

OsYSL18 is a rice iron(III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints

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Abstract Iron uptake and translocation in plants are important processes for both plant and human nutrition, whereas relatively little is known about the molecular mechanisms of iron transport within the plant body. Several reports have shown that yellow stripe 1 (YS1) and YS1-like (YSL) transporters mediate metal-phytosiderophore uptake and/or metal-nicotianamine translocation. Among the 18 YSL genes in rice (*OsYSLs*), *OsYSL18* is predicted to encode a polypeptide of 679 amino acids containing 13 putative transmembrane domains. An OsYSL18-green fluorescent protein (GFP) fusion was localized to the plasma membrane when transiently expressed in onion epidermal cells.

Electrophysiological measurements using *Xenopus laevis* oocytes showed that OsYSL18 transports iron(III)–deoxymugineic acid, but not iron(II)–nicotianamine, zinc(II)–deoxymugineic acid, or zinc(II)–nicotianamine. Reverse transcriptase PCR analysis revealed more *OsYSL18* transcripts in flowers than in shoots or roots. *OsYSL18* promoter- β -glucuronidase (*GUS*) analysis revealed that *OsYSL18* was expressed in reproductive organs including the pollen tube. In vegetative organs, *OsYSL18* was specifically expressed in lamina joints, the inner cortex of crown roots, and phloem parenchyma and companion cells at the basal part of every leaf sheath. These results suggest that OsYSL18 is an iron-phytosiderophore transporter involved in the translocation of iron in reproductive organs and phloem in joints.

Accession numbers Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number AB190926 for *OsYSL18*.

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Introduction

The most widespread human nutritional problem is iron (Fe) deficiency. There are approximately two billion anemic people worldwide, and approximately 50% of all anemia's can be attributed to Fe deficiency (Mason et al. 2001). Because plants are the primary source of food for humans, the nutritional value of plants is of central importance to human health (Grusak and Dellapenna 1999). Identification of plant mechanisms involved in the acquisition and use of Fe is a prerequisite to increasing the amount of Fe in the edible parts of plants. Identifying the transporters involved in uptake and translocation of Fe is an important aspect of this task.

Plants have evolved two distinct Fe uptake strategies (Römheld and Marschner 1986). Most plants, including dicots

and non-graminaceous monocots, take up Fe(II) from soils into root cells *via* iron-regulated transporters (IRT) after reduction from ferric [Fe(III)]-chelates by ferric reductase/oxidase (FRO) in the plasma membrane (Strategy I; Eide et al. 1996; Robinson et al. 1999). However, graminaceous plants synthesize and secrete natural Fe chelators, mugineic acid family phytosiderophores (MAs), from their roots to solubilize Fe in the rhizosphere (Strategy II; Takagi 1976, Römheld and Marchner 1986; Mori 1999). The resulting Fe(III)–MAs complexes are absorbed into the root through Fe(III)–MAs transporters in the plasma membrane. The gene encoding an Fe(III)–MAs transporter, Yellow Stripe 1 (YS1), was first isolated in *Zea mays* (Curie et al. 2001). Electrophysiological analyses revealed that YS1 functions as a proton-coupled symporter for various MAs-bound metals including Fe(III), Zn(II), Cu(II), and Ni(II) (Schaff et al. 2004). Recently, a barley homolog of YS1 (*HvYS1*) was isolated and was shown to transport Fe(III)–MAs (Murata et al. 2006).

MAs are biosynthesized from methionine (Mori and Nishizawa 1987) *via* several enzymatic reactions (Shojima et al. 1990; Mori 1999; Bashir et al. 2006). Nicotianamine (NA), the biosynthetic precursor of MAs (Shojima et al. 1989, 1990), is structurally very similar to MAs and chelates metal cations, including Fe(II) and Fe(III) (Benes et al. 1983; von Wirén et al. 1999). The biosynthetic pathway from methionine to NA is conserved in all plant species including graminaceous and non-graminaceous plants (Shojima et al. 1989), where NA may be responsible for metal homeostasis (Hell and Stephan 2003; Takahashi et al. 2003). Consistent with this notion, non-graminaceous plants also possess YS1-like (*YSL*) genes, some of which are responsible for metal-NA translocation within the plant body (DiDonato et al. 2004; Le Jean et al. 2005; Schaaf et al. 2005; Gendre et al. 2006; Waters et al. 2006; Curie et al. 2008).

Rice (*Oryza sativa* L.), one of the most important crops for world food supply, takes up Fe from the rhizosphere using the Fe(III)–deoxymugineic acid (DMA) transporter and the ferrous Fe transporter OsIRT1 (Ishimaru et al. 2006). We previously identified 18 putative YS1-like genes (*OsYSLs*) in the rice genome that exhibited 36–76% sequence similarity to the maize YS1 gene (Koike et al. 2004). We demonstrated that OsYSL2 transports Fe(II)–NA and Mn(II)–NA, but not Fe(III)–DMA (Koike et al. 2004). *OsYSL2* expression is strongly induced in Fe-deficient leaves, and is particularly dominant in the phloem cells of leaves and leaf sheaths as well as in developing seeds, suggesting its involvement in phloem transport and the translocation of mineral nutrients in grains (Koike et al. 2004). OsYSL15 transports Fe(III)–DMA, but not Fe(II)–NA, Fe(III)–NA, or Mn(II)–NA (Inoue et al. 2009). *OsYSL15* expression is strongly induced in epidermis/exodermis and phloem cells of Fe-deficient roots, suggesting its role in Fe(III)–DMA uptake from the rhizosphere and also

in phloem transport of Fe. Analysis of *OsYSL15* knockdown plants revealed that OsYSL15 is also crucial in Fe homeostasis during early growth of the seedlings (Inoue et al. 2009). The function of the other *OsYSLs* has not been investigated, except for the presence of transcripts of *OsYSL5*, 6, 7, 8, 12, 13, 14, 16, and 17 in roots and *OsYSL6*, 13, 14, and 16 in leaves (Koike et al. 2004; Inoue et al. 2009). In the present report, we demonstrate that one of the *OsYSLs*, *OsYSL18*, encodes a functional Fe(III)–DMA transporter that may be involved in DMA-mediated Fe distribution in the reproductive organs, lamina joints, and phloem cells at the base of the leaf sheath.

Materials and methods

Plant Materials

Non-transgenic and transgenic rice (*Oryza sativa* cv. Tsukinohikari) seeds were germinated on Murashige and Skoog (MS) medium and transferred to a nutrient solution (Mori and Nishizawa, 1987, Inoue et al. 2003) 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 5.5 with 1 M HCl, and the culture solution was renewed weekly. Six-week-old plants were harvested for reverse transcriptase (RT)-PCR and GUS analyses. For Fe, Zn, or Mn deficiency treatments, 4-, 3-, or 2-week-old plants were cultured without each of the specific metals for 2, 3, and 4 weeks, respectively. Flowers and seeds were obtained from soil-grown nutrient-sufficient rice plants.

RT-PCR analysis

Total RNA was isolated from rice plants grown under nutrient-sufficient or nutrient-deficient conditions using an RNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan), and the 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, Tokyo, Japan) by priming with oligo-d(T)30. The primers used for RT-PCR were *OsYSL18* forward (5'-TAAAGCTGGATGATCCTG AATTCTT) and *OsYSL18* reverse (5'-TCGCTCTAC ATGAAAAGATCAGTTC). These primers were designed to amplify the cDNA across the exons to discriminate possible genomic DNA contamination. The α -*tubulin* primers used for RT-PCR were α -*tubulin* forward (5'-TCTTCCACCCTGAGCAGCTC) and α -*tubulin* reverse (5'-AACCTTGGAGACCAGTGCAG). The sizes of the amplified fragments were confirmed by gel electrophoresis and sequencing. No genomic contamination was observed.

Transport activity of *OsYSL18* in *Xenopus laevis* oocytes

Electrophysiological measurement of *OsYSL18* transport activity using *Xenopus laevis* oocytes was performed according to Igarashi et al. (2000) and Koike et al. (2004). The entire open reading frame (ORF) of *OsYSL18* was amplified using two primers: 5'-CACCATGGAGTCGGTCGGCGACCCGCGGG-3' and 5'-AAGAATTCTAGATCATCCAGCTTTAGCCTTACTCGCCAA-3'. The amplified and verified fragment was subcloned into pENTR/D-TOPO (Invitrogen) to construct pENTR-*OsYSL18*. The entire ORF of *OsYSL18* produced by digestion with *EcoRI* and *XbaI* was inserted into the *EcoRI* and *XbaI* site of the pGEM-3zf(+) vector. The resulting plasmid, pGEMOsYSL18T, was linearized by *XbaI* digestion. Capped complementary RNA (cRNA) was synthesized in vitro with a MEGAscript SP6 kit (Ambion, Austin, TX, USA). The oocytes were prepared as described by Igarashi et al. (2000) and injected with 10 ng *OsYSL18* cRNA. The injected oocytes were incubated for 2 days and subjected to electrophysiological measurements at pH 7.5. The oocytes were positioned in a 1.0-ml recording chamber perfused with bath solution containing 96 mM NaCl, 2 mM KCl, 5 mM HEPES-NaOH solution (pH 7.5). Membrane currents were measured using the two-microelectrode voltage-clamp method with an automated Hitachi system containing a TEV-200 system (Dagan, Minneapolis, MN, USA). The oocytes were clamped at -80 mV, and steady-state currents in response to the addition of 10 μ l metal-chelate complex prepared according to Schaaf et al. (2004) (5 mM final concentration) were obtained. Each oocyte was tested with one substrate. The currents were continuously monitored and analyzed with a Mac Lab system (Adinstruments, Sydney, NSW, Australia). Six independent oocytes injected with *OsYSL18* were used to measure the currents. Substrate-induced currents were also measured in six independent water-injected oocytes as controls.

Subcellular localization of *OsYSL18*-GFP

Using the pH7FWG2 plasmid (Karimi et al. 2002) as the destination vector and pENTR-*OsYSL18* as the entry vector, an LR recombination reaction (Invitrogen) between the destination vector and the entry vector generated an expression clone containing the gene encoding cauliflower mosaic virus 35S promoter-*OsYSL18*-GFP. As a control, a non-tagged green fluorescent protein (GFP) construct CaMV35S-sGFP(S65T)-NOS3' plasmid (a generous gift from Dr. Yasuo Niwa, University of Shizuoka, Japan) was used. Onion (*Allium cepa* L.) epidermal cells were transformed using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Tokyo, Japan), and the transiently

expressed GFP fluorescence was observed using a laser-scanning confocal microscope (LSM510, Carl Zeiss, Tokyo, Japan) according to Mizuno et al. (2003).

OsYSL18 promoter-GUS analysis

A genomic sequence containing the putative promoter region of *OsYSL18* (from -3,000 to -1 bp from the translation initiation codon) was amplified by PCR using genomic DNA as a template. The primers used were: 5'-GAGAGCTAGCGCAGATAACTACTATGCTGCCCTCA-3, and 5'-GAGACTAGCCCCGGCCACTTTTCCTCCTCCTCCT-3'. The amplified and verified fragment was excised by *HindIII* and *XbaI* and was subcloned upstream of the *uidA* ORF, which encodes β -glucuronidase (GUS), in the pIG121Hm vector (Hiei et al. 1994). An *Agrobacterium tumefaciens* strain (C58) carrying the above construct was used to transform rice (*Oryza sativa* L. cv. Tsukinohikari) as described previously (Higuchi et al. 2001).

T₁ and T₂ seeds were germinated and cultured as described above and subjected to histochemical GUS analysis following the method of Jefferson et al. (1987) modified by Kosugi et al. (1991). Plant organs and sections were incubated at 37°C for 30 min to overnight in GUS reaction buffer consisting of 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc), 3 mM K₄[Fe(CN)₆], 0.5 mM K₃[Fe(CN)₆], 50 mM sodium phosphate buffer (pH 7.0), and 20% (v/v) methanol. After staining, these sections were washed in 70% ethanol for 2 days to remove the chlorophyll and then observed using an Axiophoto microscope (Carl Zeiss) following the manufacturer's instructions. For in vitro culture of pollen tubes, pollen collected just after anthesis was plated and germinated on medium containing 20% sucrose, 0.32 mM H₃BO₃, 2.04 mM CaCl₂, and 1% agarose. To prepare longitudinal and transverse sections of the basal part of the leaf sheaths, crowns were fixed in 4% formaldehyde, 5% glutaraldehyde, 0.01 M CaCl₂, and 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h on ice after staining and chlorophyll removal. Fixed samples were washed three times for 30 min each with 0.1 M sodium cacodylate and 10% sucrose, and then incubated for 2 h on ice in 1% osmium tetroxide and 0.1 M sodium cacodylate. The samples were then serially dehydrated in ethanol, acetone, and propylene oxide, and embedded in Spurr's resin. Semi-thin sections (5 μ m) were cut for observation by light microscopy.

Results

Sequence and structural organization of *OsYSL18*

Our search for YS1 homologs in the *Oryza sativa* L. ssp. *japonica* (cv. Nipponbare) rice genomic database identified 18 putative *OsYSLs* that exhibited 36–76% sequence

the topology of OsYSL18 using some web-based programs including SOSUI (<http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>), which predicted that OsYSL18 has 13 transmembrane domains (TMs) and has its N-terminus localized in the cytoplasm. The abundance of glutamic acid residues at the amino terminus of YS1 (Curie et al. 2001) is not conserved in OsYSL18. The sequence determining substrate specificity of YS1/YSL transporters as reported by Harada et al. (2007) showed low similarity between OsYSL18 and YS1 (Fig. 1b, boxed region).

Transport activity of OsYSL18 in oocytes

We examined the transport activity and substrate specificity of OsYSL18 using *Xenopus laevis* oocytes. Because previously analyzed YS1 and YSL transporters were shown to be involved in metal homeostasis and transport of DMA- or NA-chelated metals (Curie et al. 2008; Inoue et al. 2009), we investigated whether OsYSL18 was also able to transport DMA- or NA-chelated metals. OsYSL18 was heterologously expressed in the oocytes, and the substrate-induced inward currents at -80 mV in response to Fe(III)-DMA, Fe(II)-NA, Zn(II)-DMA or Zn(II)-NA were measured (Fig. 2). OsYSL18 transported Fe(III)-DMA, but did not transport Fe(II)-NA, Zn(II)-DMA, or Zn(II)-NA.

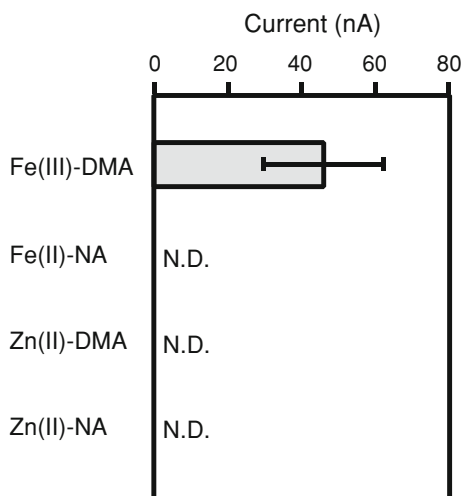


Fig. 2 Transporting activity of OsYSL18 analyzed by electrophysiological measurements using *Xenopus laevis* oocytes. The transport activities of Fe(III)-DMA, Fe(II)-NA, Zn(II)-DMA, and Zn(II)-NA were measured using the two-electrode voltage-clamp method. The oocytes were clamped at -80 mV, and steady-state currents in response to the addition of a metal-chelate complex ($10 \mu\text{l}$, 5 mM) were obtained. The data are mean \pm SE of six independent oocytes injected with OsYSL18. The same number of water-injected oocytes was used as a control. N.D., not detected

Organ-specific expression pattern of OsYSL18

To investigate the expression pattern of *OsYSL18*, RT-PCR analysis was conducted using a cDNA pool prepared from the roots, crown, shoots, or flowers of rice plants grown under nutrient-sufficient conditions (Fig. 3). After 45 cycles of PCR, *OsYSL18* transcripts were only detected from the cDNA pool prepared from flowers. By increasing the PCR to 55 cycles, *OsYSL18* transcripts were also detected in roots, crown, and shoots. No induction of *OsYSL18* expression was observed under Fe-, Zn- or Mn-deficient conditions (data not shown).

Spatial patterns of OsYSL18 expression

Subcellular localization of OsYSL18 was analyzed using GFP-fused OsYSL18 protein transiently expressed in onion epidermal cells. Cells expressing the OsYSL18-GFP fusion protein showed fluorescence in the plasma membrane which was clearly distinguishable from the non-fluorescent tonoplast and cytoplasm (Fig. 4a-f). Control experiments demonstrated that cells expressing GFP alone showed signals in the cytoplasm and the nucleus (Fig. 4g-i). Thus, OsYSL18 is a functional transporter for uptake of Fe(III)-DMA in the plasma membrane.

Cell-type specificity of *OsYSL18* expression in whole rice plants was investigated using four independent T_1 and T_2 lines harboring *OsYSL18* promoter (3.0 kb)-*GUS* constructs, all of which exhibited similar patterns of expression. As *OsYSL18* transcripts were most abundant in flowers (Fig. 3), we first examined *OsYSL18* promoter activity in flowers and developing seeds (Fig. 5). Before anthesis, the *OsYSL18* promoter was active in anthers, pollen, filaments, ovules, stigma, and vascular bundles of the lemma (Fig. 5a). Just after anthesis, expression was also observed in pollen grains and tubes (Fig. 5b, h, i). Expression in germinated pollen was further confirmed using an in vitro culture medium (Fig. 5j). In developing seeds, expression was observed in the embryonic disc and vascular bundles (Fig. 5c-f). In mature seeds, expression was dominant in the embryonic disc (Fig. 5g). *OsYSL18*

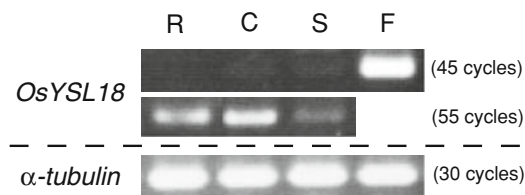


Fig. 3 RT-PCR analysis of *OsYSL18* expression. Total RNA from root (R), crown (C), shoot (S), or flower (F) grown under Fe-sufficient conditions was reverse-transcribed and amplified by PCR using primers specific to *OsYSL18* or α -tubulin for the indicated number of cycles

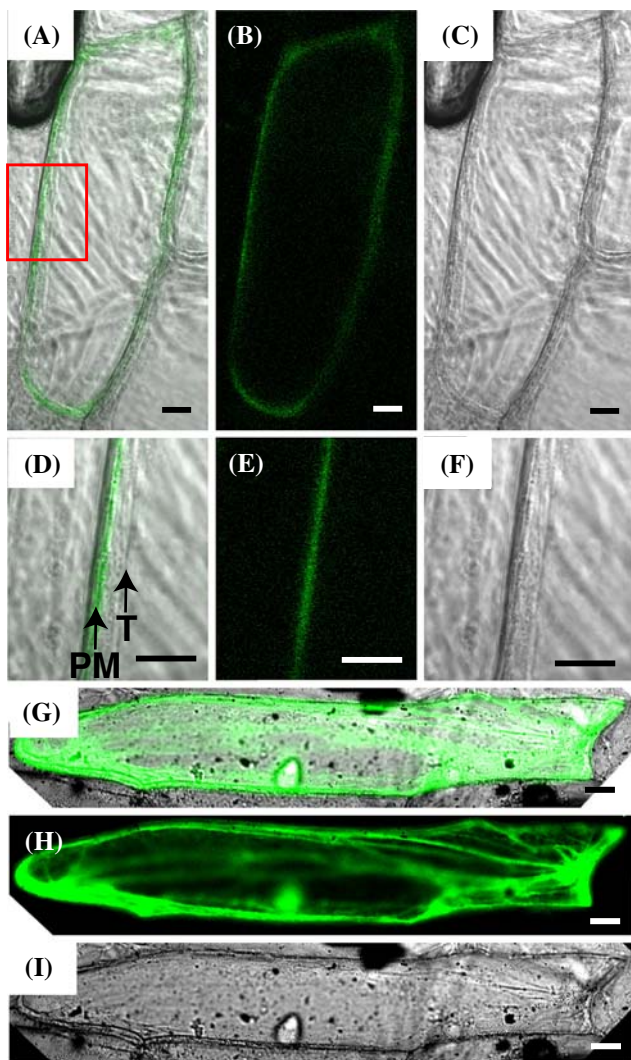


Fig. 4 Subcellular localization of OsYSL18 expression. OsYSL18–GFP fusion protein (a–f) or GFP alone (g–i) was transiently expressed in onion epidermal cells and was observed by confocal laser scanning microscopy. (a, d, g) Overlay of confocal cross-sections and transmission images. PM, plasma membrane; T, tonoplast. (b, e, h) Confocal cross-sections. (c, f, i) Transmission images. (d–f) Magnified images of the boxed area shown in (a). Scale bars 20 μ m

promoter activity was also observed in the embryonic disc of germinating seeds (data not shown).

Because *OsYSL18* transcripts were also detected in shoots, crown, and roots, although the expression level was very low in any growth condition (Fig. 3), we then investigated *OsYSL18* promoter activity in these organs. Whole-plant GUS staining revealed that *OsYSL18* expression was mostly restricted to small regions around lamina joints, crown roots, and the crown (Fig. 6a–e). Around lamina joints, observation of *OsYSL18* activity was limited to the basal parts of vascular bundles of leaf blades (Fig. 6a, b). In roots, *OsYSL18* was expressed in restricted areas of crown roots (Fig. 6c). Longitudinal sections showed expression in

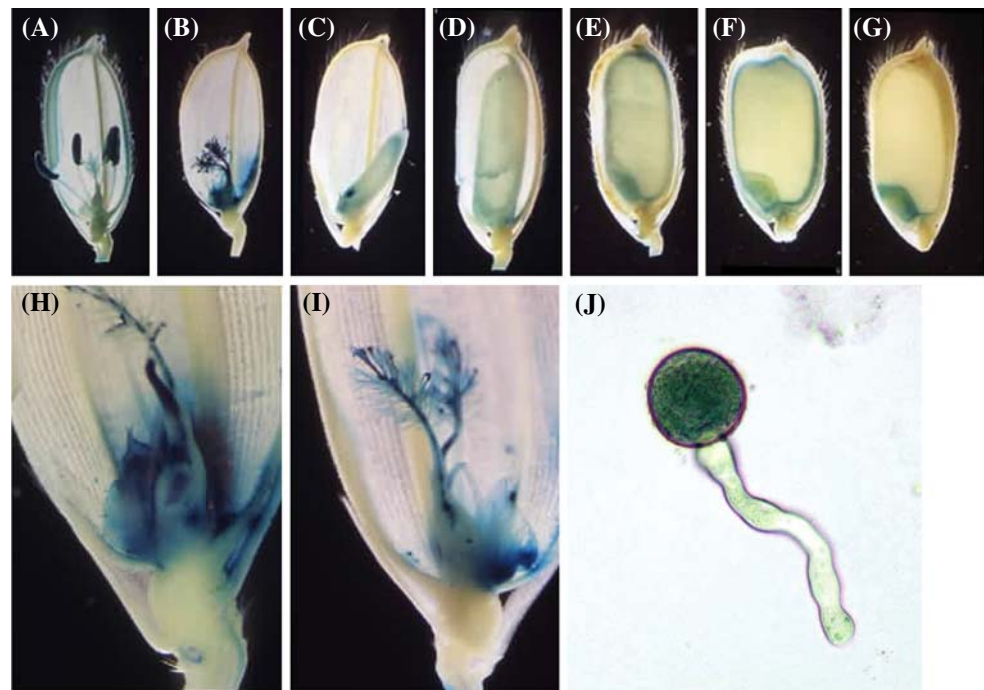
the inner cortex layer above the root tip (Fig. 6d). Around the crown, *OsYSL18* activity was observed in the basal region of the leaf sheath (Fig. 6e). Because the crown is a complex position where phloem and xylem are associated, longitudinal and transverse sections around the basal region of leaf sheaths were prepared for further analysis (Fig. 6f–k). *OsYSL18* expression was found in the basal regions of vascular bundles in every leaf sheath, but was not found in the shoot apical meristem (Fig. 6f). Expression was mainly observed in phloem cells of phloem parenchyma and companion cells (Fig. 6g–k). These expression patterns were unchanged in Fe-deficient plants (data not shown).

Discussion

OsYSL18 is a functional Fe(III)–DMA transporter but is not involved in direct Fe uptake from the rhizosphere

To date, YS1, HvYS1, and OsYSL15 have been reported as Fe–MAs transporters in maize, barley, and rice, respectively (Curie et al. 2001; Murata et al. 2006; Inoue et al. 2009). Expression of these transporters is induced by Fe deficiency in roots. Disruption of the *YS1* gene leads to leaf chlorosis due to a defect in Fe(III)–MAs uptake (von Wirén et al. 1994). HvYS1 protein is localized to root epidermal cells (Murata et al. 2006). *OsYSL15* promoter activity is dominant in epidermal and exodermal cells of Fe-deficient roots (Inoue et al. 2009). According to these data, YS1, HvYS1, and OsYSL15 may be responsible for the primary uptake of Fe–MAs from the rhizosphere. Expression of *HvYS1* and *OsYSL15* show daily fluctuations (Inoue et al. 2009; Nagasaka et al. 2009), possibly for efficient uptake of Fe coordinated with diurnal secretion of MAs (Takagi et al. 1984). In the present report we have shown that OsYSL18 is another functional Fe(III)–DMA transporter localized to the plasma membrane (Figs. 2, 4). However, the expression pattern of *OsYSL18* in roots (Figs. 3, 6) was quite different compared to that of *YS1*, *HvYS1*, or *OsYSL15*. *OsYSL18* expression in roots was extremely low and was not upregulated by Fe deficiency. *OsYSL18* promoter activity was not observed in whole roots but was specific to crown roots (Fig. 6c). Moreover, *OsYSL18* promoter activity in crown roots was restricted to the inner cortex layer (Fig. 6d), but was not obvious in epidermal and exodermal cells, where absorption of Fe(III)–DMA mainly occurs. These results suggest that OsYSL18 is a functional Fe(III)–DMA transporter, but is not involved in primary uptake of Fe(III)–DMA from the rhizosphere. Thus, the rice Strategy-II Fe uptake system would be primarily attributed to OsYSL15 but not to OsYSL18. The primary function of OsYSL18 is deduced to be translocation of Fe(III)–DMA within the plant body.

Fig. 5 Cellular localization of *OsYSL18* expression in flowers and developing seeds during maturation as observed by histochemical staining of *OsYSL18* promoter–*GUS* expression in transgenic rice plants. Before anthesis (a), just after fertilization (b), and 3 (c), 5 (d), 10 (e), 15 (f), and 30 (g) days after fertilization. (h, i) Magnified image just after fertilization. (j) Germinated pollen tube on in vitro culture medium. Shown are the results from a representative T₁ line stained overnight



Role of *OsYSL18* in fertilization

Some members of the YSL family are reported to be involved in metal movement to floral tissues mediated by NA (Curie et al. 2008). In addition to NA, DMA also exists in developing and mature rice grains (Takahashi et al. 2006; unpublished). Interestingly, knockdown mutants of *OsYSL15* exhibit severe arrest in germination and early growth, which are rescued by high iron supply (Inoue et al. 2009), suggesting that Fe in rice endosperm is stored at least partly in the form of Fe(III)–DMA to be utilized *via* *OsYSL15* during seed germination. *OsYSL18* transcripts were most abundant in flowers (Fig. 3) and *OsYSL18* promoter activity was observed in reproductive organs before and after anthesis (Fig. 5). These results strongly suggest the involvement of *OsYSL18* in Fe transport to reproductive organs. Along with *OsYSL18*, *OsYSL2* and *OsYSL15* are co-expressed in vascular bundles of the lemma and embryonic disc (Koike et al. 2004; Inoue et al. 2009). In these organs, these Fe(II)–NA and Fe(III)–DMA transporters may be cooperating in Fe translocation into seeds. Remarkably, the *OsYSL18* promoter is also active in pollen tubes (Fig. 5b, h–j). Our previous examination of other transporters involved in Fe homeostasis clarified that *OsFRDL1*, a citrate effluxer required for efficient Fe translocation (Yokosho et al. 2009), is also expressed in pollen grains (Inoue et al. 2004), whereas none of the other known Fe-related transporter genes including *OsYSL2*, *OsYSL15*, and ferrous transporter gene *OsIRT1* were expressed in pollen tubes (Koike et al. 2004; Inoue et al. 2009; Takahashi et al. unpublished). In *Arabidopsis*, two

YSL genes, *AtYSL1* and *AtYSL3*, are expressed in pollen grains, possibly mediating metal-NA translocation (Le Jean et al. 2005; Waters et al. 2006). Elongation of pollen tubes is indispensable for fertilization, during which Fe is required for various vital processes, including respiration, as well as many other enzymatic reactions. Our results strongly suggest that *OsYSL18* plays an important role in pollen function and fertilization by transporting Fe(III)–DMA into pollen cells.

OsYSL18 functions in the crown and lamina joints

In vegetative shoot organs, *OsYSL18* expression was specifically observed around the crown and lamina joints (Fig. 6a, b, e). In the crown, *OsYSL18* was expressed only in the basal parts of leaf sheath vascular bundles (Fig. 6f). Furthermore, careful observation of transverse sections revealed that *OsYSL18* expression was restricted to the phloem parenchyma and companion cells (Fig. 6g–k). This suggests that *OsYSL18* is involved in the loading of Fe(III)–DMA to phloem by transporting the metal complexes to phloem parenchyma and companion cells. Interestingly, previous tracer experiments showed that various elements supplied from roots or leaves of barley and rice first accumulate in the basal parts of the shoot and are then distributed to other plant parts, as determined in studies using ⁵⁹Fe (Mori, 1998), ⁵²Fe, ⁵²Mn, and ⁶²Zn (Watanabe et al. 2001; Tsukamoto et al. 2006, 2009), ¹¹C-methionine (Nakanishi et al. 1999; Bughio et al. 2001), ¹³N (Kiyomiya et al. 2001a), and H₂¹⁵O (Mori et al. 2000; Kiyomiya et al. 2001b; Nakanishi et al. 2002; Tsukamoto et al. 2004). As a

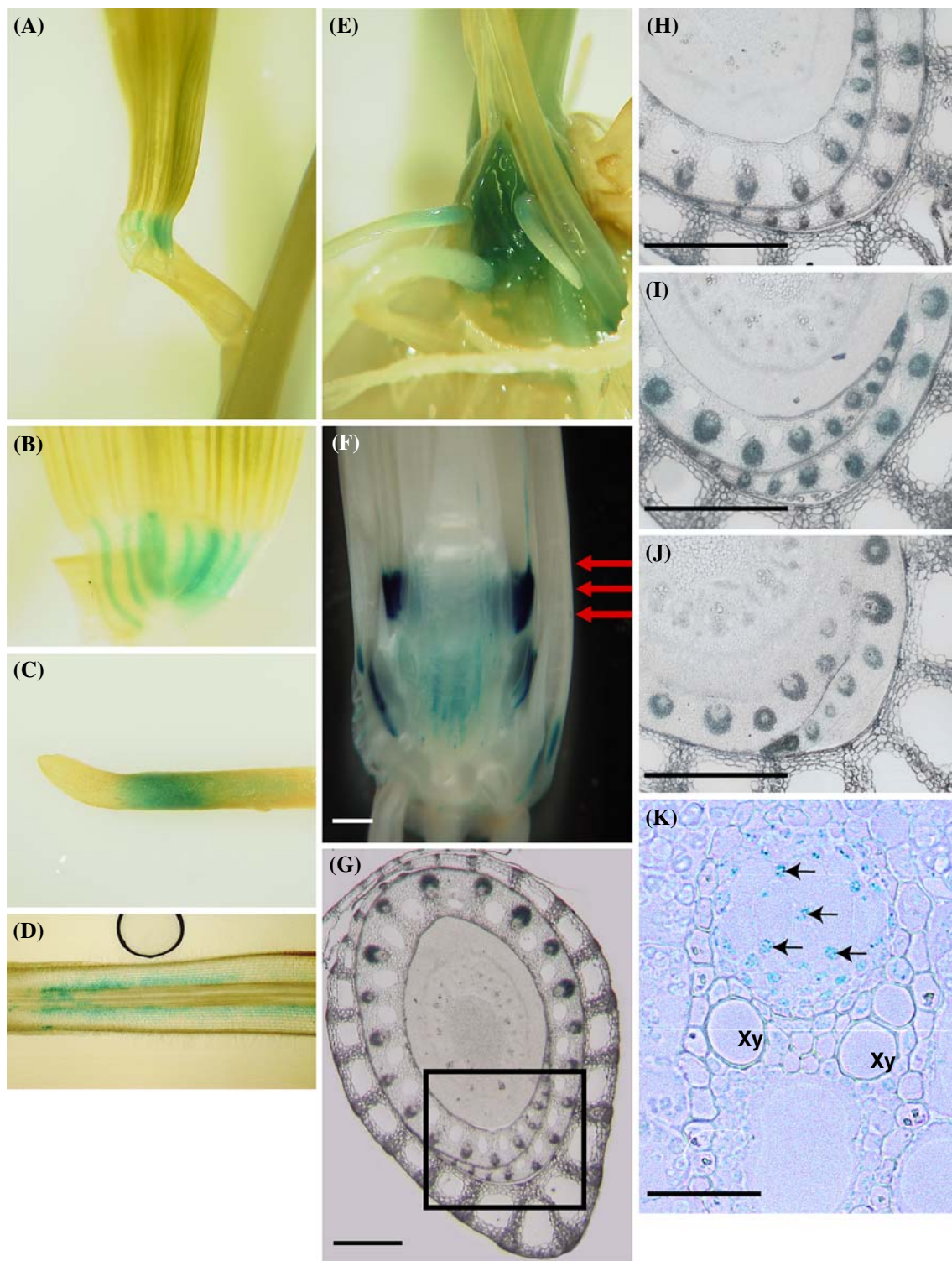


Fig. 6 Cellular localization of *OsYSL18* expression in vegetative organs as observed by histochemical staining of *OsYSL18* promoter-*GUS* expression in transgenic rice plants. **(a)** Expression in lamina joints. **(b)** Magnified image of **(a)**. **(c)** Expression in crown roots. **(d)** Longitudinal section of crown roots. **(e)** Expression in the crown. **(f)** Longitudinal section around the basal parts of the leaf sheath. *Three arrows* indicate the positions of the cross-sections shown in **(g-j)**. **(g)** Cross-section at the *upper arrow* of **(f)**. The *boxed area* shows the

magnified area in **(h)**. **(h-j)** Magnified images of cross sections at the *three arrows* of **(f)**. **(h)**, *upper arrow*; **(i)**, *middle arrow*; and **(j)** *lower arrow*. Intervals between the images are 600 μm. **(k)** Magnified image of a cross-section of a vascular bundle. *Arrow*, phloem companion cell; Xy, xylem. Shown are the results from a representative T₁ line stained for 3 h for **(a-d)**, 30 min for **(e)** and **(f)**, and overnight for **(g-k)**. Scale bars 1 mm for **(f-j)**, and 50 μm for **(k)**

result of these studies, the basal part of the shoot was designated as a discrimination center (DC), where phloem and xylem structures are complexly associated, and minerals and metabolites accumulate prior to translocation to other plant parts. Recently, Tsukamoto et al. (2009) reported that ^{52}Fe supplied as $^{52}\text{Fe(III)-DMA}$ to roots of barley is translocated mainly *via* phloem to the youngest leaf, whereas it is translocated mostly *via* xylem to older leaves, suggesting the importance of Fe transfer from xylem to phloem in the DC and/or the roots. *OsYSL18* expression was specific to the region very close to DC. Therefore, phloem loading of Fe(III)-DMA *via* *OsYSL18* might be important for Fe distribution to leaf sheaths, especially where xylem Fe translocation is not fully developed.

In lamina joints, the *OsYSL18* expression pattern was very similar to that of the crown. Expression was observed only in vascular bundles at the base of leaf blades (Fig. 6a, b). To date, there have been no reports regarding any special role of rice Fe nutrition in lamina joints. The expression of the Fe(III)-DMA transporter specific to the basal parts of vascular bundles of the leaf blades indicates that the lamina joint might also play an important role in Fe translocation in rice. Recently, Tanaka et al. (2008) reported that an *Arabidopsis* boric acid channel NIP6;1 is predominantly expressed in phloem cells of nodal regions, where xylem–phloem transfer of boric acid is proposed to occur. It is conceivable that vascular plants have developed various joint structures to facilitate xylem–phloem transfer of nutritional elements.

Involvement of DMA and NA in the long-distance transport of Fe

Among various metal chelators, the prominent importance of NA and citrate in phloem and xylem Fe translocation have been revealed by physiological, genetic, and molecular studies both in graminaceous and non-graminaceous species (Hell and Stephan 2003; Takahashi et al. 2003; Durrett et al. 2007; Curie et al. 2008; Yokosho et al. 2009). In contrast to these Fe chelators, MAs historically have been identified and characterized as substances responsible for Fe acquisition from the rhizosphere rather than internal Fe translocation. Nevertheless, various lines of evidence suggest that MAs also play roles for internal Fe distribution in graminaceous plants. Endogenous MAs were detected in shoots of barley and rice, and the amount of MAs increased dramatically under Fe deficiency (Higuchi et al. 2001). DMA was also detected in rice phloem sap (Mori et al. 1991). The genes involved in DMA biosynthesis in rice, *OsNAS1-3*, *OsNAATI*, and *OsDMASI*, are co-expressed in the phloem companion cells in roots and leaves (Inoue et al. 2003, 2008; Bashir et al. 2006). Furthermore, the *OsYSL15* transporter gene is also expressed in the phloem companion cells of roots and leaves, as well as in reproductive organs (Inoue et al. 2009). Zn might also be internally transported as a MAS-

chelated form, because Zn(II)-DMA is translocated to Zn-deficient rice leaves at a higher rate than Zn^{2+} (Suzuki et al. 2008). Expression of the newly found Fe(III)-DMA transporter gene, *OsYSL18*, in the phloem cells of phloem parenchyma and companion cells (Fig. 6) strongly supports the notion of the involvement of DMA in long-distance transport of Fe *via* phloem. Although Fe(II)-NA complexes are predicted to be more stable than Fe(III)-DMA in terms of phloem sap pH (von Wirén et al. 1999), and thus chelation is likely to change from DMA to NA in the phloem cells, MAs are likely to play roles for phloem Fe translocation as well. *OsYSL2* and *OsYSL15*, as well as *OsYSL18*, are also co-expressed in large areas of flowers and developing seeds (Fig. 5; Koike et al. 2004; Inoue et al. 2009). Thus, both Fe(II)-NA and Fe(III)-DMA also may be involved in seed Fe loading.

YSL genes consist of a large family, in which the physiological role of each member would be dependent both on its expression pattern and substrate specificity. In rice, expression of *OsYSL2* and *OsYSL15* in vegetative organs is strongly upregulated under Fe deficiency (Koike et al. 2004; Inoue et al. 2009), whereas *OsYSL18* is expressed constitutively, with no induction in response to Fe deficiency. This evidence suggests that *OsYSL2* and *OsYSL15* play important roles in Fe utilization, especially under low Fe availability, whereas *OsYSL18* is responsible for basic Fe homeostasis, which might not be fully accomplished by *OsYSL2* and *OsYSL15* under normal nutritional conditions. Expression of *OsYSL18* in specific cells, as discussed above, suggests the fundamental role of the Fe(III)-DMA transport in these cells, including pollen and phloem cells at the base of the leaf sheath.

Maize *YS1* transporter is able to transport not only Fe(III)-DMA, but also many other DMA- and NA-chelated metals, including Fe(II)-NA (Schaaf et al. 2004). Fe-deficiency-induced expression of *YS1* is evident both in roots and shoots (Curie et al. 2001; Roberts et al. 2004), suggesting that *YS1* is responsible not only for Strategy-II Fe uptake, but also for internal transport of Fe as either MAS- or NA-chelated forms. It might be interesting to explore why rice utilizes at least three *YSL* members (*OsYSL2*, *OsYSL15*, and *OsYSL18*) to accomplish Fe utilization, which might be mediated by a single *YS1* transporter in maize.

YSL family is phylogenetically composed of four subfamilies. *OsYSL18* belongs to the only subfamily with no apparent non-graminaceous members (Fig. 1a; Curie et al. 2008). This evidence, along with the result that *OsYSL18* transports Fe(III)-DMA but not Fe(II)-NA (Fig. 2), suggests the possibility that this subfamily of *YSL* transporters have been evolved to perform graminaceous-specific functions, which might be accomplished by transporting Fe(III)-MAs specifically but not Fe(II)-NA. Harada et al. (2007) reported that the outer membrane loop between the sixth and seventh transmembrane regions of *YS1* and

HvYS1 is essential for substrate specificity. They found 20 amino acid residues that maintain the helical propensity of HvYS1, which is deduced to be responsible for the more extreme substrate specificity of HvYS1 compared to YS1. Among these 20 amino acid residues, OsYSL18 possesses a sequence with little similarity to YS1 (4/20 residues; Fig. 1b, boxed region), whereas it has more similarity to HvYS1 (8/20 residues) or OsYSL15 (7/20 residues). This evidence favors strict substrate specificity of OsYSL18 (Fig. 2), which is more similar to that of HvYS1 and OsYSL15 than YS1. Because the OsYSL18 sequence shares relatively low similarity to any other YS1/YSL transporters in which substrate specificity has been determined, OsYSL18 and other members of this subfamily of YSL might form structures distinct from other well-known YS1/YSL members. Further analysis of transport activity, in combination with domain swap analysis, mutagenesis, or X-ray crystal structural analysis, of these YS1/YSL transporters should reveal the molecular mechanisms underlying substrate recognition and specificity.

The YSL family is also distantly related to the large group of oligopeptide transporters (OPT), among which an *Arabidopsis* gene (*AtOPT3*) is upregulated under Fe deficiency and is involved in Fe translocation to developing seeds (Stacey et al. 2006, 2008). Functional characterization, including substrate specificity, of *AtOPT3* and its counterparts in graminaceous species, would be beneficial for better understanding of Fe translocation and utilization in plants. Finally, manipulation of Fe(III)–DMA transporters in combination with other key components controlling Fe homeostasis will provide further understanding of the mechanisms of Fe nutrition and production of Fe-fortified rice crops.

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