Cell
PRESS

The 'ins' and 'outs' of flavonoid transport

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The sites of plant flavonoid biosynthesis, storage and final function often differ at the subcellular, cell, and even tissue and organ levels. Efficient transport systems for flavonoids across endomembranes and the plasma membrane are therefore required. However, a clear picture of the dynamic trafficking of flavonoids is only now beginning to emerge and appears to have many players. Here, we review current hypotheses for flavonoid transport, discuss whether these are mutually exclusive, highlight the importance of flavonoid efflux from vacuoles to the cytosol and consider future efforts to catch flavonoids 'in the act' of moving within and between cells. An improved understanding of transport mechanisms will facilitate the successful metabolic engineering of flavonoids for plant protection and human health.

The pieces of the flavonoid transport puzzle

Flavonoids represent one of the largest classes of plant secondary metabolites, and are involved in a multitude of physiological functions, including UV protection, insect attraction, pathogen defense, symbiosis and variation of flower color [1]. Flavonoids have been associated with many favorable agronomic traits and health benefits to humans, and their metabolic engineering is therefore an important target for plant biotechnology [2,3]. Pathways, enzymes and regulatory mechanisms of flavonoid biosynthesis have been well studied [4], yet flavonoid location and trafficking *in vivo* are still not clearly established.

There appear to be tight links between the biosynthesis, transport and cellular deposition of flavonoid compounds. Successful metabolic engineering of flavonoid production, therefore, requires a full consideration of regulatory mechanisms not only for biosynthesis, but also for transport and storage of the end products in cells and tissues. In this respect, conclusions from earlier microscopy observations of flavonoid-containing vesicles have yet to gain molecular genetic support and membrane transporter-mediated flavonoid transport has been confirmed in only a few cases [5]. Furthermore, transport across the vacuolar membrane is not unidirectional for flavonoid storage, as plant cells have mechanisms for re-mobilization of vacuolar stores of flavonoid compounds under certain physiological conditions. Parallel pathways appear to exist for the transport and storage of flavonoids at the tissue, cellular, subcellular and molecular levels, with their relative importance and modes of regulation still poorly understood. The field is however, attracting increasing attention and is set to see major advances with the application of increasingly sophisticated

genetic, biochemical and cell biological approaches. Here, we summarize recent efforts made to identify flavonoid transport mechanisms, and outline future research targets aimed at piecing together the complex puzzle linking

Glossary

ABC transporter: a large superfamily of ATP-binding cassette (ABC) proteins. They usually contain a nucleotide-binding domain and a transmembrane domain for mediating MgATP-energized transmembrane transport and/or regulation of other transporters [41]. Different subfamilies of ABC transporters have essential and diverse roles in the transport of metal ions and primary and secondary metabolites across membranes.

Anthocyanic vacuolar inclusions (AVIs): intravacuolar bodies of varying sizes containing concentrated anthocyanins. AVIs are observable in petal cells of carnation (*Dianthus caryophyllus*) and lisianthus (*Eustoma grandiflorum*), and in other anthocyanin-accumulating cells in grapevine, sweet potato (*Ipomoea batatas*), and maize. AVIs do not have membrane boundaries, but contain membrane lipids and a protein matrix bound to anthocyanins, particularly acylated anthocyanins [19].

Anthocyanoplast: initially used to describe cytoplasmic membrane-bound vesicles containing high levels of anthocyanins and regarded as anthocyanin biosynthetic sites. However, later studies confused them with anthocyanic vacuolar inclusion (AVIs), which are more widely observed inside the vacuoles of many plant species. Anthocyanoplasts are found exclusively in the cytoplasm in grapevine cells, and in protoplasts prepared from red radish (*Raphanus sativus*) seedlings.

MATE transporter: multidrug and toxic compound extrusion (MATE) family transporters. They use H^+/Na^+ gradients across membranes as a force to drive waste or toxic compounds out of the cytoplasm. MATE transporters perform conserved and basic transport functions in most prokaryotes and eukaryotes.

Pre-vacuolar compartment (PVC): an endocytic multivesicle compartment involved in ER-Golgi-vacuole vesicle trafficking. It carries proteins and other metabolites to fuse to the large central vacuole. The PVC has SNAREs or vacuolar-sorting receptors to accept precursor vesicles and then fuse to the vacuole. A specific type of PVC in lisianthus epidermal cells contains anthocyanin and was proposed to transport anthocyanins into the central vacuole (19).

Protein storage vacuole (PSV): a type of vacuole and compound organelle formed during plant seed development and maturation and containing large amounts of storage proteins. PSVs contain vacuolar-sorting receptors to recognize cargo molecules. A PSV marker co-localizes with anthocyanins, leading to the suggestion that anthocyanins are transported directly via ER-derived vesicle trafficking in a Golgi-independent manner (22).

SNAREs: soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors. These are small but abundant integral membrane proteins that mediate vesicle fusion and reside on the surface of the transport vesicle (v-SNAREs) and target membrane (**t-SNARE**s). They have a cytosolic domain called a SNARE motif, which assembles with another SNARE motif into parallel four-helix bundles within SNARE complexes and brings the transmembrane anchors and the two membrane vesicles into close proximity.

Tapetosomes: abundant organelles in the tapetum cells of anthers during the active stage of pollen maturation in Brassicaceae species. Tapetosomes originate from massive ER cisternae, which release lipid droplets that are fused into large vesicles. These lipid vesicles further fuse with ER-derived vesicles containing flavonoids. Upon tapetum cell death, tapetosomes release alkanes and oleosins in lipid droplets, along with flavonoids, to the pollen surface.

***trans*-Golgi network (TGN)**: a dynamic series of membrane compartments at the *trans*- face of the Golgi stacks. The TGN mainly processes and sorts various proteins and glycolipids at the interface of the biosynthetic and endosomal pathways. The generation and maintenance of apical and basolateral membranes relies on sorting events that occur in the TGN.

Vacuolar sorting receptors (VSRs): integral membrane proteins responsible for the proper targeting of cargo proteins to their destination compartments. VSRs are localized to ER, PVC, TGN and Golgi apparatus.

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flavonoid biosynthesis, transport and storage processes in plant cells.

Subcellular sites of flavonoid biosynthesis
The enzymes of flavonoid biosynthesis are believed to be localized on the cytosolic side of the endoplasmic reticulum (ER), organized into a multi-enzyme complex centered around the ER-associated cytochrome P450 enzymes cinnamate 4-hydroxylase, flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase [6,7]. The enzyme complexes might be brought together by protein–protein interactions on the surface of the ER [6].

Some of the enzymes of the pathway have also been shown to co-localize to the tonoplast and nucleus [6–8], consistent with the idea that flavonoid biosynthetic enzyme complexes might be dynamic to facilitate channeling or shifting of end-product biosynthesis to meet physiological requirements under different conditions [6,7]. Such enzyme complexes associated with the ER could also facilitate the transport of flavonoid products through membrane trafficking.

Subcellular distribution of flavonoids
Consistent with their diverse physiological functions, including UV protectants and modifiers of auxin transport [1,4,9], flavonoids are found in most plant cell compartments, including the cytosol, vacuole, ER, chloroplast, nucleus and small vesicles, as well as the extracellular space. Most conjugated flavonoids, such as anthocyanins and flavonol and flavone glycosides, are found primarily in the vacuole [1,4,10], where vacuolar pH and the presence of co-pigments determine anthocyanin-mediated flower coloration. The vacuole is also believed to be the site for the biosynthesis of some flavonoid derivatives. For example, catechin and epicatechin monomer units are glycosylated and then transported into the vacuole for polymerization into proanthocyanidins (PAs) in the endothelial layer of the seed coat in many species, and are finally exported to the apoplast [10]. Mutation of genes involved in PA biosynthesis or transport leads to a *transparent testa* (*tt*) phenotype. In common snapdragon (*Antirrhinum majus*) flowers, yellow aurone pigments are produced from chalcones by aureusidin synthase, a vacuolar polyphenol oxidase [11]. Increasing evidence suggests that the vacuole also acts as an important source and/or sink, regulating the homeostasis of cytosolic metal ions, metabolite precursor pools and secondary metabolites. This role requires mechanisms for both vacuolar influx and efflux.

Quercetin and kaempferol glycosides have been detected in chloroplasts, which are capable of flavonoid biosynthesis [12]. Some flavonoids and their biosynthetic enzymes are also present in the nucleus [8]. UV protection is probably a major function of flavonoids in both these compartments. In the model legume barrel medic (*Medicago truncatula*), the isoflavonoids coumestrol and 4',7-dihydroxyflavone accumulate to higher levels in the nucleus than in the cytoplasm [13], and 4',7-dihydroxyflavone might act as a signal for jasmonate-induced mobilization of vacuolar isoflavonoid glucosides for phytoalexin biosynthesis [14]. However, it is still unclear whether flavonoids are transported into the nucleus or if the nucleus is a site for their synthesis.

Some flavonoid conjugates and aglycones are secreted into the apoplastic space [15]. Flavone and flavonol aglycones have been detected in root exudates from numerous species, and flavones and isoflavones are secreted by legume roots into the rhizosphere, either to attract mutualistic microorganisms, such as nitrogen-fixing bacteria, or to provide defense against pathogens [16]. Flavonoids are also present on pollen surfaces [17].
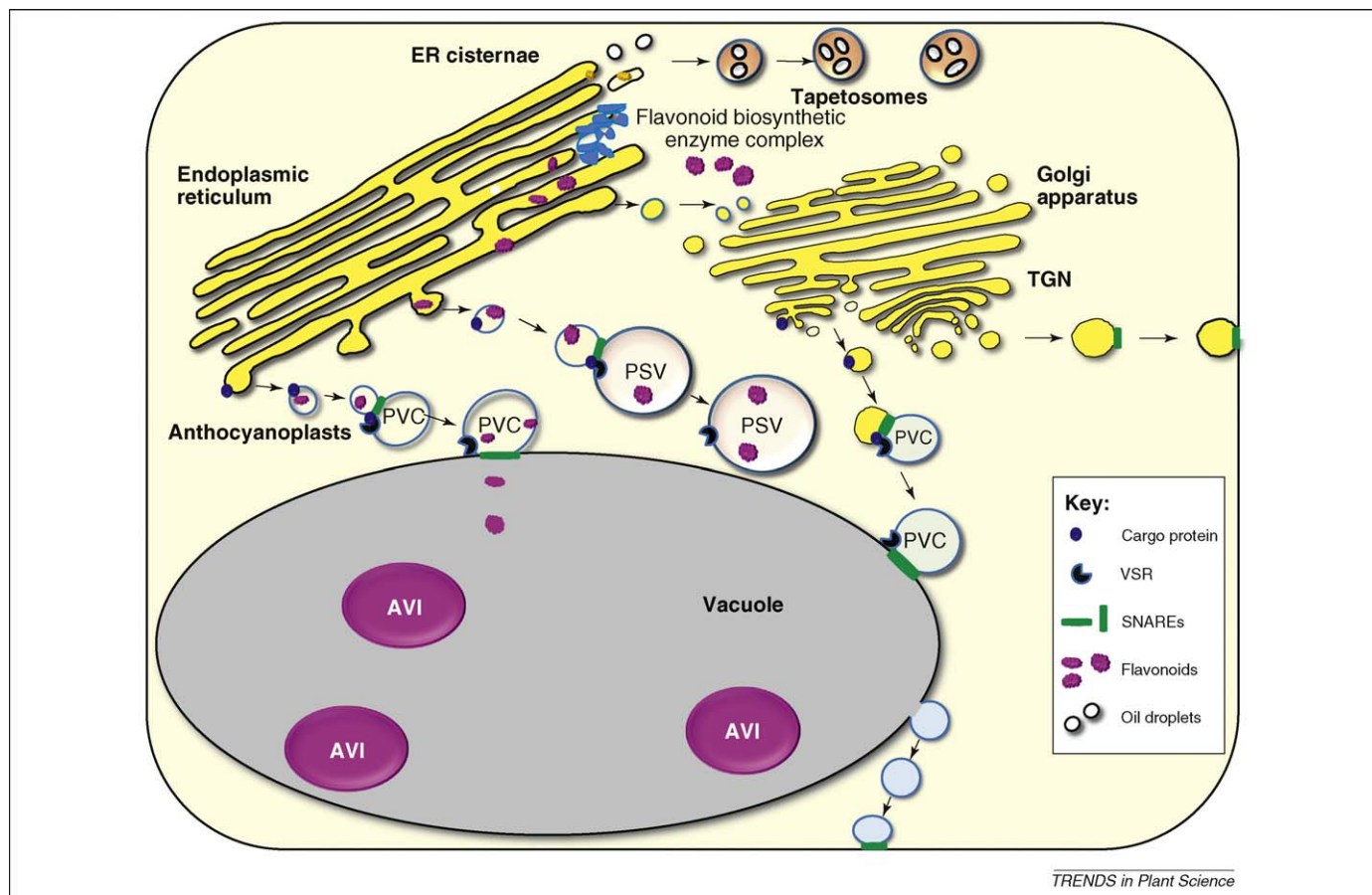
Flavonoid transport
The wide distribution of flavonoids in plant cells and their probable biosynthetic sites on the cytosolic face of the ER imply that plants have efficient flavonoid transport systems with which to deliver these metabolites across various membrane-limited compartments. However, the transport mechanisms involved in trafficking of most primary and secondary metabolites are still poorly understood [5]. Two major hypotheses have been proposed for flavonoid transport: membrane vesicle-mediated transport (Figure 1) and membrane transporter-mediated transport (Figure 2). These might not be mutually exclusive.

Vesicle trafficking-mediated flavonoid transport
The concept of vesicle-mediated flavonoid transport originated from microscopy observations. Anthocyanoplasts (see Glossary; Figure 1) were first assumed to be transport vesicles or sites of anthocyanin biosynthesis [5,18–20]. These cytoplasmic anthocyanin bodies are covered by a membrane and originate from a large number of smaller vesicle-like structures that gradually fuse together. Similar cellular structures, anthocyanic vacuolar inclusions (AVIs), are present in the vacuoles of many species and also show dynamic movement [18,19,21] (Figure 1). Although associated with specific proteins and membranous substances, AVIs are not surrounded by a membrane [18,19], and are more likely to be storage complexes than to be involved in anthocyanin transport. However, cytoplasmic vesicle-like structures containing anthocyanins (similar to anthocyanoplasts) have been observed to associate with AVIs after they have fused into the vacuole [19]. These might be transported into the central vacuole through fusion of prevacuolar compartments (PVCs) and then the central vacuole (Figure 1). In addition, anthocyanin-containing vesicle-like structures can co-localize with protein storage vacuoles (PSVs) and transport anthocyanins in a *trans*-Golgi network (TGN)-independent ER-to-PVC vesicle trafficking pathway [22] (Figure 1).

The tapetosomes in tapetal cells of *Arabidopsis* (*Arabidopsis thaliana*) anthers are specialized bodies that are structurally distinct from anthocyanoplasts. They contain ER-derived flavonoids, which are released to the pollen surface upon tapetum cell death, supporting the idea that flavonoids can be transported from the ER via cytoplasmic vesicles [17]. The ER-associated biosynthetic enzyme complex might facilitate the loading of flavonoids into the ER-derived membrane vesicles.

Early studies suggested that prevacuole-like vesicle structures containing PAs can fuse with the central vacuole and might represent a PA transport pathway

Figure 1. Proposed models for vesicle-mediated transport of flavonoids. Anthocyanoplasts have been proposed to transport anthocyanins to the central vacuole, probably by fusion into PVCs, in grapevine, red cabbage and lisianthus epidermal cells. In *Arabidopsis* leaves, a TGN-independent, ER-derived vesicle-mediated flavonoid transport directly from ER to PSVs has been proposed. In anther tapetum cells of Brassica and *Arabidopsis*, cytoplasmic vesicles called tapetosomes transport ER-derived flavonoids. The general protein secretory pathway involves trafficking of transport vesicles from the ER to the *cis*-face of the Golgi apparatus, followed by release through TGN vesicles to destinations such as the vacuole and plasma membrane. These processes involve recognition of cargo molecules by vacuolar sorting receptor (VSR)-mediated docking, and SNARE-mediated vesicle fusion. However, so far there is no genetic evidence linking this general secretory pathway to flavonoid transport. AVIs of various shapes are often found inside the vacuole without a membrane boundary; these contain membranous substances and proteins, and thus might be anthocyanin storage bodies.

(reviewed in Ref. [23]). More recently, it has been shown that *Arabidopsis* mutants with reduced PA production (*tt* mutants) also exhibit morphological defects in the central vacuole of the seed coat endothelium cells. PAs had a patchy, filamentous distribution pattern in seed of the *tds4* (leucoanthocyanidin dioxygenase) mutant, and PA storage was confined to prevacuole-like small vesicles, with no central vacuole present [24]. Similar phenotypes are observed following mutation of the *Arabidopsis TT19* glutathione *S*-transferase (GST) [25] and the *AHA10* P-type H^+-ATPase and *TT12* proton/flavonoid antiporter [26]. Therefore, either PA accumulation and vacuole biogenesis appear to be closely related, or PA precursors are transported through ER-derived vesicles to the vacuole. However, mutation of the tonoplast-localized H-ATPase *ph5* did not lead to abnormal vacuoles in petunia (*Petunia hybrida*), even though *PH5* is an ortholog of *AHA10* and is also involved in vacuolar PA accumulation in the seed coat [27].

In sorghum (*Sorghum bicolor*), colored 3-deoxyanthocyanidin phytoalexins are formed in response to fungal infection. Vesicle-like red-pigmented inclusion bodies rapidly appear in leaf epidermal cells and move to challenge sites, where they release the antimicrobial flavonoids into the cytoplasm [28]. Similar to anthocyanoplasts, these vesicles mediate a directional transport of flavonoids. Such vesicles have not yet been observed in dicots, however.

Despite the above observations, direct genetic, molecular, or biochemical evidence supporting vesicle-mediated flavonoid transport is still lacking. Many fundamental questions concerning the origin, structure, composition and behavior of anthocyanin-containing vesicles remain. If vesicle-like anthocyanin bodies are destined for the central vacuole or other membranes, they should contain coat or cargo proteins on their membranes to direct them and to be recognized by vacuolar sorting receptors (VSRs) or other protein components for vesicle trafficking (Figure 1). Currently known proteins include small GTPases, VSRs and soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), which are required for budding, targeting, docking, fusion and recycling of vesicles or trafficking proteins [29–31] (Figure 1). However, such proteins have yet to be associated with either flavonoid transport or accumulation.

The elusive role of glutathione S-transferases in flavonoid transport

Several studies point to the involvement of GSTs in anthocyanin accumulation [25,32–34]. Initially, it was

proposed that GST is required for GS-X pump type ABC transporter-mediated vacuolar sequestration of glutathione-conjugated anthocyanins, based on the observation that a multidrug resistance-associated protein (MRP)-type ABC transporter can transport glutathione-conjugates into the vacuole [32]. A similar mechanism was proposed for transport of the isoflavonoid-derived phytoalexin medicarpin [35]. However, biochemical and genetic cross-complementation studies show that the GST protein itself, rather than GST activity, is required for anthocyanin sequestration [33,36]. There are, to the best of our knowledge, no reports of naturally occurring anthocyanin-glutathione conjugates from plants, but GST can bind to anthocyanin or flavonol to form a GST-anthocyanin or GST-flavonol complex, suggesting that GSTs protect flavonoids from oxidation and/or guide them to the central vacuole [37]. It is likely that GST is one of the proteins in the flavonoid-protein complexes related to flavonoid transport. This is further supported by the observation that the *Arabidopsis tt19* GST mutant is defective in ER-tapetosome-pollen surface transport of flavonoids, in addition to exhibiting reduced PA accumulation [17,25]. Association of a GST-flavonol complex with membranes has also been demonstrated [38], providing a further piece to be fitted into the GST-mediated flavonoid transport puzzle.

Because of their close biosynthetic relatedness, anthocyanins and PAs could share similar transport mechanisms. The maize (*Zea mays*) BZ2 GST complements the petunia *AN9*-depleted anthocyanin phenotype [33], whereas petunia *AN9* complements the anthocyanin-deficient phenotype of *Arabidopsis tt19* mutants, but not the seed coat PA-deficient phenotype [25]. It has therefore been proposed that AN9 is only involved in anthocyanin binding and transport, whereas TT19 is involved in both anthocyanin and PA transport [25].

ATP binding cassette (ABC) transporters

An increasing body of genetic, biochemical and molecular biological evidence implicates ABC and multidrug and toxic compound extrusion (MATE) transporters in the transport of plant secondary metabolites, including flavonoids [39–41]. However, a full understanding of the molecular mechanisms of flavonoid transport by membrane transporters is only just beginning to emerge.

The ABC transporter family is both large and ubiquitous. The unique molecular structures of these proteins enable them to transport a range of substrates across membranes using the energy from ATP hydrolysis [42] (Figure 2). The *Arabidopsis*, rice (*Oryza sativa*), and *M. truncatula* genomes each have >120 putative ABC transporters, but only a few have been characterized. These show diverse transport activities toward metal ions, auxin, malate, defensive secondary metabolites, or xenobiotics [41]; specific examples include AtWBC12 (cutin and wax secretion) [43], AtPDR8 (pleiotropic drug resistance, exporting toxic materials to inhibit pathogen penetration) [44], AtMRP1 and 2 (glutathione conjugated and non-conjugated chlorophyll metabolites) [45,46], tobacco plasma membrane PDR-type ABC transporter (secretion of the antifungal terpenoid sclareol) [47] and Japanese gold-

thread (*Coptis japonica*) CjMDR1 (berberine alkaloid import) [48].

To date, only MRP-type ABC transporters have been implicated in flavonoid transport. Suppression of the maize tonoplast-localized ABC-type transporter ZmMRP3 results in reduced anthocyanin accumulation [39], although anthocyanin transport activity of ZmMRP3 has yet to be shown. It will be interesting to know if it can transport GST-anthocyanin conjugates. Plasma membrane ABC transporter-mediated secretion of the isoflavone genistein has been reported in soybean roots, but the gene and protein responsible are yet to be identified [49]. On the basis of inhibitor studies, MRP-type ABC transporters have also been implicated in the long-distance transport of flavonoids such as naringenin, dihydrokaempferol and dihydroquercetin via unidirectional cell–cell movement between root and shoot. This work requires additional experimental validation, but provides a first indication that ABC transporters participate in flavonoid distribution at the cell-type, tissue and organ levels [50].

MATE transporters

MATE transporters comprise a large family of transporters that are widely distributed in all living organisms, from prokaryotes to eukaryotes [51]. As secondary transporters, MATE transporters use electrochemical gradients of protons or sodium ions that are created by proton pumps, such as plasma membrane P-type ATPases, vacuolar V-type ATPases, or V-pyrophosphatase [52]. Plant MATE transporters are involved in the detoxification of xenobiotics and in the transport of a wide range of metabolites, such as cations, organic acids and secondary metabolites [53]; plant genomes therefore encode large MATE transporter families with ∼60 members [53,54].

Several *Arabidopsis* MATE transporters have been characterized. These include AtALF5 (exports toxic cations such as tetramethylammonium) [55] and AtDTX1 (confers norfloxacin, berberine and cadmium tolerance when expressed in *Escherichia coli*) [56]. The plasma membrane MATE transporter SbMATE from sorghum [57] exports citrate and confers aluminum tolerance. A berberine/proton antiporter located on the vacuolar membrane has been characterized from Japanese goldthread [58], and JAT1 is a jasmonate-inducible alkaloid transporter that can transport nicotine into the vacuole in the aerial parts of tobacco [59,60].

PA deficiency in seeds of the *Arabidopsis tt12* mutant is caused by loss of function of a MATE transporter, TT12 [61], which mediates vacuolar sequestration of cyanidin 3-O-glucoside and is localized to the tonoplast [40]. It is now known that TT12 and its ortholog MATE1 from *M. truncatula* expressed in yeast preferentially transport epicatechin 3'-O-glucoside, which is proposed to be a precursor for the biosynthesis of PAs [54] (Figure 2). This explains the *tt12* seed phenotype, and similar PA transport mechanisms could be applicable in other species. Recently, two MATE-type transporters from grapevine (*Vitis vinifera*), AM1 and AM3, were characterized as vacuolar transporters of acylated anthocyanins [62], and the tomato (*Solanum lycopersicon*) MATE transporter LaMTP77 might have a similar function [63].
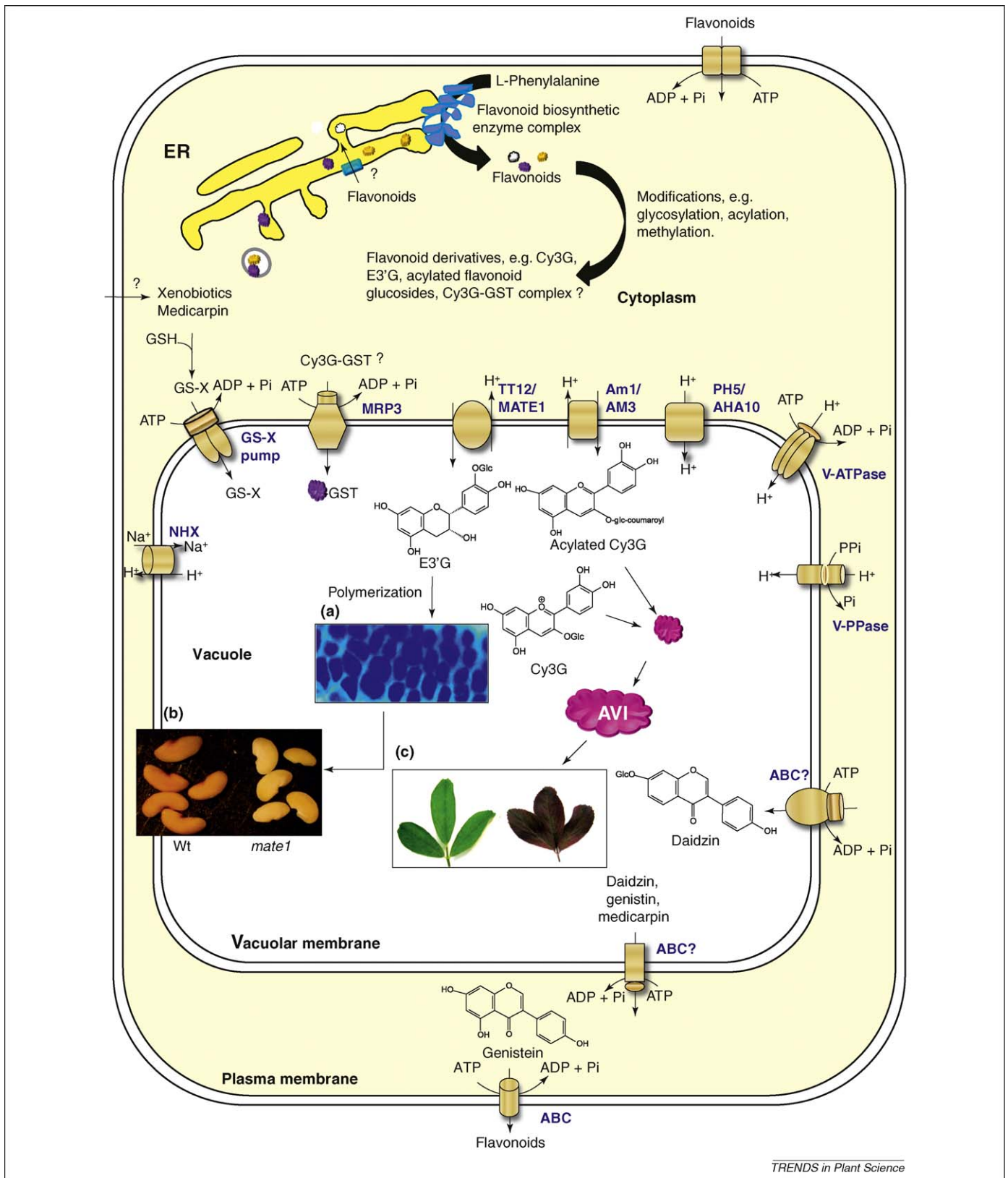
Figure 2. Schemes for membrane transporter-mediated flavonoid transport. Flavonoids are synthesized on the cytosolic face of the ER membranes by the action of biosynthetic enzyme complexes. Flavonoid aglycones are then subjected to various modifications, such as glycosylation and acylation. The vacuolar membrane MATE transporters TT12 from *Arabidopsis* and MATE1 from *Medicago truncatula* preferentially transport epicatechin 3′-*O*-glucoside (E3′G) into the vacuole for PA biosynthesis. The grapevine MATE transporters AM1 and AM3 specifically transport p-coumaroyl modified anthocyanins, such as cyanidin 3-*O*-glucoside (Cy3G), into the vacuole. MATE transporters are driven by a proton gradient that is established by the action of a V-ATPase (such as the petunia vacuolar H⁺-ATPase PH5 and possibly *Arabidopsis* AHA10) or a V-PPase. By contrast, the acidic vacuolar lumen can be neutralized by an H⁺/Na⁺ exchanger (NHX). The maize vacuolar ABC transporter MRP3 is involved in sequestration of anthocyanins into the vacuole, but its exact substrate is not yet known; it might transport an anthocyanin–GST complex. Foreign flavonoids can also be detoxified by GSH conjugation and the conjugates then transported into the vacuole by the MRP-type GS-X pump, as is the isoflavonoid phytoalexin medicarpin. Isoflavonoid glucosides such as daidzin and genistin can be transported into the vacuole by an ABC transporter, and the isoflavone aglycone genistein is transported into

Role of H+-ATPase in flavonoid transport

Given that MATE transporters are secondary transporters that use an electrochemical gradient (usually H+ in plants) as the driving force to transport other substrates across membranes, their function and activity largely depend on various types of H+-ATPases. P-type H+-ATPase can provide and maintain the H+ gradient across the plasma membrane, whereas V-type H+-ATPase or vacuolar pyrophosphatase (V-PPase) proton pumps provide and maintain H+-gradients across the vacuolar membrane [27,52] (Figure 2). Mutation of these essential enzymes should result in *tt* phenotypes or flower color changes (because anthocyanin content/composition and vacuolar pH largely determine flower color). Indeed, knockout of the P-type H+-ATPase AHA10 from *Arabidopsis* results in a *tt* seed phenotype and reduces PA content [26]. Mutation of the vacuolar membrane-localized H+-ATPase PH5 from petunia alters the pH of the vacuolar lumen and thereby changes flower color and reduces seed PA content [27]. Mutations in the petunia *AN1* and *PH4* genes also affect the pH of petal homogenates, alter flower color and reduce anthocyanin or PA production [64], further confirming that destroying the proton gradient across the tonoplast can affect MATE transporter activity for vacuolar sequestration of anthocyanins and PAs [27]. Seed coat endothelial cells in the *Arabidopsis aha10* and *tt12* mutants contain many small vacuoles instead of one large central vacuole [26,40]. Although PH5 is localized to the tonoplast [27], the exact localization of AHA10 is unclear; it was originally predicted to be a plasma membrane protein [26], but this appears inconsistent with its knock-out phenotype.

Induction of anthocyanin biosynthesis in *Medicago* spp. expressing the LAP1 MYB transcription factor is associated with the coordinated up-regulation of anthocyanin modification enzymes (glycosyltransferases and acyl transferases), a MATE-like antiporter, a Na+/H+ antiporter, and two putative ABC transporters [34]. Some or all of these molecules might be associated with vacuolar sequestration of anthocyanins.

Chemical requirements for directional flavonoid transport

Flavonoids are often subjected to multiple modifications upon biosynthesis, such as glycosylation, hydroxylation, acylation (including aromatic acylation and acylation of the attached sugar residue) and methylation. These modifications are important for increasing their solubility or stability and reducing toxicity; downregulation of cyanidin 3-*O*-glucosyltransferases, such as maize BZ1, *Arabidopsis* ANL1 or *Medicago* UGT78G1 strongly decreases anthocyanin accumulation [22,34]. Acylation of anthocyanin or apigenin 7-*O*-(6-*O*-malonylglucoside) is a prerequisite for their uptake [62], but glycosylation alone is sufficient to enable the vacuolar uptake of many flavonoids [40,54,65–69].

Some MATE transporters exhibit relatively strict substrate specificity. For example, both TT12 and MATE1 transport epicatechin 3'-*O*-glucoside with higher affinity than cyanidin 3-*O*-glucoside in yeast, and neither transports catechin 3-*O*-glucoside, flavonol glucosides such as quercetin-3-*O*-glucoside, or flavonoid aglycones [40,54]. Cyanidin 3-*O*-glucoside requires acylation before it can be transported into the vacuole by grapevine AM1 and AM3 [62]. Although flavonoid aglycones might not be suitable substrates for MATE transporters, a MATE transporter from tobacco (*Nicotiana tabacum*) can transport non-modified nicotine directly into the vacuole [59,60]; other MATE transporters, such as AtDTX1, and ABC transporters such as CjMDR1, show a relatively broad substrate spectrum [48,56].

It is not understood how plants choose between ATP-hydrolysis-dependent or H+/ Na+-gradient dependent mechanisms for transport of native metabolites or xenobiotics. The conjugation ligands, glucose or glutathione, appear to determine the transport mechanism for vacuolar uptake of herbicides [68]. The same herbicide, if conjugated with glucose, can be taken up by a proton-gradient dependent antiporter, whereas its glutathione conjugate is taken up by an ATP-energized ABC transporter. The native flavonoids saponarin (apigenin 6-*C*-glucosyl-7-*O*-glucoside) and isovitexin (apigenin 6-*C*-glucoside) are transported into the vacuole by a secondary energized proton antiporter in barley (*Hordeum vulgare*) [65,67]. However, *Arabidopsis* takes up saponarin, a foreign compound, into the vacuole using an ABC transporter [65,67]. In Japanese goldthread and meadow rue (*Thalictrum minus*) cell cultures, both ABC transporters and proton antiporters mediate the transport of berberine into the cell or vacuole, as well as out of the cell [48,58,70].

Efflux of vacuolar flavonoids

Import and export of natural products across the tonoplast or plasma membrane can be carried out by different mechanisms. For example, the import of natural products or xenobiotics into the vacuole usually occurs with conjugated forms, whereas export of the same compound does not seem to require conjugation.

How vacuolar uptake and efflux of the same flavonoid compound are controlled is a particularly interesting question, and is well exemplified by the case of the isoflavone conjugates in legume cell cultures. Chickpea (*Cicer arietinum*) cell suspension cultures constitutively accumulate isoflavone malonyl glucosides in the central vacuoles [71,72]. It is assumed that glycosylation is essential for vacuolar uptake, and that malonylation of the sugar group somehow acts to maintain the conjugate in the vacuole. Treatment of the cultures with a fungal elicitor preparation resulted in accumulation of pterocarpan phytoalexins derived from the isoflavonoid pathway; these were synthesized *de novo*, and did not utilize vacuolar stores of isoflavonoid pathway intermediates [71]. However, if the

the soybean apoplast by another ABC-type transporter. Inset pictures show **(a)** PA (blue staining) in the vacuoles in a cross-section of the *Medicago* seed coat; **(b)** *Medicago* MATE1 mutant (*mate1*) seeds with a *TRANSPARENT TESTA* phenotype as compared with wild-type (wt); and **(c)** leaves of nontransgenic alfalfa (green) and transgenic alfalfa expressing the MtLAP1 MYB transcription factor (purple), thereby overproducing a range of anthocyanins, including Cy3G. See main text for further details.

cells were treated with a fungal elicitor plus an inhibitor of phenylalanine ammonia-lyase, the first enzyme of the phenylpropanoid/(iso)flavonoid pathway, pterocarpan phytoalexin was still formed, but was derived from the mobilization of vacuolar isoflavone intermediates. The conversion of isoflavone aglycones to pterocarpans is believed to occur in the cytoplasm. Thus, when cells sense a requirement for isoflavone precursors that cannot be provided through *de novo* synthesis, vacuolar efflux is activated. This is associated with expression of glucosyl hydrolase genes [13]. The localization of these enzymes is yet to be definitively determined, so it is unclear whether a conjugate or free aglycone is the substrate for the vacuolar efflux transporter. Neither is the nature of the efflux transporter known, although isoflavone efflux across the plasma membrane occurs via an ABC-type transporter in soybean [49] (Figure 2), and methyl jasmonate-induced accumulation of medicarpin in cell suspensions of *M. truncatula* in the absence of *de novo* synthesis is accompanied by parallel depletion of vacuolar isoflavone glycoside pools and upregulation of several ABC transporter genes [13]. It also remains to be determined how the cell senses flux through the isoflavonoid pathway to determine whether to activate influx or efflux systems. Similar sensing mechanisms are likely to operate to determine the directionality of transport of other flavonoid compounds.

Vesicle-mediated efflux of vacuolar metabolites, including flavonoids, has also been proposed [5]. It has also been suggested that SNARE protein-mediated exocytosis pathways, or nondirectional secretion of cargo containing antimicrobials, are involved in the efflux of antimicrobial flavonoids into the apoplastic space, but there is as yet no direct evidence in support of this hypothesis [73].

Co-migration of flavonoids and their transporters with trafficking vesicles?

There are many pieces to the puzzle of flavonoid transport. As described above, credible evidence exists for at least three mechanisms (GST-linked, vesicle trafficking and MATE transporters, with the least equivocal evidence being available for the latter) for vacuolar uptake of flavonoids. Knockout of proteins involved in transport of PAs, such as MATE1, TT12, TT19, AHA10, or PH5, still results in a detectable level of PAs in the seed coat [25–27,40,54], suggesting that redundant mechanisms are involved in PA sequestration into the vacuole.

Can these three apparently independent mechanisms occur in parallel in a single cell type, or can the data be viewed in a more reductionist manner? Some commentators have questioned the tonoplastic localization of the MATE transporters and P-type H^+-ATPase involved in anthocyanin/PA trafficking, citing technical difficulties with cell-type specific localization of GFP-labeled transporters [74] and the possibility for dynamic movement (cycling) and/or multiple localizations of these transporters [75,76]. In the case of PAs, no enzyme has yet been implicated in their oligomerization, and chemical studies suggest non-enzymatic oligomerization at low pH. Could this low pH be provided by a vesicle-localized ATPase, such that the PAs are already undergoing oligomerization in vesicles before delivery to the vacuole? Do MATE trans-

porters function to load flavonoid conjugates into vesicles rather than directly into the vacuole? Furthermore, do these flavonoid transporters or transport-related membrane proteins co-migrate with flavonoids in trafficking vesicles?

The defective tapetosome-mediated flavonoid transport to the pollen surface in *Arabidopsis tt12* and *tt19* mutants suggests that TT12 and/or TT19 are involved in the loading of flavonoids into ER-derived tapetosomes [17]. Membrane transporters, including tonoplast targeted transporters, are themselves believed to be targeted to their destination membranes via vesicle trafficking after their synthesis on the rough ER [29,30,75,77]. The vacuolar membrane transporters contain vacuolar-sorting determinants in their sequences. Therefore, they could also carry out their transport functions on ER-derived vesicles during vesicle trafficking, if ATPase provided a proton gradient to these vesicles. Therefore, H^+-ATPases such as AHA10/PH5 and MATE transporters such as TT12/MATE1 might be functionally coupled during vesicle trafficking to the vacuole, together with flavonoids. However, this interesting hypothesis requires experimental support.

Completing the puzzle

Most of our current knowledge of flavonoid transport is related to vacuolar sequestration. Mechanisms for efflux of flavonoids from the vacuole and plant cell, uptake into the plant cell, or transport into the nucleus, chloroplast, or subcellular vesicle compartments are poorly understood, although such transport might have essential roles in plant development, growth, reproduction and defense. The question of how cells determine whether a compound is destined for influx (to the vacuole) or efflux cannot be conclusively answered until more is known about efflux transporters for secondary metabolites. In one probable model, metabolite levels in specific cell compartments are first sensed, and this activates a signal transduction pathway to regulate target transporter gene expression. Many transporter genes, such as those encoding ABC transporters, are highly regulated by various stress signals [13,44,47].

Although many questions still remain, recently developed genomics tools promise more rapid progress than seen to date. New informatics methods are facilitating prediction of transporter functions from genome sequence and microarray co-expression data [78], the discovery of the transcription factors that activate whole pathways of flavonoid biosynthesis and transport [79,80] provides an additional tool for candidate gene discovery, mutant resources are becoming available in multiple genetically tractable model systems (e.g. Ref. [81]), and combined biochemical, cell biological and genetic analysis of plant transporters is becoming routine and provides more significant insights into their functions [36,50]. However, the major challenges are likely to be in the area of cell biology, where real-time whole-cell imaging techniques will be needed to catch flavonoids 'in the act'.

Increasing the biosynthesis of flavonoids in plants is often limited by complex and yet poorly understood feedback inhibition effects. Membrane transporters might have important roles in regulating metabolic flux. Therefore, the

study of flavonoid transport will not only fill a major gap in current understanding of the complete picture of flavonoid metabolism, but will also benefit the metabolic engineering of useful flavonoid compounds in crops for improving agronomic traits and the nutritional quality of foods.

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