Chapter 2

A novel cation-dependent O-methyltransferase involved in anthocyanin methylation in grapevine

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This article has been published in Plant physiology. June 2009; 150:2057-2070

Abstract

Anthocyanins are major pigments in coloured grape berries and most of them are mono- or di-methoxylated. We report here the functional characterization of an anthocyanin O-methyltransferase (AOMT) from Vitis vinifera L. The expression pattern in two cultivars with different anthocyanin methylation profiles (Syrah and Nebbiolo) showed a peak at start ripening (véraison), when the concentration of all methylated anthocyanins begins to increase. The purified recombinant AOMT protein was active on both anthocyanins and flavonols in vitro, with Km in the micromolar range, and was dependent on divalent cations for activity. AOMT showed a preference for 3',5' methylation when a 3',4',5' hydroxylated anthocyanin substrate was tested. In order to assess its in planta activity, we performed transient expression of AOMT in tobacco leaves expressing the PAP1 transcription factor from Arabidopsis thaliana. PAP1 expression in leaves induced the accumulation of the non methylated anthocyanin delphinidin 3-rutinoside. The co-expression of PAP1 and AOMT resulted in an accumulation of malvidin 3-rutinoside. We also showed that AOMT localized
exclusively in the cytoplasm of tobacco leaf cells. These results demonstrate the ability of this enzyme to methylate anthocyanins both in vitro and in vivo, indicating that AOMT play a major role in anthocyanin biosynthesis in grape berries.

**Introduction**

Methylation by 3-adenosyl-L-methionine (SAM)-dependent O-methyltransferases (OMTs) (EC 2.1.1) is a common modification in plant secondary metabolism, which modulates the physiological properties and the chemical reactivity of phenolic groups (Zhu et al., 1994). Several classifications of plant OMTs have shown that they can be categorized into two major classes (Joshi and Chiang, 1998; Noel et al., 2003; Lam et al., 2007). In the nomenclature proposed by Noel et al. (2003), type 1 OMTs consist of homodimeric OMTs with subunit sizes of about 38-43 kDa, that do not require divalent cations for activity. This large family includes caffeic acid, flavonoid, coumarin, and alkaloid OMTs (Frick and Kutchan, 1999; Dong et al., 2003). Type 2 OMTs represent a group of lower molecular weight (23 to 27 kDa) and cation-dependent OMTs. Most members of this family have been shown to be specific for Coenzyme A (CoA) esters of phenylpropanoids, and they have been suggested to be key enzymes in lignin biosynthesis (Ye et al., 1994). Among flavonoids alone, hundreds of O-methylated compounds have been characterized, ranging from mono- to poly-methylated compounds and belonging to the chalcone, flavone, isoflavone, flavonol, and anthocyanin families (Wollenweber and Dietz, 1981; Markham, 1989; Andersen and Markham, 2006).

Anthocyanins are responsible for the characteristic red, blue, and purple colours of many plant tissues (Harborne and Williams, 2000). They play important roles as pigments of flowers and fruits to attract animals for pollination and seed dispersion, and act as protectants against UV irradiation (Winkel-Shirley, 2001). Anthocyanins contribute to the distinctive colour and the aesthetic quality of many plant-derived products such as wine. In addition to their direct role in red wine colour, anthocyanins also participate in the organoleptic and chemical attributes of wine, because of their interaction with other phenolic and aroma compounds, as well as with proteins and polysaccharides (Mazza and Miniati, 1993).

Red grape (Vitis vinifera L.) varieties usually accumulate 3-O-monoglucoside, 3-O-acetylg glucoside, and 3-O-p-coumaroylglucoside derivatives of five anthocyanidins: delphinidin, cyanidin, peonidin, petunidin and malvidin. Total amount of anthocyanins and relative abundance of single anthocyanins are extremely variable among red- to blue-skinned cultivars, however mono- and di-methylated derivatives are usually largely predominant (Mazza and Miniati, 1993). Methylation is an important aspect of grape
anthocyanin biosynthesis as anthocyanin O-methylation has a slight reddening effect, and is known to reduce the chemical reactivity of phenolic hydroxyl groups (Sarni et al., 1995). This modification is proposed to increase the stability of anthocyanins and modify their water solubility thus significantly contributing to the accumulation of colour, a pivotal quality parameter for the red wine industry. Anthocyanin methylation in grapes is affected by genetic factors, malvidin 3-glucoside being the predominant anthocyanin in most cultivars and peonidin 3-glucoside in a more restricted group of genotypes (Mattivi et al., 2006), as well as by environmental and cultural conditions (Downey et al., 2006).

Although many structural and regulatory genes involved in anthocyanin biosynthesis have been cloned from a wide variety of plant species (Mol et al., 1998; Forkmann and Heller, 1999; Winkel-Shirley, 2001), genes responsible for the formation of methylated anthocyanins remain to be characterized. Genetic analyses of anthocyanin biosynthesis in Petunia hybrid showed that two pairs of duplicate genes, Mt1/Mt2 and Mf1/Mf2, were responsible for the methylation of the anthocyanin molecule at the 3'- and 5'- positions, respectively (Wiering and de Vlaming, 1977). Genetic relationships among these 4 genes suggested that, if one or both the Mt-genes were dominant, mainly 3'-O-methylated anthocyanins (peonidin or petunidin) accumulated in the flower. The 3',5'-O-methylated malvidin accumulated as the main pigment only when one or both the Mf-genes were dominant. However, additional investigation of the relationship between the 4 methylation genes and anthocyanin O-methyltransferase activity in vitro revealed that both the Mt-enzymes and the Mf-enzymes were capable of methylating the oxygen in 3'- and the 5'-positions of the anthocyanin molecule (Jonsson et al., 1983). The same authors could then separate the 4 different anthocyanin O-methyltransferases in Petunia flower extracts, using chromatofocusing techniques (Jonsson et al., 1984). A detailed characterization of these enzymes showed that they exhibit different kinetic properties and that their combination reflects the methylation patterns of anthocyanins in flowers in different lines of Petunia hybrid. However, the genes encoding these anthocyanin O-methyltransferases from Petunia have not been cloned to date.

Bailly et al. (1997) partially purified an OMT from anthocyanin-producing grape cell suspensions. This enzyme could methylate cyanidin 3-O-glucoside, but showed no activity with cyanidin 3-p-coumaroyl-O-glucoside, suggesting that methylation occurred before acylation. Furthermore, the lack of OMT activity when delphinidin was used as a substrate indicated that more than one OMT may be involved in anthocyanin methylation in grape (Bailly et al., 1997). EST and grapevine genome sequencing (Da Silva et al., 2005; Jaillon et al., 2007) have identified a large number of putative OMTs, and expression of some of them is correlated with accumulation of methylated anthocyanins in berries (Ageorges et al., 2006; Castellarin et al., 2007). In this work, we describe the identification and the biochemical
Characterization of a novel Mg\(^{2+}\)-dependent anthocyanin O-methyltransferase from *Vitis vinifera* L.. Combining *in vitro* characterization of recombinant AOMT enzyme with *Agrobacterium*-mediated transient transformation of tobacco (*Nicotiana benthamiana*), we show that this grapevine AOMT catalyzes both 3’ and 5’ O-methylation of anthocyanins *in vitro* and *in planta*.

**Results**

**Isolation of a Candidate Anthocyanin O-Methyltransferase cDNA from Grapevine**

Recently, a high throughput transcriptomic screening identified a set of genes co-expressed during anthocyanin biosynthesis in grape (Ageorges et al., 2006). One of these genes, corresponding to the partial grape EST BQ796057, was highly similar to the multifunctional phenylpropanoid and flavonoid OMT (PFOMT) from *Mesembryanthemum crystallinum* (Ibdah et al., 2003), suggesting that this gene could be involved in anthocyanin methylation. BLAST search of *Vitis* databases using the grapevine EST BQ796057 as the query sequence identified three overlapping ESTs, named EC932040.1, EC953324.1 and CF603338.1. Their sequences were assembled in a contig and the full length cDNA was amplified by RT-PCR from mature berries (cv. Syrah). The predicted amino acid sequence of this candidate AOMT cDNA was the same in the two cultivars and corresponded to a 26.4 kDa protein, which contained a conserved domain identified as Methyltransf_3: O-methyltransferase (pfam 01596). The candidate AOMT belonged to the type 2 family of low molecular weight and cation-dependent OMTs. Most members of this family have been shown to be active with CoA esters of phenylpropanoids. However, some type 2 OMTs are also active with flavonoids and/or phenylpropanoid conjugates (Ibdah et al., 2003; Kim et al., 2006; Lee et al., 2008). The candidate AOMT presented 56 % and 54 % identity with PFOMT from *M. crystallinum* (Ibdah et al., 2003) and caffeoyl-CoA-OMT from grapevine (Busam et al., 1997), respectively (Figure. 1A). Phylogenetic analysis of selected members of the plant OMT gene family showed that the grapevine AOMT belongs to a small clade of type 2 OMT family, together with the flavonoid-OMTs PFOMT from *M. crystallinum* and OMT1 from *Arabidopsis thaliana* (Figure. 1B).
Characterization of grape methyltransferase

Figure 1. Comparison of Vitis AOMT with other OMTs. (A) The predicted amino acid sequence of AOMT from V. vinifera (VvAOMT) was aligned with the amino acid sequences of PFOMT from Mesembryanthemum crystallinum (McPFOMT) and caffeoyl-CoA-OMT from V. vinifera (VvCCoAOMT), using Clustal W. Residues shaded in grey indicate identical amino acids. (B) Phylogenetic tree of selected OMT cDNA sequences. Type 1 OMTs include caffeic acid-OMT from V. vinifera (VvCOMT, AF239740) and Nicotiana tabacum (NtCOMT, AF484252), tricetin-OMT from Triticum aestivum (TaOMT2, DQ223971), flavonoid OMTs from Catharanthus roseus (CrOMT2, AY127568) and Chrysosplenium americanum (CaOMT2, U16793; flavonol OMTs from Arabidopsis thaliana (AtFolOMT, U70424) and Oryza sativa (OsFolOMT1, DQ288259). Type 2 OMTs (in bold characters) include caffeoyl-CoA-OMT from V. vinifera (VvCCoAOMT, Z54233), N. tabacum (NtCCoAOMT, U38612) and Populus trichocarpa (PcCCoAOMT, AJ224896), flavonol OMT from O. sativa (OsFolOMT2, XM_483167), AOMT from V. vinifera (VvAOMT, accession number FJ460168), PFOMT from M. crystallinum (McPFOMT, AY145521) and flavonoid OMT from A. thaliana (AtOMT1, AY087244). The numbers beside the branches represent bootstrap values based on 500 replicates.
Characterization of grape methyltransferase

Analysis of AOMT Gene Expression during Grape Berry Development

Transcriptomic analyses have shown that AOMT gene expression was induced during anthocyanin accumulation in grape berries, leading to the hypothesis that AOMT could be involved in anthocyanin methylation (Ageorges et al., 2006; Castellarin et al., 2007). In order to gain insight into AOMT gene regulation, Syrah and Nebbiolo grape berries were harvested at different ripening stages and analysed for both anthocyanin content and AOMT gene expression (Fig. 2). No anthocyanin was detected before véraison, the onset of ripening. From this stage on, anthocyanins started to accumulate in berry skin. Malvidin accumulated in Syrah berries as the major aglycone, reaching 1.12 mg equivalent malvidin per berry (69 % of total anthocyanins) in fully mature berries (Fig. 2A). Peonidin content increased significantly during Syrah berry development, reaching 17 % of total anthocyanins (Fig. 2A). In Syrah berries, AOMT transcripts were hardly detectable before the onset of ripening (Fig. 2C). After véraison, AOMT expression level increased dramatically during the first weeks and then the expression decreased and remained stable until the end of ripening (Fig. 2C). In Nebbiolo berries, the accumulation of anthocyanins followed the same developmental pattern as in Syrah berries (Fig. 2B). In Nebbiolo the main anthocyanin was the mono-methylated peonidin 3-O-glucoside, which accumulated at concentrations about two-fold higher than the sum of petunidin 3-O-glucoside and malvidin 3-O-glucoside (Fig. 2B). However, the expression of AOMT in this variety followed the same profile as it did in Syrah (Fig. 2D). Given the fact that AOMT resembled the previously characterized caffeoyl-CoA-OMT (CCoA-OMT) (Busam et al., 1997), the expression of the CCoA-OMT gene was investigated during grape berry development, as a comparison (Supplemental Fig. S1). Unlike AOMT, the CCoA-OMT gene was strongly expressed in berries a few weeks after flowering, and then its expression decreased rapidly until véraison. During the ripening process, CCoA-OMT transcript accumulation was very weak in grape berries.

AOMT gene expression was then evaluated in several tissues of Syrah. In vegetative organs, AOMT transcripts could be detected at very low levels in young leaves (Fig. 3). Before véraison, AOMT gene expression was barely detectable in the different berry tissues, although a weak expression could be detected in seeds at green stage (Fig. 3). During ripening, AOMT transcripts were mainly detected in berry skin, where anthocyanin biosynthesis takes place. As a summary, the expression of AOMT appeared rather fruit-specific with a coincidental timing corresponding with the accumulation of anthocyanin pigments.
Figure 2. Accumulation of anthocyanins during (A) Syrah and (B) Nebbiolo berry development. The arrow indicates the véraison, the onset of ripening. Quantitative real-time PCR expression profiling of AOMT during (C) Syrah and (D) Nebbiolo berry development. Expression values have been normalized with VvEF1alpha. Key: Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin.

Figure 3. Quantitative real-time PCR expression profiling of AOMT in various grapevine organs (cv. Syrah) and in different tissues of berry at 3 stages of development (cv. Syrah). Expression values have been normalized with VvEF1alpha and expressed as relative abundance.
Characterization of Recombinant Anthocyanin O-methyltransferase

The AOMT coding sequence was cloned into the Gateway compatible entry vector pDONR207, and then transferred into the destination vector pHNGWA (Busso et al., 2005) to express the corresponding protein in *E. coli* as a His-tagged NusA fusion protein. The fusion protein was purified by metal affinity chromatography and cleaved on the resin with thrombin to yield purified recombinant AOMT (Supplemental Fig. S2). The activity of AOMT enzyme was then tested *in vitro* with a number of potential substrates (Table 1, Fig. 4), including flavan 3-ols, flavonols, anthocyanidins and anthocyanins in the presence of SAM.

![Chemical structures of some flavonoid compounds. In bold, the flavonoids assayed as substrates for AOMT. Key: glucoside](image)

**Figure 4.** Chemical structures of some flavonoid compounds. In bold, the flavonoids assayed as substrates for AOMT. Key: glucoside

**Table I.** Kinetic parameters of AOMT with potential substrates. Data are expressed as the means of triplicates assays and standard errors are indicated between brackets; n.d.: not determined.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Kcat. 10^-3 (s^-1)</th>
<th>Kcat/Km (M^-1.s^-1)</th>
<th>Specific activity (pkat.mg^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pelargonidin 3-glucoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>12 (3)</td>
</tr>
<tr>
<td>cyanidin 3-glucoside</td>
<td>43 (3)</td>
<td>90 (9)</td>
<td>1964 (240)</td>
<td>1700 (104)</td>
</tr>
<tr>
<td>delphinidin 3-glucoside</td>
<td>44 (6)</td>
<td>118 (12)</td>
<td>2470 (441)</td>
<td>2230 (133)</td>
</tr>
<tr>
<td>quercetin 3-glucoside</td>
<td>24 (3)</td>
<td>100 (5)</td>
<td>4250 (120)</td>
<td>1880 (67)</td>
</tr>
<tr>
<td>cyanidin</td>
<td>74 (2)</td>
<td>84 (2)</td>
<td>1135 (93)</td>
<td>1580 (55)</td>
</tr>
<tr>
<td>quercetin</td>
<td>33 (3)</td>
<td>112 (10)</td>
<td>3420 (80)</td>
<td>2120 (144)</td>
</tr>
<tr>
<td>myricetin</td>
<td>19 (2)</td>
<td>128 (5)</td>
<td>6830 (790)</td>
<td>2420 (89)</td>
</tr>
<tr>
<td>catechin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8 (3)</td>
</tr>
<tr>
<td>epicatechin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7 (3)</td>
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</table>
HPLC-DAD/ESI-MS was used to analyze reaction products obtained after incubation of the potential substrates without or with AOMT enzyme (Fig. 5). First, AOMT could not utilize flavan 3-ols (catechin and epicatechin) as substrates (Table 1). On the other hand, AOMT did not exhibit any methylation activity with the 4'-hydroxylated anthocyanin pelargonidin 3-O-glucoside (Fig. 5A, B). Indeed, HPLC analysis of the reaction products obtained with pelargonidin 3-O-glucoside detected one major peak, the unmodified substrate (98%) and also 2% of the corresponding aglycone, pelargonidin (Supplemental Table S1, Fig. 5A, B). On the other hand, over 50% of the cyanidin 3-O-glucoside substrate was converted into peonidin 3-O-glucoside identified by UV-visible and MS spectra (λmax 516 nm; m/z 463) (Fig. 5C, D). Delphinidin 3-O-glucoside underwent two sequential methylations on the oxygen at the 3' and 5' positions to give successively the petunidin 3-O-glucoside (λmax 525.6 nm; m/z 479) and malvidin 3-O-glucoside (λmax 527 nm; m/z 493) (Fig. 5E, F). Using anthocyanidin as substrate, AOMT was also able to methylate cyanidin leading to peonidin (λmax 523 nm; m/z 301) (Fig. 5G, H). A second product was detected (m/z 285) with an absorbance maximum at 487 nm (peak P1; Fig. 5H). This product was not identified, but may result from the unstability of anthocyanidins under experimental conditions (pH 7.5 in aqueous solution; Awika et al., 2004). In addition to anthocyanin and anthocyanidin substrates, AOMT could methylate the flavonol quercetin 3-O-glucoside to yield its 3'-O-methyl ether isorhamnetin (Fig. 5I, J). Detailed quantifications of reaction products are presented in Table S1. Taken together, these results indicated that recombinant AOMT catalysed in vitro the 3' O-methylation of anthocyanins and flavonols with a catechol B-ring (3',4' di-OH) and both 3' and 5' O-methylation for those showing a pyrogallol (3',4',5' tri-OH) B-ring.
Figure 5. Analysis of AOMT in vitro reaction products. HPLC/ESI-MS/DAD analysis of reaction products produced following incubation of potential substrates without (A, C, E, G, I) or with recombinant AOMT enzyme (B, D, F, H, J). Reactions were carried out for 60 min in a total volume of 200 µL, with 200 µM anthocyanin or flavonol substrates, 200 µM of SAM and 5 µg of purified AOMT. Substrates were the following: A, B, pelargonidin 3-glucoside; C, D, cyanidin 3-glucoside; E, F, delphinidin 3-glucoside; G, H, cyanidin; I, J, quercetin 3-glucoside. Anthocyanins were monitored at 520 nm and flavonols at 360 nm. Reaction products were identified according to their mass fragmentation, UV visible absorption spectra and retention time. The data shown are representative of three independent experiments. Key: Pl3G, pelargonidin 3-glucoside; Cy3G, cyanidin 3-glucoside; Pn3G, peonidin 3-glucoside; Dp3G, delphinidin 3-glucoside; Pt3G, petunidin 3-glucoside; Mv3G, malvidin 3-glucoside; Cy, cyanidin; Pn, peonidin; Q3G, quercetin 3-glucoside; Q, quercetin; IsoR3G, isorhamnetin 3-glucoside; IsoRh, isorhamnetin; P1, unknown product.
Kinetic analyses (Table I) confirmed that AOMT exhibited only background level of activity with pelargonidin 3-O-glucoside, catechin and epicatechin. AOMT exhibited equivalent kinetic parameters with cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, quercetin 3-O-glucoside and with the flavonol aglycones quercetin and myricetin (Table I). AOMT activity with cyanidin aglycone was slightly lower than with cyanidin 3-O-glucoside. Km values for these substrates ranged from 20 to 75 µM. Recombinant AOMT was active within a broad range of pH values, with an optimum around 7.5 on both substrates tested (cyanidin 3-O-glucoside and delphinidin 3-O-glucoside) (Supplemental Fig. S3). It retained over 25% of its activity at pH 9.4 and at pH 6.25, but activity was dramatically reduced when more acidic conditions were tested (Supplemental Fig. S3).

The influence of different divalent cations on AOMT activity was tested (Tables II, III). AOMT activity was extremely low in the absence of Mg$^{2+}$ ions, confirming the Mg dependency of AOMT (Table II). The best activity was obtained in presence of 10 mM of MgCl$_2$ (Table II). Incubation of AOMT in the presence of EDTA greatly reduced its activity with both cyanidin 3-O-glucoside and delphinidin 3-O-glucoside substrates. The presence of other divalent cations in addition to Mg$^{2+}$ strongly decreased activity in the case of Zn$^{2+}$ and Mn$^{2+}$, while little effects were observed with Ca$^{2+}$ (Table III).

Table II. In vitro AOMT activity in the presence of different MgCl$_2$ concentrations using cyanidin 3-O-glucoside as substrate and expressed as a percentage of the activity with 10 mM MgCl$_2$.

<table>
<thead>
<tr>
<th>% activity</th>
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<tbody>
<tr>
<td>MgCl2 (0 mM)</td>
</tr>
<tr>
<td>MgCl2 (2.5 mM)</td>
</tr>
<tr>
<td>MgCl2 (5 mM)</td>
</tr>
<tr>
<td>MgCl2 (10 mM)</td>
</tr>
</tbody>
</table>

Table III. In vitro AOMT activity in the presence of 10 mM MgCl$_2$ and 10 mM EDTA, ZnCl$_2$, CaCl$_2$ and MnCl$_2$ using cyanidin 3-O-glucoside and delphinidin 3-O-glucoside as substrates. Activity is expressed as a percentage of the activity with 10 mM MgCl$_2$.

<table>
<thead>
<tr>
<th>% activity</th>
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<tbody>
<tr>
<td>cyanidin 3-O-glucoside</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
</tr>
<tr>
<td>CaCl$_2$</td>
</tr>
<tr>
<td>MnCl$_2$</td>
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Characterization of Anthocyanin O-Methyltransferase Activity in Planta

Recombinant AOMT exhibited flavonol and anthocyanin O-methyltransferase activity in vitro. However, in vitro data should be taken with caution for the prediction of the physiological substrates and products of OMTs (Liu and Dixon, 2001). We used Agrobacterium-mediated transient transformation of Nicotiana benthamiana to investigate AOMT activity in planta. This approach allowed us to successfully characterize OMT activities in planta (Scalliet et al., 2006; Schmidlin et al., 2008). In order to provide AOMT with anthocyanin substrates, AOMT was co-expressed with PAP1 (Production of Anthocyanin Pigment1), an R2R3 MYB transcription factor from Arabidopsis thaliana involved in anthocyanin biosynthesis (Borevitz et al., 2000). Constitutive expression of PAP1 in A. thaliana and other plant species, including tobacco, petunia, and tomato, has been shown to induce anthocyanin accumulation, due to the coordinated up-regulation of genes in the anthocyanin biosynthetic pathway (Borevitz et al., 2000; Xie et al., 2006; Ben Zvi et al., 2008; Zuluaga et al., 2008).

No anthocyanin was detected in extracts from leaves expressing the Green Fluorescent Protein (GFP) control (Fig. 6A). As expected, Agrobacterium-mediated transient expression of PAP1 in N. benthamiana resulted in significant levels of anthocyanin accumulation in infiltrated leaves (Fig. 6B, C). The flavonoid composition of transformed tobacco leaves were then determined using both HPLC/ESI-MS/DAD and HPLC/DAD in order to compare the flavonoids produced in PAP1 leaves with those accumulated in leaves co-expressing PAP1 and AOMT. The metabolites were putatively identified from their UV-visible absorption spectra and comprehensive analyses of mass fragmentation patterns obtained by tandem MS spectroscopy were compared with those of known compounds.

Three anthocyanin pigments accumulated in tobacco leaves expressing PAP1 (Fig. 6B). Delphinidin 3-O-rutinoside was the most abundant anthocyanin amounting to approximately 96% of total anthocyanins in PAP1 leaves. The other two, petunidin 3-O-rutinoside and delphinidin 3-O-rutinoside O-hexoside were present in trace amounts (respectively peaks Pt3R, a1; Fig. 6B). Co-expression of PAP1 and AOMT resulted in a marked decrease in the delphinidin 3-O-rutinoside peak (more than 50%), together with an accumulation of malvidin 3-O-rutinoside and malvidin 3-O-glucoside (Fig. 6C). Two other discrete anthocyanins were detected in PAP1 + AOMT expressing tobacco leaves, presenting the mass-to-charge ratio of 773 and 801, respectively (peaks a2, a3; Fig. 6C). Fragmentation products (Table S2) identified delphinidin (m/z 303) and malvidin (m/z 331), as aglycone in the two compounds, respectively, with two sugar moieties attached to the anthocyanin, a rutinosine (neutral loss m/z 308) and a hexoside (neutral loss m/z 162) (Table S2). In leaves co-expressing PAP1 + AOMT, malvidin conjugates represented about 70% of total anthocyanins, and O-methylation of delphinidin conjugates was complete on both 3'
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and 5' positions, as no trace of petunidin conjugates could be detected in PAP1 + AOMT expressing tobacco leaves (Fig. 6C).

In addition to anthocyanins, two quercetin glycosides and two kampferol glycosides were identified in control tobacco leaves expressing GFP as well as in both PAP1 and PAP1 + AOMT expressing leaves (Fig. 6D, E, F). Quercetin O-rutinoside O-hexoside was the major flavonol detected in all the transformed tobacco leaves, accounting for approximately 70% of total flavonols in control leaves, and 50% in the leaves expressing PAP1. Nevertheless, the same composition in flavonol compounds were observed in leaves expressing PAP1 or co-expressing PAP1 and AOMT. Moreover, two hydroxycinnamic acids, caffeoyl quinic acid and its corresponding methylated form, feruloyl quinic acid, each with three isomers, were identified at 320 nm in 35S-GFP tobacco leaves as well as in both PAP1 and PAP1 + AOMT expressing leaves (peaks c1, c2, c3, f1, f2, f3; Fig. 6D, E, F). Two isomers of feruloyl quinic acids (f2, f3; Fig. 6D, E, F; Table S2) accumulated more in PAP1 and PAP1 + AOMT transformed leaves than in GFP control leaves. Taken together, these analyses indicated that co-expression of AOMT and PAP1 did not modify significantly the flavonol and phenolic acid profile, in comparison to leaves expressing PAP1 alone.

Subcellular Localization of AOMT

To investigate AOMT subcellular localization, a construct encoding AOMT fused to the N terminus of GFP (AOMT-GFP) was expressed in N. benthamiana using Agrobacterium-mediated transient transformation. Non-targeted GFP control and AOMT-GFP were co-expressed with PAP1 transcription factor, in order to investigate both the cellular localization and the enzyme activity of the AOMT-GFP fusion protein. Confocal microscopy imaging of tobacco cell expressing AOMT-GFP showed a cytosolic fluorescence pattern, very similar to that obtained with non-targeted GFP (Fig. 7). HPLC analysis of anthocyanins accumulated in tobacco leaves co-expressing PAP1 and AOMT-GFP (Supplemental Fig. S4) showed that the anthocyanin profile closely resembled that of leaves co-expressing PAP1 and AOMT (Fig. 6C). Taken together, these results indicated that AOMT-GFP fusion protein retained AOMT activity and was located in the cytosol of transformed cells.
Figure 6. Characterization of AOMT activity in planta using Agrobacterium-mediated transient transformation. Tobacco leaf sectors (200 mg FW) expressing GFP (A, D), PAP1 (B, E) or co-expressing PAP1 and AOMT (C, F) were excised 96 h after Agrobacterium-mediated transformation. Anthocyanin and flavonol contents were analysed using HPLC-DAD. Anthocyanins were monitored at 520 nm and flavonols at 360 nm. The data shown are representative of three independent experiments. Key: Dp3R, delphinidin 3-O-rutinoside; Pt3R, petunidin 3-O-rutinoside; Mv3G, malvidin 3-O-glucoside; Mv3R, malvidin 3-O-rutinoside; QRH, quercetin 3-O-rutinoside O-hexoside. Peak a1, delphinidin 3-O-rutinoside O-glucoside; peak a2, delphinidin 3-O-rutinoside O-hexoside; peak a3 malvidin 3-O-rutinoside O-hexoside. Peaks c1, c2, c3, isomers of caffeoyl quinic acid; peaks f1, f2, f3, isomers of feruloyl quinic acid. MS/MS spectra of Dp3R, Mv3R and Mv3G.
Figure 7. Subcellular localization of AOMT-GFP fusion protein. Transient expression of non-targeted GFP (A) and AOMT-GFP fusion protein (B) following agroinfiltration of tobacco leaves. Abaxial epidermal cells images are projections of 20 x 1µM optical sections collected by confocal laser scanning microscopy. Bars: 20 µM.

Discussion

Functional Characterization of a Candidate Anthocyanin O-Methyltransferase from Grapevine

Several recent transcriptomic studies suggested that a putative OMT could be involved in anthocyanin methoxylation in grape berries (Ageorges et al., 2006; Castellarin et al., 2007; Castellarin and Di Gaspero, 2007; Pilati et al., 2007; Cutanda-Perez et al., 2009). In order to characterize this gene, called hereafter AOMT, its cDNA was cloned from Syrah and Nebbiolo grape berries. Phylogenetic analysis of selected members of the plant OMT family showed that AOMT belonged to a subset of type 2 class of plant OMTs, and was distinct from the clade of classical CCoA-OMTs, presumably involved in lignin biosynthesis. In recent years, several OMTs active with flavonoid substrates have been characterized from different plant species, and especially OMTs catalyzing the 3' or 3', 5' O-methylation of the B-ring of flavonoid substrates. Interestingly, such OMTs have been characterized in both type 1 and type 2 plant OMT families (Muzac et al., 2000; Kim et al., 2006; Lee et al., 2008). Thus, AOMT represents a new member of a small subfamily of type 2 plant OMTs involved in the methylation of flavonoid substrates (Ibdah et al., 2003). Determination of AOMT kinetic parameters showed that Km values and specific activities for all flavonol and anthocyanin substrates tested were similar to values found for other OMTs with their primary substrate (Ibrahim et al., 1998). AOMT exhibited a strict specificity for 3’ and 5’ hydroxyl group methylation in both flavonol and anthocyanin substrates, showing no activity with pelargonidin 3-O-glucoside. Furthermore, AOMT showed roughly equivalent activity with
quercetin and quercetin 3-O-glucoside, and with cyanidin and cyanidin 3-O-glucoside, suggesting that AOMT could methylate efficiently both glycosylated and aglycone substrates. In grape berries, major anthocyanins are both glycosylated and methylated, raising the question of the order in which these two reactions occur in vivo. Ford et al. (1998) characterized an UDP glucose-flavonoid glucosyl transferase (UFGT) catalyzing the glycosylation of both anthocyanidin and flavonol substrates in vitro. However, UFGT enzyme exhibited a greatly reduced activity with malvidin aglycone compared to petunidin aglycone, even though malvidin 3-O-glucoside is usually the most abundant anthocyanin in grape berry. This suggests that anthocyanin glycosylation may occur prior methylation in grape berries.

In order to determine the subcellular localization of AOMT, an AOMT-GFP fusion protein was expressed in tobacco. Like the non-targeted GFP control, the AOMT-GFP fusion protein exhibited a clear cytosolic localization, which is very likely to reflect the physiological localization of AOMT, as discussed below. These results suggest that both glycosylation and methylation of anthocyanins take place in the cytosol, although the precise in vivo chronology of these reactions remains to be determined. Several enzymes involved in flavonoid biosynthesis have been shown to be organized into macromolecular complexes optimizing the cooperation between its members (Burbulis and Winkel-Shirley, 1999; Liu and Dixon, 2001; Winkel, 2004). The cytoplasmic localization of AOMT-GFP fusion protein has led to the suggestion that AOMT may be associated with the ER-multienzyme complexes, facilitating rapid methylation and stabilization of its product. Glycosylation and methylation reactions may take place in such complexes ensuring an efficient biosynthesis of anthocyanins in vivo, although the activity of UFGT with some anthocyanin substrates is low in vitro (Ford et al., 1998).

**AOMT Gene Expression Profile Mirrors Anthocyanin Biosynthesis in Berry**

Five major anthocyanins exist in grape berries, which differ in their hydroxylation and methylation patterns at the phenolic B-ring. These anthocyanins include the non-methylated cyanidin (3',4'-OH) and delphinidin (3',4',5'-OH), the mono-methylated peonidin (3'-OCH3 4'-OH) and petunidin (3'-OCH3 4',5'-OH), and the di-methylated malvidin (3',5'-OCH3 4'-OH), pelargonidin (4'-OH) being notably absent in all known *Vitis* genotypes (Macheix et al., 1990). The expression of the *AOMT* gene was monitored in two genotypes, characterized either by high total anthocyanin content and predominance of the di-methylated malvidin (Syrah; Mattivi et al., 2006) or by low anthocyanin content and predominance of the mono-methylated peonidin (Nebbiolo; Guidoni et al., 2008). *AOMT* gene was expressed in both varieties, exhibiting similar expression profiles, with a maximum at the onset of ripening, when methylated anthocyanins start to accumulate. Castellarin and Di Gaspero (2007)
reported similar expression profiles for this OMT gene during grape berry development in other cultivars presenting different anthocyanin composition. The evolution of the transcriptional level of AOMT through ripening and the relative abundance of methoxylated anthocyanins is compatible with a major role in anthocyanin methylation in grape varieties, irrespective of their different anthocyanin composition.

While the expression pattern and the substrate specificities of AOMT correlated well with the anthocyanin profiles found in grape berries, the situation was less clear for flavonols. Similarly to anthocyanins, di- or tri-hydroxylated flavonol compounds on B-ring (e.g. quercetin and myricetin) can be mono-methylated (quercetin to isorhamnetin and myricetin to laricitrin) or di-methylated (myricetin to syringetin). The compared study of anthocyanins and flavonols methylation patterns over different grape genotypes showed that the ratio of flavonol mono-methylation vs. total flavonol was positively correlated with the ratio of anthocyanin mono-methylation vs. total anthocyanin, and the same was true for di-methylation (Mattivi et al., 2006). These results would agree with a model where AOMT would also operate on flavonols, with the same methylation patterns as on anthocyanins. The AOMT enzymic activity measured in vitro on both substrates corroborates this hypothesis. However, in grapevine flavonols and anthocyanins accumulate at different timepoints in the berry skins: flavonols accumulate at the early stage of development and during ripening, while anthocyanins accumulate only during ripening (Downey et al., 2003). In this context, it is expected that the gene encoding the flavonol methyltransferase would have a different expression pattern from the AOMT gene. Furthermore, Castellarin and Di Gaspero (2007) have shown that AOMT gene is not expressed in Pinot gris, although this white genotype was shown to contain methylated flavonols (Mattivi et al., 2006). The possibility that an alternative flavonol–OMT is active in grape berries is thus still open.

Co-expression of PAP1 and AOMT in Tobacco Leaves Induces the Accumulation of Methylated Anthocyanins

In this work, we used tobacco leaves in which anthocyanin biosynthesis was induced by transient expression of the R2R3 MYB transcription factor PAP1 from A. thaliana to characterize the activity of AOMT in planta. Overexpression of PAP1 in transgenic plants was shown to enhance accumulation of lignin, hydroxycinnamic acid esters, and flavonoids, including various anthocyanins (Borevitz et al., 2000; Tohge et al., 2005). In the species used in our study (N. benthamiana) the most abundant anthocyanin induced by the overexpression of PAP1 was delphinidin 3-O-rutinoside. The strong accumulation of this non-methylated anthocyanin in PAP1-expressing tobacco leaves allowed the investigation of AOMT activity in vivo. The malvidin 3-O-rutinoside accumulation following co-expression of PAP1 and AOMT in tobacco leaves confirmed that AOMT alone catalysed both 3’- and 5’-
methyltransferase

Characterization of grape methyltransferase

methyltransferase

Characterization of grape methyltransferase

methylation steps of delphinidin. The predominance of malvidin 3-O-rutinoside indicates a

Characterization of grape methyltransferase

methylation steps of delphinidin. The predominance of malvidin 3-O-rutinoside indicates a
good efficiency for the double *meta* methylation *in vivo*, even if residual delphinidin was still
detected. These data confirmed the *in vitro* results that AOMT was able to catalyse two
sequential methylations of delphinidin derivatives at the 3'- and 5'- positions to yield malvidin
derivatives. However, petunidin 3-O-rutinoside was absent, suggesting a higher efficiency
for the second methylation step *in planta*, which prevented the accumulation of the mono-
methylated intermediate. In contrast to anthocyanins, co-expression of AOMT and PAP1 did
not modify significantly the flavonol and phenolic acid content in tobacco leaves, in
comparison to expression of PAP1 alone. Although AOMT was active with flavonols *in vitro*,
the tobacco leaves, which accumulated only minor amounts of flavonols, did not allow us to
confirm this activity *in vivo*.

Moreover, the co-expression of the AOMT-GFP fusion protein with PAP1
transcription factor in tobacco leaves resulted in the accumulation of methylated
anthocyanins, identical to those accumulating after co-expression of AOMT and PAP1.
These similar anthocyanin profiles indicated that the AOMT-GFP fusion protein retained
AOMT activity, and that its cytosolic localization was likely to reflect the physiological
localization of the native AOMT protein.

AOMT is Likely to Play a Major Role in Anthocyanin Methylation in Grape Berries

Previous works in petunia and grape suggested that different OMTs were responsible for the
O-methylation at the 3' and 5' positions, respectively, of anthocyanin molecules (Wiering and
de Vlaming 1977; Bailly et al., 1997). In contrast with these predictions, the AOMT described
here behaved as a bifunctional 3',5' OMT both *in vitro* and *in vivo*, suggesting that AOMT
alone would be sufficient to perform both methylation steps in grape berries. The absence of
cyanidin derivatives in *PAP1*-expressing *N. benthamiana* leaves did not allow the
investigation of AOMT activity with 3', 4'-dihydroxylated anthocyanins in the tobacco system.
However, *AOMT* gene was strongly expressed after *véraison* in Nebbiolo and in Grignolino
grapes (Castellarin and Di Gaspero, 2007) which accumulate mainly the mono-methylated
peonidin 3-O-glucoside as major anthocyanin. Given the fact that AOMT could efficiently
methylate cyanidin 3-O-glucoside *in vitro*, these results suggest that AOMT is also involved
in methylation of cyanidin derivatives in grape berries. These results can thus be
accommodated in a model where AOMT would be the major methyltransferase acting on
anthocyanins in grape berries, or the main member of a family of enzymes with similar
kinetic properties. This model would also explain the fact that among several grape cultivars
whose berry anthocyanin composition has been analysed, accumulation of petunidin in
absence of malvidin was never reported (Wenzel et al., 1987; Mattivi et al., 2006). The
predominance and preferential 3',5' methylating activity of this AOMT would also explain the
common occurrence of malvidin in the genotypes where trihydroxylated anthocyanins are available, and the existence of a positive correlation between the 3’ and 3’,5’ anthocyanin methoxylation activity as estimated by metabolomic data over 64 grape genotypes (elaborated from Mattivi et al., 2006). This model is further supported by several transcriptomic studies, which identified independently the same AOMT as a major candidate for anthocyanin methylation in grape berries (Ageorges et al., 2006; Castellarin et al., 2007; Castellarin and Di Gaspero, 2007; Pilati et al., 2007; Cutanda-Perez et al., 2009). Finally, this model does not rule out the possibility that other OMTs may also participate in anthocyanin methylation in grapevine. Analysis of the grape genome sequence (Jaillon et al., 2007; Velasco et al., 2007) shows that the AOMT gene characterized in this work belongs to a small family of 4 similar putative OMT genes. Future work will be needed to investigate the roles of the other members of this gene family and their potential role in anthocyanins and/or flavonols methylation in grapevine.

In conclusion, we characterized a grapevine anthocyanin OMT which efficiently catalyzes the methylation of anthocyanins, both in vitro and in planta. In addition, we provide evidence that AOMT is likely to play a major role in anthocyanin methylation in grape berries. Methylation tends to shift berry colour towards purple and these changes are transmitted to the wines where anthocyanins form relatively stable complexes with other grape-derived flavonoids (Heredia et al., 1998; Jensen et al., 2008). Thus, identification of AOMT constitutes an important step in understanding the biosynthesis of major anthocyanins in grape and the determination of one of the key quality parameters of red grapes and wine.

Materials and Methods

Chemicals and Radiochemicals.

S-adenosyl-L-[methyl-14C] methionine (55 mCi/mmole) ([14C]SAM) was from GE Healthcare-Amersham Biosciences. Quercetin 3-O-glucoside, pelargonidin 3-O-glucoside, cyanidin 3-O-glucoside and delphinidin -3-O-glucoside were from Extrasynthèse (Genay, France). All other chemicals and reagents were from Sigma (St. Quentin Fallavier, France).

Plant material, RNA extraction and grape anthocyanin analysis

Roots, shoots, leaves and berries were harvested from grapevine plants (Vitis vinifera L.) cv. Syrah (malvidin 3-glucoside as predominant anthocyanin) and cv. Nebbiolo (peonidin 3-glucoside as predominant anthocyanin). Syrah plants were grown in the SupAGRO-INRA vineyard (Montpellier, France) on 30 vinestocks of homogeneous vigor. Average daily
temperatures in the growing season ranged between 22.4°C (June) and 23.6°C (August), and mean relative humidity values ranged from 55.9% (June) to 67.6% (August). Nebbiolo plants were from a 15 year old experimental vineyard at Grinzane Cavour (Piedmont, Italy) on E-W oriented rows. Average daily temperatures in the growing season ranged between 17.1°C (May) and 22.8°C (August) and rainfall summed up to 411 mm in the period from May to September. Young leaves were from the third rank, counted from the apex, with mean weight of 0.3 g per leaf. Old leaves were fully expanded leaves with mean weight of 2.8 g per leaf. Berries were collected at 9 developmental stages as described previously (Terrier et al., 2005). All collected samples were quickly frozen in liquid nitrogen then ground under liquid nitrogen to a fine powder with a Dangoumau blender and stored at –80°C until use. Total RNA from Syrah tissues was extracted using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol from 200 mg starting tissue, and from Nebbiolo tissues using a modification of the Chang’s protocol (Carra et al., 2007). Grape anthocyanin analyses of Syrah berries were performed by HPLC on 200 mg of grounded frozen tissue as described in Ageorges et al. (2006). In Nebbiolo berries, anthocyanin analyses were done on 250 mg frozen tissue by HPLC following the protocol of Guidoni et al. (2002).

Cloning of AOMT cDNA and phylogenetic analysis

The full-length cDNA of AOMT was amplified from cDNA of mature berries (cv. Syrah) with high fidelity Taq Polymerase (Advantage®-HF 2 PCR kit, Clontech) using the forward primer 5’-TTTTCTTGTACGGCAGGCTTA-3’ and the reverse primer 5’-TGAGAATGGATTTAGGCTAATAGAG-3’. The amplified cDNA for AOMT was cloned into the pGEM-T easy vector (Promega) and the resulting plasmid was sequenced. OMT sequences were obtained from GenBank. Nucleic acid and protein sequences were aligned using ClustalW (Thompson et al., 1994). Phylo_win program (Galtier et al., 1996) was used to construct phylogenetic trees, using the neighbour-joining method, with 500 bootstrap replicates.

Quantitative PCR analysis of gene expression

In the case of Syrah, RNA was quantified with Ribogreen (Molecular probes) and reverse transcription was performed in triplicate from each sample from 500 ng of purified RNA using the Superscript II RT kit (Life Technologies). Triplicates were further pooled for PCR. Gene-specific primers were as follows: AOMT-F (5’-CTCTGACGGCGCTTATTA-3’) and AOMT-R (5’-CCCACACAGAGCTCTGACA-3’). Specific annealing of the oligonucleotides was controlled on dissociation kinetics performed at the end of each PCR run. The efficiency of the primer pair was measured on a plasmid serial dilution. PCR was performed in
triplicate as described in Fernandez et al. (2007). Gene transcripts were quantified upon normalization to VvEF1alpha (Terrier et al., 2005) by comparing the cycle threshold (CT) of the target gene with that of VvEF1alpha. Gene expression was expressed as mean and standard error calculated over all biological and technical replicates. In the case of Nebbiolo, the following modifications to the protocol were applied. Ten µg of RNA was reverse transcribed in duplicate using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and PowerSYBR Green (Applied Biosystems) for quantification of amplification results. The gene-specific primers for Nebbiolo were AOMT-F’ (5’-GATGAATGTCCCTGTCGATGAG-3’) and AOMT-R’ (5’-GCAAGAGCTTGGCCAAGAGA-3’).

Characterization of Recombinant AOMT
AOMT cDNA was amplified by PCR using the upstream primer 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGTTCCGCGTGGATCCATGTCCAGCTCAAGTCATAGG-3’ and the downstream primer 5’-GGGGACCACTTTGTACAAGAAAAGCTGGGTTCACTAATAGAGGCGCCCTGCAGAG-3’, and cloned into pDONR207 Gateway compatible vector (Invitrogen, Carlsbad, CA). AOMT cDNA was sequenced to verify that no mutation had been introduced, and transferred into the pHNGWA destination vector (accession number EU680842, Busso et al., 2005). AOMT enzyme was expressed as His-tagged NusA-fusion protein. Recombinant AOMT was purified using TALON metal affinity resin (Clontech) and characterized after cleavage of the NusA moiety using thrombin (GE Healthcare-Amersham). Purified recombinant AOMT (5 µg) was assayed in a final volume of 200 µl with 200 µM SAM and 200 µM anthocyanin or flavonol substrate, in 0.1 M Tris, pH 7.5, containing 10% glycerol (v/v), 10 mM MgCl₂, 14 mM 2-mercaptoethanol and 1 mg/mL bovine serum albumin. Incubations were stopped with 800 µl of methanol containing 0.2 N HCl. For kinetic studies, recombinant AOMT (200 ng) was incubated in a final volume of 25 µl as above, with the exception that 100 µM [¹⁴C]SAM (8 mCi/mmol) was used. Ranges of anthocyanin and flavonol substrate concentrations of between 3 µM and 500 µM were used for Kₘ determination. Reactions were stopped by addition of 75 µl of 2 N HCl and incubated at 95°C for 30 min for hydrolysis of glucosides. Reaction products were extracted with 100 µl of isoamyl alcohol and the incorporated radioactivity was measured by liquid scintillation. Kₘ and Vₘₐₓ values were calculated from Lineweaver-Burk plots.

The pH dependence of AOMT activity was assessed in the pH range 4.5 – 9.4 using MES (4.5 – 6.5), PIPES (6.6), HEPES (7.0 – 7.5) and Tris-HCl (7.5 – 9.4). The effect of divalent cations on enzyme activity was assessed by testing different concentrations of MgCl₂ (0, 2.5, 5, 10 mM). Metal inhibition on enzyme activity was estimated by adding to the
reaction mixture containing 10 mM MgCl₂ either CaCl₂, ZnCl₂, MnCl₂, and EDTA (final concentration 2.5 or 5.0 or 10 mM).

**Transient Expression in Tobacco**

For *Agrobacterium*-mediated transient expression, AOMT cDNA was transferred into the GATEWAY-compatible binary vector pB2GW7 (Karimi et al., 2002). A PAP1 genomic fragment from *A. thaliana* ecotype Columbia (Col-0) was amplified by PCR using the upstream primer 5'-ACCTTTTACAATTTGTTTATATTTTACG-3' and the downstream primer 5'-CAAACCTATAACAAAGCAAAACATTTGTTTATATTTTAC-3' (Borevitz et al., 2000). PAP1 gene was then reamplified using the upstream primer 5'-GGGGACAAGTTGACAAAAAGCAGGCTCAATTTGTTTATATTTTAC-3' and the downstream primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACACAAACGCAAATGTT-3' and cloned into pDONR207 vector. PAP1 gene was subsequently transferred into the GATEWAY binary vector pB2GW7 (Karimi et al., 2002). A m-GFP4-HDEL construction was used as a control (Haseloff et al., 1997). All constructs were introduced into *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation. *Nicotiana benthamiana* leaves were infiltrated with *A. tumefaciens* cultures (OD₆₀₀ 0.1 to 0.3) according to Batoko et al. (2000). Disks were punched from tobacco leaves 4 days after *Agrobacterium* infiltration and analysed for anthocyanin content.

**Subcellular localization of AOMT**

AOMT coding sequence was amplified by PCR using the upstream primer 5'-GGGGACAAGTTGACAAAAAGCAGGCTTGTTCCGCCTGGATCCATGCAGCTCAAAGTCATAGG-3' and the downstream primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACACAAACGCAAATGTT-3'. Amplified DNA fragment was first introduced into pDONR 207 (Invitrogen) and then into the GATEWAY-compatible pK7FWG2 vector (Karimi et al., 2002) in order to obtain a C-terminal GFP fusion protein (AOMT::GFP). pK7FWG2-AOMT construct was transformed into tobacco using agroinfiltration as described above. A non-targeted GFP construct (mGFP4) was used as a control (Haseloff et al., 1997). Tobacco leaves were examined 48 h after transformation using a Zeiss LSM 510 confocal imaging system attached to a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany). GFP was visualised by excitation with the 488 line of a krypton/argon laser and use of a BP 505-550 emission filter. Projections of optical sections were accomplished using LSM image-processing software (Zeiss).
Characterization of grape methyltransferase

Analysis of AOMT reaction products

The in vitro enzymatic reactions (200 µl) were diluted with 800 µl of methanol containing 0.2 N HCl. The transformed tobacco tissues were resuspended in methanol containing 0.2 N HCl (1 mg of tissue FW / ml of solvent). Samples were stirred on a Stuart Tube Rotator SB3 (Bibby Sterlin, Stone, UK) in the dark at room temperature for 1 h, centrifuged (13,000 g, 15 min, +4°C), and then dried under vacuum at +35°C for 2 h (Genevac, Ipswich, UK). Dried residues were dissolved in 100 µl of 20% methanol containing 1% HCl before HPLC analyses.

Anthocyanins and flavonols were analyzed on HPLC-DAD as previously described (Fournand et al., 2006), and further characterized with HPLC-DAD/ESI-MS. Separations were performed using a Waters Millenium HPLC-DAD system, on a (250 × 2 mm i.d.) Atlantis dC18 column (Waters; 5µm) with a guard column, operated at 30°C. Mobile phase consisted of water/formic acid (98/2, v/v) (eluant A) and water/acetonitrile/formic acid (18/80/2, v/v/v) (eluant B). Flow rate was 0.25 ml/min. The elution program was as follows: isocratic for 2 min with 0% B, 0-2% B (2-5 min), isocratic with 2% B (5-12 min), 2-3% B (12-15 min), 3-8% B (15-25 min), 8-20% B (25-40 min), 20-25% B (40-45 min), isocratic with 25% B (45-55 min), 25-65% B (55-70 min), and isocratic with 65% B (70-75 min). ESI-MS/MS analyses were performed with a ThermoFinnigan LCQ advantage mass spectrometer equipped with an electrospray source and an ion trap mass analyser controlled by the LCQ navigator software. The spectrometer was operated in the positive ion mode (source voltage, 4kV; capillary voltage, 30V; capillary temperature, 250°C; sheath gas, 50 and auxiliary gas, 10). Collision energy for fragmentation used for MS2 experiments was set at 35%. Anthocyanins, flavonols and hydroxycinnamic acids were respectively identified according to their mass fragmentation, UV visible absorption spectra and retention time. Anthocyanin quantifications were based on peak areas at 520 nm whereas quantifications for flavonols and hydroxycinnamic acids were done respectively at 360 and 320 nm.

Accession Numbers

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number FJ460168 (AOMT from Syrah). The Nebbiolo sequence differs from Syrah in two SNPs (A216 to C and T441 to C).

Acknowledgements

We are indebted to Peter Beyer (Center for Applied Biosciences, Freiburg, Germany), Bilal Camara and Florence Bouvier (IBMP, Strasbourg, France) for the use of their laboratory facilities. We thank Jérôme Mutterer (IBMP, Strasbourg) for help with confocal imaging. We thank Pascale Coste, Bernard Delnatte, Denise Hartmann, Charlotte Knichel, Jacky Misbach.
and Christian Vivant (INRA, Colmar) for assistance with plant material, Mathias de Vismes for help with tobacco transformation and Francesca Cardinale (University of Torino) for advice in production of the recombinant protein. We also thank Sandrine Viallet (INRA Montpellier) for excellent technical support in quantitative RT PCR analysis.
Supplemental Figure 1

Figure S1. Quantitative real-time PCR expression profiling of VvCCoAOMT during Nebbiolo berry development. Expression values have been normalized with VvEF1alpha and expressed as relative abundance. The arrow indicates the véraison, the onset of ripening.

Supplemental Figure 2

Figure S2. SDS-PAGE of the recombinant AOMT. Lanes: (1) protein size markers; (2) total protein extract of E. coli expressing the NusA-AOMT fusion protein; (3) purified AOMT after thrombin cleavage. The molecular weights of the markers are indicated in kDa and the arrow indicates the position of the NusA-AOMT fusion protein.
Figure S3. In vitro pH optimum of AOMT using (A) cyanidin 3-O-glucoside and (B) delphinidin 3-O-glucoside. The pH dependence of AOMT activity was assessed in the pH range 4.5 – 9.4 using MES (4.5 – 6.5), PIPES (6.6), HEPES (7.0 – 7.5) and Tris-HCl (7.5 – 9.4).

Figure S4. Anthocyanin profiles of transient transformed tobacco co-expressing PAP1 and AOMT (A), or co-expressing PAP1 and AOMT::GFP (B). The tobacco leaf sectors (200 mg FW) were excised 96 h after Agrobacterium-mediated transformation. Anthocyanin contents were analysed using HPLC-DAD. Anthocyanins were monitored at 520 nm. The data shown are representative of three independent experiments. Key: Dp3R, delphinidin 3-O-rutinoside; Mv3G, malvidin 3-O-glucoside; Mv3R, malvidin 3-O-rutinoside. Peak a3, malvidin 3-O-rutinoside O-hexoside.
Supplemental Table S1. In vitro substrate specificity of the recombinant AOMT. Reaction products were identified according to their mass fragmentation, UV visible absorption spectra and retention time. Quantifications were based on peak areas at 520 nm for anthocyanins and at 360 nm for flavonols.

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<td>465</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>quercetin</td>
<td>303</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>isorhamnetin 3-glucoside</td>
<td>479</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>isorhamnetin</td>
<td>317</td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Table S2. Flavonoid profiles in acidic MeOH-H₂O extracts of transformed tobacco leaves expressing GFP (control), PAP1 or PAP1 + AOMT.

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Rt (min)</th>
<th>[M]+ (m/z)</th>
<th>MS/MS (m/z)</th>
<th>Anthocyanins</th>
<th>GFP</th>
<th>PAP1</th>
<th>PAP1 + AOMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Delphinidin 3-O-rutinoside</td>
<td>0%</td>
<td>2.5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>611 / 465 /</td>
<td>303</td>
<td>O-hexoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a1</td>
<td>28.96</td>
<td>773</td>
<td></td>
<td>Delphinidin 3-O-rutinoside</td>
<td>0%</td>
<td>2.5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>611 / 465 /</td>
<td>303</td>
<td>O-hexoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a2</td>
<td>35.74</td>
<td>773</td>
<td></td>
<td>Delphinidin 3-O-rutinoside</td>
<td>0%</td>
<td>4.5%</td>
<td></td>
</tr>
<tr>
<td>a3</td>
<td>36.96</td>
<td>801</td>
<td></td>
<td>Delphinidin 3-O-rutinoside</td>
<td>0%</td>
<td>4.5%</td>
<td></td>
</tr>
<tr>
<td>Dp3R b</td>
<td>38.25</td>
<td>611</td>
<td>465 / 303</td>
<td>Delphinidin 3-O-rutinoside</td>
<td>0%</td>
<td>94%</td>
<td>24.2%</td>
</tr>
<tr>
<td>P13R</td>
<td>42.56</td>
<td>625</td>
<td>479 / 317</td>
<td>Petunidin 3-O-rutinoside</td>
<td>0%</td>
<td>3.5%</td>
<td></td>
</tr>
<tr>
<td>Mv3G</td>
<td>44.55</td>
<td>493</td>
<td>331</td>
<td>Malvidin 3-O-glucoside</td>
<td>0%</td>
<td>18.5%</td>
<td></td>
</tr>
<tr>
<td>Mv3R</td>
<td>46.00</td>
<td>639</td>
<td>493 / 331</td>
<td>Malvidin 3-O-rutinoside</td>
<td>0%</td>
<td>49.1%</td>
<td></td>
</tr>
</tbody>
</table>

* (m/z) rutinose = 308
* coeluted with a 3-rutinoside non identified compound (m/z=635) in PAP1+AOMT extract
**Characterization of grape methyltransferase**

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Rt (min)</th>
<th>ESI-MS (m/z)</th>
<th>Hydroxycinnamic acids</th>
<th>Tobacco leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GFP</td>
</tr>
<tr>
<td>c1</td>
<td>8.9</td>
<td>355 [M + H]+</td>
<td>C16 H18 O9</td>
<td>caffeoyl quinic acid 1</td>
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<tr>
<td>c2</td>
<td>18.2</td>
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<td></td>
<td>isomer 2</td>
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<tr>
<td>c3</td>
<td>20.4</td>
<td></td>
<td></td>
<td>isomer 3</td>
</tr>
<tr>
<td>f1</td>
<td>22.7</td>
<td>369 [M + H]+</td>
<td>C17 H21 O9</td>
<td>feruloyl quinic acid 1</td>
</tr>
<tr>
<td>f2</td>
<td>32.1</td>
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<td></td>
<td>isomer 2</td>
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<tr>
<td>f3</td>
<td>38.8</td>
<td></td>
<td></td>
<td>isomer 3</td>
</tr>
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</table>
References


Castellarin SD, Di Gaspero G (2007) Transcriptional control of anthocyanin biosynthetic genes in grapevine expressing a BAC clone encoding a myb regulator of pheoyoanthocyanin. Plant Cell Biol 8: 46


Characterization of grape methyltransferase
Characterization of grape methyltransferase


Characterization of grape methyltransferase


Wiering H, de Vlaming P (1977) Glycosylation and methylation patterns of anthocyanins in Petunia hybrida. II. The genes Mr1 and Mf2. Z Pflanzenzucht 78: 113-123


Characterization of grape methyltransferase