

# Low-affinity cation transporter (*OsLCT1*) regulates cadmium transport into rice grains

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**Accumulation of cadmium (Cd) in rice (*Oryza sativa* L.) grains poses a potential health problem, especially in Asia. Most Cd in rice grains accumulates through phloem transport, but the molecular mechanism of this transport has not been revealed. In this study, we identified a rice Cd transporter, *OsLCT1*, involved in Cd transport to the grains. *OsLCT1*-GFP was localized at the plasma membrane in plant cells, and *OsLCT1* showed Cd efflux activity in yeast. In rice plants, strong *OsLCT1* expression was observed in leaf blades and nodes during the reproductive stage. In the uppermost node, *OsLCT1* transcripts were detected around large vascular bundles and in diffuse vascular bundles. RNAi-mediated knockdown of *OsLCT1* did not affect xylem-mediated Cd transport but reduced phloem-mediated Cd transport. The knockdown plants of *OsLCT1* accumulated approximately half as much Cd in the grains as did the control plants. The content of other metals in rice grains and plant growth were not negatively affected by *OsLCT1* suppression. These results suggest that *OsLCT1* functions at the nodes in Cd transport into grains and that in a standard *japonica* cultivar, the regulation of *OsLCT1* enables the generation of “low-Cd rice” without negative effects on agronomical traits. These findings identify a transporter gene for phloem Cd transport in plants.**

heavy metals | food safety

Cadmium (Cd) is a heavy metal harmful to human health. The biological half-life of Cd in the body is estimated to be nearly 30 y (1), which leads to chronic toxicity. The adverse effect of Cd has been a worldwide concern since the outbreak of “Itai-Itai disease” in the mid-20th century in Japan that was caused by the daily consumption of Cd-contaminated rice (2, 3). Recently, the average dietary intake of Cd in Japan was estimated to be 3.0 μg Cd/kg body weight per week. This value is nearly 50% of a provisional tolerable monthly intake established by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and Contaminants (JEFCA) and higher than the tolerable weekly intake (2.5 μg Cd/kg body weight) set by the European Food Safety Authority (EFSA). Reflecting this greater Cd intake, the internal Cd level among Japanese people is high among Asians (4). Recent surveys in Sweden (5), the United Kingdom (6), and the United States (7, 8) also showed measurable internal Cd levels within the general population, indicating the importance of reducing Cd exposure in the general population (9). In Asian countries, including Japan, up to 50% of the ingested Cd comes from rice and its products (4, 10). The Food and Drug Administration’s (FDA) Total Diet Study reported that the dietary intake of Cd among Americans increased by 26% from 1990 through 2003 and that this rise was correlated with an increased consumption of rice and other grains (11).

The source of Cd in rice grains is soil. Cd is absorbed by rice roots and transported to the grains, resulting in considerable Cd accumulation even when grown on slightly or moderately Cd-polluted soil (12, 13). Transporters for mineral nutrients, such as Zn or Fe, are partly responsible for Cd transport in plants (14).

To improve the health of people who depend on rice as a staple, the establishment of a low-Cd rice cultivar is desirable. A first step in this direction is to clarify the mechanism of Cd transport in rice, including identification of the responsible molecule(s).

Recent physiological studies have advanced our understanding of how Cd is transported and accumulated in rice. The main determinant of the Cd concentration in shoot tissues is the ability to translocate Cd from root to shoot through the xylem, rather than Cd uptake by the roots (12). Quantitative trait loci (QTL) analyses have indicated several chromosomal regions controlling Cd accumulation in rice shoots; one of these loci regulates the loading of Cd into the xylem (15–19). Recently, *OsHMA3*, a rice P-type ATPase, was identified as a regulator of the xylem loading of Cd in roots (20, 21).

Despite its importance, little is known about the molecular mechanism of phloem Cd transport in plants. Phloem-mediated Cd transport to grains following xylem-mediated root-to-shoot translocation is critical for the accumulation of Cd in rice grains. Nearly 100% of the Cd in rice grains is attributable to phloem transport (22, 23). Fujimaki et al. (22) used a noninvasive live imaging technique to follow the transport of <sup>107</sup>Cd in intact rice plants. They demonstrated the importance of shoot nodes for the transfer of Cd from the xylem to the phloem. For some minerals, the contribution of transporters is crucial for this kind of re-direction of solute transport at nodal regions (24, 25). The Cd translocation ability into grains varies among rice genotypes (12). Kato et al. (26) also reported variable Cd concentrations in phloem sap among cultivars exhibiting different grain Cd levels and showed the correlation between grain and phloem sap Cd concentrations. These studies indicated the existence of transporters mediating Cd translocation into grains and suggested that regulation of the transporters can alter the level of Cd deposition in grains.

In this study, we focused on LOC\_Os06g38120 as a possible Cd transporter gene expressed in rice shoots during grain ripening. This gene, *OsLCT1*, is predicted to encode the only rice homolog of the low-affinity cation transporter 1 (*TaLCT1*) found in the wheat cDNA library. *TaLCT1* enhanced the intake of various cations, including Cd<sup>2+</sup> in yeast (27). Here, we present evidence that *OsLCT1* is a plasma membrane-localized Cd exporter involved in phloem Cd transport. By down-regulating *OsLCT1* expression, we generated rice plants with reduced grain Cd levels, demonstrating the importance of *OsLCT1* in Cd transport into grains.

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## Results

### ***OsLCT1* Is a Plasma Membrane-Localized Efflux Transporter of Cd.**

*OsLCT1* cDNA cloned from a *japonica* rice cultivar, Nipponbare, was deduced to encode 511 aa with 11 transmembrane domains (Fig. S1). The primary sequence of *OsLCT1* is 23% identical to that of *TaLCT1*. A Basic Local Alignment Search Tool (BLAST) search indicated that *OsLCT1* is a single-copy gene in the rice genome, and no homolog of *OsLCT1* was found in the *Arabidopsis thaliana* genome.

The subcellular localization of *OsLCT1* was then investigated in tobacco BY-2 cells. *OsLCT1* fused with GFP was primarily colocalized with the plasma membrane marker FM4-64, whereas in cells expressing GFP alone, green fluorescence was observed mainly in the cytosol and nucleus (Fig. 1A). This strongly suggests that *OsLCT1* is a plasma membrane protein.

*TaLCT1* enhanced the transport of various cations, including  $\text{Cd}^{2+}$ , in yeast (27). We tested whether *OsLCT1* has Cd transport activity in a yeast heterologous expression system using *WΔyef1*. This yeast strain has a defect in the gene encoding a vacuolar transporter, *Ycf1p*, which mediates the transport of a Cd–glutathione conjugate into vacuoles (28). Yeast cells were transformed with a vector containing Myc-tagged *OsLCT1* cDNA, and the expression of *OsLCT1* was confirmed by Western blot analysis using anti-Myc antibodies (Fig. S2A). *OsLCT1* expression in the yeast did not affect the Cd sensitivity of the cells (Fig. S2B). However, *OsLCT1* expression significantly reduced Cd accumulation in the cells compared with vector control cells in 10 and 20  $\mu\text{M}$  Cd treatments (Fig. 1B and Fig. S2C), sug-

gesting that *OsLCT1* is an efflux-type Cd transporter. We also analyzed the concentrations of several minerals in the cells. Yeast cells expressing *OsLCT1* showed significantly reduced Mg, Ca, K, and Mn accumulation compared with the vector control cells, with no significant difference in the concentrations of Na, Fe, Zn, or Cu (Fig. S2D). Treatment with 1 mM  $\text{CaCl}_2$  or 10 mM  $\text{MgSO}_4$  with 10  $\mu\text{M}$   $\text{CdCl}_2$  reduced the Cd transport activity of *OsLCT1*-expressing cells (Fig. S2E) compared with that under the normal Ca (0.1 mM) and Mg (1 mM) conditions (Fig. 1B and Fig. S2C).

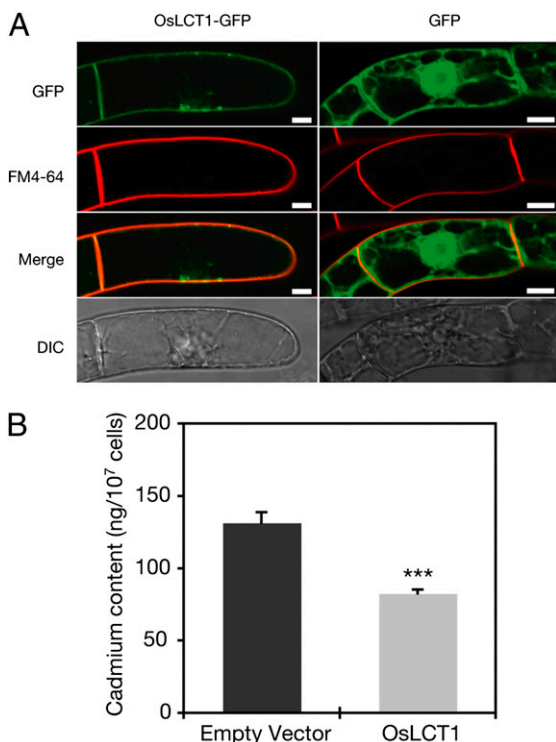
### ***OsLCT1* Is Strongly Expressed in Nodes and Leaf Blades During Grain Ripening.**

To determine the expression profile of *OsLCT1* in rice plants, we first analyzed microarray data from the rice gene expression database RiceXPro (29). The analysis of a dataset obtained from various rice tissues at different growth stages demonstrated that *OsLCT1* is strongly expressed in leaf blades during reproductive stages, rather than during vegetative stages (Fig. S3). Further analysis was conducted using real-time PCR analysis to examine the specificity of *OsLCT1* expression in more detail (Fig. 2A). In this experiment, nodes were added as a sample because they were not included in the RiceXPro microarray data. The real-time PCR analysis showed strong *OsLCT1* expression in both leaf blades and nodes during the grain-ripening stage, whereas its expression in shoots and roots in the vegetative growth stage was relatively lower (Fig. 2A).

We then examined the cell type specificity of *OsLCT1* expression by in situ hybridization in node I, the uppermost node connected to the flag leaf and the panicle (Fig. 2B–F). *OsLCT1* expression was detected specifically in vascular bundles in the middle of node I (Fig. 2B) and in the border region between node I and internode II (Fig. 2C), but expression was not observed in internode II (Fig. 2D). The accumulation pattern observed in the cross-sectioned samples was confirmed in the longitudinal sections of node I and internode II (Fig. S4B). Furthermore, high-magnification observation of the middle of node I identified the accumulation of *OsLCT1* transcripts in the cells around enlarged large vascular bundles and more strongly in the cells of diffuse vascular bundles (Fig. 2E). *OsLCT1* was also expressed in the phloem parenchyma cells surrounding the phloem of enlarged large vascular bundles (Fig. 2E). *OsLCT1* transcript accumulation in the phloem parenchyma cells was evident in the border region of node I and internode II (Fig. 2F), where the vascular bundles were not enlarged.

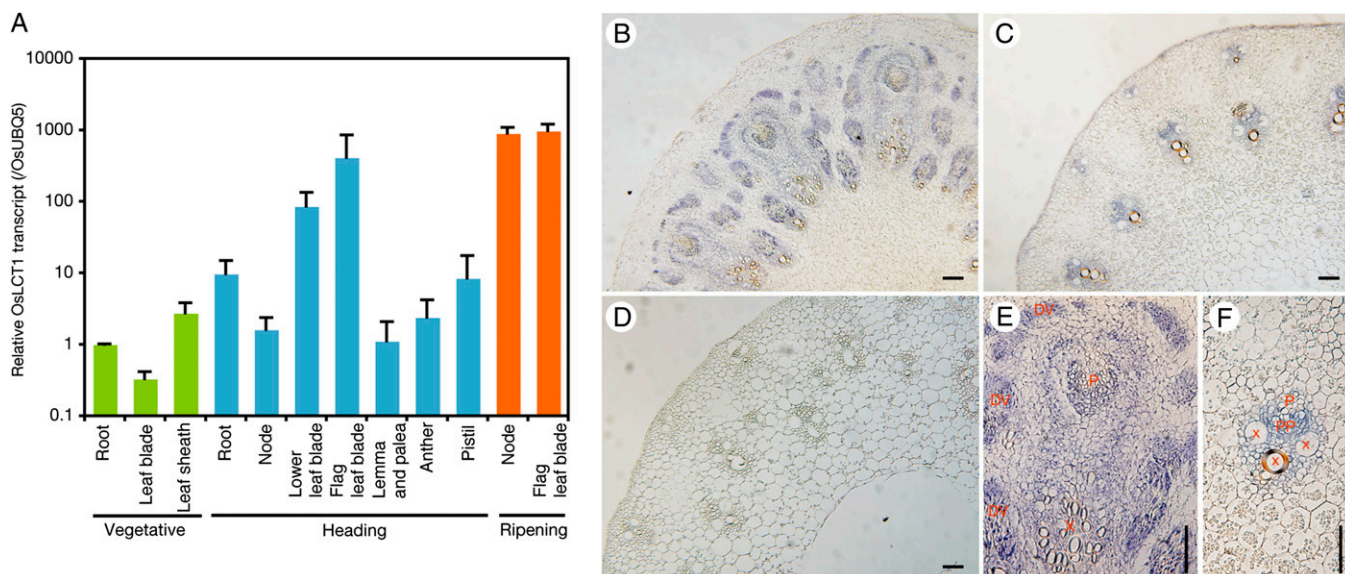
### **Down-Regulation of *OsLCT1* Decreases Grain Cd Levels in Rice Plants.**

To examine the role of *OsLCT1* in rice Cd accumulation, we generated RNAi-mediated *OsLCT1* knockdown rice plants using a *japonica* cultivar, Nipponbare. *OsLCT1* mRNA level in rice plants transformed with the RNAi vector was ~30–50% of that in vector control plants (Fig. 3A). Plants were then grown until grain ripening in soil containing 0.198 mg of Cd/kg dry weight (Table S1), which is comparable to the average Cd concentration in Japanese soils (30). Intermittent flooding was introduced throughout cultivation to enhance Cd availability in the soil and promote Cd accumulation in the grains (12). Grains were harvested from ripened plants, and histochemical Cd staining with 2-(8-quinolylazo)-4,5-diphenylimidazole (QAI) was conducted on the brown rice. QAI can specifically detect Cd in the tissue, in combination with reagents used to mask other heavy metals such as Zn and Cu (31). Less staining was observed in brown rice from the knockdown plants than in those from the control plants (Fig. 3B), suggesting less grain Cd accumulation in the knockdown line. For confirmation, the Cd content of the grains was quantified by inductively coupled plasma mass spectrometry (ICP-MS). The grains of the control plants contained nearly 0.15  $\mu\text{g}$  of Cd/g dry weight (Fig. 3C), which is greater than the average Cd concentration in brown rice produced in Japan (0.04–0.07  $\mu\text{g}/\text{g}$ )



**Fig. 1.** (A) Subcellular localization of *OsLCT1*-sGFP. *35S-OsLCT1*-sGFP (*OsLCT1*-sGFP) and *35S-sGFP* (sGFP) were introduced separately into cultured tobacco BY2 cells. FM4-64 (25  $\mu\text{M}$ ) was used as a plasma membrane marker. (Scale bars = 10  $\mu\text{m}$ .) (B) Cd transport activity of *OsLCT1*. Yeast (strain *WΔyef1*) expressing *OsLCT1* or harboring the empty vector were incubated for 75 min with arginine-phosphate medium containing 20  $\mu\text{M}$   $\text{CdCl}_2$ . The concentrations of Cd in the harvested cells were determined by ICP-MS after nitric acid digestion. Asterisks represent a significant difference from the empty vector cells ( $P < 0.001$ , *t* test). The data are presented as means  $\pm$  SD ( $n = 3$ ).





**Fig. 2.** Expression profiles of *OsLCT1* in rice. (A) Real-time PCR analysis. cDNAs were synthesized from total RNA extracted from various tissues of rice grown in a greenhouse, and the mRNA levels were quantified by real-time PCR. The data were normalized to *OsUBQ5* and are shown relative to the vegetative root sample. The data are presented as means  $\pm$  SD ( $n = 3$ ). (B–F) *In situ* hybridization of *OsLCT1* in node I. (B–D) Cross-sections of the middle of node I (B), the border region of node I and internode II (C), and internode II (D). (E and F) Enlarged images of vascular bundles in the middle of node I (E) and in the border region of node I and internode II (F). (Scale bars = 10  $\mu$ m.) DV, diffuse vascular bundles; P, phloem regions of large vascular bundles; PP, phloem parenchyma cells; X, xylem regions of large vascular bundles.

(32), suggesting that our cultivation condition reflected those that produce moderately Cd-polluted rice. Notably, under this condition, the *OsLCT1* knockdown plants showed up to 50% less Cd in brown rice than the control plants (Fig. 3C). These results suggest that *OsLCT1* is critical for the level of grain Cd accumulation when grown in soil.

***OsLCT1* Is Involved in Phloem Cd Transport.** In rice, xylem-mediated root-to-shoot Cd translocation activity (12) or phloem-mediated Cd transport (26) determine grain Cd levels. First, to examine the possibility that *OsLCT1* regulates grain Cd level by affecting xylem-mediated Cd transport, we analyzed the Cd concentration in xylem sap of the vector control and RNAi plants. Compared with the control plants, the RNAi plants showed no significant difference in root-to-shoot Cd translocation mediated by xylem (Fig. 3D).

To examine the involvement of *OsLCT1* in phloem Cd transport, phloem sap was collected by the EDTA method (33), and the level of Cd in the phloem sap was determined in addition to that of K (Fig. 3E and F). K is a major cationic element in phloem sap (34). The Cd level in phloem exudates of RNAi plants was almost half that of the control plants (Fig. 3E), although the level of K in phloem exudates did not differ significantly among tested lines (Fig. 3F). These results show the involvement of *OsLCT1* in phloem Cd transport rather than in xylem transport.

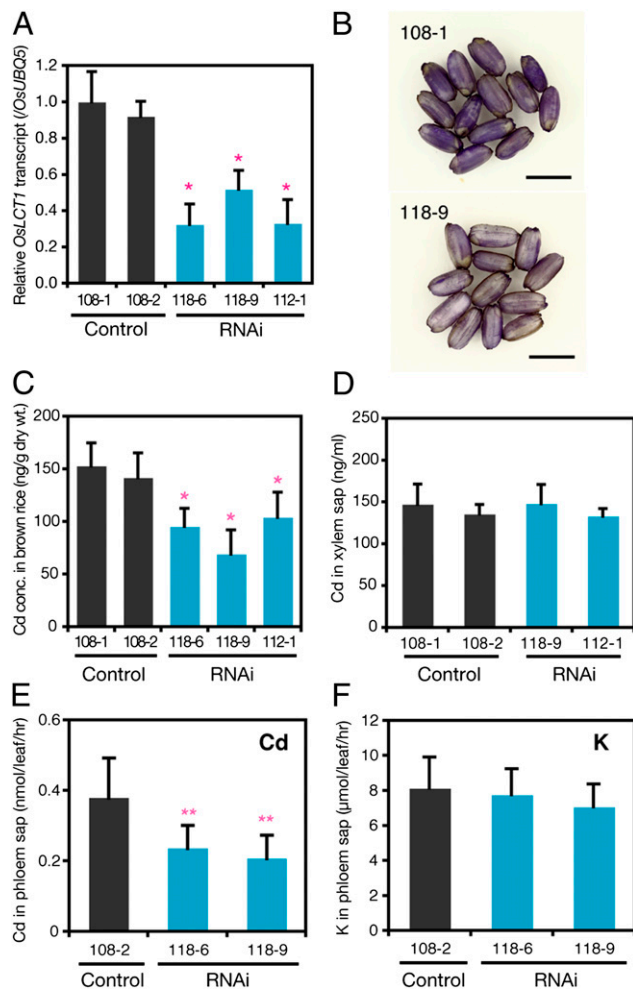
**Suppression of *OsLCT1* Expression Does Not Have Negative Effects on Growth and Grain Nutrient Accumulation in Rice Plants.** Three independent RNAi lines exhibited no apparent phenotypic difference from two vector control lines (Fig. 4A and B). No significant or consistent difference in straw weight or grain yield was observed among the three RNAi lines and two control lines (Fig. 4C and D). These results show that *OsLCT1* suppression had a negligible effect on vegetative and reproductive growth, at least under our cultivation conditions.

The yeast assay demonstrated that *OsLCT1* acts as an efflux transporter of Ca, Mg, K, and Mn, as well as of Cd (Fig. S2D),

indicating that these nutrients may be substrates of this transporter *in planta*. Thus, suppression of *OsLCT1* was expected to disturb nutrient accumulation and distribution. However, in contrast to the Cd results, no significant decrease occurred in the concentrations of minerals in the brown rice of these plants (Fig. S5A). Unexpectedly, the Fe content in the brown rice was significantly higher in the RNAi plants (Fig. S5A), and the Fe concentration in the leaf blades was significantly lower in the RNAi plants (Fig. S5B). Low Ca or Mg treatment, as well as exposure to high Na or Cd, did not affect *OsLCT1* expression (Fig. S6). These results indicate little contribution of *OsLCT1* in grain mineral accumulation except for Fe.

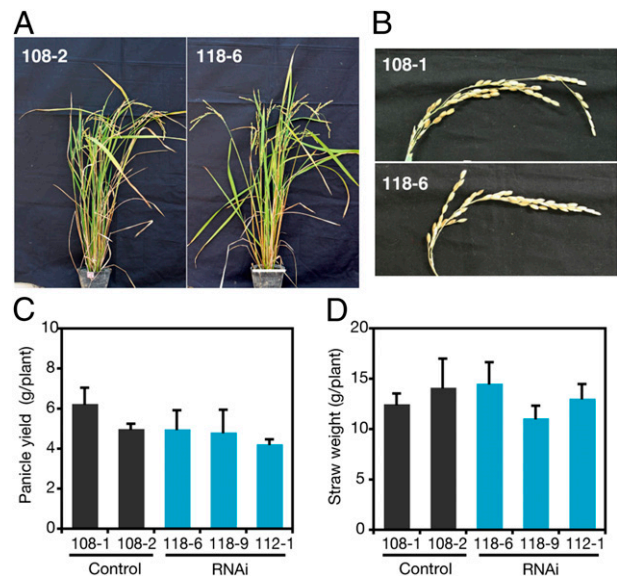
## Discussion

In this study, we characterized *OsLCT1* as a possible rice Cd transporter gene and demonstrated that *OsLCT1* is localized to the plasma membrane (Fig. 1A) and can export Cd, as well as K, Ca, Mg, and Mn (Fig. 1B and Fig. S2D). Previous reports showed that wheat *TaLCT1* also mediates the transport of various cations, such as Cd, Ca, Na, Rb, and K, but does not mediate that of Zn (27, 35). The Cd transport activity of *OsLCT1* was inhibited by high Ca or Mg levels (Fig. S2E), as with *TaLCT1* (27). These results suggest that, like *TaLCT1*, *OsLCT1* possesses cation transport activity with broad substrate specificity and is not specific to Cd transport. It is well known that  $\text{Cd}^{2+}$  and divalent cations (e.g.,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Ca}^{2+}$ ) are chemically analogous and that  $\text{Cd}^{2+}$  is transported by transporters for such ions as  $\text{Zn}^{2+}$  or  $\text{Ca}^{2+}$  (36–42). The present and previous results (27, 35) suggest that both *OsLCT1* and *TaLCT1* have a higher transport activity for Ca and Cd than for Zn. However, whereas wheat *TaLCT1* is indicated as an influx transporter (27, 35), rice *OsLCT1* appears to have efflux activity, indicating a possibility that the differentiation of rice and wheat resulted in divergent functions for *OsLCT1* and *TaLCT1*. Overall, the present results suggest that rice *LCT1* is a plasma membrane-localized cation exporter with broad substrate specificity and that *OsLCT1* possesses Cd transporter activity.



**Fig. 3.** Accumulation and transport of Cd in the RNAi-mediated knockdown rice plants of *OsLCT1*. (A) Generation of *OsLCT1* RNAi-mediated knockdown plants. The *OsLCT1* transcript was quantified by real-time PCR. Total RNAs were prepared from flag leaf blades of the plants ( $T_1$ ). The data are presented as means  $\pm$  SD ( $n = 3$ ). Asterisks represent a significant difference from both control lines ( $P < 0.05$ ;  $t$  test). (B and C) Cd accumulation in grains. *OsLCT1* RNAi and control plants ( $T_1$ ) were grown in pots containing soil until grain ripening, and the harvested grains were used for histochemical Cd staining and elemental analysis. (B) Histochemical Cd staining of brown rice by QAI. Brown rice grains were stained with QAI after dehydration and masking. (Scale bars = 5 mm.) (C) Cd concentration in brown rice of control plants and *OsLCT1* RNAi plants. The data are presented as means  $\pm$  SD ( $n = 4-6$ ). Asterisks represent a significant difference from both control lines ( $P < 0.05$ ,  $t$  test). (D) Cd concentration in the xylem sap of vector control and RNAi plants. Plants ( $T_1$ ) were grown hydroponically for 3 wk and then exposed to 0.2  $\mu$ M CdCl<sub>2</sub> for 6 h; after which, xylem sap was collected. The data are presented as means  $\pm$  SD ( $n = 4-7$ ). No significant difference was observed between the control and RNAi plants ( $P > 0.05$ ,  $t$  test). (E and F) Quantification of Cd (E) and K (F) in phloem exudates of vector control and RNAi plants. Plants ( $T_1$ ) were grown hydroponically for 2 mo with 0.2  $\mu$ M CdCl<sub>2</sub>; after which, phloem exudate was collected from the seventh and eighth leaf blades. The data are presented as means  $\pm$  SD ( $n = 8$ ).

In rice, xylem-mediated root-to-shoot Cd translocation activity (12) and phloem-mediated Cd transport activity into grains (26) are both known to determine grain Cd levels. OshMA3, a rice P-type ATPase, was demonstrated to regulate xylem-mediated translocation of Cd (20, 21). However, to date, a transporter involved in phloem Cd transport has not been identified in plants. We generated RNAi-mediated *OsLCT1* knockdown plants (Fig. 3A) and showed that *OsLCT1* is involved in phloem



**Fig. 4.** Growth of *OsLCT1* RNAi plants. (A and B) Phenotype of whole shoots (A) and panicles (B) of control (108-1 or 108-2) and RNAi plants (118-6). Representative images of the respective lines are shown. (C and D) Panicle yields and straw weight of the control and RNAi plants. The data are presented as means  $\pm$  SD ( $n = 4-6$ ). No significant difference was observed between the RNAi plants and two control lines ( $P > 0.05$ ;  $t$  test).

Cd transport rather than in xylem Cd transport (Fig. 3D and E). These results suggest that *OsLCT1* is a transporter regulating phloem Cd transport and that it, thereby, possibly regulates Cd deposition into grains. Significant reduction of grain Cd levels in RNAi plants (Fig. 3B and C) strongly supports this hypothesis.

Previous studies on Cd behavior and the structure of nodal vascular bundles in rice have suggested a pathway for Cd transport from xylem to phloem. Cd is translocated from roots to shoots by the xylem (12) and after xylem-to-phloem transfer at nodes, Cd is preferentially transported to the upper nodes and finally into a panicle rather than into leaves (22). Large vascular bundles mediate major xylem transport from roots to leaf blades, whereas diffuse vascular bundles function in transporting nutrients into the upper nodes (25, 43). These suggest intervascular Cd transfer from enlarged large vascular bundles to diffuse vascular bundles in nodes. In the case of node I, the uppermost node, large vascular bundles connect to the flag leaf and diffuse vascular bundles connect to the panicle (25, 43). Therefore, intervascular Cd transfer at node I appears to be critical for grain Cd accumulation. The fact that our *OsLCT1*-RNAi plants accumulated less Cd in grains suggests that *OsLCT1* plays a role in this intervascular Cd transport.

Cell type specificity of *OsLCT1* transcript accumulation further supports this conclusion. The several layers of cells between the enlarged large vascular bundles and diffuse vascular bundles are termed the parenchyma cell bridge, which mediates intervascular solute transport. *OsLCT1* is expressed in cells surrounding enlarged large vascular bundles (Fig. 2E). These cells appear to be part of the parenchyma cell bridge. A silicon influx transporter in rice, *Lsi6*, is also localized to the outer boundary region of enlarged large vascular bundles and mediates the first step in silicon transport across the parenchyma cell bridge (25). This transport process mediated by *Lsi6* in node I is essential for silicon accumulation in panicles (25). *OsLCT1* expressed around enlarged large vascular bundles may have a similar role in intervascular Cd transfer as *Lsi6* has for silicon. Moreover, stronger expression of *OsLCT1* was observed in the diffuse vascular bundles (Fig. 2E). Diffuse vascular bundles contain large numbers of phloem



parenchyma cells surrounded by numerous phloem sieve elements, and these tissues have been suggested to mediate active phloem transport (44, 45). In particular, in node I, diffuse vascular bundles connect to the panicle, and, thus, Cd transfer into diffuse vascular bundles in node I is likely to be crucial for Cd deposition into grains. Strong expression of *OsLCT1* in the diffuse vascular bundles of node I suggests that *OsLCT1* expression in this region is important for Cd transport into grains.

Temporal patterns of *OsLCT1* expression also support the function of *OsLCT1* in Cd transport into grains. Expression of *OsLCT1* in nodes during the grain-ripening stage is >100-fold higher than in nodes at the heading stage (Fig. 2A). Considering the fact that the grain ripening stage is a critical period for grain Cd accumulation (46), this further suggests the significance of *OsLCT1* in Cd transport into grains.

In contrast to the clear involvement of *OsLCT1* in Cd transport in rice plants, its involvement in transport of other metals is not clear, because RNAi plants did not show significant reduction in mineral nutrient contents in grains (Fig. S5A). However, the transport activity for such elements as Ca and Mg, as well as for Cd, was observed in yeast cells expressing *OsLCT1* (Fig. S2D), leaving the possibility that *OsLCT1* mediates transport of these minerals *in planta*.

## Conclusions

In the present study, we demonstrated that *OsLCT1* plays an important role in Cd loading to the phloem and grain Cd accumulation. A rice Cd transporter regulating xylem-mediated translocation of Cd has already been identified and explains the high Cd accumulation in some high Cd-accumulating cultivars (20, 21). This study demonstrates a transporter gene that is crucial for phloem Cd transport and grain Cd accumulation in a standard *japonica* cultivar. The manipulation of mineral transporters often causes adverse effects on plant growth and nutrient accumulation (39, 41, 47). However, RNAi-mediated *OsLCT1* knockdown plants showed a substantial reduction in the Cd content in brown rice, without reduction in mineral nutrients or defects in growth or grain yield under the relatively lower soil Cd condition (Fig. 4 and Fig. S5A). These results indicate the significant potential of *OsLCT1* alleles for the practical engineering or breeding of low-Cd rice plants. Considering that ~40% of the Cd intake among Japanese people originates from rice (4), a 50% reduction of the Cd content in rice by down-regulating *OsLCT1* expression will have a major impact on human Cd intake. The success of this approach with rice may be extended to other staple crops, such as wheat and maize.

## Materials and Methods

**Plant Materials.** Rice (*Oryza sativa* L., cv. Nipponbare) was used for the cloning of *OsLCT1*, expression analysis, and transformation. For in situ hybridization, the *japonica* rice cultivar Shiohari was used.

**Subcellular Localization Analysis.** The transformation of cultured tobacco BY2 cells was conducted as reported previously (48) with modifications. The cells transformed with pSU40 (*35S-LCT1-sGFP*) or pTS100 (*35S-sGFP*) were stained with 25  $\mu$ M FM4-64 just before imaging and were observed using an FV1000 laser-scanning confocal microscope (Olympus).

**Yeast Cd Transport Activity Assay.** *Saccharomyces cerevisiae* strain  $\Delta ycf1$  was used for the yeast assay.  $\Delta ycf1$  was transformed with pESC-URA (Agilent Technologies) or pSU28 (pESC-URA carrying the *OsLCT1* ORF with some modifications in the codon use). The obtained transformants were

subjected to Cd transport-activity assay according to previous reports (49, 50). After three washes with ice-cold 2 mM  $\text{CaCl}_2$ , the element concentrations in the acid-digested cells were determined by ICP-MS (model SPQ9700; SII Nano-Technology, Seiko).

**Real-Time PCR.** Total RNAs were extracted from various tissues of rice grown in a pot in a greenhouse. cDNA synthesis and real-time PCR were conducted as described previously (24). The primers used for the quantification were 5'-GAGTTCCTCGTCAGAGCTAC-3' and 5'-CAGTGCTGGATGACGAATTG-3' for *OsLCT1* and 5'-GAAGGAGGAGGAATCGAAC-3' and 5'-CTTCACAGAGGTG-ATGCTAAGG-3' for *OsUBQ5*.

**In situ Hybridization.** The ORF of *OsLCT1* was amplified by PCR and cloned into pST19 (Roche Diagnostics). The obtained plasmid was used to prepare digoxigenin-labeled antisense probe after linearization. In situ hybridization and immunological detection of the signals were carried out according to a previous report (51). The sense probe was also prepared and used as a negative control. No visible signal was observed in the sense probe-treated samples (Fig. S4A).

**Generation of *OsLCT1* Knockdown Plants.** pIG121-RNAi-DEST (52) (vector control) and pSU19 (pIG121-RNAi-DEST carrying a 527-bp fragment of the N-terminal region of the *OsLCT1* ORF) for RNAi were introduced separately into *Agrobacterium tumefaciens* strain EHA101 (Rif<sup>r</sup>) pEHA101 (Km<sup>r</sup>). Rice plants were transformed according to a previous report (53). The transcription of *OsLCT1* in flag leaf blades of each line (T<sub>0</sub> and T<sub>1</sub> generations) was evaluated by real-time PCR with *OsUBQ5* as a control. The primers used for the quantification of *OsLCT1* and *OsUBQ5* are described above.

**Soil Culture Conditions and Elemental Analysis.** A commercial soil for rice cultivation (Kumiai Chemical Industry Co.) was used in our soil culture experiment. The soil Cd, Fe, Mn, Cu, and Zn concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Varian) after extraction with 0.1 M HCl (12).

For the growth experiments, two independent vector control lines (108-1 and 108-2; T<sub>1</sub> generation) and three independent RNAi lines (118-6, 118-9, and 112-1; T<sub>1</sub> generation) were used. After confirming the T-DNA insertion by PCR, seedlings were transplanted into plastic pots filled with 0.4 kg of soil in a greenhouse under natural lighting conditions. Four grams of fertilizer (MagAmp K; Hyponex) were applied to each pot as a top dressing. Intermittent irrigation was conducted throughout the cultivation (12). Plants were harvested after grain ripening. The concentrations of heavy metals in the plant tissues were evaluated by ICP-MS (model SPQ9700; SII NanoTechnology, Seiko) after digestion with nitric acid and hydrogen peroxide (12).

**Histochemical Cd Staining.** Histochemical staining of Cd was performed according to a previous method (31) with a slight modification using QAI. Grains of vector control (108-1) and RNAi (118-9) plants harvested from pot experiments were subjected to the staining.

**Xylem and Phloem Sap Analyses.** Plant culture, Cd exposure, and xylem sap collection were carried out according to a previous report (12, 54). Phloem sap collection was conducted according to the EDTA method (33). Determination of element concentration in the sap was conducted by ICP-MS.

For further details, see *SI Materials and Methods*.

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