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To cite this version:

HAL Id: hal-02663448
https://hal.inrae.fr/hal-02663448
Submitted on 31 May 2020

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Grapevine MATE-Type Proteins Act as Vacuolar H+-Dependent Acylated Anthocyanin Transporters\textsuperscript{1[W][OA]}

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In grapevine (\textit{Vitis vinifera}), anthocyanins are responsible for most of the red, blue, and purple pigmentation found in the skin of berries. In cells, anthocyanins are synthesized in the cytoplasm and accumulated into the vacuole. However, little is known about the transport of these compounds through the tonoplast. Recently, the sequencing of the grapevine genome allowed us to identify genes encoding proteins with high sequence similarity to the Multidrug And Toxic Extrusion (MATE) family. Among them, we selected two genes as anthocyanin transporter candidates and named them \textit{anthoMATE1} (AM1) and AM3. The expression of both genes was mainly fruit specific and concomitant with the accumulation of anthocyanin pigment. Subcellular localization assays in grapevine hairy roots stably transformed with AM1:: or AM3::green fluorescent protein fusion protein revealed that AM1 and AM3 are primarily localized to the tonoplast. Yeast vesicles expressing anthoMATEs transported acylated anthocyanins in the presence of MgATP. Inhibitor studies demonstrated that AM1 and AM3 proteins act in vitro as vacuolar H\textsuperscript{+}-dependent acylated anthocyanin transporters. By contrast, under our experimental conditions, anthoMATEs could not transport malvidin 3-O-glucoside or cyanidin 3-O-glucoside, suggesting that the acyl conjugation was essential for the uptake. Taken together, these results provide evidence that in vitro the two grapevine AM1 and AM3 proteins mediate specifically acylated anthocyanin transport.

Anthocyanins are plant secondary metabolites, responsible for most of the red, blue, and purple pigmentation found in flowers, fruits, and leaves (Harborne and Williams, 2000). They are involved in plant resistance against UV light and in animal attraction for pollination and seed dissemination. In vivo, anthocyanins are accumulated in the vacuole, which offers a large storage space allowing anthocyanins to reach concentrations high enough to confer physiological and ecological advantages to plants while avoiding harmful effects (Archetti, 2000; Manetas, 2006). From a structural standpoint, anthocyanins are glycosylated derivatives of aglycone chromophores, called anthocyanidins, that differ from each other by the number of hydroxyl and methoxyl groups. Further diversity arises from the nature of the sugar(s) and from its acylation. These substitutions, catalyzed by glycosyltransferases, methyltransferases, and acyltransferases, increase the chemical stability of anthocyanins and confer to them many of their chemical and bioactive properties (Winefield, 2002). Acylated anthocyanins and especially those acylated with aromatic acids are exceptionally resistant to discoloration (Dangles et al., 1993; Cheynier et al., 2006). Grapevine (\textit{Vitis vinifera}) anthocyanins are the 3-monoglucosides of five anthocyanidins: cyanidin, delphinidin and its methylated derivatives, peonidin, petunidin, and malvidin. In addition, the glucosyl group is found acylated with acetyl, \textit{p}-coumaroyl, and, to a lesser extent, caffeoyl residues. Overall, the predominant anthocyanin in red grape cultivars is malvidin 3-O-glucoside (M3G). Nevertheless, the anthocyanin profiles show large varietal diversity and have been used as chemotaxonomy criteria (Roggero et al., 1988). In particular, the proportions of acylated anthocyanins are varietal characteristics. In grapevine berry, anthocyanins are accumulated in the skin of red cultivars. During berry development, the accumulation begins at véraison, and the anthocyanin content increases until ripening (Boss et al., 1996).

In plants, anthocyanins are part of the flavonoid biosynthetic pathway, which is one of the most intensively studied in plants (Winkel-Shirley, 2001). As a
result, most of the structural genes as well as a number of regulatory genes have now been characterized (Winkel-Shirley, 2001; Broun, 2005), but little is known about the molecular mechanisms involved in the downstream steps of the pathway, including flavonoid accumulation into the vacuole. In the cell, anthocyanins are believed to be synthesized at the cytosolic surface of the endoplasmic reticulum (ER) by a multienzyme complex, although the association of anthocyanin enzymes with the ER membrane has not been fully established (Saslowsky and Winkel-Shirley, 2001). With regard to transport from the cytoplasmic surface of the ER to the vacuole, Grotewold and Davies (2008) suggested two models, a vesicular transport (VT) model and a ligandin transporter (LT) model. The VT model may be a direct trafficking route from the ER to the vacuole involving vesicle-like structures filled with anthocyanins. Recently, in Arabidopsis (Arabidopsis thaliana) seedlings, anthocyanins were shown to be sequestered in cytoplasmic structures that resemble ER-derived vesicle-like structures (Pousta et al., 2007). In Lisianthus petals and maize (Zea mays) cell suspension, anthocyanins were accumulated as vesicle-like bodies in the cytoplasm, suggesting a transport under this form to the vacuole (Grotewold et al., 1998; Zhang et al., 2006), although the presence of membranes around such structures remains to be determined. The LT model appears to involve ligandins that bind and escort anthocyanins from the biosynthesis site to the tonoplast, where they are supposed to enter the vacuole through tonoplast transporters.

Two major mechanisms have been proposed for transport across the tonoplast, primary transport mediated by ATP-binding cassette (ABC) transporters (Lu et al., 1998; Goodman et al., 2004; Verrier et al., 2008) and secondary transport depending on the H+ gradient (Martinioa et al., 2007). Several lines of evidence indicated the involvement of primary multidrug resistance-associated protein (MRP)-type ABC transporters in the vacuolar accumulation of phenolic compounds (Klein et al., 2006). Most importantly, a maize antisense mutant of a vacuole-localized ABC subfamily member (Verrier et al., 2008), ZmMRP3, exhibited a reduction in anthocyanin production and pigment mislocalization (Goodman et al., 2004). The main secondary transport mechanism depends on a preexisting H+ gradient across the vacuolar membrane generated by V-ATPase and vacuolar H+-pyrophosphatase (Klein et al., 1996). Recently, Verweij et al. (2008) described a P-ATPase proton pump on the tonoplast involved in vacuolar acidification in petunia (Petunia hybrida) petals that likely provides the proton gradient across the membrane used to energize anthocyanin uptake. In flavonoid transport, the uptake of acylated anthocyanins into isolated vacuoles of carrot (Daucus carota) cell suspension culture was inhibited by protonophores, suggesting dependence on a proton gradient (Hopp and Seitz, 1987), which is in accordance with results in barley (Hordeum vulgare), where vacuolar flavonoid glucoside uptake was also described as a ΔpH-dependent mechanism (Klein et al., 1996). In line with this second transporter family, Multidrug And Toxic Extrusion (MATE) transporters have been identified as candidate carrier proteins for flavonoid/H+ exchange (Yazaki, 2005). The Arabidopsis gene TT12 encodes a MATE protein required for proanthocyanidin sequestration into the vacuole of the seed coat endothelium (Debeaujon et al., 2001). Marinova et al. (2007) demonstrated that TT12 is necessary for vacuolar accumulation of proanthocyanidins and has been shown to mediate anthocyanin transport in vitro. Interestingly, the MATE transporter gene MTP77 related to TT12 was overexpressed in tomato (Solanum lycopersicum) fruit, up-regulating a MYB-type transcription factor (ANT1) triggering anthocyanin hyperaccumulation (Mathews et al., 2003)

In grapevine, no experimental evidence supporting the existence of the VT and/or the LT model is available. A glutathione S-transferase (GST) whose gene expression pattern is coordinated with color development was identified in grape berries (Ageorges et al., 2006). Recently, Conn et al. (2008) showed that this GST complements the maize mutant bz2 characterized by a bronze color, resulting from a mislocalization of anthocyanins in the cytosol (Marrs et al., 1995). This result confirms the involvement of GST in vacuolar sequestration of anthocyanins. Circumstantial evidence suggests that two distinct transporters are involved in grape vacuolar anthocyanin transport: (1) a translocator homologous to the mammalian bili-translocase was identified in grape berries using an anti-bilitranslocase antibody (Braidot et al., 2008); (2) high-throughput expression analysis revealed that the ectopic expression of transcription factor VlmybA1-2 in grapevine hairy roots induced the expression of a candidate gene encoding a protein highly similar to MATE proteins (Cutanda-Perez et al., 2009). In this work, we describe the identification and biochemical characterization of two grapevine MATE transporters. The expression of these two genes was concomitant with anthocyanin accumulation in grape berry. These genes encoded proteins that are targeted to the tonoplast and were able to selectively transport acylated anthocyanins. The biochemical characterization of the transport activity suggests that these two MATE transporters are responsible for the vacuolar transport of acylated anthocyanins in grapevine.

RESULTS

Structure of AnthoMATE Genes and in Silico Analysis of Deduced Proteins

From the grapevine genome sequence (Jaillon et al., 2007) we identified 65 genes encoding proteins with high sequence similarity to MATE family proteins (Fig. 1). The phylogenetic tree was created after the alignment of full-length amino acid sequences of all identified grapevine MATEs and of the previously described plant MATEs: MTP77, the putative antho-
cyanin transporter in tomato (Mathews et al., 2003),
and TT12, the Arabidopsis proanthocyanidin precursor
transporter (Debeaujon et al., 2001). The phylogenetic
tree clearly indicated three distinct clusters, where TT12
and MTP77 are grouped in different clusters (Fig. 1).
The cluster including TT12 contained 10 grapevine MATE
proteins, where two were closely related to TT12
(GSVIVP00018841001 and GSVIVP00018839001). The
MTP77 cluster included 27 proteins and was split into
two branches. Six putative MATEs were closely related
to MTP77 (GSVIVP00037700001, GSVIVP00037698001,
GSVIVP00037696001, GSVIVP0003386001, GSVIV-
P00026941001, and GSVIVP00037697001; Fig. 1).
The three proteins presenting the highest similarity
with MTP77, a putative anthocyanin transporter
of tomato, were selected as candidates involved
in anthocyanin transport in grape berry and named
as anthoMATEs: AM1 (GSVIVT00037700001), AM2
(GSVIVT00037698001), and AM3 (GSVIVT00037696001).
Alignment of the sequences of these three proteins
showed that AM1, AM2, and AM3 exhibited 70%,
73%, and 72% nucleotide sequence identity with
MTP77, respectively, and 69%, 70%, and 67% amino
acid sequence similarity with MTP77, respectively.

All three are located in the same scaffold of the
Genoscope genome database (Fig. 2). The gene structures as predicted by the Genoscope
genome browser suggested that the genomic DNAs
of GSVIVT00037700001 (AM1), GSVIVT00037698001
(AM2), and GSVIVT00037696001 (AM3) are 3,079,
2,997, and 2,901 bp long, respectively.

From Syrah mature berries, full-length cDNAs for
AM1 and AM3 were amplified by reverse transcription-
PCR, cloned, and fully sequenced. In contrast, all our
tries to clone AM2 were not successful, suggesting
that AM2 was probably not expressed at detectable
levels in mature berry or was a pseudogene. AM1
and AM3 transcripts were 1,482 and 1,470 nucleotides
long, respectively, coding for polypeptides of 493
and 489 amino acid residues with predicted molecular
masses of 53.85 and 53.50 kD and calculated pI values
of 6.53 and 5.89, respectively (Fig. 3). The proteins
exhibited 86% identity to each other. The prediction of
transmembrane domains using the transmembrane
hidden Markov model suggested 12 putative trans-
membrane segments for AM1 and AM3. This structure
was similar to those of TT12 and MTP77 (Fig. 3). The
Pfam database predicted for both AM1 and AM3 an

Figure 1. Phylogenetic tree of the predicted amino acid sequences of grapevine MATE proteins, Arabidopsis TT12 (Q9LYT3), and
tomato MTP77 (AAQ55183). The asterisks indicate genes selected as being putative anthocyanin transporters. In the accession
numbers of grapevine sequences annotated in the Genoscope grape genome browser (http://www.genoscope.cns.fr/vitis/; data
obtained from the 8-fold coverage of the genome), “GSVIVP000” was simplified to “VvM.” Relative similarities between sequences
(proportional to horizontal branch lengths) are drawn to scale.
AnthoMATEs Are Tonoplastic Anthocyanin Transporters

Architecture with two MatE domains (PF01554). The predicted MatE domain is highly similar to the MatE domain of MTP77, while less similarity was found for TT12. Five domains (D1–D5) appeared to be particularly conserved. Interestingly, D3 was located in transmembrane segment 7, while all other conserved domains were positioned in cytoplasmic loops.

AnthoMATE Protein Localization

To examine the subcellular localization of anthoMATE proteins, the full-length AM1 and AM3 cDNAs were fused at their C termini to GFP under the control of the cauliflower mosaic virus 35S promoter. The in vivo localization of both anthoMATE proteins was performed in hairy root cultures of grapevine stably transformed with GFP fusions and, as a control, with GFP alone. Confocal microscopy analysis of hairy roots ectopically expressing the transgenes AM1::GFP and AM3::GFP was performed after plasmolysis of cells with sorbitol and counterstaining of nuclei with 4’,6-diamino-phenylindole (DAPI; Fig. 4). Epidermal cells of hairy roots expressing anthoMATE-GFP fusions exhibited intracellular membrane-bound GFP fluorescence that internally surrounded the nucleus (Fig. 4, B and C). Gentle plasmolysis separates tonoplast from the cell wall and demonstrates clearly that the GFP fluorescence surrounds the vacuole and thus localizes to the tonoplast (Fig. 4, D and E). GFP fluorescence in membrane structures attached to the nucleus was also detected for AM1::GFP and AM3::GFP compared with GFP alone, where fluorescence was observed only in the cytosol (Fig. 4). Transient expression by biolistic particle delivery of AM1::GFP fusion together with the established tonoplast marker TPK1 (Czempinski et al., 2002) fused to DsRed2 in onion (Allium cepa) bulb epidermal cells confirmed that proteins coalesce on vacuolar membranes (Supplemental Fig. S1A). We conclude that AM1 and AM3 are primarily localized to the tonoplast under our experimental conditions, even if we cannot exclude their presence on other endomembranes.

AnthoMATE Gene Expression

In order to define changes in expression associated with anthocyanin biosynthesis in grapevine berry, quantitative real-time PCR was performed on both anthoMATE genes during Syrah berry development. Both anthoMATE genes followed similar expression patterns during berry development (Fig. 5, A and B). Before the onset of ripening, AM1 and AM3 were hardly detectable in berry. After véraison, an increase in expression during the ripening stage was observed for both anthoMATE genes (Fig. 5, A and B). The expression profiles for AM1 and AM3 genes throughout berry development were correlated to the biosynthesis of anthocyanins. AnthoMATE gene expression was then evaluated in several tissues (Fig. 5, C and D). While AM1 transcript was found at low levels in young and old leaves, no AM3 expression was measured in leaves. In the other vegetative organs, both AM1 and AM3 transcripts were weakly present. Moreover, AM1 and AM3 expression was quite restricted to berry skin, where anthocyanin biosynthesis takes place (Fig. 5, C and D).

In order to investigate changes in expression in relation to anthocyanin composition in mature berries, the expression of both genes was also monitored on a set of 15 cultivars (Fig. 6). The berry samples were selected among white, pink, and red cultivars in order to maximize the phenotypic variation for anthocyanin content (Fig. 6A). Biochemical analysis showed a significant diversity in anthocyanin metabolites within the studied cultivars (Supplemental Table S1). In all cultivars, AM1 expression was observed, and the expression level was independent of the anthocyanin content and composition in the berry samples (Fig. 6C). Conversely, AM3 exhibited a higher expression in the red cultivars than in the other cultivars (Fig. 6D). When the ratio of acylated anthocyanin to total anthocyanin content in mature berry skins was compared with the expression patterns, it became evident that AM3 expression correlates with the presence of acylated anthocyanins rather than the total anthocyanin content (Fig. 6, B and D). Indeed, expression of AM3 was very weak in all white and pink cultivars, except for Roussaitis. This cultivar was the only pink cultivar containing all of the glucosylated anthocyanins plus petunidin 3-acetylglucoside and malvidin 3-p-coumaroylglucoside; all others contained only cyanidin 3-O-glucoside (C3G; Supplemental Table S1). A correlation of the level of expression of AM3 with the ratio of acylated anthocyanin to total anthocyanin content was found (Pearson test \( r = 0.75 \), significant at \( P = 0.0015 \)).

Overall, the expression of AM1 and AM3 was essentially fruit specific and concomitant with the accumulation of anthocyanin pigments. Moreover, AM3 expression in mature berry was correlated with the percentage of acylated anthocyanin contained in berry.
AnthoMATE Protein in Vitro Transport Experiments

To investigate the transport activity of anthoMATE proteins, the full-length AM1 and AM3 cDNAs were expressed in Saccharomyces cerevisiae under the control of the constitutive PMA1 promoter on a 2μ plasmid. Control experiments were performed in parallel with yeast transformed with the empty vector and with TT12 (Marinova et al., 2007). Total microsomal membrane vesicles were isolated from the transgenic yeast, since Marinova et al. (2007) demonstrated that the transgene is not only restricted to the vacuolar membrane but is also present on other endomembranes and the plasma membrane. Western blot analysis performed on the isolated membrane vesicles with anti-anthoMATE antibodies confirmed the presence of anthoMATE proteins in the yeast transformants tested (Supplemental Fig. S2A). Furthermore, the physiological tightness of independent vesicle preparations was checked through the measurement of MgATP-dependent proton pumping by quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence (Supplemental Fig. S2, B–E).

Transport experiments with various substrates were performed with a standard substrate concentration of 1 mM using the rapid filtration technique (Tommasini et al., 1996). The selected substrates were C3G, M3G, and an acylated anthocyanin mixture extracted and purified from Syrah grape berries. The acylated anthocyanin mixture contained mainly 3-p-coumaroyl-glucosylated anthocyanins and small amounts of malvidin 3-acetylglucosyl. With the different substrates, the empty vector-derived vesicles showed only a slight accumulation both in the presence and in the absence of MgATP, possibly due to an unspecific binding of substrate to membranes.

In the presence of MgATP and using either C3G or M3G, the amount of vesicle-associated substrates remained unchanged for anthoMATEs- or empty vector-derived vesicles (Fig. 7, A and B). These results suggest that, under our experimental conditions, C3G and...
M3G were not transported by either anthoMATE protein (Fig. 7, A and B).

In a second experiment, we used the acylated anthocyanin mixture as a substrate for anthoMATE-dependent transport. In the presence of MgATP, vesicles expressing AM1 or AM3 exhibited an increase in their $A_{536}$ ($\lambda_{\text{max}}$ for malvidin 3-p-coumaroylglucoside) after 1 min of transport, suggesting an uptake of this compound (Fig. 7, A and B). In the presence of MgATP, a time-dependent increase in absorption occurred when the acylated anthocyanin mixture was incubated with AM1- and AM3-containing vesicles when compared with empty vector-derived vesicles, whose absorption remained unchanged over time (Fig. 7, C and D). HPLC analysis of the anthocyanins recovered from vesicles containing anthoMATE proteins after incubation with the acylated anthocyanins showed that transport occurs in the presence of MgATP as an energy source only (Fig. 7E). Taken together, these results indicate that acylated anthocyanins were selectively transported into vesicles by both anthoMATE proteins and when energization by MgATP occurred (Fig. 8).

To validate our experimental conditions, we performed the same test using TT12-containing vesicles.
An ATP-dependent uptake of C3G by TT12-containing vesicles was observed (Supplemental Fig. S3). Using M3G or the acylated anthocyanin mixture as substrate, no transport activity could be measured with TT12-containing vesicles (Supplemental Fig. S3). The results obtained with vesicles expressing AM1, AM3, and TT12 in the uptake of different substrates, summarized in Table I, indicate that anthoMATEs mediate specifically acylated anthocyanin transport in vitro. To elucidate the type of energization mechanism, vanadate, a P-type and ABC pump inhibitor, and NH4Cl, a reagent affecting ΔpH, were used in the transport experiments with acylated anthocyanin substrate. None of the treatments altered the level of substrate bound to empty vector-derived vesicles (Table II). The addition of NH4Cl resulted in a strong decrease in the uptake of substrate, while vanadate had no observed effect, meaning that the uptake of acylated anthocyanins by anthoMATE proteins depends on a proton gradient (Table II). These data demonstrated that AM1 and AM3 act in vitro as vacuolar H+-dependent acylated anthocyanin transporters.

**DISCUSSION**

**MATE Transporters in Grapevine**

The access to the whole genome sequence of grapevine (Jaillon et al., 2007) has accelerated the identification of genes underlying traits of interest in grapevine by allowing a candidate gene approach. Our work demonstrates the biochemical function of two MATE proteins involved specifically in vacuolar transport of acylated anthocyanins. The deduced amino acid sequences indicate that the AM1 and AM3 proteins have a high degree of similarity with TT12, an Arabidopsis proanthocyanidin precursor MATE transporter, and with MTP77, a tomato putative anthocyanin MATE transporter, including five conserved regions. The anthoMATEs share similar membrane topologies with the typical secondary structure of MATE-type transporters (Omote et al., 2006). Finally, anthoMATEs share the same energization mechanism with TT12. Taken together, our data provide evidence for the hypothesis formulated by Marinova et al. (2007) that homologs of the seed-specific Arabidopsis TT12 are responsible for vacuolar anthocyanin transport in other plant parts.

In addition to anthoMATEs, we revealed a large number of virtual MATE proteins (65) close to those reported in Arabidopsis (Omote et al., 2006). Plants have a large number of MATE proteins per species, in contrast to the relatively small number in the bacterial and animal kingdoms, suggesting that MATE proteins might play an important role in the detoxification of secondary metabolites and xenobiotics in plants (Omote et al., 2006). To date, few members of the MATE family, which are localized at the tonoplast or the plasma membrane, have been functionally characterized. Some MATEs are involved in root citrate exudation in Arabidopsis, sorghum (Sorghum bicolor), and barley (Durrett et al., 2007; Furukawa et al., 2007; Magalhaes et al., 2007; Liu et al., 2009). Recently, Shoji et al. (2009) demonstrated that a MATE protein is involved in nicotine vacuolar transport in tobacco (Nicotiana tabacum). For the transport of flavonoids in grapevine, Terrier et al. (2009) recently revealed the induction of a MATE transporter (GSVIVT00018839001) after the ectopic expression of proanthocyanidin tran-
scription factors in grapevine hairy roots. This candidate is one of the most closely related to TT12 in the phylogenetic tree presented here. Further research should confirm the important role of this MATE family in grapevine. It will be interesting to elucidate if some of these grapevine MATE transporters are also localized at the plasma membrane and mediate the transport of multiple endogenous secondary metabolites and xenobiotics.

AM1 and AM3 Exhibit Overlapping Transport Functions, But Their Expression in Mature Berries Differs among Cultivars

The high similarity found for AM1 and AM3 suggests that these two genes could have significant functional overlap, especially with regard to their substrate specificity. The data presented here indicate that the expression of AM1 and AM3 in mature berries differed among cultivars. In Arabidopsis and yeast, it was demonstrated that multicopy genes showed a rapid divergence in expression to coordinately achieve more complex control of the same genetic network (Gu et al., 2002; Blanc and Wolfe, 2004). Furthermore, Blanc and Wolfe (2004) suggested that transport activity and regulatory functions are usually found to be overduplicated for better adaptability to changing environmental conditions. For example, in maize, ZmMRP4, an ABC transporter sharing extensive homology with ZmMRP3, was detected exclusively in aleurone tissues. Both of these genes are proposed to play a role in anthocyanin sequestration (Goodman et al., 2004).

Grape berries accumulate three major types of flavonoid compounds, anthocyanins, flavonols, and flavan-3-ols, at different stages of their development (Boss et al., 1996; Downey et al., 2003). The flavan-3-ols are synthesized mainly at the green stage of berry development (Downey et al., 2003), whereas anthocyanins accumulate in berry skin during ripening only (Boss et al., 1996). By contrast, the biosynthesis of flavonols occurs at two distinct periods in grape berries, the first just after flowering and the second during ripening (Downey et al., 2003). Flavanols exist only as 3-monoglycosides in grape berries without acyl substitutions (Mattivi et al., 2006), and the flavan-3-ols, which can be acylated (galloylated; Souquet...
coupled to metabolic fingerprinting. Would require a mutational or knockdown approach same or structurally different substrates in planta determine whether AM1 and AM3 transport the
AM1 is the more constitutive, base-level twin. To
ward this class of compounds is maximized, while
anthocyanins in situations where metabolic flux to-
valve" that ensures vacuolar sequestration of acylated
findings. Along this line, AM3 could be a "safety
transport functions, which is supported by our in vitro
AM1 and AM3 exhibit at least partially overlapping
biosynthesis in a coordinated manner in grape berries.
VvMYBA1 and VvMYBA2 to regulate anthocyanin
et al. (2008) identified the transcription factor
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This finding suggests differential regulation and/or
transcription of multicopy genes could be specific
for the biosynthesis of anthocyanins, flavonols, and
flavan-3-ols (Castellarin et al., 2006; Jeong et al.,
2008). Indeed, in grapevine hairy roots overexpressing
VlmybA1 (Cutanda-Perez et al., 2009), only AM3 but
not AM1 was induced in this colored tissue, suggest-
ing that AM1 might be under the control of another
transcription factor (C. Gomez, unpublished data).
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VvMYB5b and postulated that VvMYB5b acts with
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anthocyanins in situations where metabolic flux to-
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AM1 is the more constitutive, base-level twin. To
determine whether AM1 and AM3 transport the
same or structurally different substrates in planta
would require a mutational or knockdown approach
coupled to metabolic fingerprinting.

Figure 8. Quantification of MgATP-dependent uptake activity of acylated anthocyanins by AM1 and AM3. Bars show mean values of the
difference between the experiments with or without the addition of
MgATP ± confidence interval at 0.95. Values shown are averages of
eight transport experiments performed with independent vesicle prep-
arrations. Bars associated with the same letter indicate that there are no
significant differences (P > 0.05) by Student's t test.

et al., 1996), are accumulated only during the first part
of berry development when anthoMATEs are not
expressed. Thus, among these different flavonoid
compounds, anthocyanins are the only acylated
flavonoids synthesized during the induction of antho-
MATEs. The biosynthetic pathways of these flavo-
noids are probably controlled specifically, although the
regulatory network remains unclear. Moreover, in
grapevine, redundancy in flavonoid structural genes
has already been observed (Velasco et al., 2007). For
some of them, chalcone synthase, chalcone isomerase,
and flavonoid-3-hydroxylase enzymes, a divergence
in gene expression has been shown, suggesting that
transcription of multicopy genes could be specific
for the biosynthesis of anthocyanins, flavonols, and
flavan-3-ols (Castellarin et al., 2006; Jeong et al.,
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New Insights on the Anthocyanin Biosynthetic Pathway in Grapevine

AM1 and AM3 mediate the transport of antho-
cyanin-acylglucosides, while anthocyanin-glucosides
were not transported under our experimental condi-
tions. This suggests that the acyl conjugation is essen-
tial for the uptake of anthocyanin by anthoMATEs.
Transport studies with isolated vacuoles from parsley
(Petroselinum crispum) and carrot earlier indicated that
the acyl residues attached to flavonoids are important
determinants of substrate specificity (Matern et al.,
1986; Hopp and Seitz, 1987). Moreover, pH-driven conformational changes of the malonylglucosides
were suggested to be responsible for vacuolar trap-
ing of flavonoids in parsley (Matern et al., 1986). Our
data support the hypothesis that acylation reactions
may be critical to provide the correct substrates for
vacuolar deposition. This would imply that the acyl-
ation reactions take place prior to transport, and
consequently in the cytosol.

Until now, to our knowledge, no anthocyanin acyl-
transferase has been identified in grapevine. It remains
unclear if the methylation and the acylation take place
in a chronological manner into the anthocyanin path-
way. No evidence has been provided whether the enzymes are localized in the cytoplasm or inside
the vacuole. Research in other plants revealed that
the modification of anthocyanins is family or species
dependent (Tanaka et al., 2008). Two types of acyl-
transferases could be involved in anthocyanin acyla-
tion: the BAHD acyltransferases, belonging to a large
family of acyl CoA-utilizing enzymes, would be local-
ized to the cytosol (Fujiwara et al., 1998; Yonekura-
Sakakibara et al., 2000; D’Auria, 2006), and the SCPL
family, whose members are acyl Glc dependent, would
be localized to the vacuole (Noda et al., 2007; Tanaka
et al., 2008). Our results suggest that anthocyanin
acyltransferases in grape berry could be part of the
BAHD family.

Different forms of anthocyanin accumulation have
been observed as intravacuolar bodies and are referred
as anthocyanin vacuolar inclusions (AVIs) in different
plants. In flower coloration, AVIs showed a major
influence on flower color by enhancing intensity
and blueness by concentrating anthocyanins above

Table I. Uptake of different substrates into vesicles isolated from
AM1-, AM3-, or TT12-transformed yeast

AAM, Acylated anthocyanin mixture. The presence and absence of
transport activity with the corresponding substrate is indicated by +
and −, respectively.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Substrate Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3G</td>
</tr>
<tr>
<td>AM1</td>
<td>−</td>
</tr>
<tr>
<td>AM3</td>
<td>−</td>
</tr>
<tr>
<td>TT12</td>
<td>+</td>
</tr>
</tbody>
</table>

*From this work and Marinova et al. (2007).*
levels that would be impossible in vacuolar solution (Markham et al., 2000). In grapevine cell suspension, Conn et al. (2003) suggested that AVIs selectively bind acylated anthocyanins. In order to increase the level of more stable anthocyanins, the induction of AVIs may be an interesting approach. It appears that acylation may contribute to AVI formation in plants. Therefore, vacuolar acylated anthocyanin transporter might play an important role. In grapevine, it will be important to determine if a direct relation between AVI formation and anthoMATEs exists. Nevertheless, no associated boundary membrane in AVIs has yet been demonstrated.

### Anthocyanin Transport in Grape Berries May Involve Different Transport Mechanisms

AnthoMATE proteins mediate specifically acylated anthocyanin transport in vitro. This suggests that other mechanisms should be involved in the transport of the nonacylated anthocyanins predominating in grape berries. Whether MRP transporters and MATE transporters are involved in anthocyanin transport has been a matter of debate. For MATE transporters, our work indicates that acyl residues are important determinants for transport. In vitro, TT12 transported C3G in an ATP-dependent and uncoupler-sensitive manner but not the aglycones cyanidin or epicatechin (Marinova et al., 2007). In vivo, Marinova et al. (2007) proposed that TT12 transports proanthocyanidin precursors that are likely glycosylated flavan-3-ols. Omote et al. (2006) speculated that the existence of many plant MATEs might correspond to the transport of large numbers of secondary metabolites. Taken together, a specificity of MATE transporters might be speculated or suggested in flavonoid transport.

For MRP transporters, their specificity is less reported. In grapevine hairy roots expressing ectopically VilmibA1-2, a high-throughput (but nonexhaustive) transcriptomic screening did not reveal genes encoding for proteins with a structure highly similar to MRP proteins (Cutanda-Perez et al., 2009). In Arabidopsis knockout mutants, some MRPs have been identified, but none of them have resulted in flavonoidless phenotypes. AtMRP transporters exhibited an extensive range of substrates (Lu et al., 1997; Liu et al., 2001). In addition, in maize, Goodman et al. (2004) reported that the loss of ZmMRP3 function induced a mislocalization of anthocyanins, with no alteration in the anthocyanin species produced, and that the loss of ZmMRP3 function throughout the plant may negatively affect viability. All of these data suggest multiple biological roles for this family of transporters. It will be particularly important to know whether the anthocyanin transport is handled in a nonspecific manner with MRPs and in a specific manner with MATEs.

In this study, AM1::GFP and AM3::GFP protein fusions were localized at the tonoplast and in membrane structures attached to the nucleus of grapevine hairy root epidermal cells. Recently, in Arabidopsis seedlings, anthocyanins were localized in cytoplasmic structures that resemble ER bodies (Poustka et al., 2007), and in tapetum cells, flavonoids were localized in the ER network and in ER-derived tapetosomes (Hsieh and Huang, 2007). In addition, Hsieh and Huang (2007) reported that in the tapetum cells of the Arabidopsis tt12 mutant, the flavonoids were present in the cytosol and were not associated with tapetosomes, suggesting that TT12 may be also implicated in the filling of ER-derived tapetosomes and thus localized elsewhere than on the vacuolar membrane. In Marinova et al. (2007), TT12 was found to be localized exclusively to the tonoplast; however, its in vivo localization was performed on organs that do not accumulate flavonoids. Whether anthoMATE proteins are localized exclusively on the vacuolar membrane or also on the ER and in the prevacuolar compartment membrane is unclear at this time and will be further investigated. The possibility that in grape berries the lumen of the ER provides the initial site for anthocyanin accumulation suggests that the two models reviewed by Grotewold and Davies (2008), the VT and the LT model, may be involved in grape berries. In relation to the LT model, genetic evidence in maize, petunia, and Arabidopsis suggests that a specific GST is necessary for the correct anthocyanin accumulation inside the vacuole (Marrs et al., 1995; Alfenito et al., 1998; Kitamura et al., 2004). The interaction between anthocyanins and GST has not been clearly characterized. According to the LT model, the GST might escort the anthocyanin to the tonoplast. It will be important to address the question of whether a relation between the grape berry GST (Conn et al., 2008) and anthoMATEs exists.

### Table II. Effects of inhibitors on the uptake of acylated anthocyanin substrate

The uptake in the absence of inhibitors was set to 100% (for AM1). All experiments were performed in the presence of MgATP. Values were means of n independent experiments with four independent vesicle preparations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>NEV</th>
<th>AM1</th>
<th>AM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19 (n = 10)</td>
<td>100 (n = 10)</td>
<td>67 (n = 8)</td>
</tr>
<tr>
<td>Vanadate (1 mM)</td>
<td>22 ± 3 (n = 4)</td>
<td>75 ± 3 (n = 4)</td>
<td>102 ± 2 (n = 4)</td>
</tr>
<tr>
<td>NH₄Cl (5 mM)</td>
<td>20 ± 3 (n = 4)</td>
<td>15 ± 5 (n = 4)</td>
<td>21 ± 5 (n = 4)</td>
</tr>
</tbody>
</table>
In conclusion, this study reports specific transport of acylated anthocyanins mediated by vacuolar anthoMATE transporters. We suggest that the MATE proteins identified here act as acylation-dependent anthocyanin transporters in grape berry. The existence of other transport mechanisms has to be elucidated, and the characterization of their in vivo role would supply more evidence about a hypothetical relationship between the transport mechanism and the anthocyanin composition. However, it is important to note that the anthocyanin composition is different in each plant species. It will be interesting to investigate how the overall anthocyanin structures biosynthesized in one species reflect the transport mechanisms used and if the transport mechanism used is more species specific or anthocyanin structure specific.

**MATERIALS AND METHODS**

**Chemicals and Pigment Extraction**

MSG and C8G were purchased from Extrasynthes. All other chemicals were from Sigma-Aldrich.

The mixture of acylated anthocyanins was isolated from grapevine (*Vitis vinifera* ‘Syrah’) berry. At first 100 mg of each fruit was homogenized with a Dangoumau blender, and stored at –80°C. Grape skin powder was prepared from grape skin powder by extraction with methanol:water:HCl (30:70:0.2, v/v/v) twice for 3 h at room temperature. Then, after concentration of this extract under vacuum, HPLC separation was performed by means of a Gilson system equipped with a reverse-phase Microsorb C18 column (100 Å, 5 

**Glucose Analysis**

Grape juice was analyzed for glucose content by the Amplex Red Glucose/Hexokinase Assay Kit (Invitrogen). The stop-codon-less AM1, AM2, and AM3 oligonucleotides with or without 0.3 M sorbitol and mounted with the same buffer. Monoclonal antibodies against actin (Sigma-Aldrich) were used as primary antibodies. Secondary antibodies were coupled to Alexa Fluor 488 and Alexa Fluor 546 (Invitrogen). Images were taken with a confocal laser scanning microscope equipped with a 20×/0.75 NA objective. DAPI was excited at 730 nm with a Chameleon Ultra II laser, and the emission was collected between 420–470 nm.

**Phylogenetic and Sequence Analyses**

Grapevine sequences were recovered and annotated by the Genoscope genome browser (http://www.genoscope.crs.fr/vitis/; data obtained from the 8-fold coverage of the genome). Database searches for homologous sequences were performed on the BLAST server (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignment was performed with ClustalW alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

**Subcellular Localization and Confocal Scanning Microscopy**

The AM1:GFP and AM3:GFP fusions were obtained by Gateway cloning strategy (Invitrogen). The stop-codon-less AM1 and AM3 cDNAs were amplified using high-fidelity Taq Polymerase (Advantage-HF2 PCR Kit; Clontech) according to the manufacturer’s instructions. The following primers were used: AM1 forward, 5\'-CACCATGGAGACCCTGCTACCAAGC-3' and AM1 reverse, 5\'-GACATCTACCTGCTCTTCT3'- AM3 forward, 5\'-CACATGGAGACCGCTGCTACCAAGC-3' and AM3 reverse, 5\'-TACATCTGCTCATTCAATCC-3'. The resulting PCR products were transferred into pENTR/D-TOPO vector (Invitrogen) and subsequently into pHFWG2 (Karimi et al., 2002) by TOPO and LR clonase reactions according to the manufacturer’s instructions.

**Subcellular Localization of AM1::GFP and AM3::GFP fusions was investigated after stable transformation of root lines of grapevine (cv Macabeu).** Induction and culture of transformed root lines were performed as described by Torregrosa and Bouquet (1997) with the following modifications: (1) Agrobacterium *tuberculosis* A4 strain was the undamaged vector; (2) bacterial cultures were grown on semisolid MGL/B medium (5 mL 1\-1 yeast extract, 2.5 g 1\-1 Glycerol, 100 mg 1\-1 NaCl, 100 mg 1\-1 MgSO\_4\_7H\_2O and 5 g 1\-1 biotin at pH 7; Torregrosa et al., 2002) supplemented with 50 μg mL\-1 spectinomycin and then suspended with half-strength Murashige and Skoog liquid medium with 100 μM acetylseryngone (optical density at 600 nm = 0.5); and (3) extracted root tips were cultured on LGO medium (LGO as described by Torregrosa and Bouquet [1997]) solidified with 5 g 1\-1 Phytagel (Sigma) containing 200 mg 1\-1 of both augmentin and cefotaxime (Duchefa) to prevent bacteria growth. Microscopy observations were performed after three hairy root subcultures with three independent hairy root clones arising from separated induction experiments. DNA was extracted from 100 mg of fresh tissue using the DNA Plant Mini Kit (Qiagen). Transformants were checked by PCR using the hygromycin primers 5\'-GCTGAGACCTGGAGACGGTC-3' and 5\'-CAGTTTCGACATGCACCAC-3'.

For confocal microscopy analysis, young root tips were placed in 1 μM DAPI for 10 min and then rinsed in 10 mM phosphate-buffered saline, pH 7.2, with or without 0.5 mM sorbitol and mounted with the same buffer. The confocal microscope was focused on the epidermal or subepidermal cell layer. GFP was excited at 488 nm with an argon laser, and the emission was collected between 500 and 530 nm. DAPI was excited at 730 nm with a Chameleon Ultra II laser, and the emission was collected between 385 and 465 nm. The fluorescence was...
detected in multitrack configuration using the Axiovert Zeiss LSM 510 META NLO multiphotonic microscope (available at the Montpellier RIO Imaging Platform; www.mri.cnrs.fr). The specificity of signal GFP was checked by spectral analysis. Images were edited using Zeiss LSM Image Browser software and assembled for figures in Adobe Photoshop 7.0.

Preparation of Yeast Vesicles and in Vitro Transport Studies

The full-length cDNAs of AM1 and AM3 were amplified from cDNA of mature berries (Syrah) using high-fidelity Taq Polymerase (Advantage-HF2 PCR kit; Clontech) according to the manufacturer's instructions. The following primers were used: AM1 forward, 5'-GGCCGGGCACTGGAAGACGG-CGCCCTGATCAACAG3'- and AM1 reverse, 5'-AGCCGCCCTGCACAGACATCATCAGCTGCTCTTTC-3'; AM3 forward, 5'-GGCCGGGCACTGGAAGACGG-CGCCCTGATCAACAG3'- and AM3 reverse, 5'-AGCCGCCCTGCACAGACATCATCAGCTGCTCTTTC-3'. The NotI restriction site, added in all of the primers, is underlined. The amplified cDNAs for AM1 and AM3 were cloned into the pGEM-T Easy vector (Promega), and the resulting plasmids were then sequenced using M13 forward and M13 reverse primers by commercial DNA sequencing services (GATC Biotech). PCR products of AM1 and AM3 were cloned into the NotI site of the plasmid pNEV-Ura (Sauer and Stolz, 1994), resulting in pNEV-AM1 and pNEV-AM3. The constructs were sequenced to verify that no mutation had been introduced. These two constructs were then used to transform the yeast strain YPH 499 (MATa URA3-52 lys2-801 ade2-101 trp1-1α63 his3-Δ200 leu2-1) following standard procedures (Gietz and Woods, 2002). Transformants were selected on minimal synthetic dropout medium lacking uracil.

Western Blot

The isolated vesicle extracts were dissociated with SDS sample buffer containing 10% SDS and 10% mercaptoethanol and incubated in a 100°C bath for 3 min. The proteins were subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS. Western blotting of the vesicle extracts isolated was performed as described by Terrier et al. (1998). The anti-anthoMATE antibody was obtained from rabbit sera, following immunization with the peptide AALSIRVSNELGYGHPRAAK. Antibody was purchased from Proteogenix.

Yeast membrane vesicles for in vitro transport studies were isolated essentially as described by Tommasini et al. (1996) with the modifications reported by Klein et al. (2002) without the addition of bovine serum albumin essentially as described by Tommasini et al. (1996) with the modifications from Proteogenix. The isolated vesicle extracts were dissociated with SDS sample buffer containing 10% SDS and 10% mercaptoethanol and incubated in a 100°C bath for 3 min. The proteins were subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS. Western blotting of the vesicle extracts isolated was performed as described by Terrier et al. (1998). The anti-anthoMATE antibody was obtained from rabbit sera, following immunization with the peptide AALSIRVSNELGYGHPRAAK. Antibody was purchased from Proteogenix.

Yeast membrane vesicles for in vitro transport studies were isolated essentially as described by Tommasini et al. (1996) with the modifications reported by Klein et al. (2002) without the addition of bovine serum albumin into the vesicle buffer. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The following proteins were used to standardize the protein concentration: bovine serum albumin, BSA, and yeast protein. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The following proteins were used to standardize the protein concentration: bovine serum albumin, BSA, and yeast protein.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular localization of anthoMATE protein after transient expression in onion bulb epidermal cells (A) and after stable expression in grapevine (B).

Supplemental Figure S2. Analysis of vesicle fractions isolated from yeast transformants.

Supplemental Figure S3. TT12 transport activity toward M3G, C3G, and acylated anthocyanin mixture substrates.

Supplemental Table S1. Anthocyanin profiles of mature berries from a set of 15 grapevine cultivars used for expression analysis.

ACKNOWLEDGMENTS

We especially thank G. Conero for her helpful assistance for confocal microscopy. The A4 strain was introduced from the Centre Français des Bactéries Phytopathogènes d’Angers (http://www.intranet.angers.inra.fr). We thank C. Romieu for providing Gateway pH7PWG2-compatible cloning vector, F. Sauvage for technical support with western blotting, and I. Gil and G. Lopez for their assistance in the handling of the in vitro vegetal material.

Received January 13, 2009; accepted March 16, 2009; published March 18, 2009.

LITERATURE CITED


AnthoMATEs Are Tonoplastic Anthocyanin Transporters

Plant Physiol. Vol. 150, 2009 413


analyse en composantes principales et analyse factorielle discriminante. Cahiers Scientifiques RFOE 112: 277–284


