Title

Modes of secretion of plant lipophilic metabolites via ABCG transporter-dependent transport and vesicle-mediated trafficking

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Abstract

Many lipophilic metabolites produced by terrestrial plants are deposited on plant surfaces to protect them from abiotic and biotic stresses. Plant-derived lipophilic metabolites include apoplastic biopolymers, such as wax, cutin, sporopollenin, suberin, and lignin, as well as low molecular weight secondary metabolites. These secreted molecules confer adaptive toughness and robustness on plants. The mechanisms responsible for the secretion of these lipophilic metabolites remain unclear, although two pathways, mediated by transporters and vesicles, have been proposed. Recent genetic and biochemical studies have shown that ABCG transporters and membrane trafficking factors are involved in the apoplastic accumulation of lipophilic metabolites in plants. These two distinctive modes of secretion may be either exclusive or collaborative. This review describes these transporter-dependent and vesicle-mediated mechanisms underlying the secretion of lipophilic metabolites.

Keywords

lipophilic metabolite; apoplast; secretion; ABCG transporter; vesicle; cuticle; apoplastic diffusion barrier; specialized metabolite; plasma membrane; cell wall

Introduction

Terrestrial plants utilize a variety of metabolites to adapt to their environments. Many hydrophobic plant-derived metabolites are predominantly produced in outer cell layers of plant tissues and secreted onto plant surfaces, whereas hydrophilic metabolites generally accumulate in intracellular compartments, such as vacuoles. Except for primary lipids (membrane lipids and storage lipids as triacylglycerol), plant hydrophobic metabolites generally consist of lipophilic biopolymers, such as wax, cutin, sporopollenin, suberin, and lignin, as well as lower molecular weight compounds, such as volatile benzenoids, terpenoids, alkaloids, naphthoquinones, and prenylated phenols. Lipophilic biopolymers protect terrestrial plants physically and constitutively from environmental disturbances, such as water loss, UV radiation, organ fusion, and attack by bacterial and fungal pathogens, as well as functioning as apoplastic barriers that control water and solute movement [1,2]. In contrast, most low molecular weight hydrophobic compounds are specialized metabolites, which play defensive roles and communicate as signals with other plants, insect herbivores, pathogens, and pollinators.

The biosynthesis of lipophilic metabolites has been intensively investigated. Precursors

of biopolymers are synthesized in endoplasmic reticulum (ER), plastid, and cytosol [1,3-5], with further modification and polymerization occurring in apoplastic spaces [5,6]. Lipophilic specialized metabolites are also synthesized intracellularly in cytosol, ER, plastids, and peroxisomes.

Less is known, however, about the transport mechanism of these hydrophobic metabolites. Several modes of secretion have been proposed, including passive diffusion, transporter-mediated transport, vesicle-mediated transport, direct membrane contact, and transport mediated by lipid transfer proteins (LTPs) (Figure 1a) [1,2]. Genetic and biochemical studies have indicated that the two major mechanisms thought to be responsible for the secretion of lipid molecules are the ABCG transporter-mediated and vesicle-mediated pathways (Figure 1). Some metabolites, like apoplastic biopolymers in *Arabidopsis thaliana*, are transported by these two distinct modes, suggesting that ABCG transporters and vesicle-mediated transport may act in a coordinated manner to deposit lipophilic metabolites. The present review describes current knowledge about ABCG transporter-mediated and vesicle-mediated trafficking pathways required for the secretion of apoplastic biopolymers composed of aliphatic lipids and aromatic compounds, and specialized lipophilic metabolites.

ABCG transporters for lipophilic metabolite secretion

ABC proteins are a superfamily consisting mostly of transporters that mediate the primary transport of substrates across the membrane using the energy derived from the hydrolysis of ATP. Of the eight subfamilies of plant ABC proteins, one, ABCG, has been associated with the transport of lipophilic metabolites. Most of the ABCG transporters characterized to date localize to the plasma membrane (PM) and are predicted to function in exporting lipophilic molecules to apoplastic spaces (Figure 1b).

Plant cuticles, which cover aerial tissue surfaces, are composed of two lipid structures, cuticular waxes and cutins. Cutins are aliphatic polyesters deposited outside the cell wall and serve as structural backbones of cuticle. Cuticular waxes, which are embedded in cutin polyesters, consist predominantly of aliphatic very-long-chain fatty acid derivatives. The first transporter protein identified as being associated with lipophilic metabolite secretion in plant cells was *Arabidopsis* ABCG12, a half-size transporter involved in cuticular wax loading [7]. A closely related half-sized member, AtABCG11, is also involved in cuticle formation by exporting wax components and cutin monomers [8]. Such half-sized ABCG transporters function as homodimers or heterodimers to export different substrates (Figure 1b); AtABCG11 was proposed to heterodimerize with AtABCG12 and AtABCG5 to export wax precursors [9,10], whereas AtABCG11

heterodimerizes with AtABCG9 and AtABCG14 to deposit lipidic molecules required for vascular development [11]. The transport activity of cutin precursors by AtABCG11 was actually demonstrated, in which ABCG11 forms homodimers [12**]. The homologous ABCG transporters of other plants have similar roles in cuticle formation, e. g., *Medicago truncatula* SGE1 and MtABCG13 in floral tissues form heterodimers to excrete both waxes and cutins [13]. *Physcomitrella patens* PpABCG7 and *Petunia hybrida* PhABCG12 are involved in cuticular wax deposition [14,15*]. In particular for PhABCG12, the influence of cuticle thickness to volatile emission was also demonstrated, suggesting the feedback mechanism between the cuticle and transport processes [15*]. Protective cuticle formation likely resulted from the functional activities of half-type ABCG transporters conserved among land plants during evolution.

Full-size ABCG transporters have key roles in the secretion of aliphatic cutin monomers. For example, *Arabidopsis* AtABCG32 and *Hordeum spontaneum* HvABCG31 deposit cutin molecules, resulting in cuticle formation [16,17]. Two AtABCG32 homologues in *Solanum lycopersicum*, SlABCG42 and SlABCG36, have been shown to deposit aliphatic cutin monomers for cuticle formation in tomato fruits [12**]. A transport assay using *Nicotiania benthamiana* protoplasts showed that both SlABCG42 and AtABCG32 export aliphatic fatty acid derivatives, including 2-monoacylglycerol [12**].

Male gametophyte microspores are covered with pollen exine, consisting of lipidic molecules including sporopollenin, which contains saturated long aliphatic derivatives and phenolic compounds. The lipidic constituents are synthesized in anther tapetal cells and are subsequently exported to apoplastic locule and translocated to microspore surfaces [4]. Several ABCG transporters were found to be required for pollen exine formation in *Arabidopsis* and *Oryza sativa*, suggesting that these transporters function in the transport of exine-forming lipidic constituents [18,19]. AtABCG9 and AtABCG31 likely transfer steryl glycosides from tapetal cells to pollen surfaces [20], whereas AtABCG26 likely translocates polyketide derivatives as sporopollenin precursors [21].

Suberin, an aliphatic polymer ester bound to aromatic compounds, is deposited beneath cell walls as a diffusion barrier. The deposition of aliphatic suberin monomers depends on half-size ABCG transporters, such as OsABCG5 in the suberized hypodermis of rice and StABCG1 in suberized tubers of *S. tuberosum* [22,23]. Purified AtABCG1 forms homodimers, and its ATPase activity is stimulated by aliphatic suberin monomers, long chain fatty alcohols and fatty acids, findings supported by mutant analysis [24*].

Lignin is a phenolic polymer formed by oxidative coupling of monolignols that contributes to the mechanical strength of plants. Monolignols are secreted to cell walls, where they undergo polymerization by apoplastic peroxidases and laccases [6].

Monolignol transport in PM-rich vesicles of *Arabidopsis* leaves is dependent on ABC transporters [25]. In fact, full-type AtABCG29, which is expressed in vascular tissues of *Arabidopsis*, has been found to export the monolignol, *p*-coumaryl alcohol, by molecular genetics and transport assay [26]. Glucosides of monolignols are also proposed as lignin precursors. A recent computational modeling suggested that the diffusion of aglycones is supported but not their glucosides [27]. It is thus to be emphasized that chemical properties of transport substrates play important roles in the movement across the membrane.

Full-size ABCG transporters are also closely involved in the excretion of antifungal terpenes and alkaloids. For example, NbABCG1 and NbABCG2 from *N. benthamiana* are involved in the secretion of the anti-microbial sesquiterpene capsidiol, which protects against the pathogen *Phytophthora infestans* [28]. *N. tabacum* NtPDR1 has been shown to transport the endogenous diterpenes, sclareol and cembrene [29], with the ATPase activity of NtPDR1 stimulated by administration of these terpene substrates [30]. AaABCG3 from *Artemisia annua* transports the sesquiterpene *β*-caryophyllene [31], and TwPDR1 from *Tripterygium wilfordii* secretes the diterpene triptolide [32].

Secretion of the monoterpenoid indole alkaloids, catharanthine and vincamine, onto the leaf surfaces of *Catharanthus roseus* and *Vinca minor*, has been found to depend on the export activities of CrTPT2 and VmABCG1, respectively [33,34]. An indole alkaloid, camalexin, a major phytoalexin in *Arabidopsis*, is also secreted onto leaf surfaces by full-size ABCG transporters. AtABCG34 mediates camalexin secretion upon *Alternaria brassicicola* infection [35], whereas AtABCG36 and AtABCG40 secrete camalexin in response to infection with *Botrytis cinerea* [36*].

Volatile compounds are a group of low molecular weight lipophilic metabolites. A MYB-type transcription factor ODORANT1, which controls the biosynthesis of volatile organic compounds (VOCs) in *P. hybrida*, was shown to exclusively regulate the expression of *PhABCG1* in flower petals [37]. Downregulation of *PhABCG1* suggested its role in VOC emission, and a transport assay using BY-2 cells demonstrated that PhABCG1 exports methylbenzoate and benzyl alcohol, both of which are major VOCs emitted by petunia flowers [38**]. Because VOCs are emitted from the tissue surface across the cuticle, their physico-chemical properties are also important factors to influence the emission efficiency [15*].

‘Oil bodies’ in *Marchantia polymorpha* are organelles that develop in idioblastic cells, differing from typical oil bodies frequently present in plant sink organs. *M. polymorpha* ‘oil bodies’, which contain sesquiterpenes and cyclic bisbibenzyls [39], are surrounded by membranes having MpSYP12B and MpSYP13A, with the lumen of *Marchantia* ‘oil

bodies' having apoplastic properties [40,41*]. Thus, loading of those metabolites into these compartments would be equivalent to extracellular secretion. MpABCG1 is located on *Marchantia* 'oil body' membranes, with its expression regulated by two transcription factors, MpERF13 and MpC1HDZ, which are involved in the 'oil body' formation and terpenoid biosynthesis [41*,42]. These findings suggest that MpABCG1 exports sesquiterpenes into *M. polymorpha* 'oil bodies'.

Vesicle-mediated secretion of lipophilic metabolites

Lipophilic metabolites and their precursors are transported within hydrophilic cytoplasm toward the PM. A pathway should be present from the site of synthesis to the site of apoplastic accumulation across the PM. Vesicle-mediated trafficking, which sequesters these lipophilic compounds from the cytosol, is likely involved in transporting these molecules. Vesicle trafficking consists of sequential multi-step processes: vesicle assembly and formation at the donor membrane, vesicle budding, vesicle transport, vesicle tethering and membrane fusion between transport vesicles and target membrane to release vesicle cargos [43]. Each step requires the coordinated activities of many protein components with diverse molecular functions [44]. To date, only a few molecular players have been identified in plants, whereas many microscopic studies have evaluated vesicle-mediated trafficking in lipid metabolite transport (Figure 1c).

Wax secretion in *Arabidopsis* has been reported to require Golgi- and *trans*-Golgi network (TGN)-mediated trafficking [45*]. ECHIDNA is a TGN-localized protein in *Arabidopsis* that regulates multiple trafficking pathways through TGN, including secretory trafficking and vacuolar trafficking [46,47]. ECHIDNA also influences the vesicle-mediated secretion of seed coat mucilage, in which ECHIDNA and its interactive YIP4 proteins collaboratively act [48]. The *echidna* mutation, which is responsible for a defect in protein secretion, dramatically reduces wax secretion to the stem surface [45*]. The *CER11* gene encodes a phosphatase that interacts with and dephosphorylates DET3 [49], a subunit of a TGN-localized V-ATPase that regulates endosomal trafficking [50]. Similar to *echidna*, the *cer11* mutant was found to reduce wax deposition and mucilage secretion and to block protein secretion [49]. These findings suggest that CER11 might modulate the activation status of membrane trafficking components required for wax secretion. Of the eight *Arabidopsis* ADP-ribosylation factor-guanine nucleotide exchange factors (ARF-GEFs) that regulate vesicle formation and budding processes [51,52], two, GNOM-LIKE 1 (GNL1) and AtMIN7, were shown to be involved in cuticle formation [45*,53]. GNL1 is a Golgi-localized GBF-type ARF-GEF protein required for protein secretion that is also involved in cuticular wax export [45*,54]. AtMIN7 is a TGN-

localized BIG-type ARF-GEF protein involved in endosomal trafficking, as well as in cutin monomer deposition [53,55]. In addition, membrane fusion at PM is also reportedly required for wax deposition; for example, PpEXO70.3d in *P. patens* is involved in cuticle deposition on epidermal surface [56]. EXO70 is a subunit of the EXOCYST complex that is hetero-octameric and regulates vesicle tethering and membrane fusion at the PM [57].

Cutin monomers self-assemble in aqueous solutions to form lipid droplet-like micelles, termed cutinosomes [58], in both the cytoplasm and cell wall of cuticle-rich epidermal cells [59]. Electron microscopic studies in *Ornithogalum umbellatum* have suggested that cutinosomes develop from close associations among ER, lipid droplets, and microtubules; these cutinosomes subsequently pass through the cytoplasm and cell wall to fuse to cuticles [60]. Cutinosomes containing esterified cutin monomers were shown to be involved in early cuticle formation in tomato fruit [61*]. Cutinosomes may therefore structurally protect transport cargos from hydrophilic environments, including the cytoplasm and polysaccharides in the cell wall.

The secretion of pollen exine precursors is dependent on a vesicle-like system. Vesicles containing sporopollenin precursors are thought to fuse with the PM, with the release of these precursors leading to pollen wall formation [62]. Indeed, ER-derived vesicles called tapetosomes and containing flavonols, alkanes, and triacylglycerols, are present in tapetum cells of *Brassica napus* [63]. After tapetum lysis, these tapetosome contents are deposited on pollen coats [63].

Secretion of lignin precursors has been reported to require membrane vesicles. Vacuolar and microsomal vesicles prepared from a broad variety of plant species, such as *Arabidopsis*, *Populus sieboldii* × *P. grandidentata*, *Chamaecyparis obtuse*, and *Picea abies*, were shown to transport monolignol glycosides, suggesting that lignin precursors are incorporated into secretory vesicles before excretion across the PM [25,64-66]. An EXOCYST component AtEXO70A1 at the PM was shown to be crucial for the proper deposition of lignin structures in Casparian strips [67,68].

Extracellular vesicle-tubular structures (EVBs) in the root endodermis, which contain highly branched tubular networks and isolated vesicles, have been reported associated with AtEXO70A1-mediated Casparian strip formation. The attachment of EVBs to the PM has been observed in suberizing root cells, in which contained tubules and vesicles fuse with the suberin lamellae surface [69]. Treatment with brefeldin A indicated that early secretory trafficking between ER and Golgi is required for the formation of EVBs that transport putative suberin monomers, resulting in suberin lamellae formation [69].

Shikonin derivatives are red naphthoquinone pigments secreted from the root epidermis of *Lithospermum erythrorhizon*. Treatment with brefeldin A and cytochalasin D led to the

accumulation of shikonin derivatives inside these cells, suggesting that shikonin secretion depends, at least partly, on membrane trafficking machinery regulated by ARF-GEF and actomyosin cytoskeleton [70]. Lipidome analysis and *in vitro* vesicle preparation suggested that *L. erythrorhizon* cells secrete large amounts of triacylglycerols that solubilize shikonin derivatives [71*]. Triacylglycerol excretion is also observed as surface wax in *Myrica pensylvanica* fruit [72]. In both cases, secreted triacylglycerol is composed mainly of saturated fatty acids [71*,72]. Enrichment of saturated aliphatics in secreted primary lipids has also been observed in *Arabidopsis* extracellular vesicles, which are enriched in glycosylinositolphosphoceramide-type sphingolipids [73]. Because highly lipophilic metabolites like triacylglycerol are compartmentalized in lipid monolayer particles, exocytosis of those oil droplets presumably requires a complex membrane system like multivesicular bodies fused to the PM via unconventional exocytosis [74].

Conclusions and future directions

Recent investigations have extended our knowledge on the mechanisms by which lipophilic metabolites are transported to apoplastic spaces in plant cells. Secretion via ABCG transporters and transport vesicles appears to be similar to trafficking of cholesterol in mammals [75]. Apoplastic deposition of lipophilic metabolites may also be mediated by other mechanisms, such as passive diffusion, direct membrane contact between ER and PM, and exosome- and LTP-related transport (Figure 1a). These different transport modes are likely utilized for trafficking of wax, cutin, suberin, and VOCs in plant cells [1,2,76].

Better understanding of the mechanisms responsible for the secretion of lipophilic metabolites requires clarification of many biochemical aspects. Although ABCG transporters were shown to be involved in the apoplastic accumulation of lipid molecules, the detailed biochemical processes underlying transport events remain undetermined [77]. In addition to the passive diffusion of lipophilic metabolites due to their hydrophobic properties, the difficulty of heterologous expression of ABCG proteins is a hurdle to overcome in this field [78]. In some successful cases, transport assay was done in yeast [26,31,33,34] or plant host [12**,29,32,38**], however these are rather minority. The characterizations of ABCG transporters were mostly reported based on molecular genetics approaches, in which transport mechanisms remained unclear. Another important aspect is heterodimerization of half-size ABCG transporters, which affects transport substrate specificity. Arabidopsis has 28 members of half-size ABCG proteins, and the heterodimerization within this family provides a large potential to recognize vast number

of substrates to transport.

Although vesicle-mediated transport of hydrophilic flavonoid pigments into vacuoles has also been reported [79], only three membrane trafficking factors involved in flavonoid accumulation have been identified: AtEXO70B1, which is involved in anthocyanin transport [80]; and GFS9 and ECHIDNA, which are associated with the accumulation of proanthocyanidin for seed coat pigmentation [47,81]. Membrane trafficking processes are coordinated, sequential, and multi-stepped, with many involved genes being functionally redundant [44]. Thus, identification of proteins responsible for vesicle trafficking of plant specialized metabolites would be difficult.

Following the excretion of metabolites across the PM, the further dynamics of these metabolites in the cell wall remain unclear. Secreted hydrophobic molecules are deposited at native accumulation sites. For example, wax and cutin form a cuticle layer outside the cell wall, whereas suberin is located at the interface between the PM and cell wall forming suberin lamellae, while VOCs are emitted into the atmosphere. LTP proteins are thought to act as chaperones in the movement of lipophilic precursors [1,2,5,76], and phase separation has been associated with cell wall patterning [5,82*]. Possible interactions between transporter proteins and molecules involved in vesicle trafficking should also be clarified. Comprehensive understanding of the mechanisms, by which lipophilic metabolites are secreted onto plant surfaces, may provide insight into plant adaptation to terrestrial environments that support their sessile lifestyles.

Author contributions

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Conflict of interest statement

Nothing declared.

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This study showed that pollen wall patterns are formed by primexine phase separation using biophysical modeling and electron microscopic observations.

Figure Legends

Figure 1. Modes of secretion of lipophilic metabolites from plant cells.

(a) Proposed transport pathways for lipophilic metabolites in plant cells. Lipophilic metabolites and their precursors (blue circles) synthesized intracellularly can be secreted from the cells by several pathways, including by membrane transporters, vesicles-mediated excretion, lipid transfer proteins (LTPs), direct lipid transfer at membrane contact sites, and passive diffusion. ER, endoplasmic reticulum; PM, plasma membrane.

(b) ABCG transporter-dependent export of lipophilic metabolites from plant cells. Each color depicts a different ABCG protein, with heterodimers represented by two different colors. AtABCG32, SlABCG42, and AtABCG29 are full-size ABC transporters. The chemical structures of putative transport substrates are shown, in which transport activity was demonstrated, or ATPase activity of ABCG transporters was stimulated by addition of those substances. AtABCG32 and SlABCG42 have been shown to transport a monoacylglycerol (MAG: 10,16-dihydroxy hexadecanoyl-2-glycerol), a hydroxylated fatty acid (16-hydroxyhexadecanoic acid), and a dicarboxylic acid (DCA: 1,16-hexadecanedioic acid), whereas AtABCG11 homodimer transports only the MAG and the hydroxylated fatty acid, not the DCA molecule. PhABCG1 exports volatile organic compounds (VOCs), methylbenzoate and benzyl alcohol. Pi, inorganic phosphate; PM, plasma membrane.

(c) Vesicle-mediated secretion of lipophilic metabolites for apoplastic accumulation in plant cells. Purple boxes represent proteins responsible for membrane trafficking machinery involved in lipophilic metabolite transport; ECHIDNA (ECH), GNOM-LIKE 1 (GNL1), MIN7, and EXO70 family proteins. Loading of monolignol glycosides, like coniferin, into vesicles by membrane transporters is also involved in lignin precursor deposition. ER, endoplasmic reticulum; Golgi, Golgi apparatus; TGN, *trans*-Golgi network; PM, plasma membrane; EVB, extracellular vesicle-tubular structure.

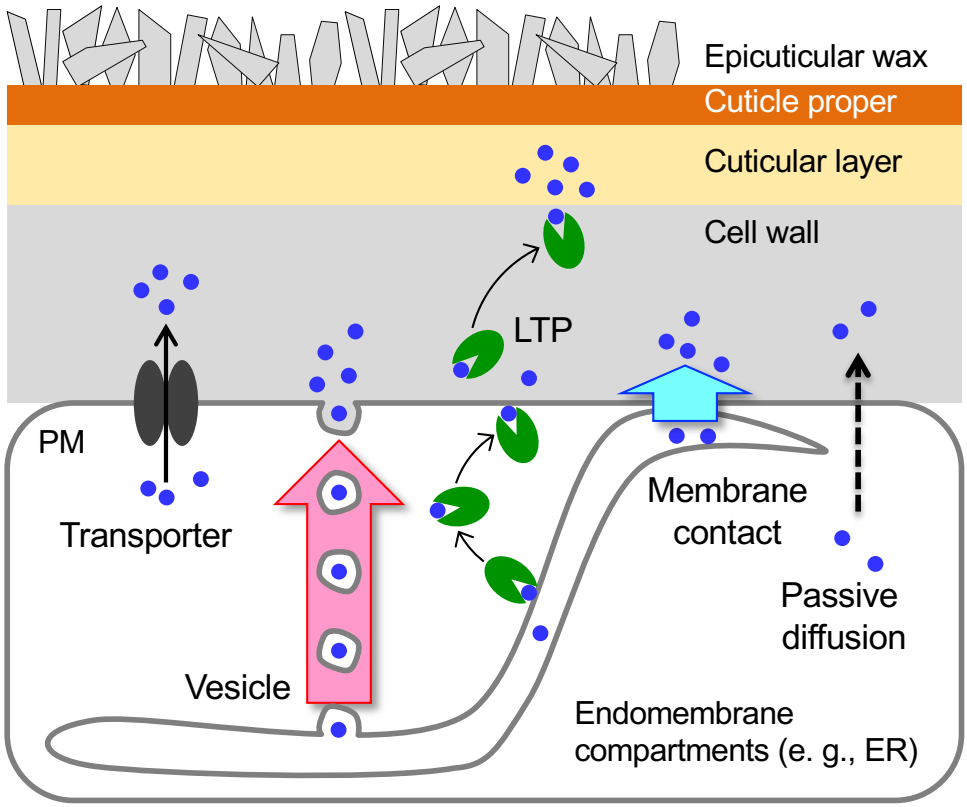

Figure 1a.
Proposed transport pathways for lipophilic metabolites in plant cells

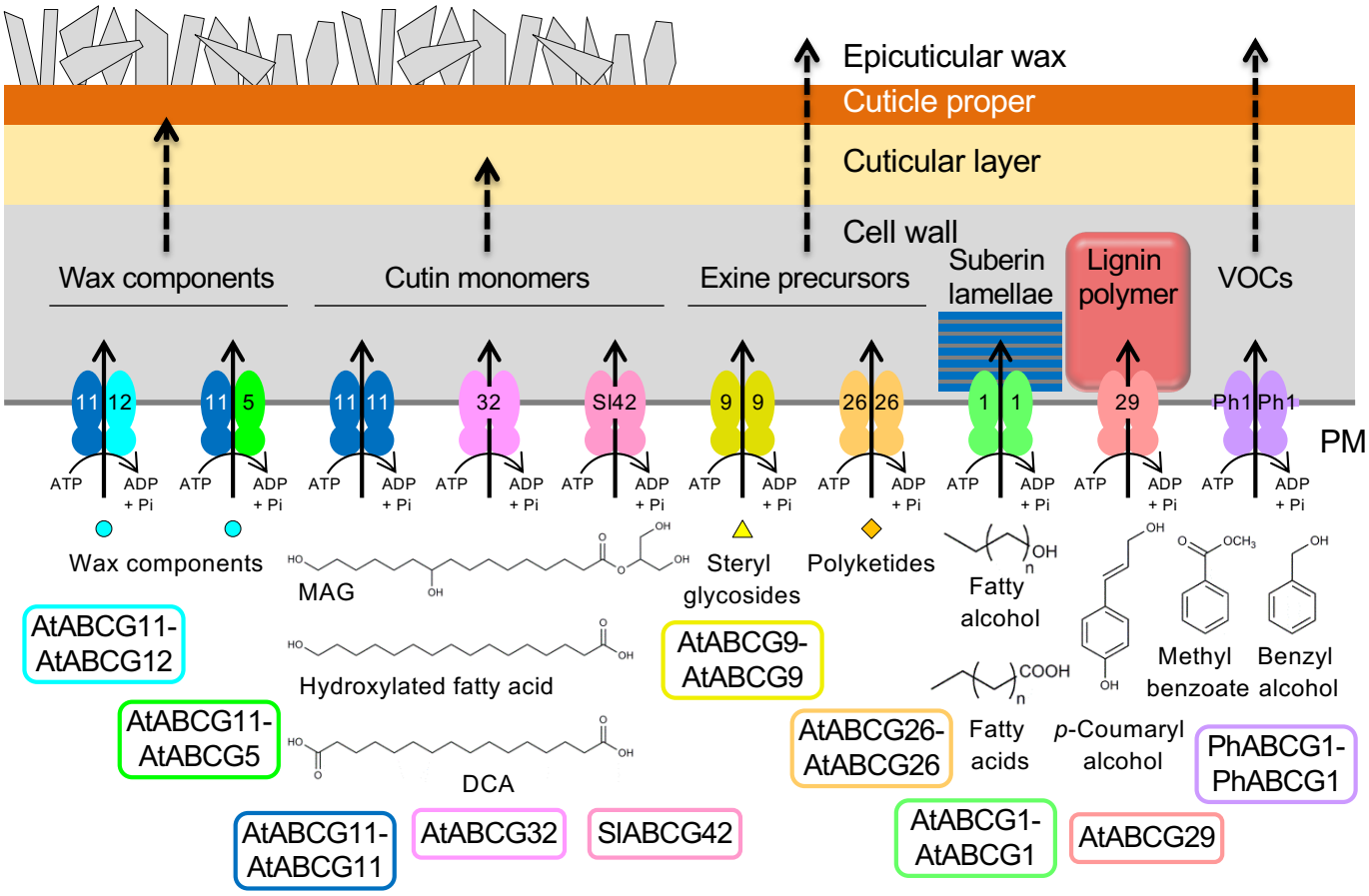

Figure 1b.
ABCG transporter-dependent export of lipophilic metabolites from plant cells

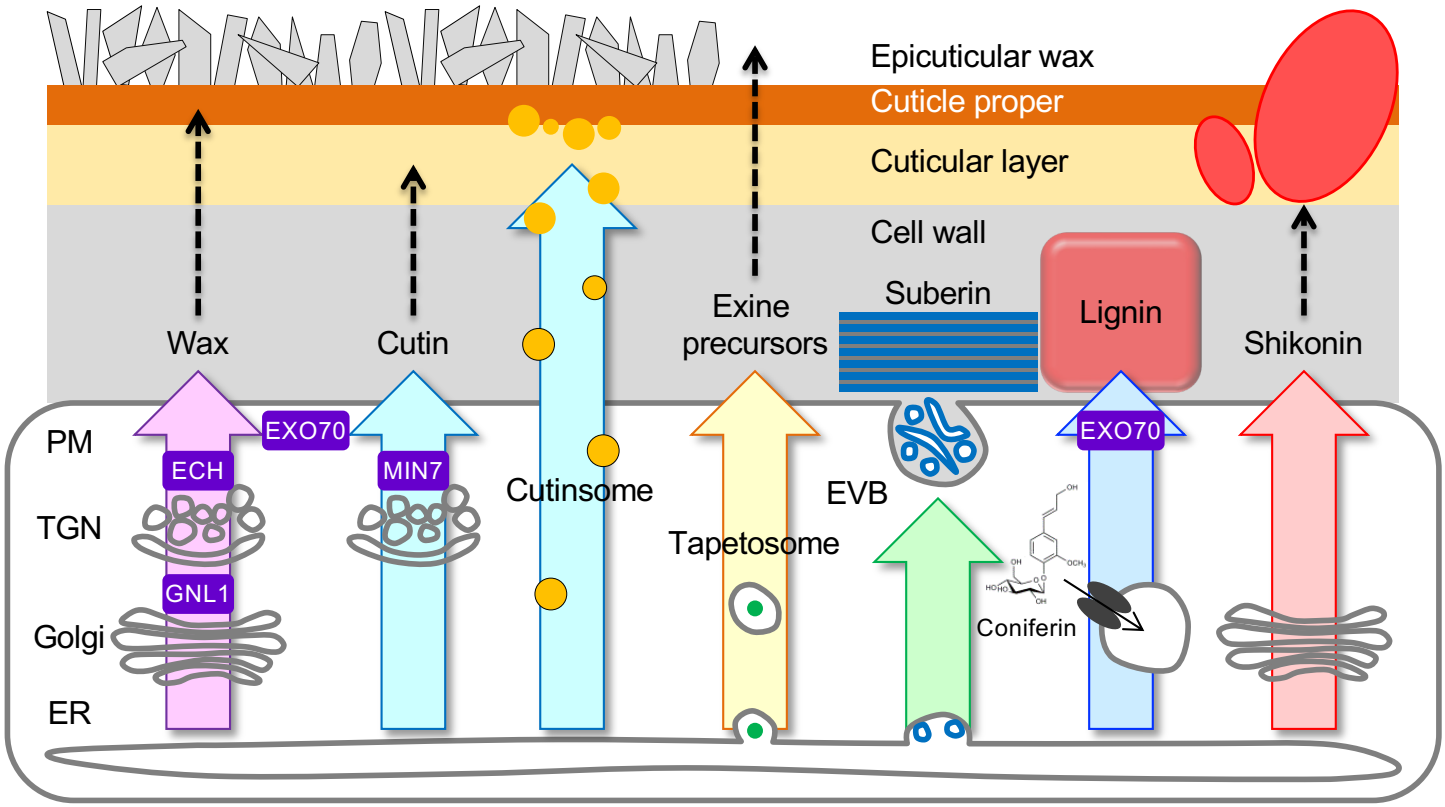

Figure 1c.
Vesicle-mediated secretion of lipophilic metabolites for apoplastic accumulation in plant cells