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Characterizing the role of rice NRAMP5 in Manganese, Iron and Cadmium Transport

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Metals like manganese (Mn) and iron (Fe) are essential for metabolism, while cadmium (Cd) is toxic for virtually all living organisms. Understanding the transport of these metals is important for breeding better crops. We have identified that OsNRAMP5 contributes to Mn, Fe and Cd transport in rice. *OsNRAMP5* expression was restricted to roots epidermis, exodermis, and outer layers of the cortex as well as in tissues around the xylem. OsNRAMP5 localized to the plasma membrane, and complemented the growth of yeast strains defective in Mn, Fe, and Cd transport. *OsNRAMP5* RNAi (OsNRAMP5i) plants accumulated less Mn in the roots, and less Mn and Fe in shoots, and xylem sap. The suppression of *OsNRAMP5* promoted Cd translocation to shoots, highlighting the importance of this gene for Cd phytoremediation. These data reveal that OsNRAMP5 contributes to Mn, Cd, and Fe transport in rice and is important for plant growth and development.

Manganese (Mn) and iron (Fe) are essential micronutrients for almost all living organisms including plants. Mn is required as a cofactor or activator for enzymes belonging to different functional groups which perform diverse functions. For example, oxalate oxidase, Mn superoxide dismutase (MnSOD), RNA polymerase, malic enzyme, isocitrate dehydrogenase, and PEP carboxykinase use Mn as a cofactor[1–4]. Mn is also important in photosynthetic oxygen evolution in chloroplasts. The oxygen evolving complex, which is a part of photosystem II in the thylakoid membrane of chloroplasts, is responsible for the terminal photooxidation of water during the light reactions of photosynthesis, and it has a metalloenzyme core containing four Mn atoms[5–7]. Mn deficiency causes interveinal chlorosis and a reduction in the content of fructans and structural carbohydrates, resulting in lax leaves[8]. Consequently, Mn-deficient plants are more susceptible to low-temperature stress and pathogen infection, leading to significant decreases in crop yield[1,9]. Similarly, Fe servers as an integral cofactor for numerous proteins. In plants, Fe is essential for chlorophyll biosynthesis and the synthesis of heme and its deficiency significantly impairs plant growth and development[10,11].

The transport of Mn is believed to share an entry route with Fe and cadmium (Cd). Fe is taken up by IRT1, a member of the Fe-regulated transporter-like protein (ZIP) gene family[12–15]. IRT1 has a relatively broad substrate spectrum and is reportedly capable of transporting other metal ions, including Mn and Cd[16]. Natural resistance associated macrophage protein (NRAMP) family members, which have been identified in many plant species, play a major role in metal homeostasis. In *Arabidopsis*, it was recently reported that AtNRAMP1 is essential for Mn uptake under Mn deficiency[17]. In addition to Mn, AtNRAMP1 transports Fe and is induced by Fe deficiency[18]. AtNRAMP6, a homolog of AtNRAMP1, does not transport Mn and Fe, and seems to affect the intracellular distribution of Cd[19].

In rice plants, little is known about Mn transport at the molecular level and the transporter for Mn uptake from soil has not been reported yet. The only reported rice Mn transporter is OsYSL2, which transports Mn- and Fe-nicotianamine (NA) complexes and contributes to phloem translocation[20,21]. On the other hand, Fe uptake

systems in rice plants are well known as Strategy II and direct ferrous Fe uptake systems. Strategy II Fe uptake systems rely on a ferric Fe chelation system through the secretion of deoxymugineic acid (DMA)[10,11,22,23]. The secreted DMA chelate Fe, and the resulting Fe(III)-DMA complex is transported by OsYSL15[23,24]. Besides using Strategy II systems, rice plants possess a direct ferrous Fe uptake system that uses the ferrous Fe transporter OsIRT1[25]. Recently we have reported that rice also secretes phenolics to increase solubility of apoplasmic Fe[26–28]. All genes related to Fe uptake are highly induced by Fe deficiency. Thus many of rice genes have the potential to complement the function of other genes for Fe transport.

Unlike Fe and Mn, Cd is a highly toxic metal for most living organisms, including humans[29]. Even at low concentrations, cumulative uptake of Cd dramatically affects cellular metabolism[30]. The effects of daily intake of Cd-contaminated rice were seen in Japanese people during the 1910–1970s, resulting in a disease termed "itai-itai"[31,32]. Cd contamination in crops is spreading all over the world, especially in countries with high industrial activity[33]. In a globalized market, Cd-contaminated food threatens not only the production areas, but also consumers living far from contaminated fields. Phytoremediation, which utilizes plants to extract soil Cd, is a promising method to decontaminate soils high in Cd. This method is cost-effective, applicable to large areas, and environmentally friendly[34]. Rice is a good candidate for Cd phytoremediation[35] due to its adaptation to a range in environmental conditions, such as flooded and drained fields. Moreover, rice has a short life cycle, a large biomass, and well-established cultivation and harvesting methods. Phytoremediation using a naturally high Cd-accumulating rice cultivar for two years showed a 38% reduction in soil Cd content and a 51% reduction in grain Cd content of successive rice crops[36,37]. Anjana Dhan is one of the highest Cd accumulating natural rice cultivars among world rice collections[38], raising the challenge to determine if its Cd accumulation capacity could be further enhanced.

Here, we show that OsNRAMP5 is a Mn, Fe and Cd transporter in rice plants. OsNRAMP5 is not regulated by Mn or Fe deficiency. The repression of OsNRAMP5 expression via RNAi lowered root and shoot Mn accumulation. In OsNRAMP5i plants, the Fe concentration was unchanged in roots, but was low in the xylem sap and shoots, and the genes related to Fe uptake and translocation were induced. OsNRAMP5 knockdown plants accumulated more Cd in shoots, making it a promising candidate for Cd phytoremediation.

Results

Identification of OsNRAMP5. Rice plants grown hydroponically in a solution containing 10 μM Cd accumulated less Mn, zinc, and copper in their roots and shoots, while Fe accumulation decreased in the shoots and increased in the roots (**Fig. 1**). Microarray analyses were performed to identify the genes responsible for the changes in metal accumulation. Our results indicate that the ferrous Fe and Cd transporters *OsIRT2* and *OsNRAMP1*, the ferrous Fe- and Mn-NA transporter *OsYSL2*, and zinc transporter *OsZIP3* were induced in the roots of plants exposed to Cd, while *OsIRT2*, *OsZIP2*, *OsZIP3*, *OsZIP8*, and the ferric Fe-DMA transporter *OsYSL15* were induced in the shoots (**Supplementary Table 1**)[21,24,25,39,40]. Among the seven members of the NRAMP family in rice, *OsNRAMP1*[40] expression was increased in roots and shoots in the presence of Cd, while *OsNRAMP5* (*Os07g0257200*) expression was decreased in both of these tissues (**Supplementary Table 1**). The root and shoot expression of other *OsNRAMP* genes did not change significantly. We next characterized *OsNRAMP5* to determine its role in Mn, Cd and Fe transport. OsNRAMP5 shares 74.5% homology with OsNRAMP1, and the expression of *OsNRAMP5* did not change in response to Mn or Fe deficiency (**Fig. 2a and b**), while the expression of *OsNRAMP5* decreased in response to the addition of Cd (**Fig. 2c**). In contrast, *OsNRAMP1* expression was upregulated by Mn deficiency in roots and shoots (**Supplementary Fig. 1a and b**).
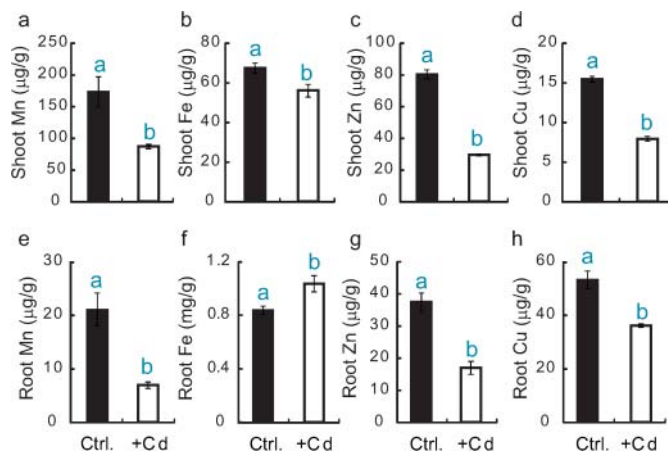
Figure 1 | Metal concentrations of rice plants grown in the presence of Cd. Shoot and root metal concentrations under control conditions (Ctrl.) or in the presence of 10 μM Cd (+Cd). (a, e) Mn, (b, f) Fe, (c, g) zinc, and (d, h) copper concentrations. Error bars represent the SD. Columns bars followed by different letters are statistically different according to analysis of variance followed by Student-Newman-Keuls (SNK) test (a, p=0.0034; b, p=0.0074; c, p=0.0000; d, p=0.0000; e, p=0.0017; f, p=0.0069; g, p=0.0005; h, p=0.0010); n=3.

OsNRAMP5 is expressed in the root epidermis, exodermis, and outer layers of the cortex. To obtain greater insight into the physiological role of OsNRAMP5, we investigated the tissue-specific localization of *OsNRAMP5* in roots through an analysis of promoter-β-glucuronidase (*GUS*) transgenic rice. GUS staining attributed to *OsNRAMP5* promoter activity was observed in the epidermis, exodermis, and outer layers of the cortex (**Fig. 2d and e**).
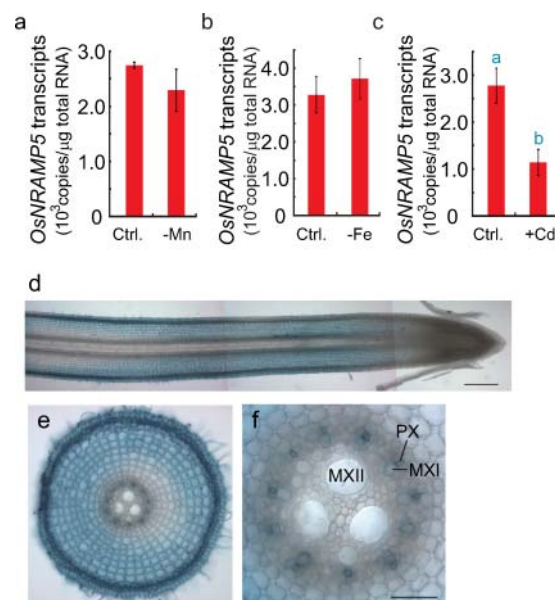

Figure 2 | OsNRAMP5 is expressed in rice roots. (a–c) Expression pattern of *OsNRAMP5* in rice grown hydroponically under Mn-deficient (-Mn) (a), Fe-deficient conditions (-Fe) (b), and in the presence of 10 μM Cd (+Cd) (c) compared to normal nutrient conditions (Ctrl.). (d–f) *OsNRAMP5* promoter GUS analysis in rice roots. Longitudinal section (d). Transverse section (e). Enlargement of part of the stele (f). MXII, metaxylem II; MXI, metaxylem I; PX, protoxylem. Scale bar = 400 μm for (d), and 50 μm for (f). Error bars represent the SD. Columns bars followed by different letters are statistically different according to analysis of variance followed by SNK test (p=0.0034); n=3.

In the stele, GUS activity was detected mainly around the xylem (**Fig. 2f**).

OsNRAMP5 is a plasma membrane-localized Mn, Fe, and Cd transporter. OsNRAMP5 fused to the N terminus of synthetic green fluorescent protein (sGFP) was transiently expressed in onion epidermal cells under control of the *cauliflower mosaic virus* (*CaMV*) *35S* promoter. OsNRAMP5-sGFP fluorescence was observed at the plasma membrane (**Fig. 3a**). To further confirm the localization, onion cells transformed with *OsNRAMP5-sGFP* were stained with plasma membrane dye FM4-64 (**Fig. 3b**) and the merged images clearly showed that OsNRAMP5 localizes to plasma membrane (**Fig. 3c**). On the other hand sGFP alone was localized to the cytoplasm and nucleus (**Fig. 3d**). The OsNRAMP5 complemented the growth of yeast strains *Δsmf1*[41] and *Δfet3Δfet4*[42], which are defective in Mn and Fe transport, respectively (**Fig. 3e and f**). In addition to Mn and Fe, OsNRAMP5 was shown to affect the growth of yeast strain *Δycf1* (**Fig. 3g**), which is defective in Cd sequestration to the vacuole and which exhibits slower growth in the presence of Cd[43]; however, OsNRAMP5 did not restore the growth of any of the yeast zinc and copper transport mutants (**Supplementary Fig. 2**). OsNRAMP1 used as a control transported Fe and Cd but not Mn in line with the previous reports[18,40]. The results clearly showed that OsNRAMP5 is localized to the plasma membrane and involved in Mn, Fe, and Cd transport. Moreover the expression around xylem indicated that OsNRAMP5 may also contribute to Mn, Fe, and Cd uptake from xylem.

OsNRAMP1 does not contribute to Mn uptake. AtNRAMP1 transports Fe and Mn, and is upregulated by Fe and Mn deficiency[17,18]. *OsNRAMP1* is induced by Fe deficiency and transports Fe, but not Mn[18,40]. To analyze the relationship between OsNRAMP1 and Mn uptake in rice plants, we developed and analyzed *OsNRAMP1* RNAi (OsNRAMP1i) plants. In OsNRAMP1i plants, *OsNRAMP1* expression was significantly downregulated (**Supplementary Fig. 1c and d**); however, there was no significant difference in Mn or Fe
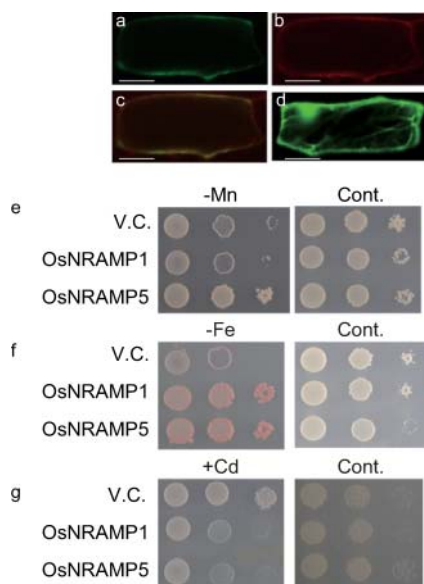
Figure 3 | OsNRAMP5 contributes to Mn, Fe and Cd transport.
(a–d) Green fluorescent protein (GFP) fluorescence in onion epidermal cells observed by confocal laser scanning microscopy (a), OsNRAMP5-sGFP fluorescence (b), fluorescence of FM4-64 and (c) overlay of FM4-64 and OsNRAMP5-sGFP. (d) Fluorescence of sGFP only. Scale bar = 50 μm. (e–g) Serial dilutions of yeast cells for *Δsmf* (Mn uptake mutant) (e), *Δfet3fet4* (Fe uptake mutant) (f), *Δycf1* (Cd-sensitive mutant) (g), transformed with empty vector (V.C.), OsNRAMP1 or OsNRAMP5.

concentration between OsNRAMP1i and wild type plants (**Supplementary Fig. 3**).

OsNRAMP5 RNAi and overexpression (OX) plants. To understand the role of OsNRAMP5 in rice plants (cultivar Tsukinohikari), RNAi knockdown plants (T5i) and OX plants of *OsNRAMP5* were developed. The *OsNRAMP5* transcript levels in T5i roots were five times lower than those in wild type, while the expression of *OsNRAMP5* in the OX lines was comparable to that in wild type plants (**Fig. 4a**). In shoots, *OsNRAMP5* expression was observed only in the OX lines (**Fig. 4b**). The growth of the T5i lines was comparable to that of wild type plants in terms of root and shoot length and SPAD reading under control conditions (**Fig. 4c and e–g**). Shoot length in the OX lines was significantly reduced compared to that in wild type plants under control conditions (**Fig. 4f**). The root and shoot Mn concentrations in the T5i plants were dramatically reduced compared to those in wild type plants (**Fig. 4h and i**). There was no significant difference in the concentration of Fe in roots (**Fig. 4j**) while the Fe concentration was significantly lower in shoots of T5i plants (**Fig. 4k**). Microarray analysis revealed that many Fe deficiency-inducible genes were upregulated in the T5i plants (**Supplementary Table 2**).

In plants grown under Mn-deficient conditions, the root and shoot length and SPAD value of the T5i lines were significantly decreased (**Fig. 4d–g**). The growth of the OX lines was also affected in terms of shoot length and SPAD value (**Fig. 4f and g**). The T5i lines accumulated less Mn than wild type plants both in roots and shoots (**Fig. 4h and i**), while the Mn concentration in the OX plants was similar to that in wild type roots (**Fig. 4h**). The Mn and Fe concentrations in xylem sap from the T5i lines were also significantly reduced compared to those in wild type plants (**Fig. 5a and b**), whereas no difference was observed in the Ca concentration (**Fig. 5c**). Under Fe-deficient conditions, there were no significant differences in plant growth, SPAD value, and metal concentrations (**Supplementary Fig. 4**).

The Mn concentration in T5i plants grown in the presence of 10 μM Cd was significantly lower in shoot and roots compared with wild type (**Fig. 6a and e**). There was no difference in Fe concentration between the T5i and wild type plants (**Fig. 6b and f**). The Cd concentration and total Cd content in the shoots were higher in the T5i plants than in wild type (**Fig. 6c and d**). On the other hand, the root Cd concentration and total Cd content of roots in the T5i plants were less than those in wild type (**Fig. 6g and h**). The total Cd content (root plus shoot) in the T5i plants was also significantly less than that in wild type plants.

Development of high Cd accumulating Anjana Dhan lines. As the OsNRAMP5i (T5i) shoots accumulated more Cd than did wild type shoots, we developed OsNRAMP5i plants (A5i) using a high Cd accumulating cultivar, Anjana Dhan[38]. There was no significant difference in shoot length between the Anjana Dhan OsNRAMP5i (A5i) and wild type plants when plants were grown in the presence of 10 μM Cd (**Fig. 7a**). *OsNRAMP5* expression in the A5i roots was 33–66% less than that in wild type when grown under normal nutrient conditions (**Fig. 7b**). At 10 μM Cd conditions, slightly less Mn accumulated in the A5i shoots and there was no difference in Fe concentration (**Fig. 7c and d**). Moreover, there was no significant difference in shoot weight; however, the shoot Cd concentration in A5i was up to 4.9 times that in wild type (**Fig. 7e and f**) and the shoot Cd content in A5i was up to 4.3 times greater than that in wild type (**Fig. 7g**).

Discussion

In rice plants, the mechanisms of Mn uptake have not been elucidated. Until now only OsYSL2, a Mn- and Fe-NA transporter, has been reported to be involved in phloem Mn transport[20,21] while the
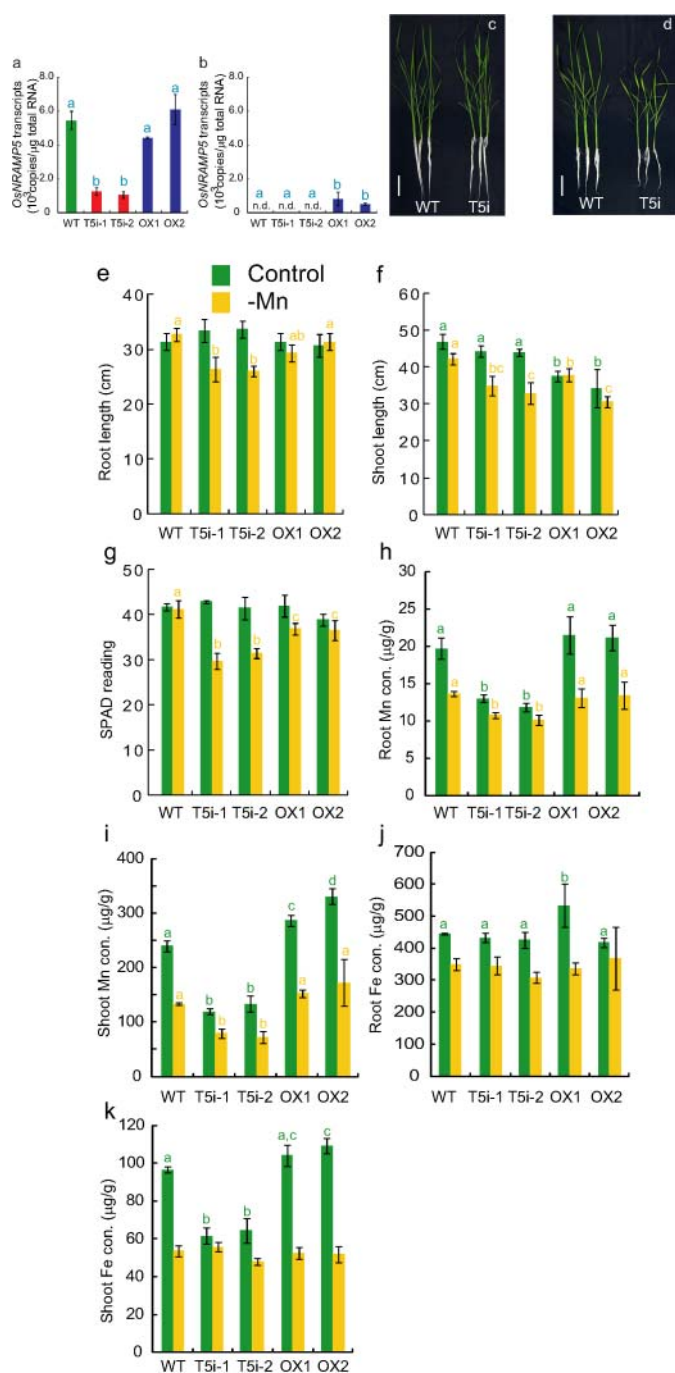
Figure 4 | Characterization of OsNRAMP5i and OsNRAMP5 OX lines.
(a, b) *OsNRAMP5* transcription in roots (a) and shoots (b) of wild type,
OsNRAMP5i (T5i-1, T5i-2), and OsNRAMP5OX (OX1, OX2) plants
(cultivar Tsukinohikari) grown in control hydroponic culture.
(c, d) Phenotypes of wild type and T5i plants grown under control (c) and
Mn-deficient conditions (d). Scale bars = 10 cm. Root length (e), shoot
length (f), SPAD value (g), root (h) and shoot (i) Mn concentrations, root
(j) and shoot (k) Fe concentrations of plant grown under control
conditions or Mn-deficient (-Mn) conditions. Error bars represent the SD.
Columns bars followed by different letters are statistically different
according to analysis of variance followed by SNK test (a, p=0.0013;
b, p=0.0003; e, p=0.0017; f, p=0.0009 (Ctrl.) & p=0.0006 (-Mn);
g, p=0.0001; h, p=0.0000 (Ctrl.) & p=0.0067 (-Mn); i, p=0.0000 (Ctrl.) &
p=0.0004 (-Mn); j, p=0.0095; k, p=0.0000; n=3.

gene involved in Mn uptake from soil has not been characterized yet.
Like OsYSL2, numerous transporters can transport more than one
metal; for example, AtNRAMP1 transports Mn and Fe[17,18,44], while
OsNRAMP1 transports Fe and Cd[18,40]. Thus, many metals compete
with each other and an abundance of one metal can disturb the
uptake of others. In this scenario, it was not surprising that Cd
showed a negative correlation with Mn absorption and accumulation
in shoots. We have previously reported 22K microarray analysis
using different concentrations of Cd and observed more significant
changes in gene expression for 10 μM Cd treatment compared with 1
μM Cd treatment[45]. In the present study, when plants were grown in
the presence of 10 μM Cd, in addition to decreased Mn accumulation
in rice plants, changes in the expression of numerous metal trans-
porters were observed. We focused on the role of OsNRAMP5 in Mn,
Fe and Cd transport. NRAMP family members are involved in Mn
transport in other organisms[17,46,47]. Although a role for rice NRAMPs
in Mn transport was previously suggested[48], the involvement of
OsNRAMP family proteins in Mn transport has not been investi-
gated. Among the seven members of the NRAMP family in rice, the
expression of *OsNRAMP1* increased in roots and shoots in the pres-
ence of Cd, while the expression of *OsNRAMP5* decreased in both
tissues (**Supplementary Table 1**). The expression of other *OsNRAMP*
family genes did not change significantly in roots and shoots. The
decreased Mn content associated with reduced *OsNRAMP5* expres-
sion suggests that gene's involved in Mn transport, and our data
confirm that OsNRAMP5 plays a role in Mn transport in rice plants.

The *Arabidopsis*, AtNRAMP1 transports Mn, in addition to Fe[17,18,44].
AtNRAMP1 is upregulated by Mn and Fe deficiency, indicating that
AtNRAMP1 is a multi-functional transporter involved in Mn and Fe
uptake. On the other hand, AtNRAMP6, the closest homolog of
AtNRAMP1, does not transport Mn[19]. In rice plants, although the
expression of OsNRAMP1 was upregulated by Fe and Mn deficiency,
it does not transport Mn (**Fig. 3e**). Moreover, no difference in Mn
concentration was observed between OsNRAMP1i and wild type
plants (**Supplementary Fig. 3**). On the other hand, OsNRAMP5 con-
tributes to Mn and Fe transport, although its expression is not upre-
gulated by Mn or Fe deficiency (**Fig. 2**). The growth of OsNRAMP5i
was comparable to that of wild type plants under normal conditions;
however, when these lines were grown under Mn-deficient conditions,
plant growth was significantly reduced (**Fig. 4**). The phenotype of
AtNRAMP1 also becomes prominent under Mn-deficient condi-
tions[17]. The reduced Mn concentration in T5i plants strongly suggested
that OsNRAMP5 contributes to Mn transport in rice.

Under normal growth conditions in rice, *OsNRAMP1*, *OsIRT1*,
and *OsYSL15* are expressed at low levels and are highly induced by
Fe deficiency[24,25,40]. On the other hand, OsNRAMP5 is a constitu-
tively expressed transporter (**Fig. 2**)[49]. OsNRAMP5 contributes to Fe
transport in addition to Mn and Cd, as revealed by yeast complement
assay (**Fig. 3e–g**). In T5i plants, the Fe concentration did not change
in roots however significant changes were observed for Fe concen-
tration in the xylem sap as well as in shoot (**Fig. 4j, k and Fig. 5b**). The
expression of genes involved in Fe uptake and translocation were
highly induced in T5i plants (**Supplementary Table 2**). The expres-
sion of NA and DMA synthesis related genes and Fe(II)/Mn(II)-NA
and Fe(III)-DMA transporter genes was also upregulated. The up-
regulations of these genes suggest that the decrease in Fe uptake by
the suppression of OsNRAMP5 would be complemented by an
increase in Fe uptake from soil through these genes, however the
root to shoot Fe transport via root xylem may not be complemented
by increased expression of these genes. These data also suggest that
OsNRAMP5 is involved in constitutive uptake of Fe, and that
OsNRAMP1, OsIRT1, and OsYSL15 play a significant role under
Fe deficiency. In rice plants, OsNRAMP5 would function jointly in
Fe uptake with these transporters.

Initially, we expected that the root and shoot Cd concentration
in the T5i plants would be low, as OsNRAMP5 contributed to Cd
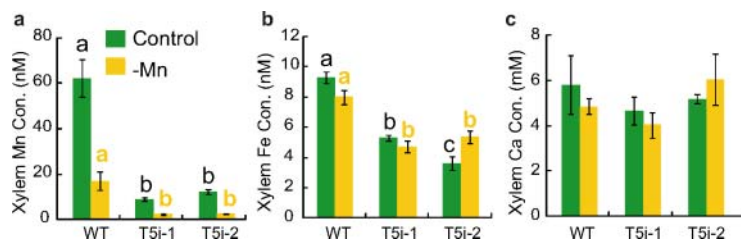
Figure 5 | Xylem sap metal concentrations in OsNRAMP5i plants. Xylem Mn (a), Fe (b), and Ca (c) concentrations in T5i plants grown under control or Mn-deficient (-Mn) conditions. Error bars represent the SD. Columns bars followed by different letters are statistically different according to analysis of variance followed by SNK test (a, p=0.0001 (Ctrl.) & p=0.0010 (-Mn); b, p=0.0000 (Ctrl.) & p=0.0001 (-Mn); n=3.

transport in yeast complementation assay (**Fig. 3g**). Unexpectedly, T5i plants accumulated small amounts of Cd in the roots but large amounts in the shoots (**Fig. 6c and g**). The total Cd content (root plus shoot) in the OsNRAMP5i plants was less than that in wild type, suggesting that OsNRAMP5 did not contribute to Cd translocation but to the uptake of Cd from the soil (**Fig. 6d and h**). The expression observed near xylem indicated the OsNRAMP5 may also be involved in Cd uptake from xylem. The role of OsHMA3 in sequestering Cd to vacuole has already been discussed[50]. It may be possible that in rice the Cd taken from xylem may be further transported into the vacuole through OsHMA3 and OsHMA3 and OsNRAMP5 may function in coordination. Cd uptake and translocation is partly mediated by the Fe uptake system in rice plants[40,51]. Induction of the genes related to Fe transport enhanced Cd translocation to the shoots in T5i plants (**Supplementary Table 2**). Expression of these genes in roots is higher in the high Cd accumulating cultivar Habataki than in the low Cd accumulating cultivar Sasanishiki[40]. Such a difference in Fe transporter expression seems to be responsible for the differences in shoot Cd accumulation between the two rice cultivars. We previously developed rice lines tolerant to a low Fe supply and with greater seed Fe content by regulating the spatial and temporal expression of different genes[21,26,52]. This led us to expect that shoot Cd translocation in rice plants could be increased by the suppression of OsNRAMP5. Indeed, this was achieved in Anjana Dhan, a natural rice cultivar that accumulates a large amount of Cd without any adverse effects on plant growth. The A5i lines accumulated up to 4.9 times more Cd than wild type. Moreover, the shoot Cd

content in A5i was up to 4.3 times higher than in wild type (**Fig. 7**). Cd phytoremediation using this efficient transgenic rice will rapidly and efficiently enable the removal of Cd from soil, leading to at least a 75% reduction in the Cd phytoremediation period[53]. This could contribute greatly to food safety and human health worldwide.

In rice plants, net photosynthesis and photosynthetic capacity correlate with the applied dose of Mn, and the optimal Mn concentration in hydroponic solutions is around 0.5–2 mg/L[54]. In this range, the Mn concentration in rice shoots is 350–896 μg/g dry weight. The Mn concentration is normally greater than the optimal concentration for rice in normal and acidic soils[54]. Therefore, controlling the expression of OsNRAMP5 may enhance photosynthesis in rice plants. Moreover, OsNRAMP5 is closely related to Fe and Cd uptake and translocation; thus, it may control the Cd concentration in rice plants. The identification of a transporter involved in constitutive Mn and Fe transport will increase both our understanding of Mn and Fe transport and of the mechanisms involved in Mn and Fe homeostasis in rice plants.

Methods

Plant material. Seeds of the *Oryza sativa* cultivars Tsukinohikari and Anjana Dhan were germinated for two weeks on Murashige and Skoog (MS) medium at 28°C under a 16-h light/8-h dark photoperiod. After germination, the seedlings were transferred to a 20-L plastic container containing a standard nutrient solution as described previously[55]. For the Cd stress treatments, four-week-old plants were transferred to a nutrient solution containing 10 μM $CdCl_2$ as described previously[45] and cultivated for two weeks. For the Mn-deficient conditions, four-week-old plants were transferred to a nutrient solution without Mn and grown for two additional weeks. For the Fe-deficient conditions, four-week-old plants were transferred to a nutrient solution
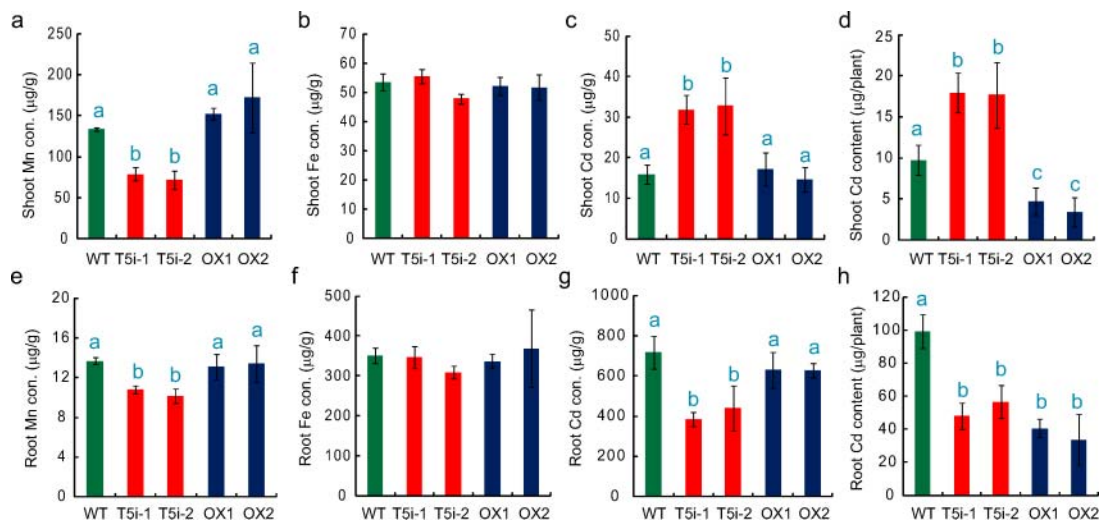
Figure 6 | Suppression of *OsNRAMP5* leads to high Cd in rice shoots. Shoot (a) and root (e) Mn; shoot (b) and root (f) Fe; shoot (c) and root (g) Cd concentrations and shoot (d) and root (h) Cd content in T5i and OX plants grown under 10 μM Cd conditions. Error bars represent the SD. Columns bars followed by different letters are statistically different according to analysis of variance followed by SNK test (a, p=0.0004; c, p=0.0005; d, p=0.0001; e, p=0.0067; g, p=0.0017; h, p=0.0001); n=3.
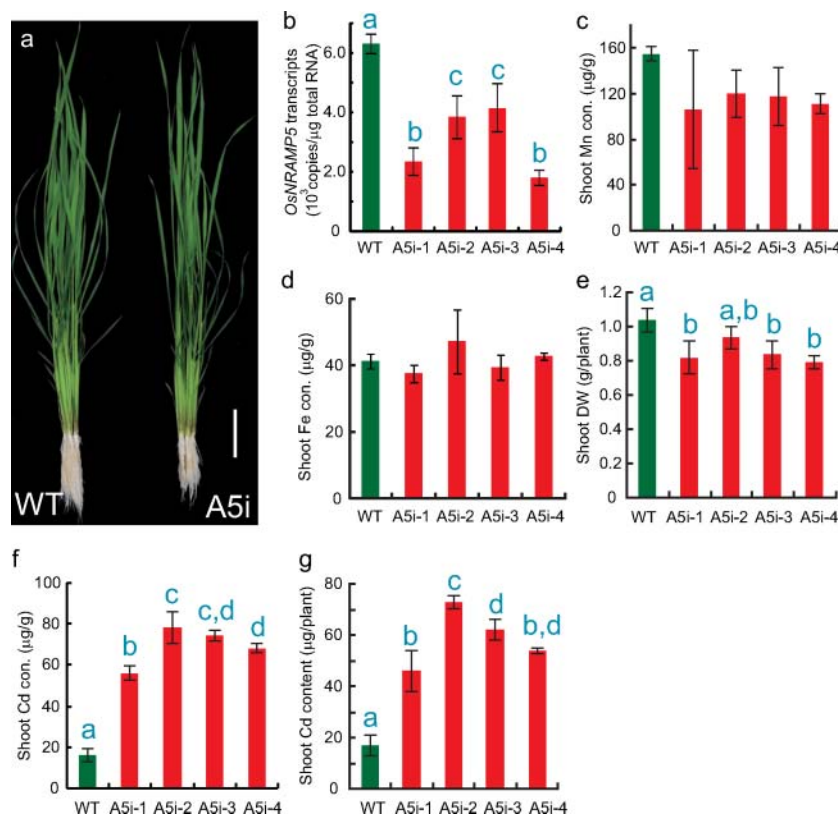
Figure 7 | OsNRAMP5i Anjana Dhan for Cd phytoremediation. (a) Phenotypes of four-week-old wild type and OsNRAMP5i (A5i-2) Anjana Dhan plants transferred to a nutrient solution containing 10 μM CdCl$_2$ and cultivated for two weeks. Scale bars = 10 cm. (b) *OsNRAMP5* transcripts in the roots of wild type and OsNRAMP5i (A5i-1, A5i-2, A5i-3, A5i-4) Anjana Dhan plants grown in normal nutrient hydroponic culture. Shoot Mn (c), Fe (d) concentration, shoot dry weight (e), shoot Cd concentration (f) and Shoot Cd content (g) of wild type and A5i plants grown under 10 μM Cd condition. Error bars represent the SD. Columns bars followed by different letters are statistically different according to analysis of variance followed by SNK test (b, p0.0000; e, p=0.0113; f, p=0.0000; g, p=0.0000); n=3.

without Fe and grown for an additional week. For the soil growth conditions, 8 ppm CdCl$_2$ was added to 500 g of bonsol (Sumitomo Chem. Co., Osaka, Japan). Two-week-old seedlings were transplanted to soil and grown in a greenhouse under natural light conditions.

Microarray and RT-PCR. Microarray analyses were done using a rice 44K custom oligo DNA microarray kit (Agilent Technology, Tokyo, Japan) as described previously[55,56]. The reproducibility of the analysis was assessed by a dye swap with two biological replicates (**Supplementary Table 1**) or three biological replicates for the T5i experiment (**Supplementary Table 2**). qRT-PCR was performed as described previously[55] using the primers *OsNRAMP5* RT forward 5′-CCGCCGTCGTCGACGCCGAGAAGGC-3′ and *OsNRAMP5* RT reverse 5′-ACACCCTTGTCGATCGATCGATCTG-3′. The *α-tubulin* primers used for qRT-PCR were *α-tubulin* forward 5′-TCTTCCACCCTGAGCAGCTC-3′ and *α-tubulin* reverse 5′-AACCTTGGAGACCAGTGCAG-3′. All experiments were performed in triplicate.

Determination of metal concentrations. Roots samples were washed with de-ionized water thrice before sampling. Leaf and root samples were dried for one week at 70°C and were digested with 3 ml of 13 M HNO3 at 220°C for 20 min using a MARS XPRESS microwave reaction system (CEM, Matthews, NC, USA) in triplicate. For xylem sap collection, rice plants were de-topped at a height of 3 cm from the root. The surface of the excised leaf sheath was gently wiped, and a tube filled with cotton was placed on the cut end. The entire length of the tube was covered with aluminum foil and the xylem sap was collected for 30 min. Forty-five rice plants were used for xylem sap collection. The xylem sap was collected in cotton and filtered with a 0.45 μm filtering column (Millipore). The extracted xylem sap was stored at −20°C until further analysis. Because the amount of xylem sap differed, 36 samples with median values of these xylem sap amounts were selected and twelve individual samples each were pooled at random into three tubes for further analysis. Xylem samples were digested with 3 ml of 13 M HNO$_3$ at 80°C for 60 min. After digestion, samples were collected, diluted to 5 ml and analyzed by ICP-AES (SPS1200VR; Seiko, Tokyo, Japan) as described previously[26,52].

Subcellular localization of *OsNRAMP5*. The full-length ORF of *OsNRAMP5* was amplified by PCR with the forward primer

5′-CACCATGGAGATTGAGAGAGAGAGCAGTG-3′ and reverse 5′-ACACCCTTGTCGATCGATCGATCTG-3′ primers, and subcloned into pH7WGF2[57]. Onion epidermal cells were transformed, stained with plasma membrane dye FM4-64 (Invitrogen, Tokyo, Japan) and observed with a LSM5 Pascal laser-scanning confocal microscope (Carl Zeiss, Tokyo, Japan) as described previously[25,55].

Yeast strains and growth media. The *OsNRAMP5* ORF was cloned into the expression vector pDR195[58] and then introduced into yeast cells using the lithium acetate method. The following strains of *Saccharomyces cerevisiae* were used: wild type parental strain (BY4741; MAT*alpha*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), ferrous Fe uptake-defective double mutant *Δfet3fet4* (MAT*alpha*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YMR058w::kanMX4*; *YMR319c::kanMX4*), Mn uptake-defective mutant *Δsmf1* (MAT*alpha*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YOL122c::kanMX4*), and Cd-sensitive mutant *Δycf1* (MAT*alpha*, *Δtrp1*, *Δhis3*, *Δleu2*, *Δura3*; *Δycf*:TRY1). *ycf1* lacks the function for compartmentalization of Cd into vacuoles[43]. For media without Fe or Mn, 10 μM bathophenanthroline disulfonic acid (BPDS) or 20 mM ethylene glycol-bis-β-aminoethylether-N,N,N',N'-tetraacetic acid (EGTA) with 50 mM 2-morpholinoethanesulfonic acid, monohydrate (MES) were added, respectively (Wako Pure Chemical Industries). For Cd treatment, 10 μM CdCl$_2$ was added. After spotting at three dilutions (optical densities at 600 nm of 0.1, 0.01, and 0.001), the plates were incubated at 30°C for five days.

Rice transformation. The 0.8-kb 5′-flanking region of *OsNRAMP5* was amplified by PCR using genomic DNA as template with the forward primer 5′-TTCCCCAAGCTTACACAAGAGGTTTTGCACACAGG -3′ and reverse primer 5′-CTCAATTCTAGATGTTGCTTCCTCTCTTAGCTTCT-3′, which contained *Hin*dIII and *Xba*I restriction sites, respectively. The amplified fragment was fused into pBluescript II SK+ and its sequence confirmed. The *OsNRAMP5* promoter was digested with *Hin*dIII and *Xba*I and the digested 0.8-kb fragment was subcloned upstream of the *uid*A ORF, which encodes β-glucuronidase, in pIG121Hm. *Oryza sativa* L. cv. Tsukinohikari was transformed and the histochemical localization of *OsNRAMP5* was observed in three independent T2 plants as described previously[25].

The plasmid pH7FWG2-*OsNRAMP5* was used to overexpress *OsNRAMP5*. To suppress *OsNRAMP1* expression, a 300-bp fragment from the 3′-UTR region of *OsNRAMP1* was amplified by PCR with the forward primer

5′-CACCTACCCTTCCAGACCGGTTACC-3′ and reverse primer 5′-GATTGTCCTTGTCCTCGCATC-3′. To suppress *OsNRAMP5* expression, a 300-bp fragment from the 3′-UTR region of *OsNRAMP5* was amplified by PCR with the forward primer 5′-GCAGTAAGAGCAAGATGGGGCCCCAC-3′ and reverse primer 5′-TTGGGAGCGGGATGTCGGCCAGGT-3′. The amplified fragment was cloned into pIG121-RNAi-DEST[21] and used for transformation.

SPAD reading. The degree of chlorosis in the youngest fully expanded leaf was determined using a SPAD-502 chlorophyll meter (Minolta Co., Tokyo, Japan).

Statistical analysis. To determine whether the observed differences between the WT and OsNRAMP5i and OX lines were statistically significant, one-way ANOVA was performed using Costat 6.400 (Cohort, USA) with a completely randomized design followed by a Student-Newman-Keuls (SNK) test ($P < 0.05$).

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Author contributions

Y.I., H.N., and N.N. designed the research, Y.I., K.B., R.T., S.I., T.A., H.N., N.N discussed the data and Y.I., K.B., R.T., H.N., N.N wrote the paper. Yeast complementation, GFP, and GUS analysis was performed by Y.I. Rice transformations (Tsukinohikari) were performed by H.S. Rice transformations (Anjana Dhan) were performed by K.S., K.O., and M.Y. Metal concentration analysis was performed by Y.I., H.S., R.T., and T.S. Microarray and expression analyses were performed by Y.I. Statistical analyses were performed by K.B.

Additional information