RESEARCH PAPER

The ABC transporter ABCG36 is required for cadmium tolerance in rice

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Abstract

Cadmium (Cd) is a highly toxic heavy metal in nature, which causes severe damage to plant growth. The molecular mechanisms for Cd detoxification are poorly understood. Here, we report that a G-type ATP-binding cassette transporter, OsABCG36, is involved in Cd tolerance in rice. OsABCG36 was expressed in both roots and shoots at a low level, but expression in the roots rather than the shoots was greatly up-regulated by a short exposure to Cd. A spatial expression analysis showed that Cd-induced expression of OsABCG36 was found in both the root tip and the mature root region. Transient expression of OsABCG36 in rice protoplast cells showed that it was localized to the plasma membrane. Immunostaining showed that OsABCG36 was localized in all root cells except the epidermal cells. Knockout of OsABCG36 resulted in increased Cd accumulation in root cell sap and enhanced Cd sensitivity, but did not affect tolerance to other metals including Al, Zn, Cu, and Pb. The concentration of Cd in the shoots was similar between the knockout lines and wild-type rice. Heterologous expression of OsABCG36 in yeast showed an efflux activity for Cd, but not for Zn. Taken together, our results indicate that OsABCG36 is not involved in Cd accumulation in the shoots, but is required for Cd tolerance by exporting Cd or Cd conjugates from the root cells in rice.

Keywords: ABC transporter, cadmium, Cd accumulation, Cd tolerance, OsABCG36, rice.

Introduction

Cadmium (Cd) is one of the naturally occurring heavy metals that is highly toxic to all organisms, including plants. Cd excess in plants can cause disruption of nutrient homeostasis, dysfunction of proteins, DNA and membrane damage, and the accumulation of toxic reactive oxygen species, resulting in significant reductions of crop growth and production (Sandalio et al., 2001; Hall, 2002; Boominathan and Doran, 2003). More seriously, Cd accumulation in the edible parts of crops potentially threatens human and animal health.
To adapt to Cd-contaminated environments, plants have evolved diverse mechanisms to cope with Cd stress, such as Cd extrusion, chelation, and sequestration (Hall, 2002; Weber et al., 2006; Lin and Aarts, 2012). Several studies have shown that ATP-binding cassette (ABC) transporters may play important roles in Cd tolerance in plants (Kim et al., 2006, 2007; Oda et al., 2011; Park et al., 2012; Brunetti et al., 2015). For example, AtABC36/AtPDR8 was reported to be essential for Cd resistance in Arabidopsis (Kim et al., 2007). This protein is localized at the plasma membrane of root cells and functions as an efflux transporter for Cd or Cd conjugates. On the other hand, two ABC-type transporters, AtABCC1 and AtABCC2, are involved in vacuolar sequestration of Cd in Arabidopsis (Park et al., 2012). Knockout of these genes resulted in hypersensitivity to Cd. Recently, another ABC-type transporter, AtABCC3, was reported to be involved in Cd tolerance by transporting phytochelatin–Cd complexes into the vacuole in Arabidopsis (Brunetti et al., 2015). In rice, there are at least 120 ABC transporters (Garcia et al., 2004), but only few of them have been characterized in terms of Cd tolerance. One of them, OsABCG43, is a Cd-inducible transporter (Oda et al., 2011). Its expression can be induced Cd in tolerant rice cultivars (Oda et al., 2011), but its exact role in Cd is unknown. Another ABCG-type transporter gene, OsABCG36/OsPDR9, was reported to be induced by Cd and was suggested to play a possible role in heavy metal stress (Moons, 2003, 2008). However, the role of OsABCG36/OsPDR9 in Cd tolerance has not been investigated. In the present study, we functionally characterized OsABCG36 in terms of its gene expression, cellular and subcellular localization, and transport activity. We also obtained two independent mutant lines of OsABCG36 by using the CRISPR/Cas9 technique and compared their Cd tolerance and accumulation with that of wild-type rice. Our results showed that OsABCG36 is involved in Cd tolerance by transporting Cd out of the root cells.

Materials and methods

Generation of OsABCG36 knockout lines

To create the OsABCG36 knockout lines, the CRISPR/Cas9 genome-targeting system was used. The pCRISPR-OsABCG36 plasmid with two OsABCG36-specific target sites was constructed as described by Ma et al. (2015). Briefly, two specific target sequences (CGCTCGGATTTCTGCCAAC and GACCTACACGGCGACAGCGCA) within OsABCG36 were selected by a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the target sequences, including protospacer adjacent motif (PAM) sequence, against the rice genome sequence. These two sequences should have a difference of at least two bases with similar non-target sequences within the PAM or PAM-proximal region and have more than five base mismatches in the PAM distal region to non-target sequences based on off-target analysis (http://skl.scau.edu.cn/off-target/). Then, target sequences were introduced into sgRNA expression cassettes by overlapping PCR, producing pU6a-OsABCG36-sgRNA or pU3-OsABCG36-sgRNA fragments. Using restriction–ligation reactions containing Bsal and T4 DNA ligase, these fragments were cloned into pYL322-GFP-OsABCG36 construct. These constructs were introduced into Agrobacterium tumefaciens strain EHA101 and transformed into the wild-type rice (Oryza sativa cv. Nipponbare). Mutation detection was carried out using primer pairs flanking the OsABCG36-specific target sites. The PCR products were sequenced directly using OsABCG36-specific primers. Homozygous mutants of OsABCG36 were selected for further phenotypic analysis as described below.

Plant materials and growth conditions

The wild-type rice (cv. Nipponbare) and two independent OsABCG36 knockout lines (osabcg36-1, osabcg36-2) were used in this study. Rice seeds were germinated in deionized water in a growth chamber for 2 days in darkness at 28 °C and then placed on a net floating on a solution containing 0.5 mM CaCl2. Seedlings were grown for 2 days at 28 °C and used for various experiments.

RNA isolation and gene expression analysis

To examine the expression pattern of OsABCG36, wild-type rice seedlings (7 days old) were exposed to different Cd concentrations (0–20 μM) for different periods of time. Root fragments (0–1 cm and 1–2 cm from the root tip) and shoots were sampled for RNA extraction, with three replicates for each sample. Total RNA was extracted using the TRIzol reagent kit (Life Technologies) according to the manufacturer’s instructions. Total RNA (1 μg) was used for first-strand cDNA synthesis using a Hiscr ipt II Q RT SuperMix Kit (Vazyme). Quantitative reverse transcription–PCR (qRT–PCR) was performed with ChamQTM SYBR Color qPCR Master Mix (Vazyme) on a StepOnePlus Real-Time PCR System (Analytik Jena AG). The primers for gene expression analysis of OsABCG36 were 5′-ATCCAGCAAGAGAGCAATG-3′ and 5′-GGTCTCATGAGGAGCAAG-3′. The primers for gene expression analysis of OsABCG37 were 5′-AACCCGCGGACGCAATCGG-3′ and 5′-TCCCCTGTGCAATTTGATGT-3′. The primers for gene expression analysis of OsABCG44 were 5′-CCTCTGAGAAGCTGCTG-3′ and 5′-GATATGGGAGATTTGAT-3′. The primers for gene expression analysis of OsABCG44 were 5′-GGTCTCATGAGGAGCAAG-3′ and 5′-AACCCGCGGACGCAATCGG-3′.

Subcellular localization of OsABCG36

To construct the GFP-OsABCG36 fusion gene, OsABCG36 cDNA was amplified from the Nipponbare cDNA by PCR using the OsABCG36-specific primers 5′-AACCGTCGAGGGAACCGGAGATCCAGAA-3′ (HindIII site in italic text) and 5′-GATATGGGAGATTTGAT-3′ (BstNI site in italic text). The amplified cDNA fragment was cloned into the pYL322-GFP vector after the GFP coding region (Ma et al., 2018), producing the GFP-OsABCG36 construct.

To construct a plasma membrane-localized fluorescence marker protein, mCherry-OsRac3 (Chen et al., 2010), we amplified the full-length cDNA sequence of OsRac3 from the Nipponbare cDNA. The primer sequences 5′-gcatggacgagctgtacaagATGGCGTCCGG-3′ and 5′-GGATCCGGAGGATTTGAT-3′ were used for amplification. The amplified cDNA fragment was cloned into the pYL322-GFP vector after the GFP coding region. The resulting fragment was then cloned in frame after the mCherry coding region into the p35S-mCherry-NosT vector by the Ω-PCR strategy (Chen et al., 2013). The plasmid DNA GFP-OsABCG36 and GFP together with a plasma membrane–localized marker, mCherry-OsRac3, or a tonoplast-localized marker, mCherry-AtTPK (Zeng et al., 2018), was introduced into rice protoplasts by polyethylene glycol–mediated transformation as previously described by Chen et al. (2011). After transformation, cells were incubated in the dark at room temperature for 12–15 h. Fluorescence images were captured using a confocal laser scanning microscope (TCS SP8; Leica).

Cell and tissue specificity of OsABCG36 expression

To investigate the cell and tissue specificity of OsABCG36 expression, we introduced the transformation vector carrying ProOsABCG36-GFP.
fission into rice (cv. Nipponbare). The promoter (2.026 kb) of *OsABCG36* was amplified by PCR from Nipponbare genomic DNA using the primers 5′-AAGCTTCATATGGGATCCATGATTGTT3′ (HindIII site in italics) and 5′-GGATCCCTAGCTCCTGGATCCATGATTGTT3′ (SalI site in italic text). Using HindIII and Sal, the amplified fragment was cloned into the pCAMBIA1300-GFP vector, carrying the GFP gene and the terminator of the nopaline synthase gene, producing the *ProOsABCG36-GFP* construct. The resulting construct was transformed into *A. tumefaciens* strain EHA101, which was introduced into the wild-type rice (cv. Nipponbare) by *A. tumefaciens*-mediated transformation.

The roots of the wild-type rice and the transgenic line carrying *ProABCG36-GFP* were sampled for immunostaining. After immunostaining using an antibody to GFP as described by Yamaji and Ma (2007), the GFP signal was observed by confocal laser scanning microscopy (TCS SP8; Leica).

**Yeast assay**

The entire open reading frame of *OsABCG36* was amplified from the wild-type rice cDNA by PCR using the primers 5′-AAGCTTCATATGGGATCCATGATTGTT3′ (HindIII site in italics) and 5′-GGATCCCTAGCTCCTGGATCCATGATTGTT3′ (SalI site in italic text). Using HindIII and Sal, the amplified fragment was cloned into the pYES2 vector (Invitrogen), producing the pYES2-*OsABCG36* construct.

The yeast strain used in this study was Δαψf1 (MATααα; Δαψf; Δhis3; Δαλn2; Δαλn3; Δαλψf::TRY1). For evaluation of Cd tolerance, pYES2-*OsABCG36* and empty vector pYES2 were transformed into the Δαψf1 mutant strain. Transformants carrying the plasmid were spotted on to solid SD-uracil medium (2% galactose) with 0, 5, 10, or 15 μM CdSO₄. The plates were photographed after incubation at 30 °C for 3 days. For evaluation of Cd efflux in yeast liquid culture, transformants were selected on uracil-deficient medium and grown in liquid SD-uracil medium (2% galactose). Cells at mid-exponential phase were harvested and transferred to SD-uracil medium (2% galactose) at pH 4.5. After cells had been cultured for 4 h, the OD_{600} of each strain was adjusted to 4.0, CdSO₄ was then added to the medium to a final concentration of 20 μM. After 4 h of incubation with shaking, cells were collected and washed once with deionized water. Cells (OD_{600}/4.0) were suspended in fresh SD-uracil medium (2% galactose) without Cd and grown at 30 °C for another 4 h. The cells were harvested every 2 h and digested with 2 M HCl. The concentration of Cd in the digest solution was measured as described below. All experiments were independently conducted at least three times.

For evaluation of Zn tolerance, the growth of yeast strain zrt1cot1 (MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; zrt1::natMX; cot1::kanMX4) at different Zn concentrations was tested on a synthetic solid medium (2% galactose, 0.67% yeast nitrogen base without metal, 2% agar at pH 6.0 with 50 mM MES). All experiments were independently conducted at least three times.

To examine the subcellular localization of OsABCG36 in yeast, the GFP-*OsABCG36* fragment from the pYL322-GFP-*OsABCG36* construct described above was cloned into the pYES2 vector, producing the pYES2-GFP-*OsABCG36* construct. The resulting construct was introduced into the Δαψf1 mutant strain according to the manufacturer’s protocols (S.c easy comp transformation kit; Invitrogen). The plasma membrane of the yeast cells was stained by CellMask™ Deep Red plasma membrane stain (Thermo Fisher Scientific) (Wang et al., 2017). The fluorescence signal was observed by confocal laser scanning microscopy (TCS SP8; Leica).

**Role of OsABCG36 in rice Cd tolerance**

The full-length open reading frame of *OsABCG36* (LOC_Os01g42380.1) was cloned based on information available in a public database (http://rice.plantbiology.msu.edu/). The cloned sequence was identical to that in the database. *OsABCG36* consists of 21 exons and 20 introns, encoding a protein of 1457 amino acids (see Supplementary Fig. S1A, B at JXB online). It belongs to the G subfamily of rice ABC transporters. Phylogenetic analysis identified homologs of OsABCG36 in Arabidopsis and rice, which are divided into half-size and full-size ABC transporters (Supplementary Fig. S1C). OsABCG36 belongs to the full-size group and shares 57% identity with AtABCG36 and OsABCG43, which are known to confer Cd tolerance in Arabidopsis and yeast, respectively (Kim et al., 2007; Oda et al., 2011).
Expression pattern of OsABCG36

The expression of OsABCG36 was detected in both the roots and shoots at a low level (Fig. 1A; Supplementary Fig. S2A). The accumulation of OsABCG36 mRNA was significantly enhanced by Cd in the root, but not in the shoot (Fig. 1A; Supplementary Fig. S2A). Furthermore, expression in roots was induced by Cd in a dose-dependent manner (Fig. 1B). The expression level was similar in the root tip (0–1 cm) and the mature zone (1–2 cm) in both the presence and absence of Cd (Fig. 1C). A time-course analysis of Cd-treated roots showed that the expression of OsABCG36 was up-regulated rapidly by Cd and reached its maximum at 3 h (Fig. 1D). These results suggested that OsABCG36 is regulated by Cd.

We also tested the response of other five OsABCG36 homologs (OsABCG32, OsABCG34, OsABCG35, OsABCG37, and OsABCG44) to Cd in rice. Among these, only OsABCG37 and OsABCG44 were expressed in roots, and their expression was not up-regulated by Cd (Supplementary Fig. S2B–D).

Tissue and cell specificity of OsABCG36 expression

To examine the tissue specificity of OsABCG36 expression in rice root, we generated transgenic rice lines expressing the
OsABCG36 pro::GFP construct. Immunostaining with an antibody to GFP showed that the signal was detected in the root cells (Fig. 2B). Furthermore, the signal in all root cells except the epidermal cells was significantly enhanced by exposure to Cd (Fig. 2C). No signal was detected in the wild-type root (Fig. 2A), indicating that the anti-GFP antibody is specific.

**Subcellular localization of OsABCG36**

Based on WoLF PSORT (https://www.genscript.com/tools/wolf-psort) analysis, OsABCG36 was predicted to be localized on the plasma membrane. To assess the subcellular localization of OsABCG36 in plant cells, we co-expressed GFP empty vector or the GFP-OsABCG36 fusion with the plasma membrane-localized marker mCherry-OsRac3 (Chen et al., 2010) and the tonoplast-localized marker mCherry-AtTPK (Zeng et al., 2018) in rice protoplasts. In contrast to GFP alone, which was widely distributed in the cytoplasm and nucleus (Fig. 3), the GFP-OsABCG36 signal was mainly co-localized with the fluorescence of mCherry-OsRac3 (Fig. 3), while it was separated from the fluorescence of mCherry-AtTPK (Supplementary Fig. S3), confirming that OsABCG36 is a plasma-membrane-localized protein.

**Transport activity for Cd in yeast**

To examine whether OsABCG36 has transport activity for Cd, we expressed it in the Cd-sensitive yeast mutant Δypl1. On SD-Gal medium without Cd, yeast cells carrying the vector control or OsABCG36 showed similar growth (Fig. 4A). However, on SD-Gal medium containing Cd, the yeast cells expressing OsABCG36 exhibited better growth than those expressing the vector control (Fig. 4A). Furthermore, we pretreated yeast cells carrying OsABCG36 or the vector control with Cd for 4 h, then transferred them to liquid medium without Cd. During the Cd pretreatment period, the OsABCG36-expressing yeast cells showed similar Cd content to those carrying the empty vector; however, the OsABCG36-expressing cells showed a significant decrease of Cd content compared with the vector control during the Cd release period (Fig. 4B). The fluorescent signal of GFP-OsABCG36 was partially colocalized with the signal of the plasma membrane marker (Supplementary Fig. S4), suggesting that at least part of OsABCG36 was localized to the plasma membrane in yeast cells. These results indicated that OsABCG36 functions as an efflux transporter for Cd in yeast.

In addition, we expressed OsABCG36 in a Zn-hypersensitive yeast mutant, Δzrt1ot1. The growth of the yeast cells expressing OsABCG36 was similar to that of cells carrying the empty vector control under treatment with Zn (Supplementary Fig. S5), suggesting that OsABCG36 likely has no transport activity for Zn in yeast.

**Cd tolerance of OsABCG36 knockout lines**

To examine the role of OsABCG36 in Cd tolerance in rice, we generated two independent knockout transgenic lines of OsABCG36 using CRISPR/Cas9 technology and compared their Cd tolerance. These mutant lines carried a deletion or insertion, which led to a frame shift and the premature termination of OsABCG36, respectively (Fig. 5A, B). In the absence of Cd, the root and shoot growth of the two OsABCG36 knockout lines was similar to that of the wild-type rice (Fig. 5C; Supplementary Fig. S6). However, the root growth of the knockout lines was more inhibited than that of the wild-type rice in the presence of 2 µM Cd (Fig. 5D, E). We also evaluated the tolerance of the knockout lines to other metals, including Cu, Zn, Al, and Pb. There were no differences in tolerance to these metals between the knockout lines and the wild-type rice (Fig. 5F; Supplementary Fig. S7). These results suggested...
that the sensitivity of the OsABCG36 knockout lines to Cd is specific.

We further compared the Cd concentration of cell sap in the root tips (0–1 cm) and mature regions (1–2 cm) in the knockout lines and wild-type plants. The OsABCG36 knockout lines showed higher Cd concentration in the cell sap in both root segments than wild-type plants (Fig. 5G). Furthermore, we conducted a Cd uptake experiment with the whole roots of the knockout lines and wild-type plants. The Cd content in the knockout lines was higher than that in the wild-type plants (Fig. 5H). These results indicated that OsABCG36 is able to transport Cd out of the cell for Cd detoxification.

**Role of OsABCG36 in Cd accumulation in rice**

To test whether OsABCG36 is also involved in Cd accumulation, we grew two knockout lines and wild-type plants under hydroponic conditions with three different Cd concentrations for 2 weeks. In the absence of Cd or in the presence of a low Cd concentration (0.1 or 1 μM), the growth of the wild-type and knockout lines was similar (Fig. 6A, C, E; Supplementary Fig. S8). There were no differences between wild-type and knockout lines in the concentration of Cd in the roots and shoots (Fig. 6D, F). However, at a higher Cd concentration (5 μM), the root length of the knockout lines was shorter than that of the wild-type rice (Fig. 6B). The dry weight of the roots was also lower than that of the wild-type (Fig. 6C). Mineral analysis showed that the concentration of Cd in roots was significantly higher in the knockout lines than in the wild-type (Fig. 6D), but the Cd concentration in shoots was similar in the knockout lines and wild-type plants (Fig. 6F). The concentrations of Zn, Cu, Fe, and Mn in the roots and shoots were also similar in all lines (Supplementary Fig. S9). Furthermore, the Cd concentration in the xylem sap was similar in all lines under long-term Cd treatment (Supplementary Fig. S10). These data indicated that the increased root Cd concentration of the knockout lines in treatments with high Cd may be attributed to failure to transport Cd out of the cell, resulting in greater Cd accumulation in the cytosol, and that OsABCG36 does not function in Cd accumulation in the aerial parts of rice.

**Discussion**

ABC transporters constitute a large superfamily of proteins in bacteria, animals, and plants (Guidotti, 1996). ABC proteins are able to transport various substrates, such as hormones, lipids, glutathione conjugates, inorganic acids, xenomolecules, and heavy metals (Rea, 2007; Verrier et al., 2008). There are more than 120 ABC members in both Arabidopsis and rice, which are classified into seven subfamilies (ABCA–ABCG) (Garcia et al., 2004). Several ABC transporters, including AtABCC1, AtABCC2, AtABCC3, and AtABCG36, have been reported to play a role in Cd tolerance by Cd extrusion or vacuolar sequestration (Kim et al., 2007; Park et al., 2012; Brunetti et al., 2015). However, the exact functions of most ABC transporters in plants are still unknown. In the present study, we functionally characterized an ABCG-type transporter gene, OsABCG36, which encodes a plasma-membrane-localized protein (Fig. 3). OsABCG36 is expressed in both roots and shoots, but only expression in the roots was rapidly and greatly up-regulated in response to Cd (Fig. 1A, D). Knockout of OsABCG36 resulted in decreased tolerance to Cd, but did not alter tolerance to other metals, including Zn, Cu, Al, and Pb (Fig. 5F; Supplementary Fig. S7). Furthermore, loss of function of OsABCG36 resulted in greater Cd accumulation in the roots and root cell sap (Fig. 5G). Although the cell sap data alone could not be used for a quantitative analysis of symplastic versus apoplastic Cd, because weakly bound Cd or Cd conjugates released from the cytosol and vacuoles may be able to make contact with the cell walls and partially bind there after the root cells are lysed by the freeze–thaw procedure, the difference was also found in the total Cd concentration of the roots (Fig. 5H). Given
Fig. 5. Sensitivity analysis of OsABCG36 knockout lines to toxic metals. (A) The two target sites of OsABCG36 used in the CRISPR/Cas9 targeting system. Black boxes represent exons and the lines between boxes represent introns. The triangles above the second and fourth exons represent the target sites in the CRISPR/Cas9 system. (B) OsABCG36 sequence of two independent CRISPR-mutated lines. The dashed boxes indicate the mutation sites. Underlines indicate the PAM sequences. WT, wild type. (C, D) Cd sensitivity of the WT and two OsABCG36 knockout lines. Germinated seeds were exposed to 0.5 mM CaCl₂ solution (pH 4.5) containing 0 or 2 μM Cd for 5 days. Scale bar=5 cm. (E) Effect of Cd on root and shoot length. (F) Effect of toxic metals on root elongation. Seedlings (5 days old) were exposed to 0.5 mM CaCl₂ solution (pH 4.5) alone or containing 30 μM Cd, 30 μM Al, 100 μM Zn, or 0.5 μM Cu for 24 h. Relative root elongation refers to (root elongation with metals)/(root elongation without metals) ×100. Data are means ±SD (n = 5). (G) Cd concentration in the root cell sap in different root segments. Roots of the WT and two OsABCG36 mutant lines were exposed to 0.5 mM CaCl₂ (pH 4.5) containing 20 μM Cd for 8 h; root tips (0–1 cm) and basal roots (1–2 cm) were excised and Cd concentration in the root cell sap was determined by ICP-MS. (H) Root Cd concentration of WT and OsABCG36 mutant lines. Seedlings (25 days old) were exposed to a nutrient solution containing 5 μM CdSO₄ for 12 h. Asterisks indicate significant differences between the WT and OsABCG36 knockout lines (*P<0.05, **P<0.01; Tukey’s test). (This figure is available in colour at JXB online.)
that OsABCG36 showed an efflux transport activity for Cd in yeast (Fig. 4B), the higher Cd accumulation in the root cell sap of the mutants is the result of loss of Cd efflux from the root cells. This may be associated with enhanced Cd toxicity in the mutants (Fig. 5D, F, 6B). The transport substrate of OsABCG36 remains to be identified in the future, but it could be ionic Cd or Cd conjugates.

Similar to most full-size G-type ABC transporters, OsABCG36 consists of two nucleotide-binding domains and two ABC2 transmembrane domains (Supplementary Fig. S1B). OsABCG36 is not the closest homolog of AtABCG36 (Supplementary Fig. S1C), but OsABCG36 functions similarly to AtABCG36 (Kim et al., 2007). Both of these proteins are efflux transporters of Cd or Cd conjugates that contribute to Cd tolerance in plants. However, OsABCG36 showed some differences from AtABCG36 in terms of expression pattern and cellular localization. For example, in the absence of Cd, the expression level of OsABCG36 was low in the root and shoot (Fig. 1A), whereas that of AtABCG36 was high (Kim et al., 2007). In the presence of Cd, the expression of OsABCG36 is rapidly and greatly induced only in the roots (Fig. 1A). Furthermore, the expression level increased with increasing external Cd concentrations (Fig. 1B), whereas that of AtABCG36 was slightly enhanced by Cd in both the roots and shoots of Arabidopsis (Kim et al., 2007). Under normal conditions, OsABCG36 was expressed in the root cells at a low level, whereas AtABCG36 was strongly expressed in the root hair and epidermis. These differences suggest that some functions of OsABCG36 and AtABCG36 diverged during evolution. Additionally, AtABCG36 has also been suggested to play a role in Pb tolerance (Kim et al., 2007). However, we found that knockout of OsABCG36 did not affect tolerance to other
metals, including Pb (Fig. 5F; Supplementary Fig. S7). Similar differences between rice and Arabidopsis have also been reported for other ABC transporters. For example, AtABCC1/2 is involved in both Cd and As tolerance in Arabidopsis (Song et al., 2010; Park et al., 2012), whereas OsABCC1 is involved only in As tolerance and not Cd tolerance (Song et al., 2014).

Detoxification of toxic metals can be achieved by a variety of mechanisms, including compartmentalization (vacuolar sequestration), chelation by metal ligands such as phytochelatins and metallothioneins, and efflux of toxic metals from the cells (Hall, 2002). The present work provides evidence that OsABCG36 is involved in transporting Cd out of the root cells (i.e., efflux). However, the effect of knockout of OsABCG36 on Cd tolerance was observed only at relatively high Cd concentrations (Fig. 5D–F, 6B). Furthermore, root elongation in the mutants was not completely inhibited in the presence of Cd (Fig. 5D–F, 6B). These results suggest that OsABCG36-mediated efflux of Cd is one of the components of Cd tolerance in rice and that its contribution is not very large.

Although knockout of OsABCG36 decreased tolerance to Cd (Fig. 5D–F, 6B), it did not affect Cd accumulation in the shoots (Fig. 6F), suggesting that OsABCG36 is not involved in Cd accumulation. In rice, several types of transporters have been shown to be involved in the uptake and distribution of Cd (Clemens and Ma, 2016; Wang et al., 2019). OsNramp5, a Nramp (natural resistance-associated macrophage protein) member, was identified as a major pathway for Cd uptake in rice (Ishimaru et al., 2012; Sasaki et al., 2012). Two P-type ATPase transporters, OsHMA3 and OsHMA2, have been reported to be responsible for the vacuolar sequestration of Cd in the root cell and root-to-shoot translocation of Cd, respectively (Ueno et al., 2010; Miyadate et al., 2011; Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013; Shao et al., 2018). At the reproductive stage, OsLCT1, a low-affinity cation transporter1, was proposed to transport Cd into rice grains (Uraguchi et al., 2011, 2014). OsHMA2 expressed in the rice nodes is also involved in Cd distribution to the grain (Yamaji et al., 2013). Among these transporters, only OsHMA3 was demonstrated to play an important role in Cd tolerance, through vacuolar sequestration of Cd in the roots (Sasaki et al., 2014). Therefore, there are at least three pathways for Cd in the root cells taken up by OsNramp5; vacuolar sequestration mediated by OsHMA3, efflux from the root cells by OsABCG36, and root-to-shoot translocation by OsHMA2. The lack of difference in shoot Cd accumulation between the mutants and wild-type rice in this study suggests that the contribution of OsABCG36 to shoot Cd accumulation is very small. One possible explanation for this is the presence of redundant genes with similar functions to OsABCG36 in rice. Phylogenetic analysis showed that OsABCG36 was closely related to OsABCG32, OsABCG34, OsABCG35, OsABCG37, and OsABCG44, and shared high identity (76–85%) with them at the amino acid level (Supplementary Fig. S1C). Among these, OsABCG37 and OsABCG44 are expressed in the roots. Recently, a comparative RNA-seq-based analysis showed that OsABCG37 and OsABCG44 were up-regulated in roots by high-Cd stress (Tan et al., 2017). These data suggested that OsABCG37 and OsABCG44 possibly play a similar function to OsABCG36 in roots although the exact roles of these genes in Cd tolerance and accumulation remain to be examined in future.

In conclusion, our results indicate that OsABCG36 localized at the plasma membrane is involved in Cd tolerance in rice through efflux of Cd out of the root cells.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Analysis of OsABCG36.

Fig. S2. Expression analysis of OsABCG36 and its homolog genes.

Fig. S3. Co-localization of OsABCG36 and AtTPK.

Fig. S4. Subcellular localization of OsABCG36 in yeast cells.

Fig. S5. The growth of zrt1tol1 transformed with empty vector pYES2 or OsABCG36.

Fig. S6. Root length of wild-type and two OsABCG36 knockout lines without Cd.

Fig. S7. Pb sensitivity of the wild-type and two OsABCG36 knockout lines.

Fig. S8. Growth of OsABCG36 knockout lines.

Fig. S9. Concentration of essential metals in the roots and shoots.

Fig. S10. Cd concentration in the xylem sap.

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Author contributions

JFM and JX designed the experiments; SF performed most of the experiments; YL, XZ, GY, DC, ZW, MS, JC, DYC, and RL participated in the research; SF and JX analyzed the data; JX wrote the paper; JFM and JX revised the paper.

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