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RESEARCH PAPER

OsZIP4, a novel zinc-regulated zinc transporter in rice

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Abstract

Zinc (Zn) is an essential element for the normal growth of plants but information is scarce on the mechanisms whereby Zn is transported in rice (*Oryza sativa* L.) plants. Four distinct genes, *OsZIP4*, *OsZIP5*, *OsZIP6*, and *OsZIP7* that exhibit sequence similarity to the rice ferrous ion transporter, *OsIRT1*, were isolated. Microarray and northern blot analysis revealed that *OsZIP4* was highly expressed under conditions of Zn deficiency in roots and shoots. Real-time-PCR revealed that the *OsZIP4* transcripts were more abundant than those of *OsZIP1* or *OsZIP3* in Zn-deficient roots and shoots. OsZIP4 complemented a Zn-uptake-deficient yeast (*Saccharomyces cerevisiae*) mutant, Δ*zrt1*,Δ*zrt2*, indicating that OsZIP4 is a functional transporter of Zn. OsZIP4-synthetic green fluorescent protein (sGFP) fusion protein was transiently expressed in onion epidermal cells localized to the plasma membrane. *In situ* hybridization analysis revealed that *OsZIP4* in Zn-deficient rice was expressed in shoots and roots, especially in phloem cells. Furthermore, *OsZIP4* transcripts were detected in the meristem of Zn-deficient roots and shoots. These results suggested that OsZIP4 is a Zn transporter that may be responsible for the translocation of Zn within rice plants.

Key words: IRT1, meristem, phloem, rice, zinc, ZIP4.

Introduction

Zinc (Zn) is an essential nutrient that plays important roles in numerous physiological processes in plants, serving as a cofactor for many enzymes and as the key structural motifs in transcriptional regulatory proteins. A deficiency of Zn, therefore, decreases growth, but excess Zn has significant toxicity to biological systems through metal-based cytotoxic reactions. Therefore, the uptake and transport of Zn must be strictly regulated. Intracellular Zn homeostasis is achieved through the co-ordinated regulation of specific transporters engaged in Zn influx, efflux, and intracellular compartmentalization.

In the *Arabidopsis thaliana* genome, a large number of cation transporters potentially involved in metal ion homeostasis have been identified (Maser *et al*., 2001). Several members of the 15 Zinc-regulated transporters, Iron-regulated transporter-like Protein (ZIP) gene family (Guerinot, 2000) have been characterized and shown to be involved in metal uptake and transport in plants (Eide *et al*., 1996; Korshunova *et al*., 1999; Vert *et al*., 2001, 2002; Connolly *et al*., 2002). The ZIP proteins have been predicted to have eight transmembrane domains with their amino- and carboxyl-terminal ends situated on the outer surface of the plasma membrane (Guerinot, 2000). These proteins vary considerably in overall length, due to a variable region between the transmembrane domains (TM)-3 and TM-4, predicted to be on the cytoplasmic side providing a potential metal-binding domain rich in histidine residues. The most conserved region of these proteins lies in a variable region that has been predicted to form an amphipathic helix containing a fully conserved histidine

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Abbreviations: IRT, iron-regulated transporter; ZIP, zinc-regulated transporters; sGFP, synthetic green fluorescent protein.

that may form part of an intramembranous metal-binding site involved in transport (Guerinot, 2000). The transport function is eliminated when the conserved histidines or certain adjacent residues are replaced by mutation (Rogers *et al*., 2000).

ZIP1, ZIP3, and ZIP4 from *Arabidopsis* restore Zn uptake to the yeast (*Saccharomyces cerevisiae*) Zn-uptake mutant, Δ*zrt1,*Δ*zrt2*, and have been proposed to play a role in Zn transport (Grotz *et al*., 1998; Guerinot, 2000). ZIP1 and ZIP3 are expressed in roots in response to Zn deficiency, suggesting that they transport Zn from the soil to the plant, while ZIP4 is expressed both in the roots and shoots, suggesting that it transports Zn intracellularly or between plant tissues (Grotz *et al*., 1998; Guerinot, 2000). ZIP2 and ZIP4 rescue yeast mutants deficient in copper (Cu) transport, and ZIP4 is up-regulated in Cu-deficient roots (Wintz *et al*., 2003). ZRT1 and ZRT2 are high- and low-affinity Zn transporters, respectively (Eide, 1998; Guerinot, 2000). The proposed role of ZIP transporters in Zn nutrition has been further supported by the characterization of homologues from a number of plant species. For example, GmZIP1 has been identified in soybean (*Glycine max*) (Moreau *et al*., 2002), and functional complementation of Δ*zrt1,*Δ*zrt2* yeast cells showed that GmZIP1 is highly selective for Zn, but not for iron (Fe) or manganese (Mn). GmZIP1 is expressed specifically in nodules, but not in roots, stems, or leaves, and the protein is localized to the peribacteroid membrane, suggesting a role in symbiosis. Ramesh *et al*. (2003) reported that OsZIP1 and OsZIP3 are also functional Zn transporters in rice (*Oryza sativa*) plants.

Previously, the *OsIRT1* gene encoding a functional Fe^{2+} transporter that is homologous to *Arabidopsis IRT1* (Bughio *et al*., 2002; Ishimaru *et al*., 2005) was isolated. *OsIRT1* was highly up-regulated in Fe-deficient roots. OsIRT1 reversed the growth defect of YH003 (Δ*ftr1*,Δ*fet4*,Δ*fre1*) on Fe-depleted media.

In this report, the OsZIP4 gene, highly homologous to OsIRT1, was isolated and characterized and it was found that *OsZIP4* encodes a Zn transporter localized to the plasma membrane and regulated by the plant's Zn status. *In situ* hybridization analysis revealed that *OsZIP4* in Zn-deficient rice was expressed in shoots and roots, especially in phloem cells and the meristem.

Materials and methods

Plant material

Oryza sativa L. cv. Nipponbare was used for the microarray, northern blot, real time-PCR, and metal concentration analyses. Seeds were germinated for 3 d at room temperature on paper soaked with distilled water. After germination, the seedlings were transferred to a Saran net floating on distilled water in a growth chamber (day: 25 °C, 14 h of light at 320 μmol photons m^{-2} s^{-1}; night: 10 h at 20 °C). After 3 d, 45 seedlings were transferred to a 20 l plastic container containing a nutrient solution with the following composition: 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.05 μM Na_2MoO_4, and 0.1 mM Fe-EDTA. The $ZnSO_4$ was omitted from the solution to induce Zn deficiency, as were the Fe, Mn, and Cu salts in order to induce deficiencies of these nutrients. The pH of the nutrient solution was adjusted daily to 5.5 with 1 M HCl, and the nutrient solution was renewed weekly. For the Zn-deficiency treatment, 2-week-old plants were transferred to nutrient solution without Zn and grown for 2 more weeks. To induce Fe, Mn, or Cu deficiency, 2-week-old plants were grown for 2 weeks in nutrient solutions without these respective nutrients.

Oligo DNA microarray analysis

A rice 22 K custom oligo DNA microarray kit (Agilent Technology, Tokyo, Japan), which contains 21 938 oligonucleotides synthesized based on the sequence data of the rice full-length cDNA project (http://cdna01.dna.affrc.go.jp/cDNA/) was used. Total RNA was extracted from shoots and roots using a RNeasy Plant Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions; the yield and RNA purity were determined spectrophotometrically. The integrity of the RNA was checked using an Agilent 2100 Bioanalyser (Agilent Technology). Total RNA (200 ng) was labelled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technology). Fluorescent-labelled targets were hybridized to Agilent rice 22 K oligo DNA microarrays. The hybridization process was performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technology). Feature Extraction software (Agilent Technology) was used for the image analysis and data extraction processes.

PCR cloning of OsZIP4

The *OsZIP4*, *OsZIP1*, and *OsZIP3* sequences, which are homologous to *OsIRT1*, were found on the Knowledge-based *Oryza* Molecular Biological Encyclopedia website (http://cdna01.dna.affrc.go.jp/cDNA/). A PCR-based cloning strategy was used to isolate *OsZIP4*, *OsIRT1*, *OsZIP1*, and *OsZIP3*. The primers used to amplify ORF were as follow; *OsZIP4* forward (5′-CACCATGGACGCCATGAGGCAGAGCACGCG), *OsZIP4* reverse (5′-TCATGCCCATATGGCAAGCAGAGACATCAT), *OsIRT1* forward (5′-CACCGAATTCCGTACGGCATGGCGACGCCGCGGACACTGGT), *OsIRT1* reverse (5′-ACAACTAATGGCGGCCGCTCACGCCCACTTGGCCATGACG), *OsZIP1* forward (5′-CACCGGCGCAAGCTTCGACCATGGCCAGGA), *OsZIP1* reverse (5′-GAAGCAAGTCTAGAACTAGGATGGATGGATC), *OsZIP3* forward (5′-CACCATGGGAGCCAAGAAGCATACCTTGCA), and *OsZIP3* reverse (5′-CTATGCCCATATGGCAAGCATTGACATCAG). The ORFs were amplified from a Zn- and Fe-deficient plant mixed cDNA library using these primers. The amplified fragment containing the *OsZIP4* coding sequence was subcloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA). This pENTR/D-TOPO entry vector containing the *OsZIP4* coding sequence was designated pENTR-*OsZIP4*. The pENTR-*OsZIP4* was confirmed by sequencing. The other amplified fragments were inserted into pENTR/D-TOPO in the same manner. Isolated ORFs were sequenced using a Thermo Sequenase Cycle Sequencing kit (Shimadzu, Kyoto) and a DNA sequencer (DSQ-2000L; Shimadzu).

Northern blot analysis

Total RNA was extracted from roots and shoots, and 10 μg per lane were electrophoresed in 1.2% (w/v) agarose gels containing 0.66 M formaldehyde, transferred to Hybond-N^+ membrane (Amersham, USA), and hybridized with probes at 65 °C according to the method of Mizuno *et al*. (2003). The amplified ORF of *OsZIP4*, *OsIRT1*, and *OsZIP1* were used to prepare probes.

Quantitative real-time-PCR of OsZIP4*,* OsZIP1*, and OsZIP3*

Total RNA was treated with RNase-free DNase I (Takara, Tokyo, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) by priming with oligo-d$(T)_{30}$. The fragment was amplified by PCR in a SmartCycler (Takara) with SYBR Green I and ExTaq™ Real-Time-PCR Version (Takara). The primers used for Real-Time-PCR were as follows; *OsZIP4* forward (5′-GCGAAAGCAACAGTGATCATG-GCGACTTTC), *OsZIP4* reverse (5′-GCAGCTCTTGGTTGCTCT-GAAGATCTCATG), *OsZIP1* forward (5′-CTCTTCAAGTTCCT-CGCCGTCCT), *OsZIP1* reverse (5′-CGGCCACGATTAATGAA-TGGGGTG), *OsZIP3* forward (5′-AATGTGCATAGCTCAACTG-CCTT), and *OsZIP3* reverse (5′-CAAAATCAAGCCTATCTGG-GA). The primers used for internal control in RT-PCR were α-*tubulin* forward, (5′-TCTTCCACCCTGAGCAGCTC) and α-*tubulin* reverse (5′-AACCTTGGAGACCAGTGCAG). There was no genomic contamination and no differences of the internal control in each deficient condition (data not shown). The sizes of the amplified fragments were confirmed by gel electrophoresis and sequencing.

Determination of metal concentrations

The plants were dried for 1 week at 65 °C. The plants (30–50 mg) were then wet-ashed with 2 ml of 11 M HNO_3 for 5 h at 150 °C. The metal concentrations were measured using inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan) at wavelengths of 238.204 (Fe), 213.856 (Zn), 293.930 (Mn), and 324.754 (Cu) nm.

Yeast strains and growth media

The following strains of the yeast *Saccharomyces cerevisiae* were used in this study: CM3260 (parent strain) *MATalpha trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52, YH003 MATalpha trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52* Δ*ftr1::URA3* Δ*fre1::HIS* Δ*fet3::TRP*, CM-SMF *MATalpha trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52* Δ*smf1::URA3*, CM-ZRT *MATalpha trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52* Δ*zrt1::URA3* Δ*zrt2::HIS3*, and FTRUNB1 *MATalpha trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52* Δ*ctr1::URA3*. Yeast disruption mutants were made by homologous recombination. Yeast cells were grown in 1% yeast extract, 2% peptone, and 2% glucose (YPD) and synthetic defined medium (SD) supplied with the appropriate amino acids. Agar (2%) was added to obtain solid plate media (Sherman, 1991). For medium deprived of Fe, Zn, or Mn, respectively, 50 μM bathophenanthroline disulphonic acid (BPDS), 0.5 M ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), or 50 mM ethylene glycol-*bis*-β-aminoethylether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Wako Pure Chemical Industries, Japan) were added. 1% yeast extract, 2% peptone, and 2% glycerol (YPG) medium were used with 10 μM Cu for Cu-deprived medium.

Functional expression in yeast

The yeast expression vector (pYH23) was kindly provided by Dr Hirotaka Yamaguchi (Kyushu National Agricultural Experiment Station) (Bughio *et al*., 2002). pYH23 has *Hin*dIII, *Pvu*II, *Pst*I, *Xho*I, *Sst*I, *Xba*I, and *Not*I sites in the *ADH1* expression cassette. The plasmid was digested with *Hin*dIII and *Xba*I, and the 1579 bp MultiSite Gateway Three-fragment (Invitrogen), containing an *att*R1 site at the 5′-end, the chloramphenicol resistance gene, the *ccd*B gene, and the *att*R2 site cassette, was inserted. This modified plasmid was designated pDESTADH as the destination vector. A subsequent attL substrate and attR substrate recombination reaction (Invitrogen) between the destination and entry vectors generated an expression clone containing the gene encoding pYH23- *OsZIP4*. The *OsZIP4* and *OsZIP3* ORFs were inserted into pYH23 in the same way. Yeast transformation was carried out using the Li-acetate transformation method (Gietz and Schiestl, 1995).

Construction of plasmid OsZIP4-sGFP *and observation of* OsZIP4-sGFP *localization*

Plasmid pUC18, containing the cauliflower mosaic virus (CaMV) 35S promoter-sGFP (S65T)-NOS3′ construct, was kindly provided by Dr Yasuo Niwa (University of Shizuoka). The construct had *Sal*I and *Nco*I sites on the 3′ side of the CaMV 35S promoter. The *Nco*I site 'CCATGG' includes the initiation codon for *sGFP*. An annealed oligomer (5′-TCGAGATATCGGTACCAGATCTGAGCTCGAGG-TCGA and 5′-CTAGTCGACCTCGAGCTCAGATCTGGTACC-GATATC) was inserted into the *Nco*I and *Sal*I site of CaMV 35S-*sGFP* (S65T)-NOS3′ to produce a new *Eco*RV (GATATC) site. The plasmid was digested with *Eco*RV, and the 1579 bp MultiSite Gateway Three-fragment (Invitrogen) was inserted. This modified plasmid was designated pDEST35S-*sGFP* as the destination vector. The ORF of *OsZIP4* was amplified using two primers: 5′-CACCATGGACGCCATGAGGCAGAGCACGCG and 5′-TGCCCATATGGCAAGCAGAGACATCATCCC. The amplified fragment containing the *OsZIP4* coding sequence was subcloned into pENTR/D-TOPO (Invitrogen). This pENTR/D-TOPO entry vector containing the *OsZIP4* coding sequence was designated pENTR-*OsZIP4*. A subsequent attL substrate and attR substrate recombination reaction (Invitrogen) between the destination and entry vectors generated an expression clone containing the gene encoding 35S-*OsZIP4-sGFP*. Onion epidermal cells were transformed using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Tokyo, Japan), and the sGFP fluorescence occurred as described by Mizuno *et al*. (2003).

In situ *localization*

Oryza sativa L. cv. Nipponbare was grown with sufficient Zn for 2 weeks and plants transferred to nutrient solution without Zn for 10 d. Tissue from these plants was fixed in 4% (w/v) paraformaldehyde for 36 h, and was then dehydrated in an ethanol series. After dehydration, the tissue was infused with paraplast and then sectioned to 10 μm and mounted on slides. The *OsZIP4* specific fragment of RT-PCR was subcloned into the pCR-TOPO vector (Invitrogen). This plasmid was used to generate sense and antisense probes for *in situ* hybridization. Sense and antisense probes were labelled with digoxigenin-11-UTP (Roche, Mannheim, Germany) according to the manufacturer's protocol. This plasmid was linearized with *Hin*dIII and transcribed with T7 RNA polymerase. After hydrolysis of the labelled probes and further tissue treatment, the slides were hybridized overnight at 42 °C and washed. The tissue was then incubated with anti-digoxigenin alkaline phosphatase conjugate (Roche) for 30 min at room temperature, and the antibody was detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate after an overnight incubation. The results were visualized using an Axiophoto microscope (Carl Zeiss, Tokyo, Japan) following the manufacturer's instructions.z

Results

Isolation and phylogenic analysis of ZIP family transporters in rice plants

A search of the computer database Knowledge-based *Oryza* Molecular Biological Encyclopedia (http://cdna01.dna.affrc.go.jp/cDNA/) enabled four distinct genes of *OsZIP* to be identified and isolated, homologous with the previously identified *OsIRT1*: *OsZIP4* (accession. no. AB126089), *OsZIP5* (accession. no. AB126087), *OsZIP6* (accession. no. AB126088), and *OsZIP7* (accession. no. AB126090). A phylogenic analysis showed that OsZIPs

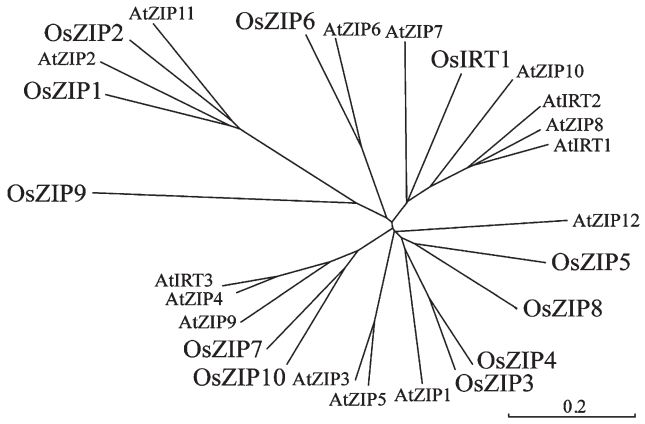

Fig. 1. Unrooted phylogenic tree for the OsIRT1, OsZIPs, AtIRTs, and AtZIPs amino acid sequences for which ORFs are confirmed. Calculations were performed using the CLUSTAL W Neighbor–Joining method and the tree was visualized with TreeView.

Table 1. *Ratio of Zn-deficiency inducible* OsZIP4, OsIRT1, *and* OsZIPs *in Zn-deficient and Zn-sufficient roots and shoots*

Microarray analysis was carried out to detect the induction of the expression of *OsZIP4*, *OsIRT1*, and *OsZIP*s in Zn-deficient roots compared with Zn-sufficient roots (–Zn Root), or in Zn-deficient shoots compared with Zn-sufficient shoots (–Zn Shoot). Data presented are the average of four biological replicates.

Gene	Root	Shoot	Accession no.
OsZIP4	4.74±0.75	20.88±5.66	AB126089
OsIRT1	2.12±0.28	1.42±0.11	AB070226
OsZIP5	1.72±0.36	3.78±0.95	AB126087
OsZIP6	0.79±0.09	1.79±0.20	AB126088
OsZIP7	1.44±0.17	4.57±1.02	AB126090
OsZIP3	1.07±0.20	0.39±0.02	AY323915

differ in their amino acid sequences and in their relationship to other members of the ZIP family (Fig. 1). The OsZIP4, OsZIP5, OsZIP6, and OsZIP7 proteins show 54, 51, 33, and 50% identity with OsIRT1, respectively. They also show 58, 49, 44, and 35% identity to ZIP1 from *A. thaliana*, respectively. The rice genome database was searched for other ZIP-like genes, and three more were found: *OsZIP8*, *OsZIP9*, and *OsZIP10*. Therefore, it appears that rice plants have OsIRT1 and ten OsZIPs.

Microarray analysis of OsZIP4, OsIRT1, OsZIP5, OsZIP6, OsZIP7, *and* OsZIP3

To understand their integrated regulation better, the expression patterns of known or potential metal transporters in rice roots and shoots in response to Zn deficiency were analysed using the 22 K microarray which contained six *OsZIP* family genes (Table 1). Of these, *OsIRT1*, *OsZIP5*, and *OsZIP4* were up-regulated in Zn-deficient roots, and *OsZIP4*, *OsZIP5*, and *OsZIP7* were up-regulated in Zn-deficient shoots. The induction ratio of *OsZIP4* by Zn deficiency was the fourth highest in shoots and the fourteenth highest in roots of the 22 K genes on this array (data not shown).

Expression patterns of OsIRT1 *and* OsZIP*s in various metal-deficient rice plants*

The abundance of *OsZIP4*, *OsIRT1,* and *OsZIP1* transcripts were confirmed by northern blot analysis under Zn-, Fe-, Mn-, or Cu-deficient conditions (Fig. 2A). The level of *OsZIP4* transcripts increased markedly in Zn-deficient shoots and roots, but a deficiency of other metals had little effect on the *OsZIP4* transcript level. In quantitative real time (RT)-PCR, the expression of *OsZIP4* was also highly induced by Zn deficiency in roots, stems, and leaves, and was the highest in young leaves (Fig. 2B).

The expression pattern of *OsIRT1* was consistent with previous results (Bughio *et al*., 2002). In contrast to previously published data for *OsZIP1* (Ramesh *et al*., 2003), however, *OsZIP1* was not up-regulated by Zn deficiency. Interestingly, *OsZIP1* was up-regulated in Cu-deficient roots (Fig. 2A). *OsZIP3* transcripts could not be detected in roots and shoots (data not shown). Consistent with northern blot analysis, RT-PCR revealed that *OsZIP1* was down-regulated by Zn deficiency. It was found that *OsZIP4* transcripts were much more abundant than those of *OsZIP1* or *OsZIP3* in Zn-deficient roots and shoots (Fig. 2C).

The time-course of *OsZIP4* expression under Zn deficiency was examined by RNA gel-blot analysis using the *OsZIP4* open reading frame (ORF) as a hybridization probe. This analysis revealed that the induction of *OsZIP4* expression in roots does not parallel that in leaves (Fig. 3). *OsZIP4* transcripts in roots were induced in 3 d and were abundant 7 d after transfer to culture lacking Zn. The corresponding values for the shoots were 5 d and 14 d. *OsZIP4* transcripts were undetectable 8 d after the resupply of Zn.

The concentrations of Zn, Mn, Fe, and Cu in Zn-deficient rice plants were determined to confirm the nutritional status of these plants. As expected, there was a significant decrease in Zn concentration (Fig. 4A, B). The Fe concentration in roots doubled in Zn-deficient roots, but there was no change in the concentrations of either Mn or Cu. In shoots, Zn deficiency caused no significant change in metal concentrations, except for the decrease of Zn (Fig. 4B). Similarly, metal concentrations of Fe-, Mn-, and Cu-deficient plants were analysed and it was confirmed that these plants were actually deficient in each metal (data not shown).

OsZIP4 *reversed the growth defect of Zn-uptake mutants of yeast*

The ability of OsZIP4 to restore the growth defect of CM-ZRT yeast strain (Δ*zrt1*, Δ*zrt2*) which possesses disrupted null mutations in the genes for the high-affinity Zn transporter was examined. The coding region of *OsZIP4* was subcloned in the yeast expression vector pYH23 under the

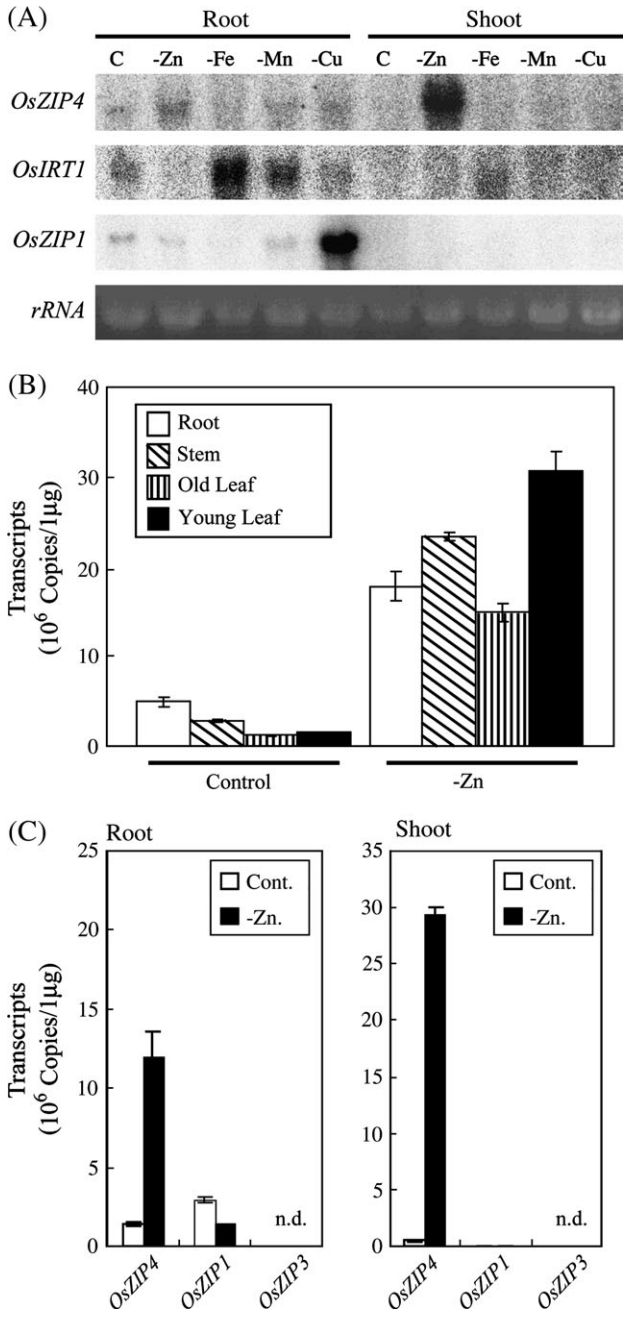

Fig. 2. Expression pattern of *OsZIP4*. (A) Northern blot analysis of the *OsZIP4*, *OsIRT1* and *OsZIP1* in the roots and leaves of rice plants grown under trace metal-deficient conditions. Total RNA (10 μg) extracted from plants grown in normal nutrient solution (control) (C) or under conditions of low zinc (–Zn), iron (–Fe), manganese (–Mn), or copper (–Cu) supply was blotted on each line. Ethidium bromide-stained rRNA is shown as a control for loading. (B) Quantification of *OsZIP4* transcripts in each plant part. Total RNA was isolated from the roots, stems, old leaves, or young leaves of control or Zn-deficient plants, and RT-PCR was performed to monitor the amplification of each gene. Values represent the mean ±SD of the number of copies of transcripts in 1 μg of total RNA of these tissues in three reactions. (C) Quantification of *OsZIP4*, *OsZIP1*, and *OsZIP3* transcripts under conditions of Zn deficiency. Total RNA was isolated from control or Zn-deficient plants, and RT-PCR was performed to monitor the amplification of each gene. The values represent the mean ±SD of the number of copies of transcripts in 1 μg of total RNA of the tissue in three reactions. n.d., not detected.

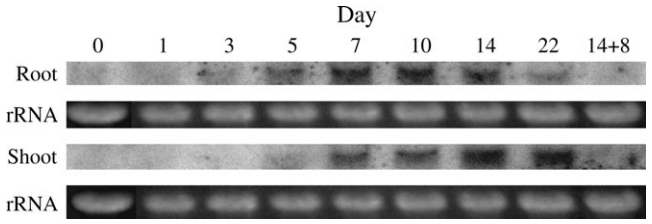

Fig. 3. Time-course of *OsZIP4* mRNA abundance patterns in response to Zn-deficient growth conditions. Rice plants were grown for 2 weeks in normal nutrient solution and then transferred to Zn-deficient culture; roots and shoots were harvested 0, 1, 3, 5, 7, 10, 14, and 22 d after the transfer. The other plants were grown for 2 weeks in normal nutrient solution, transferred to Zn-deficient culture for 14 d, and transferred a second time to Zn-sufficient culture; roots and shoots were harvested 8 d after transfer (Day 14+8). RNA samples were prepared from each tissue sample and were used to prepare RNA gel blots. The *OsZIP4* ORF was used to probe the RNA gel blot. Ethidium bromide-stained rRNA is shown as a control for loading.

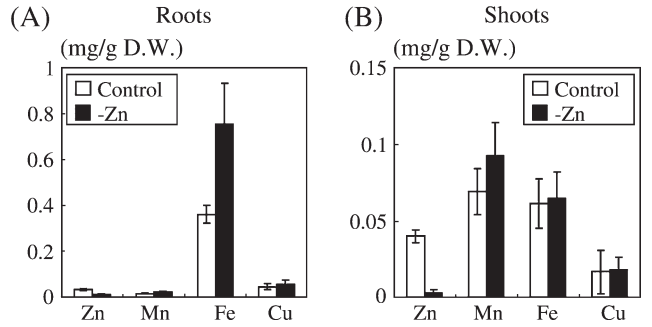

Fig. 4. Metal concentrations in roots and shoots of plants grown under conditions of Zn sufficiency or under conditions designed to induce deficiency. The concentrations of Zn, Mn, Fe, and Cu in the roots (A) and leaves (B) are expressed as mg g^{-1} dry weight and are given as the mean ±SD of three replicates of each treatment.

control of the *ADH* promoter. OsZIP3 was used as a positive control in the experiment. In a heterologous experiment using synthetic defined medium without Zn, both OsZIP4 and OsZIP3 restored the growth defect of CM-ZRT, whereas the pYH23 control did not (Fig. 5A, B).The ability of OsZIP4 to restore the growth defect of YH003 yeast strain (Δ*fet4*,Δ*fre1*,Δ*fet3*) was also studied to examine the uptake of Fe. OsIRT1 was used as a positive control in the experiment. In a heterologous experiment using synthetic defined medium without Fe, OsIRT1 restored the growth defect of YH003, whereas the pYH23 control and OsZIP4 did not (Fig. 5C). An attempt was made to complement the yeast mutant strains CM-SMF (Δ*smf1*) and FTRUNB1 (Δ*ctr1*) to examine the uptake of Mn and Cu, but no significant effects on growth were observed by the expression of OsZIP4 (data not shown).

OsZIP4 is a transporter localized to the plasma membrane

The OsZIP4 protein fused to the N terminus of synthetic green fluorescent protein (sGFP) was expressed transiently

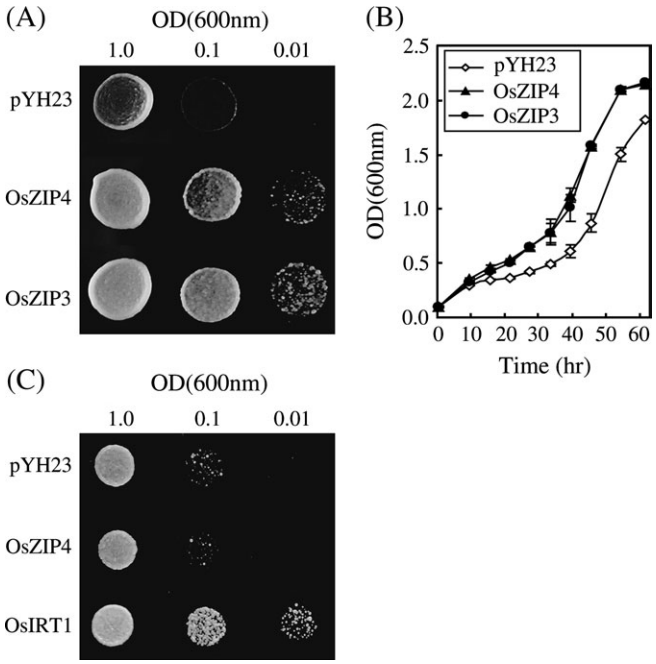

Fig. 5. Complementation of the CM-ZRT yeast mutant by OsZIP4. (A) Serial dilutions of CM-ZRT cells transformed with the empty vector (pYH23) or the vector expressing *OsZIP4* or *OsZIP3* were placed onto SD medium without Zn. (B) Quantitative growth analysis of CM-ZRT cells expressing *OsZIP4* or *OsZIP3*, or containing the empty pYH23 vector grown in SD medium. The data are the mean ±SE of three separate experiments, with a total of nine replicates. (C) Serial dilutions of YH003 cells transformed with the empty vector (pYH23) or the vector expressing *OsZIP4* or *OsIRT1* were placed onto SD medium without Fe.

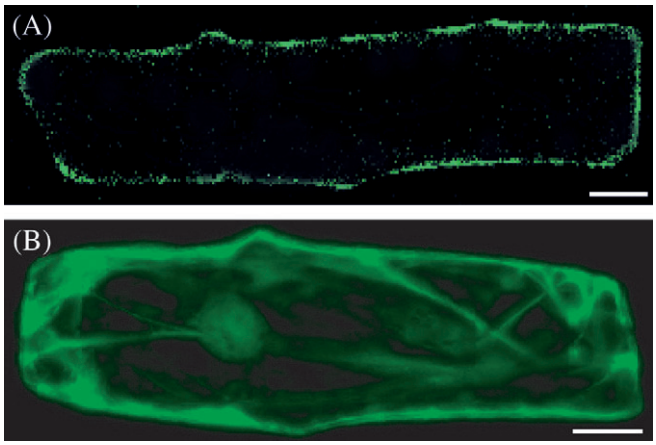

Fig. 6. Subcellular localization of transiently expressed OsZIP4-sGFP (A) or sGFP (B) fusion protein in onion epidermal cells observed using confocal laser scanning microscopy. Scale bars=20 μm.

under the control of the cauliflower mosaic virus (CaMV) 35S promoter in onion epidermis cells. The fluorescence of OsZIP4-sGFP was observed at the plasma membrane (Fig. 6A), while that of sGFP alone was localized to the cytoplasm and nucleus (Fig. 6B). This result indicates that OsZIP4 is a Zn transporter localized to the plasmamembrane.

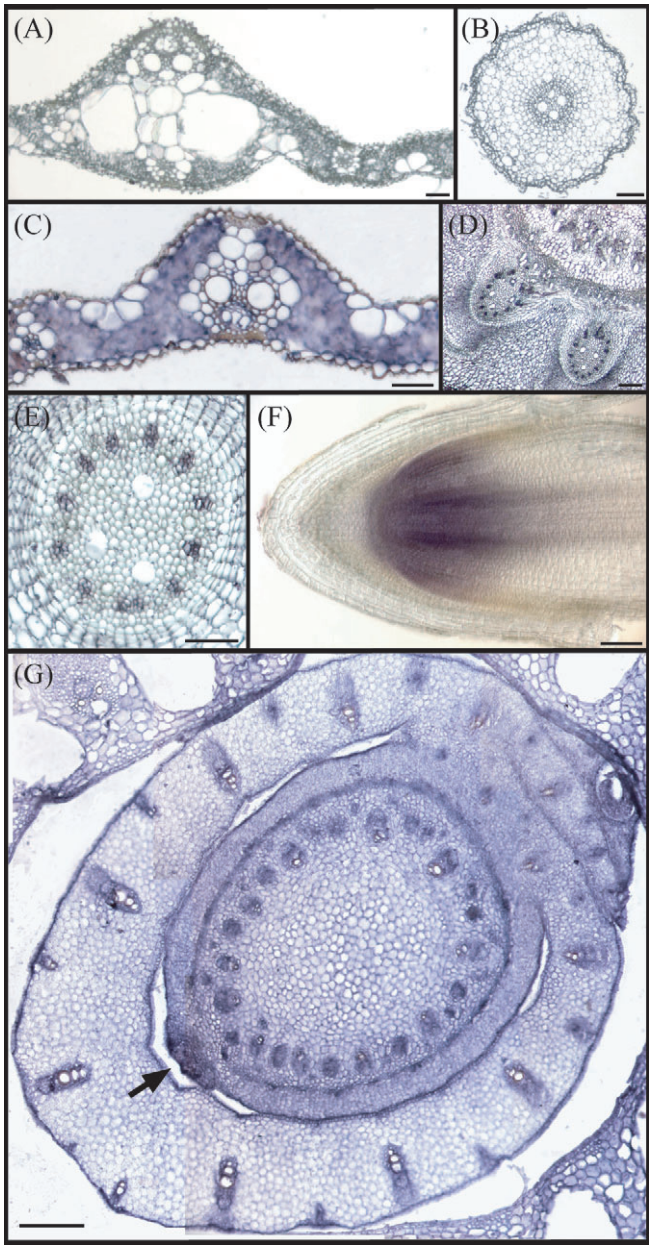

Fig. 7. *In situ* localization of *OsZIP4* transcripts. *In situ* hybridization experiments were performed on transverse sections of a Zn-deficient rice using an *OsZIP4* sense probe (A, B), or an *OsZIP4* antisense probe (C–G). Transverse sections of the leaf (A), root (B), small vascular bundle (C), stem (D), and around shoot apex (G). Arrow indicates meristem. (E), Enlargement of the stele in the root. (F), Longitudinal section of the root tip. Scale bars=800 μm for (G); 400 μm for (A), (C), and (F); 100 μm for (B), and (D); and 50 μm for (E).

OsZIP4 *is expressed in phloem cells of leaves and roots, and meristem*

In situ hybridization experiments were performed in order to localize the transcripts of *OsZIP4*. *OsZIP4* transcripts were present in all the vascular bundles and mesophyll cells of the leaves of Zn-deficient plants (Fig. 7C). In the stem, *OsZIP4* was expressed mainly in phloem cells (Fig. 7D).

In Zn-deficient roots, *OsZIP4* expression was detected mainly in the vascular bundles, especially in the phloem cells (Fig. 7E). Furthermore, strong expression was detected in the root apical meristem, and in the region of shoot meristem (Fig. 7F, G).

Discussion

These results suggest that OsZIP4 may function as a plasma membrane Zn-regulated Zn transporter responsible for the translocation of Zn. Recently, *OsZIP1* and *OsZIP3* were found to be rice Zn transporters induced by Zn deficiency (Ramesh *et al*., 2003). *OsZIP1* and *OsZIP3* expressed in the vascular bundles in shoots, and in the vascular bundles and epidermal cells in roots (Ramesh *et al*., 2003). In this experiment, however, expression of these genes was not induced by Zn deficiency (Fig. 2A, C). Moreover, the expression of *OsZIP1* was increased dramatically in Cu-deficient roots (Fig. 2A), suggesting that OsZIP1 might be related to Cu deficiency in addition to the Zn transport system. By contrast, the expression of *OsZIP4* was highly induced by Zn deficiency (Fig. 2A). The induction ratio of *OsZIP4* under Zn deficiency in a microarray analysis was the fourth highest in shoots, and the transcript level in Zn deficiency was much higher than that of *OsZIP1* and *OsZIP3*, especially in shoots (Fig. 2C). The expression of *OsZIP4* was localized in both the vascular bundles and mesophyll cells of the Zn-deficient leaves, and mainly in the vascular bundles in Zn-deficient roots (Fig. 7), suggesting that OsZIP4 may not be responsible for Zn uptake from soil, but for Zn transport within the rice plant. This expression pattern of *OsZIP4* is very similar to that of *OsYSL2*, which is a metal-nicotianamine complex transporter (Koike *et al*., 2004) and plays a role in the long-distance transport of metals in rice. It is noteworthy that the strong expression of *OsZIP4* was detected in the root apical meristem and the shoot meristem. The roles of Zn in DNA and RNA metabolism, cell division, and protein synthesis are well documented (Uchiyama *et al*., 2002). A specific high requirement for Zn in the meristem would have caused the high expression of OsZIP4 in the meristem.

The expression of *OsZIP4* in roots was increased in the early Zn-deficient stage, and the expression decreased gradually with prolonged Zn deficiency (Fig. 3). In this experiment, root and shoot growth continued for about 10 d after the start of the Zn-deficiency treatment (data not shown). Hence, it is likely that the expression of *OsZIP4* in Zn-deficient roots increased until root growth stopped. By contrast, the induction of *OsZIP4* in Zn-deficient shoots occurred after the induction of *OsZIP4* in roots, and the expression of *OsZIP4* in shoots was gradually increased by prolonged Zn deficiency. *OsZIP4* was localized in mesophyll cells in Zn-deficient shoots (Fig. 7C). Zn deficiency not only increased the expression of *OsZIP4* in young leaves, but also in old leaves. This suggests that OsZIP4 may be important for (i) the regulation of Zn supply in developing young leaves and (ii) in the long-distance transport of Zn from old to young leaves. It is possible that OsZIP4 is important in photosynthesis since carbonic anhydrase (CA) is a Zn-containing enzyme that catalyses the reversible conversion of CO_2 to HCO_3^-, and is thought to be involved in photosynthetic CO_2 accumulation. Hacisalihoglu *et al*. (2004) showed that Zn efficiency was correlated with enhanced expression and activity of CA in wheat.

In many cases, Zn-deficiency stress increases the Fe concentration in shoots, causing oxidative damage. For example, in tobacco (*Nicotiana tabaccum*) and barley (*Hordeum vulgare*), the Fe concentration in Zn-deficient plants is higher than that in control plants (Zhang *et al*., 1989; Kobayashi *et al*., 2003). However, the concentrations of Fe, Mn, and Cu in Zn-deficient rice shoots were not higher than those in the control plants (Fig. 4B). It was found that the heterologous expression of OsZIP4 in yeast had no effect on the growth of Fe- uptake mutant (Fig. 5C), Mn- or Cu-uptake mutants (data not shown). Therefore, OsZIP4 seems to be highly selective for Zn, but not for other metals. It is reported that OsZIP1 and OsZIP3 transport Zn but not Fe or Mn (Ramesh *et al*., 2003), and OsIRT1 transport Fe but not Cu (Bughio *et al*., 2002). These data suggest that ZIP family transporters in rice plants, including OsZIP4, might be transporters specific for one metal ion. If the OsZIP4 are not strictly regulated, one metal deficiency might easily cause an excess of another metal. Therefore, modifying *OsZIP4* expression in rice plants may be widely applicable to creating transgenic plants that tolerate low Zn supply, while exhibiting no toxicity toward other metals due to excessive transport.

Yellow stripe 1 (*YS1*), the gene for the transporter protein from maize (*Zea mays*), which not only transports the Fe phytosiderophore complex, but also the Zn phytosiderophore complex, has been isolated and characterized (Curie *et al*., 2001; Schaaf *et al*., 2004). OsYSLs homologous to ZmYS1 have been found in rice plants (Koike *et al*., 2004). Further study may elucidate the function of each of these metal transport proteins (OsZIPs and OsYSLs), and how the expression of these proteins is regulated developmentally and spatially. Analysis of these genes will enhance the understanding of Zn nutrition, including Zn uptake from the soil, long-distance Zn transport within the plant, and Zn homeostasis within the cell.

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