Review

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Flavonoid transport mechanisms: how to go, and with whom

Jian Zhao

National Key Laboratory of Crop Genetic Improvement, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

Subcellular flavonoid transport and its underlying regulatory mechanisms are still poorly understood, but are fascinating research frontiers in plant science. Recent studies support and further extend previous hypotheses indicating that vacuolar sequestration of flavonoids involves vesicle trafficking, membrane transporters, and glutathione S-transferase (GST). However, the question remains to be addressed of how three distinct but nonexclusive mechanisms are functionally integrated into diverse but redundant transport routes for vacuolar sequestration or extracellular secretion of flavonoids. In this review, I highlight recent progress in understanding flavonoid-transporting vesicle behavior and properties, GST and membrane transporter functions and mechanisms, and flavonoid transport substrate specificity and preference.

Flavonoid transport

Flavonoids have evolved in plants since plants colonized the land and play roles in diverse physiological processes. They are synthesized in the cytosol and transported into the vacuole for storage or to other destinations, where they can function as bioactive molecules. The different sites of flavonoid biosynthesis, storage, and function require efficient transport mechanisms for flavonoids to fulfill their biological roles. However, evidence for the subcellular distribution, transport, and storage of flavonoids has only recently emerged [1,2]. Three distinct but potentially nonexclusive mechanisms for flavonoid transport in plant cells have been proposed: vesicle trafficking, membrane transporter, and GST mediated [2]. Over the past 5 years, significant progress has been made in understanding the mechanisms for the vacuolar sequestration of flavonoids, including the functional characterization of new flavonoid transporters with novel biochemical properties and *in planta* biological functions [3–5]. Long-standing questions regarding the mechanisms by which ATP-binding cassette (ABC) transporters and GSTs function in flavonoid transport have been partly addressed [5–8]. Furthermore, GREEN FLUORESCENT SEED9 (GFS9) was characterized as the first component connecting transport of proanthocyanidins (PAs) with vesicle trafficking [9]. While these results continue to support the previously proposed hypotheses that GST-, transporter-, and vesicle trafficking-mediated flavonoid transports are not exclusive, but collaborative and well integrated [2], they have also raised intriguing new questions. In this review, I highlight these recent discoveries and re-examine newly raised questions, to clarify the mechanism of flavonoid transport from a broad contextual viewpoint to guide future research.

ABCC transporters co-transport flavonoids and glutathione

It has been suggested that ABC transporters participate in the vacuolar sequestration and/or secretion of flavonoids out of the cell [10–13]. They have also been implicated in the transport of isoflavonoids to the extracellular space for plant–microbe or symbiotic interactions in legumes, although the ABC transporters responsible for isoflavonoid trafficking between the cytoplasm, vacuole, and the apoplast are largely unknown [2,14–16] (Table 1). Genetic studies have shown that multidrug resistance-associated protein (MRP)/C-type of ABC (ABCC) transporters, such as maize MRP1, are involved in anthocyanin accumulation, with the assumption that they transport flavonoid conjugates with glutathione (GSH) [13,17]. GSTs from various plants, such as petunia (*Petunia hybrida*) ANTHOCYANIN 9 (AN9), maize (*Zea mays*) BRONZE 2 (BZ2), and Arabidopsis TRANSPARENT TESTA (TT) 19, are also essential for anthocyanin and PA accumulation [8,17,18] (Table 1). However, these GSTs do not catalyze flavonoid–GSH conjugation, and there is also no evidence for ABC-mediated transport of flavonoid–GSH conjugates [2,18]. Thus, how ABC transporters and GSTs are involved in flavonoid production has been a long-standing question. In mammalian cells, MRP transporters are involved in multidrug resistance of cancer cells by co-transporting drugs out of cells or into organelles with GSH [19]. Only recently was it shown that free GSH is strictly required for transport of an anthocyanin, malvidin 3-*O*-glucoside (M3G), into yeast vacuoles by grapevine *(Vitis vinifera)* ABCC1 [5]. *In vitro* assays demonstrated that, although ABCC1 mediated simultaneous vacuolar sequestration of both M3G and GSH, neither structural alterations of M3G nor formation of a GSH–M3G conjugate were detected during the transport process [5]. Therefore, grapevine ABCC1 is a GSH-dependent anthocyanin transporter, rather than an anthocyanin–GSH transporter.

Corresponding author: Zhao, J. (jianzhao@mail.hzau.edu.cn).
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Table 1. Transporters, GSTs, and vesicle-trafficking proteins involved in flavonoid transport[a]

Transport proteins		Plant species	Localization	Flavonoid substrates	Refs
ABC transporter	ZmMRP3	Maize (*Zea mays*)	Tonoplast	Anthocyanins	[13]
	VvABCC1	Grapevine (*Vitis vinifera*)	Tonoplast	Anthocyanins	[5]
	MtABCG10	Barrel medic (*Medicago truncatula*)	Plasma membrane	Isoflavonoids	[16]
MATE transporter	TT12	*Arabidopsis thaliana*	Tonoplast	PAs or anthocyanins	[29]
	MtMATE1	Barrel medic (*M. truncatula*)	Tonoplast/small vesicles	PAs or anthocyanins	[27]
	MtMATE2	Barrel medic (*M. truncatula*)	Tonoplast/small vesicles	Anthocyanins	[3]
	AM1	Grapevine (*V. vinifera*)	Tonoplast/small vesicles	Anthocyanins	[26]
	AM3	Grapevine (*V. vinifera*)	Tonoplast/small vesicles	Anthocyanins	[26]
	VvMATE1	Grapevine (*V. vinifera*)	Tonoplast	N.D.	[28]
	VvMATE2	Grapevine (*V. vinifera*)	Golgi complex	N.D.	[28]
GST	VvGST1	Grapevine (*V. vinifera*)	N.D.	PAs	[37]
	VvGST4	Grapevine (*V. vinifera*)	Membrane associated	Anthocyanins	[4,37]
	TT19	*A. thaliana*	Membrane associated	Anthocyanins, PAs, or flavonols	[8,22,31]
	AN9	Petunia (*Petunia hybrida*)	Membrane associated	Anthocyanins or PAs	[18]
	BZ2	Maize (*Z. mays*)	Membrane associated	Anthocyanins	[17]
	CkmGST3	Cyclamen (*Cyclamen* spp.)	N.D.	Anthocyanins	[60]
	PfGST1	Perilla (*Perilla frutescens*)	N.D.	Anthocyanins	[61]
	Fl3	Carnation (*Dianthus caryophyllus*)	N.D.	Anthocyanins	[62]
	TaGSTL1	Wheat (*Triticum aestivum*)	N.D.	Flavonols	[25]
Vesicle trafficking	GFS9	*A. thaliana*	Golgi complex	PAs	[9]
H+-ATPase	AHA10	*A. thaliana*	Tonoplast	PAs	[23,47]
	PH5	Petunia (*P. hybrida*)	Tonoplast	Protons	[63]

[a]Abbreviation: N.D., not determined.

The mechanism by which GSH is co-transported with M3G by grapevine ABCC1 remains unclear. GSH is well known for its primary role in redox homeostasis and for acting as an elicitor in stimulating flavonoid accumulation in plant cells [20,21]. A GSH-deficient mutant of *Arabidopsis thaliana*, *gsh2*, displayed defective secretory trafficking with the appearance of small vacuole-like vesicles and disappearance of the large vacuole [20]. These phenotypes are similar to those observed in *TT19*-, *Arabidopsis* H^+-*ATPase 10* (*AHA10*)-, and *GFS9*-deficient mutants, which are defective in flavonoid transport [8,9,20,22,23]. However, whether *gsh2* is also defective in flavonoid transport or is related to GSH-dependent anthocyanin transport has not yet been reported.

Subcellular localization of GSTs for flavonoid transport

GSTs that are involved in flavonoid production may not catalyze flavonoid glutathionation, but rather physically bind to flavonoids and act as flavonoid carrier proteins that facilitate flavonoid transport from the cytoplasm into the vacuole [2,6–8,24,25]. The first priority in verifying this hypothesis is to define the dynamic association of GST with flavonoids and their subcellular localizations. Using a GST–GFP fusion protein and other nondestructive cell-imaging techniques, *Arabidopsis* TT19 and GSTs from other plants were observed to be localized in the cytoplasm in immature seed coats that accumulate higher levels of PAs than of anthocyanins [6–8]. GSTs were also associated with membranes, likely the endoplasmic reticulum (ER) and the vacuole, in plant cells that produce high levels of anthocyanins [4,7,24]. The discrepancies in reported GST localization might be due to the use of different tissue types containing varying flavonoids or data interpretation. The latter is an issue because the cytosol in plants cells usually occupies a small volume compared with the central vacuole; thus, most of the cytosol is found in close association with other organelles. Conversely, the subcellular localization of GSTs may depend on the presence of anthocyanins or PAs in plant tissues. For example, a flavonoid-binding AtGSTF2 is localized near the plasma membrane (PM), but the localization is disrupted in a flavonoid-deficient *tt4* mutant [24]. Consistent with their association with membranes, VvGST and TT19 are required to transport flavonoids from small vesicles to the central vacuole [4,8]. Flavonoids and GSTs such as BZ2 can loosely associate with membranes [2,24,25]. GST may form a dynamic complex with flavonoids and vesicles for flavonoid transport [2,4,7,8,24,25]. One hypothesis is that the GST binding of GSH and flavonoids may provide ABCCs with both substrates for their co-transport [5,19] (Figures 1 and 2).

Flavonoid-dependent localization of flavonoid transporters

Flavonoid accumulation-dependent subcellular localization may be a feature of flavonoid transporters, such as grapevine multidrug and toxic compound extrusion (MATE)-type anthocyanin transporters anthoMATE1 (AM1) and AM3, VvMATE1 and 2, barrel medic (*Medicago truncatula*) MtMATE1 and 2, and *Arabidopsis* MATE transporter TT12 [3,4,26–29] (Table 1).

Hairy roots expressing AM1 or AM3 antisense constructs did not display anthocyanin-filled small vesicles, suggesting that *AM1*/*AM3* transporters were responsible for transporting acylated anthocyanins from the cytosol into the small vesicles [4,26]. AM1– and AM3–GFP were detected primarily in the tonoplast, with fewer signals in vesicles around the nucleus in hairy root lines synthesizing only low levels of anthocyanins [26]. However, AM1– and AM3–GFP mainly localized to small prevacuole-like vesicles actively moving alongside the central vacuoles in hairy