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Loss of anthocyanins and modification of the anthocyanin profiles in grape berries of Malbec and Bonarda grown under high temperature conditions

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ABSTRACT

Malbec and Bonarda are the two most widely cultivated grape varieties in Argentina, and their derived red wines are recognized worldwide, being their intense color a major quality trait. The temperature during fruit ripening conditions berries color intensity. In the main viticulture region of Malbec and Bonarda a 2–3 °C increase in temperature has been predicted for the upcoming years as consequence of the global climate change. In the present study, this predicted temperature raise was simulated under field-crop conditions, and its effect on anthocyanin pigmentation in berries of Malbec and Bonarda was monitored by HPLC analysis throughout the ripening process, in two growing seasons. Additionally, expression levels of regulatory (*MYBA1* and *MYB4*) and structural (*UFGT* and *Vv3AT*) anthocyanin genes were monitored in Malbec berry skins. Although cultivar-dependent time-course variation was observed for total anthocyanin content, in general, the berries of both cultivars grown under high temperature (HT) conditions had significantly lower total anthocyanins (~28–41% reduction), and a higher proportion of acylated anthocyanins, than their respective controls. Expression of *MYBA1* and *UFGT*, but not *MYB4*, was correlated with anthocyanin pigmentation at half ripening and harvest, whereas overexpression of the acyltransferase gene *Vv3AT* was associated with higher anthocyanin acylation in HT berries. These results suggest that color development and pigment modifications in Malbec berries under HT are regulated at transcriptional level by *MYBA1*, *UFGT*, and *Vv3AT* genes. These data contribute to the general understanding on the effect of high temperatures on anthocyanin biochemistry and genetic regulation, and may have direct implications in the production of high-quality wines from Malbec and Bonarda.

1. Introduction

Grapevine is one of the most important fruit corps worldwide and, in complement to wine industry, constitutes a socio-economic activity of great significance for wine producing countries, including Argentina. Nearly 224.707 ha of grapevine are cultivated in Argentina [1], and 71% of the production is concentrated Mendoza province. Malbec and Bonarda are the two most cultivated varieties, accounting for 58% of the Argentine surface with grapevines. The climatological characteristics of Mendoza are optimal for producing high quality grapes and wines, being Malbec and Bonarda wines from Mendoza internationally recognized. Many of their quality attributes are due to polyphenols presents in grape skin, such as anthocyanins (ANTs), which contribute greatly to the fruit and wine color.

During ripening, grape berries become red due to accumulation of anthocyanins in the skin. The concentration and composition of anthocyanins is affected by environmental factors such as temperature, exposure to light, and water availability [2–5]. Previous studies on the effect of temperature on grape berries physiology and chemical composition revealed that, in general, high tem-

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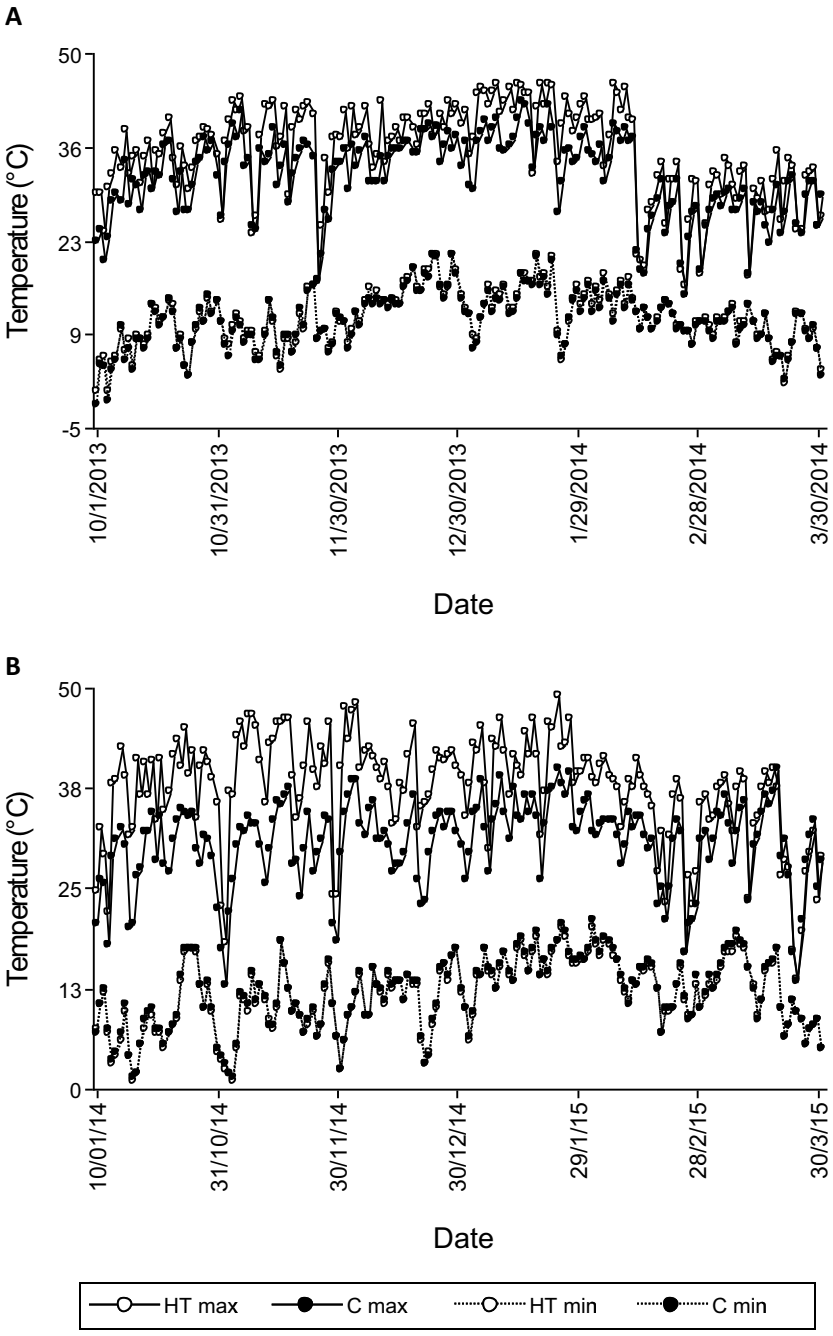

Fig. 1. Daily minimum and maximum temperatures (°C) for the high (HT) and control temperature (C) treatments during the experimental period in season 2014 (**A**) and 2015 (**B**). Error bars represent standard errors form four replicates.

peratures had a detrimental effect on berry color [6–9]. Berries of Cabernet Sauvignon and Merlot exposed to high temperatures had, in addition to reduced color, higher proportion of acylated and methoxylated anthocyanins, which are chemically-more stable [5,9,10]. Recently, an anthocyanin acyltransferease, encoded by the gene *Vv3AT*, was proposed as the enzyme responsible for the post-synthesis acylation of grape berry anthocyanins [11] However, the effects of high temperature on *Vv3AT* gene expression have not been studied.

Despite the higher relative proportion of more stable pigments observed in berries grown under high temperatures, the concomitant reduction in total anthocyanin content largely outweighs the previous benefits, and is considered a major quality problem for red grapes and wine production in warm areas.

The mechanisms underlying low anthocyanin accumulation in grape berries grown under high/increased temperatures are not clearly understood. The biosynthetic pathway and the genes involved in grapevine anthocyanin synthesis are well known. The expression of the *UFGT* gene is promoted by the transcription factor *MYBA1*, and both genes are required for color development in red grapes [12]. Yamane et al. [7] reported that reduced color development in Aki Queen red berries grown under high temperature conditions was associated with low expression of both *UFGT* and *MYBA1*. Another study, in which berries of cv. Pione (*V. vinifera x V. labrusca*) were collected at veraison and incubated in a heat chambers at 35 °C revealed reduced anthocyanin pigmentation associated with an increase in expression of *MYB4*, a putative repressor of *UFGT* [13], and a reduction in expression of

UFGT and *MYBA1*, as compared to the untreated controls at 15 °C [14]. Although the studies by Yamane et al. [7] and Azuma et al. [14] in cv Aki Queen and Pione suggest transcriptional regulation of anthocyanin pigmentation, by interaction of *MYBA1*, *UFGT* and *MYB4*, the results of Mori et al. [9] in berries of Cabernet Sauvignon exposed to increased temperatures, showing 50% reduction in anthocyanin concentration but no variation in the expression of *UFGT* and *MYBA1* suggest, conversely, that anthocyanin regulation is not at transcriptional level. Although these previous studies are not directly comparable, since very different temperature conditions and plant materials were used (including interspecific hybrids between *V. vinifera* and *V. labrusca*), they suggest that different regulatory mechanisms co-exist in grapevine, perhaps modulating color development to different extents in different genetic backgrounds. Anthocyanin regulatory mechanisms have not been investigated in the wine-producing cultivars Malbec and Bonarda.

In the last decades many investigations have focused on climate change and its worldwide potential effects on living organisms, including plants. Among the climatological variations expected, the Intergovernmental Panel on Climate Change (IPCC) [15] had predicted a thermal increases and changes on rainfall patterns. For the Argentine main viticulture region, which includes Mendoza province, the following has been predicted; a) reduced availability of irrigation water, as consequence of a decrease in snowfall; b) a 2–4 °C increase in the mean diurnal temperature during summer; and c) increased variability and intensity of summer rainfalls [16,17]. The current data suggest that climate change could deteriorate grape and wine quality by affecting color production. The effect of high temperatures on anthocyanin content and composition in Malbec and Bonarda is highly relevant for the Argentine viticulture, as these varieties give raise to the region's most emblematic and worldwide recognized Argentine wines. This information has not been elucidated yet, and might contribute to develop oenological management to maintain the wine quality.

In the present study investigates the effect of increased temperatures (Δ 2–4 °C) on anthocyanin composition and content in grape berries of Bonarda and Malbec, the two most important Argentine red-wine producing cultivars, and examined gene expression of *UFGT*, *MYBA1*, *MYB4* and *Vv3AT*.

2. Materials and methods

2.1. Plant materials, temperature conditions and experimental design

Experiments were performed in a commercial vineyard located at Ugarteche, Luján de Cuyo, Mendoza, Argentina. Vines of cv. Malbec and Bonarda were conducted on high VSP (vertical shoot position) trellis system with hail mesh and using drip irrigation. In order to increase mean diurnal temperatures on selected plants, transparent polycarbonate panels were placed from ground to cluster level, on both sides of the plants row, before sprouting to harvest (see supplementary Fig. S1). These vines (20 plants) constituted the high temperature (HT) treatment. Control plants (N = 20) were referred as the control (C) temperature treatment. Four replicates of 5 plants each were used for each temperature condition (HT and C). The temperature was monitored with iButton DS1921G 1 Wire® Thermochron® (Maxim Integrated, California, USA) thermocouples which were placed at cluster level.

Berries of Bonarda were sampled in two seasons at 50% veraison (01/30/2014 and 01/05 2015), half ripeness (02/19/2014 and 01/23/2015) and harvest (02/28/2014 and 02/23/2015). Similarly, berries of Malbec were sampled at 50% veraison (01/20/2014 and 01/05/2015), half ripeness (02/19/2014 and 01/23/2015), and harvest (03/21/2014 and 02/23/2015). Berries were cut from the plant and immediately frozen with liquid nitrogen until their storage at −80 °C in the laboratory. Phenological dates for budburst, bloom and beginning of veraison are presented in Supplementary Table 1. Soluble solids content (°Brix) and pH for 50% veraison, half ripeness and harvest are presented in Table 1.

2.2. Phenolic extraction and HPLC analysis

Grape berry skins were ground to powder in liquid nitrogen and their phenolic compounds were extracted according to Revilla et al. [18] with minor modifications. Briefly, the extracts were obtained, in darkness, by macerating 150 mg of skin powder on 1850 μl of MeOH/HCl 99:1 (v/v) (HCl 10 N) for 24 h. The extracts were then centrifuged at 4 °C and 14000 rpm, and the supernatants were recovered and stored at −20 °C, whereas the precipitates (pellets) were used for a second phenolic extraction with 1850 μl of solvent during 24 h in darkness. Equal parts of each supernatant were combined and filtered through a 45 μm pore cellulose acetate membrane (Sartorius, Göttingen, Germany), and analyzed by High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) (SPD-M10 AVP, Shimadzu Scientific Instruments, Meryland, USA).

Anthocyanins profile analysis was performed according to the protocol of the International Compendium of Analysis Methods of Musts and Wines, OIV [19]. HPLC analyses were performed using a Lichrosorb reverse-phase column (RP-18, 250 mm × 4.6 mm, 5 μm, Merck, Darmstadt, Germany). Anthocyanins were quantified at 520 nm using a calibration curve from a commercial standard of malvidin-3-glucoside chloride (Sigma, St Louis, Missouri, USA). Anthocyanin concentration was expressed as mg^{-1} of berry skin fresh weight (FW).

2.3. RNA extraction and qRT-PCR analysis

Gene expression analysis was performed by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) using total RNA from berry skins of Malbec, collected in 2014. Total RNA was isolated from approximately 400 mg of berry skin obtained from 10 berries using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) according to manufacturer's protocol, with minor modifications, namely the addition of 8.5% polyvinylpyrrolidone to the lysis solution, and two extra cleaning steps with "washing solution 1" followed by "washing solution 2". RNA concentration was measured with a spectrometer Picopet01 (Picodrop, Cambridgeshire, England). Each extraction constitutes a biological replicate.

Random primers (Invitrogen, California, USA) and M-MLV reverse transcriptase (Invitrogen, California, USA) were used for retrotranscription following the manufacturer's instructions, and using 0.5 μg of total RNA in the reaction mixture. The reactions were run in a Mastercycler Gradient 5331 (Eppendorf, Hamburg, Germany) cycler. qRT-PCR reactions were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, California, USA), using four biological replicates with two technical replicates each. The reaction mixture contained a 1/20 dilution of the retrotranscription product, 7.5 μl of SybrGreen PCR Master Mix (Applied Bisosystems, Warrington, UK), 0.3 μM of forward primer, 0.3 μM of reverse primer, and a final volume of 15 μl. The primers (Invitrogen, USA) used for the anthocyanin genes *UFGT* and *MYBA1* [20], *MYB4* [13], and *Vv3AT* [11] as well as the control/reference genes Actin-1 (*VvAct I*) [21] and Elongation factor 1-α (*EF1-α*) [22], were obtained from the respective published literature.

Table 1
Accumulated degree-days (DDs), and pH and soluble solids content (° Brix) in extracts of Malbec and Bonarda grown under HT and C conditions and from three phenological stages (veraison, half-ripeness, harvest) in seasons 2014 and 2015. Values are means of four replicates. *ns*, no statistical significances ($p < 0.05$) between treatments, for both years, according to Kruskal Wallis test.

		Bonarda						Malbec					
		Veraison		Half ripeness		Harvest		Veraison		Half ripeness		Harvest	
Treat-ment	DDs accu-mulated	pH	°Brix	pH	°Brix	pH	°Brix	pH	°Brix	pH	°Brix	pH	°Brix
2014													
HT	2005	3.4	12.7	3.38	16.88	3.61	19.35	2.87	16.15	3.55	21.38	3.73	22.77
C	1908	3.23	12.1	3.35	16.32	3.48	19.3	3.18	14.74	3.48	19	4.08	23.55
2015													
HT	2140	2.93	10.2	3.56	15.25	4.25	21.23	2.86	14.4	3.34	20.85	3.61	23.63
C	1919	2.93	10.15	3.5	14.05	4.13	20.73	3	14.9	3.41	17.93	4.03	22.4
		ns	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

2.4. Statistical analysis

Experimental design was a Latin Square with four replicates in each case. Significant differences in anthocyanin content were analyzed by mean comparisons, LSD, Fisher test for $p < 0.05$, adjusting a general linear and mixed model. Adjustments were made for heterocedasticity and for the longitudinal data correlation.

Gene relative expression data was analyzed using four biological replicates, and quantified according to Pfaffl [23]. The amplification efficiencies were calculated from raw data using LingReg PCR software [24]. Normalization of the data was done for Actin-1 and EF1-α. Significant differences were analyzed by mean comparisons were performed using LSD, Fisher test, where p values < 0.05 were considered significant, adjusting a general linear and mixed model (software InfoStat 2015).

3. Results

3.1. Temperature regimes

Mean diurnal temperature during the experiment period was 2.5–3 °C higher in the HT treatment than in the C treatment (Supplementary Fig. S2). Maximum temperatures in 2014 for HT and C treatments were 49 and 43 °C, respectively ($\Delta = 6$ °C), and in 2015, 45.5 and 40 °C ($\Delta = 5.5$ °C) (Fig. 1). Degree-days (DDs) were higher in the HT treatment for both years, with larger difference between treatments observed in 2015 (221 DDs) (Table 1). In berries of both cultivars, no statistical differences ($p < 0.05$) were found in soluble solids content and pH between the HT and C treatments.

3.2. Anthocyanin concentration and composition

Under increased temperature conditions (HT) Bonarda and Malbec berries had lower total anthocyanin concentration than their respective berries grown under natural conditions (C), especially after veraison (Fig. 2). In Bonarda berries, these differences between HT and C conditions were statistically different at harvest time, for both years 2014 ($p = 0.0305$) and 2015 ($p = 0.0091$) (Fig. 2A, B), whereas in Malbec significant variation was found only at half ripeness, in 2014 ($p = 0.0012$) and 2015 ($p = 0.0293$) (Fig. 2C, D). Such significant differences accounted for a reduction in total anthocyanin content under HT conditions of 38.6% and 40.4% in Bonarda, in 2014 and 2015, respectively, and 28% and 40.7% for Malbec, in 2014 and 2015, respectively.

Nine anthocyanin pigments, including five monoglucosides (malvidin glucoside, peonidin glucoside, petunidin glucoside, delphinidin glucoside and cyanidin glucoside) and four of their acylated derivatives (the acetylated and coumarylated forms of malvidin and peonidin glucosides) were identified and quantified by HPLC analysis. Malvidin-3-O-glucoside was the predominant anthocyanin in both cultivars, for both years. At harvest, malvidin-3-O-glucoside accounted for 45.7–54.5% and 48.3–54.1% of the total anthocyanin content in berries of Bonarda and Malbec, respectively (Table 2). Peonidin-3-O-Acetyl-glucoside was found to be at a trace level and could not be quantified in Malbec in 2015.

The relative contents (%) of glucosylated and acylated forms of anthocyanins were modified under increased temperature conditions for both cultivars. Under HT conditions, berries of both cultivars increased their relative content of acylated derivatives (and consequently reduced their proportion of glucosylated anthocyanins) at half ripeness and harvest, for the two years of the study (Fig. 3). In Bonarda berries of 2014 the percentage of acylated anthocyanins was significantly increased with HT at half ripeness (from 30.3 to 38.9%) and harvest (from 28.1 to 40.2%) (Fig. 3A). The relative increase observed in acylated anthocyanins of Bonarda berries under HT conditions (Fig. 3A) was mainly due to variation in the relative content of delphinidin, petunidin and malvidin which decreased due to the conversion to their acylated forms (Table 2). In 2015, the increase in acylated forms under HT was not significant (Fig. 3B). In Malbec, acylated anthocyanins significantly increased under HT at half ripeness, from 35.8 to 40.8%, and 28.1–31.5% in 2014 and 2015, respectively, and at harvest (from 31 to 37%) for year 2015 (Fig. 3C, D), being these changes mainly explained by the acylation of malvidin and cyanidin. Further details on the relative content of glycosylated and acylated anthocyanins for both cultivars under HT and C conditions, during fruit maturity in both growing seasons are presented in Table 2 and Supplementary Fig. S3.

3.3. Expression of anthocyanin structural and regulatory genes

The expression of the anthocyanin structural gene *UFGT*, and the transcription factors *MYBA1* and *MYB4* was measured in Malbec berry skins during fruit maturity at three ripening stages (Fig. 4). At veraison and half ripeness, the transcript levels of *UFGT* and *MYBA1* were significantly reduced in berries grown under high temperatures, and this was concomitant with a significant reduction in total anthocyanin content at half ripeness. At harvest, gene expression levels of *UFGT* and *MYBA1*, and the berries total anthocyanin content, were not statistically different between the high (HT) and control temperature (C) treatments.

Conversely, no association was found between the expression of *MYB4* and *UFGT*, or between expression of *MYB4* and anthocyanin content, for neither temperature treatments. The expression profile of the three genes is presented in Supplementary Fig. S4.

The expression of the post-synthesis anthocyanin acyltransferase gene, *Vv3AT,* was also analyzed in Malbec berry skins (Fig. 5). The transcript level of *Vv3AT* was significantly increased at veraison in HT berries, and this was associated with a higher accumulation of acylated anthocyanins later, at half ripeness. *Vv3AT* was also over-

Table 2
Concentration of nine anthocyanin pigments in berries of Bonarda and Malbec plants grown under high (HT) and control temperature (C) conditions, during fruit ripening process. **Df**, delphinidin-3-glucoside; **Cn**, cyaniding-3-glucoside; **Pt**, petunidin-3-glucoside; **Po**, peonidin-3-glucoside; **Mv**, malvidin-3-glucoside; **PoAc**, peonidin-3-O-Acetyl-glucoside; **MvAc**, malvidin-3-O-Acetyl-glucoside; **PoCu**, peonidin-3-O- Coumaryl-glucoside; **MvCu**, malvidin-3-O-Coumaryl-glucoside. Values are means ± standard errors of four replicates, expressed as malvidin-3-glucoside chloride in mg. mg^{-1} FW. Asterisks indicate significant differences ($p < 0.05$) between HT and C treatments, for a given year, phenological stage, and cultivar (DGC test).

	Bonarda					
	Veraison		Half ripeness		Harvest	
Anthocyanin/Year	HT	C	HT	C	HT	C
Df						
2014	3.52 ± 0.71	3.97 ± 0.41	3.48 ± 0.47	6.00 ± 1.44	2.79 ± 0.57*	4.96 ± 0.60
2015	11.58 ± 1.41	9.05 ± 0.25	7.33 ± 1.30	9.07 ± 1.29	3.93 ± 0.97	6.80 ± 1.35
Cn						
2014	0.53 ± 0.11	0.66 ± 0.23	0.23 ± 0.04	0.44 ± 0.17	0.21 ± 0.05	0.28 ± 0.05
2015	10.63 ± 0.71	11.71 ± 0.62	1.09 ± 0.42	1.31 ± 0.26	0.28 ± 0.10	0.61 ± 0.19
Pt						
2014	7.62 ± 0.94	8.70 ± 0.50	6.87 ± 0.47	9.81 ± 1.56	6.24 ± 0.84*	9.38 ± 0.75
2015	11.43 ± 0.90	9.90 ± 0.36	10.49 ± 0.93	12.31 ± 1.19	6.91 ± 0.97	9.66 ± 1.18
Po						
2014	5.03 ± 0.25	5.29 ± 0.78	2.47 ± 0.19	3.15 ± 0.60	2.70 ± 0.21	2.71 ± 0.27
2015	27.04 ± 2.26	31.28 ± 1.29	5.86 ± 1.11	6.48 ± 0.59	2.80 ± 0.66	4.10 ± 0.60
Mv						
2014	52.07 ± 1.12	51.72 ± 0.64	48.03 ± 0.52*	50.29 ± 0.37	47.88 ± 1.03*	54.52 ± 0.35
2015	26.11 ± 1.86	24.80 ± 1.77	45.89 ± 1.16	46.23 ± 0.80	45.74 ± 1.01	48.08 ± 0.67
PoAc						
2014	0.10 ± 0.04	0.19 ± 0.01	0.04 ± 0.01*	0.10 ± 0.01	0.02 ± 0.00*	0.07 ± 0.01
2015	0.06 ± 0.02	0.10 ± 0.02	0.07 ± 0.00*	0.12 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
MvAc						
2014	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.01	0.08 ± 0.00	0.06 ± 0.01*	0.25 ± 0.06
2015	0.05 ± 0.00*	0.03 ± 0.00	0.18 ± 0.06	0.13 ± 0.02	0.12 ± 0.02*	0.28 ± 0.04
PoCu						
2014	3.25 ± 0.03	2.86 ± 0.17	1.97 ± 0.09	1.79 ± 0.08	2.13 ± 0.13*	1.44 ± 0.03
2015	7.14 ± 1.19	7.84 ± 0.65	3.39 ± 0.21	3.49 ± 0.25	2.38 ± 0.22	2.30 ± 0.07
MvCu						
2014	27.82 ± 0.89	26.55 ± 1.90	36.86 ± 1.57*	28.34 ± 4.10	37.97 ± 2.68*	26.39 ± 1.68
2015	5.95 ± 0.16	5.30 ± 0.32	25.70 ± 2.99	20.86 ± 2.31	37.82 ± 3.83	28.13 ± 3.54
Anthocyanin/Year	Malbec					
	Veraison		Half ripeness		Harvest	
	HT	C	HT	C	HT	C
Df						
2014	3.88 ± 0.64	4.23 ± 0.54	4.54 ± 0.18	5.23 ± 0.68	3.96 ± 0.13	4.36 ± 0.28
2015	11.54 ± 0.60	11.09 ± 0.49	5.38 ± 0.13	5.42 ± 0.46	4.22 ± 0.14	4.59 ± 0.33
Cn						
2014	0.35 ± 0.06	0.24 ± 0.02	0.36 ± 0.07	0.25 ± 0.03	0.18 ± 0.02*	0.22 ± 0.01
2015	2.90 ± 0.20	2.41 ± 0.33	0.27 ± 0.01	0.33 ± 0.10	0.27 ± 0.06	0.27 ± 0.05
Pt						
2014	6.40 ± 0.55	7.16 ± 0.54	6.94 ± 0.11	8.11 ± 0.65	6.77 ± 0.05	7.36 ± 0.33
2015	13.68 ± 0.46	13.05 ± 0.17	8.23 ± 0.11	8.43 ± 0.57	6.87 ± 0.22	7.42 ± 0.31
Po						
2014	2.35 ± 0.23	1.85 ± 0.11	2.97 ± 0.41	2.41 ± 0.18	2.28 ± 0.19	2.63 ± 0.13
2015	9.19 ± 0.39	8.08 ± 0.62	2.19 ± 0.03	2.79 ± 0.34	2.58 ± 0.20	2.67 ± 0.33
Mv						
2014	37.91 ± 0.75	40.55 ± 0.39	44.95 ± 0.48*	48.92 ± 0.73	48.31 ± 0.39	50.82 ± 0.72
2015	48.03 ± 1.14	50.79 ± 0.94	52.39 ± 1.28	54.90 ± 0.79	49.11 ± 1.24*	54.08 ± 0.53
PoAc						
2014	0.09 ± 0.02*	0.24 ± 0.03	0.10 ± 0.08	0.05 ± 0.00	0.04 ± 0.00*	0.05 ± 0.00
2015	0.05 ± 0.00*	0.02 ± 0.00	ND	ND	ND	ND
MvAc						
2014	7.12 ± 0.15*	6.51 ± 0.15	5.20 ± 0.38	4.65 ± 0.20	3.84 ± 0.16	3.87 ± 0.26
2015	0.79 ± 0.03*	0.58 ± 0.01	0.84 ± 0.06*	0.63 ± 0.05	0.49 ± 0.03*	0.48 ± 0.03
PoCu						
2014	2.93 ± 0.11*	2.33 ± 0.08	2.03 ± 0.18	1.52 ± 0.07	1.66 ± 0.14	1.63 ± 0.06
2015	2.40 ± 0.09	2.23 ± 0.09	1.48 ± 0.12	1.44 ± 0.05	1.91 ± 0.14	1.53 ± 0.12
MvCu						
2014	38.98 ± 2.20	36.90 ± 1.62	32.91 ± 0.77	28.86 ± 1.19	32.96 ± 0.53	29.05 ± 1.02
2015	11.43 ± 0.56	11.75 ± 0.61	29.21 ± 1.08	26.07 ± 1.99	34.56 ± 0.99*	28.97 ± 1.43

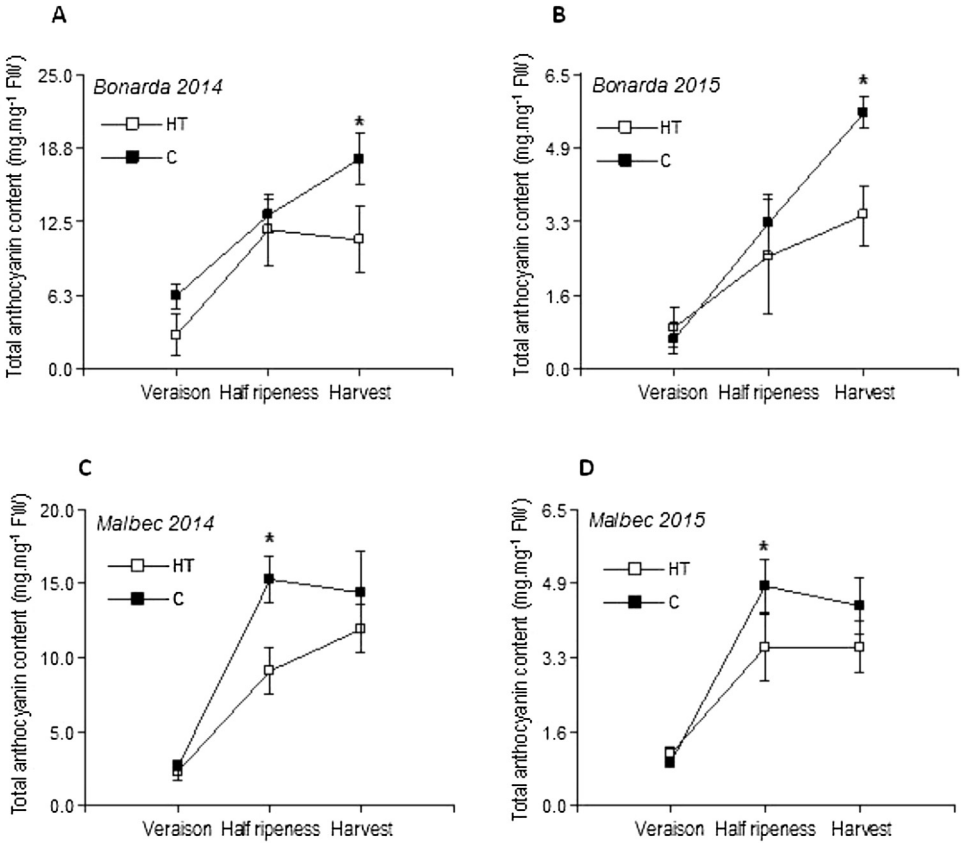

Fig. 2. The total anthocyanin concentration in Bonarda (**A** and **B**) and Malbec (**C** and **D**) in 2014 and 2015 respectively, in berries under high temperature (HT) and control temperature (C) conditions, during the experimental period. Error bars represent standard errors from four replicates. Asterisks indicate statistical significance ($p < 0.05$) between treatments according to mean comparison, LSD test.

expressed at harvest in HT berries. In both, HT and C berries, the expression level of this gene decreased from veraison to harvest, as observed in Supplementary Fig. S5.

4. Discussion

4.1. Temperature treatments

Malbec and Bonarda are the two most widely cultivated grape varieties in Argentina, and their derived red wines are recognized worldwide, being their intense color a major quality trait. Among viticulturists, it is well known that the temperature during fruit ripening is an important factor conditioning quality traits including berry color. The experiments and temperature treatments assayed in this work involved realistic field conditions, with moderate temperature differences between HT and C. These conditions are representative of the environment in Mendoza, the main viticulture region of Argentina, whereas the 2–3 °C increase in the average diurnal temperature, used in the HT treatment, is the predicted temperature raise due to climate change in this region [15–17].

4.2. Anthocyanin concentration and composition in response to high temperature

Total anthocyanin content was significantly reduced in berries of Bonarda (~40% reduction) and Malbec (28–41% reduction) grown under increased temperature conditions, but their sugar content and pH remained unaffected, in agreement with previous studies in other grape cultivars [2,8,25,26]. Decrease in total anthocyanin content due to high temperatures has been observed in Cabernet Sauvignon (~50%) and Darkridge (~30%) [8,9].

Under the experimental conditions used in this work, HT berries increased the proportion of acylated anthocyanins in both cultivars (Fig. 3). Similar results have been observed for cultivar Merlot when the cluster temperature was increased by air flow [5]. Acylation, methoxylation, and glycosylation increase anthocyanins chemical and thermal stabilities [8,10]. In the present study, the relative increase in acylated anthocyanins observed in Bonarda and Malbec berries under HT was mainly due to an increase in the coumarylated, rather than the acetylated forms of anthocyanins (Table 2). Moreover, it is clear from data obtained that the rise in acylated forms in Bonarda was mainly due to the conversion of the tri-substituted type of anthocyanins in coumarylated malvidin. Although the increase in the proportion of stable anthocyanins is, presumably, a positive consequence of the increased temperature conditions, as this may contribute to a higher color stability in grapes and wines [27], the substantial decrease in total anthocyanin content (up to ~40% decrease) observed in HT berries largely outweighs the benefits of a more stable: unstable pigments ratio.

Time-course variation of anthocyanin pigmentation in berries of Malbec and Bonarda ripening under HT and C conditions varied between the two cultivars. Although, in general, total anthocyanin content decreased by HT conditions in both cultivars and in both years assayed (Fig. 2), at harvest, Malbec berries under both HT and C had comparable – i.e., not statistically different- anthocyanin contents, whereas in Bonarda, differences in anthocyanin pigmentation between the two temperature treatments were largest – and statistically different- at this phenological stage. Thus, despite its initial drop in anthocyanin content (at half-ripeness) due to high temperatures, Malbec could overturn, to some extent, the effect of HT conditions, yielding at harvest time comparable anthocyanin

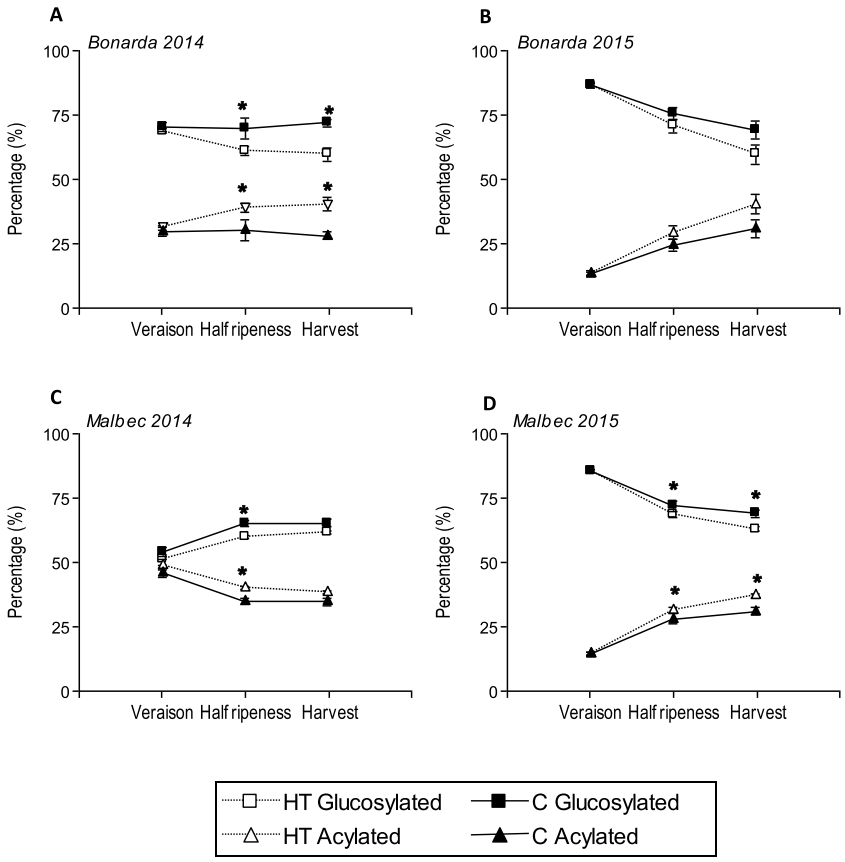

Fig. 3. Relative content (%) of acylated and glucosylated anthocyanins in berries of Bonarda (**A, B**) and Malbec (**C, D**) grown under high (HT) and control temperature (C) conditions in 2014 and 2015. Error bars represent standard errors from four replicates. Asterisks indicate statistical difference ($p < 0.05$) between treatments at a given phenological stage (LSD test).

content as the C berries. Thus, Malbec could be considered as a more adaptable cultivar for warmer regions.

A "thermal decoupling" phenomenon, consisting in a delayed anthocyanin accumulation (but not of sugar content) in berries ripened under HT, has been described in Cabernet Franc and Shiraz [28]. Whether this phenomenon may account for the fact that HT berries of Malbec had, at harvest, comparable anthocyanin content as the C berries (Fig. 2C and D) is very unlikely, since no variation in sugar content between HT and C berries was found. Additionally, the increase in the proportion of acylated anthocyanins under HT conditions remained stable until harvest, as opposed to the total anthocyanin content.

4.3. Expression of anthocyanin structural and regulatory genes in response to high temperature

In the present study, *MYBA1* and *UFGT* genes revealed changes in expression levels, in response to the thermal treatment, that were concomitant with phenotypic variations in berry color and anthocyanin content. It was detected lower *UFGT* and *MYBA1* expression level and lower anthocyanin content in HT berries compared to C, at veraison and half ripeness, suggesting that high temperatures negatively affect the expression of *MYBA1* and that the regulation of anthocyanin pigmentation is at transcriptional level. In agreement with the results obtained here, strong correlations between anthocyanin accumulation and the expression of *UFGT* and its transcriptional factor *MYBA1* have been previously reported in red grape berries [29,30].

On the other hand, the repression of *UFGT* transcription by *MYB4* was demonstrated by *in vitro* experiments in berries of the cultivar Pione (*V vinifera* x *V labrusca*) under HT conditions (15 °C increment) [14]. However, in the present study, no association between the expression levels of *MYB4* and *UFGT* was found, nor between expression levels of MYB4 and the berries anthocyanin content, suggesting that *MYB4* is not involved in the regulation of anthocyanin pigmentation in Malbec.

In addition to the transcriptional regulation by the *MYBA1*/*UFGT* genes, other regulatory mechanisms, for example, at post-transcriptional level, may also be involved in anthocyanin pigmentation under high temperature conditions. For instance, peroxidases have been found to be involved in anthocyanin degradation in berries of Sangiovese grown under high temperature conditions [7] and the peroxidase gene *VviPrx31* has been indicated as the main peroxidase involved in HT-induced anthocyanin degradation [31]. Although, the results shown in this work strongly suggest that anthocyanin regulation for Malbec berries under HT is predominantly regulated at the transcriptional level, by the interaction of *MYBA1* and *UFGT* genes.

The expression level of the acyltransferase gene, *Vv3AT*, was also modified by HT conditions, and this was associated with an increase in the relative content of acylated anthocyanins in HT berries. The higher levels of *Vv3AT* at veraison may explain the higher accumulation of acylated anthocyanins at half ripeness in Malbec berries under HT. However, the expression profile of *Vv3AT*, as well as *UFGT*, decreased continuously from veraison to harvest in HT berries (Supplementary Fig. 4 and 5), and this coincides with results obtained by Rinaldo et al. for Cabernet Sauvignon [11].

Taken together, these data indicate that heat stress, corresponding to the predicted local temperature increase, reduces anthocyanins levels in berries of Malbec and Bonarda. Addition-

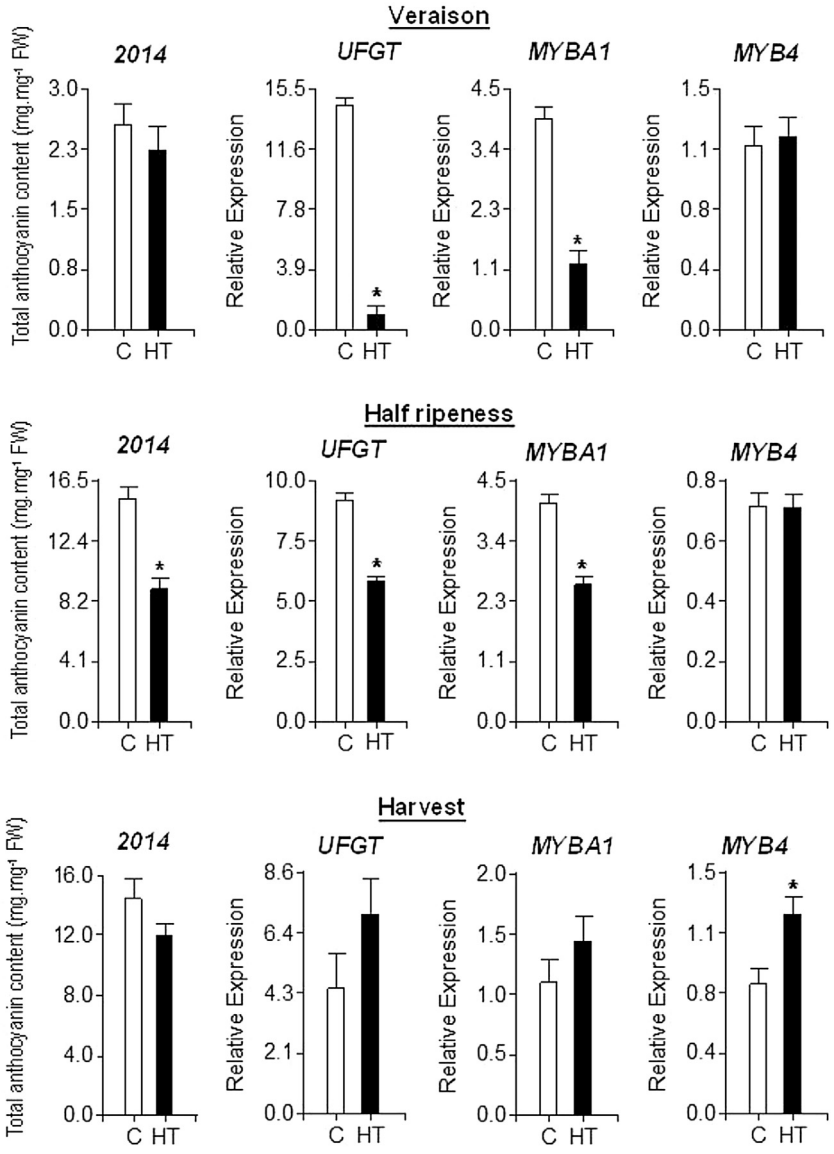

Fig. 4. Total anthocyanin content in 2014, and relative expression analysis of UDP glucose-flavonoid 3-O-glucosyltransferase (*UFGT*), *MYBA1* and *MYB4*, in berries of Malbec under high temperature (HT) and control temperature (C) conditions during the experimental period. Error bars are standard error from four biological replicates. Asterisks indicate statistical significance ($p < 0.05$) according to mean comparison, LSD test.

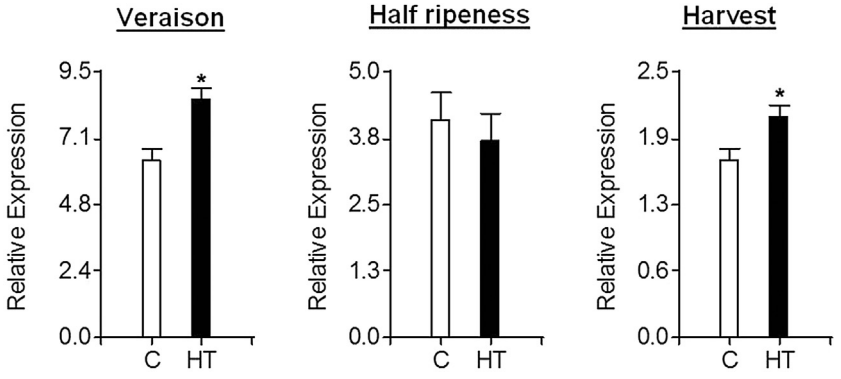

Fig. 5. Relative expression analysis of anthocyanin acyltransferase gene, *Vv3AT*, in berries of Malbec under high (HT) and control (C) temperature conditions. Error bars are standard error from four biological replicates. Asterisks indicate statistical significance ($p < 0.05$) according to mean comparison, LSD test.

ally, the anthocyanin profiles of the two varieties under high temperature shifted towards the production of acylated forms of anthocyanins. The reduction in anthocyanin pigmentation of Malbec and Bonarda implies great challenges in viticulture and oenological management in order to maintain wine quality, taking into account the central role of these cultivars for Argentine viticulture. The gene expression analysis demonstrated that *MYB4* is not responsible for the transcription down-regulation of anthocyanin synthesis in berries under high temperature regime. Instead, the expression data strongly suggest transcriptional regulation by *MYBA1* and *UFGT* genes. In addition, the acyltransferase gene *Vv3AT* was responsible for the higher accumulation of acylated anthocyanins observed in berries grown under high temperature regimes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.01.015.

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