Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (MATE) transporter in *Nicotiana tabacum*

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Alkaloids play a key role in plant defense mechanisms against pathogens and herbivores, but the plants themselves need to cope with their toxicity as well. The major alkaloid of the *Nicotiana* species, nicotine, is translocated via xylem transport from the root tissues where it is biosynthesized to the accumulation sites, the vacuoles of leaves. To unravel the molecular mechanisms behind this membrane transport, we characterized one transporter, the tobacco (*Nicotiana tabacum*) jasmonate-inducible alkaloid transporter 1 (Nt-JAT1), whose expression was coregulated with that of nicotine biosynthetic genes in methyl jasmonate-treated tobacco cells. Nt-JAT1, belonging to the family of multidrug and toxic compounds, was expressed in roots, stems, and leaves, and localized in the tonoplast of leaf cells. When produced in yeast cells, Nt-JAT1 occurred mainly in the plasma membrane and showed nicotine efflux activity. Biochemical analysis with protoproteins reconstituted with purified Nt-JAT1 and bacterial F0F1-ATPase revealed that Nt-JAT1 functioned as a proton antiporter and recognized endogenous tobacco alkaloids, such as nicotine and anabasine, and other alkaloids, such as hyoscyamine and berberine, but not flavonoids. These findings strongly suggest that Nt-JAT1 plays an important role in the nicotine translocation by acting as a secondary transporter responsible for unloading of alkaloids in the aerial parts and deposition in the vacuoles.

nicotine | vacuole

One of the largest groups of plant secondary metabolites comprises the alkaloids of which many exhibit strong biological activities. In most cases, after their biosynthesis, alkaloids are deposited in the central vacuoles of alkaloid-producing plants, sometimes after translocation from the organ where they are produced to another organ where they preferentially accumulate. However, only few transporters responsible for alkaloids or other secondary metabolites have been reported so far (1, 2), and no protein involved in vacuolar alkaloid accumulation has been identified yet.

Nicotine, a pyridine alkaloid predominantly found in *Nicotiana* species, functions as a defensive toxin by acting on the nervous system of herbivores and is produced in root tissues, where its biosynthesis increases specifically in response to attacks by pathogens, herbivores, or wounding, usually incurring jasmonate signaling. The produced nicotine is then translocated to the aerial parts of the plant and finally accumulates in the central vacuoles of the leaves (3). Accordingly, putrescine N-methyltransferase (PMT), the enzyme catalyzing the first committed step in nicotine biosynthesis, is specifically produced in the root pericycle, and not in the aerial parts (3). After nicotine is loaded into the xylem tissue, it is delivered at the leaf mesophyll cells and finally is deposited in the vacuoles (4). This translocation process implies the transport of nicotine across at least 3 different membranes. First, the nicotine produced in the root cells is exported to the apoplast by a plasma membrane-localized transporter, mainly to the xylem. Second, after the movement to the aerial tissues, nicotine is taken up at the plasma membrane of leaf cells, and finally transported to the vacuolar lumen by a tonoplast-localized transporter. However, none of these transporters has been identified yet. To discover the transporter(s) and proteins involved in nicotine accumulation in *Nicotiana* species, a cDNA-amplified fragment length polymorphism-based transcript profiling (cDNA-AFLP) had been carried out previously with Bright Yellow-2 (BY-2) cells elicited with methyl jasmonate (MeJA). In such elicited BY-2 cells, the production of nicotine biosynthetic enzymes is induced and a multidrug and toxic compound extrusion (MATE)-type transporter gene has been identified that was highly up-regulated in a coordinate manner with these biosynthetic genes (5).

Here, we characterized this MATE transporter, designated as *Nicotiana tabacum* jasmonate-inducible alkaloid transporter 1 (Nt-JAT1), as a nicotine transporter. Membrane separation and protein gel blot analysis indicated that Nt-JAT1 localized to the tonoplast in tobacco leaf cells. In *Nt-JAT1*-expressing yeast (*Saccharomyces cerevisiae*) cells, the nicotine transport activity increased. Analysis of the driving force in proteoliposomes revealed a proton antiport activity and specificity restricted to organic cations, such as the tobacco alkaloids. These data suggest that Nt-JAT1 is involved in the vacuolar accumulation of nicotine in tobacco plants, at least in the aerial parts.

**Results**

**Cloning and Sequence Analysis of Nt-JAT1.** In intact plants and also in cultivated BY-2 cells, treatment with MeJA strongly induces the biosynthesis of nicotine. Previously, a transcriptome analysis with cDNA-AFLP of MeJA-elicited tobacco BY-2 cells identified a number of nicotine biosynthetic genes, such as arginine decarboxylase, ornithine decarboxylase, aspartate oxidase, and quinolinate phosphoribosyltransferase (5). Simultaneously, several MeJA-inducible genes encoding putative transporter proteins were found, among which a MATE transporter (clone No. T401) that nicely coregulated with these nicotine biosynthetic
genes, that is, induced after 1 to 2 h and with a steadily increasing expression level (Fig. 4A). This gene was designated Nt-JAT1 and was analyzed further in detail.

Quantitative reverse-transcription (qRT)-PCR confirmed that in BY-2 cells Nt-JAT1 was strongly induced by MeJA in a manner similar to that of the PMT gene (Fig. 1B). Accordingly, a time course expression analysis in the aerial parts of 14-day-old tobacco seedlings treated with MeJA showed that the expression of Nt-JAT1 sharply increased 2 h after MeJA treatment, like that of PMT (3), and the high mRNA level was maintained for 24 h (Fig. 1C). These data confirmed Nt-JAT1 as a strong nicotine transport candidate gene.

We isolated the full-length cDNA of Nt-JAT1 from tobacco BY-2 cells by RT-PCR and 5'- and 3'-RACE. Nt-JAT1 was approximately 1.8 kb long and encoded a putative polypeptide consisting of 470 amino acids. The TMPred prediction program for transmembrane regions (http://www.ch.embnet.org/software/TMPRED.form.html) suggested that the structure of Nt-JAT1 is very similar to that of the human MATE1 with 12 transmembrane helices. Regarding the phylogenetic relationships to other plant MATE transporters, Nt-JAT1 showed a relatively high similarity (50% at amino acid level) to DTX1 of Arabidopsis thaliana, that is located in the plasma membrane and has been proposed to mediate the efflux of xenobiotics mostly by a proton-motive force (supporting information SI Fig. S1) (6).

**Expression Analysis of Nt-JAT1.** The response of Nt-JAT1 to various plant hormones, signaling molecules, and abiotic stresses was studied in 14-day-old seedlings by using RNA gel blot analysis with an Nt-JAT1-specific probe. MeJA treatments clearly up-regulated the Nt-JAT1 expression whereas benzyladenine slightly down-regulated it (Fig. 24). Sclareol, an endogenous antifungal diterpene of tobacco known to strongly induce the expression of the transporter Nicotiana plumbaginifolia pleiotropic drug resistance1 (NpPDR1) (7), had no obvious effect on the Nt-JAT1 expression. Expression analysis of Nt-JAT1 in intact plants revealed that it is expressed in all tissues tested, that is, leaves, stems, and roots (Fig. 2B).

**Nt-JAT1 is Localized to the Tonoplast in Leaves.** To investigate the subcellular localization of Nt-JAT1 in planta, polyclonal antibodies were raised against Nt-JAT1 polypeptides and protein gel blot analysis was carried out. Microsomal membranes from leaves were fractionated on sucrose density gradients and probed with Nt-JAT1 antibodies and with antibodies against several membrane marker proteins. Nt-JAT1 was cofractionated with...
vacuolar H⁺-pyrophosphatase (V-PPase), a tonoplast marker, to give a peak at the interface between 20% and 30% sucrose, whereas the plasma membrane H⁺-ATPase and membranes containing a luminal binding protein (BiP), a marker of the endoplasmic reticulum (ER), sedimento at positions different from that of Nt-JAT1 (Fig. 3). These results suggested that Nt-JAT1 was associated with the tonoplast and not with the plasma membrane or the endoplasmic reticulum in leaves.

**Nt-JAT1 Functions as a Nicotine Transporter.** To examine whether Nt-JAT1 functioned as a genuine nicotine transporter, this protein was produced in cells of the yeast strain AD12345678, which is disrupted in the major ATP-binding cassette transport-er-encoding genes that confer yeast multidrug resistance (9, 10). The cells of the Nt-JAT1 transformant and the control (transformed with the empty vector) were incubated in nicotine-containing Synthetic Dextrose (SD) medium, and the intracellular nicotine content was quantitatively analyzed by HPLC. Nt-JAT1 transformants accumulated less nicotine than control yeast cells under all concentrations tested (0.5, 1, and 3 mM) (Fig. 4A). A time course analysis (from 0 to 8 h) showed that the nicotine content in Nt-JAT1 transformants was consistently significantly lower than that of control cells (Fig. 4B), suggesting that Nt-JAT1 functioned as nicotine efflux transporter at the plasma membrane in yeast cells. Hence, an Nt-JAT1-GFP fusion protein was expressed in yeast cells to confirm that Nt-JAT1 was indeed mainly localized to the plasma membrane in yeast (Fig. 4C). In the yeast cells producing Nt-JAT1-GFP, less nicotine content was found than in control cells, indicating that the fusion protein was as active as the native Nt-JAT1 (Fig. 4D).

**Driving Force and Specificity of Nt-JAT1.** To characterize the function of Nt-JAT1 in more detail, we used a proteoliposome reconstitution system. Therefore, we overproduced Nt-JAT1 in a baculovirus system, in which the Nt-JAT1 synthesis in Sf9 cells was confirmed by protein gel blot analysis (Fig. 5A). After solubilization of the membrane fraction with octyl glucoside, Nt-JAT1 was purified through His-tag affinity chromatography and incorporated into liposomes with purified bacterial F₀F₁-ATPase (F-ATPase) with the freeze/thaw/dilution procedure (11). F-ATPase functions as a pH generator in the proteoliposome assay, we used [³⁴C]-tetraethylammonium (TEA), a typical substrate of MATE-type transporters, to measure the activity of the H⁺-coupled organic cation transporter (12). Upon the addition of ATP, the proteoliposomes facilitated the uptake of TEA in a

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**Fig. 3.** Membrane localization of Nt-JAT1 in tobacco leaves. Microsomes from tobacco leaves were fractionated with a noncontinuous gradient consisting of 0%, 20%, 30%, 34%, 38%, and 45% (wt/vol) sucrose. Membrane fractions were collected from the interfaces between different sucrose concentrations. Proteins from each interface were subjected to electrophoresis, blotted, and immunodetected with anti-Nt-JAT1 antibodies. Blots were re-probed with antibodies raised against plasma membrane (PM) H⁺-ATPase, vacuolar membrane (VM) V-PPase, and endoplasmic reticulum (ER) BiP.

**Fig. 4.** Nt-JAT1-mediated nicotine transport in yeast cells. (A) Nicotine accumulation in yeast cells. Control (white bar) or Nt-JAT1-expressing (black bar) yeast cells were incubated in half-strength SD medium, including nicotine at the indicated concentrations for 6 h. Results are mean ± SD of duplicates. Asterisks indicate statistically significant difference compared to control (Student’s t test; *, P < 0.01; **, P < 0.001; ****, P < 0.0001). (B) Time course analysis of nicotine transport in Nt-JAT1-expressing yeast cells. Control (open circles) or Nt-JAT1-expressing (filled circles) yeast cells were incubated in half-strength SD medium supplemented with 1 mM nicotine, and sampled at the times indicated. Results are mean ± SD of duplicates. Asterisks indicate statistically significant difference compared with control (Student’s t test; *, P < 0.01; **, P < 0.001; ****, P < 0.0001). (C) Localization of Nt-JAT1-GFP to yeast plasma membranes. Yeast cells expressing Nt-JAT1-GFP were grown at 30 °C to the logarithmic growth phase and observed by fluorescence microscopy. (D) Fluorescence of yeast cells transformed with Nt-JAT1-GFP, (E) bright-field contrast. (Scale bar, 5 μm.) (D) Functionality of Nt-JAT1-GFP in yeast cells. Nicotine content in control (white bar), Nt-JAT1-expressing (black bar) or Nt-JAT1-GFP-expressing (gray bar) yeast cells incubated in half-strength SD medium including nicotine for 12 h. Results are mean ± SD of duplicates. Asterisks indicate statistically significant differences (ANOVA Bonferroni test; *, P < 0.01).
time-dependent manner (Fig. 5A). Lack of Nt-JAT1 abolished the uptake activity, indicating that Nt-JAT1 was responsible for the TEA uptake.

First, the driving force was studied with this proteoliposome system. TEA uptake was strongly inhibited by azide, an F-ATPase inhibitor, and ammonium sulfate, a dissipator of the transmembrane pH gradient (ΔpH), but not of ΔΨ. Addition of 2 μM valinomycin in the presence of 65 mM KCl, which caused membrane depolarization, did not affect the TEA uptake; whereas 2 μM nigericin in the presence of KCl, which dissipates the pH gradient, clearly decreased the uptake to the level without ATP (Fig. 5B). Together, these results indicated that Nt-JAT1 mediated an electro-neutral H+/TEA exchange.

Next, we characterized the substrate specificity of Nt-JAT1 by cis-inhibition of the TEA transport with plant alkaloids and flavonoids (see Fig. S2 for structures). The TEA uptake was significantly inhibited by the representative tobacco alkaloids, nicotine and anabasine. Other exogenous alkaloids, such as berberine that had also been proposed as substrate of the Arabidopsis MATE transporter DTX1 (6) and the solanaceous alkaloid hyoscyamine, were capable of inhibiting the TEA uptake as well. In contrast, the flavonoids kaempferol and quercetin could not inhibit the TEA transporter activity by Nt-JAT1 (Table 1). These results suggested that Nt-JAT1 showed substrate specificity for alkaloids among plant secondary metabolites.

**Discussion**

Through transcript profiling analysis of MeJA-treated BY-2 cells, we isolated *Nt-JAT1* as a gene encoding a potential nicotine MATE-type transporter. Expression of *Nt-JAT1* was observed in all tobacco plant organs and the level increased by MeJA treatment in a coordinate manner with genes coding for nicotine biosynthetic enzymes. Here, we give direct evidence that Nt-JAT1 is a genuine nicotine transporter by using 2 independent methods, that is, cellular transport assays with yeast transformants and in vitro transport assays with a proteoliposome-based system.

Originally the MATE transporter family had been found in prokaryotes, but recently also in higher organisms orthologs had been identified, for example, *Arabidopsis* DTX1 and TT12 (6, 13). Here, we provide the detailed biochemical analysis of a MATE transporter from higher plants in vitro. The function of Nt-JAT1 as a secondary transporter was characterized by using proteoliposomes, into which the F-ATPase had been incorporated as a generator of ΔpH. Inhibitor experiments clearly showed that Nt-JAT1 mediated electro-neutral H+/TEA exchange. Our findings also demonstrated that this proteoliposome assay that allows formation of pH and electron potential gradients by addition of ATP is a powerful tool to study the molecular mechanism of transporters whose driving force is unknown and illustrated its value to analyze other secondary transporters in the future.

Although with regard to the substrate specificity of plant MATE transporters, *Arabidopsis* DTX1 has been shown to efflux several substrates, including plant-derived or other exogenous toxic compounds, recent studies suggested that plant MATEs might have a strict specificity for endogenous substrates (13–15).

**Table 1. Cis-inhibition of TEA uptake in Nt-JAT1 proteoliposomes**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TEA uptake, %</th>
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<tbody>
<tr>
<td>No addition</td>
<td>100.0</td>
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<tr>
<td>-ATP</td>
<td>26.1 ± 8.6</td>
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<tr>
<td>100 μM Nicotine</td>
<td>40.5 ± 6.2</td>
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<tr>
<td>100 μM Hyoscyamine</td>
<td>43.9 ± 4.7</td>
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<tr>
<td>100 μM Anabasine</td>
<td>44.6 ± 9.8</td>
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<tr>
<td>100 μM Berberine</td>
<td>55.1 ± 2.0</td>
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<tr>
<td>100 μM Kaempferol</td>
<td>92.8 ± 9.6</td>
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<tr>
<td>100 μM Quercetin</td>
<td>94.3 ± 8.4</td>
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The uptake of radio-labeled TEA (50 μM) by proteoliposomes was determined in the presence or absence of the listed compounds (100 μM) (see SI Fig. 2 for structures). Reconstituted vesicles were preincubated with the compounds before the addition of TEA. Values were expressed as percentages of radio-labeled TEA uptake under control conditions (no test substance added). The 100% control corresponds to 0.92 nmol TEA/mg protein at 5 min. Results are mean ± SD of three replicates.
For instance, the Arabidopsis TT12 transports cyanidin-3-O-glucoside, whereas citrate is transported in a highly specific manner by Arabidopsis FRD3 and the Hordeum vulgare (barley) aluminum-activated citrate transporter HvAACT1. Our results indicated that Nt-JAT1 is not exclusively specific for nicotine, the major endogenous alkaloid in tobacco, but might also recognize limited numbers of endogenous tobacco alkaloids and hyoscynamine (a tropane alkaloid) and berberine (an isquinolione alkaloid) derived from other plant species. In turn, flavonoids, such as kaempferol and quercetin, were not recognized by Nt-JAT1. Cis-inhibition was also observed with synthetic organic cations (Table S1), implying that Nt-JAT1 has a broad specificity to organic cations. However, as the physiological substrates are necessarily endogenous cations, such as pyridine alkaloids, Nt-JAT1 functions presumably actively in the sequestration of these endogenous, potentially toxic compounds (16). A similar role as “detoxification” mechanism has been proposed previously for other plant vacuolar transport systems (17). Interestingly, the human MATE1 is also a polyspecific exporter that transports nicotine and other toxic compounds (12).

As Nt-JAT1 is a H+ antiporter, the substrate is transported out of the cells in exchange of the H+ influx when it is localized to the plasma membrane, whereas when localized to the vacuolar membrane it functions as an uptake transporter because the cytosolic pH (7.2–7.5) is higher than that of the vacuolar lumen (generally 5.5). In tobacco plants, nicotine is biosynthesized in root tissue, transported upward via the xylem, and translocated into the vacuole of the leaves. In this complex and highly regulated intraorgan transport of alkaloids, Nt-JAT1 might play more than one key role, that is, not only in the final accumulation in the vacuoles of the sink organ, but also in alkaloid loading in the source organ. Its ubiquitous gene expression pattern, in both leaves and roots is in agreement with this hypothesis. Furthermore, when the membrane localization of Nt-JAT1 in tobacco roots was analyzed (in an experiment similar to that applied to leaf microsomes), surprisingly Nt-JAT1 did not cofractionate with the vacuolar membrane, but rather with the plasma membrane (data not shown). This observation suggests that Nt-JAT1 might exhibit a dual localization depending on the organ where it is produced, that is, at the tonoplast in leaves and/or at the plasma membrane in root tissues. To verify this, Nt-JAT1 accumulation was also assessed by expression of a C-terminal GFP fusion protein in different N. tabacum and N. benthamiana explants. However, due to the high background fluorescence we could not provide further support for a potential dual membrane localization of Nt-JAT1 (data not shown). Alternating subcellular localizations, caused by vesicular cycling between an endosomal compartment and the plasma membrane, have been reported for the plant PIN auxin transporters (18–20), and for the yeast general amino acid permease (21). Although the case of Nt-JAT1 needs to be further explored, the possibility that a single gene product could manage two different transport events within a plant might provide an unexpected clue as to how plants organize tightly regulated metabolite movements in their body with limited numbers of transporter genes in the genome.

Regarding the membrane transport of tobacco alkaloids, the uptake transporter for nicotine at the plasma membrane of leaf cells is still unknown. In contrast to the berberine translocation in Coptis japonica in which an ABC protein is involved in the influx at the sink tissue (22), specific transporters might not be absolutely necessary in tobacco for the uptake of nicotine at the leaf cell plasma membrane because of its hydrophobicity. Nonetheless, considering that due to the acidic condition in the apoplastic space, alkaloids are protonated and become more hydrophilic than when present in the cytosol, a specific carrier protein might be possibly responsible for the unloading of alkaloids. Further detailed investigation of (plasma membrane-localized) transporters will unravel the whole scheme of the translocation mechanism of nicotine alkaloids.

Materials and Methods

Chemicals. Nicotine and other chemicals used in this study were purchased from Wako Pure Chemicals or Nacalai Tesque.

Plant Materials. Nicotiana tabacum (cv. Samsun NN) plants were grown under 16-h light/8-h dark cycles at 25 °C. Cultured suspension cells of Nicotiana tabacum L. cv. BY-2 were maintained as described in ref. 23. To analyze the regulation of Nt-JAT1 transcripts, seeds were sown on nylon mesh (6 × 6 cm, 200-μm pore) over half-strength Linwood and Stock (LS) medium (1.5% [wt/vol] agar, pH 5.8) and further grown to 4 weeks under the same light/dark regime as described above. Roots were subjected to various treatments by gentle transfer of the mesh to new medium. After 24 h, treatments were stopped by immediate freezing of seedlings in liquid N2 to analyze the tissue-specific expression of Nt-JAT1, 1.5-month-old plants were used.

Full-length cDNA Cloning. A full-length cDNA clone of tobacco MATE Nt-JAT1 (Nucleotide Sequence Database accession AM991692) was obtained by PCR from a cDNA library of MeJA-treated tobacco BY-2 cells (24). Nt-JAT1-specific primers used for the library screening were designed based on the cDNA-AFLP tag sequence obtained previously (5) (Nucleotide Sequence Database accession CO809245): Nt-JAT1P-forward (fw), 5′-CACCACGATAACCTCGAAG-3′; Nt-JAT1P-reverse (rev) 5′-GGCCTTGTACAGTTAAG-3′.

RNA Isolation and Expression Analysis. Total tobacco RNA was prepared from 200 mg of plant material with the RNeasy plant mini kit (Qiagen). Agarose gel electrophoresis, RNA transfer onto Hybond-N’ membranes (GE-Healthcare), and hybridization with the 0.5-kb Nt-JAT1 fragment (positions +940 to +1,436) were done according to standard procedures. In a genomic DNA blot analysis, this probe enabled detection of two major bands, which, considering the tobacco amphiidiopioid, suggested that it mainly recognized Nt-JAT1 (data not shown). For the last stringent wash, 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 60 °C were used. qRT-PCR with primers elicited BY-2 cells was carried out as described in ref. 24. As a reference gene, the gene encoding ST30 was used corresponding to a constitutively expressed RNA helicase-like protein (5). Primers were constructed based on sequences from PMT (AF126812), Nt-JAT1 (AM991692), and ST30 (AM991691): PMTQ-fw, 5′-TGGATGAGCAATTCCAACA-3′; PMTQ-rev, 5′-ACACAAATCTGGCCGGATG-3′; Nt-JAT1Q-fw, 5′-AGGGCTTTGTTGTGTTATGG-3′; Nt-JAT1Q-rev, 5′-CCACAAAACACAGTTGATGC-3′; ST30Q-fw, 5′-AAACAGGCAATGCTCCTAAG-3′; and ST30Q-rev, 5′-CCTAGACGGCAACCTGCAATAC-3′.

Nt-JAT1 Antibodies. Rabbit polyclonal antibodies against Nt-JAT1 were raised by repeated injections of GST-fusion polypeptides comprising the amino acid residues M1-F31 of Nt-JAT1 (MVELPQLSKKEKQWNVDVSVLKLKT). Materials and Methods

Determination of Nt-JAT1 Localization in Tobacco. Sucrose density gradients of tobacco leaves were fractionated as described in ref. 25. For immunoblotting, proteins were treated in a denaturation buffer for 10 min at 50 °C, subjected to SDS/10% PAGE, and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). The membrane was treated with Blocking One (Nacalai) and incubated successively with primary antibodies and secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody by standard procedures. The band was visualized by chemiluminescence. Primary antibodies used for membrane marker were raised against Arabidopsis V-PPase, Arabidopsis endoplasmic reticulum BIP, and the tobacco plasma membrane H-^\text{\textsuperscript{-}}\text{ATPase}.

Functional Analysis of Nt-JAT1 in Yeast Cells. Nt-JAT1 cDNA (1.4 kb) was subcloned into the yeast expression vector pBR196 (10), and the resulting plasmid, pBR-Nt-JAT1, was used to transform the strain AD12345678 (MATa, PDR1−, ura3, his1, Δyorl3::hisG, Δyorl2::hisG, Δyorl3::hisG, Δyorl1::hisG, Δyorl1::hisG, Δyorl2::hisG, Δyorl3::hisG, and Δyorl3::hisG) (9) by the lithium acetate method. The yeast transformant was precultured in 40 mL of SD medium (−uracil), harvested at A600 = 1.0, and suspended by 40 mL half-strength SD medium (−uracil) containing nicotine at A600 = 1.0. The cells were incubated at 30 °C with shaking at 200 rpm, harvested at the indicated time by centrifugation, and washed twice with deionized cold water. Next, yeast cells were resuspended with acid-washed glass beads in buffer (50% ethanol, 50% methanol, and 0.5% acetic acid). Samples were centrifuged and the supernatant was subjected to HPLC analysis with the following specificities: mobile phase, methanol-20 mM sodium phosphate buffer (pH 7.0) (40:60);
column, 5C18-MS-II (Nacalai; 4.6 mm i.d. × 250 mm); temperature, 30 °C; flow rate, 0.6 mL/min; detection, absorbance measured at 254 nm.

The subcellular localization of NT-JAT1 in yeast was assessed with an NT-JAT1-GFP fusion construct. The cDNA fragments carrying either the coding region of NT-JAT1 or of GFP were amplified with the following primers: for NT-JAT1 (excluding the stop codon), NT-JAT1-fw1, 5′-GGAAAATTCGTACCTAGGGAGTTGCACAG-3′ and NT-JAT1-rev1 (stop codon removed) 5′-TTGCTAGCATTAGCCTCTCTCCTCGTACCTTTT-3′; for GFP, GFP-fw1 5′-AAGGAAGAATGATCTAGCAAAAGGAAAGAAGAC-3′ and GFP-rev1 5′-AAGGAAAAAAGCCGGCGCTACGTAGTTACAGTTTCATCATGTC-3′. These fragments were fused by PCR and subcloned into the pDR196 vector using the underlined EcoRI and NotI restriction sites. Sequence fidelity of the NT-JAT1-GFP was confirmed by sequencing.

**Functional Analysis of NT-JAT1 in Proteoliposomes.** Recombinant baculovirus containing NT-JAT1 cDNA was constructed with the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer’s instructions. NT-JAT1 cDNA was amplified by PCR with the primers 5′-AAAAAGCAGGCTATCATGGTAGAGGAGTTGCCACAG-3′ and 5′-AGAAAGCTGGGTCTAAGATCTTCCTTCGT-AAGGAAAAAAAGCCGGCGCTACGTAGTTACAGTTTCATCATGTC-3′, cloned into a pDONR221 vector as described in ref. 26, and subsequently to the destination vector pDEST10 to yield pDEST10-Nt-JAT1. This construct was introduced into Sf9 cells (2 × 10^7 cells per dish) at a multiplicity of infection of 1. Detailed experimental procedures used for NT-JAT1 reconstitution and transport assays are described in SI Methods.

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Supporting Information

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SI Methods

**Functional Analysis of Nt-JAT1 in Proteoliposomes.** Infected Sf9 cells (3–4 × 10⁶) were cultured for 72 h, harvested, and suspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM potassium acetate, 10% glycerol, 0.5 mM DTT, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The Nt-JAT1 protein was purified and reconstituted essentially as described (1). In brief, after disruption of the cells by sonication, the membrane fraction was centrifuged, and suspended in a buffer containing 20 mM Mops-Tris, pH 7.0, 10% glycerol, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin (∼20 mg protein/ml), and solubilized with 2% octyl glucoside (protein:detergent ratio = 1:1). After centrifugation at 260,000 × g for 30 min, the supernatant was applied on Ni-NTA Superflow resin (Qiagen), and incubated for 4 h at 4 °C. After washing with 20 mM Mops-Tris, pH 7.0, 1% octyl glucoside, 20% glycerol, and 5 mM imidazole, Nt-JAT1 was eluted with the same buffer but with 60 mM imidazole. Co-reconstitution of purified Nt-JAT1 with bacterial F-type ATPase into liposomes was carried out by the freeze-thaw method (1). Nt-JAT1 (20 μg) was mixed with F-type ATPase (90 μg) and liposomes (0.6 mg), frozen at -80 °C, quickly thawed, and diluted 30-fold with reconstitution buffer containing 20 mM Mops-Tris, pH 7.0, 0.5 mM DTT, 100 mM potassium acetate, and 5 mM Mg-acetate. Proteoliposomes were pelleted by centrifugation at 160,000 × g for 1 h at 4 °C. In control experiments, proteoliposomes were prepared without Nt-JAT1. Reconstituted proteoliposomes (10 μg protein) were suspended in a buffer consisting of 20 mM Mops-Tris, pH 7.0, 65 mM KCl, 35 mM potassium acetate, and 5 mM Mg-acetate with or without 2 mM ATP and listed ionophores. Transport assays were started by the addition of 50 μM radioactive ¹⁴C-TEA (0.5 MBq/p mol) and incubated at 30 °C. Aliquots (125 μl) were taken at the times indicated, centrifuged through a Sephadex G-50 (fine) spin column at 180 × g for 1 min; eluate radioactivity and proteins were determined (1). Selective dissipation of ΔpH or ΔΨ by the addition of ionophores was confirmed as described previously (1). Throughout the study, the results on transport assays are expressed as means ± SD (n = 3).

Bacterial F-ATPase purification, preparation of asolectin liposomes, fluorescent measurement of ΔpH and ΔΨ, protein gel blotting, SDS gel electrophoresis, and Coomassie Brilliant Blue staining were done as described (2).


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**Fig. S1.** Phylogenetic relationship of Nt-JAT1 and plant MATE family members. The amino acid sequences of MATE members were aligned with ClustalW and subjected to phylogenetic analysis provided by DDBJ. Arabidopsis MATE members are indicated as Arabidopsis Genome Initiative (AGI) codes, and reported names are circled [ALF5 (1), DTX1 (2), EDS5 (3), FRD3 (4), and TT12 (5)]. Accession numbers are as follows: LeMTP77 (Solanum lycopersicon, tomato), AY348872; LaMATE (Lupinus albus), AY631874; HvAACT1 (barley), AB302223; and SbMATE (Sorghum bicolor), EF611342.
Fig. S2. Structures of the compounds used for cis-inhibition of TEA uptake in Nt-JAT1 proteoliposomes.
Table S1. Cis-inhibition of TEA uptake in Nt-JAT1 proteoliposomes by synthetic organic cations

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TEA uptake, %</th>
</tr>
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<tbody>
<tr>
<td>No addition</td>
<td>100.0</td>
</tr>
<tr>
<td>100 µM Rhodamine</td>
<td>27.7 ± 3.9</td>
</tr>
<tr>
<td>100 µM Ethidium bromide</td>
<td>34.7 ± 1.7</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>38.3 ± 2.9</td>
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The uptake of radio-labeled TEA (50 µM) by proteoliposomes was determined in the presence or absence of the listed compounds. Reconstituted vesicles were preincubated with the compounds before the addition of TEA. Values were expressed as percentages of radio-labeled TEA uptake under control conditions (no test substance added). The 100% control corresponds to 0.92 nmol TEA/mg protein at 5 min. Values are expressed as mean ± SD (n = 3).