Development of Low-Cd Rice by mutation with Ion-beam

Satoru Ishikawa

National Institute for Agro-Environmental Sciences, Kannondai 3-1-3, Tsukuba, Ibaraki 305-8604, Japan.
*Corresponding author (E-mail: isatoru@affrc.go.jp)

Abstract: Rice (Oryza sativa L.) is a major dietary source of cadmium (Cd). However, there is currently no practical technique to substantially reduce Cd contamination of rice. Here, we report non-transgenic rice mutants from ion-beam irradiation that do not accumulate Cd in the grains and describe the mutant gene that is responsible. We found three mutants with low-Cd grains with different mutations of the same gene (OsNRAMP5). The defective transporter protein encoded by the mutant gene osnramp5 decreases root Cd influx. In Cd-contaminated paddy fields, the mutants have nearly undetectable Cd concentrations in their grains and exhibit no economically adverse traits. In addition, Cd and arsenic (As) levels in mutant simultaneously decreased under early-drainage conditions. DNA markers have been developed to facilitate marker-assisted selection of new cultivars carrying osnramp5. Our findings will help to greatly reduce Cd and As levels in paddy rice.

Key Words: Cadmium, ion-beam, arsenic, OsNramp5, rice

1. Introduction

Cadmium (Cd) is a contaminant that enters the food chain from multiple natural and industrial sources. Cd is toxic to the kidneys, and particularly to the proximal tubular cells, where it accumulates, leading to renal dysfunction (EFSA 2011). In Japan, itai-itai disease (renal osteomalacia), which is characterized by spinal and leg bone pain, is recognized as chronic toxicity induced by excess Cd in drinking water and crops (Tsuchiya 1976). To reduce the risk of Cd poisoning, the Joint FAO/WHO Expert Committee on Food Additives established a provisional tolerable monthly Cd intake of 25 µg kg⁻¹ body weight (FAO/WHO 2010), and the Codex Alimentarius Commission of the FAO/WHO established maximum Cd levels in food crops (Codex 2008). Among crops, the international criterion for rice Cd has been determined to be 0.4 mg kg⁻¹ polished rice; the value is two-fold higher than the criteria for other cereals such as wheat and maize and eight-fold higher than those for leaf vegetables. Rice is a staple food for nearly half of the world’s population, and global production and consumption of rice increased by approximately 300% from 1960 to 2011 (IRRI 2011). 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.

Cd uptake by paddy rice seems to be unique because it varies greatly depending on the soil redox potential in the paddy field. The bioavailable Cd in soil increases under oxidative conditions owing to the formation of soluble cadmium sulfate (CdSO₄) and decreases under reductive conditions because of the formation of less-soluble cadmium sulfide (CdS) (Arao et al. 2010). Therefore, unlike the upland crops, it is quite difficult to predict the rice grain Cd concentration from the soil Cd concentration and other soil chemical factors (Simmons et al. 2008). In addition, there are substantial genotypic differences in Cd accumulation in rice (Arao and Ishikawa 2006; Uraguchi et al. 2009). Generally, Cd concentrations are higher in indica-type varieties than in japonica-type ones. The genetic loci determining genotypic differences in Cd accumulation of rice have been shown by the QTL analysis for several mapping populations (Ishikawa et al. 2005; Ishikawa et al. 2010). Recently, the molecules involved in root Cd uptake (Nakanishi et al. 2006; Takahashi et al. 2011; Ishimaru et al. 2012), root vacuole sequestration of Cd (Ueno et al. 2010; Miyadate et al. 2011), root xylem loading of Cd (Satoh-Nagasawa et al. 2012), and phloem transport of Cd in the node (Uraguchi et al. 2011) have been successively found in rice, and therefore physiological and molecular processes of Cd transport in rice have been increasingly understood (Uraguchi and Fujiwara 2012). Although regulation of Cd transport by transgenic technique may enable us to reduce Cd accumulation in rice grains, commercial transgenic rice are not acceptable in Japan and many consumers fear eating the transgenic crops.

Mutations, induced by chemicals such as ethyl methane sulfonate (EMS) or ionizing radiation such as X-ray and gamma-ray, are a powerful way to explore novel mutants with the favorable traits in agriculture. Among mutagens, energetic heavy-ion beams have been recently used to generate such
mutants in higher plants because they are able to induce mutations with high frequency at a relative low dose at which virtually all plants survive, and they induce a broad spectrum of phenotypes without affecting other plant characteristics (Tanaka et al. 2010; Kazama et al. 2011). In addition, mutants produced by ion beam radiation are not transgenic plants, and are therefore more likely to be accepted by consumers, they are a practical choice for agriculture.

In the present study, we explored the rice mutants, produced by irradiation of heavy-ion beams, which are characterized by nearly non-detectable Cd in rice grain, even when cultivated in the paddy fields contaminated with high level of Cd. We also investigated the effectiveness of the low-Cd mutants to reduce simultaneously the levels of Cd and arsenic (As) in rice. Physiological, genetic, and molecular analyses were performed to identify a mutant gene responsible for low Cd in the rice mutants.

2. Materials and Methods

2.1. Production of rice mutants, growth conditions, and screening for low-Cd mutants

The 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 4000 M1 seeds were grown in a paddy field and self-pollinated, and the obtained seeds (M2) were bulked. The 2,592 M2 seedlings produced from these seeds were transplanted into plastic pots filled with Cd-contaminated paddy soil (soil Cd concentration: 1.8 mg Cd kg^{-1}). We also grew 288 Koshihikari (wild-type, WT) seedlings in the soil. All M2 and WT plants were submerged until the booting stage, and then water was withheld to increase the bioavailable Cd concentration in the soil and enhance subsequent Cd uptake (Ishikawa et al. 2011). The grains were harvested from all plants and analyzed to determine their Cd concentrations, as described below.

To evaluate Cd uptake of the three candidate mutants (lcd-kmt1, lcd-kmt2, and lcd-kmt3) selected from 2,592 plants and the WT at the vegetative seedling stage, their M3 seedlings were exposed to 20 L of half-strength Kimura B solution (Ishikawa et al. 2011) with 0.18 µM CdSO_{4} added (pH 5.2) in a Biotron (NC350, NK System, Osaka, Japan). After 4 days, the plants were harvested for metal analysis.

2.2. Field experiments

The M4 plants of three mutants and the WT were cultivated in three Cd-polluted paddy fields in different regions of Japan. The soil Cd concentrations were 1.35 mg kg^{-1} (Field A), 1.21 mg kg^{-1} (Field B), and 0.35 mg kg^{-1} (Field C) when determined by 0.1 M HCl extraction. Seedlings were transplanted into the flooded paddy fields, with a single plant per hill, spaced at 15×30 cm and with 20 plants of each mutant and the WT per row, with each genotype planted in a separate row. After 1 month, the fields were managed by means of drainage and then intermittent irrigation until grain maturity. We applied inorganic fertilizers containing N, K_{2}O, and P_{2}O_{5} using standard methods for each region. The plants were harvested at maturity and divided into grains (unpolished rice) and straw for the metal analysis.

2.3. Pot experiments

The WT and lcd-kmt1 seedlings were transplanted into 1/5000-a Wagner pots (one plant per pot) containing 3.0 kg of soil, which represented a 1:1 w/w mixture of soils that were naturally polluted with Cd and As, respectively. The Cd and As concentrations in the soil were 0.82 and 4.01 mg kg^{-1}, respectively, after the mixture, determined using 0.1 M HCl extraction for soil Cd and 1 M HCl extraction for soil As. Each of six WT and lcd-kmt1 plants were cultivated under flooded conditions until the heading stage, and then three of each group of plants were exposed to drained or continuously flooded conditions until harvest. The plants were divided into grains and straw for the Cd and As
2.4. Evaluation of agronomic traits in the mutant rice

The plants were cultivated in a non-contaminated paddy field 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 m², with a spacing of 15×30 cm. A compound fertilizer containing 8% each of N, P₂O₅, and K₂O was applied as a basal dressing at rates of 50 kg ha⁻¹ of 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, Inc., Tokyo, Japan). Agronomic traits were also measured: grain and straw yield, days to heading, plant height, culm length, and panicle number per plant. Sensory test of eating quality was conducted as follows: The cooked rice of lcd-kmt lines and WT was evaluated by a panel of 20 judges, who had been trained in the scoring of each component of eating quality. Because WT Koshihikari is the reference cultivar, all components (glossiness, smell, taste, stickiness, hardness, and overall evaluation) are score 0. The scores from the 20 judges were averaged.

2.5. Analysis of Cd and other metals (As, 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 were milled to a fine power (to pass through a 0.5-mm mesh) using a stainless-steel rotor mill (P14, Fritsch GmbH, Kastl, Germany). Sample digestion was performed as described previously (Ishikawa et al. 2005). Metal concentrations were determined by inductively coupled plasma-optical emission spectroscopy (Vista-Pro, Agilent Technologies Japan, Ltd., Tokyo, Japan) for Cu, Fe, Mn, and Zn or inductively coupled plasma mass spectroscopy (ELAN DRC-e, Perkin-Elmer Sciex, Concord, ON, Canada) for Cd and As. We used two certified standard materials 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 and NIST CRM 1568a rice flour (National Institute of Standards and Technology, Gaithersburg, MD, USA) for As.

2.6. QTL mapping and sequencing

An F₂ population derived from a cross between a high-Cd indica cultivar (Kasalath) as the female parent and a low-Cd Koshihikari mutant (lcd-kmt1) as the male parent was used for QTL mapping. The F₂ progeny (92 seedlings) were treated with 0.18 µM CdSO₄ in a hydroponic system, as described above, and then small piece of the third leaf was collected for extraction of the genomic DNA. The remaining shoots and roots were harvested to analyze the metal concentrations.

Total RNA was extracted from the roots of the WT or lcd-kmt plants using Sepasol RNA I Super (Nacalai Tesque, Inc., Kyoto, Japan) following the manufacturer’s protocol. First-strand cDNA was synthesized from 1 µg of total RNA using ReverTra Ace (TOYOBO, Co., Ltd., Osaka, Japan) and oligo(dT)20 (TOYOBO) for the reverse-transcriptase polymerase chain reaction (RT-PCR). The full-length open reading frame (ORF) of OsNRAMP5 in the WT and lcd-kmt plants was amplified by means of PCR. The amplified full-length cDNAs were sequenced using an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

2.7. Development of genetic markers

Genomic DNA was extracted from fresh leaves of the WT, lcd-kmt1, and lcd-kmt2 plants. We designed primer pairs based on the sequences in the mutation regions for lcd-kmt1 or lcd-kmt2, respectively. The reaction mixture consisted of 20 to 50 ng of template DNA, 1× KAPA2GTM Fast ReadyMix with dye (Kapa Biosystems, Boston, MA, USA), and 300 nM of each amplification primer. PCR amplifications were performed as follows: initial denaturation for 2 min at 95 °C; followed by 30
cycles of 30 sec at 95 °C, 30 sec at 58 °C, and 10 sec at 72 °C; followed by a final extension for 20 sec at 72 °C. PCR products from the WT and lcd-kmt1 plants were separated in sodium borate electrophoresis buffer in a 3% (w/v) agarose gel. The gel was stained with 1 mg/L ethidium bromide and visualized under UV light. The PCR products from the lcd-kmt2 and WT plants were digested with the restriction enzyme FastDigest FspI (Thermo Scientific) in four times the volume of the digestion buffer, followed by electrophoresis in a 1% (w/v) agarose gel.

3. Results

3.1. Isolation of low-Cd accumulating rice mutants

We irradiated 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 concentrations using 2592 M2 plants grown in Cd-polluted soil. The grain Cd concentration in wild-type (WT) Koshihikari averaged 1.73 mg kg\(^{-1}\), versus values less than 0.05 mg kg\(^{-1}\) in the three mutants. The three lcd-kmt mutants had identical phenotypes for metal concentrations. The root and shoot Cd and manganese (Mn) concentrations were significantly lower in lcd-kmt mutants than in the WT. Although the Mn concentrations were considerably lower than in the WT, there was no difference in plant growth except for lcd-kmt3. The concentrations of iron (Fe), zinc (Zn), and copper (Cu) of shoots and roots did not differ significantly between lcd-kmt mutants and the WT (Table 1). These results suggest that the lcd-kmt mutants exhibited decreased Cd uptake by their roots, and that Cd might be transported via the Mn pathway into the roots.

Table 1. Dry weights and metal concentrations in the shoots and roots of wild-type (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.

<table>
<thead>
<tr>
<th></th>
<th>Dry weight</th>
<th>Cd</th>
<th>Mn</th>
<th>Cu</th>
<th>Fe</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td>(mg kg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koshihikari</td>
<td>0.071(^{ab})</td>
<td>45.8</td>
<td>1004(^{b})</td>
<td>21.2</td>
<td>57.5</td>
<td>46.2</td>
</tr>
<tr>
<td>lcd-kmt1</td>
<td>0.081(^{b})</td>
<td>7.2</td>
<td>79.3</td>
<td>29.0</td>
<td>58.5</td>
<td>78.2</td>
</tr>
<tr>
<td>lcd-kmt2</td>
<td>0.087(^{b})</td>
<td>7.4</td>
<td>79.7</td>
<td>28.2</td>
<td>58.2</td>
<td>52.7</td>
</tr>
<tr>
<td>lcd-kmt3</td>
<td>0.057(^{a})</td>
<td>6.5</td>
<td>73.6</td>
<td>27.7</td>
<td>59.0</td>
<td>51.4</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koshihikari</td>
<td>0.024(^{ab})</td>
<td>205.4</td>
<td>113.0(^{b})</td>
<td>35.2</td>
<td>297.6</td>
<td>23.2</td>
</tr>
<tr>
<td>lcd-kmt1</td>
<td>0.026(^{b})</td>
<td>53.1</td>
<td>29.9</td>
<td>39.0</td>
<td>281.3</td>
<td>28.7</td>
</tr>
<tr>
<td>lcd-kmt2</td>
<td>0.027(^{b})</td>
<td>51.3</td>
<td>30.2</td>
<td>38.4</td>
<td>255.5</td>
<td>22.8</td>
</tr>
<tr>
<td>lcd-kmt3</td>
<td>0.019(^{a})</td>
<td>45.6</td>
<td>30.0</td>
<td>38.7</td>
<td>291.4</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Data are the means of three replicates. Within a tissue type, numbers in the same column labeled with different letters differ significantly (P < 0.05, ANOVA followed by Tukey’s test).

3.2. Grain and straw metal concentrations of lcd-kmt mutants

Field trials in three Cd-contaminated paddy fields showed that Cd concentrations in the grains (unpolished rice) of lcd-kmt1 andlcd-kmt2 were extremely low, near the limit of quantification (<0.01 mg kg\(^{-1}\)), whereas the Cd concentrations in the WT grains exceeded the maximum limit set by the Codex Alimentarius Commission (0.4 mg kg\(^{-1}\)) (Fig. 1). The straw Cd concentrations were also much lower in lcd-kmt1 and lcd-kmt2 than in the WT (Fig. 2). Similar results were observed in the Cd concentrations in grains and straws lcd-kmt3. The grain Mn concentrations in lcd-kmt mutants was approximately one-third that of the WT, and an even greater difference (nearly 30 times) was observed.
in the straw Mn concentrations. The concentrations of Cu, Fe, and Zn in grains of *lcd-kmt1* and *lcd-kmt2* were similar to those of the WT. There was no significant difference in straw Fe concentration between the WT and *lcd-kmt* mutants. Straw Zn concentrations seemed to be a little low in *lcd-kmt1* and *lcd-kmt2*.

In the pot experiment, flooded conditions significantly increased or decreased the concentrations of As or Cd, respectively, of grains and straws in both the WT and *lcd-kmt1* (Table 2). Opposite patterns of As and Cd concentrations were observed in the WT grown under the drained conditions, whereas *lcd-kmt1* showed simultaneously low levels of both Cd and As under such conditions.

**Table 2:** Grain and straw As and Cd concentrations of wild-type Koshihikari rice and the *lcd-kmt1* mutant grown in soil contaminated with both metals under different water management conditions.

<table>
<thead>
<tr>
<th>Water management</th>
<th>Cultivars</th>
<th>Grain concentration (mg kg⁻¹)</th>
<th>Straw concentration (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>As</td>
<td>Cd</td>
</tr>
<tr>
<td>Drained conditions</td>
<td>Koshihikari</td>
<td>0.17</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td><em>lcd-kmt1</em></td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>Flooded conditions</td>
<td>Koshihikari</td>
<td>1.12</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td><em>lcd-kmt1</em></td>
<td>1.15</td>
<td>ND</td>
</tr>
</tbody>
</table>

Drained conditions: The plants were cultivated under flooded conditions until heading, and under drained conditions from heading until harvest.

Flooded conditions: The plants were cultivated under continuously flooded conditions throughout the entire growth period.

ND, not detected (< 0.01 mg kg⁻¹).

### 3.3. Agronomic traits of *lcd-kmt* mutants

The mutants were cultivated in the paddy field to evaluate whether agronomic traits such as grain yield and eating quality differed between the *lcd-kmt* mutants and the WT plants. Among *lcd-kmt* mutants, the characteristics of *lcd-kmt3* apparently differed from that of the WT because of earlier conditions.
heading, smaller plant size, higher panicle numbers, and lower grain and straw yields than the WT. On the other hand, there were no apparent differences in plant morphologies and morphologies of rice grains of WT and \textit{lcd-kmt1} or WT and \textit{lcd-kmt2} (Fig. 2). In addition, no significant differences in SPAD value for chlorophyll content, plant height, culm length, and grain and straw yields were found between WT and \textit{lcd-kmt1} or between WT and \textit{lcd-kmt2} (Table 3).

<table>
<thead>
<tr>
<th>SPAD, chlorophyll content in the flag leaf determined using a SPAD meter. Values in the same column followed by different letters differ significantly ($P &lt; 0.05$, ANOVA followed by Tukey’s test).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to heading</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Koshihikari</td>
</tr>
<tr>
<td>\textit{lcd-kmt1}</td>
</tr>
<tr>
<td>\textit{lcd-kmt2}</td>
</tr>
<tr>
<td>\textit{lcd-kmt3}</td>
</tr>
</tbody>
</table>

Table 3: Comparison of agronomic traits between Koshihikari and three low-Cd mutants (\textit{lcd-kmt1}, \textit{lcd-kmt2}, and \textit{lcd-kmt3}).

3.4. Gene identification

QTL analysis revealed that the QTLs related to low shoot and root Cd and Mn concentrations were co-localized on the short arm of chromosome 7. The peaks at the maximum logarithm of odds values for all traits were found at RM3767, which was located 9.07 Mbp from the distal end of the short arm of chromosome 7. We found two genes annotated as putative heavy-metal transporters (Os07g0257200 and Os07g0258400). The genes are \textit{OsNRAMP5} (Os07g0257200) and \textit{OsNRAMP1}.
The OsNRAMP1 cDNA sequence showed no alteration of this gene in the *lcd-kmt* mutants. On the other hand, the cDNA and genomic DNA sequences of *OsNRAMP5* revealed a single-nucleotide deletion in exon IX for *lcd-kmt2* and a 433-bp insertion in the terminal portion of exon X for *lcd-kmt1*. The latter replaced the terminal 32 bp in exon X with 50 bp in *lcd-kmt1*; the remaining 383 bp of the insertion was spliced out with intron X. An NCBI BLAST search showed that the insertion-DNA sequences agreed completely with *mPingA1* sequences that have been characterized as a new class of miniature inverted-repeat transposable element–like elements in rice (Kikuchi et al. 2003). The *OsNRAMP5* open reading frame of *lcd-kmt3* was not amplified by PCR and we found an approximately 227-kbp deletion that included all of *OsNRAMP5*. *OsNRAMP5* of the WT encoded a 538 amino acid (aa) protein. The single deletion in *osnramp5-2* results in aberrant translation of 53 aa before a new stop codon at aa 358. Interestingly, the *osnramp5-1* open reading frame was completely translated, but an 11-aa region of the wild-type was replaced with 17 aa (an increase of 6 aa) at the terminal position of exon X, resulting in a 544-aa region.

### 3.4. Development of genetic marker for breeding

DNA markers that detect polymorphism in the mutated genome region can be used to develop new cultivars with the low-Cd trait. We designed primer sets to amplify the mutated region and observed different patterns of DNA fragment amplification between the WT and *lcd-kmt1* or between the WT and *lcd-kmt2* after *FspI* digestion, respectively. Since there is 443bp insertion in genomic region of *lcd-kmt1*, different PCR fragment patterns can be easily detected between *lcd-kmt1* and WT. The F1 heterozygous genotypes derived from *lcd-kmt1* x WT were detected by two bands on the gel. No different PCR fragments were observed between *lcd-kmt2* and WT. After *FspI* digestion, the PCR product of *lcd-kmt2* was cut at the specific restriction site for *FspI*, producing two fragments of the same size in the gel, whereas the PCR products of WT were not cut by this enzyme, thereby the F1 heterozygous genotypes derived from *lcd-kmt2* x WT can be detected.

### 4. Discussion

Using ion beams, new varieties of some flowers and trees have been previously commercialized, but in crops, there have not been put to practical use (Tanaka et al. 2010). By this technique, we first succeed to explore the non-transgenic rice mutants that accumulate nearly Cd-free in grains. Physiological studies in hydroponics indicated that the decreased root Cd uptake results in little amount of Cd in grains for mutants. Genetic analysis indicated that three mutants have different mutations of the same *OsNRAMP5*. This finding strongly suggests that mutation of *OsNRAMP5* greatly decrease Cd levels in rice grains. The NRAMP family of genes encodes integral membrane proteins in bacteria, fungi, plants, and animals (Cellier et al. 1995). Several NRAMP proteins functions as transporters of divalent metal ions, with broad substrate specificity (Thomine et al. 2000). In *Arabidopsis thaliana*, AtNRAMP1 is capable of transport of the multiple divalent metal ions such as Mn, Fe, and Cd. In our study, all *lcd-kmt* mutants exhibited the great reduced Mn levels in roots and shoots. Although there is a low similarity of deduced amino acid sequences between *OsNRAMP5* and AtNRAMP1, the common function for metal transport in the roots could be present.

Interestingly, three different types of mutation, which are characterized by a transposon (*mPingA1*) insertion, point-like mutation, and large DNA deletion, were found on *OsNRAMP5*. The consensus transport motif in NRAMP protein has been suggested to be involved in the interaction with ATP-coupling subunits and to be important for metal transport by the NRAMP family of transporters (Curie et al. 2000). However, this motif was transformed into a hydrophobic segment in *osnramp5-1* and was incompletely present in *osnramp5-2*. In amino acid sequence in the motif, the Gly-347 residue on *OsNRAMP5* is absolutely conserved in all members of the NRAMP family and this residue could be especially important for the metal transport activity. However, this amino acid residue was not conserved in our rice mutants. Therefore, such changes might affect Cd and Mn transport via the cell membrane in the roots.

We were most concerned whether the *lcd-kmt* mutants grown in paddy fields exhibit excellent
performance for grain Cd concentration with no significant differences in agronomic traits to the WT Koshihikari. Field trials showed that the *lcd-kmt* mutants have nearly undetectable Cd concentrations in their grains and straws. Surprisingly, there were no differences in the leaf chlorophyll content between WT and *lcd-kmt1*, although shoot Mn concentrations of *lcd-kmt* mutants drastically decreased as compared to those of WT. Rice is known to accumulate excess Mn without damage (Lidon et al. 2004), and the Mn concentration of rice shoots can be more than an order of magnitude higher than that of soybean shoots (Ishikawa et al. 2005). Presumably, rice may require less Mn for normal growth and can tolerate excess Mn induced by the reducing conditions in paddy soils.

The mutant plants of *lcd-kmt1* and *lcd-kmt2* did not exert significant negative effects on plant or grain morphology, eating quality, grain yield, or straw yield. However, *lcd-kmt3* had earlier heading and smaller plant size than the WT, presumably because of the large DNA lesions in this accession. These results indicate that *lcd-kmt1* and *lcd-kmt2* can be used as practical rice plants. Therefore, we have applied *lcd-kmt2* for rice variety registration in Japan and named it “Koshihikari Kan 1”. Moreover, we developed two DNA markers to detect polymorphism in the mutated genome region between the WT and *lcd-kmt1* and between the WT and *lcd-kmt2*. These can serve as a co-dominant marker, making it possible to detect both homozygous and heterozygous genotypes. Using the *lcd-kmt* mutants (*lcd-kmt1* and *lcd-kmt2*) and the DNA markers, the breeding programs have been launched to confer the low-Cd trait into the popular Japanese cultivars (Akitakomachi, Hinohikari, Hitomebore, etc.) which are closely related to the Koshihikari.

If rice cultivars carrying the mutant *nramp5* alleles were cultivated throughout Japan, then dietary Cd derived from rice, which accounts for about half of the Japanese diet (Watanabe et al. 2004), would decrease greatly. Reeves and Chaney (2008) pointed out the high Cd availability of rice for humans because of relatively low Fe and Zn levels in rice-based diets. Nearly Cd-free rice in this study is not needed to consider the Cd availability in rice grains, rather another issue might occur on dietary Mn intake. A formal recommended dietary allowance (RDA) has not been set for Mn because of insufficient data. Therefore, an adequate intake (AI) of Mn has been estimated based on median intake. A formal recommended dietary allowance (RDA) has not been set for Mn because of insufficient data. Therefore, an adequate intake (AI) of Mn has been estimated based on median intake in males and females (Trumbo et al. 2001). The AI of Japan (4.0 and 3.5 mg day$^{-1}$ for male and female, respectively) is as about two-times high as that of USA and Canada (2.3 and 1.8 mg day$^{-1}$ for male and female, respectively), indicating high Mn intake from diets for the Japanese. If the AI proposed by USA and Canada is sufficient to meet the Mn requirement, the decreased Mn level in the mutant rice might not greatly affect the adequate daily intake of Mn because, based on the current average rice consumption (160 g day$^{-1}$) in Japan and the grain Mn concentration (approximately 9 mg kg$^{-1}$) in the *lcd-kmt* plants, daily intake of Mn from the mutant rice would be approximately 60% of the adequate daily intake. In addition, dietary Mn is supplied from other gramineous crops, leguminous crops, vegetables, and seaweeds, so changes in its content in rice alone should not be a problem.

Another advantage of using the *lcd-kmt* mutants is that As and Cd levels in rice will simultaneously decrease under early drainage conditions, and thereby potential risks of As and Cd for rice consumption people will reduce greatly. These mutant alleles can also be introduced into *indica* cultivars, which are generally higher grain Cd concentrations than *japonica* cultivars, by means of marker-assisted selection. This technique would reduce the Cd concentrations in rice straw being fed to livestock, thereby greatly reducing bioaccumulation of Cd in meat. We therefore believe our findings represent an important tool for reducing the Cd level in paddy rice around the world (Ishikawa et al., 2012).

4. References


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