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A multidrug and toxic compound extrusion transporter mediates berberine accumulation into vacuoles in *Coptis japonica*

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ABSTRACT

Plants produce a large variety of alkaloids, which have diverse chemical structures and biological activities. Many of these alkaloids accumulate in vacuoles. Although some membrane proteins on tonoplasts have been identified as alkaloid uptake transporters, few have been characterized to date, and relatively little is known about the mechanisms underlying alkaloid transport and accumulation in plant cells. Berberine is a model alkaloid. Although all genes involved in berberine biosynthesis, as well as the master regulator, have been identified, the gene responsible for the final accumulation of berberine at tonoplasts has not been determined. This study showed that a multidrug and toxic compound extrusion protein 1 (CjMATE1) may act as a berberine transporter in cultured *Coptis japonica* cells. CjMATE1 was found to localize at tonoplasts in *C. japonica* cells and, in intact plants, to be expressed preferentially in rhizomes, the site of abundant berberine accumulation. Cellular transport analysis using a yeast expression system showed that CjMATE1 could transport berberine. Expression analysis showed that RNAi suppression of *CjbHLH1*, a master transcription factor of the berberine biosynthetic pathway, markedly reduced the expression of *CjMATE1* in a manner similar to the suppression of berberine biosynthetic genes. These results strongly suggest that CjMATE1 is the transporter that mediates berberine accumulation in vacuoles.

1. Introduction

Plants produce a vast number of secondary metabolites with high chemical diversity, enabling them to adapt to various environments. Based on their chemical features, these plant metabolites can be classified as terpenoids, phenols, and alkaloids. Alkaloids constitute a unique array of nitrogen-containing compounds, which often show strong biological activities, with some of these compounds used as human medicines and as toxins. Because most plant alkaloids are biosynthesized in a species-specific manner, most studies of the biosynthesis and transport of individual alkaloids are limited to particular plant species. A wide array of biosynthetic enzymes and associated transcription factors have been identified for some representative plant alkaloids, including nicotine, morphine and vincristine (Beaudoin and Facchini, 2014; De Luca et al., 2014; Dewey and Xie, 2013).

Berberine is a benzylisoquinoline alkaloid and the representative secondary metabolite of the perennial medicinal plant *Coptis japonica*, which belongs to the Ranunculaceae (Supplemental Fig. S1A). In intact *C. japonica* plants, berberine is synthesized in root tissues, and then translocates mainly into rhizomes, where it accumulates. Two *C. japonica* cell culture systems, the 156-S and CjY cell lines, which differ in their ability to produce berberine, have been established and used to study berberine biosynthesis and transport (Supplemental Figs. S1B and C). These cell cultures have enabled the identification of many genes involved in berberine production, including genes encoding two transcription factors, CjWRKY1 and CjbHLH1, both of which positively regulate berberine production (Kato et al., 2007; Yamada et al., 2011); all 13 genes

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encoding the enzymes involved in the biosynthesis of berberine from tyrosine (Sato, 2013); and genes encoding two membrane transporters, CjABCB1 and CjABCB2, which are involved in berberine uptake from apoplasts into cytoplasm at the plasma membrane (Shitan et al., 2003, 2013). Isolation of intact vacuoles from cultured *C. japonica* cells showed that berberine accumulated exclusively in these vacuoles (Sato et al., 1993). However, the gene responsible for the last step, i.e., vacuolar accumulation of berberine, has not yet been identified.

Our previous biochemical transport studies using vacuolar membranes of *C. japonica* suggested that berberine was transported across the tonoplast via a H^+/berberine antiporter (Otani et al., 2005). Members of the multidrug and toxic compound extrusion (MATE) transporter family have been found to mediate the secondary transport of organic substances, using a H^+ electrochemical gradient across the localized membrane as the driving force, suggesting that a member of the MATE family may be a candidate berberine transporter across the tonoplast. About 50 genes encoding MATE family members have been identified in diploid plant genomes such as *Arabidopsis*, whereas more than 100 genes have been detected in the genome of soybean, an amphidiploid plant (Li et al., 2002; Liu et al., 2016). Plant MATE transporters are involved in a wide range of biological events during plant development (Takanashi et al., 2014), with transport of secondary metabolites being among their major physiological roles. Depending on the molecular species they may transport both cationic and anionic compounds. Four MATE members have been reported to mediate nicotine accumulation into vacuoles of *Nicotiana tabacum* (Morita et al., 2009; Shitan et al., 2014b; Shoji et al., 2009), and more than 10 MATE transporters were found to be involved in vacuole uptake of anthocyanin in several dicot plants (Gomez et al., 2009; Marinova et al., 2007; Zhao and Dixon, 2009). While localized at plasma membrane, the *Arabidopsis* MATE transporter, AtDTX1 was shown to be a specific exporter of a broad range of xenobiotics and to recognize berberine as a transport substrate (Li et al., 2002). Taken together, all of these findings suggested that the MATE family may include a transporter responsible for the movement of berberine across the tonoplast of *C. japonica*.

This study demonstrates the functional characterization of a MATE transporter, CjMATE1, which was isolated from cultured *C. japonica* cells. When CjMATE1 was expressed in yeast vacuole membranes, the yeast could accumulate more berberine than the empty vector control. Moreover, the expression of *CjMATE1* in cultured *C. japonica* cells was regulated by CjbHLH1, a positive regulator of berberine biosynthesis (Yamada et al., 2011), suggesting that CjMATE1 is a protein mediating the accumulation of berberine into vacuoles.

2. Results

2.1. Survey of H^+-antiporters in EST library of cultured *C. japonica* cells

Using membrane vesicles prepared from cultured *C. japonica* cells, we previously demonstrated that berberine transport across the vacuole membrane was mediated by a H^+-antiporter (Otani et al., 2005). We therefore surveyed an EST library of cultured *C. japonica* cells to identify putative H^+-antiporter genes (Kato et al., 2007; Morishige et al., 2002; Yamada et al., 2011). Among 4242 EST sequences, 89 were presumed to encode membrane transporters (Supplemental Fig. S2). Of those, five sequences coded for putative H^+-antiporters, and 3′-RACE experiments showed that four of these sequences were partial cDNA fragments of a single gene (Supplementary Table S1). This gene was found to encode a MATE transporter, designated CjMATE1. Because we could not amplify another singlet EST, probably due to its low level of expression, we focused on functional analyses of CjMATE1.

2.2. Berberine transport activity of CjMATE1

To investigate whether this MATE transporter has berberine transport activity, a cellular transport assay was performed using a strain of *Saccharomyces cerevisiae* (Δyor1Δycf1), in which two vacuolar ATP-binding cassette (ABC) proteins involved in yeast multidrug resistance were disrupted (Decottignies et al., 1998). GFP was fused to the C-terminus of CjMATE1, and the construct was introduced into *S. cerevisiae* strain Δyor1Δycf1. Microscopic examination showed that GFP fluorescence was detected specifically at the vacuole membrane of yeast transformants (Fig. 1A).

For the transport analysis, we employed CjMATE1 without the GFP-tag to avoid any effects of the tag. CjMATE1-expressing yeast cells or control cells harboring an empty vector were incubated for 5 h in half-strength SD medium containing berberine (700 μM), and the cellular accumulation of berberine was quantitated by HPLC analysis. The berberine content was much higher in yeast cells expressing CjMATE1 than in control cells (3.5 vs. 1.8 μmol/g fresh weight), indicating that yeast cells expressing CjMATE1 can accumulate more berberine than the negative control, a finding consistent with the localization of CjMATE1 in yeast vacuole membranes (Fig. 1B). To investigate the sensitivity of CjMATE1-expressing yeast cells to berberine, these cells were spotted onto SD medium containing 0.4 or 1.0 mM berberine. However, cell

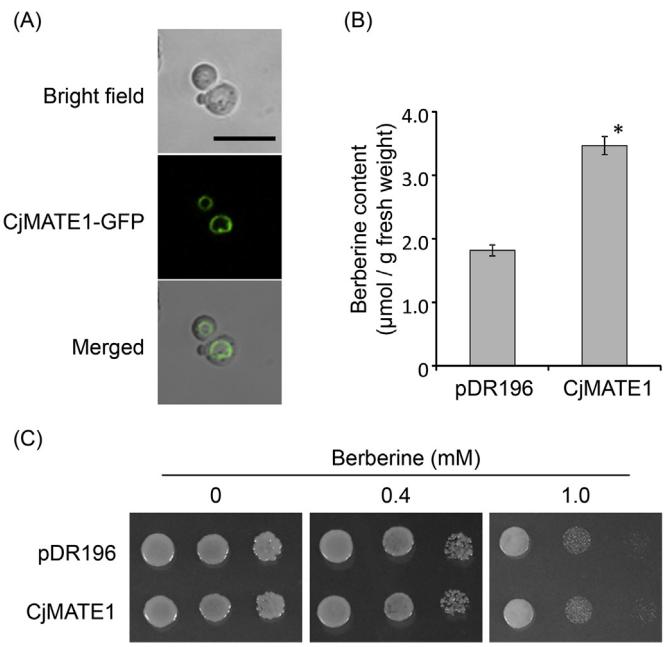

Fig. 1. Assessment of cellular transport by CjMATE1 using yeast transformants. (A) Localization of CjMATE1-GFP at the vacuolar membrane of yeast cells. Yeast cells expressing CjMATE1-GFP were grown at 30 °C to the logarithmic growth phase and observed by fluorescence microscopy. Bars = 10 μm. (B) Berberine accumulation in yeast cells. Control and CjMATE1-expressing yeast cells were incubated in half-strength SD medium containing berberine (700 μM) for 6 h. Berberine contents were quantitatively analyzed with HPLC. CjMATE1-expressing yeast cells accumulated significantly more berberine than control (*$P < 0.05$ by Student's T-test; $n = 4$). (C) Spot assay for berberine tolerance. Control (empty vector, pDR196) and CjMATE1-expressing yeast cells were incubated overnight in half-strength SD medium. The cells were diluted to OD600 = 0.5, and 10-fold serial dilutions were made. Aliquots (5 μl) of each dilution were spotted onto SD plates containing 0–1.0 mM berberine, and cells were grown for 2 days at 30 °C. No significant differences were observed between control and CjMATE1-expressing cells.

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