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MYBA2 gene involved in anthocyanin and flavonol biosynthesis pathways in grapevine

T.Q. Niu[1], Z.D. Gao[1], P.F. Zhang[1,3], X.J. Zhang[1,3], M.Y. Gao[1], W. Ji[1,3], W.X. Fan[2] and P.F. Wen[1,3]

[1]College of Horticulture, Shanxi Agricultural University, Taigu, Shanxi, China
[2]Experimental Teaching Center, Shanxi Agricultural University, Taigu, Shanxi, China
[3]Key Laboratory of Pomology, Shanxi Province, China

Corresponding author: P.F. Wen
E-mail: wenpengfei@126.com

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ABSTRACT. *MYBA2* transcription factor (Myb-related gene) affects the coloring in grapevine berry and plays an important role in the biosynthesis of anthocyanin. The *MYBA2* gene was cloned from *Vitis vinifera* L. cv. Cabernet Sauvignon and polyclonal antibodies for *VvmybA2* were prepared. The *VvmybA2* gene expression patterns were observed in seven tissues (the leaf, stem, flower, bud, root, berry, and tendril) and during the berry development stage at transcriptional and translational levels, respectively. The results indicated that the expression of *VvmybA2* was approximately 11-fold higher in the berry than that in the other six tissues, and increased rapidly from 60 days after full bloom reaching a maximum on day 80. Furthermore, both the anthocyanin content and UDP-glucose:flavonoid-3-O-glucosyltransferase (*UFGT*) gene expression levels increased rapidly 60 days after full bloom. Moreover, correlation analysis indicated that

the transcriptional and translational expression levels of the *VvmybA2* gene were significantly positively correlated with not only *UFGT* and *DFR* genes but also with the anthocyanin content during berry development. These results suggested that *VvmybA2* could not only regulate the transcription of both UFGT and DFR but also is involved in the expression of the *UFGT* gene associated with color determination in grape berries.

Key words: *VvmybA2*; MYB transcription factor; Anthocyanin; *UFGT* gene; Grapevine

INTRODUCTION

Grapevine is one of the most important economical fruit crops widely grown throughout the world. It is popular among consumers due to its rich nutrient content and flavor quality. Anthocyanins are the major compounds responsible for the color of red and black grapes and wines produced from them (Núñez et al., 2004; Ageorges et al., 2006). They also play an important role in the commercial and aesthetic value of the fruit. The anthocyanin biosynthesis pathway and the corresponding key enzymes [including chalcone synthase, flavanone-3-hydroxylase, anthocyanidin synthase (ANS), dihydroflavonol-4-reductase (DFR), and UDP-glucose:flavonoid-3-O-glucosyltransferase (UFGT)] have been investigated extensively in many higher plants, including fruit trees such as grapevine (Sparvoli et al., 1994; Jeong et al., 2004; Ageorges et al., 2006; Figueiredo-González et al., 2012), apple (Honda et al., 2002; Feng et al., 2013; Meng et al., 2015), pomegranate (Rouholamin et al., 2015), peach (Hassani et al., 2015; Liu et al., 2015), strawberry (Song et al., 2015), kiwifruit (Li et al., 2016), blueberry (Zifkin et al., 2012; Li et al., 2015), blackberry (Chen et al., 2012), pear (Fischer et al., 2007; Pierantoni et al., 2010), nectarines (Ravaglia et al., 2013), and mangosteen (Palapol et al., 2009). Although previous studies have demonstrated the role of these key enzymes in the flavonoid pathway, there is a lack of knowledge regarding the regulation mechanism of these enzymes. Recent studies revealed that the *VvUFGT* gene was expressed in the skin of red grape but not in that of the white grape, suggesting that *UFGT* is a major gene associated with the coloration of grape skin (Boss et al., 1996). Therefore, the Cabernet Sauvignon variety with red grape skin was selected as the plant material in the present study.

MYB transcription factors play the central role in the transcriptional regulation of anthocyanins (Martin and Paz-Ares, 1997). Recently, many studies related to MYB transcription factors have primarily focused on the accumulation of anthocyanin and flavonol, as well as their response to environmental factors (Takos et al., 2006; Lin-Wang et al., 2011; Xie et al., 2012; Sivankalyani et al., 2016). Overexpression of the grapevine *VvMYB5a* in tobacco induced substantial accumulation of anthocyanin and flavonol compounds and may be involved in the phenylpropanoid pathway in grapevine (Deluc et al., 2006). The expression of *VvMYBF1* during berry development correlated with the accumulation of flavonols (Czemmel et al., 2009). The *VvMYBPA1* gene could induce ectopic accumulation of proanthocyanidin in the fruit, whereas *VvMYBA2* could significantly activate only the anthocyanin-specific promoter of *VvUFGT* at approximately 600-fold (Bogset al., 2007; Kobayashi et al., 2002). Noticeably, the *VvMYBA1* and *VvMYBA2* transcription factors could specifically regulate the expression of the *UFGT* gene, which encoded an enzyme responsible for the conversion of

anthocyanidins to anthocyanins; in addition, the *MybA* genes were reported to participate in anthocyanin biosynthesis by regulating the expression of *UFGT* (Kobayashi et al., 2002). However, the mechanism by which the *VvMYBA2* gene regulates the other relevant key enzymes in the anthocyanin biosynthesis pathway remains unclear; in particular, little is known about the expression and regulation of *VvMYBA2* at the protein level in grapevine.

In the present study, we report the expression of the *VvMYBA2* gene in Cabernet Sauvignon in different tissues and during fruit development stages at transcriptional and translational levels. The relationship between *VvMYBA2* and the accumulation of anthocyanin, and the key enzymes, including UFGT, DFR, ANS, and anthocyanin reductase (ANR) in the anthocyanin pathway was analyzed. This analysis could contribute to an understanding of the role of MYB transcription factors in the regulatory mechanisms of the anthocyanin biosynthesis pathway in developing grape berries.

MATERIAL AND METHODS

Plant materials

Vitis vinifera plants were grown in a vineyard at Shanxi Agricultural University, located in Taigu County, Shanxi Province, China. For the analysis of the contents of the berries, three six-year-old grapevine plants were selected randomly, from which six grape clusters on each plant were picked from the top, middle, and bottom of the canopy in both the east and west directions of the plant, on 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110 days after full bloom. The unhealthy berries were discarded. For the expression analysis of the *VvmybA2* gene using real-time PCR, when new shoots grew eight leaves on healthy plants, samples of leaves, stems, flowers, roots, tendrils, berries, and buds were collected. All samples were quickly frozen in liquid nitrogen and maintained at 80°C until RNA isolation.

Isolation of full-length cDNA of *VvmybA2*

Total RNA was extracted from the fruit based on the method described by Wen et al. (2005). The 1st cDNA strand was synthesized according to the protocol of PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, Liaoning, China). Specific primers were designed based on the sequence of *VvmybA2* (GenBank accession No AB097924.1). The forward primer was 5'-CGCGGATCCATGAAGAGCTTAGGAGTTAG-3', and the reverse primer was 5'-CCCAAGCTTTCATTCGGTTGTGGTGACGTG-3'. The RT-PCR settings were: 94°C for 5 min, 30 cycles at 94°C for 5 s, 58°C for 30 s, 72°C for 1 min, and then 72°C for 10 min. The resultant products were separated on a 1.2% agarose gel stained with ethidium bromide.

Expression, purification, and antiserum preparation of recombinant MYBA2

cDNA fragment with restriction sites for Hind III at the 5' end and BamHI at the 3' end of *VvmybA2* gene synthesized by PCR was ligated into pEASY® - T5 Zero Cloning Vector. The recombinant vector pEASY-*VvmybA2* was generated, the plasmid extracted and introduced into the expression vector pET-30α with restriction sites for *Hin*dIII at the 5' end and *Bam*HI at the 3' end. The recombinant plasmid was then transformed into 50 μL *Escherichia coli* BL21 competent cells. The expression vector pET-VvmybA2 was generated.

Freshly prepared *E. coli* culture containing the pET-*VvmybA2* plasmid and *E. coli* BL21 were grown at 37°C for 12 h, and later inoculated into fresh Luria broth medium containing 50 μg/μL kanamycin. They were grown for 1-2 h until OD_{600} = 0.5. The expression of the fusion proteins was induced by the addition of 0.5 mM/L isopropyl β-D-1-thiogalactopyranoside at 30°C for 3 h.

The cells were harvested by centrifugation (5000 rpm, 5 min), after which they were resuspended with guanidinium lysis buffer (pH 7.8, 37°C), shaken gently for 5-10 min, and lysed by ultrasonication at 300 W, 5 s using JY92-II ultrasonic (Xinzhi Biotechnology Co., Ltd., Ningbo, China). After centrifugation (3000 *g*, 15 min) the clear supernatant was collected, filtered through a 0.45-μm filter membrane, purified by a chromatographic column with Ni-NTA agarose (Novagen Corp.), and washed twice with each of the denaturing buffers at pH 7.8, 6.0, and 5.3, respectively. The chromatographic column was purified with a denaturing elution buffer, and the purified protein was collected for SDS-PAGE analysis.

Three-month-old New Zealand white rabbits procured from the Xinglong Experimental Farm, Haidian District, Beijing, were employed for antibody production. Antiserum was collected by multi-point injection with antigen solution and Freundadjuvant (Pierce Corp.) on days 0 (600 μg/dose, six points), 21 (400 μg/dose, four points), 35 (600 μg/dose, four points) and 49 (600 μg/dose, four points), respectively. The antiserum titer was determined by indirect ELISA.

Escherichia coli DH5α (Amersham Biosciences, Piscataway, NJ, USA) strains and *E. coli* BL21 (Amersham Biosciences) strains were used for cloning and expression of the plasmid constructions, respectively. The plasmid DNA was isolated using E.Z.N.A. Plasmid Mini Kit I (Omega, Shanghai, China). The sequence analyses and homology alignments were performed using the DNAMAN (Version 4.0) software (Lynnon Biosoft, USA). Amino acid sequence similarity searches were performed using the BLASTx program on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide and transmembrane regions were predicted using SignalP 3.0 Server and TMHMM Server v. 2.0 software, respectively.

Western blotting

Total protein from different tissues of grapevine was extracted by an improved method according to Wen et al. (2008). The protein content was determined as described by Bradford (1976) and bovine serum albumin as the standard. The separation of total protein was performed using SDS-PAGE on 12% polyacrylamide gels, and electrophoresis was run on DYY-6C (Beijing Six-One Instrument Factory, Beijing, China). The proteins were transferred on to the nitrocellulose (NC) membrane in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Corp., USA). Immunological detection of proteins on the NC membrane was carried out using a primary polyclonal VvMYBA2 antibody (rabbit anti-(pET-VvmybA2) serum). The membrane was stained with 10 mL 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium in dark and double distilled water was added to terminate the reaction.

Real-time PCR

In order to determine the tissue-specific expression of *VvmybA2*, we designed specific primers to conduct real-time PCR of samples from seven tissues. RT-PCR was performed using the SYBR Premix Ex Taq II method (TaKaRa) run on an Applied Biosystems 7500 Real-Time PCR System with SYBR Green I dye. Each reaction was performed in triplicate with a

reaction volume of 25 µL. The cycling parameters were: 94°C for 10 min, 40 cycles at 94°C for 5 s, 55°C for 30 s, 72°C for 30 s. The grapevine Ubiquitin1 (GenBank Accession No. BN000705) protein was used as the standard control. The sequences of the genes were obtained from NCBI, and their primers were designed (listed in Table 1). The Excel 2003 software was used to analyze the expression profiles of the key enzymes in the process of flavonoid synthesis. The SPSS13.0 software was used for correlation analysis and analysis of variance.

Table 1. Primers of relative genes for real-time PCR.

Gene Name		Primer	Product length (bp)	GenBank Accession No.
VvUbiquitin	F	5'-GTGGTATTATTGAGCCATCCTT-3'	182	BN000705
	R	5'-AACCTCCAATCCAGTTATCTAC-3'		
VvmybA2	F	5'-GCAGGGTTGAATAGATGCCTAAA-3'	93	AB097924.1
	R	5'- CTCGTCTAATGCAAACTCTCCTCTC-3'		
VvANR	F	5'-AGAACTACAGGAGTTGGGTGAC-3'	202	DQ129684.1
	R	5'-CCTTGAATTGCTGGCTTG-3'		
VvANS	F	5'-TCCCCAGCCTGAATTGG-3'	114	EF192468.1
	R	5'-ACCCACTTGCCCTCATAGAAAA-3'		
VvDFR	F	5'-GCATGGAAGTATGCCAAGGAAA-3'	118	X75964.1
	R	5'-TCGGGGAAAGAGCAGTTATGAG-3'		
VvUFGT	F	5'-AGGATGTTTTGGAGATTGGAGTG-3'	120	X75968.1
	R	5'-TCAGATTTTCCCTCAGTTTCTTCC-3'		

Content of flavanols, flavan-3-ols, and anthocyanin

Grapevine berries were picked on 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110 days after full bloom. The content of polyphenolic flavanols was determined by the vanillin-hydrochloric acid method described by Waterhouse et al. (2000), and the contents of flavan-3-ols and anthocyanin from fruits were determined by the Ivanova et al. (2011) method and spectrophotometry, respectively.

RESULTS

Isolation and sequence analysis of the *mybA2* gene from Cabernet Sauvignon

We obtained the sequence of the *mybA2* gene from Cabernet Sauvignon based on the predicted *V. vinifera* cv. Pinot Noir *mybA2* gene sequence, using the BLAST program and the DNAMAN software. Sequence analysis indicated that the 798-bp ORF (open reading frame) encoded a stable protein with 265 amino acids, molecular weight of 31 kDa, theoretical isoelectric point of 9.67, and extinction coefficient of 48,595. BLAST searches and sequence alignment showed that the *mybA2* sequence shared 100% identity with that of *VvmybA2* (GenBank accession No. AB097924.1), more importantly, without a shift in the ORF.

Protein analysis of the *VvmybA2* gene from Cabernet Sauvignon

The analysis of the transmembrane region of the protein MybA2 employing TMHMM Server 2.0 indicated that the protein had no transmembrane region. Prediction of the signal peptide of the VvmybA2 protein by SignalP 3.0 Server indicated that the protein was non-secretory without any signal peptides.

Detection of the fusion protein by western blotting

The fusion protein pET-VvmybA2 contained His-Tag, which enabled an affinity for Ni-NTA agarose. Affinity chromatography was employed for protein purification. Figure 1 shows that the purity of the fusion protein was approximately 100%, indicating that protein purification was successful. After purification, we obtained 20 mL anti-(pET-VvmybA2) serum (6.7 mg/mL concentration determined by protein quantification) and 134 mg specific antibody.

Western blotting demonstrated that a specific reaction occurred at 35 kDa during fruit development, indicating that the anti-(pET-VvmybA2) serum had specific immunity to the VvmybA2 protein (Figure 1).

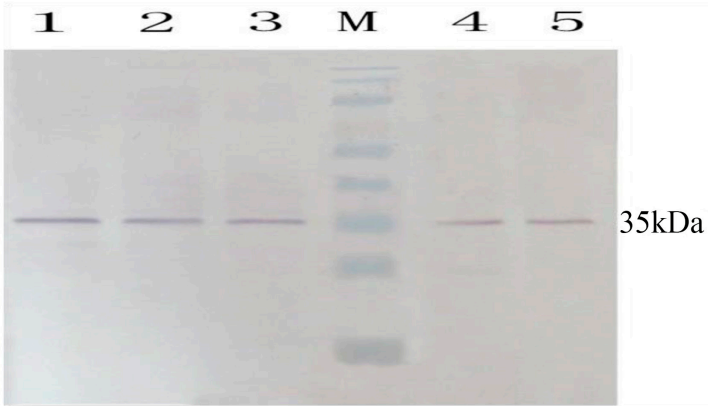

Figure 1. Detection of the pET-VvmybA2 fusion protein with rabbit anti-(pET-VvmybA2) serum using western blotting. *Lanes 1-5* = western blot of anti-(pET-VvmybA2) serum; *lane M* = marker (page-ruler prestained protein ladder).

Content of polyphenols during grapevine berry development

The images of Cabernet Sauvignon fruit during berry development are represented in Figure 2. The content of flavanols, flavan-3-ols, and anthocyanin were determined on 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110 days after full bloom (Figure 3). The contents of both flavanols and flavan-3-ols gradually declined during grapevine berry development, and the highest contents were detected during the young fruit stage, 20 days after full bloom, while the lowest were at 100 days after full bloom. In contrast, the accumulation of anthocyanin indicated a trend from low to high concentrations from 20 to 60 days after full bloom rapidly increasing to a peak on 100 days after full bloom.

Expression profiles of the *VvmybA2* gene at transcriptional and translational levels

The expression of the *VvmybA2* gene was most significant in the berry, while that of the VvMYBA2 protein was the highest in the stem, followed by that in the berry (Figure 4A and B). During berry development, the expression of the gene and protein appeared to peak at 80 and 90 days after full bloom, respectively (Figure 4C and D), both during veraison.

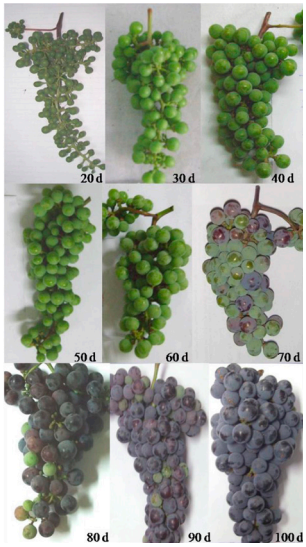

Figure 2. Development of grape berries from Cabernet Sauvignon obtained by field survey. The days are counted after the full bloom.

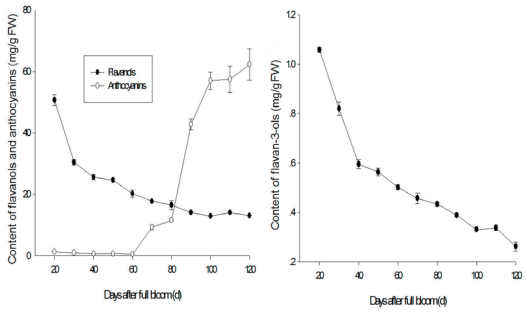

Figure 3. Content of anthocyanins, flavanols, and flavan-3-ols during grape berry development. Mean values and standard deviations were obtained from three technical and three biological replicates.

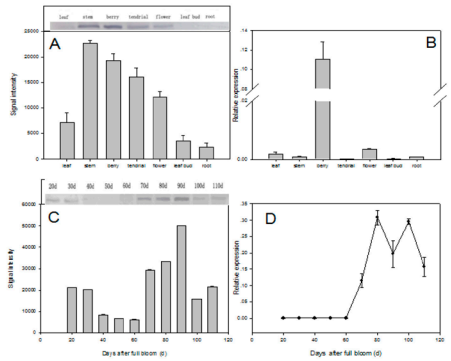

Figure 4. Contents of VvMYBA2 protein in seven organs and during different berry development stages (**A** and **C**). Expression pattern of *VvMYBA2*gene in seven organs and different berry development stages (**B** and **D**). Mean values and standard deviations were obtained from three technical and three biological replicates.

Expression profiles of the key enzymes during anthocyanin biosynthesis

ANS, ANR, DFR, and UFGT were the key enzymes in the process of anthocyanin synthesis. Figure 5 shows that the concentration of *VvUFGT* increased from day 60 after full bloom, reached the peak on day 90, and declined thereafter. The concentration of *VvANS* reached a sharp maximum at day 70 after full bloom, while that of *VvDFR* first declined, with the lowest concentration on day 60, and then increased, with the highest on day 90 after full bloom. In contrast, the expression profile of *VvANR* displayed a trend from high to low levels, with limited expression at day 60 after full bloom.

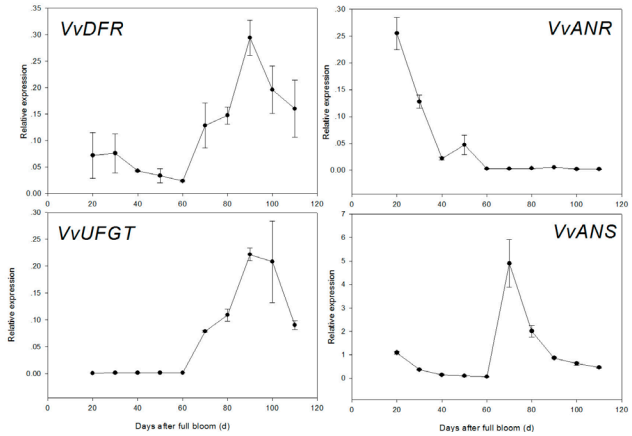

Figure 5. Expression patterns of DFR, ANS, ANR, and UFGT genes during grape berry development.

Correlation analysis

Correlation analysis was performed to illustrate the function of the *VvMYBA2* gene in the anthocyanin biosynthetic pathway. Table 2 presents the correlation analysis of *VvmybA2* gene expression at the transcriptional and translation levels with the content of flavanol, flavan-3-ols, and anthocyanins during grape berry development. A significant positive correlation was observed between the *VvmybA2* gene and its protein with the content of anthocyanin during the berry development; whereas, the *VvmybA2* gene at both transcriptional and translation levels was negatively correlated with the content of flavanol and flavan-3-ols.

Table 2. Correlation analysis of the *VvmybA2* gene at transcriptional and translation levels with the content of flavanols, flavan-3-ols, and anthocyanins during the grape berry development.

Content	VvMYBA2 protein	*VvMybA2* gene
Flavanols	-0.244	-0.632
Flavan-3-ols	-0.209	-0.654
Anthocyanins	0.415	0.762*

Data marked with *indicate $P < 0.05$.

Correlation analysis of the content of flavanol, flavan-3-ols, and anthocyanins with the expression of the key enzymes *VvDFR*, *VvANS*, *VvANR*, and *VvUFGT* during grape berry development is presented in Table 3. We observed a significant positive correlation between

the content of anthocyanins and the levels of both UFGT and DFR, while the contents of flavanol and flavan-3-ols were significantly positively correlated with that of ANR.

Table 3. Correlation analysis of the content of flavanols, flavan-3-ols, and anthocyanins with *VvDFR*, *VvANS*, *VvANR*, and *VvUFGT* gene expression during grape berry development.

	Flavanols	Flavan-3-ols	Anthocyanins
VvDFR	-0.549	-0.549	0.805**
VvANR	0.959**	0.953**	-0.453
VvANS	-0.136	-0.129	-0.038
VvUFGT	-0.648	-0.663	0.842**

Data marked with *indicate $P < 0.01$.

However, the expression levels of ANS were negatively correlated with the content of the three compounds. The correlation of *VvmybA2* gene expression at transcriptional and translational levels with that of the *VvDFR*, *VvANS*, *VvANR*, and *VvUFGT* genes during the grape berry development was analyzed and the results are presented in Table 4. These results indicated a significant positive correlation of *VvmybA2* gene expression at both transcriptional and translation levels with the levels of both UFGT and DFR, while a negative correlation was observed with that of ANR. The accumulation of VvmybA2 protein also exhibited a significantly positive correlation with the content of anthocyanin, and the expression of *VvDFR* and *VvUFGT*.

Table 4. Correlation analysis of *VvmybA2* gene expression at transcriptional and translation levels with *VvDFR*, *VvANS*, *VvANR*, and *VvUFGT* gene expression during the grape berry development.

	VvMYBA2 protein	*VvMybA2* gene
VvDFR	0.834**	0.716*
VvANR	-0.098	-0.487
VvANS	0.461	0.294
VvUFGT	0.686*	0.813**

Data marked with * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

DISCUSSION

Ripening of grapevine berry exhibited dramatic changes in gene expression and enzymatic activities at the molecular level with respect to the berry coloring, mainly depending on anthocyanin metabolism. Moreover, previous studies have indicated that the grapevine MYB transcription factor is involved in anthocyanin synthesis. The MYB gene was cloned from the Cabernet Sauvignon variety during the veraison stage, which had high similarity with *VvMYBA2* from the Pinot Noir variety. The expression of the *VvMYBA2* gene in different tissues and during fruit development stages is important to determine the anthocyanin content in grapevine fruits. Therefore, the expression of *VvMYBA2* was investigated at transcriptional and translation levels using real-time PCR and correlation analysis. The result indicated abundant expression of the *VvmybA2* gene in the berry, while it was minimal in the other six tissues, suggesting that the gene played major roles in grape berries. During fruit development, there were two peaks of *VvmybA2* gene expression: one early on day 80 and the other on day 100 after full bloom, which was similar to that of the *MybA* gene from the Kyoho variety during fruit development (Kobayashi et al., 2002).

Berry color is a crucial trait in fruits. It is well known that anthocyanins are the predominant pigment in red and black grape berries. The contents of anthocyanin, flavan-3-ols, and flavanols were investigated during fruit development. Flavan-3-ols and flavanols exhibited a downward trend from day 20 after full bloom with the lowest concentrations observed on day 100 after full bloom, similar to the expression pattern of *VvANR*. Moreover, the content of both flavanol and flavan-3-ols was significantly positively correlated with that of ANR. The *VvANR* gene is known to be expressed during early flower and berry development (Bogs et al., 2005), and is responsible for converting anthocyanidins to flavan-3-ol (Xie et al., 2003). Thus, it was suggested that *VvANR* played an important role in flavonoid biosynthesis. Anthocyanin appeared to follow an increasing trend from day 60 after full bloom, reaching a maximum on day 100, consistent with the changes observed in the anthocyanin content in Cabernet Sauvignon berries described by Wang et al. (2010). The profile of anthocyanin content coincided with the onset of anthocyanin synthesis.

In order to understand the anthocyanin biosynthesis mechanism, the expression patterns of four genes encoding the corresponding enzymes, *ANR*, *ANS*, *DFR*, and *UFGT*, were analyzed during fruit development. *VvANR* displayed expression patterns from high to low levels with minimal expression 60 days after full bloom, which differs from that of *VvANS*, *VvDRF*, and *VvUFGT*. These three genes were similar wherein, only one peak appeared on day 70 in the case of the first gene and on day 90 after full bloom in the other two. The expression patterns of *VvUFGT* were similar to that of *VvmybA2*, which was consistent with relative expression levels of *VvmybA1* and *UFGT* observed in three cultivars (Azuma et al., 2009). Based on the correlation analysis in the present study, the *VvmybA2* gene at transcriptional and translational levels was significantly positively correlated with not only the *UFGT* and *DFR* genes but also with the content of anthocyanin during the berry development. In addition, a significant positive correlation was observed between the expression of both *UFGT* and *DFR* genes and the content of anthocyanin. This correlation among *VvmybA2*, *UFGT*, and anthocyanin content suggests that *VvmybA2* is responsible for controlling the expression of the *UFGT* gene associated with color determination in grape berries, which was consistent with that of the previous studies (Kobayashi et al., 2002). Moreover, the high expression levels of *DFR* were similar to that of *VvmybA2*, suggesting that *VvmybA2* may regulate the transcription of not only *UFGT* but also *DFR*, a hypothesis supported by Jeong et al. (2004). It has also been reported that *MYB10* from nectarine positively regulated the promoters of *UFGT* and *DFR* (Ravaglia et al., 2013). In conclusion, the regulation mechanism of *VvmybA2* in the anthocyanin biosynthesis pathway needs to be further elucidated.

Conflicts of interest

The authors declare no conflict of interest.

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