



## Research article

# Characterization of a multifunctional caffeoyl-CoA *O*-methyltransferase activated in grape berries upon drought stress



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## ABSTRACT

Drought stress affects anthocyanin accumulation and modification in vegetative and reproductive plant tissues. Anthocyanins are the most abundant flavonoids in grape (*Vitis vinifera* L.) coloured berry genotypes and are essential markers of grape winemaking quality. They are mostly mono- and dimethylated, such modifications increase their stability and improve berry quality for winemaking. Anthocyanin methylation in grape berries is induced by drought stress. A few caffeoyl-CoA *O*-methyltransferases (CCoAOMTs) active on anthocyanins have been described in grape. However, no drought-activated *O*-methyltransferases have been described in grape berries yet.

In this study, we characterized *VvCCoAOMT*, a grapevine gene known to induce methylation of CoA esters in cultured grape cells. Transcript accumulation of *VvCCoAOMT* was detected in berry skins, and increased during berry ripening on the plant, and in cultured berries treated with ABA, concomitantly with accumulation of methylated anthocyanins, suggesting that anthocyanins may be substrates of this enzyme. Contrary as previously observed in cell cultures, biotic stress (*Botrytis cinerea* inoculation) did not affect *VvCCoAOMT* gene expression in leaves or berries, while drought stress increased *VvCCoAOMT* transcript in berries. The recombinant *VvCCoAOMT* protein showed *in vitro* methylating activity on cyanidin 3-*O*-glucoside. We conclude that *VvCCoAOMT* is a multifunctional *O*-methyltransferase that may contribute to anthocyanin methylation activity in grape berries, in particular under drought stress conditions.

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## 1. Introduction

Grapevine (*Vitis vinifera* L.) is the world's most important fruit crop and is cultivated under a wide range of climates. One reason for the worldwide success of grape growing is the adaptability of this species to stressful conditions, due to a large and still poorly explored array of responses to drought, heat, and chilling conditions. As concerns drought, grapes are considered relatively tolerant, due to morphological, physiological, and biochemical mechanisms that are also diversified in the many existing cultivars (Ferrandino and Lovisolo, 2014). In vineyard management, a mild drought stress is even considered beneficial as it enhances berry

quality, in particular increasing berry concentration of sugars and polyphenols (Chaves et al., 2010). The sequencing of the grape genome has opened the era of genomic investigation on the molecular reactions of grape to stress: these studies have unveiled an unexpected wealth of genes differentially regulated in response to drought (Cramer et al., 2007; Deluc et al., 2009).

Grape berries contain different classes of polyphenols: anthocyanins (in coloured varieties), flavonols, flavan-3-ols, proanthocyanidins, and non-flavonoid phenols, such as hydroxycinnamoyl tartrates (Adams, 2006). Anthocyanins are responsible for the characteristic red-blue colour of many plant tissues, where they play an important role as animal attractors for seed dispersion, as protectants against UV irradiation, and as antioxidants (Harborne and Williams, 2000). In grapevine the molecular signals controlling anthocyanin accumulation in berries have not been completely defined yet, but abscisic acid (ABA), whose concentration increases

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at the beginning of ripening (Davies et al., 1997), is a favoured candidate (Jeong et al., 2004). In the berry skins, anthocyanins are accumulated in vacuoles as 3-*O*-glucosides. All anthocyanin aglycones are hydroxylated at the 4' carbon of the B ring, but they differ in the level of further hydroxylation of the 3' and 5' carbon atoms. One or both hydroxylated carbons can then be *O*-methylated, inducing a slight reddening effect and reducing the chemical reactivity of phenolic hydroxyl groups (Sarni et al., 1995). Methylation increases anthocyanin stability and affects colour intensity and hue, both pivotal quality parameters for the red wine industry.

The response to drought stress in plants is a multifaceted process, involving among other metabolic events an increase in anthocyanin content (Sperdouli and Moustakas, 2012; Nakabayashi et al., 2014). Modifications in the concentration of anthocyanins have been recognized as responses to water stress in grape: drought increases berry anthocyanin content, and this effect is due to an increased biosynthesis (Castellarin et al., 2007a, 2007b). A further effect of drought in the grape berry is a modification of the methylation profile, as a higher concentration of methylated forms at the expense of the non-methylated ones is observed in berry skin upon long-term drought stress in the field (Castellarin et al., 2007b; Deluc et al., 2009). However, the molecular events at the base of such response to drought have not been clarified yet.

Caffeoyl-CoA *O*-methyltransferases (CCoAOMTs) have been isolated and characterized in many plants, including *Medicago sativa* L. (Inoue et al., 1998), *Nicotiana tabacum* L. (Martz et al., 1998), *Pinus taeda* L. (Li et al., 1999), *Populus* spp. (Meyermans et al., 2000), *Mesembryanthemum crystallinum* L. (Ibdah et al., 2003). Although the main role of the CCoAOMT family has been initially ascribed to lignin biosynthesis (Day et al., 2009), and to provision of feruloyl-CoA for other biosynthetic pathways (Kai et al., 2008), some CCoAOMTs also show significant activity on substrates other than CoA esters, as in the case of flavonols in *Arabidopsis* (Do et al., 2007) and ice plant (Ibdah et al., 2003). These proteins represent a subgroup of multifunctional CCoAOMTs.

A few CCoAOMTs have been studied in grape. A CCoAOMT was first isolated by Busam et al. (1997), who demonstrated the activity of the encoded protein on CoA thioesters and observed that its expression was activated in grape cell suspension cultures by biotic stress elicitors. Nevertheless, these authors did not further characterize this gene, and provided no data on its expression in berry tissues. Later on, an *O*-methyltransferase (VvAOMT) was shown, by *in vitro* and *in vivo* studies, to control anthocyanin methylation in berry skins during ripening (Huguency et al., 2009). Sequence alignment included VvAOMT within the CCoAOMT subfamily, and accordingly its activity on CoA thioesters was demonstrated *in vitro* (Lücker et al., 2010), confirming its multifunctional nature. Finally, a QTL approach led to the characterization of two further genes on the same chromosome (VvAOMT2 and 3), the former proposed to contribute to the control of anthocyanin methylation in berry skins (Fournier-Level et al., 2011).

While VvAOMT is thought to play a major role in the ripening-dependent activation of anthocyanin methylation in grape berry skin, it seems not to be responsive to drought stress (Castellarin et al., 2007a). Other, stress-responsive methyltransferases must thus contribute to the stress-dependent reshaping of the anthocyanin methylation profile. In this context, the biotic stress-responsive CCoAOMT, first isolated by Busam et al. (1997), should be a promising candidate if it was shown to respond to abiotic stress and to behave as a multifunctional CCoAOMT.

The aim of this study was to functionally characterize VvCCoAOMT and to study its role in anthocyanin methylation in grape berries. We analysed the *in vitro* activity of VvCCoAOMT on anthocyanins, demonstrating its multifunctional nature. Furthermore, we measured expression of VvCCoAOMT in berries during

development and under stressful conditions, and we show that transcript level is increased during ripening and by drought stress. Our results suggest that the encoded enzyme may significantly contribute to anthocyanin methylation in berry skin upon abiotic stress.

## 2. Materials and methods

### 2.1. Protein alignments and phylogenetic analysis

In order to establish the structure of the CCoAOMT protein family in grape, a BLAST search on the grapevine "PN40024" 12X genome draft, V1 annotation (<http://genomes.cribi.unipd.it/grape/>) was performed using VvCCoAOMT (CAA90969.1) as query. Putative caffeoyl-CoA *O*-methyltransferases characterized by score values higher than 200 were used for the following steps. Sequence identity percentages among plant OMTs and, separately, among grape CCoAOMTs, were investigated by running the MEGA 4.0 software ([www.megasoftware.net](http://www.megasoftware.net)), incorporating the BLOSUM series protein weight matrix with ClustalW alignment and setting gap opening penalty at 10 and gap extension penalty at 0.2. Phylogenetic trees were built by applying the Neighbour-Joining method with the Bootstrap analysis set at 1000 replicates. Data obtained from the alignment of VvCCoAOMT amino acid sequences were edited and marked by applying the BOXSHADE 3.21 software ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

### 2.2. Plant material and treatments

Berries of *V. vinifera* cv. Barbera (coloured, anthocyanin profile dominated by mono- and di-methylated forms) and Moscato (uncoloured) were sampled from an experimental vineyard located in Grugliasco (Piedmont, Italy, 45°03'55"N 7°35'35"E) every two weeks, starting 15 days after flowering (DAF). Véraison (onset of ripening and accumulation of skin anthocyanins) took place at 45 DAF. Gene expression was measured in the berry skins, except for ripe Barbera berries (90 DAF), where skin, pulp and seeds were analysed. Gene expression levels were also assessed in blades and petioles of fully expanded Barbera leaves, sampled at 75 DAF.

The effect of treatment with ABA was tested on cultured berries of the coloured genotype Merlot, whose anthocyanin profile is also dominated by mono- and di-methylated forms. Berry culture followed a modification of the protocol by Gambetta et al. (2010). Briefly, berries from a vineyard of the University of Udine (Italy) experimental farm (46°01'52"N, 13°13'31"E) were sampled just before véraison, surface-sterilized with 70% ethanol for 10 s, washed with 1% NaClO for 10 min, and placed, after removing the petiole with a scalpel, in glass jars with their cut surface inserted in sterile agar containing 8% sucrose and either 0 or 200  $\mu$ M ( $\pm$ )-ABA. Berries were thus incubated at 20 °C in an illuminated hood, and three replicates of two-berry samples each were taken at 4 day intervals, up to 12 days.

Barbera fully expanded leaves and ripe berries (90 DAF) were used for the induction of biotic stress with *Botrytis cinerea* Pers. The fungal pathogen was first grown on MEA (Malt Extract Agar) medium for three days at 25 °C. Plugs of colonized agar (6 mm diameter) were placed upon leaves and berries whose surface had previously been slightly injured with a sterile needle, and then incubated at 25 °C in closed and regularly moistened 20 cm Petri dishes (one leaf or 10 berries per dish). Plugs of non-inoculated MEA were placed upon control (mock-inoculated) berries and leaves. Leaf and berry samples were collected from each of three replicate Petri dishes per treatment at the beginning of incubation and respectively after 36 and 72 h for leaves, and 29, 53, and 94 h for berries. Only tissue portions close to fungal plugs were

collected, avoiding necrotic areas.

Drought stress was induced on two-year-old *V. vinifera* cv. Grenache plants grafted onto *Vitis riparia* x *Vitis berlandieri* 420A, grown in 10 L pots in a greenhouse under partially controlled climate conditions. The air temperature and relative humidity in the greenhouse ranged between 18 and 36 °C and 55–80%, respectively. Photosynthetic active radiation reached 1800  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the day/night cycle was 16–8 h. Irrigation was withheld for eight days on three pots to induce drought stress, while three pots were irrigated daily to maintain water container capacity and used as control. Water container capacity was computed following the method described by Hochberg et al. (2013), using as bulk density of our substrate 0.356  $\text{kg L}^{-1}$ . Water container capacity in irrigated pots ranged between 55 and 45% v/v (maximum capacity in our substrate 55%), while in drought-stressed pots it was lower than 30% at the last sampling date.

Leaf water potential ( $\Psi_{\text{leaf}}$ ) was measured between 10:00 and 12:00 AM on fully expanded leaves collected from primary stems at 2-day intervals, by using a Scholander-type pressure chamber (Soil Moisture Equipment Corp.). Stomatal conductance ( $g_s$ ) was assessed at the same time points on fully expanded leaves with a portable Gas Exchange Fluorescence System (GFS-3000, Heinz Walz GmbH). In the leaf chamber photosynthetic active radiation (PAR: 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and temperature (25 °C) were maintained constant. A zero-point for  $\text{CO}_2$  was set at the beginning of each experimental day. Three replicate samples (one per plant) of mature leaves and berries (75 DAF) were taken at the beginning of drought stress and after four and eight days.

All samples were quickly frozen in liquid nitrogen, ground in sterile mortars with liquid nitrogen, and stored at  $-80$  °C until use.

### 2.3. RNA extraction and quantitative PCR (qPCR) analysis of gene transcript

Total RNA was extracted from the different grape tissues (petioles, berry skin, pulp and seeds: 2 g) collected following the protocol by Carra et al., 2007 while total RNA from leaves (0.5 g) was extracted according with Gambino et al. (2008). RNA integrity and quantity were checked using a 2100 Bioanalyzer (Agilent). Only samples with a RIN (RNA Integrity Number) value higher than 7.5 were chosen for quantitative expression analyses. After a treatment with DNase I RNase-free (Fermentas), RNA was reverse transcribed in duplicate using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems); duplicates were then pooled before qPCR analysis. Gene-specific primers were designed using the Primer3 software (<http://primer3.ut.ee>). In order to confirm the induction responses to either biotic or abiotic stress at the molecular level, transcripts of a stilbene synthase [*VvSTS27*: (Vannozzi et al., 2012)] and of a dehydrin [*VvDHN1a*: (Perrone et al., 2012)] were quantified respectively. Reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems), and the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied Biosystems) was used for quantifying amplification results (Perrone et al., 2012). Three technical replicates were run for each sample. Thermal cycling conditions were as follows: an initial denaturation phase at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Specific annealing of primers was checked on dissociation kinetics performed at the end of each RT-qPCR run. Identity of amplicons was checked by sequencing when needed. Expression of target transcripts was quantified after normalization of the cycle threshold to ubiquitin (*VvUBI*). Gene expression data were calculated as transcript quantity ratios (relative quantity, RQ) by applying the 2- $\Delta\Delta\text{CT}$  method. Each analysis was performed in triplicate. Primer sequences used in qPCR experiments are reported in Tab. S1.

### 2.4. Heterologous expression of VvCCoAOMT in Escherichia coli and enzymatic assay

The full length *VvCCoAOMT* cDNA was amplified from cv. Barbera using the *VvCCoAOMT-FL* primers (Tab. S1), containing a *EcoRI* (5'-end) and a *XhoI* (3'-end) restriction site respectively. Amplification was performed using the AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen) with the following thermal cycling conditions: 95 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 68 °C for 45 s, and a final step of 68 °C for 5 min. Identity of the amplicon was checked by sequencing. The amplified fragment was digested with *EcoRI* and *XhoI*, and cloned in-frame with a N-terminal glutathione S-transferase tag into the pGEX-4T-1 vector (GE Healthcare). *E. coli* DH5 $\alpha$  competent cells were transformed for sequencing purposes, in order to verify the correct frame of the target sequence and the absence of any introduced mutation. Plasmids were then transferred into *E. coli* BL21 (DE3) protein expression-competent cells. The pGEX-4T-1 empty vector was used as negative control. Recombinant GST-*VvCCoAOMT* was extracted using the following modified version of the method described by Tolia and Joshua-Tor (2006). The glycerol stock of transformed *E. coli* BL21 was used to create a starter culture in 20 mL YTA (Yeast Triptone Agar) supplemented with ampicillin (250  $\text{mg mL}^{-1}$ ). The starter culture was incubated at 37 °C for 12–15 h, and then employed for the inoculation of 400 mL YTA supplemented with ampicillin (250  $\text{mg mL}^{-1}$ ). The culture was incubated at 37 °C until  $A_{600}$  was 0.5–0.6, and recombinant protein expression was induced by adding 4 mL of 100 mM IPTG; the incubation was continued for 2 h. Bacterial cells were harvested by centrifugation at 5000 rpm for 6 min at 4 °C, and the supernatant was discarded. After resuspension of the pellet in 24 mL of a preheated 4 M NaCl, 1 M Tris-HCl, 0.5 M EDTA solution supplemented with 0.1  $\text{mg mL}^{-1}$  lysozyme, the mixture was kept on ice for 15 min and then added with 120  $\mu\text{L}$  dithiothreitol 1 M, 240  $\mu\text{L}$  phenylmethanesulfonyl fluoride 100 mM, and 2.9 mL of 0.1  $\text{g L}^{-1}$  N-lauroylsarcosine sodium salt. Subsequently, cells were lysed by sonication until the cloudy suspension became translucent, and cell debris were precipitated by centrifugation at 5000 rpm for 30 min at 4 °C. The collected supernatant was added with 200  $\mu\text{L}$  Triton X-100 and incubated in continuous agitation at 4 °C for 1 h.

The recombinant protein was purified by a batch system using Glutathione Sepharose 4B (GE Healthcare-Amersham Biosciences) following the manufacturer's protocol. Protein concentration was determined by the Bradford method (Sigma Aldrich) according with manufacturer's instructions. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the purity of the recombinant protein.

The activity of recombinant GST-*VvCCoAOMT* (20  $\mu\text{g}$ ) was assayed on cyanidin 3-*O*-glucoside chloride and on caffeoyl-CoA. In the first case, a final volume of 150  $\mu\text{L}$  with 2.5 mM S-adenosylmethionine (Sigma), 5 mM  $\text{MgCl}_2$ , and 250  $\mu\text{M}$  cyanidin 3-*O*-glucoside chloride (Extrasynthèse) in 0.1 M Tris, pH 8.5 containing 10 mM PEG 4000, 2 mM DTT, and 14 mM  $\beta$ -mercaptoethanol was used. After incubating for 60 min at 30 °C, reactions were stopped by adding 75  $\mu\text{L}$  of 5% HCl. The activity on caffeoyl-CoA (TransMIT) was assayed in the same conditions and with the same substrate concentration. Reactions where the enzyme or the substrate were replaced with buffer were used as control blanks. The pH dependence of GST-*VvCCoAOMT* activity on cyanidin 3-*O*-glucoside and caffeoyl-CoA was assessed in the pH range between 5.4 and 9.4 using the following buffers: MES (5.4–6.25), PIPES (6.6), HEPES (6.0–7.5), and Tris-HCl (8.0–9.4). Metal inhibition of enzyme activity was determined by adding  $\text{ZnCl}_2$ ,  $\text{CaCl}_2$  or  $\text{MnCl}_2$  (10 mM) to the reaction mixture.

In order to obtain purified *VvCCoAOMT* protein, the N-terminal

GST tag of recombinant GST-VvCCoAOMT obtained by a column purification (GE Healthcare) was removed using thrombin (GE Healthcare). Kinetic studies were performed both on the recombinant GST-VvCCoAOMT fusion protein, and on VvCCoAOMT. Substrate concentrations between 0.001 and 2 mM were used. Reactions were incubated at pH 8.5 for 60 min. Kinetic calculations (determination of  $K_m$ ,  $V_{max}$  and  $K_{cat}$ ) were carried out by applying the Lineweaver–Burk transformation.

The enzymatic reaction products were analysed by HPLC-DAD. Anthocyanin separation was performed using a 1260 Infinity HPLC-DAD system (Agilent Technologies) on a Lichrocart® 250-4 HPLC-Cartridge Purospher® STAR RP-18 (5  $\mu$ m) with a guard column, operating at 30 °C. The mobile phase consisted in water: formic acid (90:10, v/v; eluent A) and methanol: formic acid: water (50:10:40, v/v/v; eluent B); flow rate was 0.8 mL min<sup>-1</sup>. For the recombinant GST-VvCCoAOMT enzyme reaction product, the elution program was as follows: 72%–55% A (0–15 min), 55%–30% A (15–35 min), 30%–10% A (35–45 min), 10%–1% A (45–50 min), 1%–72% A (50–55 min). For the VvCCoAOMT enzyme reaction product, a faster elution program was set up: 55% A (0–15 min), 55%–30% A (15–20 min), 30%–72% A (20–23 min); flow rate was 1.0 mL min<sup>-1</sup>. Hydroxycinnamoyl-CoA esters separation was performed on a Lichrocart® 250 HPLC-Cartridge Lichrospher® 100 RP-18 (5  $\mu$ m) with a guard column, operating at 30 °C. The mobile phase consisted in water: acetonitrile: formic acid (90:9.9:0.1, v/v/v; eluent A) and acetonitrile: water: acetic acid (80:19.9:0.1, v/v/v; eluent B). The elution program was as follows: 95% A (0–3 min), 95%–60% A (3–18 min), 60%–0% A (18–28 min), 0% A (28–30 min), 0%–95% A (30–32 min); flow rate was 0.5 mL min<sup>-1</sup>.

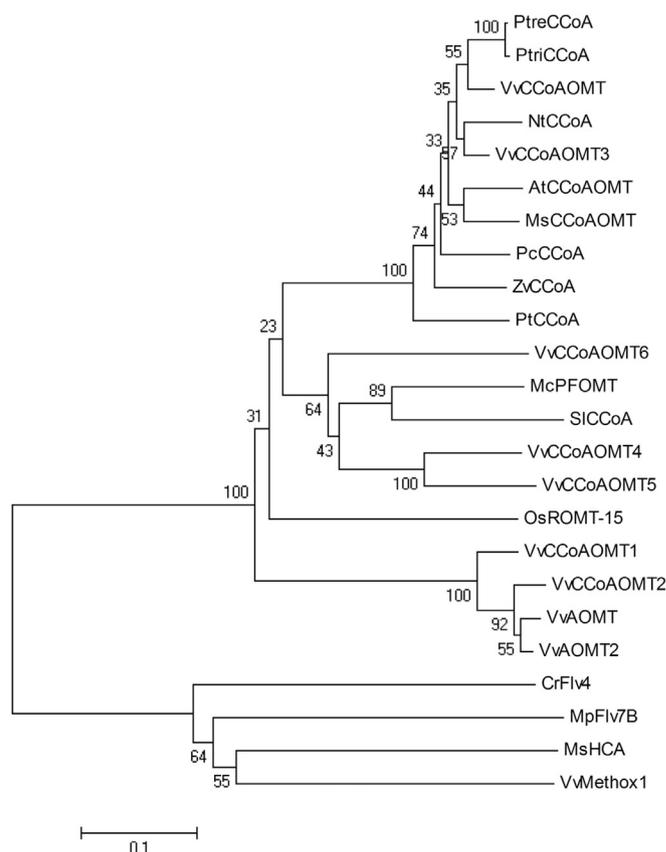
Quantitative determinations were performed using external standard calibration curves. Substrate and reaction products were identified by comparison of their retention times and UV spectra with those of standards (cyanidin 3-*O*-glucoside chloride and peonidin 3-*O*-glucoside chloride by Extrasynthèse; caffeoyl-CoA and feruloyl-CoA by TransMIT) under the same chromatographic conditions. Calibration curves with a good linearity ( $R^2 > 0.998$ ), obtained from a seven-point plot, were used to determine the concentration of reaction products.

### 3. Results

#### 3.1. Phylogenetic analysis

The interrogation of the Grape Genome Browser with the CAA90969.1 protein sequence (here referred to as VvCCoAOMT) yielded the proteins encoded by six gene models (Tab. S2) and the VvAOMT (Huguency et al., 2009) and VvAOMT2 (Fournier-Level et al., 2011) proteins. The VvCCoAOMT2 (VIT\_01s0010g03470) gene model partly corresponds in sequence to VvAOMT3 of Fournier-Level et al. (2011). Phylogenetic analysis of the family of putative VvCCoAOMT proteins (Fig. S1) showed that they group into two clades. One clade includes VvCCoAOMT (chromosome 3), VvCCoAOMT3 (chromosome 7), VvCCoAOMT4 and 5 (chromosome 11) and VvCCoAOMT6 (chromosome 12). In the second clade, VvCCoAOMT1 and 2, VvAOMT, and VvAOMT2 are all localized to chromosome 1, and enclose the anthocyanin methyltransferase cluster described by Fournier-Level et al. (2011). Phylogenetic analysis of putative VvCCoAOMT proteins together with other plant OMTs confirms that they all belong to type 2, low-MW and cation-dependent OMTs (Noel et al., 2003) (Fig. 1). Many members of this family methylate CoA esters of phenylpropanoids and are commonly called CCoAOMTs.

All VvCCoAOMTs contain characteristic dimerization domains, mostly involving hydrophobic residues, and probably form homodimers in solution. They also contain specific amino acid residues



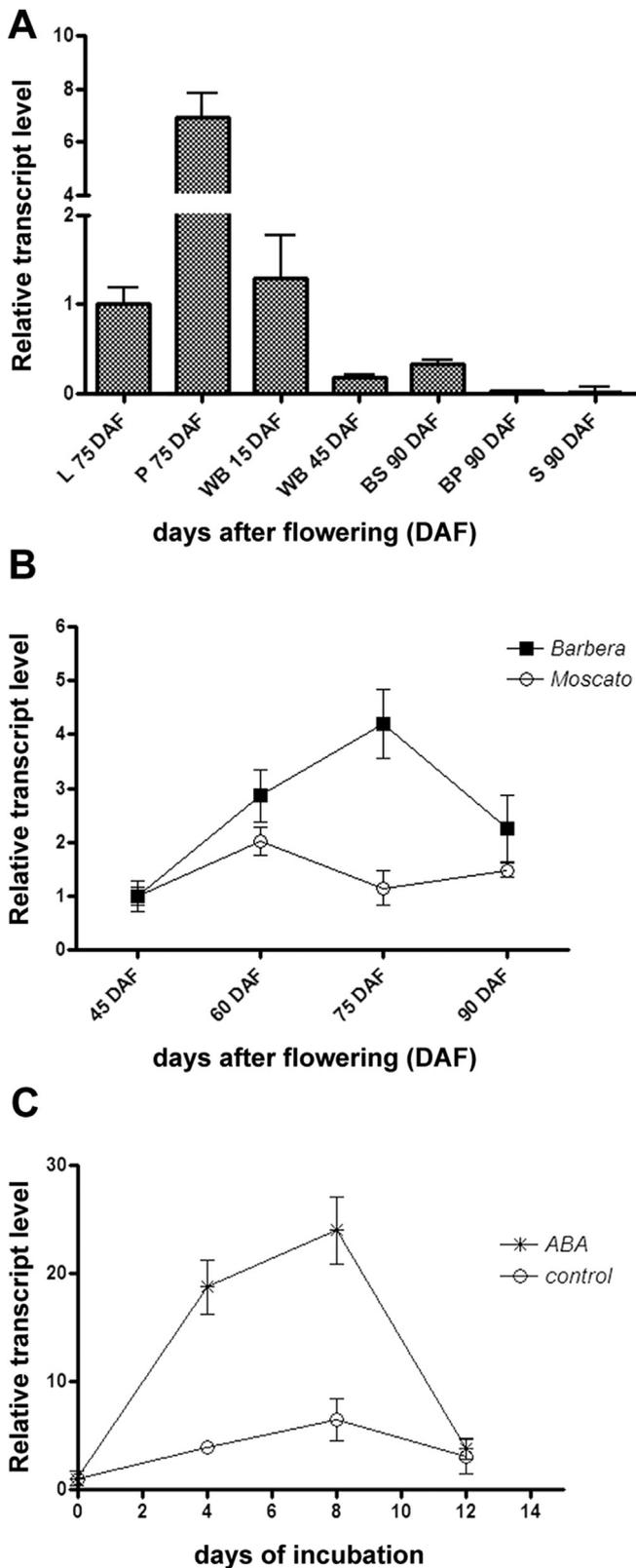
**Fig. 1.** Neighbour-joining tree of selected plant OMT proteins. Type 2 OMTs include caffeoyl-CoA OMTs from *Arabidopsis thaliana* (AtCCoAOMT; accession no. AEE30559), *Medicago sativa* (MsCCoAOMT; AAC28973), *Mesembryanthemum crystallinum* (McPFOMT; AAN61072.1), *Nicotiana tabacum* (NtCCoA; AAC49913.1), *Oryza sativa* (OsROMT15; XM\_483167), *Petroselinum crispum* (PcCCoA; AAA33851.1), *Pinus taeda* (PtCCoA; AAD02050.1), *Populus tremuloides* (PtreCCoA; AAA80651.1), *Populus trichocarpa* (PtriCCoA; CAA12198.1), *Stellaria longipes* (SiCCoA; AAB61680.1), *Vitis vinifera* (VvCCoAOMT; CAA90969.1 and VvAOMT; AC052469.1), *Zinnia violacea* (ZvCCoA; AAA59389.1). Type 1 OMTs include flavonoid 4'-*O*-methyltransferase from *Catharanthus roseus* (CrFlv4; AAR02419.1), caffeic acid 3-*O*-methyltransferase from *M. sativa* (MsHCA; AAB46623.1), flavonoid 7-*O*-methyltransferase from *Mentha x piperita* (MpFlv7B; AAR09598.1), methoxyppyrzine *O*-methyltransferase from *V. vinifera* (VvMethox1; ADJ66850.1). The significance of each node was tested using 1000 bootstrap replicates.

and the insertion loop required to bind the substrates SAM and caffeoyl-CoA as described by Ferrer et al. (2005) for a *Medicago sativa* CCoAOMT (Fig. S2).

#### 3.2. Quantification of VvCCoAOMT transcript in grape tissues and upon stress application

The transcription of VvCCoAOMT was analysed by RT-qPCR in different grape tissues, along berry ripening, upon ABA treatment of berries, and after induction of either biotic or abiotic stress.

In Barbera, VvCCoAOMT transcripts were detected in vegetative tissues (leaf blades and petioles) and in green berries (15 DAF) at higher concentrations than in berries at véraison (45 DAF). In ripe berries (90 DAF) expression was about 10 times higher in skin than in pulp or seeds (Fig. 2A). An expression study was performed in berry skins during the ripening process. In the coloured genotype Barbera, VvCCoAOMT transcript level increased after véraison, peaked at 75 DAF, and decreased thereafter (Fig. 2B). On the contrary, in the uncoloured Moscato grapes, VvCCoAOMT transcript concentration was relatively stable throughout the berry ripening



**Fig. 2.** Transcript of *VvCCoAOMT* in grape tissues during berry development and upon ABA treatment. Transcriptional changes of *VvCCoAOMT* in (A) different tissues of Barbera; (B) berry skins from véraison (45 DAF) to full ripening (90 DAF) in Barbera and Moscato; (C) berry skins of Merlot sampled before véraison and incubated on 200  $\mu$ M ( $\pm$ )-ABA. Ubiquitin (*VvUBI*) was used as the endogenous control gene for normalisation. Samples L 75 DAF (A), 45 DAF (B) and 0 days of incubation (C) were respectively used as reference for the calculation of expression ratios. Bars are standard errors

course (Fig. 2B).

In berries of Merlot sampled before véraison and treated *in vitro* with ABA, anthocyanin concentration in the skin increased to 2.28  $\mu$ g g<sup>-1</sup> after 12 days of treatment; in untreated berries, anthocyanins were not detected. The transcript level of *VvCCoAOMT* increased during the first part of the incubation period, peaked after 8 days, and then decreased; in the absence of ABA the transcript quantity was stable (Fig. 2C).

As *VvCCoAOMT* was originally reported to be activated by biotic stress elicitors in grape cell suspension cultures, we evaluated the expression response of *VvCCoAOMT* to challenge by fungal pathogens: to this aim, transcript expression was assessed in Barbera leaves and ripe berries inoculated with *B. cinerea*. A sharp increase in accumulation of stilbene synthase transcript was observed after inoculation in both organs during the experiment, in agreement with a well-known reaction of grapevine tissues to this pathogen (Bezier et al., 2002) (Fig. 3A, B). Transcript concentration of *VvCCoAOMT* in inoculated grape leaves decreased, while in berries it transiently increased, but non-inoculated controls followed a similar pattern (Fig. 3A, B).

The transcript level of *VvCCoAOMT* was also assessed upon application of an abiotic (drought) stress on whole plants. In fruit-bearing, potted Grenache plants subjected to water stress (WS), leaf water potential and stomatal conductance were lower than in irrigated plants, starting respectively from the second or the fourth day of the experiment. At the end of the experiment (eight days after withholding irrigation) both parameters were typical of drought-stressed grapevine leaves (Chitarra et al., 2014) (Fig. 4A, B). The *VvDHN1a* gene encodes a grape dehydrin protein responsive to drought stress in leaves (Perrone et al., 2012; Yang et al., 2012): as expected, its transcript concentration increased in WS leaves, confirming the physiological data, while it was not affected in berries (Fig. 4C). Transcripts of *VvCCoAOMT* showed no concentration changes in leaves upon drought, whilst they increased in berries of drought-stress plants: at the end of the experiment, the transcript level of *VvCCoAOMT* was 3 times higher than control (Fig. 4D).

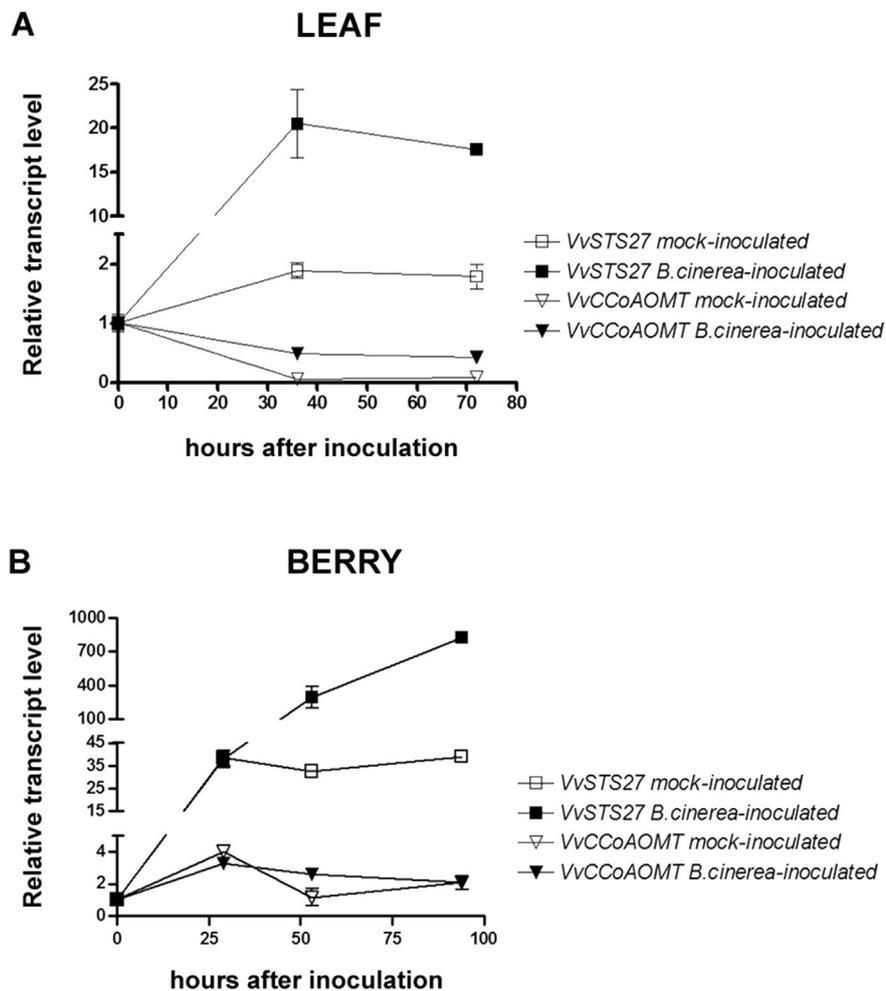
### 3.3. Characterization of *VvCCoAOMT* activity *in vitro*

The *VvCCoAOMT* coding sequence was expressed as a GST recombinant fusion protein in *E. coli*. The recombinant protein was purified (Fig. S3), and showed methylating activity on cyanidin 3-*O*-glucoside chloride. In presence of 250  $\mu$ M of cyanidin-3-*O*-glucoside chloride, the recombinant GST-*VvCCoAOMT* protein purified in batch converted about 9% of the substrate into peonidin 3-*O*-glucoside ( $\lambda_{\max}$  = 516 nm) (Fig. 5). The recombinant protein was tested under the same conditions with caffeoyl-CoA. In presence of recombinant GST-*VvCCoAOMT*, about 20% of the substrate was transformed into feruloyl-CoA ( $\lambda_{\max}$  = 346 nm). In HPLC analysis, both caffeoyl-CoA and feruloyl-CoA displayed two peaks with the same absorbance spectra, as previously reported by Rautengarten et al. (2010).

*In vitro* assay conditions were optimized using the recombinant GST-*VvCCoAOMT* protein. The optimal pH value for the methylation of cyanidin 3-*O*-glucoside chloride was 8.5. Substituting metal ions (Zn<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>) for Mg<sup>2+</sup> decreased enzyme activity on cyanidin 3-*O*-glucoside chloride below the values measured with Mg<sup>2+</sup> (Fig. S4).

Kinetic parameters were calculated both for the GST-*VvCCoAOMT* protein, and for the thrombin-cleaved *VvCCoAOMT*

of the means (n = 3). L = leaf; P = petiole; WB = whole berry; BS = berry skin; BP = berry pulp; S = seed.



**Fig. 3.** Transcript accumulation profiles in Barbera leaves and berries upon biotic stress. Expression changes of *VvSTS27* and *VvCCoAOMT* in detached leaves (A) and ripe berries (B) after inoculation with *Botrytis cinerea* (*B. cinerea*-inoculated samples) or plugs of non-inoculated MEA (mock-inoculated samples), used as negative control. Leaf samples were collected at 0, 36 and 72 h, whereas berry samples were harvested at 0, 29, 53, 94 h after inoculation. Ubiquitin (*VvUBI*) was used as the endogenous control gene for the normalisation procedure. Time at 0 h was used as the reference sample for the calculation of expression ratios. Bars are standard errors of the means ( $n = 3$ ).

protein (Table 1). The affinity to cyanidin 3-*O*-glucoside was slightly higher in the cleaved protein;  $K_m$  for caffeoyl-CoA was in the same order of magnitude than for the anthocyanin substrate. When the fusion protein was tested,  $V_{max}$  was higher for caffeoyl-CoA, than for cyanidin 3-*O*-glucoside. The cleaved protein had a higher  $V_{max}$  and a lower  $K_{cat}$  than the recombinant protein. In the presence of cyanidin 3-*O*-glucoside chloride (250  $\mu$ M), the cleaved protein transformed more than 50% of the substrate into peonidin 3-*O*-glucoside.

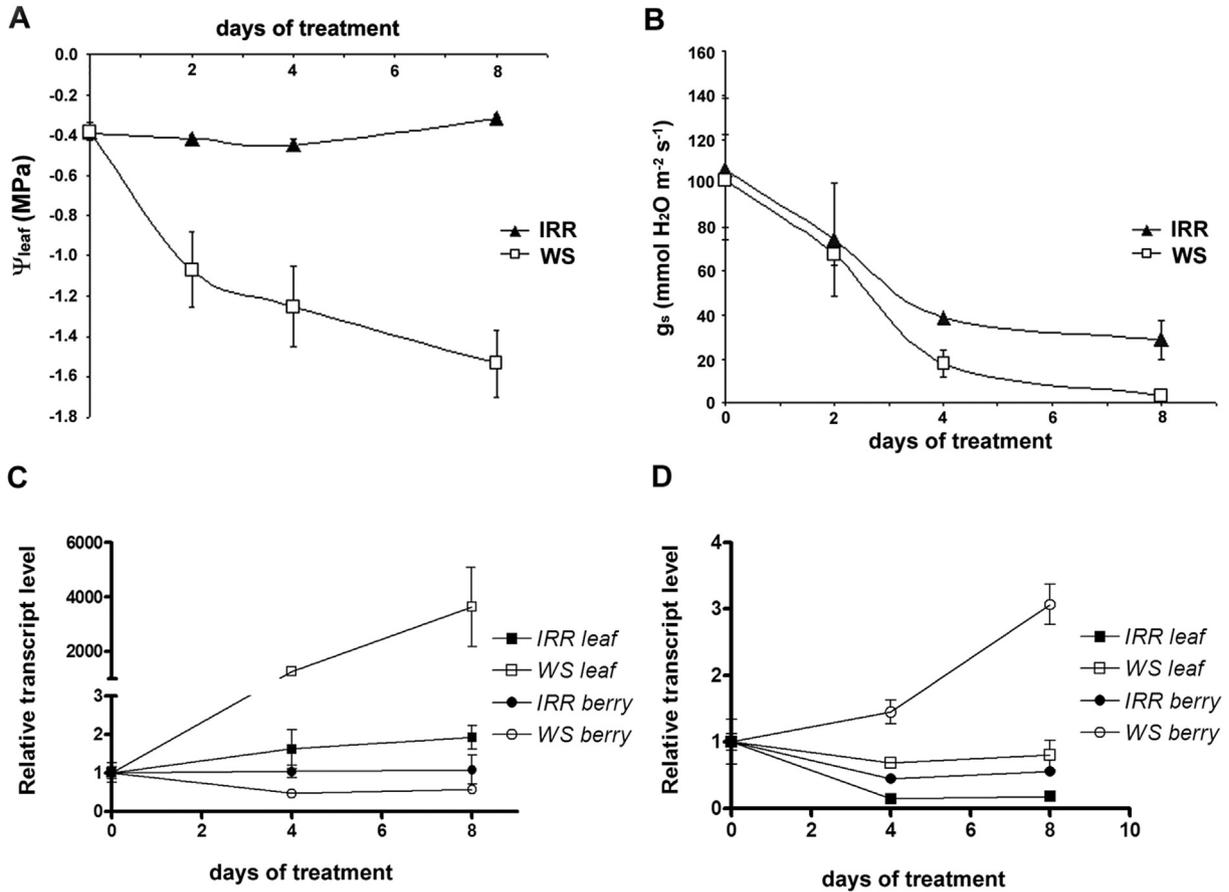
#### 4. Discussion

##### 4.1. *VvCCoAOMT* is a multifunctional enzyme active on anthocyanins

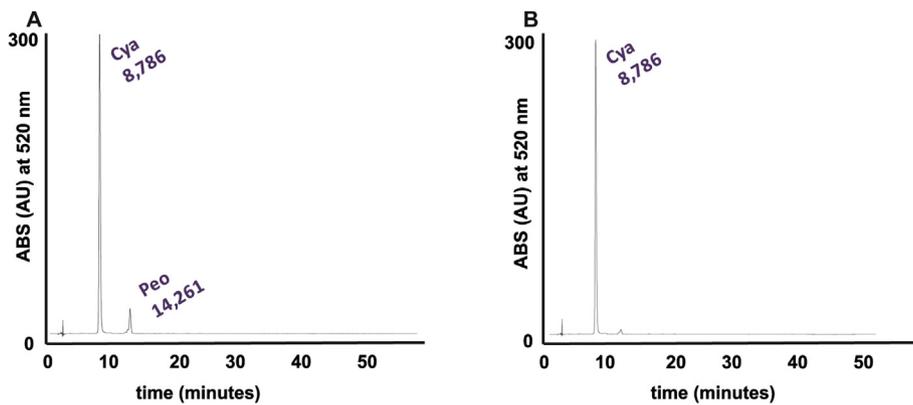
Methylation of oxygen, nitrogen and carbon is a common reaction in bacteria, fungi, plants and animals. In plants, the *O*-methylation patterns of polyhydroxylated molecules are crucial to determine their function (Ibrahim et al., 1998). Plant *S*-adenosyl-*L*-methionine (SAM)-dependent *O*-methyltransferases (OMTs; EC 2.1.1) catalyse methylation of many substrates. Over the years OMT genes have been characterized in several plant species, and different classifications have been proposed for this enzyme family (Joshi and Chiang, 1998; Lam et al., 2007). Based on their structure

and molecular weight, OMTs can be divided into two major classes (Noel et al., 2003). Type 1 OMTs consist of homodimeric enzymes with subunit sizes of 38–43 kDa, which do not require divalent cations for their activity. Conversely, type 2 OMTs represent a group of lower molecular mass (23–27 kDa), cation-dependent OMTs. The first group includes caffeic acid, flavonoid, coumarin, and alkaloid OMTs (Frick and Kutchan, 1999; NDong et al., 2003), whilst most members of the second group are specific for CoA esters of phenylpropanoids. Within the wide family of type 2 plant *O*-methyltransferases, CCoAOMTs catalyse the methylation of caffeoyl-CoA and of other cinnamic thioesters, and play a role in the biosynthesis of lignin monomers (Day et al., 2009). In plants, lignification reinforces the cell wall, and genes involved in lignin biosynthesis, CCoAOMTs included, are activated upon challenge by wall-degrading fungal pathogens (Bhuiyan et al., 2009). However, multifunctional CCoAOMTs have been described in several plants: these proteins are active also on flavonoid substrates, catalysing the 3' or 3'-5' *O*-methylation of their B ring.

By analysing the published grapevine genome, we identified six gene models that, based on the homology with orthologous proteins of the same function, putatively form a family of grape CCoAOMTs. The known genes include *VvCCoAOMT*, whose *in vitro* activity on caffeoyl-CoA has already been demonstrated (Busam et al., 1997) and *VvAOMT*, a multifunctional CCoAOMT that



**Fig. 4.** Physiological parameters and transcript accumulation profiles in Grenache leaves and berries upon water stress treatment. (A) Leaf water potential ( $\Psi_{\text{leaf}}$ ) and (B) stomatal conductance ( $g_s$ ) in leaves of potted plants normally irrigated (IRR) or water stressed (WS). (C) Transcriptional changes of *VvDHN1a* and (D) *VvCCoAOMT* genes in leaves and berries of IRR and WS plants. Ubiquitin (*VvUBI*) was used as the endogenous control gene for the normalisation procedure. Time at 0 days was used as the reference sample for the calculation of expression ratios. Bars are standard errors of the mean ( $n = 3$ ).



**Fig. 5.** HPLC-DAD separation of anthocyanin products after incubation of recombinant GST-VvCCoAOMT purified in batch with cyanidin-3-O-glucoside chloride. (A) Incubation with GST-VvCCoAOMT; (B) incubation with GST only.

**Table 1**

Kinetic parameters measured on the recombinant GST-VvCCoAOMT fusion protein, and on the thrombin-cleaved VvCCoAOMT.

	Substrate	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ ( $\text{nM s}^{-1}$ )	$K_{\text{cat}}$ ( $\text{s}^{-1}$ )
GST-VvCCoAOMT	Cyanidin 3-O-glucoside chloride	$110.3 \pm 4.0$	$4.4 \pm 0.05$	$0.0027 \pm 0.00003$
	Caffeoyl-CoA	$96.0 \pm 2.2$	$13.5 \pm 0.4$	$0.0082 \pm 0.0003$
VvCCoAOMT	Cyanidin 3-O-glucoside chloride	$82.7 \pm 20.0$	$21.3 \pm 2.2$	$0.0129 \pm 0.0014$

methylates anthocyanins and flavonols (Huguency et al., 2009). Phylogenetic analysis of grape CCoAOMTs together with other heterologous proteins shows that VvAOMT and VvCCoAOMT are part of two different clusters, both however including at least one proven multifunctional CCoAOMT: rice ROMT-15 in the first case (Lee et al., 2008), and Arabidopsis CCoAOMT in the second (Do et al., 2007). Although a limited correlation between substrate specificity and primary sequence has previously been observed for *O*-methyltransferases (Ibrahim et al., 1998; Lam et al., 2007), these observations encouraged us to test whether VvCCoAOMT is a multifunctional enzyme and has anthocyanin-methylating properties.

When tested *in vitro*, recombinant GST-VvCCoAOMT showed activity on a flavonoid substrate (cyanidin 3-*O*-glucoside chloride) and transformed about 9% of the substrate into peonidin 3-*O*-glucoside. Incubation with caffeoyl-CoA in the same conditions transformed about 20% of the substrate into feruloyl-CoA, demonstrating activity on this substrate and confirming a previous report (Busam et al., 1997). The kinetic parameters of the recombinant protein on the two substrates were in the same order of magnitude, supporting the hypothesis that VvCCoAOMT is a multifunctional enzyme.

The kinetic parameters of the thrombin-cleaved VvCCoAOMT protein are in the same order of magnitude of those observed on flavonoid substrates for other multifunctional CCoAOMTs. For instance, four recombinant proteins from different plants (*M. crystallinum*, *Stellaria longipes*, tobacco, and Arabidopsis), showed methylating activity on the flavonol quercetin with Km in the range 6.4–25  $\mu\text{M}$ , and Vmax in the range 45–560  $\text{pkat mg}^{-1}$  (Ibdah et al., 2003); similar values were observed on the flavonol myricetin for two rice CCoAOMTs (Lee et al., 2008). Grapevine VvAOMT is active on cyanidin 3-*O*-glucoside, with lower Km (43  $\mu\text{M}$ ) and higher Kcat (0.09  $\text{s}^{-1}$ ) than VvCCoAOMT (82.7  $\mu\text{M}$  and 0.0129  $\text{s}^{-1}$ , respectively). These kinetic data suggest that VvCCoAOMT may contribute to anthocyanin methylation in grape berry skins, albeit with somewhat lower efficiency than VvAOMT.

#### 4.2. Transcript of VvCCoAOMT accumulates during ripening

In order to gain a clearer picture of VvCCoAOMT functional roles, we analysed its transcript levels in different tissues, and upon natural and artificially induced berry ripening.

In vegetative tissues, VvCCoAOMT is highly expressed in leaf petioles, which are likely to contain fairly high levels of lignin, probably comparable with fruit stalks [where lignin content exceeds 150  $\text{mg g}^{-1}$  dry weight (Prozil et al., 2014)]. This agrees with a role of this protein in the methylation of caffeoyl-CoA, a reaction involved in lignin biosynthesis. Coherently, transcripts are less concentrated in leaves, where lignin is likely confined to veins.

Grape berry development follows a double-sigmoid model resulting from two consecutive stages of growth separated by a phase of slow or no growth (véraison). In particular, in the first period berries are green, firm and acidic. During this phase, organic acids, hydroxycinnamoyl esters and tannins accumulate. In Barbera berries, VvCCoAOMT transcript was more abundant in the pre-véraison stage (15 DAF) and it declined at véraison (45 DAF), suggesting a role in the methylation of compounds accumulated at early stages of fruit development. To our knowledge, no data are available on the lignin content in immature grape berries, although the skin-specific activation of cinnamoyl-CoA reductase and cinnamoyl-alcohol dehydrogenase, two genes involved in lignin biosynthesis, has been observed in ripe berries (Grimplet et al., 2007). Other potentially methylated phenolic compounds, such as flavonoids and hydroxycinnamoyl tartrates (HCTs), are accumulated in berries before véraison. Flavonols are an important class of

grape flavonoids, mainly localized in the berry skins of both white and red grapes. Flavonol biosynthesis starts at flowering and is followed by a decline until véraison; thereafter an increase in the expression of biosynthetic genes allows further accumulation (Downey et al., 2003). Several grape enzymes acting on anthocyanins were shown to be active also on flavonols both *in vitro* and *in vivo* (Ford et al., 1998; Huguency et al., 2009), and, based on its expression pattern, VvCCoAOMT could be a candidate for the biosynthesis of methylated flavonols. The content of methylated flavonols before véraison is not known, but is about 3% of total flavonols in the skin of Barbera at harvest (Mattivi et al., 2006). HCTs are the most abundant group of non-flavonoid phenols in grapes. The predominant HCTs in grape berry pulp and skins are caffeoyltartaric acid, *p*-coumaroyltartaric acid and feruloyltartaric acid (Adams, 2006). As in the case of flavonols, these compounds are accumulated both before and after véraison. The methylated feruloyl-tartaric acid is present in ripe Barbera berry skins in a range between 0.8 and 1.6% of total HCTs, which is a relatively high percentage compared to most coloured-grape varieties as many of them do not accumulate feruloyl-tartaric acid at all (Ferrandino et al., 2012).

After véraison, we detected an increase in VvCCoAOMT transcript in the coloured genotype Barbera and not in the uncoloured genotype Moscato. Since ABA is known to trigger the ripening and anthocyanin accumulation in grape berries (Pirie and Mullins, 1976; Jeong et al., 2004; Ferrandino and Lovisollo, 2014), we induced ripening with ABA treatment in incubated Merlot berries, and also in this case we observed an increase in VvCCoAOMT transcript expression. In grape skins, a similar expression pattern is typical of genes involved in anthocyanin decoration, such as the 3-*O*-glucosyltransferase UFGT (Boss et al., 1996) and the methyltransferase VvAOMT (Huguency et al., 2009), suggesting that VvCCoAOMT may contribute to anthocyanin methylation in this tissue. Nevertheless, the expression profile of VvCCoAOMT peaks earlier than what observed for VvUFGT and VvAOMT, whose transcripts increase up to the end of ripening. Also the expression profile after artificial induction of ripening with ABA mirrors the same pattern. This suggests that the action of VvCCoAOMT is concentrated in a shorter time window than that of other anthocyanin decorating enzymes.

In the second period of growth, berries soften, lose chlorophyll, and, in coloured varieties, accumulate anthocyanins in the skin. In this ripening stage, a functional role for VvCCoAOMT in methylation of flavonols and hydroxycinnamic acids seems less probable, due to the observation of no expression activation in Moscato, which contains both types of compounds at concentrations comparable with Barbera: the content of methylated flavonols in the berry skin has not been determined in this genotype yet, but it normally ranges between 2 and 5% of total flavonols in the skin of uncoloured grapes (Mattivi et al., 2006), while the concentration of feruloyl-tartaric acid in Moscato reaches 1% of total HCTs (Ferrandino et al., 2012).

#### 4.3. VvCCoAOMT is activated upon drought stress

Busam et al. (1997) showed that a line of cultured *V. vinifera* cells reacted to treatment with yeast extract with a surge of resveratrol accumulation. Yeast extract, and low concentrations of known elicitors of stress responses (salicylic acid and 2,6-dichloroisonicotinic acid), raised the expression of VvCCoAOMT in cultured cells; however, the response of this gene to stress in plant tissues was not assessed. In our study, we exposed grape berries to both biotic (inoculation with the fungal pathogen *B. cinerea*) and abiotic (drought) stress to test the hypothesis that VvCCoAOMT may be stress-inducible in the plant.

When *B. cinerea* was inoculated onto detached berries, stilbene

synthase expression increased as expected (Bezier et al., 2002). However we did not detect an increase in *VvCCoAOMT* transcript abundance. This discrepancy with the published results may be due to differences in the elicitation method applied (living fungus instead of elicitors) or the nature of the cells used: pigmented berry in the case of this study, unpigmented cell cultures, obtained from hypocotyl tissues, in the study by Busam et al. (1997). However, we did not detect expression activation also in the case of unpigmented, vegetative leaf tissues, where stilbene synthase was also activated. It is possible that the activation of *VvCCoAOMT* in tissues attacked by fungal pathogens may be limited to those actively synthesizing lignin, while activation may not be necessary in berry skins as methylated anthocyanins are not essential components of cell reactions to biotic stress. Coherently, *B. cinerea* infection has not been reported to induce accumulation of anthocyanins in grape leaves or berries. The activation of the anthocyanin biosynthetic pathway and the accumulation of anthocyanins are on the contrary observed in grape leaves upon virus (Gutha et al., 2010), phytoplasma (Margaria et al., 2014), and *Eutypa lata* (Rotter et al., 2009) infection. It will be interesting to test whether in these conditions the methylation of anthocyanins and *VvCCoAOMT* expression are also affected.

Accumulation of anthocyanins is induced in plants by abiotic stresses such as high irradiation, drought, and cold (Chalker-Scott, 1999). In grape, parallel to an increase in berry skin anthocyanin content, drought induces a profile shift towards the methylated forms at the expense of the non-methylated ones (Castellarin et al., 2007b). The enhanced methylation reported in the anthocyanin profile upon abiotic stress may increase the vacuolar antioxidant potential by favouring the biosynthesis of more stable molecules (Sarni et al., 1995).

Our experiment shows that the transcript of *VvCCoAOMT*, encoding a protein with anthocyanin methylating activity *in vitro*, increases in berries of drought-stressed grape plants. Thus *VvCCoAOMT* could significantly contribute to the increase of anthocyanin methylation activity observed upon drought. Transcript accumulation from *VvCCoAOMT4* is also activated upon drought stress (S.D. Castellarin, pers. comm.). Stress inducibility on the contrary does not seem to be a feature of *VvAOMT*, whose transcript level only slightly increases after a long-term drought treatment (Castellarin et al., 2007a). Thus activation of anthocyanin methylation upon abiotic stress could depend on a subgroup of drought-responsive *CCoAOMTs*.

Stress inducibility implies that *VvCCoAOMT* expression is activated by a drought-stress related signal. ABA is the most plausible candidate for this role, as it has been reported to increase upon drought stress in berry skins, at least partly as a consequence of increased expression of biosynthetic genes (Deluc et al., 2009). ABA activates anthocyanin biosynthesis upon osmotic stress (Loreti et al., 2008) and ABA accumulation is induced by abiotic stress in parallel to anthocyanin accumulation (Yamaguchi-Shinozaki and Shinozaki, 2006). *VvAOMT* activation is controlled by *VvMybA1*, the main transcription factor regulating anthocyanin biosynthetic genes in berry skins (Ageorges et al., 2006). Thus it can be hypothesized that transcription of *VvCCoAOMT* is under the control of other, probably ABA-dependent, stress-activated transcription factors.

## Contributions

AS conceived the study, and designed the experiments with DG, FC and SDC. DG, SP, AF, MV, CP and FR performed the experiments and/or analysed the data. AS, DG, and CP drafted the paper. All authors contributed to manuscript revision, and approved the final version.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2016.01.015>.

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