Mutations in Rice (Oryza sativa) Heavy Metal ATPase 2 (OsHMA2) Restrict the Translocation of Zinc and Cadmium

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(Received June 22, 2011; Accepted November 19, 2011)

Widespread soil contamination with heavy metals has fostered the need for plant breeders to develop new crops that do not accumulate heavy metals. Metal-transporting transmembrane proteins that transport heavy metals across the plant plasma membrane are key targets for developing these new crops. Oryza sativa heavy metal ATPase 3 (OsHMA3) is known to be a useful gene for limiting cadmium (Cd) accumulation in rice. OsHMA2 is a close homolog of OsHMA3, but the function of OsHMA2 is unknown. To gain insight into the function of OsHMA2, we analyzed three Tos17 insertion mutants. The translocation ratios of zinc (Zn) and Cd were clearly lower in all mutants than in the wild type, suggesting that OsHMA2 is a major transporter of Zn and Cd from roots to shoots. By comparing each allele in the OsHMA2 protein structure and measuring the Cd translocation ratio, we identified the C-terminal region as essential for Cd translocation into shoots. Two alleles were identified as good material for breeding rice that does not contain Cd in the grain but does contain some Zn, and that grows normally.

Keywords: Cadmium • Heavy metal ATPase • Oryza sativa • Rice • Translocation • Zinc.

Abbreviations: Ag, silver; CaMV, Cauliflower mosaic virus; Cd, cadmium; Co, cobalt; Cu, copper; EGFP, enhanced green fluorescent protein; Fe, iron; HMA, heavy metal ATPase; Mg, magnesium; Mn, manganese; Pb, lead; RT–PCR, reverse transcription–PCR; Zn, zinc.

Introduction

Contamination of soil with toxic heavy metals from mining or industrial, agricultural or military practices is a serious, worldwide public health issue (Nriagu and Pacyna 1988). Heavy metal contamination of soil reduces crop yields by interfering with plant growth and also threatens human health via accumulation of heavy metals in the food chain. Decontamination of areas by exchanging contaminated topsoil for non-contaminated soil is extremely costly. Methods to prevent plants from accumulating heavy metals, through supplying alkaline amendments to the soil rather than removing heavy metals from contaminated sites, should be feasible but are not yet established. To solve the problem of soil contamination with heavy metals, plant breeders and their collaborators have established goals to find plant hyperaccumulators of heavy metals that could be used for phytoremediation, as well as developing crop plants that do not accumulate heavy metals. To achieve these objectives, a thorough understanding of the genetic, molecular and physiological pathways of heavy metal uptake, translocation, detoxification and accumulation in plants is necessary.

Recent research has revealed that metal-transporting transmembrane proteins have crucial roles in the uptake and translocation of heavy metals (Colangelo and Guerinot 2006, Kraemer et al. 2007). Some groups of transporters act to transport heavy metals into the cytoplasm from intracellular compartments or from outside of the cell. Examples of such proteins include the natural resistance-associated macrophage protein (Thomine et al. 2000, Lanquar et al. 2005, Cailliatte et al. 2009, Cailliatte et al. 2010, Lanquar et al. 2010), the zinc-regulated transporter (ZRT), the IRT-related protein families (Grotz et al. 1998, Vert et al. 2002, Vert et al. 2009) and the Yellow Stripe 1-Like family (Curie et al. 2001).

Another group of transporters pumps the heavy metals across the plasma membrane or organellar membrane from the cytoplasm. This class of transporters in the P1B-ATPase family, known as heavy metal ATPases, includes the copper/silver (Cu/Ag) transporters and zinc/cadmium/cobalt/lead (Zn/Cd/Co/Pb) transporters. Cu/Ag transporters have been extensively characterized in Arabidopsis thaliana and include AtHMA5, AtHMA6/PAA1, AtHMA7/RAN1 and AtHMA8/
A mutant of AtHMA7 was originally characterized for its ethylene response phenotype in the presence of an ethylene receptor antagonist that has no phenotype in the wild type. From that work, AtHMA7 appears to deliver Cu and forms functional ethylene receptors (Hirayama et al. 1999, Woeste and Kieber 2000). Although information about the subcellular and tissue localization of AtHMA5, the most similar deduced protein to AtHMA7 among the Cu/Ag transporters, is not yet available, AtHMA5 is known to play a key role in Cu compartmentalization and detoxification in roots (Andrés-Colás et al. 2006). The functions of two other closely related proteins, AtHMA6 and AtHMA8, have been determined by mutant analyses; AtHMA6 functions in Cu transport through the plastid envelope, and AtHMA8 functions to transport Cu through the thylakoid membrane (Shikanai et al. 2003, Abdel-Ghany et al. 2005).

AtHMA1 was originally classified as a Zn/Cd/Co/Pb transporter, but is now also known to transport Cu. A detailed comparison of AtHMA1 with other members of the AthMA family classified this protein as a single member of a clade distinct from both the Zn/Cd/Co/Pb transporters and the Cu/Ag transporters (Seigneurin-Berny et al. 2006, Kim et al. 2009). Mutants in an appropriate background revealed that AtHMA1 is involved in Zn detoxification in the chloroplast under conditions of excess Zn (Kim et al. 2009). AtHMA2 and AtHMA4 are expressed in vascular tissues and localize to the plasma membrane (Hussain et al. 2004, Verret et al. 2004). Analyses using mutants, overexpressors and hyperaccumulators revealed that AtHMA2 and AtHMA4 are responsible for transporting Zn and Cd from roots to shoots (Eren and Arguello 2004, Hussain et al. 2004, Mills et al. 2005, Courbot et al. 2007, Hanikenne et al. 2008, Wong and Cobbett 2009). In contrast, AtHMA3 is localized in the vacuolar membrane and transports Cd, Zn, Co and Pb into the vacuoles (Gravot et al. 2004, Morel et al. 2009).

Rice (Oryza sativa), a major staple food crop for the world’s population, also contains several genes in the HMA family. Research on high-Cd-accumulating rice cultivars revealed that OsHMA3 acts at the vacuole membrane of root cells and has a crucial role in the transport of Cd into the vacuole (Ueno et al. 2009, Tezuka et al. 2010, Ueno et al. 2010, Miyadate et al. 2011). OsHMA2 is a protein with the highest similarity to OsHMA3, but little information is currently available about the function of this protein in rice (Nocito et al. 2011, Ueno et al. 2010). In this study, three allelic rice mutant lines generated by retrotransposon Tos17 insertions into the OsHMA2 gene were characterized. In contrast to OsHMA3, which limits Cd translocation from roots to shoots, OsHMA2 is essential for promoting translocation of Zn and Cd from rice roots to shoots.

Results

Retrotransposon insertion mutants of OsHMA2

To investigate the function of OsHMA2, three independent insertion lines of the retrotransposon Tos17 in the OsHMA2 gene were obtained from the Rice Genome Resource Center in Tsukuba, Japan (Miyao et al. 2003). OsHMA2 is composed of nine exons and eight introns (Fig. 1, Supplementary Fig. S1). Secondary structure prediction of membrane proteins (SOSUI; Hirokawa et al. 1998), an online bioinformatics tool, predicted that the OsHMA2 protein in the wild type has six transmembrane segments and a histidine-rich C-terminal domain (Fig. 2A, Supplementary Fig. S2). Among P1B-type ATPases known as Zn or Cd transporters and located in the...
same clade of the dendrogram (Williams and Mills 2005), AtHMA2 protein has the most similar structure to OsHMA2 (Supplementary Fig S3); the deduced protein sequences were 47.2% identical. The positions of the Tos17 insertion sites are shown in Fig. 1 and Supplementary Fig. S1. The Tos17 insertion into intron 5 was designated oshma2-1. In the other two lines, the insertions were in exon 9 at different positions. The accumulation of OsHMA2 transcripts in the mutant lines was determined by reverse transcription–PCR (RT–PCR) using the primers P99 and P101 (Fig. 1, Table 1). In spite of the insertion, RT–PCR products were obtained from total RNA in all mutants, similar to the wild type (Fig. 3). This indicates that the OsHMA2 gene was normally transcribed with Tos17 insertion and was accumulated. This prompted us to attempt to create unique OsHMA2 proteins in mutants. Thus, we analyzed the junctions of Tos17 and OsHMA2 by sequencing the cDNA to deduce the OsHMA2 proteins in the mutants.

In oshma2-1, the Tos17 insertion was in intron 5 (Supplementary Fig. S1A, B). In many cases, Tos17 in the intron is spliced out (http://www.nias.affrc.go.jp/); however, the oshma2-1 cDNA with the Tos17 insertion was not spliced out (Supplementary Fig. S1B). We cannot completely exclude the possible existence of cDNA of OsHMA2, but we have detected no evidence of Tos17-spliced out cDNA of OsHMA2 (Supplementary Fig. S4). The insertion of Tos17 into oshma2-2 and oshma2-3 was located 2,218 bp (oshma2-2) and 2,685 bp (oshma2-3) from the first ATG of the OsHMA2 cDNA (Supplementary Fig. S1A, C, D). All mutants had stop codons within the first 30 nucleotides that differ from the wild type (Supplementary Fig. S1B–D) and all aberrant versions of proteins of OsHMA2 are shorter than the normal OsHMA2 protein. The deduced OsHMA2 proteins from the full-length cDNA sequence of each allele are detailed in Supplementary Fig. S2. Only two transmembrane segments occur in oshma2-1.

**Table 1** Primer sequences

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**Fig. 2** Schematic representation of OsHMA2 proteins in the wild type and the three transposon insertion mutants based on SOSUI. Gray boxes show transmembrane segments and the gray dotted line in the C-terminal region of the wild type indicates a histidine-rich region. (A) Wild type. (B) Not only the C-terminal region but also four transmembrane segments are missing in oshma2-1. The remainder of the C-terminal end is also missing in oshma2-2. (D) Only half of the C-terminal region (170 amino acids) is missing in oshma2-3. Numbers under the letters in parentheses correspond to those in the other figures.
according to SOSUI prediction (Fig. 2B), while SOSUI predicted that all six transmembrane segments were still present in *oshma2-2* and *oshma2-3*. In *oshma2-2*, most of the C-terminal region after the last transmembrane segment is absent, and in *oshma2-3*, only part of the histidine-rich C-terminal region is absent (Fig. 2C, D).

**Mutant phenotype**

When grown in hydroponic culture, none of the *oshma2* mutants exhibited apparent phenotypes, in comparison with the wild type, although the whole plant dry weight of *oshma2-1* was reduced to 30% that of other mutants and the wild type. This reduction is mainly due to the reduced size of aerial parts of *oshma2-1* (Supplementary Fig. S5). *oshma2-2* plants and *oshma2-3* plants were fertile, whereas *oshma2-1* plants died before flowering.

**Heavy metal accumulation in *oshma2* mutants**

To confirm whether OsHMA2 functions in heavy metal uptake, translocation or accumulation, we grew wild-type and *oshma2* mutant plants under hydroponic conditions with 4.5 nM Cd. After 3 weeks, *oshma2* mutants had a significantly lower translocation ratio of Cd and Zn than the wild type, although no change occurred in the translocation ratio for other metals, including Cu, Fe, Mg and Mn (Fig. 4). The concentrations of Cd and Zn in shoots of the *oshma2* mutants were also very low (Fig. 5A, D). In contrast, the concentrations of Cd in the roots of *oshma2* mutants were not significantly different from those of the wild type (Fig. 5B), while the concentration of Zn in roots of *oshma2* mutants was higher than in the wild type (Fig. 5E). No significant differences were observed between *oshma2* mutants and the wild type in the concentration of accumulated Cd and Zn in whole plants (Fig. 5C, F). These results indicate that OsHMA2 is not responsible for the uptake of heavy metals but...
is involved in the translocation of Cd and Zn from roots to shoots. Based on the low translocation ratios for Cd and Zn, OsHMA2 seems to be a major Cd/Zn transporter in rice.

No significant differences were detected in the translocation ratio for Cd among the three oshma2 insertion mutants (Fig. 4A). Since oshma2-3, the insertion mutant lacking the histidine-rich C-terminal region (170 amino acids), had a translocation ratio for Cd similar to that of the two other mutants, the C-terminal 170 amino acids are essential for the translocation of Cd from roots to shoots by OsHMA2.

We examined the change in Cd translocation under different Cd concentrations in nutrient solution to gain more insight into the function of OsHMA2. Wild-type and oshma2-3 seedlings were grown for 3 weeks in the presence of 4.5 or 22.5 nM Cd, after which the Cd concentration in shoots and the translocation ratio of Cd were determined. When grown at the lower Cd concentration, the Cd concentration in oshma2-3 shoots was approximately 10 µg g DW⁻¹. When oshma2-3 was grown at the 5-fold higher Cd concentration, the Cd concentration in oshma2-3 shoots was 15 µg g DW⁻¹, i.e. 1.5-fold higher (Fig. 6A). In contrast, wild-type seedlings had a 3.5-fold difference in shoot Cd concentration when grown in the presence of 22.5 nM Cd compared with levels in seedlings grown in 4.5 nM Cd (Fig. 6A). The translocation ratio in oshma2-3 was negatively affected when the Cd concentration in the nutrient solution was raised 5-fold because the Cd concentration in oshma2-3 roots increased at high Cd levels to the same extent as in the wild type, whereas the increase in Cd in oshma2-3 shoots was smaller than in the wild type (Fig. 6B, C). Especially at high Cd levels, a phytochelatin–Cd complex might be synthesized in oshma2-3 roots in the same way as in the wild type. These results imply the existence of an absolute limit to Cd translocation in oshma2-3. This again confirms the importance of the C-terminal 170 amino acids for the translocation of Cd.

In terms of Zn translocation, significant differences were observed in the translocation ratio between the three retrotransposon mutants and the wild type (Fig. 4F). This implies that every domain that had been deleted from OsHMA2 in the mutants is essential. In fact, the C-terminal region after the transmembrane segment that is missing in oshma2-3 and oshma2-2, and the third to sixth transmembrane segments that are missing in oshma2-1 are all essential.

We analyzed the changes in Zn translocation under different Zn concentrations in nutrient solution, as described above for Cd. We set the concentration of Zn in nutrient solution 50-fold higher to supply excess Zn, which is an abnormal concentration in rice. In contrast to the results with an elevated Cd treatment, when the Zn content of the nutrient solution was elevated 50-fold, Zn in both wild-type and oshma2-2 shoots increased about 20-fold (Fig. 7A). Similarly, the translocation ratios of both the wild type and oshma2-2 were not affected by increasing the concentration of Zn in the nutrient solution (Fig. 7B). Hence, no significant difference was detected in the translocation of Zn under different concentrations of Zn in the nutrient solution. This suggests that other regions of OsHMA2,
in addition to the C-terminal region of OsHMA2 that is missing in oshma2-2, are sufficient for the response to elevated Zn in the nutrient solution. This is in stark contrast to the fact that the 170 amino acid C-terminal region of OsHMA2 was especially important for Cd translocation.

We next tested whether Cd and Zn translocation by OsHMA2 is competitive. We hypothesized that if translocation is competitive, the Zn or Cd translocation ratio in the wild type in the absence of other heavy metal ions would be higher than the ratio when both heavy metal ions are present in the nutrient solution. Conversely, in the oshma2 mutants, the absence of Cd or Zn should be smaller than in the wild type since OsHMA2 in the mutants does not function well. We found that not supplementing the media with one heavy metal ion did not significantly influence Zn or Cd translocation in the wild type or in the oshma2-2 mutant (Fig. 8A, B). This suggests that the concentration of Cd used (4.5 nM) does not interfere with Zn translocation via OsHMA2, and that no excess Cd is waiting to be transported when Zn is present in the nutrient solution.

**Tissue specificity of OsHMA2 expression**

The tissue specificity of OsHMA2 expression was evaluated with semi-quantitative RT–PCR (Fig. 9). OsHMA2 was expressed at low levels in leaves, shoot apices, roots and inflorescences, but a high expression level of OsHMA2 was seen in the basal and middle parts of roots.

We also investigated whether OsHMA2 is induced by Cd or Zn. Although Nocito et al. (2011) reported that OsHMA2 is induced by Cd, we observed no significant difference in OsHMA2 expression after Cd or Zn treatment (Supplementary Fig. S6).

**Subcellular localization of OsHMA2**

We analyzed transient expression and visualized the subcellular localization of OsHMA2 by bombarding the constructs into onion epidermal cells. We prepared fusion constructs
of normal OsHMA2 and the mutated version of OsHMA2 with EGFP (enhanced green fluorescent protein) under control of the Cauliflower mosaic virus (CaMV) 35S promoter (35S::OsHMA2::EGFP, 35S::oshma2-1::EGFP, 35S::oshma2-2::EGFP and 35S::oshma2-3::EGFP). Although the 35S::EGFP construct produced green fluorescence not only at the plasma membrane but also in the nuclei and cytosol, the 35S::OsHMA2::EGFP construct produced fluorescence only at the plasma membrane (Fig. 10A, B). As for mutant proteins, 35S::oshma2-1::EGFP showed the same pattern as 35S::EGFP, whereas 35S::oshma2-2::EGFP and 35S::oshma2-3::EGFP showed an identical pattern to 35S::OsHMA2::EGFP (Fig. 10C–E). This suggests that OsHMA2 is likely to be localized at the plasma membrane in rice and that the C-terminal region of OsHMA2 is not responsible for the correct subcellular localization of OsHMA2.

Heterologous assay in yeast

To confirm the function of OsHMA2, we performed a heterologous assay in yeast. The expression vector itself, vectors containing OsHMA2, and cDNA of mutants were used to transform the heterologous host Saccharomyces cerevisiae. A Zn-sensitive mutant, Δzrc1, was used for the assay of Zn tolerance, whereas a Cd-sensitive mutant, Δycf1, was used for the assay of Cd tolerance. Expression of OsHMA2 in Δzrc1 cells increased its Zn tolerance; however, expression of cDNA of oshma2-1 had no effect on Zn sensitivity (Fig. 11A). Expression of cDNA of oshma2-2 and oshma2-3 in Δzrc1 cells had the same effect on Zn sensitivity as OsHMA2 (Fig. 11A). As regards the effect of expression of cDNAs on Cd sensitivity in Δycf1 cells, the same trends were observed as for Zn sensitivity in Δzrc1 cells (Fig. 11B).
Discussion

OsHMA2 is crucial for translocation of Zn in rice

We analyzed three independently isolated mutants, and they all gave the same results but at different strengths. All mutants had a lower translocation ratio of Zn than the wild type. Especially in oshma2-1, ample Zn was present in the roots but most Zn accumulated by whole plants did not move into the shoots. This suggests that OsHMA2 is a major transporter of Zn in rice. In Arabidopsis, AthMA2 and AthMA4 are

Fig. 10 Subcellular localization of OsHMA2. Fluorescence of EGFP in onion epidermal cells expressing 35S:EGFP (A), 35S:OsHMA2:EGFP constructs (B), 35S:oshma2-1::EGFP constructs (C), 35S:oshma2-2::EGFP constructs (D) and 35S:oshma2-3::EGFP constructs (E). Bar: 100 μm.

Fig. 11 Functional assay of OsHMA2 of the wild type and mutants in yeast. Cell growth of the Δzrc1 mutant strain (A) and Δycf1 mutant strain (B) transformed with empty vector pYES2, OsHMA2 of the wild type (OsHMA2), oshma2-1, oshma2-2 and oshma2-3. The yeast cells were grown in the presence of 5 mM ZnSO4 (A) and 20 μM CdCl2 (B). The numbers at the top of the figure are the number of cells in 1 ml.
OsHMA2 mutants are blocked in Zn and Cd transport

OsHMA2 also functions as a transporter of Cd in rice

The decline in the translocation ratio of Cd in rice was not as great as in the wild type when plants were grown with excess Zn. These findings imply that regions of OsHMA2 other than the C-terminal region when it transports Zn and Cd in rice. In Arabidopsis, three genes, AtHMA3, AtHMA2, and AtHMA4, are known to transport Zn and Cd (Eren and Arguello 2004, Gravot et al. 2004, Hussain et al. 2004, Mills et al. 2005, Courbot et al. 2007, Hanikenne et al. 2008, Morel et al. 2009, Wong and Cobbett 2009). AtHMA2 and AtHMA4 are expressed predominantly in the vascular tissues of roots, leaves, and stems. The subcellular localization of AtHMA2 and AtHMA4 suggests that these proteins function to transport Zn across the plasma membrane of root vascular cells into the xylem (Hussain et al. 2004). The expression of AtHMA3 is reminiscent of that of AtHMA2 and AtHMA4; however, AtHMA3 is localized within the vacuole membrane and is thought to sequester excess Zn into the vacuole (Morel et al. 2009).

In this study, the tissue specificity of OsHMA2 was determined only by RT–PCR. However, the regions where strong expression of OsHMA2 was observed (i.e. the bottom and middle of roots) are also those with developed vascular tissue. In addition, we analyzed the subcellular localization of OsHMA2, determining that the protein is targeted to the plasma membrane, as seen in AtHMA2 and AtHMA4. Taken together with the phenotype of the oshma2 mutants, our results suggest that OsHMA2 is not the transporter for the sequestration of Zn into vacuoles for accumulation, but the transporter responsible for translocation of Zn from roots to shoots. A heterologous assay in yeast also confirmed that OsHMA2 functions as a Zn transporter. In rice, only one gene, OsHMA2, is responsible for translocation of Zn, unlike Arabidopsis, in which two genes have redundant functions (Hussain et al. 2004).

The C-terminal region of OsHMA2 is critical for translocation of Cd in rice

Most P1B-type ATPases have common structures characterized by several transmembrane segments, some of which organize transmembrane metal-binding sites, ATP-binding domains, an N-terminal metal-binding domain and a C-terminal metal-binding domain (Arguello et al. 2007). All of these structures seem to be essential and each domain has its own distinct importance in P1B-type ATPase function. In Arabidopsis, a deleted series of AtHMA2 gene constructs was introduced into an hma2,hma4 double mutant, showing that the C-terminal part of AtHMA2 does not control enzyme activity, whereas the N-terminal domain is essential in planta (Wong et al. 2009). On the other hand, experiments that complement yeast Cd- and Zn-hypersensitive mutants were performed to determine which domain of AtHMA4 is critical for enzyme activity, but the results were inconsistent. Mills et al. (2005) reported that the C-terminal region was not an absolute requirement for conferring Cd and Zn resistance in yeast, whereas Verret et al. (2005) observed that the C-terminal region (His11 stretch) of AtHMA4 was important to complement Cd and Zn hypersensitivity in yeast and functioned as a metal chelator and a regulator of enzyme activity. Experiments in planta yielded the final insight into the role of the C-terminal region of AtHMA4; Mills et al. (2010) showed that a truncated form of AtHMA4 could not complement the hma2,hma4 double mutant plants, suggesting that the C-terminal region of AtHMA4 has an important role in Cd and Zn transport in planta. Since Zn, an essential metal, often shares transporters with Cd, a toxic metal, separation of Zn and Cd transport functions is desirable (Kraemer 2009). Yet, until now, that the region of HMA genes critical for transporting Cd did not affect Zn transport was not clear.

In this study, we evaluated three OsHMA2 mutants in rice that had different degrees of protein structure alteration due to the insertion of Tos17. The comparison between the wild type and mutants for Zn translocation revealed that the C-terminal region of OsHMA2 is important. However, a significant difference was observed between the translocation ratio in each mutant, and the Zn concentration in oshma2-2 shoots increased greatly when plants were grown with excess Zn. These findings imply that regions of OsHMA2 other than the C-terminal region are also important for Zn translocation and that OsHMA2 can function to a limited extent without a C-terminal region when it transports Zn. However, the C-terminal 170 amino acids of OsHMA2 could be more critical for translocation of Cd than for translocation of Zn, since the difference in the translocation ratio between the wild type and oshma2-3 was large, a large difference did not exist among the mutants and the change in shoot Cd concentration in oshma2-3 was not as great as in the wild type when plants were grown at the higher Cd concentration. From investigation of the subcellular localization of mutant proteins, the C-terminal region of OsHMA2 is not essential for correct...
subcellular localization and seems to function via other pathways, e.g. metal binding, or the stability of the protein for translocation of Cd from root to shoot.

As for the heterologous assay, we showed that cDNA of oshma2-2 and oshma2-3 from rice had the same effect as OsHMA2 of the wild type. This suggests that the C-terminal region of OsHMA2 is not essential to complement the Cd-sensitive phenotype in yeast cells. However, here we showed the absolute requirement for the C-terminal region of OsHMA2 to translocate Cd in rice using three independent mutant alleles of OsHMA2.

Materials and Methods

Plant material

Three rice lines, NG0642, NF4888 and NF9856, carrying insertions of the Tos17 retrotransposon in the OsHMA2 gene, were kindly provided by Akio Miyao (Rice Genome Resource Center; http://www.nias.affrc.go.jp/; Hirochika et al. 1996). NG0642, NF4888 and NF9856 are referred to as oshima2-1, oshima2-2 and oshima2-3, respectively. We used cultivar Nipponbare as the wild type in this study. Seeds from the retrotransposon insertion mutants were planted in soil, heterozygous plants were grown and propagated seeds were harvested from individual plants. The methods used to investigate the genotype of each plant are described below.

The positions of the Tos17 insertions in OsHMA2 were confirmed by PCR. To detect segregation of the Tos17 insertion in OsHMA2, the Tos17 right border-specific primer P35 was used to detect all mutant alleles. The OsHMA2-specific primers P89, P93 and P951 were used for oshima2-1, oshima2-2 and oshima2-3, respectively. We used the Tos17 left-border-specific primer P144 with the OsHMA2-specific primers P90, P94 and P952 for oshima2-1, oshima2-2 and oshima2-3, respectively. To detect the corresponding PCR product from OsHMA2 to confirm no insertion, the primers P90 and P91, P93 and P94, and P951 and P952 were used for oshima2-1, oshima2-2 and oshima2-3, respectively (Table 1).

Hydroponic culture and Cd treatment

Rice seeds were surface sterilized with 1% sodium hypochlorite containing 0.1% surfactant, rinsed with water and then incubated for 24 h at 16 °C, followed by 24 h at 30 °C. Germinating seeds were transferred to Petri dishes containing wet filter paper. After culturing for 5 d in a growth chamber (14 h light at 26 °C, 10 h dark at 22 °C), each seedling was transferred to a plastic cup floating on nutrient solution (pH 5.5) containing 0.18 mM NH₄NO₃, 0.09 mM Na₂HPO₄, 0.14 mM K₂SO₄, 0.23 mM MgSO₄, 0.18 mM CaCl₂, 0.09 mM SiO₂, 22.5 μM Fe(III) EDTA, 0.78 μM CuSO₄, 0.54 μM ZnSO₄, 2.3 μM MnSO₄, 0.52 μM Na₂MoO₄ and 9.2 μM H₃BO₃. Initially, plants were grown in nutrient solution for 1 week or 1 month. After growth in normal culture, plants were moved to a nutrient solution also containing 4.5 mM Cd for 3 weeks. CdCl₂ was added every day and the nutrient solution was renewed every 2 d. In some experiments, the concentrations of Cd or Zn were increased in the nutrient solution, or Cd or Zn was completely omitted for 3 weeks.

Analysis of Cd concentration and translocation

At the end of the hydroponic culture period, the roots of the seedlings were washed in deionized water. The seedlings were divided into shoot and root tissues and dried at 105 °C for 24 h. The dried tissue samples were weighed, dried at 400 °C overnight and digested in 2 ml of 5 N HNO₃ overnight; then 8 ml of deionized water was added. The digest solutions were analyzed by inductively coupled plasma atomic emission spectrometry (Nippon Jarrell-Ash). The translocation ratio of metals from the root to the shoot was estimated as the percentage of the metals in the shoot compared with that in the whole plant.

Isolation of total RNA and RT–PCR

Total RNA was extracted from seedling roots prior to the addition of Cd to the hydroponic culture using TRIzol (Invitrogen) following the manufacturer’s protocol. First-strand cDNA was synthesized from 2 μg of total RNA using the SuperScript TM III First-Strand Synthesis System for RT-PCR (Invitrogen) in accordance with the supplier’s protocol. PCR was carried out with KOD plus (Toyobo) and the primers P99 and P101. As a standard, an actin transcript was amplified with TaKaRa Ex Taq (TaKaRa) with the primers P48 and P49 (Table 1). The PCR was carried out for 26, 27 and 28 cycles for OsHMA2 and 23, 24 and 25 cycles for actin. FLA3000 (FujiFilm) was used to measure the volume of bands to ensure that cDNAs were exponentially amplified. Each cycle consisted of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s polymerization at 72 °C.

Cloning and sequence analysis of OsHMA2

cDNAs for the wild type were amplified with KOD plus (Toyobo) and the primer sets P1046 and P96. cDNAs for mutants were also amplified with KOD plus (Toyobo) and the primer sets P1046 and P144, P35 and P102 (oshma2-1); P1046 and P102, P103 and P144 (oshma2-2); and P1046 and P102, P103 and P35 (oshma2-3). Each fragment was directly sequenced and assembled. That is, to recover the flanking sequence of Tos17 in the mutants, the Tos17 primer ANS144 was used for oshima2-1 and oshima2-2, and ANS35 was used for oshima2-3 to amplify the corresponding DNA fragments. The PCR products were sequenced and the positions of the insertions and stop codons determined for each mutant.

The protein sequences were translated from cDNA sequences. Transmembrane domains were predicted with the Internet program SOSUI engine, version 1.11.

Subcellular localization of OsHMA2

We cloned EGFP from pEFGP-N1 and pEFGP-N2 (Clontech) between the CaMV 35S promoter and the Nos terminator of
the pBI221 vector. The cDNA for OsHMA2 of the wild type was amplified from full-length cDNA fragments using KOD plus (Toyobo) and the primer sets OsHMA2F-Bgl and OsHMA2R-Kpn (Table 1). For amplification of the cDNAs of mutants, we used the primer sets P157 and P162 (oshma2-1), P157 and P161 (oshma2-2) and P157 and P160 (oshma2-3). The cDNA of the wild type was fused to the 5’ end of EGFP from pEGFP-N2. The cDNAs of mutants were fused to the 5’ end of EGFP from pEGFP-N1. The fusion constructs were introduced into onion epidermal cells by microprojectile bombardment with an IDERA II particle gun (Tanaka). GFP was visualized by fluorescence microscopy (BX5; Olympus).

Heterologous assay in yeast

The S. cerevisiae strain BY4743 (MATa/MATa his3Δ1/hi3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/+ +/lys2Δ0), a BY4743-derived strain with zrc1Δ1/zrc1Δ1 and another BY4743-derived strain with ycf1Δ1/ycf1Δ1 were used for heterologous assay of OsHMA2. OsHMA2 of the wild type and mutants was amplified with the primer sets P151 and P158 (oshma2-1), P151 and P158 (oshma2-2) and P151 and P159 (oshma2-3). The DNA fragments were digested and cloned into XbaI and BamHI sites downstream from the GAL1 (galactokinase gene) promoter in the yeast expression vector pYES2 (Invitrogen). Yeast cells were transformed with the vectors using the protocol described by Amberg et al. (2005).

The transformed yeast cells were grown overnight at 30°C in liquid SD medium (6.7 g l−1 yeast nitrogen base; Sigma) and 1.92 g l−1 yeast synthetic dropout (−ura) (Sigma) with 20 g (w/v) of glucose. The cultured cells were collected and washed three times with sterilized water. A 100 μl aliquot of the cell suspension was inoculated into 5 ml of SD medium supplemented with 2% galactose. The cultures were diluted to 107 cells ml−1 with the same medium and the cells were cultured for 16 h at 30°C. The cultures were sequentially diluted from 107 to 103 cells ml−1 with the same medium, and 4 μl of each suspension was spotted onto solidified SD medium with 2% galactose instead of 2% glucose and 5 mM ZnSO4 or 20 μM CdCl2. Plates were incubated at 30°C for 5 d.

Supplementary data

Supplementary data are available at PCP online.

Acknowledgments

The mutant lines described in this article were obtained from the Rice Genome Resource Center of the National Institute of Agrobioscience, Japan (http://www.nias.affrc.go.jp/). We thank Dr. Hiroki Rai for technical advice in the analysis of Cd concentration.

Funding

This work was supported by the Naito Foundation; the Ministry of Education, Culture, Sports, Science and Technology, Japan [grant number 21780007 to (N.S.-N.)].

References


