncharacterized, zinc uptake systems are also present in *S. cerevisiae* as demonstrated by the observation that *the zrt1 zrt2* mutant is viable [6].

5.2. Arabidopsis

Using a method similar to the one used to isolate *IRT1*, the *ZIP1*, *ZIP2*, and *ZIP3* genes of *Arabidopsis* were isolated by functional expression cloning in a *zrt1zrt2* mutant yeast strain; expression of these genes in yeast restored zinc-limited growth to this mutant [31]. Biochemical analysis of metal uptake has demonstrated that these genes encode zinc transporters. Yeast cells expressing ZIP1, ZIP2, and ZIP3 have different time-, temperature-, and concentration-dependent zinc uptake activities with apparent K_m values between 10 and 100 nM Zn(II). These values are similar to the levels of free Zn(II) available in the rhizosphere [32]. Moreover, no Fe uptake activity has been detected with any of these proteins in uptake experiments using [55]Fe. We propose that each of these three genes plays a role in zinc transport in the plant and they represent the first zinc transporter genes to be cloned from any plant species. A fourth *Arabidopsis* ZIP homolog, ZIP4, was identified in the DNA sequence databases, but its expression did not

confer zinc uptake activity in yeast. This may not be surprising as ZIP4 is predicted to have a chloroplast targeting sequence and therefore may not localize to the plasma membrane in yeast cells.

Rengel and Hawkesford [33] have previously reported the presence of a 34 kDa polypeptide that is strongly induced in a zinc-efficient variety of wheat grown under zinc deficiency. The 34 kDa polypeptide localized to the plasma membrane of roots, leading the authors to speculate that it might be a structural or regulatory component of the plasma membrane zinc transporter. The ZIP proteins we have identified to date are predicted to range in size from 36 to 39 kDa, suggesting that the wheat protein may be a member of the ZIP family. It is important to note that zinc deficiency is probably the most widespread micronutrient deficiency limiting crop production and quality in cereals such as wheat [34]. A global sampling of 190 soils from 25 countries found that 49% were low in zinc. Unlike other micronutrient deficiencies, zinc deficiency is ubiquitous: it occurs in cold and warm climates, in drained and flooded soils, and in acid and alkaline soils.

5.3. Zinc hyperaccumulating plants

Over 400 metal hyperaccumulating species of plants have been reported, of which about 16 are zinc hyperaccumulators (containing more than $10\,000$ μg zinc g^{-1} in shoot dry matter) [36]. Certain populations of *Thlaspi caerulescens* can tolerate up to $40\,000$ μg zinc g^{-1} tissue in their shoots whereas normal Zn concentration for most plants is between 20 and 100 μg g^{-1} tissue. There is great interest in hyperaccumulators because of their potential for use in extracting metals from soils, either in phytoremediation [35] or phytomining [36].

Radiotracer studies with *T. caerulescens* and a non-hyperaccumulating related species, *T. arvense*, have shown that the V_{max} for the uptake of zinc was 4.5-fold greater for *T. caerulescens* than for the non-hyperaccumulator while their K_m values were not significantly different. This suggests that zinc uptake is controlled by regulating the number of active transporters in the membrane [37]. A *T. caerulescens* gene, *ZNT1*, has been identified by functional expression cloning in a *zrt1zrt2* mutant yeast strain [39]. This gene encodes a presumptive zinc transporter

that is a member of the ZIP gene family. Northern analysis shows that the *ZNT1* transcript is very abundant in the roots and shoots of *T. caerulescens* regardless of zinc status. This is especially interesting in light of the fact that ZIP4 is also expressed in both roots and shoots of *Arabidopsis* but only when plants are zinc starved. Expressing a zinc transporter gene at all times regardless of zinc status may, in part, explain the ability of *T. caerulecens* to accumulate zinc.

5.4. Mammalian cells

Eide et al. [4] and Grotz et al. [31] had preliminarily identified two human members of the ZIP transporter family. One of these, hZIP1, is expressed in malignant prostate cell lines LN-CaP and PC-3 in a hormone-dependent manner [40]. These cell lines can accumulate high levels of zinc and the rapid uptake of zinc can be stimulated by treating cells with physiological levels of prolactin and testosterone. Taken together, these observations suggest that hZIP1 may be responsible for zinc uptake by these cells. Expression of hZIP1 was also shown to be down-regulated by zinc, as has been demonstrated for other zinc-regulated members of the ZIP family [5,6,31]. At the amino acid level, hZIP1 is most similar to the *Arabidopsis* ZIP1 protein [40]. The other human ZIP transporter, hZIP2, is expressed in uterine epithelial cells (where it is induced by growth arrest), infant brain, and like hZIP1, it is also expressed in prostate (D. Eide, personal communication). hZIP2 is 22% identical and 53% similar to IRT1. Expression of hZIP2 cells in K562 erythroleukemia cells from the CMV promoter significantly increases (3-fold) zinc uptake activity and immunofluorescence experiments using a functional epitope tagged gene demonstrate plasma membrane localization of hZIP1 [38].

5.5. Regulation of ZIP family members: transcriptional and post-translational

In all organisms, zinc uptake is tightly controlled to ensure that adequate levels of the metal are accumulated while preventing its potentially toxic overaccumulation. In yeast, this control is exerted at both the transcriptional and post-translational levels. At

the transcriptional level, expression of the *ZRT1* and *ZRT2* genes is induced more than 10-fold in zinc-limited cells [5,6]. Regulation of these genes in response to zinc is mediated by the product of the *ZAP1* gene [41,42]. ZAP1 is a zinc-responsive transcriptional activator protein that somehow senses intracellular zinc levels and translates that signal into changes in gene expression. The mechanism of this regulation is currently unknown but may involve direct binding of zinc to ZAP1, which could inhibit DNA binding or activation domain activity. *ZAP1* has been shown to bind upstream of various zinc-regulated genes such as *ZRT1* and *ZRT2* to a region termed a ZRE, for zinc responsive element. The ZRE consensus sequence (5′-ACCYYNAAGGT-3′) can be found upstream of 20 genes in the yeast genome, a number of which encode products of unknown function [42]. ZREs can function in either orientation relative to the start site of transcription. Single ZREs are active; when these elements are present in multiple copies in a promoter, they are additive rather than cooperative in their effects. The *ZAP1* gene has a single ZRE, *ZRT2* has two ZREs and *ZRT1* has three ZREs.

Post-translational regulation of ZRT1 occurs when cells are exposed to high levels of extracellular zinc [30]. Under these conditions, ZRT1 uptake activity is rapidly lost and this decrease is due to endocytosis of the ZRT1 protein and its subsequent degradation in the vacuole. Zinc-induced endocytosis of ZRT1 is a specific response to zinc and allows the rapid shutoff of zinc uptake activity thereby protecting cells from zinc overaccumulation. The regulation of protein trafficking in response to metals has also been seen for copper transporters. In cultured mammalian cells, the Menkes P-type ATPase is localized to the trans-Golgi network where it supplies the metal to secreting copper-dependent enzymes [43]. Treating cells with copper causes the protein to relocalize to the plasma membrane presumably to facilitate direct copper efflux from the cell. In yeast, the CTR1 copper transporter is endocytosed in response to copper in a manner analogous to ZRT1 [44]. However, CTR1 degradation did not require endocytosis or vacuolar proteases. SMF1 also undergoes metal dependent turnover, in response to manganese and, to a lesser extent, in response to iron [45]. Targeting of

SMF1 to the vacuole for degradation is dependent on the yeast gene product Bsd2.

Further characterization of the signal transduction pathway that controls ZRT1 endocytosis in response to zinc has established that ubiquitination is an essential part of the pathway that leads to the endocytosis of ZRT1 in response to zinc [52]. This conclusion is based on the following observations. First, ZRT1 is mono- and di-ubiquitinated in response to zinc treatment. Moreover, this ubiquitination occurs prior to endocytosis; the ubiquitinated protein accumulates in zinc treated mutant cell lines that are blocked for endocytosis. Given that transporter activity does not greatly decrease in these mutants, it appears that ubiquitin addition does not directly interfere with their zinc uptake function. Second, mutations that impair components of the ubiquitin conjugation pathway also impair ZRT1 ubiquitination and endocytosis. Specifically, mutations in the functionally overlapping UBC4 and UBC5 E2 ubiquitin conjugating enzymes and the RSP5/NPI1 E3 ubiquitin protein ligase cause defects in these processes. These components are known to provide substrate specificity to the ubiquitination process and, consistent with this, other E2 and E3 mutants have no effect on zinc-induced ZRT1 endocytosis. Third, a mutation within ZRT1 that alters a potential ubiquitination site eliminates both ubiquitination and endocytosis. Ubiquitin is conjugated to lysine residues in target proteins and lysine 195 of ZRT1 is the apparent site of ZRT1 modification. These data, combined with the studies of others showing that ubiquitin serves as a tag for endocytosis of other plasma membrane proteins, demonstrate the important role of this modification in signaling endocytosis.

In plants, we also have evidence that expression of the zinc transporters is metal responsive. *ZIP1*, *ZIP3*, and *ZIP4* mRNAs are all induced in zinc-limited plants. Furthermore, ZIP1 shows zinc-induced inactivation when expressed in yeast. That is, ZIP1-dependent zinc uptake is rapidly lost when cells are exposed to high levels of zinc (unpublished data). We are currently testing transgenic plants carrying a CaMV 35S-ZIP1 construct to see if a zinc-induced endocytosis mechanism like that affecting ZRT1 activity is also operating in plants.

6. Relationship of uptake transporters and effluxers

At this time, we have no evidence either for or against the idea that ZIP family members are involved in metal efflux. The *ZAT1* gene, encoding a protein with similarity to the mammalian zinc effluxers Znt1–4 [46] has been identified in *Arabidopsis* [47]. When *ZAT1* is overexpressed, Zn accumulates in the roots but not the shoots of transgenic *Arabidopsis* plants. The overexpressors are more Zn tolerant than wild-type plants, suggesting that ZAT1 might be involved in vacuolar sequestration of Zn.

Similar presumptive efflux transporter genes have also been cloned from *Thlaspi goesingense* [48]. These effluxers belong to the CDF family (reviewed elsewhere in this issue). The CDF family is distinguished from the ZIP family on the basis of several structural features: (1) the efflux family has six transmembrane domains whereas most members of the ZIP family are predicted to have eight transmembrane domains, (2) the efflux family has a long cytoplasmic C-terminal tail domain whereas most ZIP family members are predicted to have very short, cytoplasmic C-termini, and (3) although both the efflux family and the ZIP family members have a histidine-rich region, this region is more extensive in the efflux family members.

7. Summary and future perspectives

Why do plants have so many ZIP transporters? One lesson we have learned from yeast is that any particular metal will have two or more relatively specific transport systems: high-affinity systems that are active in metal limiting conditions and low-affinity systems that function when substrates are more abundant [49]. We also know that metals are transported from the soil into the root and then must cross both cellular and organellar membranes as they are distributed throughout the plant. Specific metal transporters may play different roles in this distribution process. Various molecular approaches ultimately can tell us not only in what tissue and cell type certain transporters are expressed but also where within a cell each is expressed. We are also now in a position to identify plant mutants carrying insertions in particular transporter genes [50,51]; this will greatly help in assigning functions. Having cloned genes in hand is also allowing us to undertake structure–function studies on the encoded proteins themselves. Finally, moving beyond how any one transporter functions, we need to keep in mind that we want to understand metal transport at the whole plant level and to use such knowledge to develop plants with enhanced mineral content as well as plants that bioaccumulate or exclude potentially toxic cations. Such understanding will require knowledge of how metal levels are sensed by plants and how metals control gene expression. Over the next 5 years, the relationship between high- and low-affinity metal transporters, between transporters responsible for metal influx and those responsible for metal efflux, and between transporters and the system(s) responsible for sensing metal levels in cells should become clearer.

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