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Molecules and Cells

Zinc Deficiency-Inducible *OsZIP8* Encodes a Plasma Membrane-Localized Zinc Transporter in Rice

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Zinc is an essential micronutrient for several physiological and biochemical processes. To investigate its transport in rice, we characterized *OsZIP8*, a rice ZIP (Zrt, Irt-like Protein) gene that is strongly up-regulated in shoots and roots under Zn deficiency. *OsZIP8* could complement the growth defect of Zn-uptake yeast mutant. The OsZIP8-GFP fusion proteins were localized to the plasma membrane, suggesting that OsZIP8 is a plasma membrane zinc transporter in rice. Activation and overexpression of this gene disturbed the zinc distribution in rice plants, resulting in lower levels in shoots and mature seeds, but an increase in the roots. Field-grown transgenic plants were shorter than the WT. Under treatment with excess zinc, transgenics contained less zinc in their shoots but accumulated more in the roots. Altogether, these results demonstrate that OsZIP8 is a zinc transporter that functions in Zn uptake and distribution. Furthermore, zinc homeostasis is important to the proper growth and development of rice.

INTRODUCTION

Zinc is an essential element that serves as a structural or catalytic cofactor for many proteins, including RNA polymerase, superoxide dismutase, alcohol dehydrogenase, carbonic anhydrase, and the Zn finger family of transcription factors (Palmer and Guerinot, 2009). Thus, cells need mechanisms for maintaining zinc homeostasis when available supplies decrease (Eide, 2009). In plants, Zn deficiency reduces growth, tolerance to stress, and chlorophyll synthesis. Approximately 30% of the world's cultivated soil is Zn-deficient. Nevertheless, excessive Zn in soil that is caused by pollution and fertilizers is also a serious problem (Kawachi et al., 2009).

Plants deliver minerals absorbed from the root to the shoot, and also re-mobilize them from senescing leaves to young developing tissues via long-distance transport systems (Park et al., 2008). For the successful maintenance of zinc homeostasis, plants greatly rely on the coordinated regulation of multiplicity by Zn transporters, including uptake transporters such as members of the zinc-regulated transporter (ZRT), iron-regulated transporter (IRT)-like proteins (ZIP), natural resistance-associated macrophage protein (NRAMP), and plant-specific Yellow-Stripe1-Like (YSL) families, as well as efflux transporters, such as those in the cation diffusion facilitator (CDF) and P_{1B}-ATPase families (Colangelo and Guerinot, 2006).

Members of the ZIP transporters are involved in transporting Zn^{2+}, Fe^{2+}, Mn^{2+}, and Cd^{2+}, and they contribute to metal-ion homeostasis by shifting these metals into the cytoplasm (Colangelo and Guerinot, 2006; Guerinot, 2000). ZIP proteins generally act at the plasma membrane either to move metals or remobilize them from intracellular compartments into the cytoplasm (Colangelo and Guerinot, 2006). Most ZIP proteins are predicted to have eight transmembrane domains but some members may have as few as five; the majority share a similar predicted topology where the amino- and carboxy termini are located on the outside surface of the plasma membrane (Gaither and Eide, 2001).

Two ZIP proteins are present in yeast: ZRT1 (a high-affinity zinc transporter) and ZRT2 (a low-affinity zinc transporter) (Zhao and Eide, 1996a; 1996b). Among the 16 ZIP proteins present in *Arabidopsis* (Maser et al., 2001), AtIRT1 was first identified through functional complementation of a yeast mutant defective in iron uptake (Eide et al., 1996). AtIRT1, a major Fe transporter at the root surface, is manifested by the lethal chlorotic phenotypes of the *irt1* mutant (Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). AtIRT2, a close homolog, is located at the intracellular vesicle and, in cooperation with AtIRT1, maintains iron homeostasis in root epidermal cells (Vert et al., 2009). AtIRT3 is a zinc and iron transporter (Lin et al., 2009). Expression of several *ZIP* genes, including *AtZIP1* to *AtZIP5*, *AtZIP9* to *AtZIP12*, and *AtIRT3*, is up-regulated under zinc-limiting conditions (Krämer et al., 2007).

Homologues of the *ZIP* genes have been characterized from several species. The soybean *GmZIP1* encodes a symbiosis-specific zinc transporter (Moreau et al., 2002). When expressed in yeast cells, GmZIP1 is highly selective for zinc, whereas Cd is the only other tested metal that is able to inhibit zinc uptake. In *Medicago truncatula*, MtZIP1 is the Zn transporter, MtZIP3 is

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the Fe transporter, MtZIP4 and MtZIP7 are Mn transporters, and MtZIP5 and MtZIP6 are Fe and Zn dual transporters (López-Millán et al., 2004). TcZNT1 from the Zn/Cd hyperaccumulator *Thlaspi caerulescens* has been cloned through functional complementation in yeast and has been shown to mediate high-affinity Zn^{2+} uptake as well as low-affinity Cd^{2+} uptake. Overexpression of *TcZNT1* increases Zn influx in roots (Pence et al., 2000). Yeast that expresses two *ZIP* genes, either *TjZNT1* or *TjZNT2* from the Ni^{2+} hyperaccumulator *T. japonicum,* shows a marked increase in Ni^{2+} tolerance (Mizuno et al., 2005).

ZIP proteins have also been studied in rice (Bughio et al., 2002; Ishimaru et al., 2005; 2006; 2007; Lee and An, 2009; Ramesh et al., 2003; Yang et al., 2009). *OsIRT1* and *OsIRT2* are expressed predominantly in the roots under low-iron conditions. When expressed in yeast cells, OsIRT1 and OsIRT2 can reverse the growth defects of a yeast Fe-uptake mutant but not of Zn-, Mn-, or Cu-uptake mutants (Bughio et al., 2002; Ishimaru et al., 2006). Yeast expressing *OsIRT1* and *OsIRT2* are more sensitive to Cd, suggesting that it is absorbed by those proteins (Nakanishi et al., 2006). When *OsIRT1* is over-expressed via the maize *ubiquitin* promoter, Fe and Zn concentrations increase in the shoots, roots, and mature seeds, and those transgenics have greater sensitivity to elevated levels of Zn and Cd, thus demonstrating that OsIRT1 transports those metals (Lee and An, 2009). OsZIP1, OsZIP3, and OsZIP4 have already been characterized as functional Zn transporters (Ishimaru et al., 2005; Ramesh et al., 2003). Expression of *OsZIP4* is induced by Zn deficiency, especially in phloem cells and meristems (Ishimaru et al., 2005). Overexpression of *OsZIP4* under the control of the CAMV *35S* promoter results in redistribution of zinc within rice plants (Ishimaru et al., 2007). *OsZIP3* is broadly expressed under normal conditions and is also slightly induced in shoots after 96 h of zinc deprivation. *OsZIP1* is induced in both roots and shoots by zinc deficiency and *OsZIP2* is also up-regulated by zinc, albeit only in the roots (Ramesh et al., 2003). Finally, the expression of *OsZIP7a* is significantly induced in roots by Fe deficiency, while that of *OsZIP8* is induced in both roots and shoots by zinc deficiency (Yang et al., 2009). When expressed in yeast cells, OsZIP7a and OsZIP8 can complement an iron uptake-deficient mutant and a zinc uptake-deficient mutant, respectively (Yang et al., 2009).

This present study examined the functioning of OsZIP8 as a plasma membrane-localized zinc transporter. We used an activation-tagging mutant and *OsZIP8*-overexpressing transgenic plants to evaluate its physiological roles in metal homeostasis.

MATERIALS AND METHODS

Plant growth

Rice seeds were surface-sterilized for 15 min in 50% (v/v) commercial bleach containing 5.25% sodium hypochlorite (NaOCl). This was followed by three washings with sterilized distilled water. The seeds were then placed on an MS agar medium containing 30 μM $ZnSO_4$, 100 μM Fe (III)-EDTA, 0.1 μM $CuSO_4$, and 10 μM $MnSO_4$ as micronutrients. For deficiency tests, seeds were germinated and grown on MS media lacking $ZnSO_4$ (Zn-deficient), Fe (III)-EDTA (Fe-deficient), $CuSO_4$ (Cu-deficient), or $MnSO_4$ (Mn-deficient). To analyze *OsZIP8* under different zinc concentrations, we grew seedlings for 7 days on MS media containing 0.0, 0.3, 3.0, 30.0, or 300.0 μM $ZnSO_4$. For time-course analyses of *OsZIP8* expression upon recovery, plants were grown under a zinc deficiency for 7 days, then transferred to a zinc-sufficient MS medium (30 μM $ZnSO_4$). Shoots and roots were harvested at 0, 0.5, 3, 4.5, 6, 12, 18, 24, 48, and 72 h after transfer. In 2007 and 2008, transgenic plants were installed and grown to maturity on paddy fields in Korea at Pohang University of Science and Technology (36°N).

RNA preparation and mRNA quantification

Shoots or roots were collected and frozen in liquid nitrogen. Total RNA was isolated with RNAiso Plus (Takara, Japan). First-strand cDNA was synthesized from 2 μg of total RNA in a 25-μl reaction mixture with M-MLV reverse transcriptase (Promega, USA). Synthesized cDNAs were used for RT-PCR and real-time PCR. Quantitative PCR analysis was performed on a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia), using a SYBR premix Ex.Taq kit (Takara Bio).The levels of *actin1* mRNA served to normalize the expression ratio for each gene. Changes in expression were calculated via the $\Delta\Delta_{Ct}$ method. Gene-specific primers included *OsZIP8*-Fw, 5′-CTCCAGCTCGGCATCAACCT-3′; *OsZIP8*-Rv, 5′-TAGGCAGGTGCACCCTCGAT-3′; *OsAct1*-Fw, 5′-GTATCCATGAGACTACATACAACT-3′; and *OsAct1*-Rv, 5′-TACTCAGCCTTGGCAATCCACA-3′.

Cellular localization of OsZIP8

A cDNA fragment spanning the entire ORF of *OsZIP8* was amplified with two primers -Z8-pgf (5′-AAAGATCTGACGAACACCACCGCCACCG-3′) and Z8-pgr (5′-AAACTAGTGGCCCATTTGGCGAGCATGG-3′) - that contained *Bgl*II and *Spe*I (underlined sequences) sites for cloning. After digestion with both sites, the fragment was inserted into the *Bgl*II/*Spe*I-linearized pCAMBIA1302 vector (CAMBIA). The resulting construct, pGA3560, contained the fusion of OsZIP8-GFP under a CaMV *35S* promoter. After confirmation by sequencing, the construct was transferred to *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method. Transgenic rice plants were generated via *Agrobacterium*-mediated co-cultivation (Lee et al., 1999). These plants were grown on a sterile solid MS and collected at 5 days after germination. Roots from plants transformed with the GFP fusion construct were examined by laser-scanning confocal microscopy (LSM 510 META; Carl Zeiss, Germany). GFP signals were detected by excitation with the 488 nm line of the argon laser and a capturing emission at 522 nm.

Isolation of T-DNA tagging mutant

Putative *OsZIP8* T-DNA tagging mutants were isolated from our rice flanking sequence-tag database (http:://www.postech.ac.kr/life/pfg). The T2 progeny of the primary mutants were grown to maturity for seed amplification. Genotypes were determined by PCR, with a set of three primers for *OsZIP8-D1* (Line 4A03374): two specific primers (F1, 5′-TGATCCCACCATTCGGTAAT-3′ and R1, 5′-GGGTACGCGTGTGAGAATTT-3′) and one T-DNA-specific primer (LB, 5′-CTAGAGTCGAGAATTCAGTACA-3′). Afterward, transcript levels for *OsZIP8* were determined by quantitative PCR analysis, using cDNA prepared from the shoots and roots of 10-day-old seedlings grown on an MS medium.

Generation of *OsZIP8*-overexpressing transgenic plants

The full-length cDNA sequence of *OsZIP8* was amplified by primers 5′-AAAAGCTTGATAGCAGCAGGAGCAGAAGA-3′ (OxF) and 5′-AAGGTACCTAGTCCCTGAGTGACGCTTGT-3′ (OxR), which contained *Sac*I and *Kpn*I sites (underlined sequences) for cloning, respectively. The PCR product was cloned into

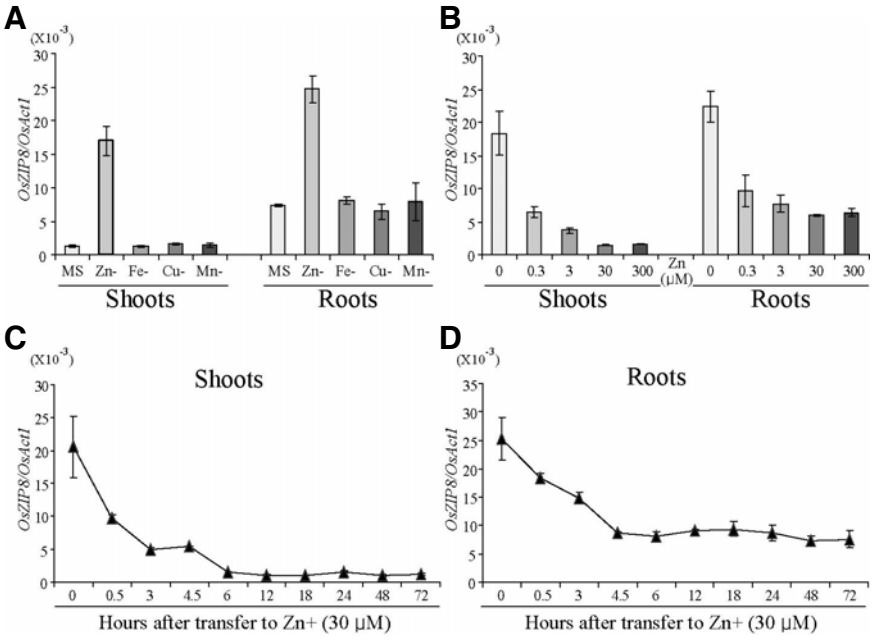

Fig. 1. Expression analysis of *OsZIP8* under micronutrient deficiencies. (A) Standard MS medium (MS) contained 100 μM Fe (III)-EDTA, 30 μM $ZnSO_4$, 0.1 μM $CuSO_4$, and 10 μM $MnSO_4$ as micronutrients. For deficiency trials, seeds were germinated and grown for 7 days in MS media lacking $ZnSO_4$ (Zn-), Fe (III)-EDTA (Fe-), $CuSO_4$ (Cu-), or $MnSO_4$ (Mn-). (B) Real-time PCR analysis of *OsZIP8* under different Zn concentrations. Seedlings were grown for 7 days on MS media containing 0.0, 0.3, 3.0, 30.0, or 300.0 μM Zn SO_4. (C) Time-course analyses of *OsZIP8* expression upon recovery in shoots and roots. Plants were grown under zinc deficiency for 7 days, then transferred to zinc-sufficient MS medium (30 μM $ZnSO_4$). Tissues were harvested 0, 0.5, 3, 4.5, 6, 12, 18, 24, 48, and 72 h after transfer. Each value is average of 3 independent experiments. Relative values are between transcript levels of *OsZIP8* and *OsAct1*. Vertical bars indicate standard deviation.

those sites between the maize *ubiquitin* promoter and the *T7* terminator of binary vector pGA1611 (Lee et al., 1999), thereby producing pGA2872. After the seedlings were transplanted to soil, we extracted and prepared their cDNA to assess *OsZIP8* transcript levels via northern blot analysis.

Determination of metal contents in plant tissues

Samples were dried for 2 days at 70°C before being weighed. Afterward, they were digested in 1 ml of 11 N HNO_3 for 3 days. Following dilution, their metal contents were determined by AAS (SpectrAA-800; Varian, USA).

RESULTS

Expression of *OsZIP8* is up-regulated by a zinc deficiency

OsZIP8 (LOC_Os07g12890) has now been isolated as a zinc transporter that reverses the growth defect of Zn-uptake mutants of yeast (Yang et al., 2009). This gene, induced in both roots and shoots by zinc deficiency, can complement that zinc-uptake yeast mutant (Yang et al., 2009). To confirm these observations and further investigate its functioning, we examined the expression pattern of *OsZIP8* under various metal-deficient statuses (Fig. 1A; Supplementary Fig. S1A). Quantitative real-time PCR analyses showed that its transcript level was increased markedly in shoots and roots that lacked Zn, whereas Fe-, Cu-, and Mn-deficiencies did not influence expression (Fig. 1A). We also measured transcripts under different zinc concentrations (Fig. 1B; Supplementary Fig. S1B), comparing performance with a control MS medium containing 30 μM Zn. Although reducing the zinc level increased expression in shoots and roots, an excessive amount (300 μM) had no effect. In response to a zinc re-supply, *OsZIP8* mRNA in the shoots declined continuously for 6 h to a very low level (Fig. 1C); this decrease was more gradual in the roots, and levels were not further reduced in that tissue after 4.5 h (Fig. 1C; Supplementary Fig. S1C).

Functional complementation analyses in yeast mutants were performed to test the metal transport activity of OsZIP8 (Supplementary Fig. S2). The *OsZIP8* cDNA was expressed *Saccharomyces cerevisiae zrt1 zrt2* double mutant (ZHY3), which possesses disrupted null mutations in the genes for the high-affinity Zn transporter, *ZRT1* and *ZRT2*, and thus the mutant is unable to grow on low-Zn media (Zhao and Eide, 1996a). *AtIRT1* was used as a positive control. Both *OsZIP8* and *AtIRT1* restored the growth defect of ZHY3, on synthetic defined medium lacking zinc, whereas the pDR195 control did not (Supplementary Fig. S2). This strongly indicates that OsZIP8 is a zinc transporter.

OsZIP8 is a plasma membrane-localized transporter

For further insight into the functioning of OsZIP8, we analyzed its subcellular distribution by expressing a fusion protein with GFP. OsZIP8 protein is predicted to be localized to the plasma membrane (http://aramemnon.botanik.uni-koeln.de).Transgenic plants were generated by *Agrobacterium*-mediated co-cultivation, using binary vector pGA3560 that encodes OsZIP8-GFP under the control of the cauliflower mosaic virus 35S promoter. As a control, we also generated transgenic plants expressing GFP alone under the control of that promoter. In plants stably transformed with these constructs, green fluorescence was observed in the seedling roots (Fig. 2). For plants transformed with the OsZIP8-GFP fusion construct, green fluorescence also was apparent in the plasma membrane, which was consistent with our prediction (Fig. 2). In contrast, fluorescence of GFP alone was observed in both cytoplasm and nucleus. Together with the yeast complementation data, this localization implied that OsZIP8 is a Zn transporter in the plasma membrane.

Production of activation tagging-mutant and *OsZIP8*-overexpressing plants

To evaluate the functions of OsZIP8 *in planta*, we isolated a putative activation tagging mutant from our rice flanking sequence-tag database (An et al., 2003; Jeon et al., 2000; Jeong et al., 2006). In Line 4A03374 (*OsZIP8-D1*), T-DNA was inserted at 1248 bp upstream from an ORF of *OsZIP8* (Fig. 3A). Homozygous plants were selected by PCR from T2 progeny, using gene-specific and T-DNA primers. Subsequent quantitative real-time PCR analysis showed that *OsZIP8* transcript

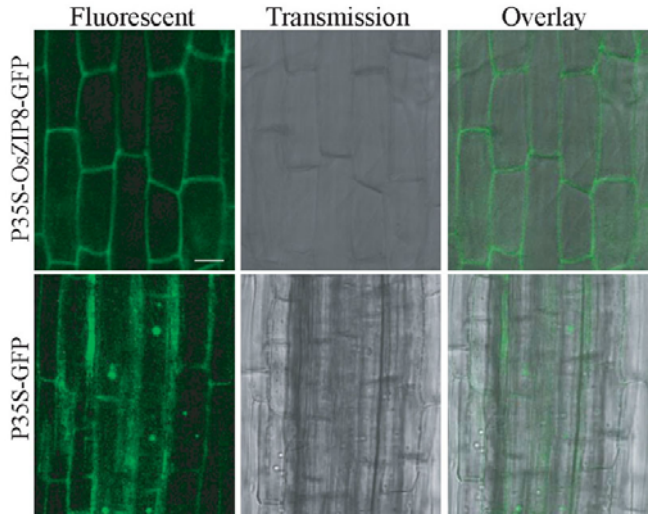

Fig. 2. Subcellular localization of OsZIP8-GFP in rice roots. Full-length *OsZIP8* cDNA was fused to *GFP* in the same reading frame and resulting construct was used for rice transformation. Repre-sentative confocal microscopic images show roots expressing OsZIP8-GFP fusion protein or GFP alone. Roots expressing *GFP* under control of CaMV *35S* promoter were used as control (P_{35S}-GFP). Fluorescence (left) and bright-field (middle) images are overlaid at right. Bars = 10 μM.

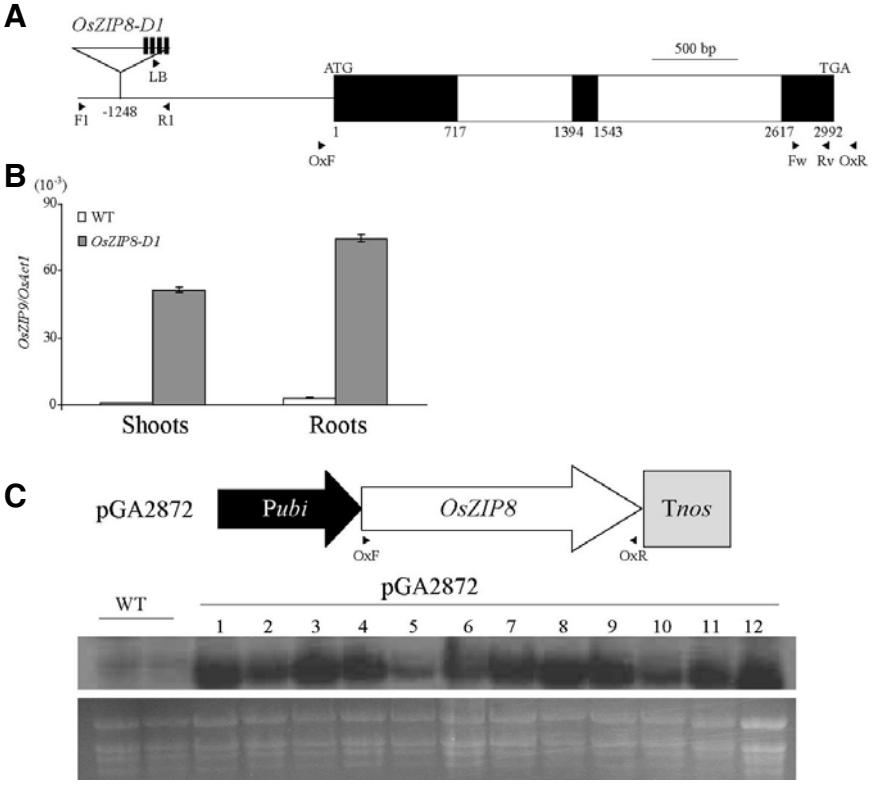

Fig. 3. Isolation of T-DNA activation-tagging mutant and generation of *OsZIP8*-overexpressing transgenic plants. (A) Schematic dia-gram of *OsZIP8* and T-DNA insertion site. Dark boxes indicate 3 exons; connecting white boxes are introns. In Line 4A03374, T-DNA was inserted into 5′ UTR region, 1248 bp away from ATG start codon. Horizontal arrows indicate primers (F1, R1, and LB) for genotyping T2 progeny. Arrows show gene-specific primers for analyzing expression level (Fw and Rv) and generating overexpressing transgenic plants (OxF and OxR). (B) Real-time analysis of *OsZIP8-D1*. *OsZIP8*-specific primers (Fw and Rv) were used with total RNA from shoot and roots of *OsZIP8-D1* and segregated WT seedlings. Transcript levels were represented by ratio between mRNA levels of *OsZIP8* and rice *actin1*. Vertical bars indicate standard deviation. (C) Generation of *OsZIP8*-overexpressing transgenic plants. Upper: Schematic diagram of pGA2872 expressing *OsZIP8*-sense transcript under control of maize *ubiquitin* promoter (P*ubi*). Lower: RNA gel blot analysis of *OsZIP8*-overxpressing transgenic plants. *OsZIP8* full-length cDNA was used as probe. Two WT and 12 independent transgenic plants were examined. rRNA levels are shown to indicate RNA amounts used for analysis.

levels were 55- and 25-fold higher in the seedling shoots and roots, respectively, compared with the WT (Fig. 3B).

To assess the functioning of *OsZIP8* further, we generated 12 transgenic plants that ectopically expressed full-length *OsZIP8* cDNA under the control of the maize *ubiquitin* promoter (Fig. 3C). Transcript levels were determined by northern blot analysis with RNA samples from leaves of independent transgenics (Fig. 3C). We selected plants #1 (Z8OX-1) and #3 (Z8OX-3) for additional analysis.

Increased expression of *OsZIP8* causes a reduction of Zn in shoots and seeds

Because *OsZIP8* is regulated by micronutrient status, we investigated this gene's role under certain deficiencies. When plants were grown on a standard MS medium the transgenics were shorter than the WT (Figs. 4A and 4B). Respective heights for *OsZIP8-D1*, Z8OX-1, and Z8OX-3 were reduced to 89%, 93%, and 91%, relative to the wild type (Fig. 4B). To assess whether elevated levels of *OsZIP8* expression affect metal distribution, we measured those concentrations in shoots and roots at the

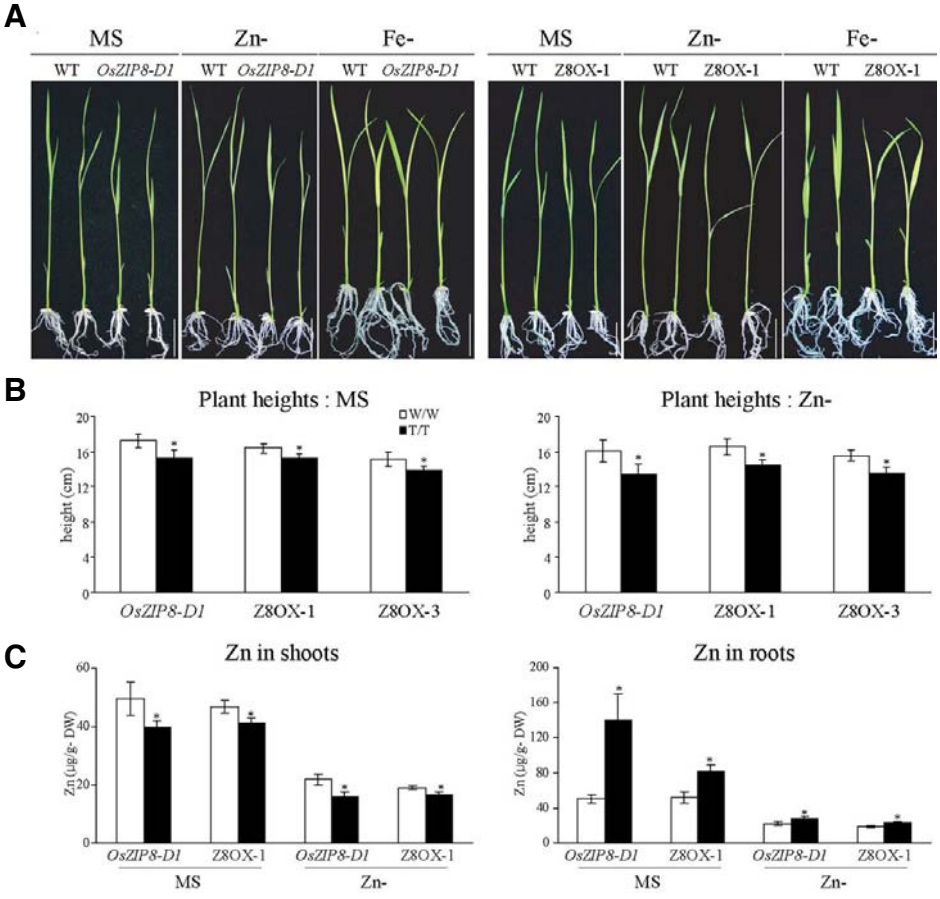

Fig. 4. Analysis of seedling phenotypes for *OsZIP8-D1*- and *OsZIP8*-overexpressing transgenics. (A) Seeds of *OsZIP8-D1* and Z8OX-1 were germinated and plants grown on standard MS media containing 100 μM Fe and 30 μM Zn (MS), or MS media without Zn (Zn-) or Fe (Fe-). Pictures were taken 8 days after germination. Bars = 5 cm. (B) Quantification of WT and *OsZIP8* mutant plants (n = 8 each) grown on control MS or Zn- media for 8 days following germination. (C) Zinc concentrations in shoots and roots from WT, *OsZIP8-D1*, and Z8OX-1 plants grown on control MS or Zn- media. Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests. *, $P < 0.05$.

seedling stage (Fig. 4C; Supplementary Figs. S3A-S3C). Compared with the WT, zinc levels in *OsZIP8-D1* and Z8OX-1 were reduced to 80% and 88% in the shoots, but increased to 278% and 160% in the roots, respectively (Fig. 4C). However, amounts of Fe, Cu, and Mn from *OsZIP8-D1* and Z8OX-1 were similar to those from the wild-type segregants (Supplementary Figs. S3A-S3C). These observations indicated that reduced heights may result from the redistribution of zinc in transgenic plants.

We also investigated whether transgenic plants are sensitive to a reduced supply of various micronutrients. Under deficient conditions, the transgenics were shorter than the WT as when grown with sufficient amounts (Figs. 4A and 4B; Supplementary Figs. S3D-S3F). When zinc was lacking, its content measured in *OsZIP8-D1* and Z8OX-1 was reduced to 73% and 88% in the shoots, but increased to 128% and 121% in the roots, respectively, compared with the WT (Fig. 4C). However, the amounts of Fe, Cu, and Mn were unchanged under that Zn deficiency (Supplementary Figs. S4A-S4C). Iron concentrations in the shoots and roots of *OsZIP8-D1* and Z8OX-1were similar to those of the WT under Fe-deficient conditions (Supplementary Fig. S4D), whereas the levels of Cu or Mn in those tissues were unchanged when either were absent (Supplementary Figs. S4E and S4F). We also measured Zn concentrations in shoots and roots under Fe-, Cu-, or Mn deficiencies (Supplementary Fig. S5). Consistent with our observations from the control and zinc-deficient tests, Zn levels were lower in the shoots but higher in the roots. All of these data demonstrated that elevated expression of *OsZIP8* influences the level of only zinc, but not Fe, Cu, or Mn.

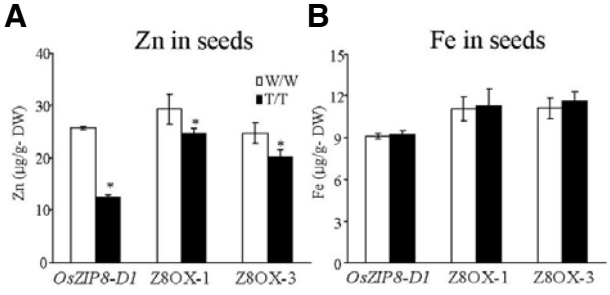

Fig. 5. Amounts of Zn (A) and Fe (B) in mature seeds from WT, *OsZIP8-D1*, Z8OX-1, and Z8OX-3 plants. Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests. *, $P < 0.05$.

Because changing the expression of *OsZIP8* altered the zinc distribution at the seedling stage, we also measured micronutrient levels in a major sink tissue, the mature seeds. Seeds from *OsZIP8-D1*, Z8OX-1, and Z8OX-3 had 48%, 84%, and 81% as much zinc as what was measured in WT seeds (Fig. 5A), whereas Fe concentrations were similar among genotypes (Fig. 5B). Levels of Cu and Mn remained unchanged in all mature seeds (Supplementary Figs. S6A and S6B).

Enhanced levels of *OsZIP8* cause zinc to accumulate under excess-Zn conditions

Previously, OsZIP8 was isolated as a functional zinc transporter in rice, as shown by complementation of Zn-uptake mutants of

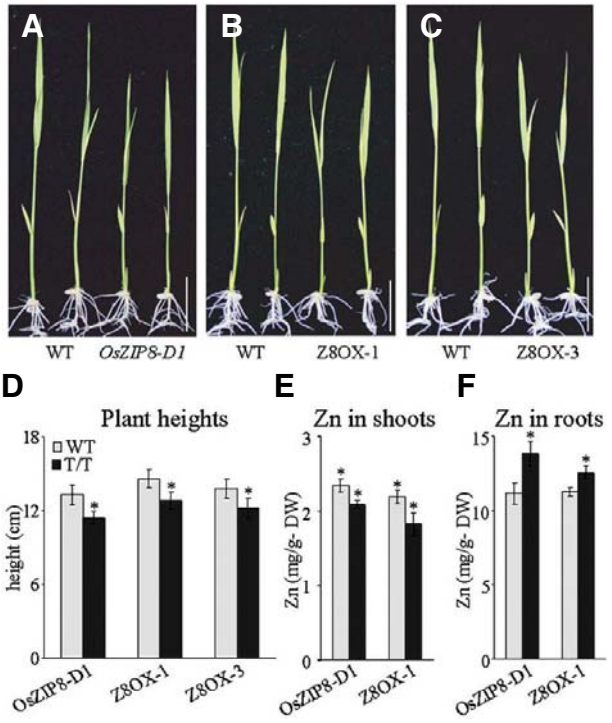

Fig. 6. Excess-Zn treatment. Seeds were germinated and plants grown for 10 days on ½MS medium supplemented with 5 mM $ZnCl_2$. Comparisons among WT and *OsZIP8-D1* (A), Z8OX-1 (B), or Z8OX-3 (C) plants. Plant heights (D) and Zn concentrations in shoots (E) and roots (F) of WT, *OsZIP8-D1*, and Z8OX-1 (n = 4). Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests. *, P < 0.05. Bars = 5 cm.

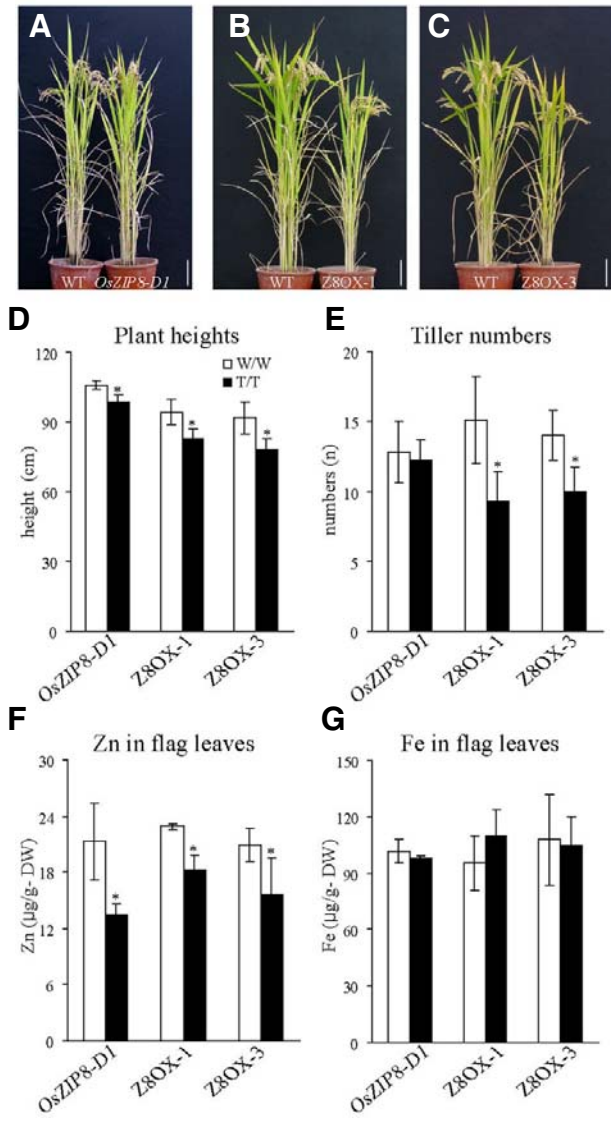

Fig. 7. Phenotypes of *OsZIP8*- activation or -overexpressing transgenic plants grown on paddy fields. Representative photographs were taken after flowering. WT was compared with *OsZIP8-D1* (A), Z8OX-1 (B), or Z8OX-3 (C). Bars = 10 cm. Plant height (D) and number of tillers (E) were recorded at flowering stage (n = 8). Zn (F) and Fe (G) contents were measured from 4 flag leaves at flowering stage. Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests. *, P < 0.05.

yeast (Yang et al., 2009) and we confirmed the Zn transport activity in yeast cells. Therefore, we tested whether it might function under treatment with excess zinc. Our earlier research had indicated that rice plants do not respond to the low concentrations (< 1 mM Zn) usually applied to *Arabidopsis* (Lee et al., 2007). Therefore, we selected 5 mM zinc as the physiological concentration for toxicity tests here. Under those conditions, transgenic plants had smaller shoots than those grown in a control MS medium (Figs. 6A-6D). Moreover, zinc concentrations in those transgenic shoots were lower than in the WT (Fig. 6E). However, the transgenic roots accumulated more Zn (Fig. 6F).

Increased expression of *OsZIP8* is associated with shorter plants and reduced zinc levels in flag leaves

To evaluate the roles of *OsZIP8* in plant development, we grew activation-mutant and over-expression transgenics on paddy fields. At the flowering stage, transgenic plants were significantly shorter than the WT (Figs. 7A-7D). The *OsZIP8*-overexpressing plants also had significantly fewer tillers than the WT, but no difference was seen when *OsZIP8-D1* plants were compared with the control (Fig. 7E). Seed yields from transgenic plants also were reduced (Table 1).

We measured Zn, Fe, Cu, and Mn concentrations in flag leaves at the flowering stage to evaluate whether elevated expression of *OsZIP8* affects metal accumulation. Transgenic plants contained less Zn (Fig. 7F), but had the same amounts of Fe, Cu, and Mn as the control (Fig. 7G; Supplementary Figs. S6C and S6D). These results suggested that zinc is important for plant growth.

DISCUSSION

In this study, we functionally characterized one of the rice ZIP transporter proteins, OsZIP8. Expression of *OsZIP8* was induced under Zn deficiency, but not when Fe, Cu, or Mn were lacking in the media. OsZIP8 was localized to the plasma membrane, as shown by the expression of the OsZIP8-GFP fusion protein in roots. These findings demonstrated that OsZIP8 is a plasma membrane-localized Zn transporter in rice.

Because plants take up micronutrients from the soil, such

Table 1. Grain yield by *OsZIP8* mutants grown on a paddy field. For yield, total spikelets and mature seeds were counted from 4 plants.

Line	Genotype	Grain yield		
		Total spikelets	Mature seeds	Fertility (%)
OsZIP8-D1	WT	1575 ± 109	943 ± 88	60 ± 1
	OsZIP8-D1	1291 ± 64	742 ± 96	60 ± 2
Z8OX-1	WT	1982 ± 148	1182 ± 87	60 ± 2
	T/T	1317 ± 194	832 ± 106	63 ± 2
Z8OX-3	WT	2061 ± 334	1196 ± 202	58 ± 5
	T/T	1646 ± 140	970 ± 80	59 ± 1

transport and regulation must operate precisely and specifically. Substrate specificity of metal transporters is very important for maintaining adequate metal homeostasis and plant growth. ZIP proteins generally contribute to cytosolic Zn import. Here, expression of *OsZIP8* was regulated by the status of Zn only, not by the levels of Fe, Mn, or Cu. In yeast, ZAP1 is the central player in plant responses to Zn deficiency (Zhao et al., 1998). This metal-responsive regulatory protein controls the expression of many genes by binding to Zn-responsive elements (ZRE) in the promoters of its target genes. In rice, two transcription factors -- IDEF1 and IDEF2 -- specifically bind the Fe deficiency-responsive *cis*-acting elements IDE1 and IDE2, respectively (Kobayashi et al., 2007; Ogo et al., 2008), indicating that *cis*-elements/transcription factor interactions are functionally associated with Fe homeostasis. Because Zn deficiency-responsive networks are largely unknown in rice, it will be interesting to identify the consensus Zn-responsive elements from several Zn deficiency-inducible genes and, thereafter, find the novel transcription factors that regulate Zn homeostasis.

Functional analysis using a Zn-uptake yeast mutant has indicated that OsZIP8 acts as a specific Zn importer in rice plants. In our study, activation-tagging and over-expression transgenics differed in their patterns of accumulation for Zn, but not for Fe, Mn, or Cu. When excess Zn was applied, transgenics accumulated more in their roots, indicating that OsZIP8 is a Zn importer.

An adequate Zn supply is important for plant growth (Cakmak, 2008; Palmgren et al., 2008). Zn deficiency is the most widespread micronutrient disorder that causes multiple symptoms in rice (Wissuwa et al., 2006). Under Zn deficiency, plants remain stunted and show substantial reduction in yields (Wissuwa et al., 2006). We observed that Zn concentrations in roots were higher in the transgenics than in WT irrespective of external Zn status, whereas Zn concentrations in shoots and seeds were lower in the transgenics compared with WT. This led to shorter plants and reduced grain yields, indicating that enhanced expression of *OsZIP8* could interrupt Zn homeostasis in rice, thus negatively influencing growth and productivity. Overexpression of *OsZIP8* raised the Zn uptake from the soil, resulting in more accumulations in root cells. However, elevation of *OsZIP8* expression might cause re-absorption of Zn to root cells that interfere with Zn movement from root cells to upper parts. A similar result was observed by overexpression of *OsZIP4* (Ishimaru et al., 2007). We postulated that other genes are needed to enhance Zn transportation to shoots and seeds.

Although phenotypes between activation tagging mutant and overexpressors were similar to each other, the relative Zn concentration in roots was higher in *OsZIP8-D1* compared with the overexpressing-transgenic plants. The *OsZIP8* OX plants showed reduction in tiller numbers, but not the *OsZIP8-D1*plants. These differences may be due to the promoters. Whereas the *ubiquitin* promoter caused ectopic overexpression, original expression patterns were maintained in *OsZIP8-D1*.

Analysis of *35S-IRT1* transgenics has revealed that, when iron is limiting, the IRT1 protein is present only in the roots even though *IRT1* mRNA is over-expressed constitutively throughout the plant (Connolly et al., 2002). This indicates that IRT1 is regulated post-transcriptionally. Furthermore, *35S-IRT1*transgenic plants show no visible morphological changes in phenotype when grown in soil or under standard conditions (Connolly et al., 2002). In our activation-tagging and over-expression transgenics, transcripts were accumulated even under metal-sufficient conditions, thereby altering plant architecture. OsZIP8-GFP fusion proteins, driven by the CaMV *35S* promoter, also were detectable in the roots when metal supplies were adequate. This demonstrated that post-transcriptional regulation does not play a significant role in our transgenic rice plants.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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