ORIGINAL PAPER

VvMATE1 and *VvMATE2* encode putative proanthocyanidin transporters expressed during berry development in *Vitis vinifera* L.

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Abstract

Key message **VvMATE1** and **VvMATE2** encode putative PA transporters expressed during seed development in grapevine. The subcellular localization of these MATE proteins suggests different routes for the intracellular transport of PAs.

Abstract Proanthocyanidins (PAs), also called condensed tannins, protect plants against herbivores and are important quality components of many fruits. PAs biosynthesis is part of the flavonoid pathway that also produces anthocyanins and flavonols. In grape fruits, PAs are present in seeds and skin tissues. PAs are synthesized in the cytoplasm and accumulated into the vacuole and apoplast; however, little is known about the mechanisms involved in the transport of these compounds to such cellular compartments. A gene encoding a Multidrug And Toxic compound Extrusion (*MATE*) family protein suggested to transport anthocyanins—named *VvMATE1*—was used to identify a second gene of the *MATE* family, *VvMATE2*. Analysis of their deduced amino acid sequences and the phylogenetic relationship with other MATE-like proteins indicated that *VvMATE1* and *VvMATE2* encode putative PA transporters. Subcellular localization assays in Arabidopsis protoplasts transformed with *VvMATE–GFP* fusion constructs along with organelle-specific markers revealed that VvMATE1 is localized in the tonoplast whereas VvMATE2 is localized in the Golgi complex. Major expression of both genes occurs during the early stages of seed development concomitant with the accumulation of PAs. Both genes are poorly expressed in the skin of berries while *VvMATE2* is also expressed in leaves. The presence of putative *cis*-acting elements in the promoters of *VvMATE1* and *VvMATE2* may explain the differential transcriptional regulation of these genes in grapevine. Altogether, these results suggest that these MATE proteins could mediate the transport and accumulation of PAs in grapevine through different routes and cellular compartments.

Communicated by P. Puigdomenech.

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Abbreviations

PA	Proanthocyanidin
PAs	Proanthocyanidins
ABA	Abscisic acid
MeJA	Methyl jasmonate
GFP	Green fluorescent protein

qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
ER	Endoplasmic reticulum
MATE	Multidrug And Toxic Compound Extrusion

Introduction

Proanthocyanidins (PAs)—also known as condensed tannins—are abundant flavonoid compounds that play important roles in the interaction between plants and their environment. They can protect plants against diseases and herbivores, and are involved in physiological functions such as seed dormancy and viability (Dixon et al. 2005; Lepiniec et al. 2006). PAs are polyphenolic polymers consisting of flavan 3-ol subunits, (+)-catechin and/or (−)-epicatechin which are synthesized via the flavonoid pathway that also produces anthocyanins and flavonols (Boss et al. 1996; Xie et al. 2003). In grapevine (*Vitis vinifera* L.), tannins are of great importance to red wine quality and are associated to organoleptic properties such as astringency and bitterness (Peleg et al. 1999; Gawel et al. 2001). These compounds play important roles during storage by the interaction with other flavonoids involved in long-term color stability (Remy et al. 2000; Escribano-Bailon et al. 2001; Wang et al. 2003). In grape fruits, PAs are present in seed and skin tissues (Kennedy et al. 2001; Kennedy and Jones 2001) and in lesser extent in the pulp (Mané et al. 2007; Verries et al. 2008). These flavonoids are actively synthesized during flower and fruit development, mainly at the green stage of berry development. In fruit skin, PAs mainly accumulate before veraison (defined as the onset of ripening in grapevine) and when anthocyanins start to be synthesized (Kennedy et al. 2001; Downey et al. 2003; Verries et al. 2008). In contrast, PA accumulation in seeds appears to continue for 1–2 weeks after veraison, suggesting that PA accumulation in seeds is independent of that in the skin (Downey et al. 2003; Bogs et al. 2005).

At the cellular level, synthesis of PA precursors and other flavonoids is believed to take place at the cytoplasmic side of the endoplasmic reticulum (ER) by an enzymatic complex and then transported for storage (Winkel 2004). Several studies have shown that PAs are stored in the vacuoles of seed coat endothelial cells during Arabidopsis early seed development (Abrahams et al. 2003; Kitamura et al. 2004) and it is likely (but not definitively proven) that PA oligomerization/polymerization occurs in the vacuole (Pourcel et al. 2005). However, the major fractions of PAs in grapes are accumulated in the apoplast in both seeds and skin, and tannins from cell walls have a higher degree of polymerization than those from the inner part of the cell (Gény et al. 2003). Because flavonoids are normally accumulated in cellular compartments other than those

where they are synthesized, their transport to the final destination is a critical process in PA biosynthesis and assembly. Even in Arabidopsis, transport of flavonoids from the ER to the vacuole is not completely understood. Currently, two proposed models for this process include vesicle trafficking and membrane transporters (Grotewold and Davies 2008; Braidot et al. 2008; Zhao and Dixon 2009; Zhao et al. 2010). In Arabidopsis seedlings, Poustka et al. (2007) observed that anthocyanins accumulate in vesicle-like structures probably derived from the ER. These structures have also been observed in grapevine hairy roots and in cell suspension cultures (Cutanda-Pérez et al. 2009; Conn et al. 2010; Gomez et al. 2011). Additionally, new evidence suggests that tannin synthesis could also take place in chloroplasts, their polymerization inside tannosomes, chloroplast-derived organelles, and their final transport to the vacuole in structures named tannosome shuttle (Wang et al. 2010; Brillouet et al. 2013). Regarding the membrane transport mechanism, two families of transporters have been shown to participate in anthocyanin and PA transport: the ATP-binding cassette (ABC) transporter family and Multidrug And Toxic Compound Extrusion (MATE) transporters. A mutation in the maize ABC-C transporter *ZmMRP3* caused a reduction in anthocyanin production and pigment mislocalization (Goodman et al. 2004). More recently, the grapevine ABCC1 protein, a tonoplast-localized ABC transporter, was shown to be involved in the transport of anthocyanidin 3-O-glucosides into the vacuole (Francisco et al. 2013). On the other hand, plant MATE proteins constitute a large family that has been involved in the transport of different organic molecules such as salicylic acid (Nawrath et al. 2002), alkaloid vacuolar transport (Shoji et al. 2009) and citrate efflux (Durrett et al. 2007; Liu et al. 2009); (Magalhaes et al. 2007); (Furukawa et al. 2007); (Yokosho et al. 2009); (Maron et al. 2010). In addition, MATE transporters have been implicated in the transport of flavonoids (Mathews et al. 2003); (Zhao and Dixon 2009; Zhao et al. 2011); (Gomez et al., 2009); (Frank et al. 2011). In *Arabidopsis*, three genes have been reported to participate in the vacuolar transport of flavan-3-ol (epicatechin). Mutations in *TT12* or *TT19* affect PA accumulation producing pale seed coats with a transparent testa phenotype due to a reduction in oxidized PAs (Debeaujon et al. 2003; Kitamura et al. 2004). TT12 exhibits a strong homology to MATE transporters (Debeaujon et al. 2001; Marinova et al. 2007) whereas TT19 to members of the multigenic glutathione S-transferase (GST) family, essential for anthocyanin and PA accumulation in *Arabidopsis*, maize and petunia (Kitamura et al. 2004, 2010). The third gene, *AHA10*, encodes a H^+-ATPase (H^+ pump) that is indirectly involved in PA metabolism by producing a H^+ gradient necessary to support the MATE's H^+/flavonoid antiporter

function (Baxter et al. 2005; Zhao et al. 2010). In grapevine, *VvGST4* is strongly associated to anthocyanin accumulation and is able to complement the maize mutant *bz2*, defective in anthocyanin transport (Conn et al. 2008). Down-regulation of *VvGST4* expression in grapevine hairy roots showed that this protein is necessary for anthocyanin transport from the ER to the tonoplast independently of flavonoid-filled vesicle trafficking (Gomez et al. 2011).

Two grapevine MATE transporters, named anthoMATEs (AMs), were shown to be involved in the transport of acylated anthocyanins in vitro. AM1 and AM3 are proteins mainly targeted to the tonoplast and membrane structures attached to the nucleus (Gomez et al. 2009). However, in tissues that accumulate anthocyanins, these proteins are closely associated to small anthocyanin-filled vesicles derived from the ER (rather than the tonoplast). AMs are also necessary for the formation of the ER-derived vesicles (Gomez et al. 2011). Grapevine genes homologous to *AHA10* have yet to be reported. Despite all these reports, little is known about the PA transport process in grapevine. Most proteins involved in flavonoid transport have been characterized only for anthocyanin transport. Ectopic expression of *VvMYBPA1* or *VvMYBPA2* in grapevine hairy roots promotes PA synthesis and induction of a set of PA biosynthetic genes. Interestingly, one up-regulated gene encodes a putative MATE transporter (Terrier et al. 2009). Because this gene is highly homologous to *TT12*, functional characterization of homologous genes in the grape genome could provide better understanding on the mechanisms of PA transport and accumulation in this species. In line with this notion, we isolated and characterized two genes, *VvMATE1* and *VvMATE2*. Sequence analysis, subcellular localization and expression pattern during berry development of these grapevine MATE members suggest that both genes encode putative PA transporters with probable involvement in different transport pathways and accumulation of PAs in *Vitis vinifera* L.

Results

VvMATE1 and VvMATE2 are members of the MATE family of transporters

To identify genes encoding putative MATE proteins in the genome of *Vitis vinifera*, a search was conducted in the Genoscope database (http://www.genoscope.cns.fr) for those presenting the highest sequence similarity to GSVIVT18839001 (named *VvMATE1*), which was described as highly homologous to *TT12* from *Arabidopsis thaliana* (Terrier et al. 2009). Only the sequence GSVIVT00018841001 (named *VvMATE2*) had an identity greater than 95 %. The full-length coding sequences of *VvMATE1* and *VvMATE2* were amplified and cloned from

cDNA of Carménère berries in the veraison stage. Their sizes were 1,521 and 1,515 bp and were predicted to encode a protein of 506 and 504 amino acid residues, respectively.

To establish the phylogenetic relationship between VvMATE1 and VvMATE2 with plant MATE-like proteins, a phylogenetic tree was created using the deduced full-length amino acid sequences of *VvMATE1* and *VvMATE2*. The analysis was performed with proteins that were described to be involved in PA transport such as *Arabidopsis* TT12 (Debeaujon et al. 2001), *Malus x domestica* MATE1 and MATE2 (Frank et al. 2011) and *Medicago truncatula* MATE1 (Zhao and Dixon 2009); and in anthocyanin transport, AM1 and AM3 from grapevine (Gomez et al. 2009) and MTP77 from tomato (Mathews et al. 2003). Proteins encoded by these grapevine genes specifically clustered with MATE proteins involved in PA transport like those of *M. domestica*, *M. truncatula*, *Arabidopsis* and *Brassica rapa* (Fig. 1) and were clearly separated from other MATE transporters participating in anthocyanin, nicotine or citrate compartmentation. Others putative transporters from *Vitis vinifera* grouped into a different clade showing high homology with NtMATE1 and NtMATE2 have been described as nicotine transport into the vacuoles of tobacco roots (Shoji et al. 2009) (Fig. 1).

VvMATE1 and VvMATE2 share 84 % amino acid sequence identity with each other and present significant similarities with other MATE proteins as determined by a BLASTP search. The most similar sequences to both grapevine proteins were *Malus x domestica* MATE1 (78 and 82 % identity, respectively) and MATE2 proteins (76 and 82 % identity, respectively) and with *Populus tricocarpa* XP-002307572 with 77 and 81 % identity, respectively. VvMATE1 and VvMATE2 are the closest sequences to the *Arabidopsis* TT12 of all MATE-like proteins found in grapevine (with a 72 and 74 % identity, respectively). Multiple alignments of VvMATE1 and VvMATE2 with MATE-like proteins revealed a strong sequence homology among them (Fig. 2). Pfam database predicted two MatE domains (PF01554) in both grapevine MATE proteins, that are similar to the MatE domain present in TT12 and other PA-transporting MATE proteins. In agreement with the structure of other MATE family members involved in PAs transport, analysis of VvMATE1 and VvMATE2 with the HMMTOP program predicted 12 transmembrane helices consistent with those observed in other MATE transporters (Debeaujon et al. 2001).

At the genomic level, both genes are located in the same scaffold in chromosome 12 (Fig. 3). Their structure reveals high conservation in the size and number of introns/exons (both containing eight exons interrupted by seven introns), similar to the structure observed in other genes that encode PA-transporting MATE proteins (Debeaujon et al. 2001; Frank et al. 2011).
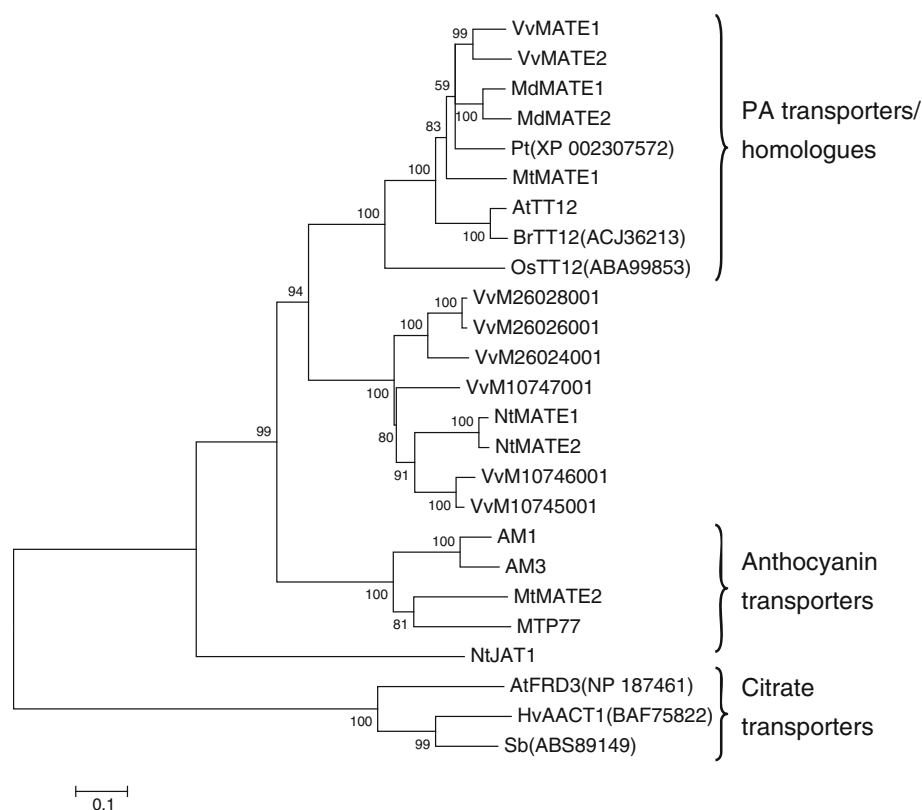
VvMATE1 is targeted to the tonoplast while VvMATE2 is localized to the Golgi complex

Due to the strong evidence that MATE proteins involved in PA transport are confined in the tonoplast, the subcellular localization of the grapevine proteins was investigated. First, a subcellular localization prediction was performed by in silico analysis of their deduced amino acid sequence. Wolf PSORT-II software (Horton et al. 2007) predicts a vacuolar location for VvMATE1, coincident with other PA transporters (Debeaujon et al. 2001; Zhao and Dixon 2009) whereas in VvMATE2 it showed a possible destination to the plasma membrane or the Golgi complex. To establish this, each cDNA was fused to GFP under the control of the CaMV 35S promoter. In addition, the vacuolar marker V-rk fused to the mCherry reporter (Nelson et al. 2007) with a red signal was used in the co-transformation of *Arabidopsis* protoplasts. Fluorescence from GFP and mCherry was observed after 24 h of incubation post transfection (Fig. 4). In the case of VvMATE1, the GFP signal overlapped with the mCherry signal from the vacuolar marker, observed in the merged images (Fig. 4c, d), strongly suggesting that this protein is localized to the tonoplast. On the other hand, co-localization assays of VvMATE2 with V-rk showed that VvMATE2–GFP did not localize to vacuoles (Supplementary Fig. 1). On the contrary, VvMATE2 was visualized as small dotty structures (with a granular pattern)

similar to Golgi structures. To confirm this, other organelle-specific markers were used. When an ER marker fused to mCherry and MATE2-GFP were co-expressed in protoplasts (Fig. 4i, j), fluorescence from GFP and mCherry did not completely overlap, but apparently VvMATE2 embedded the ER (Fig. 4k, l). However, after transforming the protoplasts with a Golgi marker fused to mCherry, the GFP signal was found to overlap with the mCherry signal (Fig. 4g, h), indicating that the VvMATE2–GFP fusion protein is targeted to the Golgi complex.

VvMATE1 and VvMATE2 are mainly expressed in seed berries

The transcriptional profiles of these putative transporter genes were determined in skin and seeds at different berry developmental stages in Carménère and associated with flavonoid synthesis. Early stages of fruit development and vegetative tissues such as leaves and roots were also analyzed. Spatial and temporal variations in expression were observed for each gene. The transcript levels of VvMATE1 were higher in seeds than in the rest of the tissues analyzed. This gene showed to be highly expressed from the time of the earliest stages of seed development; however, the highest level of transcript was observed at veraison and then decreased during ripening. Significant expression was
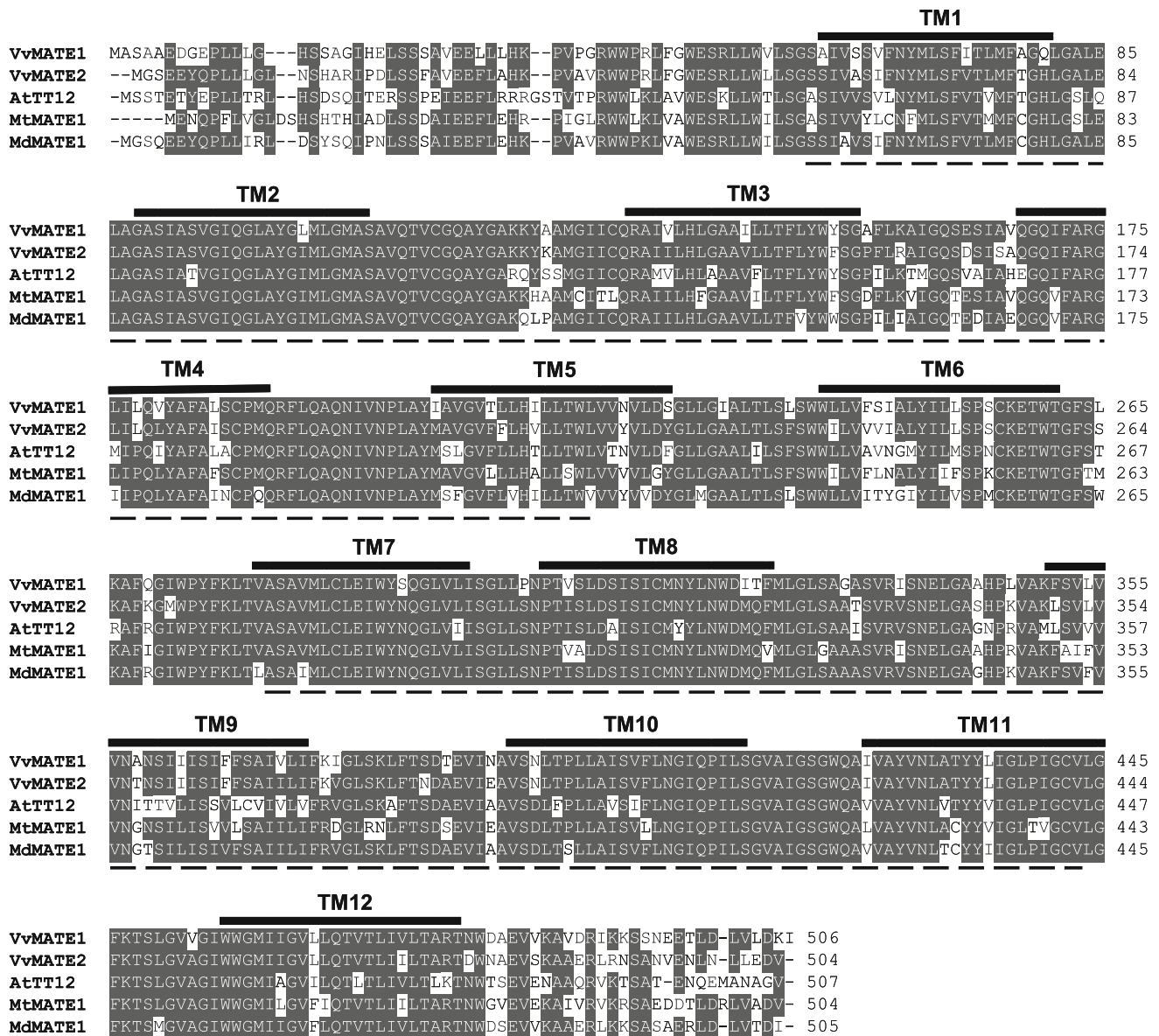
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                                                                      TM1
                                                            ━━━━━━━━━━━━━━━━━━━
VvMATE1  MASAAEDGEPLLLG---HSSAGIHELSSSAVEELLLHK--PVPGRWWPRLFGWESRLLWVLSGSAIVSSVFNYMLSFITLMBAGQLGALE  85
VvMATE2  --MGSEEYQPLLLGL--NSHARIPDLSSFAVEEFLAHK--PVAVRWWPRLFGWESRLLWLLSGSSIVASIFNYMLSFVTLMFTGHLGALE  84
AtTT12   -MSSTETYEPLLTRL--HSDSQITERSSPEIEEFLRRRGSTVTPRWWLKLAVWESKLLWTLSGASIVVSVLNYMLSFVTVMFTGHLGSLQ  87
MtMATE1  -----MENQPFLVGLDSHSHTHIADLSSDAIEEFLEHR--EIGLRWWLKLVAWESRLLWILSGASIVVYLCNFMLSFVTMMFCGHLGSLE  83
MdMATE1  -MGSQEEYQPLLIRL--DSYSQIPNLSSSAIEEFLEHK--PVAVRWWPKLVAWESRLLWILSGSSIAVSIFNYMLSFVTLMFCGHLGALE  85

               TM2                                        TM3
         ━━━━━━━━━━━━━━━━━━                        ━━━━━━━━━━━━━━━━━━━        ━━━━━━━━━━
VvMATE1  LAGASIASVGIQGLAYGLMLGMASAVQTVCGQAYGAKKYAAMGIICQRAIVLHLGAAILLTFLYWYSGAFLKAIGQSESIAVQGQIFARG 175
VvMATE2  LAGASIASVGIQGLAYGIMLGMASAVQTVCGQAYGAKKYKAMGIICQRAIILHLGAAVLLTFLYWFSGPFLRAIGQSDSISAQGQIFARG 174
AtTT12   LAGASIATVGIQGLAYGIMLGMASAVQTVCGQAYGARQYSSMGIICQRAMVLHLAAAVFLTFLYWYSGPILKTMGQSVAIAHEGQIFARG 177
MtMATE1  LAGASIASVGIQGLAYGIMLGMASAVQTVCGQAYGAKKHAAMCITLQRAIILHFGAAVILTFLYWFSGDFLVTIGQTESIAVQGQVFARG 173
MdMATE1  LAGASIASVGIQGLAYGIMLGMASAVQTVCGQAYGAKQLPAMGIICQRAIILHLGAAVLLTFVYWWSGPILIAIGQTEDIAEQGQVFARG 175

             TM4                          TM5                          TM6
         ━━━━━━━━━━━━━             ━━━━━━━━━━━━━━━━━━          ━━━━━━━━━━━━━━━━━━━
VvMATE1  LILQVYAFALSCPMQRFLQAQNIVNPLAYIAVGVTLLHILLTWLVVNVLDSGLLGIALTLSLSWWLLVFSIALYILLSPSCKETWTGFSL 265
VvMATE2  LILQLYAFAISCPMQRFLQAQNIVNPLAYMAVGVFFLHVLLTWLVVYVLDYGLLGAALTLSFSWWILVVVIALYILLSPSCKETWTGFSS 264
AtTT12   MIPQIYAFALACPMQRFLQAQNIVNPLAYMSLGVFLLHTLLTWLVTNVLDFGLLGAALILSFSWWLLVAVNGMYILMSPNCKETWTGFST 267
MtMATE1  LIPQLYAFFSCPMQRFLQAQNIVNPLAYMAVGVLLLHALLSWLVVVVLGYGLLGAALTLSFSWWILVFLNALYIIFSPKCKETWTGFTM 263
MdMATE1  IIPQLYAFAINCPQQRFLQAQNIVNPLAYMSFGVFLVHILLTWVVVYVVDYGLMGAALTLSLSWWLLVITYGIYILVSPMCKETWTGFSW 265

                   TM7                        TM8
           ━━━━━━━━━━━━━━            ━━━━━━━━━━━━━━━━━━
VvMATE1  KAFQGIWPYFKLTVASAVMLCLEIWYSQGLVLISGLLPNPTVSLDSISICMNYLNWDITFMLGLSAGASVRISNELGAAHPLVAKFSVLV 355
VvMATE2  KAFKGMWPYFKLTVASAVMLCLEIWYNQGLVLISGLLSNPTISLDSISICMNYLNWDMQFMLGLSAATSVRVSNELGASHPKVAKLSVLV 354
AtTT12   RAFRGIWPYFKLTVASAVMLCLEIWYNQGLVTISGLLSNPTISLDAISICMYYLNWDMQFMLGLSAAISVRVSNELGAGNPRVAMLSVVV 357
MtMATE1  KAFIGIWPYFKLTVASAVMLCLEIWYNQGLVLISGLLSNPTVALDSISICMNYLNWDMQVMLGLGAAASVRISNELGAAHPRVAKFAIFV 353
MdMATE1  KAFRGIWPYFKLTLASAIMLCLEIWYNQGLVLISGLLSNPTISLDSISICMNYLNWDMQFMLGLSAAASVRVSNELGAGHPKVAKFSVFV 355

             TM9                          TM10                         TM11
         ━━━━━━━━━━━━            ━━━━━━━━━━━━━━━━            ━━━━━━━━━━━━━━━━
VvMATE1  VNANSIIISIFFSAIVLIFKIGLSKLFTSDTEVINAVSNLTPLLAISVFLNGIQPILSGVAIGSGWQAIVAYVNLATYYLIGLPIGCVLG 445
VvMATE2  VNTNSIIISIFFSAIILIFKVGLSKLFTNDAEVIEAVSNLTPLLAISVFLNGIQPILSGVAIGSGWQAIVAYVNLATYYLIGLPIGCVLG 444
AtTT12   VNITTVLISSVLCVIVLVFRVGLSKAFTSDAEVIAAVSDLFPLLAVSIFLNGIQPILSGVAIGSGWQAVVAYVNLVTYYVIGLPIGCVLG 447
MtMATE1  VNGNSILISVVLSAIILIFRDGLRNLFTSDSEVIEAVSDLTPLLAISVLLNGIQPILSGVAIGSGWQALVAYVNLACYYVIGLTVGCVLG 443
MdMATE1  VNGTSILISIVFSAIILIFRVGLSKLFTSDAEVIAAVSDLTSLLAISVFLNGIQPILSGVAIGSGWQAVVAYVNLTCYYIIGLPIGCVLG 445

              TM12
         ━━━━━━━━━━━━━━━
VvMATE1  FKTSLGVVGIWWGMIIGVLLQTVTLIVLTARTNWDAEVVKAVDRIKKSSNEETLD-LVLDKI 506
VvMATE2  FKTSLGVAGIWWGMIIGVLLQTVTLIILTARTDWNAEVSKAAERLRNSANVENLN-LLEDV- 504
AtTT12   FKTSLGVAGIWWGMIAGVILQTLTLIVLTLKTNWTSEVENAAQRVKTSAT-ENQEMANAGV- 507
MtMATE1  FKTSLGVAGIWWGMILGVFIQTVTLIILTARTNWGVEVEKAIVRVKRSAEDDTLDRLVADV- 504
MdMATE1  FKTSMGVAGIWWGMIIGVFLQTVTLIVLTARTNWDSEVVKAAERLKKSASAERLD-LVTDI- 505
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Fig. 2 Alignment of the deduced amino acid sequences of VvMATE and VvMATE2 and PA-transporting MATE transporters by ClustalW. Grapevine VvMATE1 (CAO69962) and VvMATE2 (CAO69963), Arabidopsis TT12 (NP_191462), *M. truncatula* MtMATE1 (FJ858726) and apple MdMATE1 (GU064953). The predicted MATE domains (PF01554) using the Pfam database are shown in *dotted line*. Identical amino acid residues among predicted protein sequences are *shaded in black*. *Black bars* indicate the 12 transmembrane domains (TM) predicted by the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/)

also detected during flower development and fruit setting. In contrast, transcript levels of *VvMATE1* were less abundant in grape skin, similar to those found in vegetative organs (Fig. 5a). On the other hand, *VvMATE2* showed a different expression pattern than *VvMATE1*. This gene was predominantly expressed in grape seeds and leaves. The expression in seeds presented a peak at fruit setting stage following a decline at pre-veraison and then a second peak at veraison to finally decrease during seed maturity. In leaves, *VvMATE2* was found to be transcribed at high levels, being comparable with those found in seeds. Similar to *VvMATE1*, abundant transcript levels were also found in reproductive tissue and small berries but not in skin and roots (Fig. 5b).

To understand the molecular basis that may explain the differential expression of both genes, an in silico analysis of a 1,500-bp upstream regulatory region of *VvMATE1*, *VvMATE2* and *AtTT12* was performed. Five groups of common target motifs for plant MYB transcription factors, including MYB1AT (WAACCA), MYBCORE (CNGTTR), MYBPLANT (CCWACC), MYBPZM and MYBST1 (GGATA), were identified in the promoters of the three
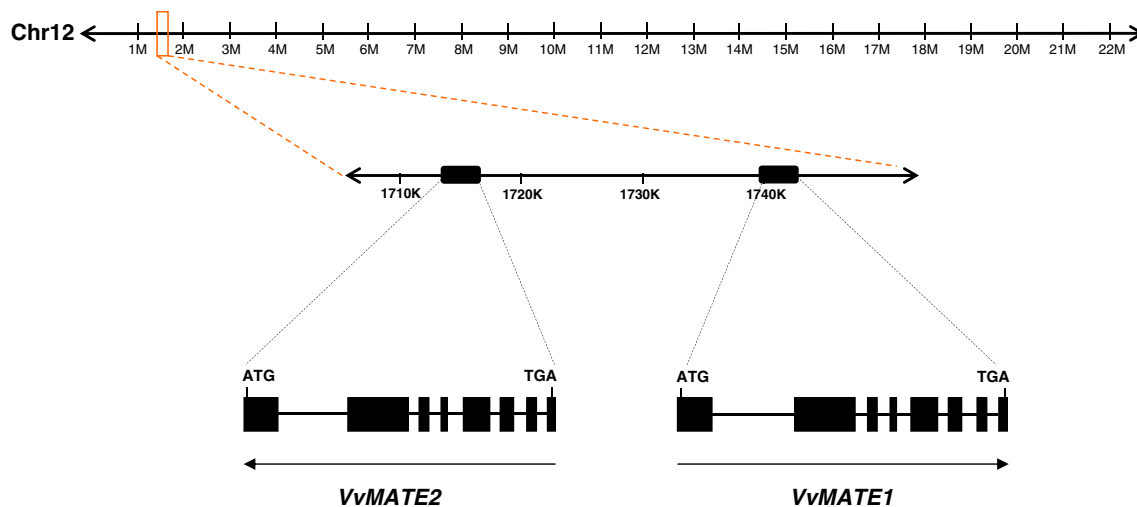
Fig. 3 Genomic structures of *VvMATE1* and *VvMATE2*. Both genes were present at the scaffold_8 at the chromosome 12 on the genoscope database (http://www.genoscope.cns.fr)
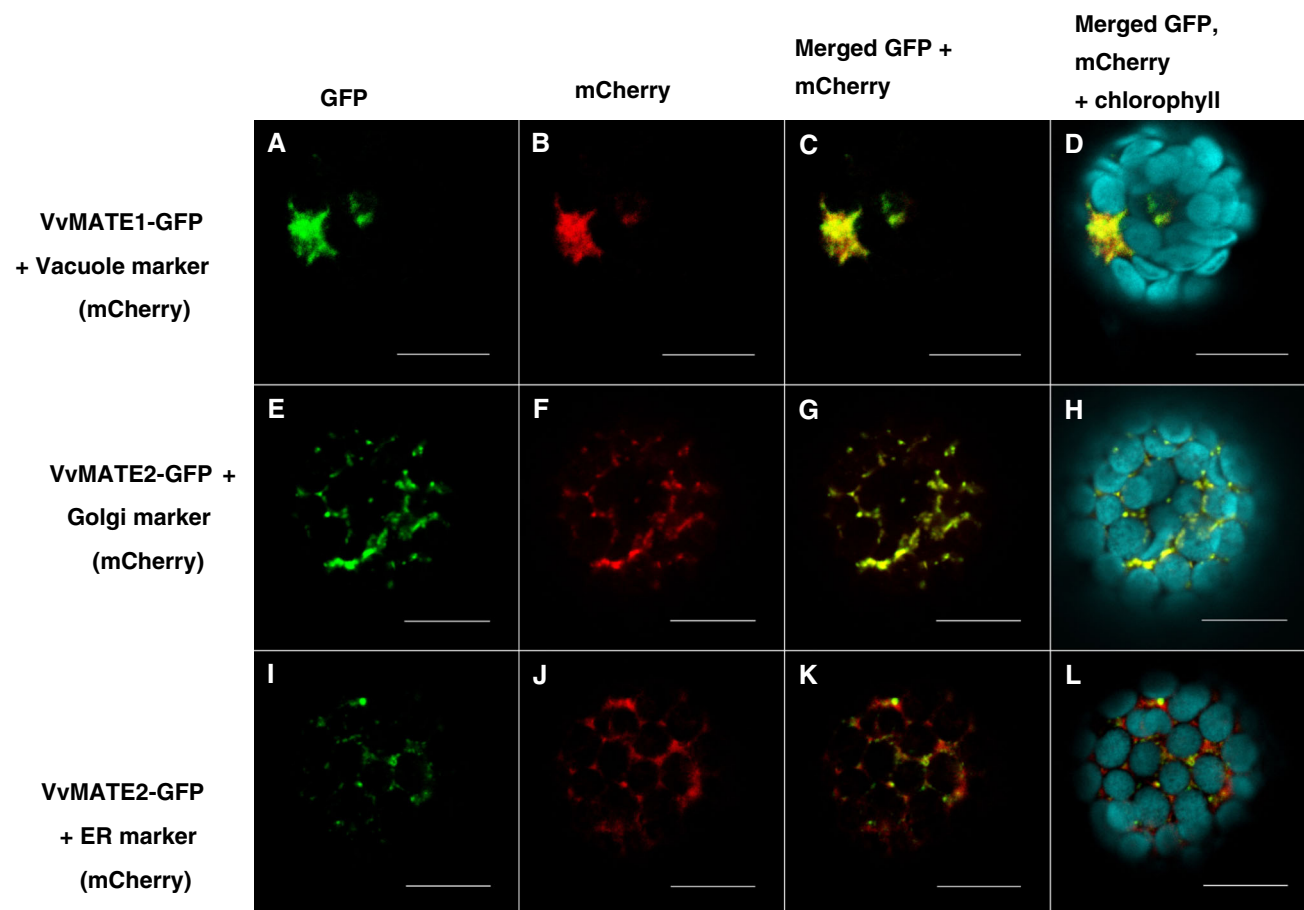

Fig. 4 Subcellular localization of VvMATE1 and VvMATE2 proteins. Fluorescent microscopy images presenting subcellular localization of VvMATE1 and VvMATE2 fusion proteins and organelle markers in transiently transformed Arabidopsis protoplasts. (**a**–**d**) Co-expression of *MATE1-GFP* with a vacuole marker (mCherry). (**e**, **f**) Co-expression of *MATE2-GFP* with the Golgi marker (mCherry). (**i**–**l**) Co-expression of *MATE2-GFP* with the ER marker (mCherry). (D, H and L) Merged fluorescence of GFP, mCherry plus chlorophyll (*light blue*) autofluorescence. *Yellow* denotes co-localization. *Bars* in all pictures are 15 μm
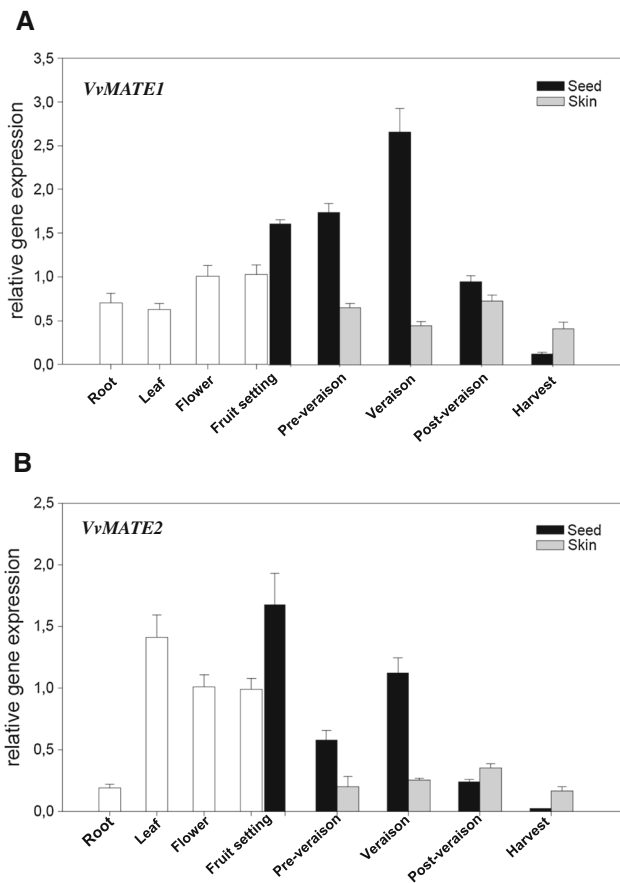
Fig. 5 Expression analysis of *VvMATE1* (**a**) and *VvMATE2* (**b**) in vegetative tissues and reproductive tissues during berry development. Seeds (*black bars*) and skins (*gray bars*) from pre-veraison were analyzed separately and only whole berries (*white bar*) and seeds at the fruit set stage were analyzed. Gene expression of *VvMATE1* and *VvMATE2* is relative to *VvGAPDH* and data are means of three biological replicates with *error bars* indicating SD

MATE genes. Additionally, the MYB4 binding motif (AACTACC), which is a target of the transcriptional repressor AtMYB4, was also identified. These elements have been related to dehydration response and to regulation of phenylpropanoid biosynthetic genes. The analysis also revealed differences in the type, number and position of the MYB target elements among the promoters. *VvMATE1* only presents the MYB1AT motif at positions −603, −1,086 and −1,317 from the translation start codon. Conversely, *VvMATE2* contains 1–3 copies of all five above-mentioned MYB target sites, whereas *TT12* presents four of them (Fig. 6). The *MYB4* binding site was more abundant in the promoter of *VvMATE2* (four copies) than in *TT12* (3 copies) and *VvMATE1* (two copies).

Furthermore, the analysis led to the identification of organ/tissue specific elements. For example, various *cis*-elements found in promoters of embryo- and seed-specific genes were present in the *TT12, VvMATE1* and *VvMATE2* promoters such as the GCN4 core, ACGT, and AACA, which are conserved in seed storage protein genes, mainly in monocot species (Müller and Knudsen 1993; Wu et al. 2000). However, the DPBF motif (ACACNNG) which is mainly embryo specific (Kim et al. 1997) was only present in the promoters of *VvMATE1* (−18, −432, −534) and *TT12* (−379) (Fig. 6). Specific elements involved in hormonal responsiveness were also detected. The *Arabidopsis TT12* promoter presents both ABRE and CGTCA elements, involved in ABA and MeJA responsiveness, respectively. In contrast, the promoter of *VvMATE1* contains only an ABRE element (at the position −872), whereas *VvMATE2* only has the cis-regulatory element involved in the MeJA responsiveness at positions −28 and −463 from the ATG codon (Fig. 6).
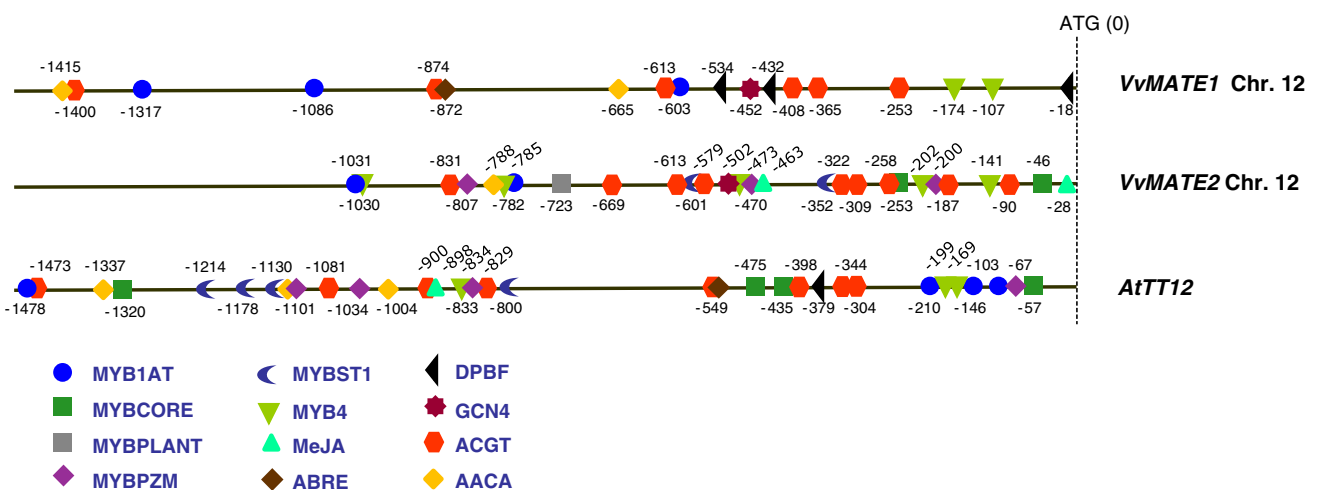

Fig. 6 In silico analysis of the promoter region of *VvMATE1*, *VvMATE2* and *AtTT12*. MYB- and MYC-binding *cis*-motifs were detected in the sense coding strand of a 1,500-bp upstream region from the start codon using the PLACE and PLANTCARE databases

Discussion

Two models have been proposed for the synthesis, transport, polymerization and accumulation of PAs in plants. In the first, the classic model: synthesis of PAs precursors is performed on the cytoplasmic face of rough endoplasmic reticulum by a multienzymatic complex (Winkel 2004), and then transported to different cellular compartments such as the vacuole, the nucleus or the apoplast, either by ER-derived vesicles or by specific membrane transporters (Debeaujon et al. 2003; Kitamura et al. 2004; Saslowsky et al. 2005; Zhao and Dixon 2009; Zhao et al. 2010). A second model, recently described, proposes that in chlorophyllous organs of Tracheophyta, including *Vitis vinifera*, the synthesis of PAs precursors occurs in chloroplasts, and their polymerization takes place inside chloroplast-derived organelles (tannosomes). Several tannosomes are transported towards the vacuole by structures called tannosome shuttle and then incorporated by invagination of the tonoplast (Wang et al. 2010; Brillouet et al. 2013). To gain insight on the PAs transport and accumulation in grapevine fruits, the characterization of two putative PAs transporter encoding genes identified in the grapevine genome was performed.

VvMATE1 and *VvMATE2* encode putative PA-transporting MATE proteins

In the *Vitis vinifera* genome, 65 virtual genes that encode MATE-like proteins have been reported, however, only few of them have been functionally characterized. The anthoMATE proteins AM1 and AM3 have been associated to acylated anthocyanin transport into the vacuole (Gomez et al. 2009, 2011. As far as we know, no MATE proteins have been characterized as PA or flavonoid transporters in grapevine. Interestingly, Terrier et al. (2009) reported the induction of GSVIVT00018839001 (named *VvMATE1* in our study) after the ectopic expression of the transcription factor VvMYBPA1 in grapevine hairy roots. In this work, we described features that point to establish the identity of VvMATE1 and a related protein, VvMATE2, as PA-transporting proteins in *Vitis vinifera*. First, VvMATE1 and VvMATE2 are the most closely related grapevine proteins to *Arabidopsis* TT12 and other known PA transporters (Fig. 2; Debeaujon et al. 2001; Zhao and Dixon 2009; Gomez et al. 2009; Frank et al. 2011). Second, the phylogenetic analysis distinguished these VvMATEs from the clade containing anthocyanin transporters (Fig. 1). Third, VvMATE1 and VvMATE2 present the two characteristic MatE domains and share similar membrane topology with PA-transporting MATE proteins (Debeaujon et al. 2001; Frank et al. 2011).

Distinct subcellular localization of VvMATE1 and VvMATE2 may imply different roles in PA accumulation

Following flavonoid synthesis in the cytoplasm, the products are stored mainly in the vacuole and in the cell wall (Kitamura 2006). Most of plant MATE proteins characterized so far appear to be targeted to the vacuolar or plasma membranes (Uhde-Stone et al. 2005; Marinova et al. 2007; Gomez et al. 2009; Zhao and Dixon 2009). The in silico prediction of the tonoplast localization of VvMATE1 was confirmed when the VvMATE1–GFP fusion protein co-localized with a tonoplast marker, strongly suggesting a role as a vacuolar transporter (Fig. 4). This result is highly consistent with the vacuole being an organelle that plays an important role in flavonoid sequestration and storage (Debeaujon et al. 2001). It was interesting to observe that the VvMATE1–GFP signal was only found in the vacuolar membrane and not in ER-derived vesicles as suggested for MATE1 of *M. trunculata* in tobacco leaf epidermal cells (Zhao and Dixon 2009). Flavonoids/PAs contained in cytoplasmatic prevacuole-like vesicles can end in the vacuole after vesicular fusion (Abrahams et al. 2002). This type of ER-derived anthocyanin-filled vesicles has also been found in grapevine hairy roots cells (Gomez et al. 2011). Subcellular localization experiments showed that grapevine AM1 and AM3 are closely associated to ER-derived vesicle structures (Gomez et al. 2011). Therefore, studying the transport activity, transport mechanism and substrate specificity of VvMATE1 and also determining its precise localization in cells that accumulate PAs in grapevine may contribute to elucidate this controversy. In contrast, localization for VvMATE2 was predicted for the plasma membrane although the in vivo assay shows a Golgi-associated VvMATE2–GFP protein fusion (Fig. 4). While this organelle localization differs from that previously observed for all other MATE proteins transporting flavonoids, is coincident with a Golgi-localized MATE transporter that has been described in *Arabidopsis* (BCD1) and is involved in organ initiation and iron homeostasis under osmotic stress (Burko et al. 2011; Seo et al. 2012).

In grape fruit, unlike anthocyanins, PAs are accumulated not only in vacuoles but also in the cell wall where they are linked to polysaccharides in both seeds and skin (Gény et al. 2003). The mechanisms involved in PA transport from vacuole and/or cytoplasm to the apoplastic space are still unknown. In this regard, the localization of VvMATE2 in the Golgi complex provides a possible exocytic route for transporting PAs to the cell wall. The Golgi complex can be an intermediary organelle for the final plasma membrane destination of the transporter. Taking these observations into account, we hypothesize that VvMATE2 could

be involved in the transport of PAs/flavonoids to the apoplast. The absence of plasma membrane-associated VvMATE2 in our approach could be explained by the use of protoplasts derived from *Arabidopsis* leaves, since cells from this tissue do not accumulate PAs in their cell wall. Therefore, its localization in cells that accumulate flavonoids in the apoplast deserves further investigation.

VvMATE1 and *VvMATE2* have different expression patterns

In grapevine, PAs are produced and accumulate in leaves and in seeds and skin of the berries (Souquet et al. 1996). PA accumulation is initiated before flowering but is most active at flowering and at fruit setting (Downey et al. 2003; Bogs et al. 2005). In contrast, the synthesis and accumulation of PAs in leaves occur throughout leaf expansion (Bogs et al. 2005). Nonetheless, PA synthesis and accumulation can be increased when the plant is under abiotic or biotic stress (Peters and Constabel 2002; Dixon et al. 2005). In this context, the expression of both *VvMATE* genes is consistent with that expected for PA transporters. *VvMATE1* transcripts were detected at high levels in reproductive tissues, specifically in seeds during early berry development. The expression profile of *VvMATE1* is in agreement with that shown for *TT12* in *Arabidopsis*, which is expressed specifically in ovules and developing seeds (Fig. 5a; Debeaujon et al. 2001). The transcriptional profile of *VvMATE1* shows a good correlation with the expression of the transcription factors *VvMYBPA1* and *VvMYBPA2*, which have been shown to induce *VvMATE1* expression (Bogs et al. 2007; Terrier et al. 2009). In spite of that, analysis of its promoter did not show the presence of a MYBCORE *cis*-element which can be recognized by PA MYB transcription factors (MYBPAs) (Akagi et al. 2009). One possibility is that *VvMATE1* might be induced by a transcription factor target of MYBPAs. On the other hand, the higher expression of *VvMATE1* in seeds, similar to *TT12*, might be supported by the presence of the DPBF element (E-box) that has been shown to be mainly embryo specific (Kim et al. 1997). In contrast, *VvMATE2* mRNA accumulates primarily in seeds and leaves (Fig. 5b) and the MYBCORE elements in its promoter can account for its expression in seeds. Similarly, its transcription profile in leaves resembles that of *VvMYBPA2* in the same tissue (Terrier et al. 2009; Fig. 6). The over-representation of MYB-binding elements in the promoter of *VvMATE2* compared to that of *VvMATE1* might be an important determinant of the differential transcriptional regulation among *MATE* genes.

Comparative analysis of the promoters of *VvMATE1*, *VvMATE2* and *TT12* suggests a possible differential hormonal regulation mediated by ABA and MeJA. The involvement of ABA in the biosynthesis and accumulation of PAs has been described in grapevine and other crops. A seasonal ABA signal and a bZIP transcription factor, DkbZIP5, which act as an ABRE-binding protein, are responsible for controlling PA levels in *Diospyros kaki* (Akagi et al. 2012). Also, exogenous ABA treatment seems to affect PA biosynthesis in the earlier developmental stages of grape fruit skin, with expression changes in at least two Myb transcription factors, *VvMYB5* and *VvMYBPA1* (Lacampagne et al. 2010). On the other hand, the MeJA signal has been associated with defense responses to pathogen and wounding, where PAs play an important role, especially in the defense against herbivores (Harborne and Grayer 1993; Peters and Constabel 2002). Therefore, a MeJA-responsive motif in the promoter of *VvMATE2*, which is also expressed in leaves, was not unexpected. However, additional examination is needed to determine the functionality of these *cis*-elements in the *VvMATE* genes.

Regarding the two models proposed to PAs transporter and accumulation in plants, results presented in this article suggest that both models coexist in grapevine. The two putative PAs transporters, VvMATE1 and VvMATE2, appear to be involved in the transport and distribution of PAs in the different compartments of the seed cells in grape fruits. In this context, our results are in agreement rather with the classic model than with the recently proposed model which does not consider specific membrane transporters for accumulation of PAs in the vacuoles or for PAs distribution to other cellular compartments as nucleus or apoplast.

Materials and methods

Plant material

Grapevine (*Vitis vinifera* L. var. Carménère) plants grown in field conditions in a commercial vineyard in the Maule Valley (Central Chile) during the 2009–2010 growing season were used in this study. Random sampling of different organs was performed including roots and leaves and starting at early flowering until mature fruit stage (from October to April). Stages to be sampled were defined according to the modified Eichhorn-Lorenz System (Coombe 1995): flowers (EL 23), setting (EL 29–31), pre-veraison (EL 33), veraison (EL 35), post-veraison (EL 36) and mature (EL 38). The skin samples were only readily separated from the rest of the berry starting at the pre-veraison stage. Leaf samples were collected from fully expanded mature leaves. All collected samples were frozen in liquid nitrogen and stored at −80 °C until their use for RNA isolation. Sampling was carried out consistently at

approximately 10:00 a.m. to discard possible oscillations in transcript levels.

RNA isolation and cDNA synthesis

RNA isolation was performed using 1–2 g of frozen tissue using the perchlorate method (5 M sodium perchlorate; 300 mM Tris–HCl pH 8; 1 % v/v SDS; 2 % v/v PEG 20,000; 8.5 p/v PVPP; 3 % v/v 2-mercaptoethanol) modified according to Davies and Robinson (1996). Three independent RNA extractions from each tissue were performed. RNA integrity was corroborated by formaldehyde agarose gel electrophoresis and their purity by OD260/280 ratio >1.95. Following DNase (DNAse I, Ambion) treatment of total RNA, first-strand cDNA synthesis was carried out from 2 µg of total RNA for each sample using oligo (dT) according to the manufacturer's instructions (AffinityScript QPCR cDNA Synthesis Kit, Stratagene, La Jolla, CA).

Phylogenetic analysis

The full-length amino acid sequences of *VvMATE1*, *VvMATE2* and other plant *MATE* were used as templates to perform a multi-alignment using the BioEdit Sequence Alignment Editor v7.0 software (Hall 1999). The potential transmembrane domains were predicted by the TMHMM program (Krogh et al. 2001). The phylogenetic tree was built from the resulting aligned sequences using the MEGA software (version 4; http://www.megasoftware.net) (Tamura et al. 2007) by the neighbor-joining method with bootstrap analysis of 1,000 replicates. The accession numbers were *Vitis vinifera* MATE1 (XP_002282907), MATE2 (XP_002282932), AM1 (ACN91542), AM3 (ACN88706); *Arabidopsis thaliana* AtTT12 (NP_191462), AtFRD3 (NP_187461); *Brassica rapa* BrTT12 (ACJ36213), *Malus domestica* MATE1 (ADO22709) and MATE2 (ADO22711); *Medicago truncatula* MATE1 (ACX37118) and MATE2 (HM856605); *Oryza sativa* TT12 (ABA99853); *Nicotiana tabacum* MATE1 (BAF47751), MATE2 (BAF47752) and JAT1 (CAQ51477); *Populus tricoparca* XP_002307572; *Hordeum vulgare* HvAACT1(BAF75822); *Sorghum bicolor* SbMATE (ABS89149).

Genetic constructions and transient expression assay in *Arabidopsis* protoplasts

The coding sequence of the target genes was amplified from cDNA of berries (cv. Carmenere) using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) and the following pair of primers: M1GFPfw: 5′-ACTCGAGATGGCCTCCGCA GCAGAG-3′ and M1GFPrv: 5′-ATCCATGGCTATCTTG TCTAGTACTAAATCCAAC-3′ for *VvMATE1*, and

M2GFPfw: 5′-ACTCGAGATGGGGTTCAGAAGAGTATC A-3′ and M2GFPrv: 5′-ATCCATGGCTACATCTTCCAA TAAGTTTAAATTCTC-3′ for *VvMATE2*. Forward and reverse primers included *Xho*I and *Nco*I restriction sites, respectively, and the reverse primers had the stop codon deleted. The resulting DNA fragments were cloned into pGEM-T vector (Promega, Madison, WI, USA) and then sequenced. The pAM1 vector used for transient transformation was derived from pGreen0029 and pA7-GFP (Katrin Czempinski, Potsdam University, Germany) vectors, carrying an enhanced version of CaMV 35S promoter. The target genes were inserted between the *Xho*I and *Nco*I sites of the pAM1 vector upstream of the *GFP* gene, which creating *VvMATE:GFP* fusions. The final constructions were also confirmed by sequencing. The following organelle markers for vacuole, ER and Golgi were used respectively: V-rk, ER-rk and G-rk (Nelson et al. 2007). All organelle markers contained the *mCherry* reporter gene. The original constructs were reduced in size by cloning them into the pAM1 vector (Ryngajllo et al. 2011) using the following restriction sites: *Hind*III and *Xma*I for the vacuole and ER marker constructs, and *Hind*III and *Xba*I for the Golgi marker construct. These constructs were used in co-transformation experiments along with *VvMATE1* and *VvMATE2*. Protoplasts obtained from 5-week-old *Arabidopsis thaliana* Col-0 plants by the tape-Arabidopsis sandwich method (Wu et al. 2009) were transformed with the different plasmids. The transformation was done as described in Wu et al. 2009 with prolonged incubation time of 15 min in PEG solution. The transformed *Arabidopsis* protoplasts were visualized 24 h after transformation using a confocal laser scanning microscope (TCS SP2/UV, Leica, Germany). The instrument was equipped with Argon and He/Ne lasers, beam splitter 488/543/633 and the following objectives: PL APO 20×, 63× W. Two different filter settings were used: (1) for GFP fluorescence, excitation with 488 nm, beam splitter DD 488/568 (double dichroic, reflects at 488 and 568 nm) and barrier filter BP 530 (band pass, 515–545 nm); (2) for mCherry, excitation at 568 nm, beam splitter DD 488/568 and barrier filter BP 590 (long pass > 590 nm). Chlorophyll autofluorescence was detected at 683–747 nm. During image acquisition, each line was scanned twice and averaged. Image analyses were performed with the Leica Confocal Software of TCS SP2 (version 2.61. build 1,537).

Gene expression analyses

Gene transcript levels were measured by quantitative PCR (qPCR) using a Stratagene Mx3000P (Agilent Technologies) system. All reactions were performed using the Brilliant SYBR Green Master Mix (Stratagene) according to the manufacturer. For each sample, qPCRs were carried out in triplicate (technical repeats) using 10 µl Master Mix,

0.5 µl of 250 nM primers, 1 µl of diluted cDNA and nuclease-free water to a final volume of 20 µl. Fluorescence was measured at the end of each amplification cycle. Amplification was followed by a melting curve analysis with continuous fluorescence acquisition during the 55–95 °C melt. The raw data were manually analyzed and expression was normalized against *VvGADPH* (accession number CN938023), which presents constitutive expression in vegetative tissues and reproductive tissues during berry development (Almada et al. 2009; Gainza-Cortés et al. 2012). The primers used for qPCR analysis were MATE1-F, 5′-GGAGATTCAAATAGAAGATGCCCAA G-3′ and MATE1-R, 5′-AACTAACCAACTTCCACCAA CTCG-3′; MATE2-F, 5′-TCTGCCTTTGAGTGAAGAAT GTAA-3′ and MATE2-R, 5′-AAGCCACAACAATACTA GAAGAAC-3′; GAPDH-Fwd, 5′-TTCCGTGTTCCTACT GTTG-3′ and GAPDH-Rev, 5′-CCTCTGAC- TCCTCCTT GAT-3′.

In silico analysis of promoters

Analysis of cis-regulatory elements in the *VvMATE1*, *VvMATE2* and *AtTT12* promoter regions was performed using the PLACE (http://www.dna.affrc.go.jp/PLACE/sig nalscan.html; Higo et al. 1999), PlantCARE (http://www. bioinformatics.psb.ugent.be/webtools/plantcare/html; Rombauts et al. 1999) and PlantPAN (http://www.plantpan.mbc. nctu.edu.tw/seq_analysis.php; Chang et al. 2008) databases.

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