Research article

Exogenous application of pectin-derived oligosaccharides to grape berries modifies anthocyanin accumulation, composition and gene expression

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ABSTRACT

Anthocyanins are secondary metabolites synthesized in grape berry skins via the phenylpropanoid pathway, with functions ranging from skin coloration to protection against pathogens or UV light. Accumulation of these compounds is highly variable depending on genetics, environmental factors and viticultural practices. Besides their biological functions, anthocyanins improve wine quality, as a high anthocyanin content in berries has a positive impact on the color, total phenolic concentration and, ultimately, the price of wine. The present work studies the effect of the pre-veraison application of pectin derived oligosaccharides (PDO) on the synthesis and accumulation of these compounds, and associates the changes observed with the expression of key genes in the phenylpropanoid pathways. To this end, pre-veraison Cabernet Sauvignon bunches were treated with PDO to subsequently determine total anthocyanin content, the anthocyanin profile (by HPLC-DAD) and gene expression (by qRT-PCR), using Ethrel and water treatments for comparison. The results show that PDO were as efficient as Ethrel in generating a significant rise in total anthocyanin content at 30 days after treatment (dat), compared with water treatments (1.32, 1.48 and 1.02 mg e.Mv-3G/g FW respectively) without any undesirable effect on berry size, soluble solids, tartaric acid concentration or pH. In addition, a significant alteration in the anthocyanin profile was observed. Specifically, a significant increase in the relative concentration of malvidin was observed for both PDO and Ethrel treatments, compared with water controls (52.8; 55.0 and 48.3%, respectively), with a significant rise in tri-hydroxylated forms and a fall in di-hydroxylated anthocyanins. The results of gene expression analyses suggest that the increment in total anthocyanin content is related to a short term increase in phenylalanine ammonia-lyase (PAL) expression, mediated by a decrease in MYB4A expression. A longer term increase in UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) expression, probably mediated by a rise in MYBA1 was also observed. Regarding the anthocyanin profile, despite the increase observed in MYB5A expression in PDO and Ethrel treatments, no changes in flavonoid 3′-hydroxylase (F-3′-H); flavonoid 3′5′-hydroxylase (F-3′5′-H) or O-methyltransferase (OMT) could be related with the profile modifications described. Overall, this study highlights that application of PDO is a novel means of altering specific grape berry anthocyanins, and could be a means of positively influencing wine quality without the addition of agrochemicals.

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1. Introduction

Anthocyanins are secondary metabolites synthesized in grape berry skins (and in some varieties in pulp or flesh) from veraison to maturity. Although their main function is berry skin coloration (red to blue color) (Boss and Davies, 2009; Kalt et al., 2003), these molecules are involved in a wide range of biological functions including antioxidant capacity, and protection against UV-light and pathogen attack (Takahama, 2004; Chalker-Scott, 1999). The

accumulation and proportion of these compounds depend on genetics (Dai et al., 2011; He et al., 2010; Rio-Segade et al., 2008), environmental factors and viticultural practices (Downey et al., 2006), with light and temperature the primary environmental factors capable of modifying the synthesis and accumulation of anthocyanins (Keller, 2010; Mori et al., 2005a,b; Roubelakis-Angelakis and Kliewer, 1986). These molecules, which are flavonoid compounds, are synthesized through the phenylpropanoid pathway (Fig. 1), which is widespread and extensively studied in different plant species. Consequently, several structural genes, encoding enzymes in the anthocyanin biosynthetic pathway, have been described (Borsani et al., 2010; Boss et al., 1996; Cultrone et al., 2010; Honda et al., 2002; Matus et al., 2008; Xie et al., 2011). Among these structural genes PAL, encoding phenylalanine ammonia-lyase (responsible for the first step in anthocyanin biosynthesis) and UFGT, encoding UDP glucose:flavonoid-3-O-glucosyltransferase (responsible for glycosylation of anthocyanidin) have been described as key genes (Fig. 1). The expression of these structural genes is in turn controlled by biosynthetic genes encoding R2R3MYB, basic helix-loop-helix (bHLH) and tryptophan-aspartic acid repeat (WDR) transcription factors (Hichri et al., 2010; Jeong et al., 2006). In grapes, several MYB-family proteins controlling various points of the phenylpropanoid pathway have been identified (Fig. 1). MYB4A transcription factor is described as an inhibitor of C4H and 4CL expression (Colquhoun et al., 2011; Jin et al., 2000) and has also been suggested as an inhibitor of PAL expression (Cavallini et al., 2015), whereas MYBA1/2 transcription factors activate the expression of UFGT and OMT (a structural gene responsible for methylation downstream of anthocyanin glycosylation) (Bogs et al., 2007; Cutanda-Perez et al., 2009; Kobayashi et al., 2002; Walker et al., 1999). Another transcription factor, MYB5A trans-activates the expression of F3′5′H (responsible for deviation of the phenylpropanoid pathway to tri-hydroxylated anthocyanin forms) (Deluc et al., 2006, 2008).

Besides their importance in plant metabolism, anthocyanins have been extensively studied due to their beneficial effect on human health (De Pascual-Teresa and Sanchez-Ballesta, 2008; Jing et al., 2008; Maletić et al., 2009) as well of being an important determinant of quality in red wines (Fanzone et al., 2012; Mori et al., 2005b). In order to increase anthocyanin synthesis and accumulation, various viticultural strategies have been developed, such as defoliation or application of chemicals, which have not always led to the desired effect and/or are expensive and time consuming (Greer and La Borde, 2006; Smart and Robinson, 1991). An innovative practice has been the application of plant cell wall derivatives (more specifically, pectin-derived oligosaccharides; PDO) in order to increase anthocyanin content in grapes. In this way, Ochoa-Villareal et al. (2011) reported that application of PDO can promote color development in Flame Seedless table grapes through anthocyanin accumulation. However, the effect of PDO on the expression of multiple structural and regulatory genes of the phenylpropanoid pathway, and their influence on wine-making varieties, has not been evaluated to date. Therefore, we examined whether application of PDO promotes anthocyanin accumulation in Cabernet Sauvignon berries, determining changes in the profile of the different anthocyanins and associating the modifications observed (either in accumulation or in the anthocyanin profile) to changes in expression of structural and regulatory genes of the
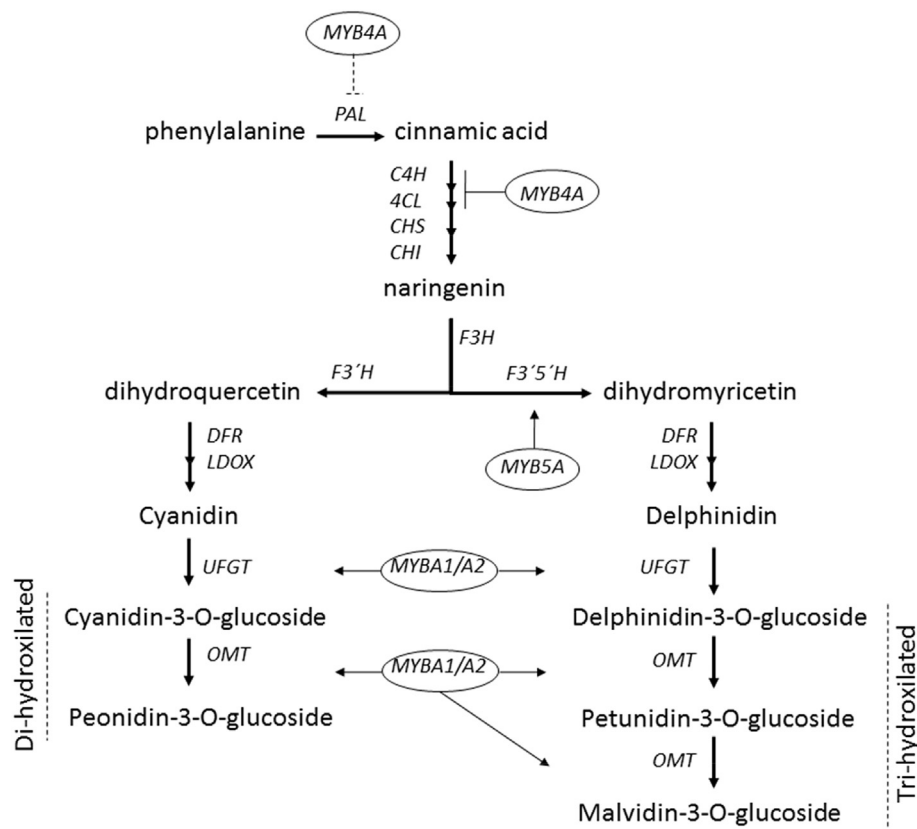
Fig. 1. Simplified overview of the phenylpropanoid pathway leading to the accumulation of di- and tri-hydroxylated anthocyanins in grape berries. Gene abbreviations: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4 hydroxylase; *4CL*, 4 coumarate:CoA ligase; *CHI*, chalcone synthase; *F3H*, flavanone 3 hydroxylase; *F3′H*, flavonoid 3′-hydroxylase; *F3′5′H*, flavonoid 3′5′-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *LDOX*, leucoanthocyanidin dioxygenase; *UFGT*, UDP-glucose flavonoid 3-O-glucosyltransferase; *OMT*, O-methyltransferase; *MYB*, R2R3-MYB transcription factor. The dashed line over *PAL* indicates a possible negative regulation by *MYB4A*.

phenylpropanoid pathway.

2. Experimental

2.1. Plant material

The following study was conducted on *Vitis vinifera* L. Cabernet Sauvignon plants located at the experimental station belonging to the Facultad de Agronomía de la Pontificia Universidad Católica de Chile located in Pirque (33°40′ S; 70°35′ W). The vines were grown on their own roots using a bilateral cordon with a vertical shoot positioning trellis system in a north-south orientation. All plants had similar growth and proper health/nutrition status. The 15 plants selected for the trial were pruned so that all possessed 12 equally-distributed clusters in an east-west exposure. Four clusters per plant were assigned to each treatment. Samples were periodically extracted and stored at −20 °C for further analysis.

2.2. PDO preparation and application

In order to obtain physiological effects, pectin-derived oligosaccharides (PDO) must have a degree of polymerization ranging from 3 to 20 galacturonic acid residues (Farmer and Ryan, 1990; Moloshok et al., 1992; Côté and Hahn, 1994; Cabrera et al., 2008). In this study, a mixture of PDO with a degree of polymerization from 3 to 6 was obtained by enzymatic digestion of polygalacturonic acid (Sigma #P3889) with endo-polygalacturonase from *Aspergillus niger* (Sigma #17389) at 37 °C for 5 min. The reaction was then stopped by boiling for 10 min. The degree of polymerization was confirmed by polyacrylamide carbohydrate electrophoresis (according to Goubet et al., 2002, 2003, 2005) and MALDI analysis (in collaboration with the Complex Carbohydrate Research Center, Athens, GA). Treatments corresponded to PDO (at 1.5 mg/mL in 50 mM sodium-acetate buffer pH 5), Ethrel (300 ppm) and water; all three contained 0.05% Tween 20, a surfactant to facilitate absorption. A buffer-alone treatment (50 mM sodium-acetate buffer pH 5; 0.05% Tween 20) was not included because preliminary tests showed that the sodium acetate had no effect on the parameters evaluated here (berry weight, pH, soluble solids, titratable acidity and TAC). Treatments were applied by direct immersion of the bunch (for 10–20 s) 5 days before the onset of *veraison* or color change.

2.3. Maturity and growth parameters

For each sampling date, berry size and weight as well as pH, titratable acidity and soluble solids (in grape juice) were performed (according to O.I.V, 2007).

2.4. Total anthocyanin content (TAC) and anthocyanin profile

Samples were subjected to hydro-alcoholic extraction (Ethanol 50% v/v, adjusted to pH 2 with HCl) following the protocol proposed by Iland et al. (1996) and the sampling and homogenization recommendations proposed by AWRI (2009). After extraction, all samples were filtered through 0.45 μm nitrocellulose membranes. Two mL aliquots were used to screen absorbance at 520 nm in a spectrophotometer (UV-1700, Shimadzu, Japan) to quantify TAC according to García-Barceló (1990). TAC was expressed as milligrams of malvidin-3G equivalent per fresh weight (mg e.Mv-3G/g FW). Anthocyanin profiling was performed according to Peña-Neira et al. (2007) using a Hitachi HPLC-DAD L-2350 system (Merck). Briefly, after injecting 150 μL, separation was achieved on a reverse phase LiChrospher 100 RP-18 column (250 mm × 4.6 mm i.d. 5 μm) (Agilent Technologies) at 20 °C. The detection was carried out at 520 nm. Solvent A was 10% formic acid in water (v/v) and solvent B was acetonitrile. The following gradient elution program was used, 0–8 min, 96% solvent A: 4% solvent B (flow rate 1.1 mL min^{-1}); 8,1–27 min, 85% solvent A: 15% solvent B (flow rate 1.1 mL min^{-1}); 27,1–40 min, 80% solvent A: 20% solvent B (flow rate 1.5 mL min^{-1}); 40,1–43 min, 70% solvent A: 30% solvent B (flow rate 1.5 mL min^{-1}); 43,1–45 min, 96% solvent A: 4% solvent B (flow rate 1.1 mL min^{-1}). All reagents were analytical grade obtained from Merck.

2.5. Nucleic acid extraction and cDNA synthesis

Total RNA was isolated from grape berries using a CTAB-spermidine extraction buffer, according to the procedure of Reid et al. (2006). For cDNA synthesis, 1 μg of total RNA was reverse transcribed with random primers using the M-MLV® reverse transcriptase (Promega) according to the manufacturer's instructions.

2.6. Quantitative comparison of gene expression

Relative transcript quantification of selected genes was performed by real time RT-PCR, using the Brilliant® SYBR® Green QPCR Master Reagent Kit (Stratagene) and the Mx3000P detection system (Stratagene) as described in the manufacturer's manual. Primers were designed according to previous reports (Table 1). A fragment of the *UBIQUITIN1* gene (99 bp; TIGR # accession TC53702) was used for normalization because its expression was stable in different samples and dates (Downey et al., 2003; Matus et al., 2010). PCR condition optimization, standard curve quantification for each gene, primer efficiency values and relative gene expression calculations were conducted according to Poupin et al. (2007). Standard quantification curves with serial dilutions of PCR products were constructed for each gene to calculate amplification efficiency according to Equation (1):

$$E = \left(10^{(-1/slope)}\right)^{-1} \tag{1}$$

The ratio between the gene of interest (GOI) and *UBIQUITIN* expression was calculated using Equation (2):

$$\frac{(1+E_{GOI})^{-\Delta Ct}}{(1+E_{UBI})^{-\Delta Ct}} = \frac{(1+E_{GOI})^{-(Ct\ GOI - Ct\ GOI\ calibrated)}}{(1+E_{UBI})^{-(Ct\ UBI - Ct\ UBI\ calibarted)}} \tag{2}$$

where E corresponds to each primer amplification value efficiency. All experiments were performed used three biological replicates and two technical replicates. Melting gradient dissociation curves were using to test reaction specificities.

2.7. Statistical analysis

TACs, anthocyanin profiles and relative gene expression data were statistically analyzed by one way ANOVA to test the significance of the effects of treatments at the different sampling dates. Tukey media comparison analysis was performed to compare the treatments. Statistical differences between means were based on the least significant method when F values were significant with $P < 0.05$.

3. Results & discussion

3.1. Berry weight and berry maturity evolution

Field-grown pre-*veraison* Cabernet Sauvignon bunches were

Table 1
Primers used for real-time quantitative PCR.

Name	TIGR # accession	Sequence	Reference
UBI	TC53702	5′-TCTGAGGCTTCGTGGTGGTA-3′(Fw)	Downey et al., 2003
		5′-AGGCGTGCATAACATTTGCG-3′(Rv)	
PAL	XM_002268737	5′-CTGGCCAAATCGAGGCTGC-3′(Fw)	Ochoa-Villarreal et al., 2011
		5′-CTTCTGCATCAGTGGATATGTG-3′(Rv)	
F-3′-H	XM_002284129.1	5′-ATTCGCCACCCTGAAATGAT-3′(Fw)	Castellarin et al., 2006
		5′-AGCCGTTGATCTCACAGCTC-3′(Rv)	
F-3′5′-H	XM_002265787.2	5′-AGAATGGGAATAGTGCTGGT-3′(Fw)	Guan et al., 2012
		5′-CCGAAAGAGAAACTGCCTT-3′(Rv)	
UFGT	AF000372	5′-GGGATGGTAATGGCTGTGG-3′(Fw)	Jeong et al., 2004
		5′-ACATGGGTGGAGAGTGAGTT-3′(Rv)	
MYB5A	AY555190	5′-GTGCAGCAGCCATCTAATGTG-3′(Fw)	Matus et al., 2009
		5′-GCAGCAGGTTCCCAGACAGT-3′(Rv)	
MYB4A	EF113078	5′-ACCGGACGTTACAACCATATC-3′(Fw)	Matus et al., 2008
		5′-TCCGTAACTGGGTTTTTCTCA-3′(Rv)	
MYBA1	AB097923	5′-TTATCGCAAGCCTCAGGACAGAAG-3′(Fw)	Yamane et al., 2006
		5′-CCCAGAAGCCCACATCAAATGGAA-3′(Rv)	
OMT	BQ796057	5′-CTCTGCAGGCGCCTCTATTA-3′(Fw)	Cutanda Perez et al., 2009
		5′-CCCAAAACAGAGTCTGGACA-3′(Rv)	

treated with PDO, using Ethrel and water treatments for comparison as described in the Experimental section. On metabolism by the plant, Ethrel is rapidly converted into ethylene, a powerful regulator of maturation (Hale et al., 1970; Szyjewicz et al., 1984; González et al., 2015). In order to discard possible effects of the treatments on berry growth and maturity, fresh weight (grams*berry^{-1}), pH, soluble solids (SS) and titratable acidity (TA) were determined. Fig. 2 shows that none of the treatments significantly affected the increase in berry fresh weight at any of the days after treatment (dat) studied, which in turn determines that maximum berry weight was reached at 30 dat (berry weight stabilization). This is relevant because after berry weight stabilization, sugar concentration, and other parameters can undergo changes related primarily to dehydration and not necessarily to metabolic processes per se (Ryan and Revilla, 2003; Yokotsuka et al., 1999). To avoid misinterpretation resulting from dehydration, all maturity parameters as well as anthocyanin concentration, anthocyanin profiles and gene expression were performed prior to berry weight stabilization (up to 30 dat). Regarding the maturity parameters evaluated, we observed that at 30 dat (Table 2) neither treatment
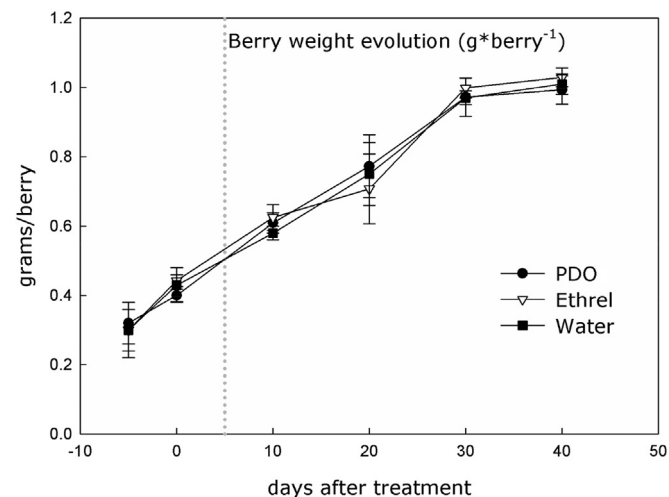
Table 2
Soluble solids (SS), pH and titratable acidity (TA) at 30 days after treatment of Cabernet Sauvignon berries with PDO, Ethrel and water. Data correspond to means (n = 4) ± se.

	SS (°Brix)	pH	TA (tartaric acid, g*L^{-1})
PDO	20.43 ± 1.36	3.69 ± 0.08	4.05 ± 0.38
Ethrel	20.95 ± 0.36	3.76 ± 0.05	4.02 ± 0.22
Water	20.10 ± 1.11	3.70 ± 0.08	4.00 ± 0.33

produced significant changes in any of these parameters (SS, pH, TA) which in turn are consistent with values reported widely in the literature (Obreque-Slier et al., 2012; Soubeyrand et al., 2014). Therefore, the results suggest that under the conditions of our study, the treatments did not significantly affect the growth or ripening of treated berries.

3.2. Effect of treatments on the total anthocyanin concentration (TAC)

Low, statistically equivalent values of TAC were observed at the beginning of the assay in all treatments (>0.03 mg e.Mv-3G/g FW) according to the pre-veraison conditions (Fig. 3). As berries ripened (10 dat) both PDO (0.21 mg e.Mv-3G/g FW) and Ethrel (0.32 mg e.Mv-3G/g FW) treatments had higher TAC than berries treated with water (0.14 mg e.Mv-3G/g FW) although only the Ethrel treatment was significant. At 20 dat, both PDO and Ethrel treatments generated a significant increase in TAC compared to the water treatment (1.17, 1.35 and 0.96 mg e.Mv-3G/g FW respectively), and this pattern of difference was maintained at 30 dat.

3.3. Effect of treatments on the anthocyan profile

Differences in TAC as well as the different dynamics of accumulation observed using the experimental conditions employed, may suggest a differential content of each type of anthocyanin. Therefore, we proceeded to evaluate the anthocyan profile by HPLC-DAD analysis. For each treatment, 15 anthocyanins were considered: delphidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) in their glycosylated (gl), acetylated (ac) and coumaroylated (cum) derivative forms (i.e., Dp3gl; Cy3gl; Pt3gl; Pn3gl; Mv3gl; Dp3acgl; Cy3acgl; Pt3acgl; Pn3acgl; Mv3acgl; Dp3cumgl; Cy3cumgl; Pt3cumgl; Pn3cumgl and Mv3cumgl). The relative concentration of each anthocyanin was calculated as the

Fig. 2. Increase in berry fresh weight of Cabernet Sauvignon treated with PDO (black filled circle), Ethrel (open inverted triangle) and water (black filled square). The dotted line indicates the date of veraison (defined as first signs of color change). Day 0 refers to the timing of the application of PDO, Ethrel or water. Data correspond to means (n = 4) ± se.
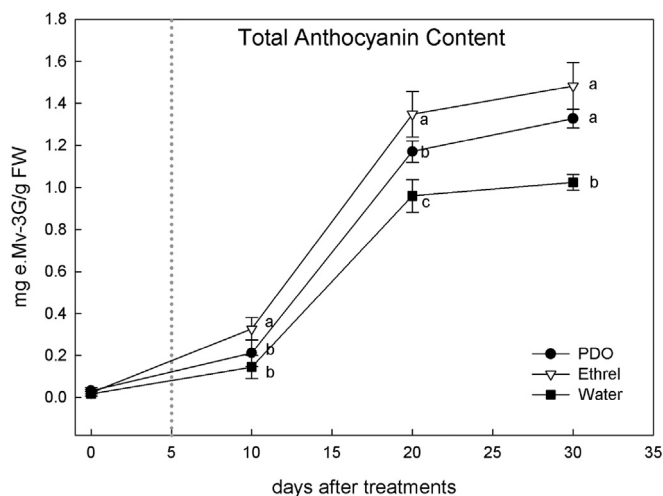
Fig. 3. Total anthocyanin content in Cabernet Sauvignon berries treated with PDO (black filled circle), Ethrel (open inverted triangle) and water (black filled square). The dotted line indicates the date of veraison (defined as first signs of color change). Data correspond to means (n = 4) $\pm$ se. A different letter at the same time point indicates significant differences ($p < 0.05$). mg e.Mv-3G/g FW; milligrams of malvidin-3G equivalent per fresh weight.

sum of its glycosylated, acetylated and coumaroylated derivative forms. Firstly we analyzed whether the differences between TAC reflect alterations in the proportions of different forms of anthocyanins present in berries. For this, we compared the contents at 20 dat (Table 3) since at this time point, all treatments presented significantly different TACs. Analysis of the data at 20 dat shows that no treatment altered significantly the abundance of the different anthocyanins, in that Cy < Pt < Dp < Pn < Mv in all cases, an inherent characteristic of this species (regardless of the variety or strain; Ortega-Regules et al., 2006). However, even though there are no changes in anthocyanin rank, we detected significant alterations in the relative concentration of each individual anthocyanin (Fig. 4). At 10 dat we observed that berries treated with Ethrel had a substantially different profile than those treated with water (higher relative concentrations of Mv and Pn and lower relative concentrations of Cy, De and Pt respectively) while berries treated with PDO displayed an intermediate behavior. At 20 dat, berries treated with PDO tended to have relative concentrations which were significantly different from those treated with water (higher concentration of Mv and lower concentrations of Cy, De, Pn and Pt) and very similar to those berries treated with Ethrel (although the former possessed lower concentrations of De and Pt). Finally, at 30 dat each treatment generated a distinct anthocyanin profile. Specifically, berries treated with PDO exhibited significantly higher relative concentrations of Mv than those treated with water but lower than those treated with Ethrel. For De, Pn and Pt, berries treated with PDO had lower relative concentrations than those treated with water but higher than those treated with Ethrel. Finally the relative concentration of Cy was the same in PDO and water treatments, but significantly lower than in those treated with
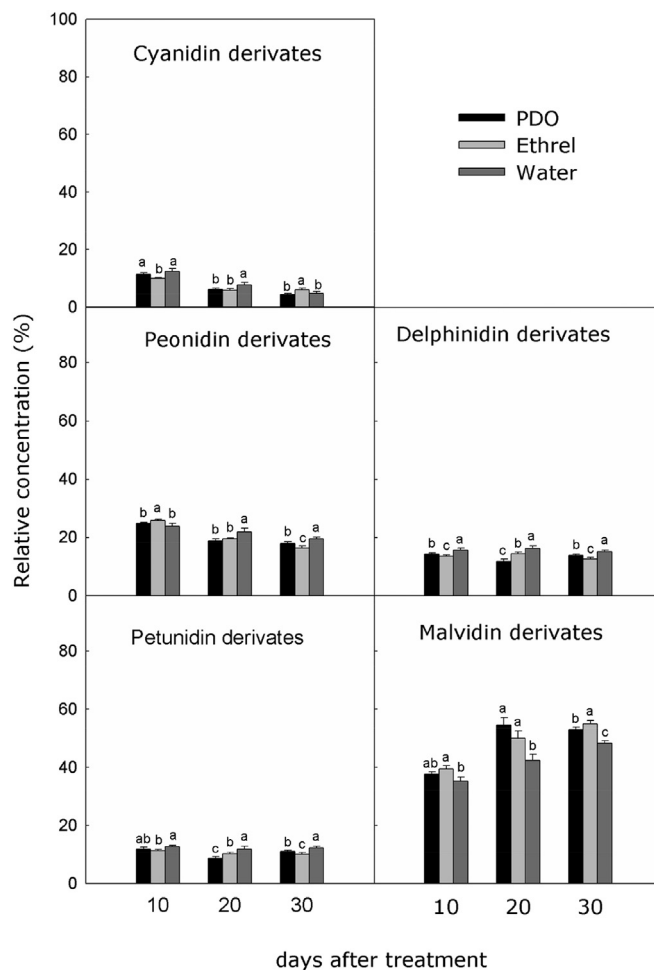

Fig. 4. Relative concentration of all types of anthocyanins at 10, 20 and 30 days after treatment of Cabernet Sauvignon berries with PDO, Ethrel and water. Data correspond to means (n = 4) $\pm$ se. Different letters within the same anthocyanin form indicate significant differences ($p < 0.05$).

Ethrel.

Taken together, it is interesting to note that equal TAC levels do not necessarily imply the same anthocyanin profile. In this way, although at 10 dat both PDO and water treatments had equal TAC, their anthocyanin profiles were significantly different, as also occurred at 30 dat for PDO and Ethrel treatments. Muñoz et al. (2014) have recently reported a similar behavior in Malbec clones with equal anthocyanin content but significantly different profiles. Another relevant aspect to note is that for any conditions in which significant increases in the relative content of Mv were determined, this was accompanied by a significant decrease in the relative content of De and Pt. A similar negative relation was observed in different Malbec clones, consistent with the fact that both De and Pt being precursors of Mv (Muñoz et al., 2014). Therefore, Mv synthesis necessarily involves the reduction of De and Pt (Boss and Davies, 2009).

Grape anthocyanins undergo a series of modifications, of which hydroxylation and methylation are the most important. With respect to hydroxylation, anthocyanins can be grouped into di-hydroxylated derivates of Cy and Pn (which tend to give red-orange hues) and tri-hydroxylated derivates of De, Pt and Mv (which tend to give violet–blue hues). Grouping anthocyanins according to their hydroxylation degree (Table 4), we observed that although initially (10 dat) the treatments are statistically

Table 3
Percentage distribution of anthocyanin forms at 20 days after treatment of Cabernet Sauvignon berries with PDO, Ethrel and water.

	Cyanidin	Petunidin	Delphinidin	Peonidin	Malvidin
PDO	6.7	9.9	13.5	15.7	54.2
Ethrel	6.1	10.9	14.3	19.7	49.0
Water	8.2	12.9	16.6	22.5	39.8

Table 4
Relative concentration of anthocyanin in Cabernet Sauvignon berries treated with PDO, Ethrel or water at 10, 20 and 30 days after treatments, grouped according to their hydroxylation degree (3-OH v/s 2-OH) and according to the presence or absence of methyl groups (met. v/s no-met.). Data correspond to means (n = 4).

		3-OH		2-OH		Met.		No-met.	
10 dat	PDO	63.7	ns	36.3	ns	74.3	b	25.7	b
	Ethrel	64.2	ns	35.8	ns	76.5	b	23.5	c
	Water	63.6	ns	36.4	ns	71.8	c	28.2	a
20 dat	PDO	74.9	a	25.1	b	82	a	18	b
	Ethrel	74.5	a	25.5	b	79.7	a	20.3	b
	Water	70.3	b	29.7	a	76.1	b	23.9	a
30 dat	PDO	77.7	a	22.3	b	81.8	ns	18.2	ns
	Ethrel	77.6	a	22.4	b	81.4	ns	18.6	ns
	Water	75.7	b	24.3	a	80.1	ns	19.9	ns

Different letters indicate significant differences ($p<0.05$). ns = not significant.

equivalent, as the berries ripened (20 and 30 dat), both PDO and Ethrel treatments generated a significant increase in tri-hydroxylated forms at the expense of the di-hydroxylated forms, as compared with the water treatment. Since hydroxylation pattern of the anthocyanins directly affects the hue and color stability (He et al., 2012), darker grapes (with greater presence of tri-hydroxylated anthocyanins) give rise to a darker wine, which in turn is a desirable attribute in wine quality (Fanzone et al., 2012). Methylation substitutions are important given the predominance of Mv which can represent up to 80% of total anthocyanins in Cabernet Sauvignon and because methylation tends to stabilize the anthocyanin structure, providing protection from further degradation (Ali et al., 2011). Grouping anthocyanins according to the presence or absence of methyl groups (Table 4), we observed that at 10 dat the relative concentrations of methylated anthocyanins were significantly different in all treatments (76.5; 74.3; and 71.8% for Ethrel, PDO and water respectively), but while the berries ripen (20 and 30 dat), the relative concentration of methylated and non-methylated anthocyanins presented no differences between treatments.

3.4. Effect of treatments on the relative expression of genes of the phenylpropanoid pathway

Given the observed changes in the TAC and anthocyanin profiles, we studied the relative expression of key genes of the phenyl-propanoid pathway. Thus, the relative expression of the structural genes *PAL*, *F-3′-H*, *F-3′5′-H*, *UFGT* and *OMT* and the regulatory genes *MYBA1*, *MYB4A* and *MYB5A* was determined by quantitative real-time PCR at 1, 10 and 20 dat in order to observe short and long term changes.

Regarding changes in TAC, we assessed whether these are related to alterations in the relative expression of the structural genes *PAL* (encoding the enzyme responsible for the first metabolic step of the phenylpropanoid pathway) and *UFGT* (encoding the enzyme responsible for stabilization of anthocyanidins by the addition of a glucose residue at position 3 of the C ring), and to the relative expression of the regulatory genes *MYB4A* (suggested negative regulator of *PAL*) (Cavallini et al., 2015) and *MYBA1* (positive regulator of *UFGT*) (Boss and Davies, 2009) (Fig. 5). Both PDO and Ethrel treatments were effective in upregulating *PAL* expression as compared with the water treatment, immediately after application (1 dat) (Fig. 5a). However, as berries ripened (10 and 20 dat), the expression levels of *PAL* decreased for all treatments (without significant differences between them). This short term activation and subsequent inhibition of *PAL* expression was expected since phenylalanine ammonia-lyase is one of the first enzymes to be activated in the phenylpropanoid pathway but, while
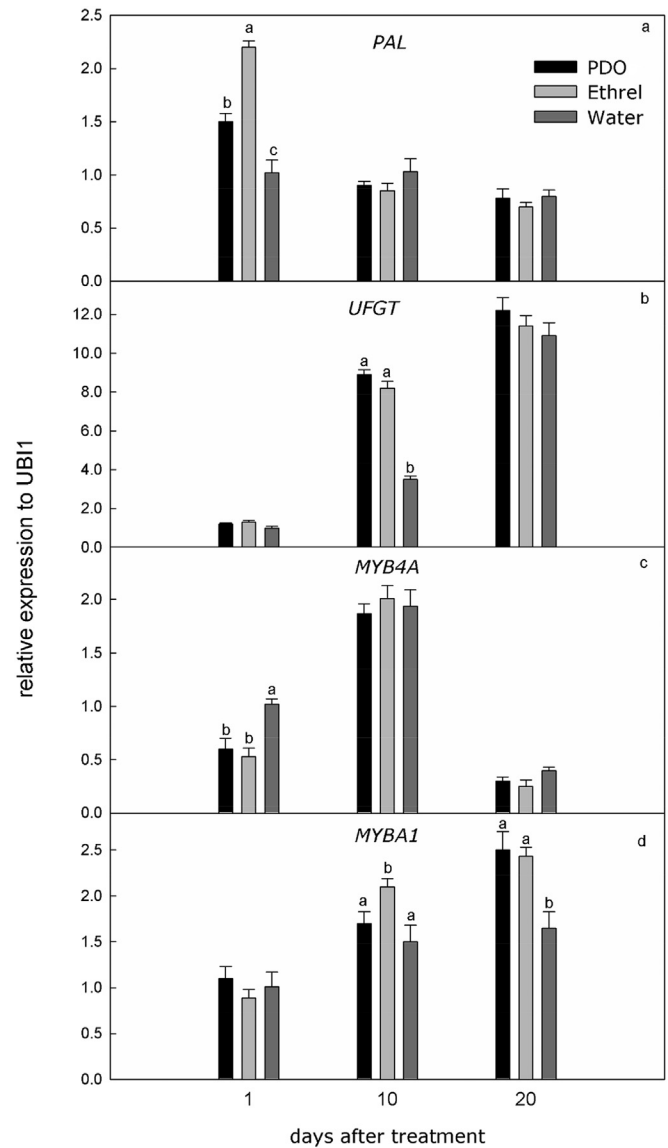
Fig. 5. Relative expression of *PAL* (a); *UFGT* (b); *MYB4A* (c) and *MYBA1* (d) at 1, 10 and 20 days after treatment of Cabernet Sauvignon berries with PDO, Ethrel and water. Data correspond to means (n = 4) ± se. Different letters indicate significant differences ($p < 0.05$).

berries ripen and acquire color, its expression subsequently decreases (Boss et al., 1996; Chen et al., 2006). The relative expression of *MYB4A* is consistent with its role as a negative regulator of *PAL* expression suggested by Cavallini et al. (2015), since we observed that both PDO and Ethrel treatments showed significantly lower expression values as compared with water controls (at 1 dat). As berries ripen, expression levels of this transcription factor tended to increase (10 dat) before falling at 20 dat, with no significant differences between treatments (which in turn is consistent with no differences in *PAL* expression observed between 10 and 20 dat). Regarding the effect of treatments on *UFGT* expression, initially none of the treatments generated significant differences in the expression level of *UFGT*. However, at 10 dat both PDO and Ethrel elevated the expression level of *UFGT* (with no difference between them), as compared with the water treatment. At 20 dat, even when an apparent increase in expression level (particularly in berries treated with PDO) was discerned, the differences were not significant. Regarding the expression level of *MYBA1* (positive regulator

of *UFGT*), no differences between treatments were observed at 1 dat; however as berries ripen both PDO and Ethrel treatments were effective in promoting expression of *MYBA1* as compared with water treatments (at 10 and 20 dat for Ethrel and only at 20 dat for PDO treatment). In this way, the short-term increase in *PAL* expression (1 dat) observed in PDO and Ethrel treatments might be related with the higher anthocyanin content described earlier. In fact, the higher efficiency of Ethrel (2.8 times) compared with PDO (1.6 times) in activating *PAL* expression (as compared with water treated berries) could explain that even by 10 dat, berries treated with Ethrel had a higher content of anthocyanin. Ochoa-Villarreal et al. (2011) reported similar results for the Flame Seedless table grape, with the difference that in their trials, both treatments (PDO and Ethrel) were equally effective in promoting expression of this gene. These authors also reported low levels of *PAL* expression as berries ripen, with no significant differences between PDO, Ethrel and water treatments. Our results suggest that this increase in *PAL* expression could be mediated by an inhibition in the expression level of *MYB4A*. Cavallini et al. (2015) have recently characterized the spatio-temporal expression of *MYB4A* and proposed a negative regulatory role for this transcription factor on *PAL* expression. In this way, the authors reported that the expression of *MYB4A* remained high during berry development with a drop at *veraison* and an increase thereafter, which is in agreement with our results. The increased level of *UFGT* expression observed at 10 dat could also be related with the increment in TAC in these berries, although this could be a direct effect of the treatments and/or the result of increased substrate availability, caused by increased *PAL* activity. The expression level of *MYBA1* is consistent with its role in promoting *UFGT* expression at 10 dat (Matus et al., 2009). However, expression levels remained significantly higher at 20 dat (when *UFGT* expression presented no differences) suggesting that the promoting effect of PDO and Ethrel on anthocyanin metabolism refers not only to structural genes, *PAL* and *UFGT*, as this transcription factor also regulates the expression of a number of other structural genes located downstream of *UFGT*, involved in anthocyanin modification and transport (Cutanda-Perez et al., 2009).

We assessed whether the changes in the anthocyan profile could be related to dynamism in the relative expression of the structural genes *F-3′-H* (the first step in the di-hydroxylated branch of the phenylpropanoid pathway), *F-3′5′-H* (the first step in the tri-hydroxylated branch), *OMT* (responsible for methylation of un-methylated anthocyanins) and of the regulatory gene *MYB5A* (positive regulator of *F-3′5′-H*; Fig. 6). We observed that the relative expression of *F-3′-H* and *F-3′5′-H* increased as the berries ripen but none of the treatments (at any of the sample dates) generated differences in the expression in these genes that may be associated with the higher contribution of tri-hydroxylated forms observed in the berries treated with PDO and Ethrel. However, measuring the expression level of *MYB5A*, we observed that during berry ripening, both PDO and Ethrel treated berries had significantly higher expression levels of this transcription factor as compared with water treated berries. Regarding *OMT*, there were no differences in expression level detected at 1 or 10 dat which could account for the higher contents of methylated anthocyanins determined at the latter time point. However, at 20 dat both PDO and Ethrel treatments were effective in promoting the expression of this gene (with a higher expression in berries treated with PDO than in those treated with Ethrel) which could be related to the higher content of methylated forms observed for the same sampling date in both of these treatments.

4. Conclusions

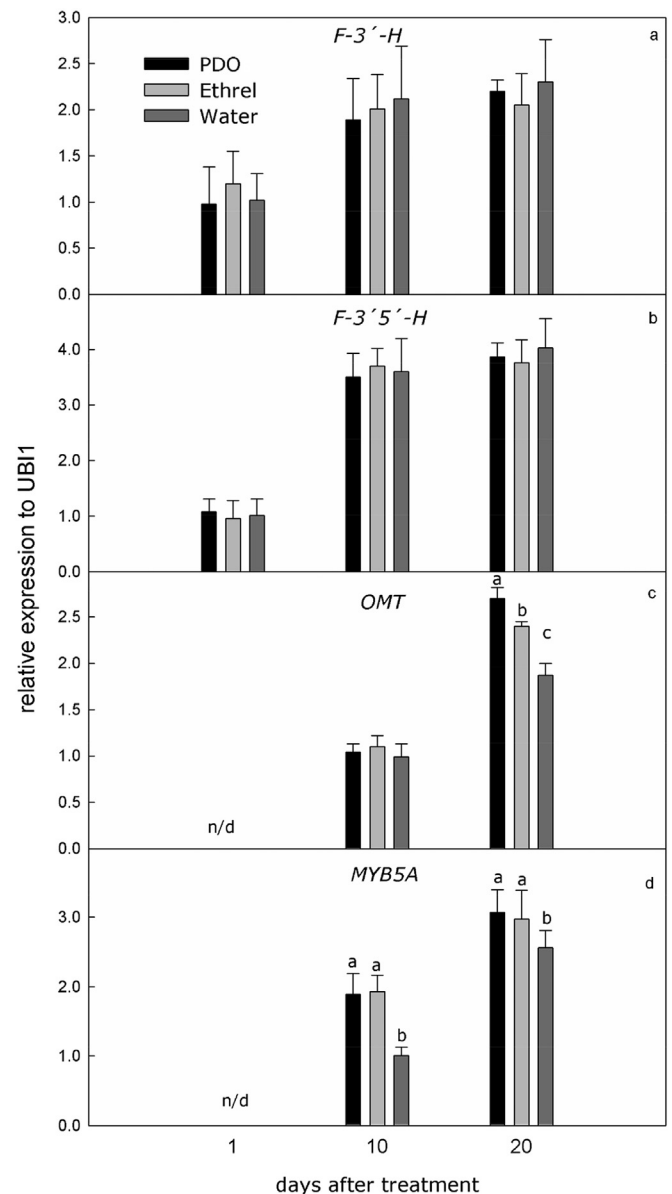In conclusion, this research presents novel information about

Fig. 6. Relative expression of *F-3′-H* (a); *F-3′5′-H* (b); *OMT* (c) and *MYB5A* (d) at 1, 10 and 20 days after treatment of Cabernet Sauvignon berries with PDO, Ethrel and water. Data correspond to means (n = 4) ± *se*. n/d: not detected. Different letters indicate significant differences ($p < 0.05$).

the modifications in total anthocyanin content and the anthocyanin profile in Cabernet Sauvignon berries in response to PDO application, in field conditions. It reveals that a single PDO application before the onset of *veraison* increases TAC to levels equivalent to those achieved by application of Ethrel, a growth regulator commonly used in other varieties for this purpose, without affecting berry quality. Furthermore, these TAC increases were accompanied by modifications of the anthocyanin profiles, especially an increase in the relative proportion of Mv accompanied by decreases in the relative proportion of Pt and Dp which in turn generated an increase in the proportion of tri-hydroxylated relative to di-hydroxylated forms. Because anthocyanins are an important part of the phenolic compounds, a group of molecules positively-related with high quality wines (Langlois et al., 2010; Sáenz-Navajas et al., 2010), the increase in TAC reported in this study through the application of PDO is an interesting alternative

management strategy for improving wine quality. The increase in tri-hydroxylated forms is expected to generate a positive impact on color, a major quality attribute of the wine (Chira et al., 2011). Such modifications in TAC and anthocyanin profile can be partly explained by a short and long term modification in the expression of key genes. Short term increases in *PAL* expression, in coordination with decreased expression of the *PAL* inhibitor, *MYB4A*, and long term increases in *UFGT* expression partly explain the observed rise in TAC, while increases in *OMT* and *MYB5A* expression could be related with the changes described in the anthocyanin profile. The fact that the temporal response in the rise in TAC after PDO and Ethrel treatments was different (the latter exerting a faster effect under these experimental conditions) and that both PDO and Ethrel treatments cause similar modifications in the relative expression of key genes in the phenylpropanoid pathway, may be interpreted that both treatments affect the synthesis and/or accumulation of anthocyanin through the induction of ethylene. However, it cannot be ruled out that the inductive effect on anthocyanins by PDO and Ethrel could be mediated by independent mechanisms. The latter has been previously-suggested by Campbell and Labavitch (1991) who noted that the application of PDO to tomato pericarp discs stimulated synthesis of ethylene and accelerated ripening. The authors concluded that some of the impact of PDO on ripening was via promotion of ethylene but also suggested that an ethylene-independent response was involved. Therefore, while the existence of a separate route of response for PDO on maturation has not been conclusively demonstrated, the independence of PDO and ethylene pathways has been established in other physiological instances, such as the induction of resistance against *Botrytis cinerea* in *Arabidopsis thaliana* (Ferrari et al., 2007). Future trials applying the blocker of ethylene perception, 1-methylcyclopropene (1-MCP) will help to dissect whether PDO exert their influence on wine grape anthocyanin metabolism via an ethylene-dependent or -independent means.

Contributions

Daniel Villegas obtained funding, performed assay, and analyzed and discussed the results. Michael Handford, José Antonio Alcalde and Alonso Pérez-Donoso performed assays, and analyzed and discussed the results. All authors wrote the manuscript, and have read and approved the final version.

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