GmZIP1 Encodes a Symbiosis-specific Zinc Transporter in Soybean*

Received for publication, July 18, 2001, and in revised form, November 8, 2001
Published, JBC Papers in Press, November 12, 2001, DOI 10.1074/jbc.M106754200

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The importance of zinc in organisms is clearly established, and mechanisms involved in zinc acquisition by plants have recently received increased interest. In this report, the identification, characterization and location of GmZIP1, the first soybean member of the ZIP family of metal transporters, are described. GmZIP1 was found to possess eight putative transmembrane domains together with a histidine-rich extra-membrane loop. By functional complementation of zrt1zrt2 yeast cells no longer able to take up zinc, GmZIP1 was found to be highly selective for zinc, with an estimated Kₘ value of 13.8 μM. Cadmium was the only other metal tested able to inhibit zinc uptake in yeast. An antibody raised against GmZIP1 specifically localized the protein to the peribacteroid membrane, an endosymbiotic membrane in nodules resulting from the interaction of the plant with its microsymbiont. The specific expression of GmZIP1 in nodules was confirmed by Northern blot, with no expression in roots, stems, or leaves of nodulated soybean plants. Antibodies to GmZIP1 inhibited zinc uptake by symbiosomes, indicating that at least some of the zinc uptake observed in isolated symbiosomes could be attributed to GmZIP1. The orientation of the protein in the membrane and its possible role in the symbiosis are discussed.

Zinc is an essential micronutrient for all organisms, including plants. More than 3% of the proteins of Saccharomyces cerevisiae and Caenorhabditis elegans are predicted to contain sequence motifs characteristic of zinc binding structural domains (1). Zinc deficiency is a widespread micronutrient deficiency limiting crop production (2). In recent years, genes encoding zinc transporters have been identified in various organisms (3–11). These studies have shed some light on zinc uptake and regulation, particularly at the plasma membrane level. However, with the exception of the recently identified Zrt3p transporter on the vacuole membrane in yeast (9), little is known about intracellular zinc transport systems, nor about the mechanisms of the transporters identified. Here we investigate zinc transport at the symbiotic interface between legumes and rhizobia, which presents an additional level of complexity.

Many legumes form a symbiosis with nitrogen-fixing soil bacteria (rhizobia) that enables the plants to utilize atmospheric N₂ for growth. Infection of the legume root by rhizobia results in the formation of specialized organs called nodules that provide the microaerobic conditions required for operation of the nitrogenase enzyme. Within the infected cells of nodules, the N₂-fixing bacteroids are enclosed in a plant membrane to form an organelle-like structure termed the symbiosome (12). The envelope of the symbiosome is called the peribacteroid membrane (PBM) and effectively controls the exchange of metabolites between the symbiotic partners. The PBM, although originating from the plasma membrane of root cells, evolves over the course of nodule organogenesis to become a new and specialized membrane containing symbiosis-specific proteins (see Ref. 13 for a review).

The principle metabolic exchange that occurs between plant and bacteroid is reduced carbon (usually malate) from the plant for fixed N₂ from the bacteroid, and specific transport mechanisms have been identified for this exchange (14). However, the bacteroids are dependent on the plant for all micronutrients, and transporters for these must also exist on the PBM. Included in these micronutrients is the essential metal zinc. Among the various transporters identified in other systems, the ZIP family of zinc transporters was first identified in Arabidopsis, and members have also been identified in other plants (see Ref. 8 for a recent review). In general, the activities of these transporters have been studied by expressing the proteins in yeast, and their activity in the parent plants has not been ascertained. Here we report the isolation of the first member of the ZIP family from soybean and localize it to the peribacteroid membrane of N₂-fixing root nodules. The ability to isolate intact symbiosomes from soybean nodules has allowed us to compare the activity of GmZIP1 in both its native membrane and in yeast.

* This research was supported by the Australian Research Council (to D. A. D.), the CNRS Programme International de Cooperation Scientifique Program 637 (to S. M., A. P.), and the Department of Energy GmZIP1 Encodes a Symbiosis-specific Zinc Transporter in Soybean*

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1 The abbreviations used are: PBM, peribacteroid membrane; LZM, low zinc medium; RACE, rapid amplification of cDNA ends; MES, 4-morpholineethanesulfonic acid; DIG, digoxigenin; SSPE, saline/sodium phosphate/EDTA; SSW, sodium salts wash buffer; TBS, Tris-buffered saline; IRT, iron-regulated transporter.

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**Materials**

The *S. cerevisiae* strains used were DY1455 (MATa ade2-101 con1-100 oc his3 leu2 trpl ura3 gal1), DEY1453 (MATa ade2 can1 his3 leu2 trpl ura3 his3:GAL1 leu2:LEU2) and ZHY3 (MATa ade6 can1 his3 leu2 trpl ura3 trpl:LEU2 zrt1::HIS3). AtT1R1 cDNA in yeast expression vector pFL61 (15) is referred to as pAtT1R1.

**PCR Cloning of GmZIP1**

GmZIP1 was cloned using PCR based on observed sequence similarity between AtT1R1 (U27590), AtT1R2 (T04324), the pea Rit1 (AF065444) and a rice EST (D49213). Near complete conservation of the amino acid sequence occurs in several short regions such as CFHQMFEGM (residues 241–249 in Rit1) and MLSMRMARWA (residues 341–348 in Rit1, underlined residues not conserved). The first set of primers corresponded to these regions, but to avoid the use of degenerate primers the codons of Rit1 from Pea (the closest relative to soybean) were used. Using these primers a partial cDNA was amplified from a soybean root nodule cDNA library (Marathon™ cDNA Amplification Kit from CLONTECH). Based on this sequence, gene-specific primers were designed (5'-RACE primer: 5'-TCT CCT TCA CTA CGA ACA TG-3' and 3'-RACE primer: 5'-CAA TAA GAC GAC TCA TGG CTG CAG CA-3'). Next, 5'- and 3'-RACE reactions were performed using a soybean root nodule cDNA library. Finally, primers designed to clone the open reading frame (5'-TTG CCT CTT TCA CTG ATC ACA TG-3') were used to amplify a full GmZIP1 open reading frame (based on sequence alignment to other ZIP genes) of 1062 bp. GmZIP1 was cloned into pFL61 yeast expression vector to give pGmZIP1.

**Symbiosome Isolation and Membrane Purification**

Soybean (*Glycine max cv. Stevens*) seeds were inoculated with *Bradyrhizobium japonicum* strain USDA 110. Plants were grown in pots as described before (19). Cells were grown in yeast extract/peptone/glucose or synthetic defined medium to select transformants. Low zinc medium was used instead of LZM or SSW. When used as the uptake buffer a 10-mM ZnCl2 solution was prepared in 0.02 N HCl. Cell number was determined by liquid scintillation counting of the filters. Competition for Zn²⁺ uptake by metal ions was measured by adding a 10-fold molar excess of iron, copper, nickel, manganese, cobalt, cadmium, or molybdenum to 20 μM ZnCl₂-labeled solution. All metals were used as their chloride salts and were of analytical reagent grade or equivalent. To study the competition for zinc uptake by metal ions, 10 mM ascorbic acid was also added to the mix, and the sulfate salt of iron was used in this case. When needed, the uptake solution was buffered with Tris-HCl for pH ranges of 7–9 or with citric acid-NaOH for pH ranges of 3–6. A stock solution of 250 mM ZnCl₂ was prepared in 0.02 N HCl. Cell number was determined by measuring the absorbance of liquid cultures at 600 nm and comparing with a standard curve.

**Northern and Southern Analysis**

Poly(A)⁺ RNA was purified from the leaves, stems, and roots as well as the nodules of various aged plants using Dynabeads oligo(dT)₅₀ (Dynal). The Northern gel was loaded with 1 μg of poly(A)⁺ RNA samples, then run, and blotted onto nylon membrane (Hybond N, Amersham Biosciences, Inc.), and the membrane baked according to standard procedures (22). GmZIP1 DNA was DIG-labeled using the PCR DIG Labeling Mix (Roche Molecular Biochemicals). Hybridization overnight at 55 °C was followed by washes (2 × 15 min at room temperature in 2 × SSC, 1% SDS and then 2 × 30 min at 68 °C in 0.1 × SSC, 1% SDS). Immunological detection of the probe was accomplished using anti-DIG antibody conjugated to alkaline phosphatase and the CDP-Star™ chemiluminescent substrate (Roche Molecular Biochemicals). Total RNAs were isolated from nodules of soybean plants using RNeasy Plant MiniKit (Qiagen). Samples containing 20 μg of RNA were denatured, separated onto a 1.2% agarose 7.4% formaldehyde gel, transferred to nylon membrane, and baked for 2 h at 80 °C. Equal loading of RNA in each lane was confirmed by visualization of ribosomal RNA bands after staining of the gel with ethidium bromide. A [³²P]-labeled riboprobe was synthesized using an in vitro transcription system kit (Promega) and Apo-linearized pGmZIP1 as template. After 12 h of hybridization at 55 °C in 50% formamide, 5 × SSPE, 0.5% SDS, 0.25% powdered milk, 10% dextran sulfate, the membrane was washed twice for 20 min at 55 °C in 2 × SSC 0.1% SDS and was then exposed to film (Biomax, Kodak).

**Preparation of Antiserum to GmZIP1 Protein**

Two peptides were selected from immunogenic regions of the GmZIP1 protein sequence, corresponding to the first 9 N-terminal amino acid residues (MKEERPLSDK) and to amino acid residues 182–196 (HGYPTPDQDSSELL) present in the loop between transmembrane domains III and IV. These two peptides are unique when searched against GenBank™. Peptides were synthesized and coupled to keyhole limpet hemocyanin as a carrier protein. Rabbits were primed and boosted three times with the mix of the two coupled peptides, following the Eurogentec Double X immunization program over a time of three months. Pre-immune serum and antiserum obtained in the

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EXPERIMENTAL PROCEDURES

Yeast Growth and Transformation

Cells were grown in yeast extract/peptone/glucose or synthetic defined media through a Percoll density gradient, as described before (19). concentrated by ammonium acetate/methanol precipitation at 4 °C. Total proteins were extracted and obtained the soybean nodule microsomes. Proteins were extracted and concentrated against GenBank™. Peptides were synthesized and coupled to keyhole limpet hemocyanin as a carrier protein. Rabbits were primed and boosted three times with the mix of the two coupled peptides, following the Eurogentec Double X immunization program over a time of three months. Pre-immune serum and antiserum obtained in the

Yeast Zinc Uptake—ZHY3 yeast strains carrying plasmid pFL61 or pGmZIP1 were grown to mid-log phase in LZW-EDTA. Cells were harvested, washed once, and resuspended in a minimal volume of LZW-EDTA. Cells were equilibrated at 30 °C for 20 min before being mixed with twice their volume of a radiolabeled Zn²⁺ solution. Uptake solution contained LZW-EDTA, pH 4.2, 20 μM ZnCl₂, and 200 nCi of [³²P]-labeled ZnCl₂ (New England Biolab). Cells were incubated in a 30 °C water bath for stated amounts of time. Aliquots were collected on glass microfiber filters (GF/F Whatman) and washed five times with 1 ml of ice-cold SSW, pH 4.2 (1 mM EDTA, 20 mM trisodium citrate, 1 mM KH₂PO₄, 1 mM CaCl₂, 5 mM MgSO₄, 1 mM NaCl, Ref. 23). ³²P-Zn²⁺ content of the cells was determined by liquid scintillation counting of the filters. Competition for Zn²⁺ uptake by metal ions was measured by adding a 10-fold molar excess of iron, copper, nickel, manganese, cobalt, cadmium, or molybdenum to 20 μM ZnCl₂-labeled solution. All metals were used as their chloride salts and were of analytical reagent grade or equivalent. To study the competition for zinc uptake by metal ions, 10 mM ascorbic acid was also added to the mix, and the sulfate salt of iron was used in this case. When needed, the uptake solution was buffered with Tris-HCl for pH ranges of 7–9 or with citric acid-NaOH for pH ranges of 3–6. A stock solution of 250 mM ZnCl₂ was prepared in 0.02 N HCl. Cell number was determined by measuring the absorbance of liquid cultures at 600 nm and comparing with a standard curve.

**Symbiosome Zinc Uptake—Isolated symbiosomes were dilute to a protein concentration of 1 mg/ml and pre-equilibrated at 30 °C for 15 min. Symbiosome aliquots were added to a double volume of assay buffer giving a final concentration of 0.3 mg/ml. Uptake experiments were conducted as described above for yeast, except that manitol medium was used instead of LZW or SSW. When used as the uptake buffer the nitritotriacetic acid was added to the manitol medium to chelate loosely bound metals. In all experiments, controls for background adherence of zinc were performed by measuring uptake at 0 °C; these values were subtracted from all of the data shown.

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Two peptides were selected from immunogenic regions of the GmZIP1 protein sequence, corresponding to the first 9 N-terminal amino acid residues (MKEERPLSDK) and to amino acid residues 182–196 (HGYPTPDQDSSELL) present in the loop between transmembrane domains III and IV. These two peptides are unique when searched against GenBank™. Peptides were synthesized and coupled to keyhole limpet hemocyanin as a carrier protein. Rabbits were primed and boosted three times with the mix of the two coupled peptides, following the Eurogentec Double X immunization program over a time of three months. Pre-immune serum and antiserum obtained in the
final bleed were purified through a HiTrap Protein A column (Amersham Biosciences, Inc.) and used at a 1:1000 dilution unless otherwise stated.

**Western Blot Analysis**

Proteins were separated on 12% polyacrylamide gels under denaturing conditions (24) and electrophoretically transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Inc.). Membranes were blocked with 1% blocking solution (Roche Molecular Biochemicals) and incubated for 1 h with a 1:1000 dilution of primary antibody. Antisera used were anti-GmZIP1 antiserum (described above) or anti-AtIRT1 antiserum. After washing off the unbound antibodies several times with 1 × TBS-Tween 20, the membranes were incubated for 1 h with a 1:20,000 dilution of sheep anti-rabbit horseradish peroxidase-conjugated IgG (Roche Molecular Biochemicals) and washed several times. Immunodetection was performed with a chemiluminescence Western blotting kit according to the supplier (Roche Molecular Biochemicals).

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**FIG. 1.** Predicted amino acid sequence of GmZIP1 and alignment with selected members of the ZIP gene family. The multiple alignment was performed with ClustalW (57). Fully conserved residues are boxed in black while semi-conservative substitutions are boxed in gray. Putative transmembrane domains for GmZIP1, as defined by TMTOP (28), are indicated by bars and Roman numerals. Arabidopsis MIPS identification numbers are as follows: IRT1 (At4 g19690), IRT2 (At4 g19680), IRT3 (At1 g0960), IRT3 (At1 g12750), IRT2 (At5 g9520), ZIP3 (At2 g32270), ZIP4 (At1 g0970), ZIP5 (At1 g05300), ZIP6 (At2 g30080), ZIP7 (GenBank™ AAD32823.1; MIPS (Munich Information Center for Protein Sequences) number not yet assigned), ZIP8 (GenBank™ AB019224; gene is incorrectly listed as a pseudogene), ZIP9 (At4 g3020), ZIP10 (At1 g31260), ZIP11 (At4 g31260), ZIP12 (At5 g62160). Other GenBank accession numbers are: Lycopersicon esculentum LeIRT1 (AF136579), LeIRT2 (AF136579) and Pisum sativum RIT1 (AF065444).
GmZIP1 Is a Member of a Zinc Transporter Family—Sequence analysis of the soybean cDNA showed that it encodes a protein of 354 amino acid residues (Fig. 1). A BLAST search on the translated protein sequence indicated strong homology with several members of the ZIP family as well as with other zinc and iron transporters. Consequently, we have named this cDNA *GmZIP1*. A phylogenetic tree obtained after compiling GmZIP1 with the sequences of 18 other known plant ZIP members (Fig. 2), revealed that GmZIP1 is most closely related to AtZIP1, AtZIP3, and AtZIP5, three recently identified zinc transporters of *Arabidopsis* (8, 25). According to the SMART (26, 27) and TMMTOP (28) predictions, GmZIP1 contains eight transmembrane-spanning regions, a very short C-terminal tail, and a predicted extracellular location of both the N- and C-terminal ends. The first 20 amino acid residues were also predicted to be part of a signal peptide. The extra-membrane loop located between putative helices III and IV is rich in histidine residues. This feature is one of the characteristics of the ZIP proteins, together with completely conserved histidine and glycine residues in helix IV, which are also present in GmZIP1 at positions 212 and 217, respectively. Moreover, amino acids 207–221 of GmZIP1 give a perfect match with the *bona fide* signature sequence of the ZIP family (29). These results, together with the presence of several putative metal ion binding sequence motifs between helices III and IV, suggest that GmZIP1 can be considered a new member of the ZIP zinc transporter family and the first one identified in soybean.
GmZIP1 Encodes a Zinc Transporter—To further characterize this protein and to establish whether it has any metal transporting capacity, the GmZIP1 cDNA was expressed in S. cerevisiae mutant strains, which are unable to grow on iron- or zinc-limited media, and their growth was monitored on plates (Fig. 3). Although the DY1455 wild type strain can grow under zinc-deficient conditions, ZHY3 cells are very sensitive to zinc deprivation because both their high (ZRT1) and low (ZRT2) affinity zinc uptake systems have been mutated (30). However, GmZIP1-expressing ZHY3 cells were able to grow on restrictive medium, indicating that GmZIP1 could encode a putative zinc transporter. A similar experiment was performed using DEY1453 cells, which lack both high (FET3) and low (FET4) affinity iron transporters and cannot grow on iron-limited media. Under the conditions tested, transformation with GmZIP1 did not restore the growth of the fet3fet4 mutant, suggesting that GmZIP1 cannot use iron as a substrate. In both sets of experiments, the mutant strains were also transformed with pAtIRT1, which has previously been shown to complement both fet3fet4 and zrt1zrt2 cells (31), as a positive control.

To quantify the transport of zinc by GmZIP1, uptake assays with $^{65}$Zn$^{2+}$ were performed in ZHY3 mutant cells. At 30 °C, zinc accumulation in GmZIP1-expressing cells was linear for at least 60 min (Fig. 4A). Cells transformed with the pFL61 empty vector, on the other hand, showed a very low level of zinc accumulation (data not shown), which presumably represented residual zinc uptake through other yeast metal ion transporters. Values obtained in these control experiments were subtracted from all the data presented. No zinc uptake could be detected when assays were conducted on ice suggesting that the zinc accumulation observed was due to an internalization of the metal rather than a nonspecific adsorption of zinc to the cell surface. An absence of uptake was also observed when cells were starved of glucose for an hour prior to starting the uptake assays (Fig. 4A). However, no change in uptake level was noticed when pH was varied from 3 to 7 (data not shown), indicating that GmZIP1 activity is not pH-dependent. pH values higher than 7 are known to lead to the formation of monovalent Zn(OH)$^+$, neutral Zn(OH)$_2$, and insoluble complexes (32) and, therefore, were not tested. We also investigated the affinity of the uptake system over a range of zinc concentrations. Uptake was followed over a 20-min period and was found to be concentration-dependent and saturable (Fig. 4B). The transport kinetic parameters, $K_m$ and $V_{max}$, were determined from Lineweaver and Burk data transformations (Fig. 4B, inset) and were estimated at 13.8 μM and 12.5 fmol per min per 10$^6$ cells, respectively.

The specificity of GmZIP1 for zinc or other metals was assessed in competition experiments performed in the presence of a 10-fold molar excess of other, non-labeled divalent cations. Among the metals tested, cadmium alone had a significant inhibitory effect on zinc uptake (Table 1). It is interesting to note that neither Fe(III) nor Fe(II) could compete with zinc, in agreement with the inability of GmZIP1 to complement the fet3fet4 mutant on iron-limited media.

Tissue-specific Expression and Localization of GmZIP in Nodules—The results presented above clearly show that the soybean GmZIP1 behaved like a zinc transporter when expressed in a heterologous system. We subsequently investigated the role of GmZIP1 in planta. Poly(A)$^+$ RNA was isolated from leaves, stems, roots, and nodules and analyzed on a Northern blot (Fig. 5). Under the conditions used, the probe detected GmZIP mRNA only in the nodules. The GmZIP transcript signal only appeared in nodules of plants 18 days and older, and the abundance of transcripts did not change between nodules of 23- and 42-day-old plants (Fig. 5). No signal was observed in roots, stems, or leaves. This tissue-specific expression suggests that GmZIP1 is a symbiotic protein that is active in mature, nitrogen-fixing nodules. Since in Arabidopsis ZIP1 and ZIP3 transcripts are only observed in roots when plants are starved of zinc (25), and since the plants used in the present...
study were grown in the presence of ample zinc, the results suggest either that the nodule-infected cell cytosol is depleted of zinc (perhaps by the bacteroids themselves) or that expression of the symbiotic gene is regulated by other factors. The answer to this question awaits further experimentation.

To analyze the presence of GmZIP1 at the protein level and to localize it within nodules, we used both an antiserum raised against AtIRT1 and a GmZIP1-specific antibody. The AtIRT1 antiserum reacted with several proteins in a microsomal membrane preparation from nodules (see “Experimental Procedures”) but only a single protein of 34 kDa on the purified PBM (Fig. 6A), which is the predicted size of GmZIP1. While we cannot eliminate the possibility that there is an IRT homologue on the PBM, which reacts with AtIRT1 antiserum, this is unlikely since the primers we used to amplify GmZIP1 from the nodule cDNA library should have amplified IRT clones also. A stronger reaction against the 34-kDa PBM protein was observed with the GmZIP1 antibody, which did not react with protein samples isolated from root microsomes or nodule microsomes isolated after removing the symbiosomes (Fig. 6B). PBM proteins isolated from plants of 4-, 6-, and 7-week-old plants reacted equally with the GmZIP1 antibody (Fig. 6C). The results shown in Fig. 6 suggest that GmZIP1 is a symbiotic isoform of a larger GmZIP family. This idea is supported by the results of the Southern blot of soybean genomic DNA (Fig. 7). The hybridization pattern seen with the GmZIP1 probe is consistent with the presence of a multigene family. No cross-reaction of the GmZIP1 antibodies with symbiosome space or bacteroid proteins was observed, nor between the PBM and the rabbit pre-immune serum (data not shown).

The results of Fig. 6 also show that the PBM preparation was not contaminated significantly by other membranes from the nodule. In this context it should be noted that we prepare PBM from purified symbiosomes that are routinely checked by microscopy and marker enzyme assays for contamination by other plant organelles and membranes (47). This contamination is negligible, largely because of the rate zonal method of purification of symbiosomes on dense Percoll gradients (19).

The presence of GmZIP1 on the PBM having been established, we further investigated the capacity of symbiosomes to take up zinc. As shown in Fig. 5B, purified symbiosomes were able to accumulate zinc, supplied as a 20 μM radiolabeled zinc chloride solution, and uptake was linear for up to 2 min. Zinc uptake by isolated symbiosomes responded to the concentration added and showed saturation kinetics (Fig. 5A). The apparent Km was 91 μM, somewhat higher than that observed with GmZIP1-expressing yeast cells. Over the concentration range tested, there was no indication of more than one transport activity. Since other, as yet unidentified, metal ion transporters could contribute to zinc uptake across the PBM, the GmZIP1 antisera was employed to confirm the involvement of GmZIP1. Isolated symbiosomes were pre-incubated with GmZIP1 antibody 30 min prior to mixing with the radiolabeled solution. The GmZIP1, resulted in a 35% (S.E. ± 11, n = 15) inhibition of zinc uptake, using a pre-incubation of the symbiosomes with the pre-immune fraction of the serum as a control (Fig. 9). This result indicates that a significant proportion of the zinc uptake observed in symbiosomes is due to GmZIP1.

The effect of divalent cations on zinc uptake by symbiosomes was analyzed by incubating the organelles with a 10-fold excess of competitor metal together with 20 μM 65Zn2+. The results...
were very similar to those obtained with yeast expressing GmZIP1; Cd$^{2+}$ was able to severely inhibit the accumulation of zinc in symbiosomes leading to a 70% decrease in 5 min (Table 1). Cu$^{2+}$ was also found to compete with zinc and caused a 30% decrease in zinc uptake. Nonetheless, the lack of complete inhibition by the GmZIP antibody, and the fact that Cu inhibited zinc uptake partially into symbiosomes but not into yeast, may reflect the presence of multiple transport systems for zinc on the PBM.

**DISCUSSION**

The PCR approach employed allowed the identification of GmZIP1, the first soybean member of the zinc- and iron-transporter family. Southern blotting indicated the presence of other members of a soybean ZIP family, but GmZIP1 was immunolocalized specifically to the peribacteroid membrane of soybean nodules. The polyclonal antibody raised in rabbit against GmZIP1 (see “Experimental Procedures”) was used to detect immunoreactive proteins isolated from peribacteroid membranes (Lane 1), nodule (Lane 2) or root microsomal fractions (Lane 3). Cross-reactions were revealed by chemiluminescence and 2-min exposure on a luminometer. GmZIP1 protein is found in the peribacteroid membrane of plants from 4–7 weeks of age. Anti-GmZIP1 antibody was used to detect GmZIP1 protein from peribacteroid membrane isolated from plants aged 4 weeks (Lane 1), 6 weeks (Lane 2), and 7 weeks (Lane 3). Luminometer exposure was for 5-min. Sizes of molecular weight markers are indicated on the right.

**FIG. 6. Immunolocalization of ZIP proteins to soybean membrane fractions.** A, AtIRT1 antibody detects several soybean ZIP proteins. AtIRT1 antibody bound to several soybean ZIP nodule microsomal proteins (Lane 1; 2-min exposure) and less strongly to a protein on the peribacteroid membrane (Lane 2; 10-min exposure). B, immunolocalization of GmZIP1 protein is specific to the peribacteroid membrane of soybean nodules. The polyclonal antibody raised in rabbit against GmZIP1 (see “Experimental Procedures”) was used to detect immunoreactive proteins isolated from peribacteroid membranes (Lane 1), nodule (Lane 2) or root microsomal fractions (Lane 3). Cross-reactions were revealed by chemiluminescence and 2-min exposure on a luminometer. GmZIP1 protein is found in the peribacteroid membrane of plants from 4–7 weeks of age. Anti-GmZIP1 antibody was used to detect GmZIP1 protein from peribacteroid membrane isolated from plants aged 4 weeks (Lane 1), 6 weeks (Lane 2), and 7 weeks (Lane 3). Luminometer exposure was for 5-min. Sizes of molecular weight markers are indicated on the right.

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**FIG. 7. Indication of a ZIP multigene family in soybean.** Southern blot analysis was performed with 5 μg of genomic DNA from soybean leaves. Genomic DNA was digested with BglII (lane 1), EcoRI (lane 2), HindIII (lane 3), or NotI (lane 4), separated on a 0.8% agarose gel, blotted onto nylon membrane, and hybridized with DIG-labeled GmZIP1 DNA. The series of molecular weight markers are shown in kilobase pairs.

**FIG. 8. Zinc accumulation in isolated symbiosomes.** A, concentration-dependent Zn$^{2+}$ uptake by symbiosomes. Five-minute uptake experiments were performed at 30 °C using substrate concentrations from 5 to 300 μM total zinc. Values from control experiments conducted on ice were subtracted. The inset graph is a Lineweaver-Burk plot of the data giving a calculated $K_m = 91 \mu$M. The data are from two independent experiments done in triplicate; the values and error bars are the mean ± S.E. ($n = 5$ or $6$), respectively. B, time-course accumulation of zinc in symbiosomes. Percoll gradient-isolated symbiosomes were incubated at 30 °C in mannitol buffer supplemented with 20 μM $^{65}$ZnCl$_2$. Experiments were performed on two independent preparations with triplicates for each data point. Average values are shown with error bars representing S.E. ($n = 6$).
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The PBM is energized by a H\(^+\)-pumping ATPase that generates a membrane potential-positive on the inside of the symbiosome (a acidic interior if permeant anions are present; see Ref. 42). In this respect, the symbiosome resembles a vacuole. Studies with tonoplast vesicles have suggested that zinc (and other divalent metal ions) enter the vacuole in exchange for H\(^+\) via an antiport mechanism (34), perhaps catalyzed by a homologue of the ZAT1 protein identified in Arabidopsis (43). It is possible that Zn/H\(^+\) antiport also occurs across the PBM. Indeed, in addition to the identified ZIP, a member of the ZAT family may also be present on the PBM since there are many similarities between the symbiosome and the vacuole of plants. However, when we tested the effect of ATP with or without permeant anions on zinc uptake by isolated symbiosomes the results were variable with, on average, a small inhibition seen in the presence of ATP. Dissipation of the membrane potential by permeant anions had no significant effect on zinc uptake (results not shown). Similar results were observed with ferric citrate uptake into isolated symbiosomes (44). In this context, it is interesting to note that a human member of the ZIP family, hZIP2, has been shown to be energy-independent with a proposed Zn/HCO\(_3\) symport mechanism (7). The proposed mechanism of zinc transport via GmZIP1 raises an interesting problem with respect to the orientation of the protein when expressed on the plasma membrane in yeast. Clearly, in this situation, GmZIP1 catalyzes uptake of zinc into the cell. However, uptake into isolated symbiosomes is equivalent to export from the plant cell. If GmZIP1 has the same physical orientation in the two membranes, which is likely considering that the secretary pathway is thought to mediate protein insertion into the PBM, then GmZIP1 must be able to catalyze bi-directional transport of zinc. This is not unusual for a carrier, but if zinc uptake into yeast is linked directly to the proton gradient then GmZIP must be able to catalyze Zn/H\(^+\) symport as well as antiport. Alternatively, zinc uptake could be linked to the membrane potential or pH gradient via other ion movements. Further experiments on the two systems may provide new insights into the mechanism of zinc transport in plants.

The difference in apparent

K\(_m\)

of zinc uptake by the two systems (13 and 91 \(\mu M\) for the yeast and symbiosome, respectively), may reflect different binding affinities on the two sides of the transporter. Although, both values still fall within the scale of a low affinity plant system (45, 46) it is also possible that the higher K\(_m\) reflects the participation of other transporters in zinc movement across the PBM. That is, the true affinity of GmZIP1 may be higher than that measured with isolated symbiosomes. The zinc concentration in the cytosol of nodule-infected cells is unknown but the calculated K\(_m\) of GmZIP1 is similar to that calculated for zinc uptake into oat root tonoplast vesicles (34). Nonetheless, it should be considered that GmZIP1 may also function to export zinc from the symbiosome in vivo.

Of a spectrum of different metals tried, GmZIP1-dependent zinc uptake in yeast was inhibited only by cadmium. This was also observed with purified symbiosomes. This inhibitory effect of cadmium on zinc uptake is not restricted to GmZIP1. Other transporters are known to present this dual zinc/cadmium uptake capacity (10), and this can probably be accounted for by the very high electronic homology between zinc and cadmium.

In the symbiosome, copper was also able to compete with zinc transport to some extent (Table I), and it is possible that in vivo GmZIP1 can transport both ions. In fact, Eckhardt et al. (48) have shown that LeIRT1 and LeIRT2 can complement a copper mutant of yeast. In this context, it is interesting to note that a putative copper/zinc superoxide dismutase, Smc02597, has been identified recently on the chromosome of Sinorhizobium meliloti (49), and superoxide dismutase enzymes are thought to play key roles in bacteroid-plant interactions (50). Nodules contain very high concentrations of iron and the inability of iron to inhibit zinc uptake may, therefore, also be an important feature of GmZIP1.

Stabilization of the metal via an electronic interaction with amino acid residues of GmZIP1 could play an important role in the specificity of the transporter. Rogers et al. (51) recently
showed that replacement of key aspartate (Asp-100 and Asp-136) residues of AtIRT1 with alanine, converted the transporter into a form only able to take up zinc, while the wild type enzyme also catalyzed iron and manganese uptake. Interestingly, unlike strains carrying the D100A allele, the strain carrying the D136A allele was no longer sensitive to 0.2 μM cadmium, indicating that the strain carrying this allele transporters less cadmium than strains carrying either the wild type IRT1 or the D100A allele. GmZIP1 has both of these conserved aspartate residues and nonetheless is unable to transport iron but is sensitive to cadmium. While the results of Rogers et al. (51) indicate that the transport of different metals are physically separable, it is also clear that substrate selectivity involves more than just a few key amino acids. Each member of this transporter family must be analyzed separately to achieve precise engineering of activities. It would certainly be interesting to perform a similar mutagenesis study on GmZIP1 and to analyze the effects of a modified specificity of the metal transporter upon the symbiosis. For this purpose, residues Asp-104 or Asp-140 of GmZIP1 could be good candidates, as they are the soybean equivalents of Asp-100 or Asp-136 in AtIRT1.

Besides being a vital micronutrient for all organisms because of its cofactor role in many enzymes, zinc is thought to play a role in signal-transduction and in gene regulation. In plants, zinc has been shown to have a major role in the regulation of genes encoding high affinity phosphate transporters in roots (52). This role is specific to zinc, as it cannot be replaced by manganese, for example, and seems very important, as this tight control of phosphate uptake is lost under zinc deficiency (52–54). In this context, it has been established that nodulation and N₂ fixation have a high phosphorus requirement. At low nitrate concentration, increasing amounts of phosphorus promote both nodule formation and N₂ fixation (55). At the microsymbiont level, the high phosphorus concentration present in nodules (20–100 mM) switches exopoly saccharide production of S. meliloti from galactoglucon (EPS II) to succinoglycan (EPS I). The lon mutant of S. meliloti, shown to constitutively express EPS II, only forms pseudo-nodules, delayed in appearance and unable to fix N₂ (56). By controlling the phosphorus status in nodules, zinc could play a critical role in nodulation and symbiosis.

Acknowledgments—We thank Dr. David Eide (University of Missouri, Columbia, MO) for providing the DEY1453 and ZHY3 yeast mutants, Dr. Emmanuel Lesuisse (Institut Jacques Monod, Paris, France) for the DY1453 yeast strain, and Joanne Castelli for expert technical assistance.

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