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Short Communication

Two B-type ATP-binding cassette (ABC) transporters localize to the plasma membrane in *Thalictrum minus*

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Abstract Alkaloids play important roles in plant defenses against herbivores and some alkaloids have medicinal uses. Medicinal alkaloids can be purified from plant tissues or produced axenically in cell culture systems. In culture, cells generally accumulate these toxic metabolites in the vacuole; however, treatment with benzyladenine (BA) induces cultured *Thalictrum minus* cells to produce the isoquinoline alkaloid berberine, which they release into the medium. A previous biochemical analysis suggested that B-type ATP-binding cassette (ABC) transporters participate in berberine efflux from cultured *T. minus* cells. In this study, we isolated full-length cDNAs of two novel B-type ABC transporter genes from *T. minus*, *Tmabcb1* and *Tmabcb2*. The encoded transporters show significant amino acid sequence identity to the *Coptis japonica* berberine transporters CjABCB1 and CjABCB2. Real-time quantitative reverse transcription PCR analyses showed that BA induces an increase in *Tmabcb1* and *Tmabcb2* mRNA levels in cultured cells. Membrane separation and immunoblot analyses indicated that these proteins localize to the plasma membrane in *T. minus* cells. These data suggest that TmABCB1 and TmABCB2 participate in berberine transport in *T. minus* cells.

Key words: ATP-binding cassette transporter, berberine, *Thalictrum minus*.

Plants produce a wide array of secondary metabolites that enable them to adapt to their environment (Croteau et al. 2000). These metabolites include alkaloids, which have diverse chemical structures and various biological activities. Some alkaloids have medicinal applications; for example, morphine is used as an analgesic and vinblastine as an anticancer drug. Stable production of these metabolites in plants requires both biosynthetic enzymes to catalyze metabolic reactions and transport proteins to move end products or biosynthetic intermediates, at the organelle, tissue, and organ levels (Shitan and Yazaki 2008; Shitan et al. 2014). Alkaloids generally accumulate to high levels in a specific organ in intact plants, e.g., strychnine in seeds of *Strychnos nux-vomica*, nicotine in leaves of tobacco, and berberine in bark of *Phellodendron amurense*. At the cellular level, sequestration into vacuoles or efflux to the apoplast separates alkaloids from the cytosol and nucleus, possibly providing a species-specific detoxification mechanism for plant cells. However, our knowledge of the membrane transport system underlying alkaloid transport remains limited.

To investigate the mechanism of alkaloid transport, we use cultured *Coptis japonica* and *Thalictrum minus* cells as models. Both cell types produce berberine, a benzylisoquinoline alkaloid that has antibacterial activity. *C. japonica* cells exclusively accumulate endogenous berberine in their vacuoles, and can also absorb exogenous berberine added to the medium (Sato et al. 1990; Sato et al. 1994). By contrast, *T. minus* cells produce berberine in response to treatment with benzyladenine (BA), and excrete the berberine into the medium (Terasaka et al. 2003a). Even in the absence of BA, *T. minus* cells can exclude exogenous berberine added to the medium (Terasaka et al. 2003a).

Plant ATP-binding cassette (ABC) transporters move various substrates, using energy obtained by ATP hydrolysis, and are involved in several physiological functions, including detoxification of xenobiotics and transport of hormones and secondary metabolites

Abbreviations: ABC, ATP-binding cassette; BA, benzyladenine.
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(Kang et al. 2011; Shoji 2014; Verrier et al. 2008; Yazaki et al. 2009). We previously demonstrated that ABC transporters function in berberine uptake by *C. japonica* cells (Sakai et al. 2002) and identified three B-type ABC transporters, CjABCB1/CjMDR1, CjABCB2, and CjABCB3. We found that CjABCB1 and CjABCB2 are preferentially expressed in the rhizome, where berberine accumulates to high levels in intact *C. japonica* plants, and are responsible for berberine uptake at the plasma membrane to facilitate berberine translocation from the root to the rhizome (Shitan et al. 2003, 2013).

Our previous biochemical studies using transport inhibitors and *T. minus* cells suggested that B-type ABC transporters mediate berberine efflux, and we identified partial sequences of putative ABC transporter genes (Terasaka et al. 2003a, 2003b). Here, we attempted to isolate full-length sequences of B-type ABC transporter genes and characterize the encoded proteins.

To obtain full-length cDNA sequences, we prepared total RNA from *T. minus* cells from the line Tm103, established in a previous study (Terasaka et al. 2003a), and subsequently performed 5′ and 3′ RACE using the FirstChoice RLM-RACE Kit (Invitrogen). We isolated two full-length cDNAs encoding B-type ABC transporters that we designated *Tmabcb1* (*Thalictrum minus abcb1*) (Accession no. LC029926) and *Tmabcb2* (Accession no. LC029927). The *Tmabcb1* and *Tmabcb2* cDNAs are each ca. 4.2 kb long and encode putative polypeptides of 1285 and 1286 amino acids, respectively. A program for predicting transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/) suggested the presence of 12 transmembrane alpha helices in both TmABCB1 and TmABCB2, similar to human ABCB1 (Ishigami et al. 2013). TmABCB1 and TmABCB2 also each have two predicted nucleotide-binding domains, which form the typical structure of a full-size B-type ABC transporter.

TmABCB1 and TmABCB2 showed 77% amino acid sequence identity. In the phylogenetic relationship of plant B-type ABC transporters, TmABCB1 and 2 belong to the same clade, termed Clade II (Geisler and Murphy 2006) (Figure 1). TmABCB1 showed high amino acid sequence identities to the berberine influx transporters CjABCB1 (78%) and CjABCB2 (75%), the auxin influx transporter AtABCB4 (70%) (Cho et al. 2007; Geisler et al. 2005; Terasaka et al. 2005), and the facultative auxin importer/exporter AtABCB21 (70%) (Kamimoto et al. 2012). Whereas, TmABCB2 also showed high identity to CjABCB1 (82%), CjABCB2 (80%), AtABCB4 (71%), and AtABCB21 (71%). Both TmABCB1 and TmABCB2 showed relatively low amino acid sequence identities to Clade I B-type ABC transporters, i.e., the auxin efflux transporters AtABCB1 and AtABCB19 (ca. 44%) (Yang and Murphy 2009), and the malate importer AtABCB14 (ca. 40%) (Lee et al. 2008).

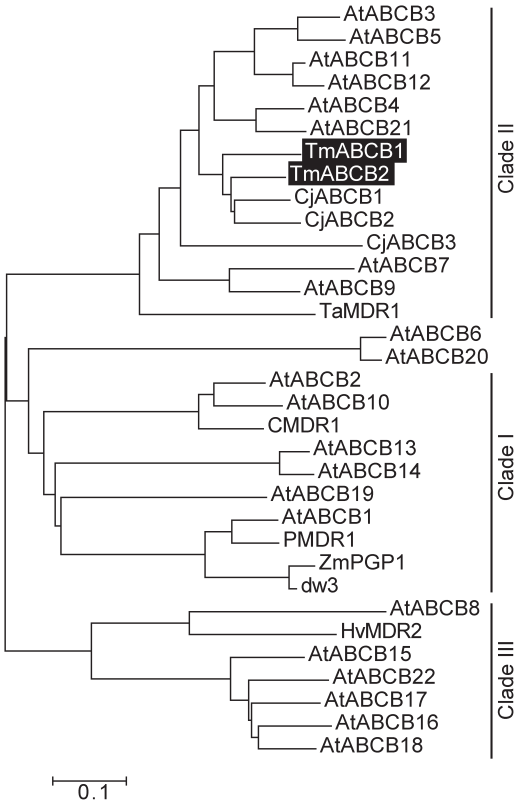

Figure 1. Phylogenetic tree of plant B-type ABC transporters. Full-length amino acid sequences of plant B-type ABC transporters from Arabidopsis, wheat, barley, potato, upland cotton, *C. japonica*, and *T. minus*, were aligned with ClustalW and subjected to phylogenetic analysis using MEGA6 (Tamura et al. 2013) with the neighbor-joining algorithm. Arabidopsis ABCB sequences were recovered according to the AGI-Code described by Verrier et al. (2008). Accession numbers BAB85651, BAB62040, BAM11098, BAM11099, AAF23176, AAD10836, CAA71179, AAR00316, and AAR10387, correspond to the transporters, TaMDR1 (wheat, *Triticum aestivum*), CjABCB1 (*Coptis japonica*), CjABCB2, CjABCB3, CMDR1 (cotton, *Gossypium hirsutum*), PMDR1 (potato, *Solanum tuberosum*), HvMDR2 (barley, *Hordeum vulgare*), ZmPGP1 (maize, *Zea mays*), and dw3 (*Sorghum bicolor*), respectively. TmABCB1 and TmABCB2 are shaded in black. The scale bar shows the number of amino acid substitutions per site.

We then investigated the expression profile of these transporter genes in *T. minus* cells using real-time quantitative reverse transcription PCR (qRT-PCR). We cultured the cells in the absence or presence of BA, as described previously (Nakagawa et al. 1984). Total RNA was prepared from cells at 9 days after inoculation, and was reverse transcribed using the ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO) according to the manufacturer's instructions. Primers were designed to amplify each gene separately and rRNA (Accession no. AF479172) was used as a control. Primer sequences were as follows: Tmabcb1-fw, ATC AAG CTA ATA AGG TTC TTT TCA TTC A; Tmabcb1-rv, TGC ATT TTC GAC ATA CAC AAT TAC A; Tmabcb2-fw, AAT CTT CAC TTA CCT AAT TTT TGG TGA TG; Tmabcb2-rv, CTG GGA TTT TGT ATT TAA GCT AAA AGA A; rRNA-fw, ACT CTC TTA AGG

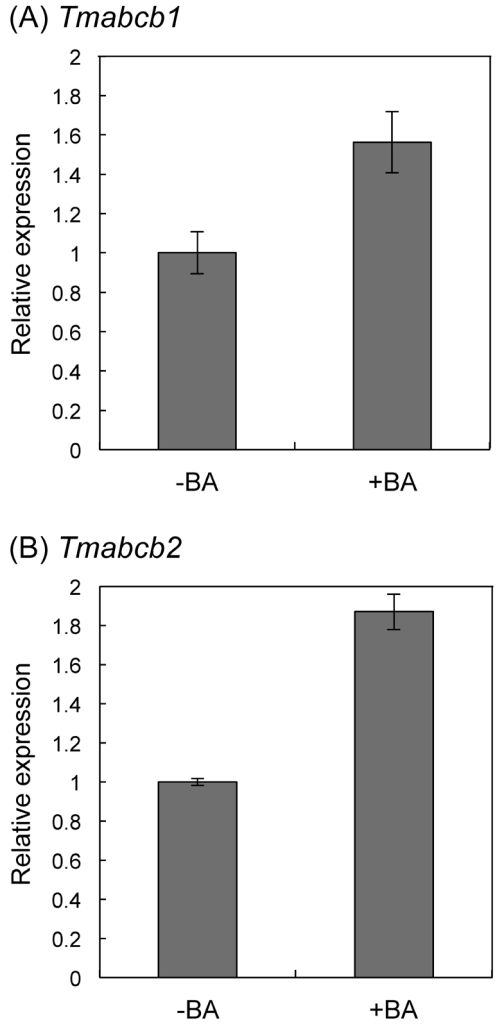

Figure 2. Relative expression of *Tmabcb1* (A) and *Tmabcb2* (B) in cultured *T. minus* cells. Expression of each gene was analyzed in cultured *T. minus* cells for 9 days in the absence (−) or presence (+) of 1 μM benzyladenine (BA) by real-time qRT-PCR and rRNA was used as an internal control. Data represent the means ± SD of three replicates.

TAG CCA AAT G; and rRNA-rv, ATT TCA CAA AGT CGG ACT AGA G. PCR was performed in a total volume of 20 μl, which contained 10 μl 2× FastStart Essential DNA Green Master (Roche), 10 pmoles of each primer, and cDNA template. The PCR program was as follows: 95°C for 10 min, 45 cycles of 95°C for 10 s, 50°C for 10 s, and 72°C for 25 s. PCR and data acquisition were performed using a LightCycler Nano instrument (Roche). This experiment showed that the transcript levels of both genes increased in response to BA (Figure 2), with *Tmabcb2* showing a slightly higher induction ratio than *Tmabcb1*. Although *T. minus* cells have berberine efflux activity even in the absence of BA (Terasaka et al. 2003a), the efflux activity might be enhanced in the presence of BA, which induces berberine biosynthesis, to efficiently export endogenous berberine. Therefore, *Tmabcb1* and *Tmabcb2* may be involved in berberine transport.

We next investigated the subcellular localization of

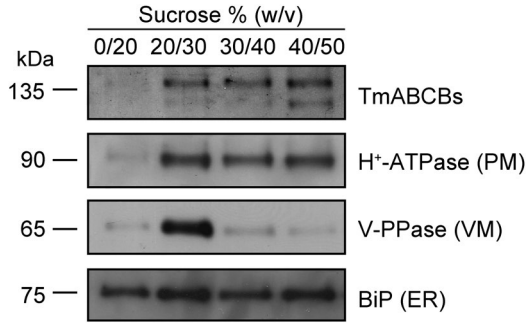

Figure 3. Plasma membrane localization of TmABCBs in *T. minus* cells. Membrane proteins fractionated by a non-continuous sucrose gradient were separated by SDS-PAGE and immunodetected with antibodies raised against TmABCBs, plasma membrane H^+-ATPase, V-PPase, and endoplasmic reticulum BiP.

these transporter proteins. Microsomal membranes from cultured cells were fractionated on sucrose density gradients, subjected to SDS-PAGE, and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore), as described previously (Shitan et al. 2003). Polyclonal antibodies against TmABCBs were raised. A keyhole limpet hemocyanin conjugate of an oligopeptide of a sequence common to both TmABCBs (*n*-SYPARPDEQIFNGC-c) was injected into a rabbit according to the standard protocol (Iwaki & Co., Ltd., Tokyo). The membrane was probed with polyclonal antibodies against TmABCBs and with antibodies against several membrane marker proteins. The anti-TmABCB antibodies detected two bands of around 130 kDa. The fractionation pattern of TmABCBs resembled that of plasma membrane H^+-ATPase and clearly differed from those of the vacuolar H^+-pyrophosphatase (V-PPase), a tonoplast marker, and membranes containing a luminal binding protein (BiP), a marker of the endoplasmic reticulum (Figure 3). These results indicate that TmABCB1 and TmABCB2 localize to the plasma membrane and not to the tonoplast or the endoplasmic reticulum.

The *Tmabcb1* and *Tmabcb2* mRNAs were induced by BA treatment, which also induces berberine biosynthesis. TmABCBs localize to the plasma membrane. These data suggest that TmABCBs mediate berberine efflux at the plasma membrane in *T. minus* cells. However, the possibility that TmABCBs might function as berberine importer cannot be denied, because both TmABCB1 and TmABCB2 had high amino acid sequence similarities to the berberine influx transporters CjABCB1 and CjABCB2, and AtABCB21, which can import or export auxin depending on the condition. This similarity suggests the possibility that TmABCBs might function in influx or efflux of berberine, or perhaps both, similar to AtABCB21. In the rhizome of *T. flavum*, a similar species of *T. minus*, the presence of berberine importer and exporter was suggested, because berberine probably

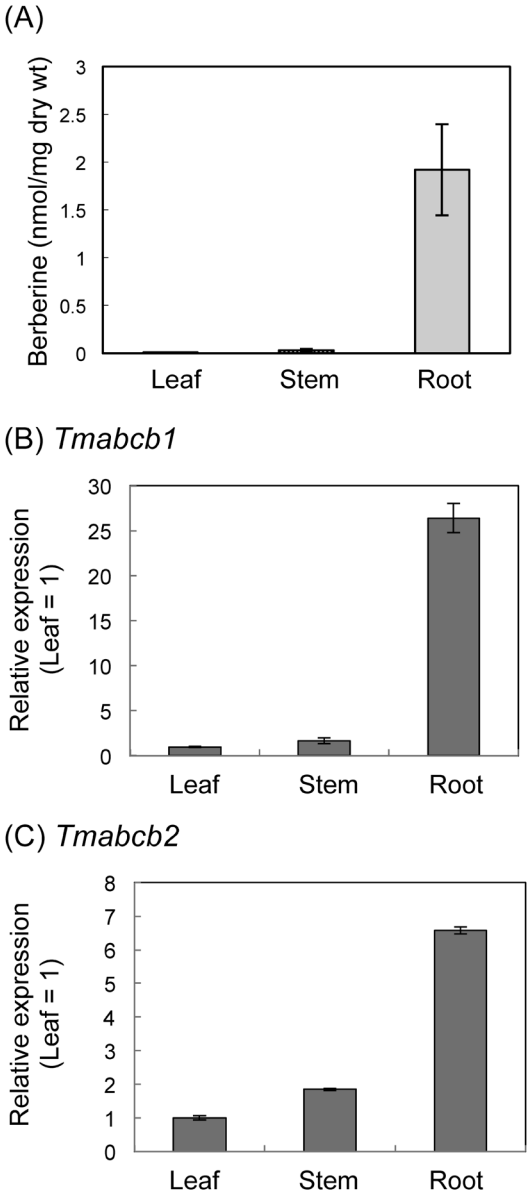

Figure 4. Root-specific accumulation of berberine and expression of *Tmabcb1* and *Tmabcb2* in intact *T. minus* plants. (A) From each dried tissue of *T. minus*, berberine was extracted using HCl-acidified methanol. The methanol extract was subjected to HPLC analysis as described previously (Shitan et al. 2005). (B) Relative expression of *Tmabcb1* (B) and *Tmabcb2* (C) in intact *T. minus* plants. Expression of each gene was analyzed by real-time qRT-PCR and rRNA was used as an internal control. Data represent the means±SD of three replicates.

moves from the apical meristem, the biosynthesis site, to the pith and cortex, the accumulation sites (Samanani et al. 2005). In intact *T. minus* plants, although rhizome was not examined due to the small size of analyzed plants, berberine accumulates to high levels in root tissues (Figure 4). Since *Tmabcb1* and *Tmabcb2* are preferentially expressed in roots (Figure 4), TmABCB1 and TmABCB2 might contribute to retention of berberine by functioning as berberine importer in this tissue. The functions of TmABCB1 and TmABCB2, however, remain to be clarified due to difficulties in subcloning the full-length cDNAs (ca. 4 kb) encoding these proteins into expression vectors, which causes many mutations during subcloning by unknown mechanisms. Further study is required to clarify their transport substrate and physiological functions.

Transport of secondary metabolites has been intensively studied in plants and several transporters have been identified (Nour-Eldin and Halkier 2013; Shitan and Yazaki 2008; Shitan et al. 2014; Zhao and Dixon 2010). Engineering plants with altered transport properties may have applications in the production of valuable compounds (Nour-Eldin and Halkier 2013). The findings presented here improve our understanding of alkaloid transport in *T. minus*. Identifying and characterizing additional alkaloid transporters, including TmABCBs, will provide further insight into alkaloid transport mechanisms in plants.

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