Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice

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Edited by Gurdev S. Khush, University of California, Davis, CA, and approved October 16, 2012 (received for review June 30, 2012)

Rice (*Oryza sativa* L.) grain is a major dietary source of cadmium (Cd), which is toxic to humans, but no practical technique exists to substantially reduce Cd contamination. Carbon ion-beam irradiation produced three rice mutants with <0.05 mg Cd·kg^{-1} in the grain compared with a mean of 1.73 mg Cd·kg^{-1} in the parent, Koshihikari. We identified the gene responsible for reduced Cd uptake and developed a strategy for marker-assisted selection of low-Cd cultivars. Sequence analysis revealed that these mutants have different mutations of the same gene (*OsNRAMP5*), which encodes a natural resistance-associated macrophage protein. Functional analysis revealed that the defective transporter protein encoded by the mutant *osnramp5* greatly decreases Cd uptake by roots, resulting in decreased Cd in the straw and grain. In addition, we developed DNA markers to facilitate marker-assisted selection of cultivars carrying *osnramp5*. When grown in Cd-contaminated paddy fields, the mutants have nearly undetectable Cd in their grains and exhibit no agriculturally or economically adverse traits. Because mutants produced by ion-beam radiation are not transgenic plants, they are likely to be accepted by consumers and thus represent a practical choice for rice production worldwide.

Cadmium (Cd), a contaminant that enters the food chain from multiple natural and industrial sources, is toxic to the kidneys, particularly to the proximal tubular cells, where it accumulates and leads to renal dysfunction (1). In Japan, *itai-itai* disease (renal osteomalacia), which is characterized by spinal and leg bone pain, is recognized as chronic toxicity caused by excess Cd in drinking water and crops (2). To reduce the risk of Cd poisoning, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives established a provisional tolerable monthly Cd intake of 25 μg·kg^{-1} body weight (3), and the Codex Alimentarius Commission of the FAO/WHO established maximum Cd levels in food crops (4). The international maximum limit for rice is 0.4 mg Cd·kg^{-1} polished rice. Rice is a staple food for nearly half of the world's population, and global production and consumption of rice increased approximately threefold from 1960 to 2011 (5). The demand for rice continues to grow, so it is necessary to produce low-Cd rice to reduce the potential risk that Cd poses to human health.

There are substantial genotypic differences in Cd accumulation in rice (6, 7), concentrations generally being higher in *indica*-type cultivars than in *japonica*-type cultivars. Genetic loci determining genotypic differences in Cd accumulation have been identified by quantitative trait locus (QTL) analysis of several mapping populations (8, 9). Recently, genes involved in Cd uptake by the root (10–13), root vacuole sequestration (14, 15), root xylem loading (16, 17), and phloem transport in the node (18) have been found in rice, so the physiological and molecular processes of Cd transport in rice are increasingly well understood (19). Although regulation of Cd transport by transgenic techniques may enable us to reduce Cd accumulation in rice grain, commercial transgenic rice is not currently acceptable in many countries, such as Japan. Many consumers fear eating food produced by transgenic plants.

Energetic heavy-ion beams have been recently used to generate mutants in higher plants because they induce mutations with high frequency at a relatively low dose (i.e., at which virtually all plants survive), and they induce a broad spectrum of phenotypes without affecting other plant characteristics (20, 21). Using this technique, unique varieties of some flowers and trees have been commercialized, but this has not yet occurred in crop plants. Mutants produced by ion-beam radiation are not transgenic, so they are more likely to be accepted by consumers.

In the present study, we report (*i*) nontransgenic rice mutants with nearly cadmium-free grain produced by irradiation with heavy-ion beams and (*ii*) the development of a DNA marker for further breeding based on the identification of the gene (*OsNRAMP5*) responsible for low Cd uptake. Field studies show that these mutants have nearly nondetectable levels of Cd in the grain, even when cultivated in paddy fields contaminated with high levels of Cd.

Results

Isolation of Low-Cd-Accumulating Rice Mutants. We irradiated seeds of the most popular Japanese temperate *japonica* rice cultivar, Koshihikari, with accelerated carbon ions. Three low-Cd mutants (*lcd-kmt1*, *lcd-kmt2*, and *lcd-kmt3*) were identified in initial screening for grain Cd concentration from among 2,592 M_2 plants grown in Cd-polluted soil. The grain Cd concentration in the three mutants was <0.05 mg·kg^{-1}, compared with an average of 1.73 mg·kg^{-1} in the WT Koshihikari parent (Fig. 1*A*). The root and shoot Cd concentrations were significantly lower in all M_3 *lcd-kmt* mutants than in the WT (Fig. 1 *B* and *C*) when the seedlings were exposed to Cd in hydroponics. The concentrations of iron (Fe), zinc (Zn), and copper (Cu) in shoots and roots did not differ significantly between the *lcd-kmt* mutants and the WT (Table S1). However, the manganese (Mn) concentration in the shoots was significantly lower in the mutants (73.6–79.7 mg·kg^{-1}) than in the WT (1,004 mg·kg^{-1}). There was no difference in plant growth among the WT and *lcd-kmt1* or *lcd-kmt2* mutants, but the growth

Author contributions: S.I., T. Arao, N.K.N., and H.N. designed research; S.I., Y.I., M.I., M.K., T. Abe, T.S., and Y.H. performed research; S.I., Y.I., M.I., T. Abe, and H.N. analyzed data; and S.I., N.K.N., and H.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The cDNA sequences reported in this paper have been deposited in the DNA Data Bank of Japan, http://ddbj.nig.ac.jp [accession nos. AB690551 (OsNRAMP5), AB690552 (osnramp5-1), and AB690553 (osnramp5-2)].

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1211132109/-/DCSupplemental.

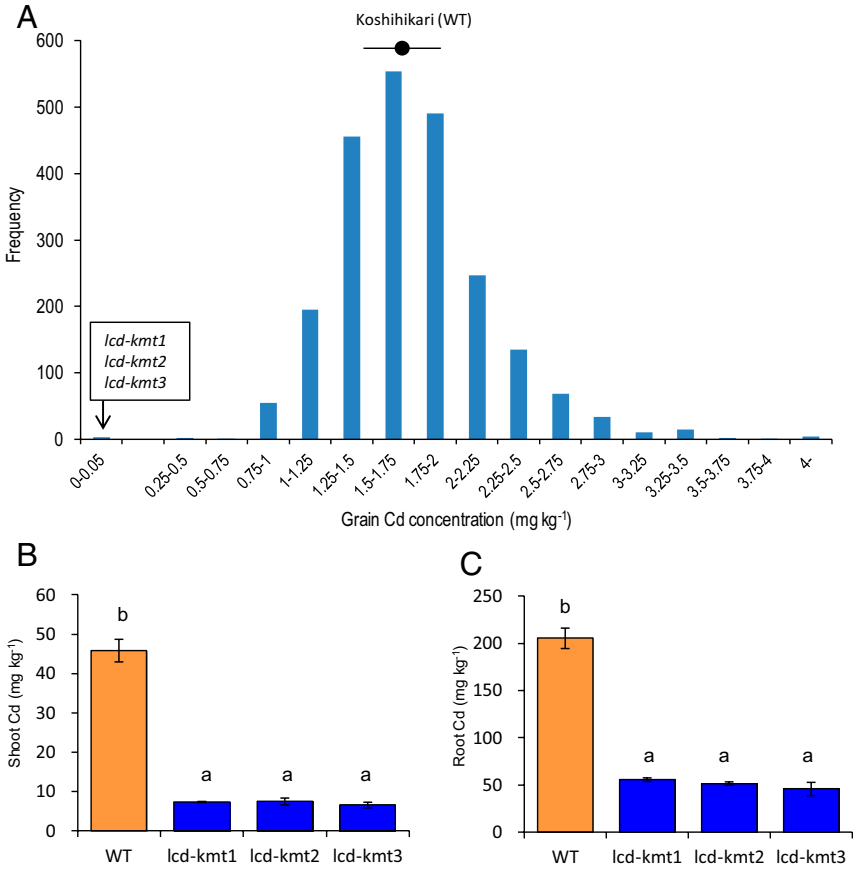

Fig. 1. (*A*) Frequency distribution of grain Cd concentration in rice mutants (2,592 M_2 plants) grown in pots filled with Cd-contaminated soil. The circle and range bar represent the mean and SD of grain Cd concentration in Koshihikari (288 plants). (*B* and *C*) Cd concentrations in the shoots and 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. Bars labeled with different letters differ significantly ($P < 0.05$, ANOVA followed by Tukey's test).

of *lcd-kmt3* was reduced (Table S1) under the sufficient Mn level in hydroponics. These results suggest that Cd might be transported via the Mn pathway into the roots.

Metal Concentrations in Grain and Agronomic Traits of Field Grown *lcd-kmt* Mutants. The M_4 *lcd-kmt* mutants and WT were cultivated together in paddy fields to evaluate their metal concentrations and agronomic traits. There were no apparent differences in plant or grain morphologies between WT and *lcd-kmt1* (Fig. 2 *A* and *B*) or between WT and *lcd-kmt2* (Fig. S1 *A* and *C*). In addition, there were no significant differences in soil plant analysis development (SPAD) value for chlorophyll content (Fig. 2*C*), grain and straw yields (Fig. 2 *D* and *E*), or eating quality (Fig. 2*F*) between WT and *lcd-kmt1*. Similar results were found between WT and *lcd-kmt2* (Table S2). This was in contrast to the *lcd-kmt3* mutant, which had significantly earlier heading, smaller plant size, higher panicle number, and lower grain and straw yields than the WT (Fig. S1 *B* and *C* and Table S2). The concentration of Cd in the grain (unpolished rice) of *lcd-kmt1* was extremely low, near the limit of detection (<0.01 $mg \cdot kg^{-1}$), whereas the Cd concentration in the WT grain exceeded the maximum limit of 0.4 $mg \cdot kg^{-1}$ (Fig. 2*G*). Indeed, the Cd concentration in *lcd-kmt1* was <3% of that in the WT. The Cd concentration in the straw was also much lower in *lcd-kmt1* than in the WT (Fig. 2*H*). Similar results were observed for Cd in grain and straw of *lcd-kmt2* and *lcd-kmt3* (Table S3).

The Mn concentration in the grain of *lcd-kmt* mutants was approximately one-third that of the WT, and an even greater difference of nearly 30-fold was evident in the Mn concentration in the straw (Table S3). The concentrations of Cu, Fe, and Zn in grains of *lcd-kmt1* and *lcd-kmt2* were similar to those of the WT and slightly higher in *lcd-kmt3* than in the WT. This was presumably because of the smaller size of *lcd-kmt3* plants. There was no significant difference in Fe concentration in the straw between the WT and *lcd-kmt* mutants, whereas that of Zn was a little lower in *lcd-kmt1* and *lcd-kmt2*.

Gene Identification. We developed an F_2 population by crossing Kasalath, an *indica*-type rice cultivar, with *lcd-kmt1* and then performed positional cloning of the gene(s) responsible for reduced Cd uptake by *lcd-kmt1*. Among the 92 F_2 individuals, 22 plants were categorized as having a similarly low shoot Cd concentration to *lcd-kmt1*, whereas 70 plants showed a relatively high shoot Cd concentration (Fig. 3*A*). The segregation ratio was not significantly different from a 1:3 low:high ratio ($\chi^2 = 0.058$, $P = 0.810$), suggesting that the low-Cd trait of *lcd-kmt1* is controlled by a single recessive gene. The gene locus associated with shoot and root Cd and Mn concentrations was localized on the short arm of chromosome 7 (Table S4). Linkage analysis showed that the gene was localized in the interval defined by the simple sequence repeat markers RM8007 and RM3635 (Fig. 3*B*). The maximum logarithm of odds values for all four traits were found at RM3767 (Table S4), which was located 9.07 Mbp from the distal end of the short arm of chromosome 7.

We found two genes, *OsNRAMP5* (Os07g0257200) and *OsNRAMP1* (Os07g0258400), annotated as putative heavy-metal transporters around RM3767 in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). The *OsNRAMP1* cDNA sequence was unchanged in the *lcd-kmt* mutants relative to the WT. On the other hand, the cDNA and genomic DNA sequences of *OsNRAMP5* revealed a single-nucleotide deletion in exon IX of *lcd-kmt2* and a 433-bp insertion in the exon X of *lcd-kmt1* (Fig. 3*C*). The latter replaced the terminal 32 bp in exon X of the WT with 50 bp in *lcd-kmt1*; the remaining 383 bp of the insertion in *lcd-kmt1* is expected to be spliced out with intron X. The inserted DNA sequence was identical to the sequence of *mPingA1*, a member of a class of miniature inverted-repeat transposable elements in rice (22). An ~227-kbp deletion that included all of

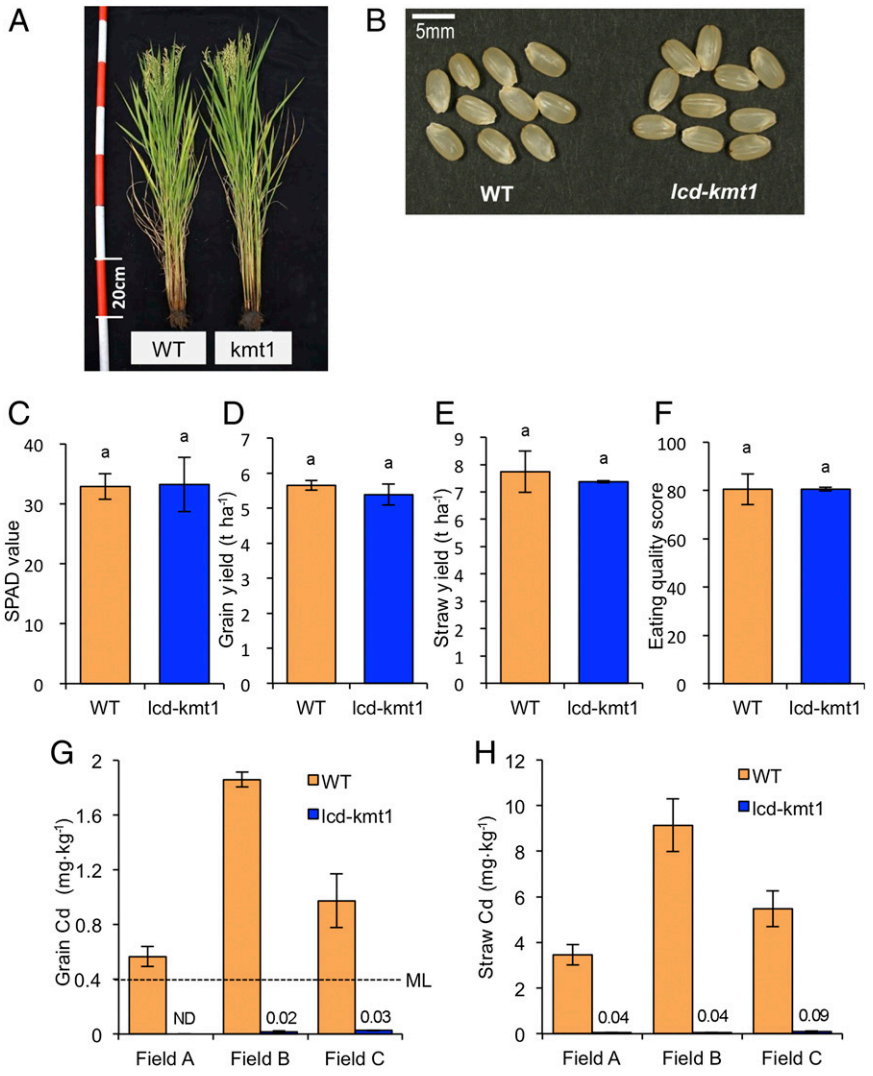

Fig. 2. Agronomic traits of Koshihikari and *lcd-kmt* mutants grown in the field. (*A*) Plant morphologies of WT Koshihikari and *lcd-kmt1*. (*B*) Morphologies of unpolished rice grains. (*C*) Chlorophyll content in the flag leaf determined using a SPAD meter. (*D*) Grain yield. (*E*) Straw yield. (*F*) Eating quality scores evaluated using a taste analyzer; values >80 are considered "good quality." No significant differences in agronomic traits or eating quality were observed between the WT and *lcd-kmt1* ($P > 0.05$, ANOVA followed by Tukey's test). (*G* and *H*) Cd concentration of unpolished rice (*G*) and straw (*H*). Plants were grown in Cd-polluted paddy fields in three regions of Japan. Data are presented as means ± SD ($n = 5$). ND, not detected; ML, maximum allowed Cd concentration for rice (Codex Alimentarius Commission).

OsNRAMP5 was found in *lcd-kmt3* (Fig. S2). On the basis of these results, we propose naming the mutant genes as *osnramp5-1* for *lcd-kmt1* (DNA Data Bank of Japan accession no. AB690552), *osnramp5-2* for *lcd-kmt2* (AB690553), and *osnramp5-3* for *lcd-kmt3*.

OsNRAMP5 (AB690551) from the WT Koshihikari is predicted to encode a 538-aa protein. The single base pair deletion in *osnramp5-2* results in aberrant translation of 53 aa before a new stop codon at amino acid 358 (Fig. S3). On the basis of the cDNA and genome sequencing data of *lcd-kmt1*, it is likely that an 11-aa region of the WT was replaced with 17 aa at the terminal position of exon X, resulting in a 544-aa protein in the osnramp5-1.

Microarray analysis showed a 2.5-fold increase in *OsNRAMP5* expression for *lcd-kmt1* compared with the WT (Table S5). The expression of other *OsNRAMP* genes did not change substantially. Moreover, marked changes in the expression of genes possibly involved in metal transport, such as *OsIRT*, *OsHMA*, and *OsLCT1*, were not found in the mutant. Rather, genes involved in the photosynthetic process were up-regulated considerably, and Fe-deficiency inducible genes were down-regulated in the *lcd-kmt1* mutant.

osnramp5-1 fused with GFP was observed at the periphery of the cells but not inside the cells (Fig. 4*A*), indicating the same localization as OsNRAMP5-GFP (10). This suggests that the mutation in osnramp5-1 did not alter the subcellular localization to the cell membrane. The growth of yeast cells expressing *osnramp5-1* was not affected by the Cd treatment (Fig. 4*B*), although the growth of transformed mutant yeast cells expressing *OsNRAMP5* was strongly impaired by Cd. This suggests that the osnramp5-1 could not transport Cd into yeast cells, whereas the WT OsNRAMP5 was able to do so. Furthermore, the mutant protein osnramp5-1 could not transport Mn and Fe, in addition to its inability to transport Cd.

Development of Genetic Markers for Breeding Low-Cd Rice. DNA markers that detect polymorphism in the region of *OsNRAMP5* would be useful for developing new cultivars with the low-Cd trait. Thus, we designed primer sets to amplify the mutated region. Different PCR fragment patterns could be readily detected between *lcd-kmt1* and WT, because there is a 433-bp insertion in *lcd-kmt1* (Fig. 4*C*). The F_1 heterozygous genotype derived from *lcd-kmt1* × WT appeared as two bands on the gel. In contrast, no differences in PCR fragment sizes were observed between the undigested PCR products of *lcd-kmt2* and WT (Fig. 4*D*). Although these two alleles differ in length by only 1 bp, the mutation created a unique FspI site in *lcd-kmt2*. FspI digestion cut the PCR product of *lcd-kmt2* (LK2) into two fragments of equal size, whereas the PCR product of WT was not cut by this enzyme. The alleles from both *lcd-kmt2* and WT could be detected in the F_1.

Using the developed genetic marker, we tested whether the mutant *osnramp5-1* allele significantly decreases Cd accumulation in F_2 plants derived from a cross between *lcd-kmt1* and Kasalath. All F_2 plants homozygous for the *osnramp5-1* allele of *lcd-kmt1* had significantly lower shoot Cd concentrations than

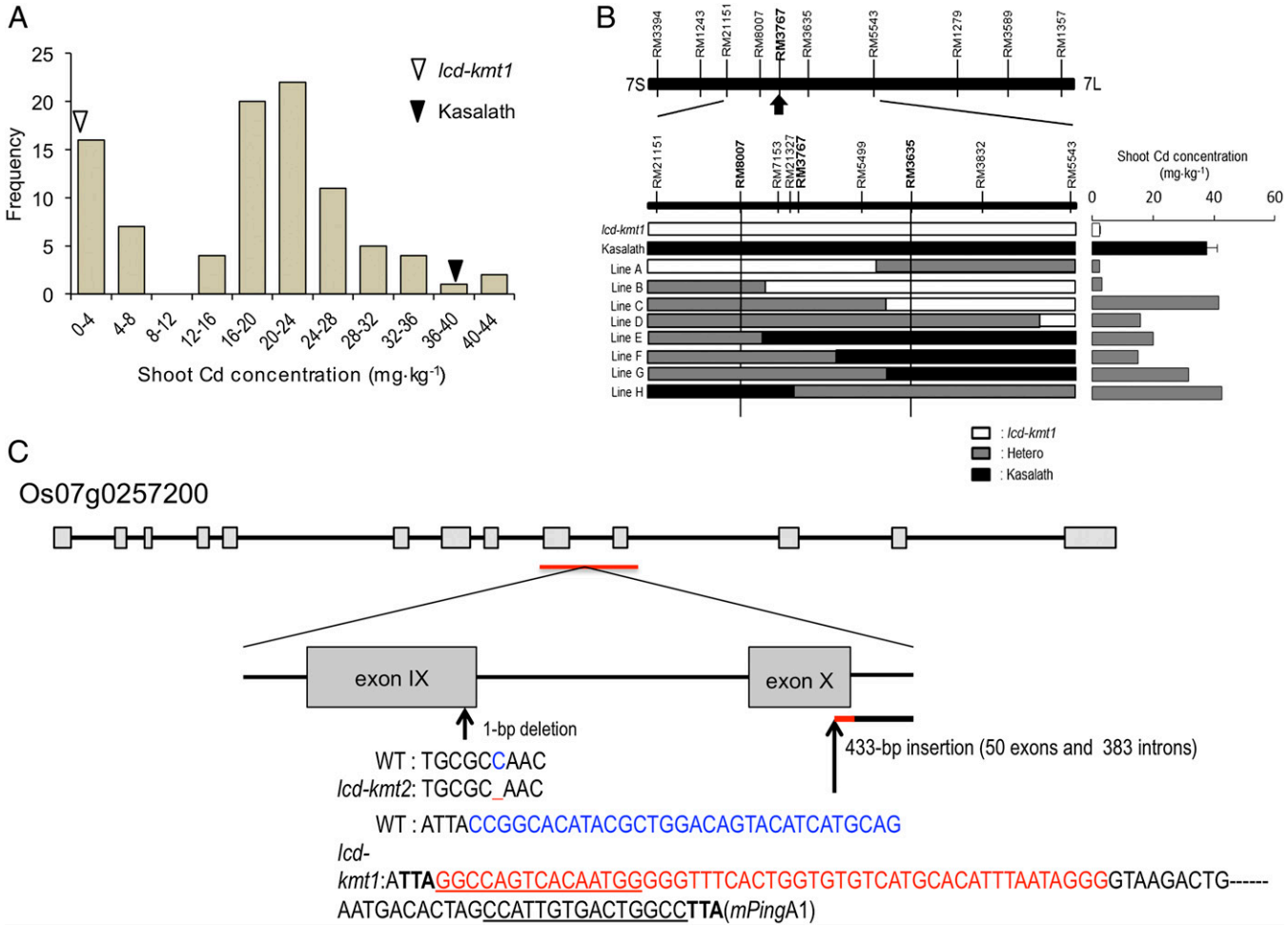

Fig. 3. Positional cloning of the gene. (*A*) Frequency distribution for shoot Cd concentration of 92 F_2 seedlings derived from a cross between *lcd-kmt1* and WT Kasalath, an *indica* cultivar. White and black triangles represent the mean shoot Cd concentration of *lcd-kmt1* and Kasalath, respectively. (*B*) Gene locus for low shoot Cd concentration on chromosome 7. Arrow indicates the peak logarithm of odds for the putative QTL gene. Graphical genotypes of F_2 plants having recombination in the candidate region (*Left*) and their shoot Cd concentrations (*Right*) are shown. White, black, and gray bars indicate regions homozygous for the *lcd-kmt1* allele, homozygous for the Kasalath allele, and heterozygous for the two alleles, respectively. (*C*) Structure of *OsNRAMP5* (Os07g0257200) and the mutation sites in *lcd-kmt1* and *lcd-kmt2*. Exons and introns are indicated by gray bars and black lines, respectively. The arrow below exon IX indicates the position of a 1-bp deletion in *lcd-kmt2* relative to the corresponding sequence in WT Koshihikari. The arrow below exon X indicates the position of a 433-bp insertion in *lcd-kmt1*. The blue WT nucleotides have been replaced by the red nucleotides in *lcd-kmt1*. The bold TTA sequences indicate 3-bp target-site duplications, and underlines indicate 15-bp terminal inverted repeats.

those homozygous for the *OsNRAMP5* allele of Kasalath and those that were heterozygous for the two alleles (Fig. 4*F*). This demonstrates that the allelic effect of *osnramp5-1* contributes to decreased Cd in rice plants. In addition, the plants homozygous for *lcd-kmt1* did not exhibit any significant decrease in their shoot dry weight (Fig. 4*E*), even if the genetic background was changed by crossing. These results indicate that introduction of the *osnramp5-1* allele into the other cultivars might not affect plant growth under the Mn-sufficient conditions.

Discussion

By using ion-beam mutagenesis, we succeeded first in producing nontransgenic rice mutants that accumulate very low Cd in grain of <3% that in Koshihikari, the most popular Japanese temperate *japonica* rice cultivar. Physiological studies in hydroponic culture demonstrated that decreased Cd uptake by roots leads to low levels of Cd in the shoot and grain of these mutants (Fig. 1). Our QTL analysis suggests that a Fe and Cd transporter gene, *OsNRAMP1*, on chromosome 7 was the most likely candidate gene, but the *OsNRAMP1* cDNA sequence was unchanged in the *lcd-kmt* mutants. Microarray analysis showed that the expressions of three other genes, *OsIRT1*, *OsIRT2*, and *OsHMA3*, previously related to Cd transport in rice, did not differ between the WT and *lcd-kmt1* mutant (Table S5). Instead, we found that the three mutant lines each had a different mutation [i.e., a transposon (*mPingA1*) insertion, a single-base pair deletion, and a large deletion], in the same gene *OsNRAMP5*, which is located near *OsNRAMP1* (Fig. 3*C* and Fig. S2). A *mPingA1* was probably activated by the ion beams (23), then transposed into a preferred insertion site (TTA) in an exon of OsNRAMP5 (22). It has been reported recently that OsNRAMP5 is involved in Mn, Fe, and Cd transport in rice roots (10, 13). Interestingly, in our previous study (10) the RNAi-induced silencing of *OsNRAMP5* in rice promoted Cd translocation to shoots, although root Cd uptake was decreased. In these *OsNRANP5*-RNAi plants, the expression of *OsNRANP5* was suppressed but the expressions of several Fe deficiency-inducible genes were up-regulated. In contrast, the expression of *osnramp5-1* present in the *lcd-kmt1* plant was increased but the expressions of Fe deficiency-inducible genes were down-regulated (Table S5). Therefore, the differential pattern in root-to-shoot Cd translocation between the RNAi-plants and *lcd-*

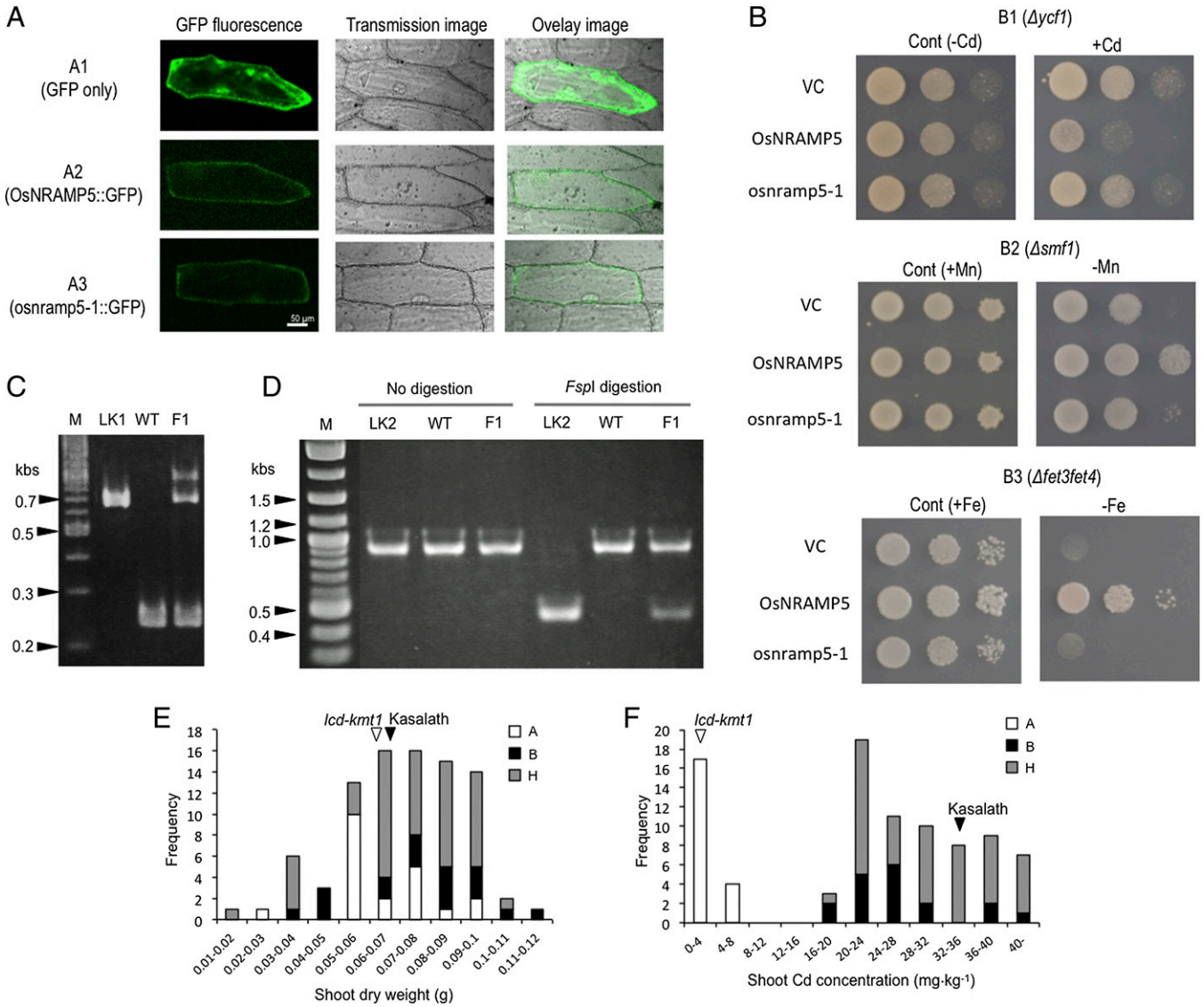

Fig. 4. (*A*) Subcellular localization of OsNRAMP5 and osnramp5-1 in transformed onion epidermal cells. (*A, 1*) GFP only; (*A, 2*) OsNRAMP5::GFP fusion protein; (*A, 3*) osnamp5-1::GFP fusion protein. (*B*) Growth of yeast cells transformed with the vector control (VC), *OsNRAMP5*, or *osnramp5-1*. Yeasts were spotted at three dilutions (optical densities at 600 nm of 0.1, 0.01, and 0.001, left to right). (*B, 1*) Growth of yeast *Δycf1* (Cd-sensitive mutant) cells; (*B, 2*) growth of *Δsmf1* (Mn-uptake mutant) yeast cells; (*B, 3*) growth of *Δfet3fet4* (Fe-uptake mutant) yeast cells. Cont, control, with (+) or without (–) the specified metal. (*C* and *D*) DNA fragments of the genomic region containing the mutation amplified by PCR. (*C*) M, size marker; LK1, *lcd-kmt1*; WT, wild-type Koshihikari; F1, F_1 progeny of *lcd-kmt1* × Koshihikari. (*D*) M, size marker; LK2, *lcd-kmt2*; WT, wild-type Koshihikari; F1, F_1 progeny of *lcd-kmt2* × Koshihikari. Where indicated, amplified samples were digested with FspI before electrophoresis. (*E*) Frequency distribution for shoot dry weight of F_2 plants derived from a cross between *lcd-kmt1* and Kasalath. Using the developed marker (*C*), the 88 F_2 plants were classified into three genotype classes: (A) those homozygous for the *osnramp5-1* allele of *lcd-kmt1*, (B) those homozygous for the *OsNRAMP5* allele of Kasalath, and (H) those that were heterozygous for the two alleles. (*F*) Frequency distribution for shoot Cd concentrations of F_2 plants used in *E*.

kmt mutants could be partly explained by the different expression of Fe deficiency-inducible genes.

Although the *osnramp5* mutant gene was expressed in the roots, the mutant transporter proteins failed to mediate uptake of Cd, Mn, and Fe in yeast (Fig. 4*B*), indicating loss of function of these metal transporters in the cell membrane. A highly conserved consensus transport motif (CTM) between transmembrane domains 8 and 9 was transformed into a hydrophobic segment in *osnramp5-1* and was truncated in *osnramp5-2* (Fig. S4). The CTM in NRAMP (natural resistance-associated macrophage protein) has been implicated in the interaction with ATP-coupling subunits and to be important for metal transport by these proteins (24). Within the CTM motif, the Gly-347 residue (based on position in OsNRAMP5) is absolutely conserved in all members of the NRAMP family. This residue could be especially important for metal transport activity because in mammalian NRAMP2, a mutant in which glycine is substituted with valine, lost NRAMP2 function in yeast (25). The Gly-347 residue is absent from the osnramp5 mutant proteins (Fig. S3). Therefore, such changes might affect Cd and Mn transport via the cell membrane in the roots.

The *lcd-kmt1* and *lcd-kmt2* mutants did not exhibit significant negative effects on plant or grain morphology, eating quality, grain yield, or straw yield (Fig. 2 and Table S2), indicating that a transposon insertion or a single base pair deletion on *OsNRAMP5* does not negatively affect agronomic traits. In contrast, *lcd-kmt3* had earlier heading and smaller plant size than the WT, presumably because of the large DNA deletions in this mutant

line (Fig. S2 and Table S2). These results indicate that *lcd-kmt1* and *lcd-kmt2* can be used directly in breeding programs.

Field trials showed that the *lcd-kmt* mutants have nearly undetectable Cd concentrations in their grain and staw (Fig. 2 *G* and *H*). Although root Cd concentrations were not measured in field conditions, root Cd uptake by *lcd-kmt* mutants is presumed to be substantially lower than in WT. If a small amount of Cd enters the root cells via other cell membrane metal transporters such as OsIRT1 (11) and OsNRAMP1 (12), a kind of "firewall" system might sequester Cd in the root vacuoles via a functional OsHMA3 transporter (14, 15). Therefore, a defective gene (*osnramp5*) working together with a functional gene (*OsHMA3*) may be responsible for the drastic decrease in grain and straw Cd concentrations in the *lcd-kmt* mutants.

Surprisingly, there were no differences in the leaf chlorophyll contents between WT and *lcd-kmt mutants* (Fig. 2*C* and Table S2), even though the shoot (straw) Mn concentration of the *lcd-kmt* mutants was markedly lower than that of the WT (Table S3), and several genes involved in the photosystem were up-regulated significantly (Table S5). Being adapted to the reducing conditions in paddy soils, rice accumulates high Mn in shoots of up to 2,000 $mg{\cdot}kg^{-1}$, an order of magnitude higher than that in soybean shoots (26), without damage (27). One T-DNA insertion line and RNAi lines of *OsNRANP5* in rice exhibited severe growth inhibition, although the Mn concentration in the straw was 100–200 $mg{\cdot}kg^{-1}$ in soil culture (13). In contrast, the *lcd-kmt* mutants did not show any adverse growth with <100 $mg{\cdot}kg^{-1}$ Mn in straw. Additionally, the F_2 plants harboring the *osnramp5-1* allele did not exhibit a significant decrease in shoot dry weight, even when their genetic background was changed by crossing. Our results indicate that rice may require less Mn for normal growth than is typically present in the shoot, and the introduction of *osnramp5-1* allele into other rice cultivars might not induce the growth inhibition under the Mn-sufficient conditions. Further investigation is needed on the effects of *osnramp5* alleles on plant growth and various agronomic traits under low-Mn conditions. The mutant *osnramp5* alleles did not significantly reduce Fe in grain and straw of the *lcd-kmt* mutants, indicating that other Fe transporters, such as OsIRT1 and OsIRT2, are more important than a functional OsNRAMP5 for Fe transport.

We have developed DNA markers that can be used to introduce the mutant *nramp5* alleles into various cultivars including *indica* type by means of marker-assisted selection. Indeed, breeding programs have been launched to transfer the low-Cd trait into other popular cultivars in Japan. These mutant alleles would also reduce the Cd concentration in rice straw being fed to livestock, thereby greatly reducing bioaccumulation of Cd in meat. We therefore believe that our findings provide an important tool for reducing the Cd levels in rice and that the risk of Cd exposure via the food chain will be greatly reduced.

Materials and Methods

Seeds of rice (*Oryza sativa* L. cv. Koshihikari) were irradiated with 320 MeV carbon ions ($^{12}C^{6+}$) at a dose of 40 Gy. Three low-Cd mutants (*lcd-kmt1*, *lcd-kmt2*, and *lcd-kmt3*) were identified according to the grain Cd concentration of 2,592 M_2 plants determined by inductively coupled plasma mass spectroscopy (ICP-MS). Positional cloning was conducted to identify the gene loci responsible for reduced Cd uptake by *lcd-kmt1*. The cDNA and genomic DNA of *OsNRAMP5* in the WT and *lcd-kmt* mutants were amplified by PCR and sequenced. Molecular methods were applied to observe the gene location in the rice plants and to analyze gene function. DNA markers were developed to assist breeding of low-Cd rice based on the sequences in the mutation regions for *lcd-kmt1* or *lcd-kmt2*. The cDNA sequences determined in this study have been submitted using the SAKURA nucleotide sequence data submission system through the Web server at the DNA Data Bank of Japan (DDBJ; http://sakura.ddbj.nig.ac.jp/) and are deposited in the DDBJ database under accession nos. AB690551 (*OsNRAMP5*), AB690552 (*osnramp5-1*), and AB690553 (*osnramp5-2*). Further details on the procedures used are available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank S. Mori, H. Hirochika, M. Yano, Y. Nagamura, K. Naito, and P. Blamey for helpful suggestions on this study; and K. Abe, H. Yamaguchi, T. Ara, T. Ibaraki, and T. Honma for help during our field experiments. This work was supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (S.I., N.K.N., and H.N.).

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