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Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese

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SUMMARY

Rice (*Oryza sativa*) is indispensable in the diet of most of the world's population. Thus, it is an important target in which to alter iron (Fe) uptake and homeostasis, so as to increase Fe accumulation in the grain. We previously isolated *OsYSL2*, a functional iron [Fe(II)]- and manganese [Mn(II)]-nicotianamine complex transporter that is expressed in phloem cells and developing seeds. We produced RNAi (OsYSL2i) and overexpression lines (OX*OsYSL2*) of OsYSL2. At the vegetative stage in an OsYSL2i line, the Fe and Mn concentrations were decreased in the shoots, and the Fe concentration was increased in the roots. At the reproductive stage, positron-emitting tracer imaging system analysis revealed that Fe translocation to the shoots and seeds was suppressed in OsYSL2i. The Fe and Mn concentrations were decreased in the seeds of OsYSL2i, especially in the endosperm. Moreover, the Fe concentration in OX*OsYSL2* was lower in the seeds and shoots, but higher in the roots, compared with the wild type. Furthermore, when *OsYSL2* expression was driven by the sucrose transporter promoter, the Fe concentration in the polished rice was up to 4.4-fold higher compared with the wild type. These results indicate that the altered expression of *OsYSL2* changes the localization of Fe, and that OsYSL2 is a critical Fe-nicotianamine transporter important for Fe translocation, especially in the shoots and endosperm.

Keywords: yellow stripe 1, endosperm, iron, manganese, nicotianamine, rice.

INTRODUCTION

Iron (Fe) is an essential micronutrient for both plants and animals. Fe deficiency is one of the most prevalent micronutrient deficiencies in the world, affecting an estimated two billion people (Stoltzfus and Dreyfuss, 1998), and is blamed for 0.8 million deaths worldwide per year (WHO, 2002). Thus, increasing the ability of plants to accumulate higher levels of Fe in grains could have a dramatic impact on human health (Guerinot, 2001; Clemens *et al.*, 2002). The target Fe concentration in polished rice is 14 $\mu g\ g^{-1}$, which is seven times higher than that in wild-type (WT) rice (Pfeiffer and Mcclafferty, 2007). Compared with WT rice, transgenic rice plants expressing soybean ferritin under the control of the rice endosperm-specific *glutelin-B1* promoter can accumulate 2.5 times more Fe in their seeds (Goto *et al.*, 1999). However, for the transgenic plants, greater accumulation of ferritin in the endosperm does not lead to increased Fe, suggesting that Fe transport to the endosperm needs to be enhanced (Lucca *et al.*, 2006). Rice endosperm without the aleurone layer, commonly known as polished rice or starch storage tissue, is eaten; however, more than 85% of the Fe in rice seeds is located in the bran, which surrounds the starch storage tissue, and is removed from the brown rice (unmilled rice without the husk) during processing (Pron-u-thai *et al.*, 2007). Therefore, efficient Fe translocation to the starch storage tissue is desirable.

Nicotianamine (NA) is an Fe chelator formed from *S*-adenosyl-L-methionine (SAM) by NA synthase (NAS). It is a structural analog of 2′-deoxymugineic acid (DMA), which is formed by NA aminotransferase (NAAT) and DMA synthase (DMAS) (Bashir *et al.*, 2006). DMA is a

phytosiderophore in rice plants that acquires Fe from the soil. The tomato (*Solanum lycopersicon*) NA-less mutant *chloronerva*, which is chlorotic, despite the presence of a high concentration of Fe in its leaves and a constitutively active Fe-uptake system in its roots, fails to set flower if not supplied with exogenous NA (Higuchi *et al.*, 1996; Stephan *et al.*, 1996; Ling *et al.*, 1999). Transgenic tobacco plants constitutively expressing the barley *NAAT* gene have a reduced NA content, resulting in young leaves with interveinal chlorosis and abnormally shaped flowers, resulting from a disturbance in Fe distribution (Takahashi *et al.*, 2003). A shortage of NA causes disorders in internal Fe transport, which leads to abnormal phenotypes, suggesting that NA is indispensable for appropriate Fe translocation in plants.

Rice plants take up Fe via the Strategy II system and direct Fe^{2+} uptake system (Ishimaru *et al.*, 2006). DMA is synthesized and released into the soil, where it chelates Fe^{3+}, and is then absorbed as Fe(III)-mugineic acid (MA) complex via specific transporters at the root surface. Maize yellow stripe 1 (*ZmYS1*) is the gene encoding the Fe^{3+}-MA transporter (von Wire'n *et al.*, 1994; Curie *et al.*, 2001). Eight *ZmYS1* homologs have been identified in the dicotyledonous plant *Arabidopsis thaliana*; however, dicotyledonous plants do not secrete MAs. Thus, it is possible that YS1-like (YSL) proteins function to transport metal-NA complexes. YSL1 from *A. thaliana* (AtYSL1) was observed on the lateral sides of the xylem parenchyma plasma membrane, but the identity of the AtYSL1 substrate was not clear in uptake experiments with yeast or *Xenopus laevis* oocytes (DiDonato *et al.*, 2004; Schaaf *et al.*, 2005). Recently, it was reported that AtYSL1 is necessary for correctly loading Fe and NA into Arabidopsis seeds (Le Jean *et al.*, 2005). In rice, the expression of *OsYSL2*, one of 18 putative *OsYSL* genes in this species, is upregulated by Fe deficiency in the shoots, with particularly strong expression in the phloem, as well as expression in developing seeds (Koike *et al.*, 2004). OsYSL2 localizes in the plasma membrane and transports Fe(II)-NA and manganese [Mn(II)]-NA, suggesting that OsYSL2 may be required for the long-distance transport of Fe(II)-NA and Mn(II)-NA through the phloem and into the grain.

In this study, we showed that a functional OsYSL2 was required to produce seeds containing Fe at WT levels, and suggest that OsYSL2 contributes to the long-distance transport of Fe into the shoot and seeds. An RNAi line of OsYSL2 (OsYSL2i) exhibited decreased Fe translocation to seeds, decreased Fe concentration in shoots and seeds, especially in the endosperm, and higher accumulation of Fe in the roots. Plants overexpressing *OsYSL2* (OX*OsYSL2*) also showed decreased Fe translocation to shoots and seeds. Moreover, *OsYSL2* expression driven by the sucrose transporter promoter increased the Fe concentration in polished rice.

RESULTS

Production and phenotype of the OsYSL2i plant

In rice, the Fe(II)-NA transporter gene *OsYSL2* expresses in shoots, particularly in the phloem, as well as in embryo and endosperm (Koike *et al.*, 2004). Understanding the role of OsYSL2 in Fe transport within rice, especially to the endosperm, is an important step in increasing the Fe content of the edible portion of rice seeds. To understand the role of OsYSL2 in rice, we sought *OsYSL2* knock-out mutant lines, but none were available in either T-DNA insertion lines or *tos17* mutant lines from the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp). Therefore, we produced *OsYSL2* knock-down lines using the RNAi method. Seven independent lines were generated, and the expression of endogenous *OsYSL2* in T_1 OsYSL2i leaves of lines 3 and 5 was suppressed, as revealed by northern blot analysis (Figure 1a). Fe concentrations in the seeds were also decreased in lines 3 and 5, consistent with the suppression of *OsYSL2* (Figure 1b). Quantitative real-time RT-PCR analysis also confirmed that OsYSL2 was significantly suppressed in the roots and shoots of line 5 in the T_2 generation, and was also suppressed to a lesser extent in the roots and shoots of line 3 (Table 1). It should be noted that the high expression of *OsYSL2* in the roots under Fe deficiency was not consistent with our previous study (Koike *et al.*, 2004), but was in line with Ogo *et al.* (2007). This discrepancy may be related to technical problems, and so we confirmed our results in several replicates.

At the early growth stage, the growth of line 5 was impaired compared with the WT, when grown under Fe-sufficient conditions. These growth defects appeared to be more significant when grown under Fe-deficient conditions (Figure 1c–e). However, there was no significant difference between line 3 and the WT, when grown under Fe-deficient or Fe-sufficient conditions (Figure 1d,e).

In the line 5 grown hydroponically, the Fe concentration was lower in the shoots and higher in the roots compared with WT plants (Figure 2a,b), whereas the Mn concentration in both the roots and shoots was lower compared with the WT (Figure 2c,d). On the other hand, line 3 accumulated Fe and Mn at the WT level, suggesting that the function of OsYSL2 in this line would not be sufficiently suppressed in the roots and shoots (Figure 2a–d).

Microarray analysis of OsYSL2i

Consistent with the quantitative real-time RT-PCR analysis, *OsYSL2* was downregulated in OsYSL2i roots compared with WT roots (Table 2). The expression of endogenous *OsYSL2* could not be detected in OsYSL2i and WT shoots, owing to the limit of detection in the microarray analysis (Appendix S1). The *OsYSL2* fragment used for RNAi was

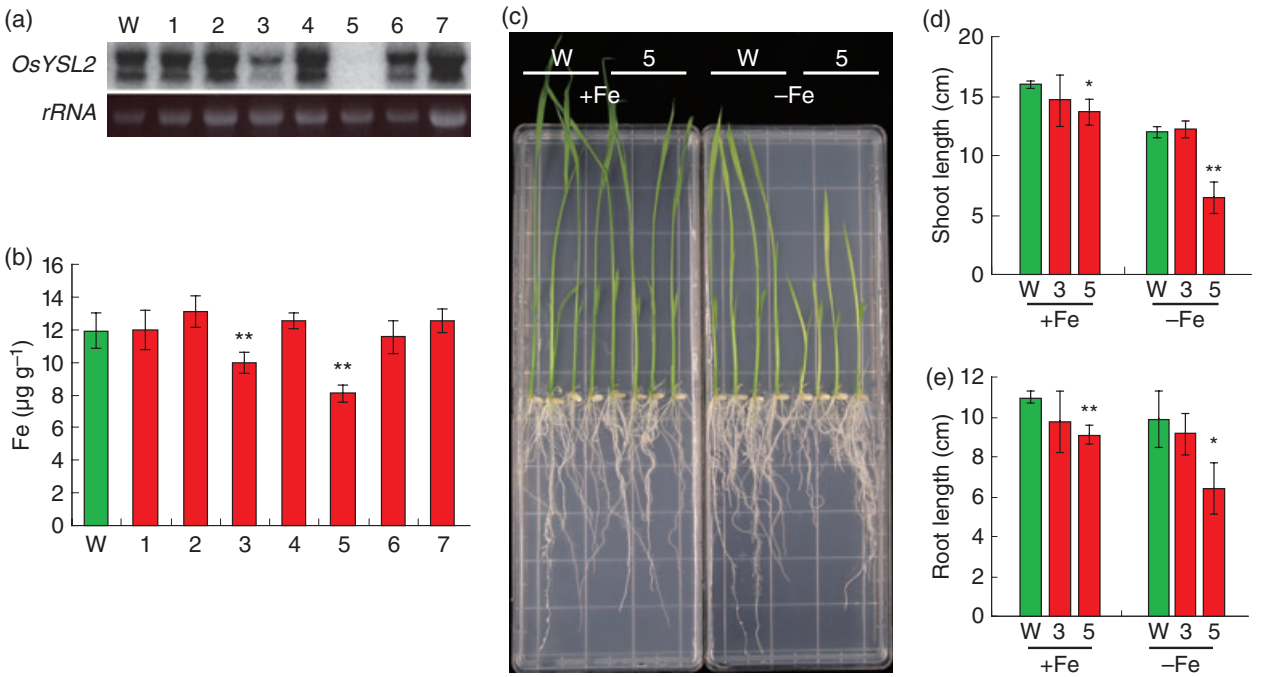

Figure 1. Expression and seed iron (Fe) concentrations of OsYSL2i and phenotype of OsYSL2i at the early growth stage.
(a) Northern blot analysis of OsYSL2 in OsYSL2i plants. Transcription of OsYSL2 in the shoots of wild-type (W) and OsYSL2i (1–7) T_1 plants grown under Fe-deficient conditions. Total RNA (10 μg) was used for northern analysis.
(b) Fe concentrations in the wild-type (W) and OsYSL2i (1–7) T_1 plants in seed grown in soil.
(c) Photograph of wild-type (W) and OsYSL2i plants (line 5) grown in Fe-sufficient (+Fe) and -deficient (–Fe) MS medium for 2 weeks. Shoot (d) and root (e) lengths in wild-type (W) and OsYSL2i plants (lines 3 and 5) grown in +Fe and –Fe MS medium for 2 weeks. The values followed by asterisks are statistically different from the wild type according to a Student's *t*-test (**P* < 0.05; ***P* < 0.01).

74% homologous with the Fe(III)-DMA transporter gene, *OsYSL15*, which is the closest homologue of *OsYSL2*. Microarray analysis revealed that *OsYSL15* was not suppressed by the *OsYSL2i* plants in the roots and shoots. Thus, the change in induction ratios of *OsYSL*s would be related to the knock-down of OsYSL2, but not to the cross suppression for the fragment. In the roots of the OsYSL2i plant, the Fe^{2+} transporter gene *OsIRT1*, Fe(III)-DMA transporter *OsYSL15*, and the vacuolar Fe transporter gene *OsNramp1* were upregulated, compared with WT roots (Table 2). SAM synthase (*OsSAMS1*), *OsNAS1* and *OsNAS2* were also upregulated in OsYSL2i roots (Table 2).

Positron-emitting tracer imaging system (PETIS) analysis of OsYSL2i

OsYSL2 is expressed at the reproductive stage, and seems to contribute to Fe accumulation in seeds (Koike *et al.*, 2004). Therefore, we performed Fe-transport experiments at the reproductive stage using PETIS, which is capable of visualizing real-time Fe translocation in plants. Compared with the WT plants, line 5 transported less Fe to the shoots and panicle under Fe-sufficient conditions (Figure 3a). Images obtained using a Bio-imaging Analyzer System (BAS) confirmed the reduced Fe transport to the panicle in the line 5, compared with the WT (Figure 3b,c). Fe accumulation in the flag leaf, leaf axis, immature and mature seeds was also reduced in line 5, compared with WT plants (Figure 3d).

Metal concentration in OsYSL2i seeds

PETIS analysis revealed that OsYSL2 contributes to Fe transport into the shoots and seeds. We measured the metal concentrations in OsYSL2i brown rice and polished rice grains to examine changes in the Fe distribution within OsYSL2i seeds, and to check the effects of *OsYSL2* knock-down on the concentrations of metals.

In line 5, which suppressed *OsYSL2* in the root, the shoot, and the ear (Table 1), the Fe concentration was 17% lower in brown rice and 37% lower in polished rice, compared with WT brown and polished rice, respectively (Figure 2e). In line 3, which suppressed *OsYSL2* mainly in the ear (Table 1), the Fe concentration was 18% lower in brown rice and 39% lower in polished rice, compared with WT brown and polished rice, respectively (Figure 2e).

OsYSL2 takes up Mn(II)-NA in addition to Fe(II)-NA (Koike *et al.*, 2004). In line 5, the Mn concentration was 29% lower in brown rice and 35% lower in polished rice, compared with WT brown and polished rice, respectively (Figure 2f). In line 3, the Mn concentration was 31% lower in brown rice and 33% lower in polished rice, respectively, compared with the WT (Figure 2f). There was no difference in the

concentration of other metals between the OsYSL2i and WT rice (data not shown).

Analysis of *OXOsYSL2* plants

We next assessed whether the overexpression of *OsYSL2* (OX*OsYSL2*) in rice plants could enhance Fe accumulation in the seeds. Twelve independent OX*OsYSL2* transgenic rice lines were examined, and quantitative real-time RT-PCR analysis confirmed the higher constitutive expression of *OsYSL2* in OX*OsYSL2* plants (lines 2 and 11; Table 1). Constitutive overexpression of *OsYSL2* decreased the Fe concentration in the brown rice and shoots (Figure 4a,b), although the Fe concentration was higher in the roots of OX*OsYSL2* plants (Figure 4c). On the other hand, the Mn concentration was increased in the brown rice and decreased in the roots of OX*OsYSL2* plants (Figure 4d–f). Nevertheless, the changes in Fe and Mn contents did not affect the growth of OX*OsYSL2* in either Fe-sufficient or Fe-deficient conditions (data not shown).

OsYSL2 driven by the OsSUT1 promoter

Sucrose is produced in photosynthetic tissues and transported via a long-distance vascular pathway to the endosperm of the seed. Suppression of the sucrose transporter *OsSUT1*, which is highly expressed in phloem cells, and around the endosperm, resulted in reduced sucrose translocation to starch storage tissue (Hirose *et al.*, 2002; Scofield *et al.*, 2002). OsSUT1 is also expressed in the mature phloem of all the vegetative tissues involved in the long-distance assimilate transport pathway during grain filling, in addition to the flag leaf blade and sheath prior to heading (Scofield *et al.*, 2007).

To enhance Fe accumulation in polished rice, *OsYSL2*, in conjunction with the 1.7-kbp sucrose transporter (*OsSUT1*) promoter, was introduced into rice plants. Six independent transformants were confirmed as harboring the *OsSUT1*-promoter *OsYSL2*, and quantitative real-time RT-PCR revealed that *OsYSL2* expression in the ears was higher than that in the WT plants (Table 3). The concentration of Fe in polished rice was higher for the plants containing *OsSUT1* promoter-driven *OsYSL2* than for WT plants: for example, the Fe concentration in polished rice of line 18 was 4.4 times higher than that in WT polished rice (Figure 5a). The Fe content of the transformed brown rice also increased in lines 2, 10, 14 and 18 (Figure 5b). Furthermore, the Fe content in polished rice was only 15% of that in brown rice for the WT plants, whereas more Fe was transported from the bran to the polished rice in the transformed plants, especially in line 18 (Figure 5b). The Mn concentration in *OsSUT1*-promoter *OsYSL2* rice also increased in both the brown rice and the polished rice (Figure 5d,e). However, there was no difference in Fe and Mn concentrations in the shoots of transgenic and WT plants (Figure 5c,f). Consistent with the expression of *OsSUT1* (Hirose *et al.*, 2002; Scofield

Table 1 Quantitative real-time RT-PCR analysis of *OsYSL2* in wild-type (WT), OsYSL2i and OX*OsYSL2* plants

	WS	WR	WE	2i-3S	2i-3R	2i-3E	2i-5S	2i-5R	2i-5E	OX2S	OX2R	OX2E	OX11S	OX11R	OX11E
+Fe	0.043 ± 0.00	0.23 ± 0.00	0.11 ± 0.03	0.032 ± 0.00	0.20 ± 0.00	n.d.	n.d.	n.d.	n.d.	25 ± 0.94	31 ± 0.19	2.5 ± 0.32	22 ± 0.41	27 ± 0.41	2.1 ± 0.28
–Fe	18 ± 0.35	14 ± 0.18	–	4.1 ± 0.97	4.5 ± 0.58	–	0.24 ± 0.00	0.090 ± 0.00	–	15 ± 0.28	21 ± 0.59	–	11 ± 0.15	18 ± 0.45	–

(10^6 copies μg^{-1} total RNA) Total RNA was isolated from the shoot (S), the root (R) and the ear (E) of WT (W), OsYSL2i (2i-3 and 2i-5) and OX*OsYSL2* (OX2 and OX11) plants grown under iron (Fe)-sufficient (+Fe) and -deficient (–Fe) conditions (WS, WR, WE, 2i-3S, 2i-3R, 2i-3E, 2i-5S, 2i-5R, 2i-5E, OX2S, OX2R, OX2E, OX11S, OX11R and OX11E), and were tested for *OsYSL2* expression by quantitative real-time RT-PCR. The values represent the mean ± SD of the number of transcripts in 1 μg of total RNA from three reactions. The root and the shoot of 4-week-old plants were sampled and the ears were sampled 8 days after fertilization. n.d., not detected; –, no sample.

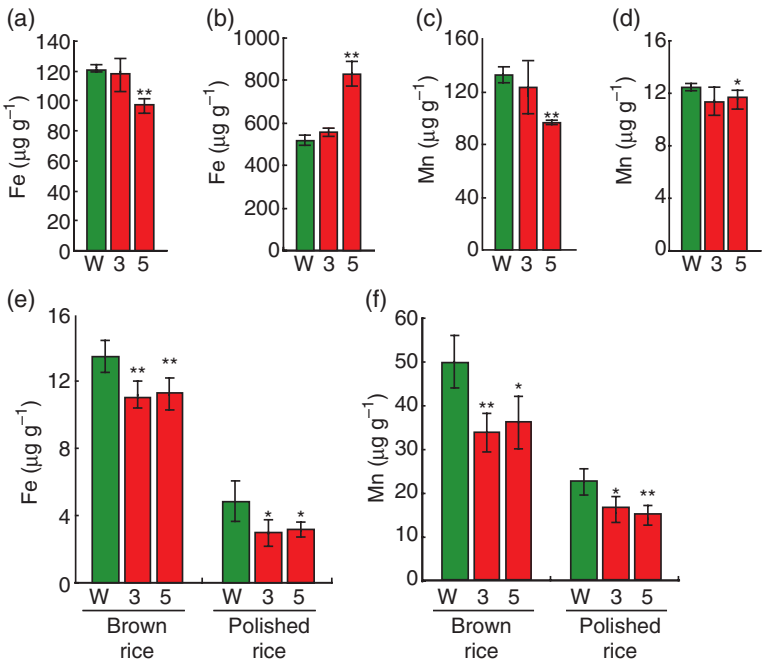

Figure 2. Iron (Fe) and manganese (Mn) concentrations of OsYSL2i plants in the vegetative stage and seeds grown in soil.
Fe concentration in the shoots (a) and roots (b) of wild-type (W) and OsYSL2i plants (lines 3 and 5) in control hydroponic culture for 4 weeks. Mn concentration in the shoots (c) and roots (d) of wild-type (W) and OsYSL2i plants (lines 3 and 5) in control hydroponic culture for 4 weeks.
(e) Concentration of Fe in the brown and the polished rice of wild-type (W) and OsYSL2i plants (lines 3 and 5).
(f) Concentration of Mn in the brown and the polished rice of wild-type (W) and OsYSL2i plants (lines 3 and 5). The values followed by asterisks are statistically different from the wild type according to a Student's *t*-test ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$). Plants were grown in soil for 26 weeks.

et al., 2002, 2007), Fe accumulation was observed in the endosperm of the rice transformed with *OsSUT1*-promoter *OsYSL2* (Figure 6a–h).

DISCUSSION

The role of OsYSL2 at various stages

During the early growth stage, OsYSL2i plants showed growth defects under Fe-sufficient and Fe-deficient conditions (Figure 1). This observation is consistent with the reported expression of *OsYSL2* mainly in the epithelium, the vascular bundle of the scutellum and the leaf primordium, suggesting that OsYSL2 is important for Fe translocation at the early growth stage (Nozoye *et al.*, 2007).

At the vegetative stage, OsYSL2i plants accumulated a high level of Fe in the roots, and a low level of Fe in the shoots (Figure 2). A low Fe level in the shoot may induce the expression of the Fe^{2+} transporter gene *OsIRT1*, the Fe(III)-DMA transporter gene *OsYSL15* and the vacuolar Fe transporter gene *OsNramp1* in the root, as all of these proteins mediate Fe uptake into cells (Table 2). The upregulation of these genes could account for the higher Fe accumulation in OsYSL2i roots (Figure 2). However, despite high Fe accumulation in the roots, the Fe concentration in the shoots was low, suggesting that Fe was not transported sufficiently because of the reduced expression of OsYSL2.

In the present study, PETIS analysis revealed that Fe translocation to the shoots and panicle of OsYSL2i plants was decreased, and the Fe concentration of OsYSL2i seeds was lower than that of WT seeds (Figures 2 and 3). In barley, it is reported that the Fe was transported from roots to young leaves via phloem (Tsukamoto *et al.*, 2009). In a young organ, such as the panicle, some Fe would be transported via the phloem; therefore, Fe(II)-NA phloem transport to the panicle would be suppressed in the OsYSL2i plant.

OsYSL2 expresses in the developing ovary 5 days after anthesis, and expression was also observed in the embryo and outer layer of the endosperm, where storage proteins and minerals accumulate (Koike *et al.*, 2004). Thus, the OsYSL2 appears to be important for long-distance transport during grain filling, particularly for Fe translocation to the endosperm, as the Fe concentration in OsYSL2i polished rice was much lower than that in WT polished rice (Figure 2).

Gene expression patterns in OsYSL2i

Compared with WT roots, the roots of OsYSL2i showed upregulation of *OsIRT1*, *OsYSL15* and *OsNramp1*. SAM synthase (*OsSAMS1*), *OsNAS1* and *OsNAS2* were also upregulated in OsYSL2i roots (Table 2). In line with the expression of *OsYSL2*, *OsIRT1* and *OsNAS1* also express in the phloem, especially in the companion cells (Inoue *et al.*, 2003; Koike *et al.*, 2004; Ishimaru *et al.*, 2006). Despite the high expression levels of *OsIRT1* and *OsNAS1* in OsYSL2i, Fe translocation to shoot and seed was reduced, which could be because of the reduced expression of OsYSL2. Thus, Fe(II)-NA translocation by OsYSL2 could not be complemented by other Fe translocation systems. These results suggest a clear role of OsYSL2 in Fe transport from root to shoot. When the expression of *OsYSL2* is altered, the Fe is not properly transported to shoots and seeds. Thus, the plant may sense Fe deficiency, and as a result upregulate the expression of *OsIRT1*, *OsYSL15* and *OsNramp1*, which are

Table 2 Gene expression patterns related to Fe homeostasis

Accession No.	Gene name	2i/W Root	2i/W Shoot	±Root	±Shoot	WRoot signal	WShoot signal
OsYSL transporters							
AK121040	*OsYSL1*	0.7	1.2	1.0	1.0	11	3
CI446246	*OsYSL2*	0.2	1.0	6.3	38.3	35	4
AK068865	*OsYSL4*	1.3	1.9	1.0	1.1	4	3
AK108750	*OsYSL5*	0.9	0.7	1.0	1.4	714	398
AK100148	*OsYSL6*	0.9	1.0	1.0	1.3	1994	1091
AK100087	*OsYSL7*	1.4	1.2	0.8	0.2	27	67
AK072347	*OsYSL8*	1.2	0.7	1.4	0.9	5	32
AK069645	*OsYSL10*	1.2	1.6	1.0	1.1	5	4
AK069437	*OsYSL12*	1.0	0.5	1.4	1.8	520	294
AK067235	*OsYSL13*	0.5	0.5	0.7	1.3	820	377
AK063464	*OsYSL15*	2.0	16.3	13.1	126.9	753	20
AK070304	*OsYSL16*	1.6	0.6	1.9	1.5	9263	7195
AK070618	*OsYSL18*	1.3	1.0	0.9	1.1	4	3
OsIRT transporters							
AK107681	*OsIRT1*	3.2	0.9	2.8	1.6	6299	306
CI162465	*OsIRT2*	0.7	0.9	3.1	26.0	2527	34
DMA metabolism and methionine cycle							
CI069897	*OsSAMS1*	72.4	16.3	0.9	0.7	91	319
AK112069	*OsNAS1*	6.6	2.1	9.2	545.5	6144	31
AK112011	*OsNAS2*	9.3	2.1	12.9	170.7	2193	14
AK070656	*OsNAS3*	1.4	1.0	2.7	0.1	4976	1497
AB206814	*OsNAAT1*	1.5	0.7	6.7	3.7	4202	2513
AK073738	*OsDMAS1*	1.6	0.8	6.5	7.7	1102	358
AK104788	*OsFDH*	1.2	1.0	3.0	1.4	2985	4151
AK119212	*OsIDI4*	1.1	1.0	3.3	1.7	14 249	10 106
AK065321	*OsDEP*	1.2	1.0	3.6	1.6	5839	6199
AK060326	*OsIDI2*	0.9	0.8	2.8	2.1	232	647
AK067649	*OsMTK1*	1.3	0.9	2.2	1.3	5701	3027
AK104481	*OsMTN*	1.5	1.0	2.7	1.1	7391	4052
AK073627	*OsAPT1*	1.0	0.9	2.2	1.7	9658	5091
Other genes regulated by Fe							
AK121534	*OsNramp1*	2.0	1.9	5.6	53.6	865	251
AK102242	*Ferritin*	1.1	0.7	0.4	0.4	8797	31049
AK068159	*OsFRO2*	1.2	2.2	0.9	8.9	5	141
AK073385	*OsIRO2*	1.8	2.6	5.9	30.4	132	5

The ratios of the inducible genes in OsYSL2i (line 5) compared with those in the wild type (WT) are presented (2i/W). The induction ratios for the WT plants were calculated as the relative increase or decrease in expression under conditions of iron (Fe) deficiency compared with that under Fe-sufficient conditions (±). The ratios are the means of two independent replicates. The root and the shoot of 4-week-old plants were sampled.

normally induced by Fe deficiency, and ultimately lead to the accumulation of more Fe in the roots.

Transcription factor *OsIRO2* is upregulated by Fe deficiency, and OsIRO2 regulates the expression of *OsNAS1*, *OsNAS2*, and genes involved in the methionine cycle under Fe-deficient conditions (Ogo *et al.*, 2007). In OsYSL2i plants, *OsNAS1* and *OsNAS2* were induced, but *OsIRO2* was not induced. The expression of *OsNAS1* and *OsNAS2* may be independent of OsIRO2 regulation in these plants.

Mn transport of OsYSL2

There are few reports on Mn transport and regulation in rice plants. In tree tobacco (*Nicotiana glauca*), Mn is commonly assumed to have low mobility in the phloem (Marschner, 1995). There are indirect evidences supporting the low mobility of Mn in Arabidopsis (Waters *et al.*, 2006; Waters and Grusak, 2008). However, it has recently been reported that the transpiration stream in barley (*Hordeum vulgare*) has little effect on Mn translocation to the youngest leaf, in contrast to the strong effect on Mn translocation in older leaves (Tsukamoto *et al.*, 2006). The data indicates that Mn is somewhat transported through the phloem as well as the xylem. In addition to Fe(II)-NA, OsYSL2 also transports Mn(II)-NA, and Mn concentrations in OsYSL2i seeds and shoots were lower than those in WT plants (Figure 2; Koike *et al.*, 2004). Thus, OsYSL2 could be involved in Mn translocation in the phloem.

Overexpression of *OsYSL2*

Although *OsYSL2* in OX*OsYSL2* plants was overexpressed in Fe-sufficient conditions, there were slight changes in Fe and Mn concentrations, and no difference in the growth between

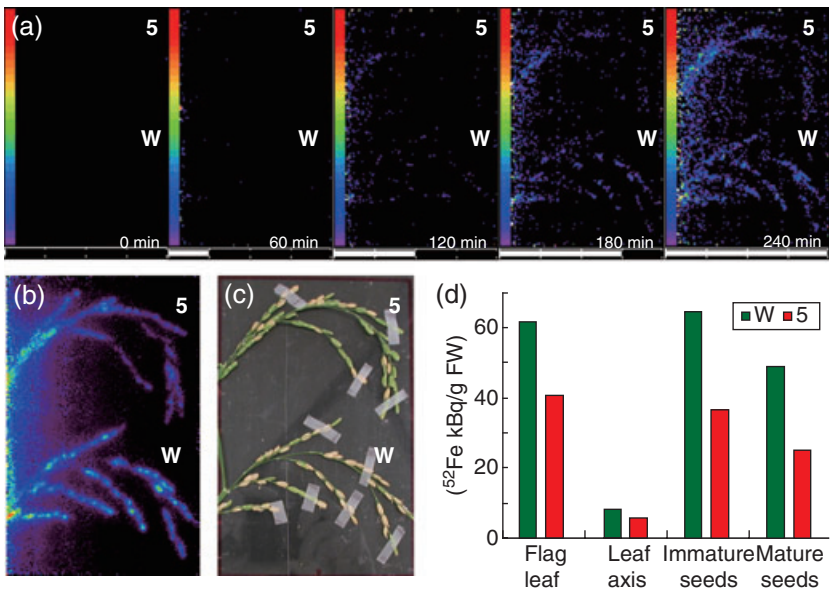

Figure 3. Iron (Fe) transloation in OsYSL2i at the reproductive stage.
(a) Fe real-time image of positron-emitting tracer imaging system (PETIS) analysis.
(b) Bio-imaging Analyzer System (BAS) image of OsYSL2i (line 5) and wild-type (W) plants. The colors, from blue through green, yellow, orange and red indicate increasing Fe uptake.
(c) Photograph of OsYSL2i (line 5) and wild-type (W) plants.
(d) Fe accumulation of OsYSL2i (line 5) and wild-type (W) plants in the flag leaf, leaf axis, immature seeds and mature seeds after 6 h of the PETIS experiment. Plants were grown in Fe-sufficient hydroponics for 17 weeks.

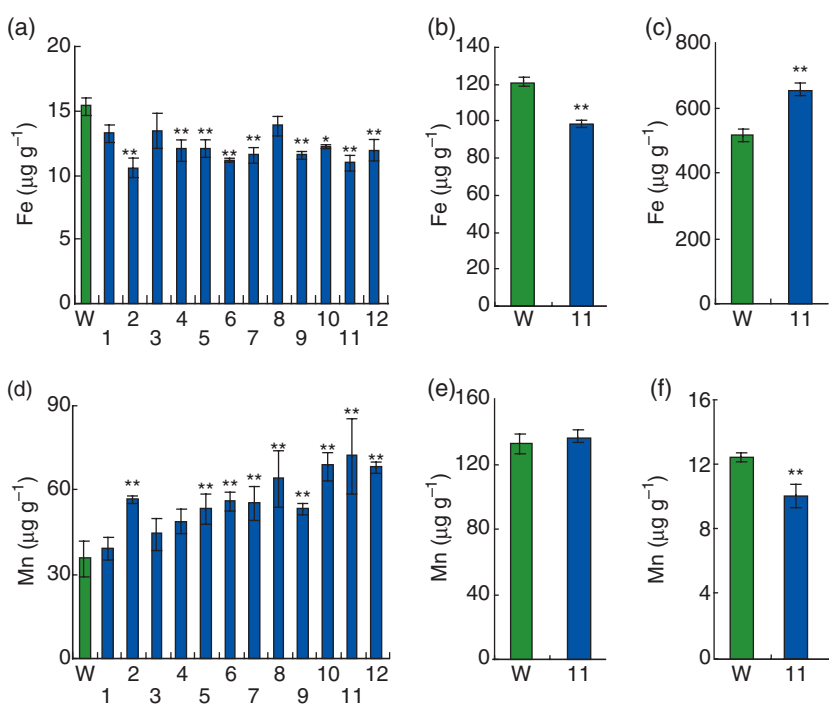

Figure 4. Iron (Fe) and manganese (Mn) concentrations in OX*OsYSL2* plants.
Fe concentration in wild-type (W) and OX*OsYSL2* seeds (a), shoots (b) and roots (c) (line 11). Mn concentration in wild-type (W) and OX*OsYSL2* (line 11) seeds (d), shoots (e) and roots (f). Plants were grown in soil (26 weeks) (a,d), and in hydroponics (4 weeks) (b,c,e,f). The values followed by asterisks are statistically different from the wild type according to a Student's *t*-test (n = 3; $*P < 0.05$; $**P < 0.01$).

WT and OX*OsYSL2* plants (Figure 4; Table 1). In Arabidopsis, analysis of the overexpression of *IRT1* transgenics revealed that IRT1 protein was present only in Fe-limited roots, although *IRT1* mRNA is expressed constitutively in such plants (Vert *et al.*, 2002). As OX*OsYSL2* plants had a strong phenotype, it may not be the case for *OsYSL2*. Surprisingly, the expression of *OsYSL2* in *OXOsYSL2* plants grown under Fe-limited conditions was less compared with *OXOsYSL2* plants grown under Fe-sufficient conditions (Table 1). It is difficult to predict the complex mechanism controlling the expression of *OsYSL2* under these conditions without extensive experiments. In this study our focus was to understand the role of OsYSL2 in Fe and Mn transport, and so we leave these questions for future studies.

Compared with WT plants, OsYSL2i and OX*OsYSL2* plants exhibited Fe concentrations that were lower in the seeds and shoots, and higher in the roots (Figures 2 and 4). In WT plants, Fe taken up from the soil is normally

Table 3 Quantitative real-time RT-PCR analysis of *OsYSL2* in the *OsSUT1*-promoter *OsYSL2* ears

	WE	1E	2E	4E	10E	14E	18E
+Fe	0.092 ± 0.010	0.21 ± 0.051	0.42 ± 0.080	0.27 ± 0.052	0.52 ± 0.12	0.69 ± 0.11	0.64 ± 0.091

(10^6 copies μg^{-1} total RNA) Total RNA was isolated from the ear (E) of wild-type (W), and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18) grown under iron (Fe)-sufficient conditions (+Fe) (WE, 1E, 2E, 4E, 10E, 14E and 18E) and tested for *OsYSL2* expression by quantitative real-time RT-PCR. The values represent the mean ± SD of the number of transcripts in 1 μg of total RNA from three reactions. The ears were sampled 8 days after fertilization.

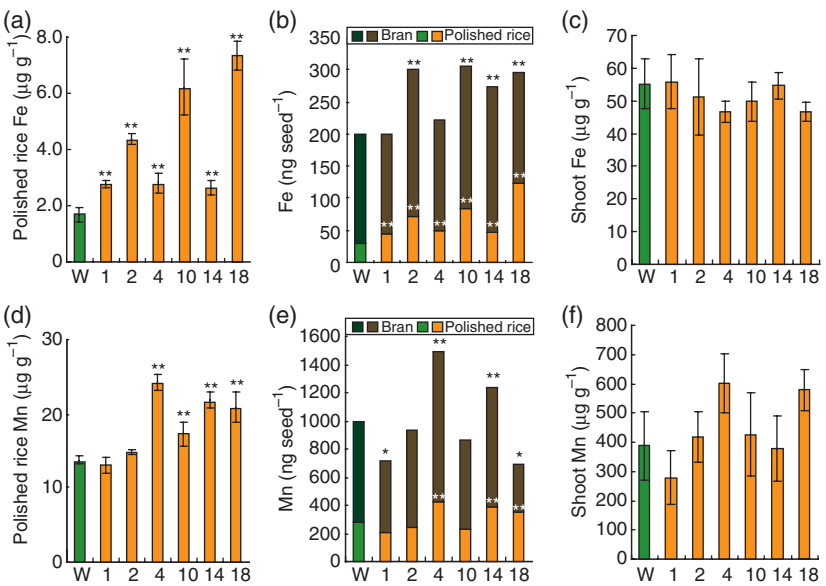

Figure 5. Iron (Fe) and manganese (Mn) content of *OsSUT1*-promoter *OsYSL2* plants.
(a) The concentration of Fe in the polished rice of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18).
(b) The Fe content in the bran and the polished rice of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18).
(c) The concentration of Fe in the shoots of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18).
(d) The concentration of Mn in the polished rice of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18).
(e) The Mn content in the bran and the polished rice of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18).
(f) The concentration of Mn in the shoot of wild-type (W) and *OsSUT1*-promoter *OsYSL2* plants (1, 2, 4, 10, 14 and 18). Plants were grown in soil for 26 weeks. The values followed by asterisks are statistically different from the wild type according to a Student's *t*-test ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$).

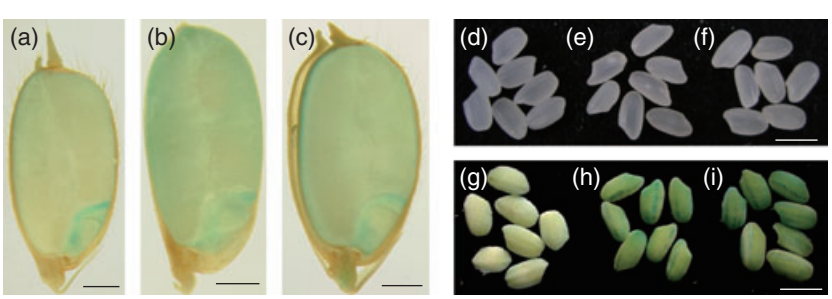

Figure 6. Iron (Fe) localization in *OsSUT1*-promoter *OsYSL2* seeds.
Fe localization in the seeds of wild-type plants (a) or *OsSUT1*-promoter *OsYSL2* lines 10 (b) and 18 (c), as determined by Perl's staining.
(d–i) Fe localization in polished rice of wild-type plants (d, g) or *OsSUT1*-promoter *OsYSL2* lines 10 (e, h) and 18 (f, i), as determined by Perl's staining. Before staining (d–f). After staining (g–i). Scale bars: 1 mm for (a–c); 5 mm for (d–i). Plants were grown in soil for 26 weeks.

translocated from the root cells through the xylem or phloem to the shoots and seeds. It seems that the roots of OX*OsYSL2* plants ectopically overexpressing *OsYSL2* absorbed Fe(II)-NA, which is normally transported to shoots and seeds, resulting in an accumulation of Fe in the roots, and a low concentration of Fe in the shoots and seeds.

A similar phenomenon has also been reported for Zn: the overexpression of the Zn transporter, OsZIP4, led to high Zn accumulation in the roots, and a low Zn concentration in the shoots and seeds (Ishimaru *et al.*, 2007a). Thus, the OsYSL2i and OXOsYSL2 plants shared the same phenotype; however, they do not share the mechanism responsible for this phenotype.

The Mn concentration was higher in OX*OsYSL2* seeds and lower in OX*OsYSL2* roots compared with the respective levels in WT plants. Thus, the Mn translocation system appears to be different for OsYSL2i and OX*OsYSL2*.

OsYSL2 driven by the *OsSUT1* promoter

The Fe concentration was lower in OsYSL2i and OX*OsYSL2* seeds compared with WT seeds. Previously, we showed that transgenic plants expressing Fe-chelate reductase under the control of the *OsIRT1* promoter were tolerant to low Fe availability in calcareous soils, suggesting that the gene could be regulated to have a desirable effect in transgenic plants (Ishimaru *et al.*, 2007b). Therefore, we investigated whether OsYSL2 could be appropriately regulated, including the temporal and spatial control of expression, to facilitate the production of transgenic rice containing high levels of Fe in the endosperm. In *OsSUT1*-promoter *OsYSL2* rice, the Fe concentration was altered mainly in the endosperm, but not in the shoots (Figure 5a,c).

Our data clearly indicated a role of OsYSL2 in transporting phloem Fe to the shoots and seeds. Moreover, we have successfully demonstrated that the seed Fe contents could be increased by appropriately regulating an Fe transporter. The expression of ferritin in the endosperm, together with *OsSUT1* promoter-driven *OsYSL2*, may facilitate the production of rice plants with even more Fe in the polished rice, which would be useful for combating Fe deficiency in humans.

EXPERIMENTAL PROCEDURES

Plant material

Oryza sativa cv. Tsukinohikari was used in all experiments. For roots and shoots of the quantitative real-time RT-PCR analysis, microarray analysis and metal concentration analysis in hydroponic culture, seeds were germinated for 3 days at 25°C on paper soaked with distilled water. After germination, the seedlings were transferred to a Saran net floating on distilled water in a growth chamber (day, 25°C, 14 h of light at 320 μmol photons m^{-2} s^{-1}; night, 10 h at 20°C). After 3 days, 45 seedlings were transferred to a 20-L plastic container containing a nutrient solution of the following composition: 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.05 μM Na_2MoO_4 and 0.1 mM Fe-EDTA. The pH of the nutrient solution was adjusted daily to 5.5 with 1 M HCl, and was renewed weekly. For the Fe-deficiency treatments, 3-week-old plants were transferred to nutrient solution without Fe, and grown for one more week. For PETIS analysis, 3-week-old plants were grown under Fe-sufficient conditions for 14 weeks.

For the seed metal concentration and the quantitative real-time RT-PCR in the ear, seeds were germinated for 2 weeks on MS medium at 28°C, under 16-h light/8-h dark conditions. After germination, the seedlings were transferred to 500 g of bonsol (Sumitomo Chem. Co., http://www.sumitomo-chem.co.jp). The soil was evenly fertilized with 3.5 g of a LongTotal-70 (Chisso-Asahi, http://www.chisso.co.jp; N : P : K, 13 : 11 : 13) and LongTotal-140 (Chisso-Asahi; N : P : K, 13 : 11 : 13) per plant. Plants were grown in a pot in a glasshouse under natural light conditions for 24 weeks. The ears for the quantitative real-time RT-PCR analysis were sampled 8 days after fertilization.

Rice transformation

A binary vector for RNAi was made by modifying pIG121Hm using Gateway technology (Hiei *et al.*, 1994). A part of the *GUS* gene (795 bp) was used as a linker. The antisense strand of the target gene was set into the 5′ side of the *GUS* linker, and the sense strand was set into in the 3′ side. A forward (5′-CAAGCTTCTAGACTCGAGTGCCTGCAGGTATCGATGAGACCA-GGAAGTCGACCCGGGATCCGAGCT-3′) and a complementary (5′-CGGATCCCGGGTCGACTTCCTGGTCTCATCGATACCTGCAGGC ACTCGAGTCTAGAAGCTTGGTAC-3′) oligonucleotide were annealed and then cloned into pBluescript SK(+) vector between the *Kpn*I and *Sac*I sites to make restriction sites (*Kpn*I–*Hin*dIII–*Xba*I–*Xho*I–*Sse*8387I–*Ban*III–*Sal*I–*Sma*I–*Bam*HI–*Sac*I). After confirmation of the sequence accuracy, the *GUS* linker, which was amplified with the primers 5′-AAAGTGTACGTATCGATGTTTGTGTGAACAA-CGAACT-3′ and 5′-TGGTTTTTGTCTCGAGCTATCAGCTCTTTAATC-GCCT-3′ was subcloned using the *Ban*III and *Sal*I sites of the modified pBluescript SK(+) vector. The Gateway RfB cassettes (Gateway vector conversion system; Invitrogen, http://www.invitrogen.com) were cloned into both sides of the *GUS* linker region in the antisense and sense orientation by insertion into the *Xho*I/*Ban*III and *Sma*I restriction enzyme sites of the vector, respectively. The whole sequence of the RfB cassettes and the *GUS* linker were cloned into the *Xba*I/*Sac*I sites of a pIG121Hm vector. Rice Actin1 promoter was subcloned between the *Hind*III and *Sal*I sites to replace the *CaMV35S* promoter of the original vector. The generated binary vector was named pIG121-RNAi-DEST.

To suppress *OsYSL2*, 300 bp of the specific region in the *OsYSL2* ORF was amplified using the primers 5′-TCTGCTGGCTTCTTTGCAT-TTTCTGTGCTCATCAA-3′ and 5′-CATGCAGATTGGTGGCTTAACC-TTAGCAAGGGAAA-3′. There is no homologous sequence of this 300-bp region used in the RNAi construct in the rice genome. The amplified fragment was cloned into pENTR/D-TOPO vector (Invitrogen), and the sequence was verified. Using an LR recombination reaction, the insert was introduced into the destination vector pIG121-RNAi-DEST.

To overexpress *OsYSL2*, the plasmid pIG121Hm was used as a backbone (Ishimaru *et al.*, 2007a). The construct had *Xba*I and *Sac*I sites on the 3′ side of the CaMV35S promoter. Adopter (*Xba*I–*Xho*I–*Sac*I) was inserted into the 3′ side of the CaMV35S promoter in pIG121Hm. The *OsYSL2* open reading frame (ORF) was amplified using the primers 5′-GAGATCTAGAATGGAAGCCGCCGCTCCCGA-GATAG-3′ and 5′-GAGACTCGAGGCTTCCGGGAGTGAACTTCATG-CAG-3′, and *OsYSL2* was digested at the *Xba*I and *Xho*I sites and subcloned to the 3′ side of the CaMV35S promoter in pIG121Hm.

To create the *OsSUT1*-promoter *OsYSL2* rice, 1.7-kbp of the promoter region of *OsSUT1* was amplified using the primers 5′-GCGTCGACGCACAACTCCATATCTCGATCC-3′ and 5′-GCGGATC-CGGCGGACGCGCCACGCACAAAC-3′, and the *OsSUT1* promoter was digested at the *Sal*I and *Bam*HI sites. The *OsYSL2* ORF was amplified using the primers 5′-GCGGATCCAGCCATGGAAGCC-

GCCGCTCCCGAG-3′ and 5′-GCGAGCTCCTAGCTTCCGGGAGT-GAACTTC-3′, and *OsYSL2* was digested at the *Bam*HI and *Sac*I sites. tNOS was digested from *CaMV35S* promoter-*sGFP*(S65T)-NOS3 with the *Not*I and *Eco*RI sites (Ishimaru *et al.*, 2005). The pBluescript SK(+) vector included the *Kpn*I and *Sac*I sites. An annealed oligomer (5′-GGTCGACGGGGGGGATCCGGGGGGAG-CTCGGGCCCGGGGGGAATTCGCGGCCGCCGGTACCAAGCT-3′and 5′-TGGTACCGGCGGCCGCGAATTCCCCCCGGGCCCGAGCTCCCCC-CGGATCCCCCCCGTCGACGGTAC-3′) was inserted between the *Kpn*I and *Sac*I sites in pBluescript SK(+) to produce *Kpn*I, *Sal*I, *Bam*HI, *Sac*I, *PsPOM*I, *Eco*RI, *Not*I, *Kpn*I, and to delete *Sac*I. tNOS fragment with *PsPOM*I and *Eco*RI, *OsYSL2* ORF with *Bam*HI and *Sac*I, and then *OsSUT1* promoter with *Sal*I and *Bam*HI were inserted into the new SK vector. *OsSUT1* promoter-*OsYSL2* ORF-tNOS was subcloned to the *Kpn*I site in pIG121Hm.

Agrobacterium tumefaciens strain (C58) carrying the above construct was used to transform rice (*O. sativa* L. cv. Tsukinohikari) following a method described previously (Ishimaru *et al.*, 2006). The T_1 seeds obtained from the transformants were germinated on MS medium containing 50 mg L^{-1} hygromycin B.

Determination of metal concentrations

Seeds, shoots or roots samples were digested with 1 ml of 13 M HNO_3 and 1 ml of 8.8 M H_2O_2 (Wako, http://www.wako-chem.co.jp) at 220°C for 20 min using MARS XPRESS (CEM, http://www.cem.com) in triplicate. After digestion, samples were collected and diluted up to a 5-ml solution, and were then analyzed by SPS1200VR ICP-AES (SEIKO, http://www.seiko.co.jp) at wavelengths of 238.204 nm (Fe) and 293.930 nm (Mn).

In order to obtain the polished rice, 30 brown rice seeds were put into a 2-ml tube and shaken vigorously for 150 s at 600 ***g***, three times, in a MULTI BEADS SHOCKER (Yasui Kikai, http://www.yasuikikai.co.jp). Rice bran and rice hulls were obtained as a by-product of the polishing process. About 10 polished rice grains from each population were digested and measured for metal concentration.

Quantitative real time-PCR of OsYSL2

Total RNA was treated with RNase-free DNase I (TaKaRa, http://www.takara-bio.com) to remove contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (ToYoBo, http://www.toyobo.co.jp) by priming with oligo-$d(T)_{30}$. A fragment was amplified by PCR in a SmartCycler (TaKaRa) with SYBR Green I and ExTaq™ RealTime-PCR Version (TaKaRa). The primers used for RealTime-PCR were as follows: *OsYSL2* forward, 5′-GAGGGACAACGGTGTCATTGCTGGT-3′; *OsYSL2* reverse, 5′-TGCAGAAAAGCCCTCGACGCCAAGA-3′. The primers used for internal control in RT-PCR were α-*tubulin* forward, 5′-TCTTCCACCCTGAGCAGCTC-3′; α-*tubulin* reverse, 5′-AACCTTG-GAGACCAGTGCAG-3′. The sizes of the amplified fragments were confirmed by agarose gel electrophoresis and sequencing.

Production of ^{52}Fe

The ^{52}Fe (half life: 8.27 h) was produced by the ^{nat}Cr (α, 4n) ^{52}Fe reaction by bombarding a 1.5-mm-thick Cr foil (natural isotopic composition, 99.9% purity; Goodfellow, http://www.goodfellow.com) with a 100-MeV α beam from the TIARA AVF cyclotron (Gunma, Japan). About 1 MBq of ^{52}Fe was produced using a beam current of 3 μA for 2 h. The radiochemical separation of ^{52}Fe from the target was carried out as described by Tsukamoto *et al.* The $^{52}Fe^{3+}$ solution lacking non-radioactive Fe was adjusted to about pH 3.0 with 1 M KOH, and the $^{52}Fe^{3+}$ was chelated with 1.12 μmol of DMA in darkness for 2 h.

Measurements using PETIS and PMPS

The positron-emitting tracer imaging system (PETIS) uses positron-emitting nuclides to monitor the movement of nutrients in plants in real time. Plant samples containing a positron-emitting nuclide are placed between two opposed two-dimensional block detectors composed of $Bi_4Ge_3O_{12}$ scintillator arrays. Two annihilation γ-rays from the decaying positrons are detected in coincidence by these detectors (Tsukamoto *et al.*, 2009). The original position of the annihilation is localized at the intersection of the object plane, with a line connecting the two detection points on the detectors. The field of view is 143 × 215.6 mm, and the spatial resolution is 2.4 mm. The resulting image is displayed on a monitor after automatic correction for a given half-life (in this case 8.27 h) and for the relative detection efficiencies within the field of view. The PETIS detectors were calibrated as follows: ^{22}Na (1-mm radius, 370 kBq) was placed at the center between the paired detectors, and radioactivity was counted using these efficiency values. The analysis of the results was performed on a Macintosh computer using the public-domain program NIH IMAGE (developed at the US National Institutes of Health, and available on the Internet by anonymous FTP at http://nih.gov or on a floppy disk from the National Technical Information Service, Springfield, VA, part number PB95-500195GEI).

Quantitative analysis by γ-ray spectrometry

After PETIS analysis or image analysis using BAS, as described below, the plants were cut into parts and analyzed for the absolute level of radioactivity from the γ-rays of decaying positrons: γ-ray spectra were measured with an ORTEC HPGe detector of 38% relative efficiency and standard electronics. The detector and the sample, placed 11 cm from the detector's surface, were housed in a lead-shielded box. The radioactivity of ^{52}Fe was determined from the peak area at 168 keV.

Conditions for ^{52}Fe translocation in plants

Fe-deficient transgenic and WT plants were supplied with 15 ml of culture solution lacking Fe in a polyethylene bag. The plants and the bags were fixed on an acrylic board and placed between a pair of PETIS detectors in a chamber at 30°C, under 65% humidity, and with a light density of 320 μmol m^{-2} s^{-1}. Both DMA-$^{52}Fe^{3+}$ (0.43 MBq, 30.7 fmol) and DMA-Fe^{3+} (0.1 mM) were added to the culture solutions. After 6 h of PETIS analysis, the plants were removed from the polyethylene bags, and the roots were gently washed for 1 min in 100 ml of a solution of 50 μM EDTA lacking Fe. The plants were then placed under a bio-imaging plate inside a cassette. After 30 min, the plate was scanned by an image analyzing system (BAS-1500; Fuji Film, http://www.fujifilm.com), and then quantitative analysis by γ-ray spectrometry was performed with a maximum value set to 100. This experiment was repeated at least two times with different plants (WT and OsYSL2i) to confirm the reproducibility of the results.

Oligo DNA microarray analysis

A rice 44K custom oligo DNA microarray kit (Agilent Technologies, http://www.home.agilent.com), which contains 43 144 unique 60-mer oligonucleotides based on the sequence data of the rice full-length cDNA project (http://cdna01.dna.affrc.go.jp/cDNA), was used. Total RNA was extracted from shoots and roots using an RNeasy Plant Kit (Qiagen, http://www.qiagen.com), following the manufacturer's instructions; the yield and RNA purity were determined spectrophotometrically. The integrity of the RNA was checked using an Agilent 2100 Bioanalyzer. Total RNA (200 ng)

was labeled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. Fluorescently labeled targets were hybridized to Agilent rice 44K oligo DNA microarrays. Microarray hybridization, scanning, and data analysis were performed as described previously. The reproducibility of the microarray analysis was assessed by a dye swap in each experiment. Genes showing a signal value >100 and a significant Student's *t*-test ($P < 0.05$) were further analyzed. The i/W ratio was calculated as (average signal values of the RNAi rice)/(average signal values of the WT rice). The OX/W ratio was calculated as (average signal values of the overexpression rice)/(average signal values of the WT rice). The ± in the WT plants was calculated as (average signal values of the WT rice under Fe-deficient conditions)/(average signal values under Fe-sufficient conditions), and these ratios were used for identifying the Fe-deficiency induction of the genes.

Genomic PCR

The primers used for genomic PCR were as follows: *OsSUT1* forward, 5′-CCCGTTGAAATAATCGCACCGTCTAG-3′; *OsYSL2* reverse, 5′-GCCCAACGAAGCTAATCGCAAACAGG-3′. A fragment was amplified by PCR with KOD plus (ToYoBo, Japan).

Iron localization

Seed and polished rice were soaked in the water overnight and cut by razor. The samples were soaked in 2% HCl (Wako) and 2% potassium hexacyano ferrate II trihydrate (Wako) for 1.5 h in seed, and for 24 h in polished rice. The stained sample was washed with the water and observed by stereoscopic microscope (AX10 canHRP; Zeiss, http://www.zeiss.com).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplemental microarray data.

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