

# Nramp5 Is a Major Transporter Responsible for Manganese and Cadmium Uptake in Rice

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Paddy rice (*Oryza sativa*) is able to accumulate high concentrations of Mn without showing toxicity; however, the molecular mechanisms underlying Mn uptake are unknown. Here, we report that a member of the Nramp (for the Natural Resistance-Associated Macrophage Protein) family, Nramp5, is involved in Mn uptake and subsequently the accumulation of high concentrations of Mn in rice. *Nramp5* was constitutively expressed in the roots and encodes a plasma membrane-localized protein. Nramp5 was polarly localized at the distal side of both exodermis and endodermis cells. Knockout of *Nramp5* resulted in a significant reduction in growth and grain yield, especially when grown at low Mn concentrations. This growth reduction could be partially rescued by supplying high concentrations of Mn but not by the addition of Fe. Mineral analysis showed that the concentration of Mn and Cd in both the roots and shoots was lower in the knockout line than in wild-type rice. A short-term uptake experiment revealed that the knockout line lost the ability to take up Mn and Cd. Taken together, Nramp5 is a major transporter of Mn and Cd and is responsible for the transport of Mn and Cd from the external solution to root cells.

## INTRODUCTION

The natural resistance-associated macrophage proteins (Nramps) constitute a large family that is evolutionarily conserved throughout organisms, including bacteria, yeast, algae, plants, and animals (Nevo and Nelson, 2006). The first *Nramp* gene, *Nramp1*, was identified in mammals and encodes an integral membrane protein recruited in phagosomes of infected macrophages (Vidal et al., 1993). Subsequent studies showed that Nramp members function as proton-coupled metal ion transporters that can transport  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Al}^{3+}$  (Colangelo and Gueriot, 2006; Nevo and Nelson, 2006; Xia et al., 2010). These proteins have been implicated in the uptake, translocation, intracellular transport, and detoxification of transition metals (Nevo and Nelson, 2006).

In the *Arabidopsis thaliana* and rice (*Oryza sativa*) genomes, there are six and seven members, respectively, and some of these have been functionally characterized. In *Arabidopsis*, Nramp1, 3, and 4 showed transport activity in yeast for Fe, Mn, and Cd (Curie et al., 2000; Thomine et al., 2000, 2003; Lanquar et al., 2005), whereas Nramp6 only transported Cd but not Fe (Cailliatte et al., 2009). Nramp2 did not show transport activity for Fe in yeast and its exact function is unknown (Curie et al., 2000). Nramp1 is localized to the plasma membrane of root cells and functions as a high-affinity transporter for Mn uptake (Cailliatte et al., 2010). Knockout of this gene resulted in


a significant reduction of growth at low Mn concentration. Both Nramp3 and Nramp4 are localized to the tonoplast in seedlings (Thomine et al., 2000; Lanquar et al., 2005) and play redundant roles in the export of Fe from the vacuole during seed germination (Lanquar et al., 2005). Therefore, the *nramp3 nramp4* double mutant displays a strong chlorotic phenotype when seeds were germinated in the absence of Fe. Recently, it was reported that Nramp3 and Nramp4 also have important roles in Mn homeostasis in adult *Arabidopsis* plants (Lanquar et al., 2010). Like Fe, they release Mn from mesophyll vacuoles to supply the Mn required in the chloroplasts. Nramp6 is targeted to a vesicular-shaped endomembrane compartment and functions as an intracellular metal transporter (Cailliatte et al., 2009). Overexpression of *Nramp6* resulted in hypersensitivity to Cd but did not change Cd concentration, suggesting that this gene is involved in the distribution/availability of Cd within cells.

By contrast, only two of the seven Nramp genes present in rice have been characterized at the molecular level. Nramp1 showed transport activity for Fe and Cd in yeast but not Mn (Curie et al., 2000; Takahashi et al., 2011). Nramp1 was localized to the plasma membrane when expressed in onion (*Allium cepa*) epidermal cells. Overexpression of *Nramp1* resulted in a slight increase in Cd in the leaves (Takahashi et al., 2011). Nramp1 is suggested to be involved in cellular Cd uptake and Cd transport within the plant, but the exact role of Os *Nramp1* in rice is unknown. On the other hand, Nramp4 (Os Nramp4) is the first transporter identified for the trivalent Al ion (Xia et al., 2010). Nramp4 shares relatively low similarity with the other Nramp members and, in contrast with other Nramp members, did not show transport activity for divalent cations, including Zn, Mn, and Fe (Xia et al., 2010, 2011). Knockout of *Nramp1* resulted in a greater reduction in Al tolerance compared with wild-type rice (Xia et al., 2010).

In addition, Nramp genes have also been cloned and characterized from other plant species, such as soybean (*Glycine*

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www.plantcell.org/cgi/doi/10.1105/tpc.112.096925

*max*), tomato (*Solanum lycopersicum*), and metal hyperaccumulator species. *Nramp3* and *Nramp4* isolated from *Thlaspi caerulescens*, a Zn/Cd hyperaccumulator, showed a higher expression level than their putative orthologs in *Arabidopsis* (Oomen et al., 2009) but had similar metal transport substrate and subcellular localization. *Nramp4* isolated from *Thlaspi japonicum*, a Ni hyperaccumulator, encodes a protein showing transport activity for Ni but not for Zn, Cd, or Mn in yeast (Mizuno et al., 2005). A soybean Nramp, DMT1 (for Divalent metal transporter1), can transport Fe, Mn, and possibly Zn and Cu (Kaiser et al., 2003). *Nramp1* and *Nramp3* from tomato are able to complement a yeast mutant defective in Mn uptake, *smf1* (Bereczky et al., 2003), suggesting their involvement in Mn transport.

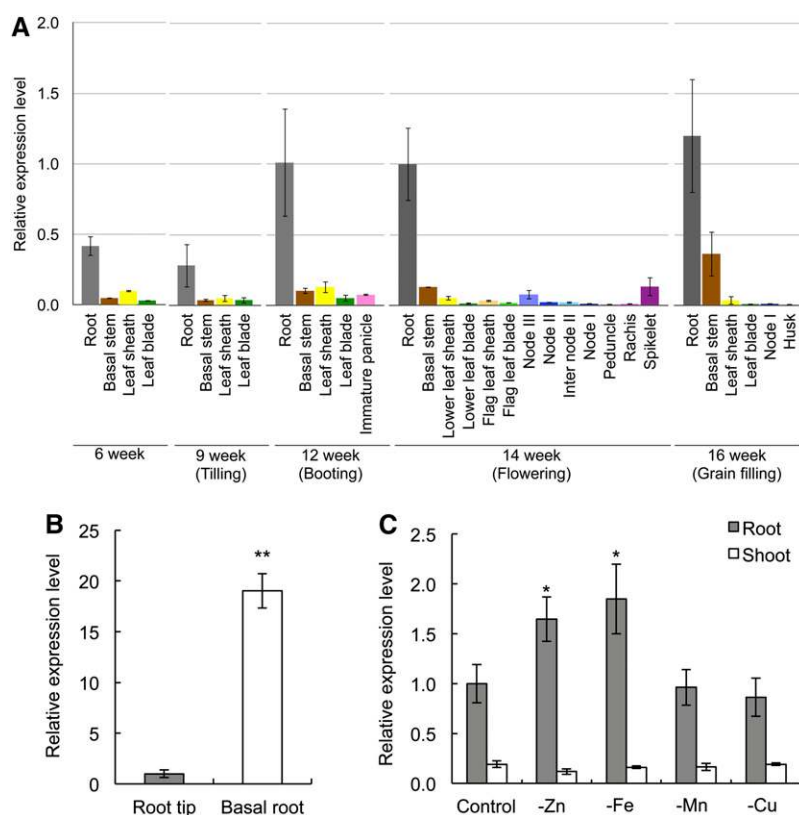
These findings indicate that Nramp members play an important role in metal transport at different cellular levels in plants. However, most studies on Nramp were conducted in yeast, and their exact physiological roles in planta are still poorly understood. In this study, we examined the function and role of an

uncharacterized Nramp member, Os *Nramp5*, in rice. We found that rice Nramp5 is a major transporter responsible for Mn uptake in the roots, which is required for high Mn accumulation in the shoots. Furthermore, we found that Nramp5 also functions as a major route for Cd entering the root cells from the external solution.

## RESULTS

### Phylogenetic Analysis of Os Nramp5

We performed 5'- and 3'-rapid amplification of cDNA ends (5'- and 3'-RACE) to clone the full-length cDNA of *Nramp5* from the rice cultivar Zhonghua 11, which was used as the wild type for the phenotypic analysis as described below. *Nramp5* from Zhonghua 11 contains 13 exons and 12 introns (see Supplemental Figure 1A online), encoding a peptide with 538 amino acids. This sequence is exactly the same as that from



**Figure 1.** Expression Pattern of *Nramp5*.

**(A)** Relative expression in various tissues at different growth stages. Rice was grown in a paddy field until ripening and various tissues were sampled. **(B)** Root spatial expression of *Nramp5*. RNA was extracted from the root tip (0 to 1 cm) or basal root region (1 to 2 cm). Asterisks indicate significant difference from the wild type at \*\* $P < 0.01$  by Student's *t* test.

**(C)** Response of *Nramp5* expression to metal deficiency. Rice was cultivated in a nutrient solution with (control) or without Zn, Fe, Mn, or Cu for 1 week. The expression level was determined by quantitative real-time RT-PCR. Expression relative to the root at flowering stage **(A)**, root tip **(B)**, and root in control condition **(C)** are shown. *HistoneH3* and *Actin* were used as internal standards. Data are means  $\pm$  sd of three biological replicates. Asterisks indicate significant difference from control condition at \* $P < 0.05$  by Dunnett's test.

[See online article for color version of this figure.]

Nipponbare (Os07g0257200), which is registered in The Rice Annotation Project Database (<http://rapdb.dna.affrc.go.jp>). Os Nramp5 exhibited 74% identity with Os Nramp1 and belonged to a distinct subgroup from that of Os Nramp1 and other Nramp monocot members (see Supplemental Figure 2 and Supplemental Data Set 1 online). Among the *Arabidopsis* members, the closest homolog of Os Nramp5 is At Nramp1, which shares 38% identity and belongs to a different subgroup (see Supplemental Figure 2 online). Using the SOSUI program (<http://bp.nuap.nagoya-u.ac.jp/sosui/>), we predicted that Os Nramp5 is a membrane protein with 10 transmembrane domains (see Supplemental Figure 1B online).

### Os Nramp5 Expression

The expression of *Nramp5* was investigated in different tissues at different growth stages. At all growth stages, *Nramp5* was mainly expressed in the roots (Figure 1A). Spatial expression analysis showed that the expression of *Nramp5* was higher in the basal root zones (1 to 2 cm from the root tip) than in the root tips (0 to 1 cm) (Figure 1B).

To investigate whether the expression of Os *Nramp5* is affected by a deficiency of essential metals, including Zn, Fe, Mn, or Cu, we exposed the seedlings to a solution that lacked each of these metals. In these plants, the expression of Os *ZIP4* (for Zinc-regulated transporters, Iron-regulated transporter-like Protein4), Os *IRT1* (for *IRON-REGULATED TRANSPORTER1*), or Os *COPT5* (for Copper transporter5), marker genes of metal deficiency (Ishimaru et al., 2005, 2006; Yuan et al., 2011), was

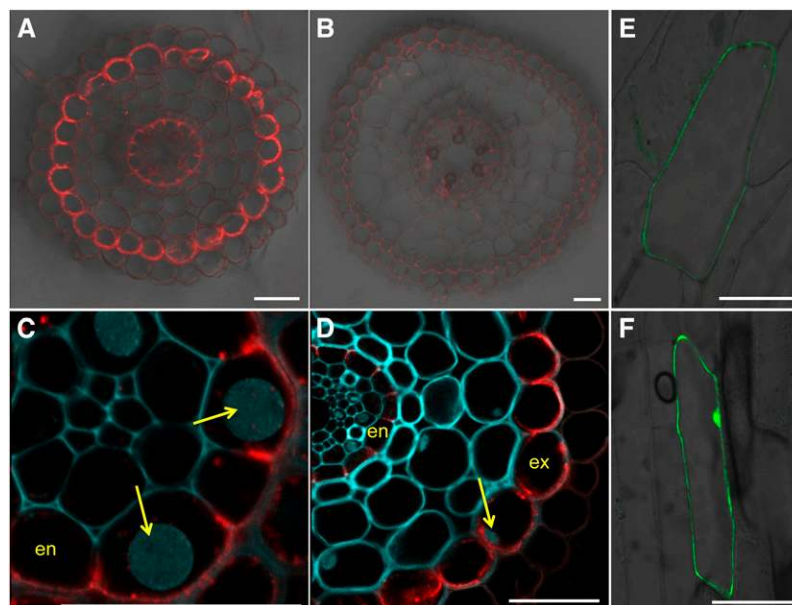
greatly induced by Zn, Fe, or Cu deficiency (see Supplemental Figure 3 online). However, the expression of Os *Nramp5* was only slightly induced in roots by a Zn and Fe deficiency and was unaffected by a Mn and Cu deficiency (Figure 1C).

### Cellular and Subcellular Localization of Os Nramp5

To determine the cellular localization of Nramp5, we performed immunostaining using an antibody against Os Nramp5. At the root mature zone, Os Nramp5 was localized to both the exodermis and endodermis (Figure 2A). The signal was not observed in the knockout mutant (Figure 2B), indicating the specificity of this antibody. Furthermore, at both exodermis and endodermis cells, Nramp5 showed polar localization at the distal side (Figures 2A, 2C, and 2D). This result is different from that of a recent article, which reported that Os Nramp5 promoter- $\beta$ -glucuronidase activity was detected in the root epidermis, exodermis, outer layers of the cortex, and around the xylem (Ishimaru et al., 2012).

Double staining with 4',6-diamidino-2-phenylindole (DAPI) for nuclei showed that Nramp5 was localized to the outermost region of the cell rather than the nuclei. Thus, Nramp5 seems to be localized to the plasma membrane (Figures 2C and 2D).

To confirm this subcellular localization, an Nramp5-green fluorescent protein (GFP) fusion was transiently introduced into onion epidermal cells and rice leaf protoplasts. Consistent with the immunostaining result (Figures 2C and 2D), the fluorescence signal of the Nramp5-GFP fusion was observed at the plasma

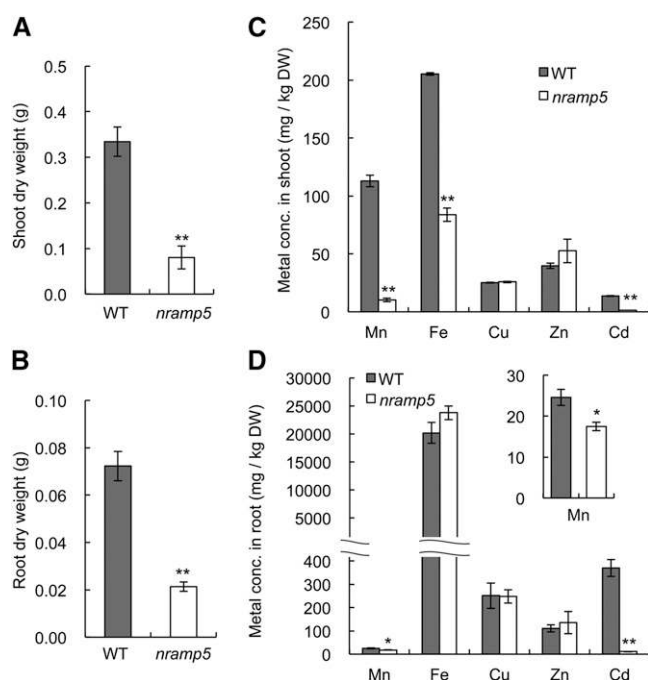


**Figure 2.** Cellular and Subcellular Localization of Nramp5.

(A) to (D) Immunostaining of roots of wild-type [(A), (C), and (D)] and knockout line *nramp5* (B) rice.

(C) and (D) High-magnification image of the endodermis (en) and exodermis (ex), respectively, costained with DAPI. Immunostaining was performed using Os Nramp5 antibody. Red represents signal from the antibody and cyan from cell wall autofluorescence. Nuclei were stained with DAPI (arrow). Bars = 20  $\mu$ m.

(E) and (F) Subcellular localization of Nramp5-GFP (E) and GFP alone (F) in the epidermal cells of onion. Bars = 100  $\mu$ m.



**Figure 3.** Phenotypic Analysis of the *Nramp5* Knockout Line.

(A) and (B) Growth of wild-type (WT) rice and the knockout line *nramp5*. (A) Shoot dry weight.

(B) Root dry weight.

(C) and (D) Mineral concentration of the shoots (C) and roots (D). Both wild-type rice and the knockout line were grown in a nutrient solution containing 0.5  $\mu$ M  $\text{MnCl}_2$ , 10  $\mu$ M  $\text{FeSO}_4$ , and 0.1  $\mu$ M  $\text{CdSO}_4$  for 3 weeks. The shoots and roots were harvested and subjected to mineral analysis by inductively coupled plasma-mass spectrometer. Data are means  $\pm$  SD of three biological replicates. Asterisks indicate significant difference from the wild type at \* $P < 0.05$  and \*\* $P < 0.01$  by Student's *t* test.

membrane in both the onion cells and rice protoplasts (Figure 2E; see Supplemental Figures 4A to 4D online). By contrast, the signal was observed in the cytosol and nuclei in cells expressing GFP alone (Figure 2F; see Supplemental Figures 4E to 4H online). All these results indicate that *Nramp5* is localized at the plasma membrane.

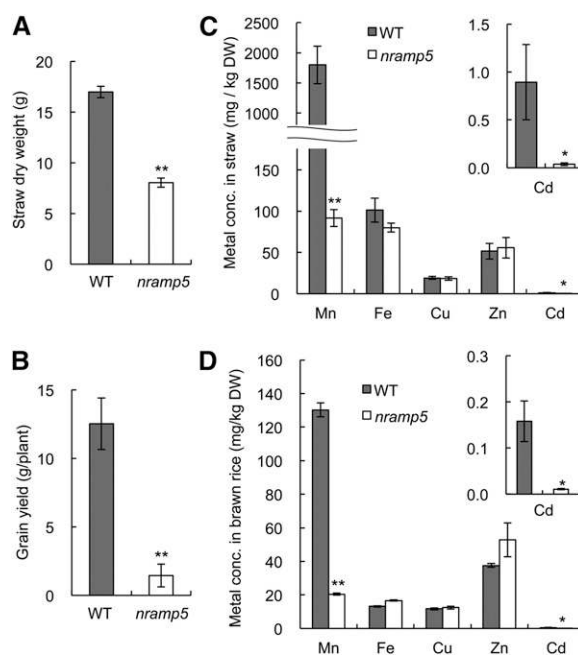
### Phenotypic Analysis of the Os *Nramp5* Knockout Line

To investigate the physiological role of *Nramp5* in the plant, we obtained a T-DNA insertion line from the Rice Mutant Database (<http://rmd.ncpgr.cn/>) (Zhang et al., 2006). The T-DNA was inserted in the 12th intron (see Supplemental Figure 1A online). *Nramp5* mRNA was not detected in this line (see Supplemental Figure 1C online), indicating that this is a knockout line of *Nramp5*.

When the knockout line was grown under normal growth conditions (half-strength Kimura B solution with 100 nM Cd, a nontoxic Cd concentration), the growth of both the shoots and roots was less than that of wild-type rice (cv Zhonghua 11) (Figures 3A and 3B). The leaves of the *Nramp5* knockout line showed severe chlorotic symptoms.

Mineral analysis of young plants grown hydroponically showed that there was no difference in the concentration of Zn and Cu in both the roots and shoots between the knockout line and wild-type line (Figures 3C and 3D); however, the Mn concentration of both the roots and shoots was remarkably lower in the knockout line than in the wild-type line. The concentration of Fe in the shoots was also lower in the mutant than in the wild-type rice (Figure 3C), but at the level of 77  $\text{mg kg}^{-1}$  dry weight (DW) $^{-1}$ , which is the level required for normal growth (Yokosho et al., 2009). The Fe concentration of the roots was similar between the wild-type rice and the knockout line (Figure 3D). Furthermore, the concentration of Cd in both the roots and shoots was lower in the knockout line than in the wild-type rice (Figures 3C and 3D).

When both lines were grown in soil until maturity under flooded conditions, the dry weight of straw in the knockout line was decreased by 53% compared with wild-type rice (Figure 4A). The grain yield of the knockout line was only 11% of the wild-type rice (Figure 4B). The concentration of Mn and Cd in the straw was lower in the mutant than in the wild type (Figure 4C). However, the Fe concentration did not differ significantly between the knockout line and the wild-type rice (Figure 4C). In the brown rice, the concentration of Mn and Cd was also significantly lower in the knockout line than in the wild-type rice (Figure 4D). The concentration of other metals, including Fe, Cu,



**Figure 4.** Growth, Yield, and Mineral Analysis of the *Nramp5* Knockout Line Grown in Soil.

(A) Straw dry weight. WT, the wild type.

(B) Grain yield.

(C) and (D) Metal concentration of the straw (C) and brown rice (D). Both wild-type rice and the knockout line were grown in soil for 4 months. Data are means  $\pm$  SD of three biological replicates. Asterisks indicate significant difference from the wild type at \* $P < 0.05$  and \*\* $P < 0.01$  by Student's *t* test.

and Zn, was similar or slightly higher in the knockout line than in the wild-type rice (Figures 4C and 4D).

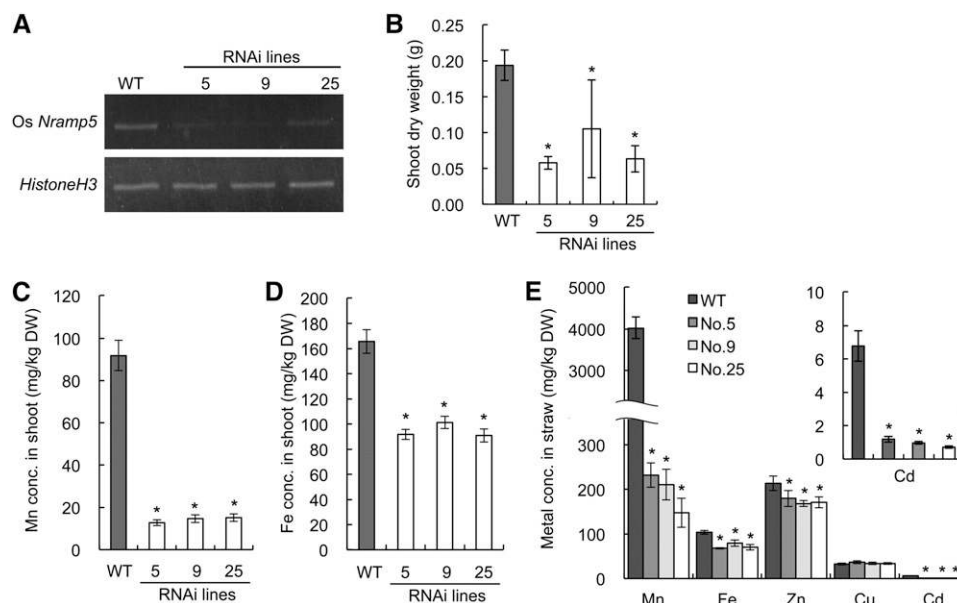
### Knockdown of *Os Nramp5* Resulted in Decreased Mn Concentration

To confirm that the phenotype observed in the T-DNA insertion line was caused by disruption of *Nramp5*, we generated transgenic lines with suppressed expression (i.e., RNA interference [RNAi] lines). The expression level of *Nramp5* in the RNAi lines was decreased compared with the wild-type rice (cv Nipponbare) (Figure 5A). Analysis with three independent lines showed that the growth of the RNAi lines was reduced (Figure 5B). The concentration of Mn in the shoots was greatly reduced in the RNAi lines compared with the wild-type rice (Figure 5C). The shoot Fe concentration was also reduced in the RNAi lines, but the extent of the decrease was less than for Mn (Figure 5D). The concentration of Zn and Cu in the roots and shoots was similar or a little bit higher in the RNAi lines than in the wild-type rice (see Supplemental Figures 5A and 5B online). Furthermore, when the knockdown lines and wild type were grown in soil until maturity under flooded conditions, the concentration of Mn and Cd in the straw was lower in the knockdown lines than in wild-type rice (Figure 5E). By contrast, the concentration of Fe, Zn, and Cu was slightly decreased or not changed in knockdown lines (Figure 5E). These results indicate that the phenotype observed in the knockout line was caused by loss of function of *Os Nramp5*.

### Increasing the Supply of Mn, but Not of Fe, Rescues the Reduced Growth of the *nramp5* Knockout Line

Since the Mn concentration was mostly affected among essential metals in the knockout line (Figures 3C and 3D), there is a possibility that the reduced growth is caused by a Mn deficiency. To test this hypothesis, the knockout line was grown hydroponically in the presence of three different Mn supply levels in the nutrient solution. At a low (0.1  $\mu$ M) Mn supply, the growth inhibition of the knockout line was more severe than at a normal (0.5  $\mu$ M) Mn supply (Figures 6A to 6E). However, at a high (5  $\mu$ M) Mn supply, the growth of the knockout line was partially restored (Figures 6C and 6F). The growth of both shoots and roots increased with an increasing Mn supply in the knockout line but not in wild-type rice (Figures 6G and 6H). These results indicate that the inhibited growth in the knockout line at low Mn supply was caused by Mn deficiency.

Mineral analysis showed that the Mn concentration was low in the shoot of the knockout line at all Mn supply levels tested, being 15.2, 5.0, and 5.9% of the wild-type rice at 0.1, 0.5, and 5  $\mu$ M Mn (Figure 7A). In wild-type rice, the shoot Mn concentration reached more than 1000 mg kg<sup>-1</sup> without showing any growth inhibition (Figures 7A and 6G), confirming that rice is highly tolerant to Mn (Sasaki et al., 2011). The concentration of Fe in the shoots did not differ between the wild-type rice and knockout line at a low Mn supply (Figure 7B). At a higher Mn supply (5  $\mu$ M), the shoot Fe concentration was lower in the mutant than in wild-type rice (Figure 7B). In the roots, the Mn



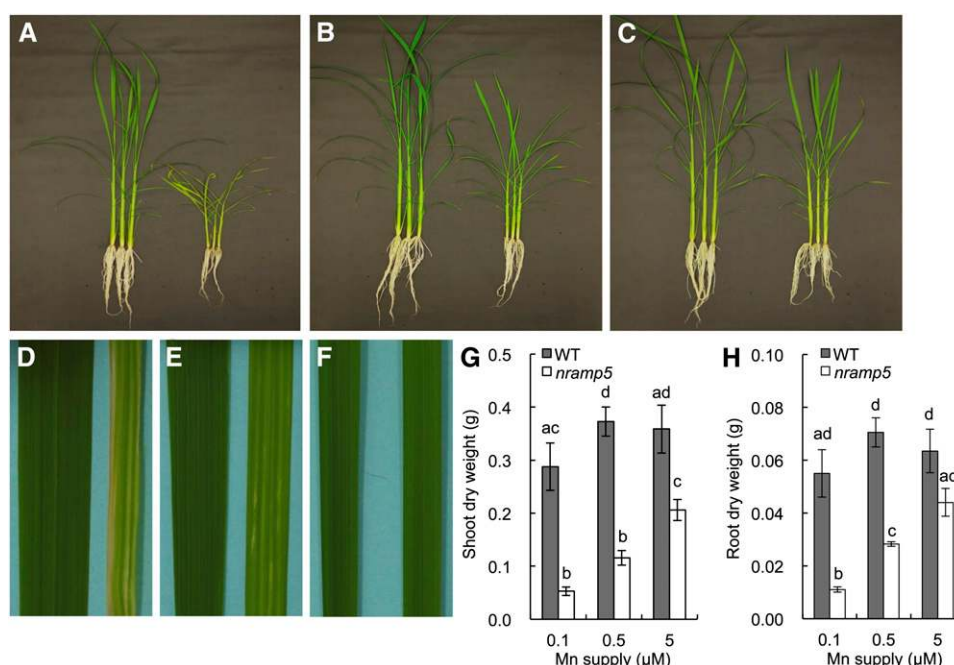
**Figure 5.** Phenotypic Analysis of the *Nramp5* Knockdown Line.

(A) Expression level of *Nramp5* in the roots of wild-type (WT) rice (cv Nipponbare) and three independent RNAi lines. PCR was run for 26 cycles.

(B) Shoot dry weight.

(C) and (D) Concentration of Mn (C) and Fe (D) in the shoots. Both wild-type rice and RNAi lines were cultivated in a nutrient solution containing 0.5  $\mu$ M MnCl<sub>2</sub> and 10  $\mu$ M FeSO<sub>4</sub> for 4 weeks. DW, dry weight.

(E) Mineral analysis of the *Nramp5* knockdown line grown in soil. Data are means  $\pm$  SD of three biological replicates. Asterisks indicate significant difference from the wild type at \**P* < 0.05 by Dunnett's test.



**Figure 6.** Rescue of the Growth Phenotype of the *Nramp5* Knockout Line by Mn Supplementation.

(A) to (F) Growth of wild-type rice (left) and the knockout line (right) grown in a nutrient solution containing 0.1 (A) and (D), 0.5 (B) and (E), or 5 (C) and (F)  $\mu\text{M}$   $\text{MnCl}_2$  and 2  $\mu\text{M}$   $\text{FeSO}_4$  for 3 weeks.

(A) to (C) Whole seedlings,

(D) to (F) The youngest leaf.

(G) and (H) Dry weight of shoots (G) and roots (H). Data are means  $\pm$  SD of three biological replicates. Different letters indicate significant difference at  $P < 0.05$  by Tukey's test. WT, the wild type.

concentration was not changed at low Mn supply but decreased at normal and high Mn supply in the knockout line (Figure 7C). The root concentration of Fe was increased in the knockout line at low and normal Mn supply but did not change at high Mn supply (Figure 7D). The increased root Fe concentration at low Mn concentration could be attributed to the inhibited root growth (Figure 6H), resulting in more Fe bound to the root surface. The concentration of Zn and Cu was similar or slightly higher or lower in the knockout line compared with wild-type rice (see Supplemental Figure 6 online). These slight changes may be attributed to the effect that a Mn deficiency had on growth (Figures 6G and 6H).

Since the shoot Fe concentration was also decreased in the knockout line, although to a lesser extent than Mn (Figure 3C), we tested whether Fe supply also affects the growth of the knockout line in the presence of a 5  $\mu\text{M}$  Mn supply. When the Fe concentration in the nutrient solution increased from 0.1 to 10  $\mu\text{M}$ , the growth of the knockout line did not improve, although the Fe concentration in the shoots increased correspondingly (Figures 8A to 8C). Interestingly, at a 0.1  $\mu\text{M}$  Fe supply, there was no difference in the shoot Fe concentration between the wild-type and knockout line (Figure 8C); however, the difference in the shoot Mn concentration was remarkable (Figure 8D). In the roots, the Mn concentration was decreased relative to the wild type at all Fe concentrations tested in the knockout line (Figure 8E), but the Fe concentration in the roots was unaffected

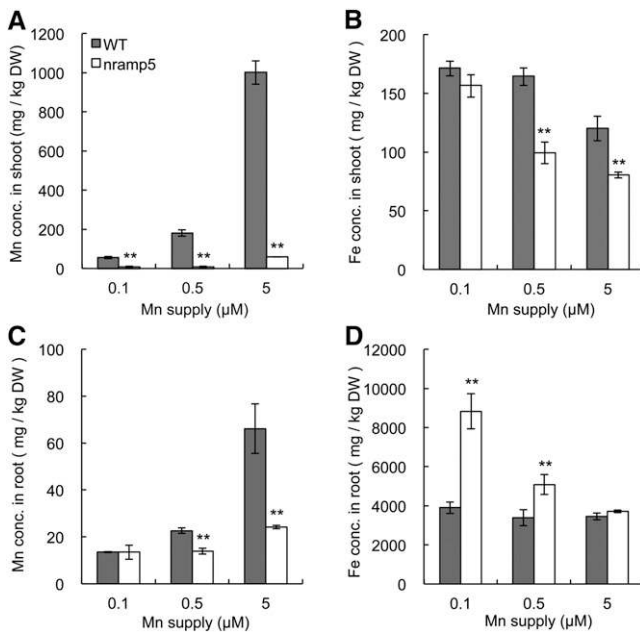
(Figures 8E and 8F). There was no large difference in the concentration of Zn and Cu in either the roots or shoots between the knockout line and wild-type rice (see Supplemental Figure 7 online). Taken together, these results indicate that the inhibited growth observed in the knockout line is caused by a Mn deficiency but not by a Fe deficiency.

### Relationship between *Os IRT1* and *Os Nramp5*

Since *IRT1* was reported to play a role in the uptake of Fe and Cd (Ishimaru et al., 2006; Nakanishi et al., 2006), we examined the expression of *IRT1* in the *nramp5* knockout mutant at different Fe concentrations. The expression of *IRT1* was upregulated by an Fe deficiency in both the wild type and the *nramp5* mutant (see Supplemental Figure 8A online). Only under Fe deficiency was the expression of *IRT1* higher in the mutant than in wild-type rice. At Fe concentrations higher than 0.1  $\mu\text{M}$ , the expression level of *IRT1* was similar in the mutant and wild-type rice (see Supplemental Figure 8A online). Since the Mn concentration in the shoot was similar in the mutant regardless of Fe concentration (see Supplemental Figure 8B online), the contribution of *IRT1* to Mn uptake is negligible, even under Fe-deficient conditions.

The uptake of Cd was also compared between wild-type rice and the *nramp5* mutant at different external Fe concentrations. Regardless of Fe supply level, the Cd concentration of both the





**Figure 7.** Concentration of Mn and Fe in the Shoots and Roots at Different Mn Supplies.

(A) and (B) Concentration of Mn (A) and Fe (B) in the shoots of wild-type (WT) and *nramp5* plants.

(C) and (D) Concentration of Mn (C) and Fe (D) in the roots of wild-type and *nramp5* plants. Both wild-type rice and the knockout line were cultivated in a nutrient solution containing 0.1, 0.5, or 5  $\mu\text{M}$   $\text{MnCl}_2$  and 2  $\mu\text{M}$   $\text{FeSO}_4$  for 3 weeks. Data are means  $\pm$  SD of three biological replicates. Asterisks indicate significant difference from the wild type at  $**P < 0.01$  by Student's *t* test.

shoot and root was lower in the mutant than in wild-type rice (see Supplemental Figure 9 online). This result indicates that the contribution of *IRT1* to Cd uptake is also negligible.

#### Short-Term Uptake Experiment for Mn and Cd

To test the transport activity for Mn and Cd in planta, we performed a short-term (30 min) uptake experiment using intact roots of both wild-type rice and the knockout line. In wild-type rice, the Mn uptake increased with increasing Mn concentration in the nutrient solution, up to 5  $\mu\text{M}$ , and reached saturation at higher Mn concentrations at 25°C (Figure 9A). By contrast, the Mn uptake in the knockout line was much lower than that of wild-type rice at all Mn concentrations tested. After subtracting the Mn uptake from that at 4°C, there was almost no Mn uptake in the mutant (Figure 9B). Based on the net Mn uptake by *Nramp5* (i.e., the uptake of wild-type rice at 25°C minus that at 4°C), the  $K_m$  and  $V_{max}$  was estimated to be 1.08  $\mu\text{M}$  and 95  $\text{mg kg}^{-1} \text{ DW h}^{-1}$ , respectively.

The Cd uptake was also higher in wild-type rice than in the knockout line at 25°C (Figure 9C). Similar to the situation for Mn, the knockout line lost its ability to take up Cd (Figure 9D). In wild-type rice, the net Cd uptake increased with increasing Cd concentrations up to 2  $\mu\text{M}$  and reached saturation at higher

concentrations (Figure 9D). The values of the  $K_m$  and  $V_{max}$  for Cd uptake were estimated to be 0.38  $\mu\text{M}$  and 60.5  $\text{mg kg}^{-1} \text{ DW h}^{-1}$ , respectively.

## DISCUSSION

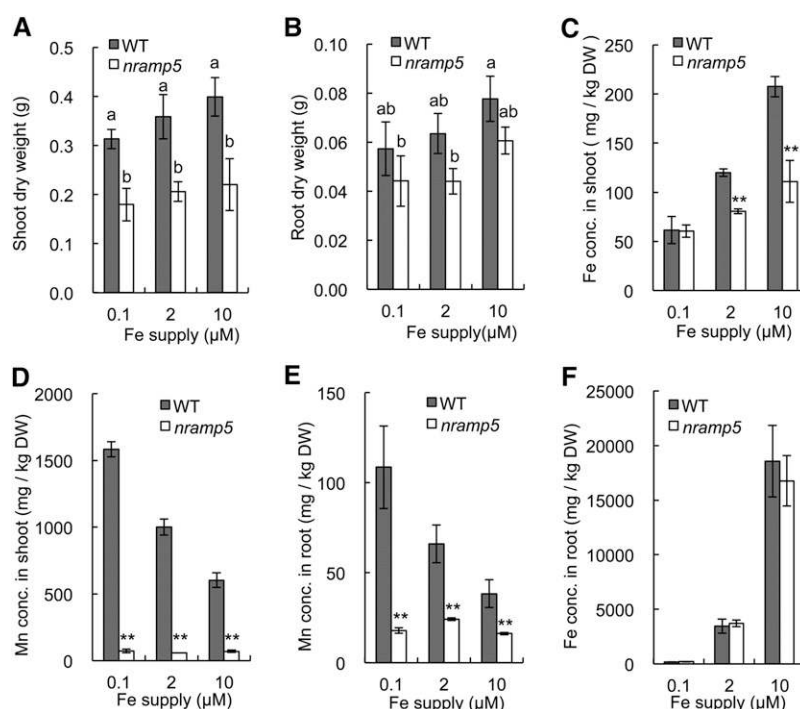
### *Nramp5* Is a Major Transporter for Mn Uptake in Rice

Mn is an essential element for plant growth; however, the molecular mechanism for Mn uptake is poorly understood compared with that of other transition metals (Pittman, 2005). In barley (*Hordeum vulgare*), *IRT1* was implicated in Mn uptake (Pedas et al., 2008). *IRT1* was localized to the plasma membrane and transported Mn as well as  $\text{Fe}^{2+}/\text{Fe}^{3+}$ , Zn, and Cd when expressed in yeast. *IRT1* expression was mildly upregulated by the deficiency of both Mn and Fe, and a Mn-efficient cultivar showed a higher expression level than a Mn-inefficient cultivar (Pedas et al., 2008). Recently, *At Nramp1* was identified as a high-affinity Mn transporter in *Arabidopsis* (Cailliatte et al., 2010). Knockout of this gene resulted in decreased Mn uptake and growth under limited Mn conditions. In this study, we found that Os *Nramp5*, which has distinct features in expression patterns and cellular localization from *At Nramp1*, functions as a major transporter for Mn uptake in rice. This conclusion is based on the following evidence: (1) Os *Nramp5* was mainly expressed in the roots and polarly localized to the plasma membrane of exodermis and endodermis cells of the roots (Figures 1, 2A, 2C, and 2D); (2) knockout or knockdown of this gene resulted in a significant reduction in Mn concentration in both roots and shoots (Figures 3C, 3D, and 5C); (3) the impaired growth of the knockout line was rescued by increasing the Mn supply, but not Fe supply (Figures 6 and 8); (4) the knockout line almost lost the ability to take up Mn (Figure 9B).

Knockout of Os *Nramp5* also resulted in a decreased concentration of Fe in the shoots (Figure 3C). This indicates that *Nramp5* is able to transport Fe in addition to Mn. However, knockout resulted in a smaller decrease in Fe than in Mn (Figure 3C). When growth was inhibited at a low Mn supply (Figure 6), the shoot Fe concentration was 156  $\text{mg kg}^{-1} \text{ DW}$  (Figure 7B), which is above the normal level of Fe required for growth (Yokosho et al., 2009). Furthermore, when the Fe concentration in the external solution decreased, growth was unaffected (Figures 8A and 8B), and the concentration of Fe in the shoot becomes similar to that of the wild type (Figure 8D). These results indicate that the uptake of Fe required for growth is mediated by other transporters and that Os *Nramp5* is responsible for additional Fe uptake. In fact, *IRT1* has been implicated in Fe uptake in rice (Ishimaru et al., 2006). Paddy soil is rich in ferrous Fe due to the reductive conditions, and it is likely that the contribution of Os *Nramp5* to Fe uptake is dispensable. This notion is supported by data from soil-grown rice (Figures 4C and 4D).

### Os *Nramp5* and *At Nramp1* Differ in Expression Pattern and Tissue and Cellular Localization

Although both Os *Nramp5* and *At Nramp1* facilitate Mn uptake in rice and *Arabidopsis*, they belong to different subgroups of the *Nramp* family (see Supplemental Figure 2 and Supplemental



**Figure 8.** Growth and Metal Concentration at Different Concentrations of Fe.

(A) and (B) Dry weight of shoots (A) and roots (B). Different letters indicate significant difference at  $P < 0.05$  by Tukey's test. WT, the wild type. (C) and (D) Concentration of Fe (C) and Mn (D) in the shoots.

(E) and (F) Concentration of Mn (E) and Fe (F) in the roots. Both the wild-type rice and knockout line were grown in a nutrient solution containing 0.1, 2, or 10  $\mu\text{M}$   $\text{FeSO}_4$  for 3 weeks. Data are means  $\pm$  SD of three biological replicates. In (C) to (F), asterisks indicate significant difference from the wild type at  $*P < 0.05$  and  $**P < 0.01$  by Student's  $t$  test.

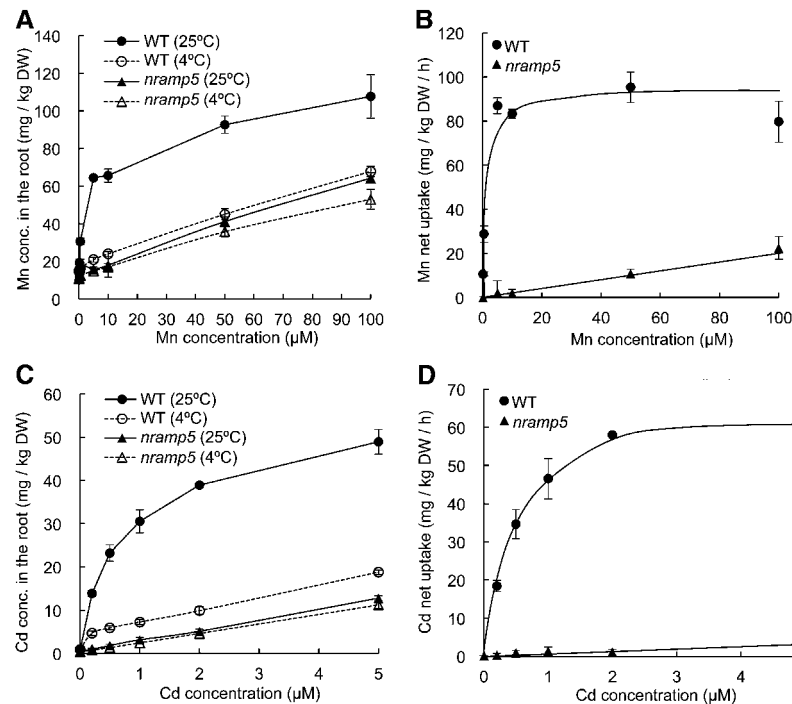
Data Set 1 online) and also differ in expression pattern and localization. The expression of *At Nramp1* is upregulated by Mn deficiency (Cailliatte et al., 2010), whereas that of *Os Nramp5* was unaffected (Figure 1C). *At Nramp1* is mainly expressed in the elongation zone of the roots. By contrast, *Os Nramp5* was mainly expressed in the mature root region (Figure 1B). At the cellular level, *At Nramp1* promoter activity was detected in all cell layers of the roots (Cailliatte et al., 2010), but *Os Nramp5* was only localized at the exodermis and endodermis (Figure 2A). Furthermore, *Os Nramp5* was polarly localized at the distal side of both cell layers (Figures 2C and 2D). The phenotype of the knockout line also differed between *At Nramp1* and *Os Nramp5*. The decreased shoot Mn concentration and growth in the *At Nramp1* knockout line was only observed under Mn-limited conditions. At higher concentrations of Mn, the growth and shoot concentration were completely rescued (Cailliatte et al., 2010). By contrast, the decreased shoot concentration of Mn was observed in the *Os Nramp5* knockout line at all concentrations of Mn tested (Figure 7). The growth of the *Os Nramp5* knockout line could not be completely rescued by a high Mn supply (Figure 6), indicating that *Os Nramp5*-mediated transport of Mn is indispensable for the healthy growth of rice. These differences between rice and *Arabidopsis* may be attributed to differences in root structure and Mn uptake ability. Different from *Arabidopsis*, rice roots have two Casparian strips, one at the exodermis and one at the endodermis (Yamaji and Ma, 2011).

Moreover, mature rice roots have a distinct structure, with a highly developed aerenchyma, and hardly have cortical cells between the exodermis and endodermis (Kawai et al., 1998). Therefore, *Os Nramp5* at the distal side of both the exodermis and endodermis facilitates the radial transport of Mn from the external solution to the stele. This is similar to the Si influx transporter *Lsi1* (for Low Silicon1) identified in rice (Ma et al., 2006). In the case of Si, an efflux transporter, *Lsi2*, localized at the proximal side of the same cell layers is required for releasing Si from the cells toward the stele (Ma et al., 2007). A similar efflux transporter, cooperating with *Os Nramp5*, is probably required for Mn uptake; however, this transporter remains to be identified in rice roots.

Paddy rice is usually cultivated under anaerobic conditions, where Mn concentration in the soil solution is high due to reduction. Rice has adapted to these environments and shows high tolerance to Mn (Sasaki et al., 2011). Furthermore, rice is able to accumulate high concentrations of Mn in the shoots. Knockout of *Os Nramp5* resulted in a remarkable reduction in shoot Mn concentration (Figures 4C and 7A), indicating that *Os Nramp5* is also responsible for high Mn accumulation.

The  $K_m$  value for Mn uptake was estimated to be 1.08  $\mu\text{M}$  (Figure 9B). This value is higher than that reported for *At Nramp1* (28 nM) and *Hv IRT1* (2 to 5 nM) (Pedas et al., 2008; Cailliatte et al., 2010), indicating that the affinity of *Os Nramp5* for Mn is lower than that for *At Nramp1* and *Hv IRT1*. This is reasonable,





**Figure 9.** Short-Term Uptake of Mn and Cd by Rice Roots.

(A) and (C) Uptake of Mn (A) and Cd (C) at 25 and 4°C. DW, dry weight; WT, the wild type.

(B) and (D) Net uptake of Mn (B) and Cd (D). The uptake was determined by exposing seedlings of both the wild-type rice and knockout line to different Mn or Cd concentrations at 25 and 4°C for 30 min. Net uptake was calculated by subtracting the apparent uptake at 4°C from that at 25°C. Data are means  $\pm$  SD of three biological replicates.

considering that Mn concentration is rich in the soil solution of paddy soil.

All these findings indicate that Os Nramp5 is not an ortholog of At Nramp1. The low similarity between Os Nramp5 and At Nramp1 suggests that Os Nramp5 is unique to rice, a Mn-accumulating species. Our results also indicate that, although Nramp members show similar transport substrate specificity in heterologous expression, often in yeast, their physiological roles in planta differ in terms of expression pattern and localization.

### Os Nramp5 Is Also Responsible for Cd Uptake in Rice

Cd is not essential for plant growth; therefore, it is assumed that no specific transporter has evolved for Cd and that Cd uses other transporters for essential metals, such as Zn and Fe, which show similar chemical characteristics (Nakanishi et al., 2006; Pendas et al., 2008). In fact, *Arabidopsis* Nramp1, Nramp3, and Nramp4 show transport activity for Fe, Mn, and Cd in yeast (Thomine et al., 2000). At Nramp1 was also reported to be responsible for Cd uptake in *Arabidopsis* (Cailliatte et al., 2010). However, it is unknown how rice takes up Cd from soil. Os IRT1, a ferrous transporter, has been implicated in Cd uptake in rice based on its transport activity for Cd in yeast (Nakanishi et al., 2006). However, the role of this gene in Cd uptake in planta has not been examined. Furthermore, the expression of Os IRT1 requires induction by Fe deficiency. Recently, a homolog of Os

Nramp5, Os Nramp1, was reported to be involved in Cd uptake (Takahashi et al., 2011), based on a slight increase in Cd in the shoot of the Os Nramp1-overexpressing line. The expression of this gene was also low in the presence of Fe, but greatly induced by Fe deficiency (Takahashi et al., 2011). Under flooded conditions, Fe is rich in soil solution; therefore, it is unlikely that Os IRT1 or Os Nramp1 plays an important role in Cd uptake due to their low expression in Fe-rich conditions. This is supported by our findings that Cd uptake was hardly affected by Fe supply level in the Os Nramp5 mutant, although the expression level of Os IRT1 was much higher in Fe-deficient plants (see Supplemental Figures 8 and 9 online). In this study, we found that Os Nramp5 transports Cd in addition to Mn and Fe (Figures 3C and 3D). Os Nramp5 is constitutively expressed in the roots throughout the growth period (Figure 1A), and knockout of Os Nramp5 resulted in almost loss of function to take up Cd (Figure 9D), resulting in low accumulation of Cd in the shoots and brown rice (Figures 3C, 4C, and 4D). These results show that Os Nramp5 is the major transporter for Cd uptake in rice.

Recently, Ishimaru et al. (2012) reported that knockdown of Os Nramp5 caused increased Cd accumulation in the shoots. However, in this study, either knockdown or knockout of Os Nramp5 resulted in a significant decrease of Cd in shoots grown in both solution and soil culture (Figures 3 to 5). This inconsistency is probably attributed to the high Cd concentration used in the earlier study. A high Cd concentration (10  $\mu$ M)

inhibits growth, which subsequently causes indirect effects. Os Nramp5 is an influx transporter of Cd at the exodermis and endodermis (Figures 2 and 9D), and it is unlikely that it also translocates Cd from the roots to the shoots as an efflux transporter.

Rice is a major source of dietary Cd; therefore, a reduction of Cd in the rice grain is an important issue for human health. Recently, a tonoplast-localized Cd transporter in rice roots, Os HMA3, was reported to be responsible for Cd accumulation by sequestering Cd into the vacuoles (Ueno et al., 2010). Although Os Nramp5 is responsible for the primary step of Cd transfer into root cells, knockout of this transporter resulted in decreased growth and yield due to loss of Mn uptake (Figure 4). Therefore, manipulating the selectivity of this transporter will be important for regulating Cd transfer from soil to the grain in future.

## METHODS

### Cloning of Full-Length Os Nramp5 cDNA

To confirm the full-length cDNA sequence of Os Nramp5, we performed 5'- and 3'-RACE (Yokosho et al., 2010). Total RNA was extracted from rice (*Oryza sativa*) roots (cv Zhonghua 11) using an RNeasy plant mini kit (Qiagen). cDNA for RACE was prepared according to the procedures described by the manufacturer (Smart RACE cDNA amplification kit; Clontech). First PCR was performed with the following primers: 5'-ACTTGGGTACTCACTCTTGCA-3' for 5'-RACE and 5'-ATCAACA-TGTACTTCTGAGCAC-3' for 3'-RACE. The first PCR products were used as template for a second PCR using primers 5'-GATGCCTCC-CTGTAACCACTC-3' for 5'-RACE and 5'-TGGCTCATCCACAACGACCT-3' for 3'-RACE. The sequence of the amplified fragments was confirmed by a sequence analyzer (ABI Prism 310 genetic analyzer; Applied Biosystems).

### Phylogenetic Analysis

Peptide sequence alignment was analyzed by ClustalW using default settings (<http://clustalw.ddbj.nig.ac.jp/>). The phylogenetic tree was constructed using the neighbor-joining algorithm by MEGA4 software (<http://megasoftware.net/>) after ClustalW alignment with 1000 bootstrap trials. Transmembrane domains were predicted with SOSUI version 1.11 (<http://bp.nuap.nagoya-u.ac.jp/sosui/>).

### Expression Pattern of Os Nramp5

Rice (cv Nipponbare) was grown in a paddy field from mid June to the end of September. Different tissues, including root, basal stem, lower leaf sheath, lower leaf blade, flag leaf sheath, flag leaf blade, node III, node II, internode I, node I, peduncle, rachis, spikelet, and husk, were sampled at different growth stages. For the root spatial expression experiment, different regions (0 to 1 cm and 1 to 2 cm from the apex) of seminal root of 5-d-old seedlings were sampled. The effect of metal deficiency on Os Nramp5 expression was investigated by exposing the seedlings (14 d old) to a nutrient solution without Fe, Zn, Cu, or Mn for 7 d, and the roots and shoots were then harvested separately.

All samples were frozen in liquid nitrogen for RNA extraction. Extraction of total RNA was the same as described above. One microgram of total RNA was used for first-strand cDNA synthesis using a SuperScript II kit (Invitrogen) following the manufacturer's instructions. The expression was determined with SsoFast EvaGreen Supermix (Bio-Rad) on real-time PCR machine CFX384 (Bio-Rad). The primer sequences for RT-PCR of Os Nramp5 were 5'-CAGCAGCAGTAAGAGCAAGATG-3' and

5'-GTGCTCAGGAAGTACATGTTGAT-3'. The metal deficiency inducible genes primer sequence for RT-PCR were 5'-CATGTCCGTCATGGCCA-AGT-3' and 5'-TGTCTGCAGCTGATGATCGAG-3' for Os *IRT1*, 5'-TCA-CTGAGGCCGTCGTCGAATCAGG-3' and 5'-ACGACAAGTGCAGTCA-GCTGT-3' for Os *ZIP4*, and 5'-GCTGTCTCGCTCGTCATGGT-3' and 5'-CGCACACACAAACATCAACAA-3' for Os *COPT5*. *HistoneH3* and *Actin* were used as an internal control, with primers 5'-AGTTTGGT-CGCTCTCGATTTCG-3' and 5'-TCAACAAGTTGACCACGTCACG-3' for *HistoneH3* and 5'-GACTCTGGTGATGGTGTGACG-3' and 5'-GGCTG-GAAGAGGACCTCAGG-3' for *Actin*, and normalized relative expression was calculated by the  $\Delta\Delta$ cycle threshold method using CFX Manager software (Bio-Rad). The amplification efficiency of Os Nramp5 was 94.2%. Data shown are from three different plants in a single experiment, but all experiments were repeated at least twice.

### Tissue and Subcellular Localization of Os Nramp5

The synthetic peptide MEIERESSERGSISWRASA-C (positions 1 to 19 of Os Nramp5) was used to immunize rabbits to obtain antibodies against Os Nramp5. The obtained antiserum was purified through a peptide affinity column before use. Roots (10-d-old seedlings) of wild-type rice and the knockout line were used for immunostaining Os Nramp5 as described previously (Yamaji et al., 2008). Fluorescence of the secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) was observed with a confocal laser scanning microscope (LSM700; Carl Zeiss). Double staining with DAPI for nuclei was also performed to investigate the subcellular localization.

To examine the cellular localization of Os Nramp5 in onion epidermal cells, the open reading frame of Os Nramp5 was amplified by PCR using primers 5'-TTCCGGAATGGAGATTGAGAGAGAGAGCA-3' and 5'-TTC-CGGACTACCTTGGGAGCGGGATGTC-3'. After sequence confirmation, this clone was inserted into the cauliflower mosaic virus 35S GFP vector. The resulting plasmid was designated Os Nramp5-GFP. Gold particles with a diameter of 1  $\mu$ m coated with Os Nramp5-GFP or GFP alone were introduced into onion epidermal cells using particle bombardment (PDS-1000/He particle delivery system; Bio-Rad) using 1100 p.s.i. rupture disks.

The subcellular localization was further investigated by introducing Os Nramp5-GFP into rice leaf protoplasts. The rice leaf protoplasts were prepared from 4-week-old cv Nipponbare seedlings grown hydroponically and were used for transformation by the polyethylene glycol method as described previously (Chen et al., 2006). The GFP signal was observed with a confocal laser scanning microscope (LSM700; Carl Zeiss).

### Phenotypic Analysis of the Os Nramp5 Knockout Line

We obtained a T-DNA insertion line of Os Nramp5 from the Rice Mutant Database (<http://rmd.ncpgr.cn/>) (Zhang et al., 2006). Homozygous lines generated from this line were screened by PCR using Os Nramp5-specific primers (5'-GAGATTAATCATGTATTGCG-3' and 5'-TTTAACGGGTATC-GACTGAT-3') and a T-DNA left border primer (5'-AACGTCCGCA-TGTGTTATTAAG-3'). Seeds of wild-type rice (cv Zhonghua 11) and the T-DNA insertion homozygous line were soaked in deionized water overnight at 30°C in the dark and then transferred to a net floating on a 0.5 mM CaCl<sub>2</sub> solution. After 7 d, the seedlings were transferred to a 3.5-liter plastic pot containing half-strength Kimura B solution, pH 5.6, containing 0.18 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.27 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.09 mM KNO<sub>3</sub>, 0.18 mM Ca (NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 0.09 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5  $\mu$ M MnCl<sub>2</sub>·4H<sub>2</sub>O, 3  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 1  $\mu$ M (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.4  $\mu$ M ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.2  $\mu$ M CuSO<sub>4</sub>·5H<sub>2</sub>O, 10  $\mu$ M FeSO<sub>4</sub>, and 100 nM CdSO<sub>4</sub>. This solution was renewed every 2 d. The plants were grown in a greenhouse at 25 to 30°C under natural light. After 21 d, the roots and shoots were harvested and subjected to mineral analysis. All experiments were conducted with three biological replicates.

To compare the growth of the knockout line with wild-type rice in soil culture, both lines were grown in soil until ripening in a greenhouse at 25 to

30°C under natural light. After 4 months under flooded conditions, the plants were harvested and subjected to investigation of grain yield and mineral analysis. For mineral analysis, straw and grain were dried at 70°C for 3 d and then subjected to mineral determination as described below. All experiments were conducted with three biological replicates.

To investigate the effect of different concentrations of Mn on growth, seedlings of both wild-type rice and the knockout line prepared as described above were grown in the nutrient solution containing 0.1, 0.5, or 5  $\mu\text{M}$  Mn as  $\text{MnCl}_2$  in the presence of 2  $\mu\text{M}$  Fe as  $\text{FeSO}_4$ . The effect of a different Fe supply on growth was investigated by growing the plants in a nutrient solution containing 0.1, 2, or 10  $\mu\text{M}$  Fe in the presence of 5  $\mu\text{M}$  Mn. The solution was renewed every 2 d. After 21 d, the roots were washed with distilled water three times and separated from the shoots. The samples were dried at 70°C for 3 d and subjected to mineral determination as described below.

#### Expression of *Os IRT1* at Different Fe Concentrations

Seedlings of both wild-type rice and the knockout line as prepared above were grown in the nutrient solution with different concentrations of Fe (0, 0.1, or 10  $\mu\text{M}$ ) as  $\text{FeSO}_4$  for 7 d, and then the roots and shoots were harvested separately. The roots were frozen in liquid nitrogen for RNA extraction. Methods for the extraction of total RNA and cDNA synthesis were the same as described above. Part of the samples was subjected to metal analysis as described below.

#### Cd Concentration of the *Os Nramp5* Knockout Line and Wild-Type Rice Supplied with Different Concentrations of Fe

Seedlings of both wild-type rice and the knockout line were grown in a nutrient solution with different concentrations of Fe (0.1, 2, or 10  $\mu\text{M}$ ) as  $\text{FeSO}_4$  for 3 weeks and then transferred to the same solution in the presence of 0.1  $\mu\text{M}$  Cd. After 4 d, the roots were washed with distilled water three times and separated from the shoots. The samples were dried at 70°C for 3 d and subjected to mineral determination as described below.

#### Phenotypic Analysis of the *Os Nramp5* Knockdown Line

To generate the hairpin RNAi construct, we cloned a 200-bp fragment (1 to 200 bp from the 5'-untranslated region) of *Os Nramp5* cDNA as inverted repeats into the pANDA vector (Miki and Shimamoto, 2004) under control of the maize (*Zea mays*) ubiquitin1 promoter and subsequently transformed the vector into *Agrobacterium tumefaciens* (strain EHA101). The primer sequences used for amplifying a 200-bp fragment of *Os Nramp5* cDNA were 5'-AAAAAGCAGGCTGTCACTACCACCTCTCTT-3' and 5'-AGAAAGCTGGGTAGCAGCTGATCATCTGCGTCG-3'. For phenotypic analysis, both wild-type rice and three independent RNAi lines were cultivated in a nutrient solution containing 0.5  $\mu\text{M}$   $\text{MnCl}_2$  for 4 weeks. After the 4 weeks, the shoots were harvested and subjected to mineral analysis. The roots were frozen in liquid nitrogen for RNA extraction. Extraction of total RNA and cDNA synthesis were performed as above. The expression level of *Os Nramp5* in the RNAi lines was determined by PCR using Takara Ex Taq (Takara). PCR was run for 26 cycles. The primer sequences for PCR were 5'-ATGGAGATTGAGAGAGAGAGCA-3' and 5'-CTACCTTGGGAGCGGGATGT-3'. *HistoneH3* was used as an internal control and was amplified with primers 5'-GGTCACTTGTTGATTCCC-CTCT-3' and 5'-AACCGCAAAATCCAAGAACG-3'.

To compare the metal concentration of the knockdown line with that of wild-type rice in soil culture, both lines were grown in soil until ripening in a greenhouse at 25 to 30°C under natural light. After 4 months under flooded conditions, straw was harvested for mineral determination as described above.

#### Short-Term Uptake Experiment

To examine the transport activity for Mn and Cd in planta, we performed a short-term (30 min) uptake experiment using intact plants of both wild-type rice and the knockout line. The seedlings (28 d old) were exposed to a nutrient solution without Mn for 1 week and then subjected to a uptake solution containing various concentrations of Mn (0, 0.1, 0.5, 5, 10, 50, and 100  $\mu\text{M}$ ) or Cd (0, 0.1, 0.5, 1, 2, and 5  $\mu\text{M}$ ) at 25 and 4°C. After 30 min, the roots were washed three times with 5 mM  $\text{CaCl}_2$  and separated from the shoots. The roots were dried at 70°C for 3 d and used for mineral determination as described below.

#### Determination of Metals in Plant Tissues

The dried samples were digested with concentrated  $\text{HNO}_3$  (60%) at a temperature of up to 140°C. The metal concentration in the digest solution was determined by atomic absorption spectrometry (Z-2000; Hitachi) and inductively coupled plasma-mass spectrometer (7700X; Agilent Technologies) after dilution.

#### Statistical Analysis of Data

Data were analyzed using Student's *t* test, Dunnett's test, or Tukey's test. Significance was defined as  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*).

#### Accession Number

Sequence data from this article can be found in the GenBank/EMBL databases under accession number AB698459 for *Nramp5*.

#### Supplemental Data

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

**Supplemental Figure 1.** Gene Structure and Knockout Line of *Os Nramp5*.

**Supplemental Figure 2.** Phylogenetic Tree of *Nramp* Proteins in the Plant Kingdom.

**Supplemental Figure 3.** Expression Pattern of Metal Deficiency-Inducible Genes.

**Supplemental Figure 4.** Subcellular Localization of *Os Nramp5* in Rice Leaf Protoplast.

**Supplemental Figure 5.** Phenotypic Analysis of *Os Nramp5* Knockdown Line.

**Supplemental Figure 6.** Concentration of Zn and Cu in the Shoots and Roots at Different Mn Supply.

**Supplemental Figure 7.** Metal Concentration of Shoots and Roots at Different Fe Supply.

**Supplemental Figure 8.** Expression of *Os IRT1* and Mn Accumulation in the *Os Nramp5* Mutant at Different Fe Concentrations.

**Supplemental Figure 9.** Cd Concentration at Different Fe Supply Concentrations.

**Supplemental Data Set 1.** Amino Acid Sequences Used to Generate the Phylogeny Presented in Supplemental Figure 2.

#### ACKNOWLEDGMENTS

The research was supported by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (22119002 and 24248014 to J.F.M.).

and by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics for Agricultural Innovation, QTL-4005, to J.F.M.). We thank Changyin Wu (National Center of Plant Gene Research, Huazhong Agricultural University, Wuhan, China) for providing the T-DNA insertion line.

## AUTHOR CONTRIBUTIONS

J.F.M. and N.Y. designed the research. A.S., N.Y., K.Y., and J.F.M. performed the experiments, analyzed the data, and wrote the article.

Received February 12, 2012; revised March 29, 2012; accepted April 26, 2012; published May 15, 2012.

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