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Role of vacuolar transporter proteins in plant secondary metabolism: *Catharanthus roseus* cell culture

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Abstract Here the current status of knowledge on some well-characterized transporters located in the vacuolar membrane is reviewed. As different cellular compartments and even different cells may be involved in certain steps of a biosynthetic pathway, the regulation of the flux is not only dependent on structural genes encoding enzymes catabolizing certain steps but also transport has a major regulatory function. The aim of the present review is to give an overview of the present knowledge of transport of secondary metabolites in plants, and to use this information in the context of our knowledge about *Catharanthus roseus* alkaloid biosynthesis. This should lead to further insight in the possible role of various transporters in the regulation of the biosynthesis of these alkaloids.

Keywords *Catharanthus roseus* · Secondary metabolism · Vacuole · Tonoplast · Transporter

Abbreviations

ABC	ATP-binding cassette transporter
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
DCCD	*N,N'*-dicyclohexylcarbodiimide
MDR	multidrug resistance protein
MRP	multidrug resistance related protein
PDR	pleiotropic drug resistance proteins
TIA	terpenoid indole alkaloids
V-ATPase	vacuolar H^+-ATPase
V-PPase	vacuolar pyrophosphatase

Role of transport in biosynthesis of secondary metabolites in plants

Plants synthesize a bewildering array of compounds with a variety of physiological roles that are collectively referred to as secondary metabolites or natural products. The compartmentation of synthesis, degradation, and storage of secondary metabolites is achieved by a series of integrated processes controlled mainly by the membranes by virtue of their permeability properties and the different physico-chemical conditions present in the different compartments

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separated by the membranes. Most secondary metabolites are toxic to the plant itself and necessitate effective mechanisms for detoxification and/or compartmentation. Once secondary metabolites have been transported to their storage sites, they may interact with chemicals and/or proteins to form longer-lasting structures, or be degraded by catabolic enzymes and/or chemical reactions for recycling in other metabolic pathways. The most common storage compartment in the cell for secondary metabolites is the vacuole. This compartmentation may improve the efficiency of their production and avoids harmful effects in the cells (Guern et al. 1987; Luckner et al. 1980; Matile 1987; Wink 1997a, b). In this review, we will particularly focus on the transport through the vacuolar membrane, i.e., on the intracellular transport.

The central vacuole is the largest compartment of a mature plant cell and may occupy more than 90% of the total cell volume. It represents a very large storage space. However, recent results indicate that besides the large central vacuole, several small vacuoles may exist in a plant cell (Jauh et al. 1999; Paris et al. 1996). Molecules stored mainly in the vacuoles include: inorganic salts, such as sodium, potassium, calcium, magnesium, chloride, nitrate and water, enabling the plant to reach a large size and surface area (Blumwald and Gelli 1997; Matile 1987; Martinoia 1992). The vacuole also serves as an internal reservoir of metabolites and nutrients and takes part in cytosolic ion homeostasis (Boller and Wiemken 1986; Guern et al. 1987; Leigh 1997; Matile 1987; Martinoia 1992).

Many transport systems for secondary metabolites such as cardenolides, anthocyanins and indole alkaloids have been investigated in the tonoplast of various plants (Blom et al. 1991; Deus-Neumann and Zenk 1984, 1986; Hopp and Seitz 1987; Martinoia et al. 2000; Mende and Wink 1987; Otani et al. 2005; Renaudin 1989). Three principal mechanisms for vacuolar accumulation of secondary metabolites have been proposed: H^+-antiport and electrogenic uniport, ion and conformational trapping, and directly energized mechanisms catalyzed by ATP-Binding Cassette (ABC) transporters (Klein et al. 1996; Li et al. 1995). Of these, the first two mechanisms depend on a preexisting H^+-electrochemical potential difference across the vacuolar membrane, generated by V-ATPase and V-PPase (Rea and Sanders 1987; Rea and Poole 1993; Taiz 1992; Sze et al. 1999; Zhen et al. 1997). Alternatively, or in addition, the secondary metabolites may passively equilibrate across the vacuolar membrane and become trapped in the vacuole by protonation, altered configuration due to the acidic environment, isomerization, complexation with ions, binding to phenolics or to the tonoplast, or interaction with other vacuolar constituents, and even crystallization (Böhm and Franke 1982; Constabel et al. 1980; Endress 1994; Hopp and Seitz 1987; Kurdjian 1982; Matile et al. 1970; McCaskill et al. 1988; Nagakawa et al. 1984; Pradier et al. 1988; Rataboul et al. 1985; Renaudin 1981, 1989; Renaudin and Guern 1982).

The two proton pumps located in the plant vacuolar membranes: V-ATPase and V-PPase were found to be important for vacuolar uptake of most solutes (Table 1). These proton pumps are expressed differently amongst plants and tissues (Maeshima 2000; Rea and Poole 1993). Both pumps catalyze electrogenic H^+-translocation from the cytosol into the vacuole to establish an inside-acid pH gradient (ΔpH) and an inside-positive electric potential difference ($\Delta \psi$). This generated electrochemical gradient can be utilized to accumulate cations by a proton antiport mechanism of anions due to the membrane potential difference. The one known mechanism for the transport of secondary metabolites that is not obligatorily dependent on the V-ATPase and V-PPase, has the most recently been discovered: MgATP-energized transport by an ATP-binding cassette (ABC) transporter (Martinoia et al. 2001; Rea et al. 1998; Rea 1999). Progress in characterizing the role of ABC transporters in plants is very rapid, after the isolation of ABC genes. Sequence and expression information together with detailed biochemical studies led to new insight on the functions of these transporters. The fact that plant alkaloids such as vincristine, taxol, scopolamine and berberine are often substrates for, or inhibitors of MDR proteins suggests a role of plant ABC transporters in synthesis and compartmentation of these compounds (Horio et al. 1988; Maeng et al. 2002; Walle and

Table 1 Reported characterized of V-ATPase and V-PPase transporters in plant

Transporter	Species	Transported molecules	References
V-ATPase	*Beta vulgaris*	Glucosylated p-hydroxycinnamic acid	Bartholomew et al. (2002)
	Beta vulgaris	Salicylic acid 2-*O*-*β*-D-glucose	Dean and Mills (2004)
	Catharanthus roseus	Inorganic phosphate	Massonneau et al. (2000)
	Coptis japonica	Berberine	Otani et al. (2005)
	Daucus carota	Anthocyanin	Hopp and Seitz (1987)
	Fumaria capreolata	(*S*)-Reticuline	Deus-Neumann and Zenk (1986)
	Hordeum vulgare	Esculin	Werner and Matile (1985)
	Hordeum vulgare	Saponarin, isovitexin	Klein et al. (1996)
	Lupinus polyphyllus	Lupanin	Mende and Wink (1987)
	Melilotus alba	*O*-coumaric glucoside	Rataboul et al. (1985)
	Petroselinum hortense	Apigenin-7-(6-*O*-malonyl) glucoside	Matern et al. (1986)
V-PPase	*Calendula officinalis*	3-*O*-monoglucoside of oleanolic acid	Szakiel and Zowska 2002
	Vitis vinifera	Malate, tartrate	Terrier et al. (1998)

Walle 1998). There is increasing evidences to support this hypothesis, for example, studies of alkaloids transport in *Thalictrum minus* cells (Terasaka et al. 2003) and *Coptis japonica* cells (Yasaki et al. 2001). Several examples of characterized plant MDR-, MRP- and PDR-type ABC transporters are summarized in Table 2.

Diversity of vacuolar transporters

Recent investigations have shown that the energetics and kinetics of the uptake mechanism depend not only upon the source of secondary metabolite but also the plant species from which the vacuoles were isolated. More than one type of transporter/channel exists at the vacuolar membrane, responsible for specific endogenous compounds and/or compounds synthesized by different plants. The transport of secondary metabolites and/or xenobiotics across the vacuolar membrane is determined by the ligand to which they are conjugated. Polyphenolic acids (Regnault-Roger et al. 2004), flavonols (Jones and Vogt 2001), monoterpenes (Sefton et al. 1994), sesquiterpenes (Castillo et al. 1999), flavonoids (Geibel 1995) and hydroxybenzoic acids (Klick and Herrmann 1988; Yazaki et al. 1995) accumulate as both aglycones and glycosides, whilst alkaloids rarely accumulate as glycosides (Warzecha et al. 1999). Szakiel and Janiszowska (2002) demonstrated that two endogenous oleanolic acid monoglycosides distinctly differed in their mechanism of transport to the vacuole in *Calendula officinalis* cells. The monoglucoside is transported by an active proton pump whereas the corresponding monoglucuronide is taken up by a passive, carrier-mediated process (Szakiel and Janiszowska 2002).

Martinoia et al. (2001) speculated that naturally occurring glucosylated plant secondary metabolites enter the vacuole by H^+-antiport, whereas complex glucosides such as glucosylated xenobiotics are either transported into vacuoles or enter via ABC transporters, possibly MRP-subclass glutathione conjugate pumps. This has been supported by the results of comparative analyses of flavone glucoside uptake into barley and *Arabidopsis* vacuoles. The transport of saponarin into barley vacuoles occurs by an H^+-antiporter but is directly energized by ABC-type transporters in the non-saponarin producing plant, *Arabidopsis* (Fragne et al. 2002). Similar findings have been reported, for a glucosylated herbicide and for glucosylated *p*-hydroxycinnamic acid that are transported into vacuoles isolated from red beet (*Beta vulgaris*) by the V-ATPase pump, whereas the glutathionated herbicide and glutathionated *p*-hydroxycinnamic acid are transported by an ABC transporter (Bartholomew et al. 2002). Recently, Dean and Mills (2004) suggested that salicylic acid 2-*O*-*β*-D-glucose (SAG) was transported into the tonoplast vesicles isolated from soybean (*Glycine max* L.) via an ABC transporter but in red beet cells, the vacuolar uptake of SAG occurs through a H^+-antiporter mechanism.

Table 2 Reported characteristics of putative plant MDR-, MRP- and PDR-type ABC transporters

ABC transporter genes	Species	GenBank Accesion number	Predict subcellular localization	Known or putative transported molecules	References
MDR subfamily					
AtMDR1	*Arabidopsis thaliana*	AAD31576	Plasma membrane	Xenobiotics, auxins	Sanchez-Fernandez et al. (2001), Thomas et al. (2000), Windsor et al. (2003)
AtMDR2	*Arabidopsis thaliana*	AL161564	Plasma membrane	Xenobiotics	Dudler and Sidler (1998)
AtMDR4	*Arabidopsis thaliana*	AAC34225	Plasma membrane	Auxin	Santeliaet al. (2005)
CjMDR1	*Coptis japonica*	AB043999	Plasma membrane	Berberine	Yazaki et al. (2001)
TaMDR1	*Triticum aestivum*	AB055077	Plasma membrane	Aluminum	Sasaki et al. (2002)
PMDR1	*Solanum tuberosum*	U52079	Plasma membrane	Calcium ?	Wang et al. (1996)
MRP subfamily					
AtMRP1	*Arabidopsis thaliana*	AC025295	Tonoplast	GS-X	Lu et al. (1997)
AtMRP2	*Arabidopsis thaliana*	AC003096	Tonoplast	GS-X, glucuronide, chlorophyll catabolite	Lu et al. (1998), Liu et al. (2001)
AtMRP3	*Arabidopsis thaliana*	AP000375	Tonoplast	GS-X, chlorophyll catabolite, glucuronide	Tommasini et al. (1998)
AtMRP4	*Arabidopsis thaliana*	AF243509	Plasma membrane	Xenobiotics	Sanchez-Fernandez et al. (1998) Klein et al. (2004)
AtMRP5	*Arabidopsis thaliana*	AC002411	Tonoplast	GS-X, glucuronide	Gaedeke et al. (2001)
TaMRP1	*Triticum aestivum*	AY064479	Tonoplast	Xenobiotics	Theodoulou et al. (2003)
ZmMRP3	*Zea mays*	AY609318	Tonoplast	Anthocyanin	Goodman et al. (2004)
PDR subfamily					
SpTUR2	*Spirodela polyrrhiza*	CAA94437	Plasma membrane	Diterpenoid sclareol	Smart and Fleming (1996) van den Brule et al. 2002
NpABC1	*Nicotiana plumbaginifolia*	AJ404328	Plasma membrane	Plant defense molecules	Jasinski et al. (2001)
NtPDR1	*Nicotiana tabacum*	AB075550	Plasma membrane	Plant defense molecules	Sasabe et al. (2002)
AtPDR12	*Arabidopsis thaliana*	AAF71978	Plasma membrane	Diterpenoid sclareol, plant defense molecules	Campbell et al. (2003), Lee et al. (2005)
OsPDR9	*Oryza sativa*	AY271618	Plasma membrane	Plant defense molecules	Moons (2003)

The results of these studies indicate that it is the identity of the ligand, glucose, glutathione or glucuronide to which the parent compound is attached that primarily determines which carrier the conjugate interacts with. A consequence of this is that xenobiotics and endogenous plant secondary metabolites can share common carriers if they are conjugated to the same ligand, even if they bear little structural resemblance to each other (Bartholomew et al. 2002).

Terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*

Catharanthus roseus(L.) G. Don, Madagascar periwinkle, is one of the most extensively investigated medicinal plants. The pharmaceutical importance of this plant is due to the presence of two antitumor alkaloids, vincristine and vinblastine, as well as the compounds ajmalicine and serpentine that improve blood circulation in the

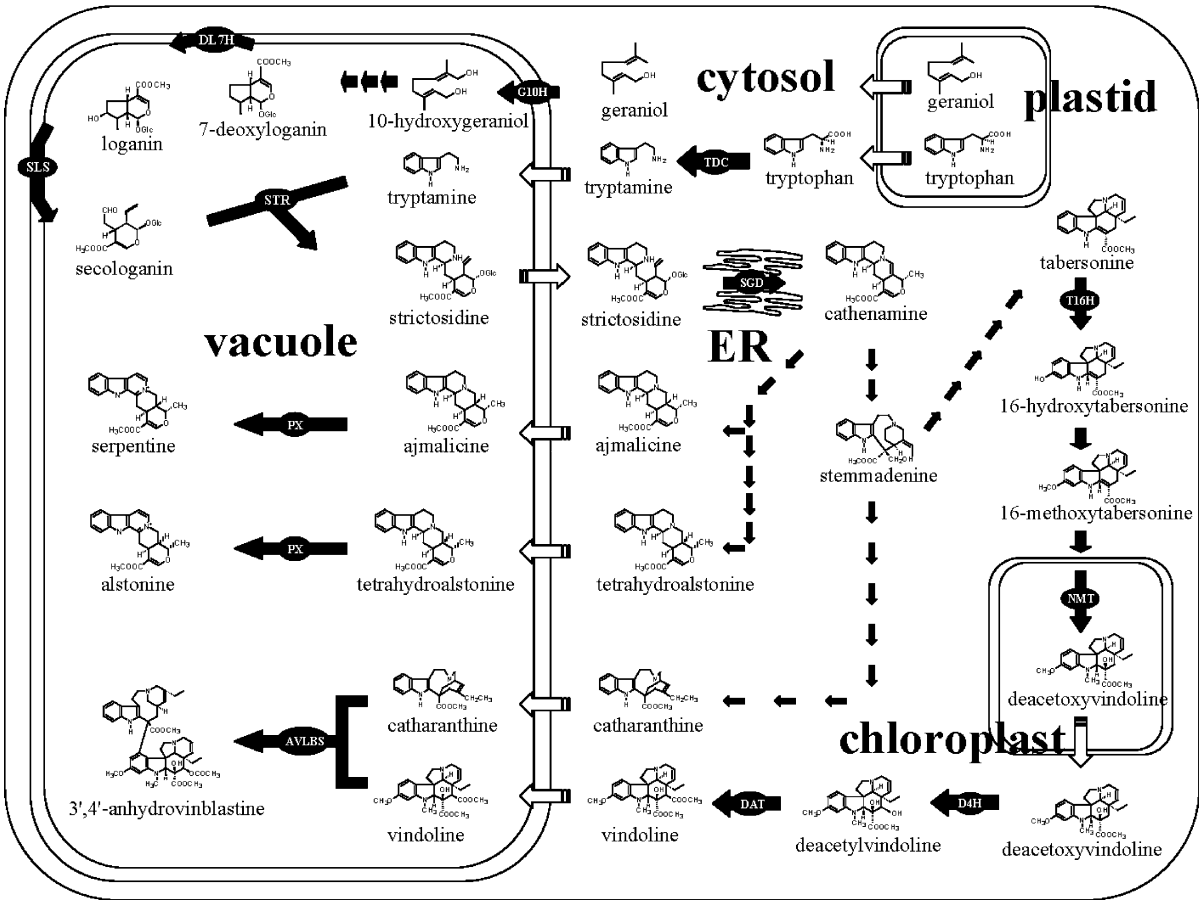

Fig. 1 Subcellular compartmentation of terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* leading to the corynanthe, aspidosperma and iboga type of alkaloids, which are derived from the central intermediate strictosidine. Cathenamine is converted to ajmalicine and tetrahydroalstonine by several enzymatic steps. Stemmadenine is transformed to catharanthine and vindoline and coupled enzymatically to yield dimeric indole alkaloids like vinblastine. G10H, Geraniol-10-hydroxylase; DL7H, Deoxyloganin 7-hydroxylase; SLS, secologanin synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, Strictosidine glucosidase; T16H, Tabersonine 16-hydroxylase; D4H, desacetoxyvindoline 4-hydroxylase; NMT, *N*-methyltransferase; DAT, deacetylvindoline 4-*O*-acetyltransferase; AVLBS, Anhydrovinblastine synthase. G10H, DL7H and SLS are assumed to be localized in the tonoplast membrane. Solid lines represent single reactions, dashed lines represent multiple reactions. Solid lines represent single reactions, dashed lines represent multiple reactions. Empty arrow indicate the transport across subcellular membranes

Table 3 Enzymes specifically involved in *Catharanthus* alkaloid biosynthesis

Enzyme	Abbreviation	Predicted subcellular localization	References
Trytophan decarboxylase	TDC	Cytosol	De Luca et al. (1989)
Deoxyloganin 7-hydroxylase	DL7H	Microsome/tonoplast	Irmler et al. (2000)
Secologanin synthase	SLS	Microsome/tonoplast	Irmler et al. (2000)
Geraniol 10-hydroxylase	G10H	Provacuolar membrane	Madyastha et al. (1977)
Strictosidine synthase	STR	Vacuole	McKnight et al. (1991)
Strictosidine β-D-glucosidase	SGD	Tonoplast/ER	Stevens et al. (1993), Geerling et al. (2000)
Tabersonine 16-hydroxylase	T16H	Microsome	Schröder et al. (1999)
N-methyltransferase	NMT	Thylakoid membrane	Dethier and De Luca (1993), De Luca and Cutler (1987)
Desacetoxyvindoline 4-hydroxylase	D4H	Cytosol	Vázquez-Flota et al. (1997)
Deacetylvindoline 4-*O*-acetyltranferase	DAT	Cytosol	St-Pierre et al. (1998)
16-hydroxytabersonine *O*-methyltransferase	16HT-OMT	?	Cacace et al. (2003)
Anhydrovinblastine synthase	AVLBS	Vacuole	Sottomayor et al. (1998)

brain (Verpoorte et al. 1998). Although the biosynthetic pathway for the production of terpenoid indole alkaloids (TIA) in *C. roseus* has been studied and more than 10 genes encoding specific enzymes have been discovered and partially characterized (Fig. 1, Table 3), the regulation of the pathway has been difficult to characterize.

The TIA biosynthesis pathway often involves multiple organellar compartments, resulting in transport limitation and sequestered pools of metabolites (Burlat et al. 2004; Kutchan 2005; St-Pierre et al. 1999; Verpoorte et al. 1997). The early steps of the terpenoid pathway (leading to geraniol) and the tryptophan pathway are expected to occur in plastids (Poulsen and Verpoorte 1991; Zhang et al. 2001). The conversion of tryptophan to tryptamine by tryptophan decarboxylase (TDC) occurs in the cytosol (De Luca and Cutler 1987; Stevens et al. 1993). Recent data have shown that two enzymes involved in secologanin biosynthesis are located in microsomes of *C. roseus* cells (Irmler et al. 2000). Deoxyloganin 7-hydroxylase (DL7H) catalyzes the conversion of 7-deoxyloganin to loganin and secologanin is then synthesized from loganin by secologanin synthase (SLS). Contin et al. (1999a, b) suggested that the conversion of loganin to secologanin may take place in or at the vacuoles. Since strictosidine synthase (STR) is localized in the vacuole, tryptamine must be transported across the tonoplast before coupling to secologanin can occur (McKnight et al. 1991). Geraniol-10-hydroxylase (G10H), which catalyzes an early step in secologanin biosynthesis, is associated with provacuolar membranes (Collu et al. 2001; Madyastha et al. 1977). Strictosidine glucosidase (SGD), the enzyme catalyzing the deglucosylation of strictosidine, was suggested to be at least partly bound to the external face of the tonoplast (Stevens et al. 1993). However, *in vivo* localization studies showed that SGD is associated with the endoplasmic reticulum (ER; Geerlings et al. 2000). The P_{450}-dependent monooxygenase tabersonine 16-hydroxylase (T16H), which is involved in the C-16 hydroxylation of tabersonine, was also shown to be associated with the microsome (Schröder et al. 1999). The enzyme possibly catalyzing the next step, 16-hydroxytabersonine *O*-methyltransferase (16HT-OMT), has been studied (Cacace et al. 2003). A *N*-methyltransferase (NMT) catalyzes the third-to-last step in vindoline biosynthesis and was found to be associated with thylakoid membranes (De Luca and Cutler 1987; Dethier and De Luca 1993). However, chloroplast development is apparently not necessary since NMT activity was also detected in etiolated seedlings. The last two steps in vindoline biosynthesis, catalyzed by desacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline-4-*O*-acetyltransferase (DAT), occur in the cytosol (De Luca and Cutler 1987; St-Pierre et al. 1998; Vazquez-Flotä et al. 1997). Vindoline must then be channeled back to the vacuole where 3′,4′-anhydrovinblastine synthase

(AVLBS), a basic peroxidase-like enzyme, necessary for coupling vindoline to catharanthine is localized (Sottomayor et al. 1998). None of the enzymes leading to catharanthine has been described. Overall, subcellular compartmentation of alkaloid metabolism is thus quite complex with biosynthetic enzymes localized in the cytosol, vacuole, provacuole, tonoplast membrane, endoplasmic reticulum, chloroplast stroma, thylakoid membranes and specific vesicles (Facchini 2001).

The compartmentation of these enzymes effectively sequesters toxic alkaloids and their biosynthetic intermediates away from the cytosol. The transport of alkaloid end-products or pathway intermediates into the storage site vacuoles may use specific transporters of different classes. Thus, alkaloid-specific transporters may be needed to deliver alkaloids to their proper accumulation sites. The subcellular trafficking of pathway intermediates also creates an important level of metabolic regulation that could not occur if enzymes and substrates diffused freely in the cytosol.

Transport of terpenoid indole alkaloids across the tonoplast membrane in *C. roseus*

Many TIA biosynthetic enzymes, including G10H, STR, DL7H, SLS, and AVLBS, are associated with the vacuole. Although the vacuolar transport of some TIA, such as ajmalicine, serpentine, catharanthine and vindoline, has been studied (Blom et al. 1991; Deus-Neumann and Zenk 1984; Guern et al. 1987; Meijer et al. 1993; Renaudin 1981, 1989; Renaudin and Guern 1982), the mechanisms involved in channeling pathway intermediates to specific subcellular compartments are still poorly understood. Since TIA biosynthesis was found to be related to the morphological changes of the vacuole (Neuman et al. 1983), the storage capacity of the vacuole may be a limiting factor in the production of a compound by the plant cell (Zenk 1978). Thus, it seems of interest to elucidate whether the vacuole plays a role in the metabolism of these compounds. Possible indole alkaloids and precursors transported into the vacuole isolated from *C. roseus* are discussed below.

Tryptamine

Tryptamine biosynthesis seems to occur in the cytosol (De Luca and Cutler 1987; Stevens et al. 1993). After overexpression of an enzyme converting tryptophan into tryptamine (TDC) in *C. roseus* cell cultures, no increase on alkaloid production was observed, suggesting that TDC is not the rate-limiting step in the alkaloid biosynthesis (Canel et al. 1998). Alkaloid accumulation was not enhanced when secologanin and tryptamine were added to the transgenic *C. roseus* cell cultures overexpressing the TDC and STR. Different compartmentation and subcellular relocation of intermediates could explain this effect (Stevens et al. 1993). The transport of this intermediate into the vacuoles is slightly inhibited by agents that collapse or prevent the formation of an H^+ gradient and are inhibited by bafilomycin A1, a known vacuolar-H^+ ATPase inhibitor, suggests that the accumulation of this compounds is via V-ATPase transporters (Roytrakul 2004).

Secologanin

The iridoid glucoside secologanin is derived from the triose phosphate/pyruvate pathway (Contin et al. 1998). As the conversion of loganin to secologanin seems to occur in the vacuoles, where secologanin accumulates (Contin 1999a, b), it is tempting to speculate that SLS and maybe also DL7H are located at the vacuolar membrane. It would be interesting to know whether the conversion of 7-deoxyloganin to secologanin catalyzed by DL7H and SLS or the uptake of secologanin into vacuoles is the limiting factor in TIA production. In most cases, glucosylated compounds are transported into the vacuole by H^+-antiporter mechanism (Martinoia et al. 2000), such as isovitexin and saponarin (Klein et al. 1996), oleanolic acid glucoside (Szakiel and Janiszowska 1992) and esculin (Werner and Matile 1985). A study of the uptake mechanism of this glucoside precursor into the vacuoles isolated from *C. roseus* showed an involvement of ABC transporter (Roytrakul 2004). This finding showed a new property of the vacuolar transport of the endogenous glucoside.

Strictosidine

The central intermediate strictosidine (Nagakura et al. 1978; Stöckigt and Zenk 1977) may be an interesting model for studying vacuolar transport in *C. roseus* because this glucoside is synthesized in the vacuole where it is stored. The release of strictosidine was demonstrated in transgenic tobacco cell suspension upon feeding of secologanin (Hallard et al. 1997). Thus, it may be concluded that the storage of strictosidine inside the vacuole is specific to plant species. The vacuolar accumulation of strictosidine was effected by vanadate, NH_4Cl, CCCP and DCCD implied the regulation by an ABC transporter and a proton symport (Roytrakul 2004).

Ajmalicine

Ajmalicine is actively transported into isolated vacuoles against a concentration gradient with a K_m value of 1.67 μM suggesting that this alkaloid is removed very efficiently from the cytosol (Blom et al. 1991; Deus-Neumann and Zenk 1984). The stimulatory effect of ATP on ajmalicine uptake is counteracted by KNO_3(Blom et al. 1991), the specific inhibitor of the V-ATPase transporter. Ajmalicine accumulates inside the vacuole by conversion into the charged serpentine, and an ion-trap is created to retain the alkaloid more efficiently in the vacuole (Blom et al. 1991). The rate of ajmalicine uptake was 81 pmol/10^6 vacuole/90 min (Deus-Neumann and Zenk 1984). CCCP, gramicidine and NH_4Cl as well as bafilomycin A1, had a negligible effect on the accumulation of ajmalicine in the presence of MgATP (Roytrakul 2004). The inhibition of MgATP-dependent accumulation exerted by vanadate (Roytrakul 2004). These results strongly imply that the ajmalicine transporter is belonging to the ABC transporter family.

Tetrahydroalstonine

Tetrahydroalstonine freely diffuses across the tonoplast in its neutral nonprotonated form and may be accumulated by an ion-trap in its charged protonated form like ajmalicine. The recent data (Roytrakul 2004) indicated that tetrahydroalstonine transport into the *Catharanthus* vacuole is weakly energized by MgATP, inhibited by vanadate suggesting that an ABC transporter may be involved, but with less efficiency than for ajmalicine.

Stemmadenine

Stemmadenine has been proposed as an intermediate leading to catharanthine and vindoline (Qureshi and Scott 1968a, b) indicating a possible branchpoint in the biosynthesis of alkaloids. However, the biosynthetic pathway from cathenamine to tabersonine through stemmadenine is still not characterized. Stemmadenine was efficiently taken up by isolated vacuoles irrespective of the presence of MgATP (Roytrakul 2004). Vanadate inhibited the accumulation of this alkaloid in the presence of MgATP into *C. roseus* vacuoles (70% inhibition). Thus, stemmadenine may enter the vacuole by an vanadate-sensitive transporter which is not energized by ATP (Roytrakul 2004).

Catharanthine

Uptake of catharanthine in *Catharanthus* vacuoles was 31 pmol/10^6 vacuole/90 min, with an apparent K_m value of 2.5 μM (Deus-Neumann and Zenk 1984). Catharanthine may be subjected to rapid turnover in *C. roseus* cells to unknown metabolites causing low levels of dimeric alkaloids in undifferentiated cell cultures. Recent finding showed that the carrier responsible for the accumulation of catharanthine is a member of ATP-energized ATP-binding cassette (ABC) transporters (Roytrakul 2004).

Vindoline

Transport of vindoline across the tonoplast has been characterized as an active, energy-requiring mechanism (Deus-Neumann and Zenk 1984; McCaskill et al. 1988), which is sensitive to the temperature and pH of the surrounding medium, stimulated by K^+ and Mg^{2+}, and inhibited by DCCD and Cu^{2+} (Deus-Neumann and Zenk 1984). Deus-Neumann and Zenk (1984) determined a K_m value of 1.5 μM for vindoline, while

the rate of transport was 199 pmol/10^6 vacuole/90 min. Our observations (Roytrakul 2004) indicated that vindoline is transported via an ABC transporter. However, vindoline may be accumulated by an ion-trap mechanism and coupled with catharanthine forming anhydrovinblastine inside the vacuoles.

Transport of other metabolites through the tonoplast membrane in *C. roseus* cell culture

Inorganic orthophosphate

Inorganic orthophosphate (Pi) might function as a regulatory factor of cytoplasmic pH, a non-competitive inhibitor of the H^+-pumps of both the plasma membrane and tonoplast (Mimura et al. 2000). Transport of Pi across the tonoplast membrane of *C. roseus* was strongly stimulated in the presence of Mg-ATP and Mg-pyrophosphate (Mg-PPi), and inhibited by bafilomycin A1 and concanamycin which are potent inhibitors of the vacuolar H^+-ATPase (Massonneau et al. 2000). In the presence of PPi, the tonoplast-bound inorganic pyrophosphatase from *C. roseus* cells is able to create a proton-gradient which can drive the synthesis of ATP from ADP and Pi. Proton gradient and ATP synthesis were suppressed by the protonic ionophore gramicidin D (Dupaix et al. 1989). This electrochemical gradient by the H^+-pumps is found to be the driving force of Pi uptake, whereas the Δ pH plays only a minor role (Massonneau et al. 2000).

Glyphosate

Glyphosate is distributed between the cytosolic and the vacuolar compartments, but the greater part is localized in the cytosol. The major elements increasing the cellular glyphosate uptake were Ca^{2+}, Mg^{2+}, and iron (Morin et al. 1997). Recently, Tilquin et al. (2000) demonstrated that glyphosate is taken up in *C. roseus* suspensions culture by an Fe/glyphosate co-transport, stimulated by the action of Ca^{2+}. This co-transport system was found to occur with different degrees of efficiency in various plant cell suspensions, indicating that this glyphosate uptake process can be considered to be a general mechanism in plant cells.

Malate

This organic anion accumulates in the central vacuole of most plant cells. Malate has several important roles in plant vacuoles such as the maintenance of charge balance and pH regulation, as an osmolyte involved in the generation of cell turgor, and as a storage form of CO_2. The transport rate was strongly stimulated when the pH of the incubation medium was decreased from pH 7.0 to 5.0 (Dietz 1992). Moreover, treatment with a pH 2 solution resulted in a gradual decrease of the malate content, indicating the operation of a biochemical pH regulation mechanism (Mimura 2000). Several lines of evidence (saturation kinetics, action of malate analogs and protein modifiers) support the concept that malate transport is mediated by a protein carrier which could be implicated in the uptake process of the protonated form. The malate transported in the vesicles was released by lowering the external malate concentration. The release was prevented by the anion transport inhibitor (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid or DIDS) indicating the reversibility of the carrier (Bouyssou et al. 1990).

Calcium ions

The vacuole serves as a primary pool of free calcium ions in plant cells and the vacuole is a major source of Ca^{2+} for intracellular calcium signaling (Maeshima 2001). The Ca^{2+}/H^+ antiporter together with the Ca^{2+}-ATPase plays a key role in vacuolar Ca^{2+} accumulation. Zhao et al. (2001) have demonstrated that calcium influx plays a critical role in alkaloid biosynthesis in normal and elicited-*C. roseus* cell cultures. Further studies are needed to elucidate a functional relationship between Ca^{2+} transporters localized on the *Catharanthus* tonoplast and regulation of TIA biosynthesis.

Conclusions and perspectives

Plant vacuoles are dynamic and can change morphologically and functionally to suit the needs of the cell. Rapid progress is being made in the areas of tonoplast transport and its regulation. The challenge is to merge views of vacuole function and biochemistry into a clear, unified picture that encompasses the dynamic nature of the vacuole.

An understanding of the biological processes that permit the synthesis and accumulation of terpenoid indole alkaloids in plants has advanced considerably over the past decade. This rapid progress has been facilitated by the availability of an impressive collection of alkaloid biosynthetic genes. These tools, combined with recent developments in plant genomics and proteomics, will promote efforts to identify more genes involved in the TIA pathways. TIA pathways are highly regulated and involve cell-, tissue-, development-, and environment-specific controls. Elaboration of the subcellular compartmentation of enzymes in TIA biosynthesis gives intriguing new views on the complexity of plant metabolism. Our emerging knowledge of the biochemistry, molecular biology, and cell biology of alkaloid biosynthesis will also lead to exciting opportunities to engineer TIA metabolism in transgenic plants.

Although this review has not answered how secondary metabolites are transported across the vacuolar membrane in *C. roseus* cells, principally due to a shortage of available information, it has highlighted which transporters, located in the tonoplast membrane, need to be considered.

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