Plant Biotechnology 27, 39–46 (2010)

Original Paper

Isolation of multidrug resistance associated protein like gene from lead hyperaccumulator common buckwheat and its lead detoxification ability

Takafumi Mizuno[1,*], Masahiro Nakagawa[1,a], Hiroshi Ono[1,b], Daisuke Sugiura[1,c], Hideo Tamura[2], Hitoshi Obata[1]

[1] Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan; [2] Biotechnology Group, Energy Application R&D Center, Chubu Electric Power Co., Nagoya, Aichi 459-8522, Japan
* E-mail: tmizuno@bio.mie-u.ac.jp Tel: +81-59-231-9607 FAX: +81-59-231-9684

Received July 2, 2009; accepted October 13, 2009 (Edited by K. Yazaki)

Abstract A multidrug resistance associated protein homolog gene, named *FeABCC1*, was isolated from a commercial brand of common buckwheat that has high lead tolerance and accumulation ability. The deduced amino acid sequence of FeABCC1 had 64% identity with AtMRP3 (AtABCC3) of *Arabidopsis thaliana*, and was predicted as integral membrane protein with 12 transmembrane domains. FeABCC1 expression was induced in the shoot of common buckwheat treated with lead. The yeast transformed with *FeABCC1* showed a marked increasing in lead tolerance with the gene expression, and accumulated more lead compared to control. Not similar to AtMRP3, FeABCC1 expression did not effect to cadmium tolerance in yeast, and inhibition of glutathione synthesis by buthionine sulfoximine was not effect lead tolerance. These data suggests that FeABCC1 has some role for lead detoxyfication in common buckwheat independently to glutathione chelating.

Key words: Common buckwheat, FeABCC1, glutathione, hyperaccumulator, lead, multidrug resistance associated protein.

Contamination of soil by lead often occurs in areas such as clay pigeon shooting sites and industrial factories. In Japan, more areas are polluted by lead than by any other metal. Phytoremediation of soil contaminated with lead and other toxic metals is a topic of investigation, and a few trials have been carried out using large biomass plants and chelator chemicals (Kumar et al. 1995; Chaney et al. 2007). On the other hand, lead hyperaccumulating plants, which can accumulate lead to a concentration greater than 1000 mg kg^{-1} dry weight in their shoots, have been sought to achieve effective decontamination of soil. Several lead hyperaccumulators have been found, such as *Sesbania drummondii* (Sahi et al. 2002) and *Athyrium yokoscense* (Kamachi et al. 2005), and their capacities for the depuration of polluted soil have been tested.

Common buckwheat (*Fagopyrum esculentum* Moench) has also been used for phytoremediation of lead contaminated soil (Tamura et al. 2005). Plants of this species can accumulate lead up to 8000 mg kg^{-1} dry weight in their shoots without concomitant treatment with chelator chemicals. With other favorable characteristics such as its superior growth rate in soil poor in nutrients, this plant is one of the most suitable plants for the practical phytoremediation of lead-contaminated soil (Honda et al. 2007).

In contrast to the progress in the practical use of these plants for phytoremediation, the mechanisms of lead transport remain largely unelucidated. Recently, several experiments have shown that increased lead tolerance and accumulation in *Arabidopsis thaliana* are associated with the expression of certain genes. AtPDR12 (AtABCG40) was identified as the plasma membrane transporter that exports Pb(II) out of the membrane (Lee et al. 2005), and AtHMA3 was reported as a transporter allowing Cd/Zn/Co/Pb vacuolar storage (Morel et al. 2009). Furthermore, overexpression of the yeast multidrug resistance associated protein (MRP) homolog ycf1 in *Arabidopsis thaliana* resulted in a 1.5- to 2-fold lead accumulation (Song et al. 2003). It is speculated that homologs of these genes also play a role in plants where high levels of metal accumulation occur.

Abbreviations: MRP, multidrug resistance associated protein; BSO, buthionine sulfoximine

[a] Present address: AJINOMOTO GENERAL FOODS, INC., Shinjuku, Tokyo 169-0073, Japan

[b] Present address: Tokai Pickling Co., Ltd, Toyohashi, Aichi 440-8530, Japan

[c] Present address: Koshii Woods Industry, Osaka 559-0026, Japan

This article can be found at http://www.jspcmb.jp/

The MRPs, which nomenclaturally identified as ABCC (Verrier et al. 2008), constitute a subfamily of the ATP-binding cassette (ABC) transporter family, which preferentially transports highly water-soluble and negatively charged substrates, particularly glutathione-conjugated materials. In plant molecular biology, the ABCC is one of the most thoroughly analyzed of the ABC transporters. Among the 15 homologs of the ABCC transporter genes found in the *A. thaliana* genome (Kolukisaoglu et al. 2002), AtMRP3 can transport glutathione-conjugated cadmium to the vacuoles (Tommasini et al. 1998) and it is transcriptionally upregulated by Cd(II) stimulation (Bovet et al. 2003). Except for these reports, there have been very few studies on the relationship between metal tolerance/storage in plants and MRP homologs. Furthermore, until date, we do not know the role of MRPs in lead detoxification in the lead hyperaccumulating plants.

We chose the lead hyperaccumulating common buckwheat as a model to investigate the significance of ABCC proteins in lead tolerance/accumulation in plants. The aim of the present study was to clarify whether an *ABCC*-like gene(s) exists in common buckwheat, and to investigate its transcriptional regulation by Pb(II) stimulation as well as its ability to detoxify Pb(II) in yeast cells. Furthermore, the participation of glutathione conjugation in metal transport by FeABCC1 was investigated.

Materials and methods

Plant material and growth condition

Seeds of common buckwheat (*F. esculentum* Moench; commercial name, Milky Way) were purchased from Sakata Seed Corporation (Kanagawa, Japan). They were germinated on sterilized barmicurait in a growth chamber at 23°C with a scheduled (16 h day/8 h night) artificial light period.

Cloning of FeABCC1 cDNA

Leaves of 2-week-old common buckwheat plants (about 1 g) were frozen in liquid nitrogen and pulverized. Total RNA was isolated with Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Ten micrograms of total RNA was used to make the cDNA template, using M-MLV reverse transcriptase (Takara, Shiga, Japan) and oligo d(T)-anchor primer, 5′-GACCACGCGTATCGATGTCG-AC$(T)_{16}$V (V: A, C, or G).

Two PCR primers designed for gene fragment amplification of the MRP homolog were constructed, based on the conserved DNA sequence of putative ABCC clones from *A. thaliana* AtMRP3 (AtMRP3, DDBJ/EMBL, GenBank Accession No. AAC49791; AtMRP1, AAB67319), *Saccharomyces cerevisiae* (YCF1, P39109), *Rattus norvegicus* (RtCMOAT, AAC42087), and *Homo sapiens* (human ABCC1, P33527)

The sequences of the primers constructed were as follows: MRP5′-1 (sense primer), 5′-GGAATTGTTGGRAGGACAGG-3′ (R: A or G) and MRP3′-1 (antisense primer), 5′-AGCAGTKGCTTCATCAAGMAC-3′ (K: G or T; M: G or T). DNA fragments amplified by PCR were subcloned with TA cloning vector pCR2.1-Topo (Invitrogen, Carlsbad, CA), and amplified by transformation into *Escherichia coli*. The gene was sequenced with an automated DNA sequencer (Model 377A; Applied Biosystems Japan, Tokyo, Japan). The whole sequence of the cloned MRP homolog (*FeABCC1*) was determined by 5′ and 3′ rapid amplification of cDNA ends (RACE) using a 5′/3′ RACE Kit (Roche Diagnostics GmbH, Mannheim, Germany), including RT-PCR using an oligo d(T)-anchor primer and poly(A) addition to the 3′-end of the cDNA with terminal deoxyribonucleotide transferase, and was confirmed by sequencing three independent clones of whole *FeABCC1* cDNA. The *FeABCC1* sequence determined in this study was submitted to the SAKURA nucleotide sequence data submission system at the DNA Data Bank of Japan (DDBJ) through the WWW server (http://www.ddbj.nig.ac.jp/Welcome-e.html), and was deposited in the DDBJ database under the Accession number AB306326. Homologous sequences were searched within the DDBJ/EMBL/GenBank database using BLAST (Altschul et al. 1990), and DNA/protein sequences were aligned using the CLUSTAL W multiple sequence alignment program (Thompson et al. 1994). TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used for predicting transmembrane helices.The SMART (Simple Modular Architecture Research Tool) program (http://smart.embl-heidelberg.de/) was used to identify and annotate the genetically mobile domains and analyze their architecture (Schultz et al. 1998, Letunic et al. 2006).

Expression analysis of FeABCC1 in Pb(II)-stimulated common buckwheat

Common buckwheat seeds were germinated and grown for 7 days in pots containing vermiculite at 22°C in total darkness. Plants were then transferred to a hydroponic medium (MGRM) (Fujiwara et al. 1992) and cultured for 7 days in a glasshouse at an average temperature of 20–25°C. Lead stimulation was performed for 24 h by supplementation with 1 mmol L^{-1} $Pb(NO_3)_2$, leaves were then collected and used for RT-PCR analysis.

RNA isolation was performed as described above. Total RNA (5 μg) isolated from lead-stimulated and non-stimulated plants was used for reverse transcription using an oligo d(T)-anchor primer. The transcription level of FeABCC1 was compared between the lead-stimulated and non-stimulated conditions with partial FeABCC1 amplification (354 bp) using primer sets MRP5′ (5′-TAAAACAACCTTGATACAGGC-3′) and MRP3′ (5′-ATCTTCGTCTTCTTGAGGAGT-3′), and Takara Ex TaqT^{TM} (Takara, Otsu, Japan). Fragment amplifications by PCR were performed in a 25-μl reaction mixture with a thermal cycle set (one step of 5 min at 94°C; 28 cycles of 1 min at 94°C, 60°C, and 72°C, each; and one step of 5 min at 72°C)

The gene coding for actin was used as an internal control. Sequences of primers used for partial actin cDNA (762 bp) were as follows: actin 5′, 5′-ATTGGGATGACATGGAGAA-GATTTG-3′ and actin 3′, 5′-CACTTCATTATGGAGTTATA-GGTGG-3′. Amplified cDNAs were subjected to agarose gel electrophoresis and detected by ethidium bromide staining. The amounts of amplified cDNAs were quantified using the NIH

Image program developed at the US National Institutes of Health (http://rsb.info.nih.gov/nih-image/) following picture scanning of the gels and image inversion using the IvanView program (http://www.ivanview.com/).

Construction FeABCC1 expression yeast

The cadmium-sensitive *Saccharomyces cerevisiae* BY4741 mutant Δycf1 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YDR135c::kanMX4*), lacking the MRP-like vacuolar ABC transporter yeast cadmium factor 1, which is sensitive to lead (Song et al. 2003) was obtained from EUROSCARF (Frankfurt, Germany). YNB-Ura$^-$ medium lacking specific nutrients was used for the selection and maintenance of the yeast cells transformed with plasmids (Mizuno et al. 2005).

Full-length *FeABCC1*, with appropriate restriction enzyme sites attached at the 5′/3′ termini, was amplified by PCR and was ligated into pKT10-Gal-HA-BS. Yeast cells were transformed with the constructed plasmid pKT10-*FeABCC1*, which can produce FeABCC1 with the GAL4 promoter, by the lithium acetate method (Kaiser et al. 1994). The transformants were then selected on 13 g l^{-1} agar plates containing yeast nitrogen base medium (no amino acids contained, pH 6.0) supplemented with histidine, leucine, methionine (YNB-Ura$^-$ medium), and 20 g l^{-1} glucose. The recombinant yeast carrying FeABCC1 was detected by PCR using primer sets of 5′-CTTGGTACCATGGAACCC-3′ (forward) and 5′-TCCG-TCGACTTAGAGATG-3′ (reverse).

Pb(II) tolerance and accumulation in yeast

Δycf1 cells transformed with empty or constructed vectors were precultured with YNB-Ura$^-$ at 30°C, 150 rpm, until OD_{600}=0.6, and the tolerance/accumulation experiments were performed as explained below.

In plate culture experiments, cells were diluted to OD_{600}=0.1 and 0.01 in distilled ultrapure water and spotted (5 μl) on agar plates of YNB-Ura$^-$ medium containing 0–50 μmol l^{-1} $Pb(NO_3)_2$, supplied with 20 g l^{-1} galactose or glucose. Then, the plates were incubated at 30°C for 72 h. In liquid culture experiments, precultured cells were diluted to OD_{600}=0.001 with YNB-Ura$^-$ medium containing 20 g l^{-1} galactose and 10 μmol l^{-1} $Pb(NO_3)_2$ and cultured at 30°C with shaking at 150 rpm. Cell growth was determined from the OD_{600} values. In the Pb(II) accumulation experiments, liquid culture was performed as described above for 4 days. Collected cells were washed twice with ice-cold 1.5 μmol l^{-1} sodium tartrate (Hall and Williams 2003), followed by drying at 80°C for 12 h. After decomposition of the dried sample in a mixture of nitric acid and perchloric acid, lead content was quantitatively analyzed using an atomic absorption spectrometer (Type AA6500S; Shimadzu, Kyoto, Japan). All experiments were replicated at least three times, and Student's *t*-test was performed to evaluate the significance of the difference between control yeast cells and cells expressing *FeABCC1*.

To elucidate the participation of glutathione conjugation in lead detoxification, cell growth was also studied in the presence of 200 μmol l^{-1} buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-Cys synthase (Griffith 1982).

Cd(II) tolerance and accumulation in yeast

The Cd(II) tolerance and accumulation ability of FeABCC1-expressing Δycf1 cells was investigated in the same way as described for the Pb(II) experiments, using YNB-Ura$^-$ medium plates containing 0–60 μmol l^{-1} $CdCl_2$ (for plate culture) and 10 μmol l^{-1} $CdCl_2$ (for liquid culture). All experiments were replicated at least four times.

Results

Sequence characteristics of FeABCC1

We successfully amplified several cDNA fragments by PCR using a cDNA template of common buckwheat and degenerate primers for MRP homologs, and found a sequence fragment of MRP homolog among them. Using 5′ and 3′ RACE analysis, we successfully cloned and determined the sequences of one cDNA (4065 bp) containing an open reading frame of 3843 bp. The deduced amino acid sequence of this gene, designed FeABCC1, comprised 1281 amino acids and showed the highest residue identity (64%) to *A. thaliana* AtMRP3. The amino acid length of the N-terminal region of FeABCC1 was 230 residues shorter than that of AtMRP3, but almost the same as that of the maize MRP homologue ZmMRP2(ZmABCC2) (Figure 1A). There was 33% amino acid identity between FeABCC1 and YCF1, which have been reported to confer Pb(II) resistance (Song et al. 2003) and 30–64% amino acid identity with other MRP homologs belonging to the ABCC family of the ABC superfamily (Figure 1B).

Using the TMHMM program for hydrophobic domain prediction, we detected two highly hydrophobic transmembrane domains (TMD)—TMD1 and TMD2—each containing five to seven putative transmembrane helices (Figure 1C). In addition, two AAA (ATPases associated with a variety of cellular activities) domains, which were predicted to be nucleotide-binding domains (NBD), were suggested by the SMART program. These domains were found between TMD1 and TMD2 (amino acid positions 444–617) and the C-terminal region (1059–1244). The order TMD-NBD-TMD-NBD is a typical topology for the MRP subfamily of ABC transporters (Martinoia et al. 2002).

Expression of FeABCC1 is elevated by Pb(II) treatment

A primer set that amplified a part of *FeABCC1* (bp positions 3216–3569 from start codon) was constructed and tested by RT-PCR. The PCR product was detected as an apparent single band on agarose gel electrophoresis, and no other sequence without *FeABCC1* was detected by further sequencing the amplified cDNA. Without Pb(II) treatment, the *FeABCC1* transcript was expressed in the shoots but was not detected in the roots. With Pb(II) stimulation, however, the transcription level of *FeABCC1* in the shoots was upregulated about 1.7-fold, and was slightly detected in the roots (Figure 2).

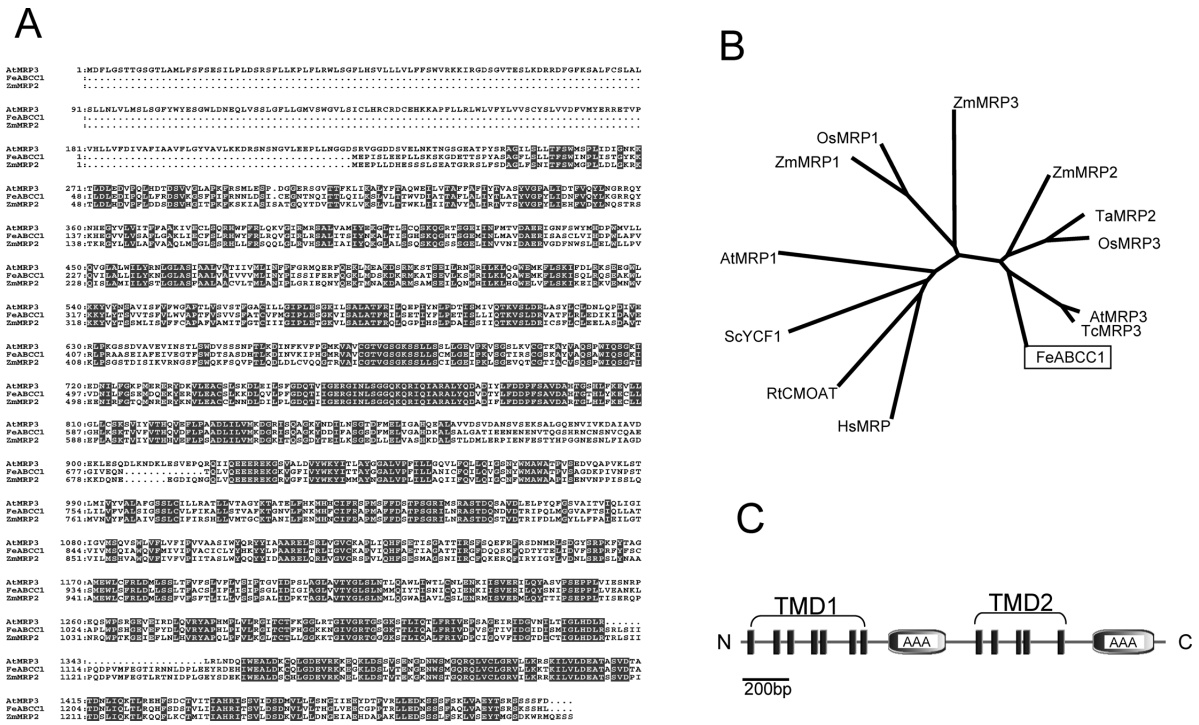

Figure 1. Characteristics of the FeABCC1 sequence. (A) Multiple alignments of plant MRP transporters. Conserved peptides in each ABCC from *Arabidopsis thaliana* (AtMRP3), FeABCC1, and *Zea mays* (ZmMRP2) are indicated by white letters on a colored background. (B) Unrooted dendrogram of ABCC proteins from plants (*Fagopyrum esculentum*, *A. thaliana*, *Z. mays*, *Oryza sativa*, *Triticum aestivum*, and *Thlaspi caerulescens*), human (*Homo sapiens*), rat (*Rattus norvegicus*), and yeast (*Saccharomyces cerevisiae*). The GenBank/DDBJ/EMBL Accession numbers of the MRPs shown in A and B are as follows: FeABCC1, BAF63397; AtMRP1(AtABCC1), NP174329; AtMRP3(AtABCC3), NP187915; ZmMRP1(ZmABCC1), AAO72316; ZmMRP2(ZmABCC2), AAO72318; ZmMRP3(ZmABCC3), AAT37905; OsMRP1(OsABCC1), CAD59603; OsMRP3(OsABCC3), BAB62557; HsMRP, P33527; RtCMOAT, NP036965; ScYCF1, P39109. (C) Scheme of the sequence characteristics of FeABCC1. N and C indicate the amino- and carboxyl-terminus, respectively. TMD and AAA indicate the highly hydrophobic transmembrane domains and the domains of ATPases associated with a variety of cellular activities, respectively. The positions of 12 putative transmembrane helices are shown as rods.

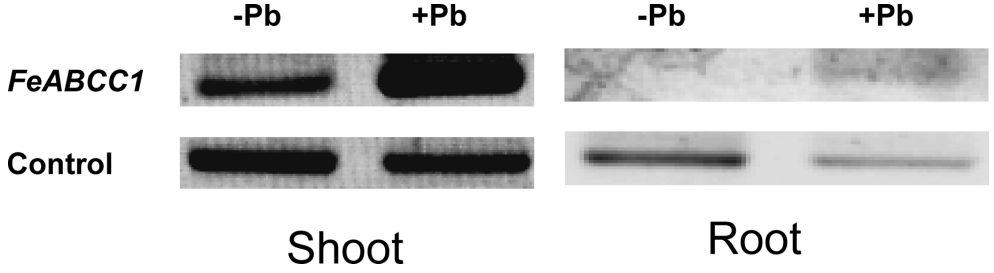

Figure 2. Pb(II)-induced elevation of FeABCC1 expression detected by RT-PCR. Total RNA was extracted from the shoots and roots of 2-week-old common buckwheat plants without (−Pb) and with (+Pb) 10 μM $Pb(NO_3)_2$. Actin was used as the control.

FeABCC1 confers resistance to and allows accumulation of Pb(II) but not Cd(II) in yeast

We examined the viability of recombinant yeast on plates with various concentrations of Pb(II). When *FeABCC1* expression was suppressed by glucose, Δycf1 containing pKT10-FeABCC1 showed growth inhibition the same as that in the control with increasing Pb(II) concentration. In plates containing 20 g l^{-1} galactose, the inducer of *FeABCC1*, growth of control and *FeABCC1*-expressing Δycf1 cells was not inhibited at up to a concentration of 35 μmol l^{-1} Pb(II). Growth of control yeast cells was strongly inhibited from 40 μmol l^{-1} Pb(II), in contrast to FeABCC1-expressing yeast cells, which maintained growth up to 45 μmol l^{-1} Pb(II) (Figure 3A).

Alleviation of growth inhibition by *FeABCC1* expression was also seen in yeast cells during liquid culture (Figure 3B). Faster growth of FeABCC1-expressing yeast compared with the control was observed from 48 h of culture in YNB-Ura$^-$ medium containing 10 μmol l^{-1} Pb(II) and galactose. After 72 h of culture, the OD_{600} value of FeABCC1-expressing yeast reached 0.408, which was about 1.68 times the control value (0.243).

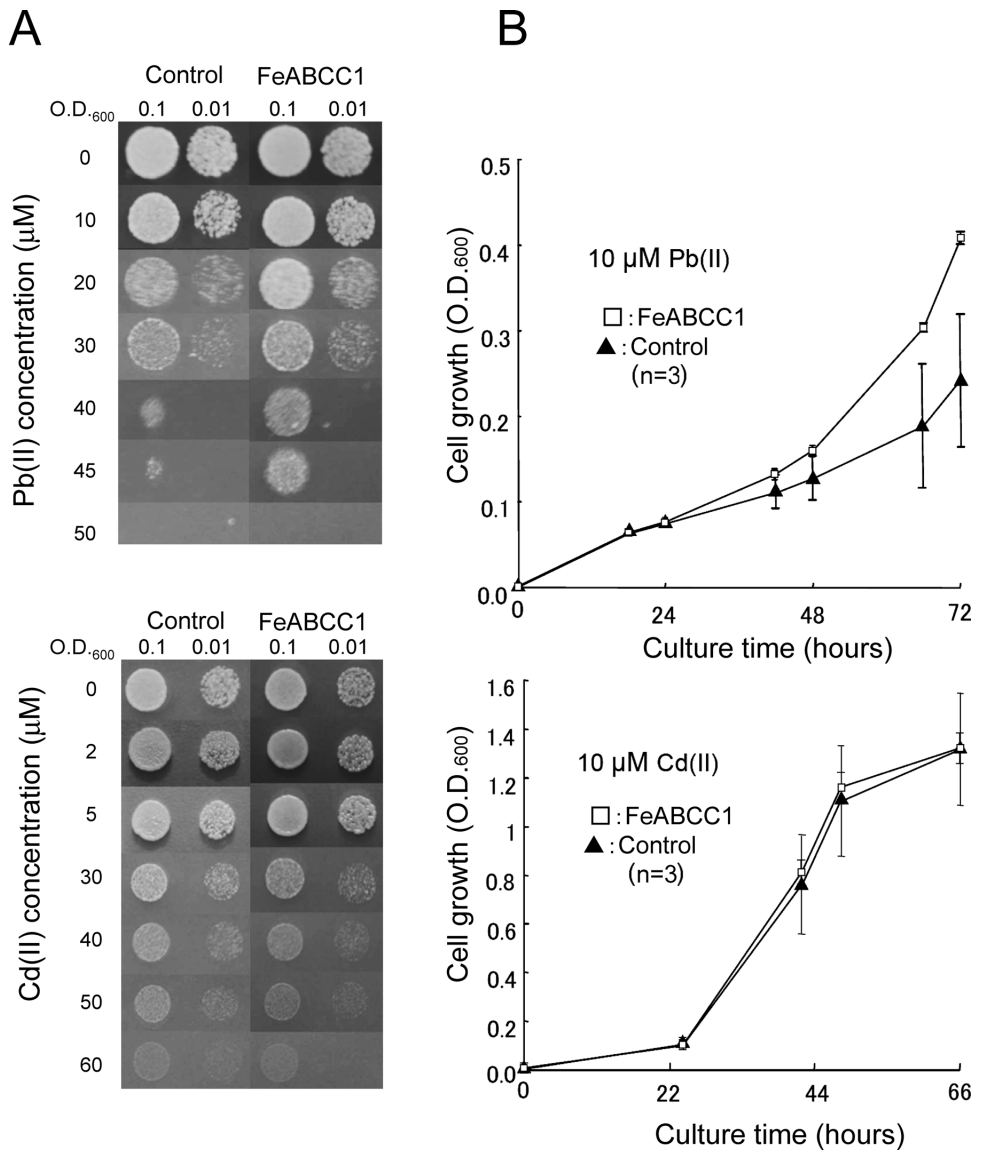

Figure 3. Growth inhibition of *FeABCC1*-expressing yeast cells. (A) Growth on lead- or cadmium-contaminated agar medium or in liquid medium. Precultured control (left) and *FeABCC1*-expressing yeast cells were diluted to optical density (O.D. 600)=0.1 or 0.01, and 5 μl of each sample was spotted on YNB-Ura$^-$ medium plates containing 0–50 μmol l^{-1} $Pb(NO_3)_2$ or 0–60 μM $CdCl_2$, incubated at 30°C for 36 h. Three independent tests were carried out to confirm the results. (B) Growth in liquid medium containing 10 μmol l^{-1} lead or cadmium. Cell growth was determined by the OD_{600} values.

FeABCC1 expression increased the accumulation of lead in yeast (Figure 4). *FeABCC1*-expressing yeast cells accumulated lead to about 1.4 times (65.9 ± 8.19 μg g^{-1} dry weight) the level in control yeast cells (46.3 ± 11.9 μg g^{-1} dry weight).

Contrary to our expectation that FeABCC1, like AtMRP3, would also confer Cd(II) resistance on yeast, *FeABCC1* expression could not rescue the high cadmium sensitivity of Δycf1 mutants, and did not increase cadmium accumulation (Figures 3 and 4). *FeABCC1* expression induced by supplementation of the medium with galactose did not result in suppression of the growth inhibition induced by Cd(II); growth was similar to that on Cd(II)-containing YNB-Ura$^-$ plates (Figure 3A). Furthermore, both control and *FeABCC1*-expressing yeast multiplied at the same rate in liquid medium containing 10 μmol l^{-1} Cd(II) (Figure 3B). In this culture condition, the Cd(II) concentration of FeABCC1-expressing yeast cells reached 83.3 ± 3.41 μg g^{-1} in 4 days, but this accumulation level was not significantly different from that in control yeast cells (86.8 ± 5.43 μg g^{-1}) (Figure 4).

Lead tolerance conferred by FeABCC1 expression is independent of glutathione synthesis

To protect themselves from the toxicity of heavy metals,

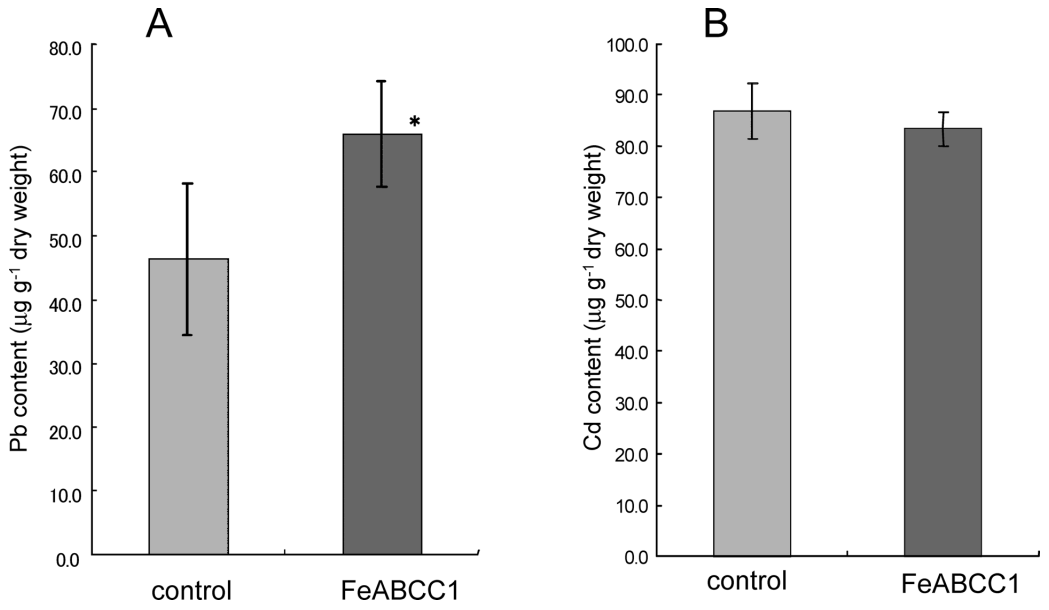

Figure 4. Lead and cadmium accumulation in *FeABCC1*-expressing yeast cells. Cells were cultured for 4 days in medium supplemented with 10 μM $Pb(NO_3)_2$ (A) or $CdCl_2$ (B). Data are expressed as mean±standard error (n=3). * $P<0.05$ *vs*. control.

plants produce several kinds of chelator peptides such as glutathione and phytochelatin. The chelator-conjugated metals are excreted from the cytoplasm through the plasma membrane, or sequestrated to vacuoles through the vacuole membrane, by ABC transporters such as MRP and PDR. AtMRP3 confers cadmium detoxification ability through sequestration of glutathione-conjugated Cd(II) into the vacuoles . This mechanism was confirmed with experiments using BSO, a well-known inhibitor of γ-glutamylcysteine synthetase (Griffith 1982). To verify the participation of glutathione in lead tolerance and accumulation in *FeABCC1*-expressing yeast, the lead tolerance and accumulation test described above was investigated again in experiments wherein the glutathione synthesis was inhibited.

Experiments were performed as described in Figure 3, but with 200 μmol l^{-1} BSO in the medium and without additional supplementation. Compared with the results shown in Figure 3, the growth of both control and *FeABCC1*-expressing yeast cells was suppressed; however, lead tolerance and accumulation was increased because of *FeABCC1* expression. Control and *FeABCC1*-expressing yeast cells showed similar growth up to a concentration of 20 μmol l^{-1} Pb(II), but only the *FeABCC1*-expressing yeast cells survived on the medium containing 40 μmol l^{-1} Pb(II) (Figure 5A). In liquid medium containing BSO and 10 μmol l^{-1} Pb(II), the OD600 value of control and *FeABCC1*-expressing yeasts was suppressed until 48h of culture; however, only the *FeABCC1*-expressing yeast recovered to 0.433 ± 0.170, about three times the control value (0.149 ± 0.021). Lead accumulation in FeABCC1-expressing yeast cells was

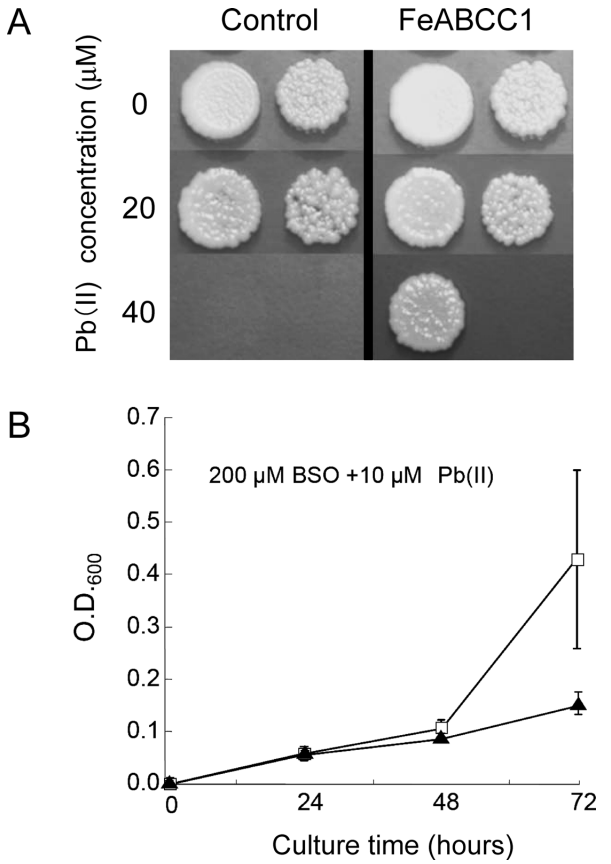

Figure 5. Effect of inhibition of glutathione synthesis on the growth of FeABCC1-expressing yeast cells in medium containing lead. Cell culture using YNB-Ura$^-$ medium in agar plates (A) or liquid medium (B) was performed as shown in Figure 3B except for the addition of 200 μmol l^{-1} buthionine sulfoximine (BSO). Three independent tests were carried out to confirm the results shown in (A), and the cell growth determinations shown in (B) were performed four times. Data are expressed as mean±standard error.

1.3 times greater ($185.8 \pm 46.6\ \mu g\ g^{-1}$ dry weight) than that in control yeast cells ($139.4\ \mu g\ g^{-1}$ dry weight). These results suggest indirectly that the lead tolerance conferred by *FeABCC1* expression is independent of the chelating effect of glutathione.

Discussion

Common buckwheat originated in the high mountainous regions of China and Tibet (Ohnishi and Matsuoka 1996; Konishi et al. 2005) and is well known as a short-season crop that grows well even on poor-quality soil. These characteristics help the common buckwheat plant to acquire nutrients, including various trace elements, and transport them aggressively for rapid seed germination and growth in severe climates and poor quality soil. At the same time, this transport system also bears the risk of absorbing metals present in the soil and accumulating them in its tissues, despite their toxicity toward these plants. As Tamura et al. (2005) reported, the lead content in the shoots of common buckwheat is higher than that in the roots when cultivated in soil heavily contaminated with lead. This is a distinguishing characteristic of this plant, because lead in the soil shows low bioavailability to plants, and usually the transport of lead to the shoots is severely limited. This phenomenon suggests that common buckwheat possesses effective systems for the uptake and transport of metals, including essential and non-essential, in addition to a powerful metal detoxification system(s), and that these systems together result in this plant accumulating lead to extraordinary levels. However, this idea has not been investigated and demonstrated sufficiently, and we have no data on the accumulation of metals other than lead in common buckwheat.

In this study, we have shown that a newly isolated MRP homolog in a lead hyperaccumulating plant provides yeast with strong tolerance of Pb(II). To our knowledge, this is the first report that is associated with a high level of tolerance to Pb(II) but not with resistance to Cd(II). Until date, there have not been any reports on high levels of accumulation of metals other than lead in common buckwheat; nevertheless, the results presented here indicate that this species may have a mechanism of dealing with toxic metals that operates at high levels of mineral nutrient uptake.

Furthermore, our results also suggest the importance of lead transport by FeABCC1 during lead accumulation in the aboveground parts of common buckwheat, where highly active cells need to be protected. For the purpose of determination FeABCC1 localization in yeast cells, we constructed a yeast expression system in which FeABCC1 was fused with green fluorescent protein (GFP) at its N- or C-terminus, and observed them under a fluorescence microscope. However, FeABCC1–GFP was found in both the plasma and vacuole membranes of the yeast cells and we could not determine the exact site within the cells where FeABCC1 acted in the detoxification of lead. Furthermore, we also performed the prediction of subcellular localization of FeABCC1 using on-line computer program WoLF PSORT (Horton et al. 2007), but FeABCC1 was predicted to locate both plasma and vacuole membranes. From the observations that the expression of FeABCC1 was associated with increased lead accumulation in the yeast cells and reports that the *A. thaliana* MRP homolog AtMRP3 is located on the vacuole membrane (Tommasini et al. 1998), FeABCC1 is speculated to locate on the vacuole membrane and is responsible for the transport of lead to the vacuole, but we have not gain a certain evidence on this.

Glutathione is a well-known peptide that has a role in the detoxification of toxic materials in plant cells (Martin 2003). A human MRP homolog, ABCC1, has been investigated for its ability to recognize substrates, and has been reported to transport many kinds of drugs in a glutathione-conjugated form (Deeley and Cole 2006). In *A. thaliana*, cadmium is transported by AtMRP3 in a glutathione-conjugated form (Tommasini et al. 1998). It has also been speculated that lead detoxification in FeABCC1-expressing yeast cells is related to glutathione conjugation, but results have failed to demonstrate this. The lower growth rate on plates containing Pb(II) and BSO (Figure 5) compared with those without BSO (Figure 3) is thought to be attributed to BSO, because glutathione has various roles in the activities of cells, and inhibition of its synthesis directly affects cell growth (Martin 2003). Hence, although at present we do not know how lead is transported, we have demonstrated here that the lead tolerance associated with FeABCC1 is independent of the glutathione conjugation of the metal.

The findings reported in this study make an interesting point that two typical toxic heavy metals—lead and cadmium—do not possess the same detoxification and sequestration mechanism in cells of common buckwheat. As shown in Figure 1, in plants such as *A. thaliana* and *O. sativa*, several kinds of MRP homolog were found in their genome, and we also recognized the existence of other MRP homologs by genomic Southern blot analysis using a partial fragment of *FeABCC1* as a probe (data not shown). FeABCC1 may work in harmony with other homologs for the detoxification, by various means, of harmful metals taken up together with other essential nutrients.

We have reported the participation of MRP homolog FeABCC1 in lead tolerance and accumulation in yeast, and its characteristic difference from AtMRP3. We are now constructing a line of *FeABCC1*-expressing *A. thaliana* plants. In future, we plan to report on the function of FeABCC1 in lead detoxification in these

plants, its intercellular localization, and the significance of lead detoxification.

Acknowledgements

This work was financially supported by Chubu Electric Power Co., and supported in part by a Grant-in-Aid for Scientific Research on Priority Areas "Nutrient uptake and transport in plant identification of molecules responsible for transport and their regulation mechanisms" to H.O. and T.M. (No. 18056009) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman D (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410

Bovet E, Eggmann T, Meyalan-Bettex M, Polier J, Kammer P, Marin E, Feller U, Martinola E (2003) Transcript levels of AtMRPs after cadmium treatment: induction of AtMRP3. *Plant Cell Environ* 26: 371–381

Chaney RL, Angle JS, Broadhurst CL, Peters CA, Tappero RV, Sparks DL (2007) Improved understanding of hyperaccumulation yields Commercial phytoextraction and phytomining Technologies. *J Environ Qual* 36: 1429–1443

Deeley RG, Cole SP (2006) Substrate recognition and transport by multidrug resistance protein 1 (ABCC1). *FEBS Lett* 580: 1103–1111

Fujiwara T, Hirai MY, Chino M, Komeda Y, Naito, S (1992) Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. *Plant Physiol* 99: 263–268

Griffith OW (1982) Mechanism of action, metabolism and toxicity of buthione sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J Biol Chem* 107: 1067–1073

Hall JL, Williams LE (2003) Transition metal transporters in plant. *J Exp Bot* 54: 2601–2613

Honda M, Tamura H, Kimura T, Kinoshita T, Matsufuru H, Sato T (2007) Control of lead polluted leachate in a box-scale phytoremediation test using common buckwheat (*Fagopyrum esculentum* Moench) grown on lead contaminated soil. *Environ Technol* 28: 425–431

Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. *Nucl Acids Res* 35: 585–587

Kaiser C, Michaelis S, Mitchell A (1994) Lithium acetate yeast transformation. In: *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, New York, pp 133–134

Kamachi H, Komori I, Tamura H, Sawa Y, Karahara I, et al. (2005) Lead tolerance and accumulation in the gametophytes of the fern *Athyrium yokoscense*. *J Plant Res* 118: 137–145

Kolukisaoglu HU, Bovet L, Klein M, Eggmann T, Geisler M, Wanke D, Martinoia E, Schulz B (2002) Family business: the multidrug-resistance related protein (MRP) ABC transporter genes in *Arabidopsis thaliana*. *Planta* 216: 107–119

Konishi T, Yasui Y, Ohnishi O (2005) Original birthplace of cultivated common buckwheat inferred from genetic relationships among cultivated populations and natural populations of wild common buckwheat revealed by AFLP analysis. *Genes Genet Syst* 80: 113–119

Kumar PBAN, Dushenkov V, Motto H, Raskin I (1995) Phytoextraction: The use of plants to remove heavy metals from soils. *Environ Sci Technol* 29: 1232–1238

Lee M, Lee K, Lee J, Noh EW, Lee Y (2005) AtPDR12 Contributes to lead resistance in *Arabidopsis*. *Plant Physiol* 138: 827–836

Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P (2006) SMART 5: domains in the context of genomes and networks. *Nucl Acids Res* 34: 257–260

Martin MN (2003) Biosynthesis and metabolism of glutathione in plant. *Genet Eng (NY)* 25: 163–188

Martinoia E, Klein M, Geisler M, Bovet L, Forestier C, Kolukisaoglu U, Muller-Rober B, Schulz B (2002) Multifunctionality of plant ABC transporters—more than just detoxifiers. *Planta* 214: 345–355

Mizuno T, Usui K, Horie K, Nosaka S, Mizuno N, Obata H (2005) Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni^{2+}-transport abilities. *Plant Physiol Biochem* 43: 793–801

Morel M, Crouzet J, Gravot A, Auroy P, Leonhardt N, Vavasseur A, Richaud P (2009) AtHMA3, a P1B-ATPase Allowing Cd/Zn/Co/Pb Vacuolar Storage in Arabidopsis. *Plant Physiol* 149: 894–904

Ohnishi O, Matsuoka Y (1996) Search for the wild ancestor of buckwheat II. Taxonomy of Fagopyrum (Polygonaceae) species based on morphology, isozymes and cpDNA variability. *Genes Genet Syst* 71: 383–390

Sahi SV, Bryant NL, Sharma NC, Singh SR (2002) Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. *Environ Sci Technol* 36: 4676–4680

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. *Proc Natl Acad Sci USA* 95: 5857–5864

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. *Nat Biotechnol* 21: 914–919

Tamura H, Honda M, Sato T, Kamachi H (2005) Pb hyperaccumulation and tolerance in common buckwheat (*Fagopyrum esculentum* Moench). *J Plant Res* 118: 355–359

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res* 22: 4673–4680

Tommasini R, Vogt E, Fromenteau M, Hortensteiner S, Matile P, Amrhein N, Martinoia E (1998) An ABC-transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. *Plant J* 13: 773–780

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, et al. (2008) Plant ABC proteins—a unified nomenclature and updated inventory. *Trends Plant Sci* 13: 151–159