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Review

Alkaloid transporters in plants

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Abstract Plants produce a multitude of secondary metabolites, including alkaloids with biological activities, and many alkaloids have been used for medicinal purposes. The biosynthetic enzymes and genes involved in alkaloid metabolic pathways exhibit divergent localizations, implying that alkaloid metabolites, including pathway products and intermediates, travel from organelle to organelle, cell to cell, and organ to organ. Biochemical studies have indicated that specific transporters move these metabolites. Indeed, molecular and cellular approaches have identified alkaloid transporters of the ATP-binding cassette (ABC) protein, multidrug and toxic compound extrusion (MATE), and purine permease (PUP) families. Interestingly, some of these transporters were found to be required for the efficient biosynthesis of alkaloids in plants. Here, we provide an updated inventory of alkaloid transporters and discuss the possibility of genetically manipulating the expression of these transporters to increase the accumulation of valuable alkaloid compounds.

Key words: ABC transporter, alkaloid, MATE, PUP, secondary metabolites, transporter.

Introduction

Plants produce a wide array of secondary or specialized metabolites, to adapt to their changing environments (Croteau et al. 2000). Alkaloids contain nitrogen and are usually alkaline secondary metabolites with diverse chemical structures and biological activities (Iwasa et al. 1998; Steppuhn et al. 2004). Their bioactive properties make alkaloids important players in plant defense responses against microbes, insects, and other herbivores. Humans use bioactive alkaloids as medicines (Croteau et al. 2000); for instance, morphine is widely used as an analgesic and vincristine as an anticancer drug. Given their potential medicinal applications, alkaloids have long attracted the attention of phytochemists and pharmacologists.

Intensive studies have led to the chemical identification of valuable alkaloids and have elucidated the enzymes and genes involved in their biosynthesis. Molecular and cellular studies of alkaloid biosynthesis have revealed that enzymes and metabolites related to the same alkaloid biosynthetic pathways do not necessarily co-localize at the tissue, cellular, and subcellular level, implying that alkaloids and their biosynthetic intermediates are dynamically transported in plant tissues (Shitan and Yazaki 2007; Verma et al. 2012). Three modes of alkaloid transport exist: inter-organ, inter-cellular, and intra-cellular (Shitan and Yazaki 2007). In organ-to-organ transport, alkaloids synthesized in source organs are translocated to sink organs, which frequently correspond to the plant parts used for medicinal purposes. For example, berberine translocates from the root to the rhizome in *Coptis japonica*, senecionine *N*-oxide translocates from the root to the inflorescence via the phloem in *Senecio* species, and nicotine translocates from the root to the leaf via the xylem in *Nicotiana* species (De Luca and St Pierre 2000). Biosynthesis sometime occurs in multiple cell types within an organ, and transporters move pathway intermediates between the cells. In *Papaver somniferum* (opium poppy), thebaine, a biosynthetic intermediate of morphine, is thought to move from sieve elements to laticifers before being further metabolized to morphine in the laticifers (Beaudoin and Facchini 2014). Vinblastine formation has been proposed to occur in different cell types in the leaves of *Catharanthus roseus*, and pathway intermediates are assumed to move from the internal phloem-associated parenchyma (IPAP) to the epidermis, and finally to laticifers and idioplasts (De Luca et al. 2014). Alkaloids and their intermediates also move among different organelles, such as the nucleus, endoplasmic reticulum (ER), plastid, and vacuole (Shitan and Yazaki

Abbreviations: ABC, ATP-binding cassette; IPAP, internal phloem-associated parenchyma; MATE, multidrug and toxic compound extrusion; MIA, monoterpenoid indole alkaloids; NBD, nucleotide-binding domain; PUP, purine permease; TMD, transmembrane domain; VIGS, virus-induced gene silencing.

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Table 1. Alkaloid transporters in plants.

Gene name	Accession No.	Plant Spp.	Tissue expression	Subcellular localization	Transport substrate	Expression in heterologous host	Ref.
CjABCB1/ CjMDR1 (ABC)	AB043999	*Coptis japonica*	Rhizome, flower, bud, peduncle, petiole especially high in rhizome	PM (MF and WB)	berberine, 4-nitroquinoline *N*-oxide	*Xenopus* oocyte Yeast cellular transport (*Saccaromyces cerevisiae*)	Yazaki et al. 2001 Shitan et al. 2003 Shitan et al. 2013
CjABCB2 (ABC)	AB674325	*Coptis japonica*	Rhizome	PM (MF and WB)	berberine, 4-nitroquinoline *N*-oxide	Yeast cellular transport (*S. cerevisiae*)	Shitan et al. 2013
CjABCB3 (ABC)	AB674326	*Coptis japonica*	not detected	not examined	berberine	Yeast cellular transport (*S. cerevisiae*)	Shitan et al. 2013
CrTPT2 (ABC)	KC511771	*Catharanthus roseus*	Flower, leaf, stem, root, especially high in epidermal cells in young leaf	PM (GFP)	catharanthine	Yeast cellular- and vecicle-transport (*S. cerevisiae*)	Yu and Luca 2013
NtMATE1 (MATE)	AB286961	*Nicotiana tabacum*	Root	VM (MF and WB, GFP, IM)	nicotine, hyoscyamine, scopolamine	Yeast cellular transport (*S. cerevisiae*)	Shoji et al. 2009
NtMATE2 (MATE)	AB286962	*Nicotiana tabacum*		not examined	not examined		Shoji et al. 2009
Nt-JAT1 (MATE)	AM991692	*Nicotiana tabacum*	Leaf, stem, root	VM in leaf cells (MF and WB)	nicotine, anabasine, hyoscyamine, berberine	Yeast cellular transport (*S. cerevisiae*), proteoliposome transport	Morita et al. 2009
Nt-JAT2 (MATE)	AB922128	*Nicotiana tabacum*	Leaf	VM (GFP)	nicotine, anabasine, anatabine, hyoscyamine, scopolamine, berberine	Yeast cellular transport (*S. cerevisiae*)	Shitan et al. 2014
NtNUP1 (PUP)	GU174267	*Nicotiana tabacum*	Leaf, root, especially high in root tip	PM (GFP)	nicotine, anabasine, anatabine, atropine, kinetin, vitamin B6	Yeast cellular transport (*S. pombe* and *S. cerevisiae*)	Hildreth et al. 2011 Kato et al. 2014

Note: PM, plasma membrane; VM, vacuolar membrane; MF, microsomal fraction; WB, western blot; GFP, green fluorescent protein; IM, immunoelectron microscopy.

2007; Verma et al. 2012).

Elucidating the mechanisms underlying alkaloid transport in plants is important for understanding the *in planta* formation and accumulation of this class of compounds, and is necessary for developing plants with improved alkaloid production. In this review, we summarize our current knowledge of alkaloid transporters in plants and discuss their physiological roles (Table 1).

Transport systems

Three distinct mechanisms are proposed for alkaloid transport: simple diffusion followed by membrane trapping, transporter-mediated membrane transport, and vesicle-mediated transport (Shitan and Yazaki 2007, 2013).

Membrane trapping was first proposed to function in the vacuolar accumulation of alkaloids (Matile 1976). Alkaloids, weakly basic organic compounds, are thought to be capable, in a limited fashion, of penetrating the tonoplast by simple diffusion. Once incorporated into vacuoles, alkaloids are readily protonated in the acidic lumen, becoming membrane-impermeable hydrophilic forms that remain trapped inside the vacuoles. This mechanism, which largely depends on the chemical nature of alkaloids, seems applicable to most alkaloids; however, a few exceptions to this model have been reported (Deus-Neumann and Zenk 1986; McCaskill et al. 1988).

Biochemical analyses using intact vacuoles suggested that energy-dependent and alkaloid-specific carriers localized at the tonoplast mediate active membrane transport (Deus-Neumann and Zenk 1984, 1986). The recent identification of vacuolar alkaloid transporters supports this notion (Morita et al. 2009; Shoji et al. 2009; Shitan et al. 2014).

The vesicle-mediated transport model is supported by microscopic observation of alkaloid-containing small vesicles, which are believed to deliver alkaloids to vacuoles by fusion between the membranous compartments. It remains unclear whether the small vesicles originate from the ER or other organelles and how alkaloids are incorporated into these vesicles. Benzylisoquinoline alkaloids, including berberine in *Berberis* species (Amann et al. 1986; Bock et al. 2002), and sanguinarine in cultured cells of opium poppy (Alcantara et al. 2005), are thought to undergo vesicle-mediated transport.

These three mechanisms occur in plant cells, but different mechanism would contribute to different alkaloids depending on the chemical properties of the alkaloids and on the plant species. Both transporter- and vesicle-mediated transport have been proposed to underlie the vacuolar accumulation of anthocyanins, a class of plant flavonoid pigments (Petrussa et al. 2013; Zhao and Dixon 2010).

Transporter families

The plant alkaloid transporters that have been reported to date belong to the ATP-binding cassette (ABC) protein, multidrug and toxic compound extrusion (MATE), and purine permease (PUP) families (Figure 1).

ABC proteins occur widely in prokaryotes and eukaryotes, forming a large superfamily (Higgins 1992). Plant genomes contain numerous genes encoding ABC proteins (Kang et al. 2011; Martinoia et al. 2002; Shoji 2014; Verrier et al. 2008); for instance, the *Arabidopsis thaliana* genome contains approximately 120 genes that encode ABC proteins, and similar numbers of ABC family members occur in other species (Cakir and Kilickaya 2013; Garcia et al. 2004; Jasinski et al. 2009; Sugiyama et al. 2006). Most ABC proteins contain transmembrane domains (TMDs) and nucleotide-binding domains (NBDs), or ABC domains, that function in ATP binding and hydrolysis (Figure 1). As primary transporters, ABC proteins couple energy directly obtained through ATP hydrolysis with substrate translocation. ABC proteins from plants are mostly grouped into eight subfamilies (ABCA-G and ABCI; ABCH is absent in plant genomes), based on sequence similarity and thus on the arrangement of TMDs and NBDs. Although plant ABC subfamilies were initially named after human and microbial prototypes, e.g., multidrug resistance (MDR) and pleotropic drug resistance (PDR), researchers of plant ABC proteins have proposed a unified nomenclature (Verrier et al. 2008), which was followed in this review. In addition to alkaloid transport, ABC proteins play diverse roles in plant growth and adaptation to the environment, as reviewed elsewhere (Kang et al. 2011; Shoji 2014; Verrier et al. 2008; Yazaki et al. 2009).

MATE transporters are membrane proteins with twelve transmembrane α-helixes and function as antiporters that couple substrate movement with energy stored as H^+ or Na^+ gradients across membranes (Figure 1) (Omote et al. 2006). MATE transporters were originally identified in bacteria as transporters critical for multidrug resistance (Morita et al. 1998), and similar roles in detoxification were subsequently identified for MATEs mainly from mammals (Otsuka et al. 2005). MATEs also occur in plants, and plant MATEs, like their counterparts in bacteria and mammals, transport xenobiotic compounds. For example, AtDTX1 from Arabidopsis mediates the efflux of various compounds, including two exogenous alkaloids berberine and palmatine (Li et al. 2002). MATEs involved in xenobiotic detoxification accept a wide range of substrates, but some MATEs have narrower substrate specificities;

for example, some MATEs from plants accept citrate, salicylic acid, or flavonoids as substrates (Durrett et al. 2007; Serrano et al. 2013; Zhao and Dixon 2010). Recent research has revealed the functions of many MATEs in plants (Shoji 2014; Takanashi et al. 2014; Yazaki et al. 2008).

Arabidopsis PUP1, a founding member of the PUP family (Jelesko 2012), is a transporter protein with ten transmembrane α-helixes (Figure 1). The Arabidopsis genome contains 21 genes encoding proteins structurally related to AtPUP1, all in the same family. Initially identified as a protein that complements a yeast mutant deficient in adenine transport, AtPUP1 acts as a H^+/substrate symporter that mediates the transport of purine ring-containing compounds, such as adenine, cytosine, and cytokinins, from the apoplast to the cytoplasm (Burkle et al. 2003; Gillissen et al. 2000). A study on the expression of *AtPUP1* and its closest homolog *AtPUP2* in Arabidopsis tissues suggested the involvement of these transporters in the long-distance transport of adenine and cytokinins (Burkle et al. 2003). Recent work expanded the repertory of AtPUP1 substrates by showing that AtPUP1 also transports the pyridine ring-containing vitamin B6 (pyridoxine) and its derivatives (pyridoxamine and pyridoxal) (Szydlowski et al. 2013).

Berberine transport in *Coptis japonica*

The rhizome of the perennial medicinal plant *Coptis japonica* (Ranunculaceae) contains high amounts of the benzylisoquinoline alkaloid berberine, which has antibacterial activity (Iwasa et al. 1998), and the *C. japonica* rhizome has long been used to treat gastrointestinal infections in Asian countries. Plants may accumulate anti-microbial berberine in the rhizome for defense purposes, as the rhizome has rich stores of starch and therefore is vulnerable to soil-borne pathogens (Shitan and Yazaki 2007). As the genes underlying berberine biosynthesis are preferentially expressed in the root (Ikezawa et al. 2003), alkaloid formation is assumed to occur in the roots, and not in the rhizome. This spatial separation between the biosynthesis and accumulation of the alkaloid suggests that berberine undergoes inter-organ translocation from the root to the rhizome. Berberine transport has been analyzed in cultured *C. japonica* cells that were originally induced

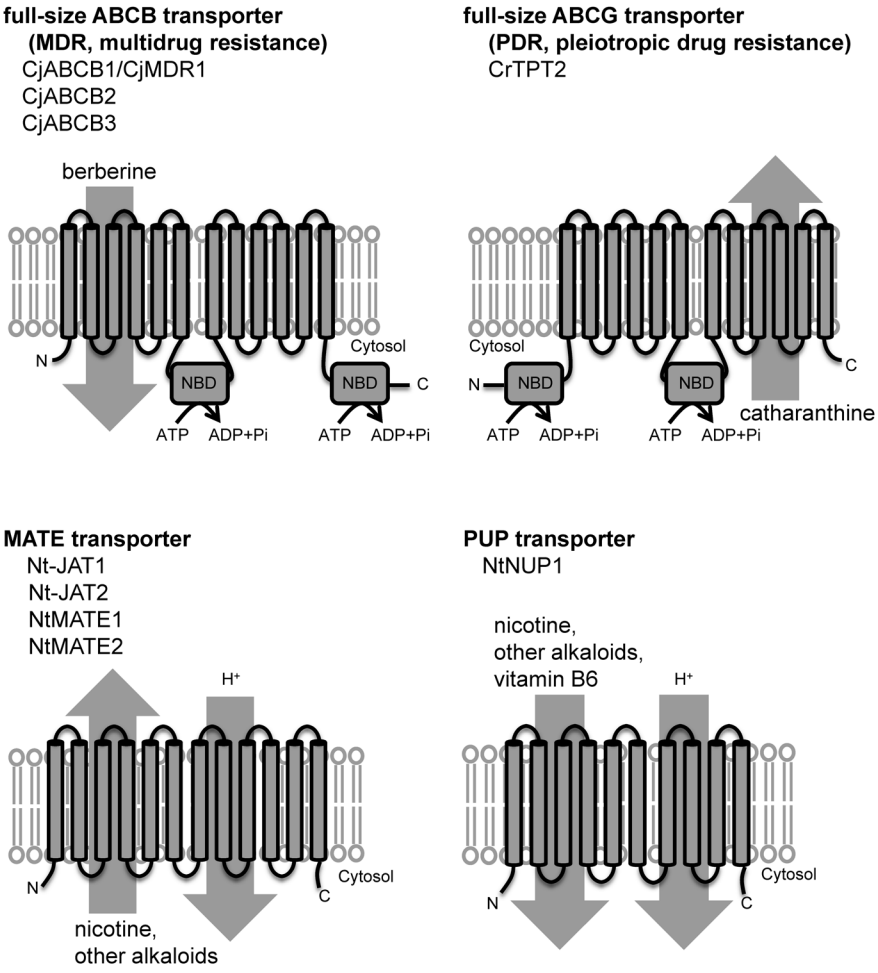

Figure 1. Putative structures and function of full-size ABCB, full-size ABCG, MATE and PUP transporters.

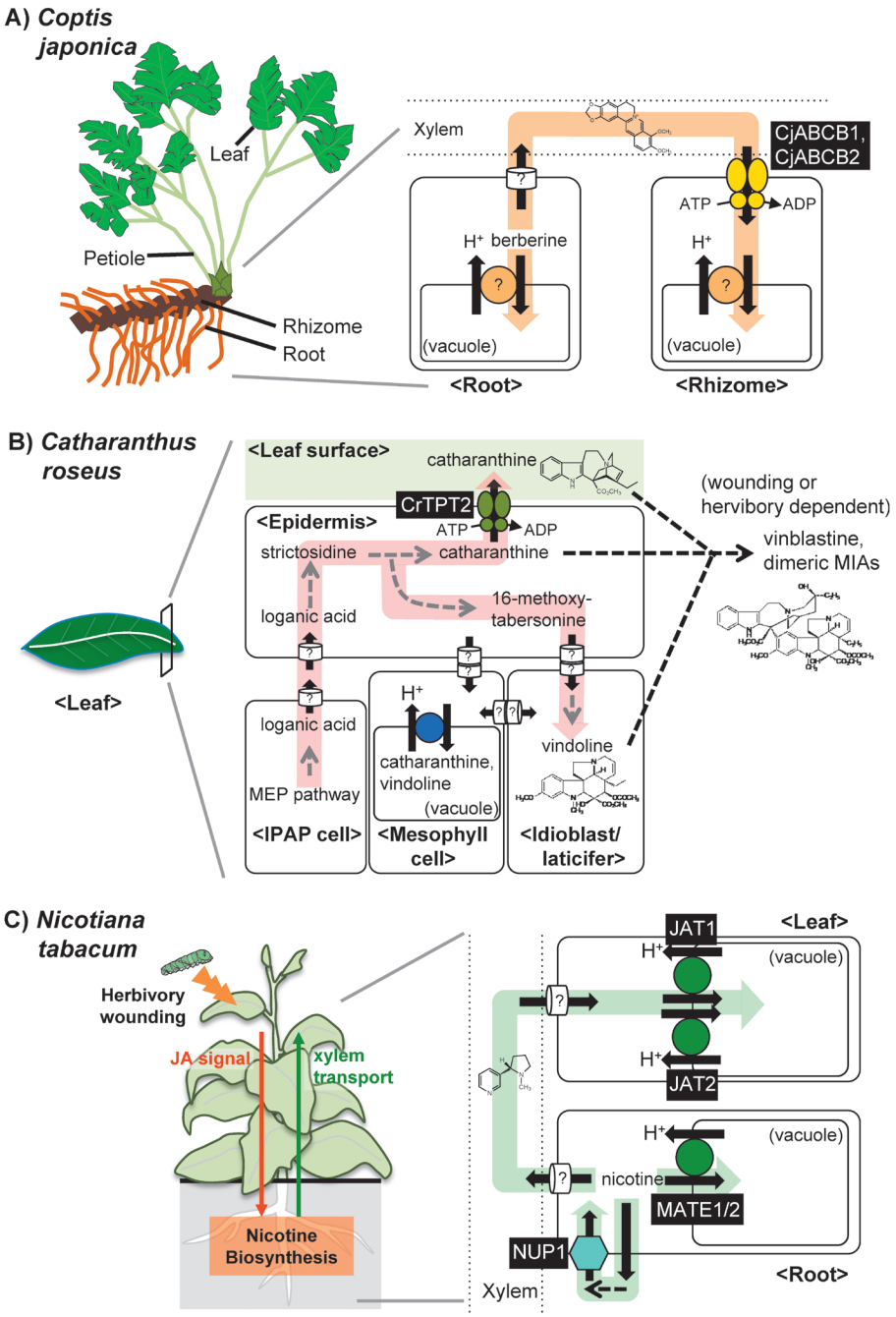

Figure 2. (A) Berberine transport in *Coptis japonica*. Berberine is thought to be synthesized in the root and then translocated to the rhizome via the xylem. Plasma membrane-localized ABC transporters, CjABCB1 and CjABCB2, function in berberine uptake from the xylem in the rhizome. Once imported into the cytosol, berberine is rapidly transported to the vacuolar lumen via an H^+/berberine antiporter. (B) Monoterpenoid indole alkaloid (MIA) transport in the leaf tissue of *Catharanthus roseus*. Early steps of MIA biosynthesis occur in the internal phloem-associated (IPAP) parenchyma, and a resulting monoterpenoid intermediate, loganic acid, moves to the epidermis via an unknown mechanism. In the epidermis, loganic acid is converted into strictosidine, a key intermediate of the MIA pathway, and then to catharanthine and other monomeric MIAs. Catharanthine formed in the epidermis is exported to the cell surface by plasma membrane-localized CrTPT2. Vindoline formation occurs in internal leaf cells such as laticifers and idioblasts. In mesophyll cells, MIAs are presumed to be sequestered into the vacuole by a H^+/MIAs antiporter. (C) Nicotine transport in *Nicotiana tabacum*. Nicotine biosynthesis occurs in the root and is elicited by the jasmonate signal. NtMATE1 and NtMATE2 are required for vacuolar sequestration of nicotine in root cells. Plasma membrane-localized NUP1 facilitates the uptake of apoplastic nicotine into the cytoplasm and also affects nicotine metabolism in the root. In the leaf, Nt-JAT1 and Nt-JAT2 transport nicotine into the vacuole. Nicotine accumulation in aerial parts is essential for plant defense against insects and other herbivores.

from rootlets. These *C. japonica* cells actively take up exogenous berberine added to the culture medium and sequester the berberine into the vacuole (Sato et al. 1990; Sato et al. 1994). Berberine uptake depends on the level of ATP and is significantly decreased by inhibitors of B-type ABC transporters, including human ABCB1, also known as multidrug resistance1 (MDR1) or P-glycoprotein, suggesting the involvement of ABCB transporters in this process (Sakai et al. 2002). Three genes encoding ABCB transporters, *CjABCB1*/*CjMDR1*, *CjABCB2*, and *CjABCB3*, were cloned from *C. japonica* cultured cells (Shitan et al. 2003, 2013a; Yazaki et al. 2001). All three ABCBs mediated berberine influx when expressed in *Xenopus* oocytes or yeast. At the time of the report (Shitan et al. 2003), CjABCB1 was the only eukaryotic ABCB protein known to mediate inward substrate movement; however, new research has identified plant ABCBs with influx activity and ABCBs that conditionally mediate influx (Kamimoto et al. 2012; Lee et al. 2008; Shitan et al. 2013a; Terasaka et al. 2005; Yang and Murphy 2009). CjABCB1 and CjABCB2, which share 82% amino acid identity, localize to the plasma membrane and their encoding genes are preferentially expressed in xylem tissues of the rhizome. CjABCB1 and CjABCB2 are thought to be involved in the long-distance transport of berberine, which is produced in the roots and loaded into the xylem. This pair of highly similar proteins is thought to function at the plasma membrane to unload berberine from the xylem into the rhizome (Figure 2A) (Shitan et al. 2003, 2013a).

CjABCB1 could be used in genetic engineering strategies aimed at improving alkaloid metabolism and accumulation. When *CjABCB1* was down-regulated in transgenic *C. japonica* plants, berberine accumulation decreased, probably due to reduced CjABCB1-dependent influx of berberine (Shitan et al. 2005). Overexpression of *CjABCB1* in the cultured cells of *C. roseus* (Madagascar periwinkle), which does not produce berberine, but does biosynthesize monoterpenoid indole alkaloids (MIAs), significantly increased the accumulation of the MIAs ajmalicine and tetrahydroalstonine, when the cells were fed with these alkaloids. These MIAs might be recognized by CjABCB1 and transported into *C. roseus* cells (Pomahacova et al. 2009).

Once incorporated into cells, berberine is sequestered into the vacuole. A biochemical analysis using tonoplast vesicles isolated from *C. japonica* cells revealed that berberine accumulates in the vacuole via a H^+/berberine antiport mechanism (Otani et al. 2005). Thus, proton gradient-driven transporters are thought to transport berberine across the tonoplast (Figure 2A). A tonoplast-localized MATE transporter with berberine transport activity, recently isolated from a *C. japonica* cDNA library, is a candidate for the vacuolar transporter (K. Takanashi, personal communication).

MIA transport in *Catharanthus roseus*

The medicinal and ornamental plant *C. roseus* produces a variety of MIAs, such as vindoline, catharanthine, vinblastine, and vincristine. Many bioactive MIAs have uses as pharmaceuticals. For example, the dimeric MIAs vinblastine and vincristine, which derive from the monomeric MIAs catharanthine and vindoline, are used as anticancer drugs (Cragg and Newman 2005). Since the production of these clinically important dimeric MIAs is quantitatively limited in plants, there is much interest in developing biotechnological strategies to improve MIA production. Recent advances in transcriptomics and proteomics have led to the identification of genes and enzymes involved in MIA biosynthesis. Detailed analyses of the expression patterns of MIA biosynthesis genes indicated that at least four cell types in the leaf, i.e., IPAP, epidermal cells, laticifers, and idioblasts, are involved in MIA biosynthesis (De Luca et al. 2014; Verma et al. 2012), as illustrated in Figure 2B. This indicated the existence of mechanisms for intercellular trafficking of pathway intermediates, i.e., loganic acid from the IPAP to the epidermis and 16-methoxytabersonine from the epidermis to the laticifers and idioblasts. However, the underlying molecular mechanisms have yet to be explored.

Spatial separation of the two monomeric MIAs required for the formation of vinblastine and vincristine explains the limited accumulation of dimeric MIAs in plants. Catharanthine is synthesized in the leaf epidermis and then secreted to the leaf surface, where it acts as a defensive toxin against insects, while vindoline is produced and accumulates in inner cells, laticifers and idioblast. Dimeric MIA formation likely occurs mainly after wounding or hervivory-induced leaf damage, which mixes the separated catharanthine and vindoline (Figure 2B) (Roepke et al. 2010).

Identification of the full-size ABCG transporter CrTPT2 provided insight into how catharanthine formed in leaf epidermal cells is exported to its surface (Yu and De Luca 2013). CrTPT2 belongs to the G subfamily of ABC proteins, which include various members involved in disease resistance, cuticle formation, and hormone transport (Kang et al. 2011; Ko et al. 2014; Shoji 2014; Zhang et al. 2014). Transport assays using yeast cellular and vesicular transport systems showed that CrTPT2 mediated the ATP-dependent transport of catharanthine, but not other MIAs. *CrTPT2* is annotated as a transporter gene in the leaf epidermis-enriched transcript database (Murata et al. 2008), and *CrTPT2* is expressed predominantly in the leaf epidermis. Treatment with methyl jasmonate (MeJA) or catharanthine induces *CrTPT2* expression. In *C. roseus* plants, virus-induced gene silencing (VIGS) of *CrTPT2* resulted in decreased deposition of catharanthine at the leaf surface, along with

increased accumulation of catharanthine and increased dimerization with vindoline, but no change in vindoline content in leaf tissues. The altered distribution and accumulation of catharanthine and its dimerized form in the leaves could be explained by decreased catharanthine export caused by silencing of *CrTPT2* (Figure 2B). The finding that dimeric MIAs increase in *CrTPT2*-silenced plants may provide a strategy for increasing the production of dimeric MIAs.

MIA biosynthetic enzymes localize to multiple organelles, such as the nucleus, ER, cytosol, plastid, and vacuolar lumen, indicating that alkaloid biosynthetic intermediates undergo dynamic intracellular trafficking (Guirimand et al. 2011; Verma et al. 2012). The transport mechanisms underlying the vacuolar accumulation of intermediates are being investigated using biochemical approaches (Carqueijeiro et al. 2013). In *C. roseus* mesophyll cells, MIAs stored in the vacuole include catharanthine, vindoline, and probably also α-3′,4′-anhydrovinblastine, a dimer of catharanthine and vindoline, and a precursor of vinblastine and vincristine. Uptake of MIAs into intact vacuoles and tonoplast vesicles prepared from mesophyll cells was stimulated by ATP and strongly inhibited by NH_4^+, which abolishes the proton gradient established across membranes. By contrast, vanadate, an inhibitor of ABC transporters, had no significant effect on MIA uptake. Moreover, the addition of MIAs dissipated the proton gradient established across the tonoplast. These results suggest the involvement of a proton antiporter in the uptake of MIAs into mesophyll vacuoles (Figure 2B) (Carqueijeiro et al. 2013).

Nicotine transport in *Nicotiana tabacum*

Nicotine is a well-known pyridine alkaloid produced in *Nicotiana* species (Solanaceae family), including *N. tabacum* (tobacco), and is found in cigarettes and other tobacco products. Because of its potent neurotoxicity, nicotine plays an important role as a defense compound against insect herbivores in tobacco (Steppuhn et al. 2004). In tobacco, insect attacks strongly induce nicotine accumulation via jasmonate signaling (Shoji and Hashimoto 2013). Nicotine accumulates in nearly all parts of tobacco plants, but its biosynthesis occurs specifically in the roots (Shoji and Hashimoto 2013). Once produced in the root, nicotine is translocated to the aerial parts via the xylem (Baldwin 1989), and stored at high concentrations in the vacuoles of leaf cells (ca. 60 mM) (Lochmann et al. 2001). A classic grafting experiment initially revealed the root-to-shoot translocation of nicotine (Solt and Dawson 1958), but the molecular mechanism underlying this process has remained elusive.

Nt-JAT1 (*jasmonate-inducible alkaloid tranporter1*), which encodes a MATE transporter (Morita et al. 2009), was initially identified through transcriptome profiling as a gene up-regulated by MeJA coordinately with nicotine biosynthetic genes in tobacco Bright Yellow-2 cultured cells (Goossens et al. 2003). Functional analyses using yeast cellular transport and proteoliposome systems reconstituted with purified Nt-JAT1 (expressed in Sf9 cells) and bacterial F_0F_1-ATPase showed that Nt-JAT1 functions as an H^+/nicotine antiporter. Furthermore, Nt-JAT1 transports alkaloids other than nicotine, such as hyoscyamine and berberine, but not flavonoids, suggesting its preference for alkaloids. *Nt-JAT1* mRNA is expressed in leaves, stems, and roots. In leaf cells, Nt-JAT1 localizes to the tonoplast and might thus play a role in the vacuolar sequestration of nicotine (Figure 2C) (Morita et al. 2009). Very recently, another MATE transporter was isolated and designated Nt-JAT2. This MATE transporter localizes to the tonoplast and transports nicotine. Since Nt-JAT2 is specifically expressed in leaves and MeJA treatment induces its expression in younger and older leaves, Nt-JAT2 could contribute to the protection of organs that are vulnerable to insect herbivory by accumulating nicotine in the vacuole (Shitan et al. 2014).

Nicotine also accumulates to some extent in the roots, where it forms. Two homologous MATE transporters, NtMATE1 and NtMATE2 (96% amino acid sequence identity), were reported to be responsible for the vacuolar accumulation of nicotine in the roots (Shoji et al. 2009). *NtMATE*s were down-regulated in the roots of a regulatory mutant that shows a low-nicotine phenotype. *NtMATE* mRNAs are predominantly expressed in the roots and, to a lesser extent, in the flowers. Localization analyses using GFP-fusion proteins and immunogold electron microscopy revealed that NtMATE1 localizes to the tonoplast. RNAi-mediated suppression of *NtMATE* genes in transgenic tobacco plants mitigated the inhibition of root growth by nicotine exogenously supplied to the growth medium, possibly by altering the in planta homeostasis of nicotine. A yeast transport assay suggested that NtMATE1 functions as a proton-dependent antiporter that transports nicotine and other alkaloids. These results suggest that NtMATE1 and NtMATE2 transport nicotine into root vacuoles (Figure 2C) (Shoji et al. 2009). NtMATE-mediated transport is considered to contribute mainly to the vacuolar retention of nicotine, as nicotine is thought to be formed in the vacuolar lumen (Kajikawa et al. 2011).

Tobacco NUP1 (nicotine uptake permease1) is a plasma membrane-localized nicotine transporter of the PUP family (Hildreth et al. 2011). *NUP1* was initially identified as a gene that is regulated by two nicotine-controlling loci (Kidd et al. 2006), like other genes involved in nicotine biosynthesis and transport. *NUP1* transcripts are abundant in the roots, especially in

root tips actively synthesizing nicotine, and are less abundant in the leaves (Hildreth et al. 2011). AtPUP1, an Arabidopsis homolog of NUP1, mediates the uptake of purine bases and the phytohormone cytokinine (Burkle et al. 2003; Gillissen et al. 2000), but tobacco NUP1 promotes nicotine uptake when expressed in fission yeast (*Schizosaccharomyces pombe*) (Hildreth et al. 2011). The down-regulation of *NUP1* in tobacco hairy roots reduced the intracellular accumulation of nicotine and increased nicotine levels in the culture medium, indicating that NUP1 affected nicotine metabolism and distribution. The altered distribution of nicotine might reflect the inhibition of *NUP1*-dependent cellular uptake of nicotine. It remains to be determined whether and, if so, how the nicotine transport activity of NUP1 is related to the decrease of total nicotine accumulation. Tobacco plants regenerated from *NUP1*-suppressed hairy roots had lower nicotine levels in both the roots and leaves, in line with the results of the hairy root analysis. Surprisingly, the regenerated tobacco plants showed an increased rate of root elongation, suggesting that NUP1 has a novel and unknown role in root growth. The recent identification of vitamin B6 as a transport substrate of NUP1 (Kato et al. 2014), similar to AtPUP1 (Szydlowski et al. 2013), suggests that the less-understood roles of NUP1 in regulation of nicotine metabolism and root growth may be unrelated to its nicotine transport activity. It will be interesting to determine whether the role of NUP1 as a vitamin B6 transporter is related to the phenotypes observed in the *NUP1*-supressed tobacco hairy roots and plants.

The wild *Nicotiana* species *N. alata* shows a unique pattern of alkaloid accumulation, providing interesting insight into nicotine translocation (Pakdeechanuan et al. 2012). In *N. alata*, nicotine and its derivatives accumulate in the roots, but not in the shoots; by contrast, these alkaloids occur in the roots and shoots in most other *Nicotiana* species, including the closely-related *N. langsdorffii*. Alkaloid loading into xylem in the roots, or a step before that, is assumed to be blocked in *N. alata*, according to a grafting experiment between *N. alata* and *N. langsdorffii*, which showed that rootstocks from *N. alata* caused alkaloid deficiency in the shoots, and that the xylem sap of *N. alata* had no detectable alkaloids. Interspecific crossing between *N. alata* and *N. langsdorffii* revealed the dominance of the non-translocation phenotype of *N. alata* over that of *N. langsdorffii*. The molecular mechanism underlying this phenomenon has yet to be revealed. A number of plants produce and store medicinal compounds in their roots, without moving these compounds to their shoots. Deciphering the mechanism of the non-translocation phenotype of *N. alata* might shed light on how such medicinal plants retain the synthesized secondary metabolites in their roots.

Concluding remarks and future perspectives

Recent studies of alkaloid biosynthesis have provided detailed information on the expression of genes, the localization of enzymes, and the accumulation of metabolites involved in alkaloid biosynthesis, and have shed light on the movement of alkaloids and their biosynthetic intermediates in plants. Alkaloid transport has further been examined by the biochemical characterization of membrane transport systems, and alkaloid transporters of the ABC, MATE, and PUP families have been identified (Table 1). The alkaloid transporters also play important roles in plant defense responses against herbivores and microorganisms, reflecting the involvement of alkaloids in plant defenses. ABC and MATE family transporters have been shown to be widely distributed and to be functionally important transporters of secondary metabolites, including not only alkaloids, but also flavonoids and terpenoids (Crouzet et al. 2013; Shoji 2014; Yazaki et al. 2008, 2009; Zhao and Dixon 2010). The molecular elucidation of alkaloid transporters could contribute to our understanding of a wide range of transport mechanisms.

How can we isolate the genes responsible for alkaloid transport? Most of the alkaloid transporter genes identified so far, including those encoding CrTPT2, Nt-JAT1, Nt-JAT2, NtMATE1, NtMATE2, and NUP1, were originally identified, usually through large-scale transcriptome analyses, based on their expression patterns, which correlated well with those of relevant biosynthetic enzymes. Recent advances in genomics and transcriptomics promise to provide increasing amounts of genetic information, and databases that specialize in medicinal plants, such as Phytometasyn (http://www.phytometasyn.ca), Medicinal Plant Metabolomics Resource (http://www.medicinalplantgenomics.msu.edu), and Transcriptome Project of Medicinal Plants (http://www.uic.edu/pharmacy/MedPlTranscriptome/), promise to enable researchers to access, synthesize, and integrate the emerging data. Selecting appropriate candidates by searching databases is the first, key step towards isolating plant alkaloid transporters.

After selection of candidates, the second step in isolating alkaloid transporters is characterization of their transport activities using heterologous systems, such as *Xenopus* oocytes, yeast cells, and tobacco Bright Yellow-2 cultured cells (Table 1). This step is critically important and sometimes limiting, and so technical breakthroughs that facilitate the analysis of heterologously expressed membrane proteins are keenly awaited. The yeast system, which has been adapted for the functional characterization of many plant alkaloid transporters, offers the following advantages: i) it is easy to implement with standard laboratory equipment, ii) it is a eukaryotic

system and therefore suitable for functionally expressing plant proteins, and iii) it is capable of measuring the activities of both primary and secondary transporters, which transport substrate by directly using energy of ATP or PPi hydrolysis or using electrochemical gradients of protons or ions, respectively, and so should be a first choice for such examinations (Shitan et al. 2013b).

As shown for CjABCB1, CrTPT2 and NtNUP1, altering the expression of alkaloid transporters influences the accumulation, and sometimes also the production, of relevant alkaloids (Hildreth et al. 2011; Pomahacova et al. 2009; Shitan et al. 2005; Yu and De Luca 2013). However, the mechanistic details underlying these phenomena remain unclear. These findings have prompted interest in engineering of alkaloid transport as an alternative strategy to metabolic engineering. Transport engineering involves genetic manipulation of metabolite transport systems, mainly targeting membrane transporters. Transformation protocols have begun to be established for medicinal plants, making the introduction of transporter genes possible. VIGS is a promising method to knock down gene functions, and can be applied to a wide range of plants. Metabolic engineering or synthetic approaches that target not only biosynthetic enzymes but also transporters would facilitate the production of valuable compounds in plants.

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