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Characterization of *Coptis japonica* CjABCB2, an ATP-binding cassette protein involved in alkaloid transport

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Abstract

Higher plants produce a large number of secondary metabolites. Among these are the alkaloids, a group of small nitrogen-containing molecules. Alkaloids often have strong biological activity that protects alkaloid-producing plants from herbivores, and often accumulate to high concentrations in a specific organelle of a particular organ in the producing plant. However, our knowledge of the membrane transport mechanism of alkaloids is still limited. *Coptis japonica*, a perennial ranunculaceous plant, produces the benzyloquinoline alkaloid berberine. This alkaloid, though biosynthesized in the root tissues, accumulates in the rhizome, suggesting translocation of the molecule via xylem. In this study, we isolated a novel ATP-binding cassette (ABC) protein gene of B-type, *Cjabcb2*, from *C. japonica*. Northern analysis showed that *Cjabcb2* was preferentially expressed in the rhizome, which is the sink organ of berberine. Functional analysis of CjABCB2 using yeast suggested that CjABCB2 transports berberine in an inward direction. Membrane separation and *in situ* hybridization data indicated that CjABCB2 might be involved in the translocation of berberine from the root to the rhizome by transporting berberine at the plasma membrane of cells around the xylem of rhizome.

Keywords: ABC transporter, Alkaloid, Berberine, *Coptis japonica*, CjABCB2, Translocation

1. Introduction

Higher plants produce a large number of alkaloids which have diverse chemical structures and biological activities. Some of them are used as medicines, such as anticancer drugs, analgesics, and antimalarial drugs (Croteau et al., 2000; Kutchan, 1995; Zenk and Juenger, 2007). Due to their strong antimicrobial and antifungal activities, as well as their other cytotoxicities, alkaloids in plants are presumed to be endogenous chemical barriers that protect the plants from pathogens or herbivores. For instance, berberine, a yellow benzyloquinoline alkaloid, which is conventionally used as a bitter stomachic and an anti-diarrhetic in many countries, shows strong antimicrobial activity toward both Gram-positive and Gram-negative bacteria as well as other microorganisms.

It is often observed that a particular alkaloid preferentially accumulates in a particular organ in medicinal plants, rather than being equally distributed around the whole plant body (Maier et al., 1999). In some cases, alkaloids translocate from a source organ to a sink organ where they accumulate to a constant level. For example, nicotine is synthesized in root tissues in *Nicotiana* species, but mainly accumulates within the vacuoles of leaves (Hashimoto Yamada 1994). Moreover, the movement of biosynthetic intermediates of alkaloids from a specific cell layer to other cell layers has also been suggested (Facchini and De Luca, 2008; Kutchan, 2005; Weid et al., 2004). Details of the movement of alkaloids within the organelles in a cell are summarized elsewhere (Shitan and Yazaki, 2007). Membrane transport of alkaloids is a key biological event that drives movement of alkaloids among compartments, but the mechanisms of membrane transport, as well as the accumulation machinery of alkaloids in plants, has remained largely unknown despite several decades of research.

A simple model for alkaloid accumulation in plant vacuoles is the ion-trap mechanism (Matile, 1976). In this model, alkaloids, which are in a lipophilic state in the neutral pH of cytosol (ca. pH 7.2), can freely pass through the tonoplast by simple diffusion. In the acidic condition of the vacuolar lumen (e.g. pH 5.5), however, they are immediately protonated to form a hydrophilic ion, which is incapable of permeating through the membrane, resulting in the trapping of alkaloid molecule inside. In this model, alkaloid movement takes place by one-way diffusion, in which transporter molecules are not necessary. Contrary to this model, studies by Dr. Zenk's group and also by other independent research groups have demonstrated that the vacuolar transport of alkaloids is managed by specific carriers that can recognize stereochemistry of transport substrates in an energy-requiring manner (Deus-Neumann and Zenk, 1984, 1986; Mende and Wink, 1987). These reports suggest the involvement of a proton-antiport carrier system. A third mechanism proposed for vacuolar transport of alkaloids is via a directly energized transporter, the ATP-binding cassette (ABC) transporter, which might also be responsible for the transport of other secondary metabolites (Klein et al., 2000). Recent progress in this field has identified several transporters for alkaloids. For instance, Nt-JAT1 (*Nicotiana tabacum* jasmonate-inducible alkaloid transporter1) and NtMATE1, belonging to the MATE (multidrug and toxic compound extrusion) transporter family, are involved in vacuolar transport of alkaloids in *N. tabacum* (Morita et al., 2009; Shoji et al., 2009). For the vacuolar accumulation of alkaloid, a vesicle-mediated mechanism has also been proposed (Amann et al., 1986; Bock et al., 2002; Galneder et al., 1988). However, our knowledge of the number and type of transporters responsible for the transport and translocation of the many divergent alkaloids in plants is still very limited. Hence,

further identification and characterization of alkaloid transporters is of critical importance for progress in the field.

In this study, we have used *Coptis japonica* (Ranunculaceae) as a model plant to isolate transporters for endogenous alkaloids. *C. japonica* is a perennial medicinal plant grown in Asian countries, and produces berberine as its main alkaloid. Berberine accumulates to high levels in the rhizome of *C. japonica*, while genes coding for the biosynthetic enzymes of berberine are specifically expressed in the root tissues (Ikezawa et al., 2003), suggesting that berberine translocates from the root to the rhizome via transporter molecules. A cell culture system of *C. japonica* that efficiently produces and accumulates berberine has been maintained for many years. It has been demonstrated in cultured cells that berberine exclusively accumulates in the vacuoles (Sato et al., 1992). The *C. japonica* cell cultures can also actively take up berberine exogenously added to the culture medium, and the administered berberine is also sequestered in the vacuolar lumen (Sakai et al., 2002). The cell culture system of *C. japonica* is thus a powerful tool for the biochemical analysis of alkaloid transport and is a suitable source for isolation of transporter cDNAs. Our previous study showed that the uptake of berberine by *C. japonica* cells depends on the endogenous ATP level, and several inhibitors of human ABCB1 (MDR1), a representative ABC transporter responsible for multidrug resistance of cancer cells, significantly suppresses berberine uptake from the medium (Sakai et al., 2002). We further clarified that a B-type ABC protein CjMDR1, which is now called CjABCB1 according to the unified nomenclature (Verrier et al., 2008), functions as a berberine transporter at the plasma membrane (Shitan et al., 2003).

In this paper, we have isolated two cDNAs for novel ABCB proteins, CjABCB2 and 3, which have significant amino acid similarities with CjABCB1, from cultured cells of *C. japonica*. We have mainly focused on the characterization of *Cjabcb2*, and demonstrated that CjABCB2 may be mediating the translocation of berberine from root to the rhizome in plant.

2. Results

2.1. Cloning and Sequence Analysis of *Cjabcb2* and *Cjabcb3*

Our previous study suggested that an ABC protein of B-type was involved in berberine uptake in cultured *C. japonica* cells (Sakai et al., 2002). To obtain relevant genes encoding ABCB proteins, degenerate primers corresponding to the highly conserved amino acid sequences for nucleotide binding folds (NBFs) of ABC proteins were designed. By a combination of RT-PCR, 5' and 3' RACE, we obtained two full-length cDNAs of ABCB proteins, which were designated *Cjabcb2* and 3 (*Coptis japonica abcb*). Both *Cjabcb2* and 3 were ca. 4.2 kb in length and coded for putative polypeptides composed of 1,292 and 1,288 amino acids, respectively. Hydropathy analysis with the program on the 'DAS' transmembrane prediction server (<http://www.sbc.su.se/~miklos/DAS/>) suggested that the topologies of CjABCB2 and 3 are very similar to that of human ABCB1, i.e. twelve transmembrane helices and two NBFs representing full-size ABC proteins. In the phylogenetic relationship of B-type plant ABC transporters, both CjABCB2 and 3 belong to Clade II (Fig. 1) (Geisler and Murphy, 2006). In this clade, two ABC proteins have already been characterized; CjABCB1 of *C. japonica* functions as a berberine influx transporter and AtABCB4 (AtPGP4) is involved in auxin transport in Arabidopsis root (Cho et al., 2007; Santelia et al., 2005; Shitan et al., 2003; Terasaka et al., 2005). CjABCB2 showed strong amino acid sequence similarities to CjABCB1 (82%) and AtABCB4 (70%), whereas CjABCB3 showed lower similarity to CjABCB1 (61%) and AtABCB4 (59%) than CjABCB2.

2.2. *Cjabcb2* and *Cjabcb3* expression in intact plant and cultured cells of *C. japonica*

The expression of *Cjabcb2* and *3* in the cultured *C. japonica* cells and various organs of intact plants were analyzed by northern blot hybridization. In the cultured *C. japonica* cells, the expression level of *Cjabcb2* remained almost constant (Fig. 2A), as did the berberine content in the cells (Fig. 2B), while the mRNA level appeared to be slightly higher in the growth phase than in the stationary and lag phase. By contrast, *Cjabcb3* expression in cultured cells gradually increased in the growth phase up to 14 days after inoculation and then decreased (Fig. 2A).

In intact plants, a clear, tissue-specific preference of *Cjabcb2* expression was observed. Higher expression of *Cjabcb2* mRNA was detected in rhizome, the main accumulation site of berberine (Ikezawa et al., 2003) (Fig. 3), whereas almost no detectable transcripts were seen in other organs. These results reveal a clear correlation between the gene expression of *Cjabcb2* and the accumulation of the alkaloid in plant organs. *Cjabcb3* expression was, however, almost undetectable in intact plants, suggesting that the contribution of this gene to the specific berberine accumulation in rhizome is not large. Thus, further analysis was performed only with *Cjabcb2*.

2.3. *CjABCB2* functions as a berberine influx pump

Because *Cjabcb2* expression showed a correlation to the berberine accumulation in intact plant, we investigated whether *CjABCB2* had the ability to transport berberine. To characterize the transport function of *CjABCB2*, we used *Saccharomyces cerevisiae* AD12345678 strain, which shows high drug sensitivity due to the deletion of several ABC protein-related genes (Decottignies et al., 1998). An expression construct of *CjABCB2* downstream of the strong constitutive promoter PMA1 was introduced into the yeast strain. Since berberine is cytotoxic to many unicellular organisms like bacteria

and yeast, we analyzed the function of CjABCB2 by estimating yeast growth in the presence of berberine and several other drugs for comparison (Fig. 4). CjABCB2 transformant appeared to be more sensitive to berberine, although the difference was small. A larger difference in drug sensitivity between CjABCB2 transformant and the empty vector control was found with 4-nitroquinoline *N*-oxide (4-NQO), which has quinoline moiety in its structure and a substrate of yeast pleiotropic drug resistance (PDR)-type ABC protein, SNQ2 (Servos et al., 1993). These data suggested that 4-NQO was recognized as a substrate of CjABCB2 and taken up into cells, resulting in increased sensitivity to 4-NQO in yeast. However, cycloheximide, a substrate of another yeast ABC protein PDR5, showed no clear difference between CjABCB2-expressing and control yeast cells, which indicated that substrates of CjABCB2 were not as broad as those of yeast PDR5 or human ABCB1. From the structure similarity of berberine and 4-NQO, it is presumed that isoquinoline or quinoline derivatives are preferable substrates for CjABCB2.

2.4 CjABCB2 was localized at the plasma membrane

To investigate the subcellular localization of CjABCB2 in *C. japonica* cells, we prepared polyclonal antibodies against a peptide sequence of CjABCB2. These antibodies reacted with a 140 kDa protein in yeast cells expressing CjABCB2, indicating that these antibodies recognized CjABCB2 (Fig. 5A). For subcellular localization analysis of CjABCB2, microsomal membranes were first fractionated by sucrose density gradient centrifugation and then a western blot was performed. As shown in Fig. 5B, CjABCB2 co-fractionated with the plasma membrane H⁺-ATPase, peaking at 30/40% sucrose fraction. In contrast, vacuolar H⁺-pyrophosphatase

(V-PPase), a marker of tonoplast, peaked at the 0/20% sucrose fraction, and membranes containing luminal binding protein (BiP), a marker of endoplasmic reticulum (ER), formed sediment at positions different from CjABCB2. These results suggested that CjABCB2 was associated with the plasma membrane and not with the tonoplast or ER.

The aqueous two-phase partitioning method was also performed to confirm the plasma membrane localization of CjABCB2 (Fig. 5C). CjABCB2 and H⁺-ATPase were highly enriched in the upper-phase fraction (U) and greatly depleted in the lower-phase fraction (L). V-PPase, tonoplast marker, was enriched in the lower-phase fraction as logically expected. Bip, ER marker, was partly detected in the upper-phase fraction, but was also detected in the lower-phase, unlike H⁺-ATPase. Taken together, these results indicated that CjABCB2 was localized at the plasma membrane.

2.5. In situ localization of Cjabcb2 mRNA

To identify the physiological role of CjABCB2 *in planta*, *in situ* hybridization was performed using an antisense RNA probe prepared from *Cjabcb2* cDNA. We analyzed two organs, rhizome and root, corresponding to the sink organ for berberine accumulation and the source organ for its biosynthesis, respectively. In the rhizome section, weak signals for the *Cjabcb2* mRNA were observed with the antisense probe around the vascular tissue, particularly xylem cells (Fig. 6A), whereas no signal was observed with the sense probe as a negative control (Fig. 6B). Almost no signals were detected in root sections with either antisense or sense probe (Fig. 6 C and D). Berberine may translocate from the root to the rhizome via several transporters, i.e., efflux from source cells and uptake at sink cells both at plasma and vacuolar membranes,

and these results suggest that an ABC transporter, CjABCB2, may participate in the accumulation of berberine in rhizome by acting as the berberine influx transporter.

3. Discussion

Recently there have been reports of the involvement of ABC proteins in the transport of secondary metabolites; these reports are summarized in review articles (Yazaki, 2005, 2006). The maize ABCC (multidrug resistance associated protein, MRP)-type transporter, ZmMRP3 was required for the vacuolar accumulation of anthocyanin (Goodman et al., 2004). For plant pathogen resistance, *Nicotiana plumbaginifolia* ABCG (PDR)-type ABC transporter, NpPDR1 plays an important role by excreting sclareol, an antifungal diterpenoid compound in *Nicotiana* species, to the leaf surface (Jasinski et al., 2001). Wax is a complex mixture of lipophilic metabolites, which protect plants from drought stress, and half-size ABC proteins, AtABCG11 (AtWBC11) and AtABCG12 (AtWBC12), ABCG (white-brown complex, WBC)-type proteins, are involved in the secretion of wax components on the plant surface (Bird et al., 2007; Luo et al., 2007; Pighin et al., 2004; Ukitsu et al., 2007). These findings suggest that a number of ABC transporters are involved in the membrane transport of endogenous metabolites in plant body.

We isolated in this study two cDNAs, *Cjabcb2* and *Cjabcb3*, which belong to ABC protein of B-type. Functional and expression analyses suggested that CjABCB2 was involved in the unloading of berberine in the rhizome, i.e., berberine is biosynthesized in root tissues and translocates upward, probably via xylem transport, whereas in the rhizome the berberine is trapped by plasma membrane-localized CjABCB2 and accumulates in rhizome tissue. Since CjABCB1, the closest member of CjABCB2, was shown to play a similar function in berberine transport (Shitan et al., 2003), these two ABC proteins may function in a cooperative manner for berberine accumulation in rhizome. Since the rhizome is an important sink organ for starch as

well as berberine in the *C. japonica* plant, the accumulation of berberine in rhizome has a clear benefit, as the anti-microbial alkaloid, acting as a chemical defense, can protect the carbohydrate-rich sink tissue from attack by soil pathogens. The expression of CjABCB1 is, however, observed not only in rhizome but also in petiole, peduncle, and flower (Yazaki et al., 2001). By contrast CjABCB2 is expressed only in rhizome (Fig. 3), suggesting that CjABCB2 is specialized for berberine accumulation in rhizome, whereas CjABCB1 might be responsible for the berberine accumulation in other tissues of low berberine content.

For characterization of plant ABC proteins, selection of a suitable expression system is crucial. In the case of CjABCB2, although we first tried to express it in *Xenopus* oocyte, we did not observe accumulation of the protein in this heterologous host (data not shown). These data suggested that CjABCB2 was not compatible with *Xenopus* oocyte, even though it has high amino acid identity (82%) with CjABCB1, whose expression has been successfully performed in oocytes (Shitan et al., 2003). As a second choice of heterologous expression, we selected a yeast system and were able to characterize CjABCB2 function. Other plant ABCB proteins, AtABCB1 (AtPGP1) and AtABCB4 (AtPGP4) were expressed and characterized in Hela cells, yeast cells, and BY-2 cells, to demonstrate the transport function of auxin (Cho et al., 2007; Geisler et al., 2005; Santelia et al., 2005; Terasaka et al., 2005). Yeast vesicle transport analyses were demonstrated for ABCC members as well; of these members, AtABCC1 (AtMRP1), 2 and 3 had transport activity for glutathione conjugate (Lu et al., 1998; Tommasini et al., 1998), and AtABCC5 (AtMRP5) could transport estradiol-17-(β -D-glucuronide) (Gaedeke et al., 2001). Sulfonyleurea-binding activity of AtABCC5 was shown by HEK293 cell expression system (Lee et al., 2004). Other ABC

proteins were characterized directly in mutant plants, in which ABC protein expression was modified by T-DNA insertion, antisense or overexpression (Goodman et al., 2004; Pighin et al., 2004). In general, however, few plant ABC proteins have been biochemically characterized, probably due to the difficulty in finding an appropriate heterologous host. To characterize ABC protein function, it would be very important to screen several heterologous expression systems.

The final intracellular accumulation site of berberine is vacuole in *C. japonica*. For vacuolar alkaloid accumulation, Dr. Zenk's group predicted a highly specific carrier-mediated and energy-dependent proton antiport system as early as the 1980s. (Deus-Neumann and Zenk, 1984, 1986). Our biochemical analysis in *C. japonica* suggested that vacuolar berberine transport is indeed mediated by an H^+ /berberine antiporter (Otani et al., 2005). As an H^+ /substrate antiporter family, MATE-type transporters have recently been reported to transport various secondary metabolites, which were reviewed previously (Yazaki et al., 2008). We have shown that Nt-JAT1, a tobacco MATE transporter, is involved in nicotine translocation and vacuolar accumulation in tobacco leaves (Morita et al., 2009). In fact, though it is known that MATE-type transporters, functioning as H^+ /substrate antiporters, serve an important membrane protein family responsible for transporting various endogenous metabolites (Gomez et al., 2009; Marinova et al., 2007; Maron et al., 2010), it is still unknown whether or not the vacuolar berberine transporter is a member of the MATE-type proteins. Further studies on vacuolar transporters in *C. japonica* are necessary in order to understand the system responsible for transport and sequestration of berberine in vacuoles of the cells of this plant.

4. Conclusions

We isolated a novel ABC protein, CjABCB2, from the berberine-producing plant *C. japonica*. CjABCB2 was specifically expressed in the rhizome, where berberine accumulates to high levels. This protein localized to the plasma membrane and functioned as a berberine influx transporter. *In situ* hybridization showed that *Cjabcb2* was expressed specifically around xylem tissue of rhizome. These data suggested that CjABCB2 was involved in the berberine translocation by unloading the protein in the rhizome. Transport of secondary metabolites including alkaloids is a hot topic of late (Shitan and Yazaki, 2007; Yazaki, 2005; Yazaki et al., 2009), and the present findings may contribute at least partly to an understanding of the molecular mechanism of alkaloid transport in plants.

5. Experimental

5.1. Cultured Cells

High berberine-producing cultured *C. japonica* cells, which were originally induced from rootlets of *C. japonica* Makino var. *dissecta* (Yamabe) Nakai, were maintained as described previously (Sato and Yamada, 1984).

5.2. Chemicals

Berberine and other chemicals used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) or Nakalai Tesque (Kyoto, Japan).

5.3 Isolation of full-length *abcb*-like gene from cultured *C. japonica* cells

Total RNA prepared from *C. japonica* cells using RNeasy Plant Mini Kit (Qiagen) was reverse-transcribed with M-MLV reverse transcriptase (NEB). Nested PCR was carried out with Taq DNA polymerase (Takara), using the DNA-RNA hybrid as a template, and two sets of degenerate primers designed from the highly conserved amino acid sequences in the nucleotide-binding fold (NBF) which is the domain responsible for the ATP-binding property of ABC transporters; Fwd-primer1, 5'-MARACIYTIGCHYTIGTIGG-3' (n- (Q/K)TTLALVG -c) and Rv-primer1, 5'-GCRCTXHTXGCYTCRTCXARXAG-3' (n- LLDEATSA -c): Fwd-primer2, 5'-GGITGYGGIAARWSISIGT-3' (n- GCGKS(T/S)V -c) and Rv-primer2, 5'-GCDATXCKYTGYTTYTGXCCXCC-3' (n- GGQKQRIA -c). As the PCR products, two 320 bp fragments for the NBF domain of ABCB-type transporter were isolated. Using two specific primers corresponding to the internal sequences of these DNA fragments, RT-PCR was performed again using a heat-stable DNA polymerase of

high-fidelity KOD (TOYOBO), resulting in the amplification of a DNA fragment (2.2 kb) which was significantly similar to known ABCB-like transporters. To obtain full-length cDNA clones, 5' and 3' RACE using a cDNA amplification kit (Clontech) were used. The accession numbers of *Cjabcb2* and *Cjabcb3* are AB674325 and AB674326, respectively.

5.4. Organ-specific expression

Total RNAs were isolated from root, rhizome, petiole, leaf, peduncle, bud, flower, and seed of the intact plant of *C. japonica*. Aliquots of 10 µg of RNA were loaded on formamide-containing agarose gel, electrophoresed and blotted onto a nylon membrane. As the hybridization probe, 3'-UTR of *Cjabcb2* or *Cjabcb3* (200 bp) were used. As the load control, 18S rRNA was used.

5.5. Time-course of gene expression and berberine contents

Time-course of *Cjabcb2* or *Cjabcb3* expression in cultured cells of *Coptis japonica* was analyzed by northern hybridization. The cultured cells were harvested 2, 5, 9, 14, 21 days after inoculation, from which poly (A)⁺ RNA was isolated and loaded on formamide-containing agarose gel. As the hybridization probe, 3'-UTR of *Cjabcb2* or *Cjabcb3* (200 bp) were used. ATP synthase (β subunit) was used to analyze the loading amount. Cell growth was monitored and berberine content in the cells was quantified by HPLC analysis. For HPLC analysis, alkaloids were extracted from cells with HCl-acidified methanol until the cells lost their yellow color. The supernatant was subjected to HPLC analysis: mobile phase, 50 mM tartaric acid solution containing 10 mM SDS - acetonitrile - methanol (100 : 100 : 25); column, TSK-GEL ODS-80TM

(TOSOH, 4.6 i.d. X 250 mm); temperature, 40°C; flow rate, 1.2 ml/min; detection, absorbance measured at 260 nm.

5.6. Peptide antibody against CjABCB2

A KLH (keyhole limpet hemocyanin) conjugate of an oligopeptide of CjABCB2, at position 665 (n- CSRGSSGVGNSSRKS –c), was injected into rabbit according to the standard protocol (Kurabo Industries, Japan). After the fourth boost, antiserum was recovered and used for immunoblot analysis without further purification.

5.7. Functional analysis of CjABCB2 using Yeast

Cjabcb2 cDNA (4.0 kb) was subcloned into yeast expression vector pDR196, kindly provided by Dr. W. Frommer (Carnegie Institution, Stanford, USA), at the *Not I* site. The resulting plasmid, pDR-CjABCB2, was used to transform strain AD12345678 (*MATa*, *PDR1-3*, *ura3*, *his1*, *Δyor1::hisG*, *Δsnq2::hisG*, *Δpdr5::hisG*, *Δpdr10::hisG*, *Δpdr11::hisG*, *Δycf1::hisG*, *Δpdr3::hisG*; and *Δpdr15::hisG*) (Decottignies et al., 1998) by the lithium acetate method (Yazaki et al., 2002), and then selected by SD medium (-uracil). The drug sensitivity of yeast transformants was tested by spotting SD- cultures (-uracil) onto agar plates containing various compounds. Five µl of the transformant diluted to same density $A_{600}=0.5$ was spotted onto each plate and growth was monitored after incubation of the cells for 44 h at 25°C. The drugs, berberine and cycloheximide, used in this study were dissolved in water, while 4-nitroquinoline *N*-oxide was dissolved in acetone.

5.8. Isolation of Plasma Membranes

Plasma membranes were purified from a microsomal fraction of *C. japonica* cells by partitioning in an aqueous polymer two-phase system and sucrose gradient fractionation as described previously (Larsson et al., 1987; Otani et al., 2005). Protein electrophoreses and electrophoretic transfer was performed as described previously (Shitan et al., 2003). Antibodies used for immunodetection were against CjABCB2, vacuolar H⁺-pyrophosphatase from Arabidopsis (V-PPase), endoplasmic reticulum BiP (BiP), and plasma membrane H⁺-ATPase (H⁺-ATPase).

5.9. *In Situ* hybridization

Root and rhizome were fixed on ice in freshly prepared solutions of 2 and 4% formaldehyde in 50% ethanol and 10% acetic acid, respectively, for 3 h. The samples were dehydrated through an ethanol series, and embedded with Paraplast plus (OXFORD). Sections 10- to 20- μ m thick prepared with a microtome (RM 2155, Leica) were hybridized with the digoxigenin-labeled antisense or sense RNA probe of *Cjabcb2*, which is a 178-bp fragment containing the 3'-untranslated region. *In situ* hybridization and the detection procedures were performed as described previously (Yamada et al., 1995).

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Figure Legends

Fig. 1. Phylogenetic tree of the plant ABCB-type ABC transporters

The unrooted phylogenetic tree is based on a multiple alignment of the peptide sequences of ABCBs from *Arabidopsis*, wheat, barley, potato, upland cotton, and *Coptis japonica*, produced by the ClustalW program (<http://align.genome.jp/>). The resultant tree was produced using TREEVIEW. *Arabidopsis* ABCB sequences were recovered according to the AGI-Code described by Verrier et al. (2008). Accession numbers BAB85651, BAB62040, AAF23176, AAD10836, CAA71179, AAR00316, and AAR10387, correspond to the transporters, TaMDR1 (wheat, *Triticum aestivum*), CjABCB1 (*Coptis japonica*), CMDR1 (cotton, *Gossypium hirsutum*), PMDR1 (potato, *Solanum tuberosum*), HvMDR2 (barley, *Hordeum vulgare*), ZmPGP1 (maize, *Zea mays*), dw3 (*Sorghum bicolor*), respectively.

Fig. 2. Northern hybridization of *Cjabcb2* and 3 (A), and berberine accumulation in cultured *C. japonica* cells (B)

The cultured cells were harvested 2, 5, 9, 14, 21 days after inoculation, 3 µg of poly (A)⁺ RNA was isolated and loaded on formamide-containing agarose gel. As the hybridization probe, 3'-untranslated region of *Cjabcb2* (200 bp) and *Cjabcb3* (200 bp) were used. ATP synthase (β subunit) was used as the load control. Berberine content in the cells was monitored by HPLC analysis.

Fig. 3. Northern analysis of *Cjabcb2* in intact *C. japonica* plant

Organ-specific expression of *CjABCB2* in the intact plant and cultured cells of *C. japonica* detected by Northern hybridization (probe: 200 bp fragment of *Cjabcb2* 3'-untranslated region). Total RNA (10 µg) was isolated from each organ and separated on formamide-containing agarose gel. As the load control, 18S rRNA was used.

Fig. 4. Functional characterization of CjABCB2 using yeast cells

Yeast strain AD12345678 was transformed with pDR196 (empty vector) or pDR-CjABCB2. Each transformant was pre-cultured overnight in SD medium (-uracil). The cultures were diluted to A₆₀₀=0.5. Five µl was spotted onto an SD medium (-uracil) plate containing Ber (berberine, 3.5 µM), 4-NQO (4-nitroquinoline *N*-oxide, 0.11 µg/ml) or Cyc (cycloheximide, 0.0175 µg/ml) and incubated for 44 h at 25°C.

Fig. 5. Plasma membrane localization of CjABCB2 in *C. japonica* cells

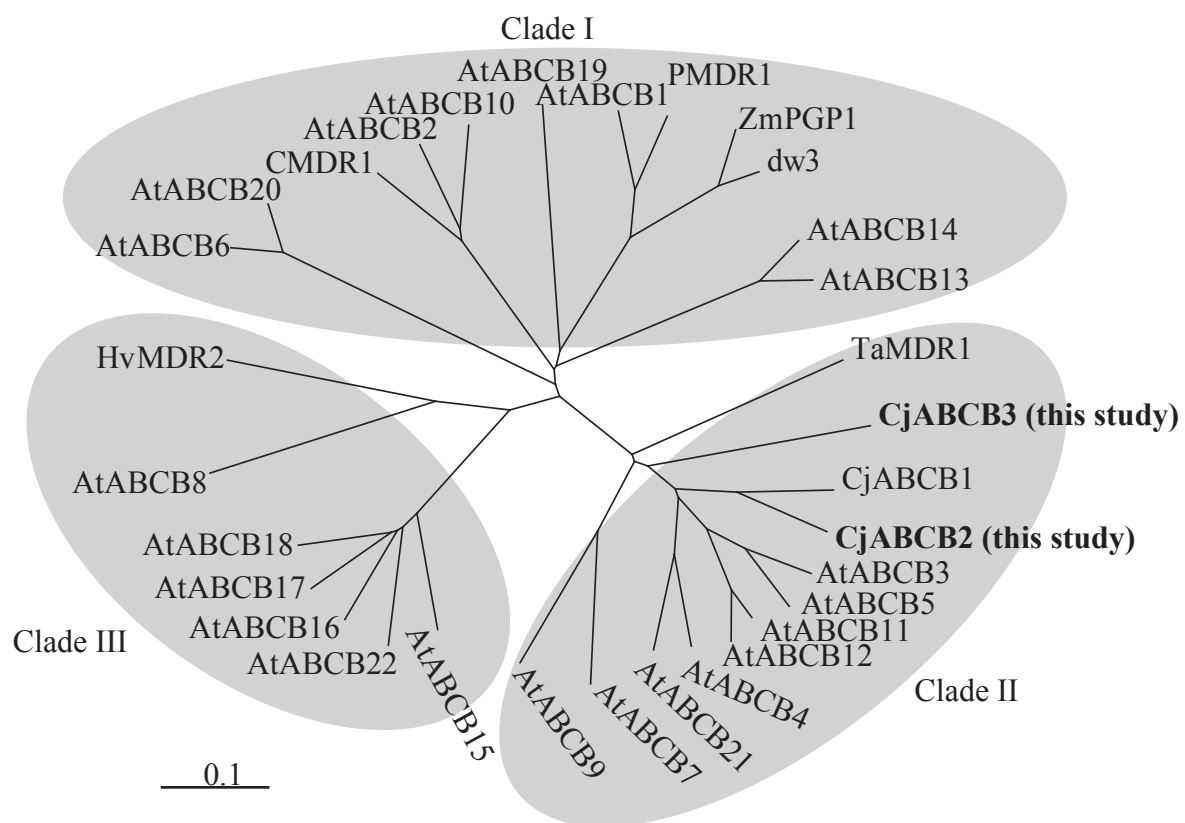
(A) Microsomal fraction proteins (40 µg) of *C. japonica* cells and membrane proteins (20 µg per lane) of yeast cells with pDR196 (vector control), or pDR196-CjABCB2 were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was probed with anti-CjABCB2 polyclonal antibodies.

(B) Immuno-detection of CjABCB2 in *C. japonica* plasma membranes. Fractionation of total microsomes from *C. japonica* cells on a non-continuous sucrose gradient consisting of 0, 20, 30, 40, and 50% (wt/vol) sucrose. Membrane fractions from the interfaces between different sucrose concentrations were collected and immuno-detected with anti-CjABCB2 antibodies. Blots were probed with antibodies raised against CjABCB2, plasma membrane H⁺-ATPase (H⁺-ATPase), vacuolar H⁺-pyrophosphatase (V-PPase), and endoplasmic reticulum BiP (BiP).

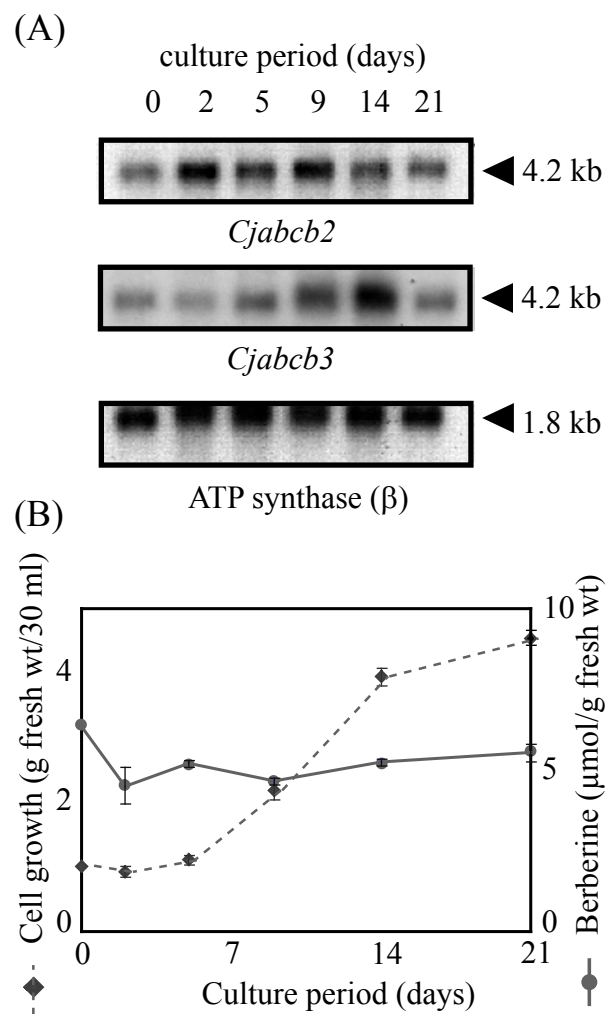
(C) Microsomal membranes (Mic) from *C. japonica* cells were fractionated by the aqueous two-phase partitioning method into an upper phase (U) enriched with plasma membranes and a lower phase (L) containing intracellular membranes. Proteins from each fraction (10 µg per lane) were immuno-blotted. The same membrane was re-probed with antibodies against the indicated marker proteins.

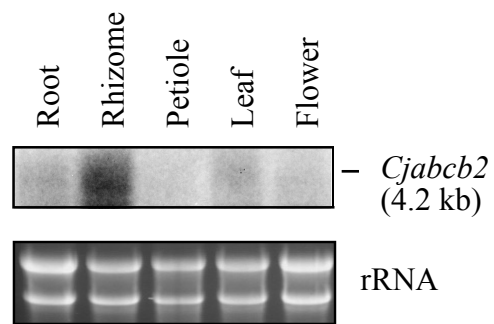
Fig. 6. *In situ* hybridization of *Cjabcb2* in the rhizome and root of *C. japonica*

(A, B) *In situ* hybridization using a digoxigenin-labeled antisense (A) and sense (B) probe in the rhizome. (C, D) *In situ* hybridization using a digoxigenin-labeled antisense (C) and sense (D) probe in the root. BF, bast fiber; C, cortex; E, epidermis; M, medulla; Ph, phloem; VB, vascular bundle; X, xylem. Bars, 500 µm.

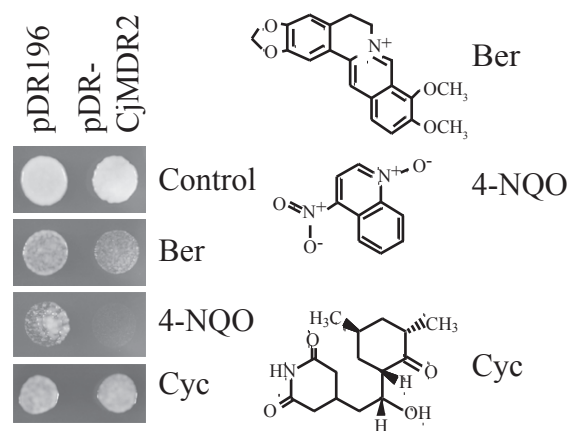


Shitan et al. Fig. 1

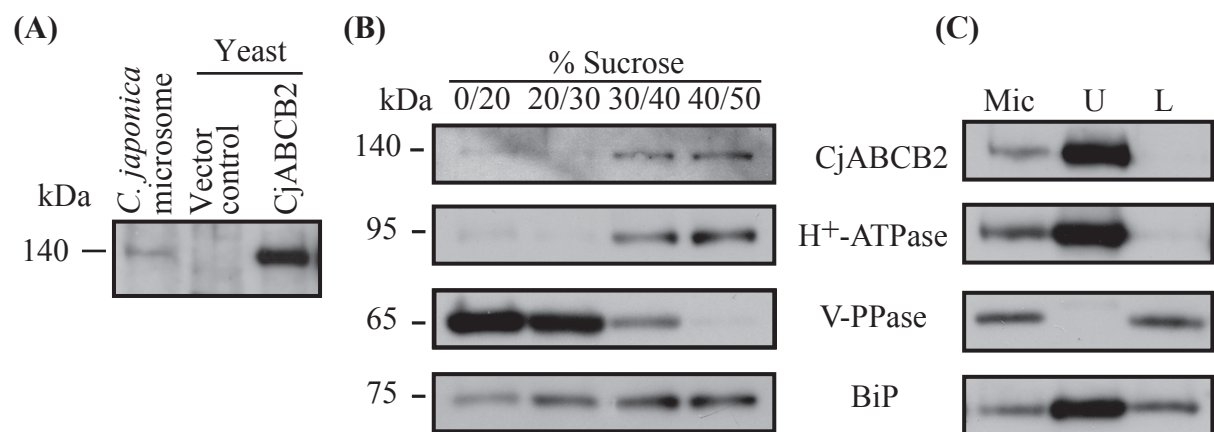




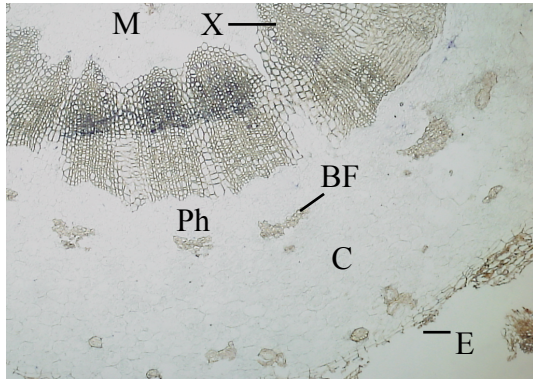
Shitan et al. Fig. 3



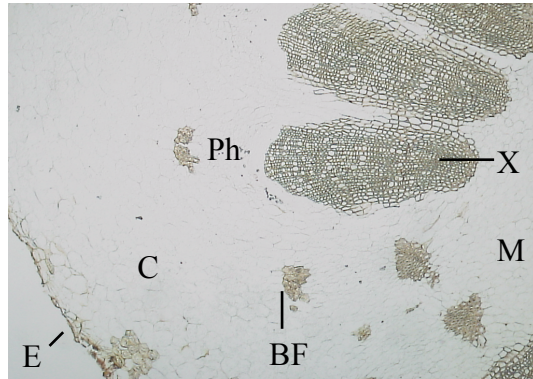
Shitan et al. Fig. 4



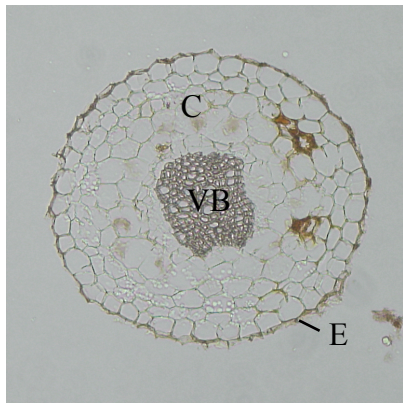
A



B



C



D

