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

Takafumi Mizuno1,*, Masahiro Nakagawa1,a, Hiroshi Ono1,b, Daisuke Sugiura1,c, Hideo Tamura2, Hitoshi Obata1

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 detoxification 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 ABC is one of the most thoroughly analyzed of the ABC transporters. About 15 homologs of the ABC 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*; commercial name, Milky Way) were purchased from Sakata Seed Corporation (Kanagawa, Japan). They were germinated on sterilized barmicurial 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) and was frozen in liquid nitrogen and pulverized. Total RNA was used to make the cDNA template, using oligo d(T)-anchor primer. The transcription level of FeABCC1 was measured by RT-PCR amplification 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 mM Pb(NO3)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 plants with partial FeABCC1 amplification (354 bp) using primer set MRP5′ (5′-TCCTCGTCTTCTGGGTGAGG-3′) and MRP3′ (5′-ATCTTCGTCTTCTGAAGGT-3′), and Takara Ex Taq™ (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′-TAAAACAACCTTGATACAGGC-3′ and actin 3′-ATTGGGATGACATGGAGAA-CACTTCATTATGGAGTTATA-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’-TCCGTCGACTTAGAGATG-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 ABC 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 (Martinioa 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).
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 OD₆₀₀ value of FeABCC1-expressing yeast reached 0.408, which was about 1.68 times the control value (0.243).
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,
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 4. Lead and cadmium accumulation in FeABCC1-expressing yeast cells. Cells were cultured for 4 days in medium supplemented with 10 μM Pb(NO₃)₂ (A) or CdCl₂ (B). Data are expressed as mean ± standard error (n=3). * P<0.05 vs. control.

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 ± 46.6 μg g⁻¹ dry weight) than that in control yeast cells (139.4 μg 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, ABC1, 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