A Member of the Heavy Metal P-Type ATPase OsHMA5 Is Involved in Xylem Loading of Copper in Rice[1][W][OPEN]

Fenglin Deng, Naoki Yamaji, Jixing Xia, and Jian Feng Ma*

Institute of Plant Science and Resources, Okayama University, Kurashiki 710–0046, Japan

ORCID ID: 0000-0003-3411-827X (J.F.M.).

Heavy metal-transporting P-type ATPase (HMA) has been implicated in the transport of heavy metals in plants. Here, we report the function and role of an uncharacterized member of HMA, OsHMA5 in rice (*Oryza sativa*). Knockout of *OsHMA5* resulted in a decreased copper (Cu) concentration in the shoots but an increased Cu concentration in the roots at the vegetative stage. At the reproductive stage, the concentration of Cu in the brown rice was significantly lower in the mutants than in the wild-type rice; however, there was no difference in the concentrations of iron, manganese, and zinc between two independent mutants and the wild type. The Cu concentration of xylem sap was lower in the mutants than in the wild-type rice. *OsHMA5* was mainly expressed in the roots at the vegetative stage but also in nodes, peduncle, rachis, and husk at the reproductive stage. The expression was up-regulated by excess Cu but not by the deficiency of Cu and other metals, including zinc, iron, and manganese, at the vegetative stage. Analysis of the transgenic rice carrying the *OsHMA5* promoter fused with green fluorescent protein revealed that it was localized at the root pericycle cells and xylem region of diffuse vascular bundles in node I, vascular tissues of peduncle, rachis, and husk. Furthermore, immunostaining with an antibody against OsHMA5 revealed that it was localized to the plasma membrane. Expression of *OsHMA5* in a Cu transport-defective mutant yeast (*Saccharomyces cerevisiae*) strain restored the growth. Taken together, OsHMA5 is involved in loading Cu to the xylem of the roots and other organs.

Plants require nutrient elements to maintain normal growth and development. A number of different transporters, such as Cation Diffusion Facilitator, Natural resistance-associated macrophage protein, ATP-Binding Cassette, Zinc- and Iron-regulated-like Protein, and P-type ATPase, have been reported to be involved in the uptake, translocation, distribution, and homeostasis of nutrients (Hall and Williams, 2003; Krämer et al., 2007; Palmer and Guerinot, 2009). Among them, heavy metal-transporting P-type ATPase (HMA), the P_{1B} subfamily of the P-type ATPase superfamily, has been implicated in heavy metal transport (Williams and Mills, 2005; Grotz and Guerinot, 2006; Argüello et al., 2007; Burkhead et al., 2009). There are eight and nine members of P_{1B}-ATPase in *Arabidopsis thaliana* and rice (*Oryza sativa*), respectively (Williams and Mills, 2005). They are divided into two groups: zinc (Zn)/cadmium (Cd)/cobalt/lead (Pb) and copper (Cu)/silver transporters (Williams and Mills, 2005). AtHMA1 to AtHMA4 in *A. thaliana* and OsHMA1 to OsHMA3 in rice belong to the former group, while AtHMA5 to AtHMA8 and OsHMA4 to OsHMA9 belong to the latter group, although AtHMA1 has also been shown to transport Zn, Cu, and calcium (Axelsen and Palmgren, 2001; Williams and Mills, 2005; Seigneurin-Berny et al., 2006; Moreno et al., 2008; Kim et al., 2009).

All members of HMAs in *A. thaliana* have been functionally characterized. AtHMA1 is involved in delivering Cu to the stroma, exporting Zn^{2+} from the chloroplast, or as a Ca^{2+}/heavy metal transporter to the intracellular organelle (Seigneurin-Berny et al., 2006; Moreno et al., 2008; Kim et al., 2009). AtHMA2 and AtHMA4 localized at the pericycle are partially redundant and responsible for the release of Zn into the xylem (xylem loading) as well as Cd (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009; Wong et al., 2009), while AtHMA3 localized at the tonoplast plays a role in the detoxification of Zn/Cd/cobalt/Pb by mediating them into the vacuole (Morel et al., 2009; Chao et al., 2012). On the other hand, AtHMA5 is involved in the Cu translocation from roots to shoots or Cu detoxification of roots (Andrés-Colás et al., 2006; Kobayashi et al., 2008). AtHMA6 (PAA1, for P-type ATPase of Arabidopsis1) localized at the chloroplast periphery has been proposed to transport Cu over the chloroplast envelope, whereas AtHMA8 (PAA2) localized at the thylakoid membranes most likely transports Cu into the thylakoid lumen to supply plastocyanin (Shikanai et al., 2003; Abdel-Ghany et al., 2005). Finally, AtHMA7 (RESPONSIVE-TO-ANTAGONIST1) is responsible for delivering Cu to ethylene receptors and Cu homeostasis in the seedlings (Hirayama et al., 1999; Woeste and Kieber, 2000; Binder et al., 2010).

By contrast, only three out of nine P-type ATPase members have been functionally characterized in rice.

[1] This work 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 (grant no. 22119002 to J.F.M.) and by the Ohara Foundation for Agriculture Research.

* Address correspondence to maj@rib.okayama-u.ac.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jian Feng Ma (maj@rib.okayama-u.ac.jp).

[W] The online version of this article contains Web-only data.

[OPEN] Articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.113.226225

OsHMA2 was recently reported to be involved in the root-shoot translocation of Zn and Cd (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013). Furthermore, OsHMA2 at the node is required for preferential distribution of Zn to young leaves and panicles (Yamaji et al., 2013). OsHMA3 is localized to the tonoplast of the root cells and responsible for the sequestration of Cd into the vacuoles (Ueno et al., 2010; Miyadate et al., 2011). On the other hand, OsHMA9 was mainly expressed in vascular tissues, including the xylem and phloem (Lee et al., 2007). The knockout lines accumulated more Zn, Cu, Pb, and Cd, suggesting its role in the efflux of these metals from the cells (Lee et al., 2007).

Some members of P-type ATPase have also been identified in other plant species, including barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), *Thlaspi caerulescens* (*Noccaea caerulescens*), and *Arabidopsis halleri*. HvHMA1 from barley might be involved in mobilizing Zn and Cu during the stage of grain filling (Mikkelsen et al., 2012). HvHMA2 from barley and TaHMA2 from wheat showed similar functions as OsHMA2 in rice (Mills et al., 2012; Tan et al., 2013). AhHMA3 in *A. halleri*, a Zn hyperaccumulator, is probably involved in high Zn accumulation (Becher et al., 2004; Chiang et al., 2006). Furthermore, AhHMA4 for Zn translocation showed a higher expression level (Chiang et al., 2006; Hanikenne et al., 2008). On the other hand, TcHMA3 from ecotype Ganges of *T. caerulescens*, a Cd hyperaccumulator, plays an important role in the detoxification of Cd by sequestering Cd into the vacuole of the leaves (Ueno et al., 2011). High expression of TcHMA4 (NcHMA4) was also reported in *T. caerulescens* (Bernard et al., 2004; Papoyan and Kochian, 2004; Craciun et al., 2012).

In this study, we investigated the function and role of an uncharacterized member of P-type ATPase in rice, OsHMA5. We found that OsHMA5 is involved in the xylem loading of Cu at both the vegetative and reproductive growth stages.

RESULTS

Phylogenetic Analysis of *OsHMA5*

The full-length coding region of *OsHMA5* (Os04g0556000) was amplified by PCR from complementary DNA (cDNA) of rice roots (cv Nipponbare) using primers designed according to the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). The sequence obtained was exactly the same as that registered in the database. *OsHMA5* contains six exons and five introns (Supplemental Fig. S1A), encoding a peptide of 1,002 amino acids. Prediction with both the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/) and HMMTOP (http://www.enzim.hu/hmmtop/) showed that OsHMA5 is a membrane protein with eight transmembrane domains and a long hydrophilic loop between transmembrane domains 6 and 7 (Supplemental Fig. S1B). OsHMA5 contains all of the structural features of P-type ATPases, including two GMTCxxC motifs (GMTCAAC, amino acids 83–89; GMTCTSC, amino acids 161–167) and the CPC(x)6P motif (CPCALGLATP, amino acids 637–646; Supplemental Fig. S1B).

Phylogenetic analysis showed that OsHMA5 shares 72% identity with AtHMA5 (Supplemental Fig. S2). Among the nine HMA members in rice, the closest homolog of OsHMA5 is OsHMA4, which shares 57% identity (Supplemental Fig. S2).

Expression Pattern of *OsHMA5*

At the vegetative stage, *OsHMA5* was mainly expressed in the roots (Fig. 1A). The expression level was hardly affected by the deficiency of metals, including Zn, iron (Fe), manganese (Mn), and Cu (Fig. 1A), while the expression was slightly increased by a high Cu concentration (Fig. 1B). Spatial expression analysis showed that the expression of *OsHMA5* was much higher in the basal root zones (1–2 cm from the apex) than in the root tips (0–1 cm; Fig. 1C). Furthermore, with the help of laser microdissection, the expression was only detected in the central cylinder of the roots, including pericycle and inner tissues, but not in the outer tissues, including cortex and epidermis (Fig. 1D).

The expression of *OsHMA5* was also investigated in different tissues of rice grown in a field through the whole growth period. At the vegetative growth stage, *OsHMA5* was highly expressed in the roots, but at the reproductive growth stage, the higher expression was also found in nodes, peduncle, rachis, and husk (Fig. 1E).

Tissue Specificity of *OsHMA5* Expression

To examine the tissue and cell specificity of *OsHMA5* expression, transgenic rice carrying the *OsHMA5* promoter fused with GFP was generated. Immunostaining with an antibody against GFP detected the signal only in the pericycle cells of the roots at the vegetative stage (Fig. 2A). At the reproductive stage, the signal was observed in the xylem region of diffuse vascular bundles in node I, parenchyma cells of vascular tissues in peduncle, husk, and rachis (Fig. 2, B–G).

Cellular and Subcellular Localization of OsHMA5

The localization of the OsHMA5 protein in the seminal roots of rice was investigated by immunostaining using an antibody against OsHMA5. Similar to the promoter-GFP expression pattern (Fig. 2A), OsHMA5 protein was localized at the root pericycle cells (Fig. 3A). No signal was observed in the two knockout lines described below (Fig. 3, B and C), indicating the specificity of the antibody. Costaining with 4′,6-diamidino-2-phenylindole (DAPI) showed that the fluorescence signal of OsHMA5 was located at peripheral region of the cells outside the nuclei. This localization was

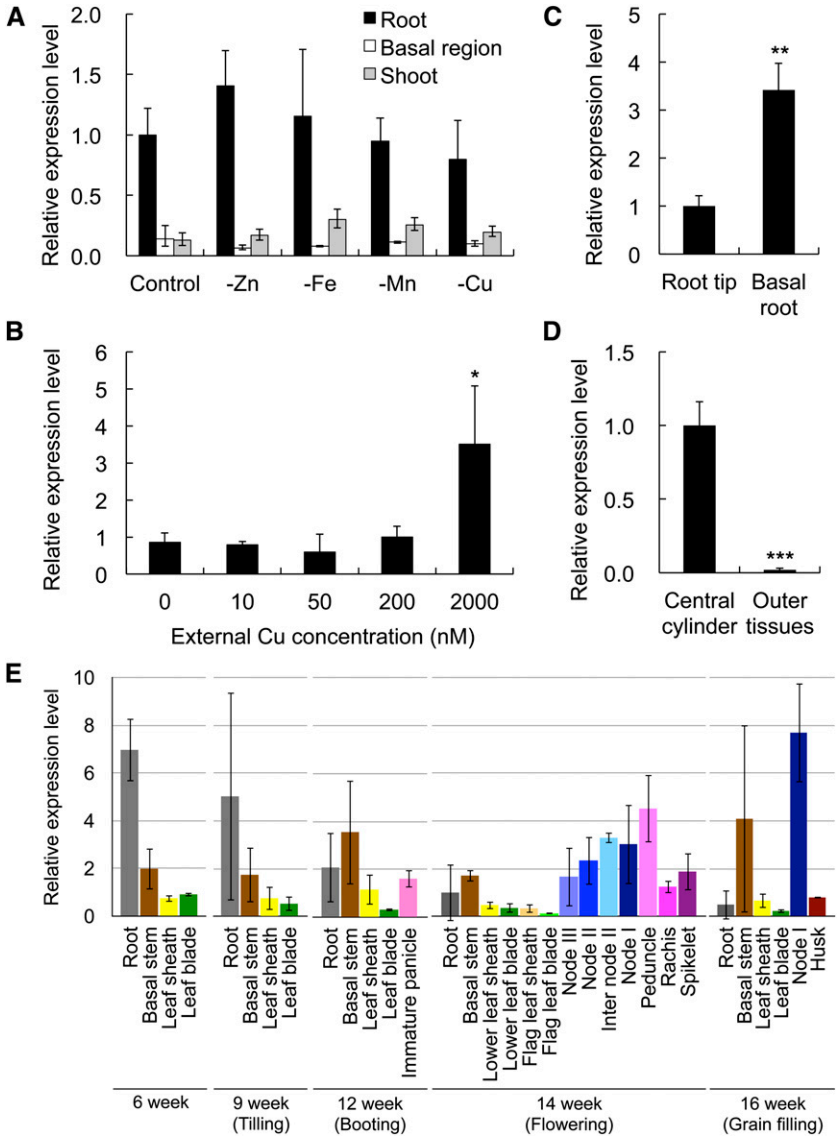

Figure 1. Expression pattern of *OsHMA5* in rice. A, Organ-dependent expression of *OsHMA5* at the vegetative stage. Plants were exposed to a nutrient solution with or without Zn, Fe, Mn, or Cu for 7 d. B, *OsHMA5* expression in response to Cu. Seedlings were exposed to different Cu concentrations for 1 d. C, Root spatial expression of *OsHMA5*. Different segments (0–1 and 1–2 cm) were excised. D, Tissue-specific expression of *OsHMA5*. Different tissues were separated by laser microdissection. E, Expression level of *OsHMA5* in different organs at different growth stages. Samples were taken from rice grown in a paddy field. The expression level was determined by real-time RT-PCR. Relative expression level is shown. Error bars represent SD of three independent biological replicates. Statistical comparison was performed by Tukey's test: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

similar to OsHMA2, a plasma membrane-localized transporter of Zn and Cd (Yamaji et al., 2013), suggesting that OsHMA5 was also localized to the plasma membrane (Fig. 3, D–F).

Phenotypic Analysis of the *OsHMA5* Knockout Lines

To investigate the physiological role of OsHMA5 in rice, we obtained two independent *Tos17* retrotransposon insertion lines from the Rice Mutant Panel. *Tos17* was inserted in the second (NF8524) and fifth (NE6050) exons (Supplemental Fig. S1A). No transcript of *OsHMA5* was detected in the roots of two homozygous lines (Supplemental Fig. S1C), indicating that they are knockout lines of *OsHMA5*.

When the wild-type rice and two mutants were grown in a nutrient solution at the vegetative stage, no difference in growth was observed between them (Fig. 4A). Mineral analysis showed that the Cu concentration was significantly lower in the shoots but higher in the roots of the mutants compared with the wild-type rice (Fig. 4, B and C). However, there was no consistent difference in Mn, Fe, and Zn concentration of both roots and shoots between the wild-type rice and two mutants. The concentration of Zn was slightly higher in the roots only in one mutant (Fig. 4C).

The grain yield was 30% lower in the mutants than in the wild-type rice when grown in a field until ripening (Fig. 5A), and the fertility was decreased in the mutants (Fig. 5B). The concentration of Cu in the brown rice was decreased by 57% compared with wild-type rice (Fig. 5C), whereas no decrease of other metals, including Fe, Mn, and Zn, was found between the wild-type rice and mutants (Fig. 5C). These results consistently indicate that transport of Cu rather than other metals was altered in the knockout lines.

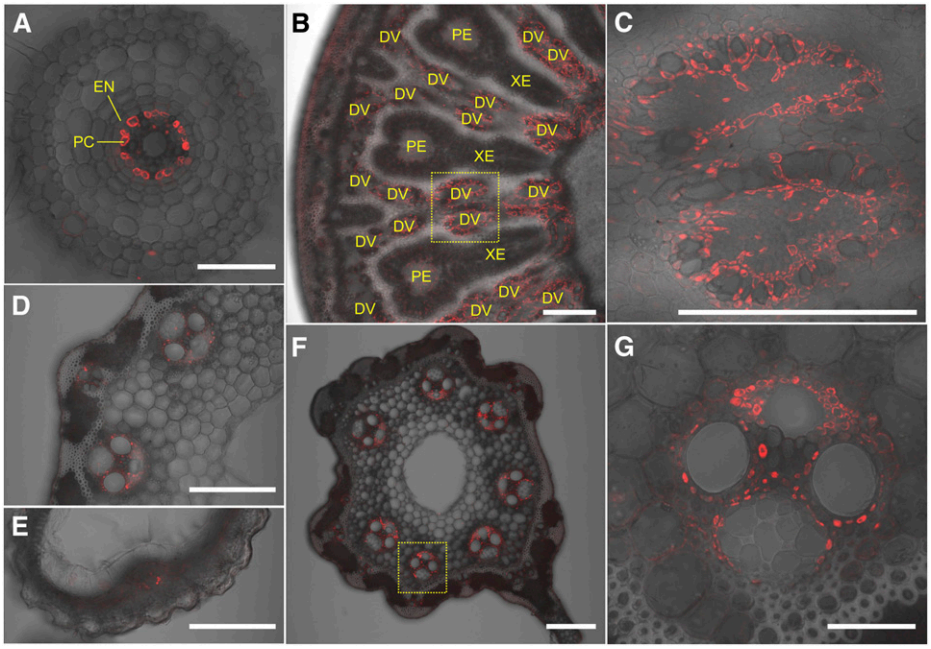

Figure 2. Tissue specificity of *OsHMA5* expression. Immunostaining of p*OsHMA5*-GFP transgenic rice with an anti-GFP antibody was performed in different organs, including root (A), node I (B and C), peduncle (D), husk (E), and rachis (F and G). Areas boxed with broken lines in B and F are magnified in C and G, respectively. Red color shows signal from GFP antibody detected with a secondary antibody. DV, Diffuse vascular bundles; EN, endodermis; PC, pericycle; PE and XE, phloem and xylem region of enlarged vascular bundles, respectively. Bars = 50 μm (A and G) and 200 μm (B–F).

The Knockout of *OsHMA5* Resulted in Decreased Root-Shoot Translocation of Cu

To further dissect the role of OsMHA5 in Cu transport, we exposed the seedlings to different concentrations of Cu ranging from 10 to 2,000 nM for 20 d. The total Cu uptake was similar between the wild-type rice and mutants at each Cu concentration (Fig. 6A); however, the Cu concentration was lower in the shoots and higher in the roots in two independent mutants than in the wild-type rice at each Cu concentration (Fig. 6, B and C), resulting in a lower root-shoot translocation of Cu in the mutants (Fig. 6D). These results indicate that OsHMA5 is involved in the translocation of Cu from the roots to the shoots.

To confirm this role of OsHMA5, we determined the concentration of Cu in the xylem sap. A time-course experiment showed that the Cu concentration in the xylem sap decreased in the mutants at 2 h and later after exposure to Cu compared with the wild-type rice (Fig. 7A). A dose-response experiment showed that the Cu concentration in the xylem sap increased with increasing external Cu concentrations in the wild-type rice (Fig. 7B). However, the Cu concentration in the

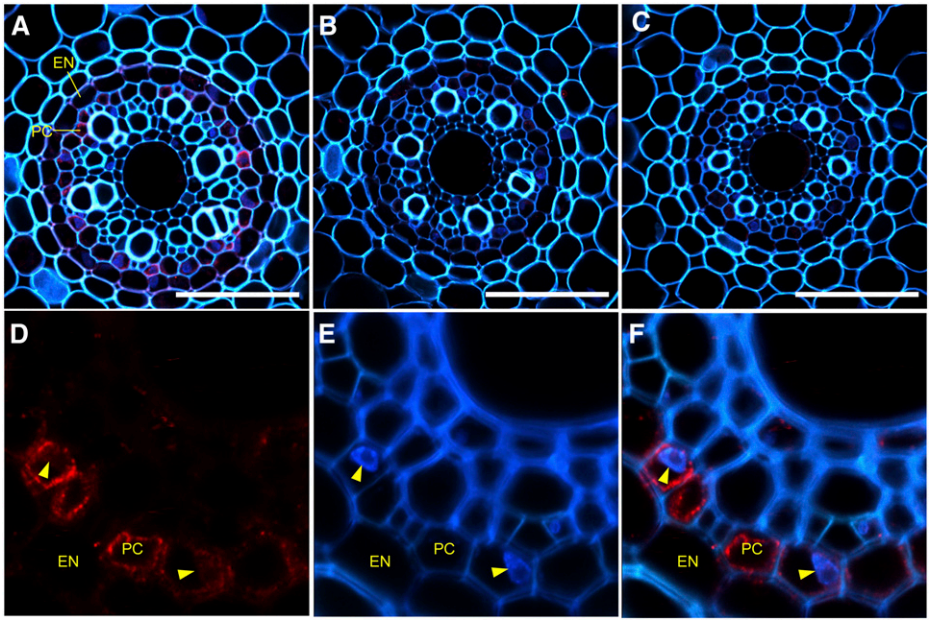

Figure 3. Localization of OsHMA5 in rice roots. Immunohistochemical staining of OsHMA5 with anti-OsHMA5 polyclonal antibody was performed in the roots of wild-type rice (A and D–F) and two knockout lines, NE6050 (B) and NF8524 (C). Magnified images of the stele in the wild type are shown in D to F. The signal of anti-OsHMA5 antibody (D; red), autofluorescence of cell wall and nuclei stained by DAPI (E; blue), and the merged image (F) are shown. The nucleus is indicated by yellow arrowheads. EN, Endodermis; PC, pericycle. Bars = 50 μm.

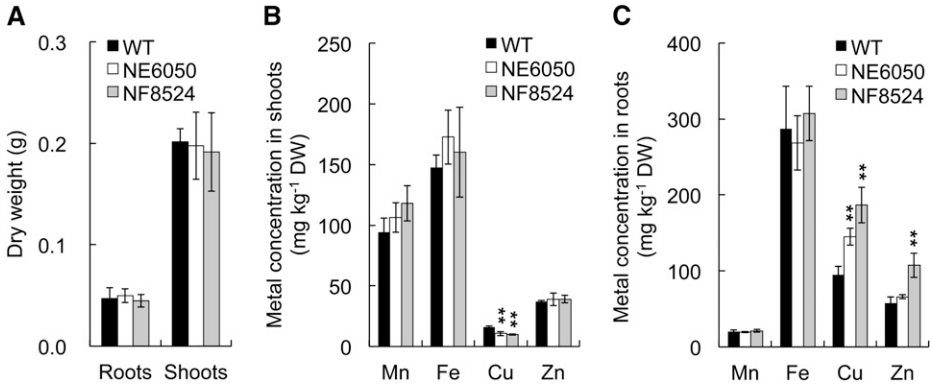

Figure 4. Growth and Cu concentrations of the *OsHMA5* mutants. A, Dry weight of roots and shoots. B and C, Metal concentrations in the shoots (B) and roots (C) of the *OsHMA5* knockout lines and wild-type rice (WT). Both wild-type rice and the knockout lines (NE6050 and NF8524) were cultivated in a nutrient solution containing 200 nM $CuSO_4$ for 16 d. Asterisks above the bars indicate significant differences (**$P < 0.01$) between the wild type and the knockout lines by Tukey's test. Error bars represent SD of three independent biological replicates. DW, Dry weight.

xylem sap was lower in the mutants than in the wild-type rice, especially at higher Cu concentrations (Fig. 7B).

Cu Tolerance in OsHMA5 Mutants

Since AtHMA5, a homolog of OsHMA5 in *A. thaliana*, is involved in Cu detoxification (Andrés-Colás et al., 2006; Kobayashi et al., 2008), we compared the Cu tolerance at different Cu concentrations between the wild-type rice and *OsHMA5* knockout lines by measuring root elongation (24 h). The root elongation was inhibited similarly by different Cu concentrations in wild-type rice and two mutants. At 1,000 nM Cu, the root elongation of all lines almost stopped (Fig. 8). There results indicate that knockout of *OsHMA5* did not affect the tolerance to Cu.

Transport Activity of OsHMA5 in Yeast

To test whether OsHMA5 has transport activity for Cu, we performed a complementation test by heterologously expressing *OsHMA5* in a yeast (*Saccharomyces cerevisiae*) mutant (Δ*ccc2*) that has been used in many studies (Kobayashi et al., 2008). Ccc2 is a Cu-transporting P-type ATPase that delivers Cu to the multicopper oxidase Fet3P, required for high-affinity Fe uptake at the plasma membrane (Yuan et al., 1995). Therefore, the Δ*ccc2* mutant is not able to grow under Fe-deficient conditions. Under Cu- or Fe-sufficient conditions, the Δ*ccc2* mutant cells expressing *OsHMA5* or not showed no difference in growth (Fig. 9). However, under Fe-limited conditions, Δ*ccc2* mutant cells expressing *OsHMA5* were able to restore growth (Fig. 9), but the vector control was not. The growth was also tested in the wild-type yeast strain (BY4741) as a positive control. The growth was similar between the yeast expressing *OsHMA5* or not under either Fe-limited or Fe-sufficient conditions (Fig. 9). These results provided direct evidence of Cu transport mediated by OsHMA5 protein in yeast.

DISCUSSION

P-type ATPases have been implicated in the transport of metals such as Zn, Cd, Pb, and Cu in different plant species (Williams and Mills, 2005; Grotz and

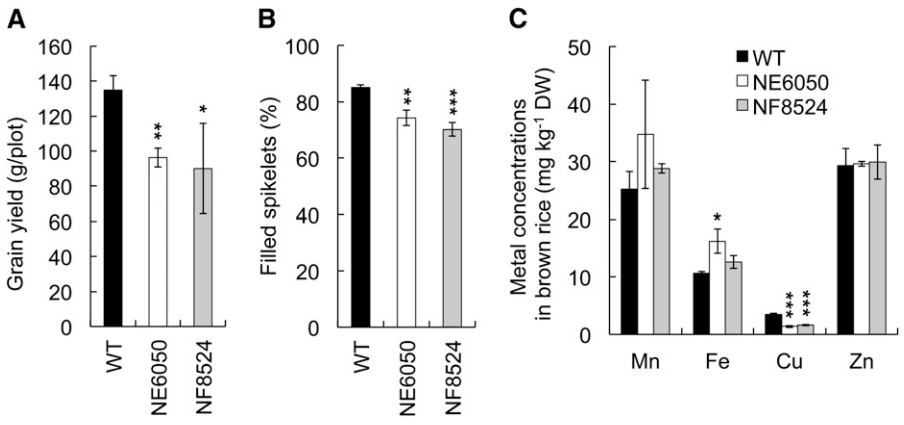

Figure 5. Grain yield and mineral analysis of the *OsHMA5* knockout lines. A, Grain yield. B, Fertility. C, Metal concentration of brown rice. The *OsHMA5* knockout lines (NE6050 and NF8524) and wild-type rice (WT) were grown in a paddy field. Asterisks above the bars indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) between the wild type and the knockout lines by Tukey's test. Error bars represent SD of three independent biological replicates. DW, Dry weight.

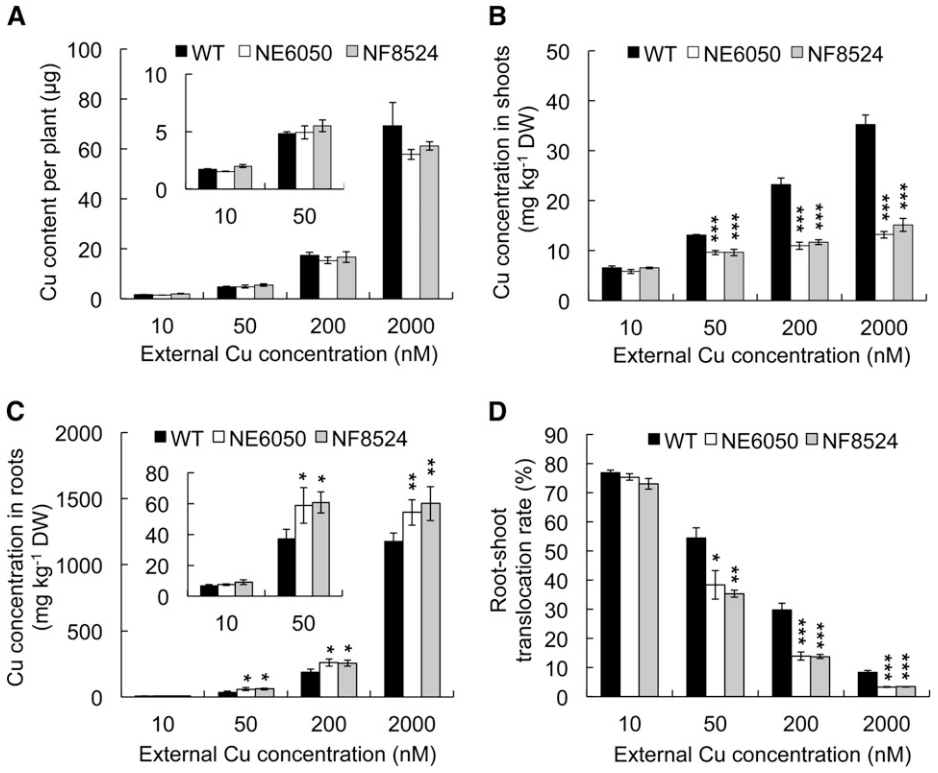

Figure 6. Uptake and translocation of Cu in *OsHMA5* knockout lines and wild-type rice. Both wild-type rice (WT) and the knockout lines (NE6050 and NF8524) were cultivated in a nutrient solution containing 10, 50, 200, or 2,000 nM $CuSO_4$ for 20 d. Total Cu uptake (A), Cu concentrations in the shoots (B) and roots (C), and the translocation rate of Cu from roots to shoots (D) are shown. Asterisks above the bars indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) between the wild type and the knockout lines by Tukey's test. Error bars represent SD of three independent biological replicates. DW, Dry weight.

Guerinot, 2006; Argüello et al., 2007; Burkhead et al., 2009). The functional characterization of OsHMA5 in this study showed that it transports Cu and is involved in the xylem loading of Cu at the both vegetative and reproductive growth stages (Figs. 4–7).

Xylem loading is an important step for the translocation of nutrients from the roots to the shoots. A number of transporters for xylem loading have been identified. For example, AtHMA2 and AtHMA4 in *A. thaliana* and OsHMA2 in rice are responsible for xylem loading of Zn as well as Cd (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009; Wong et al., 2009; Yamaji et al., 2013). PHO1 in *A. thaliana* is involved in the transfer of inorganic phosphate from roots to shoots (Poirier et al., 1991; Chiou and Lin, 2011). BOR1 was reported to be a boron transporter for xylem loading in *A. thaliana* (Takano et al., 2002). OsHMA5 was localized at the pericycle cell layer of root mature zones (Figs. 1–3), which is adjacent to the xylem vessel. Knockout of *OsHMA5* did not affect total Cu accumulation but resulted in decreased Cu concentration of the shoot and xylem sap (Figs. 4B, 6, A and B, and 7) and increased root Cu concentration (Figs. 4C and 6C). These findings indicate that xylem loading of Cu is mediated by OsHMA5 in rice roots.

At the reproductive stage, *OsHMA5* was also expressed in the xylem region of diffuse vascular bundles in node I, parenchyma cells of vascular tissues in peduncle, husk, and rachis (Fig. 3, B–G). It is likely that OsHMA5 also plays an important role in releasing Cu from these tissues into the xylem connected to the grains. This is supported by the finding that knockout of *OsHMA5* resulted in decreased Cu concentration in the grains (Fig. 5C). The grain yield was also decreased in the knockout lines (Fig. 5A), probably due to a deficiency of Cu. At the vegetative growth stage, although the Cu concentration in the shoots of the knockout lines was also decreased in the mutants (Fig. 4B), the growth of the mutants was not significantly affected (Fig. 4A). This is probably because the Cu requirement for plant growth is very low at the vegetative stage. In fact, the

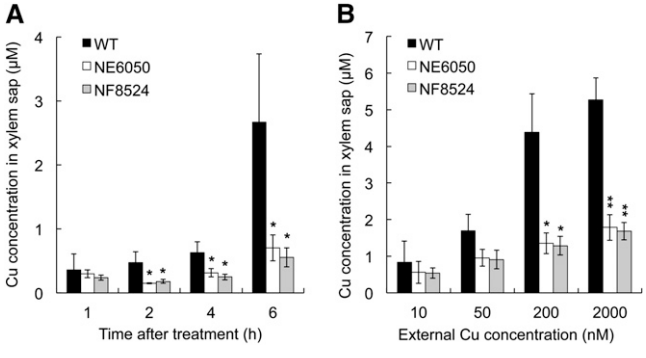

Figure 7. Characterization of Cu in xylem sap. A, Time-dependent change of Cu in the xylem sap. B, Dose response of Cu in the xylem sap. Xylem sap was collected from the wild-type rice (WT) and the knockout lines (NE6050 and NF8524) exposed to 200 nM Cu for different times (A) or to different Cu concentrations (B). Data are means of three biological replicates. Asterisks above the bars indicate significant differences (*$P < 0.05$ and **$P < 0.01$) between the wild-type rice and the knockout lines by Tukey's test.

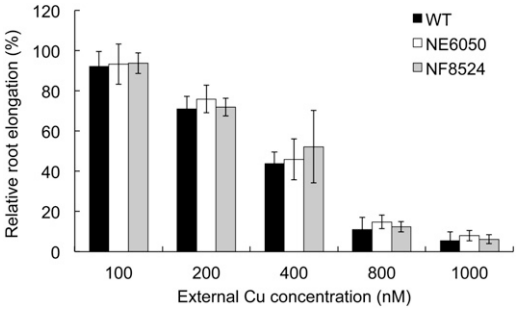

Figure 8. Cu tolerance in wild-type rice and *OsHMA5* knockout lines. Seedlings of the wild-type rice (WT) and two *OsHMA5* knockout lines (NE6050 and NF8524) were exposed to a solution containing different concentrations of Cu (0, 100, 200, 400, 800, and 1,000 nM) for 24 h. The root length was measured before and after the treatment. Data are means ± SD ($n = 12$). There was no significant difference at $P < 0.05$ by Tukey's test.

shoot Cu concentration in the mutant was higher than the deficiency level limit (5 mg kg^{-1}; Fig. 4B). At the reproductive stage, higher Cu may be required for pollen development and fertility. Furthermore, not only xylem loading of Cu from the roots, but also loading from other organs (node I, peduncle, husk, and rachis), seems to be necessary to meet the higher demand of Cu in the grains.

OsHMA5 belongs to the Cu^+/Ag^+ transporter group (Supplemental Fig. S2). This suggests that the chemical form of Cu for xylem loading is Cu^+ rather than Cu^{2+} or other forms. This is supported by a recent study showing that the majority of Cu in the roots is present as Cu^+ species (Ryan et al., 2013). Cu uptake in the roots is likely mediated by a member of COPPER TRANSPORTER (COPT), a homolog of the yeast Cu transporter CTR1 (Sancenón et al., 2003; Yuan et al., 2011). COPT is a transporter for Cu^+. The dominant form of Cu in the xylem sap is 2′-deoxymugineic acid-Cu, whereas Cu in the phloem sap is bound to several compounds, such as nicotianamine, His, and others, in rice (Ando et al., 2012). Recently, OsYSL16 was found to be a Cu-nicotianamine transporter, which is responsible for delivering Cu from the old tissues to the developing young tissues and seeds through phloem transport (Zheng et al., 2012). These findings suggest that plants use different types of transporters for uptake, translocation, and distribution of Cu with different forms.

The closest homolog of OsHMA5 in *A. thaliana*, AtHMA5, has been reported to be involved in Cu detoxification (Andrés-Colás et al., 2006; Kobayashi et al., 2008). *AtHMA5* was mainly expressed in roots and flowers, and its expression was greatly up-regulated by high Cu concentrations (Andrés-Colás et al., 2006). However, although the expression of *OsHMA5* was also induced by high Cu (Fig. 1B), the induction was very weak (only 2-fold). Knockout of *AtHMA5* or mutations in the conserved domains resulted in increased sensitivity to high Cu (Andrés-Colás et al., 2006; Kobayashi et al., 2008). However, since the tissue and cell specificity of the localization of AtHMA5 proteins are still unknown, it is difficulty to directly conclude whether AtHMA5 is also involved in the xylem loading of Cu like OsHMA5. Furthermore, the phenotype is also different between knockout lines of *AtHMA5* and *OsHMA5*. Knockout of *AtHMA5* resulted in increased Cu accumulation in both roots and shoots (Andrés-Colás et al., 2006), whereas knockout of *OsHMA5* resulted in increased Cu accumulation in the roots but decreased Cu accumulation in the shoots (Figs. 4 and 6). Knockout of *AtHMA5* greatly affects the tolerance to high Cu (Andrés-Colás et al., 2006), but the Cu-induced inhibition of root elongation at higher Cu concentrations was similar between the wild-type rice and *OsHMA5* mutants (Fig. 8). Therefore, the roles of AtHMA5 and OsHMA5 may differ; AtHMA5 is involved in Cu detoxification, whereas OsHMA5 is basically responsible for xylem loading of Cu, although further comparative work is required. Most Cu is retained in the rice roots, and a small part of the translocation of Cu into the shoots may not contribute to Cu tolerance.

Among rice P-type ATPases characterized, OsHMA2 transports Zn and Cd (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013), OsHMA3 is involved in Cd accumulation (Ueno et al., 2010; Miyadate et al., 2011), and OsHMA9 is responsible for the transport of Zn, Cu, Pb, and Cd (Lee et al., 2007). Knockout of *OsHMA5* only resulted in altered Cu concentration, but not other metals, including Zn, Fe, and Mn (Fig. 4). These facts suggest that OsHMA members have different transport substrate selectivity. It would be interesting to examine this metal specificity in the future. They also show different tissue, cellular, and subcellular specificity of localization. OsHMA2 and OsHMA5 are similarly localized to the plasma membrane of pericycle cells in the roots (Figs. 2 and 3; Yamaji

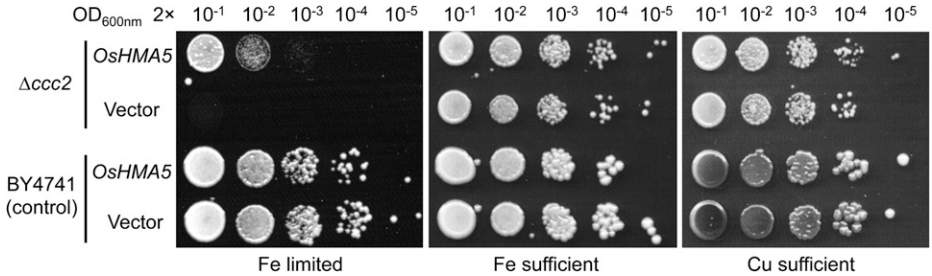

Figure 9. Transport activity of OsHMA5 in yeast. Yeast strains BY4741 and Δ*ccc2* transformed with empty vector pYES2 (Vector) or *OsHMA5* were cultivated on Fe-limited, Fe-sufficient, and Cu-sufficient plates as indicated. Eight microliters of cell suspension with an OD_{600nm} of 0.2 and four serial 1:10 dilutions were spotted and incubated at 30°C for 3 d.

et al., 2013), but OsHMA2 is mainly localized in the phloem region in node I (Yamaji et al., 2013), while OsHMA5 is localized in the xylem region. On the other hand, OsHMA3 is localized to the tonoplast of all root cells (Ueno et al., 2010), but OsHMA9 is localized to the plasma membrane in vascular bundles and anthers (Lee et al., 2007). The localization specificity of these HMA members may determine their physiological roles in metal transport in plants.

In conclusion, OsHMA5 is a transporter for xylem loading of Cu at both vegetative and reproductive stages of rice.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two independent *Tos17*-inserted mutants (NE6050 and NF8524) of rice (*Oryza sativa*) were obtained from the Rice Mutant Panel (http://tos.nias.affrc.go.jp/). The homozygous lines were isolated by PCR using *OsHMA5*-specific primers (5′-CTCCTCACATAATTACATGGCAAC-3′ and 5′-TCAACGGCCCAGTTTTGGGCT-3′) and a reverse primer of *Tos17* (5′-GGTGAAAAGGACAGTGGAGCA-3′).

Seeds of wild-type rice (cv Nipponbare) and the *Tos17* insertion homozygous mutants were germinated in tap water in the dark at 30°C for 2 d and then transferred to a net floating on a 0.5 mM $CaCl_2$ solution. The seedlings used in following experiments were grown in a glasshouse under natural light at 25°C to 30°C using one-half-strength Kimura B solution, containing 200 nM $CuSO_4 \cdot 5H_2O$, 0.09 mM KH_2PO_4, 0.27 mM $MgSO_4 \cdot 7H_2O$, 0.18 mM $(NH_4)_2SO_4$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2 \cdot 4H_2O$, 3 μM H_3BO_3, 0.5 μM $MnCl_2 \cdot 4H_2O$, 1 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4 μM $ZnSO_4 \cdot 7H_2O$, and 20 μM Fe(III)-EDTA. The pH of the nutrient solution was adjusted to 5.6 with 1 N NaOH, and the solution was renewed every 2 d. All experiments were performed with three biological replicates.

RNA Isolation and Full-Length Open Reading Frame Cloning

The total RNA was extracted from rice roots (cv Nipponbare) using an RNeasy plant mini kit (Qiagen), treated with DNase I (Invitrogen) and then converted to cDNA by using a SuperScript II kit (Invitrogen) following the manufacturer's instructions. The full-length open reading frame of *OsHMA5* was amplified by PCR using primers 5′-ATGGCGGCGAGCACTCGA-3′ and 5′-TCAACGGCCCAGTTTTGGGCT-3′, which were designed according to the annotated information of Os04g0556000 in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). The sequence of the amplified cDNA was confirmed by a sequence analyzer (ABI Prism 3100; Applied Biosystems).

Phylogenetic Analysis

For phylogenetic analysis, the HMA family proteins in *Arabidopsis thaliana* and rice were aligned with the ClustalX program (Thompson et al., 1997), and then maximum parsimony analysis was performed with MEGA software (http://www.megasoftware.net/) using the neighbor-joining method with 1,000 bootstrap trials (Tamura et al., 2007).

Phenotypic Analysis of the Knockout Lines

For phenotypic analysis, 14-d-old seedlings of both wild-type rice and knockout lines were exposed to various $CuSO_4 \cdot 5H_2O$ concentrations (0, 10, 50, 200, and 2,000 nM). After exposure for 20 d, the roots were washed with 0.5 mM $CaCl_2$ three times and harvested from the shoots separately. Xylem sap for dose response experiment was also collected at the same time (details were the same as below).

The wild-type rice and two knockout lines were also grown in a paddy field from June to October 2011. At harvest, grain yield per plot containing four plants each was recorded. The fertility was investigated by soaking the seeds in 8.5% (w/v) NaCl solution and calculated as follows: number of sinking seeds/total number of seeds × 100.

Determination of Metals in Plant Tissues and Xylem Sap

The shoots and roots harvested were dried at 70°C for 3 d and then subjected to digestion with concentrated HNO_3 at a temperature up to 140°C. The metal concentration in the digested solution was determined by inductively coupled plasma-mass spectrometry (7700X; Agilent Technologies) after dilution.

Xylem sap was collected from 20-d-old seedlings exposed to different Cu concentrations at different times. The shoots (2 cm above the roots) were excised with a razor, and then the xylem sap was collected with a micropipette for 1 h after decapitation of the shoot. The Cu concentration of the xylem sap was determined as described above after being diluted with 5% HNO_3.

Gene Expression Analysis

To examine the expression pattern of *OsHMA5* at the vegetative stage, the seedlings (cv Nipponbare) prepared above were exposed to a solution without Cu, Fe, Zn, or Mn (Zheng et al., 2012). After 1 week, roots, shoots, and basal regions (2 cm above the roots) were sampled and subjected to RNA extraction. For spatial expression analysis, different root segments (0–1 and 1–2 cm) of 4-d-old seedlings were sampled for RNA extraction. To investigate the dose response of *OsHMA5* expression to Cu, 6-d-old seedlings of wild-type rice grown in Cu-deprived medium were exposed to various Cu concentrations (0, 10, 50, 200, and 2,000 nM) for 1 d, and the whole roots were sampled for RNA extraction. To investigate the expression pattern of *OsHMA5* at different growth stages, different tissues from plants (cv Nipponbare) grown in a paddy field were taken as described by Yamaji et al. (2013).

The tissue-specific expression of *OsHMA5* was examined with laser microdissection according to the described methods (Fujii et al., 2012). The segments at 1.75 to 2.25 cm from the root tip of 4-d-old seedlings of cv Nipponbare were collected for the tissue sections. The central cylinder (pericycle and inner tissues) and outer tissues (cortex and epidermis) were separated using a Veritas Laser Microdissection System LCC1704 (Molecular Devices) and used for total RNA extraction.

The expression of *OsHMA5* was investigated by quantitative real-time reverse transcription (RT)-PCR (Mastercycler realplex4; Eppendorf) using the *HistoneH3* gene as the internal control. The primer sequences for RT-PCR were 5′-AAGGTGGAGAGTATAATGGTGAC-3′ and 5′-CCTTCCGGCCGACTGAAGTTC-3′ for *OsHMA5* and 5′-GGTCAACTTGTTGATTCCCCTCT-3′ and 5′-AACCGCAAAATCCAAAGAACG-3′ for *OsHistoneH3*. The expression data were normalized, and the relative expression was calculated by the ΔΔcycle threshold method. All the experiments were performed with three biological replicates.

Transgenic Rice Carrying *OsHMA5* Promoter-GFP

To investigate the tissue and cellular specificity of *OsHMA5* expression, we amplified the 2,152-bp region upstream of the initiation codon of *OsHMA5* by PCR from cv Nipponbare genomic DNA using primers 5′-GGTACCCTCGATCGGATTAGCTCCCATGT-3′ (*Kpn*I site underlined) and 5′-GGATCCTGGCAGCGTGTTCGCGTGACCCC-3′ (*Bam*HI site underlined). Using *Kpn*I and *Bam*HI, the amplified fragment was cloned into pPZP2H-lac carrying GFP and the terminator of the nopaline synthase gene (Fuse et al., 2001), producing the *OsHMA5* promoter-GFP construct. This construct was subsequently introduced into *Agrobacterium tumefaciens* (strain EHA101). Callus was induced from mature embryos of rice cv Nipponbare for *A. tumefaciens*-mediated transformation. Transformation was performed according to the protocol of Chen et al. (2003).

Immunohistological Staining

Transgenic lines carrying *OsHMA5* promoter-GFP were used for immunostaining. Different organs at different growth stages were sampled, and the immunostaining was carried out using an antibody against GFP (A11122; Molecular Probes) as described previously (Yamaji and Ma, 2007).

Immunostaining was also performed with an antibody against OsHMA5 in the wild-type rice and mutants. An antiserum was raised in rabbit against a synthetic peptide corresponding to residues 862 to 880 (CKPEQKAEKVKELQSAGRTV; the C at the N terminus was required for the peptide synthesis) of OsHMA5. The

antibody was purified through the peptide affinity column before use. 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.

Cu Tolerance Evaluation

To compare the Cu tolerance, 3-d-old seedlings were exposed to a 0.5 mM $CaCl_2$ solution (pH 5.6) containing 0, 100, 200, 400, 800, and 1,000 nM $CuSO_4 \cdot 5H_2O$ for 24 h. The root length of each seedling was measured with a ruler before and after the treatments, and relative root elongation (root elongation with Cu/root elongation without Cu × 100) was calculated. Ten replicates were made for each treatment.

Transport Activity of OsHMA5 in Yeast

The yeast (*Saccharomyces cerevisiae*) mutant Δ*ccc2* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ccc2::LEU2*), created from the wild-type strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), was used as the host stain in this study. Briefly, the sequence of *CCC2* (NM_001180578) in the genome of BY4741 was replaced by *LEU2* gene (NM_001178665) using a modified homologous recombination method (Brachmann et al., 1998).

To construct the yeast expression vector pYES2-OsHMA5, primers 5′-GGATCC-AAAAAAATGGCGGCGAGCACTCGAG-3′ and 5′-AGGGCCCTCAACGGCC-CAGTTTTGGGCT-3′ with *Bam*HI and *Psp*OMI restriction sites (underlined), respectively, were used to obtain the full-length cDNA of *OsHMA5* with additional 5′-ScKozak sequence. The amplified product was ligated into pYES2 vector (Invitrogen), which was first digested with *Bam*HI and *Not*I. After being sequenced for confirmation, the plasmid pYES2-OsHMA5 and empty pYES2 vector were introduced into BY4741 and Δ*ccc2* competent cells, respectively, according to the manufacturer's protocol (S.c. EasyComp Transformation Kit; Invitrogen).

The fresh yeast clone of control and pYES2-OsHMA5 was inoculated in 5 mL of conventional synthetic medium without uracil overnight at 30°C. The cells were collected and washed with sterile deionized water and then grown in 5 mL of conventional synthetic medium without uracil containing 2% (w/v) Gal for 24 h at 30°C. After being washed two times, the cells were resuspended in 5 mL of Fe-limited medium [0.17% (w/v) yeast nitrogen base without $CuSO_4$ and $FeCl_3$ (BIO 101 Systems), 0.2% (w/v) dropout mix without uracil, 2% (w/v) Gal, 1% (w/v) raffinose, 50 mM MES (pH 6.1), and 1 mM 3-(2-pyridyl)-5,6-bis (4-sulfophenyl)-1,2,4-triazine disodium salt] and incubated at 30°C for 18 h. The yeast cells were washed three times, and the optical density at 600 nm (OD_{600nm}) was adjusted to 0.2 with sterile distilled water. Ten-fold dilutions of these cell suspensions were made gradually (OD_{600nm} = 0.2, 0.02, 0.002, 0.0002, and 0.00002), and 8 μL of each was spotted on three different plates (Fe-limited, Fe-sufficient, and Cu-sufficient), respectively (Southron et al., 2004). The plates were incubated at 30°C for 3 d and photographed.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's test. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

The sequence of *OsHMA5* for this article can be found in the GenBank/EMBL databases under accession number AB840272.

Supplemental Data

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

Supplemental Figure S1. Structure of the *OsHMA5* gene and its predicated protein.

Supplemental Figure S2. Phylogenetic tree of rice and *A. thaliana* HMA proteins.

ACKNOWLEDGMENTS

We thank the Rice Genome Resource Center for providing *Tos17* insertion lines. We also thank Ikuko Yonamine for preparing the Δ*ccc2* mutant.

Received August 5, 2013; accepted September 22, 2013; published September 24, 2013.

LITERATURE CITED

Abdel-Ghany SE, Müller-Moulé P, Niyogi KK, Pilon M, Shikanai T (2005) Two P-type ATPases are required for copper delivery in *Arabidopsis thaliana* chloroplasts. Plant Cell **17:** 1233–1251

Ando Y, Nagata S, Yanagisawa S, Yoneyama T (2012) Copper in xylem and phloem saps from rice (*Oryza sativa*): the effect of moderate copper concentrations in the growth medium on the accumulation of five essential metals and a speciation analysis of copper-containing compounds. Funct Plant Biol **40:** 89–100

Andrés-Colás N, Sancenón V, Rodríguez-Navarro S, Mayo S, Thiele DJ, Ecker JR, Puig S, Peñarrubia L (2006) The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J **45:** 225–236

Argüello JM, Eren E, González-Guerrero M (2007) The structure and function of heavy metal transport P1B-ATPases. Biometals **20:** 233–248

Axelsen KB, Palmgren MG (2001) Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol **126:** 696–706

Becher M, Talke IN, Krall L, Krämer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J **37:** 251–268

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004) A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. FEBS Lett **569:** 140–148

Binder BM, Rodríguez FI, Bleecker AB (2010) The copper transporter RAN1 is essential for biogenesis of ethylene receptors in *Arabidopsis*. J Biol Chem **285:** 37263–37270

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115–132

Burkhead JL, Reynolds KA, Abdel-Ghany SE, Cohu CM, Pilon M (2009) Copper homeostasis. New Phytol **182:** 799–816

Chao DY, Silva A, Baxter I, Huang YS, Nordborg M, Danku J, Lahner B, Yakubova E, Salt DE (2012) Genome-wide association studies identify heavy metal ATPase3 as the primary determinant of natural variation in leaf cadmium in *Arabidopsis thaliana*. PLoS Genet **8:** e1002923

Chen S, Jin W, Wang M, Zhang F, Zhou J, Jia Q, Wu Y, Liu F, Wu P (2003) Distribution and characterization of over 1000 T-DNA tags in rice genome. Plant J **36:** 105–113

Chiang HC, Lo JC, Yeh KC (2006) Genes associated with heavy metal tolerance and accumulation in Zn/Cd hyperaccumulator *Arabidopsis halleri*: a genomic survey with cDNA microarray. Environ Sci Technol **40:** 6792–6798

Chiou TJ, Lin SI (2011) Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol **62:** 185–206

Craciun AR, Meyer CL, Chen J, Roosens N, De Groodt R, Hilson P, Verbruggen N (2012) Variation in HMA4 gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. J Exp Bot **63:** 4179–4189

Fujii M, Yokosho K, Yamaji N, Saisho D, Yamane M, Takahashi H, Sato K, Nakazono M, Ma JF (2012) Acquisition of aluminium tolerance by modification of a single gene in barley. Nat Commun **3:** 713

Fuse T, Sasaki T, Yano M (2001) Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotechnol **18:** 219–222

Grotz N, Guerinot ML (2006) Molecular aspects of Cu, Fe and Zn homeostasis in plants. Biochim Biophys Acta **1763:** 595–608

Hall JL, Williams LE (2003) Transition metal transporters in plants. J Exp Bot **54:** 2601–2613

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature **453:** 391–395

Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell **97:** 383–393

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. Plant Cell **16:** 1327–1339

Kim YY, Choi H, Segami S, Cho HT, Martinoia E, Maeshima M, Lee Y (2009) AtHMA1 contributes to the detoxification of excess Zn(II) in Arabidopsis. Plant J **58:** 737–753

Kobayashi Y, Kuroda K, Kimura K, Southron-Francis JL, Furuzawa A, Kimura K, Iuchi S, Kobayashi M, Taylor GJ, Koyama H (2008) Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in Arabidopsis. Plant Physiol **148:** 969–980

Krämer U, Talke IN, Hanikenne M (2007) Transition metal transport. FEBS Lett **581:** 2263–2272

Lee S, Kim YY, Lee Y, An G (2007) Rice P_{1B}-type heavy-metal ATPase, OsHMA9, is a metal efflux protein. Plant Physiol **145:** 831–842

Mikkelsen MD, Pedas P, Schiller M, Vincze E, Mills RF, Borg S, Møller A, Schjoerring JK, Williams LE, Baekgaard L, et al (2012) Barley HvHMA1 is a heavy metal pump involved in mobilizing organellar Zn and Cu and plays a role in metal loading into grains. PLoS ONE **7:** e49027

Mills RF, Peaston KA, Runions J, Williams LE (2012) HvHMA2, a P_{1B}-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. PLoS ONE **7:** e42640

Miyadate H, Adachi S, Hiraizumi A, Tezuka K, Nakazawa N, Kawamoto T, Katou K, Kodama I, Sakurai K, Takahashi H, et al (2011) OsHMA3, a P1B-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. New Phytol **189:** 190–199

Morel M, Crouzet J, Gravot A, Auroy P, Leonhardt N, Vavasseur A, Richaud P (2009) AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. Plant Physiol **149:** 894–904

Moreno I, Norambuena L, Maturana D, Toro M, Vergara C, Orellana A, Zurita-Silva A, Ordenes VR (2008) AtHMA1 is a thapsigargin-sensitive Ca^{2+}/heavy metal pump. J Biol Chem **283:** 9633–9641

Palmer CM, Guerinot ML (2009) Facing the challenges of Cu, Fe and Zn homeostasis in plants. Nat Chem Biol **5:** 333–340

Papoyan A, Kochian LV (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance: characterization of a novel heavy metal transporting ATPase. Plant Physiol **136:** 3814–3823

Poirier Y, Thoma S, Somerville C, Schiefelbein J (1991) Mutant of Arabidopsis deficient in xylem loading of phosphate. Plant Physiol **97:** 1087–1093

Ryan BM, Kirby JK, Degryse F, Harris H, McLaughlin MJ, Scheiderich K (2013) Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms. New Phytol **199:** 367–378

Sancenón V, Puig S, Mira H, Thiele DJ, Peñarrubia L (2003) Identification of a copper transporter family in *Arabidopsis thaliana*. Plant Mol Biol **51:** 577–587

Satoh-Nagasawa N, Mori M, Nakazawa N, Kawamoto T, Nagato Y, Sakurai K, Takahashi H, Watanabe A, Akagi H (2012) Mutations in rice (*Oryza sativa*) heavy metal ATPase 2 (OsHMA2) restrict the translocation of zinc and cadmium. Plant Cell Physiol **53:** 213–224

Seigneurin-Berny D, Gravot A, Auroy P, Mazard C, Kraut A, Finazzi G, Grunwald D, Rappaport F, Vavasseur A, Joyard J, et al (2006) HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem **281:** 2882–2892

Shikanai T, Müller-Moulé P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of *Arabidopsis*, functions in copper transport in chloroplasts. Plant Cell **15:** 1333–1346

Southron JL, Basu U, Taylor GJ (2004) Complementation of *Saccharomyces cerevisiae ccc2* mutant by a putative P_{1B}-ATPase from *Brassica napus* supports a copper-transporting function. FEBS Lett **566:** 218–222

Takahashi R, Ishimaru Y, Shimo H, Ogo Y, Senoura T, Nishizawa NK, Nakanishi H (2012) The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. Plant Cell Environ **35:** 1948–1957

Takano J, Noguchi K, Yasumori M, Kobayashi M, Gajdos Z, Miwa K, Hayashi H, Yoneyama T, Fujiwara T (2002) Arabidopsis boron transporter for xylem loading. Nature **420:** 337–340

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol **24:** 1596–1599

Tan J, Wang J, Chai T, Zhang Y, Feng S, Li Y, Zhao H, Liu H, Chai X (2013) Functional analyses of TaHMA2, a P(1B)-type ATPase in wheat. Plant Biotechnol J **11:** 420–431

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:** 4876–4882

Ueno D, Milner MJ, Yamaji N, Yokosho K, Koyama E, Clemencia Zambrano M, Kaskie M, Ebbs S, Kochian LV, Ma JF (2011) Elevated expression of *TcHMA3* plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. Plant J **66:** 852–862

Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF (2010) Gene limiting cadmium accumulation in rice. Proc Natl Acad Sci USA **107:** 16500–16505

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett **576:** 306–312

Williams LE, Mills RF (2005) P(1B)-ATPases: an ancient family of transition metal pumps with diverse functions in plants. Trends Plant Sci **10:** 491–502

Woeste KE, Kieber JJ (2000) A strong loss-of-function mutation in *RAN1* results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell **12:** 443–455

Wong CK, Cobbett CS (2009) HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. New Phytol **181:** 71–78

Wong CK, Jarvis RS, Sherson SM, Cobbett CS (2009) Functional analysis of the heavy metal binding domains of the Zn/Cd-transporting ATPase, HMA2, in *Arabidopsis thaliana*. New Phytol **181:** 79–88

Yamaji N, Ma JF (2007) Spatial distribution and temporal variation of the rice silicon transporter Lsi1. Plant Physiol **143:** 1306–1313

Yamaji N, Xia JX, Mitani-Ueno N, Yokosho K, Feng Ma J (2013) Preferential delivery of zinc to developing tissues in rice is mediated by P-type heavy metal ATPase OsHMA2. Plant Physiol **162:** 927–939

Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc Natl Acad Sci USA **92:** 2632–2636

Yuan M, Li X, Xiao J, Wang S (2011) Molecular and functional analyses of COPT/Ctr-type copper transporter-like gene family in rice. BMC Plant Biol **11:** 69

Zheng L, Yamaji N, Yokosho K, Ma JF (2012) YSL16 is a phloem-localized transporter of the copper-nicotianamine complex that is responsible for copper distribution in rice. Plant Cell **24:** 3767–3782