Zinc is an essential mineral for a wide variety of physiological and biochemical processes. To understand zinc transport in cereals, we identified putative zinc transporters in gene databases. Three full-length cDNAs were identified and characterized from rice (Oryza sativa). Two of the cDNAs partially complemented a yeast (Saccharomyces cerevisiae) mutant deficient in zinc uptake at low concentrations. The two transporters showed many similarities in function but differed in ionic selectivity and pH optimum of activity. Expression patterns also differed between the two genes. One gene was broadly expressed under all conditions, and the other gene was mainly induced by zinc deficiency to higher levels in roots than in leaves. Although the timing of expression differed between the two genes, localization of expression overlapped in roots. Comparisons of the protein sequences, ionic selectivity, and gene expression patterns of the two transporters suggest that they may play different roles in the physiology of the whole plant.

An appreciation for the importance of zinc in molecular biology, structural biology, and nutritional sciences has been rapidly growing over the past ten years (Berg and Shi, 1996). Zinc plays many essential unique biological roles in part because it possesses unique chemical characteristics. It is well known that a vast array of proteins use zinc for stabilizing their structures in a functional form (Christianson, 1991). In many cases, zinc interacts with cysteines and histidines in proteins. The most conspicuous examples of such interactions are the zinc finger transcription factors, which require the binding of zinc for activation of transcription (Alberts et al., 1998). Zinc is well suited for its role in protein structure because it is a borderline acid and therefore can interact strongly with ligands, it is not redox active, and it is relatively labile and therefore undergoes ligand exchange reactions rapidly.

Studies of zinc uptake in biology are critical because zinc is essential for all organisms, and human zinc deficiency ranks third in importance after iron and vitamin A deficiency (Hambidge, 2000). Food zinc content is very important because the supplementation of minerals is often difficult to achieve, particularly in developing countries. Therefore, it has been suggested that increased levels of zinc in staple foods may play a role in reducing zinc deficiency (Ruel and Bouis, 1998; Graham et al., 1999; Welch and Graham, 1999). Because zinc plays multiple roles in plant biochemical and physiological processes, even slight deficiencies will cause a decrease in growth, yield, and zinc content of edible parts. Therefore, it is essential to understand the molecular details of how plants take up, translocate, and store zinc.

Zinc is taken up from soils by root membrane transport mechanisms. The selectivity of these transporters determines whether other divalent cations are imported at the same time as zinc. Recent advances have revealed that plant genomes contain several gene families involved in the transport of divalent micronutrients (Maser et al., 2001). Some of these transporters have broad substrate specificity (Korshunova et al., 1999), but the range of specificities in plant zinc transporters is unknown because their functional characterization is lagging behind the gene discovery. There appears to be at least one family of transporters in plants that should be relatively specific for iron and zinc uptake; however, the precise metal ions that these transporters take up may vary according to the composition of metal ions in the environment (Eide et al., 1996; Grotz et al., 1998; Pence et al., 2000; Eckhardt et al., 2001; Vert et al., 2001; Moreau et al., 2002). Although the molecular identity of specific zinc transporters has become evident in plants, we know little about how the structur...
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From the consensus tree, it appears that OsZIP1 and likely model for a single unrooted phylogenetic tree. Sus brought these trees into the most statistically 100 phylogenetic trees from the alignments. Econsen was used. Eprotpars was then used to create Madison, WI) was used to generate an initial align-
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pea (Pisum sativum), Arabidopsis, and Thalaspi caerulescens. For the most part, the expression of genes encoding the plant ZIP transporters appears to be induced by zinc or iron deprivation (Eide et al., 1996; Grotz et al., 1998). Guerinot, 2000; Pence et al., 2000; Eckhardt et al., 2001; Vert et al., 2001; Moreau et al., 2002), and humans (Gaither and Eide, 2000; Gaither and Eide, 2001b) has been studied. In plants, ZIP transporters have been described mainly from dicots, including pea (Pisum sativum), Arabidopsis, and Thalaspi caerulescens. For the most part, the expression of genes encoding the plant ZIP transporters appears to be induced by zinc or iron deprivation (Eide et al., 1996; Grotz et al., 1998). Some of the ZIP family members, such as IRT genes, are constitutively expressed, but expression levels increase with iron deprivation (Eckhardt et al., 2001; Vert et al., 2001).

In this study, we present data showing novel differential selectivities and differential expression of two zinc transporters from rice (Oryza sativa). Our studies focused on rice because it is the model cereal system, and a wealth of full-length cDNAs are available from expressed sequence tag (EST) projects. Our interest in cereals was also based on their importance as world food crops and the differences in micronutrient uptake mechanisms that may exist between certain monocot species and dicot species (Welch, 1995; von Wiren et al., 1996).

RESULTS

Phylogenetic analysis showed that OsZIP1 and OsZIP3 differ significantly in their amino acid sequence and in their relationship to other members of the ZIP family (Fig. 1). Twenty-nine zinc transporter proteins were aligned from Arabidopsis, tomato, T. caerulescens, rice, soybean, and yeast. To determine the relationships in the primary amino acid sequences between the different transporters, Pileup (GCG, Madison, WI) was used to generate an initial alignment. To create 100 different possible alignments, SeqBoot was used. Eprotpars was then used to create 100 phylogenetic trees from the alignments; Econsensus brought these trees into the most statistically likely model for a single unrooted phylogenetic tree. From the consensus tree, it appears that OsZIP1 and OsZIP2 are closely related to AtZIP2 and MzZIP. However, it appears that OsZIP3 is not closely related to any of the other previously characterized zinc transporters with the exception, perhaps, of GmZIP1.

Complementation tests were performed using the ZHY3 mutant (Zhao and Eide, 1996a). As a positive control, cells expressing AtZIP1 and AtZIP3 were dropped onto solid medium containing 1 mM ZnCl2 and 150 μM ZnCl2 (Fig. 2A). These strains grew under both conditions. Cells expressing the rice cDNA clones OsZIP1 and OsZIP3 grew well on 1 mM ZnCl2 and weakly at 150 μM ZnCl2. The cells containing the empty vector pYES2 or expressing the cDNA OsZIP2 grew only on medium containing 1 mM ZnCl2. To confirm the weak complementation of ZHY3 by OsZIP1 and OsZIP3, cells were grown in liquid medium containing 1 mM ZnCl2. Quantitative analysis of growth clearly showed that the cells expressing OsZIP1 and OsZIP3 grew at a faster rate than cells expressing OsZIP2 or containing the empty vector pYES2 (Fig. 2B). The weak complementation and the faster growth rates suggested the possibility that OsZIP1 and OsZIP3 encoded functional zinc transporters. OsZIP1 and OsZIP3 also weakly complemented the yeast mutant defective in manganese uptake (smf1; Supek et al., 1996), but not the yeast mutant fet3fet4 defective in iron uptake (Dix et al., 1994).

To directly measure the uptake rates of zinc into the ZHY3 cells, we used 65Zn as a tracer for uptake.

Figure 1. Unrooted tree that highlights the relationship among the zinc transporter proteins in different plant species. At, Arabidopsis; Gm, soybean (Glycine max); Tc, T. caerulescens; Or, rice; Sc, S. cerevisiae; Mt, Medicago truncatula; Ps, pea; Le, tomato (Lycopersicon esculentum). GenBank identification numbers are provided next to each protein.
At pH 4.7, we found that the cells expressing OsZIP1 had significantly higher uptake rates than the other strains tested (Fig. 3A). As a control at that pH, we tested the ZHY3 cells expressing AtZIP1 and found uptake rates similar to those that had been measured previously (Grotz et al., 1998). To determine whether a more neutral pH would alter uptake rates, we tested uptake at pH 6.0. Under these conditions, we observed a reduced uptake rate in the control cells expressing AtZIP1 (Fig. 3B, inset) and the strain expressing OsZIP2. In contrast, we found significantly higher uptake rates in the cells expressing OsZIP3; such rates were not observed at pH 4.7.

The effect of competing divalent cations on the uptake of zinc in strains expressing OsZIP1 or OsZIP3 was determined by measuring zinc uptake in the presence of a 10-fold excess of several divalent cations (Fig. 4). These experiments were completed at pH 4.7 for OsZIP1 and pH 6.0 for OsZIP3. In general, the uptake of zinc in cells expressing OsZIP3 was less inhibited by competing divalent cations than by cells expressing OsZIP1. In the case of a 10-fold increase in zinc, we found that zinc uptake was reduced more in cells expressing OsZIP3. Overall, it appeared that the OsZIP3 transporter was more selective for zinc uptake than for other divalent cations. Particularly striking was the effect of added cadmium. The zinc uptake in cells expressing OsZIP1 was potently inhibited by cadmium, whereas there was virtually no effect in the cells expressing OsZIP3.

To further demonstrate the effects of cadmium on the different yeast strains, we conducted a quantitative growth study. Because we found that even low concentrations of cadmium inhibited the growth of ZHY3 expressing OsZIP1, we grew the cells to approximately mid log phase and then added cadmium (Fig. 5). Several concentrations of cadmium were tested, but only the lowest concentration of cadmium
tested is shown in Figure 5. The concentration of 10 μM Cd2+ only reduced the growth of the strain expressing OsZIP1; higher concentrations of Cd2+ also slightly reduced the growth of the other strains. These quantitative growth experiments strongly suggest that cadmium was more inhibitory to the yeast cells expressing OsZIP1 than the cells expressing OsZIP3, OsZIP2, or the cells containing the empty vector.

To determine whether the effects of cadmium were due to a blockade of zinc uptake or due to cadmium uptake and the ensuing toxicity, we used inductively coupled plasma to analyze cells that had been grown in the presence of cadmium for internal content. Those analyses confirmed that the cells expressing OsZIP1 took up three to four (±5%) times more cadmium than the cells containing the empty pYES2 vector or cells expressing OsZIP3.

To study the kinetics of zinc uptake, we measured uptake rate as a function of external zinc concentration. The affinity for zinc for both transporters was similar: between 16 and 18 μM (Fig. 6). Interestingly, the V_max for uptake was twice as high for the cells expressing OsZIP1 than for the cells expressing OsZIP3. The higher V_max for OsZIP1 (Fig. 6A) was consistent with higher uptake rates measured as a function of time (Fig. 3).

In addition to differences in ionic selectivity, clear differences emerged in the expression of genes encoding these transporters. We found differences in the level of gene expression and in how gene expression was controlled. In the case of OsZIP3, the transcript could be detected in total RNA present in roots (Fig. 7A) and shoots (Fig. 7B) of plants that were grown in zinc-replete conditions. Only a slight induction in gene expression was observed in shoots after 96 h of zinc deprivation. In contrast, the OsZIP1 transcript was of lower abundance and could only be detected in poly(A)+ mRNA. Expression of OsZIP1 was only induced in roots and in shoots 24 to 96 h after zinc deprivation (Fig. 7, C and D). After the plants were deprived of zinc, the OsZIP2 transcript was visible in the poly(A)+ mRNA fraction of roots and, to a lesser extent, in shoots. Although the genes were differentially expressed, the transcripts localize to the same cells in the root (Fig. 8, A, B, E, and F).
Only OsZIP1 could be detected in leaves, and those transcripts were mainly found in the cells that made up the vascular bundles (Fig. 8, C and D). In the stem, OsZIP3 was detected in the vascular bundles and epidermal cells (Fig. 8, G and H).

**DISCUSSION**

Several genes encoding zinc transporters have now been cloned, and the encoded proteins have been functionally characterized (Gaither and Eide, 2001a). Except for OsIRT1, most of these transporters come from dicot plants (Bughio et al., 2002). The study of micronutrient uptake of iron and zinc in monocots is particularly important because the Graminaceous monocot species have different strategies for iron uptake (Mori, 1999) and perhaps for zinc uptake (von Wiren et al., 1996). In addition, the monocot cereals rice, wheat (*Triticum aestivum*), and corn (*Zea mays*) are the major world food crops. It is for these reasons that we embarked on a study to characterize zinc transporters from rice.

**Protein Structure**

In searching gene databases, we identified at least four ESTs for what appeared to be rice zinc transporters or clones belonging to the ZIP family, which also include iron transporters. Comparison of the protein sequences of 29 ZIP transporters from a range of plant species and yeast suggested that there are distinct structural differences between the two rice transporters that we were able to functionally char-
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General Functional Characteristics

The cDNAs OsZIP1 and OsZIP3 partially complemented the ZHY3 yeast mutant (Zhao and Eide, 1996a) defect in growth on low-zinc medium, which suggests that they may be zinc transporters. Uptake experiments provided direct evidence that these cDNAs encode transporters that are capable of transporting zinc. The uptake rates of OsZIP1 and OsZIP3 were lower than those of AtZIP1. This may account for the partial complementation of the yeast mutant. Zinc uptake activity was clearly evident in the cells expressing OsZIP1 at pH 4.7 and in cells expressing OsZIP3 at pH 6.0. We were not able to detect zinc uptake in the cells expressing OsZIP2. Northern-blot analysis of yeast cells expressing these three cDNAs indicated that transcript from OsZIP2 was barely detectable (data not shown), and, therefore, that the lack of complementation may have been due to lack of gene expression. Zinc uptake activity in Arabidopsis ZIP transporters has also been found to be pH dependent. Most of the AtZIP transporters have higher zinc uptake rates at more acidic pH (4.0). However, the pH optimum of AtZIP2 from Arabidopsis (Grotz et al., 1998) was 6.0, similar to the pH optimum measured for OsZIP3. Although OsZIP1 shows a high degree of homology to the AtZIP2 protein from Arabidopsis (Grotz et al., 1998), it has a different pH optimum for zinc transport activity. The dependence of uptake activity on pH has also been observed with many other ion transporters, such as the mannitol transporter in celery (Apium graveolens; Noiraud et al., 2001) and the human zinc transporter hZIP2 (Gaither and Eide, 2000).

The lag time for zinc uptake observed in the cells expressing the rice transporters contrasted the absence of lag in cells expressing AtZIP1. This lag could not be explained by changes in the pH of the medium by the yeast cells. The lag could have been due to some posttranslational modification in response to zinc, which caused an up-regulation of the transporter.

Ionic Selectivity

The ionic selectivity of ion transporters is a very important characteristic for plants because it impinges on the plant’s tolerance and uptake of toxic minerals. Zinc binds to many different types of proteins (Christianson, 1991; Alberts et al., 1998); the different amino acid motifs that may function as zinc-binding sites have been identified mainly in soluble proteins (Alberts et al., 1998). In a majority of cases, His and Cys are key residues in binding zinc. Very little is known about how zinc is bound to membrane transport proteins, transported across the membrane, and released to the cytoplasm during the transport process. Studies on how zinc and cadmium bind to transport mechanisms in plants will be important for increasing crop yields on zinc-deficient soils, boosting the micronutrient density of foods (Kochian and Garvin, 1999; Schachtman and Barker, 1999; Welch and Graham, 1999; Guerinot and Salt, 2001), and creating crop plants that are better able to filter out toxic ions from contaminated soils.

Recent progress has identified several different classes of zinc transporter molecules, but the structures that determine the metal-binding characteristics of these proteins have not yet been elucidated (Gaither and Eide, 2001a). In Arabidopsis, there are 15 members in the ZIP family, several of which have been characterized at the molecular and functional level (Maser et al., 2001). Only small differences between zinc and cadmium transport have been demonstrated thus far among the ZIP transporters. For example, zinc transport through AtZIP2 appears to be almost fully blocked by cadmium, whereas AtZIP1 and AtZIP3 are partially blocked by cadmium (Grotz et al., 1998), as is the case for GmZIP1 (Moreau et al., 2002). These small differences in selectivity contrast the large differences in selectivity that we found between two zinc transporters in rice: OsZIP1 and OsZIP3. OsZIP1-mediated zinc uptake was strongly inhibited by cadmium, magnesium, and zinc, whereas OsZIP3-mediated zinc uptake was strongly inhibited by zinc and magnesium and, to a lesser extent, by calcium. The addition of NaCl reduced zinc uptake rates in both yeast strains. In the case of the cells expressing OsZIP3, as little as 100 μM NaCl reduced uptake rates by 50% (data not shown). In tests done on solid medium, we found that iron, manganese, and copper had no effect on the growth of the yeast strains used (data not shown). Therefore, these ions were not tested in the competition studies. Remarkably, cadmium had no effect on the zinc uptake activity mediated by OsZIP3. These results are supported by our growth studies, which show that cells expressing OsZIP3 take up less cadmium. These cells’ growth was also less inhibited by cadmium. A difference in cadmium permeability, shown by growth tests on solid medium, was also found between AtIRT1 and AtIRT2 (Vert et al., 2001).
In a recent study, single amino acid substitutions altered the ionic selectivity of the IRT1 transporter (Rogers et al., 2000). The change in one amino acid reduced the cadmium permeability and maintained zinc uptake but abolished iron and manganese uptake. The identification of this single amino acid substitution D136A provides the first localization of a putative cadmium-binding site in the ZIP proteins, of which IRT1 is a member. Although the characterized ZIP transporters differ in selectivity, the rice OsZIP3 is to our knowledge, the first zinc transporter to be found that is highly selective for zinc over toxic ions such as cadmium.

**Gene Expression**

Gene expression patterns for members of the ZIP family have been widely reported. In a recent paper, it was shown that the transcript levels of IRT1 do not necessarily correlate with the levels of protein produced (Connolly et al., 2002). Many of the ZIP family genes are induced upon starvation of zinc or iron. Similar to our findings for the rice genes OsZIP3 and OsZIP1, some of these transcripts were found in roots and leaves (Pence et al., 2000). However, the expression of many ZIP family members is mainly restricted to roots (Eide et al., 1996b; Grotz et al., 1998; Eckhardt et al., 2001; Vert et al., 2001, 2002; Bughio et al., 2002) and nodules (Moreau et al., 2002). It is also interesting to note that the expression of OsZIP1 was not detectable under control conditions and was primarily localized to the roots. In contrast, OsZIP3 was expressed under control conditions, but gene expression was slightly up-regulated when zinc was removed from the medium. OsZIP3 was also expressed in roots and leaves, but expression was much higher in leaves. These results suggest that OsZIP1 is primarily involved in metal uptake when rice is deprived of zinc or other metals, and that OsZIP3 is constitutively active and involved in overall cell zinc homeostasis, particularly in leaves.

Transcripts from both genes were localized to the epidermis and stele of roots of zinc-deprived plants. These results suggest that the transporters play similar roles but are expressed under different conditions. In leaves, OsZIP1 transcripts were localized to the cells containing the vascular tissue. This suggests a role for this transporter in zinc absorption or transfer from the vascular tissue.

Resources generated from EST projects allowed us to identify several putative zinc transporters from an agronomically important crop. Using a yeast mutant, we have provided a thorough functional analysis of two of these transporters. One transporter, OsZIP1, shows a broad substrate specificity for divalent cations and has inducible gene expression. The other transporter, OsZIP3, appears to be more selective for zinc, is not permeable to toxic cadmium, and has more constitutive expression. The link between differences in ionic selectivity and gene expression patterns provide important suggestions for analyzing the physiological function of these two zinc transporters in the future.

**Materials and Methods**

** Yeast Strains and cDNA Information **

*Saccharomyces cerevisiae*

strain ZHY3 (Zhao and Eide, 1996a) was used to study the function of rice zinc transporters. The yeast strain ZHY3 was grown on LZN (Zhou and Eide, 1996a) or yeast nitrogen base (YNB) medium supplemented with 1 mm ZnCl2 and 2% (w/v) Gal. Three cDNA clones were identified by searching GenBank with amino acid sequences from Arabidopsis ZIP transporters. The cDNA clones were obtained from the Japanese Rice Genome Research Program. Clones were sequenced and appeared to be full length.

** Complementation **

For the complementation experiments, OsZIP1 (AY302058), OsZIP2 (AY302059), and OsZIP3 (AY329153) cDNAs from rice (*Oryza sativa*) were directionally cloned into the *Kpn* I sites of the yeast expression vector pYES2. These constructs were introduced into the yeast strain ZHY3, and the transformants were selected on LZM medium supplemented with 1 mm ZnSO4 and lacking uracil. The cDNAs *AiZIP1* and *AiZIP3* in pFL61 (Minet et al., 1992) were transformed into the yeast strain ZHY3 and used as positive controls, whereas ZHY3 containing the empty plasmid pYES2 was used as a negative control. All of the yeast strains were grown overnight in LZN with 1 mm ZnSO4. The OD600 of the cultures was adjusted to 0.1, and 5 μL of 10X serial dilutions were spotted onto LZN plates containing 150 μm or 1 mm ZnSO4 to test for complementation.

** Growth Experiment **

Yeast strain ZHY3 expressing the cDNAs OsZIP1, OsZIP2, and OsZIP3 and containing the empty plasmid pYES2 was used in the growth experiments. A 5-μL aliquot of YNB supplemented with 1 mm ZnCl2 was inoculated with a single colony of a yeast strain grown on plates containing the same medium and was then grown overnight at 30°C in an orbital shaker. All of the yeast strains were inoculated in 5 mL each of YNB medium supplemented with indicated concentrations of ZnCl2 (1 mm) at an optical density of 0.08 from overnight cultures. To demonstrate the effect of cadmium, cadmium chloride (0.01 mm) was added to the cultures at mid-log phase of growth. The optical density of the cultures was sampled periodically, with three replicates for each data point. Experiments were replicated with the same results several times; only the final experiment is shown.

** Uptake Experiments **

Zinc uptake assays were performed as described by Eide and Guarente (1992) except that 65ZnCl2 (NEN Life Science Products, Boston) and LZM-EDTA (LZN without EDTA) were substituted for 59FeCl3 and low-iron medium-EDTA (low-iron medium minus EDTA). The yeast strain ZHY3 with the empty pYES2 vector and strains expressing the cDNAs OsZIP1, OsZIP2, and OsZIP3 were grown to mid-log phase in LZN supplemented with 1 mm ZnCl2 for uptake experiments. Exponentially growing cells were harvested, and pellets were washed twice in ice-cold assay buffer LZN-EDTA and resuspended in 0.1 ml of the original volume culture in assay buffer. Cell suspensions were kept on ice before use. Uptake solutions were prepared by diluting ZnCl2 to the specified concentrations in the uptake buffer (LZN-EDTA). To measure uptake, 50 μL of cell suspension was added to 450 μL of uptake buffer containing the isotope. Cell suspensions were transferred to a heating block at 30°C. Tubes containing the cells were vortexed every two to 3 h. The samples were vortexed, and yeast cells were collected on a nylon membrane (0.45 μm) using vacuum. The membranes were then washed with 10 mL of ice-cold 1 mm EDTA, 20 mm Tri-sodium citrate, 1 mm KH2PO4, 1 mm CaCl2, 5 mm MgSO4, and 1 mm NaCl. Cell-associated 65Zn was measured with a scintillation counter (LS 380; Beckman).
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In Situ Localization

Rice cv Taipei 309 was grown in hydroponic culture as described above for 26 d with 16 h of light per day (200 μmol m⁻² s⁻¹) at 22°C. Plants were transferred into hydroponic medium without zinc for 96 h and were then harvested. Tissue from these plants was fixed in 4% (w/v) paraformaldehyde–hydroxy tive for 36 h, and then was dehydrated in an ethanol series. After dehydration, tissue was infused with paraplast and then sectioned to 8 μm and mounted on slides. Probes from the third end of each of the cDNAs were labeled using the DIG system and protocol (Roche, Mannheim, Germany). After in situ hybridization of the tissue, the slides were hybridized overnight at 55°C and washed. The tissue was then incubated with anti-DIG AP conjugate (Roche) for 2 h at room temperature, and the antibody was detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate after an overnight incubation. Slides were air dried, and results were visualized using a microscope (Eclipse 800; Nikon, Melville, NY) and were documented with a Retiga Q-imaging CCD camera (Burrnaby, Canada).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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LITERATURE CITED
