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RESEARCH PAPER

The tonoplast-localized transporter OsHMA3 plays an important role in maintaining Zn homeostasis in rice

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Abstract

In order to respond to fluctuating zinc (Zn) in the environment, plants must have a system to control Zn homeostasis. However, how plants maintain an appropriate level of Zn during their growth and development is still poorly understood. In this study, we found that OsHMA3, a tonoplast-localized transporter for Zn/Cd, plays an important role in Zn homeostasis in rice. Accessions with the functional allele of *OsHMA3* showed greater tolerance to high Zn than those with the non-functional allele based on root elongation test. A ^{67}Zn-labeling experiment showed that accessions with loss of function of *OsHMA3* had lower Zn accumulation in the roots but similar concentrations in the shoots compared with functional *OsHMA3* accessions. When exposed to Zn-free growing medium, the concentration in the root cell sap was rapidly decreased in accessions with functional *OsHMA3*, but less dramatic changes were observed in non-functional accessions. A mobility experiment showed that more Zn in the roots was translocated to the shoots in accessions with functional *OsHMA3*. Higher expression levels of *OsZIP4*, *OsZIP5*, *OsZIP8*, and *OsZIP10* were found in the roots of accessions with functional *OsHMA3* in response to Zn deficiency. Taken together, our results indicate that OsHMA3 plays an important role in rice roots in both Zn detoxification and storage by sequestration into the vacuoles, depending on Zn concentration in the environment.

Keywords: OsHMA3, vacuolar sequestration, ZIP transporter, Zn distribution, Zn root-to-shoot mobility, Zn tolerance.

Introduction

Zinc (Zn) is an essential microelement for plant growth and development. As the second most abundant transition metal in plants after iron, Zn is the only metal represented in all enzyme classes and performs both catalytic and structural roles in a large number of proteins that regulate various important biological and metabolic processes (Broadley *et al.*, 2007, 2011; Palmgren *et al.*, 2008). In most crop plants, the typical requirement of Zn in leaves for adequate growth is 15–20 mg kg^{-1} dry weight (Marschner, 2012). When Zn is deficient, plants develop specific symptoms, characterized by necrosis of the root apex, leaf chlorosis, reduction in leaf size, and shortening of internodes, ultimately causing suppression of growth (Broadley *et al.*, 2007, 2011). On the other hand, excess Zn causes inhibition of root elongation, leaf chlorosis, stunted growth, and reduced yield (Boawn and Rasmussen, 1971; Chaney, 1993; Broadley *et al.*, 2007, 2011; Kawachi *et al.*, 2009; Sinclair and Krämer, 2012). During the growth of a plant, the Zn concentration in the environment may vary from deficient to toxic levels. Therefore, maintaining the cytosolic Zn homeostasis in plant cells is essential and important for their healthy growth and development (Broadley *et al.*, 2007; Olsen and Palmgren, 2014).

In order to respond to differing Zn concentrations in the soil, plants have developed a sophisticated network of membrane transport systems for optimizing its uptake, sequestration, root-to-shoot translocation, distribution, and redistribution between different tissues and organs. To date, three Zn-transporter gene families have been reported in plants, encoding ZIPs (zinc-regulated transporters, iron-regulated transporter-like proteins), MTPs (metal tolerance proteins), and HMAs (heavy metal ATPases) (Olsen and Palmgren, 2014). In rice, Zn uptake was believed to be mediated by OsZIP1 and OsZIP3 (Ramesh *et al.*, 2003; Bashir *et al.*, 2012), both of which partially complement uptake in yeast at low Zn concentration. However, more recently it has been reported that OsZIP3 in rice is required for unloading Zn from the xylem of enlarged vascular bundles and for preferential distribution to the developing tissues in shoot, rather than for uptake from the soil to roots (Sasaki *et al.*, 2015). *OsZIP1* is expressed in the epidermis and vascular tissues of roots and leaves (Ramesh *et al.*, 2003; Bashir *et al.*, 2012), but its contribution to the uptake of Zn remains further investigation. After uptake, some of the Zn seems to be sequestered into the vacuoles by OsHMA3 (Ueno *et al.*, 2010), while some is loaded to the xylem by OsHMA2 for subsequent translocation to the shoots (Yamaji *et al.*, 2013). OsHMA2 and OsHMA3 share 66.6% similarity, but show different tissue and subcellular localization: OsHMA3 is localized to the tonoplast of all root cells (Ueno *et al.*, 2010), while OsHMA2 is localized to the plasma membrane of root pericycle cells (Yamaji *et al.*, 2013).

Distribution of Zn to the different above-ground organs of the plant is mediated by at least two transporters, OsZIP3 and OsHMA2. Both *OsZIP3* and *OsHMA2* are highly expressed in the nodes, a hub for mineral element distribution in rice (Yamaji and Ma, 2014, 2017). OsZIP3 is localized in the intervening parenchyma cells of the xylem and in the xylem transfer cells of enlarged vascular bundles (EVBs) (Sasaki *et al.*, 2015), while OsHMA2 is localized at the phloem region of both EVBs and diffuse vascular bundles (DVBs) (Yamaji *et al.*, 2013). Thus, OsZIP3 is responsible for unloading Zn from the xylem of EVBs, while OsHMA2 is responsible for loading it to the phloem of DVBs and EVBs (Yamaji *et al.*, 2013). In the leaves, Zn seems to be sequestered into the vacuoles by OsMTP1, a tonoplast-localized Zn transporter (Yuan *et al.*, 2012; Menguer *et al.*, 2013) that is highly expressed in mature leaves and stem. In addition to these transporters, OsZIP1, OsZIP4, OsZIP5, OsZIP6, and OsZIP8 also show transport activity for Zn when expressed in heterogeneous systems (Ramesh *et al.*, 2003; Ishimaru *et al.*, 2005; Yang *et al.*, 2009; Lee *et al.*, 2010a, 2010b; Kavitha *et al.*, 2015), but their exact roles in rice are still unknown.

Some of these transporter genes respond to changes in Zn in the environment at the transcript level. For example, the expression of *OsZIP4*, *OsZIP5*, and *OsZIP8* is up-regulated by Zn deficiency (Suzuki *et al.*, 2012). However, some genes such as *OsZIP3* and *OsHMA2* are expressed constitutively (Yamaji *et al.*, 2013; Sasaki *et al.*, 2015); it is unknown whether these transporters also respond to changes in Zn at the protein level. Thus, the mechanisms underlying Zn homeostasis are still poorly understood.

In the present study, we investigated the role of OsHMA3 in Zn homeostasis in rice. *OsHMA3* was initially isolated and identified as a key gene responsible for low cadmium (Cd) accumulation in rice shoots and grains through map-based cloning from a cross-population of high- and low Cd-accumulating accessions (Ueno *et al.*, 2010; Miyadate *et al.*, 2011). Differences in OsHMA3 between high and low Cd-accumulating accessions result from one amino acid substitution at position 80 (Ueno *et al.*, 2010), resulting in loss-of-function of Cd transport in the high Cd-accumulating cultivar even though both accessions have similar expression levels and tissue and subcellular localization of OsHMA3. Knockdown of *OsHMA3* increases Cd concentrations in the shoot, but the Zn concentration in the shoot is not altered compared with the wild-type. Furthermore, when *OsHMA3* is overexpressed under the control of the *ubiquitin* or *OsHMA2* promoter, not only Cd, but also Zn in the roots and the root cell sap are increased (Sasaki *et al.*, 2014; Shao *et al.*, 2018). Several genes related to Zn transporters are up-regulated in *OsHMA3*-overexpression lines (Sasaki *et al.*, 2014). These results suggest that the OsHMA3 localized at the tonoplast is also a transporter for Zn in addition to Cd, and that the constant Zn concentration in the shoots is maintained by the up-regulation of Zn transporters. However, this suggestion raises questions regarding the original role of OsHMA3 in native rice. We therefore investigated native OsHMA3 in rice by comparing accessions with the functional *OsHMA3* allele with those with a loss-of-function allele in order to determine its roles in tolerance to Zn toxicity and in re-utilization of Zn.

Materials and methods

Plant material and growth conditions

Four accessions of rice (*Oryza sativa*) were used, namely Nipponbare, Taichung 65, Jarjan, and Anjana Dhan. We also used the *TCM213* mutant, which was isolated from seeds of Taichung 65 mutated by *N*-methyl-*N*-nitrosourea and has a loss of function of *OsHMA3* (Shao *et al.*, 2017). Nipponbare and Taichung 65 have the functional *OsHMA3* allele, while Jarjan and Anjana Dhan have the loss-of-functional allele (Ueno et al., 2009a, 2009b, 2010). Seeds were soaked in water at 30 °C in the dark for 2 d and then transferred to a net floating on a 0.5 mM $CaCl_2$ solution. After 5 d, the seedlings were transferred to a half-strength Kimura B solution (pH 5.6) and grown in a greenhouse at 25–30 °C under natural light as described previously (Che *et al.*, 2016). Solutions were renewed every 2 d and three biological replicates were conducted for each experiment (one or two plants for each replicate in different pots). Each experiment described below was repeated at least three times independently.

Evaluation of Zn tolerance

Seeds of all five accessions were soaked in water at 30 °C in the dark for 2 d and then transferred to a net floating on a 0.5 mM $CaCl_2$ solution. After 2 d, seedlings were transferred to 0.5 mM $CaCl_2$ solutions containing 0, 0.04, 0.4, 4, or 40 μM $ZnSO_4$ for 24 h. The root length of 6–10 plants was measured before and after the treatment and the relative root elongation (root elongation +Zn/root elongation –Zn × 100) was calculated to evaluate the Zn tolerance of each accession.

Dose-dependent accumulation of Zn in accessions with different OsHMA3 *alleles*

To investigate the role of the different *OsHMA3* alleles in Zn accumulation, 18-d-old seedlings of the four accessions were grown in a half-strength Kimura B solution containing 0, 0.04, 0.4, or 4 μM $ZnSO_4$. Inductively coupled plasma (ICP)-MS analysis showed that the solution for the 0 μM treatment actually contained 0.005 μM Zn due to

contamination from other chemicals. The solution was renewed every 2 d. After treatment for 2 weeks, the roots were washed three times with 5 mM $CaCl_2$ and separated from the shoots before determination of Zn by ICP-MS. The root-to-shoot translocation ration of Zn was calculated as: (Zn content in shoots/total Zn content) × 100.

Collection of root cell sap

For analysis of root cell sap, 18-d-old seedlings of the four cultivars were grown in a half-strength Kimura B solution containing 0, 0.04, 0.4, or 4 μM $ZnSO_4$ as described above. Roots were washed three times with 5 mM $CaCl_2$ for and excised with a razor. After blotting with a paper towel, the roots were placed on a filter in a tube and frozen at −80 °C until use. After thawing at room temperature, the cell sap was obtained by centrifugation at 15 000 *g* for 20 min (Sasaki *et al.*, 2014).

Determination of short-term distribution of ^{67}Zn

To compare the short-term distribution of Zn in different organs of the four accessions, a ^{67}Zn-labeling experiment was conducted. Seedlings (12-d-old) grown in half-strength Kimura B solution containing 4 μM $ZnSO_4$ were transferred to a similar solution but with 4 μM $^{67}ZnCl_2$. After 24 h, the roots were washed three times with 5 mM $CaCl_2$ and were separated from the shoots. Different organs including the shoot basal region (0.5 cm from the root–shoot junction), different leaves, and tiller were sampled for determination of ^{67}Zn.

Determination of re-utilization of Zn in the roots

Seedlings (12-d-old) of the four accessions that had been labeled with 4 μM ^{67}Zn for 1 d were transferred to half-strength Kimura B solution without Zn. At 0 h, 6 h, 24 h, 3 d, and 7 d after transfer, the roots were sampled for cell sap collection as described above. To examine the mobility of ^{67}Zn, similar seedlings labeled with 4 μM ^{67}Zn for 1 d were transferred to a solution free of Zn for 14 d and different organs were sampled at 0 d and 14 d. The mobility of ^{67}Zn in an organ was calculated as (Content at 14 d − Content at 0 d)/Content at 0 d and expressed as a percentage.

Determination of Zn and other mineral concentrations

All tissue samples collected were dried in an oven at 70 °C for 3 d. After recording the dry weight, samples were subjected to digestion with HNO_3 as described previously (Zheng *et al.*, 2012). The concentrations of Zn and other mineral elements in the digestion solution and root cell sap were determined by ICP-MS (Agilent 7700). The concentrations of ^{67}Zn and ^{66}Zn were determined using the isotope mode.

Gene expression analysis

Seedlings (12 d old) of the four accessions were exposed to either 0 μM (−Zn treatment) or 0.4 μM (+Zn treatment) in half-strength Kimura B solution for 3 d before sampling. For RNA extraction, the plants were divided into roots, the shoot basal region (0.5 cm, including basal nodes), and the rest of the shoot. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) and converted to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo) following the manufacturer's protocol. The cDNAs were amplified using SsoFast EvaGreen Supermix (Bio-Rad) and quantitative real-time PCR was performed on a Bio-Rad CFX384 using specific gene primers for *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10*. *OsHistoneH3* was used as an internal standard. The relative expression was normalized using the $2^{-\Delta\Delta Ct}$ method using the CFX Manager software (Bio-Rad). The primers are listed in Supplementary Table S1 at *JXB* online.

Results

Role of OsHMA3 in tolerance to high Zn

To test whether OsHMA3 is involved in Zn detoxification, we compared root elongation of four rice accessions with different *OsHMA3* alleles at different Zn concentrations. The root elongation over 24 h was not inhibited by Zn up to 4 μM in Nipponbare and Taichung 65 with the functional *OsHMA3* allele (Fig. 1). In contrast, elongation was reduced by Zn from 0.04 μM in Jarjan and Anjana Dhan with loss of function of *OsHMA3* allele. At 4 μM, the relative root elongation was decreased to 69.2% and 74.1% in Jarjan and Anjana Dhan, respectively. At 40 μM Zn concentration, relative root elongation was decreased to 65.6% and 78.3% in Taichung 65 and Nipponbare, respectively, while it was decreased to 49.4% and 45.5% in Jarjan and Anjana Dhan, respectively. Similar results were obtained with the *TCM213* mutant and its wild-type Taichung 65, with the mutant displaying a significantly lower relative root elongation rate under Zn treatment (Supplementary Fig. S1). These results indicated that functional *OsHMA3* is involved in detoxification of high Zn in the roots.

Accumulation patterns of Zn in different accessions with either the functional or non-functional allele of OsHMA3

The four rice accessions were grown in different external Zn concentrations ranging from 0–4 μM. At low external concentrations (0 μM and 0.04 μM), the Zn concentrations in the roots of Nipponbare and Taichung 65 with the functional allele were slightly higher than those of Jarjan and Anjana Dhan with the loss-of-function allele, and these differences became significant at high external concentrations (0.4 μM and 4 μM; Fig. 2A). In contrast, there were no significant differences in the shoot Zn concentration among the four cultivars at any of the external concentrations tested, although the shoot Zn increased with increasing external Zn concentration (Fig. 2B). Similar results were also observed in the *TCM213* mutant compared with wild-type Taichung 65. At high external concentrations (≥0.4 μM), the Zn concentration in the roots of

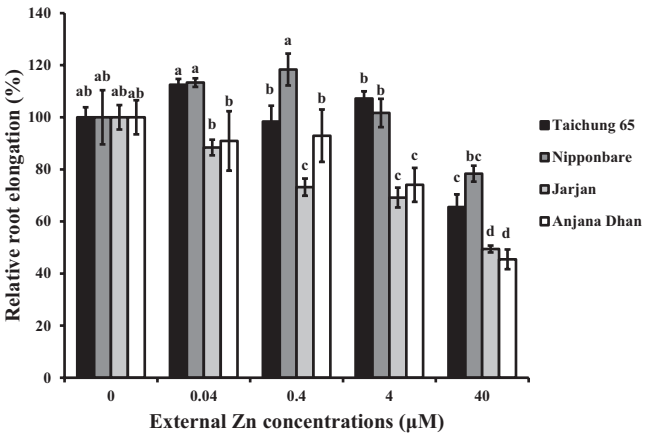

Fig. 1. Tolerance to Zn toxicity in four rice accessions with different *OsHMA3* alleles as indicated by root elongation. Seedlings (4 d old) of two accessions with the functional *OsHMA3* allele (Nipponbare and Taichung 65) and two with the loss-of-function allele (Jarjan and Anjana Dhan) were transferred to 0.5 mM $CaCl_2$ solutions containing 0, 0.04, 0.4, 4, or 40 μM Zn for 24 h. Root length was measured before and after the treatment and relative root elongation was calculated as: (root elongation +Zn/root elongation −Zn) ×100. Data are means (±SD) of 6–10 biological replicates. Different letters indicate significant differences as determined by Tukey's test ($P<0.05$).

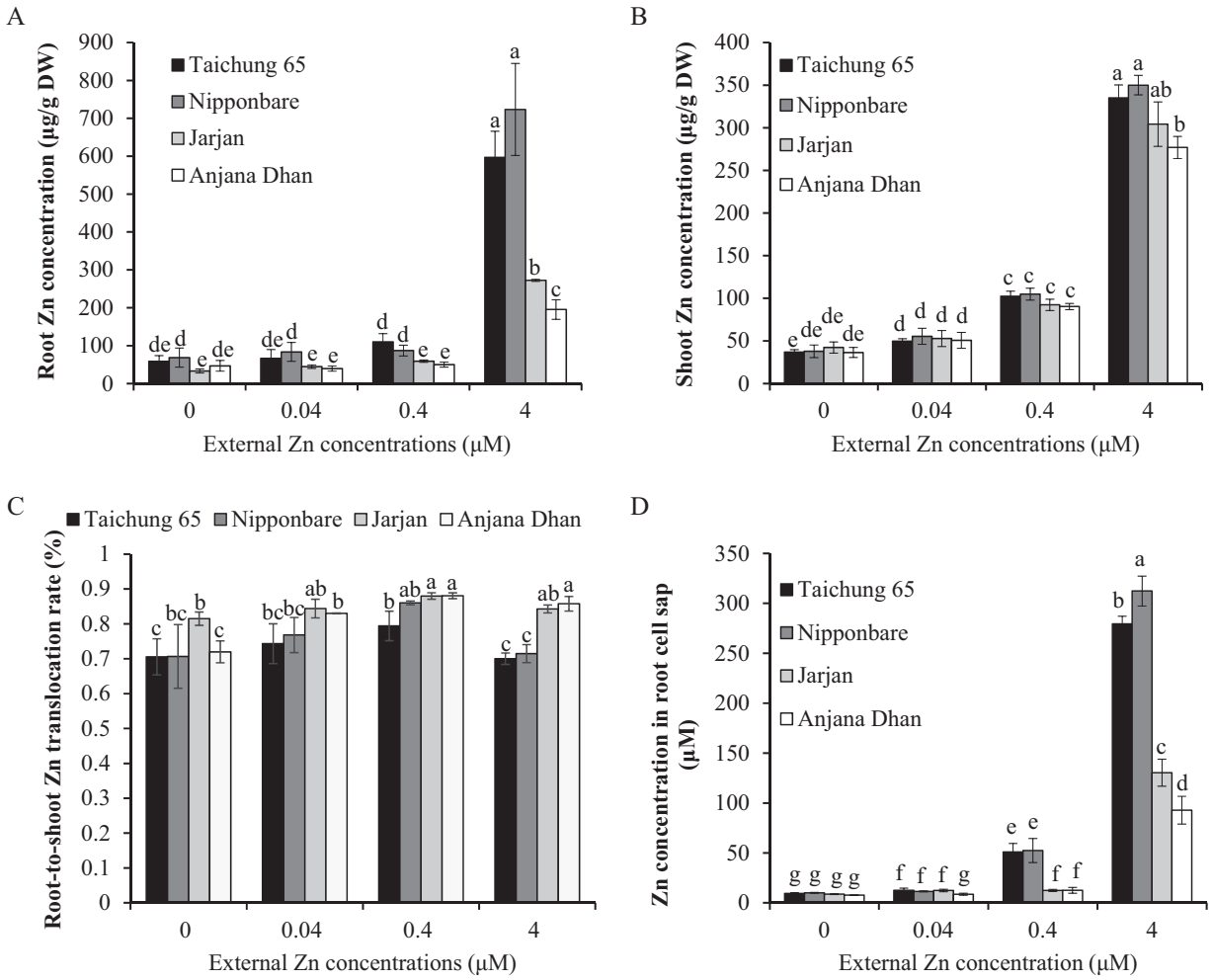

Fig. 2. Patterns of Zn accumulation in four rice accessions with different *OsHMA3* alleles. Zn concentration in (A) the roots and (B) the shoot. (C) Root-to-shoot Zn translocation and (D) Zn concentration in the root cell sap. Seedlings (18 d old) of two accessions with the functional *OsHMA3* allele (Nipponbare and Taichung 65) and two with the loss-of-function allele (Jarjan and Anjana Dhan) were grown in half-strength Kimura B solutions containing 0, 0.04, 0.4, or 4 μM Zn for 2 weeks. The Zn concentration was determined by ICP-MS. Data are means (±SD) of three biological replicates. Different letters indicate significant differences as determined by Tukey's test ($P<0.05$).

TCM213 were significantly lower than in Taichung 65, and there were no differences in the shoots (Supplementary Fig. S2A, B). The root-to-shoot translocation ratio was similar in all the accessions at low Zn concentrations (Fig. 2C), but at high Zn concentrations Jarjan and Anjana Dhan showed higher ratios than Nipponbare and Taichung 65 (Fig. 2C). No large differences were found in the concentrations of other mineral elements, except for P, Mn, and Ca (Supplementary Fig. S3).

Analysis of the root cell sap showed that there were almost no differences in the Zn concentrations among the four accessions at low external concentrations (0 μM and 0.04 μM) (Fig. 2D). However, at higher concentrations (0.4 μM and 4 μM), Zn concentrations in the root cell sap in Nipponbare and Taichung 65 were more than double those in Jarjan and Anjana Dhan. These results indicated that loss of function of *OsHMA3* altered the accumulation patterns of Zn in the roots and shoots.

To confirm these results, we performed a short-term (24 h) labeling experiment with a stable isotope. Significantly lower ^{67}Zn concentrations were found in the roots of Jarjan and Anjana Dhan compared with Nipponbare and Taichung 65, but no differences were found in the shoot basal region or in the leaves (Fig. 3A). Less ^{67}Zn was distributed to the roots of Jarjan and Anjana Dhan compared with Nipponbare and Taichung 65 (Fig. 3B). There were generally no differences in the distribution in the shoots, except that less ^{67}Zn was distributed to leaf 4 and more was distributed to leaf 6 in Nipponbare and Taichung 65 compared with Jarjan and Anjana Dhan (Fig. 3B). These differences may be attributed to differences in leaf size (results not shown).

Re-utilization of Zn in the roots of different accessions with or without the functional allele of OsHMA3

In order to examine the role of OsHMA3 in re-utilization of Zn in the roots, the four accessions were labeled with ^{67}Zn for 24 h before being exposed to a solution without Zn, and the ^{67}Zn in the root cell sap was monitored. At day 0, the ^{67}Zn concentration was much higher in Nipponbare and Taichung 65 than in Jarjan and Anjana Dhan (Fig. 4A). The concentration decreased with time under Zn deficiency in Nipponbare and Taichung 65, but that it did not vary much in Jarjan and Anjana Dhan. By day 7, the Zn concentration in the root cell

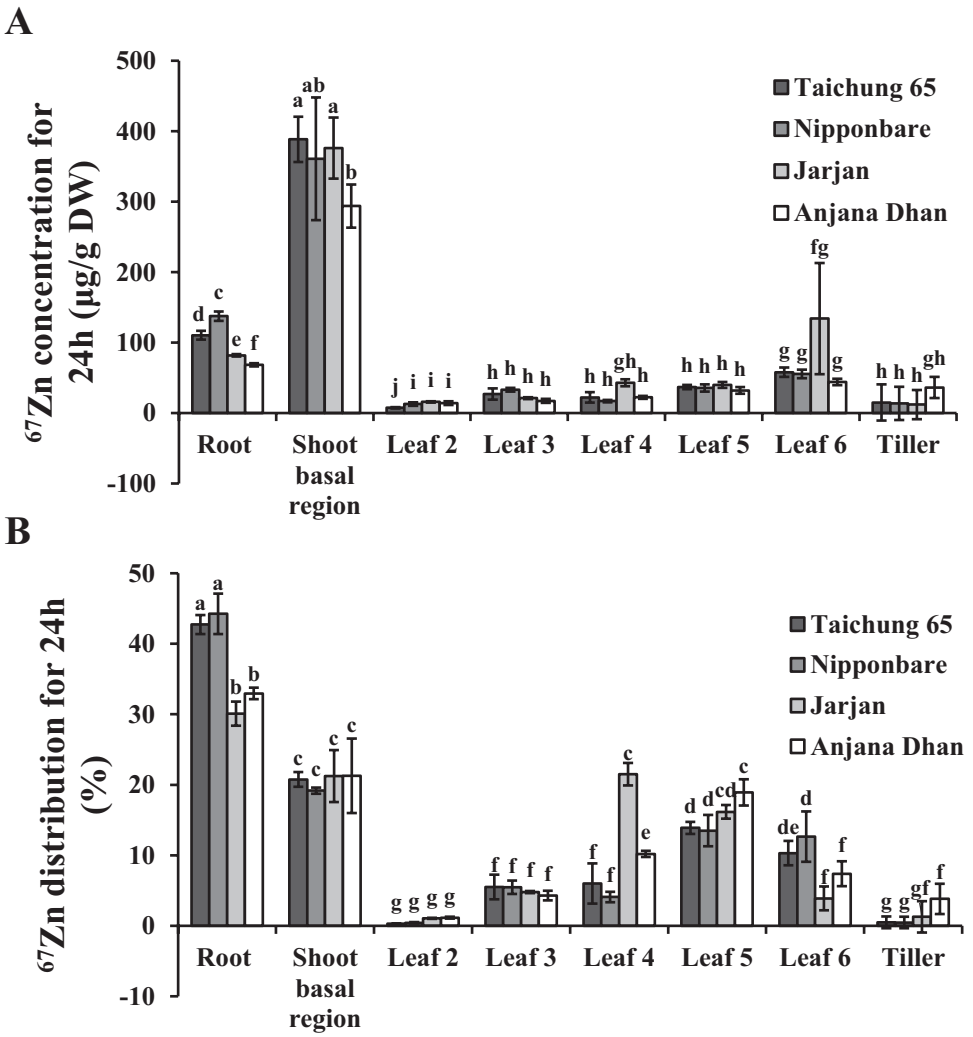

Fig. 3. Short-term distribution of ^{67}Zn in different organs of four rice accessions with different *OsHMA3* alleles. (A) ^{67}Zn concentrations and (B) distribution of ^{67}Zn in different organs. Seedlings (12 d old) of two accessions with the functional *OsHMA3* allele (Nipponbare and Taichung 65) and two accessions with the loss-of-function allele (Jarjan and Anjana Dhan) were grown in half-strength Kimura B solutions containing 4 μM ^{67}Zn for 24 h. Concentrations of ^{67}Zn were determined by ICP-MS using the isotope mode. Data are means (±SD) of three biological replicates. Different letters indicate significant differences as determined by Tukey's test (P<0.05).

sap had declined from 160.6 μM to 112.9 μM in Taichung 65 and from 163.6 μM to 115.5 μM in Nipponbare, whilst in contrast it had only declined from 107.0 μM to 96.8 μM in Jarjan and from 99.3 μM to 83.1 μM in Anjana Dhan (Fig. 4B). These results suggested that more Zn in the root cell sap could be transported out to the shoots under Zn deficiency in the accessions possessing the functional allele of *OsHMA3*.

To further examine the mobility of Zn, the accessions were labeled with ^{67}Zn for 24 h and then exposed to Zn-free solution for 14 d. Three new leaves and one tiller appeared during this period. The total Zn content was similar between the plants before and after Zn-deficiency treatment (Fig. 5A), indicating that there was no loss of ^{67}Zn. About 26–27% of the total ^{67}Zn was mobilized from the roots in Nipponbare and Taichung 65, in contrast to 14–18% from Jarjan and Anjana Dhan (Fig. 5B). There were almost no changes in the ^{67}Zn content in the shoot basal region and the older leaves (2–6) of Nipponbare and Taichung 65 at the end of the treatment. In Jarjan and Anjana Dhan, ^{67}Zn in the shoot basal region was decreased by 6.4–8.6% of and it was decreased by 3.1–6.3% in leaf 4. Similar ^{67}Zn mobility was found in all the cultivars in the new leaves (7–9) and tillers (Fig. 5B).

In addition, we labeled the *TCM213* mutant and Taichung 65 wild-type with ^{67}Zn for 24 h and then transferred the plants to a Zn-free solution for 7 d. Similar to the results for the accessions, 15.4% of the total ^{67}Zn was mobilized from the roots in the wild-type, in contrast to 6.3% mobility in the mutant (Supplementary Fig. S4). The wild-type showed almost no change in ^{67}Zn content in the shoot basal region and older leaves (2–4), whereas in the mutant the ^{67}Zn content of the shoot basal region was decreased by 6.2%, and it was decreased by 2.4% and 2.6% in leaf 4 and leaf 5, respectively.

OsZIP *gene expression in different cultivars with or without the functional* OsHMA3 *allele*

To investigate the molecular mechanisms behind different mobility of Zn in the different accessions, we compared

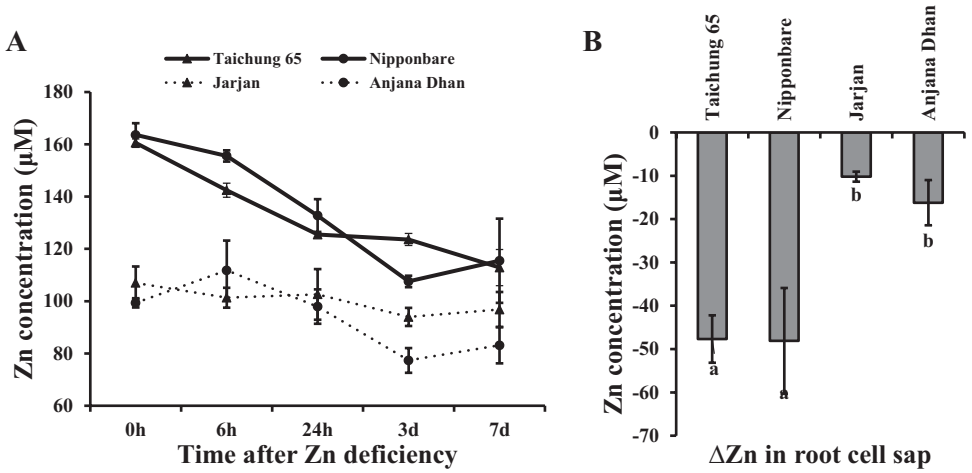

Fig. 4. Changes in Zn concentrations in the root cell sap of four rice accessions with different *OsHMA3* alleles. (A) Time-course of changes in Zn concentrations in the root cell sap. (B) Difference in Zn concentration in the root cell sap after Zn deficiency for 7 d. Seedlings (12 d old) of two accessions with the functional *OsHMA3* allele (Nipponbare and Taichung 65) and two accessions with the loss-of-function allele (Jarjan and Anjana Dhan) were grown in half-strength Kimura B solution with 4 μM Zn for 1 week and then transferred (at time 0 h) to a new solution without Zn. Zn concentration was determined by ICP-MS. Data are means (±SD) of three biological replicates. Different letters indicate significant differences as determined by Tukey's test (P<0.05).

the expression of *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* in the roots, shoot basal region, and the rest of the shoots. Under the +Zn treatment, the expression levels of all the genes were of similar magnitude (Fig. 6). There was some variation in expression in the roots in the +Zn treatment, but all showed significant increases under Zn deficiency (Fig. 6A) and, with the exception of *OsZIP9*, there was greater up-regulation in Nipponbare and Taichung 65 than in Jarjan and Anjana Dhan. In the other parts of the plant, only the expression of *OsZIP5* and *OsZIP8* was consistently higher in the accessions with the functional *OsHMA3* allele (Fig. 6B, C).

Discussion

OsHMA3 is required for tolerance to high external Zn concentrations

OsHMA3 is a tonoplast-localized protein in rice roots (Ueno *et al.*, 2010). It functions in limiting the root-to-shoot translocation of Cd by sequestering it into root vacuoles, resulting in low accumulation in the shoots and grains (Ueno *et al.*, 2010; Sasaki *et al.*, 2014). Previous studies have shown that overexpression of *OsHMA3* in Nipponbare results in consistently higher Zn concentrations in roots, suggesting that OsHMA3 also participates in transporting Zn into the root vacuoles (Ueno *et al.*, 2010; Sasaki *et al.*, 2014). Since the expression of *OsHMA3* in native rice accessions is low (Ueno *et al.*, 2009a, 2009b, 2010), the original role of OsHMA3 in Zn homeostasis remains unknown. In our present study, we first examined the role of OsHMA3 in detoxification of high Zn by comparing rice accessions with different *OsHMA3* alleles. The root elongation test clearly showed that loss of function of *OsHMA3* resulted in enhanced sensitivity to high Zn in the accessions tested (Fig. 1; Supplementary Fig. S1), and accessions with the functional *OsHMA3* allele accumulated more Zn in the roots and in the root cell sap (most of which is from the vacuoles) compared with accessions without the functional allele (Fig. 2A, D; Supplementary Fig. S2A). This suggested that OsHMA3 is required for tolerance to high Zn in the roots through sequestration into the vacuoles to maintain the proper Zn concentration in the root cytosol. Furthermore, over-expression of *OsHMA3* also increases tolerance to Cd toxicity in rice (Sasaki *et al.*, 2014), indicating that OsHMA3 localized at the tonoplast transports both Cd and Zn into to the vacuoles. Since Cd is not an essential element for plant growth and its concentration in natural soils is low, the original role of OsHMA3 could have been to sequester Zn to vacuoles in the roots of rice.

Vacuolar Zn sequestered by OsHMA3 is an important source for remobilization under deficiency conditions

Plant vacuoles function as a storage site for nutrients (Marty, 1999), and are important as a source under nutrient deficiency conditions. In the rice accessions with the functional allele of *OsHMA3* (Nipponbare and Taichung 65), the root and root cell sap contained more Zn compared with those with loss of function of the allele (Jarjan and Anjana Dhan) (Fig. 2A, D). We also found that more Zn was mobilized from the roots of Nipponbare and Taichung 65 than from those of Jarjan and Anjana Dhan under deficiency conditions (Fig. 5B). After 7 d with Zn deficiency, the concentration in the root cell sap was decreased by 29.7% in Nipponbare and Taichung 65 but Jarjan and Anjana Dhan only showed a decrease of 9.6–16.5% (Fig. 4). These results indicated that Zn sequestered by OsHMA3 in the roots provided an important source for the shoot under conditions of Zn deficiency. The movement of Zn from the vacuole for subsequent translocation to the shoots requires an efflux transporter, but it has not been identified yet. A member of the ZIP transporter family, ZRT3, has been reported in yeast as a tonoplast-localized transporter that is responsible for

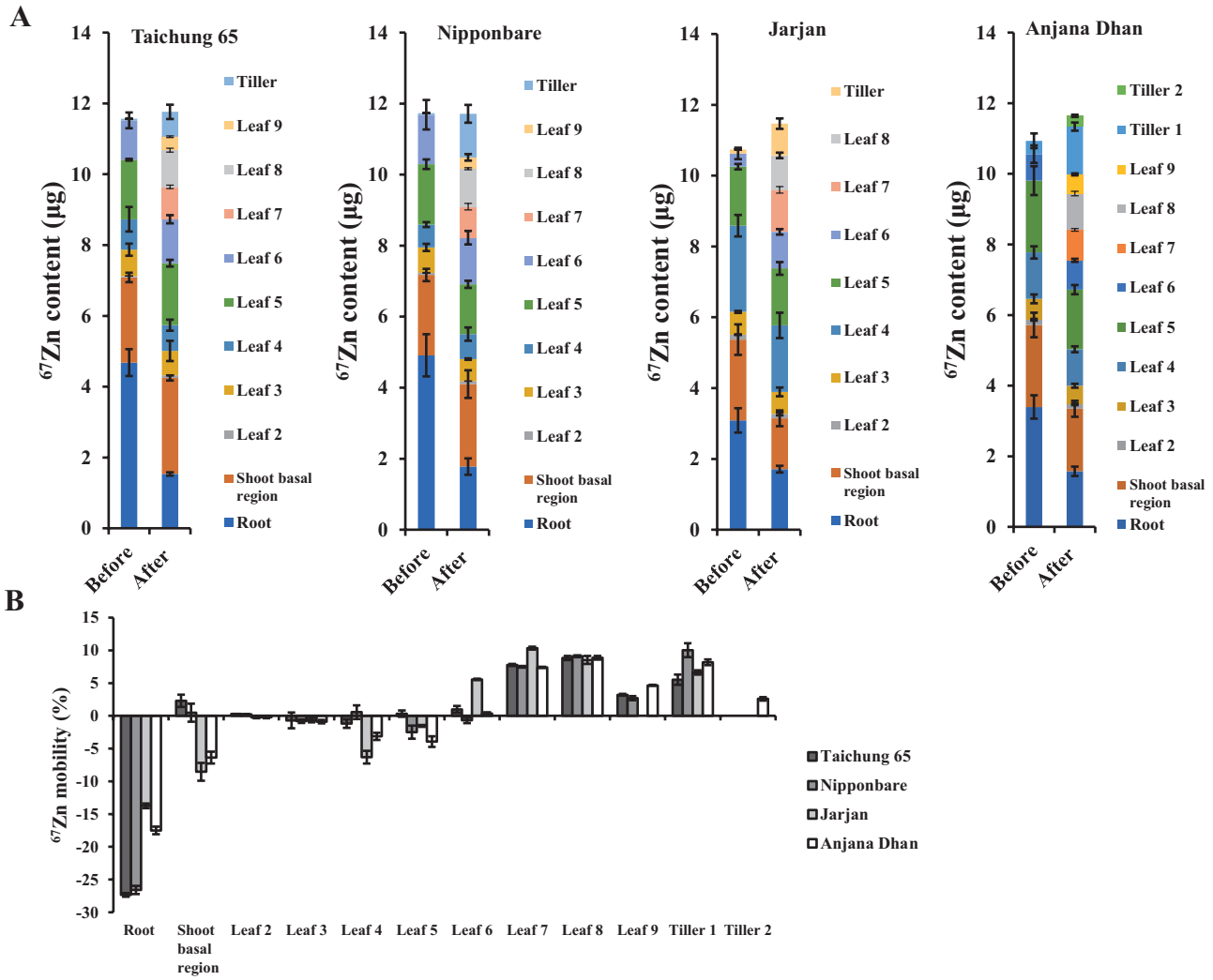

Fig. 5. Mobility of ^{67}Zn in four rice accessions with different *OsHMA3* alleles after Zn deficiency. (A) Total ^{67}Zn accumulation in plants before and after Zn-deficiency, (B) mobility of ^{67}Zn in different organs after Zn deficiency for 14 d. Seedlings (12 d old) of two accessions with the functional *OsHMA3* allele (Nipponbare and Taichung 65) and two accessions with the loss-of-function allele (Jarjan and Anjana Dhan) were grown in a half-strength Kimura B solution with 4 μM ^{67}Zn for 24 h and then transferred to a Zn-free solution for 14 d. ^{67}Zn was determined by ICP-MS using the isotope mode. Mobility was calculated as the difference in ^{67}Zn accumulation before and after Zn deficiency, and expressed as a percentage of the initial (before) value. Data are means (±SD) of three biological replicates. Different letters indicate significant differences as determined by Tukey's test (P<0.05).

the efflux of stored Zn from the vacuole to the cytosol during the transition from Zn-replete to Zn-limiting conditions (MacDiarmid *et al.*, 2000).

Although the mobility of Zn in the roots of the rice accessions with loss of function of *OsHMA3* was lower, the young leaves contained similar concentrations compared to those of the cultivars with the functional allele (Fig. 5B). This could be due in part to mobility of Zn from the basal nodes. Rice basal nodes contain much higher Zn than other tissues (Fig. 3A; Yamaji *et al.*, 2013; Sasaki *et al.*, 2015), so they could also serve as a source under deficiency conditions. Our results suggested that in order to satisfy the Zn demand for the growth of new leaves under deficiency, Nipponbare and Taichung 65 mostly depended on movement from the roots, while Jarjan and Anjana Dhan depended on movement from the basal nodes. In accessions with the functional *OsHMA3* allele it seems that Zn in the roots will be re-utilized first.

In spite of the loss of function of *OsHMA3*, Jarjan and Anjana Dhan contained similar Zn concentrations to Nipponbare and Taichung 65 in the shoots at all the external Zn concentrations tested (Fig. 2B). In a previous study examining overexpression of *OsHMA3*, it was found that the maintenance of the Zn concentration in the shoots was achieved by up-regulation of the expression of *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* in the roots (Sasaki *et al.*, 2014). We therefore also compared the expression levels of these genes in the four accessions with different *OsHMA3* alleles. Under Zn deficiency, the expression levels of *OsZIP4*, *OsZIP5*, *OsZIP8*, and *OsZIP10* in the roots were significantly higher in Nipponbare and Taichung 65 than in Jarjan and Anjana Dhan, but there were no clear differences in expression of *OsZIP9* between the cultivars (Fig. 6A). There were no differences in the expression of these genes in the shoot basal region and in the rest of the shoot except for *OsZIP5* and *OsZIP8* (Fig. 6B, C). While there are no reports

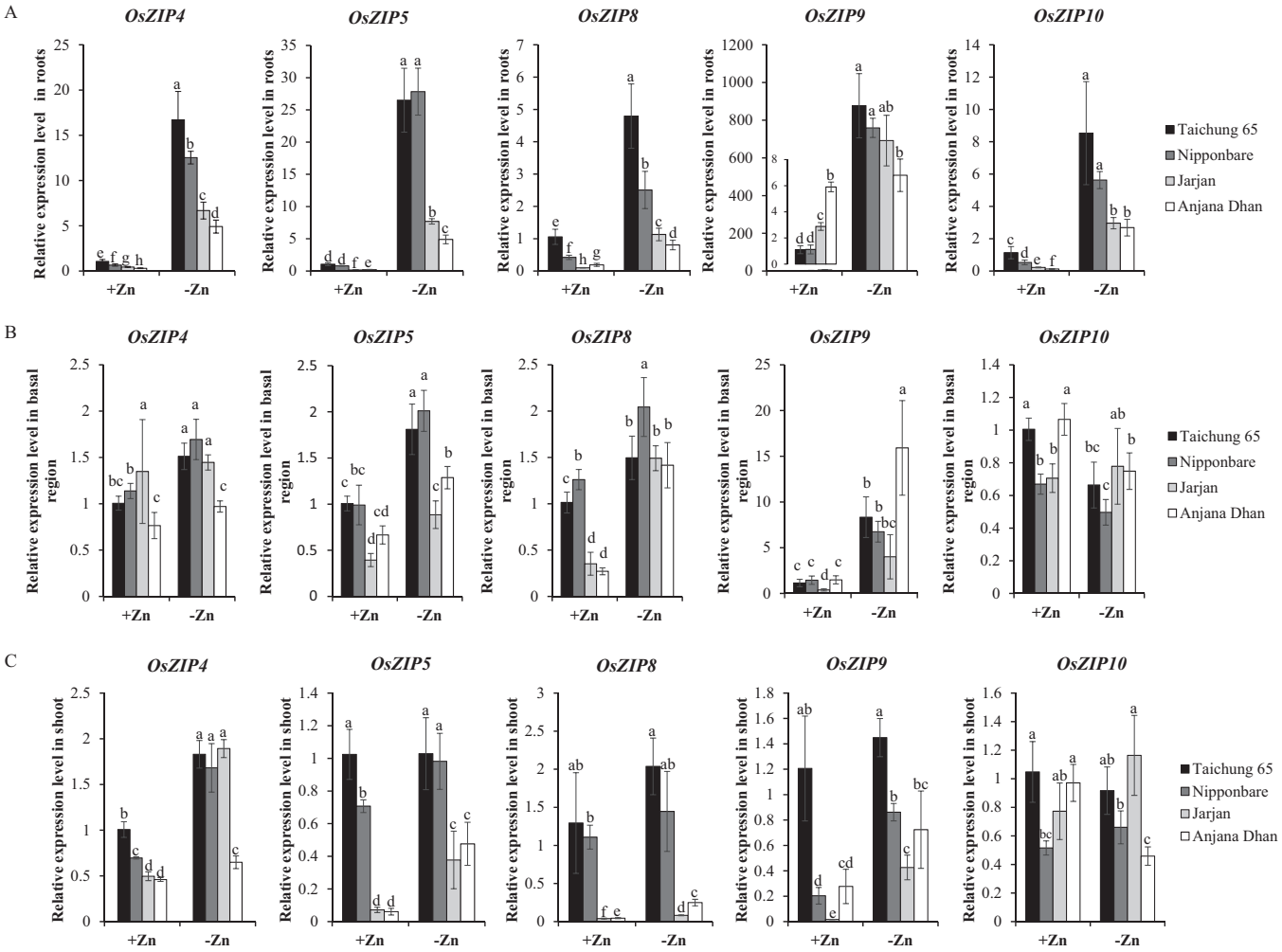

Fig. 6. Expression levels of some *OsZIP* genes in four rice accessions with different *OsHMA3* alleles under Zn-sufficient (+Zn) and Zn-deficient (–Zn) conditions. Expression levels of *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* in (A) the roots, (B) the shoot basal region, and (C) the rest of the shoots were determined by quantitative RT-PCR. Seedlings (12 d old) were grown in a half-strength Kimura B solution with 0 μM or 4 μM Zn for 3 d. *Histone H3* was used as the internal standard. Expression levels relative to Taichung 65 at +Zn are shown. Data are means (±SD) of three biological replicates. Different letters indicate significant differences as determined by Tukey's test ($P<0.05$).

concerning the exact role of OsZIP10 in Zn uptake or distribution, several studies have shown that OsZIP4, OsZIP5, and OsZIP8 are localized to the plasma membrane and are highly induced by Zn-deficient conditions (Ishimaru *et al.*, 2005; Lee *et al.*, 2010a, 2010b). Studies of heterogonous expression in a yeast mutant deficient for Zn-uptake and overexpression in rice have suggested that OsZIP4, OsZIP5, and OsZIP8 are Zn transporters that function in its uptake and distribution (Ishimaru *et al.*, 2007; Lee *et al.*, 2010a, 2010b), although their exact roles in rice need to be further investigated. Our results suggested that the accumulation of Zn in the shoots in the accessions with the functional *OsHMA3* allele is the result of up-regulation of these genes related to Zn transport.

Conclusions

Our results show that OsHMA3 in native rice functions as a transporter for sequestering Zn into the vacuoles of root cells and it plays an important role in maintaining Zn homeostasis. At high external Zn concentrations, OsHMA3 is required for detoxification, while under deficiency conditions Zn sequestered by OsHMA3 is an important source for the growth of developing tissues.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primer sequences used in gene expression analysis.

Fig. S1. Tolerance to Zn toxicity in the *TCM213* mutant and the wild-type Taichung 65.

Fig. S2. Root and shoot Zn concentrations in the *TCM213* mutant and the wild-type Taichung 65.

Fig. S3. Concentrations of various mineral elements in rice cultivars with different *OsHMA3* alleles.

Fig. S4. Mobility of ^{67}Zn in the *TCM213* mutant and wild-type Taichung 65 following a Zn-deficiency treatment.

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