Characterization of Vacuolar Transport of the Endogenous Alkaloid Berberine in Coptis japonica

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Alkaloids comprise one of the largest groups of plant secondary metabolites. Many of them exhibit strong biological activities, and, in most cases, they are accumulated in the central vacuole of alkaloid-producing plants after synthesis. However, the mechanisms involved in alkaloid transport across the tonoplast are only poorly understood. In this study, we analyzed the vacuolar transport mechanism of an isoquinoline alkaloid, berberine, which is produced and accumulated in the vacuole of cultured cells of Coptis japonica. The characterization of berberine transport using intact vacuoles and a tonoplast vesicle system showed that berberine uptake was stimulated by Mg/ATP, as well as GTP, CTP, UTP, and Mg/pyrophosphate. Berberine uptake was strongly inhibited by NH4 and bafilomycin A1, while vanadate, which is commonly used to inhibit ATP-binding cassette transporters, had only a slight effect, which suggests the presence of a typical secondary transport mechanism. This is contrary to the situation in the plasma membrane of this plant cell, where the ATP-binding cassette transporter is involved in berberine transport. Model experiments with liposomes demonstrated that an ion-trap mechanism was hardly implicated in berberine transport. Further studies suggested that berberine was transported across the tonoplast via an H+/berberine antiporter, which has a Km value of 43.7 μM for berberine. Competition experiments using various berberine analogs, as well as other classes of alkaloids, revealed that this transporter is fairly specific, but not exclusive, for berberine.

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the ATP-binding cassette (ABC) transporter, is present on the tonoplast and might be responsible for the transport of secondary metabolites (Klein et al., 2000). However, there have been few detailed biochemical analyses of the vacuolar membrane transport of endogenous alkaloids.

To study the mechanism of alkaloid transport in plant vacuoles, we have been using a yellow isoquinoline alkaloid, berberine, which was the first alkaloid to have its biosynthesis fully described at the enzyme level (Zenk, 1995). Berberine is stable and widely used as an antibacterial and antimalaria drug in many countries (Yamamoto et al., 1993; Iwasa et al., 1998). While it is one of the most widely occurring alkaloids in many plant families, we used cell cultures of Coptis japonica (Ranunculaceae) as a model system. C. japonica is a perennial medicinal plant grown in Asian countries, and berberine is highly accumulated in its rhizome as its main alkaloid. Cultured C. japonica cells produce berberine and accumulate it exclusively in the vacuole (Sato et al., 1990, 1994). Moreover, exogenous berberine added to the culture medium is actively taken up by C. japonica cells (Sato et al., 1990, 1994), and is also transported into the vacuole and stably accumulated in the lumen (Sato et al., 1993; Sakai et al., 2002). Our previous studies to identify the berberine transporter in C. japonica cells showed that CjMDR1, a multidrug-resistance protein-type ABC transporter, was involved in the transport of berberine at the plasma membrane (Shitan et al., 2003). However, it is still unclear how berberine is transported across the tonoplast.

In this study, we analyzed the vacuolar transport mechanism of berberine by using intact vacuoles and tonoplast vesicles of C. japonica cells. Berberine transport into tonoplast vesicles was stimulated by Mg/ATP and was inhibited by NH$_4^+$ and bafilomycin A1, but not by vanadate, which suggested that uptake involved an H$^+$/berberine-antiporter.

RESULTS

Uptake of [$^3$H]Berberine by Isolated Vacuoles

To clarify the mechanism of the vacuolar uptake of berberine, we first isolated intact vacuoles from 2-week-old C. japonica suspension cultures. Vacuoles of C. japonica cells appear to be bright yellow because of the high concentration of endogenous berberine, which reaches more than 72 mM. Their diameter was 11 to 28 µm and the average volume was calculated to be 4.9 µL. The vacuolar pH of C. japonica cells was approximately 5.5. These isolated vacuoles were incubated with radiolabeled berberine in an assay buffer of pH 7.8, which mimicked the endogenous state of vacuoles in terms of the ΔpH across the tonoplast. Incubated vacuoles were passed through a silicon oil layer by centrifugal filtration and [$^3$H]berberine in the vacuoles recovered from the bottom layer was estimated by scintillation counting. Burst vacuoles, which were prepared by sonication in the presence of Triton, were used as a negative control. As a result, the clear uptake of berberine by intact vacuoles was observed in the absence of Mg/ATP, and berberine uptake was somewhat stimulated by Mg/ATP, but not by Mg/ADP and Mg/ATP-γ-S, a nonhydrolyzable analog of ATP (Fig. 1). This suggests that berberine is apparently taken up by intact vacuoles, probably due to the preexisting ΔpH in the assay condition (incubation buffer, pH 7.8), and that ATP enhances berberine transport into the vacuole, whereas the effect is seemingly indirect. However, further detailed analyses of berberine uptake using various inhibitors were hampered because of the instability of the vacuoles, i.e. it was very difficult to perform the assays using solvents such as dimethyl sulfoxide (DMSO). For this reason, we decided to further analyze berberine transport using tonoplast vesicles.

ATP-Dependent Uptake of Berberine by Tonoplast Vesicles

We purified tonoplast vesicles by fractionating microsomes of C. japonica cells on a discontinuous Suc density gradient. Vanadate-sensitive ATPase and
KNO₃-sensitive ATPase activities were measured as marker enzymes for plasma membrane and tonoplasts, respectively. Tonoplast-rich membrane vesicles were recovered at 0/20% Suc fraction (Fig. 2A). The enrichment of the tonoplast in this fraction was also confirmed by immunodetection of vacuolar pyrophosphatase (V-PPase), a tonoplast marker, whereas plasma membrane H⁺-ATPase and luminal binding protein (BiP), an ER marker, sedimented at positions different from V-PPase (Fig. 2B). The tonoplast vesicle fraction showed clear [³H]berberine uptake, while other fractions, which contain a large portion of plasma membrane and ER, did not show the uptake activity even in the presence of ATP (Fig. 2C). These results suggested that the tonoplast vesicles had the berberine transport activity, which was not observed in plasma or ER membranes. Using the tonoplast vesicles, we measured the time course of [³H]berberine uptake (Fig. 2D). Rapid uptake was observed in the presence of Mg/ATP, whereas no uptake was seen in the absence of Mg/ATP, which indicates that Mg/ATP is required for berberine uptake in this assay system. This berberine transport activity was reproducibly observed among each preparation of the tonoplast vesicles, i.e. the uptake within the range between 12 and 18 pmol/µg protein was observed for 5 min in the presence of Mg/ATP.

We also investigated the pH dependency of berberine uptake by this assay method. The tonoplast vesicles were incubated in reaction mixtures of different pH values within the range of physiological conditions, from pH 6.5 to 8.0. Figure 3 shows that berberine uptake rose as pH increased from 6.5 and had an optimum pH around 7.5, which is in the common cytosolic pH.

Next, we investigated whether the effect of energization on the vacuolar transport of berberine was exclusive to Mg/ATP, or if it could be substituted by other nucleotides. Figure 4 shows that, although some of these effects were only slight, Mg/GTP, Mg/CTP, and Mg/UTP could each stimulate berberine uptake (23%, 14%, and 9% of Mg/ATP, respectively), while the addition of Mg/AMP had no effect, confirming that the hydrolysis of these nucleotide triphosphates was necessary for berberine uptake. Notably, Mg/pyrophosphate was also able to stimulate berberine uptake.

Figure 2. Time-dependent uptake of berberine into tonoplast vesicles. A, Tonoplast vesicles were prepared by Suc gradient fractionation. Vanadate-sensitive ATPase (■) and KNO₃-sensitive ATPase (○) activities were measured as marker enzymes for plasma membrane and tonoplast content, respectively. B, Plasma membrane H⁺-ATPase, V-PPase, and BiP were immunodetected to confirm the purity of tonoplast vesicles. C, Each membrane fraction was incubated with 50 µM of [³H]berberine in the presence (●) or absence (○) of 5 mM Mg/ATP. D, Tonoplast vesicles (0/20% Suc fraction) were incubated with 50 µM of [³H]berberine in the presence (●) or absence (○) of 5 mM Mg/ATP. Berberine uptake was monitored at 25°C, as described in “Materials and Methods.” Data are an average of three replicates and are presented as pmol/µg protein.
uptake to about 35% the amount of Mg/ATP, suggesting the involvement of a transport mechanism that depends on the H\(^+\)-gradient across the tonoplast.

Table I shows a comparison of berberine uptake in the presence of various inhibitors. The addition of NH\(_4\)Cl, which abolishes the ΔpH across the membrane, inhibited berberine uptake by 80%, and the V-type ATPase inhibitor bafilomycin A1 reduced uptake by 68%. On the other hand, vanadate, which is a typical inhibitor of ABC transporters, exhibited only 10% inhibition, which excludes the possible involvement of an ABC transporter as the main transport mechanism of berberine into vacuoles of \textit{C. japonica}.

Basic compounds, such as amines and alkaloids, may be trapped by membrane vesicles via a differential pH shift, i.e. when the inside of a membrane vesicle is acidic, an amine compound that permeates through the lipid layer under the neutral condition of the cytosol is protonated under the acidic conditions inside vesicles, which forms a polar quaternary amine that cannot permeate back across the lipid layer and is thus trapped inside (ion-trap mechanism). Since the quaternary nitrogen atom of berberine is part of an aromatic six-member ring structure, berberine does not take protonation form, the positive charge remaining stable under physiological conditions (structure is shown in Table II), and the permeability of berberine through the lipid bilayer is thought not to be very high. To exclude the possibility of an ion-trap mechanism for the transport and accumulation of berberine by vacuoles, we measured the uptake of berberine into liposomes that contain a stable pH gradient (interior acidic) and compared it to the uptake of a model compound, methylamine, which is transported into vacuoles by an ion-trap mechanism (Churchill and Sze, 1983). In the presence of the pH gradient, methylamine was taken up and accumulated in liposomes, i.e. 6-fold accumulation was observed compared to that in the absence of the pH gradient. Berberine accumulation with the pH gradient was 1.6-fold higher than that without the pH gradient (Fig. 5).

The reason for the marginal difference in berberine accumulation in liposomes between in the presence and absence of the pH gradient is not clear. One possible explanation is that membrane potential or berberine solubility might be affected by NH\(_4\)Cl, which altered hydrophobic interaction between berberine and liposome, resulting in lower accumulation in the absence of the pH gradient. Using the tonoplast vesicles, on the other hand, berberine accumulation with ATP was approximately 4-fold higher than that without ATP, as shown in Figure 2D. Taken together, berberine was unlikely to be accumulated by an ion-trap mechanism, but rather a specific carrier was necessary for berberine transport in the \textit{C. japonica} tonoplast.

**Kinetic Determinants and Competitive Inhibition of Vacuolar Transport of Berberine**

The uptake of berberine by tonoplast vesicles exhibited \(K_m\)-type saturation kinetics, as shown in Figure 6. The \(K_m\) and \(V_{max}\) values were calculated to be 43.7 μM and 13.5 pmol/μg protein per min, respectively.

Next, we carried out a competitive inhibition experiment in the berberine uptake assay system. Table II shows the effects of berberine analogs, as well as nicotine and vinblastine, on [\(^3\)H]berberine uptake by tonoplast vesicles. A protoberberine-type alkaloid, reticuline, and another berberine analog, palmatine, inhibited berberine uptake, whereas norlaudanosoline, tetrahydropalmatine, and a pyrrolidine-type alkaloid, nicotine, did not show competitive inhibition. Interestingly, vinblastine, which is an indole alkaloid and shows no structural resemblance to berberine, also inhibited berberine uptake. This result suggests that the antiporter shows a preference for protoberberine-type alkaloids, but is not exclusive to berberine.

**DISCUSSION**

Biosynthetic reactions of alkaloids occur in various organelles in plant cells, which may vary according
Vacuolar Transport of Alkaloid

**Table I. Effects of various inhibitors on ATP-dependent berberine uptake**

Tonoplast vesicles were incubated in the presence of 50 \( \mu \text{M} \) \( [3\text{H}] \)berberine and the compounds listed. Results are mean \( \pm \text{SD} \) of three replicates. Asterisk indicates statistically significant difference compared to + ATP (*, \( P < 0.05 \); **, \( P < 0.01 \)).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Berberine Uptake (%)</th>
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<tbody>
<tr>
<td>+5 mM MgATP</td>
<td>100</td>
</tr>
<tr>
<td>+5 mM MgATP + NH(_4)^+ (10 mM)</td>
<td>20.0 ( \pm ) 6.0**</td>
</tr>
<tr>
<td>+5 mM MgATP + bafilomycin A1 (1 ( \mu \text{M} ))</td>
<td>32.0 ( \pm ) 26.0*</td>
</tr>
<tr>
<td>+5 mM MgATP + vanadate (1 mM)</td>
<td>90.0 ( \pm ) 8.0</td>
</tr>
</tbody>
</table>

to the type of alkaloid involved, e.g. quinolizidine alkaloids of legumes appear to be biosynthesized in mesophyll chloroplasts of green leaves (De Luca and St. Pierre, 2000). However, the final intracellular destination of most biosynthesized alkaloid molecules is vacuoles. In plants, four major uptake mechanisms have been proposed for secondary metabolite translocation across the tonoplast, i.e. \( \text{H}^+ \) have been proposed for secondary metabolite translocation, whereas \( \text{H}^+ \) antiporter contributes to retain these intermediates inside such vesicles, since the antiporter showed relatively broad substrate specificity (Table II). When these vesicles fuse to the large central vacuole, the membrane of vesicles is integrated into the tonoplast. Molecular cloning of an \( \text{H}^+ \)/berberine antiporter and its characterization should provide further information to prove this hypothesis about the mechanism of berberine accumulation in \( C. \text{japonica} \) cells.

It is not yet clear why \( C. \text{japonica} \) cells have two different types of transporters for berberine; i.e. ABC transporter (CjMDR1) at the plasma membrane (Shitan et al., 2003) and \( \text{H}^+/\text{berberine} \) antiporter at the tonoplast, but the difference in their \( V_{\text{max}} \) might offer one possible explanation. Compared to the antiporter at the tonoplast, CjMDR1 appears to pump berberine relatively slowly (the \( V_{\text{max}} \) of CjMDR1 for berberine is 0.08 nmol/mg protein) to the cytosol from the apoplast (Shitan et al., 2003), whereas the transport across the tonoplast by the \( \text{H}^+/\text{berberine} \) antiporter seems to be more rapid (the \( V_{\text{max}} \) of the antiporter is 13.5 nmol/mg protein). If this difference in transport efficiency is valid for \( C. \text{japonica} \) cells in vivo, the rapid accumulation in vacuoles via precursor-accumulating vesicles (Mitsuhashi et al., 2001). The antiporter mechanism for berberine transport demonstrated in this article may not conflict with vesicle-mediated transport. Precursors of berberine are more hydrophobic than berberine and the \( \text{H}^+/\text{berberine} \) antiporter contributes to retain these intermediates inside such vesicles, since the antiporter showed relatively broad substrate specificity (Table II). When these vesicles fuse to the large central vacuole, the membrane of vesicles is integrated into the tonoplast. Molecular cloning of an \( \text{H}^+ \)/berberine antiporter and its characterization should provide further information to prove this hypothesis about the mechanism of berberine accumulation in \( C. \text{japonica} \) cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Berberine Uptake (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Reticiline</td>
<td>27.9 ( \pm ) 17.4**</td>
</tr>
<tr>
<td>Norlaudanosoline</td>
<td>107.1 ( \pm ) 17.4</td>
</tr>
<tr>
<td>Palmitine</td>
<td>62.8 ( \pm ) 5.8**</td>
</tr>
<tr>
<td>Tetrahydropalmatine</td>
<td>100 ( \pm ) 27.9</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>48.8 ( \pm ) 23.3*</td>
</tr>
<tr>
<td>Nicotine</td>
<td>76.7 ( \pm ) 57.1</td>
</tr>
</tbody>
</table>

![Diagram](image-url)
transport by the antiporter would largely contribute to the detoxification of this cytotoxic alkaloid; i.e., the cytosolic concentration of berberine in *C. japonica* cells can be kept at a low level by the scheme shown in Figure 7. Therefore *C. japonica* cells are capable of safely accumulating berberine in vacuoles even if they are cultured in the presence of 750 μM berberine in the medium, which is highly toxic to tobacco (*Nicotiana tabacum*) and other plant cells, but not to *C. japonica* cells (Sakai et al., 2002).

In bacteria, several antiporters that accept berberine as their substrate have been reported, although Na⁺ is the counterpart instead of H⁺, and their roles as drug efflux pumps for the purpose of detoxification have been demonstrated. For example, NorM from *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*, YdhE, which are both multidrug and toxic compound extrusion (MATE)-type transporters, efflux several toxic compounds, including berberine, and confer multidrug resistance to these microorganisms (Xu et al., 2003). In plants, a plasma membrane-localized MATE-type transporter of Arabidopsis (*Arabidopsis thaliana*), AIDTX1, which also recognizes berberine as an exogenous substrate (Li et al., 2002), has been reported so far as an H⁺/berberine antiporter of *C. japonica*, although the MATE family in Coptis may be different from these known MATE members since the former may only be responsible for the endogenous alkaloid, not other exogenous compounds.

Plant alkaloids are often translocated from the source organ to a sink organ (Hashimoto and Yamada, 1994; Hartmann, 1999). Berberine in *C. japonica* is also translocated from root tissues to rhizome, which involves transport across several membranes, i.e., at the plasma membrane in root and rhizome and at the tonoplast in cortex cells of rhizome. Together with our previous work on CjMDR1 at the plasma membrane, this study on the tonoplast should help to clarify the entire molecular mechanism of the translocation of alkaloids in plants.

**MATERIALS AND METHODS**

**Chemicals**

Chemicals used in this study were purchased from Wako Pure Chemicals (Osaka) or Nakalai Tesque (Kyoto).

![Figure 5](image-url) Berberine uptake into liposomes in the presence and absence of a pH gradient. A, [¹⁴C]Methylamine uptake into liposomes. B, [³H]Berberine uptake into liposomes. A pH gradient was generated as described in “Materials and Methods.” Liposomes were incubated at 25°C for 10 min with 20 μM [¹⁴C]methylamine (approximately 1 μCi/mL) or 50 μM [³H]berberine, and uptake was determined as described for the measurement of berberine transport. To destroy the pH gradient, NH₄Cl (10 mM) was added to the incubation mixture. Results are mean ± SD of three replicates. Asterisk indicates statistically significant difference (*, *P* < 0.05; **, *P* < 0.01).

![Figure 6](image-url) Uptake of [³H]berberine into tonoplast vesicles shows *Kₘ*--type saturation kinetics. A, Tonoplast vesicles were incubated in the presence of [³H]berberine. B, Representative saturation experiments are illustrated as a Hanes-Woolf plot. *Kₘ* value measured in three independent experiments ranged between 10 and 50 μM.
Vacuolar Transport of Alkaloid

Figure 7. Proposed model for berberine transport and accumulation. Exogenous berberine is taken up relatively slowly by CjMDR1, an ABC transporter, while the H+/berberine antiporter effluxes berberine very rapidly from the cytosol to the vacuole via a proton gradient formed by V-ATPase and V-PPase, resulting in a low berberine concentration in the cytosol.

Cultured Cells

High berberine-producing cultures of Coptis japonica, which were originally induced from the rootlets of C. japonica Makino var. dissecta (Yamabe), were maintained as described (Sato and Yamada, 1984). Two-week-old cells were used for the experiments.

Isolation of Vacuoles

Protoplasts were isolated from cultured cells by the procedure of Sato et al. (1990). Isolation of vacuoles from protoplasts was carried out by the procedure of Sato et al. (1992). Isolated purified vacuoles were used immediately for the uptake experiment.

Preparation of Tonoplast Vesicles

The tonoplast vesicles used in this study were prepared from C. japonica cells by the procedure of Rocha Facanha and de Meis (1998) with some modifications. Cells were collected and homogenized in 2 mL/g of ice-cold homogenizing buffer (10% [v/v] glycerol, 0.5% [w/v] polyvinylpolypyrrolidone, 5 mM EDTA, and 0.1 mM Tris-HCl adjusted to pH 7.6 and autoclaved). Prior to use, 150 mM KCl, 3.3 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride were added to the buffer and the mixture was stirred for 1 min. The following modifications were also applied: Homogenate was strained through Miracloth (Merck, Rahway, NJ) instead of cheesecloth; 20%, 30%, 40% sucrose solutions instead of water. Isolated membrane vesicles were used for the experiments.

Preparation of [3H]Berberine

[3H]Berberine (specific activity, 7.4 mCi/nmol) was prepared by labeling berberine with Na[B3H]4. First, 25 mg of cold NaBH4 were added to 10 mL of Na[B3H]4 (Amersham Biosciences, Buckinghamshire, UK) for dilution, which was dissolved in 100 MU of 0.2% NaOH. To this solution was slowly added sodium dihydrogen phosphate (50 mg dihydrate), and the mixture was stirred for 8 h. Next, 100 mg I2 were added to oxidize the tetrahydroberberine and the mixture was stirred for 1 h. Excess I2 was decomposed with 120 mg NaHSO3 by stirring for 30 min. The berberine iodide that precipitated in the solution was recovered by filtration, and berberine chloride was formed by treatment with 200 MU AgCl. The yielded berberine chloride was further purified by preparative thin-layer chromatography, using the solvent system by the procedure of Ikuta and Yokawa (1988). Berberine was recovered by elution with methanol, which was evaporated to dryness and dissolved in water for transport assay.

Measurement of Berberine Transport

Uptake of [3H]berberine by intact vacuoles was measured by the centrifugal filtration technique through silicone oil as described in the literature (Heldt and Sauer, 1971) with some modifications. Isolated vacuoles were incubated with 1 MU [3H]berberine at 25°C in vacuole resuspension buffer (0.7 M sucrose, 0.2 mM glycinebetaine, 1 mM NaH2PO4, 2 mM HEPES-NaOH, 1 mg/mL bovine serum albumin, pH 7.8) for 20 min. As the negative control, burst vacuoles prepared by sonication in the presence of 2% Triton-X were used. Filtering centrifugation was carried out at room temperature using 400-μL plastic tubes. The tube contained the (from bottom) 50 μL bottom buffer (25% Percoll, 0.7% bovine serum albumin, 2% Triton-X), 150 μL silicone oil (SHSSH, Toray Dow Corning Co. Ltd., Tokyo), and 70 μL vacuoles in incubation buffer, and was centrifuged at 6,000 g for 1 min. The contents of the plastic tube were then frozen in liquid N2, the bottom layer was recovered by cutting off the tube with a knife, and radioactivity was estimated by a scintillation counter (Beckman Instruments, Fullerton, CA).

Transport Assay with Liposomes

Liposomes having transmembrane pH gradients were prepared by the procedure of Mayer et al. (1990) with some modifications. Briefly, asolectin (Wako Pure Chemicals) was dissolved in 300 mM citrate buffer (pH 5.5) at a concentration of 5 mg/mL. Multilamellar vesicles were subjected to five freeze-thaw cycles (freezing in liquid N2 and thawing at 40°C). Transmembrane pH gradients were established by passing the vesicles through a Sephadex G-25 column (PD-10) equilibrated in 20 mM HEPES containing 150 mM NaCl (pH 7.5). Liposomes (1.25 mg/mL) were incubated at 25°C for 10 min with 20 μM [14C]methylamine (approximately 1 μCi/mL) or 50 μM [3H]berberine. The uptake of methylamine or berberine by liposomes was measured by the spin-column assay, as described above, for the measurement of berberine transport.
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LITERATURE CITED


