Supporting Information

Ishikawa et al. 10.1073/pnas.1211132109

SI Materials and Methods

Production of Rice Mutants, Growth Conditions, and Screening for Low-Cd Mutants. Husked seeds of rice (*Oryza sativa* L. cv. Koshihikari) were irradiated with 320 MeV carbon ions ($^{12}C^{6+}$) from an azimuthally varying field cyclotron (Japan Atomic Energy Agency, Takasaki, Gunma, Japan) at a dose of 40 Gy. Approximately 4,000 M_1 seeds were grown in a paddy field and self-pollinated, and the resultant seeds (M_2) were bulked. The 2,592 M_2 seedlings and 288 Koshihikari (WT) seedlings were transplanted into plastic pots filled with 300 g of Cd-contaminated paddy soil (soil Cd concentration: 1.8 mg $Cd{\cdot}kg^{-1}$). All plants were submerged until the booting stage, and then water was withheld to increase the bioavailable Cd concentration in the soil (1). The grains were harvested from all plants and analyzed to determine their Cd concentration as described below.

Hydroponic Experiments. To evaluate Cd uptake of the three candidate mutants (*lcd-kmt1*, *lcd-kmt2*, and *lcd-kmt3*) selected from the M_2 plants, the resultant M_3 and WT seedlings were grown hydroponically in 20 L of half-strength Kimura B solution (2) with 0.18 μM $CdSO_4$ added (pH 5.2) in a Biotron (NC350; NK System) at 30 °C and 70% humidity, with a 16-h photoperiod. After 4 d, the plants were harvested for metal analysis (see below).

Field Experiments. M_4 plants of the three mutant lines and the WT were cultivated in Cd-polluted paddy fields with soils classified as Gleysols in three regions of Japan. The soil Cd concentration was 1.35 $mg{\cdot}kg^{-1}$ (field A), 1.21 $mg{\cdot}kg^{-1}$ (field B), and 0.35 $mg{\cdot}kg^{-1}$ (field C) as determined by 0.1-M HCl extraction (the method stipulated by the *Agricultural Land-Soil Pollution Prevention Law* in Japan). Seedlings were transplanted into the flooded paddy fields, one plant per hill, spaced at 15 cm × 30 cm. Each genotype was planted in a separate row of 20 plants. After 1 mo, the fields were drained and then irrigated intermittently until grain maturity. We applied inorganic fertilizers containing N, K, and P using standard methods for each region. The plants were harvested at maturity and divided into grains (unpolished rice) and straw for metal analysis as described below.

Evaluation of Agronomic Traits in the Mutant Rice. The plants were cultivated in a paddy field not contaminated with Cd at the experimental field of the National Institute for Agro-Environmental Sciences under conventional intermittent irrigation until grain maturity. The planting density was 22.2 hills per m^2, with a spacing of 15 cm × 30 cm. A compound fertilizer containing 8% (wt/wt) each of N, P, and K was applied as a basal dressing at rates of 50 $kg{\cdot}ha^{-1}$ N, P, and K. The chlorophyll contents in the flag leaf at the booting stage were determined using a SPAD meter (SPAD-502Plus; Konica Minolta Sensing). Agronomic traits were also measured: grain and straw yields, days to heading, plant height, culm length, and panicle number per plant. The eating quality scores were evaluated according to several factors, including the amylose and protein contents, by using a taste analyzer (RCTA11A; Satake Corporation).

Analysis of Cd and Other Metals (Cu, Fe, Mn, and Zn). The grain samples were air-dried, and other samples (shoots and roots) were oven-dried at 70 °C. The mature shoot samples (straw) were milled to a fine power to pass through a 0.5-mm mesh using a stainless-steel rotor mill (P14; Fritsch). Sample digestion was performed as described previously (3). Metal concentrations were determined by inductively coupled plasma-optical emission spectroscopy (Vista-Pro; Agilent Technologies Japan) for Cu, Fe, Mn, and Zn or inductively coupled plasma mass spectroscopy (ELAN DRC-e; Perkin-Elmer Sciex) for Cd. We used a certified standard material to calibrate the concentrations of the metals in the rice samples: NIES CRM no. 10 rice flour (National Institute for Environmental Studies, Tsukuba, Japan) for Cd, Cu, Fe, Mn, and Zn.

Positional Cloning. An F_2 population derived from a cross between a high-Cd *indica* cultivar (Kasalath) and a low-Cd Koshihikari mutant (*lcd-kmt1*) was used for positional cloning. The F_2 progeny consisting of 92 individuals (14-d-old seedlings) were grown for 4 d in a hydroponic system with 0.18 μM $CdSO_4$ as described above, and then a small piece of the leaf was collected for extraction of the genomic DNA. The remaining shoots and roots were harvested for metal analysis. Leaf DNA was extracted using the method of Xu et al. (4) and a genetic linkage map constructed using 93 simple sequence repeat (SSR) markers (5). Moreover, four SSR markers (RM7153, RM21327, RM5499, and RM3832) were added in the interval between RM8007 and RM5543, which are located near the target gene. The linkage order and genetic distances between markers were calculated using MAPMAKER/EXP version 3.0 (www.broadinstitute.org/ftp/distribution/software/mapmaker3/). Quantitative trait locus (QTL) analysis was performed by means of composite interval mapping using QTL Cartographer version 2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm).

Cloning and Sequencing of *OsNRAMP5*. Total RNA was extracted from the roots of WT or *lcd-kmt* plants using Sepasol RNA I Super (Nacalai Tesque) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (Toyobo) and oligo(dT)20 (Toyobo) for the RT-PCR. The full-length ORF of *OsNRAMP5* in the WT and *lcd-kmt* plants was amplified by means of PCR using primer pair A (5′-CACCATGGAGATTGAGAGAGAGAGCAGTG-3′ and 5′-ACACCCTTGTCGATCGATCGATCTG-3′), which was designed according to the sequence data of Nipponbare gene Os07g0257200 in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). The amplified full-length cDNAs were cloned into the pENTR/D-TOPO vector (Invitrogen) and then sequenced using the BigDye Terminator Cycle Sequencing Kit 3.1 in the forward and reverse directions using universal M13 primers (Invitrogen) and primer pair B (5′-GCAAGTCGAGTGCGATCGTG-3′and 5′- CGCCGATGATGGAGACGATG-3′) in an ABI 3130xl genetic analyzer (Applied Biosystems). Genomic DNA extracted from the roots of the WT and *lcd-kmt* plants was amplified by means of PCR using primer pair B, and the resultant fragments were directly sequenced as described above. Multiple amino acid alignments were performed using ClustalW (www.ch.embnet.org/software/ClustalW.html) and displayed using BOXSHADE version 3.21 software (www.ch.embnet.org/software/BOX_form.html) hosted on the Web server of the Swiss Institute of Bioinformatics. The hydropathicity plot was created with the ProtScale software hosted on the proteomics server of the Swiss Institute of Bioinformatics (http://web.expasy.org/protscale/) according to the method of Kyte and Doolittle with a window size of nine (6).

Microarray. Seedlings (14 d old) of WT and *lcd-kmt1* were transferred to nutrient solution (*Hydroponic Experiments*, above)

and grown for 3 wk. Total RNA was extracted from the whole roots and labeled with Cy-3 and Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification kit. Microarray analysis was performed using a rice 44K oligo-DNA microarray according to the manufacturer's protocol (Agilent Technologies Japan; G2519F#15241). The hybridized microarrays were scanned using an Agilent Microarray Scanner, and the imaging data were analyzed by Agilent Feature Extraction software. Genes showing fold changes, which were calculated according to the differences in signal intensity of *lcd-kmt1* and WT, >2 or <0.5 and with *P* values <0.05 were considered to be significantly up- or down-regulated, respectively.

Subcellular Localization of *OsNRAMP5*. The full-length ORFs of *OsNRAMP5* and *osnramp5-1*, which were derived from WT and *lcd-kmt1* plants, respectively, were subcloned into pH7WGF2 using the LR recombination reaction (7). Onion epidermal cells were transformed using a Biolistic PDS-1000/He particle delivery system (Bio-Rad) and observed with an LSM5 Pascal laser-scanning confocal microscope (Carl Zeiss) (8).

Transport Activity in Yeast Cells. The ORFs of *OsNRAMP5* and *osnramp5-1* were cloned into the expression vector pDR195 (9) and then introduced into yeast cells using the lithium acetate method. The following strains of yeast (*Saccharomyces cerevisiae*) were used: WT strain (BY4741, *MATa*, *his3Δ1;leu2Δ0; met15Δ0; ura3Δ0*), the Mn uptake-defective mutant *Δsmf1* (*MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOL122c::kanMX4*), the ferrous iron uptake-defective double mutant *Δfet3fet4* (*MATa/MATalpha ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::His3/fet3–2::HIS3 fet4-1::LEU2/fet4-1::LEU2*), and the Cd-sensitive mutant *Δycf1*(*MATalpha trp1-63 leu2-3, 112 gcn4-101 his3-609 ura3-52 ycf1::TRP1*), which lacks the function for compartmentalization of Cd into vacuoles (10). To produce medium deficient in Fe or Mn, 10 μM bathophenanthroline disulfonic acid or 20 mM ethylene glycol-bis-β-aminoethylether-*N*,*N*,*N*',*N*' tetraacetic acid with 50 mM 2-morpholinoethanesulfonic acid monohydrate were added, respectively (Wako Pure Chemical). For the Cd treatment, 10 μM $CdCl_2$ was added to the medium. After spotting the yeast at three dilutions (optical densities at 600 nm of 0.1, 0.01, and 0.001), the plates were incubated at 30 °C for 5 d.

Development of Genetic Markers. Genomic DNA was extracted from fresh leaves of WT, *lcd-kmt1*, and *lcd-kmt2* plants. We designed primer pair C (5′-TTCAGAACGTGCTGGGCAAGTCG-3′ and 5′-ACGGATTAACAAATTAATTATGTGGCAG-3′) and primer pair D (5′-TATATTCAGCCTGGGCAGATCGAG-3′ and 5′-TGATGTACTGTCCAGCGTATGTGC-3′) according to the sequences around the mutated regions in *lcd-kmt1* or *lcd-kmt2*, respectively. The reaction mixture consisted of template DNA, 1× KAPA2GTM Fast ReadyMix with dye (Kapa Biosystems), and 300 nM of each amplification primer. PCR products from the WT and *lcd-kmt1* plants were separated in sodium borate electrophoresis buffer in 3% (wt/vol) agarose gel (11) (LE analytic grade; Promega). To distinguish between the WT and *lcd-kmt2* alleles, PCR products from *lcd-kmt2*, WT, and F_1 plants were digested with FastDigest FspI (Thermo Scientific), followed by electrophoresis in 1% (wt/vol) agarose gel.

The genetic marker, which was developed to distinguish between *OsNRAMP5* and *osnramp5-1*, was used for genotyping of F_2 progeny derived from a cross between Kasalath and *lcd-kmt1*. The 88 F_2 seedlings were treated with 0.18 μM $CdSO_4$, and then a small piece of the leaf was collected for extraction of the genomic DNA as described above. The remaining shoots were harvested for Cd analysis.

Statistical Analysis. All metal concentrations and agronomic traits were compared using one-way ANOVA. Significant differences in mean values were evaluated using Tukey's test. All calculations were performed using IBM SPSS Statistics (IBM Japan).

1. Ishikawa S, et al. (2010) A major quantitative trait locus for increasing cadmium-specific concentration in rice grain is located on the short arm of chromosome 7. *J Exp Bot* 61(3):923–934.
2. Ishikawa S, et al. (2011) Real-time imaging and analysis of differences in cadmium dynamics in rice cultivars (*Oryza sativa*) using positron-emitting ^{107}Cd tracer. *BMC Plant Biol* 11:172.
3. Ishikawa S, Ae N, Yano M (2005) Chromosomal regions with quantitative trait loci controlling cadmium concentration in brown rice (*Oryza sativa*). *New Phytol* 168(2): 345–350.
4. Xu X, Kawasaki S, Fujimura T, Wang CT (2005) A protocol for high-throughput extraction of DNA from rice leaves. *Plant Mol Biol Rep* 23(3):291–295.
5. McCouch SR, et al. (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res* 9(6):199–207.
6. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157(1):105–132.
7. Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci* 7(5):193–195.
8. Ishimaru Y, et al. (2006) Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant J* 45(3):335–346.
9. Rentsch D, et al. (1995) NTR1 encodes a high affinity oligopeptide transporter in Arabidopsis. *FEBS Lett* 370(3):264–268.
10. Li ZS, et al. (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc Natl Acad Sci USA* 94(1):42–47.
11. Brody JR, Kern SE (2004) Sodium boric acid: A Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36(2):214–216.

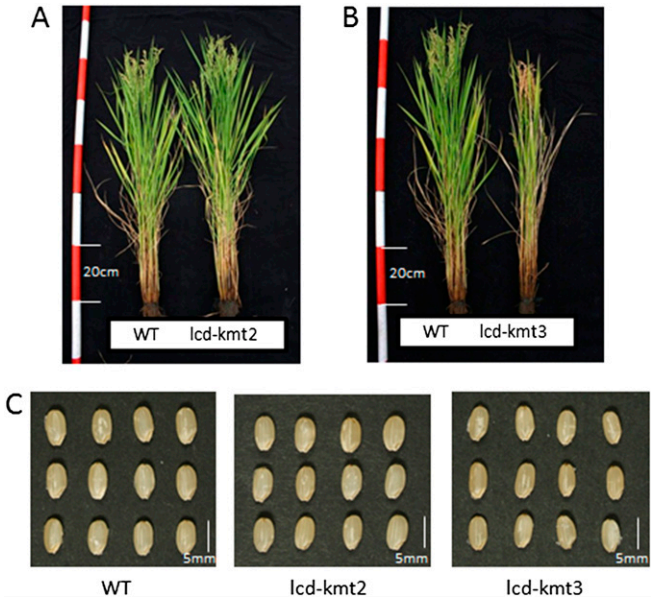

Fig. S1. (*A*) Plant morphologies of Koshihikari (WT) and *lcd-kmt2*. (*B*) Plant morphologies of Koshihikari (WT) and *lcd-kmt3*. (*C*) Morphologies of unpolished rice grains in the WT, *lcd-kmt2*, and *lcd-kmt*3.

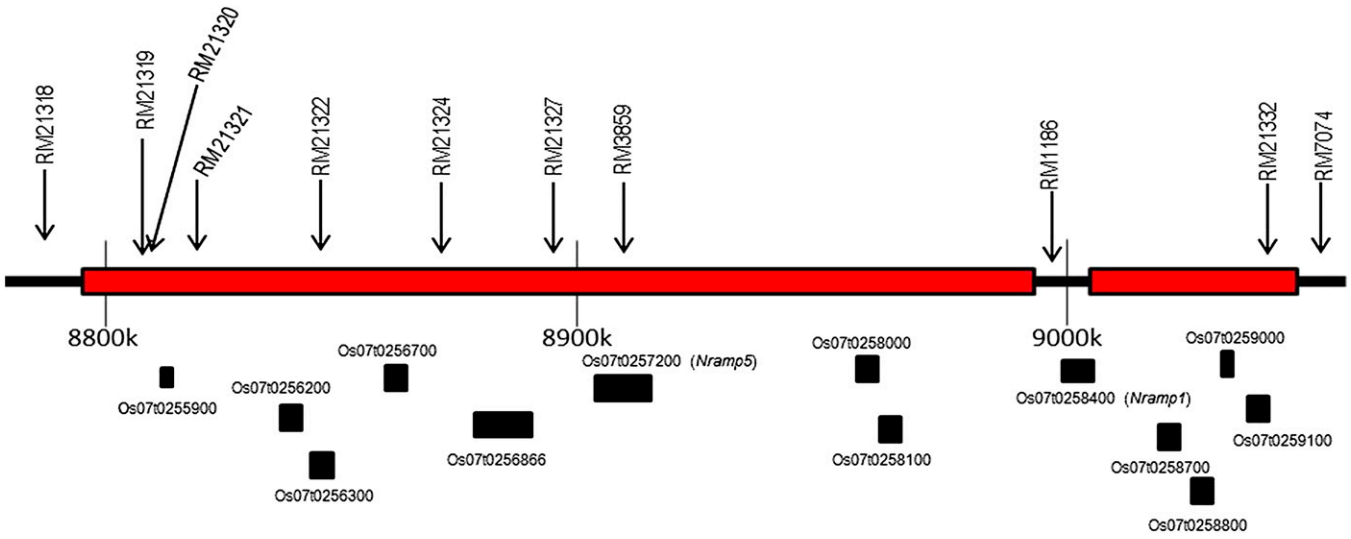

Fig. S2. Regions deleted in *lcd-kmt3* relative to WT Koshihikari. Red bars show the genomic regions deleted by ion-beam irradiation. The regions were identified by using the SSR markers indicated at the top of the figure. Black bars at the bottom indicate annotated ORFs (both exons and introns) in the deleted region (data from http://rapdb.dna.affrc.go.jp/).

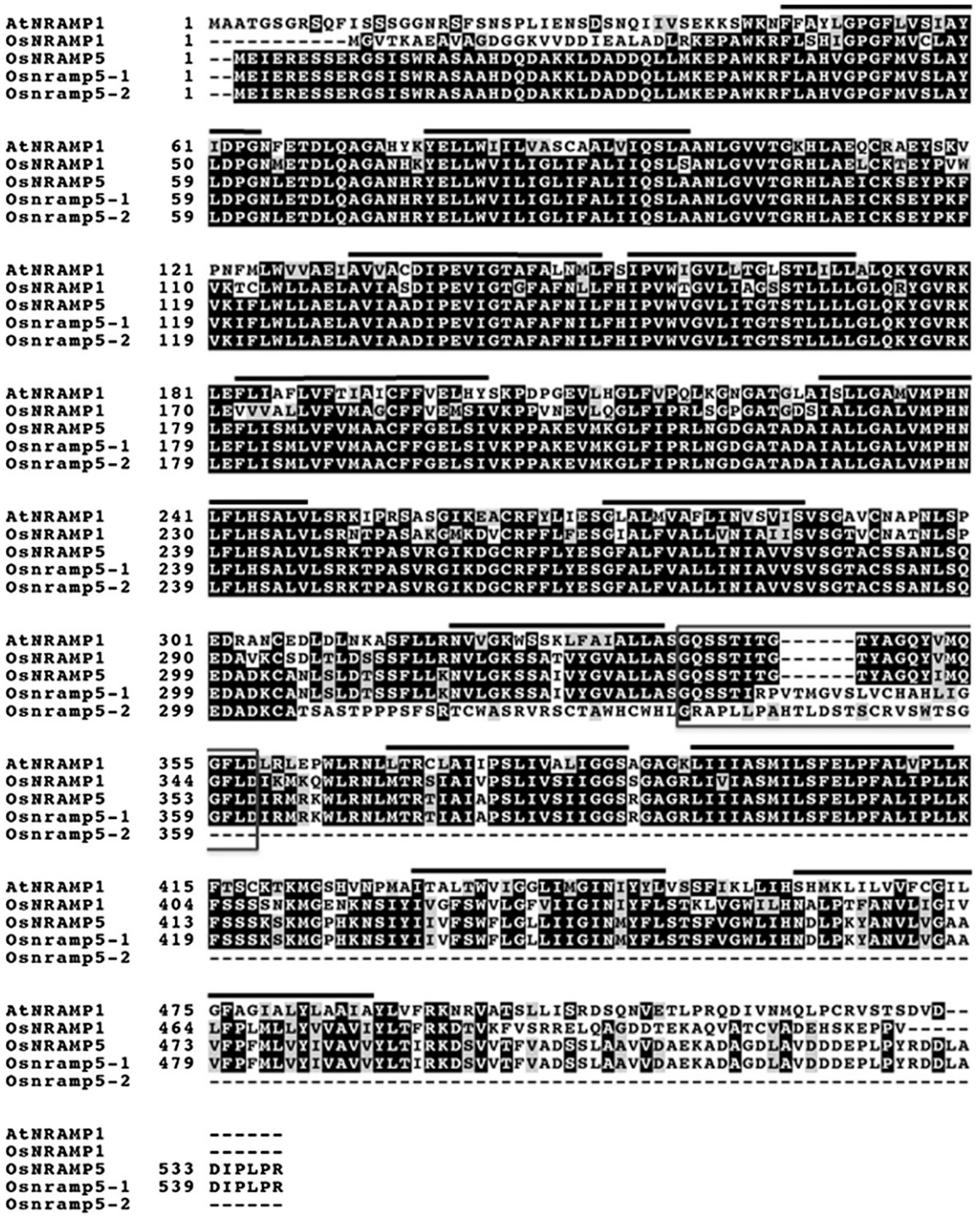

Fig. S3. Multiple alignment of the deduced acid sequences of *AtNRAMP1*, *OsNRAMP1*, *OsNRAMP5*, *osnramp5-1*, and *osnramp5-2*. Multiple amino acid alignments were performed using ClustalW (www.ch.embnet.org/software/ClustalW.html) and displayed using version 3.21 of BOXSHADE software (www.ch.embnet.org/software/BOX_form.html) hosted on the Web server of the Swiss Institute of Bioinformatics. Lines above the sequences indicate the positions of predicted transmembrane domains. The consensus transport motif between transmembrane domains 8 and 9 is boxed. DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory accession nos.: *AtNRAMP1*, BT029300; *OsNRAMP1*, AK121534; *OsNRAMP5*, AB690551; *osnramp5-1*, AB690552; *osnramp5-2*, AB690553.

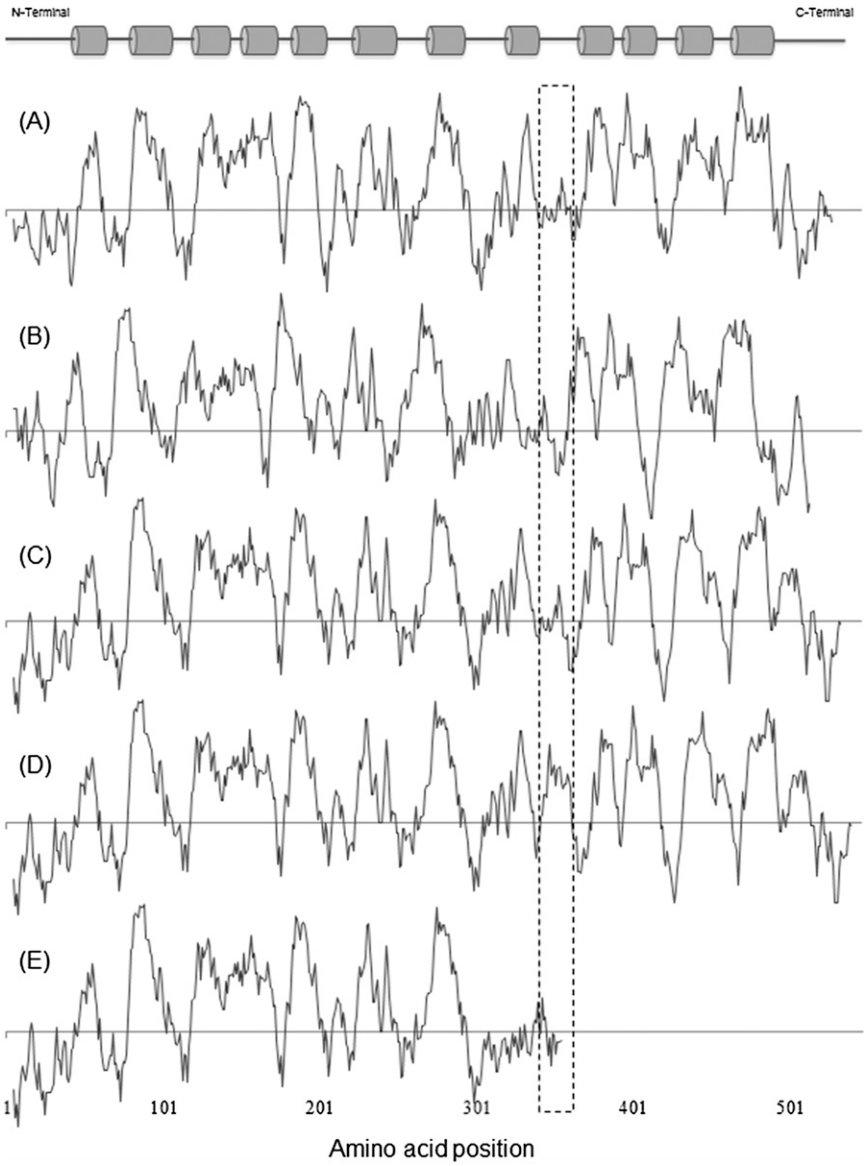

Fig. S4. Alignments of the hydropathicity profiles of the predicted amino acid sequences of (*A*) AtNRAMP1, (*B*) OsNRAMP1, (*C*) OsNRAMP5, (*D*) osnramp5-1, and (*E*) osnramp5-2. The hydropathicity plot was created with ProtScale software hosted on the proteomics server of the Swiss Institute of Bioinformatics (http://web.expasy.org/protscale/), according to the method of Kyte and Doolittle (6) with a window size of nine. The positions of the predicted 12 transmembrane domains (gray cylinders) are indicated immediately above the hydropathicity profiles, and the horizontal line in the plot represents a hydropathicity of 0. The dashed box represents the consensus transport motif.

Table S1. Dry weight and metal concentrations in the shoots and roots of WT Koshihikari and of three low-Cd Koshihikari mutants (*lcd-kmt1, lcd-kmt2,* and *lcd-kmt3*) grown in hydroponic culture containing 0.18 μM Cd

Plant part	Type	Dry weight(g)	Cd (mg·kg^{-1})	Mn (mg·kg^{-1})	Cu (mg·kg^{-1})	Fe (mg·kg^{-1})	Zn (mg·kg^{-1})
Shoot	Koshihikari	0.071^{a,b}	45.8^{b}	1,004^{b}	21.2^{a}	57.5^{a}	46.2^{a}
	lcd-kmt1	0.081^{b}	7.2^{a}	79.3^{a}	29.0^{b}	58.5^{a}	78.2^{a}
	lcd-kmt2	0.087^{b}	7.4^{a}	79.7^{a}	28.2^{b}	58.2^{a}	52.7^{a}
	lcd-kmt3	0.057^{a}	6.5^{a}	73.6^{a}	27.7^{b}	59.0^{a}	51.4^{a}
Root	Koshihikari	0.024^{a,b}	205.4^{b}	113.0^{b}	35.2^{a}	297.6^{a}	23.2^{a}
	lcd-kmt1	0.026^{b}	53.1^{a}	29.9^{a}	39.0^{a}	281.3^{a}	28.7^{a}
	lcd-kmt2	0.027^{b}	51.3^{a}	30.2^{a}	38.4^{a}	255.5^{a}	22.8^{a}
	lcd-kmt3	0.019^{a}	45.6^{a}	30.0^{a}	38.7^{a}	291.4^{a}	25.0^{a}

Data are the means of three replicates. Within a tissue type, numbers in the same column labeled with different letters indicate significant difference at $P < 0.05$ by Tukey's test.

Table S2. Comparisons of agronomic traits between Koshihikari and three low-Cd mutants (*lcd-kmt1*, *lcd-kmt2*, and *lcd-kmt3*)

Plant	SPAD	Time to heading (d)	Plant height (cm)	Culm length (cm)	Panicle number (plant^{-1})	Grain yield (t ha^{-1})	Straw yield (t ha^{-1})	Eating quality (score)
Koshihikari	32.9^{a}	85.4^{b,c}	103.4^{b}	86.5^{b}	13.2^{a}	5.65^{b}	7.74^{b}	80.5^{a}
lcd-kmt1	33.2^{a}	84.8^{b}	105.3^{b}	88.8^{b}	13.4^{a}	5.39^{b}	7.38^{b}	80.5^{a}
lcd-kmt2	32.9^{a}	86.8^{c}	104.0^{b}	87.4^{b}	13.7^{a}	5.57^{b}	7.43^{b}	79.0^{a}
lcd-kmt3	30.6^{a}	70.4^{a}	91.7^{a}	73.1^{a}	16.3^{b}	4.23^{a}	4.78^{a}	80.0^{a}

SPAD, chlorophyll content in the flag leaf determined using a SPAD meter. Values in the same column followed by different letters indicate significant difference at $P < 0.05$ by Tukey's test.

Table S3. Metal concentrations of grain and straw in Koshihikari and the three low-Cd mutants (*lcd-kmt1*, *lcd-kmt2*, and *lcd-kmt3*) grown in a paddy field contaminated with Cd (field B)

Plant part	Type	Cd (mg·kg^{-1})	Mn (mg·kg^{-1})	Cu (mg·kg^{-1})	Fe (mg·kg^{-1})	Zn (mg·kg^{-1})
Grain	Koshihikari	1.86^{b}	30.9^{b}	4.72^{a}	14.2^{a}	34.4^{a}
	lcd-kmt1	0.02^{a}	9.39^{a}	4.40^{a}	14.7^{a}	34.4^{a}
	lcd-kmt2	0.02^{a}	9.06^{a}	4.22^{a}	15.8^{a}	31.4^{a}
	lcd-kmt3	0.02^{a}	11.1^{a}	7.34^{b}	19.3^{a}	35.4^{a}
Straw	Koshihikari	9.14^{b}	714^{b}	3.57^{a}	71.7^{a}	43.3^{a}
	lcd-kmt1	0.04^{a}	24.9^{a}	4.04^{a}	64.5^{a}	28.1^{b}
	lcd-kmt2	0.07^{a}	24.5^{a}	4.35^{a}	50.6^{a}	32.4^{b}
	lcd-kmt3	0.06^{a}	34.8^{a}	6.84^{b}	107^{a}	37.0^{a,b}

Data are the means of five replicates. Values for a metal in the same column and same tissue labeled with different letters indicate significant difference at $P < 0.05$ by Tukey's test.

Table S4. QTLs for shoot and root Cd and Mn concentrations in a F_2 population derived from a cross between *lcd-kmt1* and Kasalath

Trait	Chromosome	SSR marker*	LOD†	Additive effect‡	$r^{2\S}$
Shoot Cd concentration	7	RM3767	28.9	−10.3	0.69
Root Cd concentration	7	RM3767	11.8	−43.3	0.33
Shoot Mn concentration	7	RM3767	28.1	−481	0.65
Root Mn concentration	7	RM3767	33.1	−102	0.73

*SSR marker closest to location of peak logarithm of odds (LOD) score.
†Maximum LOD score.
‡A negative value for the additive effect indicates that the *lcd-kmt1* allele decreased the phenotypic value (i.e., the Cd or Mn concentration).
$^{\S}r^2$ represents the proportion of the phenotypic variance explained by each QTL.

Table S5. Comparisons of gene expression between the WT and *lcd-kmt1* mutant using microarray analysis

Gene locus	Gene names and descriptions	Signal (WT)	Signal (*lcd-kmt1*)	Fold change (*lcd-kmt1*/WT)
OsNRAMP family				
Os07g0258400	OsNRAMP1	13.0	7.3	0.56
Os03g0208500	OsNRAMP2	233.9	223.5	0.96
Os06g0676000	OsNRAMP3	1,776.8	1,648.8	0.93
Os02g0131800	OsNRAMP4	703.1	749.6	1.07
Os07g0257200	OsNRAMP5	474.0	1,173.1	2.47
Os01g0503400	OsNRAMP6	117.1	139.3	1.19
Os12g0581600	OsNRAMP7	469.7	483.2	1.03
OsZIP family				
Os03g0667500	OsIRT1	451.7	455.7	1.01
Os03g0667300	OsIRT2	33.1	14.7	0.44
Os01g0972200	OsZIP1	2,210.6	2,159.7	0.98
Os03g0411800	OsZIP2	3,350.1	4,004.6	1.20
Os04g0613000	OsZIP3	2.2	2.5	1.10
Os08g0207500	OsZIP4	498.2	403.3	0.81
Os05g0472700	OsZIP5	1,351.8	1,675.7	1.24
Os05g0164800	OsZIP6	284.0	254.0	0.89
Os05g0198400	OsZIP7	460.9	424.0	0.92
Os07g0232800	OsZIP8	592.6	624.8	1.05
Os05g0472400	OsZIP9	13.2	10.6	0.80
Os06g0566300	OsZIP10	7.5	2.7	0.35
Os05g0316100	OsZIP11	476.6	493.9	1.04
OsHMA family				
Os06g0690700	OsHMA1	159.0	144.0	0.91
Os06g0700700	OsHMA2	7.6	11.7	1.53
Os07g0232900	OsHMA3	1,161.3	1,310.5	1.13
Os02g0196600	OsHMA4	1,120.6	1,341.9	1.20
Os04g0556000	OsHMA5	51.2	38.7	0.76
Os02g0172600	OsHMA6	2.3	2.4	1.07
Os08g0486100	OsHMA7	275.9	304.2	1.10
Os03g0178100	OsHMA8	82.0	66.4	0.81
Os06g0665800	OsHMA9	8,133.0	8,391.6	1.03
Os06g0579200	OsLCT1	2.5	2.6	1.06
Up-regulated genes in *lcd-kmt1*				
Os03g0432100	Similar to Pyruvate, phosphate dikinase 2	3.6	39.0	10.93
Os02g0197600	Chlorophyll a/b-binding protein type III (fragment)	2.0	18.1	8.98
Os03g0592500	Photosystem II type II chlorophyll a/b binding protein (fragment)	2.1	17.0	7.90
Os01g0600900	Chlorophyll a-b binding protein 2, chloroplast precursor (LHCII type I CAB-2) (LHCP)	58.0	431.1	7.43
Os12g0420400	Photosystem I reaction center subunit XI, chloroplast precursor (PSI- L) (PSI subunit V)	7.0	46.7	6.67
Down-regulated genes in *lcd-kmt1*				
Os03g0307300	OsNAS1	6,804.5	22.2	0.003
Os03g0307200	OsNAS2	4,249.2	67.3	0.02
Os02g0306400	OsNAAT1	394.6	91.2	0.23
Os03g0237100	OsDMAS1	119.7	35.2	0.29