Overexpression of the OsZIP4 zinc transporter confers disarrangement of zinc distribution in rice plants

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Abstract

Zinc (Zn), an essential nutrient in cells, plays a vital role in controlling cellular processes such as growth, development, and differentiation. Although the mechanisms of Zn translocation in rice plants (Oryza sativa) are not fully understood, it has recently received increased interest. OsZIP4 is a Zn transporter that localizes to apical cells. Transgenic rice plants overexpressing the OsZIP4 gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter were produced. The Zn concentration in roots of 35S-OsZIP4 transgenic plants was 10 times higher than in those of vector controls, but it was five times lower in shoots. The Zn concentration in seeds of 35S-OsZIP4 plants was four times lower compared with vector controls. Northern blot analysis and quantitative real-time reverse transcription-PCR revealed transcripts of OsZIP4 expression driven by the CaMV 35S promoter in roots and shoots of 35S-OsZIP4 plants, but levels of endogenous OsZIP4 transcripts were low in roots and high in shoots compared with vector controls. Northern blot analysis and quantitative real-time reverse transcription-PCR revealed transcripts of OsZIP4 expression driven by the CaMV 35S promoter in roots and shoots of 35S-OsZIP4 plants, but levels of endogenous OsZIP4 transcripts were low in roots and high in shoots compared with vector controls. Microarray analysis revealed that the genes expressed in shoots of 35S-OsZIP4 plants coincided with those induced in shoots of Zn-deficient plants. These results indicate that constitutive expression of OsZIP4 changes the Zn distribution within rice plants, and that OsZIP4 is a critical Zn transporter that must be strictly regulated.

Key words: OsZIP4, rice, seed, zinc transporter.

Introduction

The variety of roles that zinc (Zn) plays in cellular processes is a good example of the diverse biological utility of metal ions. Zn is involved in protein, nucleic acid, carbohydrate, and lipid metabolism. In addition, Zn is critical in the control of gene transcription and the coordination of other biological processes regulated by proteins containing DNA-binding Zn-finger motifs (Rhodes et al., 1993), RING fingers, and LIM domains (Vallee et al., 1993). Several molecules associated with DNA and RNA synthesis are also Zn metalloenzymes, such as RNA polymerases (Wu et al., 1992), reverse transcriptases, and transcription factors (Wu and Wu, 1989). Zn is a non-redox-active ion and is therefore targeted to transcription factors and other enzymes involved in DNA metabolism, as the use of redox-active metal ions for these tasks could lead to radical reactions and nucleic acid damage. However, these processes must be tightly regulated to ensure that the exact amount of Zn is present at all times. Although it is an essential nutrient, Zn can be toxic if excess amounts are accumulated. The precise cause of Zn toxicity is unknown, but the metal may bind to inappropriate intracellular ligands or compete with other metal ions for enzyme active sites or transporter proteins. In order for Zn to play such a varied role in cells, and because it cannot passively diffuse across cell membranes, it must be transported into the intracellular compartments of a cell where it is required for these Zn-dependent..
processes. A group of proteins called Zn transporters is dedicated to the transport of Zn across biological membranes.

The Zn-regulated transporter, iron (Fe)-regulated transporter-like protein (ZIP) family of Zn and Fe transporters occurs in plants, bacteria, fungi, and humans (Gaither and Eide, 2001). Previous studies have investigated the functions of several members of the ZIP family in Arabidopsis thaliana (Eide et al., 1996; Guerinot, 2000; Vert et al., 2002), soybean (Glycine max; Moreau et al., 2002), and rice (Oryza sativa; Bughiio et al., 2002; Ramesh et al., 2003; Ishimaru et al., 2005, 2006). For the most part, the expression of genes encoding the plant ZIP transporters appears to be induced by Zn or Fe deficiency (Eide et al., 1996). Some of the ZIP family members, such as IRT genes, are constitutively expressed, but expression levels increase with Fe deficiency (Vert et al., 2002).

Five ZIP transporter genes have been reported for rice, OsIRT1, OsIRT2, OsZIP1, OsZIP3, and OsZIP4. OsIRT1 and OsIRT2 are ferrous iron transporters (Ishimaru et al., 2006). OsZIP1 is primarily associated with metal uptake, and OsZIP3 with overall Zn homeostasis, especially in leaves (Ramesh et al., 2003). OsZIP4 may be involved in the translocation of Zn, particularly in the meristem (Ishimaru et al., 2005).

In this study, transgenic rice plants overexpressing OsZIP4 under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35S-OsZIP4) were prepared. The 35S-OsZIP4 transgenic plants accumulated Zn mainly in the roots, rather than in shoots. The Zn concentration in the roots of transgenic plants was lower than that in plants transformed with vector control (VC). Although the transcription level of OsZIP4 expression driven by the CaMV 35S promoter was high, the level of endogenous OsZIP4 transcripts was low in roots compared with shoots. Microarray analysis revealed that the genes expressed in shoots of the 35S-OsZIP4 plants coincided with those induced in shoots of Zn-deficient non-transgenic plants.

**Materials and methods**

**Plant material**

Transgenic rice T1 or T2 seeds were germinated for 2 weeks on Murashige and Skoog (MS) medium at 28 °C under 16 h light/8 h dark conditions. Plants were then transferred to a 20.1 plastic container containing a nutrient solution with the following composition: 0.7 mM K2SO4, 0.1 mM KCl, 0.1 mM KH2PO4, 2.0 mM Ca(NO3)2, 0.5 mM MgSO4, 10 μM H3BO3, 0.5 μM MnSO4, 0.2 μM CuSO4, 0.5 μM ZnSO4, 0.05 μM Na2MoO4, and 0.1 mM Fe-EDTA. The pH of the nutrient solution was adjusted daily to 5.5 with 1 M HCl and was renewed weekly. The ZnSO4 was omitted from the solution to induce Zn deficiency, whereas 100 μM ZnSO4 was added to induce Zn excess, and 2-week-old plants were transferred to these solutions and were grown for a further 2 weeks. Five-week-old plants were used for microarray analysis, quantitative real-time reverse transcription (RT)-PCR, and measurement of metal concentration.

**Rice transformation**

The plasmid pG121Hm was used as a backbone (Ishimaru et al., 2007). The construct had XbaI and SalI sites on the 3′ side of the CaMV 35S promoter. The plasmid pENTR-OsZIP4 (Ishimaru et al., 2005) containing OsZIP4 was digested at XbaI and SalI sites and subcloned in the 3′ side of the CaMV 35S promoter in pG121Hm. An Agrobacterium tumefaciens strain (C58) carrying the above construct was used to transform rice (O. sativa L. cv. Tsukinohikari) following the method of Ishimaru et al. (2007). The T1 seeds obtained from the transformants were germinated on MS medium containing 50 mg l−1 hygromycin B.

**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, Piscataway, NJ, USA), and hybridized with probes at 65 °C according to the method of Ishimaru et al. (2005). The amplified open reading frame (ORF) of OsZIP4 (Ishimaru et al., 2005) was used to prepare probes.

**Quantitative real time-PCR of OsZIP4**

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-(dT)15. The fragment was amplified by PCR in a SmartCycler (Takara) with SYBR Green I and ExTaq™ RealTime-PCR (Takara). The primers used for real-time PCR were as follows; OsZIP4 forward (5′-GGCGGAAGCACAAGTCATGCGGACTTTTC-3′), OsZIP4 reverse (5′-GCAGCTCTTGGTTGGCTCTGGAAGATCATC-3′), OsZIP4 total forward (5′-CAAAACCTTGCAGCTACAAACTAAGCTC-3′), and OsZIP4 total reverse (5′-GCAGCTCTTGGTTGGCTCTGGAAGATCATC-3′). The primers used for internal control in RT-PCR were 18s-tubulin forward, (5′-TCTTCAACCCCTTGCGACGTC-3′) and 18s-tubulin reverse (5′-AACCTTGAGACGCAGTCGAC-3′). There was no genomic contamination and no differences in the internal control in each sample (data not shown).

The sizes of the amplified fragments were confirmed by gel electrophoresis and sequencing.

**Oligo DNA microarray analysis**

A rice 22K custom oligo DNA microarray kit (Agilent Technology, Tokyo, Japan), which contains 21 938 oligonucleotides 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 an RNaseasy 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 Bioanalyzer. Total RNA (200 ng) was labelled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. Fluorescently labelled targets were hybridized to Agilent rice 22K custom 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’s Feature Extraction software was used for the image analysis and data extraction processes.

**Determination of metal concentrations**

Plants were dried for 1 week at 65 °C. The plants (30–50 mg) were then wet-ashed with 2 ml of 11 M HNO3 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.

Results

Analysis of phenotypes and metal concentration in 35S-OsZIP4 plants

35S-OsZIP4 transgenic rice plants were constructed, and 56 independent transgenic lines were identified based on segregation ratios and PCR analysis of genomic DNA (data not shown). The T1 seeds of a 35S-OsZIP4 plant were harvested, grown hydroponically, and analysed to gain information about the role of 35S-OsZIP4. A 50% reduction in plant height and root length was observed in the transgenic plants compared with the VCs (Fig. 1A–C). Some black spots, which were similar to those in Zn-deficient shoots, were also observed on old leaves of 35S-OsZIP4 plants.

The metal concentration was measured in 35S-OsZIP4 plants because the phenotype of the plants suggested that OsZIP4 may affect Zn distribution in rice plants. The Zn concentration in shoots of the transgenic plants was five times lower than that of VCs (Fig. 1D), confirming that symptoms observed in leaves of 35S-OsZIP4 plants were due to Zn deficiency. Unexpectedly, the transgenic plants accumulated 10 times more Zn in their roots compared with the VCs (Fig. 1E). The Fe concentration in 35S-OsZIP4 plants was 1.5 times higher than that of VCs in shoots and was 1.2 times higher than that of VCs in roots (Fig. 1D, E). The manganese and copper concentration in 35S-OsZIP4 plants was not significantly different from that of VCs (Fig. 1D, E).

Expression pattern of OsZIP4 in 35S-OsZIP4 plants

To examine the steady-state OsZIP4 mRNA levels in the transgenic plants, two independent lines of 35S-OsZIP4 plants were selected and RNA gel blot analysis was performed using OsZIP4 cDNA as a probe. RNA was isolated from the roots and shoots of T2 plants grown under Zn-sufficient conditions. As expected, 1.5 kb OsZIP4 mRNA was expressed at high levels in both shoots and roots of the two transgenic lines (Fig. 2A, B). Moreover, endogenous expression of 1.8 kb OsZIP4 mRNA was observed in shoot tissue of both transgenic lines. No endogenous expression was detected in the roots of either transgenic line. In the VCs, 1.8 kb OsZIP4 mRNA was not expressed in the shoots, but was expressed at low levels in roots, as shown previously (Ishimaru et al., 2005). Consistent with northern blot analysis, quantitative real-time RT-PCR revealed that endogenous 1.8 kb OsZIP4 mRNA was down-regulated in roots, and up-regulated in shoots (Table 1).
Microarray analysis of 35S-OsZIP4

To clarify the regulation of 35S-OsZIP4 (line 34), the expression patterns were analyzed by 22K microarray analysis. In 35S-OsZIP4 shoots, 236 genes were induced, of which 188 are normally induced by Zn deficiency in wild-type plants (Fig. 3). In 35S-OsZIP4 roots, 235 genes were induced, of which 102 genes were down-regulated by Zn deficiency in VC plants. Consistent with the RNA gel blot analysis and quantitative real-time RT-PCR, endogenous OsZIP4 was up-regulated in the roots and down-regulated in the shoots (Table 2; see Supplementary Table S1 at JXB online).

Analysis of Zn concentration in 35S-OsZIP4 seeds

As the expression of OsZIP4 in Saccharomyces cerevisiae leads to the uptake of Zn (Ishimaru et al., 2005), it was expected that overexpression of OsZIP4 in rice plants would cause enhanced Zn accumulation in seeds. When the metal contents in T1 seeds of the transgenic plants were analysed, the Zn concentration of 35S-OsZIP4 seeds was found to be 52% lower than in seeds harvested from a VC (Fig. 4A). The Zn concentration in T2 seeds was also four times lower in 35S-OsZIP4 plants than in the VCs (Fig. 4B).

Discussion

It is vital to maintain the appropriate Zn concentrations in plant cells because plants may suffer from Zn deficiency or toxicity, ultimately leading to abnormal growth. Therefore, the Zn concentration must be strictly regulated, especially in root and shoot meristems, as Zn is an essential cofactor for many enzymes. Previously, the hypothesis that OsZIP4 may be involved in Zn uptake in the phloem and meristem was proposed and data were provided to support this (Ishimaru et al., 2005). Here, it

Table 1. Quantitative real-time RT-PCR analysis of OsZIP4

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<th></th>
<th>+ZnS</th>
<th>–ZnS</th>
<th>VCS</th>
<th>34S</th>
<th>22S</th>
<th>+ZnR</th>
<th>–ZnR</th>
<th>VCR</th>
<th>34R</th>
<th>22R</th>
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<tr>
<td>Endogenous 1.8 kb OsZIP4 mRNA</td>
<td>0.62±0.16</td>
<td>37±1.9</td>
<td>0.56±0.051</td>
<td>36±4.8</td>
<td>28±1.5</td>
<td>2.1±0.37</td>
<td>6.8±1.1</td>
<td>1.4±0.14</td>
<td>0.088±0.017</td>
<td>0.086±0.018</td>
</tr>
<tr>
<td>Total OsZIP4 transcripts</td>
<td>0.59±0.15</td>
<td>35±1.5</td>
<td>0.37±0.059</td>
<td>1200±110</td>
<td>1100±110</td>
<td>2.9±0.18</td>
<td>10±0.79</td>
<td>1.9±0.12</td>
<td>230±35</td>
<td>1500±91</td>
</tr>
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a 10⁶ copies/1 μg total RNA.
Ramesh et al. (2004) reported that overexpression of ZIP1, an Arabidopsis Zn transporter, leads to 2-fold higher Zn concentrations in seeds and shoots of transgenic wheat. In the present study, 35S-OsZIP4 plants accumulated 10 times more Zn in their roots compared with the VCs. In contrast, the Zn concentration in seeds and shoots of 35S-OsZIP4 plants was low compared with VCs (Fig. 1D).

In response to Zn deficiency, the gene encoding mugineic acid family phytosiderophores is induced, resulting in increased production and secretion of phytosiderophores in barley (Suzuki et al., 2006). Microarray analysis showed that nicotianamine synthase as well as nicotianamine aminotransferase were up-regulated in 35S-OsZIP4 shoots (see Supplementary Table S1 at JXB online). It is speculated that nicotianamine or deoxymugineic acid synthesized in 35S-OsZIP4 shoots may be involved either in the long-distance transport of Zn or in increasing the availability of Zn in the cell. Talke et al. (2006) also reported that ZIP family genes, nicotianamine synthase genes, and Zn efflux genes in Arabidopsis halleri are highly up-regulated in Zn-deficient conditions, and Zn hyperaccumulation in A. halleri involves enhanced partitioning of Zn from roots into shoots.

Transgenic tobacco overexpressing ferritin have increased leaf Fe concentrations and root Fe³⁺-chelate reductase activity, which is part of the Strategy I Fe uptake system (van Wuytswinkel et al., 1998). This transgenic tobacco shows higher Fe storage capacity, and excessive Fe sequestration disturbs the Fe metabolism in shoots, driving shoot physiology toward Fe-deficient conditions. As a consequence, the Fe uptake system is active in transgenic tobacco roots, suggesting that differences in the Fe concentrations between shoots and roots could affect each other. In the present study, 35S-OsZIP4 shoots showed Zn deficiency, and the genes induced by Zn deficiency were expressed in shoots. However, genes induced by Zn deficiency were repressed in 35S-OsZIP4 roots, which accumulated high levels of Zn, suggesting that the Zn concentrations between shoots and roots could not be under the control of integrated pathways.

Specificity of plant transporters may be achieved by differential regulation at transcriptional and post-transcriptional levels, as shown for IRT1 and metal transporters in other eukaryotes (Gunshin et al., 1997; Connolly et al., 2002). The 35S-OsZIP4 plants also overexpressed OsZIP4. As the antibodies to determine the OsZIP4 protein levels were not available, the metal concentration in 35S-OsZIP4 plants was measured to gain a better understanding of metal homeostasis. The 35S-OsZIP4 roots accumulated high levels of Zn even under Zn-sufficient conditions, suggesting the possibility that OsZIP4 is expressed and not regulated at the post-transcriptional level, or that the regulation may be masked.

Fig. 3. Microarray analysis of 35S-OsZIP4. The ratios of 2-fold more up-regulated genes in 35S-OsZIP4 compared with vector controls (VCs) are presented (‘OX/V up’). The ratios of 2-fold more down-regulated genes in 35S-OsZIP4 plants compared with VCs are presented (‘OX/V down’). The 2-fold higher or lower induction ratios of non-transgenic plants were calculated as the relative increases (‘-Zn/+Zn up’) or decreases (‘-Zn/+Zn down’) in expression under conditions of Zn deficiency compared with the expression under control conditions. The ratios are the means of two independent replicates.

has been demonstrated that an apparent phenotype reflecting Zn distribution, as assessed by Zn concentrations in the roots and shoots, OsZIP4 expression, and expression patterns of the genes involved in Zn homeostasis, was manifested in 35S-OsZIP4 plants even under normal growth conditions. The transgenic plants accumulated more Zn in the roots, although they collected little Zn in the shoots and exhibited symptoms of Zn deficiency. Under conditions of excess Zn, non-transgenic rice accumulated Zn not only in the roots but also in the shoots (data not shown). Therefore, it was a distinct characteristic of 35S-OsZIP4 plants that the Zn concentration was low in shoots, whereas the roots accumulated excess Zn. In wild-type plants, Zn taken up from the soil was normally translocated through the root cells and transported to the xylem or phloem for transport to shoots. In 35S-OsZIP4 plants, OsZIP4 driven by the 35S promoter in roots may, however, be involved in reabsorption of Zn which is normally transported to shoots, leading to the Zn accumulation in roots and Zn deficiency in shoots. These data indicate that OsZIP4 is essential for Zn distribution and must be tightly regulated.
by the activity of the strong and constitutive CaMV 35S promoter. IRT1 contains two intracellular loop lysine residues that could serve as binding sites for ubiquitin, and IRT1 mutated in these two lysine residues is not regulated at the post-transcriptional level (ML Guerinot, personal communication). On the other hand, OsZIP4 does not contain two such intercellular loop lysine residues, suggesting that OsZIP4 might be not regulated at the post-transcriptional level.

These results indicated that OsZIP4 is a Zn transporter responsible for the translocation of Zn within rice plants and may need to be tightly regulated. It responds to changes in Zn concentration in rice plants.

**Supplementary data**

The following supplementary material is available at JXB online.

**Table S1.** Summary of the microarray analysis in 35S-OsZIP4. The ratios of inducible genes in 35S-OsZIP4, compared with vector controls, are presented (OX/V). The induction ratios of non-transgenic plants were calculated as the relative increases or decreases in expression under conditions of Zn deficiency compared with the expression under control conditions (–Zn/+Zn). The ratios are the means of two independent replicates.
Acknowledgements

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