RESEARCH PAPER

The OsNRAMP1 iron transporter is involved in Cd accumulation in rice

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Abstract

Cadmium (Cd) is a heavy metal toxic to humans and the accumulation of Cd in the rice grain is a major agricultural problem, particularly in Asia. The role of the iron transporter OsNRAMP1 in Cd uptake and transport in rice was investigated here. An OsNRAMP1:GFP fusion protein was localized to the plasma membrane in onion epidermal cells. The growth of yeast expressing OsNRAMP1 was impaired in the presence of Cd compared with yeast transformed with an empty vector. Moreover, the Cd content of OsNRAMP1-expressing yeast exceeded that of the vector control. The expression of OsNRAMP1 in the roots was higher in a high Cd-accumulating cultivar (Habataki) than a low Cd-accumulating cultivar (Sasanishiki) regardless of the presence of Cd, and the amino acid sequence of OsNRAMP1 showed 100% identity between Sasanishiki and Habataki. Over-expression of OsNRAMP1 in rice increased Cd accumulation in the leaves. These results suggest that OsNRAMP1 participates in cellular Cd uptake and Cd transport within plants, and the higher expression of OsNRAMP1 in the roots could lead to an increase in Cd accumulation in the shoots. Our results indicated that OsNRAMP1 is an important protein in high-level Cd accumulation in rice.

Key words: Cadmium, metal transporter, OsNRAMP1, phytoremediation, rice.

Introduction

Cadmium (Cd) is one of the most toxic heavy metals, and causes serious health problems in humans. Cd accumulates in the human body through the food chain. Accumulation of Cd in rice grain has become a growing threat to agriculture in Asia in recent years because rice is a dietary staple for Asians and a major source of Cd. In Japan, Cd intake from rice accounts for about one-half of the intake from food (Food Safety Commission, 2008). The Codex Alimentarius Commission established a Cd content of 0.4 mg kg⁻¹ polished rice grain as the limit for food safety and human health (CODEX, 2006). Thus, new technologies to reduce the Cd content in rice grains are urgently required to reduce the Cd risk.

One method of reducing Cd in rice grain is to modify the genes involved in Cd uptake or translocation. Although the mechanism underlying the uptake and translocation of Cd in plants is not completely understood, some iron (Fe) transporters take up Cd. Ferrous Fe transporters, OsIRT1 and OsIRT2, take up Cd (Nakanishi et al., 2006), and over-expression of OsIRT1 has been shown to increase Cd accumulation (Lee and An, 2009). Arabidopsis over-expressing AtIRT1 and AtIRT2 also showed increased Cd accumulation (Connolly et al., 2002; Vert et al., 2009).

The natural resistance-associated macrophage proteins (NRAMPs) consist of a large family of integral membrane proteins, and NRAMP genes have been identified in several plant species (Williams et al., 2000; Bereczky et al., 2003; Kaiser et al., 2003; Mizuno et al., 2005; Xiao et al., 2008; Oomen et al., 2009; Wei et al., 2009). The NRAMP family
of metal transporters function as Fe transporters. AtNRAMP1 expression is induced by Fe deficiency in the roots and is considered to control Fe homeostasis in plants (Curie et al., 2000). AtNRAMP3 and AtNRAMP4 are also induced by Fe starvation and mobilize vacuolar Fe (Thomine et al., 2000, 2003; Lanquar et al., 2005). Yeast expressing AtNRAMP1, AtNRAMP3, AtNRAMP4, and AtNRAMP6 showed increased sensitivity to Cd, and the over-expression of AtNRAMP3, AtNRAMP4, and AtNRAMP6 in Arabidopsis conferred hypersensitivity to Cd (Thomine et al., 2000; Lanquar et al., 2004; Cailliatte et al., 2009). These results suggest that NRAMP proteins also mediate Cd transport. Rice has seven NRAMP genes (Narayanan et al., 2007), and OsNRAMP1 is involved in Fe uptake function in yeast (Curie et al., 2000).

In rice, there is genotypic variation in the Cd levels of grains and shoots, with Cd accumulation in both shoots and grains being generally greater in indica rice cultivars (including Habataki, Jarjan, Anjana Dhan, and Cho-ko-koku) than in japonica cultivars (including Sasanishiki, Nipponbare, and Tsukinohikari; Arao and Ae, 2003; Ishikawa et al., 2009; Uraguchi et al., 2009). Uraguchi et al. (2009) showed that the Cd concentration of xylem sap was higher in indica cultivars. These results suggest that xylem-mediated Cd translocation from roots to shoots is a major factor determining Cd accumulation levels in shoots and grains of rice. Recently, quantitative trait loci (QTL) for Cd concentration in rice were identified on chromosome 7 (Tezuka et al., 2009; Ueno et al., 2009; Ishikawa et al., 2010). The responsive gene for this QTL of Cho-ko-koku and Anjana Dhan was identified as OsHMA3 (Miyadate et al., 2011; Ueno et al., 2010). Ueno et al. (2010) reported that the amino acid at position 80 in OsHMA3 is critical for the function, and that mutation of this amino acid caused loss of function to sequester Cd into vacuoles in root cells, resulting in the high translocation of Cd from the roots to the shoots in Anjana Dhan. According to this model, Cd concentration in the cytoplasm is higher in the root cells of high Cd-accumulating cultivars than in low Cd-accumulating cultivars. However, the difference in Cd uptake in the root cells between high and low Cd-accumulating cultivars has not been clarified.

In the present study, the role of OsNRAMP1 in Cd uptake and accumulation in rice was investigated using japonica and indica rice cultivars.

Materials and methods

Plant materials and growth conditions

Rice (Oryza sativa L. cv. Sasanishiki, Nipponbare, Tsukinohikari, Habataki, Jarjan, Anjana Dhan, and Cho-ko-koku) seeds were germinated for 3 d at room temperature on paper soaked with distilled water. After germination, seedlings were transferred to a net floating on distilled water and grown for 7 d at room temperature. Transgenic rice T1 seeds were germinated on MS medium containing 50 mg l\(^{-1}\) hygromycin B and grown for 2 weeks at 28 °C. Seedlings were transferred to a 20 l plastic container and cultivated hydroponically in a greenhouse (30 °C, natural light). The composition of the nutrient solution was described previously by Ishimaru et al. (2006). The nutrient solution was adjusted every day to pH 5.5 with 1 M HCl and changed once per week. For Cd stress treatments, 2-week-old plants were transferred to a nutrient solution containing 0.2 or 1 μM CdCl\(_2\) and cultivated for an additional week. For Fe deficiency treatments, 3-week-old plants were transferred to a nutrient solution without Fe and cultivated for another 1 week.

Plant metal content

Harvested plants were washed with tap water and rinsed with distilled water, then blotted using paper towels. The plants were then separated into leaves and roots and dried at 70 °C for 1 week. Sample digestion was performed as described previously by Ishimaru et al. (2010), except that the digestion time and temperature were changed to 20 min at 230 °C. Metal concentrations were measured using inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan). Three biological replicates were used for each treatment.

Cloning of OsNRAMP1 and OsHMA3

Total RNA was extracted from rice using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and then reverse-transcribed using an oligo(dT) primer and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The complete open reading frame (ORF) of OsNRAMP1 was obtained by PCR using the primers HindIII-Forward 5’-CGACTAAGCTTAAAGACGCC-CACTTAGTG-3’ and Xhol-Reverse 5’-CCGGTCTA-GAAGGCTACTACGGTGCT-3’, which were designed based on sequence information from the RAP-DB database (http://rapdb.dna.affrc.go.jp). A partial fragment of OsHMA3 was obtained by PCR using forward 5’-CACCATGGCCCAGGAGATTAGGGCG-3’) and reverse (5’-GGCGTGTAAGTACTTTGC GCAC-3’) primers.

Subcellular localization of OsNRAMP1

The OsNRAMP1 ORF without the stop codon was cloned into the plant transient expression vector pH7FWG2 (Karimi et al., 2002) using the Gateway system (Invitrogen) to generate a fusion construct with green fluorescent protein (GFP) as an additional C-terminal domain under the control of the CaMV 35S promoter. The OsNRAMP1:GFP fusion construct or an untagged GFP construct was transiently expressed in onion epidermal cells transformed by DNA particle bombardment. After 8 h, fluorescent cells were imaged by confocal microscopy (LSM5Pascal; Carl Zeiss, Göttingen, Germany). To induce cell plasmolysis, onion epidermal cells were soaked in 20% sucrose solution for 20 min before imaging.

Yeast growth conditions and 109Cd uptake analysis

The OsNRAMP1 ORF was inserted into the expression vector pDR195 (Rentsch et al., 1995), then introduced into yeast strain yepl using the lithium acetate method. The yepl mutant lacks the function for the compartmentalization of Cd into vacuoles (Li et al., 1997). Yeast yepl cells transformed with the empty pDR195 vector were used as controls. The transformed yeast cells were grown in synthetic defined (SD) medium and spotted onto SD agar containing 10 μM CdCl\(_2\).

For the 109Cd uptake experiment, transformed yeast cells were amplifed in SD medium. A 109Cd uptake assay was performed as described previously (Eide et al., 1992), except that 109CdCl\(_2\) was substituted for 55FeCl\(_3\). Cell samples were collected by centrifugation using Ultrafree MC (pore size 0.45 μm; Millipore, Billerica, MA, USA) and washed twice. The yeast 109Cd content was measured using an AutoWell Gamma System (ARC-300; Aloka, Tokyo, Japan).
Analysis of OsNRAMP1 expression
Total RNA prepared from the shoots or roots of the plants was reverse-transcribed as described above. Quantitative RT-PCR was then performed using the Smart Cycler System (Takara, Shiga, Japan). Amplification was performed using forward (5'-GGATTCTCCTGGGTGCTGGGTT-3') and reverse (5'-GCAACAATCTACTCCATGGGCC-3') primers with SYBR Premix Ex Taq (Perfect Real Time; Takara); α-tubulin was used as an internal standard (Ishimaru et al., 2006). The products were subcloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Serially diluted vector was also amplified to calculate the OsNRAMP1 copy number.

Genomic DNA extraction
Leaves were ground in liquid nitrogen, and suspended in extraction buffer (200 mM TRIS-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS). After incubation for 1 h at room temperature, the supernatant was transferred to a new tube and the DNA was purified by 2-propanol precipitation.

Rice transformation
OsNRAMP1-overexpressing rice was produced using a pH7FWG2-OsNRAMP1 vector described in the section dealing with subcellular localization of OsNRAMP1. Agrobacterium-mediated transformation of rice (cv. Tsukinohikari) was performed as described previously (Higuchi et al., 2001). Eight independently transformed over-expressing rice plants (35S-rice) were generated, and two lines showing high levels of OsNRAMP1 expression in the whole plant were selected (see Supplementary Fig. S1 at JXB online).

Results
OsNRAMP1 is localized to the plasma membrane and functions in Cd uptake
The ORFs of OsNRAMP1 from three rice cultivars (Nipponbare, Sasanishiki, Habataki) were isolated (Fig. 1). There was no difference in nucleotide or amino acid sequence among the three cultivars.

The subcellular localization of OsNRAMP1 was investigated using an OsNRAMP1:GFP fusion protein. Green fluorescence was observed at the periphery of the cells but not inside the cells (Fig. 2A–C). Plasmolysis of the cells was performed to confirm the plasma membrane localization of the protein (Fig. 2D, E).

The growth of yeast expressing OsNRAMP1 was impaired compared with that of yeast transformed with the empty vector in the presence of 10 µM Cd (Fig. 3A). Furthermore, with 1 µM Cd treatment for 30 min, the Cd content of the OsNRAMP1-expressing yeast was 1.4 times higher than that of the control (Fig. 3B).

In the presence of 1 µM Cd, the growth of OsNRAMP1-overexpressing rice was significantly inhibited in both shoots and roots (Fig. 4A, B). The growth of OsNRAMP1-overexpressing rice was also inhibited under the control condition (Fig. 4A, B). The Cd concentration in the leaves of 35S-rice was significantly higher than that of non-transformed rice (WT; Fig. 4C). On the other hand, the concentrations of other essential metals (Fe, Zn, Mn, Cu) were not changed in the presence of 1 µM Cd, except for the

Metal concentrations in the roots and the leaves of Sasanishiki and Habataki
In the presence of 1 µM Cd, the Cd concentrations in the leaves and the roots of Habataki were 1.8 times and 1.2 times higher than those of Sasanishiki, respectively (Fig. 5A, B). Fe concentration in the leaves was decreased in both Sasanishiki and Habataki by Cd treatment, whereas those in the roots remained essentially unchanged (Fig. 5C, D). In the presence of Cd, the concentrations of the other essential metals (Zn, Mn, Cu) also decreased in the leaves and roots of Sasanishiki and Habataki (see Supplementary Fig. S3 at JXB online).

Expression analysis of OsNRAMP1 in different cultivars
The level of OsNRAMP1 expression in the roots was increased in both Sasanishiki and Habataki in the presence of 1 µM Cd (Fig. 6A). The levels of OsNRAMP1 expression in the roots of Habataki were higher than those of Sasanishiki in the presence of 0 (control), 0.2 and 1 µM Cd. In addition, a higher expression of OsNRAMP1 in roots was also observed in other Cd-accumulating indica cultivars, including Jarjan, Anjana Dhan, and Cho-ko-koku, compared with the low Cd-accumulating japonica cultivars, Nipponbare and Tsukinohikari (Fig. 6B). Expression of OsNRAMP1 in shoots was increased only in Sasanishiki in the presence of Cd (Fig. 6A).

Under conditions of Fe deficiency, the expression of OsNRAMP1 was increased in both the shoots and roots of Sasanishiki and Habataki; the expression levels in the two cultivars were almost identical (Fig. 6C).
Analysis of the promoter sequence

To gain an insight into the differential expression of OsNRAMP1 among cultivars, the 2 kb 5′-flanking regions of OsNRAMP1 from Sasanishiki and Habataki were isolated and compared. Seventeen nucleotides were substituted, and one nucleotide gap and one nucleotide insertion were found in Habataki (Fig. 7A). Furthermore, 406 bp (from the translational initiation codon in Sasanishiki) were missing in Habataki (Fig. 7A). This 400-bp deletion was also found in the 5′-flanking region of other high Cd-accumulating cultivars, including Jarjan, Anjana Dhan, and Cho-ko-koku, whereas it was not found in low Cd-accumulating cultivars, including Nipponbare and Tsukinohikari (Fig. 7B).

Amino acid sequence of OsHMA3 in different cultivars

Part of OsHMA3 was isolated from Sasanishiki and Habataki, and the predicted amino acid sequences were compared (Fig. 8). The substitution from Arg to His at position 80 was observed only in Anjana Dhan and Cho-ko-koku, and the amino acid sequence of OsHMA3 from Habataki was the same as those of the low Cd-accumulating cultivars, Sasanishiki and Nipponbare, at this position.

Discussion

In this study, OsNRAMP1 was isolated and characterized from the low Cd-accumulating rice cultivar Sasanishiki and the high Cd-accumulating cultivar Habataki. OsNRAMP1 was localized to the plasma membrane (Fig. 2), and OsNRAMP1-expressing yeast accumulated larger amounts of Cd than the vector control (Fig. 3B). Furthermore, over-expression of OsNRAMP1 in rice increased Cd accumulation in the leaves (Fig. 4C). These results indicated that OsNRAMP1 participates in cellular Cd uptake and Cd transport within the plant.

AtNRAMP1, which shows high homology with OsNRAMP1, has been reported to act as a Mn transporter (Cailliatte et al., 2010). However, OsNRAMP1 did not complement the Mn-deficient phenotype of the yeast smf1 mutant (data not shown), which lacks the Mn2+ uptake function (Supek et al., 1996).

Fe-deficient rice absorbed larger amounts of Cd than Fe-sufficient rice, suggesting that the induced expression of Fe transporters in response to Fe deficiency contributes to Cd uptake in rice (Nakanishi et al., 2006). Among the major Fe transporters induced by Fe deficiency, OsIRT1 and OsIRT2 take up Cd (Nakanishi et al., 2006). Another Fe transporter family, the OsYSLs, has been identified in rice, and OsYSL2 and OsYSL15 were shown to be strongly induced by Fe deficiency (Koike et al., 2004; Inoue et al., 2009). OsYSL2 may not contribute to Cd uptake in the roots. OsYSL2 transports Fe-nicotianamine and Mn-nicotianamine, and is responsible for the internal transport of Fe and Mn (Koike et al., 2004). By contrast, OsYSL15 transports Fe-deoxymugineic acid (DMA) and is responsible for Fe uptake from the soil (Inoue et al., 2009). Nakanishi et al. (2006) reported that the growth of yeast...
expressing OsYSL15 under Cd stress did not change significantly in the presence of DMA. Meda et al. (2007) reported that the Cd-DMA complex was weak compared with Fe-DMA, and the specificity of Cd-DMA transport by ZmYS1 was very low (Schaaf et al., 2004), suggesting that OsYSL15 is not involved in Cd transport.

OsNRAMP1 could participate in Cd uptake or transport in rice in addition to OsIRT1 and OsIRT2. Cailliatte et al. (2010) discussed the possibility of Mn acquisition in the roots of Arabidopsis by AtIRT1 and AtNRAMP1, and suggested that these transporters have different affinity constants for Mn and act in different tissues in the roots. They also reported that AtIRT1 is expressed exclusively in the external layers of the absorption zone of the root, whereas AtNRAMP1 is more widespread along the roots including the inner layers. Thus, it is possible that OsNRAMP1, OsIRT1, and OsIRT2 also function in different tissues in the roots. OsIRT1 was highly expressed in the epidermis and the cortex around the endodermis in the roots, and considered to function in Fe2+ and Cd uptake from the soil (Ishimaru et al., 2006; Nakanishi et al., 2006). The Cd concentration of OsIRT1-overexpressing rice was higher than that of the wild type in both the roots and the shoots (Lee and An, 2009). On the other hand, the Cd concentration in the leaves of OsNRAMP1-overexpressing rice was higher than that of the wild type, whereas the Cd concentration in the roots was lower (Fig. 4C, D). This result suggests that Cd translocation from the roots to the shoots was highly activated in addition to the increase in Cd uptake from the rhizosphere in OsNRAMP1-overexpressing rice.

Furthermore, the growth of OsNRAMP1-overexpressing rice was significantly inhibited compared with WT, indicating that ectopic expression of OsNRAMP1 could disturb metal distribution in rice, and suggesting that OsNRAMP1 may play an important role in Cd distribution in rice. It is possible that OsNRAMP1 transports Cd into the cytoplasm in the inner layers of the roots following Cd uptake from the rhizosphere by OsIRT1, and participates in the control of Cd availability for loading into the xylem. Further detailed analysis to determine the tissue localization of OsNRAMP1 will clarify its role in Cd acquisition and translocation.

The level of OsNRAMP1 expression was higher in the roots of high Cd-accumulating indica cultivars than in low Cd-accumulating japonica cultivars (Fig. 6B). As the OsNRAMP1 of Sasanishiki and Habataki showed 100% identity at the amino acid level, the transport ability of OsNRAMP1 does not differ between the low and high Cd-accumulating cultivars. Thus, it is possible that differences in the expression level of OsNRAMP1 in the roots are responsible for the observed differences in Cd accumulation among these cultivars. Comparative analysis of the putative promoter region of OsNRAMP1 revealed a 406 bp deletion in Habataki (Fig. 7A) that was also found in other high Cd-accumulating cultivars (Fig. 7B). Fe deficiency treatment induced expression of OsNRAMP1 in both Sasanishiki and Habataki (Fig. 6C). The core sequences for binding to known transcription factors related to Fe deficiency, including OsIRO2, IDEF1, and IDEF2 (Ogo et al., 2006, 2008; Kobayashi et al., 2007), do not exist within the deleted 406 bp domain, although there are two IDE2 core sequences in the 2 kb 5'-flanking regions of OsNRAMP1 from Sasanishiki and Habataki. Thus, induction by Fe deficiency was unaffected by the deletion of this region.
However, it is possible that other cis-acting elements, which do regulate the expression of OsNRAMP1, exist within this 406 bp region. Three putative DNA-binding domains for MYB transcription factors (GGATA, CNGTT) were identified in this region. Some MYB proteins act as negative regulators of gene expression (Chinnusamy et al., 2007; Matsui et al., 2008), and some MYB genes have been reported to be responsive to Cd (Chen et al., 2006). The difference in OsNRAMP1 expression between Sasanishiki and Habataki may be dependent on these transcription factors.

Recently, QTL for Cd accumulation of Habataki was also identified on chromosome 7 (Ishikawa et al., 2010), but the responsive gene for this QTL has yet to be identified. OsNRAMP1 is located in this QTL region, and the amino acid change for the loss of function of OsHMA3 was not observed in Habataki (Fig. 8). Thus, the OsHMA3 mutation is apparently not the reason for Cd accumulation in the shoots of Habataki. The loss of function of OsHMA3 is believed to lead to an elevated Cd concentration in the cytoplasm and higher loading into the xylem. These observations indicate that Cd content in the root cells is an important factor for Cd translocation from the roots to the shoots and Cd accumulation in the shoots. It seems likely that higher levels of OsNRAMP1 expression in the roots of indica cultivars were responsible for higher Cd uptake in the root cells. Our results suggest that OsNRAMP1 is an important protein for Cd higher accumulation in rice.

**Supplementary data**

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Expression of OsNRAMP1 in 35S-rice.
 Supplementary Fig. S2. Metal concentrations of the roots and leaves of 35S-rice grown in the absence (control) or presence of 1 μM CdCl₂.

 Supplementary Fig. S3. Metal concentrations of the roots and leaves of Sasanishiki and Habataki grown in the absence (control) or presence of 1 μM CdCl₂.

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