OsYSL6 Is Involved in the Detoxification of Excess Manganese in Rice$^{1}$$^{[W]}$[OA]

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Yellow Stripe-Like (YSL) proteins belong to the oligopeptide transporter family and have been implicated in metal transport and homeostasis in different plant species. Here, we functionally characterized a rice (Oryza sativa) YSL member, OsYSL6. Knockout of OsYSL6 resulted in decreased growth of both roots and shoots only in the high-manganese (Mn) condition. There was no difference in the concentration of total Mn and other essential metals between the wild-type rice and the knockout line, but the knockout line showed a higher Mn concentration in the leaf apoplastic solution and a lower Mn concentration in the symplastic solution than wild-type rice. OsYSL6 was constitutively expressed in both the shoots and roots, and the expression level was not affected by either deficiency or toxicity of various metals. Furthermore, the expression level increased with leaf age. Analysis with OsYSL6 promoter-green fluorescent protein transgenic rice revealed that OsYSL6 was expressed in all cells of both the roots and shoots. Heterologous expression of OsYSL6 in yeast showed transport activity for the Mn-nicotianamine complex but not for the Mn-mugineic acid complex. Taken together, our results suggest that OsYSL6 is a Mn-nicotianamine transporter that is required for the detoxification of excess Mn in rice.

A number of different transporters have been reported to be involved in the uptake, short- and long-distance transport, intracellular transport, distribution, and homeostasis of essential metals (Hall and Williams, 2003; Palmer and Guerinot, 2009). One group of these transporters is Yellow Stripe-Like (YSL) proteins. YSLs belong to the oligopeptide transporter family but have low similarity with other plant oligopeptide transporter members (Yen et al., 2001). Since the first YSL transporter (ZmYS1) for uptake of the ferric-phytosiderophore complex was identified in maize (Zea mays); Curie et al., 2001), eight and 18 members similar to ZmYS1 have been found in the Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa) genomes, respectively (Curie et al., 2009). Functional analysis of some YSL members has shown that they are not only involved in Fe uptake but are also involved in the transport and homeostasis of other transition metals such as zinc (Zn), nickel (Ni), manganese (Mn), and copper (Cu; Curie et al., 2009).

YSL members differ in their expression patterns, localization, and transport substrate specificity. ZmYS1, HvYS1 from barley (Hordeum vulgare), and OsYSL15 from rice transport the Fe(III)-phytosiderophore complex and are localized in the plasma membrane of epidermal cells in the roots (Curie et al., 2001; Murata et al., 2006; Inoue et al., 2009; Ueno et al., 2009). The expression of these transporter genes is greatly enhanced by iron (Fe) deficiency. These transporters are mainly involved in the primary uptake of Fe from the rhizosphere to the root cells, although ZmYS1 and OsYSL15 are also expressed in the shoots (Curie et al., 2001; Murata et al., 2006; Inoue et al., 2009; Lee et al., 2009). Although OsYSL18 also transports Fe(III)-deoxymugineic acid (DMA), it is expressed more in the flowers and probably responsible for the translocation of Fe in reproductive organs and phloem in joints (Aoyama et al., 2009).

By contrast, other members including OsYSL2 and AtYSL2 transport Fe(II)- and/or other metal-nicotiamine (NA) complexes, but not Fe(III)-phytosiderophore complex (DiDonato et al., 2004; Koike et al., 2004; Schaaf et al., 2005). OsYSL2 transports Fe(II)-NA complex and Mn-NA complex and is mainly expressed in the phloem cells of the vascular bundles, especially in the companion cells of Fe-deficient leaves (Koike et al., 2004). The expression of OsYSL2 is greatly induced by Fe deficiency in the leaves but not in the roots. OsYSL2 is suggested to be involved in the phloem transport of Fe and Mn, including the translocation of Fe and Mn into the rice grains (Koike et al., 2004). AtYSL2 is expressed in many cell types in both roots and shoots (DiDonato et al., 2004; Schaaf et al., 2005). AtYSL2 showed transport activity in yeast for Cu-NA and Fe (II)-NA in one study (DiDonato et al., 2004) but not in another study (Schaaf et al., 2005). Interestingly, the expression of AtYSL2 was down-regulated by Fe deficiency (DiDonato et al., 2004; Schaaf et al., 2005). AtYSL2, therefore, is suggested to be involved in the lateral movement of metals in the vasculature or in Fe transport.
and Zn homeostasis (DiDonato et al., 2004; Schaaf et al., 2005). A recent study showed that AtYSL1 and -3 are able to transport Fe(II)-NA (Chu et al., 2010). Furthermore, AtYSL3 but not AtYSL1 also transports Fe(III)-phytosiderophore in yeast, although Arabidopsis does not synthesize phytosiderophore. The expression patterns of AtYSL1 and AtYSL3 are similar to AtYSL2, which is down-regulated by Fe deficiency but up-regulated by Fe excess (Le Jean et al., 2005; Waters et al., 2006). AtYSL1 and -3 are responsible for the mobilization of micronutrients such as Mn, Zn, Cu, and Fe from leaves and for loading of Fe-NA into the seeds (Le Jean et al., 2005; Waters et al., 2006).

Three YSLs were also isolated from a metal hyperaccumulator, Thlaspi caerulescens, and one of them, TcYSL3, is an Fe/Ni-NA influx transporter (Gendre et al., 2007), which is suggested to be involved in the entry of Ni-NA into the symplastic transport in the roots for delivery to the xylem and unloading of the Ni-NA complexes from the xylem in the leaves. More recently, HvYSL5 from barley was characterized (Zheng et al., 2011). The expression of this gene is strongly induced in the root by Fe deficiency and shows diurnal rhythm. HvYSL5 is localized at all root cells, but the transport substrate and exact function of this protein are unknown. All these findings indicate that YSLs have diverse functions in the transport of various metals.

In this study, we examined the function of an uncharacterized YSL member, OsYSL6 in rice. We found that, different from other YSL members, OsYSL6 is required for tolerance to excess Mn in rice.

RESULTS
Structure of the OsYSL6 Gene
OsYSL6 contains seven exons and six introns (Supplemental Fig. S1A), encoding a protein with 678 amino acids. OsYSL6 shares 81% identity and 83%
similarity with HvYSL5, the closest homolog in the YSL family (Zheng et al., 2011). Prediction with the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) showed that OsYSL6 is a membrane protein with 12 transmembrane domains (Supplemental Fig. S1B).

Phenotype of the OsYSL6 Knockout Line

To investigate the role of OsYSL6, we obtained a Tos-17 insertion line (NC6189; osysl6). Tos-17 was inserted in the sixth exon (Supplemental Fig. S1A). No transcript of OsYSL6 was detected in this line (Supplemental Fig. S1C), indicating that this is a knockout line of OsYSL6.

We first investigated the response of the wild type (cv Nipponbare) and the osysl6 knockout line to the deficiency and excess of various metals. When the plants were subjected to different Fe concentrations ranging from deficiency (0.05 μM) to excess (1,000 μM), both the wild-type rice and the knockout line showed Fe deficiency-induced chlorosis in the Fe deficiency condition (Supplemental Fig. S2A) and reduced growth in the Fe excess condition (Supplemental Fig. S2D). However, there was no difference in growth between the wild-type rice and the knockout line at all Fe concentrations tested (Supplemental Fig. S2). The difference in growth was also not observed between two lines treated with other metals, including Zn and Cu (data not shown). However, when the plants were subjected to different Mn concentrations, the growth of osysl6 did not differ from the wild-type rice at Mn concentrations up to 100 μM but was reduced at 1,000 μM (Fig. 1). At 1,000 μM Mn treatment, the fresh weight of the shoots and roots in the knockout line was 58% and 53%, respectively, of the wild type (Fig. 2).
older true leaf of the knockout line exhibited necrosis, a typical Mn toxicity symptom (Fig. 1F), which was not observed in the wild-type rice (Fig. 1E).

Complementation Test

To confirm that disruption of OsYSL6 was responsible for the increased Mn sensitivity in the osysl6 mutant observed above (Figs. 1 and 2), we conducted a complementation test by introducing a DNA fragment containing the OsYSL6 promoter region, the entire open reading frame, and the 3’ untranslated region into the knockout line by Agrobacterium tumefaciens-mediated transformation. Analysis with two independent transgenic lines (lines 1 and 2) showed that there was no difference in the growth among transgenic lines, the wild type, and the osysl6 mutant at 0.5 μM Mn (Fig. 3A). At 1,000 μM Mn, although the growth of all lines was inhibited, the inhibition was alleviated in the transgenic lines compared with the knockout line (Fig. 3B). These results indicated that OsYSL6 is a responsible gene for the phenotype observed in the mutant.

Tissue Mineral Analysis

We compared the concentrations of Mn, Fe, Cu, and Zn in the roots and shoots of wild-type rice and the knockout line exposed to different Mn concentrations. The Mn concentrations in both the roots and shoots increased with increasing external Mn concentrations (Fig. 4, A and C), but there was no significant difference between wild-type rice and the knockout line at all Mn concentrations tested, although the growth of the knockout line was inhibited at 1,000 μM Mn (Figs. 1 and 2). The concentration of Fe tended to decrease with increasing Mn concentrations in the external solution in both the roots and shoots, but there was no difference between wild-type rice and the knockout line.
Differences in the concentrations of Cu and Zn were also not found (Supplemental Fig. S3). Since the Mn toxicity symptom appeared in the old leaf (Fig. 1), we then determined the concentrations of Mn, Fe, Cu, and Zn in the oldest true leaf of both wild-type rice and the knockout line. The Mn concentration in this leaf reached as high as 4,000 mg kg\(^{-1}\), but there was no significant difference between the two lines (Supplemental Fig. S4A). Differences in the Fe, Zn, and Cu concentrations between the two lines were also not observed (Supplemental Fig. S4, B–D).

**Apoplastic and Symplastic Mn Concentrations**

We compared Mn concentrations in the apoplast and symplast of the old leaf between wild-type rice and the knockout line according to the method of Fuhrs et al. (2010). Purity check showed that the activity of malic dehydrogenase in the apoplastic solution was only 1% of the total activity, indicating that the apoplastic solution was hardly contaminated by symplastic solution. The knockout line showed a 3-fold higher Mn concentration in the apoplastic solution than wild-type rice (Fig. 5A). By contrast, the Mn concentration in the symplastic solution of the knockout line was lower than that of wild-type rice (Fig. 5B), being 57% of the wild-type rice.

**Expression Pattern of OsYSL6**

The expression level of OsYSL6 in both the shoots and roots was determined by absolute quantitative real-time reverse transcription (RT)-PCR. OsYSL6 showed 9.1 \(\times\) 10\(^3\) and 7.1 \(\times\) 10\(^3\) copies ng\(^{-1}\) RNA in the shoots and roots, respectively (Fig. 6A).

A dose-response experiment with different Mn concentrations showed that OsYSL6 expression was not induced by either Mn excess or deficiency in both the roots and shoots (Fig. 6, B and C). The expression did not respond to the deficiency of Zn, Fe, Mn, and Cu in both the roots and shoots (Fig. 6, D and E). These trends were unchanged when the expression was normalized based on two other internal standards (Actin and Ubiquitin; Supplemental Figs. S5 and S6). These results indicate that OsYSL6 was constitutively expressed in the shoots and roots.

The expression of OsYSL6 in different leaves was further investigated in plants treated with 500 \(\mu M\) Mn. The expression level increased with leaf age: old leaf (leaf 1) showed higher expression, while young leaf (leaf 5) showed lower expression (Fig. 7A). This trend is similar to Mn concentration in the different leaves (Fig. 7B).

**Figure 7.** Expression levels of OsYSL6 and Mn concentrations in different leaves. A, Expression pattern of OsYSL6 in leaves 1, 3, and 5 (numbered from the bottom). B, Mn concentrations in different leaves. Seedlings (cv Nipponbare) were grown in a nutrient solution containing 500 \(\mu M\) MnCl\(_2\) for 3 weeks. The expression level was determined by real time RT-PCR and Mn by atomic absorption spectrometry. Expression levels relative to leaf 5 are shown. Data are means of three biological replicates.

**Apoplast and Symplast of OsYSL6 Expression**

We investigated the tissue expression pattern of OsYSL6 by using GFP reporter constructs. The expression was constitutively observed in the shoots and roots (Fig. 8A and B). Immunostaining of the leaves showed a similar expression pattern (Fig. 8C and D). Immunostaining was performed by using an antibody against GFP. Bars = 100 \(\mu m\).
Tissue Expression Profile of OsYSL6

To investigate the tissue expression profile of OsYSL6, we introduced the OsYSL6 promoter region fused with GFP into wild-type rice (cv Nipponbare) by Agrobacterium-mediated transformation. The localization of GFP was examined by immunostaining using an anti-GFP antibody. In OsYSL6 promoter-GFP transgenic rice, the GFP signal was observed in all cells of both the roots and leaves (Fig. 8, A and C). No fluorescence signal was detected in the wild-type rice (Fig. 8, B and D).

Transport Activity of OsYSL6 in Yeast

Since the knockout line of OsYSL6 only showed a clear phenotype at high Mn concentration (Figs. 1 and 2), we tested the transport activity of this protein for Mn complexed with NA and mugineic acid in a yeast mutant (Δsmf1) defective in Mn uptake (Supek et al., 1996). When gene expression was induced in the presence of Gal, the yeast carrying OsYSL6 showed higher transport activity for Mn-NA complex compared with vector control at 20 min and thereafter (Fig. 9A). However, this activity disappeared when gene expression was not induced in the presence of Glc (Fig. 9B). The OsYSL6-expressing yeast did not show transport activity for Mn-DMA complex (Fig. 9C). These results suggested that OsYSL6 is a transporter for Mn-NA complex.

DISCUSSION

OsYSL6 Is a Mn-NA Transporter Required for the Detoxification of Excess Mn

Among 18 members of YSLs in rice, OsYSL6 forms a separate cluster with OsYSL5 (Curie et al., 2009; Zheng et al., 2011). Knockout of OsYSL6 only resulted in increased sensitivity to high Mn concentration (Figs. 1 and 2) but did not affect the sensitivity to either deficiency or toxicity of other metals, including Fe, Cu, and Zn (Supplemental Fig. S2). These results revealed that, different from other members characterized, OsYSL6 is involved in the detoxification of excess Mn in rice.

OsYSL6 shows distinct expression patterns, cell specificity of localization, and transport substrate specificity. OsYSL6 is expressed at high levels in both the roots and shoots (Fig. 6A). Unlike other YSL members, whose expression is up- or down-regulated by Fe deficiency, OsYSL6 is constitutively expressed in both the roots and shoots, irrespective of deficiency or excess treatment with metals (Fig. 6, B–E; Supplemental Figs. S5 and S6). Although HvYSL5 is the closest homolog of OsYSL6 (Zheng et al., 2011), they also show different expression patterns. Unlike OsYSL6 (Fig. 6), HvYSL5 is only expressed in the roots, not in the shoots, and the expression is greatly induced by Fe deficiency (Zheng et al., 2011), suggesting a different function from OsYSL6. Interestingly, the expression increased with leaf age (Fig. 7A). The older leaf accumulates more Mn than the young leaf (Fig. 7B). How-

Figure 9. Transport activity of OsYSL6 in yeast. Time-dependent uptake of Mn into yeast cells is shown. Yeast strain Δsmf1 transformed with empty vector pYES2 or OsYSL6 was cultivated in the presence of 2% Gal (A and C) or Glc (B) for 2 h and then exposed to a solution containing 5 μM Mn-NA (A and B) or Mn-DMA (C) for 20, 40, 60, or 120 min. Mn concentration was determined by atomic absorption spectrometry. Data are means of three biological replicates.
ever, it is unclear whether increased expression is associated with high Mn concentration in the old leaves.

OsYSL6 is expressed in all cells of the roots and shoots (Fig. 8). This cell specificity of localization is also different from other YSL members. For example, HvYSL1 and ZmYSL1 are localized at root epidermal cells (Murata et al., 2006; Ueno et al., 2009). OsYSL2 is mainly localized in the phloem cells of the vascular bundles (Koike et al., 2004). These differences in their cell specificity of localization suggest that they play different roles in different cells.

Heterologous expression of OsYSL6 in yeast indicates that this protein is able to transport Mn-NA complex, but not Mn-DMA complex (Fig. 9). So far, only OsYSL2 was reported to have transport activity for Mn-NA complex in *Xenopus laevis* oocytes (Koike et al., 2004). However, the role of this gene in rice Mn nutrition is unknown. The similarity between OsYSL2 and OsYSL6 is only 54%. The mechanism controlling transport substrate specificity is unknown and remains to be examined in the future.

Possible Mechanisms of OsYSL6 in the Detoxification of Excess Mn

Rice is usually cultivated under flooded conditions, where Mn concentration in soil solution is very high due to reduction. To adapt these environments, rice has developed strategies to accumulate and tolerate high Mn in leaves. For example, barley shows Mn toxicity symptoms when it accumulates 150 µg g⁻¹ dry weight, whereas rice can accumulate Mn to more than 5,000 µg g⁻¹ dry weight without showing any toxicity symptoms (Vlamis and Williams, 1964). In this study, when the wild-type rice was grown at 1,000 µM Mn, the Mn concentration was about 5,000 µg g⁻¹ in the shoot (Fig. 4), but no Mn toxicity symptom was observed (Fig. 1), supporting high Mn tolerance in rice. In Arabidopsis, several transporters such as cation/Mn²⁺ transporter and the cation diffusion facilitator family have been reported to be involved in the detoxification of excess Mn (Hirschi et al., 2000; Schaaf et al., 2004). However, the role of this gene in rice Mn nutrition is unknown. The similarity between OsYSL2 and OsYSL6 is only 54%. The mechanism controlling transport substrate specificity is unknown and remains to be examined in the future.

In conclusion, OsYSL6 is a Mn-NA transporter that is required for the detoxification of high Mn in both the roots and shoots. Knockout of this gene causes the accumulation of Mn in the apoplast, resulting in Mn toxicity.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

We obtained a Tos-17 insertion line (NC6189) of *OsYSL6* from the Rice Genome Resource Center of Japan. Seeds of the wild-type rice (*Oryza sativa* ‘Nipponbare’) and the Tos-17 insertion homozygous line (designated *osyl6* hereafter) were soaked in deionized water overnight at 30°C and then transferred to a net floating on a 0.5 mM CaCl₂ solution. After 7 d, the seedlings were transferred to a 3.5-L plastic pot containing half-strength Kimura B solution (pH 5.6) containing 0.18 mM (NH₄)₂SO₄, 0.27 mM MgSO₄·7H₂O, 0.09 mM KNO₃, 0.18 mM Ca(NO₃)₂·4H₂O, 0.09 mM KH₂PO₄, 0.5 mM K₂HPO₄, 0.5 mM MnCl₂·4H₂O, 3 mM H₂BO₃, 1 mM (NH₄)₆Mo₇O₂₄·4H₂O, 0.4 mM ZnSO₄·7H₂O, 0.2 mM CuSO₄·5H₂O, and 10 µM FeSO₄. This solution was renewed every 2 d. These seedlings were grown in a greenhouse at 25°C to 30°C under natural light. All experiments were conducted with three biological replicates and repeated at least twice.

**Phenotypic Analysis of the Knockout Line**

The seedlings described above of both wild-type rice and the knockout line were grown in the nutrient solution with different concentrations of Fe (0.05–1,000 µM), Mn (0.05–1,000 µM), Zn (0–4 µM), and Cu (0–2 µM). The solution was renewed every 2 d. After 21 d, the roots were washed with distilled water three times and separated from the shoots with a razor. The fresh weight of both the roots and shoots was recorded. The samples were dried at 70°C for 3 d and used for mineral determination as described below. In a separate experiment, the seedlings were exposed to 1,000 µM Mn for 21 d and the oldest true leaf was sampled for analysis of metals.
Complementation Test

For the complementation test of the osys6 knockout line, we amplified a 7,000-bp DNA fragment containing the OsYSL6 promoter region (2,164 bp), the OsYSL6 genomic DNA (4,196 kb), and the 3' untranslated region (640 bp) from the Nipponbare genomic DNA by PCR. The primers used were 5'-TGTCAGACCTGTCGCCGCTACG-3' and 5'-TGACTCCAGGCAACAATTGCCTTCCTACG-3'. After sequence confirmation, this clone was inserted into the pZP2Phlac vector and transformed into Agrobacterium tumefaciens (strain EHA101). Calluses derived from the Tos-17 insertion line were transformed by Agrobacterium-mediated transformation. Growth of 1,000 μM Mn was compared between two independent transgenic lines, the wild-type line, and the Tos-17 insertion line as described above.

Determination of Metals in Plant Tissues

The dried samples were digested with concentrated HNO₃ (60%) at a temperature up to 140°C. The metal concentration in the digest solution was determined by atomic absorption spectrometry (Z-2000; Hitachi) after dilution.

Extraction of Apoplastic and Symplastic Solutions of Leaf Blades

Extraction of apoplastic and symplastic solutions of the leaf blades was performed according to Fuhrs et al. (2010). Briefly, 10 leaf blades from each pot were cut from plants exposed to 500 μM Mn for 3 days and washed three times with distilled water. The leaves were then infiltrated with distilled water by centrifugation at 8,000 g for 5 min. The infiltrated leaf blades were transferred to a centrifugil filter device and centrifuged at 8,000 g for 5 min to obtain the apoplastic solution. The leaves were then frozen at −80°C for 4 h. To collect symplastic solution, the frozen leaf blades were thawed at room temperature for a short time and then centrifuged at 14,000 g for 5 min. The Mn concentration in the apoplastic and symplastic solutions was determined as described above.

To check the purity of the apoplastic solution, the activity of malic dehydrogenase in the apoplastic and symplastic solutions was determined according to Bergmeyer and Bernt (1974).

Expression Pattern

The expression levels of OsYSL6 in the roots and shoots were determined by absolute real-time RT-PCR. Both the roots and shoots of rice grown under normal conditions were sampled for RNA extraction. A 10-ng portion of the first-strand cDNA was used as the template. The primers used were 5'-GTTTGTCTGGAGGAGGTTAAG-3' and 5'-GATCTGACCTTCCGCG-3'. To generate a standard curve for absolute quantification, a series of dilutions (from 1 × 10⁻¹ to 10⁻⁹ ng) of plasmids was made and then subjected to real-time PCR. Threshold cycle values for each sample were converted into absolute copy numbers using the standard curves.

To investigate the response of OsYSL6 to different metal deficiencies, the seedlings (14 d old) were grown in a nutrient solution without Fe, Zn, Cu, or Mn for 7 d and then the roots and shoots were sampled for RNA extraction. The effects of different Mn concentrations on OsYSL6 expression were investigated by exposing the seedlings (14 d old) to 0.05, 0.5, 100, or 1,000 μM Mn for 21 d. True leaves 1, 3, and 5 (numbered from the bottom) were also sampled for leaf age-dependent expression analysis. All samples were frozen in liquid nitrogen for RNA extraction. Total RNA was extracted by using an RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using the SuperScript II kit (Invitrogen), following the manufacturer’s instructions, with an oligo(dT)₁₅ primer. Expression was determined with SYBR Premix Ex Taq (Takara) on Mastercycler ep realplex (Eppendorf). The primer sequences for RT-PCR of OsYSL6 were 5'-GTTTGTCTGGAGGAGGTTAAG-3' and 5'-GATCTGACCTTCCGCG-3'. These primers contained Xho and NotI sites to facilitate cloning of the amplified cDNA. The fragment was first cloned into the pGEM-T vector (Promega). After sequence confirmation, the OsYSL6 cDNA with BamHI and Xhol was excised for cloning into pYES2 (Invitrogen). The resulting plasmid or the empty vector pYES2 was introduced into yeast strain smf7 (MATa his3Δ2 metSΔ50 ura3Δ0 YOL122::KanMX4) according to the manufacturer’s protocols (9C Easy Comp Transformation Kit; Invitrogen). Transformants were selected on uracil-deficient medium and grown in synthetic complete (SC-uracil) yeast medium containing 2% Glc, 0.67% yeast nitrogen base without amino acids (Difco), 0.2% appropriate amino acids, and 2% agar at pH 6.5. One colony was selected in each transformation strain and grown in the liquid SC-uracil medium. For measurement of Mn uptake, cells at midexponential phase were harvested and transferred to a minus-metal medium (BIO 101 Systems). Gene expression was induced by 2% Gal or suppressed by 2% Glc as a control for 2 h before the uptake experiment. The cells at optical density of 3.0 were exposed to 5 μM Mn-NA or Mn-DMA. At 20, 40, 60, and 120 min of incubation with gentle shaking, cells were harvested by centrifugation and washed three times with deionized water (MilliQ; Millipore). After drying at 70°C, the samples were digested with 2 N HCl. Mn concentration was determined as described above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB673449.

Immunohistological Staining

To investigate the cellular localization of OsYSL6, we introduced a construct consisting of the promoter (2.14 kb) of OsYSL6 fused with GFP to rice (cv Nipponbare). The 2.14-kb promoter region from the initiation codon of OsYSL6 was amplified by PCR from Nipponbare genomic DNA using primers 5'-GGATCCCTATGCTGCTGATAGA-3' and 5'-CTCCAGCGGGAAATCGCTTCCTCCTC-3'.

Using KmPl and Xhol, the amplified fragment was cloned into pZP2Phlac carrying GFP and the terminator of the napaline synthase gene, producing the OsYSL6 promoter-GFP construct. The construct was transformed into Agrobacterium (strain EHA101). Calluses derived from the wild type (cv Nipponbare) were transformed by Agrobacterium-mediated transformation.

Immunostaining was performed with roots and leaves of the wild-type rice and the transgenic plant carrying OsYSL6 promoter-GFP as described previously (Yamaji et al., 2008) using an antibody against GFP (A11122; Molecular Probes).

Transport Activity of OsYSL6 in Yeast

The cDNA fragment containing an entire OsYSL6 open reading frame was amplified by RT-PCR using the primers 5'-GGATCCAAATTGCGGTGCGCGGGACAG-3' and 5'-CTCCAGCTCAGCGTAAAGGGATGCGTGA-3'. These primers contained BamHI and Xhol sites to facilitate cloning of the amplified cDNA. The fragment was first cloned into the pGEM-T vector (Promega). After sequence confirmation, the OsYSL6 cDNA with BamHI and Xhol was excised for cloning into pYES2 (Invitrogen). The resulting plasmid or the empty vector pYES2 was introduced into yeast strain smf7 (MATa his3Δ2 metSΔ50 ura3Δ0 YOL122::KanMX4) according to the manufacturer’s protocols (9C Easy Comp Transformation Kit; Invitrogen). Transformants were selected on uracil-deficient medium and grown in synthetic complete (SC-uracil) yeast medium containing 2% Glc, 0.67% yeast nitrogen base without amino acids (Difco), 0.2% appropriate amino acids, and 2% agar at pH 6.5. One colony was selected in each transformation strain and grown in the liquid SC-uracil medium.

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Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Structure and Tos-17 insertion line of OsYSL6.

**Supplemental Figure S2.** The response of the wild type and knockout line to the Fe deficiency or excess.

**Supplemental Figure S3.** Concentration of Zn and Cu in the shoots.

**Supplemental Figure S4.** Comparison of metal concentration in the oldest true leaf between wild-type rice and OsYSL6 knockout line.

**Supplemental Figure S5.** Effect of different Mn concentrations on OsYSL6 expression.

**Supplemental Figure S6.** Response of OsYSL6 expression to metal deficiency.

**Supplemental Figure S7.** Subcellular localization of OsYSL6-GFP fusion protein.

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LITERATURE CITED


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