

RESEARCH PAPER

Low cadmium (LCD), a novel gene related to cadmium tolerance and accumulation in rice

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Abstract

The contamination of food crops by cadmium (Cd) is a major concern in food production because it can reduce crop yields and threaten human health. In this study, knockout rice plants (*Oryza sativa*) tagged with the gene trap vector pGA2707 were screened for Cd tolerance, and the tolerant line *lcd* was obtained. The *lcd* mutant showed tolerance to Cd on agar plates and in hydroponic culture during early plant development. Metal concentration measurements in hydroponically grown plants revealed significantly less Cd in the shoots of *lcd* plants compared with wild-type (WT) shoots. When cultured in the field in soil artificially contaminated with low levels of Cd, *lcd* showed no significant difference in the Cd content of its leaf blades; however, the Cd concentration in the grains was 55% lower in 2009 and 43% lower in 2010. There were no significant differences in plant dry weight or seed yield between *lcd* and wild-type plants. LCD, a novel gene, is not homologous to any other known gene. LCD localized to the cytoplasm and nucleus, and was expressed mainly in the vascular tissues in the roots and phloem companion cells in the leaves. These data indicate that *lcd* may be useful for understanding Cd transport mechanisms and is a promising candidate rice line for use in combating the threat of Cd to human health.

Key words: Accumulation, cadmium, rice, tolerance.

Introduction

Cadmium (Cd) is a heavy metal that has been widely released into the environment. Once released into the environment, Cd enters the biogeochemical cycle and tends to accumulate in soils and sediments, where it becomes available to rooted plants. Cd uptake is harmful to most plants; it can reduce plant growth, and even kill plants in extreme cases.

Critical problems related to environmental exposure to Cd were reported in Japan in the early 20th century; people consuming contaminated food and water developed 'itai-itai disease', which caused renal abnormalities and weak bones (Horiguchi *et al.*, 2010). To suppress Cd pollution, most industrialized nations have set strict limits on Cd exposure, and in 2004 the Joint FAO/WHO Expert Committee on Food Additives, in association with the Codex Alimentarius

Commission, established allowable limits for Cd levels in food; for rice, the limit was set at 0.4 mg kg⁻¹. The mechanisms that control Cd accumulation in the edible parts of plants are poorly understood. For the general population, the major route of Cd exposure is through food (Nordic Council of Ministers, 2003). Rice grains contaminated with Cd represent a major risk to the health of more than half of the world's population, which depends on rice as a basic staple.

Several mechanisms related to Cd tolerance in plants have been identified, one of which involves complexing with metal-chelating peptides, such as metallothionein (MT), glutathione (GSH), and phytochelatins (PCs) (Grill *et al.*, 1995; Howden *et al.*, 1995; Cobbett *et al.*, 1998). In addition, transporter proteins play major roles in Cd

tolerance and accumulation in many organisms. In *Arabidopsis thaliana*, the P-type ATPases AtHMA2/AtHMA4 and the ABC transporter AtPDR8, from the pleiotropic drug resistance (PDR) family, which was previously reported to be involved in pathogen resistance, are involved in Cd detoxification by mediating Cd efflux from cells (Das *et al.*, 1997; Li *et al.*, 1997; Eren *et al.*, 2004; Kobae *et al.*, 2006; Stein *et al.*, 2006). In rice, the ZIP family transporters OsIRT1 and OsIRT2, which are iron (Fe) transporters, and OsZIP1, which is a zinc (Zn) transporter, have been reported to transport Cd (Korshunova *et al.*, 1999; Nakanishi *et al.*, 2006). Positron-emitting tracer imaging system (PETIS) experiments have shown the importance of the phloem system in Cd distribution in rice and transport to the grains (Fujimaki *et al.*, 2010).

With the goal of improving food safety, the current study aimed to identify genes related to Cd tolerance and accumulation in rice through the screening of a library of T-DNA-tagged rice lines. In this study, the discovery of a Cd-tolerant knockout mutant that accumulates lower concentrations of Cd in its grains without significant differences in plant biomass and grain yield is reported. In this mutant, the T-DNA was inserted into the first intron of *OsLCD*; the gene's possible roles in the mutant are discussed. A non-transgenic mutant in which *OsLCD* was knocked out was also found; this showed reduced Cd concentrations in its grains. This mutant is a promising candidate for the mitigation of the Cd threat to human health.

Materials and methods

Plant materials

Oryza sativa var. japonica cv. Hwayoung was used for Cd tolerance analyses, metal concentration measurements, and microarray analyses of the *lcd* mutant. To screen for Cd tolerance, seed stocks of mutant lines in which pGA2707 T-DNA was inserted were germinated and grown for 10–15 d in a growth chamber (16 h of light/8 h of dark at 28 °C) on Murashige and Skoog (MS) medium containing 50 mg l⁻¹ hygromycin and 1.0 mM CdSO₄. In the Cd sensitivity analyses, mutant and wild-type (WT) seeds were germinated on MS medium with or without hygromycin, respectively, for 3 d in a growth chamber (16 h of light/8 h of dark at 28 °C) then transferred to MS medium containing 1.0 mM CdSO₄ without hygromycin and grown for another 10 d under the same conditions.

For the metal concentration measurements and microarray analyses, mutant and WT seeds were germinated and grown for 7 d on MS medium with or without hygromycin, respectively, then transferred to 20.0 l plastic containers containing a nutrient solution composed of 2 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.7 mM K₂SO₄, 0.1 mM KCl, 0.1 mM KH₂PO₄, 0.1 mM Fe(III)-EDTA, 10 μM H₃BO₃, 0.5 μM MnSO₄, 0.5 μM ZnSO₄, 0.2 μM CuSO₄, and 0.01 μM (NH₄)₆Mo₇O₂₅. The culture solution was adjusted to pH 5.0–5.5 with 1 N KOH. After 7 d, the plants were transferred to containers containing the same nutrient solution with or without the addition of 10 μM CdSO₄ and grown for another 14 d.

Inverse PCR (iPCR) (Hui *et al.*, 1998; Spertini *et al.*, 1999) was performed to isolate the T-DNA flanking sequences. A 1 μg aliquot of genomic DNA was digested with 10 U of *Pst*I in 50 μl for 10 h. Following heat inactivation of the enzyme, the samples

were precipitated with ethanol and dissolved in 50 μl of ligation buffer, then ligated at 8 °C for 12 h using 1 U of T4 DNA ligase (Boehringer Mannheim/Roche, Basel, Switzerland). Nested PCR was performed to amplify the right and left flanking sequences of the T-DNA. For the first PCR, ~2% of the ligated DNA was incubated with 5 μM primer (GUS2R, TTGGGGTTTCTACAGGACGTAAC, and HPH4R, CCATGTAGTGTATTGACC-GATTC for the right border; HPH1F, GATCGTTATGTTATCGGCACTT, and Ptb3R, GGTGAATGGCATCGTTT-GAA for the left border) in a 25 μl solution containing dNTPs and 0.1 U of ExTaq polymerase (Takara, Shiga, Japan). PCR was performed with an initial 5 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 4 min, followed by a final 10 min at 72 °C. A 0.1 μl aliquot of the product was then used as template for the second PCR with the primers GUS1R, CAAGTTAGTCATGTAATTAGCCAC, and HPH3R, TCGTCTGGCTAAGATCGGCCGCA for the right border; and HPH2F, AGTGCTTGACATTGGGGAATTG, and Ptb2R, ACAAGCCGTAAGTGCAAGTG for the left border under the same conditions. The products were separated by 1.0% (w/v) agarose gel electrophoresis, eluted with a GeneClean III Kit (Q-Biogene, Carlsbad, CA, USA), and sequenced using a 3130 Genetic Analyzer (Applied Biosystems).

RT-PCR

Total RNA was isolated from *lcd*, *lcd-tos17*, and their respective WT plants grown under normal conditions. Total RNA was treated with RNase-free DNase I (Takara Bio, Otsu, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesized from 2 μg of RNA using SuperScript II reverse transcriptase (Toyobo) by priming with oligo-d(T)₃₀, the final volume was adjusted to 100 μl, and 2 μl from each sample was used for subsequent reactions. Fragments were amplified by PCR. The *LCD* primers used for RT-PCR were as follows: *LCD* forward 5'-CTATGATTTTCATCGGATCTACCGACTG-3' and *LCD* reverse 5'-CTAAGAACCAAAAACCTCCTAACAGGAG-3'. For *LCD* RT-PCR, the annealing temperature was set to 65 °C (20 s). The α -tubulin primers used for RT-PCR were as follows: α -tubulin forward 5'-TCTTCCACCCTGAGCAGCTC3' and α -tubulin reverse 5'-AACCTTGGAGACCAGTGCAG-3'. For α -tubulin RT-PCR, the annealing temperature was set to 55 °C (30 s).

Metal concentration measurements

Roots and shoots were harvested and dried overnight at 80 °C. Dried samples (50–100 mg of roots; 100–200 mg of shoots) were digested in 3 ml of 13 M HNO₃ at 220 °C for 20 min with MARS Xpress (CEM, Matthews, NC, USA). After digestion, the samples were diluted to a volume of 5 ml and analysed by inductively coupled plasma atomic emission spectrometry (model SPS1200VR; Seiko, Tokyo, Japan) at the following wavelengths: 226.502 (Cd), 238.204 (Fe), 213.856 (Zn), 293.930 (manganese, Mn), and 324.754 (copper, Cu) nm.

Subcellular localization of LCD

The full-length open reading frame (ORF) of *LCD* (951 bp) was amplified with forward and reverse primers as follows: 5'-CACCATGGTGGAAACCACAGTTG-3' and 5'-TTTTTTTCAAGGCCACTTCCAGA-3'. The full-length *LCD* ORF was cloned into pENTR/TOPO (Invitrogen) and then subcloned into pH7WGF2 (Karimi *et al.*, 2002) under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter using the LR recombination reaction (Invitrogen) (Ishimaru *et al.*, 2009). Onion epidermal cells were transformed using a Biolistic PDS-1000/He particle delivery system (Bio-Rad).

Histochemical analysis

The 1.5 kb 5' upstream region of the *LCD* gene was amplified using KOD plus polymerase at an annealing temperature of 65 °C by the forward primer 5'-GGGGGGAAGCTTCGGACTATAA-TATGTTGTGTGTA-3' and the reverse primer 5'-GGGGGGTCTAGATTATAAGTTCCATTAACAAGTT-3' which contain *Hind*III and *Xba*I restriction sites. The amplified fragment was fused into the *pBluescript*IISK+ vector, and its sequence was confirmed. The LCD promoter was digested with *Xba*I and *Hind*III, and the digested 1.5 kb fragment was subcloned upstream of the *uidA* ORF, which encodes β -glucuronidase (GUS), in the pGI21Hm vector. *Oryza sativa* L. cv. Tsukinohikari was transformed (Ishimaru *et al.*, 2006). Rice plants were grown in culture solution in a greenhouse for 3 weeks after germination on MS medium, and the histochemical localization was observed in four different independent T₂ plants. Roots and stems of transgenic plants were cut with a scalpel into ~1 cm sections. The sections were embedded in 4% agar and then cut in sections of 80–130 μ m using a DTK-100 vibrating microtome (Dosaka EM Co. Ltd, Kyoto, Japan). The sections were incubated at 37 °C for 15 min in GUS reaction buffer {1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, 3 mM K₄[Fe(CN)₆], 0.5 mM K₃[Fe(CN)₆], 50 mM sodium phosphate (pH 7.0), and 20% (v/v) methanol}. Following staining, the sections were washed in 70% ethanol until observation. GUS staining was observed using an Axiophoto microscope (Carl Zeiss), following the manufacturer's instructions.

Oligo-DNA microarray analysis

A rice 44k oligo DNA microarray (Agilent Technology, Tokyo, Japan) containing 45 151 60-mer oligonucleotides based on the sequence data of the rice full-length cDNA project (<http://cdna01.dna.affrc.go.jp/cDNA/>) was used. Total RNA was extracted from shoots and roots using an RNeasy Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The yield and RNA purity were determined spectrophotometrically. The integrity of the RNA was checked using an Agilent 2100 Bioanalyzer. Total RNA (200 ng) was labelled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. Fluorescently labelled targets were hybridized to Agilent rice 44K oligo DNA microarrays. Microarray hybridization, scanning, and data analysis were performed following the manufacturer's instructions. The reproducibility of the analyses was assessed by a dye swap in each experiment.

Results

Isolation of a Cd-tolerant mutant from a library of T-DNA-tagged lines

WT plants (cv. Hwayoung) were sown on MS medium at different concentrations of CdSO₄ to find the optimum Cd²⁺ concentration in the medium for the selection of Cd-tolerant mutants. Addition of CdSO₄ caused some complications in the MS culture medium. First, high concentrations of salts bind to agar, preventing it from hardening. To solve this issue, the concentration of agar in the medium was raised from 0.8% to 1.2% to make the MS medium hard enough for proper plant culture. Secondly, usually addition of salts to the culture displaces the Fe from Fe-EDTA, and then addition of high concentrations of CdSO₄ would displace almost all the Fe from Fe-EDTA; however, this did not seem to affect the selection efficiency of the screening. A concentration of 1.0 mM CdSO₄ was found to slow shoot and root growth development visibly in the WT plants and

was consequently used to select for Cd-tolerant mutants (Supplementary Fig. S1 available at *JXB* online).

A pool of rice T-DNA mutants in which the plasmid pGA2707 (Spertini *et al.*, 1999) was randomly inserted into the rice genome, resulting in knockout mutants, was screened for mutants exhibiting Cd tolerance. A Cd-tolerant mutant, named *lcd* (low Cd), was selected from 3993 independent T-DNA mutant lines among ~9500 seeds. The *lcd* mutant grew better than WT plants on MS agar plates containing 1.0 mM CdSO₄ and had longer shoots (Fig. 1A, B). The Cd concentration in the shoots of the mutant was ~30% less than in the shoots of the WT plants (Fig. 1C).

The T-DNA in *lcd* was inserted into the first intron of *OsLCD*, leading to knockout

The T-DNA insertion in the *lcd* mutant was located using iPCR. A sequence analysis of the T-DNA-flanking region in *lcd* revealed that the T-DNA was inserted 811 bp upstream of the initiation codon in *OsLCD* (*O. sativa* LCD) on chromosome 1 (Fig. 2A). The gene was identified as locus Os01g0956700, according to the Rice Annotation Project (RAP; <http://rapdb.dna.affrc.go.jp>) of the International Rice Genome Sequencing Project (IRGSP; <http://rapdb.dna.affrc.go.jp/E/IRGSP/index.html>). T-DNA insertion usually leads to gene disruption. Therefore, RT-PCR was performed to confirm that *OsLCD* was knocked out in the mutants. *OsLCD* expression was observed in both the roots and shoots of WT (Hwayoung) plants, but not in *lcd*, confirming the knockout of *OsLCD* (Fig. 2B).

Tos17 lines are mutants generated through retrotransposon insertion (Hirochika *et al.*, 1996) available to order for research purposes (<http://tos.nias.affrc.go.jp/>). A *tos17*

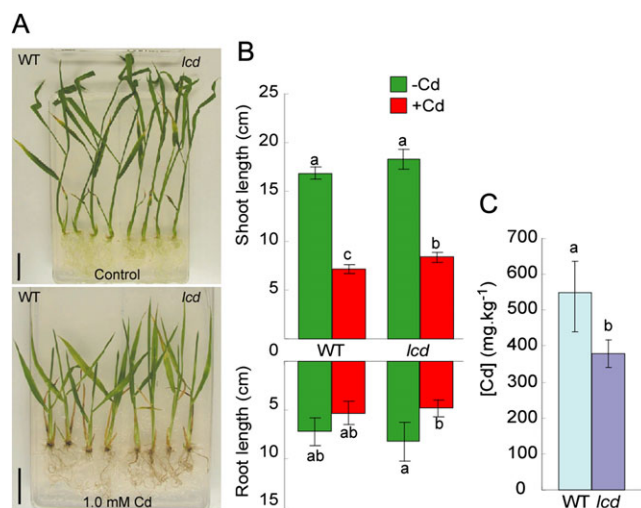


Fig. 1. Selection of the Cd-tolerant mutant line *lcd*. (A) WT and *lcd* mutants were grown in MS medium with or without 1.0 mM Cd for 10 d. (B) Root and shoot length of the WT and *lcd* in both conditions. (C) Cd concentration in shoots of the WT and *lcd* from plants grown in MS medium in both conditions. Bars=2 cm. Values followed by different letters are statistically different according to Student–Newman–Keuls test ($n=4$).

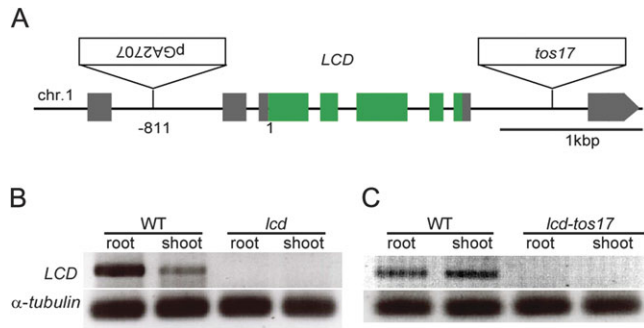


Fig. 2. Insertion positions and *LCD* expression in the mutants. (A) Schematic representation of *LCD* and the insertion positions of the T-DNA and the *tos17* fragment. (B and C) Total RNA was extracted from roots and shoots for *lcd/lcd-tos17* and their respective WT, and RT-PCR was performed.

mutant (cv. Nipponbare) with the retrotransposon fragment inserted into *OsLCD* was found. RT-PCR performed on both WT (Nipponbare) and *lcd-tos17* mutant plants confirmed that *OsLCD* was expressed in the WT plants but was knocked out in the *lcd-tos17* mutant plants (Fig. 2C).

Reduced Cd accumulation in the shoots of the *lcd* mutant

The selected mutants, *lcd* and *lcd-tos17*, and their WT versions, Hwayoung and Nipponbare, respectively, were germinated on MS plates then transferred to nutrient solution with or without 10 μ M CdSO₄. Under control conditions (no Cd), the *lcd* mutant had longer roots than did the WT (Hwayoung), but no visible differences were observed between the *lcd-tos17* mutant and Nipponbare (Fig. 3A, B, D, E). When grown in the presence of Cd, the *lcd* mutant had longer roots and shoots compared with the WT (Hwayoung); this phenotype was not observed, however, in the *lcd-tos17* mutant.

Metal concentrations were measured in the roots and shoots of *lcd*, *lcd-tos17*, and WT plants after growth in the presence of Cd. Less Cd accumulated in the shoots of *lcd* and *lcd-tos17* compared with their respective WT versions (Fig. 3C, F). More Cd accumulated in the roots of *lcd* than in the roots of Hwayoung; however, no significant difference in Cd accumulation was observed in the roots of *lcd-tos17* compared with its WT (Fig. 3D, F). The concentrations of other metals, including Zn, Mn, Fe, and Cu, were also measured. In *lcd* shoots, decreased metal concentrations were observed in the Zn, Mn, and Fe concentrations, while in *lcd* roots, significant differences were observed only for increased Zn and decreased Fe. In the case of *lcd-tos17*, increased metal concentrations were found for the shoot Zn and Mn, while no differences were observed for root metal concentrations (Supplementary Fig. S2 at JXB online).

Subcellular localization of *OsLCD*

A fusion protein construct [*OsLCD*::green fluorescent protein (GFP)] was transiently expressed in onion (*Allium cepa*) bulb

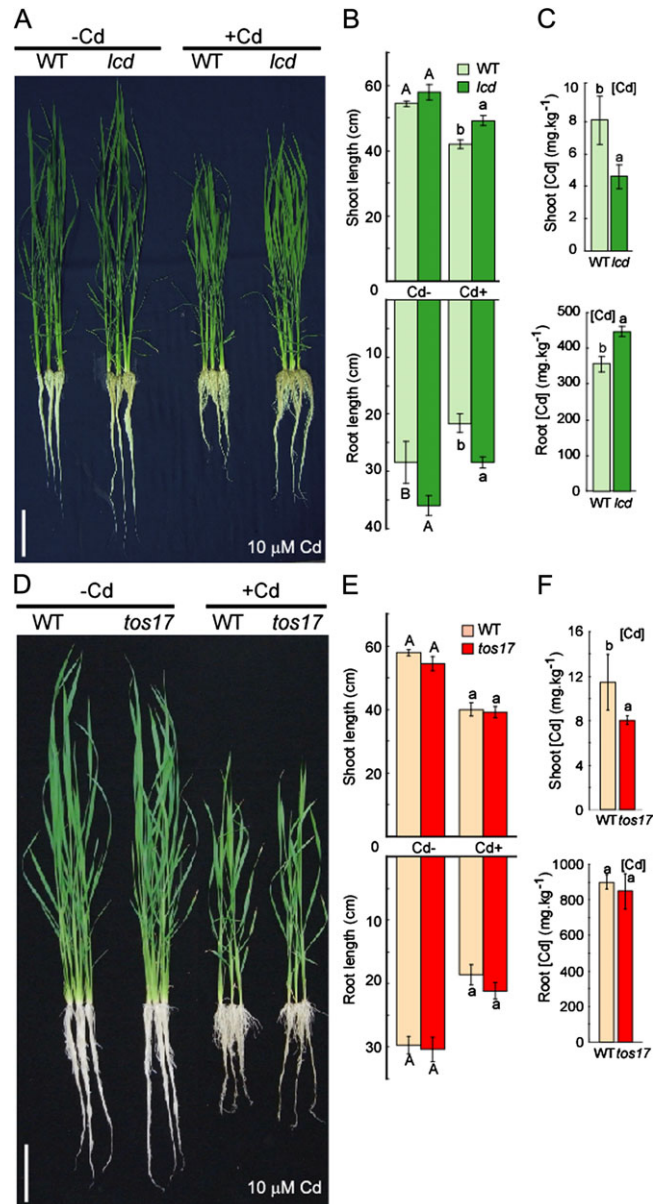


Fig. 3. *lcd* and *lcd-tos17* phenotype in response to Cd. (A and D) WT and *lcd/lcd-tos17* were grown in nutrient solution for 1 week then grown in nutrient solution with or without 10 μ M Cd for another week. (B and E) Root and shoot lengths of WT and *lcd/lcd-tos17* in both conditions ($n=3$). (C and F) Cd concentration of roots and shoots ($n=3$). Bars=10 cm. Values followed by different letters are statistically different according to Student–Newman–Keuls.

epidermal cells. *OsLCD*::GFP fluorescence was detected in the cytoplasm and nucleus (Fig. 4).

Tissue expression of *OsLCD*

RT-PCR showed that *OsLCD* was expressed in both the roots and shoots of WT plants (Fig. 2B, C). To determine more accurately where *OsLCD* was expressed, GUS expression under control of the *OsLCD* promoter was examined in roots and shoots. In roots, GUS activity was localized to

the central region (Fig. 5A, longitudinal section), which represents the root vascular bundle (Fig. 5B and C, transverse section). In leaves, GUS activity was observed mainly in the phloem companion cells (Fig. 5D). These data suggest that *OsLCD* affects Cd transport within plants as part of the plant transport system.

Field-grown *lcd* grains accumulated less Cd

To determine the effects of knocking out *OsLCD* on field-grown plants, *lcd* (T-DNA) and Hwayoung (WT) plants were grown in a restricted experimental field where the soil was

artificially contaminated (Cd-salt amendment for experiment purposes) with Cd (average of 3.6 ppm Cd) under continuous flooded conditions until harvest in 2009. The *lcd* mutant plants showed normal growth with no significant differences in panicle formation or seed appearance compared with the WT (Fig. 6A, B). There was also no difference in plant biomass, represented by leaf dry weight, grain yield, or grain weight (Fig. 6C–E). There was no significant difference in the Cd concentration in *lcd* mutant and WT leaves (Fig. 6F); however, the grains of the *lcd* plants accumulated 55% less Cd compared with the WT without significant changes in the Fe and Zn concentrations (Fig. 6G; Supplementary Fig. S3 at

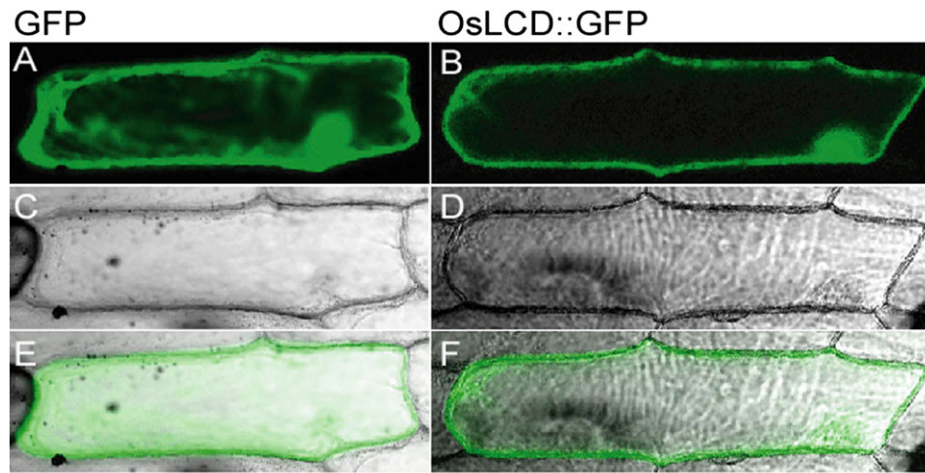


Fig. 4. Subcellular localization of OsLCD. The OsLCD::GFP fusion protein was transiently expressed in onion (*Allium cepa*) epidermal cells and fluorescence was analysed. (A and B) GFP fluorescence. (C and D) Transient imaging. (E and F) Merged image. Bars=50 μ m.

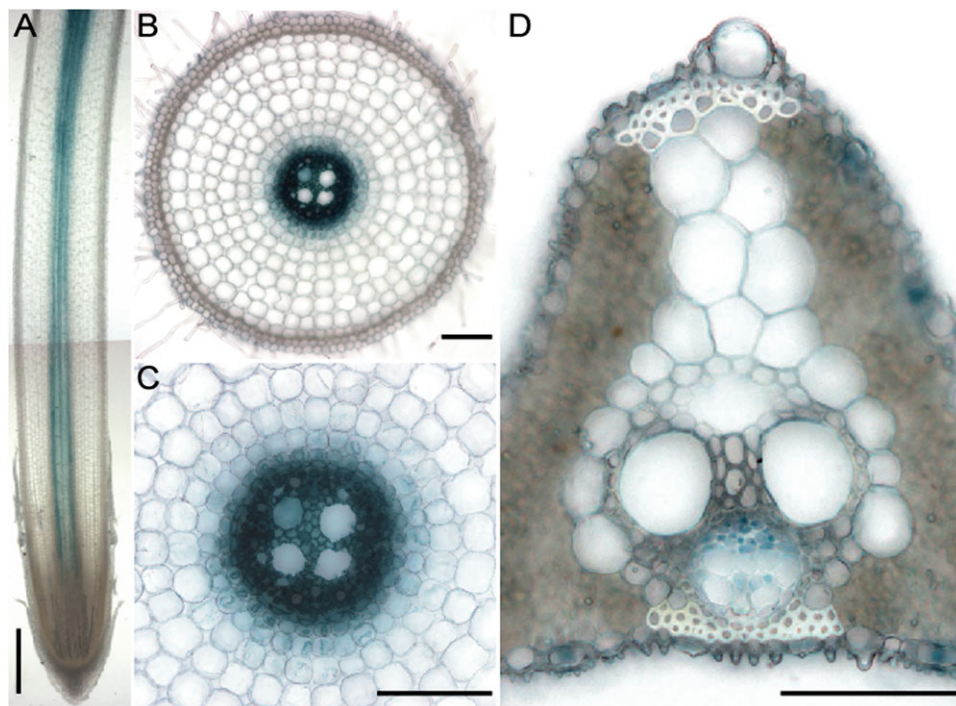


Fig. 5. Tissue expression analysis of OsLCD. *OsLCDp-GUS* plants were germinated and cultured in nutrient solution for 2 weeks and analysed for GUS staining. (A) Longitudinal section of a root. (B) Cross-section. (C) Amplified view of the cross-section. (D) Leaf cross-section. Bars=500 μ m in A, 100 μ m in B and C, and 50 μ m in D.

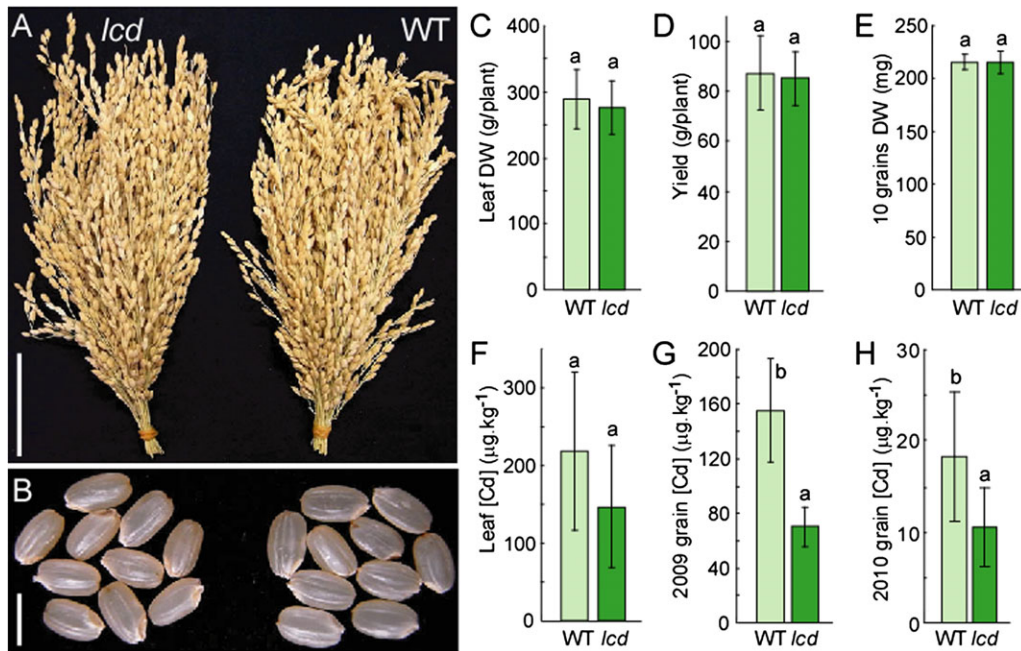


Fig. 6. Field experiment. Photo of panicles (A) and grains (B). (C) Leaf dry weight (DW). (D) Grain yield per plant. (E) DW of 10 grains. (F) Leaf Cd concentration. (G) Grain Cd concentration in *lcd* and the WT from the field experiment in 2009 ($n=6$). (H) Grain Cd concentration in *lcd* and the WT from the field experiment in 2010 ($n=25$). Bars=5 cm in A and 5 mm in B. Values followed by different letters are statistically different according to Student–Newman–Keuls test.

JXB online). To confirm these results, the field experiment was repeated in 2010 (average of 0.89 ppm Cd) with a greater number of plants. Again, the *lcd* mutant accumulated 43% less Cd in its grains than did the WT (Fig. 6H).

Transcriptome analysis of the *lcd* mutant

To examine the mechanisms of Cd tolerance and accumulation in *lcd* and *lcd-tos17*, genome-wide mRNA levels were analysed in the mutants and their respective WT versions using a 44k oligonucleotide DNA chip. In *lcd*, 475 genes in both the roots and shoots showed a >2-fold change in expression relative to WT plants under normal growth conditions, while in the *lcd-tos17* mutants, 42 genes showed a similarly high expression level. In the *lcd* plants, 325 genes, including *LCD* itself, showed a >50% decrease in expression relative to WT plants in both the roots and shoots under normal growth conditions, while in the *lcd-tos17* mutants, only 70 showed such a decrease in expression.

Supplementary Table S1 at *JXB* online lists the genes that were down-regulated (ratio <0.5) in the roots and shoots of the *lcd* and *lcd-tos17* mutants compared with their respective WT plants. Among these 24 genes, a dehydrin gene belonging to a group of genes previously reported to be related to abiotic stress (Nylander *et al.*, 2000) showed very strong down-regulation, particularly in the roots. Supplementary Table S2 also lists the genes that were up-regulated (ratio >2) in both the *lcd* and *lcd-tos17* mutants compared with their respective WT plants. Among the up-regulated genes, an ABC transporter (multidrug resistance protein 1 homologue; Os01g0533900) showed increased expression in both roots and shoots.

A previous report proposed that the transporter genes PDR, MDR, MRP, MATE, CDF, OsHMA5, and OsIRT were involved in the early response to Cd stress (3 h exposure at 1 μM Cd²⁺) (Ogawa *et al.*, 2009). Table 1 shows the expression ratios in *lcd* and *lcd-tos17* of the transporter genes proposed to be involved in the Cd stress response by Ogawa *et al.* (2009). WT plants exposed to 10 μM Cd²⁺ for 2 weeks showed enhanced expression levels of OsPDR9, OsPDR17, OsPDR16, MDR, MRP, and MATE efflux family proteins in response to Cd in the roots, in agreement with Ogawa *et al.* (2009). However, OsPDR1 showed reduced expression in the roots and enhanced expression in the shoots. Microarray analysis of these genes in *lcd* and *lcd-tos17* revealed that none of these genes had significantly different expression levels relative to the WT.

Discussion

Through screening of T-DNA insertion mutants, a Cd-tolerant mutant, *lcd*, was identified which accumulated less Cd in its shoots compared with WT plants. The T-DNA in *lcd* was inserted in the first intron of *OsLCD* (Os01g0956700). Currently, the function of *OsLCD* is unknown; thus, no information about putative domains and motifs could be found.

The *lcd* mutant, when grown in hydroponic culture, accumulated more Cd in its roots than did the WT, but accumulated less Cd in the shoots, suggesting that *OsLCD* is involved in Cd partitioning in rice. On the other hand, the *lcd-tos17* mutant showed some phenotype discrepancies in

Table 1. Transporter genes possibly involved in Cd stress response

Locus	Description	Signal (WT; HW)		<i>lcd</i> /WT		<i>tos</i> /WT		Cd+/Cd- (WT; HW)	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Os01g0609300	OsPDR9	27	200	1.0	0.8	1.5	1.3	20.3	1.3
Os08g0544400	OaPDR1	261	20	1.0	1.0	0.8	0.7	0.1	4.5
Os08g0384500	OsPDR17	82	65	1.2	0.8	1.3	1.2	5.0	1.2
Os01g0609900	OsPDR8	8361	469	1.0	0.9	1.1	1.1	1.9	0.9
Os01g0342700	OsPDR16	45	32	1.1	0.6	1.0	1.5	2.1	1.4
Os03g0667500	OsiRT1	22 169	891	0.9	1.0	0.6	0.8	0.8	1.3
Os07g0232900	OsHMA3	10 083	949	1.1	1.0	0.8	0.8	0.4	1.3
Os01g0695800	MDR	108	272	1.1	1.1	1.0	1.0	16.7	1.6
Os04g0209200	MRP	186	156	1.3	1.3	1.1	1.0	2.5	0.7
Os03g0571900	MatE (PEZ1)	252	702	0.9	0.9	1.2	0.9	4.1	1.2

Signal, signal intensity in WT for roots and shoots; *lcd*/WT, ratio of *lcd* in relation to WT (cv. Hwayoung) in roots and shoots; *tos*/WT, ratio of *lcd-tos17* in relation to WT in roots and shoots; Cd+/Cd-, ratio between 10 μ M Cd²⁺ treatment and control in the WT (cv. Hwayoung).

root Cd accumulation and accumulation profiling of other metals compared with *lcd*, but a similar decrease in Cd accumulation in shoots, which might be the result of the differences in expression of genes related to metal homeostasis in the genetic backgrounds of each mutant (Supplementary Table S3 at *JXB* online). The Cd partitioning hypothesis is supported by the OsLCDp-GUS analysis, which indicated that *OsLCD* was expressed in tissues related to transport, for example root vascular bundles and leaf phloem companion cells. A previous report (Fujimaki *et al.*, 2010) discussed the importance of phloem in Cd distribution within plants and subsequent transport to the grains. The present data support this previous report by showing that the knockout of a gene, the normal expression of which was localized to phloem companion cells, resulted in altered Cd distribution in the plant body and reduced the grain Cd concentration. Tian *et al.* (2010) reported that the knockout of a gene encoding a phloem companion cell-specific protein that is normally secreted into phloem sieve elements affected the distribution of certain metals in the plant, most probably by altering the translocation of these metals. Although the function of LCD is unclear, it may be related to Cd transport in a similar manner, affecting its translocation/solubility inside plants.

In WT plants, exposure to Cd leads to less accumulation of Zn in the roots and shoots, suggesting that Cd interfered with Zn accumulation; this phenomenon was also observed in the *lcd* mutant (data not shown). Cd and Zn have similar physical and chemical properties; therefore, Cd may compete with Zn for various biological ligands (Azpiroz-Leehan *et al.*, 1997), resulting in less Zn accumulation. In the *lcd* mutant, Zn showed the same distribution pattern as Cd; namely, greater accumulation in the roots and less accumulation in the shoots compared with the WT under control and high Cd conditions, which suggests that the natural function of LCD is related to Zn transport. However, when *lcd* plants were grown in soil as opposed to hydroponic culture with a low Cd concentration, there were no significant differences from the WT in the shoot Cd, Zn, Mn, and Fe concentrations.

The grains of *lcd* mutant plants grown in soil had lower Cd concentrations relative to the WT, but showed no significant differences in Zn, Mn, and Fe relative to the WT. These observations suggest that the *lcd* mutant exhibits different behaviours with respect to Cd translocation in hydroponic versus soil culture. This may be a function of the bio-availability of Cd, which is lower in a soil culture system.

Sequence analysis of OsLCD showed no homology with other known proteins, suggesting that this protein may be specific to rice. Microarray analysis of *lcd* and *lcd-tos17* mutant plants confirmed the knockout of *OsLCD* (Supplementary Table S1 at *JXB* online). Microarray analysis also revealed 24 genes that were down-regulated in both *lcd* and *lcd-tos17*, with strong down-regulation of a dehydrin gene, particularly in the roots. Dehydrins present a distinct group of stress response proteins, the so-called late embryogenesis abundant (LEA) proteins. Among the genes that were up-regulated in both *lcd* and *lcd-tos17*, an ABC transporter (multidrug resistance protein 1 homologue; Os01g0533900) showed increased expression in both roots and shoots (Supplementary Table S2). It was the only transporter protein found with a changed expression level in both mutants and may be related to the Cd accumulation phenotype of *lcd* and *lcd-tos17*; however, a more detailed analysis of the ABC transporter gene is required to understand the possible relationship(s) between its gene product, LCD, and the Cd phenotype. Ogawa *et al.* (2009) found that a number of transporters, including PDR, MDR, MRP, MATE, CDF, OsHMA3, and OsiRT, were induced under Cd stress and may be involved in the Cd stress response (Table 1). These transporters showed no significant changes in expression in *lcd* and *lcd-tos17*, indicating that the Cd phenotype presented by the mutants is not directly related to these previously reported transporters; therefore, another pathway remains to be elucidated.

In summary, LCD is a soluble protein expressed in plant vascular tissues, the absence of which causes alterations in Cd transport within plants. Although the mechanisms involved in the *lcd* mutant phenotype are unclear, these

data indicate that *lcd* might be a useful mutant line for research aimed at understanding Cd transport in plants and is a promising candidate rice line for use in combating the threat of Cd to human health.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. WT seeds were sown in MS medium containing Cd concentrations of 0, 0.25, 0.5, 1.0, 1.5, and 2.0 mM for 7 d.

Figure S2. Root and shoot metal concentration.

Figure S3. Field grain and leaf metal concentration.

Table S1. Down-regulated (ratio ≤ 0.5) genes in microarray analysis in both shoots and roots of *lcd*.

Table S2. Up-regulated (ratio ≥ 2) genes in microarray analysis in both shoots and roots of *lcd*.

Table S3. Differences in gene expression in different genetic backgrounds of rice.

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