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A Plant Plasma Membrane ATP Binding Cassette–Type Transporter Is Involved in Antifungal Terpenoid Secretion

Michał Jasiński,[a] Yvan Stukkens,[a] Hervé Degand,[a] Bénédicte Purnelle,[a] Jacqueline Marchand-Brynaert,[b] and Marc Boutry[a,1]

[a] Unité de Biochimie Physiologique, Université Catholique de Louvain, Croix du Sud 2-20, B-1348 Louvain-la-Neuve, Belgium

[b] Unité de Chimie Organique et Médicinale, Université Catholique de Louvain, Place Louis Pasteur 1, B-1348 Louvain-la-Neuve, Belgium

ATP binding cassette (ABC) transporters, which are found in all species, are known mainly for their ability to confer drug resistance. To date, most of the ABC transporters characterized in plants have been localized in the vacuolar membrane and are considered to be involved in the intracellular sequestration of cytotoxins. Working on the assumption that certain ABC transporters might be involved in defense metabolite secretion and their expression might be regulated by the concentration of these metabolites, we treated a *Nicotiana plumbaginifolia* cell culture with sclareolide, a close analog of sclareol, an antifungal diterpene produced at the leaf surface of *Nicotiana* spp; this resulted in the appearance of a 160-kD plasma membrane protein, which was partially sequenced. The corresponding cDNA (*NpABC1*) was cloned and shown to encode an ABC transporter. In vitro and in situ immunodetection showed NpABC1 to be localized in the plasma membrane. Under normal conditions, expression was found in the leaf epidermis. In cell culture and in leaf tissues, *NpABC1* expression was strongly enhanced by sclareolide and sclareol. In parallel with *NpABC1* induction, cells acquired the ability to excrete a labeled synthetic sclareolide derivative. These data suggest that NpABC1 is involved in the secretion of a secondary metabolite that plays a role in plant defense.

INTRODUCTION

The ATP binding cassette (ABC) superfamily of membrane transporters is found in all prokaryotic and eukaryotic species. All members of this family are involved in the active transport of many chemically and structurally unrelated compounds and use ATP hydrolysis as a source of energy (Higgins, 1992). Several of these proteins have been identified by their ability to confer drug resistance, hence their designation as multidrug resistance (MDR) or pleiotropic drug resistance (PDR) proteins. For example, the human MDR1 (Chen et al., 1986), which is also designated P-glycoprotein, is implicated in resistance of cell lines to antitumor drugs (Ueda et al., 1987), PDR5 is a multidrug transporter found in *Saccharomyces cerevisiae* (Balzi et al., 1994), and *Candida* PDR1 (CDR1) and CDR2, identified in the yeast *Candida albicans*, contribute to fungicide resistance (Prasad et al., 1995; Sanglard et al., 1997). An analog of human MDR1 also has been found in prokaryotes (van Veen et al., 1996). Other ABC transporters were characterized by their ability to secrete internal compounds; this is the case with the *S. cerevisiae* protein STE6, which is involved in the transport of a mating pheromone peptide (Kuchler et al., 1989), and mammalian MDR2, which acts as a phospholipid translocator (Ruetz and Gros, 1994).

Multidrug resistance–related proteins (MRPs) are ABC proteins that can be distinguished from the proteins described above by their possession of an N-terminal hydrophobic extension and an internal regulatory domain. In mammals, the MRPs HmMRP1 (Cole et al., 1992) and cMOAT (Buchler et al., 1996) transport glutathione *S*-conjugates and are implicated in the detoxification process. MRPs are also referred to as GS-X pumps (Ishikawa, 1992).

ABC proteins possess a characteristic modular structure consisting of one or two copies of two basic structural elements, a hydrophobic transmembrane domain (TMD), usually with six membrane-spanning α helices, and a cytosolic domain involved in ATP binding, which is known as the nucleotide binding fold (NBF) (Hyde et al., 1990). In the majority of eukaryotic ABC transporters, the core domains are contiguous on a single polypeptide in a "forward" TMD1-NBF1-TMD2-NBF2 orientation (e.g., MDR1, STE6) or a "reverse" NBF1-TMD1-NBF2-TMD2 orientation (e.g., PDR5). The ATP binding domain from a *Salmonella typhimurium*

[1] To whom correspondence should be addressed. E-mail boutry@fysa.ucl.ac.be; fax 32-10-473872.

ABC protein was crystallized and shown to have an L-shaped structure with two arms, one of which contains most of the residues required for ATP binding, whereas the other arm interacts with membrane-bound parts of the protein (Hung et al., 1998).

Observation of glutathione conjugate uptake into plant vacuoles (Martinoia et al., 1993) triggered the identification of vacuolar ABC transporters in plants. They belong to MRPs or GS-X pumps and are considered to be involved in the intracellular sequestration of toxins (reviewed in Coleman et al., 1997; Rea et al., 1998; Rea, 1999; Theodoulou, 2000). Among several MRP genes identified in Arabidopsis, four have been characterized in more detail. *AtMRP1*, *AtMRP2*, and *AtMRP3* encode functional GS-X pumps and transport xenobiotics and endogenous substances, such as chlorophyll catabolites, into the vacuole (Lu et al., 1997, 1998; Tommasini et al., 1998). Expression of *AtMRP4* was found to be induced by herbicide safeners (Sánchez-Fernández et al., 1998). In barley, the vacuolar uptake of glucoside derivatives has been characterized and suggested to be catalyzed by an ABC transporter (Klein et al., 1996).

Less is known about plant plasma membrane ABC transporters. Within the MDR family, the Arabidopsis AtPGP1, which is localized in the plasma membrane (Dudler and Hertig, 1992; Sidler et al., 1998), is known to be involved in hypocotyl cell elongation. Heterologous expression in yeast has suggested that this enzyme might transport ATP (Thomas et al., 2000). Other MDR-like ABC proteins have been isolated from potato (Wang et al., 1996) and barley (Davies et al., 1997), but their substrates and subcellular localization are unknown. TUR2 from the aquatic plant *Spirodela polyrrhiza* has been shown to be induced by environmental stress, including high salt and low temperature. The TUR2 transcript is upregulated by abscisic acid and cycloheximide (Smart and Fleming, 1996). By analogy to its closest fungal homolog (PDR5), TUR2 is thought to be an efflux pump for cytotoxic compounds, but its localization and transport properties are unknown. Finally, an ABC transporter from alfalfa (Fowler et al., 1998) is induced by auxin analogs; again, its localization and substrates are unknown.

At least 49 putative ABC transporter genes are found within the Arabidopsis genome database (Davies and Coleman, 2000). However, for many of these genes, protein localization is unknown, and because the translocated substrates might vary considerably for the different proteins, there are no clues regarding the functions of these putative transporters. Therefore, we wanted to develop a functional search method for ABC transporters involved in secondary metabolite transport. In this article, we report the cloning of *NpABC1*, an ABC transporter gene from *Nicotiana plumbaginifolia*. NpABC1 expression is induced by sclareol, a labdane-type diterpene found on the leaf surface of *Nicotiana* spp that has fungitoxic properties and also affects plant growth (Bailey et al., 1975; Cutler et al., 1977). In situ localization indicated that NpABC1 is present in the plasma membrane. In addition, our study suggested that plant plasma membrane transporters might be implicated in the secretion of endogenous metabolites that play a role in defense against biotic stress.

RESULTS

The Terpenoid Sclareolide Induces the Synthesis of a 160-kD Protein

To functionally identify plant plasma membrane ABC transporters, we assumed that the expression of some of these might be induced or enhanced when their substrate concentrations increase within the cell. Therefore, we treated *N. plumbaginifolia* cell cultures with increasing concentrations of various secondary metabolites. In addition, to more readily identify the putative induced ABC transporters, we labeled newly synthesized proteins by adding $^{35}SO_4^{2-}$. Finally, to restrict our search to plasma membrane ABC transporters, we compared the SDS-PAGE protein profiles of phase partition–enriched plasma membrane fractions from treated and untreated samples. An interesting response was found using the terpenoid sclareolide, which is a close analog of sclareol (Figure 1), an antifungal metabolite found on the leaf surface of *Nicotiana* spp (Bailey et al., 1975). Twelve hours after sclareolide addition, we detected a strongly labeled band with a molecular mass of 160 kD, a size compatible with a full ABC transporter (Figure 2A). To determine the actual amount of this protein, we performed the same experiment on a large scale in the absence of radioactive labeling but using Coomassie blue staining, which showed the presence of a 160-kD band specific to the sclareolide-treated culture (Figure 2B). We then determined the partial amino acid sequence of this protein, which we named NpABC1.

Isolation and Characterization of *NpABC1*

Direct N-terminal sequencing of NpABC1 prepared by preparative gel electrophoresis failed, suggesting in vivo or in vitro N-terminal modification. Therefore, we obtained sequence information for several NpABC1 peptides obtained by subtilisin or trypsin cleavage and reverse phase HPLC. Two of these sequences, IHQPSID and EFAEAY, were found to be homologous with conserved sequences within the ABC transporter superfamily. On the basis of these sequences, we designed degenerate primers, which we used to obtain a 1.3-kb cDNA fragment by reverse transcription–polymerase chain reaction (PCR) of RNA prepared from sclareolide-treated cells. The most upstream sequence of the cDNA was obtained by rapid amplification of cDNA ends–PCR. The full-length cDNA contained a 4311-bp open reading frame predicted to encode a protein of 1436 amino

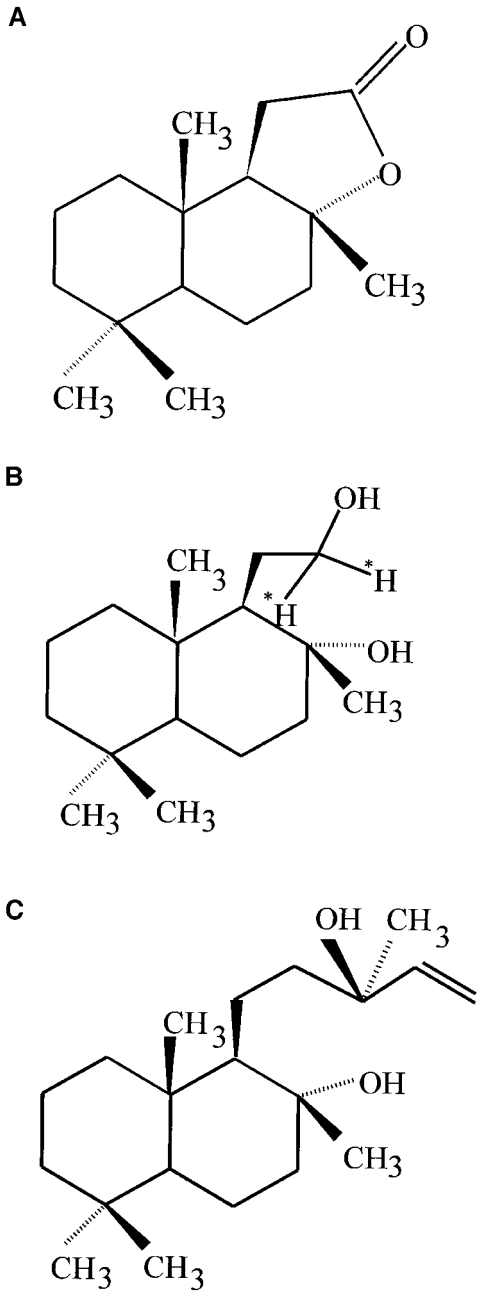

Figure 1. Chemical Structures of the Terpenoids Used in This Study.

(A) Sclareolide.

(B) ^{3}H-Decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol. The asterisks denote the two protons that can be labeled after reduction of the lactone ring.

(C) Sclareol.

acid residues with a size of 161,848 D, in good agreement with the results of SDS-PAGE (Figure 2B). The translation initiation codon could be identified unambiguously, because it possessed a good context sequence, AAAATGG (Joshi et al., 1997; Lukaszewicz et al., 2000), and was preceded by an in-frame stop codon.

NpABC1 has a typical domain structure consisting of two halves, one hydrophilic domain containing NBF and one hydrophobic domain containing TMD, present in PDR5-like reverse orientation (Figure 3). The NBF region, consisting of ~200 amino acids, is the most conserved feature of ABC proteins, is involved in ATP binding, and is made up of the highly conserved Walker A and B motifs (Walker et al., 1982) as well as a sequence known as the ABC signature (Shyamala et al., 1991). According to the Kyte and Doolittle (1982) algorithm, each of the two hydrophobic NpABC1 domains contains six transmembrane-spanning α helices. A homology search using the BLAST program (Altschul et al., 1997) showed the closest homologs of NpABC1 to be a putative ABC transporter (AC013453) from Arabidopsis (70% identity) and TUR2 from *S. polyrrhiza* (69% identity; Smart and Fleming, 1996). In fungi, the closest homologs are PDR5 (28% identity; Balzi et al., 1994) and SNQ2 (25% identity; Servos et al., 1993) from *S. cerevisiae*.

Induction of *NpABC1* Expression

To follow the induction of *NpABC1* expression, we performed RNA gel blot analysis using a probe corresponding to the cDNA 3′ untranslated region. Three hours after sclareolide addition, we observed a strong band of ~4.9 kb, which had almost disappeared after 12 hr (Figure 4A). As a control, the signal detected with a probe for a constitutive mitochondrial ATP synthase subunit (*atp2-2*) did not vary.

To follow NpABC1 expression at the protein level, a six-histidine–tagged 93-residue polypeptide corresponding to a region downstream of the NpABC1 C-terminal NBF (Figure 3) was expressed in *Escherichia coli*, purified on a nickel affinity column, and used to immunize rabbits and affinity purify the anti-NpABC1 antibodies. By protein gel blot analysis, these recognized a single band, the size of which corresponded to that of NpABC1 (Figure 2C). Analysis of a microsomal fraction from control cells revealed a faint band at ~160 kD, the intensity of which was strongly increased after sclareolide treatment. In contrast to the *NpABC1* transcript, the amount of this protein remained high for at least 12 hr after sclareolide addition (Figure 4B). As a control, the amount of plasma membrane H^{+}-ATPase did not vary during this time. Testing at lower sclareolide concentrations showed that 20 μM was enough to induce NpABC1 (Figure 4C).

Although sclareolide provides a very convenient means of inducing NpABC1 expression, it is not a major diterpene produced in *Nicotiana* spp. Therefore, we examined the effects of sclareol, which is structurally very close to sclareolide, is one of the most abundant diterpenes of the leaf

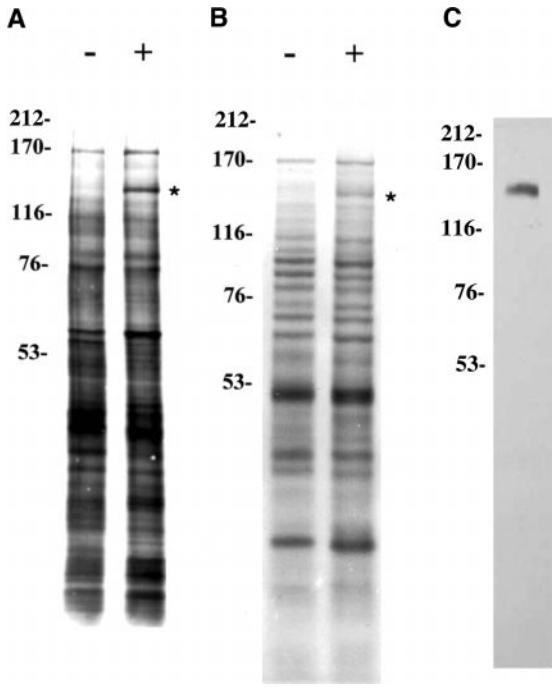

Figure 2. Sclareolide Induces Expression of a Plasma Membrane Protein.

(A) Cells grown in medium with a reduced amount of exogenous sulfur were treated (+) or not treated (−) with 500 μM sclareolide, labeled with $^{35}SO_4^{2-}$, and harvested 12 hr after sclareolide addition. Plasma membranes were prepared, and the proteins were electrophoresed and blotted onto a polyvinylidene difluoride membrane that was subjected to autoradiography.

(B) As in **(A)**, except that the cells were grown in normal medium and the proteins were stained with Coomassie blue.

(C) Protein blot analysis of a microsomal fraction prepared from *N. plumbaginifolia* cells grown for 24 hr in the presence of 500 μM sclareolide. Proteins (2 μg) were electrophoresed, blotted, and immunodetected using anti-NpABC1 antibodies.

The asterisks indicate the induced protein. The positions of molecular mass markers (in kilodaltons) are indicated.

surface, and is reported to confer antimicrobial and growth-regulating activity (Bailey et al., 1975; Cutler et al., 1977; Kennedy et al., 1992). We found that the addition of 20 μM sclareol to a *N. plumbaginifolia* cell culture induced NpABC1 expression (Figure 4D).

Finally, we determined whether sclareol induction of NpABC1 expression in culture cells recapitulates a biological event occurring in the plant. In situ immunolocalization in tissues requires embedding, and NpABC1 antibodies did not recognize the corresponding antigens after this rough treatment. Protein gel blot analysis of a microsomal fraction isolated from *N. plumbaginifolia* leaves revealed almost no NpABC1 expression in untreated leaves. However, when the epidermis was peeled off, NpABC1 was clearly detected in this tissue (Figure 4E). After sclareol infiltration, NpABC1 expression was enhanced, confirming the induction of NpABC1 by this terpene (Figure 4E).

NpABC1 Is Localized in the Plasma Membrane

Although NpABC1 was detected in a plasma membrane–enriched fraction (Figure 2), we could not exclude the possibility that this was contaminated with another membrane fraction. As an initial approach to localizing NpABC1 at the subcellular level, we compared the NpABC1 signal in a microsomal fraction and in a phase partition–purified plasma membrane fraction and found that the enrichment of NpABC1 in the plasma membrane fraction was similar to that seen for H^+-ATPase, a plasma membrane marker (Figure 5A). We also submitted a microsomal fraction to equilibrium density centrifugation on a sucrose gradient. NpABC1 and the plasma membrane H^+-ATPase peaked in the same fractions, whereas membranes containing binding protein (BiP), an endoplasmic reticulum marker, and tonoplast intrinsic protein (TIP), a vacuolar marker, sedimented at a different position (Figure 5B). As a third approach, we performed in situ immunolocalization with culture cells because these do not require embedding, which was found to destroy antigenic epitopes. We used a suspension culture of a closely related species, *N. tabacum* line Bright Yellow 2, because the vacuole can be readily stained by neutral red. In control cells not treated with sclareolide, a low and nonspecific background was seen in the whole cell using anti-NpABC1 antibodies (Figures 5C and 5D). After sclareolide addition to the cell culture, a clear signal appeared around the cell (Figures 5E and 5F) similar to that seen using antibodies against H^+-ATPase, the plasma membrane marker (Figures 5G and 5H), or that identified for a plasma membrane Ca^{2+}-ATPase in Bright Yellow 2 cells (Chung et al., 2000). Some internal labeling was also found for NpABC as well as for H^+-ATPase, reflecting invagination of the plasma membrane or internal vesicles belonging to the secretory pathway. The absence of NpABC1 from the vacuolar membrane (tonoplast), which was evidenced by the subcellular fractionation (Figure 5B), was confirmed here, because the vacuole appeared fragmented into several large vesicles that could not be misidentified as the plasma membrane (Figure 5I). As another control, antibodies against mitochondrial ATP synthase labeled mitochondria and not the plasma membrane (Figure 5J).

Efflux of the ^{3}H-Sclareolide Derivative Correlates with *NpABC1* Expression

The cloning approach we used was based on the assumption that expression of an ABC transporter (in this case, NpABC1) might be induced when the concentration of the transported substrate is increased. To this point, we had shown that sclareolide or sclareol induced NpABC1 expres-

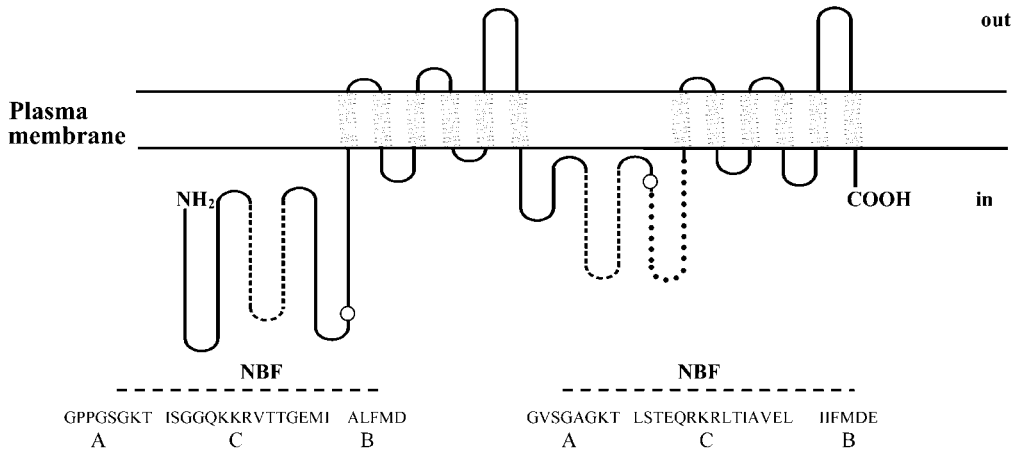

Figure 3. Putative Topology of NpABC1.

The topology is based on that generally proposed for ABC transporters (Higgins, 1992). The protein is made up of two halves, each of which consists of one hydrophilic and one hydrophobic domain. Both hydrophilic domains contain an NBF domain (dashed lines). The sequences corresponding to Walker's A and B motifs and the ABC signature (C) are shown. Putative transmembrane segments are indicated (zigzag lines), as well as the region from Phe-1057 to Asp-1150 used to raise polyclonal antibodies (dotted line). The peptides sequenced and used to design the degenerated primers for PCR are shown as open circles.

sion, but not that it was transported. To address this matter, because the lactone ring in sclareolide is probably not essential for the induction of *NpABC1* expression because it is not present in sclareol, we prepared a radioactive sclareolide derivative by transforming the lactone ring into a diol (Figure 1B). We first demonstrated that the unlabeled derivative was able to induce NpABC1 expression as well as sclareolide (Figure 6A). Then, using a mixture of sclareolide and the labeled derivative, we simultaneously monitored the induction of NpABC1 expression by protein gel blotting (Figure 6B) and the amount of labeled sclareolide derivative that accumulated within the cells (Figure 6C). Because diterpenes are soluble in both hydrophilic and hydrophobic media (Guo and Wagner, 1995), they can cross biological membranes and equilibrate rapidly within cells; however, during the course of our experiment, we observed a decrease in cell-associated radioactivity (Figure 6C) that correlated with the increase in NpABC1 levels (Figure 6B), suggesting that the induced NpABC1 was involved in transporting the labeled diterpene back out of the cell. The decrease of cell-associated radioactivity reached a plateau, which might reflect the equilibrium between entry by passive diffusion and exit by active extrusion catalyzed by NpABC1. An additional possibility is that terpenoids are highly soluble in membranes and might continue to accumulate at the outer leaflet of the plasma membrane. Eight hours after induction, we divided the culture in halves. To deplete the level of ATP, we treated half with the ATP synthesis inhibitor cocktail (NaF, a glycolysis inhibitor, as well as NaN_3, and carbonylcyamide m-chlorophenylhydrazone, mitochondrial electron chain and phosphorylation inhibitors) used by Rawyler et al. (1999) and found that the cell-associated radioactivity increased rapidly (Figure 6C), indicating that sclareolide extrusion is linked to an energetic process.

Using another approach, we compared the labeled sclareolide derivative associated with control cells and cells that had been incubated for 12 hr with unlabeled sclareolide. The terpenoid-pretreated cells showed less accumulation than the untreated cells, and this difference was removed by ATP synthesis inhibitors (Figure 6D).

DISCUSSION

Our functional screening for ABC transporters was based on the assumption that increasing the cellular concentration of some metabolites would induce or upregulate the expression of an ABC transporter involved in secreting these chemicals. Using this approach, *N. plumbaginifolia* suspension cell culture proved to be very convenient, because it provides a homogenous sample in which cells are in direct contact with the surrounding medium and should respond evenly. This approach worked well with sclareolide and resulted in the identification of *NpABC1*.

The deduced sequence for NpABC1 is typical of ABC transporters and contains the highly conserved Walker A and B motifs. Compared with most fungal homologs, NpABC1 and TUR2 from the aquatic plant *S. polyrrhiza* have an additional stretch of 25 amino acid residues between the Walker A motif and the ABC signature. Crystallographic study of the ATP binding domain of a bacterial ABC protein

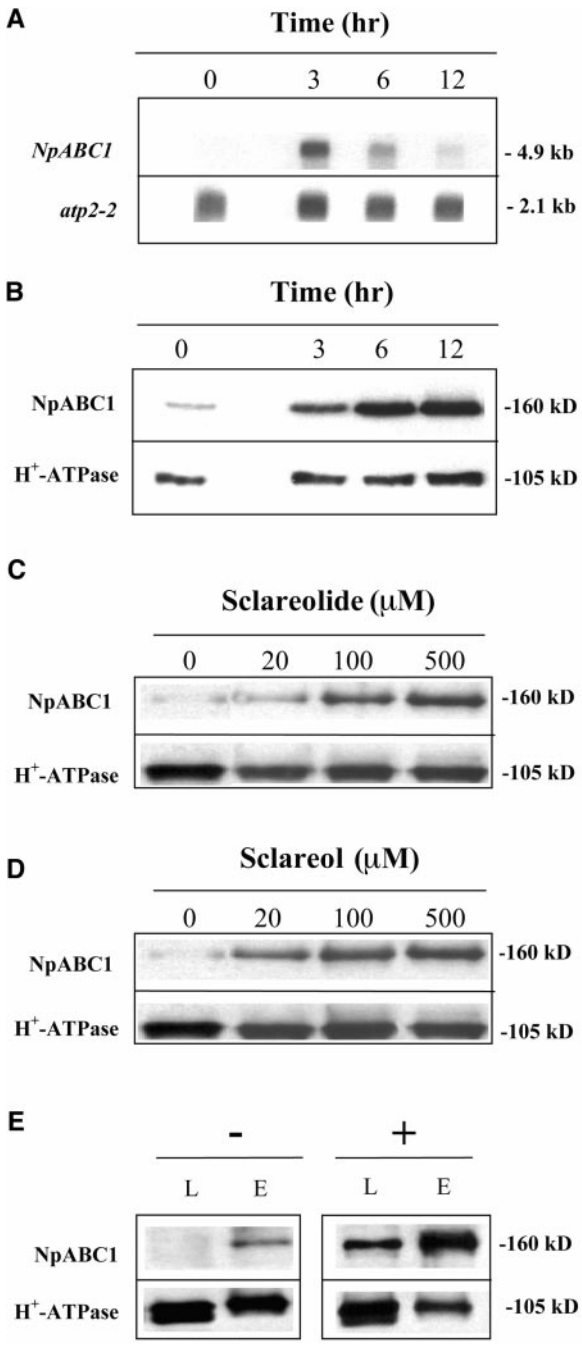

Figure 4. Induction of *NpABC1* Expression by Sclareolide and Sclareol.

(A) Time course of the induction of *NpABC1* transcripts by sclareolide in cell culture. Total RNA was isolated from a *N. plumbaginifolia* cell culture treated with 500 μM sclareolide for the indicated times, and 10-μg samples were electrophoresed, blotted, and hybridized with a ^{32}P-labeled probe corresponding either to the 3′ untranslated region of the *NpABC1* cDNA or to *atp2-2*, which was used as a control.

(Hung et al., 1998) has shown that the ATP binding subunit forms an L shape, and the α helices between the Walker A motif and the ABC signature have been suggested to play a role as a signaling domain (Holland and Blight, 1999). Functional differences between NpABC1 and fungal homologs therefore might be linked to regulatory aspects.

NpABC1 transcript levels were high 3 hr after sclareolide addition and then decreased, suggesting that the inducing signal (possibly the internal concentration of this terpene) was reduced to a level less than that needed for induction. In contrast, the protein concentration remained fairly stable for up to 12 hr (Figure 4B) or even 96 hr (data not shown) after treatment. The simplest explanation for the induction of *NpABC1* expression by sclareol or sclareolide is that a regulatory system activates the expression of the transporter, which pumps a metabolite out of the cell when its concentration within the cell reaches a certain level and before it becomes toxic. The hypothesis of an indirect effect, that is, that cell poisoning causes a general stress response that activates several genes, including *NpABC1*, cannot be excluded because the initial screening (Figure 1) used a sclareolide concentration (500 μM) that slowed cell growth. However, the observation that 20 μM sclareol or sclareolide, the lowest concentrations tested, induced NpABC1 but had no effect on cell growth suggests a more direct effect of the diterpenes on the induction of NpABC1 expression. It is clear that any progress regarding the induction mechanism involved would greatly benefit from the identification (e.g., through gene reporter approaches) of *cis*-regulating sequences and *trans*-activating factors involved in the transcriptional activation of *NpABC1*. In this regard as well, cell culture represents a very convenient tool for deciphering the transducing pathway from sclareol to gene expression.

(B) Time course of NpABC1 induction by sclareolide in cell culture. The *N. plumbaginifolia* cell culture was treated with 500 μM sclareolide, and samples were taken at the indicated times for the preparation of a microsomal fraction. The microsomal proteins (10 μg) were subjected to SDS-PAGE and protein gel blot analysis using anti-NpABC1 or anti–H$^+$-ATPase antibodies.

(C) Protein gel blot analysis of the induction of NpABC1 by varied sclareolide concentrations in *N. plumbaginifolia* cell culture. A microsomal fraction was isolated 12 hr after treatment, and samples (10 μg) were electrophoresed and immunodetected as described in **(B)**.

(D) Protein gel blot analysis of NpABC1 induction by varied sclareol concentrations in cell culture. A microsomal fraction was isolated 12 hr after treatment, and samples (10 μg) were electrophoresed and immunodetected as described in **(B)**.

(E) Expression of NpABC1 in leaf tissues. Leaves of *N. plumbaginifolia* were infiltrated (+) or not infiltrated (−) with 200 μM sclareol for 18 hr. Microsomal fractions were prepared from the entire leaf (L) or the peeled epidermis (E), and proteins (5 μg) were electrophoresed and immunodetected as described in **(B)**.

The sizes of the bands (in kilobases or kilodaltons) are indicated at right.

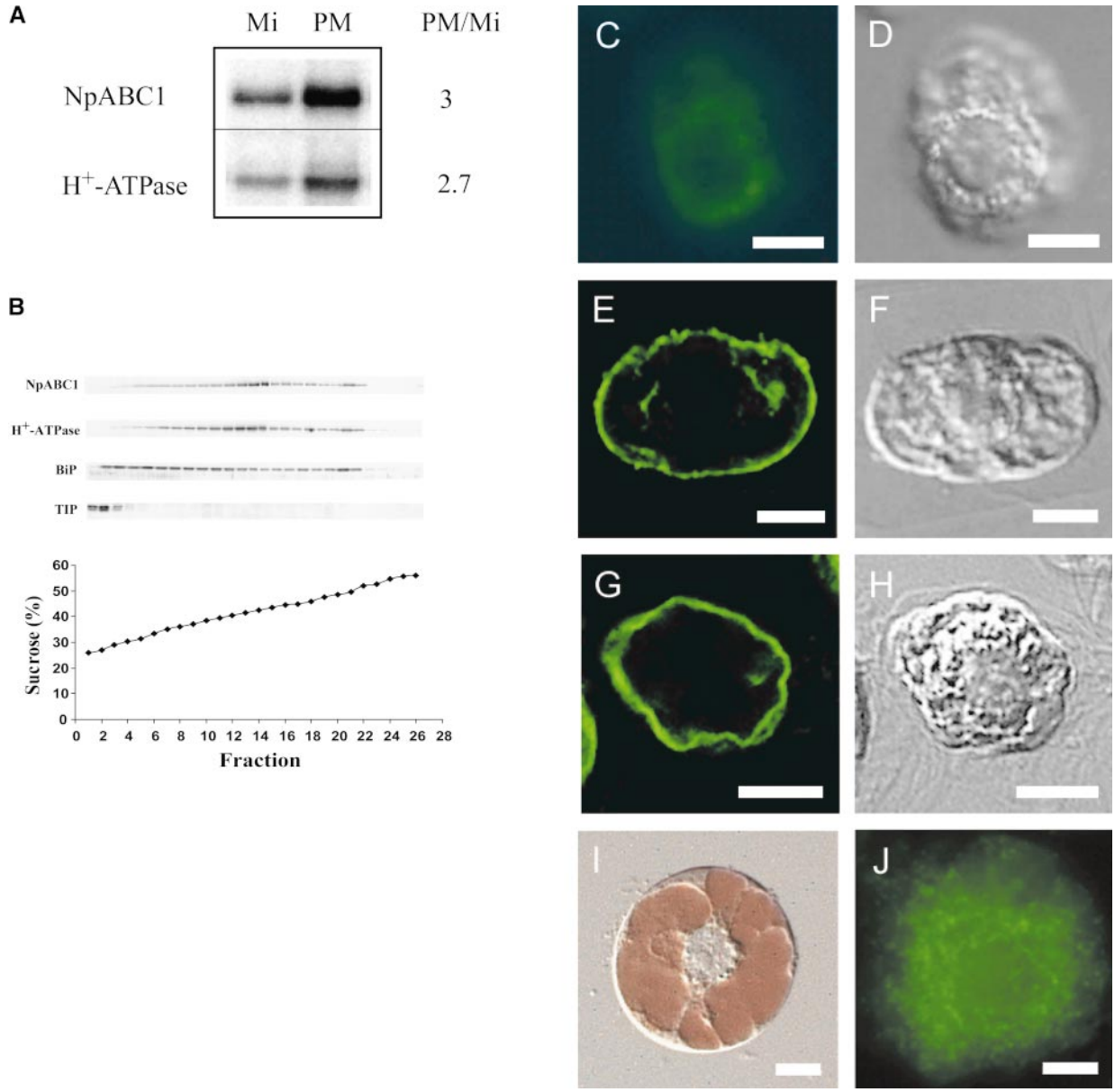

Figure 5. NpABC1 Is Localized in the Plasma Membrane.

(A) Protein gel blot analysis of microsomal (Mi) and plasma membrane (PM) fractions prepared from cells grown for 24 hr in the presence of 500 μM sclareolide. Protein samples (5 μg) were electrophoresed, blotted, and immunodetected using anti-NpABC1 or anti–H^+-ATPase antibodies and ^{125}I-labeled protein A. The quantitative analysis was performed using an image analysis system (Bio-Rad). PM/Mi indicates the signal ratio for the two membrane fractions.

(B) A microsomal fraction prepared from a *N. plumbaginifolia* cell culture grown for 24 hr in the presence of 200 μM sclareolide was centrifuged on a sucrose density gradient. Proteins were electrophoresed, blotted, and immunodetected using antibodies against NpABC1, H^+-ATPase (plasma membrane marker), BiP (endoplasmic reticulum marker) and TIP (tonoplast marker). The sucrose concentration of the gradient fractions was measured with a refractometer and is shown below.

(C) In situ immunodetection of a protoplast from control *N. tabacum* line Bright Yellow 2 (not treated with sclareolide) with primary antibodies against NpABC1 and secondary fluorescein-conjugated antibodies.

(D) Nomarski image of the protoplast shown in **(C)**.

(E) Protoplast from a cell treated for 18 hr with 500 μM sclareolide and immunodetected as described in **(C)**.

(F) Nomarski image of the protoplast shown in **(E)**.

(G) Protoplast from a cell treated with sclareolide as described in **(E)** and immunodetected with primary antibodies against H^+-ATPase.

(H) Nomarski image of the protoplast shown in **(G)**.

(I) Nomarski image of a protoplast from a cell treated as described in **(E)** and stained for 20 min with neutral red.

(J) Protoplast from a cell treated with sclareolide as described in **(E)** and immunoblotted with primary antibodies against mitochondrial ATP synthase β. Bars in **(C)** to **(J)** = 10 μm.

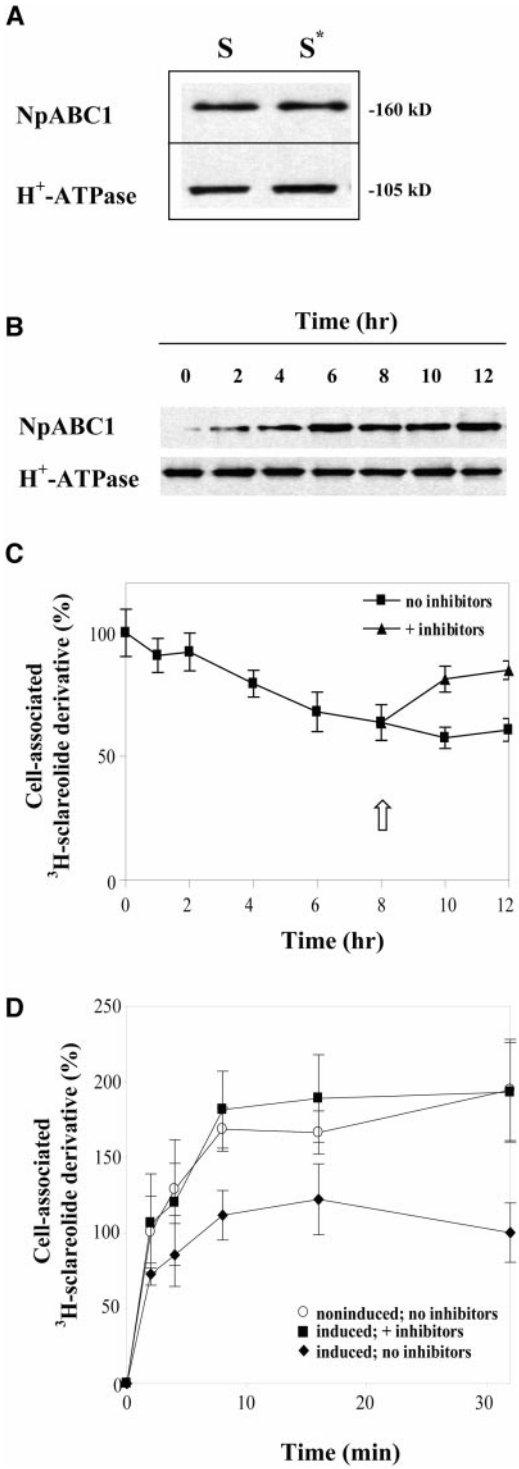

Figure 6. Efflux of the ^{3}H-Labeled Sclareolide Derivative Correlates with *NpABC1* Expression.

(A) Protein gel blot analysis of NpABC1 induction by 100 μM sclareol (S) and 100 μM ^{3}H-decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol (S*) in cell culture of *N. plumbaginifolia*. Microsomal fractions were prepared 12 hr after induction. Samples (10 μg) were subjected to SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and analyzed using anti-NpABC1 or anti-H$^+$-ATPase antibodies. The sizes of the bands (in kilodaltons) are indicated at right.

(B) Protein gel blot analysis of NpABC1. The *N. plumbaginifolia* cell culture was treated with a mixture of 100 μM sclareolide and 4 μM labeled sclareolide derivative, and samples were taken at the indicated times for the preparation of a microsomal fraction, which was subjected to SDS-PAGE and protein gel blot analysis using anti-NpABC1 or anti-H$^+$-ATPase antibodies.

(C) Amount of ^{3}H-decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol inside the *N. plumbaginifolia* cells as a function of time. Cells were treated as described in **(B)**, and then, at the indicated times, samples (closed squares) were filtered and the cell-associated radioactivity was counted. After 8 hr (arrow), ATP synthesis inhibitors (5 mM NaF, 2 mM NaN_3, and 1 μM carbonylcyanide m-chlorophenylhydrazone) were added to half of the cell culture (closed triangles), and incubation continued for an additional 4 hr. The values represent the means of four independent experiments ±SD. Sclareol equilibrates in the cell within 1 min. The 100% value therefore corresponds to the cell-associated radioactivity 1 min after the addition of the ^{3}H-sclareolide derivative to the cell culture.

(D) Cells were treated with 100 μM sclareolide (closed diamonds and closed squares) or not treated (open circles) for 12 hr and washed, and then the ^{3}H-sclareolide derivative (4 μM) was added either alone (closed diamonds and open circles) or together with the same ATP synthesis inhibitors described in **(C)** (closed squares). Cell samples were taken at the indicated times and counted. The values represent the means of six independent experiments ±SD. The 100% value corresponds to the radioactivity associated at the end of the experiment with induced cells not treated with inhibitors (closed diamonds).

The facts that NpABC1 is expressed in the leaf epidermis, that its expression is enhanced by sclareol, and that this chemical is found at the leaf surface suggested that NpABC1 actually transports diterpenoids. To demonstrate this point, we took advantage of the fact that these compounds are highly soluble in both hydrophobic and hydrophilic media (Guo and Wagner, 1995) and so permeate rapidly through biological membranes, allowing us to follow the exclusion of an added labeled sclareolide derivative that is identical to sclareol except for a shorter lateral substitution. As induction of NpABC1 expression increased, the amount of cell-associated radioactivity decreased, suggesting that the induced NpABC1 was involved in extruding the labeled terpenoid. This effect was blocked when the cells were poisoned with ATP synthesis inhibitors, indicating that an energy-dependent process was involved. Although exclusion was not complete, this probably reflects the high solubility of terpene compounds in membranes.

Localization of NpABC1 to the plasma membrane was demonstrated by the similar behavior of this transporter and of the plasma membrane H$^+$-ATPase, using three different approaches: plasma membrane enrichment by phase parti-

tion, equilibrium density centrifugation, and in situ detection. This does not exclude that NpABC1 might also be present in internal membranes such as secretory vesicles, but in this case it should represent a minor fraction.

At this stage, we cannot formally exclude the possibility that another diterpenoid-induced ABC transporter was responsible for secretion of the labeled sclareolide derivative, but this is unlikely. Indeed, antibodies obtained against an NpABC1 region outside the highly conserved ATP binding domain recognized a strong band after sclareol induction. Only proteins closely related to NpABC1 are expected to be detected because only one from the >60 predicted ABC proteins from Arabidopsis showed an identity superior to 65% within this region. In addition, besides the two peptide sequences used to clone NpABC1, four other ABC-like sequences were obtained (LMDXLAGR, QEIYVGPLGR, AAYISQHDL, and PVFY), and they all fit the predicted NpABC1 sequence whereas, except for the last one, none of them displays 100% identity to any of the predicted ABC transporters from Arabidopsis. Therefore, we conclude that NpABC1 or a very closely related transporter is involved in sclareolide transport.

Constitutive expression of *NpABC1* in cell cultures or heterogeneous expression, for example, in yeast, will be required to determine directly the substrates of this enzyme, especially because ABC transporters usually have a broad range of substrates. In addition, in the case of both plant cell and yeast cultures, transport also can be tested easily by determining the resistance to toxic metabolites of an NpABC1-expressing strain.

Although TUR2 from *S. polyrrhiza* is the closest characterized homolog of NpABC1 (69% identity), these two proteins do not seem to be functionally equivalent. The transported substrates for TUR2 are not known, but the expression of its corresponding gene is induced by abscisic acid, which, although it belongs to the terpenoid class, has no effect on *NpABC1* expression (data not shown). Similarly, the fact that the yeast PDR5 is one of the closest fungal homologs of NpABC1 should not be considered as proof that they share the same pattern of substrates.

A clear disadvantage of the use of cell cultures is that this model does not provide any information on the physiological roles of *NpABC1* in the plant. One clue comes from the observation that *NpABC1* expression is strongly enhanced by sclareol, a major diterpene that is toxic for various organisms and that is found in Solanaceae (Bailey et al., 1975), Labiatae (Souleles and Argyriadou, 1997), and Gymnospermae (Pietsch and Konig, 2000) species. It inhibits the growth of rust fungi (Bailey et al., 1975) and various *Staphylococcus* spp (Demetzos et al., 1999) and induces apoptosis in human leukemia cell lines (Dimas et al., 1999). It also inhibits the growth of wheat coleoptiles (Cutler et al., 1977), indicating that it also has an effect on plant cells. Sclareol toxicity at high concentration was confirmed by the observation that the growth of *N. plumbaginifolia* cells was reduced progressively when the sclareol concentration in the external medium was increased up to 500 μM (data not shown). It is important, therefore, for the cell to develop an efficient exclusion system. Although vacuolar sequestration is a well-known method of detoxification in plant cells, the localization of NpABC1 in the plasma membrane, as shown by both in vitro and in vivo immunolocalization, indicates that in this case cell extrusion is involved. In the absence of added sclareol, there was low expression of *NpABC1* in the leaf; however, expression was concentrated in the epidermis. This is in agreement with the observation that sclareol is a major component of the leaf surface of *Nicotiana* spp, in which it is thought to protect the plant against fungal diseases (Bailey et al., 1975). Sclareol biosynthesis occurs essentially in the trichomes (Guo and Wagner, 1995), to which *NpABC1* expression might be restricted.

In conclusion, the functional approach we have developed has led to the isolation of *NpABC1*, a gene whose expression is induced by the diterpenoid sclareol. The encoded protein was localized to the plasma membrane, and there is evidence that it participates in the extrusion of this antimicrobial terpenoid. Thus, in addition to a detoxification role already described for vacuolar enzymes, plant ABC transporters also are involved in plant defense.

METHODS

Plant Material

The *Nicotiana plumbaginifolia* suspension cell culture (Barfield et al., 1985), was maintained in darkness at 25°C in 1-liter Erlenmeyer flasks on a rotary shaker at 100 rpm. Every 14 days, 20 mL of culture was transferred to a new flask containing 200 mL of medium (4.4 g/L Murashige and Skoog medium [ICN, Costa Mesa, CA], 3% sucrose, 0.5 mg/L 2,4-D, 0.5 mg/L 6-benzylaminopurine, 2.5 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.05 mg/L calcium pantothenate, 0.5 mg/L nicotinic acid, and 50 mg/L *myo*-inositol, pH 5.7).

Induction of NpABC1 Expression in Plant Suspension Cell Culture and Leaves of *N. plumbaginifolia*

Twelve days after culture dilution, sclareolide or sclareol (Aldrich), solubilized in DMSO, was added to the culture flask to give a final concentration of 20 to 500 μM. Control cultures were treated with equal volumes of DMSO. For leaf treatment, sclareol (200 μM) was infiltrated through stomata of 3-month-old *N. plumbaginifolia* leaves using a syringe without a needle.

In Vivo Labeling of Proteins with ^{35}S

Cells (5-mL cultures) were grown for 12 days in the medium described above in which $MgSO_4$ was replaced by 350 mg/L $MgCl_2$. Three hours after sclareolide addition, 20 μCi/mL $^{35}SO_4^{2-}$ (1000 Ci/mmol; Amersham) was added to the medium, and incubation was continued for an additional 9 hr.

Membrane Preparation

For mini-preparations after in vivo labeling, cells (2.5 mL) were harvested 12 to 60 hr after sclareolide addition and suspended in 800 μL of homogenization buffer (250 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 7 g/L polyvinylpolypyrrolidone, 0.1% DTT, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL each of leupeptin, pepstatin, aprotinin, antipain, and chymostatin). They were then ground at 4°C in a mill (Braun Biotech; Melsungen, Germany) for 2 min in the presence of 500 mg of glass beads (0.8 mm diameter), and the homogenate was centrifuged at 9000*g* for 10 min at 4°C. The resulting supernatant was centrifuged at 14,000*g* for 30 min, giving a pelleted microsomal fraction from which plasma membranes were purified by partitioning in an aqueous two-phase system (1 mL of phase mixture), as described by Larsson et al. (1987). For large-scale preparations (1-liter cultures), cells were harvested 12 hr after sclareolide addition, washed with 0.2% Na_2EDTA and 0.15% KCl, pH 5.7, suspended in 80 mL of homogenization buffer, and ground as described above. The homogenate was centrifuged at 12,000*g* for 10 min , the resulting supernatant was centrifuged at 67,000*g* for 30 min, and plasma membranes were purified by partitioning in the two-phase system (27 mL of phase mixture).

SDS-PAGE and Protein Digestion

For preparative purposes, plasma membrane proteins (1 mg) were solubilized for 1 hr at 4°C in 2% (w/v) octylglucoside (Boehringer Mannheim), 80 mM Tris-HCl, pH 6.8, and the protease inhibitors mentioned above. The soluble fraction was recovered by centrifugation at 20,000*g* for 30 min at 4°C, diluted in Laemmli buffer (Laemmli, 1970), incubated for 15 min at 37°C, and loaded onto SDS–polyacrylamide gels. After Coomassie Brilliant Blue R 250 staining, gel slices containing the bands of interest were excised, washed with water, and equilibrated for 2 hr at 25°C in 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, and 2 mM Tris-HCl, pH 6.8, and then proteins were eluted and concentrated using a low-melting-point agarose–based gel system (Rider et al., 1995). The agarose slice containing the concentrated protein was melted at 56°C in 100 μL of 0.1 M Tris-HCl, 7 M urea, and 2 mM $CaCl_2$, pH 8.6, and then 1 μg of subtilisin A (Fluka, Milwaukee, WI) was added and digestion was performed overnight at 37°C. Alternately, digestion was performed with 1 μg trypsin (Boehringer Mannheim) in 100 mM Tris-HCl, pH 8.5, 10% acetonitrile (Boehringer Mannheim), and 1% Triton X-100 (Calbiochem, La Jolla, Ca). Generated peptides were separated by reverse phase HPLC. Selected peptides were sequenced by automated Edman degradation using an Applied Biosystems (Foster City, CA) pulsed liquid phase sequencer (model 477A).

Cloning and Sequencing of *NpABC1* cDNA

The sequences of two selected peptides were used to synthesize degenerate oligonucleotide primers. The primers for peptide 1 (IHQPSID) were MJ1 (5′-AT[A/T/C]CA[C/T]CA[A/G]CC[A/T/C/G]-[A/T][C/G][A/T/C/G]ATTGA-3′), MJ2 (5′-AT[A/T/C]CA[C/T]CA[A/G]CC-[A/T/C/G][A/T][C/G][A/T/C/G]ATCGA-3′), and MJ3 (5′-AT[A/T/C]CA-[C/T]CA[A/G]CC[A/T/C/G][A/T][C/G][A/T/C/G]ATAGA-3′), whereas the primer for peptide 2 (EFAEAY) was MJ4 (5′-GA[A/G]TT[C/T]GC[A/T/G/C]GA[A/G]GC[A/T/G/C]TA-3′).

Three hours after sclareolide addition, total RNA was extracted from 4 g of *N. plumbaginifolia* cells and purified on a CsCl cushion, as described by Kingston (1997). Poly(A) RNA was purified using the PolyA-Tract mRNA isolation system IV (Promega). Using oligo(dT), mRNA was reverse transcribed as described previously (Beverley, 1992) and amplified by polymerase chain reaction (PCR) in the presence of oligo(dT) and primer MJ1, MJ2, or MJ3 (3′ end PCR fragment). A 1356-bp amplification product was cloned into the pGEM-T-easy vector (Promega) and sequenced using the Big Dye terminator sequencing kit (Perkin-Elmer) and an ABI PRISM 377 DNA sequencer (Applied Biosystems). Starting with the degenerate primer MJ4 and oligonucleotide 5′-GTCACTCGTACCAGGTCGCGGCAC-GC-3′, which was designed using the results of 3′ end PCR, a full-length cDNA was obtained using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Two independent clones containing the entire cDNA (named *NpABC1*) were sequenced in both directions, using either vector primers or specific oligonucleotides designed using the previously obtained sequence.

RNA Gel Blot Analysis

Three hours after sclareolide addition to cell cultures, total RNA was isolated using the Trizol reagent (Gibco BRL), then a 10-μg sample was electrophoresed on a 1.2% (w/v) agarose-formaldehyde gel and transferred to a Hybond N^+ nylon membrane (Amersham International) (Sambrook et al., 1989). Blots were hybridized with a 125-bp ^{32}P-radiolabeled probe corresponding to the *NpABC1* 3′ untranslated region, amplified, and labeled during PCR with the oligonucleotides 5′-TTATGAGTAACAATTAC-3′ and 5′-GTGTTTAATTTGCTG-TTG-3′. As a control, blots were hybridized with a 469-bp PCR fragment obtained with the primers 5′-CGTCAAATGAATGAG-3′ and 5′-CTGTACCCACGAGC-3′ from cDNA clone *atp2-2* encoding the β subunit of mitochondrial ATP synthase (Boutry and Chua, 1985). After hybridization, the blots were washed with 1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for 15 min at 65°C.

Antibody Preparation

The 279-bp DNA fragment encoding Phe-1057 to Asp-1150 of NpABC1 was amplified by PCR using primers, one of which allowed the insertion of a six-histidine tag at the C-terminal end, and the PCR reaction product was inserted into the *Escherichia coli* expression vector pGEX-KG (Guan and Dixon, 1991). The protein of interest was produced in *E. coli*, purified on a nickel–nitrilotriacetic acid agarose resin (Qiagen, Valencia, CA), and used to immunize rabbits. The polyclonal antibodies produced were purified by affinity chromatography on the *E. coli*–produced protein coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia Biotech).

Protein Gel Blotting

For immunoblotting, proteins were solubilized in an SDS cocktail containing protease inhibitors, subjected to SDS-PAGE (7% gel), and then transferred electrophoretically to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA), which was

then blocked with 3% nonfat milk powder in 20 mM Tris-HCl, 137 mM NaCl, and 0.5% Tween 80, pH 7.6. The membrane was then incubated with primary rabbit antibodies specific for either NpABC1 or a plasma membrane H^+-ATPase (Morsomme et al., 1998) and then with anti–rabbit alkaline phosphatase–conjugated secondary antibodies (Boehringer Mannheim), binding of which was detected by chemiluminescence. For quantitative analysis, the secondary antibodies were replaced with ^{125}I-labeled protein A, the signal was detected using a phosphorimager, and the bands were quantified by image analysis (Molecular Analyst; Bio-Rad).

In Situ Immunolocalization

Cells (4-day-old cultures) of *N. tabacum* line Bright Yellow 2 (Nagata et al., 1981) were centrifuged at 100*g* for 10 min, suspended in 15 mL of digestion buffer (0.55 M sorbitol, 0.6% cellulase, and 0.2% MACEROZYME R-10 [Yakult Honska, Tokyo, Japan]), and incubated for 4 hr at 25°C on a rotary shaker at 100 rpm, generating protoplasts that were centrifuged at 73*g* for 10 min, washed three times (4 mM $CaCl_2$, 80 mM KCl, 8% mannitol, 2 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, and 0.1% BSA), and fixed for 2 hr at 20°C in 3.7% formaldehyde, 100 mM K_2HPO_4/KH_2PO_4, pH 6.5, and 0.5 mM $MgCl_2$. Primary antibodies were used at a dilution of 1:20, and secondary antibodies (fluorescein goat anti–rabbit IgG; Molecular Probes, Eugene, OR) were used at a dilution of 1:100. For vacuole staining, protoplasts were incubated for 20 min with 1 mg of neutral red and washed. Samples were analyzed using a microscope (Bio Rad MRC-1024 or Leica DM R).

Fractionation of Organelles

The micosomal membrane fraction (300 μg) was resuspended in 5 mM KH_2PO_4, 3 mM KCl, and 1 mM EDTA, pH 7.6, and layered onto a 4-mL sucrose gradient (30 to 56% [w/w]) prepared in the same medium. After centrifugation at 100,000*g* for 18 hr at 2°C, fractions (100 μl) were collected, and proteins were separated by SDS-PAGE and transferred to nitrocellulose filters (Millipore, Bedford, MA). Immunodetection of NpABC1, H^+-ATPase, the endoplasmic reticulum binding protein (BiP) (Höfte and Chrispeels, 1992), and the tonoplast intrinsic protein (TIP) (Barrieu et al., 1998) was performed as described above.

Labeling of Sclareolide

^{3}H-Decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol was obtained by reduction of sclareolide with ^{3}H-$NaBH_4$ according to a general procedure for lactone transformation into diols (Santaniello et al., 1982) adapted for our purpose. A mixture of sclareolide (250 mg, 1 mmol; Aldrich), polyethylene glycol 400 (5 mL), $NaBH_4$ (114 mg, 3 mmol; Merck), and ^{3}H-$NaBH_4$ (0.75 mg, 0.02 mmol, 13 Ci/mmol; Amersham) was stirred, and the solution was slowly brought to 80°C under argon and maintained at this temperature for 5 hr. The solution was then cooled to room temperature and acidified cautiously with 2 N HCl (10 mL). Extraction with diethyl ether (3 × 10 mL), washing of the organic layers with water (3 × 20 mL), drying over ^{3}H-$NaBH_4$, and concentration gave the labeled diol (chemical yield, 200 mg, 80%, 2 mCi/mmol), the purity of which was checked by silica gel thin-layer chromatography (silica gel 60 F_{254}; Merck) (elution with CH_2Cl_2/ethyl acetate, 4:1; R_F of diol = 0.25; R_F of lactone = 0.95). The structure of the similarly prepared unlabeled diol was confirmed by NMR (data available on request).

Measurement of Transport of ^{3}H-Decahydro-2-Hydroxy-2,5,5,8a-Tetramethyl-1-Naphthalene Ethanol

A 9-day-old *N. plumbaginifolia* cell culture was treated with a mixture of 100 μM sclareolide and 4 μM ^{3}H-decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol (~2 mCi/mmol), and 1-mL samples were harvested at various times between 0 and 12 hr. A microsomal fraction was prepared, and proteins were immunoblotted as described above. In parallel, 1-mL samples were vacuum filtered through prewetted Millipore cellulose nitrate filters (pore size, 45 μm), washed with 1 mL of ice-cold fresh culture medium containing 100 μM sclareolide, rinsed twice with 1 mL of ice-cold water containing 100 μM sclareolide, and then dried, and the retained radioactivity was measured by liquid scintillation counting (Beckman LS 1701). ATP synthesis inhibitors (5 mM NaF, 2 mM NaN_3, and 1 μM carbonylcyamide m-chlorophenylhydrazone) were added to half of the cell suspension after 8 hr of incubation. In a second approach, a 200-mL cell culture was incubated with 100 μM sclareolide for 12 hr, and the cells were washed twice with growth medium without sclareolide, resuspended in 200 mL of growth medium, and divided into 1-mL aliquots. The labeled sclareolide derivative (4 μM) was added, either alone or in combination with the ATP synthesis inhibitors described above, and incubation continued for 0 to 32 min. One-milliliter samples were filtered, and the retained radioactivity was measured as described above. The value observed at time 0 was considered to represent nonspecific binding and was deduced from the values obtained for the other time points.

Accession Number

NpABC1 sequence data have been submitted to EMBL under the accession number AJ404328.

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