

SI Materials and Methods

Plant materials and growth conditions. A F₂ population, derived from a cross between a high Cd-accumulating cultivar (Anjana Dhan) as a female parent and a low Cd-accumulating cultivar (Nipponbare) as a male parent of rice (*Oryza sativa* L.), was used for fine mapping. The F₂ plants were grown hydroponically as previously described (1), in a temperature-controlled greenhouse (30°C, 12 h day/ 22°C, 12 h night) under natural sunlight for 11 to 14 days and then subjected to Cd treatment. Transgenic plants were pre-cultured on gel for about 100 days after introduction of each plasmid (2). The transgenic seedlings were cultivated in a nutrient solution for 1 to 3 weeks and then subjected to Cd treatment. Cd treatment was performed by exposing the seedlings to a nutrient solution containing 50 nM CdSO₄ for ten days. The treatment solution was changed once every two days. For soil culture, the plants were grown in a moderately Cd-contaminated soil (1.5 Cd mg kg⁻¹ soil) without flooding for 5 months. After treatments, the shoots including leaf blades and leaf sheaths, roots and brown rice (de-husked grains) were harvested for determination of Cd and other metals by flame atomic absorption spectrometry (Z-2000; Hitachi, Tokyo, Japan).

Delimitation of candidate genomic region of the QTL. A two-steps procedure was applied to delimit the candidate genomic region of the QTL. First, plants having recombination between SSR markers RM21238 and RM7153 were screened from 965 F₂ plants. Second, we screened plants with recombination between RM21251 and RM21275, from 808 F₂ plants. To determine precise position of recombination occurring in plants selected, further genotyping was carried out by using the new markers located in the interval. To obtain informative simple sequence repeat (SSR) markers, we surveyed SSR motif (3) in the candidate region of the QTL. We also designed one Indel and two CAPS markers based on Nipponbare reference sequence. Primer information of those markers newly obtained is listed in Table S4. Association between relative Cd accumulation and marker genotypes was investigated to delimit a candidate genomic region of QTL.

Cloning of *OsZIP8* and *OsHMA3*, and plasmid construction. To obtain the sequences of 5' and 3' ends of *OsZIP8a* ORF, DNA fragment was amplified from genomic DNA using primer sets, 5'- CCGAGCGACTAATCCAAGG-3' and

5'-TCAAACCCACAAAGGTAGGC-3', which were designed on the basis of genomic DNA sequence in Nipponbare. The cDNA fragment containing an entire ORF of *OsZIP8n* was amplified using primers 5'-TAAGCTTAAAAATGAGGACGAACACCA-3' and 5'-TGCATGCTAGGCCCATTTGGCGA-3, and of *OsZIP8a* was amplified using primers 5'-TAAGCTTAAAAATGCGGACGAACACC-3' and 5'-TGCATGCTAGGCCCATTTGGCGA-3. For the positive control, the cDNA fragment containing an entire ORF of *AtNramp4* was amplified by RT-PCR using primers 5'-GGATCCGAAATATGTCGGAGACTGATAGAG-3' and 5'-TCACTCATCATCCCTCTGTGGT-3' from Arabidopsis. Each cDNA fragments were subcloned into pYES2 vector at optimal restriction sites, and resulting plasmids were introduced into yeast strain.

To clone *OsHMA3* from each cultivar, we extracted total RNA from the roots using an RNeasy plant mini kit (Qiagen, <http://www.qiagen.com/>). After the reaction of DNase I (Invitrogen, <http://www.invitrogen.com/>), the total RNA was converted to cDNA using the protocol attached to SuperScript II (Invitrogen). The open reading frame (ORF) of *OsHMA3n* was amplified by RT-PCR using primer sets, 5'-ATGGCCGAAAGGATGAGGCG-3' and 5'-TCATCCTTTCACCTTCACCGGAG-3', designed according to the sequence information of Os07g0232900 in the Rice Annotation Project Database (<http://rapdb.dna.affrc.go.jp/>). The full-length *OsHMA3a* cDNA was generated by the RACE method from total RNA of Anjana Dhan seedlings (SMART™ RACE cDNA amplification kit; Clontech, <http://www.clontech.com/>) using gene-specific primers, 5'-TGCCAATGTCCTTCTGTTCCCA-3' for 5'-RACE and 5'-TCCATCCAACCAAACCCGGAAA-3' for 3'-RACE, designed according to the sequence of Os07g0232900. The entire cDNAs were subcloned into the pGEM-T Easy vector (Promega, <http://www.promega.com/>) and sequenced using a Big-Dye sequencing kit (Applied Biosystems, <http://www.appliedbiosystems.com/>) with gene-specific primers on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems).

Sequence analysis and phylogenetic tree. Sequence alignment was analyzed by ClustalW (<http://clustalw.ddbj.nig.ac.jp/>). Transmembrane domains were predicted

with internet-programs SOSUI ver. 1.11 (<http://bp.nuap.nagoya-u.ac.jp/sosui/>). Homology of amino acid sequences was analyzed using a web-site NPS@ (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). The phylogenetic tree was constructed by MEGA 4 software (released from <http://www.megasoftware.net/>) after ClustalW alignment.

Transgenic plants. To generate the hairpin RNAi construct, we cloned a 511-bp fragment (893 to 1407 bp from transcriptional start) of *OsHMA3n* cDNA as inverted repeats into the pANDA vector under control of maize ubiquitin1 promoter (4).

To generate a construct carrying ubiquitin promoter, we amplified *OsHMA3n* and NOS terminator, *OsHMA3n* cDNA by PCR from pGEM-T Easy-*OsHMA3* plasmid using primer set, 5'-AGGATCCATGGCCGAAAGGATGAGG-3' and 5'-TGGATCCGCAACATCATCCTTTCACCTTCACC-3'. The fragment was cloned into pANDA vector, and then excised together with maize ubiquitin1 promoter followed by subcloned into pPZP2H-lac binary vector (5).

To construct a translational *pOsHMA3-GFP* fusion, we amplified 2-kb of upstream region (-34 to -2094 bp from the translational start codon) of *OsHMA3* gene by PCR from Nipponbare genomic DNA using primers, 5'-ATCTAGAAGCATAAAAGAATAGAGCCGTGGAC-3' and 5'-ATCTAGAATGCAAGTGGGGATCAAGGA-3'. The promoter was cloned into the *Xba*I site of GFP and NOS terminator in pUC18 vector. The construct carrying the *pOsHMA3n-GFP* and NOS terminator were subcloned into pPZP2H-lac binary vector.

All constructs were introduced into rice calluses derived from Nipponbare by means of *Agrobacterium*-mediated transformation (2). The GFP signal was observed by confocal laser microscopy (LSM700; Carl Zeiss).

To do complementation test, a 6.8 kb DNA fragment harboring 2.1 kb promoter and full length of *OsHMA3n* was amplified by PCR using Nipponbare genomic DNA as a template. Primer pairs for the amplification of the two DNA fragments were 5'-atctagaAGCATAAAAGAATAGAGCCGTGGAC-3' and 5'-GGATGCGTCAATCAGTTTACCA-3', 5'-GGCACAATGAACTTTGACGGT-3' and 5'-CTCTTCTGGACAAGCTTCCTTAATC-3', respectively. The two DNA fragments were firstly cloned into pTA2 vector and then fused together using enzyme *Afl*III. The fused 6.8 kb DNA was then inserted into a binary vector pPZP2H-lac (5) and

transformed into calluses from Anjana Dhan cultivar by *Agrobacterium tumefaciens* (strain EHA101)-mediated method (2).

Expression pattern. To investigate expression pattern of *OsHMA3* genes, we extracted RNA from the shoots and roots of both cultivars (12-d-old). Spatial expression of *OsHMA3* was examined by excising the roots at different segments (0-1 cm, 1-2 cm, and 2-3 cm) of rice exposed to 0 or 1 μM CdSO_4 for 24 hours, followed by RNA extraction. The expression levels were analyzed using Thunderbird™ qPCR Mix (Toyobo, <http://www.toyobo.co.jp/>) with the following primer pairs: 5'-TCCATCCAACCAAACCCGGAAA -3' and 5'-TGCCAATGTCCTTCTGTTCCCA -3' for *OsHMA3*. *Histone H3* was used as an internal standard with the primers; 5'-GGTCAACTTGTTGATTCCCCTCT-3' and 5'-AACCGCAAAATCCAAAGAACG-3'. Data were collected in accordance with the 7500 Real Time PCR System (Applied Biosystems). ΔCt method was used to evaluate the relative quantities of each amplified product. Amplification efficiency of the real time PCR was checked by standard curve using diluted plasmid DNA as template (efficiency = 1.96 for both *OsHMA3n* and *OsHMA3a*).

Immunohistological staining. The synthetic peptide C-CAKTMNSGEVKG (positions 993-1004 of *OsHMA3n*) was used to immunize rabbits to obtain antibodies against *OsHMA3*. The obtained antiserum was purified through a peptide affinity column before use. The roots of both cultivars (10-d-old seedlings) were used for immunostaining of *OsHMA3* protein with a 1:300 dilution as described previously (6). Fluorescence of secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) was observed with a confocal laser scanning microscopy (LSM700; Carl Zeiss).

Construction and transient expression analysis of a GFP-*OsHMA3* fusion. The ORF of *OsHMA3n* and *OsHMA3a* cDNA fragments were amplified using primers 5'-ATCCGGAATGGCCGAAAGGATGAGGC-3' and 5'-TTCCGGATCCTTTCACCTCACC GGAG-3'. The *OsHMA3* fragment was ligated to the 3' end of GFP carrying linker sequence, which encodes seven additional amino acid (SGGGGGG), and placed under the control of the CaMV 35S promoter in pUC18 (Takara). The resulting plasmid (pGFP-*OsHMA3*) or GFP alone was coated with

1 μm gold particles and introduced into onion epidermal cells with or without DsRed-HDEL, an ER marker, using particle bombardment (PDS-1000/He particle delivery system, Bio-Rad, <http://www.bio-rad.com/>) using 1100 psi pressure disks. GFP fluorescence was observed using confocal laser microscopy (LSM700; Carl Zeiss).

Western-blot analysis. Eight grams of whole roots harvested from OsHMA3*n* over-expressing lines, which were grown hydroponically for 137 days, were homogenized in 60 mL of ice-cold homogenizing buffer composing of 100 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5% (w/v) polyvinylpyrrolidone, 5 mM EDTA, 3.3 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol. After filtration, the homogenates were centrifuged at 8,000 g at 4°C for 10 min to yield the supernatant and centrifuged again under the same conditions. The supernatants were then ultracentrifuged at 100,000 g for 40 min. The pellets were resuspended in 1 mL of resuspension buffer containing 10 mM Tris-HCl, pH 7.6, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, and 1/100 volume of Protease Inhibitor Cocktail for plant cell and tissue extracts (Sigma-Aldrich). The suspended solution was then fractionated with discontinuous sucrose gradients (20 to 60% sucrose in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM DTT) by ultracentrifugation at 100,000 g for 120 min. The fractionated membranes were recovered by ultracentrifugation at 100,000 g for 40 min. Each pellet was resuspended in 100 mL resuspension buffer supplemented with 1/100 volume of Protease Inhibitor Cocktail for plant cell and tissue extracts (Sigma-Aldrich) and 1 mM DTT.

Equal amounts of samples were mixed with same volume of sample buffer containing 100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 0.008% (w/v) bromophenol blue, and 0.12 mM DTT. The mixture was allowed to incubate at 65°C for 10 min and SDS-PAGE was run using 5% to 20% gradient polyacryl-amide gels (ATTO, Tokyo, Japan). The transfer to polyvinylidene difluoride membrane was performed with a semidry blotting system, and the membrane was treated with the purified primary rabbit anti-OsHMA3 (100-times dilution), anti- γ -TIP (1,000-times dilution) and anti-H⁺-ATPase polyclonal antibodies. ECL peroxidase labeled anti-rabbit antibody (10,000-times dilution; GE Healthcare, <https://www2.gehealthcare.com/>) was used as a secondary antibody, and an ECL Plus western blotting detection system (GE Healthcare) was used for detection via

chemiluminescence.

Functional analysis in yeast. *Saccharomyces cerevisiae* reference strain BY4741 (Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) and mutant strains Δ *zrc1* (Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; YMR243c::kanMX4) and Δ *cot1* (Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; YOR316c::kanMX4) were purchased from Euroscarf (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html>). *OsHMA3a/n* genes were amplified by PCR from the plasmids prepared above using primer sets, 5'-AAAGCTTAAAAATGGCCGGAAAGGA-3' and 5'-TGAATTCATCCTTTCACTTCACCG-3'. The fragment containing the ORF was inserted into the *Hind*III and *Eco*RI sites of a yeast expression vector, pYES2.

To construct chimeric genes, we first amplified N-terminal fragments containing 1531 bp from the transcription start site from each pGEM-T Easy-*OsHMA3* plasmid using primer sets, 5'-AAAGCTTAAAAATGGCCGGAAAGGA-3' and 5'-TGTAGATGTGCTTTCCATGGATCTCTCCAT-3'. We also amplified C-terminal fragments from 1501 bp to transcription end using primer sets, 5'-ATGGAGAGATCCATGGAAAGCACATCTACA-3' and 5'-TGAATTCATCCTTTCACTTCACCG-3'. The chimeric genes were amplified from the combination of PCR fragments, N-terminal *OsHMA3a* and C-terminal *OsHMA3n* or N-terminal *OsHMA3n* and C-terminal *OsHMA3a*, using primer sets, 5'-AAAGCTTAAAAATGGCCGGAAAGGA-3' and 5'-TGAATTCATCCTTTCACTTCACCG-3'. The fragments were cloned into pGEM-T Easy vector, excised at *Hind*III and *Eco*RI site, and then subcloned into pYES2 vector.

For evaluation of zinc and cobalt tolerance in yeast, *OsHMA3n*, *OsHMA3a* or *AtHMA3* gene were constructed into pYES2 vector and transformed into a mutant yeast strain Δ *zrc1* or Δ *cot1*. The transformed yeast was selected on a SD medium without uracil (Ura). Positive clones were cultured in glucose-containing SD-Ura liquid media to the early log phase for growth assays. Five microliters of the cell suspension with an OD value of 0.5 and four serial 1:10 dilutions were spotted on SD-Ura plates containing 2% glucose or galactose, and with or without 4 mM ZnSO₄ or 2.5 mM CoCl₂. The yeast was grown on the plates at 30°C for 3 d for the comparison. For evaluation of cadmium tolerance, plasmids were transformed into both wild-type

BY4741 and $\Delta ycf1$ mutant strains. Positive clones were cultured and spotted on SD-Ura plates containing 0 or 2 μM CdCl_2 for $\Delta ycf1$ mutant strain, and 0 or 20 μM CdCl_2 for the wild-type BY4741. The yeast was grown on the plates at 30°C for 3 d for the comparison.

For site-directed mutagenesis analysis, to substitute amino acid at position 80 from Arg to His, we amplified cDNA fragments containing either N-terminal or C-terminal ends using primer sets, 5'-ATGGCCGGAAAGGATGAGGCG-3' and 5'-GACGACGACGGTGCGGGACGCCACGACGAC-3' (for N-terminal), or 5'-GTCGTCGTGGCGTCCCGCACCGTCGTCGTC-3' and 5'-TCATCCTTTCACTTCACCGGAG-3' (for C-terminal), respectively. The fragments were used as templates to amplify ORF of *OsHMA3a*-containing mutation using primers 5'-ATGGCCGGAAAGGATGAGGCG-3' and 5'-TCATCCTTTCACTTCACCGGAG-3'. To substitute amino acid at position 638 from Val to Ara, we amplified cDNA fragments using primer sets, 5'-ATGGCCGGAAAGGATGAGGCG-3' and 5'-CGCCACGTCCGCCGCCAGCGCAGCCG-3' (for N-terminal) or 5'-CGGCTGCGCTGGCGGCGGACGTGGGCG-3' and 5'-TCATCCTTTCACTTCACCGGAG-3' (for C-terminal), and the ORF was subsequently amplified as described above. The plasmid containing a single mutation was used as the template to substitute amino acids at the two positions. The metal sensitivity assay was performed as described above. After 3 days of incubation at 30°C, the plates were photographed.

References

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suppression of gene function in rice. *Plant Cell Physiol* 45:490-495.

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6. Yamaji N, Ma JF (2007) Spatial distribution and temporal variation of the rice silicon transporter Lsi1. *Plant Physiol* 143:1306-1313.

OsZIP8n	1	MRTN--TTATVLLAAVALLLATAARGDGGDGGCGKEDAAAGRDRARARGLKIAAFFSIL
OsZIP8a	1	MRTNTTTTATVLLAAVALLLATAARGDGGDGGCGKEDAAAGRDRARARGLKIAAFFSIL
OsZIP8n	59	VCGALGCGLPSLGRHVPALRPDGDVFFLVKAFAAGVILATGFIHILPDAFDNLTDDCLPA
OsZIP8a	61	VCGALGCGLPSLGRHVPALRPDGDVFFLVKAFAAGVILATGFIHILPDAFDNLTDDCLPA
OsZIP8n	119	GGPWKEFPFAGFGAMVGAIGTLVVDTLATGYFTRALSKKDAATAAAVADEEKQSAAATQQ
OsZIP8a	121	GGPWKEFPFAGFGAMVGAIGTLVVDTLATGYFTRAQSKKDA--AAVADEEKQSAAATTQ
OsZIP8n	179	HNH HNH HVVG DGGGGGEEHEGQVHVHTHATHGHAHGSSALVAAVGEDDKETTLRHRVIS
OsZIP8a	179	QHN HH --YVVG DGGGG--EEHEGQVHVHTHATHGHAHGSSALVAAVGEDDKETTLRHRVIS
OsZIP8n	239	QVLELGIVVHSVIIIGISLGASQNPETIKPLVVALSFHQMFEGMGLGGCIVQAKFKVRSIV
OsZIP8a	236	QVLELGIVVHSVIIIGISLGASQNPETIKPLVVALSFHQMFEGMGLGGCIVQAKFKVRSIV
OsZIP8n	299	TMV LF CLTTPVGI AV GVG ISS VYNESSPTALVVEGILNSVAAGIL IY MALVDLLAEDFM
OsZIP8a	296	TMV LF CLTTPVGI AV GVG ISS VYNESSPTALVVEGILNSVAAGIL IY MALVDLLAEDFM
OsZIP8n	359	NPRVQSKGKLQLGINLAML AG GLMSMLAKWA
OsZIP8a	356	NPRVQSRGKLQLGINLAML AG GLMSMLAKWA

Fig. S1. Alignment of two allelic proteins (OsZIP8a and OsZIP8n) from Anjana Dhan and Nipponbare.

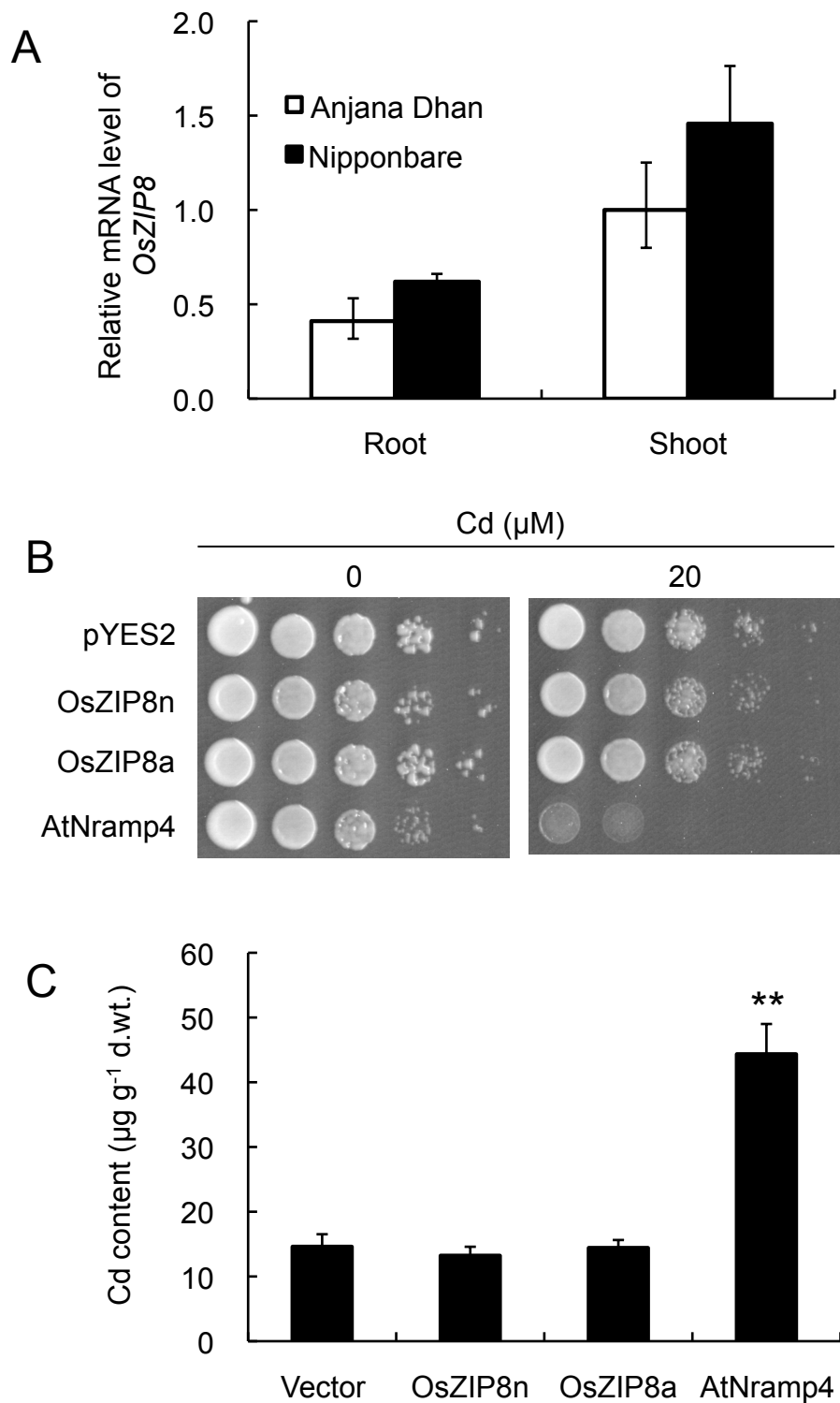


Fig. S2. Functional analysis of *OsZIP8*. (A) Expression of *OsZIP8* in different tissues of both Anjana Dhan and Nipponbare. Expression relative to Anjana Dhan shoot was shown. (B) Expression of *OsZIP8* on Cd tolerance in yeast. *AtNramp4* was used as a positive control. (C) Cd uptake in yeast expressing *OsZIP8* from different cultivars, *AtNramp4* and empty vector. Data are means of three biological replicates.

OsHMA3a	1	MAGKDEAEGL EARLLLLPPEAAAEPTRCGGDGGGGGRKRKKT YLDVLGVCCSAEVALV
OsHMA3n	1	MAGKDEAEGL EARLLLLPPEAAAEPTRCGGDGGGGGRKRKKT YLDVLGVCCSAEVALV
OsHMA3a	61	ERLLAPLDGVRVVS VVVAS ⁸⁰ HTVVVEHDPAAAPESVIVKALNKAGLEASVRAYGSSGVVSR
OsHMA3n	61	ERLLAPLDGVRVVS VVVASHTVVVEHDPAAAPESVIVKALNKAGLEASVRAYGSSGVVSR
OsHMA3a	121	WPSPIVASGVLLTASFFEWLFPPLQCLAVA AVVAGAPPMVRRGFAAASRLSLDINVLML
OsHMA3n	121	WPSPIVASGVLLTASFFEWLFPPLQCLAVA AVVAGAPPMVRRGFAAASRLSLDINVLML
OsHMA3a	181	IAVSGALCLGDYTEAGAI VFLFTTAEWLET LACTKASAGMSSLMGMLPVKAVIATTGEVV
OsHMA3n	181	IAVAGALCLGDYTEAGAI VFLFTTAEWLET LACTKASAGMSSLMGMLPVKAVIATTGEVV
OsHMA3a	241	SVRDVRVGDVVAVRAGEIVPVDGVVVDGQSEV DERSLITGESFPVPKQPHSEVWAGTMNLD
OsHMA3n	241	SVRDVRVGDVVAVRAGEIVPVDGVVVDGQSEV DERSLITGESFPVPKQPHSEVWAGTMNFD
OsHMA3a	301	GYIAVRTTALAENSTVAKMERLVEAAQNSRSKMQRLIDSCAKYYTPAVVVVAAGVALIPA
OsHMA3n	301	GYIAVRTTALAENSTVAKMERLVEAAQNSRSKTQRLIDSCAKYYTPAVVVVAAGVALIPA
OsHMA3a	361	LLGADGLEQWKLALVMLVSVCPCALVLPVASF CAMLRAARMGIFIKGGDVL ESSLGEI
OsHMA3n	361	LLGADGLEQWKLALVMLVSA ^V CPCALVLPVASF CAMLRAARMGIFIKGGDVL ESSLGEI
OsHMA3a	421	RAVAFDKTGTITRGEFSIDSFHLVGDHKVEMD HLLYWIASIESKSSHPMAAALVEYAQSK
OsHMA3n	421	RAVAFDKTGTITRGEFSIDSFHLVGDHKVEMD HLLYWIASIESKSSHPMAAALVEYAQSK
OsHMA3a	481	SIQPNPENVA DFQIYPGEGIYGEIHGKHIYIGNRRTLARASSPQTIQEMGEMIKGVSIGY
OsHMA3n	481	SIQPNPENVGDFRIYPGEGIYGEIHGKHIYIGNRRTLARASSPQSTIQEMGEMIKGVSIGY
OsHMA3a	541	VICDGELAGVFSLSDDCRTGAAEAIRELGSLGIKTVMLTGDS SAAATHAQGQLGAVMEEL
OsHMA3n	541	VICDGELAGVFSLSDDCRTGAAEAIRELGSLGIKSVMLTGDS SAAATHAQGQLGGVMEEL
OsHMA3a	601	HSELLPEDKVR LVDGLKARFGPTMMVGDGMNDAAALAVADVGVSMGISGSAAAMETSHAT
OsHMA3n	601	HSELLPEDKVR LVSGLKARFGPTMMVGDGMNDAAALAAADVGVSMGISGSAAAMETSHAT
OsHMA3a	661	LMSSDVLRVPEAVRLGR ⁶³⁸ ARRTIAVNVAGSVAVKA AVLALAAAWRPVLWAAVLADVGTCL
OsHMA3n	661	LMSSDVLRVPEAVRLGRARRTIAVNVAGSVAVKA AVLALAAAWRPVLWAAVLADVGTCL
OsHMA3a	721	LVVLNSMTLLREKWKGGAKEDGACRATARSLAMRSQLAPDSQAPNAAAAAAGREQTNGC
OsHMA3n	721	LVVLNSMTLLREEWKGGAKEDGACRATARSLVMRSQLAADSQAPNAADAGAAGREQTNGC
OsHMA3a	781	RCCPKPSMSPDHSVVIDIPAGGEHQEERPA ^{VII} AAAVVAKCCGGGGGE-----
OsHMA3n	781	RCCPKPGMSPEHSVVIDIRADGERQEERPA ^{VII} AAAVVAKCCGGGGGEGIRCGASKKPTATVV
OsHMA3a	826	-----GIGCGASKKPTATAVVA ^{VIII} KCCGG
OsHMA3n	841	VAKCCGGGGGGEGTRCGASKNPATAAVVAKCCSGGGGEGIGCGASKKPTATAVVA ^{VIII} KCCGG
OsHMA3a	848	GGEGTRCGASKRPATAAVVAKCCGGGGGGEGTGCGASKRSPPAEGSCSGGEGGTNGVGRCC
OsHMA3n	901	GGEGTRCAASKKPATAAVVAKCCGGGGGGEGTGCGASKRSPPAEGSCSGGEGGTNGVGRCC
OsHMA3a	908	TSVKRPTCCDMGAADVSDSSPETAKDCRNARCCAKTMNSGEVKG
OsHMA3n	961	TSVKRPTCCDMGAAEVSDSSPETAKDCRNARCCAKTMNSGEVKG

Fig. S3.

Alignment of two allelic proteins (OsHMA3a and OsHMA3n) from Anjana Dhan and Nipponbare. Positions 80 and 638 are marked by green box. Transmembrane domains were underlined with blue line. Typical modifies were boxed with red.


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AhHMA3
AhHMA3 800 CSEETRKAVGDWVSLSSCKKSSHYKHDLKMKGGSSGCCANKSEKVEEVAKSCCEKPKOMESAGDCKSSHFEEKKHAEEITVLPVOMIGOALETTELQTKETTKTRCCDNKEKAKKKGL
AhHMA4 800 CSEKTRKSEGDWVSLSSCKKSSHYKHDLKMKGGSSGCCASKNEKGEVVAKSCCEKPKOMESAGDCKSSHFEEKKHAEEITVLPVOMIGOALETTELQTKETTKTRCCDNKEKAKKKGL
AhHMA2 784 CAEPYDLGHGHDSSGCCDSKS00PH0HEVOY00SCHNKPSGLDSGCCGGKS00PH0HEL00SCHDKPSGLDIGTGRKHEGSSTLWNLFGBAKEELKVLVN
OshMA2 801 ITNKACHDGNHHCADTSNLHDTKKHDCHGHESTCKEELNALPPTNDHACHGHEHSHCEEPVALHSTGENACHEHEHHCDEPISHGADKHACHDHEVHEHHCDEEQTPHTADL
OshMA3a 826 GIGCCGASKKPTATAVAWAKCCGGGGEGTRCGASKRPAATAVAWAKCCGGGGEGTRCGASKRSPAEGSSGGGGGGTNGV
OshMA3n 837 ATVVVAKCCGGGGGGEGTRCGASKRPAATAVAWAKCCGGGGEGTRCGASKRSPAEGSSGGGGGGTNGV
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AhHMA3
AhHMA3 920 LLSSEDTSYLEKGVLLKDEGNCKSAGCOKTGTVKQSCCHEKAPLDEITKLVSGNTEGEVGEQTDEIKIEGDCKSSGCSDEKOTIGEITLASEEADSTDCSSG-----CQMDKEEVTQICGL
AhHMA4 920 LLSSENTPYLEKGVLLKDEGNCKSSGENMGTVKQSCCHEKGCSDEKOTIGEITLASEEITDDQDCSSGCCVNEGTVKQSFDEKKHSVLVEKGLDMETGFCCDAKLVCCGNTEGEVKEQCRL
AhHMA2 884 GFCSSPADLAITSLKVKSDSHCKSN-----
OshMA2 921 HPCHDHDNLEVEEVKDCHAEPPHHNHCCHEPHDOVKNDTHPVQEHSISIEESSDHHEHHNHEEHAEDCG-----
OshMA3a 904 GRCGTSVKRPTCDMGADVSDSSPETAKDCRNARCCAKTMSGEVKG-----
OshMA3n 957 GRCGTSVKRPTCDMGAEVSDSSPETAKDCRNARCCAKTMSGEVKG-----
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AhHMA3
AhHMA3 1036 ETEGGGDCKSHCCGTGLTOEGSSKLGNVETAOS-----GGCGTVKVSSSOSCTSSTDLVLSDLOVTKDEHESSHGAVKVETCCKVKIPEACAPECKEKERHSGKSCCRSVAKKEFCSHRH
AhHMA4 1040 EIKKEEHCKSGCCGEEIOTGEITLVSEETIESTNCSTGCCVDKEEVTOTCHEKPASLVWSGLEVKDEHESSHRAVKVETCCKVKIPEACASKRDRAKRHSGKSCCRSVAKKEFCSHRH
AhHMA2 909 -----CSSRERCHHGSNCCRSVAKESSHDH
OshMA2 994 -----HHPKPKDCAPPTDCISRNCSNTSKGKDICSSLHRDHTSOASRCCRSVAKKEFCSHRH
OshMA3a -----
OshMA3n -----
-----
AhHMA3
AhHMA3 1152 HHHHHHHHHHVSA-----
AhHMA4 1160 HHHHHHHHHHVSA-----
AhHMA2 935 HHTRAHGVGTLKEIVIE-----
OshMA2 1053 CCSHIVKLPEIVE-----
OshMA3a -----
OshMA3n -----

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Fig. S4.

Alignment of HMA3-like proteins in *Arabidopsis thaliana* (AtHMA2, 3 and 4), *Arabidopsis halleri* (AhHMA3 and 4) and rice (OshMA2, OshMA3a and OshMA3n). Positions 80 and 638 in OshMA3a/n are marked by green box. Conserved sequence of HMA domain is marked by orange box.

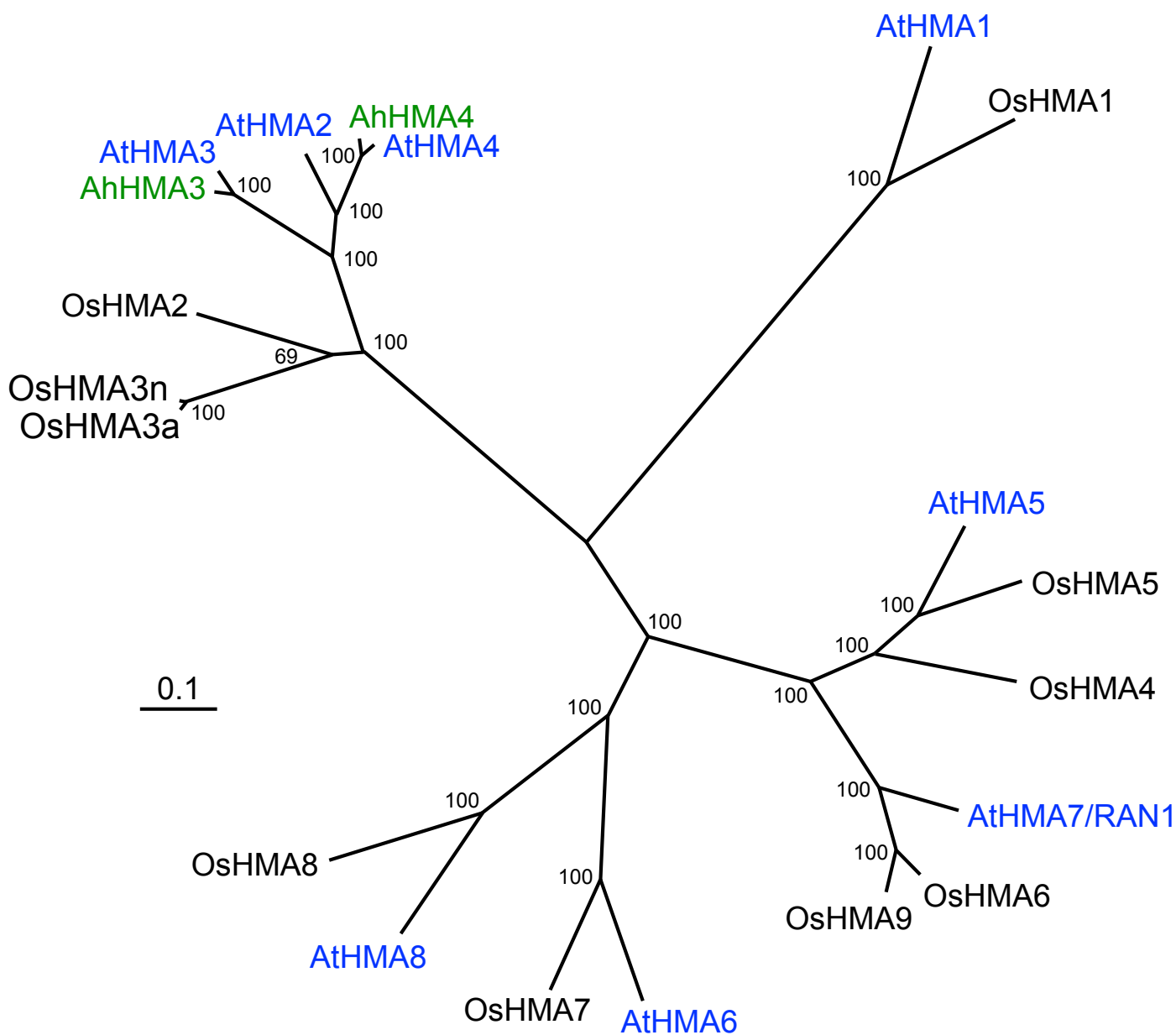


Fig. S5. Phylogenetic relationship of HMA proteins in rice (black), *Arabidopsis thaliana* (blue), and *Arabidopsis halleri* (green). Bootstrap values from 1000 trials are indicated. The 0.1 scale shows substitution distance.

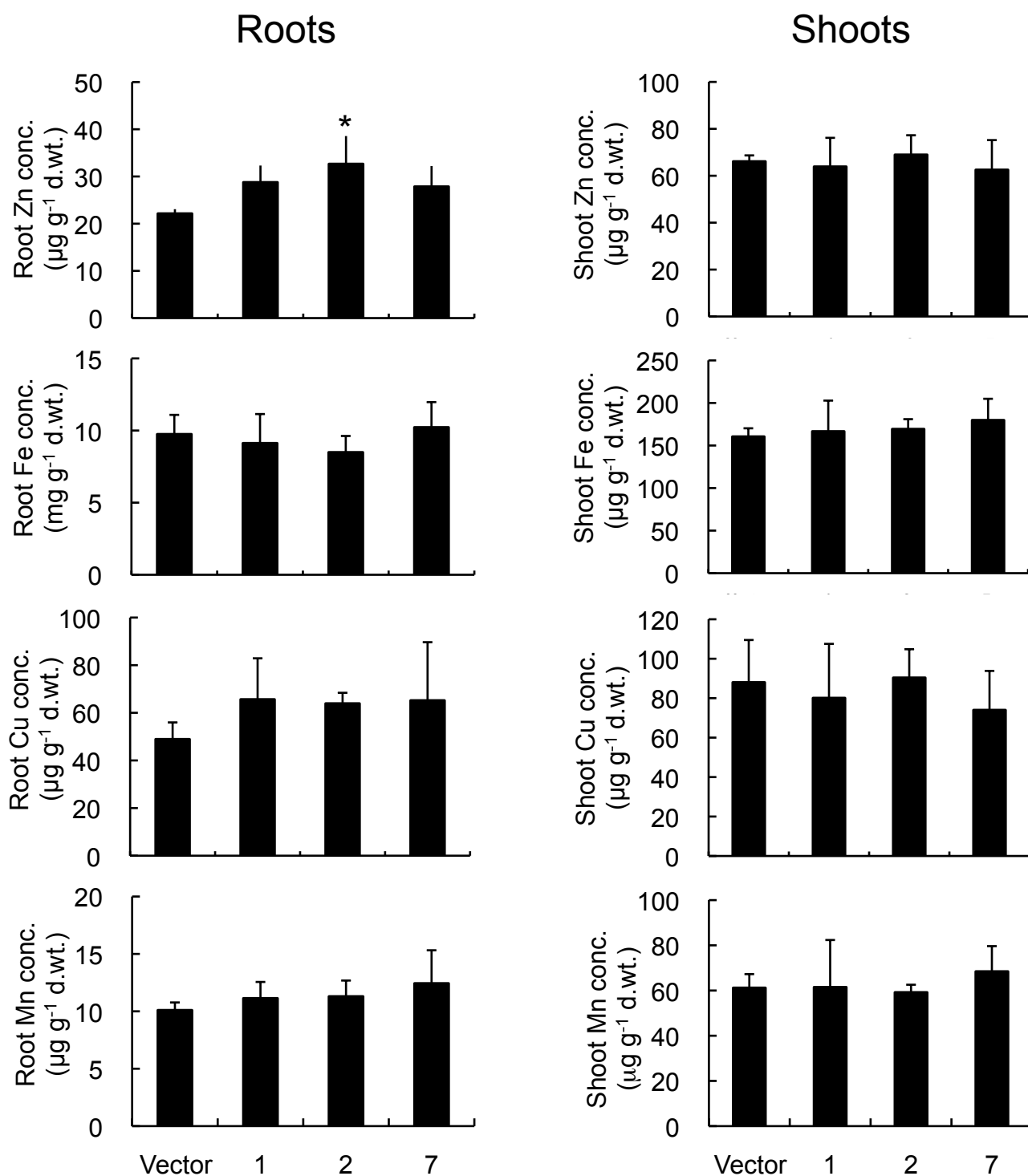


Fig. S6. Concentration of micronutrients in three independent transgenic lines carrying *OsHMA3n* and vector control line of Anjana Dhan background. The plants were exposed to 50 nM Cd for ten days. Data are means \pm SD of four biological replicates. * $p < 0.05$; Dunnett's t-test.

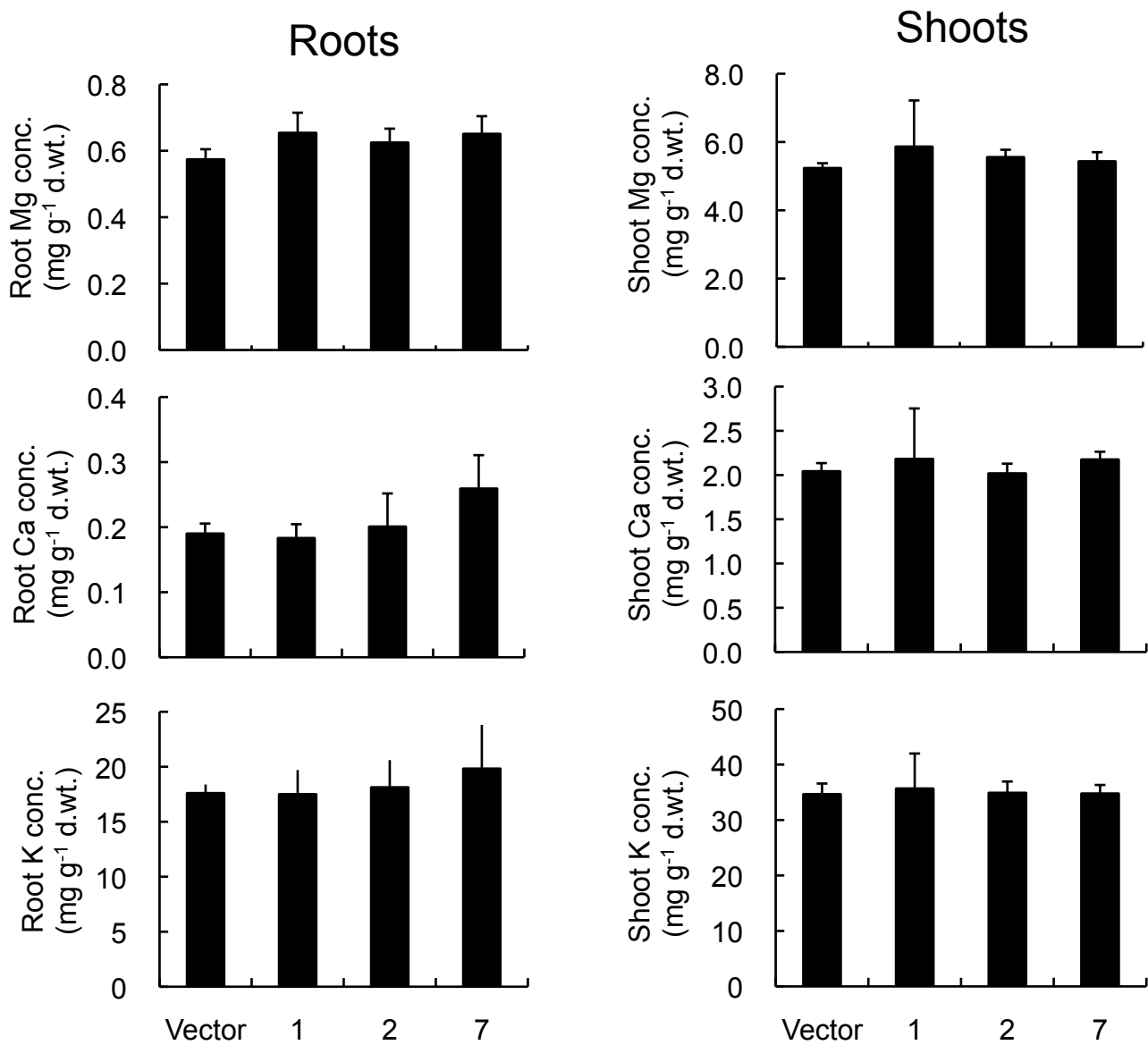


Fig. S7. Concentration of macronutrients in three independent transgenic lines carrying *OsHMA3n* and vector control line of Anjana Dhan background. The plants were exposed to 50 nM Cd for ten days. Data are means \pm SD of four biological replicates.

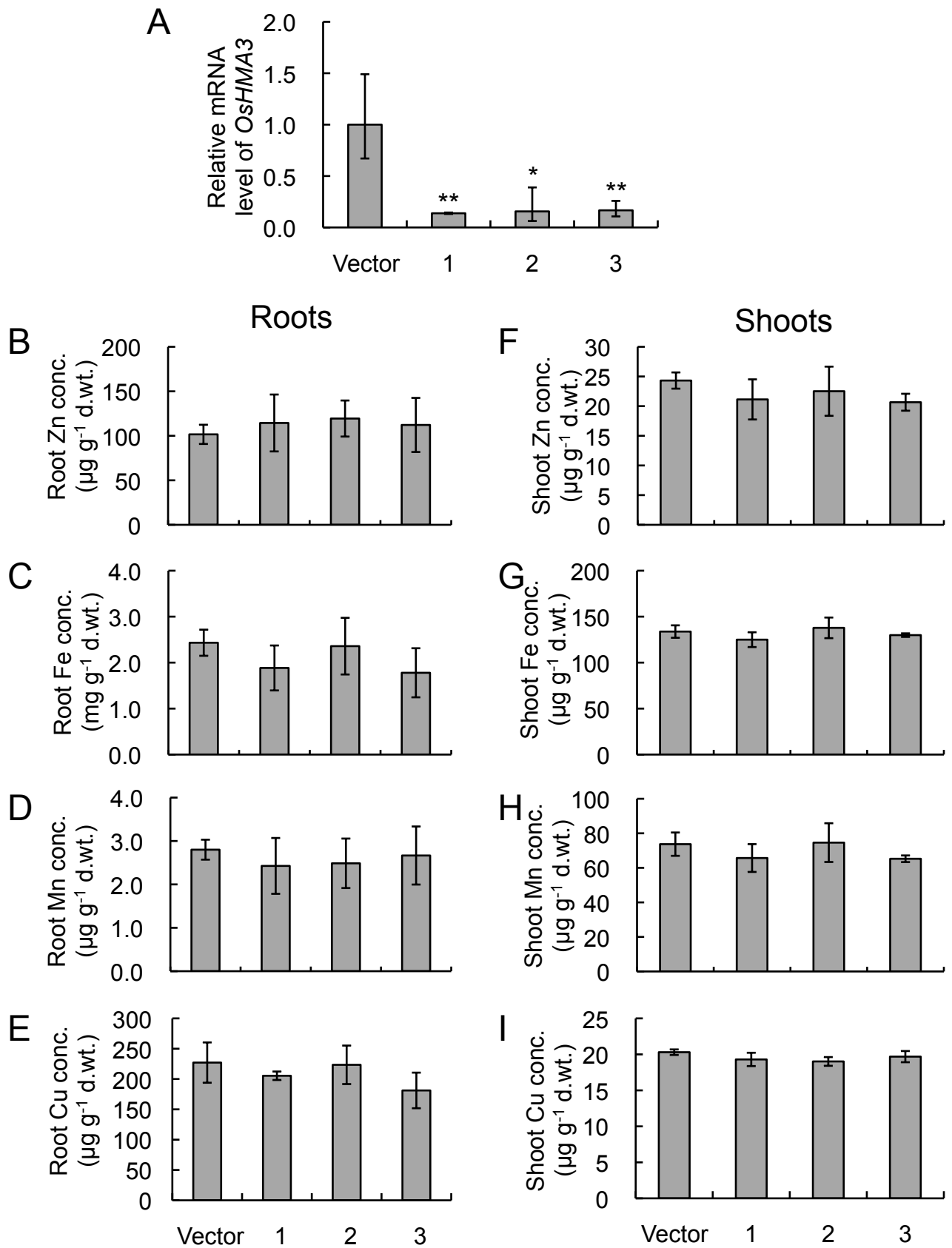


Fig. S8. Expression level (A) of *OsHMA3* and concentration of macronutrients in the roots and shoots of three independent RNAi and vector control line of Nipponbare background (B-I). The plants were exposed to 50 nM Cd for ten days. Expression relative to the vector control was shown. Data are means \pm SD of three biological replicates * $p < 0.05$, ** $p < 0.01$; Dunnett's t-test.

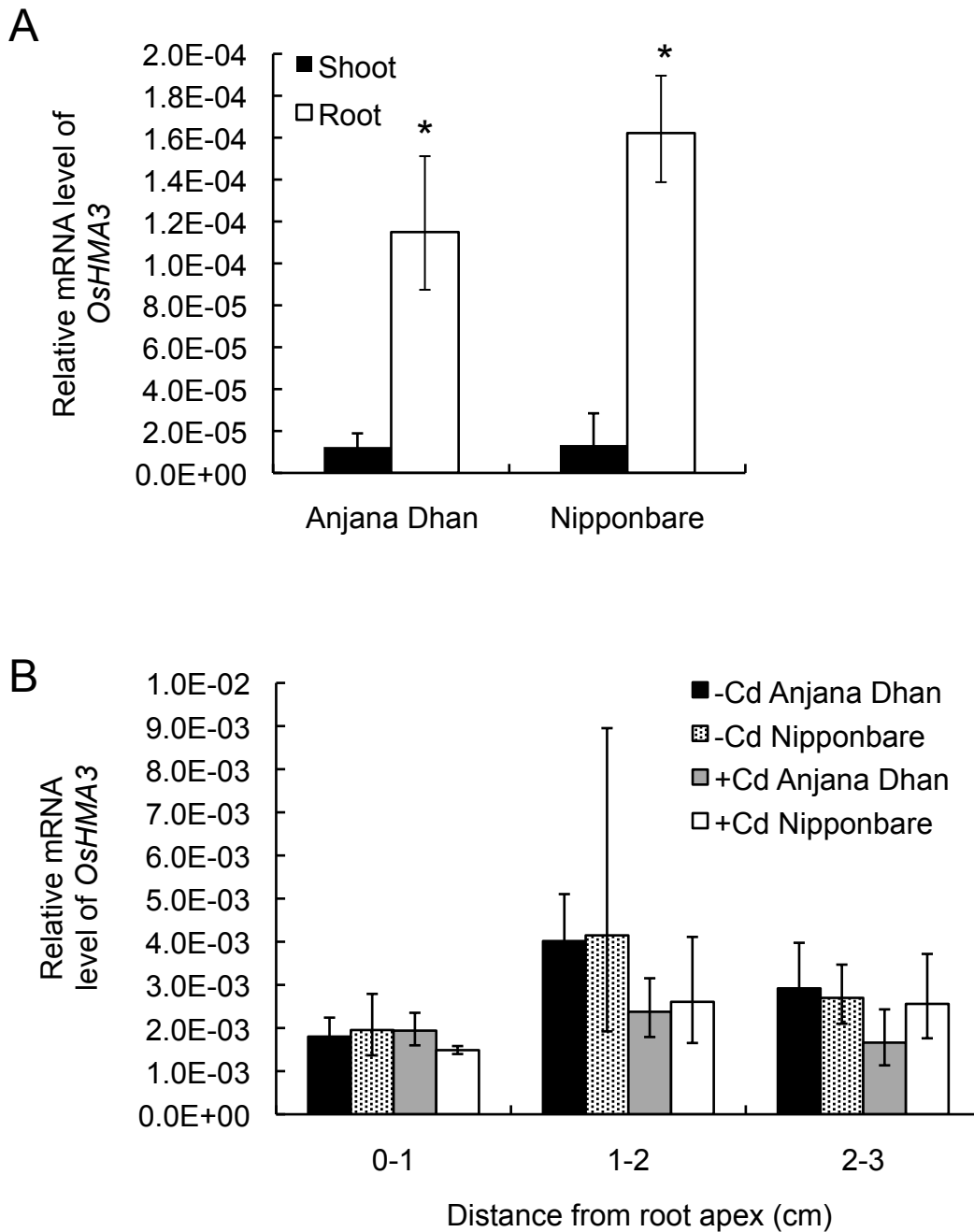


Fig. S9. Expression patterns of allelic Cd transporter genes.

(A) Expression of two allelic genes of *OsHMA3* in the roots and shoots of Nipponbare and Anjana Dhan. (B) Spatial expression of two allelic genes in different root segments of Nipponbare and Anjana Dhan. Seedlings were exposed to 0 or 1 μM CdSO_4 for 24 hours and then the roots were excised at 0-1, 1-2, and 2-3 cm. The expression level was determined by quantitative real time RT-PCR. *Histone H3* was used for internal control. Expression relative to the *Histone H3* expression level are shown. Error bars represent $\pm\text{SD}$ (n=3). * $p < 0.05$; Dunnett's t-test.

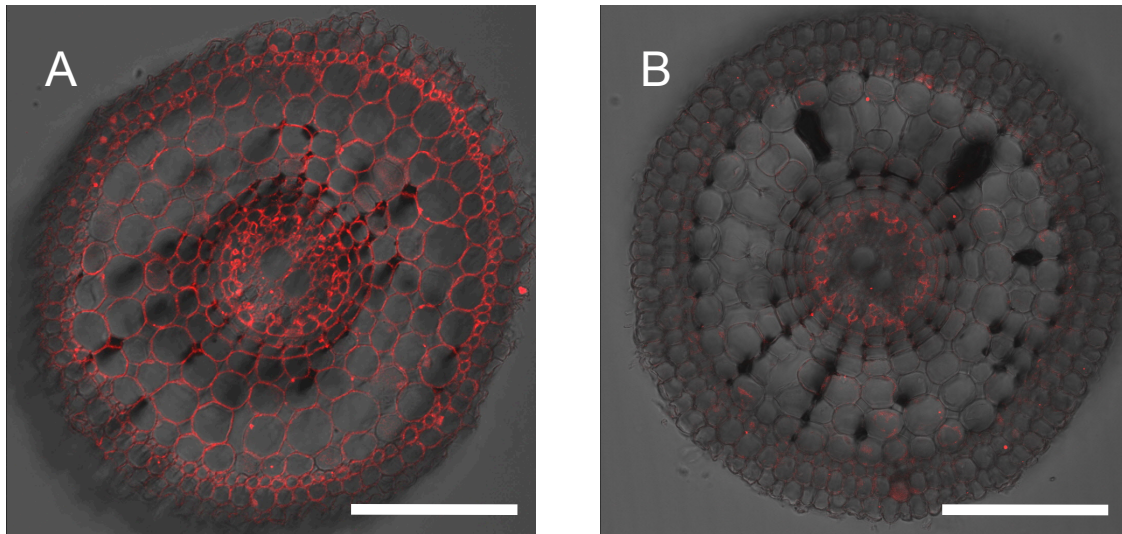


Fig. S10. Cellular localization of OsHMA3 in the roots of overexpressing (A) and knockdown lines (B) generated from Nipponbare. Immunostaining was performed using an antibody specific to OsHMA3. bar, 100 μ m.

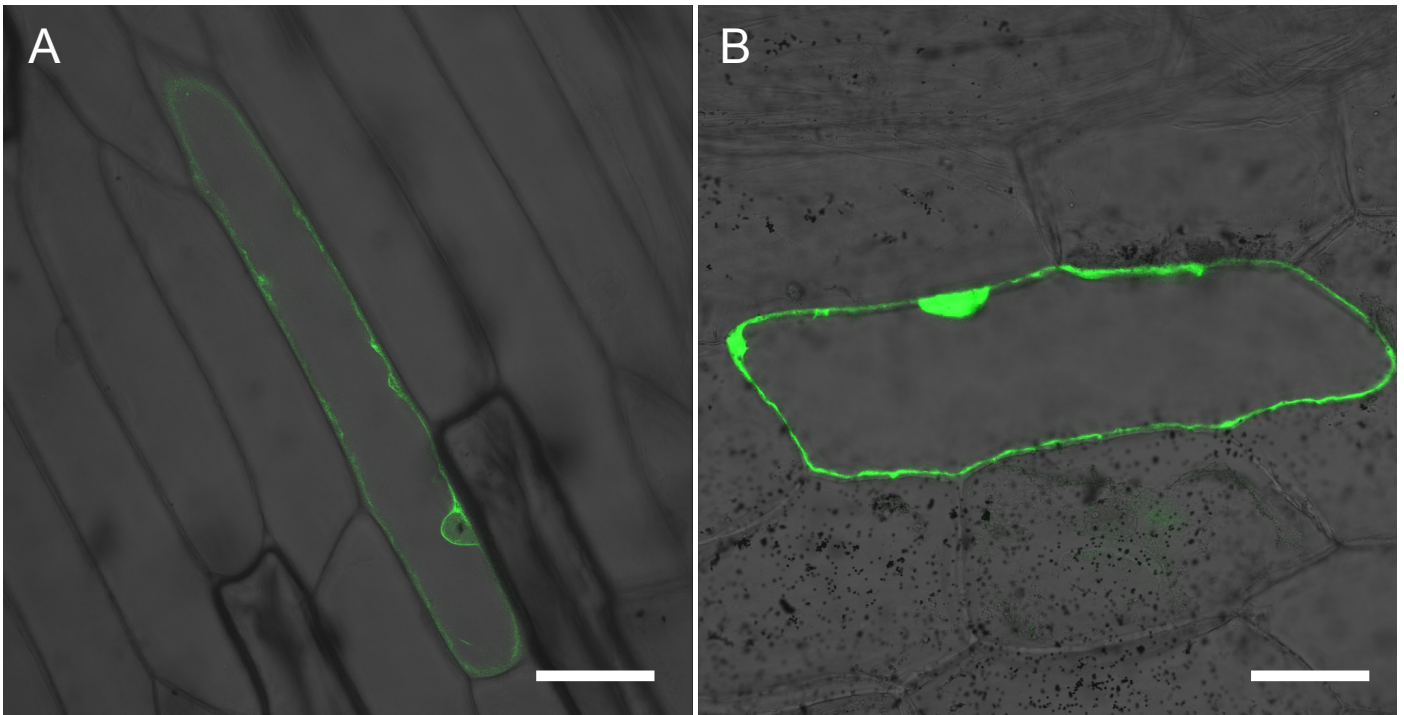


Fig. S11. Subcellular localization of OsHMA3 from Anjana Dhan. Fluorescence of GFP in onion epidermal cells expressing GFP-OsHMA3a fusion (*A*) or GFP alone (*B*) as a control. bar, 100 μ m.

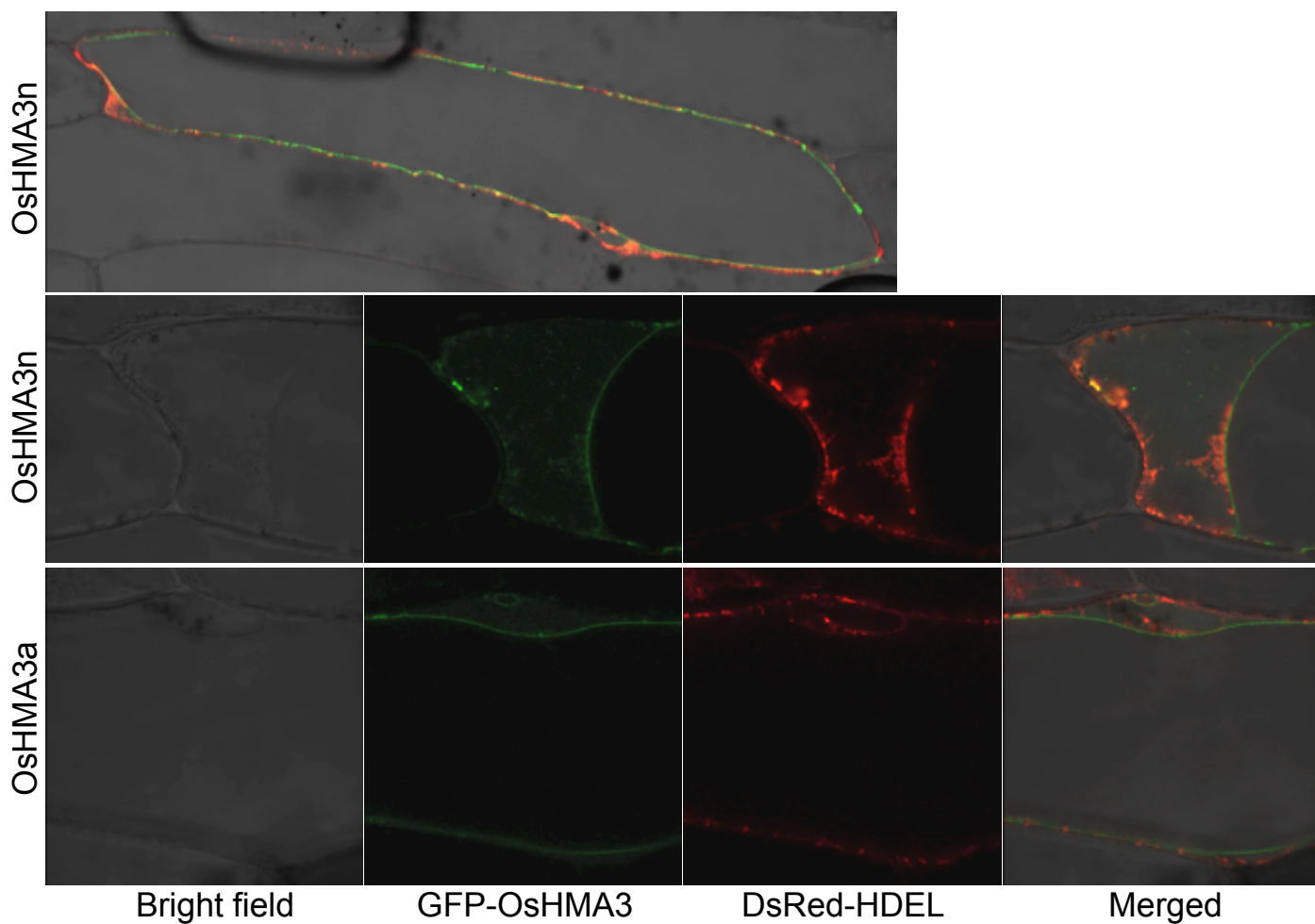


Fig. S12. Subcellular localization of OsHMA3 co-expressed with ER marker DsRed-HDEL. Fluorescence of GFP in onion epidermal cells expressing GFP-OsHMA3n/a fusion (green) and ER marker DsRed-HDEL (red).

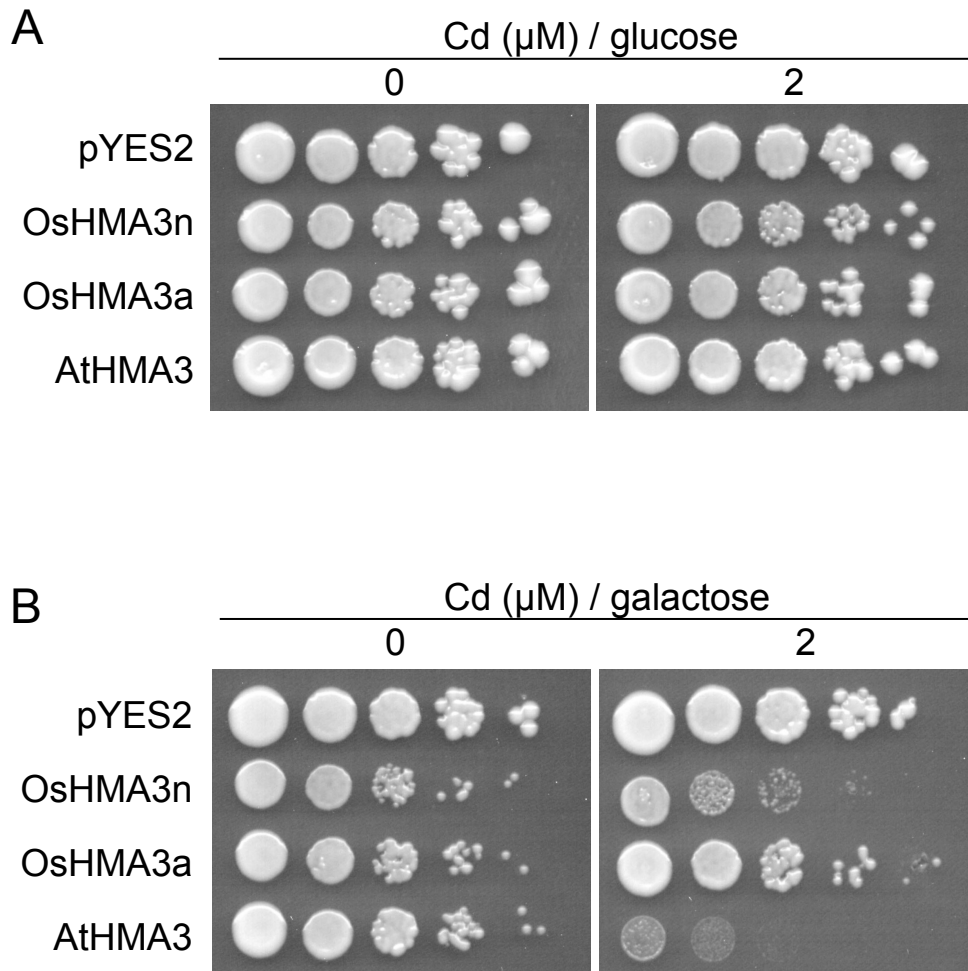


Fig. S13.

Growth of $\Delta ycf1$ cells transformed with empty vector pYES2, *OsHMA3n*, *OsHMA3a* and *AtHMA3* in the presence of glucose (A) or galactose (B). The yeast was grown for three days on a plate with or without 2 μM Cd.

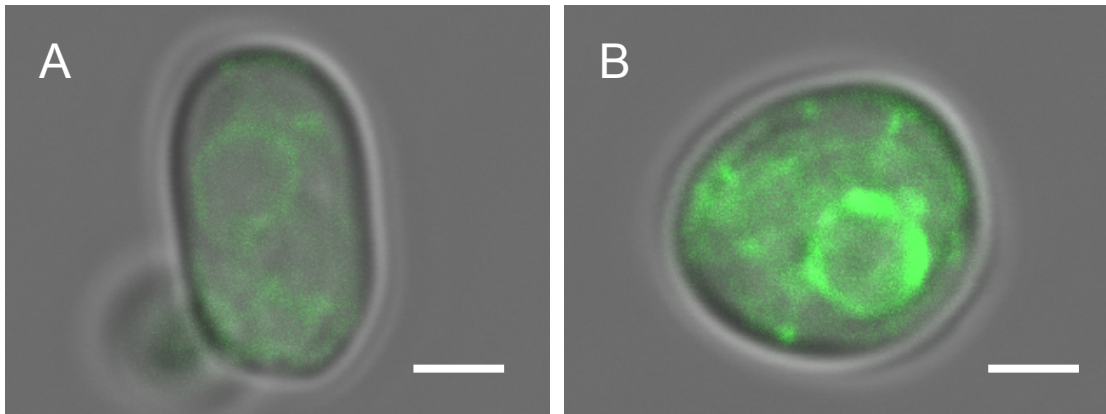


Fig. S14.
Subcellular localization of OsHMA3n-GFP (A) and OsHMA3a-GFP (B) in yeast.

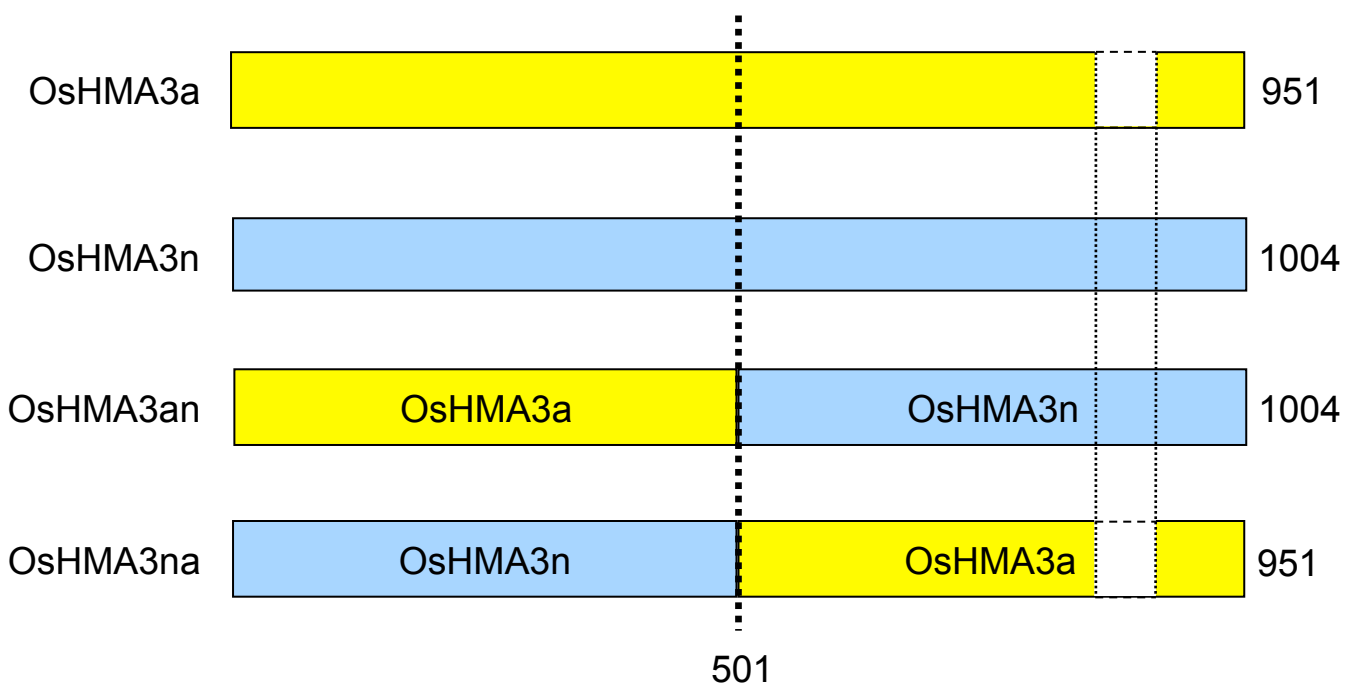


Fig. S15.

A schematic presentation of chimera proteins between OsHMA3n and OsHMA3a.

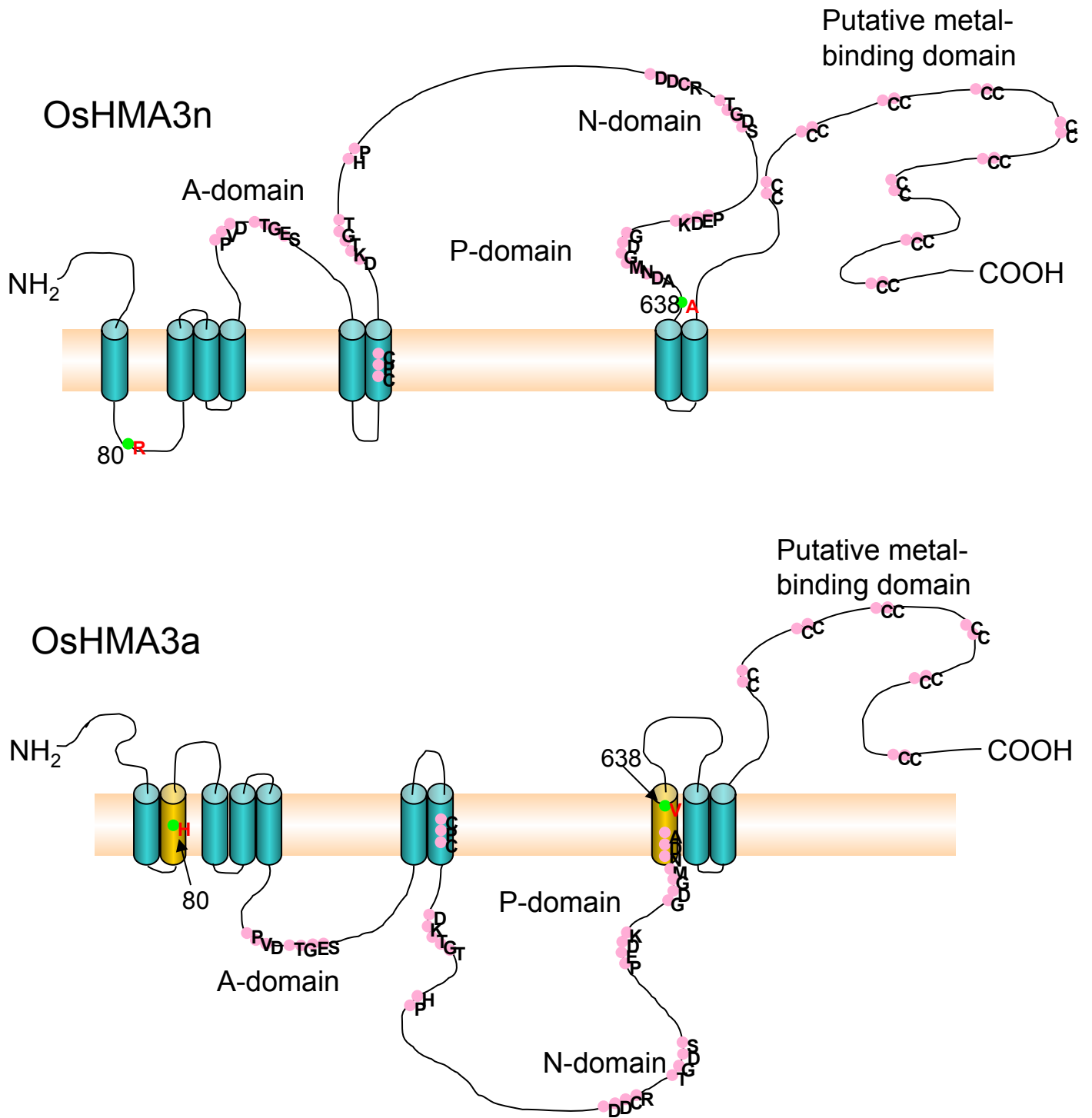


Fig. S16.

Transmembrane domains of OsHMA3n and OsHMA3a proteins predicated by SOSUI. Typical motifs are shown.

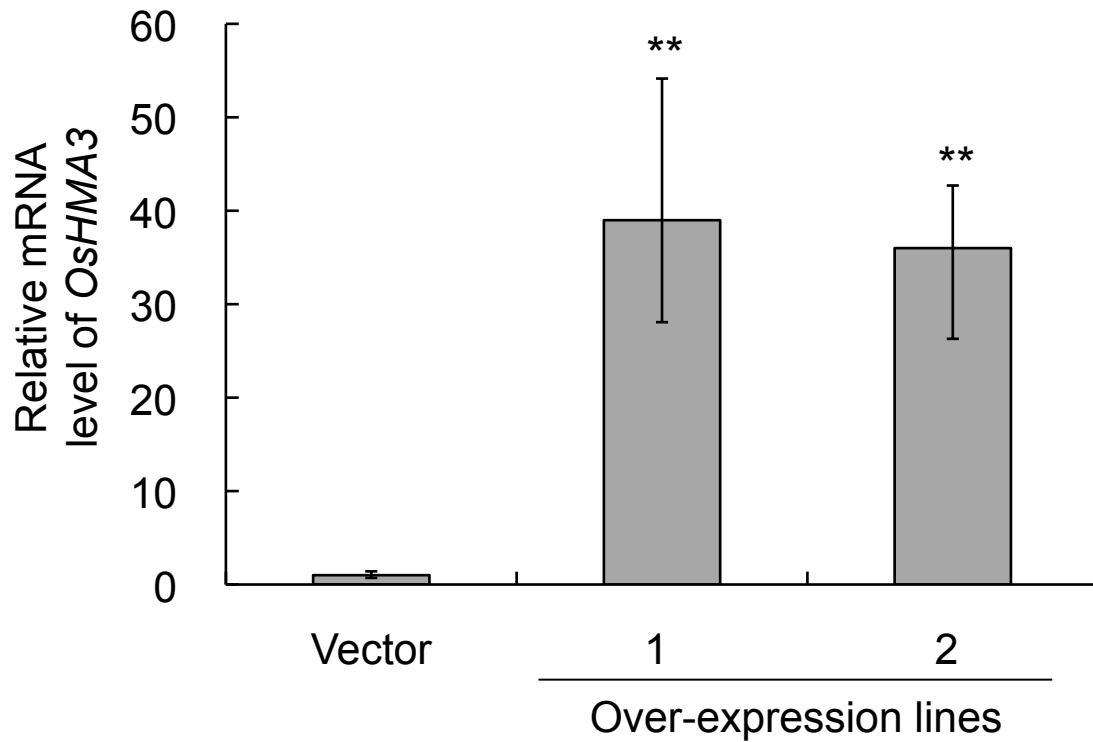


Fig. S17. Expression level of *OsHMA3n* of two independent over-expressing and vector control lines. Expression relative to vector control was shown. Data are means \pm SD of three biological replicates. ** $p < 0.01$; Dunnett's t-test.

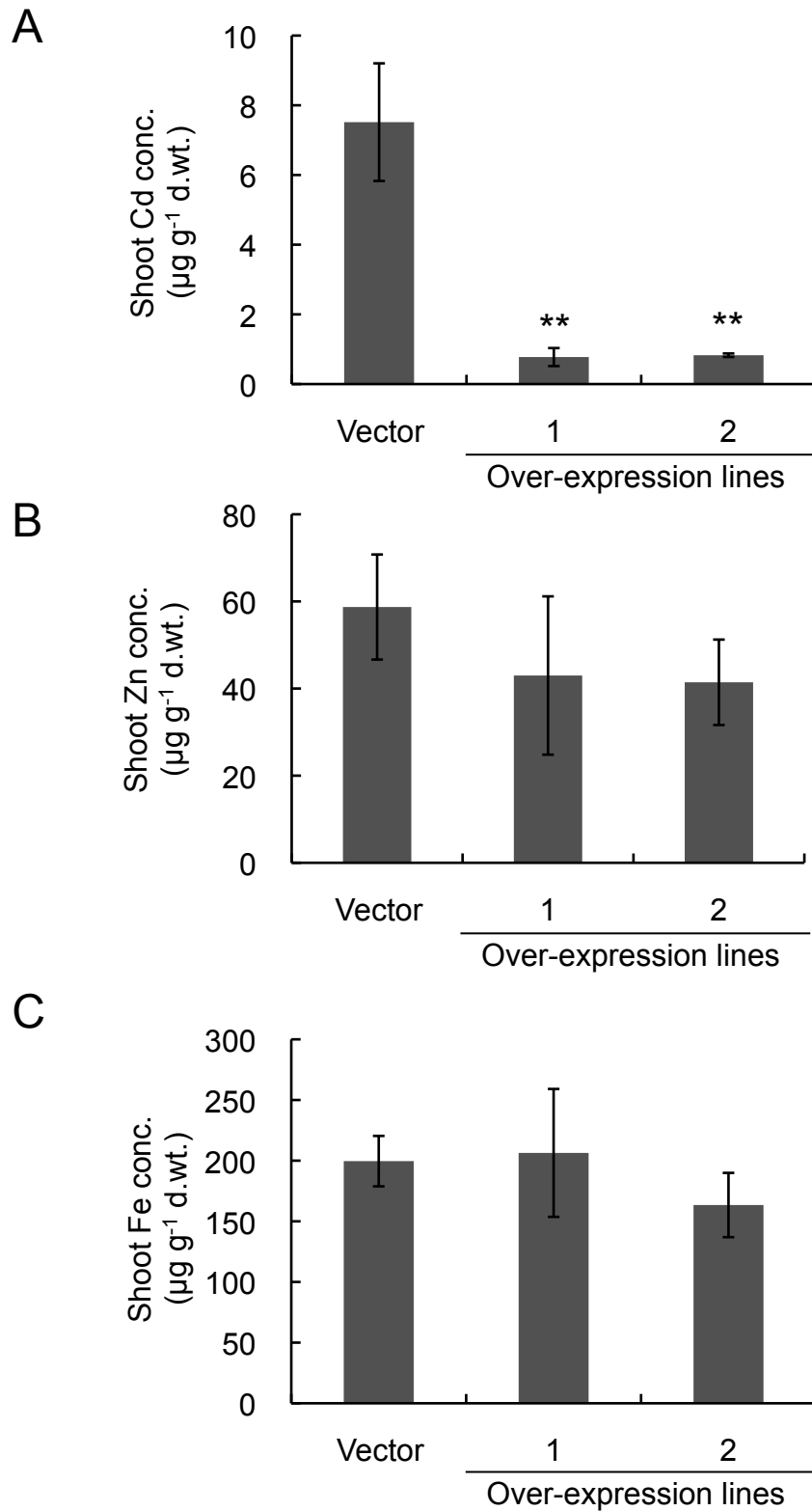


Fig. S18. Cd (A), Zn (B) and Fe (C) concentration of shoots of two independent overexpressing and vector control lines. All lines were grown in a Cd-contaminated soil for five months. Data are means \pm SD of three biological replicates. ** $p < 0.01$; Dunnett's t-test.

Table S1. Genotypes of 7 SSR markers on chromosome 7 in 67 recombinants and their relative Cd accumulation.

Recombinant plant No.	Genotypes of SSR markers on chromosome 7 (Physical distance (bp) from distal end of short arm of chromosome 7 (IRGSP Build 5))							Relative Cd accumulation (%)
	RM21238 (6785202)	RM21251 (7152467)	RM21260 (7304039)	RM21268 (7561641)	RM21275 (7643698)	RM8006 (7718177)	RM7153 (8640613)	
2	A	A	A	A	A	A	H	136.3
6	A	A	A	A	A	A	H	99.1
9	A	A	A	A	A	A	H	90.1
10	A	A	A	A	A	A	H	96.9
33	A	A	A	A	A	A	H	100.6
36	A	A	A	A	A	A	H	74.8
37	A	A	A	A	A	A	H	107.8
64	A	A	A	A	A	A	H	83.4
56	A	A	A	A	A	A	H	59.9
29	A	A	A	A	A	H	H	90.8
3	A	A	A	A	H	H	H	108.6
27	A	A	A	A	H	H	H	92.6
8	A	A	H	H	H	H	H	30.2
40	A	A	H	H	H	H	H	48.0
13	A	H	H	H	H	H	H	27.3
15	A	H	H	H	H	H	H	33.7
16	A	H	H	H	H	H	H	30.7
24	A	H	H	H	H	H	H	35.2
28	A	H	H	H	H	H	H	24.1
32	A	H	H	H	H	H	H	41.0
38	A	H	H	H	H	H	H	50.9
39	A	H	H	H	H	H	H	41.8
41	A	H	H	H	H	H	H	61.2
44	A	H	H	H	H	H	H	50.1
48	A	H	H	H	H	H	H	28.1
50	A	H	H	H	H	H	H	44.0
54	A	H	H	H	H	H	H	36.3
57	A	H	H	H	H	H	H	40.0
5	B	B	B	B	B	B	H	13.2
25	B	B	B	B	B	B	H	17.7
43	B	B	B	B	B	B	H	29.8
49	B	B	B	B	B	B	H	16.4
61	B	B	B	B	B	H	H	20.5
35	B	B	B	B	H	H	H	17.3
59	B	B	B	B	H	H	H	24.7
20	B	B	H	H	H	H	H	37.9
11	B	H	H	H	H	H	H	32.6
30	B	H	H	H	H	H	H	40.4
42	B	H	H	H	H	H	H	48.8
46	B	H	H	H	H	H	H	24.7
67	B	H	H	H	H	H	H	37.2
31	H	A	A	A	A	A	A	118.8
34	H	A	A	A	A	A	A	86.0
45	H	A	A	A	A	A	A	99.1
55	H	A	A	A	A	A	A	108.6
17	H	B	B	B	B	B	B	28.5
26	H	B	B	B	B	B	B	17.8
52	H	B	B	B	B	B	B	32.6
58	H	B	B	B	B	B	B	25.3
60	H	B	B	B	B	B	B	18.4
21	H	H	A	A	A	A	A	81.5
66	H	H	B	B	B	B	B	31.3
14	H	H	H	H	H	A	A	37.9
63	H	H	H	H	H	A	A	60.9
4	H	H	H	H	H	B	B	32.9
1	H	H	H	H	H	H	A	21.9
18	H	H	H	H	H	H	A	41.4
22	H	H	H	H	H	H	A	37.9
23	H	H	H	H	H	H	A	35.4
51	H	H	H	H	H	H	A	35.1
62	H	H	H	H	H	H	A	33.1
65	H	H	H	H	H	H	A	34.7
7	H	H	H	H	H	H	B	27.7
12	H	H	H	H	H	H	B	30.7
19	H	H	H	H	H	H	B	34.3
47	H	H	H	H	H	H	B	33.2
53	H	H	H	H	H	H	B	45.4

A, B and H indicate homozygous of Anjana Dhan allele, homozygous of Nipponbare allele and heterozygous, respectively.

Table S2. Results of QTL analysis using 67 plants which recombinations occurred in the candidate genomic region of the QTL.

Marker	Position	LOD	a	d	PVE(%)
RM21238	0.00	3.11	17.61	0.38	19.0
	0.02	3.72	19.63	-4.66	25.9
	0.04	6.72	34.40	-24.99	81.8
	0.06	10.44	35.14	-24.57	82.8
	0.08	13.17	35.72	-24.13	83.5
	0.10	15.32	36.15	-23.75	84.0
	0.12	17.07	36.46	-23.44	84.4
	0.14	18.52	36.66	-23.20	84.6
	0.16	19.72	36.80	-23.00	84.8
	0.18	20.67	36.87	-22.83	84.8
	0.20	21.36	36.87	-22.67	84.7
	0.22	21.69	36.80	-22.49	84.5
	0.24	21.06	36.67	-22.06	83.3
	RM21251	0.25	20.76	36.51	-21.64
0.27		27.61	36.83	-21.98	84.7
RM21260	0.29	28.58	36.80	-21.90	84.7
RM21268	0.29	28.57	36.80	-21.90	84.7
	0.31	27.16	36.78	-22.27	84.4
RM21275	0.32	18.70	36.40	-19.67	71.9
	0.34	20.32	36.32	-23.16	83.5
RM8006	0.36	13.84	33.08	-16.88	60.7
	0.38	17.72	35.51	-23.77	82.9
	0.40	18.08	35.68	-24.05	83.2
	0.42	17.81	35.69	-24.31	83.3
	0.44	17.17	35.60	-24.58	83.3
	0.46	16.23	35.44	-24.88	83.2
	0.48	15.00	35.22	-25.25	83.0
	0.50	13.43	34.94	-25.69	82.8
	0.52	11.40	34.60	-26.22	82.5
	0.54	8.66	34.23	-26.81	82.1
	0.56	2.22	20.10	-1.06	18.4
	0.58	1.73	15.97	5.63	11.5
RM7153	0.59	0.08	-1.99	-0.40	0.1

All genetic parameters were calculated by QTL Cartographer ver 2.5

(BASTEN et al. 2005) with 2 cM interval. LOD: Log-likelihood value, a:

additive effect of Anjana Dhani allele on relative Cd accumulation, d:

dominance effect, PVE: Percent of phenotypic variance explained by QTL.

Table S3.

Annotation of candidate genes in the QTL region of chromosome 7

Locus ID	Annotation
Os07g0231900	Peptidase, trypsin-like serine and cysteine domain containing protein
Os07g0232200	Similar to Flavohemoprotein b5/b5R variant
Os07g0232300	Conserved hypothetical protein
Os07g0232800	OsZIP8
Os07g0232900	OsHMA3
Os07g0233300	Similar to Nucleic acid binding protein-like

Table S4. Primer sequences of SSR markers and newly designed Indel and CAPS markers used in the linkage mapping. For CAPS markers, target SNPs, restriction enzymes (RE) used to detect polymorphisms

Marker name	Forward (5'---3')	Reverse (5'---3')	Marker type
RM21251	TTAGCTACCCTCAACAAGAGCATTGG	TGCCAGGTTCTGTTGGATAAAGG	SSR
RM21260	CTGCACAACCAGGAGAAATTAAGC	CTGACCACCTAGCTTGCCCTACC	SSR
RM21261	CCTCCATTTCAGCCACCACC	CAGAGTACGGCTGATTGACTGC	SSR
RM21263	CGTGATTGCTAAGAAACCCTTCG	TGGTCGCCAGAGATAAGTATCAGC	SSR
RM21264	CAGACGATGACGATGACTGC	ACAGCCTGCTTCCCTCTCTCC	SSR
RM21265	TCGTGCATGCCATCTAAATA	GAAAAATCAACGGCGTCAAATA	SSR
RM21268	GCAAACCTAGCAAGTAGCAAGAACG	GAGTGCCTGTGTATATAGGATACG	SSR
RM21275	ATCGATCAAGCTCCGTATCATGG	TGTCGTAGCCTCCCAATCACC	SSR
OSHMA3-29	TGAAAGTAAACAGCAGGATAGGGG	TGCTTACCGAACAAGAAGACTG	SNP
OSHMA3-30	ATCCTATGGCATTACTGGTTCAA	CCAACAAGATACAAGTGGGAAGA	SNP
OSHMA3-25	CTAACTCCAGCCGTCACC	CATTGAAGCATGTGCTAATCAC	CAPS marker (NsiI digestion)