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

Mihoko Otani2, Nobukazu Shitan2, Kyoko Sakai, Enrico Martinoa, Fumihiko Sato, and Kazufumi Yazaki*

Division of Integrated Life Science, Graduate School of Biosciences (M.O., F.S.), and Division of Applied Life Sciences, Graduate School of Agriculture (K.S., F.S.), Kyoto University, Kyoto 606–8502, Japan; Laboratory of Plant Gene Expression, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji 611–0011, Japan (N.S., K.Y.); and Zurich Basel Plant Science Center, University of Zurich, Plant Biology, CH–8008 Zurich, Switzerland (E.M.)

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.

Alkaloids, which are nitrogen-containing low-Mr substances, are a major class of plant secondary metabolites that show a wide variety of chemical structures and biological activities. Some are used in both modern and traditional medicine. For instance, vincristine and taxol are widely used as anticancer drugs and morphine is an indispensable analgesic in clinical medicine. They also play important roles in plants as endogenous biological barriers to protect against pathogens or herbivores due to their strong antimicrobial, antifungal (Iwasa et al., 1998; Mahady et al., 2003), and anti-insect activities (Steppuhn et al., 2004). The importance of alkaloids to both humans and plants has attracted the attention of many researchers. Many systems for the production of alkaloids have been established (Kutchan, 1995) and biosynthetic pathways have been actively studied (Facchini, 2001). However, the transport and accumulation mechanisms of these alkaloids in plant cells are still largely unknown.

On the other hand, alkaloid-producing plant cells seem to be insensitive to their own metabolites, probably because they have a detoxification mechanism to prevent the cytotoxicity of alkaloids. However, such plant detoxification of secondary metabolites is not well understood. One possible explanation is the compartmentation of alkaloids into the plant vacuole. Many alkaloids are presumed to be synthesized in the cytosol and on the endoplasmic reticulum (ER) and then transported through the tonoplast to be sequestered in the vacuolar matrix. A simple model for alkaloid accumulation in plant vacuoles is the ion-trap mechanism (Matile, 1976). According to this model, alkaloids, which are in a lipophilic state in the neutral pH of cytosol, can freely pass through the tonoplast by simple diffusion. However, under the acidic conditions inside the vacuole, they are protonated to form hydrophilic cations, and therefore become unable to permeate through the membrane and are trapped inside. Contrary to this hypothesis, several studies have demonstrated that the vacuolar transport of alkaloids was managed by specific carriers in an energy-requiring manner, which may involve a proton-antipor transport carrier system (Deus-Neumann and Zenk, 1984, 1986; Mende and Wink, 1987; Wink and Mende, 1987). A third possible mechanism was recently proposed; i.e. a directly energized transporter,
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 pL. 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/mg 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.
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 NH₄Cl, 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 *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 NH₄Cl, 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 *C. japonica* tonoplast.

**Kinetic Determinants and Competitive Inhibition of Vacular 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 $[^3H]$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
The ion-trap mechanism was not applicable to berberine accumulation in this plant, but rather a certain carrier-mediated transport mechanism is involved. Based on these results, we conclude that berberine was transported across the tonoplast via an H\(^+\)/berberine antiporter, which uses the proton gradient formed by the two vacuolar proton pumps, vacuolar H\(^+\)-ATPase and PPase, localized at the tonoplast (Fig. 7).

When the subcellular localization of biosynthetic enzymes of berberine is considered, another transport mechanism may be possible for endogenously biosynthesized berberine. The terminal steps of berberine biosynthesis are reportedly located exclusively in specific vesicles that are observed in Berberis and other berberine-producing plant cell cultures (Amann et al., 1986; Galneder et al., 1988). These vesicles are described as highly specific and unique compartments, which are probably derived from the ER, and later fuse with the central vacuole (Bock et al., 2002). A similar mechanism was also proposed for another isoquinoline alkaloid, sanguinarine, by a recent study using cultured opium poppy (Papaver somniferum) cells (Alcantara et al., 2005). According to this scheme, endogenous berberine is thought to be synthesized in these specific vesicles and transported to the vacuole by a vesicle transport mechanism. This mechanism seems to be very similar to that of storage protein 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 H\(^+\)/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 H\(^+\)/berberine antiporter and its characterization should provide further information to prove this hypothesis about the mechanism of berberine accumulation in C. japonica cells.

It is not yet clear why C. japonica cells have two different types of transporters for berberine; i.e. ABC transporter (CjMDR1) at the plasma membrane (Shitan et al., 2003) and H\(^+\)/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 H\(^+\)/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. japonica cells in vivo, the rapid

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Berberine Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>+5 mM MgATP</td>
<td>100</td>
</tr>
<tr>
<td>+5 mM MgATP + NH(_4)(^+) (10 mM)</td>
<td>20.0 ± 6.0**</td>
</tr>
<tr>
<td>+5 mM MgATP + bafilomycin A1 (1 mM)</td>
<td>32.0 ± 26.0*</td>
</tr>
<tr>
<td>+5 mM MgATP + vanadate (1 mM)</td>
<td>90.0 ± 8.0</td>
</tr>
</tbody>
</table>

### Table II. Effects of various compounds, including protoberberine alkaloids and other alkaloids, on berberine uptake

<table>
<thead>
<tr>
<th>Compound</th>
<th>Berberine Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Reticuline</td>
<td>27.9 ± 17.4**</td>
</tr>
<tr>
<td>Norlaudanosoline</td>
<td>107.1 ± 17.4</td>
</tr>
<tr>
<td>Palmatine</td>
<td>62.8 ± 5.8**</td>
</tr>
<tr>
<td>Tetrahydropalmatine</td>
<td>100 ± 27.9</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>48.8 ± 23.3*</td>
</tr>
<tr>
<td>Nicotine</td>
<td>76.7 ± 57.1</td>
</tr>
</tbody>
</table>

**Table II.** Effects of various compounds, including protoberberine alkaloids and other alkaloids, on berberine uptake

Tonoplast vesicles were incubated with 50 μM [\(^3\)H]berberine and various compounds in the absence (control) or presence of 250 μM of the compounds indicated. Results are mean ± SD of three replicates. Asterisk indicates statistically significant difference compared to control (*, P < 0.05; **, P < 0.01).
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+. 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*), AtDTX1, which also recognizes berberine as an exogenous substrate (Li et al., 2002), has been reported so far as an H+/berberine antiporter that acts as a detoxifier. Sequence data of these known antiporters can be used to clone the 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](https://plantphysiol.org)

**Figure 5.** 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](https://plantphysiol.org)

**Figure 6.** Uptake of [3H]berberine into tonoplast vesicles shows $K_m$-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. $K_m$ value measured in three independent experiments ranged between 10 and 50 μM.
**Preparation of [3H]Berberine**

[3H]Berberine (specific activity, 7.4 mCi/mmol) was prepared by labeling berberine with NaB[3H]I. First, 25 mg of cold NaBH4 were added to 10 mL of NaB[3H]I (Amersham Biosciences, Buckinghamshire, UK) for dilution, which was dissolved in 100 μL of 0.2% NaOH. To this solution was slowly added berberine hydrochloride (50 mg) dissolved in water, which was stirred for 8 h. Next, 100 mg LiI were added to oxidize the tetrahydro-berberine and the mixture was stirred for 1 h. Excess LiI was decomposed with 120 mg NaHSO4, 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 mg AgCl. The yielded berberine chloride was further purified by preparative thin-layer chromatography, using the solvent system by the procedure of Ikuta and Toka (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 centrifuged filtration technique through silicone oil as described in the literature (Heldt and Sauer, 1971) with some modifications. Isolated vacuoles were incubated with 1 μmol [3H]berberine at 25°C in vacuole resuspension buffer (0.2 M Glycin betaine, 1 mM NaH4PO4, 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 (from the bottom) 50 μL bottom buffer (25% Percoll, 0.7 M sorbitol, 2% Triton-X), 150 μL silicone oil (SH550:SH556 77:23; Toray Dow Corning Co. Ltd, Tokyo), and 70 μL vacuoles in incubation buffer, and was centrifuged at 6,000g 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 μL 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 8.0 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%, and each fraction recovered from interfaces was resuspended with resuspension buffer (10% v/v glycerol, 1 mM EDTA, and 10 mM Tris-HCl adjusted to pH 7.6 and autoclaved) instead of water. Isolated membrane vesicles were stored at 80°C in resuspension buffer containing 10 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Vanadate-sensitive ATPase and KNO3-sensitive ATPase activities were measured as marker enzymes for plasma membrane and tonoplastic contents, respectively. For the berberine uptake, the membrane vesicles obtained from 0/20% Suc fraction were used as the tonoplast vesicles. The purity of tonoplastic vesicles was checked by immunodetection. The antibodies used for immunodetection were against plasma membrane H+-ATPase, vacuolar H+-pyrophosphatase from Arabidopsis (Arabidopsis thaliana), and ER luminal BiP (see “Acknowledgments” for the sources of antibodies).

**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 Melis (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 8.0 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%, and each fraction recovered from interfaces was resuspended with resuspension buffer (10% v/v glycerol, 1 mM EDTA, and 10 mM Tris-HCl adjusted to pH 7.6 and autoclaved) instead of water. Isolated membrane vesicles were stored at −80°C in resuspension buffer containing 10 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Vanadate-sensitive ATPase and KNO3-sensitive ATPase activities were measured as marker enzymes for plasma membrane and tonoplastic contents, respectively. For the berberine uptake, the membrane vesicles obtained from 0/20% Suc fraction were used as the tonoplast vesicles. The purity of tonoplastic vesicles was checked by immunodetection. The antibodies used for immunodetection were against plasma membrane H+-ATPase, vacuolar H+-pyrophosphatase from Arabidopsis (Arabidopsis thaliana), and ER luminal BiP (see “Acknowledgments” for the sources of antibodies).
ACKNOWLEDGMENTS

We thank Dr. Yoshinori Moriyama of Okayama University and Dr. Eduardo Blumwald of the University of California for their helpful technical advice regarding the transport assay. We are also grateful to Dr. M. Boutry, Université Catholique de Louvain, for providing anti-H\(^{\prime}\)ATPase antibodies, Dr. M. Sato, Kyoto University, for anti-V-PPase antibodies, and Dr. N. Koizumi, Nara Institute of Science and Technology, for anti-BiP antibodies. Received April 19, 2005; revised May 19, 2005; accepted May 19, 2005; published July 15, 2005.

LITERATURE CITED


