The ZIP Transporter Family Member OsZIP9 Contributes To Root Zinc Uptake in Rice under Zinc-Limited Conditions

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Zinc (Zn) is an important essential micronutrient for plants and humans; however, the exact transporter responsible for root zinc uptake from soil has not been identified. Here, we found that OsZIP9, a member of the ZRT–IRT-related protein, is involved in Zn uptake in rice (Oryza sativa) under Zn-limited conditions. OsZIP9 was mainly localized to the plasma membrane and showed transport activity for Zn in yeast (Saccharomyces cerevisiae). Expression pattern analysis showed that OsZIP9 was mainly expressed in the roots throughout all growth stages and its expression was upregulated by Zn-deficiency. Furthermore, OsZIP9 was expressed in the exodermis and endodermis of root mature regions. For plants grown in a hydroponic solution with low Zn concentration, knockout of OsZIP9 significantly reduced plant growth, which was accompanied by decreased Zn concentrations in both the root and shoot. However, plant growth and Zn accumulation did not differ between knockout lines and wild-type rice under Zn-sufficient conditions. When grown in soil, Zn concentrations in the shoots and grains of knockout lines were decreased to half of wild-type rice, whereas the concentrations of other mineral nutrients were not altered. A short-term kinetic experiment with stable isotope $^{67}$Zn showed that $^{67}$Zn uptake in knockout lines was much lower than that in wild-type rice. Combined, these results indicate that OsZIP9 localized at the root exodermis and endodermis functions as an influx transporter of Zn and contributes to Zn uptake under Zn-limited conditions in rice.

Zinc (Zn) is an essential micronutrient for plant growth and development (Broadley et al., 2012). Zn plays structural and catalytic roles in large number of proteins. However, Zn deficiency is the most widely occurring micronutrient deficiency in crops worldwide, which has been a limiting factor of crop production on millions of hectares of arable land, especially in alkaline soil (Barker and Pilbeam, 2015). Furthermore, this deficiency also results in Zn deficiency in humans because Zn in edible parts of crops is our primary source of Zn intake. Therefore, it is important to understand the molecular mechanisms of Zn transport and regulation in crops for enhancing crop tolerance to Zn deficiency and preserving Zn accumulation in edible plant parts.

The predominant form of Zn in soil solution is the divalent cation (Zn$^{2+}$) in most soils, although it may be present as Zn(OH)$^+$ at high pH. The transport of Zn from soil to different organs and tissues have been proposed to be mediated by different transporters such as members of the Zn-regulated transporter, the iron-regulated transporter-like proteins (ZRT–IRT-related protein [ZIP] family), the yellow-stripe1–like (YSL) family, the heavy metal ATPases (HMAs), and the cation diffusion facilitator (CDF; Grotz et al., 1998; Guerinot, 2000; Sinclair and Krämer, 2012). Among them, several members of the ZIP family have been implicated in uptake and transport of Zn. ZIP transporters were first identified in yeast (Saccharomyces cerevisiae) and Arabidopsis (Arabidopsis thaliana; IRT; Eide et al., 1996; Zhao and Eide, 1996). Homologous ZIP proteins are present in many plant species. For example, there are 15 members in Arabidopsis (Milner et al., 2013), 17 in rice (Oryza sativa; Chen et al., 2008), 14 in wheat (Triticum aestivum; Evens et al., 2017), 12 in barley (Hordeum vulgare; Tiong et al., 2014), and 23 in common bean (Phaseolus vulgaris; Astudillo et al., 2013). Most ZIP proteins have 309 to 470 amino acids and are predicted to have eight transmembrane domains and a similar membrane topology in which the amino- and carboxy-terminal ends of the proteins are located on the outside surface of the plasma membrane (Guerinot, 2000). Based on transport assays mainly in yeast mutants, ZIP transporters show broad substrate transport activity; in addition to transporting Zn and iron (Fe), they also transport manganese (Mn),
cadium (Cd), and cobalt (Co), although some members only transport Zn (Korshunova et al., 1999; Waters and Sankaran, 2011; Milner et al., 2013). The ZIP genes also show different expression patterns; some are only expressed in the roots (Bughio et al., 2002; Ishimaru et al., 2006), whereas others are expressed in different tissues (Ishimaru et al., 2005; Yang et al., 2009; Lee et al., 2010a, 2010b; Kavitha et al., 2015; Sasaki et al., 2015). The response of ZIP genes to different Zn concentrations differs between members, but most ZIP genes reported are upregulated by Zn-deficiency (Ishimaru et al., 2005; Yang et al., 2009; Lee et al., 2010a, 2010b; Kavitha et al., 2015). Furthermore, two basic-region Leu-zipper (bZIP) transcription factors, bZIP19 and bZIP23, are reported to be involved in regulation of ZIP expression (Assunção et al., 2010; Inaba et al., 2015).

Rice is a staple food for half of the global population, and therefore it provides an important source of dietary Zn intake in rice-eating populations. However, the transport system of Zn in rice has not been well understood. Several ZIP members have been functionally characterized in terms of transport activity, expression patterns, and ectopic expression analysis. OsZIP1, OsZIP3, OsZIP4, OsZIP5, OsZIP7a, and OsZIP8 showed influx transport activity for Zn in yeast (Ramesh et al., 2003, Ishimaru et al., 2005; Yang et al., 2009; Lee et al., 2010a, 2010b; Tan et al., 2019). However, OsZIP2 in yeast and OsZIP6 in Xenopus oocytes did not show transport activity for Zn (Ramesh et al., 2003; Kavitha et al., 2015). Rice ZIP genes also show different expression patterns; OsZIP1, OsZIP4, OsZIP5, OsZIP6, OsZIP7a, and OsZIP8 are expressed in both the roots and shoots (Ramesh et al., 2003; Ishimaru et al., 2005; Yang et al., 2009; Lee et al., 2010a, 2010b; Tan et al., 2019). Furthermore, the expression of OsZIP2 and OsZIP6 in Xenopus oocytes was not induced by Zn-deficiency, whereas OsZIP1 and OsZIP3 are constitutively expressed (Suzuki et al., 2012; Sasaki et al., 2015). OsZIP8 showed much higher expression, although expression efficiency was significantly upregulated after Zn deficiency, but not by Cu- or Mn-deficiency, whereas OsZIP1 and OsZIP3 are constitutively expressed (Suzuki et al., 2012; Sasaki et al., 2015).

RESULTS

Cloning of OsZIP9

To examine whether OsZIP9 is able to transport Zn, we expressed it in Zn uptake-defective yeast cells (ZHY3) under control of the Gal-inducible promoter. A time-course experiment with stable isotope 67Zn showed that in the presence of Glc (no OsZIP9 expression), there was no difference in Zn accumulation (Δ67Zn) between vector control and yeast expressing OsZIP9 (Fig. 1A). However, when the expression of OsZIP9 was induced by the presence of Gal, yeast expressing OsZIP9 showed much higher Δ67Zn compared with the empty vector control (Fig. 1B).

To examine the transport specificity of OsZIP9 for metals, we compared the transport activity for Fe, copper (Cu), and Zn using respective stable isotopes, specifically 63Zn, 65Cu, or 57Fe, in wild-type yeast cells (BY4741). In the presence of Gal, OsZIP9 transported only Zn and not Fe or Cu (Fig. 1C).

Expression Pattern Analysis of OsZIP9

The expression pattern of OsZIP9 was investigated in plants grown in either soil or nutrient solution by reverse-transcription quantitative PCR (RT-qPCR). In samples derived from rice grown in the field, OsZIP9 was found to be mainly expressed in the roots at all growth stages (Fig. 2A). In samples from hydroponically cultivated rice, the expression of OsZIP9 in the roots was strongly induced by Zn-deficiency, but not by Cu- or Mn-deficiency (Fig. 2B). OsZIP9 expression was also induced by Fe-deficiency, but to a lesser extent. Time-dependent expression analysis showed that OsZIP9 expression was significantly upregulated after 1 d and further increased after 3 d of Zn deficiency (Supplemental Fig. S3A). However, 1 d of Fe deficiency did not induce OsZIP9 expression, although expression
induction was observed after 3 d of Fe deficiency (Supplemental Fig. S3B).

We also investigated the spatial expression pattern of OsZIP9 in different root regions. The expression of OsZIP9 was very low in the root tip region (0–0.5 cm from the root tip; Fig. 2C). However, higher expression was detected in root mature regions (>1.0 cm).

Tissue Specificity of OsZIP9 Expression

To investigate the tissue specificity of OsZIP9 expression, we generated transgenic lines carrying the promoter of OsZIP9 fused with GFP. Immunostaining using GFP antibody showed that the signal was very weak in both the root tip (0.2 cm from the root tip) and mature region (1.5 cm from the root tip) of plants supplied with Zn (Fig. 3, A and D). However, in Zn-deficient roots, ZIP9 was strongly expressed at the exodermis and endodermis of the root mature region (Fig. 3, E, G, and H). The signal in the root tip of Zn-deficient plants was also weak, which is consistent with the spatial expression pattern of OsZIP9 (Fig. 2C). No signal was detected in wild-type plants (Fig. 3, C and F), indicating the specificity of the antibody.

Subcellular Localization of OsZIP9

Subcellular localization of OsZIP9 was investigated by transiently expressing a GFP-OsZIP9 fusion in rice protoplasts and onion (Allium cepa) epidermal cells. In rice protoplasts expressing GFP alone, the GFP signal was detected in the cytoplasm and nuclei (Supplemental Fig. S4, A–D). However, in protoplasts expressing GFP-OsZIP9, the GFP signal was mainly localized to the peripheral membrane of the cells, although some signal was also detected in the endomembrane (Supplemental Fig. S4, E–H). Similar results were obtained in onion epidermal cells (Supplemental Fig. S4, I–L). To further confirm OsZIP9 subcellular localization, we performed double staining using DAPI and an OsZIP9 antibody. In the roots of plants exposed to Zn conditions for 4 d, OsZIP9 was localized to the periphery of the cells, outside of the nuclei stained by DAPI (Supplemental Fig. S4, M–P). No signal was detected in the knockout line (Supplemental Fig. S4Q). Taken together, these results indicate that OsZIP9 is most likely localized to the plasma membrane.

Phenotypic Analysis of OsZIP9 Knockout Lines in Hydroponic and Soil Culture

To investigate the role of OsZIP9 in Zn transport, we generated OsZIP9 knockout lines by the CRISPR/Cas9 technique. We obtained two independent knockout lines with different target positions (oszip9-1 and oszip9-2): one (oszip9-1) with a 1-bp deletion at the first exon, and the other (oszip9-2) with a 1-bp insertion at the second exon (Supplemental Fig. S2B).

We first grew the wild-type rice and two independent knockout lines in a nutrient solution containing different Zn concentrations (0.02, 0.2, or 2 μM). At 0.02 μM Zn, growth of the two knockout lines was obviously inhibited compared with wild-type rice (Fig. 4A). New leaves showed typical Zn-deficiency symptoms in the knockout lines, but not in the wild-type rice (Fig. 4D). The shoot
fresh weight of the knockout lines was 65% of the wild-type rice (Fig. 4E), although the root fresh weight did not differ between different lines (Fig. 4F). However, at 0.2 and 2 μM Zn, growth was similar between wild-type rice and the knockout lines (Fig. 4, B, C, E, and F).

We then compared mineral element profiles in the roots and shoots of wild-type rice and the knockout lines exposed to different Zn concentrations. At 0.02 μM Zn, both the concentration and content of Zn in the roots and shoots were significantly lower in the knockout lines than in wild-type rice (Fig. 5). At 0.2 μM Zn, shoot Zn concentration and content were lower in the knockout lines than in wild-type rice, but root Zn concentration and content were similar between different lines. However, Zn concentration and content in both the roots and shoots of the different lines were comparable at 2 μM Zn (Fig. 5). There was no difference in the concentrations of calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), Fe, Cu, and Mn in the roots of wild-type rice and the knockout lines (Supplemental Figs. S5, A–D, and S6, A–C); however, the knockout mutants showed higher concentrations of Ca, Mg, Fe, Cu, and Mn in the shoots at 0.02 μM Zn, but not at 0.2- and 2 μM Zn (Supplemental Figs. S5, E and F, and S6, D–F). Moreover, the contents of these elements except Fe were similar between the different lines at all Zn concentrations tested (Supplemental Figs. S7, E and F, and S8, D–F), indicating that the higher concentrations observed at 0.02 μM Zn were caused by decreased growth. The shoot concentration and content of K were slightly decreased in the knockout lines, whereas those of P were not altered compared with wild-type rice (Supplemental Figs. S5, G and H, and S7, G and H).

Figure 2. Expression pattern of OsZIP9. A, Growth stage- and organ-dependent expression of OsZIP9. Samples of various organs were taken from rice grown in the field at different growth stages. B, Response of OsZIP9 expression to metal deficiency. Rice seedlings were grown in the one-half strength Kimura B solution with or without Cu, Zn, Fe, or Mn for 3 d. C, Spatial expression pattern of OsZIP9 in roots. Different root segments (0–0.5, 0.5–1.0, 1.0–1.5, 1.5–2.0, 2.0–2.5, and 2.5–3.0 cm from the root tip) were collected from roots of 5-d-old seedlings. The expression level of OsZIP9 was determined by RT-qPCR. Histone H3 (A and B) and Actin (C) were used as internal controls. The expression relative to root at 6 weeks (A), control condition (B), and the root segment of 2.5 to 3.0 cm (C) are shown. Data are means ± so of three biological replicates. Statistical comparison was performed by ANOVA followed by Tukey-Kramer's test. Different letters indicate significant difference (P < 0.01).
When grown in soil until maturity, the knockout lines accumulated less than half the amount of Zn in wild-type rice in straw and brown rice grain (Fig. 6). However, the concentrations of other elements, including Cu, Fe, and Mn, in straw and brown rice were comparable between wild-type rice and the knockout lines, except that the concentration of Mn in straw was slightly increased in the knockout lines compared with wild-type rice (Fig. 6). We also compared accumulation of Cd and arsenic (As) in straw and brown rice. No difference in the accumulation of these two toxic elements was found in either straw or brown rice between wild-type rice and the OsZIP9 knockout lines (Supplemental Fig. S9). Combined, these results indicate that OsZIP9 is a specific transporter for Zn in rice roots.

Short-Term Uptake Experiments with Stable Isotope $^{67}$Zn

To confirm whether Zn uptake was altered in the knockout lines, we performed a short-term (24-h) labeling experiment with stable isotope $^{67}$Zn. After the exposure of Zn-deficient plants to 0.4 $\mu$M $^{67}$Zn for 24 h, the OsZIP9 knockout lines accumulated much less $^{67}$Zn (as $\Delta^{67}$Zn) in both the roots and shoots compared with wild-type rice (Fig. 7A). The $\Delta^{67}$Zn uptake in the knockout lines was 41% of wild-type rice (Fig. 7B); however, there was no difference in the root-to-shoot translocation of $\Delta^{67}$Zn between the different lines (Fig. 7C). To confirm these results, we also used an OsZIP9 RNA interference (RNAi) line, which showed ~80% reduction in OsZIP9 expression compared to wild-type rice (Supplemental Fig. S10A). Similar to the knockout lines, the $\Delta^{67}$Zn concentration in both the roots and shoots was lower in the RNAi line than in wild-type rice (Supplemental Fig. S10B). The $\Delta^{67}$Zn uptake in the RNAi line was 66% of that in wild-type rice (Supplemental Fig. S10C), whereas the root-to-shoot translocation was similar between the RNAi line and wild-type rice (Supplemental Fig. S10D).

Furthermore, we performed a kinetic uptake experiment with $^{67}$Zn in Zn-deficient plants at 4°C and 25°C. At 4°C, there was no difference in $\Delta^{67}$Zn uptake (30 min) between wild-type rice and the knockout lines (Fig. 7D). However, at 25°C, the $\Delta^{67}$Zn uptake was higher in wild-type rice than in the knockout lines, although the uptake increased with increasing $^{67}$Zn concentrations in the nutrient solution in all lines (Fig. 7D). The net uptake of $\Delta^{67}$Zn calculated was significantly higher in wild-type rice than in the knockout lines (Fig. 7E). Knockdown of OsZIP9 also significantly reduced the net uptake of $\Delta^{67}$Zn.
Together, these results support that OsZIP9 contributes to Zn uptake in rice roots.

**DISCUSSION**

Based on analyses of expression pattern, transport activity in heterologous systems, and ectopic expression, a number of transporters have been suggested to function in root Zn uptake, such as AtZIP2, AtIRT1, and AtIRT3 in Arabidopsis (Vert et al., 2002; Lin et al., 2009; Palmer and Guerinot, 2009; Milner et al., 2013); OsZIP1 and OsZIP3 in rice (Ramesh et al., 2003); and HvZIP7 in barley (Tiong et al., 2014). However, the exact transporter for Zn uptake in roots has not been identified in plants (Olsen and Palmgren, 2014). In this study, we functionally characterized OsZIP9 in rice in terms of growth stage- and organ-dependent expression pattern, subcellular localization, transport activity in both yeast and A to C, Phenotype of the wild-type (WT) rice and two OsZIP9 knockout lines (oszip9-1 and oszip9-2). Scale bars = 10 cm. D, Zn-deficiency symptom of new leaf. Scale bar = 2.5 cm. E and F, Fresh weight of shoots (E) and roots (F). The plants were grown in a nutrient solution containing 0.02 (A and D), 0.2 (B), and 2 μM (C) Zn for 17 d. Data in E and F are means ± SD of three biological replicates. Statistical comparison was performed by ANOVA followed by Tukey-Kramer’s test. Different letters indicate significant difference ($P < 0.01$).
and knockout/knockdown lines, tissue-specificity of localization, and detailed phenotypic analysis of knockout lines growing in both nutrient solution and soil. We revealed that OsZIP9 contributes to Zn uptake under Zn-limited conditions, especially in soil. This conclusion is supported by several lines of evidence: (1) OsZIP9 is localized to the plasma membrane (Supplemental Fig. S4); (2) OsZIP9 shows transport activity for Zn (Fig. 1); (3) OsZIP9 is mainly expressed in the roots through the whole growth period (Fig. 2A); (4) OsZIP9 expression is induced by Zn-deficiency (Fig. 2B); (5) OsZIP9 is expressed at the exodermis and endodermis of mature root region (Figs. 2C and 3); (6) Knockout or knockdown of OsZIP9 results in remarkably decreased Zn uptake at low Zn concentration in nutrient solution, but not at high Zn concentrations (Figs. 5 and 7, Supplemental Fig. S10); and (7) knockout of OsZIP9 decreases Zn uptake from soil (Fig. 6).

OsZIP9 showed transport activity for Zn in yeast, but not for Fe and Cu (Fig. 1). Transport activity for Mn was not tested in yeast because a stable isotope of Mn was not available. However, no difference in Mn accumulation was found between wild-type rice and knockout lines of OsZIP9 (Supplemental Fig. S8). Furthermore, OsNramp5 is reported to mediate Mn uptake in rice (Sasaki et al., 2012). Therefore, it is unlikely that OsZIP9 contributes to Mn uptake.

Rice roots are characterized by two Casparian strips at both the exodermis and endodermis (Enstone et al., 2002). Furthermore, mature roots have a highly developed aerenchyma in which almost all of the cortex cells between the exodermis and endodermis are destroyed. Therefore, rice has developed an efficient uptake system for mineral elements, which is mediated by the cooperation of influx and efflux transporters expressed at both the exodermis and endodermis of the root.

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**Figure 6.** Comparison of metal accumulation between wild-type (WT) rice and two independent OsZIP9 knockout lines grown in soil. A and B, Metal concentrations in the straw (A) and brown rice (B). Both the wild-type rice and two independent OsZIP9 knockout lines were grown in soil under flooded conditions until maturity. The concentration of different metals was determined by ICP-MS. Data are means ± SD of three biological replicates. Statistical comparison was performed by ANOVA followed by Tukey-Kramer’s test. Different letters indicate significant difference (P < 0.01). All data for each element were compared with the wild-type rice. DW, Dry weight.

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**Figure 7.** Short-term labeling experiment with 67Zn. A, Concentration of Δ67Zn in the roots and shoots. B, Uptake of Δ67Zn. C, Root to shoot translocation of Δ67Zn. The wild-type (WT) rice and two independent OsZIP9 knockout lines grown in 0.02 μM Zn conditions for 17 d were exposed to a solution containing 0.4 μM Zn for 24 h. D and E, Kinetic study of 67Zn uptake. Seedlings grown in Zn-deficient solution for 7 d were exposed to a solution containing different concentrations of 67Zn for 30 min at 25°C or 4°C. Net uptake (E) was calculated by subtracting the apparent uptake at 4°C from that at 25°C. Data are means ± SD of three biological replicates. Different letters and asterisks indicate significant difference (P < 0.01). Statistical comparison was performed by ANOVA followed by Tukey-Kramer’s test. DW, Dry weight.
OsZIP9 is localized in the exodermis and endodermis of the roots. Expression of OsZIP9 at the exodermis and endodermis in the root mature region supports its importance in Zn uptake (Figs. 2C and 3). Because OsZIP9 likely functions as an influx transporter based on yeast transport assay results (Fig. 1), an efflux transporter for cooperative Zn transport with OsZIP9 is required for efficient Zn uptake, which remains to be identified in the future.

Knockout or knockdown of OsZIP9 resulted in decreased Zn uptake only under Zn-limited conditions, but not under Zn-sufficient conditions in nutrient solution (Fig. 5; Supplemental Fig. S10), suggesting that OsZIP9 functions as a high-affinity transporter for Zn. This is in contrast to AtZIP9 and AtZIP12 in Arabidopsis, whereby knockout of AtZIP9 and AtZIP12 only affects Zn uptake at high Zn concentrations and not at low Zn concentrations (Inaba et al., 2015). In paddy soil, the Zn concentration in soil solution is very low (Wang et al., 2020). In fact, OsZIP9 plays an important role in Zn uptake from soil because knockout mutants of OsZIP9 exhibited significant decreases in Zn accumulation in both the straw and brown rice under flooded conditions (Fig. 6). This is also supported by higher expression of OsZIP9 under flooded conditions compared to upland conditions (Wang et al., 2020). Because knockout of OsZIP9 did not completely abolish Zn uptake even under Zn-limited conditions (Fig. 5), other unidentified transporters may also be involved in Zn uptake in rice. One candidate is OsZIP1, because it is also highly expressed in the roots (Ramesh et al., 2003), although its exact role in Zn uptake remains to be examined. Furthermore, transporters functioning at high Zn concentrations also require characterization in the future.

The expression of OsZIP9 was also induced by Fe-deficiency to some extent, although the extent of expression induction was not as high as that caused by Zn deficiency (Fig. 2B). However, expression induction by Zn deficiency occurred earlier than by Fe deficiency (Supplemental Fig. S3), suggesting that induction by Fe deficiency was caused by indirect effects, although the exact mechanism is unknown. In OsZIP9 knockout mutants, higher Fe accumulation in the shoots was observed at low Zn supply in nutrient solution (Fig. 6). Because the mutant plants suffered from Zn deficiency at low Zn concentrations (Figs. 4 and 5), some genes related to Fe uptake in the roots may have been induced. However, in soil culture, knockout of OsZIP9 did not affect Fe accumulation in the shoots (Fig. 6), due to high Fe concentration in soil solution of paddy soil (Wang et al., 2020).

Identification of OsZIP9 in this study provides further understanding of the Zn transport system in rice. Zn in soil is first taken up by OsZIP9 localized at the exodermis and endodermis of the roots and other uncharacterized transporters (Fig. 3). Zn is partially sequestered by OsHMA3 localized at the tonoplast in root cells (Cai et al., 2019) and the remaining Zn is translocated to the shoot by OsHMA2 localized at the pericycle (Yamaji et al., 2013). OsZIP7 was also implicated in Zn xylem loading although its exact role remains to be examined (Tan et al., 2019). At the node, Zn is preferentially delivered to developing organs such as new leaves and grains by OsZIP3 and OsHMA2. OsZIP3 is localized to xylem transfer cells in enlarged vascular bundles (EVBs) of the nodes and responsible for unloading of Zn from the xylem of EVB (Sasaki et al., 2015), whereas OsHMA2 is localized at the phloem region of both EVBs and diffuse vascular bundles and is responsible for loading Zn to the phloem of diffuse vascular bundles and EVBs (Yamaji et al., 2013). However, some missing transporters, such as Zn efflux transporter(s) in root and node, remain to be identified in future investigations to gain a holistic understanding of the Zn transport system in rice. This will contribute to breeding rice cultivars with high tolerance to Zn deficiency and with high Zn accumulation in the grain.

In conclusion, OsZIP9 identified in this study is a transporter for Zn and it contributes to root Zn uptake in soil.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Seeds of the wild-type rice (*Oryza sativa* ‘Nipponbare’), two independent CRISPR/Cas9 OsZIP9 knockout lines (T2), one RNAi line, and transgenic lines (T2) carrying the promoter of ZIPS fused with GFP were soaked in water in the dark at 30°C. After 2 d, the germinated seeds were placed on a plastic net floating on a 0.5 mM CaCl2 solution in a 1.2-L plastic pot. The seedlings (7-d-old) were transferred to a 3.5-L plastic pot containing one-half-strength Kimura B solution (0.4 μM of Zn at pH 5.6; Ma et al., 2002). The nutrient solution was exchanged every 2 d. All plants were grown in a controlled greenhouse at 25°C to 30°C, under natural light.

**Cloning of Full-Length cDNA of OsZIP9**

The full-length open-reading frame (ORF) of OsZIP9 was amplified by PCR using primers listed in Supplemental Table S1, which were designed based on a putative cDNA clone (Os05g0472400) in the Rice Annotation Project Database (http://rapdb.dna.afrc.go.jp/), with a putative translational start and stop site. Total RNA was extracted from rice roots (‘Nipponbare’) using a RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com) and then converted to cDNA using the protocol supplied by the manufacturer of ReverTra Ace qPCR RT Master Mix with genomic DNA remover (TOYOBO). The amplified cDNA was cloned into pGEM-T vector (Promega; https://www.promega.com/) and the sequence was confirmed by an ABI Prism 3130 sequencer by (Applied Biosystems; http://www.appliedbiosystems.com/).

**Phylogenetic Analysis**

The alignment was performed with the tool ClustalW using default settings (http://clustalw.ddbj.nig.ac.jp/), and the phylogenetic tree was constructed using the neighbor-joining algorithm with the software MEGA v.6.0 (Tamura et al., 2013). Bootstrap support was calculated (1,000 replications).
Transport Activity Assay of OsZIP9

The OsZIP9-pGEM constructed as above was introduced into pYES2 vector (Invitrogen) through restriction sites of BamHI and XhoI under the control of Gal-inducible promoter, followed by introducing it into a wild-type yeast (Saccharomyces cerevisiae) strain (BY4741; MATa his3Δ1 leu2Δ2 trpl3Δ1 ura3D1 lys2Δ800). In a time-course experiment, ZnCl2 expressing OsZIP9 or empty vector was grown in the SC(-Uracil) medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) Gal, 0.2% (w/v) appropriate amino acid, and 2% (w/v) agar at pH 6.0 for selection. Yeast samples were first incubated in SC(-Uracil) liquid medium with 50 μg of M55S containing 2% (w/v) Gal or 2% (w/v) Gal (as a negative control) for 2 h, followed by washing three times with sterilized Milli-Q water (EMD Millipore). The yeast cells were then exposed to a solution containing 5 μM of stable isotope 65ZnCl2 (97% enrichment; Taiyo Nippon Sanso). At 0, 20, 40, 60, and 120 min of incubation with shaking at 30°C, the yeast cells were harvested by centrifugation (2,300g, 5 min). The yeast pellet was washed three times with 5 μM of CaCl2 solution and then digested by 2% (v/v) HCl for the determination of metals as described below.

To examine the transport activity for Zn, Fe, and Cu, the wild-type yeast cells (BY4741) expressing OsZIP9 or empty vector were prepared as above and then cultured for 4 h in the presence of 2% (w/v) Gal for gene induction, followed by exposure to a solution containing 5 μM of each stable isotope including 65ZnCl2 (97% enrichment), 64CuCl2 (99.7% enrichment), or 65FeCl3 (96.1%, 65Fe). 65FeCl3 was prepared from 65FeC13 by reduction with ascorbic acid. These stable isotopes were purchased from Taiyo Nippon Sanso. After incubation with shaking for 2 h at 30°C, the yeast cells were harvested by centrifugation (2,300g, 5 min) and subjected to determination of metals as described below.

Expression Analysis of OsZIP9

To investigate the expression pattern of OsZIP9 in different organs at different growth stage, we used the same cDNA samples collected in the field as described in Sasaki et al. (2015).

To investigate the response of OsZIP9 expression in roots to metal deficiency, 20-d-old seedlings (cv Nipponbare) were grown in the one-half strength Kimbera B solution with or without Mn, Fe, Cu, or Zn for 3 d. To further examine the time-dependent response, seedlings (20-d-old) were exposed to –Fe or –Zn for 1 and 3 d, and root samples were taken for expression analysis as described below.

For spatial expression analysis, different root segments (0-0.5, 0.5-1.0, 1.0-1.5, 1.5-2.0, 2.0-2.5, and 2.5-3.0 cm from the root tip) were excised from the roots of 5-d-old seedlings.

Samples taken were immediately frozen in liquid nitrogen and then subjected to total RNA extraction using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized by ReverTra Ace qPCR RT Kit (TOYOBO) or SuperScript II (Invitrogen) according to the manufacturer’s instructions. The expression analysis of OsZIP9 was determined with SsoFast EvaGreen Supermix (Bio-Rad) or KOD polymerase (Toyobo). Gene expression was determined with the ΔΔCt method. The primer sequences used were listed in Supplemental Table S1.

Generation of Transgenic Rice Lines

For generation of the transgenic lines carrying the promoter of ZIP9 fused with GFP, the promoter region of OsZIP9 (3,001 bp) was first amplified with PCR using the primers shown in Supplemental Table S1. The amplified region was introduced into the pGEM-T easy vector. After confirmation of the sequence, the plasmid was introduced into pZP22H-lac vector including GFP by KpnI and BamHI, followed by vector transfer to calluses (cv Nipponbare) via Agrobacterium tumefaciens-mediated transformation (Hiei et al., 1994).

OsZIP9 knockout lines were generated by using CRISPR/Cas9 using the plant expression vector of Cas9 (pU6gRNA) and single-guide RNA expression vector (pZDgRNA_Cas9ver.2_HPT) as described in Che et al. (2017). Twenty bases upstream of the PAM motif were selected as candidate target sequences (Supplemental Fig. S2A). Two targets of OsZIP9 were selected. The primers for target sequences in the ORF region of OsZIP9 are listed in Supplemental Table S1. The derived constructs were transformed into calluses as described above.

To genotype the resultant mutants, genomic DNA was extracted from leaves of transgenic rice plants. PCR amplifications were carried out using primer pairs flanking the designed target sites as listed in Supplemental Table S1. The PCR products (~500 bp) were sequenced directly using internal specific primers, of which the binding positions are desirably at ~200-bp upstream of the target sites. Two homologous knockout lines without Cas9 were selected and the T2 generation was used in the following phenotypic analysis.

An RNAi line was generated according to Miki and Shimamoto (2004) using the primers listed in Supplemental Table S1. The expression level of OsZIP9 in the RNAi line was investigated as described above.

Immunostaining Analysis for Transgenic Lines Carrying OsZIP9 Promoter-GFP

To investigate the tissue-specificity of OsZIP9 expression, immunostaining was performed in the transgenic lines (T2) carrying OsZIP9 promoter-GFP by using an antibody against GFP (Thermo Fisher Scientific). Two-week-old plants grown in one-half strength Kimura B solution were exposed to a solution containing 0.4 μM of Zn, or not, for 5 d. Cross sections from the root tip (0.2 cm from the tip) and mature region (1.5 cm from the root tip) were prepared and the method for immunostaining was the same as described in Yamaji and Ma (2007). The signal of fluorescence was observed with a TCS SP8× confocal laser scanning microscope (Leica Microsystems).

Subcellular Localization of OsZIP9

Subcellular localization of OsZIP9 was investigated by transiently expressing GFP-OsZIP9 fusion into rice protoplasts and onion (Allium cepa) epidermal cells. The ORF of OsZIP9 was amplified by PCR from rice (Nipponbare) root cDNA using primers with the BotGA and N0ff sites (Supplemental Table S1). The ORF was fused with a linker (SSCGCG) and then inserted into the cauliflower mosaic virus 35S GFP vector at the N terminus according to Sasaki et al. (2012). Rice protoplast transformation was performed by the polyethylene glycol method as described in Chen et al. (2006). The same plasmid with DoRed was transformed into onion epidermal cells as per the method described in Yokosho et al. (2016). The GFP signal was observed with a TCS SP8× confocal laser scanning microscope (Leica Microsystems).

We also performed double staining by using DAPI as a nuclei marker and an OsZIP9 antibody for further confirmation of the subcellular localization. The synthetic peptide (DASSHHDHERGN) was used to immunize rabbits to obtain antibodies against OsZIP9. The antiserum was purified through a peptide affinity column. The roots of wild type and the knockout line exposed to –Zn for 4 d were used for the immunostaining. The method for immunostaining and secondary antibody incubation were the same as described in Yamaji and Ma (2007). The fluorescence signal was observed through confocal laser scanning microscopy (TCS SP8×; Leica Microsystems).

Phenotypic Analysis of OsZIP9 Knockout Lines

The wild-type rice and two independent OsZIP9 knockout lines (T2, oszip9-1, oszip9-2) generated by CRISPR/Cas9 were used for phenotypic analysis. In a hydroponic solution, seedlings (19-d-old) grown in a 3.5-L plastic pot were transferred to a 1.2-L, plastic pot (one plant for each line) with a nutrient solution containing different Zn concentrations: 0.02, 0.2, and 2 μM. The treatment solution was renewed every 2 d. After 17 d, the plants were photographed. The roots were washed with 5 μM of CaCl2 three times and separated from the shoots. The fresh weights of the roots and shoots were recorded. The concentrations of mineral elements in the roots and shoots were determined as described below.

For soil culture, both wild-type rice and two independent knockout lines were grown in a pot containing 3.5 kg of soil collected from a field at the Institute of Plant Science and Resources, Okayama University, under flooded conditions. Tap water was supplied daily and a 2-cm water layer was maintained on the topsoil. Plants were grown in a temperature-controlled glasshouse (~22°C to 30°C) under natural light. At the ripening stage, the plant was harvested and separated into straw and brown rice. The concentrations of mineral elements were determined as described below.

Short-Term Uptake Experiment with Stable Isotope 67Zn

Seedlings (wild type, knockout lines, RNAi line) grown in 0.02 μM Zn for 17 d were exposed to a solution containing 0.4 μM of 67Zn. After 24 h, the roots were washed and separated from the shoots as described above.
A kinetic study of Zn uptake was performed by exposing the seedlings (wild type, knockout lines, RNAi line) grown in – Zn solution for 7 d to different δ6Zn concentrations in the range of 0 to 2 μM at 25°C and 4°C. After 30 min, the roots were washed three times with 5 mM of CaCl2 and harvested for element determination as described below.

**Determination of Metals in Plant and Yeast Samples**

The roots and shoots were dried at 70°C for at least 3 d before being digested by HNO3 (60% [w/v]) as described in Sasaki et al. (2012). The concentrations of mineral elements in digestion solutions derived from plants and yeast were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, model no. 7700X; Agilent Technologies). The concentrations of 67Zn, 65Cu, and 63Zn, 65Fe, and 65Cu (net Zn, Fe, or Cu increase) were calculated according to Yamaji et al. (2013).

**Statistical Analyses**

Statistical comparison using the software SPSS Statistics 19 (IBM) was performed by ANOVA, followed by Tukey-Kramer’s test.

**Accession Number**

Accession number of OsZIP9 is registered as LC521921 in the GenBank/ European Molecular Biology Laboratory databases (https://www.ncbi.nlm.nih.gov/genbank/).

**Supplemental Data**

The following supplemental materials are available.

Supplemental Figure S1. Sequence analysis of OsZIPs.

Supplemental Figure S2. Mutated sequences of OsZIP9 gene in CRISPR/Cas9 mutants.

Supplemental Figure S3. Time-dependent response of OsZIP9 to Zn- and Fe-deficiency in the roots.

Supplemental Figure S4. Subcellular localization of OsZIP9.

Supplemental Figure S5. The concentrations of macro-elements in the roots and shoots.

Supplemental Figure S6. Concentrations of Fe, Mn, and Cu in the roots and shoots.

Supplemental Figure S7. Contents of macro-elements in the roots and shoots.

Supplemental Figure S8. Contents of Fe, Mn, and Cu in the roots and shoots.

Supplemental Figure S9. Concentrations of Cd and As in straw and brown rice.

Supplemental Figure S10. Effect of knockdown of OsZIP9 on Zn uptake and accumulation.

Supplemental Table S1. List of primers used in this study.

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