RESEARCH PAPER

Overexpression of OsHMA3 enhances Cd tolerance and expression of Zn transporter genes in rice

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Abstract

As a member of the heavy metal ATPase (HMA) family, OsHMA3 is a tonoplast-localized transporter for Cd in the roots of rice (Oryza sativa). Overexpression of OsHMA3 selectively reduces Cd accumulation in the grain. Further characterization in the present study revealed that overexpression of OsHMA3 also enhances the tolerance to toxic Cd. The growth of both the roots and shoots was similar in the absence of Cd between an OsHMA3-overexpressed line and vector control, but the Cd-inhibited growth was significantly alleviated in the OsHMA3-overexpressed line. The overexpressed line showed higher Cd concentration in the roots, but lower Cd concentration in the shoots compared with the wild-type rice and vector control line, indicating that overexpression of OsHMA3 enhanced vacuolar sequestration of Cd in the roots. The Zn concentration in the roots of the OsHMA3-overexpressed line was constantly higher than that of vector control, but the Zn concentration in the shoots was similar between the overexpressed line and vector control. Five transporter genes belonging to the ZIP family were constitutively up-regulated in the OsHMA3-overexpressed line. These results suggest that shoot Zn level was maintained by up-regulating these genes involved in the Zn uptake/translocation. Taken together, overexpression of OsHMA3 is an efficient way to reduce Cd accumulation in the grain and to enhance Cd tolerance in rice.

Key words: OsHMA3, Cd, overexpression, Oryza sativa, Zn transporter, tolerance.

Introduction

Rice (Oryza sativa) as a staple food is a major source of dietary intake of cadmium (Cd), which accounts for more than 40% of total Cd intake in Japan (Shimbo et al., 2001). Itai-itai disease, which is mainly due to kidney tubule damage, was caused in the past by taking rice with high Cd (Horiguchi et al., 1994). Therefore, control of Cd accumulation in rice grain is a very important issue of food safety, especially in areas with high Cd contamination.

Recently great progress has been made in understanding molecular mechanisms of Cd accumulation in rice (Clemens et al., 2013). The uptake of Cd in the roots is mediated by OsNramp5, a plasma-membrane-localized transporter belonging to the Nramp family (Sasaki et al., 2012). OsNramp5 is expressed in the roots through the whole growth period, and is polarly localized at the distal side of the exodermis and endodermis (Sasaki et al., 2012). Knockout of OsNramp5 resulted in decreased Cd uptake and Cd accumulation in the grain (Sasaki et al., 2012; Ishikawa et al., 2012). However, as OsNramp5 is also a major transporter responsible for Mn uptake, knockout of this gene caused reduced growth and yield owing to Mn deficiency (Sasaki et al., 2012), although this effect was not observed in another study (Ishikawa et al., 2012). In addition to OsNramp5, OsIRT1 and OsIRT2, belonging to the ZIP transporters, and OsNramp1 have also been proposed to contribute to Cd uptake, but their actual contribution is unknown (Clemens et al., 2013).
Cd taken up into the cells is sequestered into the vacuoles by OsHMA3, a member of HMA family (Ueno et al., 2010; Miyadate et al., 2011). OsHMA3 is localized to the tonoplast of all root cells (Ueno et al., 2010). Mutation of OsHMA3 through single amino acid substitution resulted in high Cd accumulation in the grain in some high Cd-accumulating rice cultivars such as Anjana Dhan and Jarjan (Ueno et al., 2010; Miyadate et al., 2011; Ueno et al., 2011). This allelic variation in OsHMA3 accounts for a major QTL for Cd accumulation detected on chromosome 7 (Ueno et al., 2009). On the other hand, overexpression of OsHMA3 reduced Cd accumulation in the grain, but did not affect the concentration of Fe and Zn (Ueno et al., 2010).

The root-to-shoot translocation of Cd was mediated by OsHMA2, a homologue of OsHMA3 (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013). However, differently from OsHMA3, OsHMA2 is localized at the plasma-membrane of root pericycle cells (Yamaji et al., 2013). Knockout of OsHMA2 resulted in marked reduction of Cd in the shoots and grain (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013), but also caused reduction of the growth and grain yield (Yamai et al., 2013). This is because OsHMA2 is also a Zn transporter, which is responsible for preferential distribution of Zn to the developing tissues. At the vegetative and reproductive growth stages, OsHMA2 is also localized at the phloem region of the nodes, which is involved in the inter-vascular transfer of Zn and Cd (Yamaji et al., 2013). Another transporter, OsLCT1, also mediates xylem-to-phloem transfer of Cd (but not Zn) in node I of rice (Uraguchi et al., 2011). OsLCT1 was expressed in both enlarged vascular bundles and diffuse vascular bundles of the node. Knockdown of OsLCT1 also resulted in decreased Cd in the phloem sap and Cd accumulation in the grain (Uraguchi et al., 2011).

Identification of transporters involved in Cd accumulation as described above indicates that Cd is transported through transporters for essential metals such as Zn and Mn. Therefore, manipulation of these transporters will also affect the uptake and distribution of these essential metals, causing growth inhibition. However, overexpression of OsHMA3 only affected Cd accumulation in the grain, but not Fe and Zn (Ueno et al., 2010). To understand the underlying mechanisms, in the present study we further characterized an OsHMA3-overexpressed line. We found that overexpression of OsHMA3 enhanced the tolerance to Cd toxicity. Furthermore, we found that although OsHMA3 is also responsible for vacuolar sequestration of Zn, the Zn level in the shoot of OsHMA3-overexpressed line is maintained by up-regulating five ZIP genes implicated in Zn uptake and translocation.

![Image](https://example.com/image.png)

**Fig. 1.** Effect of overexpression of OsHMA3 on Cd tolerance in rice. (A) Phenotype of OsHMA3 overexpressed line (OX), vector control line (VC), and non-transgenic wild-type rice (WT, cv. Nipponbare). (B) Root dry weight of the three lines. (C) Shoot dry weight of the three lines. All lines were cultivated in one-half strength Kimura B solution containing 0, 100, and 1000 nM Cd for 22 d. Data are means±SD of three biological replicates. Statistical comparison was performed by one-way ANOVA followed by the Tukey’s test. All data were compared with the wild type, vector control, and overexpression line in each treatment (*P<0.05 and **P<0.01).
Materials and methods

Plant materials and growth conditions

Seeds of wild-type rice (WT, cv. Nipponbare), an empty vector line and an OsHMA3 overexpressed (OX) line prepared before (Ueno et al., 2010), were germinated in the dark at 30 °C for 2 d. The germinated seeds were transferred on a net floating on a 0.5 mM CaCl₂ solution in a 1.5 L plastic container. The solution was changed once every 2 d. After growth for 7–10 d at 25 °C, seedlings with similar size were transferred into a 3-L pot containing half-strength Kimura B solution (Zheng et al., 2012). The nutrient solution was changed once every 2 d. The plants were grown in a closed green house with natural light at 25 °C. All experiments were repeated at least once with 3–4 biological replicates for each.

Evaluation of Cd tolerance

Seedlings (24-d-old) were exposed to one-half strength Kimura B solution containing 0, 100, and 1000 nM Cd in a 1.2-L pot. The solution was renewed every 2 d. After 22 d, the roots were washed with 5 mM CaCl₂ for three times and separated from the shoots. The samples were dried in an oven at 70 °C for at least 3 d. After the dry weight of the roots and shoots was recorded, the samples were subjected to digest for mineral analysis as described below. Three biological replicates (one plant for each) were made for each treatment.

Kinetic study of Cd and Zn uptake

To investigate time-dependent uptake of Cd, the seedlings (38-day-old) were exposed to a one-half-strength Kimura B solution containing 500 nM Cd in a 1.2-L pot. The roots were sampled at 1, 3, 6, 12, and 24 h after the exposure with four biological replicates (one plant for each). For investigation of Zn uptake, seedlings (27-day-old) were exposed to a half-strength Kimura B solution containing 500 nM ZnCl₂ with four biological replicates (one plant for each). The ZnCl₂ stable isotope was purchased from Taiyo Nippon Sanso Corporation (Tokyo, Japan). At 0.5, 1, 3, 6, 12, and 24 h, the roots were sampled.

Fig. 2. Concentration of Cd and Zn in the roots and shoots of the OsHMA3 overexpressed line. An OsHMA3 overexpressed line (OX), vector control line (VC), and non-transgenic wild-type rice (WT, cv. Nipponbare) were grown in one-half strength Kimura B solution containing 0, 100, and 1000 nM Cd for 22 d. The concentration of Cd (A, B) and Zn (C, D) in the roots (A, C) and shoots (B, D) was determined with ICP-MS. Data are means ± SD of three biological replicates. Statistical comparison was performed by one-way ANOVA followed by the Tukey’s test. All data were compared with the wild type, vector control and overexpression line in each treatment (*P < 0.05 and **P < 0.01).
Root cell sap extraction, sample digest, and mineral determination

The frozen samples were placed in ultra free-MC centrifugal filter units (0.2 µm, Millipore) at room temperature. After thawing for a short-time, the tubes were centrifuged at 20 400 g for 10 min to obtain the root cell sap.

The dried root and shoot samples were digested with HNO₃ as described before (Zheng et al., 2012).

The concentration of Zn, Fe, Mn, Cu, Cd, Ni, Pb, and Co in the cell sap and digest solution was determined by ICP-MS (Agilent 7700). The concentration of ⁶⁷Zn was determined with an isotope mode.

Expression analysis of ZIP genes

To compare expression of genes related to Zn transport, samples of both OsHMA3-overexpressed line and vector control were taken from the roots of 28-day-old seedlings exposed to 0 or 200 nM Cd for 24 h with three biological replicates. Total RNA was extracted by using an RNeasy Plant Mini Kit (Qiagen), which was converted to cDNA followed by DNase I treatment using the protocol supplied by the manufacturers of SuperScript II (Invitrogen). The cDNAs were amplified by SsoFast EvaGreen Supermix (Bio-Rad). The expression of ten ZIP genes was determined by quantitative real-time PCR using the primers listed in Supplementary Table S1 on CFX384 (Bio-Rad). The expression data were normalized by Histone H3 and Actin as internal standards, and relative expression was calculated by the comparative cycle threshold method using CFX Manager software (Bio-Rad).

Results

Overexpression of OsHMA3 enhanced Cd tolerance

Two independent OsHMA3-overexpressed lines were used to evaluate Cd accumulation in brown rice (Ueno et al., 2010). Both lines showed similar expression level of OsHMA3 and phenotype (Cd accumulation). In the present study, one line was selected to further investigate the effect of overexpression of OsHMA3 on Cd tolerance and other traits.

![Graphs showing Cd accumulation in roots and shoots of WT, VC, and OX lines with various Cd concentrations](https://academic.oup.com/jxb/article-abstract/65/20/6013/2485383)

**Fig. 3.** Concentration of Cu, Fe, and Mn in the roots and shoots. An OsHMA3 overexpressed line (OX), vector control line (VC), and non-transgenic wild-type rice (WT, cv. Nipponbare) were grown in one-half strength Kimura B solution containing 0, 100, and 1000 nM Cd for 22 d. The concentration of Cu (A, B), Fe (C, D), and Mn (E, F) in the roots (A, C, E) and shoots (B, D, F) was determined with ICP-MS. Data are means±SD of three biological replicates. Statistical comparison was performed by one-way ANOVA followed by the Tukey’s test. All data were compared with the wild type, vector control and overexpression line in each treatment (*P<0.05 and **P<0.01).
In the absence of Cd, similar growth was observed among wild-type rice (WT), vector control (VC), and the overexpressed line (OX) (Fig. 1A–C). At 100nM Cd, although the growth of the shoots of OX was slightly better than that of WT and VC, there was no significant difference among the three lines. However, at 1000nM Cd, the leaves of WT and VC showed severe chlorosis (Fig. 1A) and the dry weight of the shoots and roots were lower than OX (Fig. 1B, C). There was no difference in the growth between WT and VC at either Cd concentration, indicating that transformation did not affect the growth itself.

Mineral analysis showed that the Cd concentration in the roots was much higher in the OX than in the WT and VC at both lower and higher Cd concentrations (Fig. 2A). However, the Cd concentration in the shoots was significantly lower in the OX than in the WT and VC (Fig. 2B). Surprisingly, the Zn concentration in the roots of OX was always much higher than that of WT and VC (Fig. 2C), but the Zn concentration in the shoots was similar among the three lines under the same treatment, although high Cd (1000nM Cd) slightly decreased Zn concentration in all lines (Fig. 2D). There was no significant difference in the concentration of Cu, Fe, and Mn in the shoots and roots among the three lines in the absence of Cd (Fig. 3). However, in the presence of Cd, the concentration of Cu and Mn of both the roots and shoots was lower in the WT and VC than in the OX (Fig. 3A, B, E, F), but there was no difference in the Fe concentration of the roots and shoots among the three lines (Fig. 3C, D).

**Kinetics of Cd and Zn uptake**

To further investigate the effect of OsHMA3 overexpression on the Cd uptake, a time-dependent change of Cd concentration in the root cell sap was monitored. Root cell sap mainly contains vacuolar sap; therefore, it could reflect Cd/Zn transport into the vacuoles indirectly. Up to 12 h after exposure to 500nM Cd, there was no difference in the Cd concentration of the root cell sap among two lines (Fig. 4A). However, at 24 h after Cd exposure, the Cd concentration in the cell sap was significantly higher in the OX than in the WT (Fig. 4A).

As Zn concentration was much higher in the roots of the OsHMA3-overexpressed line (Fig. 2C), the Zn concentration in the root cell sap was also determined. The Zn concentration in the root cell sap was always higher (2.1–4.0 times) in the OX than in the WT (Fig. 4B).

To examine how fast Zn newly taken up is accumulated in the roots, a labelling experiment with $^{67}$Zn stable isotope was performed. Compared with the WT, the OX showed a higher $^{67}$Zn accumulation from 6 h after the exposure to $^{67}$Zn (Fig. 5). At 24 h, OX accumulated more than three times $^{67}$Zn more in the roots than the WT.

**Uptake of other divalent metals**

To further examine whether overexpression of OsHMA3 also affects the uptake of other divalent metals, we exposed both WT and OX to Pb, Ni, and Co for 24 h. The shoot Ni concentration was slightly lower in OX than in WT (Fig. 6A–C), but there was no difference in the concentration of Pb and Co between OX and WT. In the roots, the concentration of Ni and Pb was significantly higher in the OX than in the WT, and the concentration of Pb was higher in the OX than in the WT.

Fig. 4. Time-dependent change of Cd and Zn in the root cell sap. Seedlings of an OsHMA3 overexpressed line (OX) and non-transgenic wild-type rice (WT, cv. Nipponbare) were exposed to 500nM Cd for different times. Concentration of Cd (A) and Zn (B) in the root cell sap was determined with ICP-MS. Data are means±SD of four biological replicates. Asterisks indicate significant difference from WT at *P<0.05 and **P<0.01 by Student’s t-test.

Fig. 5. Time-dependent accumulation of $^{67}$Zn in the roots. Seedlings of an OsHMA3 overexpressed line (OX) and non-transgenic wild-type rice (WT, cv. Nipponbare) were exposed to 500nM $^{67}$Zn for different times. The concentration of newly accumulated Zn ($\Delta$Zn) was calculated by $^{65}$Zn/$^{67}$Zn ratio determined with isotope mode of ICP-MS. Data are means±SD of four biological replicates. Asterisks indicate significant difference from WT at **P<0.01 by Student’s t-test.
slightly higher in the OX than in the WT, but no difference in the Co concentration was found between the two lines (Fig. 6D–F).

**Expression of ZIP genes in the OsHMA3-overexpressed line**

To understand the mechanisms responsible for the increased Zn in the roots, but unchanged Zn in the shoots of OX, we compared the expression level of ten genes belonging to the ZIP family between the vector control and the overexpressed line. Among them, the expression level of OsZIP4, OsZIP5, OsZIP8, OsZIP9, and OsZIP10 was significantly higher in the overexpressed line than in the vector control irrespectively of Cd treatment (Fig. 7), whereas there was no significant difference in other ZIP gene expression.

**Discussion**

**Overexpression of OsHMA3 enhanced the Cd tolerance in rice**

OsHMA3 is mainly expressed in the roots at low level and the expression is not induced by Cd in wild-type rice (Ueno et al., 2010). Overexpression of OsHMA3 under control of the maize ubiquitin1 promoter resulted in reduced accumulation of Cd in the shoots of brown rice, but did not alter the concentration of iron and zinc (Ueno et al., 2010). In the present study, we found that overexpression of OsHMA3 also enhanced the tolerance to toxic Cd (Fig. 1). This effect could be attributed to the decreased Cd concentration in the shoots of OX (Fig. 2). Cd displaces essential metals such as Zn, Fe, and Ca from a number of metalloproteins owing to their chemical similarity (Verbruggen et al., 2009). Cd also binds to sulfhydryl residues of constituent proteins or enzymes because of its high affinity for sulfur, resulting in the dysfunction of these proteins. Therefore, high Cd concentration in the shoots causes growth inhibition. When exposed to 1000 nM Cd, the shoot Cd concentration in the OX was 20 mg kg⁻¹, whereas that in the WT was higher than 100 mg kg⁻¹ (Fig. 2B).

The decreased Cd concentration in the shoots of OX is caused by enhanced sequestration of Cd into the vacuoles in the roots. OsHMA3 is a tonoplast-localized transporter for Cd (Ueno et al., 2010). Loss of function of OsHMA3 results in low accumulation of Cd in the roots, but high accumulation in the shoots (Ueno et al., 2010). By contrast, overexpression of functional OsHMA3 enhances vacuolar sequestration of Cd in the roots, resulting in accumulation of Cd (Fig. 2B). This enhanced vacuolar sequestration also increases tolerance of the roots to Cd (Fig. 1B).

**Zinc homeostasis is maintained by up-regulating ZIP genes in the OsHMA3-overexpressed line**

Overexpression of OsHMA3 did not affect the Zn concentration in the shoots and brown rice (Fig. 2D, Ueno et al., 2010).
but increased Zn concentration in the roots (Fig. 2C). Similarly to Cd, the Zn concentration in the root cell sap was always higher in OX than in the WT (Fig. 4). Furthermore, a labelling experiment with $^{65}$Zn showed that Zn was accumulated in the roots as fast as Cd in the overexpression line (Fig. 5). These results indicate that OsHMA3 is also able to transport Zn into the vacuoles in addition to Cd when it is overexpressed. OsHMA3 is usually expressed at a low level in natural rice cultivars. Therefore, loss of function of OsHMA3 did not affect the Zn concentration much in the roots (Lee et al., 2010b). These findings suggest that translocation of Zn to the shoots is compensated by up-regulating these ZIP genes, which are involved in Zn uptake/translocation/distribution in the OsHMA3-overexpressed line.

If OsHMA3 is also responsible for sequestration of Zn into the vacuoles in the roots of an OsHMA3-overexpressed line, the question arises how the overexpression line is maintained at a similar Zn level to that of WT in the shoots (Fig. 2D). This is different from Cd because overexpression of OsHMA3 decreased Cd concentration in the shoots although both Cd and Zn were increased in the roots of the overexpression line (Fig. 2A, B). Zn as an essential element is required for many biological processes (Krämer et al., 2007). Therefore the homeostasis of Zn is strictly regulated at different steps including uptake, translocation, and distribution (Olsen and Palmgren, 2014). Many transporters belonging to ZIP (ZRT-IRT-like protein), MTP (metal tolerance protein) and HMA (heavy metal ATPase) families have been proposed to be involved in Zn transport (Olsen and Palmgren, 2014). Expression analysis revealed that five genes (OsZIP4, OsZIP5, OsZIP8, OsZIP9, and OsZIP10) belonging to the ZIP family were constantly up-regulated in the overexpression line in both the absence and presence of Cd (Fig. 7). Although the exact roles of these ZIP genes are unknown, OsZIP4 and OsZIP8 have been proposed to be involved in Zn uptake/translocation (Ishimaru et al., 2005; Lee et al., 2010a; Bashir et al., 2012) and OsZIP5 was implicated in Zn distribution (Lee et al., 2010b). These findings suggest that translocation of Zn to the shoots is compensated by up-regulating these ZIP genes, which are involved in Zn uptake/translocation/distribution in the OsHMA3-overexpressed line.
A homologue of OsHMA3 in *Arabidopsis*, AtHMA3 shows transport activity for Pb and Cd when expressed in a yeast mutant (Gravot et al., 2004). Ectopic overexpression of AtHMA3 improved plant tolerance to Cd, Co, Pb, and Zn (Morel et al., 2009). However, different from OsHMA3, overexpression of AtHMA3 resulted in increased accumulation of Cd in *Arabidopsis* shoot (Morel et al., 2009). On the other hand, AhHMA3 identified from a Zn-hyperaccumulating plant, *Arabidopsis halleri*, shows transport activity for Zn (Becher et al., 2004). We found that OsHMA3 is able to transport Cd and Zn in its overexpressed line (Figs 4 and 5), although the transport activity for Zn and Co was not detected when expressed in yeast (Ueno et al., 2010). Overexpression of OsHMA3 only gave slight effect on uptake of Pb and Co (Fig. 6). The concentration of Ni in the overexpression line of OsHMA3 was also increased in the roots, but decreased in the shoots (Fig. 6). This trend is similar to that of Cd although more changes was observed for Cd (Fig. 2). There is a possibility that OsHMA3 also transports Ni, although the affinity may be lower compared with Cd. The mechanism underlying the different transport subtype specificity and affinity of HMA3 from different plant species remains to be examined in future.

The concentration of Fe in both the roots and shoots was unaffected by overexpression of OsHMA3 (Fig. 3). Rice takes up Fe as either ferrous iron or as an Fe–mugineic acid complex (Ishimaru et al., 2006). After it is taken up, Fe may be complexed with ligands, which could not be transported by OsHMA3. By contrast, the concentration of Cu and Mn was somewhat affected by overexpression of OsHMA3 (Fig. 3A, B, E, F). However, different from Zn, there was no difference in the concentration of Cu and Mn between OX and VC in the absence of Cd (Fig. 3A, B, E, F). Furthermore, unlike Zn, the concentration of Cu and Mn was also increased in the roots of OX. This increase may be caused indirectly by Cd-inhibited growth although this needs to be further examined (Fig. 1).

In conclusion, overexpression of OsHMA3 enhanced the tolerance to Cd toxicity by increasing sequestration of Cd into vacuoles of root cells and subsequently decreasing translocation of toxic Cd to the shoots. OsHMA3 is also able to transport Zn, but the Zn concentration in the shoots is maintained by up-regulating ZIP genes involved in Zn uptake/translocation/distribution in the OsHMA3 overexpressed line. Our results indicate that overexpression of OsHMA3 is an efficient way to reduce Cd accumulation in the grain and to enhance Cd tolerance in rice because unlike knockout of OsNramp5 and OsHMA2 there was no negative effect on growth.

**Supplementary data**

**Supplementary Table S1.** Primer sequences for ZIP genes.

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**References**


