A loss-of-function allele of *OsHMA3* associated with high cadmium accumulation in shoots and grain of *Japonica* rice cultivars

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ABSTRACT

Excessive cadmium (Cd) accumulation in rice poses a risk to food safety. OsHMA3 plays an important role in restricting Cd translocation from roots to shoots. A non-functional allele of *OsHMA3* has been reported in some *Indica* rice cultivars with high Cd accumulation, but it is not known if *OsHMA3* allelic variation is associated with Cd accumulation in *Japonica* cultivars. In this study, we identified a *Japonica* cultivar with consistently high Cd accumulation in shoots and grain in both field and greenhouse experiments. The cultivar possesses an *OsHMA3* allele with a predicted amino acid mutation at the 380th position from Ser to Arg. The haplotype had no Cd transport activity when the gene was expressed in yeast, and the allele did not complement a known nonfunctional allele of *OsHMA3* in F_1 test. The allele is present only in temperate *Japonica* cultivars among diversity panels of 1483 rice cultivars. Different cultivars possessing this allele showed greatly increased root-to-shoot Cd translocation and a shift in root Cd speciation from Cd-S to Cd-O bonding determined by synchrotron X-ray absorption spectroscopy. Our study has identified a new loss-of-function allele of *OsHMA3* in *Japonica* rice cultivars leading to high Cd accumulation in shoots and grain.

Key-words: Allelic variation; Cadmium; Cd speciation; *Oryza sativa* (Rice); OsHMA3; Translocation

INTRODUCTION

Cadmium (Cd) is one of the most toxic heavy metals. Exposure to elevated levels of Cd can cause renal dysfunction, osteoporosis and cancers (Nawrot *et al.* 2006; Nordberg 2009; Åkesson *et al.* 2014). Due to a relatively easy transfer of Cd from soil to food crops, food accounts for about 90% of the Cd exposure in the general non-smoking population (Clemens *et al.* 2013). In particular, rice (*Oryza sativa*) is a major source of dietary Cd intake for populations consuming rice as the staple food (Watanabe *et al.* 2004; Meharg *et al.* 2013). As a result of soil contamination, soil acidification and growing of rice cultivars high in Cd accumulation, considerable proportions of rice produced in some areas of Southern China contain levels of Cd exceeding the Chinese food safety limit of 0.2 mg kg^{-1} for rice grain (Williams *et al.* 2009; Du *et al.* 2013; Zhao *et al.* 2015). A number of studies have reported the link between elevated Cd intake, with rice being the most important source, and adverse health effects in areas impacted by Cd contamination in China (e.g. Jin *et al.* 2002; Jin *et al.* 2004; Zhang *et al.* 2014). To develop strategies to minimise the transfer of Cd from soil to the food chain requires a better understanding of the mechanisms controlling Cd uptake and translocation in plants.

Recent studies have revealed a number of genes playing important roles in Cd uptake and translocation in rice and other plant species. The entry of Cd into rice root cells is mediated mainly by the manganese (Mn) transporter OsNramp5 (Natural Resistance-Associated Macrophage Protein 5); knockout mutants of *OsNramp5* lost >90% of the Cd absorption ability of the wild-type plants (Sasaki *et al.* 2012; Yang *et al.* 2014). Ishikawa *et al.* (2012) isolated a number of rice mutants low in Cd accumulation by screening a mutant library and linked the phenotype to mutations of *OsNramp5*. They showed that the mutants grown in Cd-contaminated soils contained low concentrations of Cd in their grains and exhibited no agriculturally or economically adverse traits. In contrast, the studies by Sasaki *et al.* (2012)

and Yang *et al.* (2014) showed that the T-DNA insertion mutants of *OsNramp5* suffered from Mn deficiency and growth impairment under the conditions of low Mn supply.

The P_{1B}-type Heavy Metal ATPase 3 (HMA3) has been shown to play an important role in Cd tolerance and accumulation in shoots of *Arabidopsis thaliana* (Morel *et al.* 2009; Chao *et al.* 2012) and Cd translocation from roots to shoots in rice (Ueno *et al.* 2010; Miyadate *et al.* 2011). This transporter is localized at the tonoplast mediating the transport of Cd from the cytoplasm into the vacuoles. AtHMA3 is also able to transport other metals including Zn, Co and Pb (Morel *et al.* 2009). *OsHMA3* is mainly expressed in rice roots and the expression is not responsive to Cd exposure (Ueno *et al.* 2010). Silencing *OsHMA3* resulted in increased Cd translocation from roots to shoots, whereas overexpression of *OsHMA3* produced the opposite effect (Ueno *et al.* 2010; Sasaki *et al.* 2014). Large genotypic variations in shoot and grain Cd concentrations have been reported in rice (Arao & Ae 2003; Ueno *et al.* 2009; Pinson *et al.* 2015). These variations were found to correlate closely with the Cd concentration in the xylem sap, suggesting that the root-to-shoot translocation is a key process controlling Cd accumulation in rice shoots and grain (Uraguchi *et al.* 2009). A number of *Indica* rice cultivars, such as Anjana Dhan, Cho-Ko-Koku and Jarjan, accumulate high levels of Cd in their shoots and grain due to a high translocation of Cd from roots to shoots. Genetic analysis revealed that this phenotype is controlled by a recessive allele located at the chromosome 7 (Ueno *et al.* 2010; Miyadate *et al.* 2011; Ueno *et al.* 2011a). Map-based cloning showed that the allele represents a loss-of-function *OsHMA3*, resulting in a weakened vacuolar sequestration of Cd in roots and an enhanced Cd translocation to shoots (Ueno *et al.* 2010; Miyadate *et al.* 2011). Further analysis using heterologous expression in yeast showed that the mutation of the 80^{th} amino acid residue from Arg to His is responsible for the loss of function of the OsHMA3 protein in the high Cd cultivar Anjana Dhan (Ueno *et al.* 2010), as well as in the cultivars Cho-Ko-Koku and Jarjan because they share the same

coding sequence as that of Anjana Dhan. Similarly, hypofunctional alleles of *AtHMA3* were found to be associated with high Cd accumulation in leaves among different natural accessions of *A. thaliana* (Chao *et al.* 2012).

OsHMA2 is localized in the pericycle cells inside the stele of rice roots and in the phloem region of enlarged and diffuse vascular bundles in the nodes (Yamaji *et al.* 2013). OsHMA2 functions as a plasma membrane transporter for Zn and Cd to facilitate their translocation from roots to shoots and the distribution to the panicle in the nodes (Satoh-Nagasawa *et al.* 2012; Takahashi *et al.* 2012; Yamaji *et al.* 2013). Mutation in *OsHMA2* leads to lower Cd and Zn concentrations in the reproductive tissues of rice (Satoh-Nagasawa *et al.* 2012; Takahashi *et al.* 2012; Yamaji *et al.* 2013). OsLCT1 (Low Affinity Cation Transporter 1) is another transporter that is involved in Cd distribution to rice grain (Uraguchi *et al.* 2011). OsLCT1 is localized at the plasma membrane in the phloem parenchyma cells in rice nodes and acts as an efflux-type transporter to facilitate Cd loading into the phloem sieve tube. Knocking down *OsLCT1* was found to decrease Cd concentration in rice grain by half (Uraguchi *et al.* 2011).

Cultivated rice contains two main subspecies, *Indica* and *Japonica*, with the former being grown mainly in tropical and subtropical South and Southeast Asia and the latter in temperate East Asia, upland areas of Southeast Asia and high elevations in South Asia. In general, *Indica* rice tends to have a higher Cd accumulation in shoots and grain than *Japonica* rice (Arao & Ae 2003; He *et al.* 2006; Uraguchi & Fujiwara 2013). The reason for this difference is not understood. Among *Japonica* cultivars, there is still large genotypic variation (Ueno *et al.* 2009) with some cultivars accumulating high Cd concentrations in grain (Pinson *et al.* 2015), but there are few studies investigating the genetic basis underlying this variation. Understanding the genetic basis for genotypic variation is important for the strategy of breeding low Cd accumulating crops. In the present study, we identified a number

of high Cd accumulating *Japonica* cultivars and showed that this phenotype was caused by a new loss-of-function allele of *OsHMA3*.

MATERIALS AND METHODS

Plant materials, field and pot experiments

Twenty rice cultivars were selected from a panel of 533 cultivars of diverse genetic background that has been used for genome-wide association (GWA) mapping of agronomic traits (Chen *et al.* 2014). Cultivars are coded in the present study as their entry number in the GWA panel and their names and the subspecies information are provided in Supporting Information Table S1. These cultivars were grown in two paddy fields in China in 2013, including a Cd-contaminated soil in Hunan province and a non-contaminated soil in Jiangsu province. Soil Cd concentration was 2.0 and 0.2 mg kg^{-1}, respectively, in the Hunan and Jiangsu field sites, and soil pH was 5.6 and 7.2, respectively. Each cultivar was grown in triplicates in a randomized block design with six rows of ten plants in each replicate. Paddy water management, fertilizer applications and crop protection followed the local farming practices. In 2014, the 20 cultivars were also grown in a pot experiment inside a net enclosure in Nanjing with 3 replicates for each cultivar. An uncontaminated paddy soil (total Cd 0.3 mg kg^{-1}, pH 7.3) was collected from Nanjing and amended with 2 mg Cd kg^{-1} as $CdCl_2$. Each pot was filled with 2.5 kg soil. Soil was maintained at moist but aerobic conditions during plant growth to increase Cd availability. Plants were harvested at maturity for analysis of the concentrations of Cd and other metals in grain and straw.

Following the above experiments and sequencing of *OsHMA3*, a number of cultivars representing different *OsHMA3* alleles were selected from the GWA panel and other sources

for further experiments. A high Cd accumulating cultivar (line No. 35, a *Japonica* cultivar) was crossed to Anjana Dhan, an *Indica* cultivar possessing a non-functional *OsHMA3* allele (Ueno *et al.* 2010). The F_1 progeny was used in the subsequent experiments.

Hydroponic experiments

A series of hydroponic experiments were conducted to investigate the uptake and root-to-shoot translocation of Cd in selected rice cultivars representing different *OsHMA3* alleles. Seeds were germinated in deionized water in the dark at 37°C for 3 d and transferred to a net floating on deionized water for further 3 d. The seedlings were transferred to a growth room with a light intensity of 250 μmol m^{-2} s^{-1}. Temperature was maintained at 28°C during the day and 25°C during the night with a 12 h photoperiod. Plants were grown in a ½ strength Kimura B nutrient solution with the following composition (μM): 90 KH_2PO_4, 270 $MgSO_4$, 180 $(NH_4)_2SO_4$, 90 KNO_3, 180 $Ca(NO_3)_2$, 3 H_3BO_3, 0.5 $MnCl_2$, 1 $(NH_4)_6Mo_7O_{24}$, 0.4 $ZnSO_4$ and 20 Fe(III)-EDTA. The pH value of the nutrient solution was adjusted to 5.6 with 0.1 M NaOH, and the solution was renewed every 3 d. After pre-culture for 20 d, plants were used in a series of experiments with different concentrations of Cd (0.05 – 0.5 μM Cd, added as $CdCl_2$) or different length of Cd exposure. Each treatment was replicated in 3 or 4 times depending on the experiment. At the end of the experiment, roots were washed with deionized water for three times and blotted dry. Roots and shoots were separated and dried at 60°C for 3 d before analysis. In some of the experiments, xylem sap was collected before plants were harvested. Shoots were cut at 2 cm above the stem base at 2 h after the light period started in the morning. The xylem exudate was collected for 1 h into a clean cotton ball covered with a plastic wrap. The exudate was span out of the cotton ball by centrifugation and diluted 4 times with 2% HNO_3 prior to measurement of Cd concentration.

Cd uptake kinetics was determined in selected cultivars in a short-term uptake experiment at either 25 ^{o}C or 4 ^{o}C (ice-cooled). Four 20-d-old seedlings of each cultivar were transferred to 1 litre basal solution containing 0.5 mM $CaCl_2$ and 2 mM MES (pH 5.6). After 12 h, seedlings were exposed to 0, 0.2, 0.5, 1, 2 or 5 μM $CdCl_2$ in the basal solution for 20 min under continuous aeration with four replicates per treatment. Roots were rinsed with ice-cold basal nutrient solution for 5 min and then with deionized water for 3 times.

Determination of Cd and other metals in plant tissues

Dry plant tissues were ground to fine powder and digested with 5 ml HNO_3/$HClO_4$ (85:15 v/v) in a heating block. Blanks and a certified reference material (rice grain) were included in the digestion for quality control. The concentrations of Cd and other metals were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Total DNA extraction and analysis of *OsHMA3* sequences

To determine the coding sequences of *OsHMA3* of the cultivars used in the present study, total DNA was extracted from 12-d-old rice shoots. Four fragments covering the full-length of *OsHMA3* were amplified by PCR using four primer sets (Supporting Information Table S2). The primers were designed on the basis of the genomic DNA sequence in Nipponbare according to the information of Os07G12900 in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Functional analysis of *OsHMA3* alleles in yeast

Saccharomyces cerevisiae reference strain INVSc1 (Mata; his3Δ1; leu2; trp1-289; ura3-52) and SEY6210 (MATα; leu2-3,112; ura3-52; his3-Δ200; trp1-Δ901; suc2-Δ9; lys2-801) were obtained from Changsha Yingrun Biotechnology Co. Total RNA was extracted from the roots of five rice cultivars representing different *OsHMA3* alleles using a plant RNA extraction kit (BioTeke). After the reaction with DNase I (TAKARA), the total RNA was converted to cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme). The cDNA fragment containing the entire open reading frame (ORF) of *OsHMA3* was amplified by PCR with the primer set listed in Supporting Information Table S2. The fragments containing the ORF were inserted into the *HindIII* and *EcoRI* sites of the yeast expression vector pYES2.0 (Changsha Yingrun Biotechnology Co.) and transformed into the wild-type yeast INVSc1 strain, and wild-type SEY6210 strain and *ycf1* mutant (a Cd-sensitive mutant). The transformed yeast was selected on a SD medium without uracil (SD-U). Positive clones were cultured in SD-U liquid media with 2% glucose to the early log phase, enriched and washed with sterile water for three times. Five microliters of the cell suspension with an initial OD value of 1.0 and four serial 1:10 dilutions were spotted on SD-U plates containing 0, 5, or 10 μM $CdCl_2$ (for INVSc1 strain), or 0, 15 or 30 μM CdCl2 (for SEY6210 strain and *ycf1* mutant) in the presence of 2% glucose or galactose. The plates were incubated at 30°C for 3 d before the growth phenotypes were evaluated.

To determine Cd accumulation in yeast, the transformed yeast strains were cultured in SD-U liquid media containing 2% glucose to the log phase, enriched by centrifugation and washed with sterile water for 3 times. The cells were transferred to SD-U liquid media containing 2% galactose with an initial OD value of 0.2. After incubation for 3 h, the media were amended with 5 μM $CdCl_2$ and the cells were cultured for 12 h. The cells were washed

with a cold (4°C) EDTA solution (10 μM, pH 5.0) twice and then with deionized water twice, and freeze dried. The cells were digested in 5 ml HNO_3 in a microwave oven prior to Cd determination by ICP-MS.

Detection of two loss-of-function alleles of *OsHMA3* in the GWA panel

Mutations at the 80th (Ueno *et al.* 2010) and 380th (the present study) amino acid residues are associated with a loss of function of OsHMA3. The frequencies of these two alleles in the GWA panel of 533 rice accessions were determined using Derived Cleaved Amplified Polymorphic Sequences (dCAPS) assay with the markers listed in Supporting Information Table S2. The fragment was subject to enzyme-assisted digestion through Hpy188I and then detected by polyacrylamide gel electrophoresis.

The expression level of *OsHMA3*

Twelve-d-old seedlings of four cultivars representing different alleles of *OsHMA3* were grown in the ½ Kimura B nutrient solution containing 0 or 1 μM $CdCl_2$ for 12 h. Total RNA was extracted from the roots and converted to cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme). The *OsHMA3* expression level was quantified using an AceQ™ qPCR SYBR® Green Master Mix (Vazyme) with the primer set listed in Supporting Information Table S2. Histone H3 was used as an internal standard. Data were collected in accordance with the CFX96 Real-Time PCR Detection System (Bio-Rad).

Subcellular localisation of OsHMA3

The ORF of *OsHMA3* from the cDNA fragment of Nipponbare or cultivar No. 35 was amplified using primers 5'-AAAAAGCAGGCTTCATGGCCGGAAAGGATGAGGCGGAA-3' and 5'-AGAAAGCTGGGTTCATCCTTTCACTTCACCGGAGTTCAT-3'. The *OsHMA3* fragments were recombined into a N-YFP plasmid (pEarlyGate 104). The resulting constructs or empty vector were coated with 1 μm gold particles and bombarded into onion epidermal cells to analyze transient expression. YFP fluorescence was observed using confocal laser microscopy (LSM750; Carl Zeiss).

Cadmium speciation by X-ray absorption spectroscopy (XAS)

Cd speciation in roots and shoots of three cultivars representing functional and non-functional *OsHMA3* was determined using Cd *K*-edge X-ray absorption near edge structure (XANES) spectroscopy. Twenty-day-old seedlings were grown in nutrient solution containing 1 μM $CdCl_2$ for 3 d. Roots were immersed in 10 mM $Ca(NO_3)_2$ for 5 min to desorb apoplastic Cd and then washed with deionized water three times. Roots and shoots were blotted dry, frozen in liquid nitrogen and freeze dried. The Cd XANES data were collected using a liquid helium cryostat at the XAS Beamline at the Australian Synchrotron, Melbourne. The spectra were collected in fluorescence mode with a 100-element solid-state Ge detector. To prepare samples, ca. 1-2 g freeze dried tissues were ground under liquid nitrogen using an agate mortar and pestle, and the homogenized tissues were pressed as tablets using a hand pellet press. Eleven aqueous standard compounds were prepared by mixing $Cd(NO_3)_2$ with various ligands. In all instances, the final concentration of Cd was 1 mM, together with 1%

polygalacturonate (the main component of cell walls) or 50 mM of one of nine ligands: cysteine, citrate, glutathione, histidine, methionine, malate, phytate, phytochelatin 2, and succinate, with pH being adjusted to 6.0 using 0.1 M NaOH. An aqueous solution of $Cd(NO_3)_2$ (1 mM) was also analysed. All aqueous standards were mixed in 30% glycerol to avoid ice crystal formation. The XANES spectra of rice samples (2-5 scans per sample) and standards (two scan per standard) were energy normalized using Athena software. Principal component analysis (PCA) of the normalized sample spectra was used to estimate the likely number of species contained in the samples, while target transformation was used to identify relevant standards for linear combination fitting (LCF) of the sample spectra. For the LCF, the fitting energy range was –30 to +100 eV relative to the Cd K-edge, and a maximum of three standards was permitted for each fit.

Statistical analysis

Data were analysed using one-way ANOVA followed by Turkey's HSD test ($P < 0.05$).

RESULTS

Genotypic variation in Cd accumulation

To test the range of variation and the stability of Cd accumulation across different environments, 20 rice cultivars were grown in two field experiments and a soil pot experiment. Grain Cd concentration varied by 9.4, 12.6 and 50.9 fold, respectively, in the three experiments (Fig. 1A, C, E), whilst straw Cd concentration varied by 5.5, 5.4 and 22.6

fold, respectively (Fig. 1B, D, F). Analysis using the Spearman's rank correlation showed a highly significant ($r = 0.69$, $P < 0.001$) correlation between grain Cd concentrations in the two field experiments. The correlation between the field and pot experiments was weaker but still significant ($r = 0.41 – 0.51$, $P < 0.05$). Cultivar No. 35 had the highest grain Cd concentration among all lines in all three experiments, whereas cultivars No. 32 and 172 were consistently low across the three experiments. Other cultivars were more variable in the ranking across the three experiments. There were significant correlations between grain and straw Cd concentrations in each experiment, but the slope of the regression line varied by 9 fold (Supporting Information Fig. S1). The slope was the largest in the experiment at the uncontaminated field site and the lowest at the Cd-contaminated field site, suggesting a possible saturation in the Cd translocation from straw to grain when the availability of Cd in the soil was high.

Compared with Cd, the variations in the concentrations of Zn in grain and straw among the 20 cultivars were considerably smaller (2.6 – 4.0 fold and 2.3 – 2.8 fold, respectively, in the three experiments; Supporting Information Fig. S2). There were no significant correlations between the concentrations of Cd and Zn in grain or in straw.

The 20 rice cultivars were also grown in a hydroponic experiment with 0.5 μM Cd to test the variation in the distribution of Cd between roots and shoots (Fig. 1G, H). Compared with the field and soil pot experiments, the variations in shoot and root Cd concentrations were relatively small, by 4.3 and 2.5 fold, respectively. Cultivar No. 35 had the highest concentration of Cd in shoots, which is consistent with it being the highest Cd accumulator in grain in the field and pot experiments. In contrast, this cultivar had the lowest Cd concentration in roots. Among all rice cultivars, there was no significant correlation between shoot and root Cd concentrations (Supporting Information Fig. S3).

Polymorphism in the OsHMA3 protein coding sequence

Previous reports have identified a recessive loss-of-function allele of *OsHMA3*, leading to excessive Cd accumulation in rice shoots and grain in a number of *Indica* cultivars (Ueno *et al.* 2010; Miyadate *et al.* 2011). The coding regions of *OsHMA3* of the 20 rice lines studied above were therefore sequenced. Based on the *OsHMA3* sequences, seven protein coding haplotypes could be predicted (Fig. 2A, Supporting Information Fig. S4). Ten of the 20 rice cultivars have the same haplotype as that of the Nipponbare OsHMA3 (designated as type I), which is known to be functional (Ueno *et al.* 2010). None of the *OsHMA3* sequences of the 20 rice lines has the same loss-of-function allele reported for the high Cd varieties Anjana Dhan (the protein coding haplotype designated as type VIII here) (Ueno *et al.* 2010). Compared with the functional type I OsHMA3, the high Cd cultivar No. 35 has a single amino acid mutation at the 380th position with Ser being substituted by Arg (designated as type II). Types III and IV also have a single amino acid change compared to type I, whereas types V to VII have multiple changes in amino acid residues. Types I to V are present predominantly in *Japonica* cultivars, whereas types VI to VIII are present mostly in *Indica* cultivars (Supporting Information Table S1). Prediction using the SOSUI programme (http://bp.nuap.nagoya-u.ac.jp/sosui/) showed no changes in the transmembrane domains (TM) among OsHMA3 haplotypes I to VII (Fig. 2B), which is in contrast to the non-functional type VIII with a predicted extra TM and a mislocation of the ATP-binding domain to the vacuolar side resulting from the Arg to His substitution at the 80th position (Fig. 2C). The 380th amino acid that was mutated in type II was predicted to be located near the inner surface (the vacuolar side) of the TM6 (Fig. 2B).

To assess the frequency of the type II and VIII alleles of *OsHMA3* in the GWA diversity panel, we used dCAPS markers targeting the SNPs coding for the 380th and 80th amino acid

residues, respectively. Among the 533 genetically diverse rice cultivars (Chen *et al.* 2014), four cultivars had the 380th mutation allele, all belonging to temperate *Japonica* with the origin of the variety being either China or Uzbekistan (Supporting Information Table S1). Only one cultivar (No. 483) had the 80th mutation allele, which belonged to the Aus group and originated from Pakistan. Sequence analysis confirmed that these two alleles were the same as those in cultivar No. 35 and Anjana Dhan, respectively.

We further analysed the frequency of the type II allele in a sequenced set of 950 worldwide rice accessions reported by Huang *et al.* (2012). Seven accessions were found to possess the type II allele, all belonging to temperate *Japonica* subspecies.

Heterologous expression of different *OsHMA3* alleles in yeast

To investigate the activity of Cd transport, 5 different alleles of *OsHMA3* were chosen to be expressed in yeast with the *GAL1* promoter in the pYES2 vector. The alleles tested included the *OsHMA3* sequences coding for type I, II, IV, V and VIII. In the presence of glucose, no difference in the Cd sensitivity was found among the yeast strains expressing either the vector control or different alleles of *OsHMA3* (Fig. 3A). In the presence of galactose, which induced gene expression, the yeast strains expressing the *OsHMA3* alleles coding for types I, IV and V were more sensitive to Cd than the vector control, whereas those expressing the alleles coding for types II and VIII showed a similar Cd sensitivity to the vector control (Fig. 3B). Despite multiple amino acid substitutions in the haplotype V, it did not appear to affect its Cd transport activity. Types I, II and VIII alleles were also expressed in the Cd-sensitive yeast mutant *ycf1*. Similar to the experiment with the wild-type yeast, expression of type I allele in *ycf1* increased its Cd sensitivity, whereas expression of either type II or VIII allele had no effect (Supporting Information Fig. S5). The results for the haplotypes I (Nipponbare,

functional) and VIII (Anjana Dhan, 80th mutation) were consistent with the report by Ueno *et al.* (2010).

Cd accumulation in yeast was also determined. The yeast strains expressing the *OsHMA3* alleles coding for types I, IV and V accumulated significantly more Cd than the vector control, whereas those expressing the alleles coding for types II and VIII accumulated similar Cd concentration to the vector control (Fig. 3C). The results suggest that the haplotypes I, IV and V were functional, whereas the haplotypes II and VIII were inactive.

F_1 allelic test

To confirm that the *OsHMA3* allele of cultivar No. 35 (coding for haplotype II) causes high Cd accumulation in shoots, No. 35 was crossed with Anjana Dhan which has a recessive mutation in the 80th amino acid in OsHMA3 resulting in a high Cd accumulation phenotype (Ueno *et al.* 2010). F_1 plants, the two parental lines and Nipponbare were grown in hydroponic culture with 0.1 μM Cd for 7 d. Cultivar No. 35 and Anjana Dhan showed similar Cd distribution phenotype, with a much higher shoot Cd concentration and a much lower root Cd concentration than those of Nipponbare (Fig. 4). Seven F_1 plants from the No. 35 / Anjana Dhan cross showed the same phenotype as the two parental lines, indicating that the allele coding for haplotype II in cultivar 35 was not able to complement the loss-of-function *OsHMA3* allele of Anjana Dhan and is therefore also a non-functional allele.

Subcellular localisation of OsHMA3

Ueno *et al.* (2010) showed that OsHMA3 is localised to the tonoplast for both type I and VIII alleles. To investigate if mutation at the 380th position (type II) affects the subcellular

localisation of OsHMA3, fusion proteins of YFP-OsHMA3 (type I and II) were transiently expressed in onion epidermal cells. For both types, the fluorescence signal appears to be associated with endomembrane, possibly including tonoplast, with the nucleus being outside of the signal (Supporting Information Fig. S6).

Cd translocation from roots to shoots

To further investigate the Cd translocation phenotype, two to four cultivars possessing the *OsHMA3* alleles coding for the haplotypes I, II and VIII, respectively, were grown hydroponically and exposed to 0.5 μM Cd for 7 d. Cd concentrations in roots of the type I cultivars were significantly higher than those of type II and VIII, whereas shoot Cd concentrations of type II and VIII cultivars were significantly higher than those of type I cultivars (Fig. 5). Type VIII cultivars also had a higher shoot Cd concentration than type II cultivars in this experiment. The shoot to root Cd concentration ratio followed the order of type VIII > type II > type I. On average, the ratio of type II and VIII was 5 and 10 fold, respectively, of that of type I. The concentrations of Cd in xylem sap were also higher in type II and VIII than in type I (Fig. 5). By contrast to Cd, there was no systematic difference between the three haplotypes in Zn translocation from roots to shoots (Supporting Information Fig. S7).

To investigate the difference in Cd translocation from roots to shoots across a range of environmentally realistic Cd concentrations, rice cultivars representing OsHMA3 haplotypes I, II, V and VIII were grown hydroponically and exposed to different Cd concentrations for 3 d. Although root and shoot Cd concentrations generally increased with the increasing Cd concentration in the external medium, there were clear differences between different types (Fig. 6). The type II and VIII cultivars had lower Cd concentrations in roots than the type I

and V cultivars, whereas the opposite was true for shoot Cd concentration. At 0.05 and 0.1 μM Cd, the type II and VIII cultivars accumulated similar concentrations of Cd in shoots, whereas at higher concentrations of Cd (0.2 and 0.5 μM Cd), shoot Cd concentration in the type VIII cultivar was higher than that in the type II cultivar. The ratio of shoot to root Cd concentration was markedly higher in the type II and VIII cultivars than in the type I and V cultivars. At 0.5 μM Cd, the ratio was also significantly higher in type VIII than that in type II. The pattern of xylem sap Cd concentration was similar to that of shoot Cd concentration, with the type II and VIII cultivars having significantly higher concentrations than the type I and V cultivars.

A time-course experiment was conducted where plants were exposed to 0.1 μM Cd for 1 to 5 d. Cd concentrations in roots increased with time, but more slowly in the type II and VIII cultivars than in the type I and V cultivars (Fig. 7). In contrast, Cd concentration in shoots increased rapidly in the type II and VIII cultivars, but only slowly in the type I and V cultivars. Similarly, the ratio of shoot to root Cd concentration and xylem sap Cd concentration increased linearly with time in the type II and VIII cultivars, but remained at low levels and changed little in the type I and V cultivars during the time course. In this experiment, there was no significant difference between the type II and VIII cultivars in the four parameters described above, which was consistent with the results obtained at 0.1 μM Cd in the concentration-dependent experiment (Fig. 6).

Cd uptake kinetics

Cd uptake kinetics in four cultivars representing different *OsHMA3* alleles were determined in a short-term (20 min) experiment at either 25 ^{o}C or 4 ^{o}C (Supporting Information Fig. S8). At 4 ^{o}C, Cd accumulation in roots was small in all cultivars and increased linearly with

solution Cd concentration, suggesting that the accumulation represents apoplastic binding of Cd. There were no significant differences in apoplastic binding of Cd between cultivars. At 25 ^{o}C, Cd accumulation in roots increased with the external Cd concentration in a hyperbolic pattern consistent with the Michalies-Menten kinetics. K_m varied from 2.1 to 4.1 μM, whilst V_{max} varied from 210 to 332 mg Cd kg^{-1} root dry weight h^{-1}. The V_{max} of cultivar No. 483 (type VIII) was approximately 60% larger than that in other three cultivars, suggesting that this cultivar has a higher rate of Cd uptake in the high external Cd range.

***OsHMA3* expression**

Quantitative RT-PCR was used to determine the expression level of *OsHMA3* in the roots of different rice cultivars. There were no significant differences in *OsHMA3* expression between the four cultivars tested, representing types I, II, V and VIII, in either the absence or the presence of Cd (Fig. 8). The presence of Cd tended to decrease the expression of *OsHMA3*, but the effect was significant only in cultivars No. 124 (type V) and 483 (type VIII).

Cd speciation using XANES

A loss of function of OsHMA3 may lead to changes in Cd speciation in plant tissues. This possibility was investigated using synchrotron based X-ray absorption near edge structure (XANES). Rice plants representing the OsHMA3 haplotypes I, II and VIII were treated with 1 μM Cd for 3 d and the Cd XANES spectra of root and shoot tissues were obtained (Fig. 9A, B). The spectra could be fitted with a combination of Cd complexed with thiol (S-bound) or carboxylate (O-bound) compounds. In the rice line possessing a functional allele of *OsHMA3* (haplotype I, represented by cultivar No. 32), all of the Cd in roots was found to be

complexed with thiol compounds (Fig. 9C). In contrast, in the two rice cultivars with loss of function alleles of *OsHMA3* (haplotypes II and VIII, represented by cultivars 35 and 483, respectively), between 10 and 15% of the Cd in roots was complexed with carboxylate groups with the remainder being complexed with thiol compounds. In the shoot tissues, approximately 60 – 80% of the Cd was complexed with thiol compounds and the remainder being complexed with carboxylate groups (Fig. 9D), and there was not systematic difference between cultivars with the functional or loss of function alleles.

DISCUSSION

Previous studies have identified a recessive loss-of-function allele of *OsHMA3* leading to high Cd accumulation in the shoots and grain of a number of *Indica* rice cultivars (Ueno *et al.* 2010; Miyadate *et al.* 2011; Ueno *et al.* 2011a). Using heterologous expression in yeast, Ueno *et al.* (2010) further demonstrated that a mutation at the 80th position of amino acid (R80H) was responsible for the loss of function in this allele, whereas the deletion of the 826th – 878th amino acid residues in the C terminus had no impact on its Cd transport activity. Among the seven alleles of *OsHMA3* present in the 20 rice cultivars investigated initially in our study, we have identified a new loss-of-function allele that is present in a number of temperate *Japonica* rice cultivars and is also associated with high Cd accumulation in the shoots and grain. This allele is not present in any of the *Indica* cultivars in a GWA diversity panel of 533 rice cultivars (Chen *et al.* 2014) or in a sequenced set of 950 worldwide rice varieties (Huang *et al.* 2012). Compared with the functional allele represented by the *OsHMA3* of Nipponbare, this new allele has a single SNP in the coding region leading to a mutation in the 380th amino acid from Ser to Arg (Fig. 2A). Heterologous expression in both the wild-type and *ycf1* mutant of yeast showed that the OsHMA3 haplotype (type II) coded by the No. 35 allele was

not active in Cd transport, resulting in the same Cd sensitivity and accumulation phenotypes in yeast as the vector control and the strain expressing the known non-functional allele of Anjana Dhan (type VIII) (Fig. 3). When expressed in yeast, both the functional (type I) and non-functional (type VIII) OsHMA3 proteins have been previously shown to be expressed but mislocalised to the ER (Ueno *et al.* 2010). In the case of functional OsHMA3, this mislocalisation resulted in increased Cd sensitivity rather than decreased sensitivity that would be expected if the protein were to be localised to the tonoplast (Ueno *et al.* 2010). Further evidence for the type II allele being non-functional came from the cross between No. 35 and Anjana Dhan, showing that the No. 35 allele could not complement the Anjana Dhan allele in the F_1 offspring (Fig. 4).

Because Arg is positively charged within the physiological pH range, a mutation from Ser to Arg is expected to change the charge characteristics of the transporter protein. Prediction of the transmembrane domain using the SOSUI programme suggests that the 380th amino acid residue of OsHMA3 is located at the membrane surface facing the vacuolar side (Fig. 2B). A positively charged Arg located at this position may therefore block the entry of cationic Cd^{2+} via charge repulsion. Mutation at the 380th position does not affect the subcellular localisation of OsHMA3 (Supporting Information Fig. S6), as has been demonstrated for the type VIII allele of OsHMA3 (Ueno *et al.* 2010).

When rice cultivars with different alleles of *OsHMA3* were grown in hydroponic experiments, the cultivars possessing both the type II and VIII alleles under different genetic backgrounds exhibited consistent phenotypes of decreased Cd concentration in roots, increased Cd concentrations in the xylem sap and shoots and a greatly increased shoot to root Cd concentration ratio (Figs. 5-7). These phenotypes can be explained by the loss of function of OsHMA3 in transporting Cd into the vacuoles in roots for sequestration (Ueno *et al.* 2010). By contrast, the Cd translocation phenotype was not related to the expression level of

OsHMA3 in rice roots (Fig. 8, also Ueno *et al.* 2010), as has also been found in *A. thaliana* (Chao *et al.* 2012). Cultivars with the type VIII allele appeared to have a greater shoot Cd accumulation than those with the type II allele at relatively high concentrations (0.2 – 0.5 μM) of external Cd (Figs. 5 and 6). However, this difference may be caused by other factors besides *HMA3*. Short-term uptake kinetics showed that a type VIII cultivar used in the experiments happened to have a greater Cd uptake than other cultivars in the high Cd concentration range (Supporting Information Fig. S8). The fact that the type II allele does not complement the loss-of-function type VIII allele (Ueno *et al.* 2010) in the F1 test (Fig. 4) and the similar phenotype observed when both alleles were expressed in yeast (Fig. 3) suggest that the type II allele is also a loss-of-function allele rather than a partial loss-of-function allele.

HMA3 proteins presumably transport Cd into the vacuoles in the ionic form. Cd inside the vacuoles is mostly complexed with thiol compounds, such as phytochelatins (PCs), which may further transform into high molecular weight (HMW) complexes by incorporating sulphide (Rauser 1995; Cobbett & Goldsbrough 2002). PCs or PC-Cd complexes can be transported into the vacuoles via AtABCC1/2 transporters in *A. thaliana* (Song *et al.* 2010; Park *et al.* 2012), although OsABCC1 does not confer Cd tolerance in rice (Song *et al.* 2014). Previous studies using synchrotron X-ray absorption spectrometry have shown that most Cd in the roots of *Brassica junceae* and rice was bonded to S (Salt *et al.* 1995; Yamaoka *et al.* 2010). This was confirmed in the present study for the rice cultivar possessing a functional allele of *OsHMA3*. However, the loss of function of *OsHMA3* resulted in a noticeable shift in the Cd speciation in roots from Cd-S bonding to a mixture of Cd-S and Cd-O bonding (Fig. 9). This shift may arise from a decreased Cd sequestration in the vacuoles, leaving some Cd to form weaker complexes with O-ligands in the cytoplasm, which are likely to be more available for efflux towards the xylem for root-to-shoot translocation. Because *OsHMA3* is

mainly expressed in rice roots (Ueno *et al.* 2010), its loss of function would not impact on the Cd speciation in the shoots.

The Arabidopsis AtHMA3 is able to transport Cd, Zn, Co and Pb (Morel *et al.* 2009), and NcHMA3 from *Noccaea caerulescens* can transport Cd and Zn (Ueno *et al.* 2011b). A recent study by Sasaki et al. (2014) showed that overexpression of *OsHMA3* increased the accumulation of both Cd and Zn in rice roots, supporting the notion that OsHMA3 is able to transport both Cd and Zn into the root vacuoles. However, unlike the Cd translocation phenotype, there was no systematic difference in the root-to-shoot translocation of Zn between rice cultivars possessing either functional or non-functional alleles of *OsHMA3* (Supporting Information Fig. S7). Similarly, silencing or overexpressing of *OsHMA3* had little effect on Zn accumulation in rice shoots or grain (Ueno *et al.* 2010; Sasaki *et al.* 2014). A likely explanation is that the homeostasis of Zn, an essential micronutrient, is tightly regulated, and an alteration in the expression level or the function of *OsHMA3* could be compensated by other transporters, as suggested by Chao *et al.* (2012) for *A. thaliana*. For example, Sasaki *et al.* (2014) showed that the expressions of five *ZIP* genes possibly involved in Zn transport were upregulated in the transgenic rice overexpressing *OsHMA3*. These results support the conclusion that OsHMA3 impacts mainly the vacuolar sequestration and subsequent root-to-shoot translocation of Cd, not Zn, in rice.

Both the type II and type VIII loss-of-function alleles of *OsHMA3* are rare in a GWA panel that includes 533 rice lines of diverse genetic diversity. The type II allele was found in four cultivars of temperate *Japonica* rice, whilst only one *Aus* cultivar had the type VIII allele. Additionally, 7 varieties belonging to temperate *Japonica* were found to have the type II allele in a worldwide collection of 950 accessions (Huang *et al.* 2012). The geographical distributions of the two alleles are also different, with the type II allele being present mainly in Chinese cultivars grown in the temperate region along the Yangtze River and the type VIII

allele being distributed in south Asia (cultivar No. 483 from Pakistan and Anjana Dhan from Nepal). GWA is not able to identify these rare alleles because of the low statistical power for associating phenotype with rare genotype. It is interesting to note that GWA was successfully used to identify *AtHMA3* as a key determinant of natural variation in shoot Cd accumulation in *A. thaliana* (Chao *et al.* 2012). In that study the hypofunctional alleles of *AtHMA3* were found to be more common than the hyperfunctional alleles with a frequency ratio of about 2:1. It is speculated that hypofunctional alleles of *AtHMA3* may confer adaptive advantages in restricting Zn sequestration in root vacuoles and allowing more Zn to be transported to the shoots in Zn-deficient environment, whereas the hyperfunctional alleles of *AtHMA3* may confer Cd tolerance in the Cd-enriched environment (Chao *et al.* 2012). The adaptive advantages of OsHMA3 loss-of-function alleles, if any, are unknown. Nevertheless, it is remarkable that not only is a significant portion of variation in Cd accumulation in both rice and *A. thaliana* driven by variation in HMA3 but also that the type of variation is conserved (Ueno *et al.* 2010; Chao *et al.* 2012; also the present study). It is the variation in the protein with amino acid polymorphisms affecting the transport activity rather than in the expression of the gene. This suggests that selection is acting to modulate the same regions of the HMA3 protein across plant species.

Although it is generally thought that *Indica* cultivars tend to accumulate more Cd in shoots and grain than *Japonica* cultivars (Uraguchi & Fujiwara 2013), the present study has shown that some *Japonica* cultivars can also accumulate very high concentrations of Cd in grain (Fig. 1). This high accumulation is caused by the loss of function of OsHMA3, but the allele identified in the present study is different from that previously reported for high Cd accumulating *Indica* cultivars (Ueno *et al.* 2010; Miyadate *et al.* 2011). Molecular markers can be used to screen these alleles to identify cultivars with high Cd accumulation genotypes. These genotypes can then be excluded in breeding programmes to avoid high Cd

accumulation in rice grain. Alternatively, the high Cd genotypes can be selected for phytoremediation of Cd-contaminated paddy soil (Murakami *et al.* 2009).

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REFERENCES

Åkesson A., Barregard L., Bergdahl I.A., Nordberg G.F., Nordberg M. & Skerfving S. (2014) Non-renal effects and the risk assessment of environmental cadmium exposure. *Environmental Health Perspectives*, **122**, 431-438.

Arao T. & Ae N. (2003) Genotypic variations in cadmium levels of rice grain. *Soil Science and Plant Nutrition*, **49**, 473-479.

Chao D.-Y., Silva A., Baxter I., Huang Y.S., Nordborg M., Danku J., . . . Salt D.E. (2012) Genome-wide association studies identify heavy metal ATPase3 as the primary determinant of natural variation in leaf cadmium in *Arabidopsis thaliana*. *PloS Genetics*, **8**, e1002923.

Chen W., Gao Y.Q., Xie W.B., Gong L., Lu K., Wang W.S., . . . Luo J. (2014) Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. *Nature Genetics*, **46**, 714-721.

Clemens S., Aarts M.G.M., Thomine S. & Verbruggen N. (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends in Plant Science*, **18**, 92-99.

Cobbett C. & Goldsbrough P. (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, **53**, 159-182.

Du Y., Hu X.F., Wu X.H., Shu Y., Jiang Y. & Yan X.J. (2013) Affects of mining activities on Cd pollution to the paddy soils and rice grain in Hunan province, Central South China. *Environmental Monitoring and Assessment*, **185**, 9843-9856.

He J.Y., Zhu C., Ren Y.F., Yan Y.P. & Jiang D. (2006) Genotypic variation in grain cadmium concentration of lowland rice. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde*, **169**, 711-716.

Huang X., Zhao Y., Wei X., Li C., Wang A., Zhao Q., . . . Han B. (2012) Genome-wide association study of flowering time and grain yield traits in a worldwide collection of rice germplasm. *Nature Genetics*, **44**, 32-U53.

Ishikawa S., Ishimaru Y., Igura M., Kuramata M., Abe T., Senoura T., . . . Nakanishi H. (2012) Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 19166-19171.

Jin T.Y., Kong Q.H., Ye T.T., Wu X.W. & Nordberg G.F. (2004) Renal dysfunction of cadmium-exposed workers residing in a cadmium-polluted environment. *Biometals*, **17**, 513-518.

Jin T.Y., Nordberg M., Frech W., Dumont X., Bernard A., Ye T.T., . . . Nordberg G.F. (2002) Cadmium biomonitoring and renal dysfunction among a population environmentally exposed to cadmium from smelting in China (ChinaCad). *Biometals*, **15**, 397-410.

Meharg A.A., Norton G., Deacon C., Williams P., Adomako E.E., Price A., . . . Islam M.R. (2013) Variation in rice cadmium related to human exposure. *Environmental Science & Technology*, **47**, 5613-5618.

Miyadate H., Adachi S., Hiraizumi A., Tezuka K., Nakazawa N., Kawamoto T., . . . Akagi H. (2011) OsHMA3, a P(1B)-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. *New Phytologist*, **189**, 190-199.

Morel M., Crouzet J., Gravot A., Auroy P., Leonhardt N., Vavasseur A. & Richaud P. (2009) AtHMA3, a P(1B)-ATPase allowing Cd/Zn/Co/Pb aacuolar storage in Arabidopsis. *Plant Physiology*, **149**, 894-904.

Murakami M., Nakagawa F., Ae N., Ito M. & Arao T. (2009) Phytoextraction by rice capable of accumulating Cd at high levels: Reduction of Cd content of rice grain. *Environmental Science & Technology*, **43**, 5878-5883.

Nawrot T., Plusquin M., Hogervorst J., Roels H.A., Celis H., Thijs L., . . . Staessen J.A. (2006) Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *Lancet Oncology*, **7**, 119-126.

Nordberg G.F. (2009) Historical perspectives on cadmium toxicology. *Toxicology and Applied Pharmacology*, **238**, 192-200.

Park J., Song W.-Y., Ko D., Eom Y., Hansen T.H., Schiller M., . . . Lee Y. (2012) The

phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. *Plant Journal*, **69**, 278-288.

Pinson S.R.M., Tarpley L., Yan W.G., Yeater K., Lahner B., Yakubova E., . . . Salt D.E. (2015) World-wide genetic diversity for mineral element concentrations in rice grain. *Crop Science*, **55**, 1-18.

Rauser W.E. (1995) Phytochelatins and related peptides - structure, biosynthesis, and function. *Plant Physiology*, **109**, 1141-1149.

Salt D.E., Prince R.C., Pickering I.J. & Raskin I. (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiology*, **109**, 1427-1433.

Sasaki A., Yamaji N. & Ma J.F. (2014) Overexpression of OsHMA3 enhances Cd tolerance and expression of Zn transporter genes in rice. *Journal of Experimental Botany*, **65**, 6013-6021.

Sasaki A., Yamaji N., Yokosho K. & Ma J.F. (2012) Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell*, **24**, 2155-2167.

Satoh-Nagasawa N., Mori M., Nakazawa N., Kawamoto T., Nagato Y., Sakurai K., . . . Akagi H. (2012) Mutations in rice (*Oryza sativa*) Heavy Metal ATPase 2 (OsHMA2) restrict the translocation of zinc and cadmium. *Plant and Cell Physiology*, **53**, 213-224.

Song W.-Y., Yamaki T., Yamaji N., Ko D., Jung K.-H., Fujii-Kashino M., . . . Ma J.F. (2014) A rice ABC transporter, OsABCC1, reduces arsenic accumulation in the grain. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 15699-15704.

Song W.Y., Park J., Mendoza-Cozatl D.G., Suter-Grotemeyer M., Shim D., Hortensteiner S., . . . Martinoia E. (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 21187-21192.

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

Ueno D., Kono I., Yokosho K., Ando T., Yano M. & Ma J.F. (2009) A major quantitative trait locus controlling cadmium translocation in rice (*Oryza sativa*). *New Phytologist*, **182**, 644-653.

Ueno D., Koyama E., Yamaji N. & Ma J.F. (2011a) Physiological, genetic, and molecular characterization of a high-Cd-accumulating rice cultivar, Jarjan. *Journal of Experimental Botany*, **62**, 2265-2272.

Ueno D., Milner M.J., Yamaji N., Yokosho K., Koyama E., Zambrano M.C., . . . Ma J.F. (2011b) Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of Thlaspi caerulescens. *Plant Journal*, **66**, 852-862.

Ueno D., Yamaji N., Kono I., Huang C.F., Ando T., Yano M. & Ma J.F. (2010) Gene limiting cadmium accumulation in rice. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 16500-16505.

Uraguchi S. & Fujiwara T. (2013) Rice breaks ground for cadmium-free cereals. *Current Opinion in Plant Biology*, **16**, 328-334.

Uraguchi S., Kamiya T., Sakamoto T., Kasai K., Sato Y., Nagamura Y., . . . Fujiwara T. (2011) Low-affinity cation transporter (OsLCT1) regulates cadmium transport into rice grains. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 20959-20964.

Uraguchi S., Mori S., Kuramata M., Kawasaki A., Arao T. & Ishikawa S. (2009) Root-to-shoot Cd translocation via the xylem is the major process determining shoot and grain cadmium accumulation in rice. *Journal of Experimental Botany*, **60**, 2677-2688.

Watanabe T., Shimbo S., Nakatsuka H., Koizumi A., Higashikawa K., Matsuda-Inoguchi N. & Ikeda M. (2004) Gender-related difference, geographical variation and time trend in dietary cadmium intake in Japan. *Science of the Total Environment*, **329**, 17-27.

Williams P.N., Lei M., Sun G.X., Huang Q., Lu Y., Deacon C., . . . Zhu Y.G. (2009) Occurrence and partitioning of cadmium, arsenic and lead in mine impacted paddy rice: Hunan, China. *Environmental Science & Technology*, **43**, 637-642.

Yamaji N., Xia J., Mitani-Ueno N., Yokosho K. & Ma J.F. (2013) Preferential delivery of zinc to developing tissues in rice is mediated by P-type Heavy Metal ATPase OsHMA2. *Plant Physiology*, **162**, 927-939.

Yamaoka W., Takada S., Takehisa H., Hayashi Y., Hokura A., Terada Y., . . . Nakai I. (2010) Study on accumulation mechanism of cadmium in rice (*Oriza sativa* L.) by micro-xrf imaging and x-ray absorption fine structure analysis utilizing synchrotron radiation. *Bunseki Kagaku*, **59**, 463-475.

Yang M., Zhang Y.Y., Zhang L., Hu J., Zhang X., Lu K., . . . Lian X.M. (2014) OsNRAMP5 contributes to manganese translocation and distribution in rice shoots *Journal of Experimental Botany*, **65**, 4849-4861.

Zhang W.L., Du Y., Zhai M.M. & Shang Q. (2014) Cadmium exposure and its health effects: A 19-year follow-up study of a polluted area in China. *Science of the Total*

Environment, **470**, 224-228.

Zhao F.-J., Ma Y., Zhu Y.-G., Tang Z. & McGrath S.P. (2015) Soil contamination in China: Current status and mitigation strategies. *Environmental Science & Technology*, **49**, 750-759.

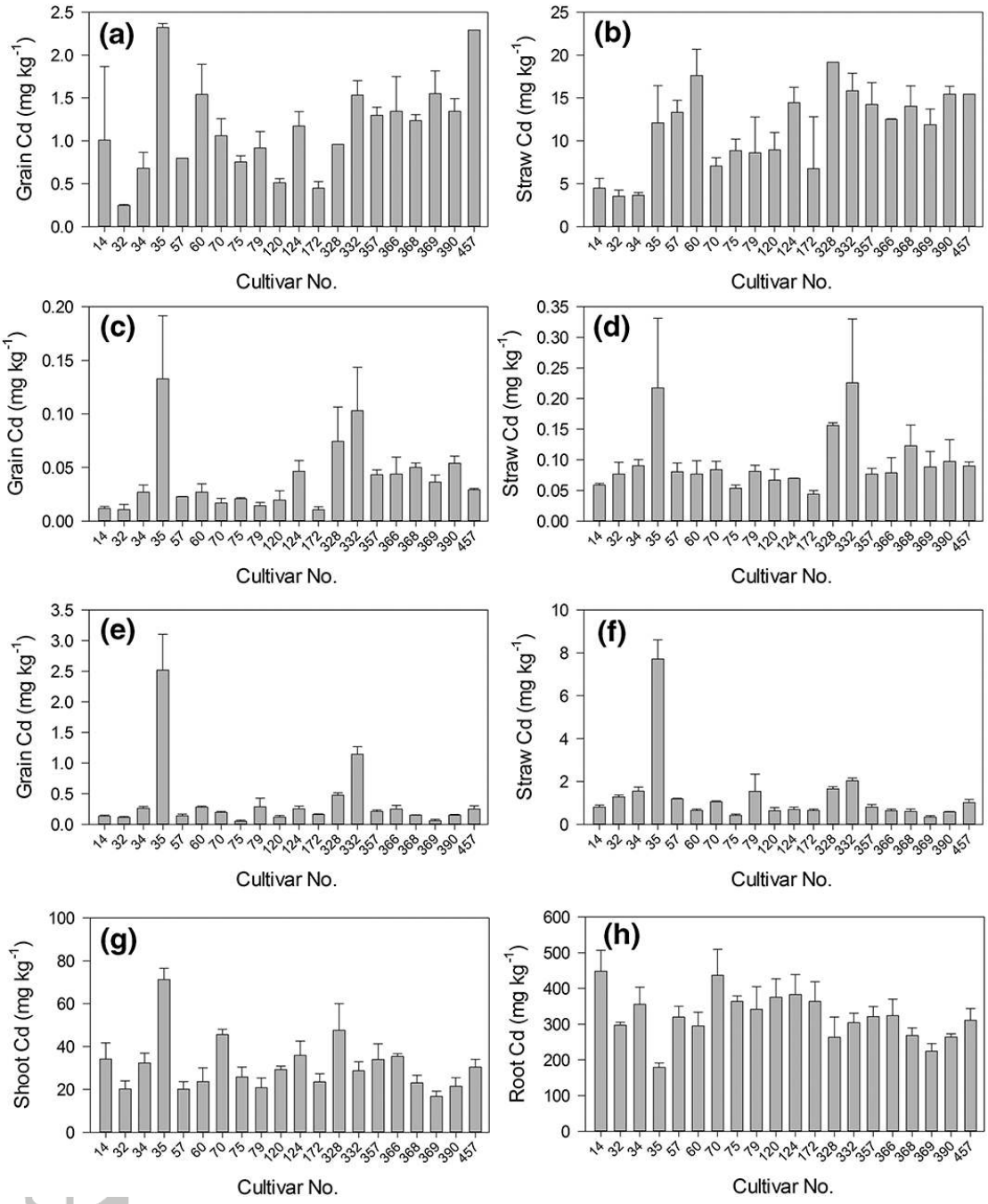

FIGURE 1. Cd concentrations in rice grain (A, C, E), straw (B, D, F), shoot (G) and root (H) of 20 cultivars grown in field experiments at a Cd-contaminated soil (A, B) or a uncontaminated soil (C, D), a pot experiment amended with 2 mg Cd kg^{-1} soil (E, F) or in a hydroponic experiment with 0.5 μM Cd (G, H). Data are means ± SD (n = 3 biological replicates).

(a)

OsHMA3 haplotype	Amino Acid Position												Cultivars*
	80	299	380	550	614	638	678	728	736	752	775	826-878	
I	R	F	S	V	S	A	C	T	G	V	E		Nipponbare, 32, 34, 57, 60, 70, 75, 120, 172, 328, 332
II	R	F	R	V	S	A	C	T	G	V	E		35
III	R	F	S	V	S	A	C	T	G	V	D		457
IV	R	F	S	V	S	A	C	T	C	V	E		14, 79
V	R	L	S	I	G	A	R	T	G	A	E		124, 357, 368, 369
VI	R	L	S	I	G	A	R	T	C	A	E		390
VII	R	L	S	I	G	A	R	P	C	A	E		366
VIII	H	L	S	V	D	V	R	T	G	A	E	Deletion	Anjana Dhan, CCG, 483

* See Supporting Information Table S1 for cultivar names and subspecies information.

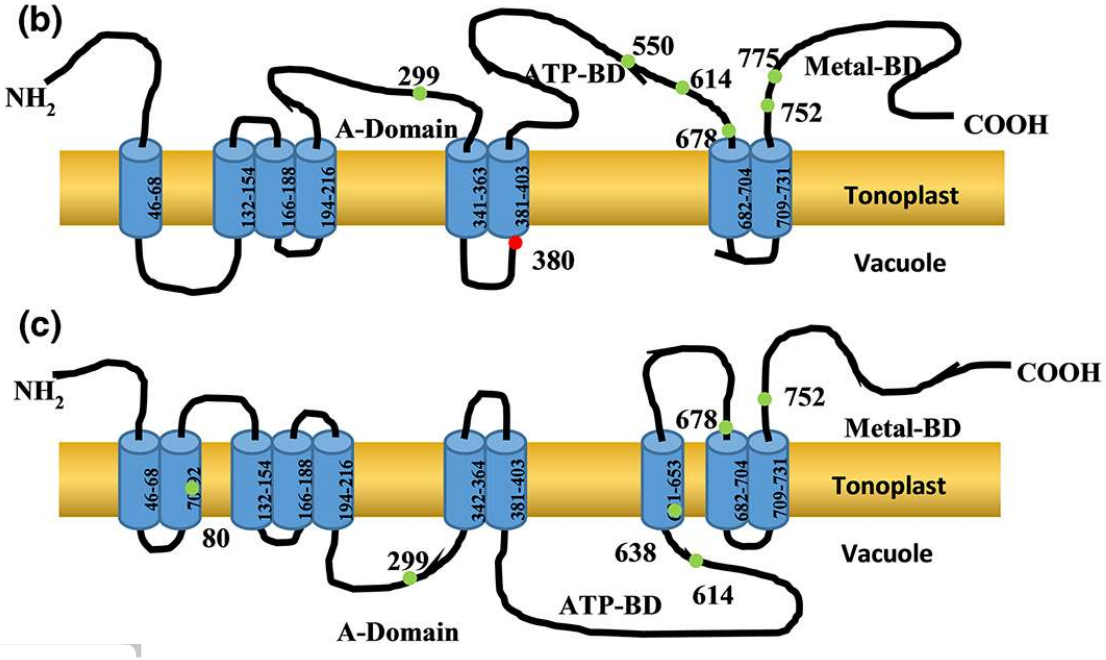

FIGURE 2. OsHMA3 haplotypes present in the rice cultivars used in the present study (A) and predicted transmembrane domains of OsHMA3 (B, C). (B) Haplotypes I – VII; (C) Haplotype VIII. Prediction was made by using the SOSUI programme (http://bp.nuap.nagoya-u.ac.jp/sosui/).

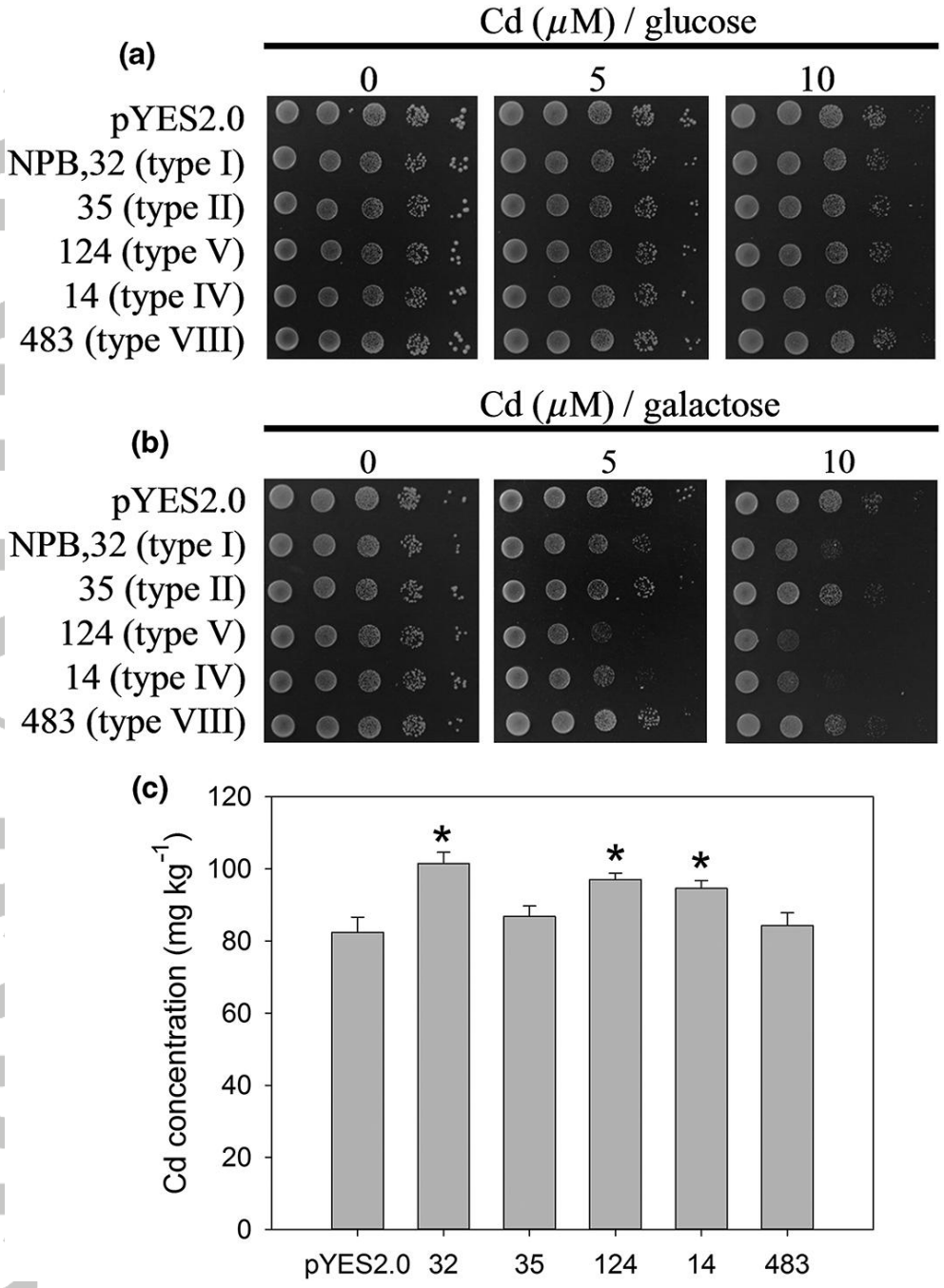

FIGURE 3. Functional assay of *OsHMA3* by heterologous expression in yeast. Yeast strains expressing the empty vector pYES2.0 or different alleles of *OsHMA3* were grown in SD-U medium containing different Cd concentrations in the presence of glucose (A) or galactose (B), and Cd concentration of yeast cells expressing the empty vector or different *OsHMA3* alleles after exposure to 5 μM Cd for 24 h (C). Data in (C) are means ± SD (n = 3 biological replicates). Different letters indicate significant difference. NPB, Nipponbare.

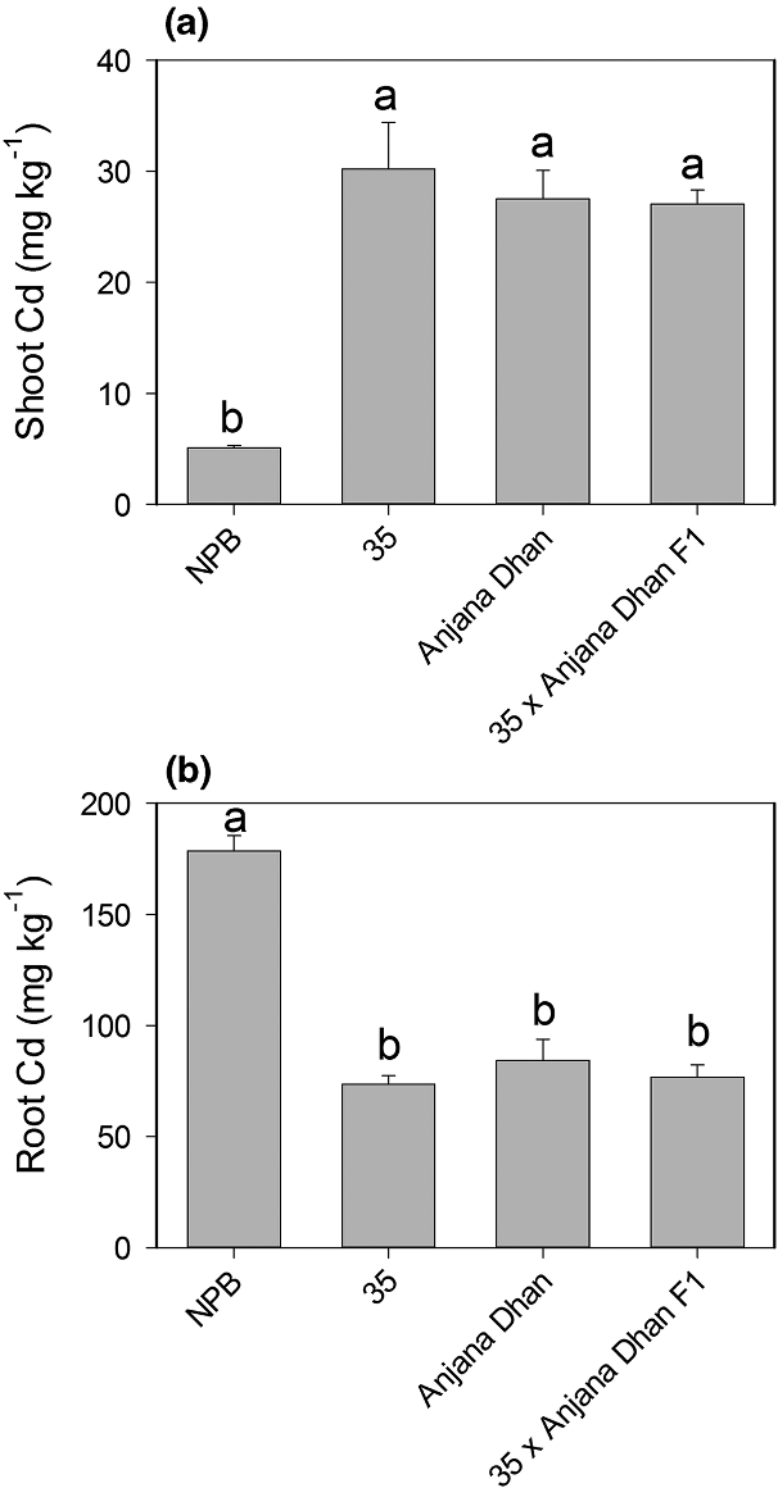

FIGURE 4. F_1 allelic test. Cd concentrations in shoots (A) and roots (B) of Nipponbare (NPB), line No. 35, Anjana Dhan and 35 x Anjana Dhan F_1 offspring grown in 0.1 μM Cd for 7 days. Data are means ± SD (n = 4 or 7 biological replicates for parental lines and F1 offspring, respectively). Different letters indicate significant difference.

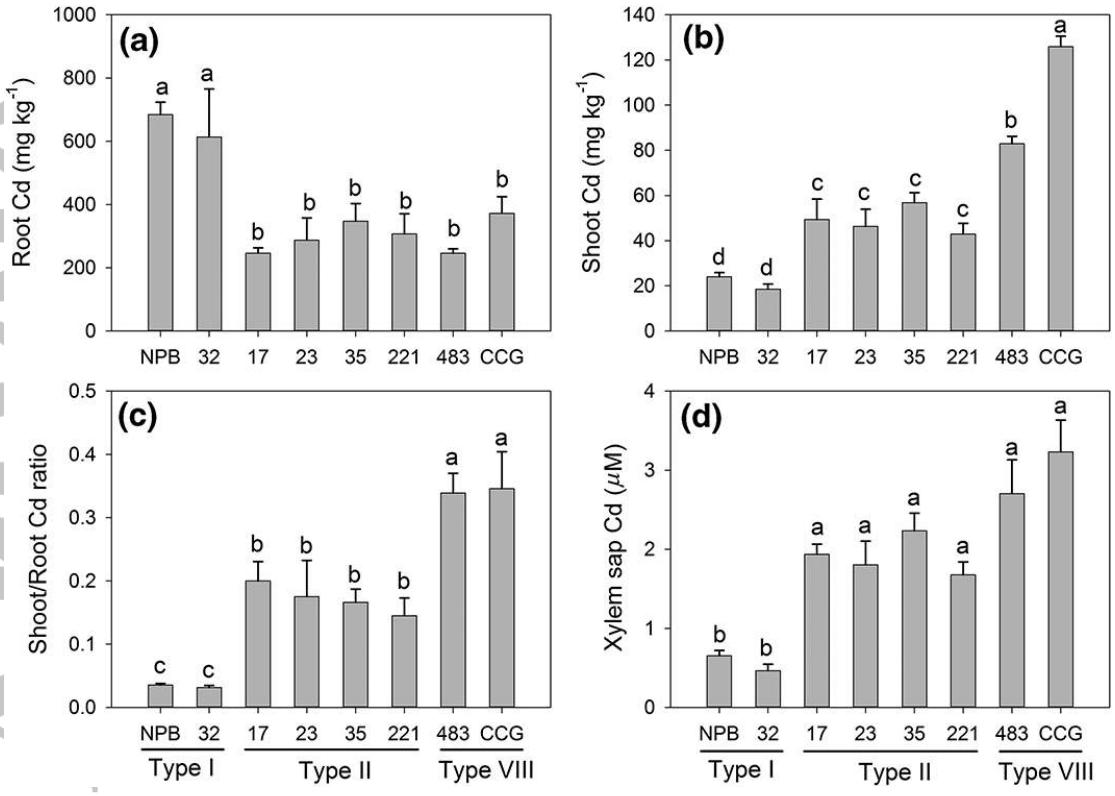

FIGURE 5. Cd concentrations in roots (A), shoots (B), xylem sap (D) and the ratio of shoot to root Cd concentration (C) in different rice cultivars representing type I, II and VIII alleles of *OsHMA3*. Plants were grown in 0.5 μM Cd for 7 days. Data are means ± SD (n = 4 biological replicates). Different letters indicate significant difference.

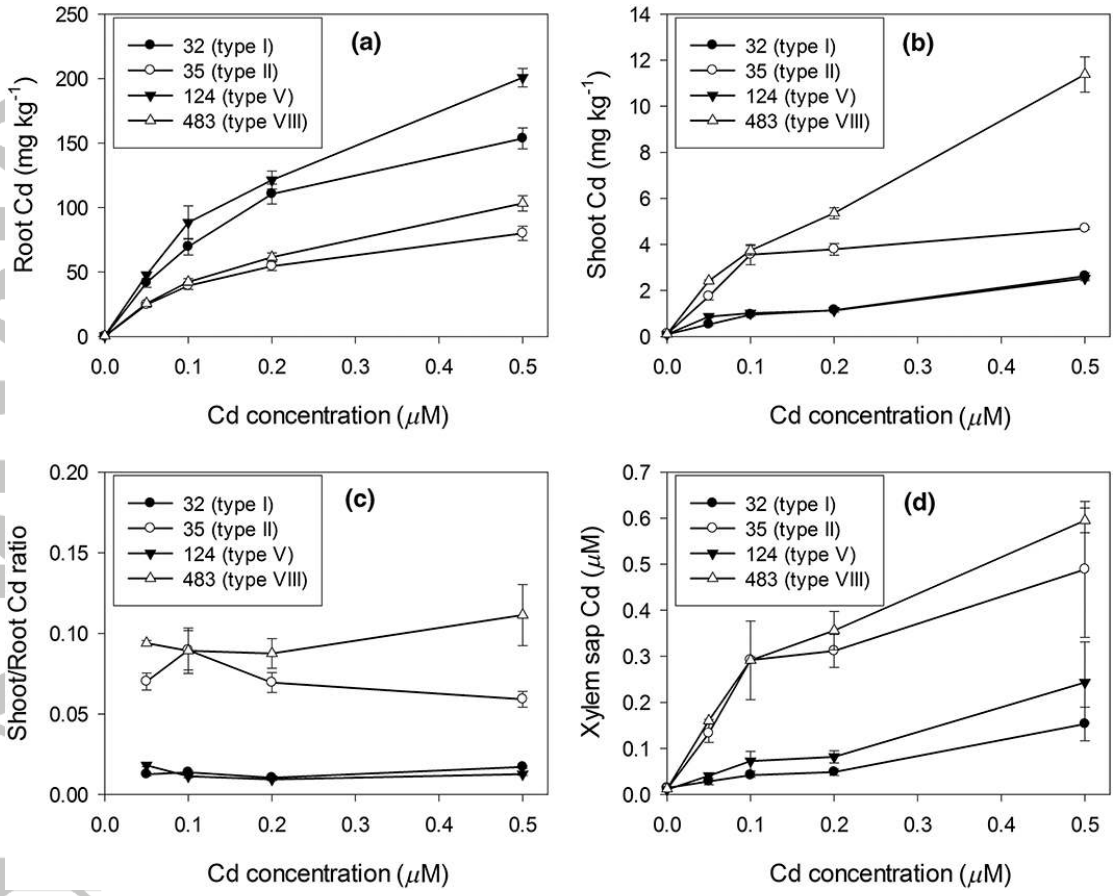

FIGURE 6. Concentration-dependent Cd uptake and translocation. Cd concentrations in roots (A), shoots (B), xylem sap (D) and the ratio of shoot to root Cd concentration (C) in different rice cultivars representing type I, II, V and VIII alleles of *OsHMA3*. Plants were grown in 0 - 0.5 μM Cd for 3 days. Data are means ± SD (n = 4 biological replicates).

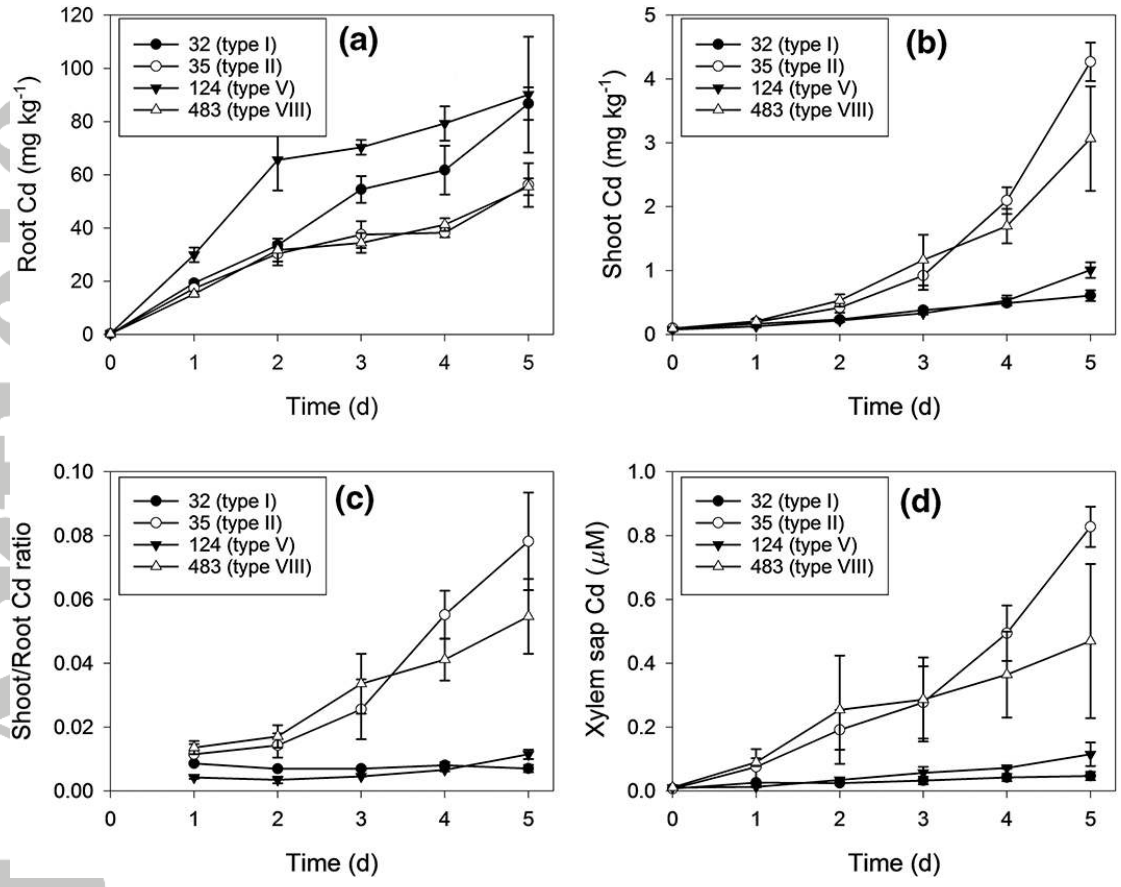

FIGURE 7. Time-course of Cd uptake and translocation. Cd concentrations in roots (A), shoots (B), xylem sap (D) and the ratio of shoot to root Cd concentration (C) in different rice cultivars representing type I, II, V and VIII alleles of *OsHMA3*. Plants were grown in 0.1 μM Cd for 1 - 5 days. Data are means ± SD (n = 4 biological replicates).

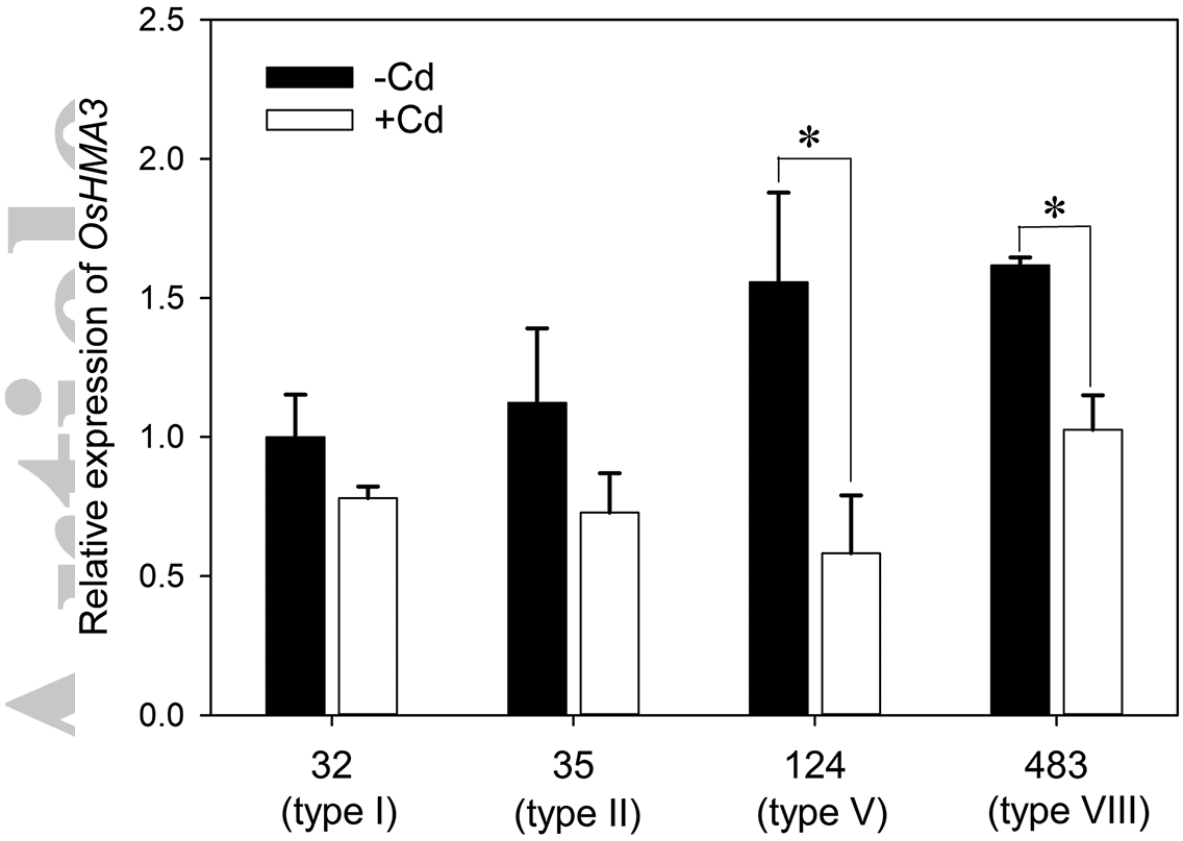

FIGURE 8. Relative expression of *OsHMA3* in rice roots of different cultivars representing type I, II, V and VIII alleles of *OsHMA3* with or without exposure to 1 μM Cd for 12 h. Data are means ± SD (n = 3 biological replicates). * indicates significant difference.

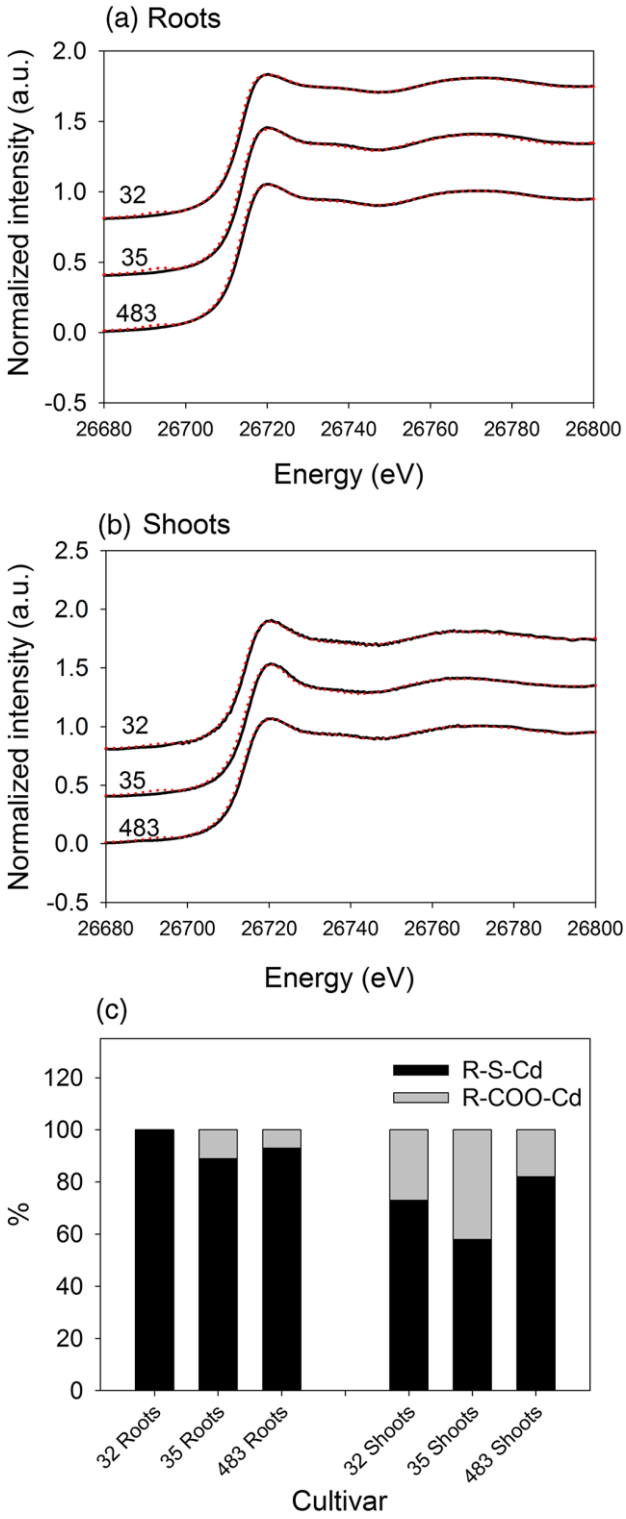

FIGURE 9. Cd speciation in rice roots and shoots by synchrotron X-ray absorption spectroscopy. X-ray absorption near edge structure (XANES) spectra of roots (A) and shoots (B), and the proportions of Cd bonded to S or O ligands in roots and shoots (C) of three rice cultivars representing type I, II and VIII alleles of *OsHMA3*.