OsFRDL1 expressed in nodes is required for distribution of iron to grains in rice

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Abstract
Iron (Fe) is essential for plant growth and development, but the molecular mechanisms underlying its distribution to different organs are poorly understood. We found that OsFRDL1 (FERRIC REDUCTASE DEFECTIVE LIKE 1), a plasma membrane-localized transporter for citrate, was highly expressed in the upper nodes of rice at the reproductive growth stage. OsFRDL1 was expressed in most cells of enlarged vascular bundles, diffuse vascular bundles, and the interjacent parenchyma cell bridges of uppermost node I, as well as vascular tissues of the leaf blade, leaf sheath, peduncle, rachis, husk, and stamen. Knockout of OsFRDL1 decreased pollen viability and grain fertility when grown in a paddy field. Iron was deposited in the parenchyma cell bridges, a few of the cell layers of the parenchyma tissues outside of the bundle sheath of enlarged vascular bundles in node I in both the wild-type rice and osfrdl1 mutant, but the mutant accumulated more Fe than the wild-type rice in this area. A stem-fed experiment with stable isotope $^{57}$Fe showed that the distribution of Fe in the anther and panicle decreased in the knockout line, but that in the flag leaf it increased compared with the wild-type rice. Taken together, our results show that OsFRDL1 expressed in the upper nodes is required for the distribution of Fe in the panicles through solubilizing Fe deposited in the apoplastic part of nodes in rice.

Key words: Apoplastic Fe, citrate efflux, $^{57}$Fe distribution, node, rice, transporter.

Introduction
Iron (Fe) is an essential element for plant growth and development because it is involved in the transport of electron in many metabolic processes such as respiration and photosynthesis, and is required as a cofactor of numerous enzymes (Hell and Stephan, 2003). The requirement for Fe for normal growth is between 50 mg kg$^{-1}$ and 150 mg kg$^{-1}$ in plant shoot dry weight depending on the plant species (Marschner, 1995), but different tissues also have different Fe requirements; for example, shoot apices require more Fe than other tissues for their active growth. Therefore, Fe in soil must be first taken up by the roots, followed by translocation from the roots to the shoots, and finally distributed to different tissues depending on their requirement. These processes are thought to be mediated by different transporters (Thomine and Vert, 2013), but only a few of them have been identified.

In dicots, Fe uptake is mediated by IRT1 (IRON REGULATED TRANSPORTER 1), a transporter for ferrous iron (Fe$^{2+}$) (Eide et al., 1996; Vert et al., 2003). IRT1 is localized at the root epidermal cells and its expression is induced by Fe deficiency. In contrast, in gramineous plants,
Fe is taken up in the form of an Fe$^{3+}$–phytosiderophore complex, which is mediated by a transporter belonging to the YELLOW STRIPE/YELLOW STRIPE1-LIKE (YSY) family. The expression of YSI in maize, HvYSI in barley, and OsYSYL15 in rice is also induced by Fe deficiency (Curie et al., 2001; Murata et al., 2006; Inoue et al., 2009). Furthermore, HvYSI is also localized at the epidermal cells of barley roots (Murata et al., 2006). Rice seems to have uptake systems mediated by both OsIIRT1 and OsYSYL15 (Ishimaru et al., 2006; Inoue et al., 2009), although their contribution to the uptake differs with soil conditions. It is speculated that in upland soil, OsYSYL15-mediated uptake is predominant, while OsIIRT1 plays a major role in Fe uptake in paddy soil, where ferrous Fe is rich. In addition, OsNramp5 (natural resistance-associated macrophage protein 5) and OsNramp1 also show transport activity for Fe$^{2+}$, although their contribution to total Fe uptake is relatively small (Takahashi et al., 2011; Ishimaru et al., 2012; Sasaki et al., 2012).

After the uptake, in Arabidopsis, part of Fe will be sequestered into the vacuoles through VIT1 (vacuole iron transporter 1) (Kim et al., 2006) and IREG2/FPN2 (IRON REGULATED PROTEIN 2/ferroportin 2) (Schaaf et al., 2006; Morrissey et al., 2009) localized at the tonoplast of epidermal cells, while another part of Fe is loaded to the xylem by AtIREG1/FPN1, a plasma membrane-localized transporter at the pericycle cells (Morrissey et al., 2009). In the same cells, AtNramp3 and AtNramp4 are also expressed, which pump Fe out of the vacuoles in Arabidopsis (Thomine et al., 2003; Lanquar et al., 2005). OsVIT1 and OsVIT2 in rice are also implicated in vacuolar sequestration of Fe (Zhang et al., 2012).

Several studies have shown that a citrate efflux transporter belonging to the multidrug and toxic compound extrusion (MATE) family is required for the efficient translocation of Fe from the roots to the shoots. AtFRD3 (FERRIC REDUCTASE DEFECTIVE 3) in Arabidopsis, OsFRDL1 in rice, GmFRD3 in soybean, and HvAACT1 (Aluminum Activated Citrate Transporter 1) in barley are localized at the root pericycle cells and are responsible for releasing citrate to the xylem (Durrett et al., 2007; Rogers et al., 2009; Yokosho et al., 2009; Fujii et al., 2012). Knockout of these genes results in precipitation of Fe in the root stele and leaf chlorosis in Arabidopsis and rice (Durrett et al., 2007; Yokosho et al., 2009). In addition to citrate, several other chelators are also reported to be involved in internal mobilization of Fe in plants, including nicotianamine, deoxymugineic acids, and phenolic compounds. Release of phenolic acid to the xylem is mediated by PEZ1 (phenolics efflux zero 1) (Ishimaru et al., 2011), while deoxymugineic acid is transported by TOM2 (transporter of mugineic acid 2) (Nozoye et al., 2015).

Iron loaded to the xylem will be distributed to different organs and tissues depending on their demand, but the molecular mechanism underlying Fe distribution is poorly understood. Recently, in rice, it has been shown that nodes play an important role in the distribution of mineral elements (Yamaji and Ma, 2014). Several transporters required for the intervacular transfer at the nodes from the enlarged vascular bundle to the diffuse vascular bundle have been identified. In particular, transporters at the uppermost node I at the reproductive stage are most important for delivering mineral elements to the seeds. For example, three Si transporters (Lsi2, Lsi3, and Lsi6) localized in the different cell layers of node I are involved in the preferential distribution of Si to the pani-

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**Materials and methods**

**Plant materials and growth condition in a paddy field**

A Tos-17 insertion line (osfrdl1) identified previously (Yokosho et al., 2009), pOsFRDL1 (promoter of OsFRDL1)-GFP (green fluorescent protein) transgenic rice, and its wild type (Oryza sativa, cv. Nipponbare) were used in this study. Seeds were germinated in tap water for 2 d in the dark at 30 °C and then transferred to a net floating on a 0.5 mM CaCl$_2$ solution in a 1.2 liter pot. After 7 d, the seedlings were cultured in a nutrient solution (1/2 strength Kimura B solution) containing macronutrients (NH$_4$O (0.18 mM), MgSO$_4$$\cdot$7H$_2$O (0.27 mM), KNO$_3$ (0.09 mM), Ca(NO$_3$)$_2$$\cdot$4H$_2$O (0.18 mM), and K$_2$HPO$_4$ (0.09 mM), and micronutrients MnCl$_2$$\cdot$4H$_2$O (0.5 μM), H$_2$BO$_3$ (3 μM), (NH$_4$)$_2$MoO$_4$$\cdot$4H$_2$O (1 μM), ZnSO$_4$$\cdot$7H$_2$O (0.4 μM), CuSO$_4$$\cdot$5H$_2$O (0.2 μM), and Fe-EDTA (20 μM). The pH of this solution was adjusted to 5.6 and the nutrient solution was renewed every 2 d.

Seedlings (3 weeks old) of both the wild type and osfrdl1 were transplanted in a paddy field at the experimental farm of Okayama University in mid June, 2013. Each plot (0.7 × 0.7 m) contained eight seedlings, and four replicates were made for each line. At the end of October, plants were harvested and used for investigation of yield components and mineral determination.

**Determination of yield components**

At harvest, the panicle number per plant was recorded. After air-drying, the spikelet number per panicle was counted from 10 panicles in each line. The percentage of filled spikelets (fertility) was determined using 300 spikelets in a salt solution with a specific gravity of 1.06 (number of sinking spikelets/total number of spikelets×100). After air-drying, the straws and the filled spikelets were weighed and the 1000-grain weight of filled grains was calculated according to Tamai and Ma (2008). Total filled grain weight per plant (grain yield per plant) was calculated from panicle number×grain number per panicle×fertility (%)/1000×grain weight (g).

**Determination of metal concentration in different organs**

Harvested plants were separated into different organs including brown rice, husk, rachis, flag leaf blade, flag leaf sheath, node I, and the remaining part of the shoots (straw). Samples were dried at 70 °C in an oven for several days and weighed. Digestion of samples was performed with concentrated nitric acid ([60% (w/v)] at 140 °C. The metal concentrations in the digestion solution were determined by inductively coupled plasma mass spectrometry (ICP-MS) (7700; Agilent, http://www.agilent.com/).
Expression pattern of OsFRDL1 at the reproductive growth stage

To investigate the expression of OsFRDL1 in different organs at the reproductive stage, different organs were sampled from the plants grown in the paddy field as described above at the flowering stage. Total RNA was extracted using an RNaseasy plant mini kit (Qiagen, Hilden, NRW, Germany) and then converted to cDNA after DNase I treatment using a SuperScript II kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. The expression level of OsFRDL1 was determined by Sso Fast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), and quantitative real-time PCR was performed on CFX384 (Bio-Rad) using primers: OsFRDL1 (forward) 5′-TCACCAATGCTAGGGCCTGC-3′ and (reverse) 5′-AACCACCGGAAACACCCCTG-3′; Histone H3 (forward) 5′-AGTTTGGTGCTCTCGATTTTG-3′ and (reverse) 5′-TCAACAGGTGACCAGCTACAG-3′; and Actin (forward) 5′-GACTCTGTATGTTCTGACG-3′ and (reverse) 5′-GGCTGGAAAGAGACCTCAGG-3′. Both Histone H3 and Actin were used as internal standards, and relative expression was calculated by the ddCT method using CFX Manager software (Bio-Rad).

Cell specificity of OsFRDL1 expression

To investigate the cell specificity of OsFRDL1 expression, we generated transgenic rice carrying the OsFRDL1 promoter fused with GFP. The 2 kb promoter region of OsFRDL1 (~2001 to −1 bp from the transcriptional start site) was amplified by PCR using primers 5′-AGAGCCTCAATCTAATCGTACTAGACTG-3′ and 5′-TGTCGACGTCTTCGCTCGT-3′ from Nipponbare genomic DNA. The fragment was then subcloned into the binary vector pHZ2H-sGFP (Fuse et al., 2001) to generate pOsFRDL1-GFP using the cloning sites SacI and SalI. This construct was subsequently introduced into Agrobacterium tumefaciens (strain EHA101). Callus was induced from mature embryos of rice cultivar Nipponbare. Agrobacterium-mediated transformation was performed according to the protocol of Hiei et al. (1994).

Immunostaining with an antibody against GFP was performed with the transgenic plants according to Yamaji and Ma (2007). The transgenic line and wildtype were grown in the nutrient solution as described above. At the flowering stage, the flag leaf blade and sheath, node I, peduncle (internode I), rachis, husk, and anther were sampled and subjected to immunostaining. The signal was observed with a confocal laser scanning microscope (LSM700; Carl Zeiss).

Pollen grain viability staining

Pollen grains from both the wild type and osfrdl1 grown in the paddy field as described above were sampled before flowering for viability testing and measurement of anther and pistil size before flowering. Three spikelets from one plant were randomly sampled from a total of four plants. Photographs of pollen were taken under an optical microscope (VHX2000; KEYENCE), and the size of the anther and pistil was measured from the image data. The pollen grains were stained with 1% KI–I2 solution for 5 min at room temperature. Photographs were taken under an optical microscope (CKX41 with CCD camera FX630; Olympus). The pollen viability ratio was calculated as follows: pollen stained with purple color/total pollen in the screen×100. Four biological replicates were performed.

Short-term stem-feeding experiment with 57Fe, rubidium, and strontium

Both the wild type and osfrdl1 were cultivated in the paddy field as described above. At the flowering stage, the plants were cut at internode III (3 cm from node II) with a razor blade. A 5 µM concentration of 57FeCl3 (96.1% 57Fe, TRACE Sciences International) with 1 µM rubidium (Rb) and 1 µM strontium (Sr) in 0.5 mM CaCl2, was fed from the cut end in a growth chamber at 25 °C. 80% relative humidity. After incubation for 24 h, the flag leaf, node I, and panicle were harvested separately and dried at 70 °C in an oven for several days. Concentrations of 57Fe, Rb, and Sr in each part were determined by ICP-MS using isotope mode after digestion as described above.

Results

Expression pattern of OsFRDL1 at the flowering stage

OsFRDL1 is mainly expressed in the roots at the vegetative growth stage of rice (Inoue et al., 2004; Yokosho et al., 2009). To investigate the tissue specificity of OsFRDL1 expression at the reproductive stage, we took different organs of rice grown in a paddy field at the flowering stage and determined their expression level by a quantitative reverse transcription–PCR (RT–PCR). At the flowering stage, in addition to its expression in the roots, higher expression of OsFRDL1 was found in the above-ground parts including nodes, rachis, peduncle, spikelet, and leaves (Fig. 1). The expression levels of the rachis and uppermost node in particular were higher than that of other organs (Fig. 1).

To examine the cell specificity of OsFRDL1 expression, we generated transgenic rice carrying the OsFRDL1 promoter (2 kb) fused with GFP. Immunostaining with an antibody against GFP showed that OsFRDL1 promoter activity was detected at the vascular tissue of the leaf blade of the transgenic line (Fig. 2A, B). However, the signal was not detected in the leaf of wild-type rice (Fig. 2C), indicating the specificity of the antibody used. In addition to this specificity, we confirmed that the signal was detected in vascular tissue in the roots (data not shown), which is the same as previous results by immunostaining with an anti-OsFRDL1 antibody and by GUS (β-glucuronidase) staining in OsFRDL1 promoter–GUS transgenic rice (Inoue et al., 2004; Yokosho et al., 2009). The GFP signal was also observed in the vascular tissues of the leaf sheath (Fig. 2D), peduncle (Fig. 2E), rachis (Fig. 2F), and husk (Fig. 2G). These results for the leaf blade and flower are consistent with the previously seen tissue-specific expression pattern (Inoue et al., 2004). The expression of the OsFRDL1 promoter was also observed in the vascular tissues of the filament but not in the anthers (Fig. 2H). In node I, the expression of the OsFRDL1 promoter was detected in enlarged vascular bundles, diffuse vascular bundles, and the intervening parenchyma tissues (parenchyma cell bridges) (Fig. 2I, J).
Knockout of OsFRDL1 resulted in significant reduction of grain yield

To investigate the role of OsFRDL1 at the reproductive growth stage, we grew a Tos-17 retrotransposon insertional line (osfrdl1) identified previously (Yokosho et al., 2009) and its wild-type rice in a paddy field until ripening. A previous study showed that osfrdl1 (ND8025) is a knockout line without expression of OsFRDL1 (Yokosho et al., 2009). There was no difference in the biomass of the straw between osfrdl1 and the wild type (Fig. 3A). However, the grain yield was decreased by ~60% in osfrdl1 compared with...
Functional analysis of OsFRDL1 at the reproductive stage

among yield components, there was no difference in the panicle number and 1000-grain weight of filled grains between osfrdl1 and the wild type (Fig. 3C, D), but the grain number per panicle was slightly less in the mutant than in the wild type (Fig. 3E). The most significant difference between osfrdl1 and the wild type was observed in the percentage of filled grains (fertility); the fertility of osfrdl1 was decreased by ~55% compared with the wild type (Fig. 3F, G).

To examine whether the reduced fertility results from pollen viability, we performed KI–I2 staining. The amount of normal pollen grains stained with purple color was more in the wild type than in the mutant (Fig. 4A, B), while more abnormal pollen grains stained with weak red color were observed in the mutant (Fig. 4B). As a result, the pollen viability was 20% lower in osfrdl1 than in the wild type (Fig. 4C). Furthermore, the size of the anther was smaller in osfrdl1 compared with the wild type, but there was no difference in the size of the pistil (Fig. 4D–G).

Metal concentration in different organs of rice grown in a paddy field

The concentration of Fe and other metals in different organs was compared between the wild type and osfrdl1 grown in a paddy field. A higher Fe concentration in the flag leaf sheath and blade was found in osfrdl1 than in the wild type (Fig. 5A), while in node I, the Fe concentration was lower in the mutant than in the wild type. There was no difference in the Fe concentration in other organs including remaining straw, peduncle, rachis, husk, and brown rice in the two lines (Fig. 5A). No difference was found in the concentration of Mn and Zn in all organs except brown rice for Zn and Mn, and peduncle for Zn between the wild type and osfrdl1 (Fig. 5B, C). However, the Cu concentration in all organs was higher in the mutant than in the wild type (Fig. 5D).

Fe deposition staining of node I

We examined Fe deposition in node I of both osfrdl1 and the wild type by Perls’ blue staining. In both lines, Fe was deposited in parenchyma cell bridges; a few cell layers next to the apoplastic barrier at the bundle sheath of enlarged vascular bundles (Fig. 6). This deposition was located between the enlarged vascular bundle and diffuse vascular bundle (Fig. 6). The signal of Fe deposition at these cell layers was stronger in osfrdl1 than in the wild type (Fig. 6).

Fig. 3. Phenotypic analysis of the osfrdl1 mutant grown in a paddy field. Both the osfrdl1 mutant and its wild-type (WT) rice (cv. Nipponbare) were cultivated in a paddy field until ripening. (A) Dry weight of straw, (B) grain yield per plant, (C) panicle number per plant, (D) 1000-grain weight of filled grains, (E) grain number per panicle, (F) percentage of filled spikelets. Error bars represent ±SD of eight biological replicates. (G) Panicles at harvest. Asterisks above the bars indicate significant differences (*P<0.05; **P<0.01) compared with the wild-type rice.
Knockout of OsFRDL1 altered Fe distribution at the flowering stage

OsFRDL1 is involved in the translocation of Fe from the roots to the shoots (Yokosho et al., 2009). To exclude its role in the distribution of Fe, we performed a short-term stem-feeding experiment with the $^{57}$Fe stable isotope at the flowering stage. Ferric $^{57}$Fe was fed to the cut end of internode III (3 cm from node II) with Rb and strontium Sr as markers of phloem and xylem transport, respectively (Kuppelwieser and Feller, 1991). The results showed that the distribution of newly absorbed $^{57}$Fe ($\Delta^{57}$Fe) to the flag leaf was increased, but that to the panicles was decreased in osfrdl1 compared with the wild type (Fig. 7A). Although the Fe staining intensity in osfrdl1 was stronger than that in the wild type (Fig. 6), there was no difference in $^{57}$Fe distribution of the node between the wild type and osfrdl1 (Fig. 5A). This difference could be attributed to short exposure (24h) to $^{57}$Fe. On the other hand, there was no difference in the distribution of Rb and Sr to the flag leaf and panicles between the two lines (Fig. 7B, C). Taken together, these results indicate that OsFRDL1 in node I plays an important role in the distribution of Fe at the reproductive growth stage.

Discussion

OsFRDL1 is a plasma membrane-localized efflux transporter for citrate (Inoue et al., 2004; Yokosho et al., 2009). At the vegetative growth stage of rice, OsFRDL1 is mainly expressed in the pericycle cells of mature root regions and mediates release of citrate to the xylem, which is required for the translocation of Fe from the roots to the shoots (Inoue et al., 2004; Yokosho et al., 2009). In the present study, we found that OsFRDL1 is also involved in the distribution of Fe to panicles at the reproductive growth stage. OsFRDL1 showed higher expression in node I and other reproductive organs (Fig. 1). Knockout of this gene resulted in increased Fe deposition in node I and decreased distribution of Fe to the panicles (Figs 5–7). These alterations may be associated with decreased pollen viability and grain fertility (Figs 3, 4).

Iron plays essential roles in pollen development (Lanquar et al., 2005; Roschztartditz et al., 2011; Schuler et al., 2012). To deliver Fe to the pollen and other reproductive organs efficiently in gramineous plants, an intervascular transfer of Fe from the enlarged vascular bundles to the diffuse vascular bundles is required in the nodes (Fig. 8; Yamaji and Ma, 2014). However, transporters involved in this process have not been identified. Iron in the xylem could form complexes with citrate (Tiffin, 1970; Durrett et al., 2007; Yokosho et al., 2009; Rellán-Alvarez et al., 2010), phytosiderophore (Kakei et al., 2009; Nozoye et al., 2015), and phenolic compounds (Ishimaru et al., 2011), but the Fe–citrate complex is the dominant form because the citrate concentration in the xylem is much higher than that of other chelators.
Functional analysis of OsFRDL1 at the reproductive stage

Fig. 5. Metal concentration in different organs at the reproductive growth stage of rice. Both the wild type (WT) and the osfrdl1 mutant were grown in a paddy field until ripening. After harvest, different organs were separated and subjected to determination of Fe (A), Mn (B), Zn (C), and Cu (D). Error bars represent ±SD of four biological replicates. Asterisks above the bars indicate significant differences (*P<0.05; **P<0.01) compared with the WT rice.

Fig. 6. Iron deposition in node I with Perls’ blue staining. Both the wild type (WT; A, B) and the osfrdl1 (C, D) mutant were grown in a paddy field until the flowering stage. Node I was sliced and subjected to Perls’ blue staining. Blue color shows Fe deposition. Scale bars=100 μm. EVB, enlarged vascular bundle; DVB, diffuse vascular bundle; bs, bundle sheath of enlarged vascular bundle; pcb, parenchyma cell bridge.
For preferential distribution of Fe to the reproductive organs through intervascular transfer, the Fe–citrate complex transported to the upper nodes through an enlarged vascular bundle must be unloaded first to the xylem parenchyma cells and then loaded to the xylem of the diffuse vascular bundle (Fig. 8). In the osfrdl1 mutant, due to decreased citrate in the

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**Fig. 7.** Distribution of Δ^{57}Fe, rubidium (Rb), and strontium (Sr) to different organs at the reproductive growth stage. Both the wild type (WT) and osfrdl1 were cultivated in a paddy field until the flowering stage. The plants were cut at internode III below node II and fed with 5 µM ^{57}FeCl₃, 1 µM Rb, and 1 µM Sr in a 0.5 mM CaCl₂ solution. After 24 h, the flag leaf, node I, and panicle were separately harvested and subjected to determination of ^{57}Fe, Rb, and Sr. The distribution ratios of Δ^{57}Fe (A), Rb (B), and Sr (C) in different organs were calculated. Error bars represent ±SD of three biological replicates. Asterisks above the bars indicate significant differences (*P<0.05; **P<0.01) compared with the WT rice.

**Fig. 8.** Schematic presentation of the role of OsFRDL1 in node I. The Fe–citrate complex in the xylem of enlarged vascular bundle in node I is unloaded by an unidentified influx transporter to the xylem parenchyma cells. Fe is then symplastically transported to the bundle sheath cells, where an apoplastic barrier is located. Part of Fe is released from the bundle sheath cells and/or parenchyma cell bridge through an unidentified efflux transporter and deposited in the apoplast of the parenchyma cell bridge as an Fe pool. OsFRDL1 localized at most cells of node I mediates release of citrate from the cells, which solubilizes apoplastic Fe deposited at the parenchyma cell bridge. The Fe–citrate complex is finally re-loaded to the diffuse vascular bundle for subsequent loading of Fe to the panicle.
xylem, less Fe–citrate will be unloaded from the xylem. As a result, more unchelated Fe will be transported to the flag leaf following the transpiration, resulting in higher Fe in the flag leaf of the mutant. After Fe–citrate is loaded from the xylem, citrate is again required for mobilization of Fe between two vascular bundles; therefore, knockout of OsFRDL1 resulted in more Fe precipitation in the parenchymal cell bridge.

Recently, an apoplastic barrier was found to be located on the bundle sheath of the enlarged vascular bundles (Yamaji et al., 2015). Therefore, Fe in the xylem parenchyma cells must thus be sympatlymphically transported to the bundle sheath cells (Fig. 8). We found that Fe was heavily deposited in the parenchyma cell bridges next to this apoplastic barrier (Fig. 6), indicating that part of Fe is transported out again from the bundle sheath cells and/or parenchyma cell bridges to the apoplast. This Fe deposition seems to be an Fe pool, which is necessary to avoid excess Fe concentration in the symplast (Curie and Briat, 2003). Therefore, to utilize this Fe for subsequent transport of Fe to the panicles in rice, a solubilization process by suitable chelating molecules is required. OsFRDL1 expressed in node I seems to be involved in this solubilization process by releasing citrate to the apoplastic space. This is supported by the finding that more Fe was deposited in the parenchyma cell bridge of node I in osfrd1 (Fig. 6). Furthermore, knockout of OsFRDL1 resulted in decreased distribution of Fe to the panicles, but increased distribution to the flag leaf and higher Fe concentration in the flag leaf (Figs 5, 7). This role of OsFRDL1 in rice is similar to that of AtFRD3 in Arabidopsis. In addition to its role in root to shoot translocation of Fe, a recent study showed that AtFRD3 also plays an important role in proper Fe transport to the embryo and pollen (Roschztardtz et al., 2011). AtFRD3 is expressed in the seeds and flowers of Arabidopsis, and knockout of this gene results in a defect of early germination and almost complete sterility (Roschztardtz et al., 2011). In addition to the expression of OsFRDL1 in nodules, it was also expressed in the vascular tissues of other organs including the leaf, peduncle, rachis, husk, and filament (Fig. 2). Similar to its role in nodules, OsFRDL1 in these tissues is probably also involved in the solubilization of apoplastic Fe. These findings suggest that both AtFRD3 in Arabidopsis and OsFRDL1 in rice have similar role in solubilizing apoplastic Fe in reproductive organs.

Under Fe-limited conditions, knockout of OsFRDL1 resulted in a significant decrease of Fe concentration in the shoots (Yokosho et al., 2009). However, no difference was found in Fe concentration of straw between the wild type and osfrd1 when grown in a paddy field (Fig. 5). This inconsistency may be attributed to the Fe concentration in the hydroponic culture solution or soil solution. In paddy fields, ferrous Fe in soil solution is very high due to soil reduction, resulting in higher Fe accumulation in the mutant shoot. In fact, when the osfrd1 mutant was grown hydroponically in a high Fe concentration, the Fe concentration of the shoots also significantly increased (Yokosho et al., 2009). Therefore, the reduced pollen viability and grain fertility in the mutant is not due to the decreased root to shoot translocation of Fe, but to the incorrect distribution of Fe to different organs at the flowering stage. Among other essential metals, there was almost no difference in the accumulation of Zn and Mn between the wild type and osfrd1 mutant (Fig. 5), but the Cu concentration in all organs was higher in the mutant than in the wild type. The reason for this increased Cu accumulation in the mutant shoots is unknown. One possibility is that internal Fe deficiency in the mutant due to incorrect Fe distribution at the reproductive stage may induce expression of some unknown Cu uptake transporter genes. Studies showed that there was crosstalk between Fe and Cu in plants (Bernal et al., 2012; Perea-Garcia et al., 2013; Waters et al., 2014). In rice, the expression of OsYSL16, which is a Cu-nicotianamine transporter gene responsible for delivering Cu from the old tissues to the young tissues through phloem transport, was up-regulated by Fe deficiency in the roots (Zheng et al., 2012). The expression of OsCOPT (COPPER TRANSPORTER) 2, 5, and 7 in the shoots was also induced by Fe deficiency although their exact role remains to be investigated (Yuan et al., 2011). In Arabidopsis, knockout of AtFRD3 resulted in increased accumulation of Mn and Co (Roschztardtz et al., 2011).

In conclusion, in addition to its role in translocation of Fe from the roots to shoots, OsFRDL1 also plays an important role in distribution of Fe to the reproductive organs by releasing citrate to solubilize apoplastic Fe in the vascular tissues of rice.

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