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Overexpression of *OsABCG48* Lowers Cadmium in Rice (*Oryza sativa* L.)

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Abstract: Cadmium pollution threatens food safety and security by causing health issues and reducing farmland availability. Engineering genetic changes in crop plants to lower Cd accumulation can be a cost-effective approach to address this problem. Previously, we reported that a rice line, 2B, which expresses a truncated version of *OsO3L2* had reduced Cd accumulation throughout the plant, including in seed. However, downstream events caused by expression of this gene were not known. In this study, RNA-seq was used to identify differentially expressed genes between the wild type and 2B rice with or without Cd treatment, leading to the study of an ABC transporter gene, *OsABCG48* (ATP-Binding Cassette transporter G family member 48). Heterologous expression of *OsABCG48* conferred tolerance to Cd in *Schizosaccharomyces pombe*, *Arabidopsis* and rice. Moreover, overexpressing *OsABCG48* in rice lowered root Cd accumulation that was associated with more extensive lateral root development. These data suggest that *OsABCG48* might have applications for engineering low-Cd rice.

Keywords: ABC transporter; Cd stress; RNA-seq; expression profiling; rice (*Oryza sativa*)

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1. Introduction

Cadmium (Cd) is one of the most concerning environmental pollutants in the world. As a nonessential metal, it replaces essential metals to cause ion homeostasis disruption, protein dysfunction, DNA and plasma membrane damage and reactive oxygen species accumulation [1–5]. Cd can lead to brittle bones and has a long biological half-life (~10–30 years) in the kidney, such that even low-dose intake from chronic exposure can cause kidney failure [6]. A major route of Cd intake is through the food chain; although farm animal bones and internal organs are not a major part of the human diet, especially in developed countries, plant-derived Cd is a major source [7,8]. Cd from contaminated soil can be transferred to humans via plant uptake, and Cd soil pollution has been a concern for food security and public health. Cd dietary intake is mainly from leafy vegetables and especially cereals, since the latter are consumed at greater quantities. Cd contamination in rice and durum wheat has been reported on many occasions, and in the case of rice, it has been a publicized problem in many parts of East and South East Asia, especially since rice provides the major calorie intake [9–14].

Although the remediation of soil could solve the problem at its source, neither soil cleaning nor replacement has been an affordable option. Soil replacement over the course of 30 years in Japan's Toyama Prefecture, the site where Cd pollution caused the "Itai-itai disease" in the 1940s [14], costed JPY 40.7 billion for 863 hectares, or JPY 47 million (~USD 438,000) per hectare [15]. Compared with soil remediation, a more cost-effective means to address the issue of Cd dietary intake is to develop crop plants that can reduce Cd

accumulation in edible parts. Toward this goal, the engineering of lower Cd accumulation in rice has been reported by several groups.

Several genes that have been engineered to lower Cd accumulation encode ion transporters. A manganese (Mn) transporter, *OsNramp5*, has been shown to affect Cd accumulation in root, straw and grain [16]. However, unless the soil has sufficient Mn, reducing the production of *OsNramp5* also causes Mn deficiency. With certain down-regulated alleles of *OsNramp5*, Cd uptake in rice decreased without affecting grain yield, although the grain Mn nutrient was reduced to a third of its normal level [17]. The P1B-type ATPase transporter *OsHMA3* was found to affect Cd translocation from roots to above ground tissues [18]. Overexpression of *OsHMA3* reduced Cd in dehusked (brown) rice through sequestration of Cd into root vacuoles. The *OsLCT1* (Low-affinity Cation Transporter 1) transporter moves Cd from the xylem through phloem to grain, and plants with lower production of *OsLCT1* showed reduced grain Cd accumulation of ~50% but had increased grain Fe by ~33% [19].

There are also non-transporter genes involved in Cd accumulation in rice. Plants that fail to express *LCD* (Low CdMium) showed about a 55% reduction in grain Cd when grown in soil with low Cd contamination [20]. More recently, this laboratory reported engineering the production of full-length or truncated versions of *OsO3L2* and *OsO3L3* that lowered grain Cd [21,22]. These proteins are members of the OXS3 (OXidative Stress 3) family, where in *Arabidopsis thaliana*, OXS3 is needed for tolerance to metal and oxidative stress [23] and plays a role in regulating stress-induced flowering time [24].

In this study, we describe an RNA-seq analysis on the rice line 2B, which overproduces a truncated form of *OsO3L2* that has been shown previously to reduce Cd accumulation in rice [21]. After comparing line 2B to the wild type (WT) without or with Cd, we focused our study on *OsABCG48*, a gene involved in Cd tolerance and accumulation in rice that belongs to the ABC transporter G family.

2. Materials and Methods

2.1. Plant Culture and Treatment

Seeds from rice (*Oryza sativa japonica* L.) cultivar Zhonghua11 and its derivative line 2B were surface sterilized with 5% sodium hypochlorite, washed with distilled water 3 times and germinated for 2 days in the dark at 30 °C. After germination, seedlings were transferred to polyethylene containers with 1/2 Kimura B nutrient solution (pH 5.6). The Kimura B nutrition solution contained (NH₄)₂SO₄, MgSO₄·7H₂O, KNO₃, Ca(NO₃)₂·4H₂O and KH₂PO₄ and micronutrients MnCl₂·4H₂O, H₃BO₃, (NH₄)₆Mo₇O₂₄·4H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O and Fe-EDTA (All reagents purchased from Sigma-Aldrich, St. Luis, MO, USA). After incubation for 2 days in the dark at 30 °C, and 12 days under long-day conditions (16 h light/8 h dark) at 28 °C, the 14-day-old seedlings were transferred to new nutrient solution for 12 h without or with 75 µM Cd. Afterwards, whole seedlings were washed 3 times to remove surface Cd and frozen in liquid nitrogen for RNA extraction. Each rice line with or without Cd treatment was sampled in duplicate, giving rise to 8 samples for RNA-seq analysis.

For Cd tolerance assay in rice, 2-day-old seedlings of the wild type and three *OsABCG48* overexpression lines were cultured in 1/2 Kimura B nutrient solution (pH 5.6) containing 0 or 2 µM Cd for 5 days. The root and shoot lengths of each seedling were measured and photographed with 3 biological replicates. For the Cd uptake experiments, rice seedlings were cultured with nutrient solution for 28 days and then treated with 2 µM Cd for 12 h, refreshed with new solution every week. Roots and shoots of seedlings were sampled and dried at 80 °C in an oven to constant dry weight, followed by digestion with HNO₃ in the Microwave Sample Preparation System (Multiwave3000, Anot Paar, Austria). The Cd concentration was determined by ICP-MS (Agilent 7700X, Agilent Technologies, Santa Clara, CA, USA).

2.2. RNA-seq Library Construction, Sequencing and Data Analysis

WT and line 2B seedlings were grown on 1/2 Kimura B nutrient solution (pH 5.6) for 14 days and then treated with 0 or 75 μM CdCl_2 for 12 h. Whole seedlings were collected and sent for sequencing by Illumina HiSeqTM PE150 (Illumina, San Diego, CA, USA) and an index of the reference genome (MSU Rice Genome Annotation Project Release 7.0) was built using Bowtie (v2.2.3), and paired-end clean reads were aligned to the reference genome using TopHat (v2.0.12) by Novogene (Beijing, China), where total RNA isolation, library construction, sequencing, assessment of RNA-seq quality, screening of DEGs (Differentially Expressed Genes) and their expression analysis (heat map) were conducted. Sequencing was set to obtain 6–10 G clean bases for each sample. FPKM (fragments per kilobase of transcript sequence per million base pairs sequenced) was used to calculate the expression level for transcripts [25]. Identification of DEGs was conducted by the DESeq R package (v1.18.0) in the R (v3.5.1) environment with adjusted p -values < 0.05 , whereas probability p_{adj} values were adjusted using Benjamin and Hochberg's approach for controlling the false discovery rate [26]. Heat maps were created with the pheatmap R package (v1.0.10), using the $\lg(\text{FPKM} + 1)$ values for clustering. RNA-seq data were deposited in the NCBI Sequence Read Archive database under the accession numbers SAMN16930351 to SAMN16930358. Portions of the RNAs used for RNA-seq were kept for subsequent qRT-PCR (quantitative Reverse Transcription Polymerase Chain Reaction) verification.

2.3. qRT-PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for qRT-PCR analysis. cDNA synthesis was carried out with total RNA using a PrimeScriptTM RT reagent kit with a gDNA Eraser (Takara, China). SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, China) was used for qRT-PCR on a LightCycler[®] 480 II/96 qPCR System (Roche, Rotkreuz, Switzerland) following a reaction protocol of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s. *OsActin-1* and *AtUBQ5* were used as reference genes for rice and *Arabidopsis*, respectively. Primers listed in Table S1 were designed using Sigma-Aldrich qRT-PCR primer tools (St. Luis, MO, USA). The 14-day-old rice and 21-day-old *Arabidopsis* plants were grown as described in Sections 2.1 and 2.5, respectively.

2.4. Cd Tolerance Assay in *S. Pombe*

Full-length cDNAs of selected DEGs were from qRT-PCR and cloned into pART1 by in-Fusion HD cloning (Clontech, Shiga, Japan). After verification by DNA sequencing, expression constructs were transformed into *S. pombe* strain JS23 (h^+ , *ura4.294*, *leu1.32*) by the LiAc method [27,28]. For assessing Cd tolerance, *S. pombe* cells in 10-fold serial dilutions were spotted onto EMM (Edinburgh Minimal Medium) + uracil media agar plates containing different concentrations of CdCl_2 (Sigma) and incubated at 30 °C for 5 to 7 days.

2.5. Expression of *OsABCG48* in Plants

Coding sequences of *OsABCG48* and *OsHIP43* were inserted separately into pSUPER1300 by in-Fusion HD cloning (Clontech) and introduced into *Agrobacterium tumefaciens* GV3101 for transformation into *Arabidopsis* ecotype Columbia-0 (Col-0) by the floral dip method [29]. Putative T1 transformants were screened in 1/2 MS plates containing 50 mg mL^{-1} of hygromycin. T1 plants were transplanted on soil for harvesting T2 seeds for PCR confirmation of the *OsABCG48* transformation. T3 individuals from T2 self-fertilization were used to deduce T2 homologous lines based on Mendelian segregation. The T-DNA insertion mutant of *AtABCG34* (SALK_082843) was obtained from the Arabidopsis Biological Resource Center. The *Arabidopsis* root growth assay and transgene expression analysis were performed on vertically placed plates containing 1/2 strength MS with or without CdCl_2 (0, 25, 50, 75 and 100 μM), MnSO_4 (0, 25, 50, 75, 100 and 125 μM) or CuSO_4 (0, 12.5, 25, 37.5, 50 μM) for 21 days.

For rice transformation, the *Agrobacterium*-mediated method was used [30], and regenerated plants were selected on 1/2 MS medium containing 100 mg L^{-1} of hygromycin. For

the *OsABCG48* overexpression analysis, seedlings were grown in 1/2 Kimura B nutrient solution (pH 5.6) containing 0 or 2 μ M Cd for 5 days, as for the Cd tolerance assay.

3. Results

3.1. RNA-Seq Analysis and Identification of Differentially Expressed Genes

Previously, we described that the rice line 2B that expresses the truncated form of *OsO3L2* was able to reduce the overall Cd content in rice [21]. As *OsO3L2* is an OXS3 (OXidative Stress 3) protein family member that resides in the nucleus and co-localizes with histone H2A [21,23], we speculated that *OsO3L2* might be a regulator of downstream genes. To determine the genes that were associated with Cd tolerance and low Cd accumulation in the 2B genotype, we conducted a genome-wide analysis of transcripts using high-throughput RNA-seq. Eight libraries were constructed, with two replicates of the following four sample types: (1) wild type without Cd (WT – Cd), (2) wild type with Cd (WT + Cd), (3) line 2B without Cd (2B – Cd) and (4) line 2B with Cd (2B + Cd).

RNA-seq revealed a total number of 1420 DEGs using $P_{adj} < 0.05$ as a cutoff on 42,211,728–61,852,692 clean reads. As depicted in the heat map in Figure 1A, the DEGs were grouped into six clusters (cluster I to VI) based on the similarity of their gene expression profiles. The comparison between the WT and 2B columns shows that regardless of Cd treatment, 2B plants showed down-regulated DEGs in clusters I and III, but upregulated DEGs in clusters V and VI. Since these four clusters comprise ~65% of the total DEGs, the 2B genotype itself contributed a major change in gene expression. As for changes caused by Cd, both WT and 2B plants showed Cd-upregulated genes (~45% of the total DEGs) in clusters II and V, and Cd-down-regulated genes in clusters III and IV (23% of the total DEGs), but the DEG changes in clusters III and V (~33% of the total DEGs) were the combined effects of both the 2B genotype and Cd treatment.

The comparisons among the four treatment groups are shown in the Venn diagram in Figure 1B. Cd caused 5.4-fold more DEGs (729, blue) (Table S2) in WT than in 2B plants (135, green) (Table S3). Comparing between 2B and WT, only 275 DEGs (purple) were found in the absence of Cd (Table S4), but 616 DEGs (yellow) were found when treated with Cd (Table S5). Of particular interest was the 561 DEGs unique to the WT – Cd vs. WT + Cd comparison as well as the 50 DEGs unique to the 2B – Cd vs. 2B + Cd comparison. The 10-fold higher DEGs in WT plants (561 vs. 50) could possibly indicate that more DEGs were needed to mitigate the damage caused by the higher accumulation of Cd, as though 2B plants might have already been primed to better tolerate Cd stress. Based on this hypothesis, those genes primed to better tolerate Cd stress in 2B plants would be among the 275 DEGs between WT and 2B in the absence of Cd (Figure 1C). Since 150 of those genes were common between WT + Cd and 2B + Cd, this leaves 125 DEGs unique to the 2B genotype prior to Cd treatment. Cd treatment in 2B plants caused 135 DEGs (Figure 1D), but 73 of them were common in the Cd treatment of WT; therefore, only 62 were unique to the 2B genotype. These comparisons suggest that 187 DEGs (125 prior to Cd stress; 62 after Cd stress) could be the focus in finding the genes responsible for the 2B low-Cd phenotype.

3.2. Verification of DEGs Possibly Related to Ion Transportation

Since numerous studies have identified transporter genes to be involved in Cd accumulation, including *OsNramp5*, *OsHMA3*, *OsLCT1*, *OsLCD*, *CAL1* and *OsCd1* [16–20,31,32], we were particularly entertained by the possibility that the low Cd accumulation in 2B plants might be due to DEGs in metal ion transport. Based on this consideration, eight DEGs potentially involved in substrate transport were selected, including *WAT1* (Walls Are Thin 1)-related genes OS10G0199500 and OS04G0422300, *ABCG* (ABC transporter G family) genes OS11G0587600 (*OsABCG48*) and OS07G0522500 (*OsABCG43*), probable aquaporin genes OS01G0975900 (*OsTIP1*) and OS09G0541000 (*OsPIP2-7*), a *HIPP* (Heavy metal-associated Isoprenylated Plant Protein) gene OS04G0661100 (*OsHIPP43*) and an *nsLTP* (non-specific Lipid-Transfer Protein) gene OS03G0793900 (*OsnsLTP2*) (Figure 2A). According to Gene Ontology (<http://www.geneontology.org>), *WAT1* and *ABCG* genes

encode drug/metabolite transporters. The aquaporin genes also have probable transporter activities, *OsHIPP43* encodes a heavy metal-associated domain that might function in metal ion transport and *OsnsLTP2* has been speculated to have ion transmembrane transporter activity. To verify the RNA-seq results (Figure 2A), qRT-PCR was conducted on these eight genes (Figure 2B–I). However, the qRT-PCR data could not confirm that expression of OS10G0199500 (*WAT1*-related gene) was higher in 2B than in WT without Cd treatment (Figure 2A,B), and they failed to detect differential expression of OS07G0522500 (*OsABCG43*) between WT and 2B under Cd stress (Figure 2A,E). For OS04G0422300, a statistically significant difference was found only between 2B – Cd and WT + Cd (Figure 2C). Aside from these three, the expressions of the other five genes were confirmed by qRT-PCR and were advanced for further analysis.

Figure 1. Expression profiling on rice altered by 2B expression and/or Cd treatment. (A) Heat map shows a one-dimensional hierarchical cluster analysis of 1420 DEGs (differentially expressed genes). Clusters I to VI produced according to the lg (FPKM + 1) values, % of total shown. (B) Venn diagram shows four comparison groups. (C) Comparison of WT + Cd vs. 2B + Cd and WT – Cd vs. 2B – Cd. (D) Comparison of WT – Cd vs. WT + Cd and 2B – Cd vs. 2B + Cd. Number of genes in each partition with up or down arrows indicates up- or down-regulation, respectively.

Figure 2. RNA-seq and qRT-PCR comparisons of candidate transporter genes. (A) DEGs with transporter-associated activity identified by RNA-seq. Green to red color scale indicates down- to upregulation. (B–I) qRT-PCR of gene expression normalized to *OsActin-1*. Data (bar and error bar) are shown as mean $\pm$ SD ($n = 3$). Unpaired Student's *t*-test used for statistical significance (* $p < 0.05$, ** $p < 0.01$) of samples indicated on top of connecting lines, or by comparison to WT – Cd indicated on top of error bar.

3.3. Heterologous Expression of *OsABCG48* Conferred Tolerance to Cd in *S. pombe* and *Arabidopsis*

Since the production of the OsO3L2 protein and its truncated 2B peptide is known to enhance oxidative stress tolerance in *S. pombe* [21], we cloned these five genes into vector pART1 and transformed it into fission yeast. Enhanced Cd tolerance was observed only for OS04G0661100 (*OsHIPP43*) and OS11G0587600 (*OsABCG48*) (Figure 3A). We next tested whether *OsHIPP43* and *OsABCG48* could enhance Cd tolerance in *Arabidopsis*. Each gene

was expressed from the strong mannopine synthase-derived promoter *Super_{pro}*, and after *Agrobacterium*-mediated transformation, six *OsHIPP43*- and nine *OsABCG48*-expressing lines were obtained. Two randomly chosen lines of each were tested on a range of CdCl_2 concentrations (0 μM , 25 μM , 50 μM , 75 μM and 100 μM). Although T3 homozygous seedlings of two *OsHIPP43*-transformed lines showed *OsHIPP43* expression (Figure S1A), they did not show enhanced tolerance to Cd. For the two *OsABCG48* expression lines, *OsABCG48*-a and *OsABCG48*-b, they grew about 22% to 23% longer roots than the WT control in 50 μM of Cd, but statistically significant differences were not observed with lower or higher Cd concentrations (Figure 3B,C). We also tested a knockout line (SALK_082843) of *AtABCG34*, the most closely related *OsABCG48* ortholog in *Arabidopsis* (Figure S2). The two *OsABCG48* expression lines grew 44% to 45% longer roots than the *Atabcg34* knockout line (Figure 3B,C). qRT-PCR verified that this mutant lacked *AtABCG34* expression, while detectable *OsABCG48* transcription was found in the *OsABCG48* expression lines (Figure S1B–D). Since the manganese transporter Nramp5 in rice can mediate Cd transport [16], and HIPPs can lower the kinetic barrier for Cu transfer [33,34], we also tested Mn and Cu tolerance. However, no significant difference was found between the WT and the *OsABCG48*-expressing line (Figure S3). This indicates that *OsABCG48* specifically confers tolerance to Cd and not to Cu or Mn.

Figure 3. Expression of *OsABCG48* confers tolerance to Cd in *S. pombe* and *Arabidopsis*. (A) Dilution (10-fold) bioassay of *S. pombe* strains on EMM + uracil agar plates without or with CdCl_2 (0.5 mM); EV is empty vector control. (B) Cd tolerance assay of *OsABCG48* expression lines of *Arabidopsis*. (C) Root length recorded from 21-day-old seedlings germinated and grown in medium without or with 50 μM CdCl_2 . Figure 3B is from a representative experiment among three with similar results. *Atabcg34* T-DNA knockout line (SALK_082843) used as negative control. Data (bar and error bar) are shown as mean $\pm$ SD ($n = 13$ to 19); statistical analysis by unpaired Student's *t*-test compared to WT (** $p < 0.01$); scale bar = 1 cm.

3.4. Overexpressing *OsABCG48* Enhances Cd Tolerance and Lowers Cd Accumulation in Rice

To explore a possible relationship between the tissue-specific expression and function of *OsABCG48*, paddy-grown rice was sampled after two weeks of flowering. The qRT-PCR assay showed that *OsABCG48* was expressed in the nodes, stems and seeds and

more so in the roots and leaves (Figure 4A). Along with the data from RiceXPro (<http://ricexpro.dna.affrc.go.jp> accessed on 30 September 2020) that *OsABCG48* showed the highest expression in the roots (Figure S4), it seems most probable that *OsABCG48* has the highest activity in the roots.

Figure 4. Expression of *OsABCG48* in rice normalized to *OsActin-1*. (A) Field-grown WT rice two weeks after flowering. (B) Seven-day-old T2 seedlings from *OsABCG48*-transformed lines C6.2, E8.3 and A6.3. Data (bar and error bar) are shown as mean $\pm$ SD ($n = 3$); statistical analysis by unpaired Student's *t*-test compared to WT (* $p < 0.05$, ** $p < 0.01$).

Using the same *Arabidopsis* expression constructs, we conducted *Agrobacterium*-mediated transformation on eight embryogenic rice calluses (Table S6). Multiple T0 plants were regenerated from each callus. Of the T0 plants derived from the six calluses that produced seeds, 7 out of 16 T0 plants (A5, C4, C8, F2, F7, G1 and G6) were likely to be single-locus based on resistance to sensitive segregation data from germination on hygromycin-containing plates. In the sampling of 88 out of 359 hygromycin-resistant clones, 74% were PCR-positive for the *Superpro-OsABCG48* junction (Table S6). These seven lines were preserved to produce homozygous T3 and T4 seeds for testing of grain Cd. However, for the other T0 plants deemed to harbor multiple *OsABCG48* loci, we could afford to use them to test the effect from overexpression of *OsABCG48*. T2 seedlings from three multi-loci lines (A6.3, C6.2 and E8.3) were found to show ~5-fold higher expression of *OsABCG48* than WT plants (Figure 4B). While these T2 seedlings did not show a difference in primary root and shoot lengths in the absence of Cd, the presence of Cd reduced primary root and shoot lengths by about half in both WT and *OsABCG48* overexpression lines, but the primary root lengths of three overexpressing lines were 12% to 17% longer than WT after a 5-day treatment with 2 μ M CdCl₂ (Figure 5A–D). Aside from the primary root length, differences were also found on lateral roots, especially on those developed from the primary root as each of the three overexpressing lines grew three- to four-fold more and longer lateral roots than the WT when treated with Cd (Figure 5B,E). It is possible that more and longer lateral roots in the *OsABCG48* overexpression plants had compensated for the Cd-repressed growth of primary roots and hence contributed to Cd tolerance.

Most importantly, we want to know whether overexpression of *OsABCG48* could lower Cd accumulation, like the overexpression of *OsO3L2* or its truncated fragment 2B [21,22]. T2 seedlings of lines A6.3, C6.2 and E8.3 were germinated and cultured hydroponically for 28 days before transplanting into the same solution containing 2 μ M Cd for another 12 h. Roots and shoots were then harvested, dried and subjected to ICP-MS analysis. The results showed that the root Cd of these T2 seedlings varied from 46.9 to 70.9 mg/kg, or 39% to 60% less root Cd than the 116 mg/kg in the WT control (Figure 5F). Although a lower Cd content was not observed in shoots, the values were under 0.2 mg/kg, suggesting that the 12-h

treatment might not have been long enough for Cd to reach above ground tissues. Since *OsNramp5*, *OsHMA3*, *OsLCT1* and *OsO3L2* were reported to affect Cd accumulation, we measured their expressions in line C6.2, but a significant difference was not found between WT and the T2 seedlings of C6.2 (Figure S5). This shows that the low Cd accumulation in the *OsABCG48*-overexpressing seedlings was independent of a change in expressions of those four genes.

Figure 5. Expression of *OsABCG48* confers tolerance to Cd and lowers Cd accumulation in T2 rice. Two-day-old T2 seedlings from T1 lines (C6.2, E8.3, A6.3) were grown in 1/2 Kimura B solution (pH 5.6) for 5 days (A) without Cd or (B) with 2 μM Cd, with insert showing 4-fold magnified view of lateral roots from primary roots 2 cm from root tip. (C,D) Root and shoot lengths recorded from (A) and (B), respectively. (E) Number and length of lateral roots derived from primary roots shown as the insert in (B). (F) Cd accumulation in 28-day-old T2 seedlings from T1 lines (C6.2, E8.3 and A6.3) treated with 2 μM Cd for 12 h. Data (bar and error bar) are shown as mean ± SD ($n = 3$ for root length and Cd concentration, and 15 for lateral roots length); statistical analysis by unpaired Student's *t*-test compared to WT (* $p < 0.05$, ** $p < 0.01$); scale bar = 5 cm.

4. Discussion

The RNA-seq analysis revealed 1420 DEGs caused by genotype and treatment, but due to limited resources to investigate the numerous candidates, we focused on those likely involved in metal ion transport. The testing of five candidate genes in the fission yeast narrowed the search to two genes, *OsABCG48* and *OsHIPP43*, which were able to enhance Cd tolerance in *S. pombe*. For *OsABCG48*, this might not be surprising since it is an ortholog of *OsABCG43* (Figure S2) that had previously been reported to enhance Cd tolerance in the budding yeast [35]. According to the transmembrane analysis by TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/> accessed on 30 September 2020), *OsABCG48* is similar to most full-size G-type ABC transporters, consisting of two conserved transmembrane domains (Figure S2). As with other ABC transporters, it is likely to be involved in substrate translocation across the lipid bilayer [36]. There are also several other ABC transporters that have been reported to be associated with Cd transport, such as *AtABCC1*, *AtABCC2*, *AtABCC3*, *AtPDR8* and *OsABCG36* [37–40].

Since *OsABCG48* enhanced Cd tolerance in *Arabidopsis* and rice as well as reduced root Cd accumulation in rice seedlings, *OsABCG48* overexpression appears to mimic the same phenotype observed with seedlings overexpressing *OsO3L2* [22]. This suggests that *OsO3L2* might be an upstream regulator of *OsABCG48*, and this possibility is strengthened by the fact that *OsO3L2* overexpression upregulated *OsABCG48* expression (Figure 2D), but not vice versa (Figure S5). According to phylogenetic analysis, *OsABCG40* is also a closely related member in the same clade as *OsABCG43* and *OsABCG48* (Figure S2). However, the RNA-seq data did not identify *OsABCG40* as a DEG, and thus we excluded it from this investigation. As for *OsABCG43* that was identified in the RNA-seq assay, not only were we unable to confirm differential expression by qRT-PCR but we were also unable to amplify its full-length cDNA from rice seedlings, as all our PCR products lacked ~500 bp of the 5' end of its coding sequence. Hence, despite its homology with *OsABCG48*, we did not investigate its ability to confer Cd tolerance in heterologous hosts.

Given that *OsABCG48* might function independently of previously identified transporters that can recognize Cd as a substrate (*OsNramp5*, *OsHMA3* and *OsLCT1*) (Figure S5), *OsABCG48* could be in a different Cd transport pathway. However, future studies are needed to determine whether *OsABCG48* is indeed capable of transporting Cd, and if so, whether low Cd accumulation is due to reduced Cd into, or higher Cd out of, plant roots. Moreover, even if we could ascertain that *OsABCG48* is an important determinant, it does not rule out the possibility of other DEGs contributing to the *OsO3L2*-mediated low-Cd phenotype, or miRNAs not identified by our RNA-seq but which may also regulate Cd-responsive genes including the ABCG family member *AtPDR8* [41].

5. Conclusions

RNA-seq led to discovering that *OsABCG48* can enhance Cd tolerance in *S. pombe*, *Arabidopsis* and rice. Moreover, *OsABCG48* overexpression in rice lowered root Cd accumulation that may be due to promoting lateral root development. From the view of biotechnology, this study adds a potential new tool to modify rice for reduced dietary intake of a nonessential but toxic metal. However, since the *OsABCG48* overexpression lines tested thus far have multiple loci, practical engineering for a stable trait awaits further experiments on T3 and T4 homozygous seeds from single-locus (and single-copy) lines. It would also be interesting to speculate that since overexpression of *OsABCG48* could reduce Cd accumulation, the down-regulation of *OsABCG48* might be expected to increase Cd accumulation. If so, that could be useful for phytoremediation, like mutant cases of the *CAX1* gene from *Brassica rapa* and *OsABCG36* [40,42].

Supplementary Materials: The following are available online at <http://www.mdpi.com/xxx/s1>. Figure S1: Verification of *OsHIPP43-a*, *OsHIPP43-b*, *OsABCG48-a*, *OsABCG48-b* and SALK_082843 in *Arabidopsis*. Figure S2. Phylogenetic analysis of G subfamily of ABC proteins from *Arabidopsis* and rice and transmembrane analysis of *OsABCG48*. Figure S3: *OsABCG48* expression lines tested for Mn

or Cu tolerance in *Arabidopsis*. Figure S4: Relative expression of *OsABCG48* in rice organs/tissues. Figure S5: Expression of *OsNRAMP5*, *OsHMA3*, *OsLCT1* and *OsO3L2* in *OsABCG48* overexpression line C6.2. Table S1: Primers sequences. Tables S2–S5: DEGs from WT – Cd vs. WT + Cd, 2B – Cd vs. 2B + Cd, 2B – Cd vs. WT – Cd and 2B + Cd vs. WT + Cd, respectively. Table S6: Transformation of *OsABCG48* in rice.

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