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A Trafficking Pathway for Anthocyanins Overlaps with the Endoplasmic Reticulum-to-Vacuole Protein Sorting Route in *Arabidopsis* and Contributes to the Formation of Vacuolar Inclusions

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ABSTRACT

Plants produce a very large number of specialized compounds that must be transported from their site of synthesis to the sites of storage or disposal. Anthocyanin accumulation has provided a powerful system to elucidate the molecular and cellular mechanisms associated with the intra-cellular trafficking of phytochemicals. Benefiting from the unique fluorescent properties of anthocyanins, we show here that, in Arabidopsis, one route for anthocyanin transport to the vacuole involves vesicle-like structures shared with components of the secretory pathway. By co-localizing the red fluorescence of the anthocyanins with GFP markers of the endomembrane system in Arabidopsis seedlings, we show that anthocyanins are also sequestered to the endoplasmic reticulum (ER) and to ER-derived vesicle-like structures targeted directly to the protein storage vacuole in a Golgi-independent manner. Moreover, our results indicate that the vacuolar accumulation of anthocyanins does not depend solely on glutathione S-transferase (GST) activity or ATP-dependent transport mechanisms. Indeed, we observed a dramatic increase of anthocyanin-filled sub-vacuolar structures, without a significant effect on total anthocyanin levels, when we inhibited GST activity, or the ATP-dependent transporters with vanadate, a general ATPase inhibitor. Taken together, these results provide evidence for an alternative novel mechanism of vesicular transport and vacuolar sequestration of anthocyanins in Arabidopsis.
INTRODUCTION

The accurate delivery and sequestration of chemically reactive and potentially toxic metabolites poses a significant challenge for plant cells, which can simultaneously accumulate hundreds of different phytochemicals, derived from both primary and secondary metabolism. Establishing the cellular and molecular mechanisms that participate in the trafficking of phytochemicals within and between plant cells poses an important biological problem, with significant implications for the engineering of plant metabolism.

Anthocyanins are one of the major classes of plant pigments and serve multiple eco-physiological functions (Grotewold, 2006). Anthocyanins are synthesized from the general phenylpropanoid pathway, by the action of a metabolon loosely associated with the cytoplasmic face of the endoplasmic reticulum (ER), and likely forming a multi-enzyme complex (Winkel-Shirley, 1999; Winkel, 2004). Once synthesized, anthocyanins accumulate in a large central vacuole; this localization being necessary to prevent oxidation (Marrs et al., 1995) and for anthocyanins to function as pigments. In vivo anthocyanin coloration is significantly affected by factors that influence vacuolar pH (Yoshida et al., 1995), the presence of co-pigments (Forkmann, 1991) and the formation of anthocyanic vacuolar inclusions (AVIs) (Markham et al., 2000). Thus, anthocyanins (or anthocyanin precursors) need to be transported from the cytoplasmic surface of the ER to the vacuole.

Over the past few years, several factors that affect the proper sequestration of anthocyanins have been identified. Perturbation in the modifications of the core anthocyanidin skeleton, required for uptake by the transporters, leads to accumulation of the flavonoid in the cytoplasm. In maize, impairment of the UDP-glucose:cyanidin 3-O-glucosyltransferase gene bronzel1 (BZ1) suppresses anthocyanin accumulation (Larson and Coe, 1977; Fedoroff et al., 1984). Mutations in the maize BZ2 gene, which encodes a glutathione S-transferase (GST), prevents the vacuolar localization of anthocyanins, and brown oxidation products accumulate (hence the Bronze2 name) (Marrs et al., 1995). Similarly, the Petunia AN9 gene encodes a GST and, despite the low identity between AN9 and BZ2, BZ2 complements AN9 mutants (Alfenito et al., 1998). Interestingly, the GST enzymatic activity of AN9 is not required for the AN9-dependent vacuolar sequestration of anthocyanins, suggesting that AN9/BZ2 serve as “ligandins”, most likely for the stabilization, but possibly also for escorting anthocyanins (e.g., cyanidin 3-glucoside, C3G) from the ER to the tonoplast (Mueller et al., 2000). The
identification of the maize tonoplast-localized Multidrug Resistance-Associate Protein, ZmMRP3, induced by the C1 and R anthocyanin regulators (Bruce et al., 2000), provides an additional player in a model involving carrier and transporter proteins in the trafficking of anthocyanins from the ER surface to the vacuole (Goodman et al., 2004). In Arabidopsis, TRANSPARENT TESTA 19 (TT19) mutations affect both anthocyanin accumulation in vegetative tissues and proanthocyanidin (PA) accumulation in seed coats. TT19 encodes a GST, and AN9 complements the anthocyanin but not the PA defect of the TT19 mutant (Kitamura et al., 2004). While TT19 and AN9/BZ2 may function similarly by stabilizing/escorting anthocyanins, the TT19 mutant has a distinctive phenotype in the seed coat, where PA precursors accumulate in cytoplasmic membrane-wrapped structures (Kitamura et al., 2004). This contrasts with the phenotype of mutations in the TT12 locus, encoding a MATE transporter involved in PA vacuolar sequestration, in which the PA precursors are evenly distributed in the cytoplasm (Debeaujon et al., 2001).

Plant cells contain at least two different types of vacuolar compartments (Paris et al., 1996), which are most often referred to as the lytic and the protein storage vacuoles (PSV). PSVs can be compound organelles, evidenced by the presence in tobacco seeds of a sub-vacuolar membrane-bound compartment containing organic acids and proteins (Jiang et al., 2001). The secretory pathway is responsible for the vacuolar transport of proteins through the interaction of specific sorting signals in the proteins and vacuolar sorting receptors. The major route of vacuolar protein transport is from the ER through the trans-Golgi network (TGN) complex, a route that is shared among all eukaryotes (Neumann et al., 2003; Vitale and Hinz, 2005). However, a direct trafficking route from the ER to the vacuole exists in plants, which was first identified for the transport of proteins targeted to the PSV by large vesicles known as precursor-accumulating (PAC) vesicles (Hara-Nishimura et al., 1998). Spindle-shaped ER bodies (Matsushima et al., 2003) provide additional possible vehicles for the transport of proteins, rubber or oil from the ER to the vacuole, by a mechanism resembling autophagy (Herman and Schmidt, 2004). Whether ER bodies are involved in the transport of PAs or anthocyanins from the ER to the vacuole remains unclear, but the localization of Arabidopsis flavonoid biosynthetic enzymes to large electron-dense cytoplasmic structures and to the tonoplast (Saslowsky and Winkel-Shirley, 2001) suggests that mechanisms other than cytoplasmic flavonoid carrier proteins are at play in the sub-cellular trafficking of anthocyanins.
Most significant in highlighting a vesicular transport for flavonoids is the recent description of the tapetosomes as ER-derived structures that store ER-derived flavonols for their delivery to the Brassica pollen surface upon tapetal cell death (Hsieh and Huang, 2007).

Taking advantage of unique red fluorescent and colored properties of anthocyanins, we describe here the co-localization of anthocyanins with vesicle-like structures containing a protein marker (GFP-Chi) for the protein storage vacuole in Arabidopsis. Consistent with a trans-Golgi network independent ER-to vacuole vesicular transport of anthocyanins, Brefeldin A (BFA), a Golgi-disturbing agent (Dinter and Berger, 1998) has no effect on the accumulation of anthocyanins, and the red fluorescent anthocyanins are detected in ER compartments identified by the GFP-HDEL marker. We describe the accumulation of anthocyanins in the vacuole in neutral red (NR)-staining sub-vacuolar compartments. In sharp departure from what has been observed in other plants, the treatment with ABC transport inhibitors does not significantly decrease the amount of anthocyanins. However, vanadate, a fairly general inhibitor of ATPases including ABC transporters, induces a dramatic increase of anthocyanin-filled sub-vacuolar structures. Our results indicate that Arabidopsis cells accumulating high levels of anthocyanins utilize components of the protein secretory trafficking pathway for the direct transport of anthocyanin pigments from the ER to the vacuole, and provide evidence for the existence of novel sub-vacuolar compartments for their storage.

RESULTS

Induction of Anthocyanin Accumulation in Arabidopsis Seedlings

To induce high anthocyanin levels in young seedling, we grow seeds for 2-3 days under high light conditions in plain liquid sucrose media without a nitrogen source (Anthocyanin Inductive Condition, see MATERIAL & METHODS). If tt5 seedlings are grown in similar conditions (Fig. 1A), no pigmentation is observed because of the absence of the chalcone isomerase enzyme encoded by the TT5 locus (Shirley et al., 1992). However, if the product of CHI, naringenin (50 - 200 µM), is added to the media, high levels of anthocyanins are observed (Fig. 1A) within 8-10 hours, reaching a maximum at about 24 hrs (see below). The addition of naringenin further increases (1.5-2 fold) the anthocyanin accumulation levels of wild type seedlings grown in Anthocyanin Inductive Conditions (Fig. 1B). In addition, because the inductive conditions suppress chlorophyll synthesis in the cotyledons, there was no background
color interference with the anthocyanins. These results indicate that the treatment of wild type or tt5 seedlings grown under Anthocyanin Inductive Conditions with naringenin provides a good system for high levels of anthocyanin production in *Arabidopsis*.

**Novel Fluorescent Properties of *Arabidopsis* Anthocyanins**

The fluorescence provided by the ring-stacking interaction of flavonol and flavone aglycones with diphenylboric acid (DPBA) has been utilized to investigate the localization of several flavonoids (Buer and Muday, 2004; Peer and Murphy, 2006; Vargo et al., 2006; Hsieh and Huang, 2007). However, DPBA does not fluoresce with anthocyanins, prompting us to seek another mean for the cytoplasmic visualization of these compounds.

To determine whether anthocyanins fluoresce in a spectral range that would allow the visualization of these compounds in the presence of GFP markers of the endomembrane trafficking system, we investigated the fluorescence properties of *Arabidopsis* anthocyanins. Mutant tt5 seedlings grown in Anthocyanin Inductive Conditions, in the absence of naringenin showed no fluorescence in the red channel when excited at 488 and 544 nm of the argon-ion and helium-neon lasers, respectively (Emission >565 nm, Fig. 5A). However, when incubated in the presence of naringenin, the tt5 seedlings displayed strong fluorescence in the red channel (Fig. 2A). Two mutants, tt6 and tt3, that block anthocyanin production downstream of the step catalyzed by TT5, were tested for the accumulation of fluorescence. The absence of red fluorescence in naringenin-treated tt6 (not shown) and tt3 seedlings (Fig. 2A) indicates that the fluorescence was not due to naringenin itself or to a metabolic by-product of naringenin, but rather a consequence of the presence of a flavonoid after the enzymatic step catalyzed by DFR. Leucocyanidin, however, showed no fluorescence (not shown). Similar red fluorescence was observed in wild type seedlings, grown in Anthocyanin Inductive Conditions, both in the presence or absence (not shown) of naringenin (Fig. 2A, Ler).

To demonstrate that the red fluorescence was due to the anthocyanidins/anthocyanins and not to another pathway intermediate, acid-hydrolyzed methanol extracts from wild type (Ler) and tt5 seedlings were separated on a cellulose TLC plate. As previously described (Dong et al., 2001), a single spot corresponding to cyanidin was observed which was absent in tt5 seedlings (Fig. S1A). Under the UV light (~254 nm), this spot fluoresces red. The cyanidin spot of the TLC plate was imaged using confocal laser scanning microscopy using the same
excitation and emission wavelengths as used for the microscopy of the seedlings. Cyanidin-loaded cellulose fluoresced red when excited at 488/544 nm and visualized using the long pass emission filter of 565LP. No fluorescence was observed using the 515-530 nm emission filter. The blank sample, a cellulose spot below the origin, did not fluoresce (Fig. S1B).

To conclusively prove that the red fluorescence observed during microscopy did come from the anthocyanin/anthocyanidin, we measured the emission spectra from the cyanidin spot isolated from the TLC plate using fluorescence spectrophotometry. The cyanidin spot was extracted from the cellulose plate using 95% ethanol. Absorption and fluorescence spectra (Fig. 2B) were obtained. The absorption maximum of cyanidin in ethanol was established to be 547 nm and the emission maxima was 595 nm, at the excitation wavelength of 544 nm. The 595 nm emission maxima was observed irrespectively of the excitation wavelength, which ranged from 280 to 544 nm (data not shown), with no peaks appearing in the 500 – 530 nm wavelengths, corresponding to the GFP emission spectrum. Finally, to confirm that the main red fluorescent compounds in cells expressing anthocyanins correspond to anthocyanins themselves, methanolic extracts of PAP1-D (Borevitz et al., 2000) plants were separated by reverse-phase HPLC and the absorption (530 nm) and fluorescence spectra (excitation at 540 nm and emission at 620 nm) were compared (Fig. 2C). The two spectra show a very good correspondence, indicating that all the major Arabidopsis anthocyanins (Tohge et al., 2005) fluoresce red. Together, these results conclusively prove that anthocyanins fluoresce in a range compatible with the utilization of GFP as a marker to follow sub-cellular trafficking pathways.

Anthocyanins Share a Golgi Independent, Vesicular Trafficking Pathway with Proteins Targeted to the PSV

The plant secretory system involves multiple pathways for the transport of proteins to the vacuole (Carter et al., 2004), and green fluorescent protein (GFP) fusion markers (Chalfie et al., 1994) permit to distinguish between them (Neuhaus, 2000; Di Sansebastiano et al., 2001). To establish whether the ER or ER bodies are a possible initial site of anthocyanin accumulation, as previously suggested for maize (Grotewold et al., 1998) and recently described for flavonols in Brassica and Arabidopsis tapetum cells (Hsieh and Huang, 2007), Arabidopsis seedlings transformed with GFP-HDEL (Haseloff et al., 1997), where HDEL correspond to an ER-retention signal sequence, were grown under Anthocyanin Inductive Conditions, with (+ N) or
without (-N) naringenin (Fig. 3). Intact seedlings (Fig. 3A - H) and protoplasts (Fig. 3I - P) were directly observed by confocal laser scanning microscopy. The GFP-HDEL marker provided green fluorescence to spindle-shaped ER bodies in the cotyledonal cells (Fig. 3A, I). After incubating the seedlings for 24 h with naringenin, the number of green fluorescent bodies increased and the bodies appeared more dilated (Fig. 3, compare A and E). The red channel showed the presence of red fluorescence occupying most of the cell, which correlated with the vacuolar pigmentation provided by the anthocyanins. In addition, red fluorescence was observed in spindle-shaped bodies, the number and size of which dramatically increased in seedlings treated with naringenin (Fig. 3, compare B and F). These spindle-like structures were clearly outside the large central vacuole, further evidenced by 3-D reconstructions from the confocal images (not shown). Observation under both the red and green channels showed the co-localization of the red and green fluorescence in the ER bodies, but not of the vacuolar anthocyanins (Fig. 3C, G, K, O). The very strong red fluorescence of the anthocyanins in the vacuole, which occupy more than 80% of the cellular volume in these cotyledon epidermal cells, made it difficult to establish whether there was any red anthocyanin fluorescence in the cytoplasm that was not associated with the GFP-HDEL marker. Interestingly however, not all the GFP-HDEL marker was found to be associated with the red fluorescence, suggesting either the presence of two populations of ER bodies, some filled with anthocyanins and others not, or that the levels of red fluorescence in green- but not red-fluorescing bodies was below the level of detection. Taken together, these findings indicate that at least a part of the red fluorescing anthocyanins co-localize with the GFP-HDEL marker in ER bodies.

To investigate the possible transport route of anthocyanins from the ER bodies to the vacuole, we utilized intact plants (Fig. 4A – D) or isolated protoplasts (Fig. 4E – H) of transgenic Arabidopsis lines expressing vacuolar sorting signals fused to GFP. The GFP-Chi marker, corresponding to a fusion of the green fluorescence protein to the C-terminal vacuolar sorting determinant (VSD) from the barley chitinase A protein, is targeted to pH-neutral protein storage vacuoles directly from the ER in a Golgi-independent manner (Di Sansebastiano et al., 1998, 2001; Fluckiger et al., 2003). Arabidopsis seedlings transgenic for 35S::GFP-Chi grown in Anthocyanin Inductive Conditions for 3 d show green fluorescence provided by GFP-Chi in discrete structures that could correspond to the endoplasmic reticulum and to small peripheral vacuoles (Fig. 4A). These seedlings accumulated anthocyanins in the epidermal cells of the
cotyledons in the form of uniform vacuolar red fluorescence and in discrete cytoplasmic structures (Fig. 4B, an example indicated by the arrow) that often co-localized with the green GFP fluorescence (Fig. 4A - C). Consistent with previous findings (Di Sansebastiano et al., 1998; Fluckiger et al., 2003) that showed that chloroplast-poor cells failed to accumulate GFP-Chi in the large central vacuole, we did not observe a co-localization of red- and green fluorescence in the central vacuole (Fig. 4C, G). However, this could also be a consequence of the more acidic pH of the vacuole affecting the GFP fluorescence, and not necessarily that the GFP-Chi doesn’t accumulate there. Similarly to what we observed for GFP-HDEL (Fig. 3), not all the small structures that accumulated GFP-Chi accumulated fluorescent anthocyanins.

δ-TIP was previously shown to localize to vegetative storage protein- and pigment-accumulating vacuoles (Jauh et al., 1999). Consistent with this, we observed that δ-TIP-marked vacuoles accumulated anthocyanins as seen in the co-localization of red anthocyanin fluorescence in vacuoles with δ-TIP-GFP in epidermal cells (Fig. 4I – L), and protoplasts (Fig. 4Q – T).

To explore whether anthocyanins would also co-localize with components of the secretory pathway that utilize the trans-Golgi network for transport from the ER to the vacuole, we utilized Arabidopsis lines expressing an N-terminal vacuolar sorting determinant from the barley aleurain fused to GFP (Ale-GFP) (Di Sansebastiano et al., 2001). Epidermal cells of 35S::Ale-GFP expressing seedlings grown under anthocyanin inductive conditions (Fig. 4M - P) showed small green fluorescent bodies, likely corresponding to lytic vacuoles (Fluckiger et al., 2003), and smaller punctuated structures marked with Ale-GFP peripheral to the large central vacuole (Fig. 4M). No co-localization of Ale-GFP and red fluorescence was observed (Fig. 4O), suggesting that anthocyanins follow the direct ER to vacuole route, rather than going through the Golgi pathway.

To conclusively establish that the observed vesicular trafficking of anthocyanins did not involve the TGN, we investigated the effect of Brefeldin A (BFA), a Golgi-disturbing agent (Driouich et al., 1993; Satiat-Jeunemaitre et al., 1996), on the accumulation of anthocyanins and the formation of the AVIs. After incubating 2.5 days-old tt5 seedlings with BFA (10 mg/ml) for one hour, we added 100 µM naringenin, and measured the amount of anthocyanins that accumulated after 24 h. No difference was observed in the levels of anthocyanins when comparing BFA-treated and non-treated seedlings, nor did we observe any effect of BFA on the
formation of the AVIs (Fig. 5A, yellow line). Consistent with the BFA treatment affecting the TGN-dependent transport, and providing evidence that BFA was effective in disturbing the TGN under the conditions tested, we observed that the green fluorescence furnished by Ale-GFP was significantly different after BFA treatment, indicating a likely retention in ER-like structures (Fig. S2). In addition, the protein sorting inhibitor Sortin 1, which interferes with the TGN-dependent vacuolar transport of proteins (Zouhar et al., 2004), had no effect on the ability of tt5 seedlings grown under Anthocyanin Inductive Conditions to accumulate pigments or form AVIs when complemented with naringenin (not shown). Taken together, these results indicate that anthocyanins can utilize a TGN-independent vesicular transport from the ER to the vacuole that at least in part overlaps with protein trafficking pathways to the protein storage vacuole.

**Anthocyanin Accumulating Sub-Vacuolar Structures in Arabidopsis**

The normally low anthocyanin pigment accumulation of Arabidopsis vegetative green tissues is dramatically enhanced in PAP1-D plants, resulting from the over-expression of the PAP1 R2R3-MYB anthocyanin regulator (Borevitz et al., 2000; Tohge et al., 2005). Yet, the PAP1-D pigmentation phenotype is usually not observed until plants are 2-3 weeks old. The microscopic observation of pigmented tissues in the PAP1-D plants revealed, in a fraction of the pigmented epidermal cells, the presence of small anthocyanin inclusions that appeared as rounded spherical structures, apparently within the large central vacuole (Fig. 6A, PAP1-D). At a much lower frequency, similar structures were also observed in mature wild type Landsberg erecta (Ler) plants grown under high light conditions (Fig. 6B, Ler).

Neutral red (NR) provides a vital vacuolar stain which diffuses through membranes, but which is trapped in the acidic vacuolar compartment by protonation (Ehara et al., 1996; Di Sansebastiano et al., 1998). Staining of PAP1-D leaves with NR showed the presence of NR-staining bodies in over 70% of the epidermal cells. These NR-staining bodies were similar in shape and size to the anthocyanin inclusions, but were present in wild type in a larger number of cells (Fig. 6B, Ler). These NR-staining structures (but not anthocyanin inclusions) were also found, although at a lower frequency, in tt5 plants grown under either normal or Anthocyanin Inductive Conditions (Supplementary Fig. S3 and Fig. 6B, tt5).

To determine whether the anthocyanin inclusions were inside the vacuole or whether they corresponded to a separate NR-staining acidic compartment, vacuoles were isolated from PAP1-
plants (see MATERIAL & METHODS). The NR-staining and anthocyanin-accumulating bodies were always observed inside the large central vacuole (Fig. 6C, D), indicating that they most likely correspond to sub-vacuolar structures. For clarity purposes and to avoid introducing one additional name for these structures, we will refer to them here as anthocyanic vacuolar inclusions or AVIs. The anthocyanin pigmentation of the AVIs was more intense than in the rest of the vacuole (Fig. 6C), indicating that anthocyanins, although present in the vacuolar sap, were enriched in the AVIs. Similarly, NR was preferentially sequestered in these sub-vacuolar compartments, staining these structures darker than the surrounding vacuole (Fig. 6D). Taken together, these results demonstrate the presence in Arabidopsis of novel AVI-like structures that accumulate anthocyanins, and suggest that they are either more acidic than the rest of the vacuolar sap (and hence are likely membrane bound), that they contain compounds with affinity for NR [such as other phenolics (Stadelmann and Kinzel, 1972)], or a combination of both.

Participation of ABC Transporters and GSTs on AVI Formation

Vanadate significantly reduces anthocyanin accumulation in maize cells (Marrs et al., 1995). To investigate the effect of vanadate in the accumulation of anthocyanins and in the formation of AVIs in Arabidopsis, tt5 seedlings were grown in Anthocyanin Inductive Conditions for 2.5 days and treated with 1 mM vanadate one hour prior to the addition of 100 μM naringenin. While anthocyanins take longer to accumulate in the vanadate-treated seedlings compared to the untreated control (Fig. 5A), a delay explained by a reduced naringenin uptake in the first 15 hours (Fig. 5B), there was little difference in anthocyanin accumulation between vanadate-treated and control tt5 seedlings with naringenin after 24 hrs (Fig. 5A). However, when seedlings were observed under the microscope, a dramatic increase in the number of AVIs was noticed in vanadate-treated, compared to control seedlings (Fig. 7, compare A - B and C - D). Nearly every cell contained AVIs, clearly visible even in the absence of NR. The bathochromic shift of the AVIs (from purple-red to bluish, Fig. 7D) reflects the alkalinization of the vacuole, and the incubation of the vanadate-treated seedlings for a short time in diluted acid conditions rapidly restores a bright pink color to the vacuole (not shown). The addition of vanadate did not result in an immediate alkalinization of the media in which the seedlings were grown. In contrast, when vanadate was added to tt5 seedlings in the absence of naringenin, no significant difference in the number of sub-vacuolar structures staining with NR was observed (not shown),
suggesting that the observed increase in sub-vacuolar structures by vanadate is dependent on the presence of anthocyanins. From these results, we conclude that anthocyanins can accumulate in Arabidopsis even in the presence of inhibitors of ABC transporters, and that the inhibition of ABC transporters results in the increased number of AVIs, suggesting that their formation (or filling) does not require ATP-energized transporters.

A major function of plant ABC transporters, particularly from the multidrug resistance-associated protein family (MRP), is to pump conjugates of potentially toxic compounds with glutathione (GSH) to the vacuole (Klein et al., 2006). To establish the participation of GSH or GSTs in the accumulation of anthocyanins and in the formation of AVIs, we treated tt5 seedlings grown in Anthocyanin Inductive Conditions with 100 µM naringenin and with 1 mM buthionine sulfoximine (BSO), which depletes cellular GSH levels, or with 0.1 mM 1-chloro-2-4-dinitrobenzene (CDNB), a common GST substrate that saturates the enzymes, decreasing the activity on other substrates. Similarly as observed with vanadate, both treatments resulted in a significant increase in the accumulation of AVIs, but without the bathochromic shift (Fig 6E – H). Neither the CDNB nor the BSO treatments resulted in a significant effect in the total levels of anthocyanins (not shown). Taken together, these findings indicate that the inhibition of the synthesis or transport of glutathionated compounds to the vacuole results in an increase in the formation of AVIs without an obvious effect on total anthocyanin accumulation.

DISCUSSION

Despite the fundamental importance for plants to properly transport and sequester phytochemicals, little is known about the molecular and cellular mechanisms involved in these processes. Taking advantage of novel anthocyanin red auto-fluorescence properties in combination with protein markers for the secretory pathway, we describe here a TGN-independent ER to vacuole vesicular anthocyanin trafficking route shared with proteins targeted to the PSV. We also uncover the presence of novel Arabidopsis anthocyanin-accumulating sub-vacuolar structures that resemble the anthocyanoplasts/AVIs present in the pigmented tissues of many other plant species.

Establishing trafficking pathways for anthocyanins has been complicated by the fact that the color of the compounds depends on the proper conditions (pH and modifications) furnished by the vacuole. Anthocyanins extracts from red cabbage (Brassica oleracea) were previously
shown to fluoresce with peaks at 363nm, 434nm, and 519nm (Drabent et al., 1999). Our studies, however, identified significant fluorescence in vivo for total anthocyanins and for individual pigments above 565nm (Fig. 2), making this fluorescence compatible with the visualization of GFP. The difference in our results with those previously reported is likely a consequence of the red cabbage extracts containing complex mixtures of anthocyanins with other phenolics and proteins. Indeed, when the red cabbage extracts were subjected to a chromatographic separation, one of the peaks [peak 10, (Drabent et al., 1999)] displayed a significant fluorescence increase in the 550-650nm range (Drabent et al., 1999). Our results, demonstrating that anthocyanins can have fluorescence properties compatible with GFP visualization, pave the way for similar co-localization studies to be carried out in other plants. Auto-fluorescence provides a significant advantage over the use of flavonoid stains such as DPBA, as it can be visualized in vivo, without disturbing the cellular organization.

Taking advantage of the fluorescent properties of anthocyanins, we exposed a trafficking mechanism for these compounds from the ER to the vacuole that involves membrane-bound structures that initially contain the ER marker GFP-HDEL (Fig. 3). The shape and induction of these structures in GFP-HDEL-expressing plants make them likely candidates for being ER-bodies (Matsushima et al., 2003), which correspond to ER-derived cytoplasmic structures proposed to be transferred to the vacuole by mechanisms that include autophagy (Herman and Schmidt, 2004). We established that the red-fluorescing anthocyanins co-localized with the PSV targeted marker GFP-Chi (Fig. 4), which uses a TGN-independent ER-to-vacuole trafficking mechanism. The TGN-independent vesicular trafficking of anthocyanins was further confirmed by the observation that anthocyanin accumulation is insensitive to BFA (Fig. 5A), and that the red fluorescence did not co-localize with a marker (Ale-GFP) that utilizes a TGN-dependent pathway (Fig. 4). These results suggest that anthocyanins may “hitchhike” on the protein secretory pathway for transport from the ER to the tonoplast. It is however unclear whether the accumulation of anthocyanins in GFP-HDEL containing structures precedes their localization in the GFP-Chi vesicles, or whether these reflect two separate mechanisms by which anthocyanins can reach the vacuole in membrane-bound structures. However, the co-localization of the red-fluorescing anthocyanins with the GFP-Chi marker (Fig. 4), which did accumulate in the ER and in ER-derived structures (Fluckiger et al., 2003), highlights that the presence of the pigments in ER bodies was unlikely driven by the expression of GFP-HDEL, which sometimes results in ER
body formation, possibly because of ER-retention or retardation of the fusion proteins (Herman and Schmidt, 2004). The visualization of anthocyanins in the ER bodies could have been furnished by a higher concentration of the pigment in the dilated ER. Anthocyanin fluorescence was not detected in the thin reticulate cortical ER, which could be a consequence of either low signal, below the detection limits, or to anthocyanins accumulating in only specific domains of the ER. This latter possibility would be consistent with the apparent exclusion of anthocyanins from some of the ER bodies (Fig. 3).

In many plant species, anthocyanins accumulate in the vacuole in discrete structures described by a variety of names (Pecket and Small, 1980; Nozzolillo and Ishikura, 1988; Nozue et al., 1993; Kubo et al., 1995; Markham et al., 2000; Conn et al., 2003; Irani and Grotewold, 2005; Zhang et al., 2006). We found here that intra-vacuolar anthocyanin-accumulating inclusions are also present in Arabidopsis, particularly in cells induced to accumulate high anthocyanins levels, either as a consequence of the expression of the PAP1 regulator, or by the addition of the pathway intermediate, naringenin. These inclusions stained heavily with NR (Fig. 5), a vital dye that gets trapped by protonation into acidic compartments. Generally, the number of NR-staining intra-vacuolar bodies present was larger than the structures heavily pigmented with anthocyanin (Fig. 6B). This may indicate that NR stained all the AVIs, but only those with high levels of anthocyanins were visible in the absence of NR. Alternatively, there might be different types of subvacuolar structures, only some of them capable of accumulating anthocyanins. Interestingly, however, in vanadate-, BSO- or CDNB-treated seedlings, most of these sub-vacuolar structures were filled with anthocyanins (Fig. 7, see below). NR-staining structures were also found in plants lacking anthocyanins, such as for example tt5 mutants. This suggests that the formation of these structures may not be triggered by the accumulation of anthocyanins. More likely, once anthocyanins reach the vacuole, they enter pre-existing NR-staining bodies resulting in the characteristic coloration of the AVIs.

To investigate the possibility that an autophagic mechanism (Marty, 1978) is involved in the formation of the sub-vacuolar structures, we looked into whether a mutation in the ATG7 locus (atg7-1, in the Ws genetic background) affects the formation of the NR-staining structures or the formation of the AVIs. ATG7 encodes the Arabidopsis E1-like ATP-dependent activating enzyme required for autophagy (Doelling et al., 2002), previously known as APG7 (Klionsky et al., 2003). We could not detect any significant difference in the number of NR-staining sub-
vacuolar structures or AVIs (under Anthocyanin Inductive Conditions) between *atg7-1* and *Ws* (not shown). However, *Ws* seedlings accumulated less anthocyanins and had significantly lower number of AVIs, when compared to Ler or Col seedlings (not shown), indicating that natural variation among accessions influence the physiology of these sub-vacuolar compartments, something that needs to be taken into consideration when comparing mutants. To further eliminate a possible role of autophagy, we investigated the effect of 3-methyladenine (3-MA), a potent autophagy inhibitor in animal (Seglen and Gordon, 1982) and plant (Takatsuka et al., 2004) cells, on anthocyanin accumulation and AVI formation. 3-MA functions by inhibiting the PI3K enzyme, necessary for the nucleation of pre-autophagic structures (Thompson and Vierstra, 2005). The treatment of three day-old *tt5* seedlings grown in Anthocyanin Inductive Conditions with 10 mM 3-MA and 100 µM naringenin resulted in similar anthocyanin levels and number of AVIs as control *tt5* seedlings treated only naringenin (not shown), yet affected, as expected, the distribution of the GFP-Chi marker (Supplementary Fig. S3). These results led us to conclude that a classical autophagic mechanism is unlikely to be involved in the formation of the AVIs.

The existence of a vesicular-type transport of anthocyanins from the ER to the vacuole provides an alternative to models that involve AN9/BZ2-like GST carrier proteins and/or tonoplast transporters for the cytoplasmic and tonoplast trafficking of these compounds, respectively (Alfenito et al., 1998; Mueller et al., 2000; Mueller and Walbot, 2001; Goodman et al., 2004). Interestingly, while the *tt19* mutation completely abolishes anthocyanin and PA accumulation (Kitamura et al., 2004), perturbing the formation or vacuolar uptake of glutathione conjugates (GS-X) with CDNB or BSO, or inhibiting ABC transporters with vanadate increases the number of AVIs (Fig. 7), without a significant effect on anthocyanin accumulation (Fig. 5). This is in sharp contrast to what has been previously found in maize, where the vanadate treatment phenocopies the *bz2* mutation with respect to anthocyanin accumulation (Marrs et al., 1995), suggesting distinct mechanisms of action of TT19 and ATP-energized transport mechanisms. While *bz2* mutants accumulate brown pigments in the cytoplasm (Marrs et al., 1995), *tt19* mutants lack significant amount of pigments, even under Anthocyanin Inductive Conditions in the presence of 200 µM naringenin (not shown). Similarly, the petals of Petunia *an9* mutants are colorless (Mueller et al., 2000). These differences could suggest that distinct biochemical products result in maize, Petunia and *Arabidopsis* from the blockage in the
BZ2/AN9/TT19 steps. However, this possibility is unlikely, given that the proteins seem to be largely exchangeable between different plant species with regards to anthocyanin accumulation (Alfenito et al., 1998; Mueller et al., 2000; Larsen et al., 2003; Kitamura et al., 2004). Alternatively, it is possible that TT19 has additional functions than those proposed for BZ2. This could explain the ability of BZ2 to complement the anthocyanin deficiency phenotype of \textit{tt19} mutants, but not PAs, and the presence of vesicles filled with PA precursors in seed coat endothelial cells (Kitamura et al., 2004; Kitamura, 2006). Further highlighting an additional role of TT19, flavonoids could not be transported across the ER membrane to then form part of the tapetosomes in \textit{tt19} \textit{Arabidopsis} tapetum cells (Hsieh and Huang, 2007).

The results presented here provide a new perspective with regards to the ER-to vacuole trafficking and the vacuolar sequestration of anthocyanin pigments, and maybe of other vacuole-targeted phenolics compounds as well. While our results do not rule out the existence of other mechanisms for the transport of anthocyanins to the vacuole, such as the interplay of GSTs and tonoplast transporters (Goodman et al., 2004), they highlight the existence of a vesicular transport of anthocyanins with properties shared with the secretory pathway. Cellular, molecular and genetic tools are becoming increasingly available in \textit{Arabidopsis} to further dissect the mechanisms by which anthocyanins are transported and sequestered in the vacuole.

**MATERIAL & METHODS**

**Plant Materials and Growth Conditions**

GFP-HDEL (Haseloff, 1999), GFP-Chi (Di Sansebastiano et al., 1998), and Ale-GFP (Di Sansebastiano et al., 2001) were used as GFP-expressing lines. \textit{Arabidopsis} chalcone isomerase (\textit{tt5-1}), flavanone 3-hydroxylase (\textit{tt6}), dihydroflavonol reductase (\textit{tt3}), and \textit{PAP-1D} seeds were obtained from the ABRC (Columbus, Ohio). For induction of anthocyanins in seedlings (Anthocyanin Inductive Conditions), seeds were surface-sterilized and plated in water containing 3% sucrose. After 2 days of stratification at 4°C, seeds were germinated for 2 – 4 days at 25°C ± 2°C in continuous cool white light (GE F30T12-CW-RS) at ~100 ±10 µmol m$^{-2}$ s$^{-1}$ on a rotatory shaker at 100 rpm. For naringenin treatments, seedlings were allowed to grow for 2.5 days and then naringenin (Aldrich) was added to a final concentration of 100µM or 200µM from a 100mM stock (in ethanol). Treatments with the various chemicals were carried out after seedlings were germinated for 2.5 days (unless otherwise indicated). The seedlings...
were pre-incubated with each inhibitor (Sigma, St Louis, MO) for 1 hr at 25 ± 2 °C before the addition of naringenin. Only 3-MA was added 12 hrs before the naringenin treatment. The final concentrations were 1 mM for vanadate (Stock solution 1M sodium ortovanadate in water), 10 µg/ml for BFA (Stock solution 10mg/ml in ethanol) and 10 mM for 3-MA (Stock solution 1M in water). Each treatment was done at least in triplicate. Soil-sown seeds were stratified at 4°C for 2 days and transferred to a growth chamber at 22°C±2 with a 16-h-dark/8-h-light photoperiod.

Anthocyanin Extraction, Analysis and Quantification.

After the different treatments, seedlings were harvested, rinsed with water and lyophilized for 2 days. Dry weight was measured and 50% methanol was added to get a final suspension of 50 µg/µl (w/v). Two volumes of acidic methanol (1% HCl in 50% MeOH) were added and absorption read at 520nm using a Cary 50 UV-VIS spectrophotometer (Varian, Inc. USA) in 40 µl quartz microcuvettes. The fluorescence spectra of anthocyanins were determined on a Flex station spectrofluorometer (Molecular Devices, CA), with readings taken at 10 nm intervals. Aglycones were obtained by boiling the methanolic extracts containing 1 M HCL for 20 min. For TLC experiments, the anthocyanidins were extracted by adding one quarter of the original volume of isoamyl alcohol and separated on TLC plates (5730/6, Merck, EM Science, Germany) in a pre-saturated chamber with water:formic acid:HCl (10:30:3).

The HPLC analysis of flavonoids and anthocyanins was carried out by separating 20 µl of the methanolic extract on a C-18 column using a Waters Alliance® 2695 Separations module equipped with a 2996 Photodiode Array Detector and a fluorescence detector (Waters Corporation, Milford, MA). Flavonoids were separated using Solvent A: 5% formic acid in water; Solvent B: 5% formic acid in acetonitrile; 0 to 30 min, 95% A to 70% A, linear gradient; 30 to 35 min, 70% A to 95% A, linear gradient. Chromatograms and spectra were extracted and analyzed with the Empower™ software (Waters Corporation, Milford, MA).

Protoplast and Vacuole Isolation

Plant tissue (0.4g) was sliced into pieces with a razor blade and incubated for 2 hrs at 25 °C in the solution containing 2% (w/v) cellulase Onozuka R-10 (KARLAN, Santa Rosa, CA) and 1% (w/v) macerozyme R-10 (KARLAN, Santa Rosa, CA) dissolved in 4 ml of enzyme incubation medium (0.8 M mannitol, 60 mM MES and 5 mM MgCl2, pH 5.5). Digested tissues
were filtered through one layer of Miracloth (Calbiochem, CA, USA). The protoplasts were centrifuged at 600 rpm in a swing bucket centrifuge (Marathon 21000R, Fisher Scientific, USA) for 10 min at 4°C. Vacuole isolation was then performed as previously described (Di Sansebastiano et al., 1998).

**Microscopy**

Neutral red (NR, Sigma) was dissolved in water and used at final concentration 1 mg/ml. Seedlings, protoplasts and vacuoles were incubated with NR for 20 min at room temperature. For quantifying the number of AVIs, the same area of abaxial epidermal cells of cotyledons was always observed, or cells were counted in the entire abaxial surface. The samples were examined using a PCM-2000/Nikon Eclipse600 laser scanning microscope (Nikon, Tokyo, Japan) equipped with argon and green He-Ne laser (Ex 488, 544). To visualize GFP and anthocyanins, a 515/30 nm band pass emission filter (EM515/30HQ) and 565nm long pass filter (E565LP) were used, respectively. Light microscopy observations were made with a Nikon Eclipse 600 microscope equipped with Nomarski differential interference contrast optics (DIC). Images were captured and processed with a SPOT 2 Slider charge-coupled device camera and the associated software (Diagnostic Instruments, Sterling Heights, MI). All images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

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FIGURE LEGENDS

Figure 1. Chemical complementation of tt5 mutants with naringenin. A, Three days old tt5 and wt (Ler) seedlings grown in a 3% sucrose water solution, in continuous white light, in the absence or presence of 100 µM naringenin. B, Spectrophotometric measurement (530 nm) of anthocyanin content of tt5 and wt (Ler) seedlings in the absence (-N) or presence (+N) of 100 µM naringenin.

Figure 2. Auto-fluorescence properties of Arabidopsis anthocyanins. A, Auto-fluorescence of anthocyanins in tt5, tt3 and wt (Ler) Arabidopsis epidermal cells, in the absence (-N) or presence (+N) of 100 µM naringenin visualized by confocal laser scanning microscopy. B, Absorption and fluorescence spectra of an ethanolic extract of cyanidin (see supplementary Fig. S1 for TLC). C, Reverse-phase HPLC chromatograms of anthocyanins extracted from PAP1-D plants showing an overlay of the absorption (Abs 530nm) and fluorescence signals (Ex/Em, 540nm/620nm).

Figure 3. Anthocyanins co-localize with an endoplasmic reticulum (ER) marker. A-E, Co-localization of GFP-HDEL with red anthocyanins in epidermal cells of Arabidopsis seedlings grown in the absence (-N) or presence (+N) of 100 µM naringenin. The GFP-HDEL marker (green fluorescence) is retained in the ER of epidermal cells of Arabidopsis, and showed prominent spindle shaped ER bodies (A and E). The red auto-fluorescence, provided by the anthocyanins (B and F), filled the central vacuole with a faint co-localization in the ER bodies (C and G). Twenty-four hrs after naringenin treatment, the number of ER bodies increased (E-G), with a concomitant increase in the intensity of anthocyanin autofluorescence in the ER bodies as seen distinctly in the red chanel (F) and co-localized with GFP-HDEL in the merge (G). D and H, Bright field of the same images on the left. I-P, Isolated protoplasts of GFP-HDEL seedlings treated without (-N) and with (+N) 100 µM naringenin. The fluorescence patterns for GFP-HDEL marker (I, K, M and O) and the anthocyanins (J, K, N and O) in the protoplasts showed similar trends as the epidermal cells of the intact seedlings. The ER bodies with the co-localized anthocyanins were clearly visible in the cytoplasm (M, N and O). P and L, Bright field of the images on the left. Scale bar = 10 µm.
**Figure 4.** Anthocyanins localize with GFP-Chi labeled vesicles, accumulate in δ-TIP vacuoles and do not share the Golgi-dependent route marked by Ale-GFP vesicles to the vacuole. Confocal laser scanning microscopy images of cotyledonary epidermal cells (A-D, I-L, M-P) and protoplasts (E-H, Q-T) isolated from 3 days old seedlings of the various endomembrane GFP marker lines treated with naringenin for 12 hrs. Epidermal cells show numerous small GFP-Chi labeled vesicles (A, C), anthocyanin red fluorescence in the central vacuole (B, C) which co-localized with GFP fluorescence in the GFP-Chi vesicles (B and C, marked with arrow). Protoplasts isolated from the GFP-Chi seedlings showed a similar pattern where the anthocyanins colocalized in the GFP-Chi vesicles, which were clearly visible in the cytoplasm (E - G, marked with arrows). δ-TIP- GFP labels the tonoplast (I, K) of anthocyanin filled vacuoles (J, K). Protoplasts from δ-TIP-GFP seedlings showed anthocyanins in the central vacuole (R - T) and the presence of a round sub-vacuolar AVI that did not fluoresce (R – T, marked with an arrow). No co-localization of anthocyanins and Ale-GFP vesicles is observed (M - P). Scale bar = 10 µm.

**Figure 5.** Effect of the transport inhibitors vanadate and BFA on anthocyanin accumulation in naringenin-complemented tt5 seedlings. A, Time profile of anthocyanin accumulation evaluated by spectrophotometric measurement at 530 nm at various times (indicated in the x-axis) after the treatment with 1 mM vanadate (blue) or 10 µg/ml BFA (yellow). The red curve corresponds to the untreated control. Anthocyanin contents reached a plateau after 24 hrs. B, Uptake profile of naringenin from the media in tt5 seedlings in the same samples evaluated in A.

**Figure 6.** *Arabidopsis* sub-vacuolar inclusions accumulate anthocyanins (AVIs) and stain with neutral red (NR). A, Epidermal cells of two-weeks old PAP1-D plants with AVIs. Staining with NR revealed the presence of sub-vacuolar structures with similar staining as the vacuolar sap. B, AVI (red bar, pigmentation provided by the accumulation of anthocyanins) and NR-staining sub-vacuolar structures (purple bars) accumulated in wild type and tt5 seedlings untreated (-N) or treated with 100 µM naringenin (+N). See Supplementary Fig. S3 for images representing the cells from which the data was obtained. C, AVIs in isolated vacuoles from PAP1-D plants. D, The isolated vacuoles of PAP1-D showed strong NR uptake by sub-vacuolar structures when
compared to the sap, suggesting that they corresponded to acidic, membrane bound sub-vacuolar compartments. Scale bars = 10 µm.

**Figure 7.** AVIs are formed in the presence of transport inhibitors. **A-H,** AVI formation in *tt5* seedlings treated with naringenin, together with inhibitors affecting ABC transporters (vanadate, Na$_3$VO$_4$, C - D), cellular GSH levels (BSO, E - F), or GST enzymatic activity (CDNB, G - H). Similar areas of the cotyledon (A, C, E and G) were observed to avoid variance due to positional and development effects. The vacuoles of vanadate-treated cells were more alkaline, reflected in the bluish hue of the anthocyanins, whereas cells treated with BSO or CNDB accumulate more AVIs without the same effect on pH. Seedlings were observed 24 hrs after the addition of naringenin and the various inhibitors. Scale bars = 10 µm.
A

- naringenin

100 µM naringenin

B

![Bar chart](chart.png)

- **tt5-N**
- **tt5+N**
- **WT-N**
- **WT+N**

**A530 (mg dry tissue/ml)**

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Fig 1

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