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ORIGINAL PAPER

Knockout of *OsHMA3* in an *indica* rice increases cadmium sensitivity and inhibits plant growth

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Abstract

Cadmium (Cd) is a heavy metal that poses harm to both plants and humans. OsHMA3, a member of the heavy metal ATPase (HMA) family, plays a crucial role in sequestering Cd into the vacuoles of roots, thereby limiting its accumulation in rice grains. However, the response of rice plants to Cd under complete loss-of-function of *OsHMA3* remains unclear. In this study, we successfully generated *OsHMA3* null mutants in an *indica* variety 93 − 11 using CRISPR/Cas9 technology. A Cd resistance experiment revealed that the *Oshma3* mutants exhibited increased sensitivity to Cd compared to the wild-type at a tested concentration of 10 μM $CdCl_2$. Furthermore, the seedlings of *Oshma3* mutant lines displayed inhibited plant growth in the presence of 1 μM Cd, specifically suppressing aboveground growth. As expected, knockout lines of *OsHMA3* showed lower Cd accumulation in roots but higher concentrations in shoots compared to wild-type plants, highlighting the role of *OsHMA3* in root-to-shoot Cd translocation. We further performed RNA sequencing analysis on wild-type and *Oshma3* plants under control and Cd treatment conditions and found that differentially expressed genes were mainly enriched in metal ion binding, integral component of the membranes, and biosynthesis pathways for secondary metabolites triggered by exposure to Cd. When grown in a paddy field, the *Oshma3* mutants exhibited shorter plant height, lower seed setting rate, and higher Cd accumulation in grains compared to wild-type plants. Our results indicate that knockout of *OsHMA3* in the 93 − 11 variety increases sensitivity to Cd and inhibits plant growth.

Keywords Cadmium · *OsHMA3* · Loss-of-function · Sensitivity · *Indica* rice variety

Introduction

Cadmium (Cd) is a highly toxic heavy metal that poses significant risks to both plants and humans (Soni et al. 2024). In plants, Cd accumulation can have multiple direct and indirect effects on growth and disrupt various physiological functions (Haider et al. 2021; Tang et al. 2023). For humans, even small amounts of Cd exposure, well below the limits for acute toxicity, can lead to disease due to its long-term bioaccumulation in the human body (Uraguchi and Fujiwara 2012; Clemens et al. 2013; Clemens and Ma 2016). However, the contamination of Cd in agricultural soils has become a serious issue in recent years, primarily attributed to the widespread practice of intensive mining and industrial activities (Zhao et al. 2015; Shi et al. 2019). As a consequence, crops grown in these contaminated soils tend to accumulate significant levels of Cd in their edible parts, leading to a substantial Cd intake for humans through their diet (Wang et al. 2019). Rice is a major crop in China and holds significant importance in the Chinese population's diet (Song et al. 2017). Nevertheless, it is pertinent to acknowledge that rice has also been identified as the primary dietary source of Cd for the Chinese population (Zhao et al. 2015; Song et al. 2017). Thus, it is crucial to understand the mechanisms that control Cd uptake and translocation in rice.

Huijing Yan and Xiaozhen Jiao contributed equally to this work.

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The accumulation of Cd in rice grains is a complex process that involves various steps such as root uptake, xylem loading, root-to-shoot translocation, and phloem transport (Zhao et al. 2022). Several divalent transition metal transporters in rice, including those for manganese (Mn), iron (Fe), and zinc (Zn), have been identified as crucial players in mediating the uptake and translocation of Cd (Liu et al. 2020a). For instance, OsNRAMP5, a member of the Natural Resistance-Associated Macrophage Protein (NRAMP) family, is mainly responsible for the transport of Mn and is also involved in Cd uptake (Sasaki et al. 2012; Yu et al. 2022). Another NRAMP family member, OsNRAMP1, also contributes to the uptake of both Mn and Cd (Takahashi et al. 2011; Chang et al. 2020). Furthermore, the major facilitator family protein OsCd1 and the Zn transporters OsZIP9 and OsZIP5 have been identified as key players in Cd uptake in rice (Yan et al. 2019; Tan et al. 2020). The root-to-shoot translocation of Cd is a critical process that governs the accumulation of Cd in above-ground tissues and is controlled by OsHMA2 (Takahashi et al. 2012; Satoh-Nagasawa et al. 2012; Yamaji et al. 2013). *OsHMA2* encodes a P_{1b}-type ATPase metal pump localized in the plasma membranes of pericycle cells in the roots and the phloem region in the nodes (Yamaji et al. 2013). Loss-of-function of *OsHMA2* has been shown to significantly reduce the translocation of both Cd and Zn from the roots to shoots (Satoh-Nagasawa et al. 2012). Various transporters have also been identified to regulate the Cd xylem-to-phloem transfer process, ultimately affecting the concentration of Cd in grains. For instance, OsLCT1, a low-affinity cation transporter, is highly expressed in leaf blades and nodes and helps transport Cd to the grains (Uraguchi et al. 2011). Another transporter, OsZIP7, plays a role in the translocation of Zn and Cd in rice by regulating their distribution from roots to shoots (Tan et al. 2019). The cation/Ca exchanger OsCCX2 is involved in the intervascular transfer of Cd in rice nodes, and the knockout of this transporter leads to a decrease in Cd accumulation in rice grains (Hao et al. 2018).

Among the transporters identified to be involved in Cd accumulation in rice, OsHMA3 acts as a key determinant in minimizing Cd levels in grains by regulating the rate of Cd translocation from roots to shoots (Clemens and Ma 2016; Zhao et al. 2022). The first reported occurrence of *OsHMA3* being responsible for Cd accumulation in rice was identified in a mapping population obtained from a cross between a high and low Cd-accumulating cultivar (Ueno et al. 2010). *OsHMA3*, whcih encodes a P_{1B}-type of ATPase, influences root-to-shoot Cd translocation in rice by facilitating efflux into vacuoles (Miyadate et al. 2011). The function of *OsHMA3* was found to be absent in high-Cd cultivars in both studies (Ueno et al. 2010; Miyadate et al. 2011). Subsequently, new loss-of-function alleles of *OsHMA3* have been identified in specific rice germplasm resources (Yan et al. 2016; Sui et al. 2019; Sun et al. 2019). Further research has revealed that variations in the promoter region of *OsHMA3* play a role in the differential accumulation of Cd in grain between *Indica* and *Japonica* rice (Liu et al. 2020a). Additionally, *OsHMA3* also has an important role in maintaining Zn homeostasis in rice (Cai et al. 2019). Overexpressing *OsHMA3* not only reduces Cd transportation from roots to shoots but also enhances rice tolerance to Cd stress (Sasaki et al. 2014; Lu et al. 2019). Utilizing the *OsHMA2* promoter to regulate the expression of *OsHMA3* has been shown to effectively decrease Cd accumulation in rice grains (Shao et al., 2018).

Although several naturally occurring loss-of-function alleles of *OsHMA3* have been extensively studied in previous research (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019; Sun et al. 2019), the creation of an artificially induced loss-of-function mutant of *OsHMA3* has not been reported thus far. Therefore, in this study, we successfully generated *OsHMA3* null mutants in the *indica* variety 93 – 11 utilizing CRISPR/Cas9 technology. Our results indicate that the knockout of *OsHMA3* lines dramatically increases the susceptibility of rice to Cd and inhibits plant growth even with low Cd exposure.

Materials and methods

Vector construction and plant transformation

The genomic sequence of *OsHMA3* was downloaded from the Rice Genome Annotation Project website. To confirm the presence of any SNP at the designated target site in the transformed 93 – 11 variety, primers were designed to amplify a fragment that encompassed 379 bp upstream and 248 bp downstream of the target site for Sanger sequencing. The CRISPR-Cas9 vector for disrupting *OsHMA3* was constructed utilizing the isocaudomer ligation method, as outlined in a previous study by Wang et al. (2015). The annealed HMA3g++/HMA3g– oligonucleotides were ligated into the SK-sgRNA vector that had been digested with AarI. Subsequently, the sgRNA of *HMA3* (digested with KpnI and BglII) was assembled into the pC1300-Ubi:Cas9 binary vector (digested with KpnI and BamHI) to obtain the vector pC1300-Ubi:Cas9-sgRNA^{OsHMA3}. The binary vector was then introduced into *Agrobacterium tumefaciens* (Strain EHA105) and transformed into calluses derived from the 93 – 11 variety generate transgenic lines by Wuhan Boyuan Biotech Company. All the primers for vector construction are listed in Table S1.

Detection of mutations

The genomic DNA of transgenic plants was extracted from approximately 50 mg of fresh leaf tissue using the CTAB method. PCR was carried out with KOD FX DNA polymerase (Toyobo, Japan) to amplify the fragments surrounding the *OsHMA3* target site. The primers HMA3-F and HMA3-R can be found in Table S1. Mutations in the mutants were analyzed using the Hi-TOM method as described in Liu et al. (2019).

Field experiments

The *OsHMA3* mutants, along with 93 – 11 plants, were cultivated in transgenic paddy fields at the China National Rice Research Institute in Hangzhou, China during the summer season. Each line was replicated three times in a randomized plot design, with six rows of plants per plot, totaling approximately 36 plants per line. Rice cultivation practices followed local protocols for field management. For the investigation of agronomic traits, six individual plants from each line were cultivated until maturity. The following agronomic traits were assessed: seed setting rate, plant height, tiller number per plant, panicle length, primary panicle branch number, secondary panicle branch number, grain number per panicle, flag leaf length and flag leaf width. Mature seeds from each line were used to determine grain metal element concentrations.

Hydroponic experiments

Seeds were soaked in deionized water at 37 °C in the dark for 2 days, followed by transferring them to a net floating on deionized water for an additional 5 days. The seedlings were then cultured in a half-strength Kimura B nutrient solution (pH 5.4). The composition of the solution, measured in millimolars (mM), included 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 solution was renewed every 2 days. The plants were cultivated in a greenhouse under natural sunlight, with temperatures reaching 30 °C during the day and dropping to 25 °C at night (Liu et al. 2020a). After reaching 21 days of age, the plants were exposed to the half-strength Kimura B nutrient solution (pH 5.4) containing 0 and 1 μM $CdCl_2$ for 10 days, with the solution being renewed every 2 days. Following the treatment, plants were washed with distilled water and roots and shoots were harvested separately for Cd measurement. Furthermore, five individual plants for each line were sampled and their root length, shoot length, root weight, and shoot weight were measured.

Evaluation of resistance to Cd

Ten seedlings of 93 – 11 and *Oshma3* mutants were exposed to a 0.5 mM $CaCl_2$ solution containing 0 and 10 μM $CdCl_2$ at pH 4.5 for 48 h. Relative root elongation (RRE) was used to evaluate the resistance to Cd (Liu et al. 2016).

Determination of metals in plant tissues

Metal concentrations were measured in Rice Product Quality Supervision and Inspection Center, Ministry of Agriculture and Rural Affairs, China National Rice Research Institute. Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) was employed (Instrument type: *XSeries 2*) to determine concentrations of Cd, Fe, copper (Cu), Zn, and Mn according to method for determining multiple elements in food (National Food Safety Standard, GB 5009.268–2016, China). The dried samples were digested with a mixture of HNO_3 (85%) and $HClO_4$ (15%) at a gradient temperature (60 °C for 1 h, 120 °C for 1 h, 150 °C for 1 h, and up to 190 °C) (Liu et al. 2020b).

RNA-seq and data analysis

The 21-day-old plants were exposed to the half-strength Kimura B nutrient solution containing 0 and 1 μM $CdCl_2$ for 2 days. The roots of both the 93 – 11 and *Oshma3-1* mutant were ground into a fine powder using a mortar and pestle in liquid nitrogen. Total RNA was then extracted from the samples using TRIzol reagent (Invitrogen, 15,596,026) and sent to AZenTa life sciences (Suzhou, China) for RNA-seq library construction and sequencing on an Illumina HiSeq 2000 platform. Gene ontology (GO) analysis was performed using GOseq (Young et al. 2010) and pathway enrichment analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes database (Kanehisa et al. 2008).

Results

Genome editing of *OsHMA3* in the *indica* variety 93 – 11

The *OsHMA3* gene has a full length of 3,015 base pairs (bp), distributed in 7 exons and 5 introns, encoding a Cd/Zn-transporting ATPase consisting of 1,005 amino acids (AA). Among natural cultivars, there are at least eight protein-coding haplotypes of the OsHMA3 transporter (Yan et al. 2016), with the *indica* variety 93 – 11 sharing the same protein haplotype as the functional type V OsHMA3 (Liu et al. 2020a). To disrupt the *OsHMA3* gene in the *indica* variety 93 – 11, we utilized the CRISPR/Cas9 genome editing system to

target a specific site within exon 2 of the gene (Fig. 1A). Subsequently, we successfully constructed the CRISPR-Cas9 vector pC1300-Ubi:Cas9-sgRNA^{OsHMA3} (Fig. 1B) and introduced it into 93 − 11 using *Agrobacterium*. In the T_0 generation, a total of ten independent transgenic plants were obtained, two of which exhibited homozygous mutations designated as *hma3-1* (1 bp insertion) and *hma3-2* (10 bp deletion), respectively (Fig. 1C). As shown in Fig. 1D, both mutations resulted in premature termination of *OsHMA3* transcription and ultimately encoded severely truncated proteins. These mutant lines were propagated to the T_1 generation, and their seeds were harvested for subsequent experiments.

Knockout of *OsHMA3* leads to increased sensitivity to Cd

The overexpression of *OsHMA3* has been shown to significantly increase tolerance to toxic Cd (Sasaki et al. 2014; Lu et al. 2019). However, the response of loss-of-function mutants of *OsHMA3* to Cd is not well understood. To compare the Cd resistance phenotype of *Oshma3* mutants with the WT, we exposed roots of both the WT and the mutants to Cd concentrations of 0 and 10 µM. In the absence of Cd, the root elongation of the mutants was similar to that of the WT (WT, 26.0 ± 5.5 mm/48 h; *hma3-1*, 27.6 ± 3.3 mm/48 h; *hma3-2*, 24.8 ± 4.1 mm/48 h). However, at 10 µM Cd, there was an inhibition of 67% and 65% of the root elongation in the *hma3-1* and *hma3-2* mutants, respectively, whereas the inhibition of the WT was 57% (Fig. 2). These results revealed that the knockout of *OsHMA3* made the plants more sensitive to Cd compared to the WT.

We further investigated the growth of *Oshma3* seedlings under low Cd concentration conditions. When the plants were grown under normal conditions, no noticeable diferences in seedling growth were observed between WT and *Oshma3* mutants (Fig. 3A). However, the presence of 1 µM Cd dramatically inhibited the plant growth of *Oshma3* lines compared to WT plants, particularly affecting the aboveground part of *Oshma3* mutants (Fig. 3B). Quantitative analyses confirmed that the root length, shoot length, and shoot weight of *Oshma3* plants were significantly lower than those of WT plants when grown on 1 µM Cd (Fig. 3C-F). These results indicate that the *Oshma3* mutants are sensitive to Cd.

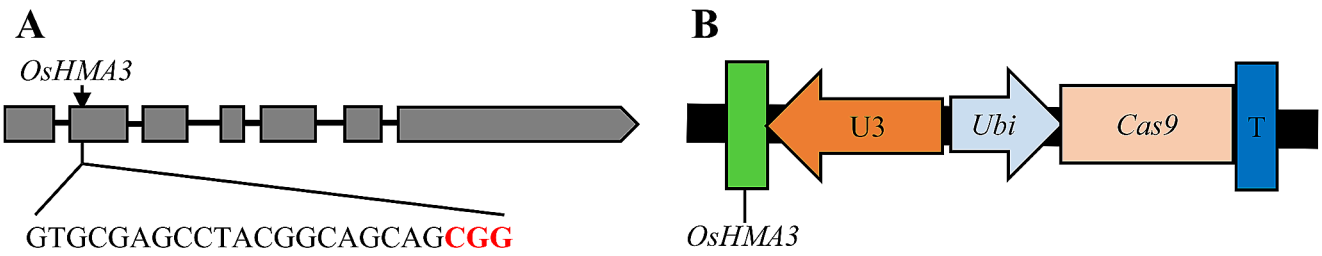
Oshma3 mutants display increased Cd accumulation in shoots

As *OsHMA3* regulates the rate of Cd translocation from roots to shoots (Miyadate et al. 2011), we further explored the impact of *OsHMA3* loss of function on Cd accumulation in roots and shoots. Under normal conditions, the *Oshma3* mutants exhibited significantly lower levels of Cd accumulation in the roots, while exhibiting much higher Cd concentrations in the shoots compared to the WT plants (Fig. 4A). In the presence of 1 µM Cd, both WT and *Oshma3* plants accumulated higher levels of Cd in both roots and shoots; however, the *Oshma3* lines had much higher Cd concentrations in the shoots than the WT plants (Fig. 4B). The Cd concentrations in WT and *Oshma3* plants were consistent with their seedling growth phenotype, indicating that the knockout of *OsHMA3* resulted in increased Cd accumulation in shoots, thus leading to a more severe growth inhibition compared to WT plants. Additionally, the accumulation of other divalent metals, including Zn, Cu, Fe, and Mn, was compared between WT and *Oshma3* mutants. As shown in Fig. 4, the *Oshma3* mutants had much higher Zn and Cu concentrations in the roots, but showed no significant difference in the shoots under both 0 and 1 µM Cd conditions when compared to the WT plants. Meanwhile, Fe and Mn showed no significant difference in both roots and shoots between WT and *Oshma3* mutants.

Transcriptional expression of *Oshma3* significantly altered by Cd exposure

To investigate the impact of *OsHMA3* loss of function on gene expression, we performed RNA sequencing experiments. In total, 36.4 ~ 47.2 million reads were obtained from the roots of WT and *Oshma3-1* plants grown in 0 and 1 µM Cd. Among them, 31.4 ~ 41.2 million reads were mapped to the reference genome (*Oryza sativa* L. *indica* 93 − 11 AA genome), with an average mapping rate of 87.2%. After filtering, the Q30 values ranged from 90.08 to 92.05%, while the GC values ranged from 49.77 to 51.05% for all four samples (Table S2). These findings indicate that the transcriptome data collected were of high quality, suitable for further bioinformatics analysis.

Using the above data, we evaluated the abundance value of gene expression in each sample using 'fragments per kilobase of exon model per million mapped reads' (FPKM). The FPKM boxplots revealed a high overall abundance of gene expression in all samples, and the transcript levels showed strong reproducibility among biological replicates (Fig. 5A). To identify differentially expressed genes (DEGs), we set a two-fold change in expression level as the threshold. In WT roots, 1,350 DEGs (645 upregulated and 705 downregulated) were observed after exposure to Cd, whereas in *Oshma3-1* roots, 2,767 DEGs (1,919 upregulated and 848 downregulated) were identified following Cd exposure. Moreover, we detected 614 DEGs (234 upregulated and 380 downregulated) between WT and *Oshma3-1* without Cd, but 1,662 DEGs (1,149 upregulated and 477 downregulated) between them in the presence of Cd (Fig. 5B;



C

Code	Alleles	Sequence	Mutation
Wild type	-	GTGCGAGCCTACGGCAGCAGCGG	-
hma3-1	a1	GTGCGAGCCTACGGCAGgCAGCGG	+1 bp
	a2	GTGCGAGCCTACGGCAGgCAGCGG	+1 bp
hma3-2	a1	GTGCGAGC----------AGCGG	-10 bp
	a2	GTGCGAGC----------AGCGG	-10 bp

D

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WT       MAGKDEAEGLEARLLLLPPEAAAEEPTRCGGGDGGGGGRKRKKTYLDVLGVCCSAEVALVERLLAPLDGVRVVSVVVASRTVVVEHDPAAAPESAIVKALNKAGLEASVRAYGSSGVVSR   120
hma3-1   MAGKDEAEGLEARLLLLPPEAAAEEPTRCGGGDGGGGGRKRKKTYLDVLGVCCSAEVALVERLLAPLDGVRVVSVVVASRTVVVEHDPAAAPESAIVKALNKAGLEASVRAYGRQ....R   116
hma3-2   MAGKDEAEGLEARLLLLPPEAAAEEPTRCGGGDGGGGGRKRKKTYLDVLGVCCSAEVALVERLLAPLDGVRVVSVVVASRTVVVEHDPAAAPESAIV.....................   97
Consensusmagkdeaeglearllllppeaaaeeptrcgggdgggggrkrkktyldvlgvccsaevalverllapldgvrvvsvvvasrtvvvehdpaaapesaiv

WT       WPSPYIVASGVLLTASFFEWLFPPLQCLAVAAVVAGAPPMVRRGFAAASRLSLDINVLMLIAVAGALCLGDYTEAGAIVFLFTTAEWLETLACTKASAGMSSLMGMLPVKAVIATTGEVV   240
hma3-1   RGQPVAEPVHRRLRR.......PPHGVLLRVALPS..PAVPGRGGRRRRR...........AADGAPWVRRGEPA.........VARHQRPHAHRRRRRALF........RRLHGGRRHRL   200
hma3-2   ..........................................................................................................................   97
Consensus

WT       SVRDVRVGDVVAVRAGEIVPVDGVVVDGQSEVDE.RSLTGESFPVPKQPHSEVWAGTMNFDGYIAVRTTALAENSTVAKMERLVEAAQNSRSKTQRLIDSCAKYYTPAVVVVAAGVALIP   359
hma3-1   PLHHRRMARDAGLHKGERR...DVVVDGHAAGEGGHRDDGRGCQRARRPRGRRRRGQG.....................RRDRPRRRRGGRRPERGRREEPHRRVLPGAEAAALRGLGRHN   297
hma3-2   ..........................................................................................................................   97
Consensus

WT       ALLGADGLEQWWKLALVMLVSACPCALVLSTPVASFCAMLRAARMGIFIKGGDVLESLGEIRAVAFDKTGTITRGEFSIDSFHLVGDHKVEMDHLLYWIASIESKSSHPMAAALVEYAQS   479
hma3-1   EL........................................................................................................................   299
hma3-2   ..........................................................................................................................   97
Consensus

WT       KSIQPNPENVGDFRIYPGEGIYGEIHGKHIYIGNRRTLARASSPQSTQEMGEMIKGVSIGYVICDGELAGVFSLSDDCRTGAAEAIRELGSLGIKSVMLTGDSSAAATHAQGQLGGVMEE   599
hma3-1   ..........................................................................................................................   299
hma3-2   ..........................................................................................................................   97
Consensus

WT       LHSELLPEDKVRLVSGLKARFGPTMMVGDGMNDAAALAAADVGVSMGISGSAAAMETSHATLMSSDVLRVPEAVRLGRCARRTIAVNVAGSVAVKAAVLALAAAWRPVLWAAVLADVGTC   719
hma3-1   ..........................................................................................................................   299
hma3-2   ..........................................................................................................................   97
Consensus

WT       LLVVLNSMTLLREEWKGGAKEDGACRATARSLVMRSQLAADSQAPNAADAGAAGREQTNGCRCCPKPGMSPEHSVVIDIRADGERQEERPAEAAVVAKCCGGGGGEGIRCGASKKPTATV   839
hma3-1   ..........................................................................................................................   299
hma3-2   ..........................................................................................................................   97
Consensus

WT       VVAKCCGGGGGGEGTRCGASKNPATAAVVAKCCSGGGGEGIGCGASKKPTATAVVAKCCGGGGEGTRCAASKKPATAAVVAKCCGGDGGEGTGCGASKRSPPAEGSCSGGEGGTNGVGRC   959
hma3-1   ..........................................................................................................................   299
hma3-2   ..........................................................................................................................   97
Consensus

WT       CTSVKRPTCCDMGAAEVSDSSPETAKDCRNGRCCAKTMNSGEVK                                                                          1003
hma3-1   ..........................................                                                                            299
hma3-2   ..........................................                                                                            97
Consensus
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Fig. 1 Genome editing of *OsHMA3* in the *indica* variety 93 – 11. (**A**) *OsHMA3* gene structure and gRNA target site. The protospacer adjacent motif sequence is highlighted in red. (**B**) The structure of CRISPR-Cas9 vector targeting *OsHMA3*. (**C**) Two CRISPR/Cas9 lines of *OsHMA3* in the 93 – 11 variety. Inserted or deleted nucleotides are indicated by red lowercase letters and dashed lines, respectively. (**D**) Amino acid sequence alignment of OsHMA3 protein of WT and mutants

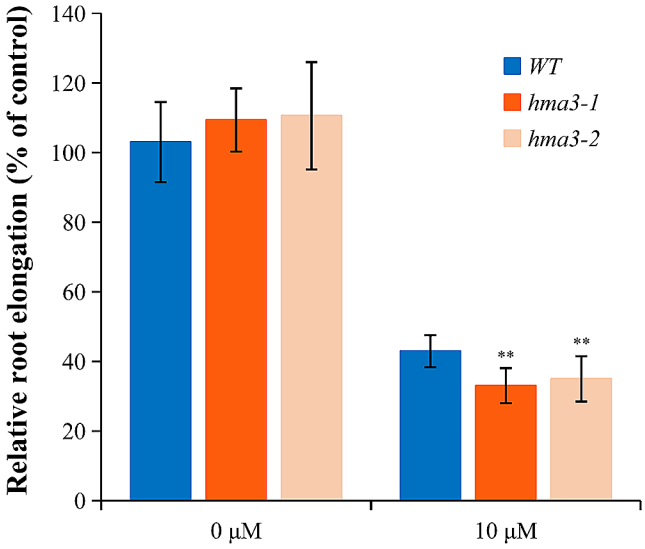

Fig. 2 Sensitivity to Cd. Seedlings of wild-type (WT) and *Oshma3* mutants were exposed for a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 and 10 μM Cd for 48 h. Data are means ± SD ($n = 10$). Relative root elongation (RRE) was used to evaluate their sensitivity to Cd. ** indicates a significance level at 1% (Student's *t*-test)

Table S3-S6). The dramatically increased number of DEGs between WT and *Oshma3-1* after Cd exposure suggests that *Oshma3-1* was more responsive to Cd at the transcriptomic level compared to WT, which was also consistent with their distinct responses to Cd exposure based on morphology. Heatmaps confirmed the notable changes in transcriptional expression of the *Oshma3* mutant after exposure to Cd, with a majority of genes showing upregulation (Fig. 5C). Furthermore, there were 539 DEGs that were shared between the two comparisons of 'WT -Cd Vs. +Cd' and '*Oshma3-1* -Cd Vs. +Cd', suggesting that these genes are likely to specifically respond to Cd. Additionally, we found 142 DEGs that were shared between the two comparisons of 'WT Vs. *Oshma3-1* -Cd' and 'WT Vs. *Oshma3-1* + Cd', indicating that these genes are key genes directly affected by the loss of function of *OsHMA3* (Fig. 5D).

We conducted additional gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to identify functional information of the DEGs obtained from the two comparisons, 'WT Vs. *Oshma3-1* -Cd' and 'WT Vs. *Oshma3-1* + Cd'. The GO

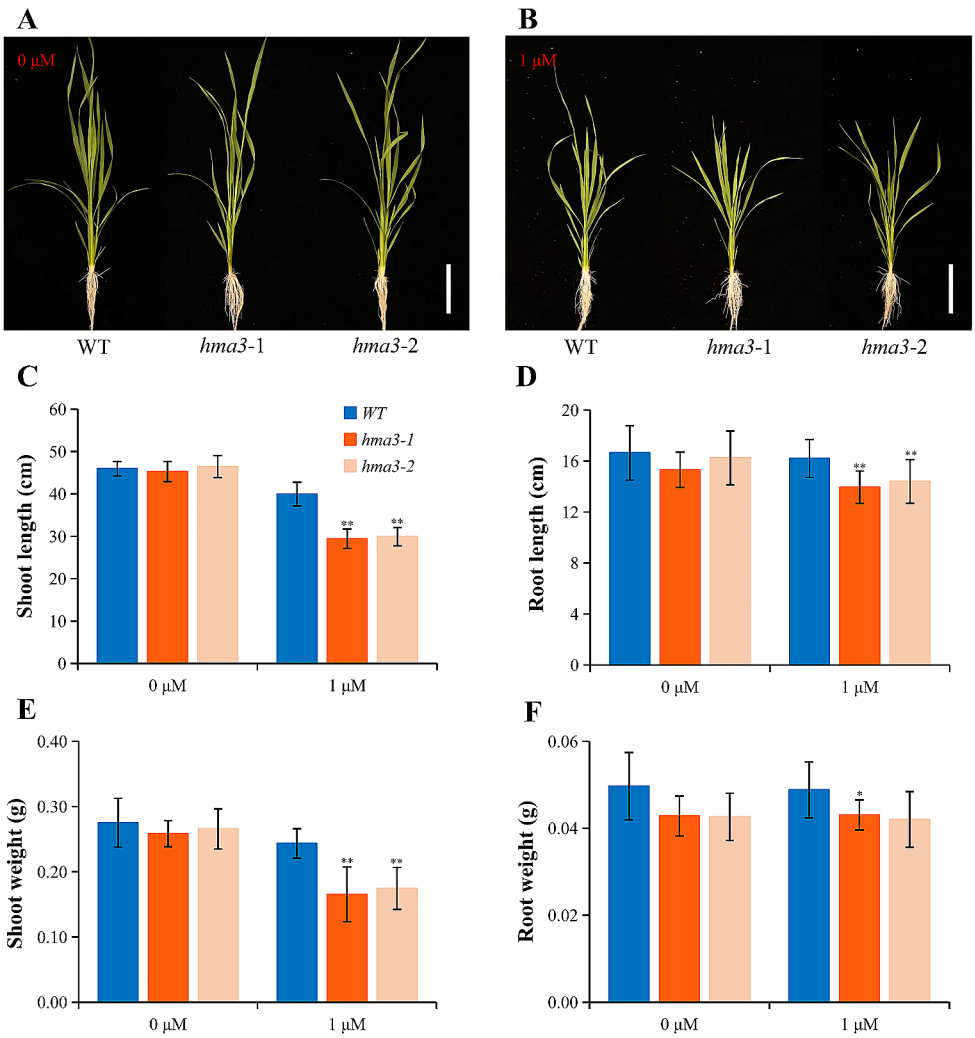

Fig. 3 Growth phenotype of the *OsHMA3* knockout mutants grown in the presence of Cd. (**A**-**B**) Phenotypes of wild-type (WT) and *Oshma3* mutants grown under control (**A**) and 1 μM Cd (**B**) for 7 d. (**C**-**D**) Weight of root (**C**) and shoot (**D**) of WT and *Oshma3* seedlings. (**E**-**F**) Length of root (**E**) and shoot (**F**) of WT and *Oshma3* seedlings. Scale bar, 10 cm. Data are means ± SD ($n = 5$ biological replicates). * and ** indicate a significance level at 5% and 1%, respectively (Student's *t*-test)

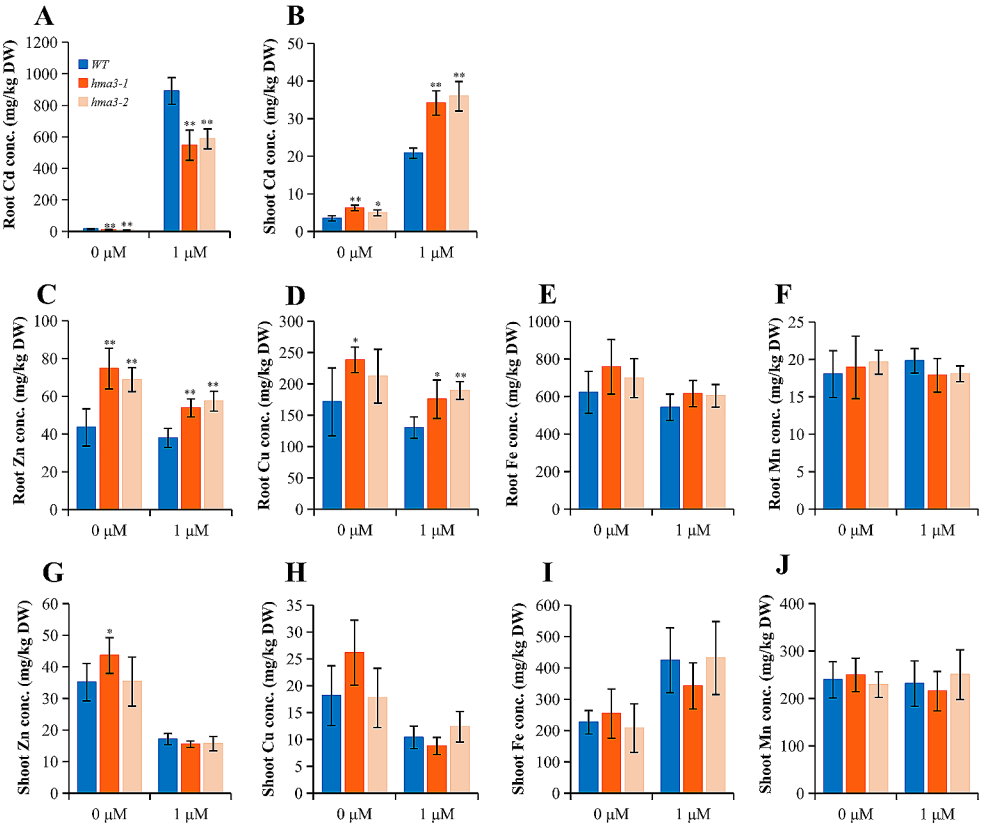

Fig. 4 Metal concentrations in the root and shoot. (**A-B**) Concentrations of Cd in root (**A**) and shoot (**B**) of WT and *Oshma3* mutants. (**C-J**) Concentrations of Zn (**C**, **G**), Cu (**D**, **H**), Fe (**E**, **I**), and Mn (**F**, **J**) in root and shoot of WT and *Oshma3* mutants. The seedlings (21-d-old) were grown under control and 1 μM Cd for 7 d. Data are means ± SD ($n = 5$ biological replicates). * and ** indicate a significance level at 5% and 1%, respectively (Student's *t*-test)

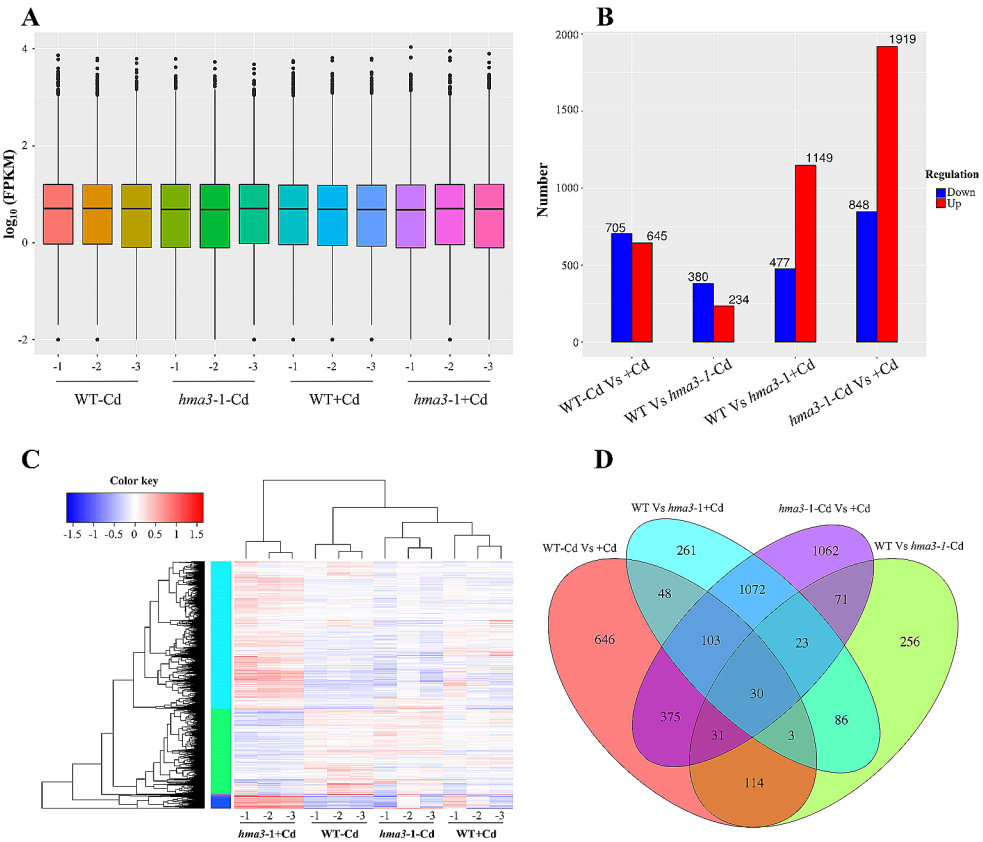

Fig. 5 Differentially expressed genes identified for wild-type (WT) and *Oshma3* mutant with or without the presence of Cd. (**A**) Transcript expression boxplots of WT and *Oshma3* plants treated with -Cd (0 μM) and + Cd (1 μM). (**B**) Number of differentially expressed genes in each comparison. (**C**) Cluster analysis of differentially expressed genes. Red represents highly expressed genes, and blue represents poorly expressed genes. (**D**) Venn diagrams for shared differentially expressed genes in all comparisons

enrichment analysis revealed that the DEGs identified between WT and *Oshma3-1* in the absence of Cd were significantly enriched in 5 GO terms (each consisting of more than 10 genes). These terms included molecular functions such as 'DNA binding' and 'sequence specific DNA binding transcription factor activity', cellular component 'extracellular region', and biological processes 'defense response' and 'defense response to fungus' (Fig. 6A). However, in the presence of Cd, the number of significantly enriched GO terms for the DEGs between WT and *Oshma3-1* increased to 24, and there was a substantial increase in the number of genes. The most enriched GO terms in molecular function changed to 'metal ion binding', and in cellular component changed to 'nucleus' (Fig. 6B). In the KEGG pathway enrichment analysis, we found that the DEGs from the 'WT Vs. *Oshma3-1* -Cd' comparison significantly enriched 15 pathways, and the most enriched pathways were 'metabolic pathways' and 'biosynthesis of secondary metabolites' (Fig. 6C). Furthermore, when Cd was present, both the number of DEGs and their expression changes in the 'biosynthesis of secondary metabolites' pathway were significantly increased (Fig. 6D).

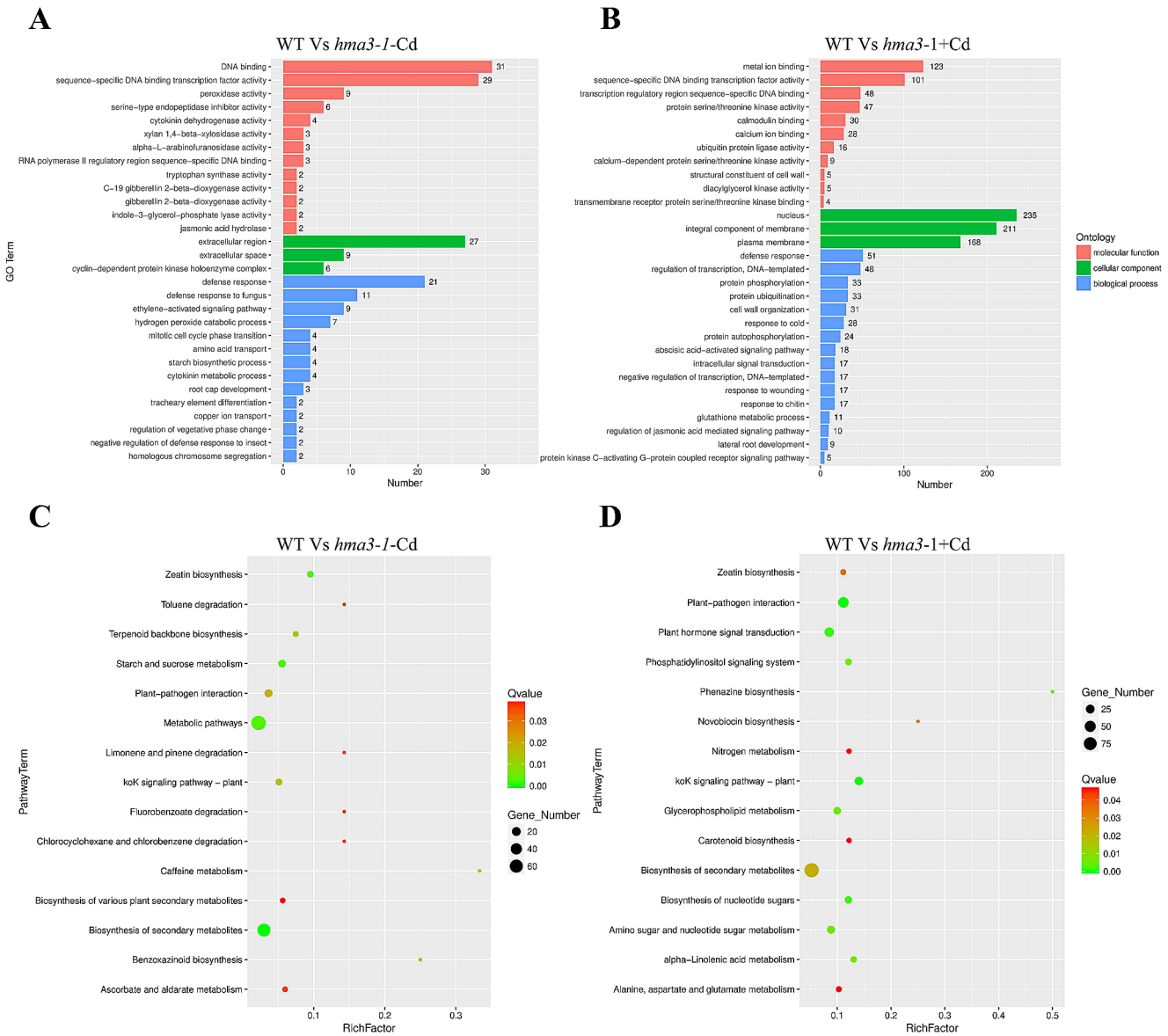

Fig. 6 GO and KEGG enrichment analysis of differentially expressed genes for wild-type (WT) and *Oshma3* mutant with or without the presence of Cd. (**A-B**) GO enrichment analysis of differentially expressed genes between WT and *Oshma3 plants* treated with 0 μM Cd (**A**) and 1 μM Cd (**B**). (**C-D**) KEGG enrichment analysis of differentially expressed genes between WT and *Oshma3 plants* treated with 0 μM Cd (**C**) and 1 μM Cd (**D**)

Oshma3 mutants exhibit low fertility and high grain Cd accumulation

To assess the impact of the loss function of *OsHMA3* on plant growth and grain Cd accumulation in paddy fields, we conducted a planting experiment with both the WT and *Oshma3* mutants in a paddy field. The soil in this field had a Cd content of 0.65 ± 0.15 mg/kg ($n = 3$) and a pH of 5.83 ± 0.17 ($n = 3$). Compared to the WT, the *Oshma3* mutants displayed reduced plant height at maturity. Furthermore, the fertility of the *Oshma3* mutants was significantly reduced, with a seed setting rate of 53.1 ± 5.0% and 50.9 ± 4.8% for *Oshma3-1* and *Oshma3-2*, respectively, compared to the WT (80.0 ± 3.2%) (Fig. 7A-D). However, there were no significant differences in agronomic traits between the WT and *Oshma3* mutants, which included tiller number per plant, panicle length, primary panicle branch number, secondary panicle branch number, grain number per panicle, flag leaf length, and flag leaf width (Fig. 7E-K).

Following the harvest of seeds, the concentration of Cd in the grains was analyzed. The grain Cd content in WT plants was found to be 363.3 ± 68.2 μg/kg ($n = 6$). In contrast, the grain Cd content in *Oshma3* mutants showed a significant increase, reaching 1658.64 ± 214.65 μg/kg and 1208.28 ± 67.06 μg/kg for *Oshma3-1* and *Oshma3-2*, respectively, when compared to WT plants (Fig. 8A). Additionally, other divalent metals such as Zn, Cu, Fe, and Mn were also measured in the grains. The results revealed that the concentrations of Zn and Cu were significantly elevated in the grains of *Oshma3* mutants as compared to WT plants (Fig. 8B-D).

Discussion

OsHMA3, which encodes a P_{1B}-type ATPase, plays a crucial role in regulating the translocation of Cd from roots to shoots and affects the concentration of Cd in rice grains through its involvement in Cd efflux into vacuoles in root cells (Sasaki et al. 2014; Lu et al. 2019). Although a few naturally occurring *OsHMA3* loss-of-function alleles have been reported (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019; Sun et al. 2019), the impact of genome-edited loss-of-function of *OsHMA3* in a conventional rice variety

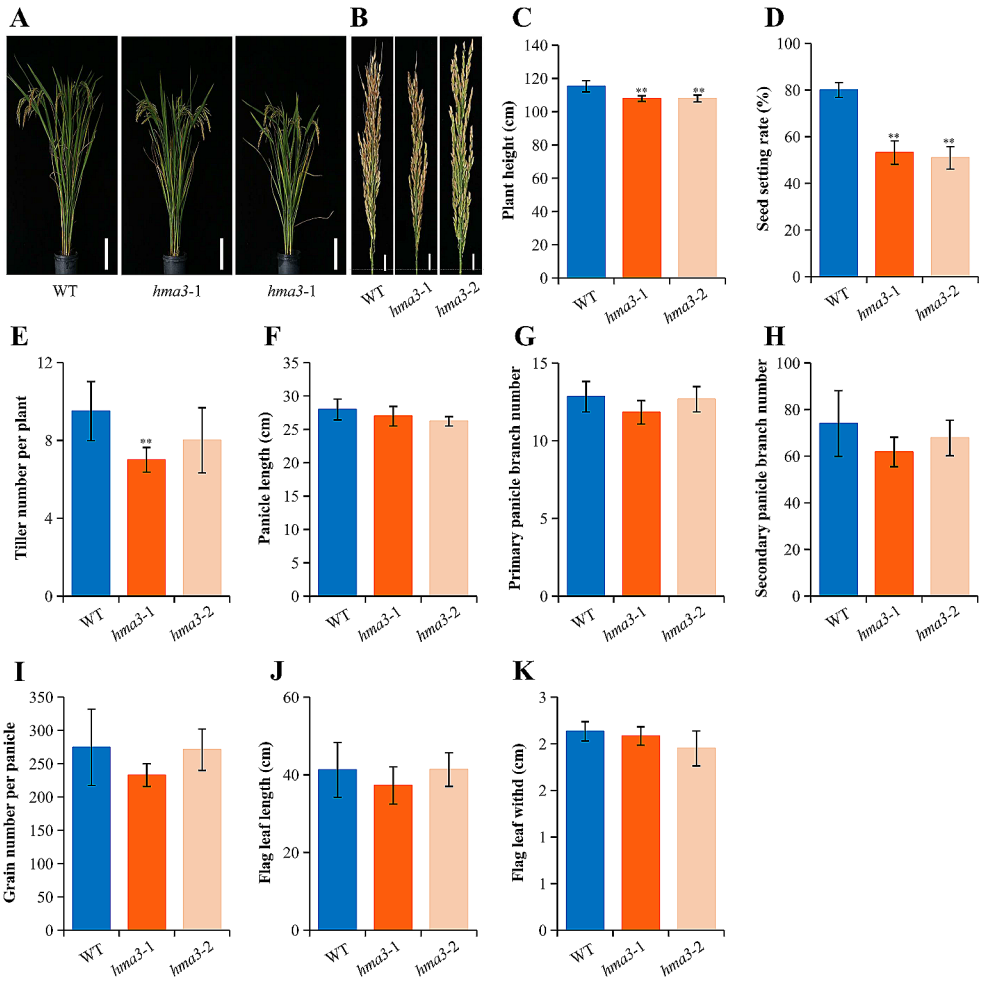

Fig. 7 Phenotypic comparison between wild-type (WT) and *Oshma3* mutants. (**A-B**) Plant (**A**) and panicle (**B**) morphology of WT and *Oshma3* mutants. (**C-K**) Plant height (**C**), seed setting rate (**D**), tiller number per plant (**E**), panicle length (**F**), primary panicle branch number (**G**), secondary panicle branch number (**H**), grain number per panicle (**I**), flag leaf length (**J**) and flag leaf width (**K**) of WT and *Oshma3* mutants. Scale bars, 25 cm (**A**) or 2 cm (**B**). Data are means ± SD ($n = 6$ biological replicates). ** indicate a significance level at 1% (Student's *t*-test)

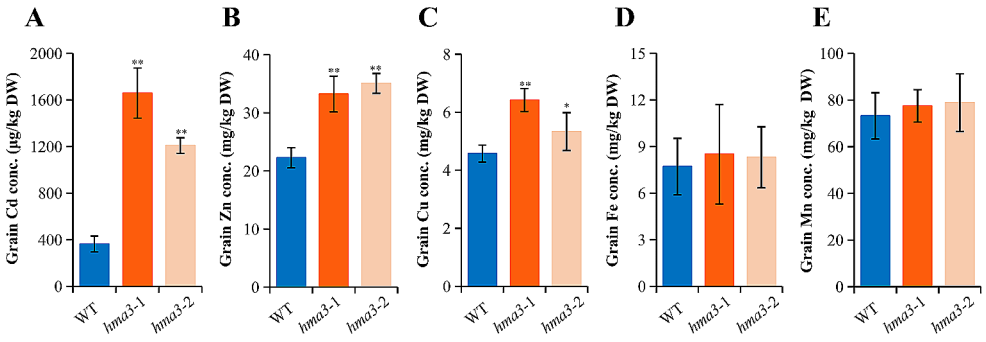

Fig. 8 Metal concentrations in grain of the wild-type (WT) and *Oshma3* plants grown in a paddy field. (**A-G**) Grain concentration of Cd (**A**), Zn (**B**), Cu (**C**), Fe (**D**), and Mn (**E**). Data are means ± SD ($n = 6$ biological replicates). * and ** indicate a significance level at 5% and 1%, respectively (Student's *t*-test)

has not been explored to date. In this study, we utilized CRISPR/Cas9 technology to create a knockout of *OsHMA3* in the *indica* variety 93 – 11. Consistent with the phenotype observed in naturally occurring loss-of-function alleles of *OsHMA3* (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019; Sun et al. 2019), the genetically engineered *OsHMA3* loss-of-function mutants exhibited reduced Cd accumulation in roots but increased Cd accumulation in shoots and grains compared to the WT plants. These findings provide additional evidence supporting the functional role of *OsHMA3* in the 93 – 11 genetic background.

Previous studies have demonstrated that overexpression of *OsHMA3* enhances rice's tolerance to Cd stress (Sasaki et al. 2014; Lu et al. 2019). In our study, we found that the loss-of-function of *OsHMA3* in the 93 – 11 variety increased its sensitivity to Cd exposure, confirming this conclusion through a Cd resistance experiment. We also observed that *Oshma3* mutant seedlings exhibited normal growth in the absence of Cd, but experienced inhibited growth under low Cd conditions. In field tests, the *Oshma3* mutants also exhibited decreased plant height and significantly reduced fertility compared to the WT. The decreased plant growth and fertility in *Oshma3* mutants can be attributed to high Cd accumulation in aboveground tissues, which has a toxic effect on rice plants. Interestingly, such effects have not been documented in earlier studies on natural loss-of-function *OsHMA3* alleles (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019; Sun et al. 2019). One possible explanation is that these natural mutations lack appropriate controls for comparison. Additionally, it is conceivable that rice varieties with high Cd accumulation may have evolved Cd-tolerant mechanisms to protect against the detrimental effects of Cd on plant growth.

OsHMA3 is a highly specific transporter for Cd, and it is believed to have a potential role in Zn transport as well (Ueno et al. 2010; Miyadate et al. 2011; Satoh-Nagasawa et al. 2013; Sasaki et al. 2014). In this study, knockout mutants of *OsHMA3* in the 93 – 11 rice variety showed similar shoot Zn content compared to the WT, but exhibited increased Zn concentrations in the roots and grains. This suggests that the absence of *OsHMA3* may affect the transport or distribution of Zn in rice. However, when the functional allele of *OsHMA3* from Nipponbare and the loss of function allele from Anjana Dhan were expressed in yeast, neither allele affected Zn sensitivity in the *Δzrc1* mutant strain (Ueno et al. 2010; Yan et al. 2016), suggesting that OsHMA3 does not directly transport Zn. These findings are further supported by results from *OsHMA3* RNA interference (RNAi) and overexpression lines in the Nipponbare variety (Satoh-Nagasawa et al. 2013; Sasaki et al. 2014). The *OsHMA3* RNAi plants showed a higher translocation ratio of Zn compared to the control (Satoh-Nagasawa et al. 2013), while the *OsHMA3*-overexpressed line resulted in an increase in root Zn concentration but a decrease in shoot Zn concentration (Sasaki et al. 2014). This could be linked to the modulation of expression levels of ZRT-IRT-like protein (ZIP) family genes, which are responsible for Zn uptake and translocation (Sasaki et al. 2014; Lu et al. 2019). Evidence suggests that five ZIP family genes including *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* were consistently up-regulated in the overexpression line, regardless of the presence or absence of Cd (Sasaki et al. 2014).

Mineral analysis from a previous study by Sasaki et al. (2014) indicated that there was accumulation of Cd in both the roots and shoots of *OsHMA3* overexpression lines in the Nipponbare background, a typical *japonica* rice variety, under 0 μM Cd conditions. In contrast, our current research on *Oshma3* mutants in the background of the *indica* variety 93 – 11 has shown relatively high concentrations of Cd in the absence of added Cd, under normal conditions at 0 μM Cd. Several potential factors could explain the observed high Cd accumulation in our study. Firstly, it is possible that Cd was present in the water used for preparing the Kimura B nutrient solution. Secondly, there may have been traces of Cd in the reused containers. Additionally, the potential impact of Cd pollution during sample preparation should be considered. Moreover, it is important to note that the seeds of the *indica* rice variety may inherently possess higher Cd content compared to the *japonica* variety, as suggested by Liu et al. (2020a). While these factors may have influenced the accurate phenotype of the *Oshma3* mutants, they do not affect the overall conclusion of our study.

Conclusions

The *OsHMA3* gene is known to have a pivotal role in the regulation of Cd accumulation in rice. Through the use of CRISPR/Cas9 technology, we were able to generate loss-of-function mutants of *OsHMA3* in the indica variety 93 − 11. The resulting mutants exhibited increased sensitivity to Cd exposure. This study has expanded our knowledge on the functions of *OsHMA3* in rice, shedding light on its importance in the plant's response to heavy metal stress.

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Author contributions C.L.L, and W.H.L managed the project. H.J.Y, X.Z.J, Y.Y.C, and H.L performed the experiments. H.J.Y, and C.L.L analyzed the data and wrote the manuscript. C.L.L revised the manuscript.

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Data availability All datasets for this study are included in the manuscript and the supplementary file.

Declarations

Competing interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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