Rice OsYSL15 Is an Iron-regulated Iron(III)-Deoxymugineic Acid Transporter Expressed in the Roots and Is Essential for Iron Uptake in Early Growth of the Seedlings

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Gymnastic plants take up iron through YS1 (yellow stripe 1) and YS1-like (YSL) transporters using iron-chelating compounds known as mugineic acid family phytosiderophores. We examined the expression of 18 rice (Oryza sativa L.) YSL genes (OsYSL1-18) in the epidermis/exodermis, cortex, and stele of rice roots. Expression of OsYSL15 in root epidermis and stele was induced by iron deficiency and showed daily fluctuation. OsYSL15 restored a yeast mutant defective in iron uptake when supplied with iron(III)-deoxymugineic acid and transported iron(III)-deoxymugineic acid in Xenopus laevis oocytes. An OsYSL15-green fluorescent protein fusion was localized to the plasma membrane when transiently expressed in onion epidermal cells. OsYSL15 promoter-β-glucuronidase analysis revealed that OsYSL15 expression in roots was dominant in the epidermis/exodermis and phloem cells under conditions of iron deficiency and was detected only in phloem under iron sufficiency. These results strongly suggest that OsYSL15 is the dominant iron(III)-deoxymugineic acid transporter responsible for iron uptake from the rhizosphere and is also responsible for phloem transport of iron. OsYSL15 was also expressed in flowers, developing seeds, and in the embryonic scutellar epithelial cells during seed germination. OsYSL15 knockdown seedlings showed severe arrest in germination and early growth and were rescued by high iron supply. These results demonstrate that rice OsYSL15 plays a crucial role in iron homeostasis during the early stages of growth.

Iron is essential for virtually all living organisms. Iron deficiency is the most widespread human nutritional problem in the world. There are two billion anemic people worldwide, and ~50% of all anemia cases can be attributed to iron deficiency (1). In plants, iron plays a key role in electron transfer in both photosynthetic and respiratory reactions in chloroplasts and mitochondria. Although abundant in mineral soils, iron is sparingly soluble under aerobic conditions at high soil pH. Consequently, plants grown on calcareous soils often exhibit severe chlorosis because of iron deficiency, which is a major agricultural problem resulting in reduced crop yields (2).

Higher plants have two strategies for the uptake of oxidized Fe(III) from the rhizosphere (3). All higher plants except graminaceous plants take up iron by using ferric-chelate reductases to reduce ferric iron to Fe(II), which is absorbed by ferrous iron transporters (strategy I (4–6)). Alternatively, graminaceous plants secrete iron chelators called mugineic acid family phytosiderophores (MAs4) from their roots to solubilize iron in the rhizosphere (strategy II (3, 7, 8)). These chelating MAs have six coordination sites (three −COOH, two −NH, and one -OH) that bind to iron and are thought to form octahedral Fe(III) complexes. Gramineous plants then take up the iron as Fe(III)-MAs complexes from the rhizosphere through specific transporters (9, 10).

The biosynthetic pathway for MAs in graminaceous plants has been elucidated (8, 11–14). Methionine, the precursor of MAs (11), is converted to 2′-deoxymugineic acid (DMA) via several enzymatic reactions. Whereas rice (Oryza sativa L.) and maize (Zea mays L.) secrete DMA, other species, including barley (Hordeum vulgare L.) and rye (Secale cereale L.), further hydroxylate DMA to other MAs.

The production and secretion of MAs markedly increase in response to iron deficiency. The secretion of MAs in barley follows a distinct diurnal rhythm with a peak just after sunrise or initial illumination (15). The diurnal rhythm of the secretion of MAs in rice has not been fully characterized.

The genes that encode the biosynthetic enzymes in the reaction pathway converting S-adenosylmethionine to MAs have been isolated and characterized in barley (HvNASI-7, NASHORI-2, HvNAAT-A, and -B, and HvDMASI (16–19)).

4 The abbreviations used are: MA, mugineic acid family phytosiderophore; DMA, 2′-deoxymugineic acid; GFP, green fluorescent protein; GUS, β-glucuronidase; VC, vector control; MS, Murashige and Skoog medium; RT, reverse transcription; ORF, open reading frame; MES, 4-morpholineethanesulfonic acid; NA, nicotianamine; TM, transmembrane domain; IDEF, iron deficiency-responsive element-binding factor; CBS, circadian clock-associated 1-binding site.
and rice (OsNAS1–3, OsNAAT1, and OsDMAS1 (19–21)). Expression of these genes is strongly induced in response to iron deficiency. In rice, histochemical analysis of promoter-β-glucuronidase (GUS) transformants revealed that OsNAS1, OsNAS2, OsNAAT1, and OsDMAS1 share highly similar expression patterns, with significant expression in all cells of iron-deficient roots especially in the companion and pericycle cells (19, 21, 22), where DMA is thought to be synthesized.

Cloning of the maize YSI (yellow stripe 1) gene (10) led to the identification of the specific transporters responsible for uptake of iron-chelated MAs complexes from the rhizosphere into root cells. The maize ysi1 mutant is defective in Fe(III)-MAS uptake (23). YSI expression is increased in both roots and shoots under conditions of iron deficiency, but it is not strongly affected by zinc or copper deficiency (10, 24). Schaaf et al. (25) investigated the transport properties of YSI1 in Xenopus oocytes by electrophysiological analysis. YSI1 functions as a proton-coupled symporter for various DMA-bound metals including Fe(III), Zn(II), Cu(II), and Ni(II). YSI also transports nicotianamine (NA)-chelated Ni(II), Fe(II), and Fe(III) complexes. Recently, a barley homolog of YSI1 (HvYS1) has been identified (26). In contrast to YSI1, HvYS1 is highly specific for Fe(III)-MAs while demonstrating a low transport activity for MAs chelated to Zn(II), Cu(II), Ni(II), or Co(II). Non-graminaceous plants also possess YSI1-like (YSL) genes that encode transporters considered to play important roles in internal metal homeostasis by transporting metal-NA complexes, as non-graminaceous plants synthesize NA but not MAs (27–31).

Our previous search for YSI homologs in the rice genome data base identified 18 putative OsYSL genes (32). Among these are OsYSL2 transports Fe(II)-NA and Mn(II)-NA but not Fe(III)-MAs (32). OsYSL2 expression is strongly induced in iron-deficient leaves with particularly strong expression in the phloem cells of the leaves and leaf sheaths. These results suggest that OsYSL2 functions as an Fe(II)-NA transporter responsible for the phloem transport of iron (32). To date, no rice OsYSL with a transport activity for Fe(III)-MAS has been identified. Here we describe that OsYSL with a transport activity for Fe(III)-MAs has been identified. OsYSL15 for transport of iron during early seedling growth is essential for the phloem transport of iron. An essential role of the Fe(III)-DMA transporter, whose expression pattern strongly suggested by the results presented here.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Nontransgenic and transgenic rice seeds were germinated on Murashige and Skoog (MS) medium and transferred to nutrient solution (11, 22) in a greenhouse with 30 °C light/25 °C dark periods under natural light conditions. The pH of the culture solution was adjusted daily to 5.3 with 1 M HCl. For micronutrient deficiency treatments, 3-week-old plants were transferred to nutrient solution without iron, zinc, manganese, or copper and grown for 3 more weeks. Time course expression analysis was carried out as described previously (33). Plants were grown under iron-deficient conditions for 2 weeks and were harvested at 3-h intervals after the lights were turned on (0 h). The lights were turned off at 14 h and were turned on again at 24 h. Flowers and seeds were obtained from iron-sufficient rice plants.

**Laser Microdissection and Expression Analysis**—Laser microdissection was used to examine tissue-specific expression (34). Iron-sufficient and iron-deficient roots were fixed by a 10-min infiltration of 3:1 ethanol:acetic acid into the tissues under vacuum on ice. The vials containing the samples in the fixative were then gently mixed on a rotator at 4 °C for 1 h. This fixation step was repeated twice with a fresh solution of fixative. The fixed samples were then transferred to 10% sucrose solution, and the tissues were infiltrated with this solution under vacuum for 10 min on ice, followed by gentle mixing on a rotator at 4 °C for 1 h. After replacement with a fresh sucrose solution, the vials were gently mixed overnight on a rotator at 4 °C. The fixed samples were then embedded in diethyl pyrocarbonate water, frozen in a dry ice hexane bath, and stored at −80 °C. The tissues were sectioned into 20-µm thick slices in a cryostat (Leica CM1850) and mounted on polyethylene foil pretreated with a tissue-adhesive solution of 0.1% poly-L-lysine (Sigma) at −30 °C. The foil was attached to a glass slide and dried at room temperature.

The sections were laser-microdissected with a Leica AS LMD (Leica Microsystems). Target tissues were selected and dissected from the sections (Fig. 1, a–d). Twenty sections of the epidermis/exodermis, cortex, and stele were dissected and separately collected in 0.5-ml sample tubes. To avoid contamination, we confirmed the identity of the tissues in each tube at every step.

Total RNA was extracted from the microdissected samples using an RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer’s instructions. The amount of total RNA was measured fluorometrically using a RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR) with 485 nm excitation and 530 nm emission wavelengths according to the manufacturer’s instructions. Total RNA (15 ng) was used to synthesize first-strand cDNA using oligo(dT) primer and avian myeloblastosis virus reverse transcriptase XL (Takara, Japan). One-twentieth of the resulting cDNA sample was used in a 25-µl PCR with the specific primers shown in supplemental Table S1. As a control, rice actin-specific primers were used. The PCR products were analyzed by electrophoresis in agarose gels.

**Yeast Complementation**—The following strains of Saccharomyces cerevisiae were used in this study: CM3260 (parent strain) MATa trp1-63 leu2-3, 112 gen4-101 his3-609 ura3-52, and DEY1453 (fet3 fet4 mutant) MATa/MATa ade2+/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::His3/fet3-2::His3 fet3-4::LEU2/fet4-1::LEU2. Yeast cells were grown in YPD (1% yeast extract, 2% peptone and 2% glucose) or SD medium supplied with the appropriate amino acids. Agar was added to 2% for solid plate media. Fe(III)-DMAS complexes were prepared as described previously (25); Fe(III)-DMA was prepared by mixing appropriate amounts of a 10 mM FeCl3 solution, pH < 2, and MES/Tris buffer (pH 7.5) and 100 mM DMA for 2 h at room temperature. The chelate solution was filtered through an Amicon ultrafilter MC 0.22-µm filter unit (Millipore) to remove precipitated iron.

The yeast expression vectors pDR195 and pDR195-YS1 (29) were the kind gifts of Dr. Nicolaus von Wirén (University of...
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Hohenheim, Germany). Restriction sites (XbaI, BamHI, and Xhol) were generated in the multicloning site using the following oligonucleotide linkers: 5’-GTCGACTCTAGAGATCCCTCGAGTAAGATCT-3’ and 5’-AGATCTTCACTCGAGGATCTCTTACTCTTAGATGCCGAC-3’. For complementation assays, single colonies of transformed yeast using the lithium acetate transformation method (35). Yeast cells were transformed using the lithium acetate transformation method (35). Yeast cells were transformed using the lithium acetate transformation method (35).

Vector to form pDR195-OsYSL15. Yeast cells were transformed using the lithium acetate transformation method (35).

OsYSL15/H11032 CCGCTAAGCGAGATCGACGC-3’ and the reverse primer 5’-GAGAGAAGCCCTTGTAGCATCGTACCTCGAG-3’.

The amplified and verified fragment was excised using HindIII and XbaI and subcloned upstream of the uidA ORF, which encodes GUS, in the pIG121Hm vector (38). An Agrobacterium tumefaciens strain (C58) carrying the above construct was used to transform rice (O. sativa L. cv. Tsukinohikari) as described previously (20). T1 seeds were germinated and cultured as described above and subjected to GUS expression analysis as described previously (22). Histochemical staining during seed germination was carried out as described previously (39).

Generation and Characterization of OsYSL15 Knockdown Rice—To suppress OsYSL15 expression, a 209-bp fragment of the OsYSL15 gene was amplified by PCR with the primers OsYSL15f 5’-CACCTGGAAGCTAAGAGGTAGTGT-GTT-3’ and OsYSL15r 5’-ATGCCAACACTAAAACAAATTCTT-CAGT-3’, and the amplified fragment was cloned into a Gateway pENTR/D-TOPO cloning vector (Invitrogen). The verified fragment was transferred into pIG121-RNAi-DEST (40) by an LR clonase reaction (Invitrogen), and used for rice transformation.

Transgenic OsYSL15 knockdown (OsYSL15i) seeds were germinated on standard MS medium, iron-free MS medium, or MS medium with 10% (v/v) of Tetsuriki-aqua (containing ~5 mM Fe(II)-citrate; Aichi Steel, Aichi, Japan (41)). For quantitative RT-PCR analysis of OsYSL15, seeds were first germinated on MS medium with 10% (v/v) of Tetsuriki-aqua for 6 days and then transplanted to iron-free MS medium for 6 days.

RESULTS

Microdissection Expression Analysis of OsYSLs in Root Tissues—We previously used TBLASTN to search the rice (O. sativa L. ssp. Japonica cv. Nipponbare) genomic data base, resulting in 18 potential OsYSL genes (32). To estimate possible functions of the 18 OsYSL genes, we performed RT-PCR in the epidermis/exodermis, cortex, and stele of iron-sufficient and -deficient roots (Fig. 1). Tissues were isolated using laser microdissection (34) (Fig. 1, a–d). Expression of OsYSL15 and OsYSL16 was induced in the epidermis/exodermis and stele of iron-deficient roots (Fig. 1e). Expression of OsYSL5, OsYSL6, OsYSL7, OsYSL14, and OsYSL17 was detected in all tissues of both iron-sufficient and -deficient roots. Expression of OsYSL8 was down-regulated by iron deficiency, and expression of OsYSL12 was detected in the cortex and stele under both iron-sufficient and -deficient conditions. Expression of OsYSL13 was detected in both iron-sufficient and -deficient cortex. No expression of OsYSL1, OsYSL2, OsYSL3, OsYSL4, OsYSL9, OsYSL10, OsYSL11, and OsYSL18 was detected in any of the tissues analyzed. We chose to focus on the OsYSL15 gene because its expression was strongly up-regulated in the root epidermis under conditions of iron deficiency, indicating that its encoded protein is likely to play a role in iron uptake from the rhizosphere.

Sequence and Protein Structure of OsYSL15—The OsYSL15 cDNA has a 199-bp open reading frame (ORF). The gene is located on one arm of chromosome 2 and has seven exons, as do YSL1 and OsYSL2 (10, 32). Notably, the length of each exon of OsYSL15 is comparable with the exons of OsYSL2. OsYSL15
OsYSL15 Transports Fe(III)-DMA Complexes—A yeast complementation assay using a fet3 fet4 mutant defective in high and low affinity iron uptake (43) was used to identify the transport activity of OsYSL15. OsYSL15 rescued the growth defect of the fet3 fet4 mutant on SD medium containing Fe(III)-DMA complexes (Fig. 3a). Maize YS1 also rescued the yeast strain from the growth defect, but the control vector did not, consistent with previous reports (10, 25). Introduction of OsYSL15 or YS1 had no effect on yeast grown on normal or iron-minus SD medium without Fe(III)-DMA (Fig. 3, b and c).

Electrophysiological studies with X. laevis oocytes were used to further confirm substrates transported by OsYSL15. OsYSL15 was heterologously expressed in the oocytes, and the substrate-induced inward currents at −80 mV were measured in response to Fe(III)-DMA or several related compounds (Fig. 3d). OsYSL15 transported Fe(III)-DMA but did not transport Fe(II)-NA, Fe(III)-NA, or Mn(II)-NA complexes. Consistent results were observed under different clamping voltages (data not shown).

Regulation of OsYSL15 Expression by Micronutrient Deficiencies and Daily Fluctuations—The upstream sequence of OsYSL15 was searched for known cis-acting elements. Among the elements related to iron deficiency response in rice plants (44–47), OsYSL15 possessed an IDE1-binding site (CATGC) (45) at 277 to 273 bases upstream from the putative translation start site and multiple IDE2-binding core sites (CA(A/C)G(T/C)(T/C/A)(T/C/A)) (46) within 1.5-kb 5′-upstream. Also, a circadian clock-associated 1-binding site (CBS: AAAAAATCT), which regulates circadian expression (48), was identified at 331 to 323 bases upstream from the putative translation start site.

To examine whether OsYSL15 expression is regulated by micronutrient deficiencies or in diurnal fashion, we performed Northern blot and quantitative RT-PCR analysis (Fig. 4). Consistent with previous reports (32) and laser microdissection analysis (Fig. 1), OsYSL15 expression was strongly up-regulated in iron-deficient roots but not in leaves (Fig. 4). In contrast, zinc, manganese, or copper deficiency treatments did not induce OsYSL15 either in leaves or roots (Fig. 4a). In iron-deficient roots, expression of OsYSL15 showed a daily fluctuation (Fig. 4b). The transcript level increased around the period of illumination onset, and decreased thereafter to the lowest level at the start of darkness.

Spatial and Temporal Patterns of OsYSL15 Expression—Subcellular localization of OsYSL15 was analyzed using green fluorescent protein (GFP)-fused OsYSL15 protein transiently expressed in onion (Allium cepa L.) epidermal cells. Cells expressing the OsYSL15-GFP fusion protein demonstrated fluorescence in the plasma membrane (Fig. 5a). Control experiments using cells expressing GFP alone showed fluorescence in the cytoplasm and the nucleus (Fig. 5d). Thus, OsYSL15 is a functional transporter for uptake of Fe(III)-DMA through the plasma membrane.
The OsYSL15 promoter (1.5 kb) fused to GUS and introduced into rice was used to investigate cell specificity of OsYSL15 expression in whole rice plants. Five independent T2 plants harboring the OsYSL15 promoter-GUS were grown under iron-sufficient or -deficient conditions. All five transgenic lines showed similar patterns of OsYSL15 expression. OsYSL15 promoter activity was detected in the central cylinder of iron-sufficient roots (Fig. 5c). At higher magnifications, promoter activity was apparent only in phloem cells (Fig. 5d). Longitudinal sectioning of iron-sufficient rice roots showed that the staining was observed in specific regions of the phloem cells (Fig. 5g, arrows). Occasionally, some of the exodermal cells of iron-sufficient roots were also weakly stained (data not shown).

In iron-deficient roots, the OsYSL15 promoter was active in all tissues, including epidermis, exodermis, cortex, lateral roots, and the central cylinder (Fig. 5e). Especially strong staining was observed in phloem cells (Fig. 5f). Strong GUS activity was detected in the cells surrounding metaxylem in the regions where lateral roots emerge in iron-deficient roots (Fig. 5e and f). Longitudinal sections showed no detectable promoter activity in the root cap cells (Fig. 5h).

In shoots, the OsYSL15 promoter was weakly active in companion cells and in some of the xylem parenchyma of iron-sufficient leaves (Fig. 5i). Iron deficiency treatment did not change expression levels or localization (Fig. 5j).

The OsYSL15 promoter was also active in flowers and developing seeds. Promoter activity was dominant in the vascular bundles of spikelets, stamens, pistils, and husk prior to anthesis (Fig. 5k). After fertilization, expression was observed in the husk, spikelets, and pistil (Fig. 5l). Staining was observed in the dorsal vascular bundles of developing ovals 3, 5, and 10 days after anthesis (Fig. 5, m–o). Expression was observed in the embryo and dorsal vascular bundles 15 days after anthesis (Fig. 5p). Characteristic expression was observed in the scutellum and endosperm 30 days after fertilization (Fig. 5q).

OsYSL15 Knockdown Plants Exhibit Severe Arrest in Germination and/or Early Seedling Growth—To further characterize the function of OsYSL15, we developed 40 lines of transgenic rice OsYSL15 knockdown plants with RNA interference expression suppression. These transformants (OsYSL15i plants) grew healthily during adult phase, exhibiting slight retardation compared with vector controls (VC) and only slight repression of OsYSL15 expression (data not shown). During seed germination and early seedling development, however, OsYSL15i lines exhibited severe growth defects when grown on standard MS medium. Quantitative RT-PCR experiments revealed that expression of OsYSL15 was severely decreased to between 3 and 24% compared with control seedlings (Fig. 6a). Expression of close homologs of OsYSL15, including OsYSL2, OsYSL16 and OsYSL9, was not largely affected in these OsYSL15i seedlings (supplemental Fig. S2).

Nine days after sowing, average shoot lengths of OsYSL15i plants grown on standard MS medium were suppressed to between 5% (transgenic line 3) and 20% (transgenic line 17)
OsYSL15i plants grown on iron-free MS medium displayed slightly more severe growth defects, with average shoot lengths suppressed to between 7% (transgenic line 4) and 17% (transgenic line 17) of those of control plants (Fig. 6, c and f). These growth defects were significantly restored when OsYSL15i plants were grown on a medium supplemented with 0.5 mM Fe(II)-citrate. Average shoot lengths of OsYSL15i plants ranged between 30% (transgenic line 4) and 72% (transgenic line 5) of controls when grown on this high-iron medium (Fig. 6, d and g). Suppression of OsYSL15 expression was correlated with seedling growth to some extent. The least suppressed transgenic line (line 17) showed the best growth on standard and iron-free MS medium (Fig. 6, a–c), whereas the most suppressed transgenic line (line 4) showed the least recovery of growth on high-Fe(II) medium (Fig. 6, a and d). OsYSL15i plants on standard MS medium turned brown and died less than 21 days after sowing (data not shown).

To gain further insight into the relationship between OsYSL15 expression and seed germination, we monitored OsYSL15 expression during seed germination using OsYSL15 promoter-GUS seeds. The OsYSL15 promoter was gradually activated during seed germination (Fig. 6, h–k). The promoter was active in the scutellum from 0 to 3 days after sowing (Fig. 6, a–c), whereas the most suppressed transgenic line (line 4) showed the least recovery of growth on high-Fe(II) medium (Fig. 6, a and d). OsYSL15i plants on standard MS medium turned brown and died less than 21 days after sowing (data not shown).
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FIGURE 5. Subcellular and cellular localization of OsYSL15 expression. a and b, subcellular localization of transiently expressed OsYSL15-GFP fusion protein (a) and GFP (b) in onion epidermal cells. Scale bars = 50 μm. c–q, histochemical staining of OsYSL15 promoter-GUS expression in transgenic rice plants grown under iron sufficiency (c, d, g, i, and k–q) or iron deficiency (e, f, h, and j). c and e, root transverse sections. d and f, enlarged portion of the stele. g and h, longitudinal root section. Arrows in g indicate the sites of expression. i, iron-sufficient leaf. j, iron-deficient leaf. Scale bars, 100 μm for c and e; 50 μm for d, f, i, and j; and 500 μm for g and h. k–q, expression in flowers and developing seeds during maturation. Before anthesis (k), after fertilization (l), and 3 (m), 5 (n), 10 (o), 15 (p), and 30 (q) days after fertilization.

DISCUSSION

OsYSL15 Is an Iron-regulated Fe(III)-DMA Transporter Expressed in the Uptake Site of Iron from the Rhizosphere—Strategy II iron uptake in graminaceous plants typically has three steps as follows: 1) biosynthesis of MAs in roots, 2) MAs secretion to the rhizosphere, and 3) uptake of MAs-chelated Fe(III) through the membrane of root cells (3). To identify the rice counterpart of YSL for Fe(III)-MAs uptake, we analyzed the expression patterns of the 18 OsYSL genes in microdissected root tissues, leading to the prediction that the OsYSL15 gene, which is strongly up-regulated in the root epidermis under conditions of iron deficiency (Fig. 1), is responsible for strategy II-based iron uptake from the rhizosphere. Analysis of OsYSL15 transport activity demonstrated an ability to transport Fe(III)-DMA (Fig. 3), strongly supporting the hypothesis that rice takes up Fe(III)-DMA from the rhizosphere using an OsYSL15 transporter under iron-deficient conditions.

OsYSL15 promoter-GUS analysis revealed strong expression in the epidermal and exodermal cells of iron-deficient roots (Fig. 5, e and h) where absorption of Fe(III)-DMA complexes mainly occurs. In these cells, DMA is produced and thought to be secreted into the rhizosphere to solubilize oxidized Fe(III). Elongation and division zones at about 5–40 mm from the root tip are the dominant site for iron uptake in barley roots (9). Spatial expression patterns of OsYSL15 (Fig. 5) as well as those of OsNAS1, OsNAS2, OsNAAT1, and OsDMAS1 (19, 21, 22) correlate well with the putative site of DMA production and Fe(III)-DMA absorption from the rhizosphere.

The secretion of MAs in barley follows a distinct diurnal rhythm (15). A peak in secretion occurs just after initial illumination and ceases within 2–3 h. Although diurnal secretion of MAs from rice roots is not obvious because of the much lower levels of secreted MAs compared with barley, rice genes involved in DMA biosynthesis, including OsNAS1, OsNAS2, and OsNAAT1, exhibit diurnal changes in expression (33). Transcript levels of these genes increase prior to illumination and gradually decrease after illumination. Promoter regions of these genes possess CBS or related evening element-like sequences (33). Here we report the presence of a CBS element in the promoter region of OsYSL15 and daily fluctuation in OsYSL15 expression (Fig. 4b), with increased expression during the period of illumination onset. These results suggest that OsYSL15 may function in Fe(III)-DMA uptake coordinated with diurnal biosynthesis and secretion of DMA. In rice roots, DMA would be synthesized preferentially at night and secreted in the morning. OsYSL15 is thought to be responsible for effective uptake of Fe(III)-DMA complexes in the daytime. Similar daily fluctuation in expression is also observed for barley HvYSL1 gene (49).
Biosynthesis and secretion of DMA is strongly induced under conditions of iron deficiency. Similarly to the genes responsible for DMA biosynthesis, OsYSL15 was transcriptionally up-regulated by iron deficiency (Fig. 1 and Fig. 4a).

Recently we found that an iron deficiency-inducible basic helix-loop-helix transcription factor, OsIRO2, positively regulates the iron deficiency-induced expression of OsYSL15 as well as the genes responsible for DMA biosynthesis, including OsNAS1, OsNAS2, OsNAAT1, and OsDMAS1 (40). Because OsYSL15 possesses no OsIRO2-binding core sites (CACGTGG; 47) within its functional 1.5-kb 5'-upstream, OsYSL15 is thought to be regulated indirectly by OsIRO2. Recent identification of iron deficiency-responsive element-binding factors (IDEFs) revealed that IDEF1 and IDEF2, which specifically bind to IDE1 and IDE2, respectively, regulate iron homeostasis in rice (45, 46). The presence of an IDEF1-binding site and multiple IDEF2-binding core sites in the OsYSL15 promoter suggests that OsYSL15 expression is regulated by multiple pathways in the transcriptional network involving IDEF1, IDEF2, OsIRO2, and other transcription factors.

Along with Fe(III)-DMA uptake through OsYSL15, rice plants also take up Fe^{2+} ion as an iron source via the OsIRT1 transporter (50). Transgenic rice plants carrying reconstructed ferric-chelate reductase gene exhibited higher iron uptake rate and iron deficiency tolerance (51), clarifying significance of ferrous iron uptake system in rice. Cheng et al. (52) reported that a rice naat1 mutant defective in producing DMA is lethal under aerobic conditions but is able to grow under waterlogged condition or when Fe(II) is supplied. These results suggest that relative importance of Fe(III)-DMA uptake through OsYSL15 versus Fe^{2+} uptake through OsIRT1 is strongly dependent on growth conditions. Because our OsYSL15 knockdown plants showed only slight gene repression during the adult phase, further characterization of other knockdown or knock-out plants, possibly by using an inducible RNA interference technique, will show the important role of OsYSL15 in iron uptake from the rhizosphere.

Possible Involvement of OsYSL15 in Long Distance Transport of Iron—DMA is present in both rice shoots and roots (20), as well as rice phloem sap (53). Additionally, the genes involved in DMA biosynthesis, including OsNAS1–3, OsNAAT1, and OsDMAS1, are markedly expressed in phloem companion cells in rice roots and shoots (19, 21, 22). Concomitant expression of OsYSL15 in the phloem cells in roots (Fig. 5, c–f) supports the idea that OsYSL15 may function in the long distance transport of Fe(III)-DMA complexes from roots to shoots via phloem. Recent analysis revealed that Zn(II)-DMA is translocated to zinc-deficient rice leaves at higher rate than Zn^{2+} (54), suggesting an important role of DMA in metal distribution within the plant in addition to metal absorption from the rhizosphere.

OsYSL15 and OsYSL2 may coordinately function in long distance transport of iron in the phloem; Fe(III)-DMA and Fe(II)-NA may coexist in the cells and be transported to shoots through these

**FIGURE 6. Significance of OsYSL15 expression during germination.** a, quantitative RT-PCR analysis confirming suppressed OsYSL15 expression in roots of OsYSL15 knockdown (OsYSL15i) seedlings. Relative transcript levels compared with VC seedlings shown as mean ± S.D. (n = 3). b–d, shoot lengths of OsYSL15i and control seedlings 9 days after sowing on standard MS medium (b), iron-free MS medium (c), or MS medium supplemented with −0.5 mM of Fe(II)-citrate by Tetsuriki-aqua fertilizer (d). Data are shown as mean ± S.D. (n = 2–5 for OsYSL15i and n = 12–17 for VC). e, seedlings of VC and OsYSL15i (line 4) 7 days after sowing on standard (e) or iron-free (f) MS medium. g, seedlings of VC and OsYSL15i (line 1) 9 days after sowing on MS medium supplemented with −0.5 mM Fe(II)-citrate by Tetsuriki-aqua. h–k, histochemical staining of OsYSL15 promoter-GUS transfectants during seed germination. Fully mature seeds (h), and 1 (i), 2 (j), and 3 (k) days after sowing. S, scutellum; V, ventral scale; B, bud scale.
transports. OsYSL2 is strongly expressed in phloem companion cells in iron-deficient shoots, and its encoded protein transports Fe(II)-NA complexes (32). Phloem possesses a pH of around 8.0 (55), an environment in which Fe(II)-NA complexes are predicted to be more stable than Fe(III)-DMA (56). High amounts of endogenous NA are present in iron-deficient rice shoots (20). Reduction of Fe(III) and chelate exchange steps are needed for conversion of Fe(III)-DMA to Fe(II)-NA complexes. Two rice genes putatively encoding ferric-chelate reductase (OsFRDs) in the plasma membrane (50) or NADPH-dependent ferric-chelate reductase proteins in the cytoplasm (57) may reduce ferrous ions of Fe(III)-DMA complexes. Chelation is likely to change from DMA to NA in the phloem cells as the Fe(II)-NA complex is relatively stable at high pH (56).

In contrast to phloem cells, OsYSL15 is not notably expressed in root pericycle cells adjacent to the protoxylem (Fig. 5, c–f) where genes involved in DMA biosynthesis, including OsNAS1–3, OsNAAT1, and OsDMAS1, are markedly expressed (19, 21, 22). Thus, OsYSL15 might not be the primary transporter for transport of Fe(III)-DMA complexes in xylem. Because OsYSL5, -6, -7, -12, -14, and -17 are also expressed in the root stele (Fig. 1), these OsYSLs may be involved in xylem loading/unloading of DMA- or NA-chelated metals. Because xylem is apoplastic, efflux-type transporters for metals and/or metal-chelator complexes should also be needed for long distance transport, in addition to influx-type transporters, including YSLs. Recently, an Arabidopsis transporter of the multidrug and toxin efflux family, FRD3, has been shown to be involved in citrate efflux into xylem, which is needed for efficient iron transport within the plant (58). A rice FRD3-like gene, OsFRDL1, is expressed in root pericycle cells adjacent to the protoxylem and metaxylem (59). Thus, OsFRDL1, in addition to as yet unknown iron efflux transporters, might be responsible for long distance transport of iron via xylem.

Role of OsYSL15 during Seed Germination and Early Seedling Growth—OsYSL15 knockdown plants exhibited severe arrest in seed germination and/or early seedling growth, which was even more severe in iron-deficient medium and was greatly rescued by high concentrations of Fe(II) (Fig. 6, a–g). These results indicate that OsYSL15-mediated iron transport is essential for seed germination. OsYSL15 is highly expressed in the embryonic scutellar epithelium cells during seed germination (Fig. 6, h–k). Thus, OsYSL15 may mediate Fe(III)-DMA transport from endosperm through the epithelial layer. The epithelial layer in the dorsal portion of the scutellum faces the endosperm and functions in the absorption of storage reserves from it (60). Total iron content in the endosperm is higher than that in the embryo (61). During seed maturation, OsYSL15 is expressed in vascular bundles, embryo, and endosperm (Fig. 5, k–q). OsNAS1, OsNAS2, OsNAAT1, and OsDMAS1 are also expressed in maturing endosperm, where high accumulation of DMA also occurs.5 This DMA would be used to form Fe(III)-DMA complexes during seed germination. In addition, OsYSL15 may play a role in absorption of Fe(III)-DMA complexes directly into the embryo from outside the plant (e.g. soil). OsYSL15 was expressed in the ventral scale and bud scale cells of 3-day-old seedlings (Fig. 6k). OsNAS1, OsNAS2, OsNAS3, and OsNAAT1 are expressed in the germinating embryo (39), where they might contribute to synthesis and secretion of DMA for iron absorption from the soil during seed germination.

In Arabidopsis, some metal transporters are important in early growth. irt1 mutants defective in primary Fe2+
transport at the root surface are able to develop even though they are strongly chlorotic (6). A double mutant of the divalent metal transporters Nramp3 and Nramp4 exhibits germination arrest; these transporters play a role in mobilization of vacuolar iron during seed germination (62). Another mutant seedling defective in vacuolar membrane transporter VIT1 grows poorly on high pH soil (63). In contrast to the roles of these free-metal transporters, OsYSL15 transports Fe(III)-DMA complexes specifically (Fig. 3), suggesting that DMA itself is the essential chelator for rice seed germination. Consistent with this notion, Cheng et al. (52) reported that a rice naat1 mutant defective in producing DMA is unable to survive longer than 20 days after germination when supplied with Fe(III)-citrate as the sole iron source. During 2 days after sowing, the ferrous transporter gene OsIRT1 is expressed in the vascular bundle of the scutellum and the leaf primordium but not in the embryonic scutellar epithelium cells (39). Thus, DMA appears to play a crucial role in iron absorption from the endosperm. Phytic acid constitutes ≥1% of seed dry weight and is typically deposited in seeds as mixed phytate or phytin salts of potassium and magnesium, although these salts also contain other mineral cations such as calcium, Fe(III), and zinc (64–66). DMA strongly chelates ferric iron (56) and is thought to be effective in recovering the Fe(III) from Fe(III)-phytate deposits in the endosperm, an unavailable form of iron. Similarly, in humans, high phytate diets significantly decrease the availability of iron (67, 68). Although iron content in rice seeds is not high compared with other human dietary sources, increasing DMA and Fe(III)-DMA complexes in the endosperm should improve iron bioavailability. Manipulation of OsYSL15 expression in combination with genes involved in DMA biosynthesis, other transporter genes, and/or the genes involved in iron storage could pave the way for development of iron-enriched crops for human health, as well as providing sustainable food production in adverse soils.

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