ELSEVIER

Transporters of secondary metabolites

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The membrane transport of plant secondary metabolites is a newly developing research area. Recent progress in genome and expressed sequence tag (EST) databases has revealed that many transporters and channels exist in plant genome. Studies of the genetic sequences that encode these proteins, and of phenotypes caused by the mutation of these sequences, have been used to characterize the membrane transport of plant secondary metabolites. Such studies have clarified that membrane transport is fairly specific and highly regulated for each secondary metabolite. Not only genes that are involved in the biosynthesis of secondary metabolites but also genes that are involved in their transport will be important for systematic metabolic engineering aimed at increasing the productivity of valuable secondary metabolites *in planta*.

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Current Opinion in Plant Biology 2005, **8**:301–307

This review comes from a themed issue on
Physiology and metabolism
Edited by Toni Kutchan and Richard Dixon

Available online 8th April 2005

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2005.03.011

Introduction

Plants produce a large number of secondary metabolites, which are classified into several groups according to their biosynthetic routes and structural features. To achieve their function, such as protection against UV light or pathogens, they are generally accumulated in specific tissues or cell-types in which subcellular localization is highly regulated. Secondary metabolites are often transported from source cells to neighboring cells, or even further to other tissues or remote organs. Recent progress in molecular biology has enabled us to study transporter proteins for these natural products in plants. In this review, I introduce the development of this research field over the past few years, providing an overview of proteins that are involved in the membrane transport of secondary metabolites.

The function of vacuole in secondary metabolism

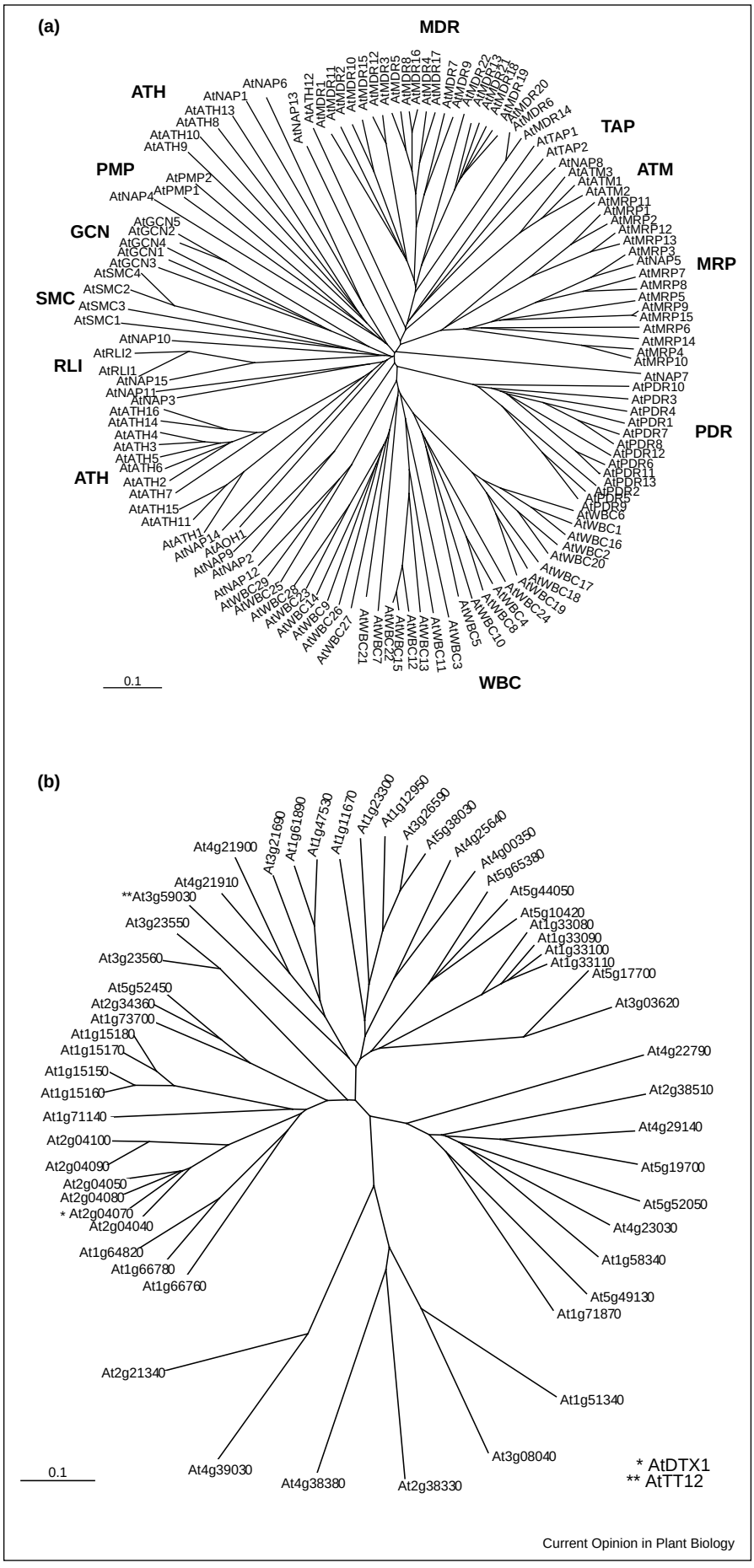
Storage vacuoles, which often occupy 40–90% of the inner volume of plant cells, play a pivotal role in the accumulation of secondary metabolites in plants. The accumulation of secondary metabolites in vacuoles has at least two positive roles: the sequestration of biologically active endogenous metabolites inside the cells and the protection of such metabolites from catabolism [1]. Two major mechanisms are proposed for the vacuolar transport of secondary metabolites: H^+-gradient-dependent secondary transport via H^+-antiport and directly energized primary transport by ATP-binding cassette (ABC) transporters [2].

ABC transporters constitute a large protein family that is found in a range of organisms from bacteria to humans. Because of intensive studies on the roles of ABC transporters in multidrug resistance in animal cancer cells, it had long been believed that they exhibit broad substrate specificity. Recent studies have demonstrated that the function of ABC transporters is not restricted to detoxification processes [2]. Furthermore, they have been found to be involved in many specific biological activities, such as cell signaling, that have strict substrate specificity [3,4], as well as in other divergent physiological functions [5,6]. It was suggested recently that the ABC transporter family might have evolved according to the need to transport specific substrates in each organism, and not as drug efflux pumps ([7]; Figure 1a).

Alkaloids

The mechanism for the long-distance transport of alkaloids is well elucidated in Solanaceae. Nicotine biosynthetic enzymes are expressed specifically in the root tissues, which is advantageous for the xylem transport of nicotine [8•]. The transporter that is involved in the translocation of nicotine has no yet been identified, but a multidrug resistance protein (MDR)-like transport activity was measured in the Malpighian tubules of tobacco hornworm, *Manduca sexta* [9].

Plant alkaloids are often effluxed by ABC transporters in microorganisms and herbivorous insects, but only a few of these transporters are currently known to be responsible for alkaloid transport *in planta*. Recent studies show that the uptake of an isoquinoline alkaloid, berberine, by *Coptis japonica* cells is mediated by an ABC transporter [10], and *CjMDR1* was cloned as a candidate gene for this MDR transporter [11]. Functional analyses of CjMDR1 using *Xenopus* oocytes showed that this protein recognized berberine as its substrate and transported it in an inward direction [12•], although most eukaryotic ABC transporters are known to function as efflux carriers. Because berberine is biosynthesized in root tissues, this alkaloid is translocated to the rhizome where it is trapped


(a)
MDR
ATH
PMP
GCN
SMC
RLI
ATH
TAP
ATM
MRP
PDR
WBC
0.1
(b)
0.1
* AtDTX1
** AtTT12
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by the plasma-membrane-localized CjMDR1, resulting in its accumulation in the rhizome. An ABC transporter has been shown to be involved in the secretion of berberine from cultured *Thalictrum minus* cells into the culture medium [13,14]. Interestingly, the identified ABC transporter shares high amino acid sequence similarity with CjMDR1. The regulatory mechanism that determines the direction of transportation remains to be clarified.

Berberine can also be transported by a H^+-antiport mechanism in heterologous plants. A detoxifying efflux carrier, AtDTX1 belonging to the MATE (multidrug and toxic compound extrusion) family, has been identified in *Arabidopsis* [15]. MATE is a large gene family with at least 56 members encoded by the *Arabidopsis* genome [16]. AtDTX1 is localized at the plasma membrane and mediates the efflux of exogenous toxic compounds, including berberine, by a proton-motive force (Figure 1b).

In *Berberis*, the terminal steps of berberine biosynthesis take place exclusively in specific vesicles, which are presumably derived from the endoplasmic reticulum (ER) and later fused with the central vacuole [17]. This scheme fits plants that produce and accumulate berberine in the same cells, but the carrier-mediated mechanism is appropriate for plant species, such as *C. japonica*, whose sink and source organs are distant.

Another important isoquinoline alkaloid, morphine, is accumulated in the large membranous vesicles of latex in opium poppy. Immunofluorescence analyses using antibodies that are specific for five enzymes of alkaloid formation in opium poppy have been recently reported [18]. In the capsule and stem, both *O*-methyltransferases and an *O*-acetyltransferase are found predominantly in parenchyma cells within the vascular bundle, whereas codeinone reductase is localized to laticifers, suggesting that pathway intermediates must be transferred between these locations. Another group reported that three morphine biosynthetic enzymes are localized in the sieve elements of opium poppy [19]. Deus-Neumann and Zenk's early work on indole alkaloids [20] indicated that their vacuolar transport was energy-requiring, although no transporter gene for indole alkaloid has yet been isolated to my knowledge.

Phenols

In plants, glucosidation plays a key role in the detoxification of endogenous secondary metabolites and xenobiotics, with their glucosides often accumulating in the vacuoles. Multidrug resistance-associated protein (MRP)-type ABC transporters are reported to be involved in the vacuolar sequestration of such glucosides, in addition to that of glucuronides and glutathione conjugates [21].

The involvement of MRP in the transport of such phenolic glucosides was suggested in the *bronze-2* (*bz2*) mutant of maize [22]. *BZ2* encodes a glutathione *S*-transferase, and *bz2* mutants are defective in the accumulation of anthocyanin in the vacuole. Because MRPs have substrate preference for glutathione conjugates, and as their transport activity is often stimulated in the presence of glutathione, the involvement of MRP in the vacuolar transport of anthocyanin was presumed. Similar results were also reported in dicots, such as *Arabidopsis* [23] and carnation [24]. Further direct evidence of the involvement of MRP in anthocyanin accumulation was provided by Goodman *et al*. [25••] who found that the maize ABC transporter, ZmMRP3, is localized in tonoplast and required for anthocyanin transport.

An H^+-gradient-dependent transport has also been found to have a role in anthocyanin accumulation. The *Arabidopsis* mutant *transparent testa12* (*tt12*) shows strongly reduced proanthocyanidin deposition in the vacuoles of endothelial cells [26]. The gene product of *TT12* is a secondary transporter-like protein belonging to the MATE family, suggesting that this protein is responsible for the vacuolar transport of anthocyanin via an H^+-antiport mechanism. A recent paper reported the presence of a similar MATE-protein in tomato [27].

In plants, endogenous or exogenous substrates can be transported by different transporter proteins using different mechanisms. For example, the uptake of the main barley flavonoid, saponarin (an apigenin glucoside), into barley vacuoles occurs via H^+-antiport, whereas the transport of saponarin into the vacuoles of *Arabidopsis*, a heterologous plant that does not produce this metabolite, displays characteristics that are typical of an ABC transporter [28]. Similarly, a herbicide glucoside, hydroxyprimisulfuron-glucoside, is taken up by barley vacuoles via a directly energized primary transport mechanism [29].

The dependence of membrane transport on the presence of a glucose or glutathione ligand was investigated with a sulfonylurea herbicide, chlorsulfuron, using vacuolar membrane vesicles purified from red beet (*Beta vulgaris*) [21]. The glutathione conjugate of the chlorsulfuron analog appeared to be transported by an ABC transporter,

(Figure 1 Legend) Phylogenetic relationship of ABC proteins and MATE family members in *Arabidopsis*. The amino-acid sequences of **(a)** ABC proteins and **(b)** MATE members were aligned using ClustalW and subjected to phylogenetic analysis provided by EMBL. The nomenclature of ABC proteins is according to Sánchez-Fernández *et al*. [49]. The abbreviations of each ABC protein subfamily are as follows: AOH, ABC1 homolog; ATH, ABC2 homolog; ATM, ABC transporter of mitochondria; GCN, general control non-repressible; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NAP, non-intrinsic ABC protein; PDR, pleiotropic drug resistance protein; PMP, peroxisomal membrane protein; RLI, RNase L inhibitor; SMC, structural maintenance of chromosome; TAP, transporter associated with antigen processing; WBC, white-brown complex protein. MATE members are indicated as *Arabidopsis* Genome Initiative (AGI) codes.

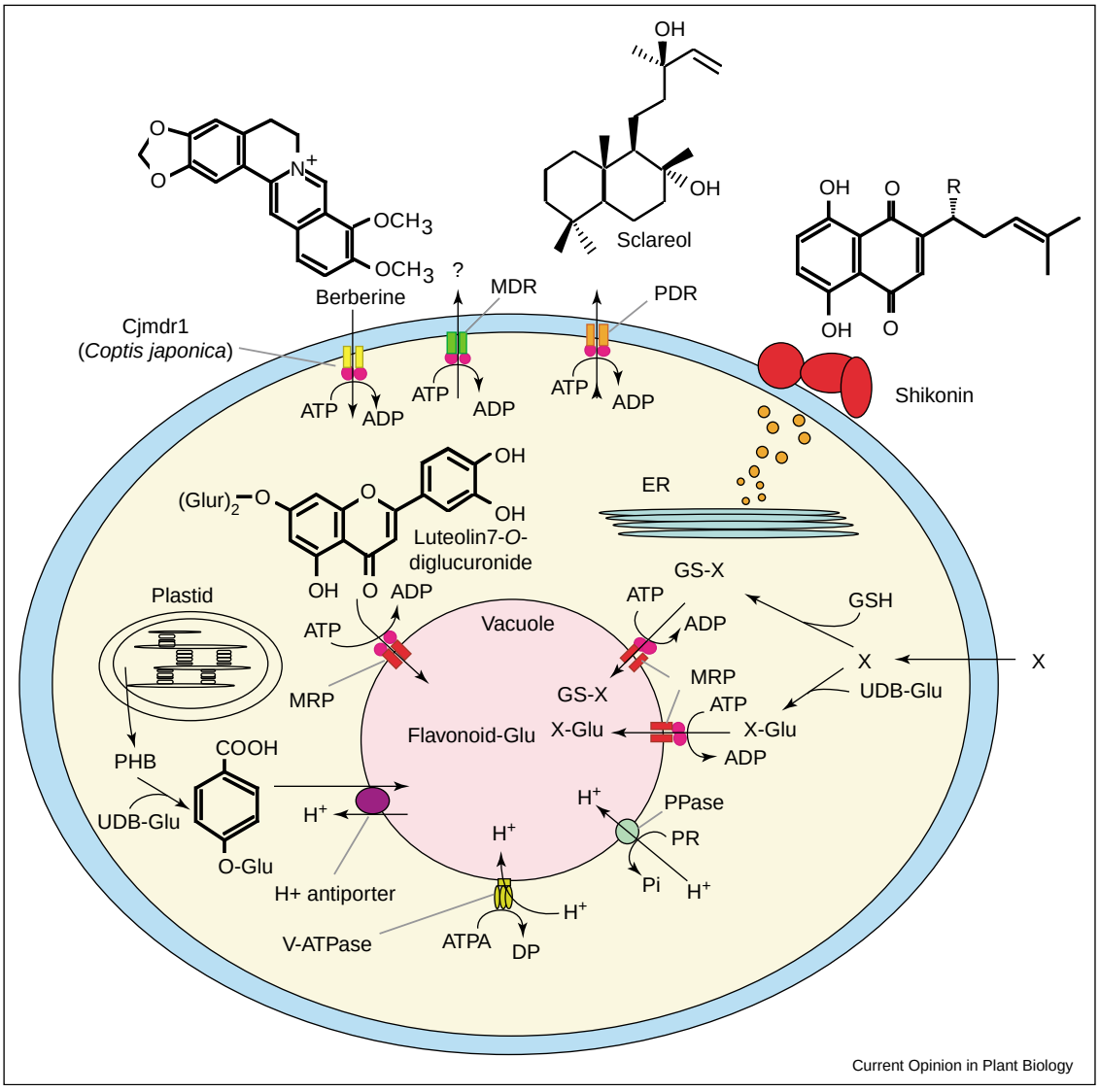
whereas the phenol glucosides of *p*-hydroxycinnamic acid and *p*-hydroxybenzoic acid were apparently transported by a H^+-gradient-dependent transport mechanism. When cinnamic acid was conjugated with glutathione, however, it was transported into the vesicles via a GS-X pump, which is defined as MRP [30]. These data suggest that the sugar moiety is a preferred 'tag' recognized by the secondary transporters, whereas the glutathione moiety is preferred by MRP proteins functioning as GS-X pumps [31,32].

Sucrose transport is known to be mediated by a H^+-symporter. A survey of the substrate specificity of the sucrose transporter AtSUC2 of *Arabidopsis* has provided a new insight into secondary metabolite transporters in plants [33•]. In the assay using AtSUC2 expressed in *Xenopus* oocytes, large inward currents were observed for eight glucosides, including arbutin and salicin, out of the 24 sugars and glucosides analyzed. This suggests that a sucrose transporter of low substrate specificity might participate in transporting glucosides, adding to its main function of sucrose transportation into the phloem.

Terpenoids

There are many reports on the emission of volatile terpenes, such as the emission of mono- and sesquiterpene from the flowers of *Arabidopsis* [34] and snapdragon [35], and from the leaves of woody plants [36]. Emissions of volatile terpenoids are also dramatically induced by insect attacks on maize leaves [37] and cotton flower buds [38], where biosynthetic genes are strongly induced by

Figure 2



Model of transport processes for secondary metabolites in a plant cell.

such attack. It is widely accepted that the emission of volatile terpenoids is regulated at the level of transcription of biosynthetic enzymes, and it is widely believed that no transporter or permease is needed for their emission.

In recent years, there have been attempts to metabolically engineer monoterpene biosynthesis. For example, in *Arabidopsis* [39] and tobacco [40,41], one or several of the genes involved in monoterpene biosyntheses were expressed to successfully produce monoterpenes in homologous and heterologous plants. A portion of the monoterpenes that were produced in this way were emitted from the leaves of transgenic plants, presumably by simple diffusion because of their high volatility and lipophilicity. Detailed physicochemical analyses have been made to determine how the emission of volatiles is regulated [42]. However, there is no strong evidence to deny the existence of a broad-specificity permease that facilitates the emission of volatile terpenoids.

A unique plasma-membrane-localized ABC transporter for the transport of diterpenes, pleiotropic drug resistance (PDR) protein, was first identified in the leaves of *Nicotiana plumbaginifolia* [43]. PDR seems to be involved in the efflux of an endogenous antifungal diterpene, sclareol. Its orthologs have also been found in *Arabidopsis* [44], and *Spirodela polyrrhiza* [4], and these proteins were strongly induced by elicitor treatment, suggesting that they are directly involved in the pathogen resistance processes by transporting diterpene metabolites.

Vesicle transport

Little is known about the transport mechanism for lipophilic secondary metabolites, such as triterpenes and phytosterols. One model of lipophilic secondary metabolite transportation is the shikonin production system in *Lithospermum erythrorhizon* cell and hairy root cultures [45]. After their biosynthesis in the ER, shikonin derivatives, which are red naphthoquinones, are accumulated in the red granules that are attached to the cell surface [46]. There, the intracellular movement of shikonin is managed via vesicle transport, and two hypotheses are presented for the mechanism of this transport: first, direct transfer of lipids from ER to the plasma membrane and, second, Golgi-mediated exocytosis, as proposed for cuticular wax transport [47].

Another model of vesicle transport has been reported in maize BMS (Black Mexican Sweet) cells. The induction of the *P1* transcription factor caused the accumulation of green auto-fluorescent bodies in the maize cytoplasm, which later appeared to fuse with the plasma membrane and thus be secreted out of the cytoplasm to the cell wall [48•]. This system will provide material that might be applicable for the study of vesicle-mediated transport of secondary metabolites.

Conclusions

A schematic drawing of transport processes of secondary metabolites is shown in Figure 2. The molecular analysis of the membrane transport of plant secondary metabolites is a fairly new field in plant biology. The dispersed localizations of both the end-products discussed in this review and their biosynthetic enzymes indicate that biosynthetic intermediates might move among organelles during the biosynthesis of secondary metabolites [49–51]. Although simple diffusion might be sufficient for hydrophilic intermediates, transporters that are localized in organelles might take part in the regulation of transportation, especially that of lipophilic intermediates [52]. Metabolic engineering has become a popular means of increasing the production of secondary metabolites by overexpressing biosynthetic enzymes [53]. The introduction of accumulation mechanisms by engineering transport systems should be an effective way to increase the production of secondary metabolites.

Acknowledgements

I thank the Ministry of Education, Culture, Sports, Science and Technology of Japan (No.s 00L01605 and 15031217) and the Uehara Memorial Foundation for supporting my work by providing research grants.

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