Bunch Shading During Different Developmental Stages Affects the Phenolic Biosynthesis in Berry Skins of ‘Cabernet Sauvignon’ Grapes

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ABSTRACT. The effect of bunch shading during early development (before the onset of ripening) and/or during ripening on the phenolic composition of grape skins (Vitis vinifera L. cv. Cabernet Sauvignon) was examined. Shading during early development resulted in decreased proanthocyanidin (PA) concentrations. The PA concentrations decreased during ripening, and the decrease of the concentrations was lower in berries shaded during early development than that in the exposed berries. Thus, no significant effect of shading during early development was observed at harvest. Shading during ripening did not influence this decline in the PAs. On the other hand, shading during early development induced changes in the composition such as a decrease of the trihydroxylated subunits within PAs, which agreed with the relative decrease of VvF3 5′H expression. The anthocyanin concentrations were remarkably reduced when the bunches were shaded during ripening, which was in accordance with the decreased transcription of several anthocyanin biosynthetic genes and transcriptional factors. Shading during early development did not influence the anthocyanin concentrations at harvest; however, it decreased the proportion of trihydroxylated anthocyanins. Thus, shading during early development also had an influence on the compounds biosynthesized during ripening.

Black and red grapes produce highly diverse phenolic compounds in berries, largely in skin and seedcoat. Proanthocyanidins (PAs), condensed tannins, are polymers of flavan-3-ol units (e.g., catechin, epicatechin, and epigallocatechin). PAs accumulate in the berry skins and seeds. Other flavonoids, anthocyanins and flavonols, are found only in the skins. Hydroxycinnamates are found in the flesh as well as the skins. These compounds are important as a result of their contribution to the color, bitterness, and astringency of red wine as well as to the color of table grapes (Cheynier, 2005; Cheynier et al. 1999; Vidal et al., 2003). Moreover, their potential human health benefits provide incentive to many researchers to study the regulation of the contents and composition in grape and wine (Jackson, 2000a).

Each class of flavonoid, anthocyanins, flavonols, and flavan-3-ols, is biosynthesized by a one-step enzyme reaction branched from the common flavonoid pathway (Fig. 1). Flavonoid 3′-hydroxylases (F3′H) and flavonoid 3′,5′-hydroxylases (F3′,5′H) catalyze hydroxylation at the 3′ and 3′, 5′ positions of the B-ring of flavonoids, respectively. Thus, these enzymes are presumed to control the branching points of the parallel pathways producing the compositionally different flavonoids with a B-ring hydroxylation pattern. The transcription of VvF3′H and VvF3′,5′H was reported to correlate with the composition of some flavonoids in grape (Castellarin and Gaspero, 2007; Jeong et al., 2006). An additional gene, VvCYTB5, was identified as a candidate for modulating F3′H and F3′,5′H activity and may also be required for the hydroxylation of the B-ring of flavonoids (Bogs et al., 2006). Generally, the enzymatic amounts and activities involved in flavonoid biosynthesis are presumed to be regulated predominantly at the level of transcription (Davies and Schwinn, 2003).

Grape is a nonclimacteric fruit, and berry development consists of two successive sigmoidal growth periods (Stages I and III) separated by a lag phase (Stage II) (Coome, 1976). The period of transition from Stage II to Stage III is called veraison, when the metabolism in berries changes markedly toward ripening (Deluc et al., 2007). Most PAs and hydroxycinnamates are biosynthesized before veraison, whereas anthocyanins are biosynthesized after it. Flavonols are biosynthesized during two distinct periods, the first of which is around flowering and the second during ripening of the developing berries (Downey et al., 2003a; Fujita et al., 2006). Thus, biosynthesis of each class of phenolics appears to be under a different control system, although a large part of its biosynthetic pathway is shared by the other phenolics. The regulation of the structural genes in the phenylpropanoid and flavonoid pathway by the complex of DNA-binding R2R3 MYB transcription factors, basic helix loop helix, and other classes of transcription factors was found in several plant species (Baudry et al., 2004; Hartmann et al., 2005; Weissshaar and Jenkins, 1998). In grapes, recently some regulators belonging to the MYB transcription superfamily were reported to regulate specifically the different branches of the flavonoid pathway: VvMYBA1 is a putative regulator of the PA pathway (Bogs et al., 2007), whereas VvMYB1 and VvMYBA2 regulate the anthocyanin pathway (Kobayashi et al., 2004; Walker et al., 2007) and VvMYB5a might be involved in the control of a number of different branches of the phenylpropanoid pathway (Deluc et al., 2006).

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Grape skin phenolics are sensitive to environmental factors such as light, heat, water relations, nutrients, and viticultural practices (Downey et al., 2006; Jackson and Lombard, 1993), consistent with the possible role of phenolics as ultraviolet-absorbing compounds and scavengers of active oxygen species (Landry et al., 1995; Nagata et al., 2003).

Bunch exposure or shading is regarded as one of the most influential practices because increased or decreased light exposure particularly on fruit bunches significantly influences the accumulation of anthocyanins and the total phenolics in grape berries (Jackson and Lombard, 1993; Morrison and Noble, 1990; Rojas-Lara and Morrison, 1989). Artificial bunch shading imposed by black shadecloth reduced the transcription of some structural genes on the biosynthetic pathways of several phenolics such as PAs, anthocyanins, and flavonols in grape berry skins, concomitant with the decreased contents of these phenolics (Fujita et al., 2006, 2007; Jeong et al., 2004). Bunch exposure induced the changes in the composition toward a higher proportion of B-ring trihydroxylation within PAs and anthocyanins compared with those in bunches shaded by lightproof boxes (Cortell and Kennedy, 2006; Downey et al., 2004).

The effect of bunch shading during specific stages of berry development (Stages I and II and/or Stage III) on fruit growth and composition in the berry skins of ‘Cabernet Sauvignon’ was previously reported (Dokoozlian and Kliwer, 1996). Anthocyanin and phenolic concentration were highest in the berry skins exposed to light throughout berry development and lowest in those shaded throughout berry development. Light exposure during Stages I and II appeared necessary for maximum anthocyanins during Stage III, although grape berries do not accumulate anthocyanins during this stage. However, the effect on the detailed phenolic profile in the berry skins has not been characterized.

The maximum PA concentration in the shaded berries during Stages I, II, and III was much lower than exposed berries around veraison; however, the decrease in the concentrations in shaded berries during Stage III was smaller than that in exposed berries, resulting in no appreciable difference at the harvest stage (Downey et al., 2004; Fujita et al., 2007). Whether shading during Stage III influences these decreases in concentration remains to be determined.

The first objective of this research was to examine the effects of bunch shading during different stages of berry development
Materials and Methods

Plant material and shading treatment. A field experiment was conducted in 2006 using eight vines of ‘Cabernet Sauvignon’ grown in an experimental vineyard in Higashi-Hiroshima, Japan. Vines were trained on a Guyot trellising system (Jackson, 2000b), in which shoots were trained upward and each vine carried ≈20 bunches of grapes. Eight vines were grouped into four sets of two vines to provide four biological replicates. Half of the bunches on each vine were randomly selected and shaded 1 week after anthesis; and, at veraison, 9 weeks after flowering (WAF), half of the bunches in each treatment were randomly selected and shaded until harvest while the others were exposed to natural sunlight. Thus, four treatments—light exposure during Stages I, II, and III (LL); light exposure during Stages I and II and then shading during Stage III (LS); shading during Stages I and II and then light exposure during Stage III (SL); and shading during Stages I, II, and III (SS)—were carried out with four biological replicates. Four layers of black shadecloth (≈8 × 8 threads/cm²) were applied on the bunches for shading, which reduced the light intensity to ≈9% of that of the exposed bunches during daytime on clear, sunny days. A frame of plastic netting was placed inside the bags of black shadecloth to prevent contact between the bags and the bunches. In addition, the bags were fastened to the shoots using wires to prevent any contact between the bags and the bunches.

Two hundred berries from the bunches of each of four treatments were randomly collected at various stages of berry development and ripening. The berry weight was recorded before the berry dissection. The berries were then manually peeled with a scalpel to eliminate any flesh. The berry skins were immediately frozen in liquid nitrogen and stored at –80 °C until use. Soluble solids concentration of the juice was measured using a digital refractometer (Attago, Tokyo, Japan), and titratable acidity was determined by titration of 10 mL juice with 0.1 N NaOH to an end point of pH 8.2 and expressed as grams tartaric acid per liter.

Extraction, fractionation, and quantification of skin monomeric phenolics and proanthocyanidins. Skin phenolics were extracted from berries with a modified method of Downey et al. (2003b). For the analysis of monomeric phenolics, 0.3 g of ground powder was extracted with 12 mL of 2% (v/v) formic acid in 70% methanol for 24 h. The concentrations and compositions of monomeric phenolics were determined using reversed-phase high-performance liquid chromatography described previously (Koyama et al., 2007). Each phenolic determined was expressed as the concentration in fresh tissues (milligrams per gram of skin fresh weight).

The cumulative expression of VvMYB5a, VvMYB5a, and VvMYB5a was calculated as the area below the expression curves during time series from 7 to 12 WAF using the method described by Castellarin et al. (2007a).

Statistical analysis. For the samples taken during Stages I and II, the t test was carried out for each sampling time to test the significant difference between the shaded and exposed berry skins. For the samples taken during Stage III, two-way analysis...
of variance (ANOVA) was applied for each sampling time to test the significant effects of the two factors: shading during Stages I and II and shading during Stage III. The SPSS statistical package (SPSS, Chicago, IL) was used for every statistical analysis.

Results

Berry Development and Ripening. Berry weights in the bunches shaded during Stages I and II were lower than those in the exposed berries from the initial sampling time (4 WAF) and were not restored through subsequent light exposure during Stage III (Table 2).

The soluble solids concentrations of the berries shaded during Stages I and II were slightly but significantly higher than those of the exposed berries at harvest (Table 2). The titratable acidity in the berries shaded during Stages I, II, and III (SS) was slightly higher than in the others at harvest.

Analysis of the Proanthocyanidin Pathway in Berry Skins. Significant amounts of PA in the berry skins had already accumulated at 4 WAF, maximized around veraison (9 WAF), and decreased gradually throughout Stage III (Fig. 2A). The PA concentrations in the berries shaded during Stages I and II were lower than those in the exposed berries at 7 and 9 WAF. At 9 WAF, the PA concentrations were 25% lower in the shaded berries than in the exposed berries. By 12 WAF, PA concentrations in LL and LS berries declined more than and did not differ from those in SL and SS berries. PA concentrations further declined by 18 WAF with no difference among these treatments. With regard to the effect of light condition during Stage III, PA concentrations declined similarly between exposed (LL and SL) and shaded (LS and SS) berries.

VvANR and VvLAR2 are known to function at the branching points of the flavonoid pathway, leading to the synthesis of catechin and epicatechin, respectively. Recent research indicated that catechin and epicatechin act not only as initiating units, but also as the precursors of extension units for the synthesis of PAs (Dixon et al., 2005; Marles et al., 2003; Xie and Dixon, 2005). When significant amounts of PAs accumulated, the levels of expression of both genes were high. At 4 WAF, the mRNA levels (Fig. 2B–C) were the highest during this study period and diminished to a trace level at veraison (9 WAF) when the PA concentrations showed a maximum level (Fig. 2A). The expression of these genes during Stage III was not determined because the low levels of mRNA expression of these genes during the period were previously reported (Bogs et al., 2005; Fujita et al., 2007). The expression of VvLAR2 in the berry skins was unaffected by light condition. The expression of VvLAR1, the other leucoanthocyanidin-reductase isogene, was only a trace level and, therefore, is not shown. On the other hand, VvANR transcript levels at 4 WAF tended, although not statistically different, to be repressed in skins of berries from the shaded bunches as compared with exposed bunches (Fig. 2C).

VvMYBPA1, a putative regulator of PA synthesis in grape berry, was transcribed in two phases, similar to the common genes on flavonoid biosynthetic pathway (Boss et al., 1996). In LL and LS berries, the second peak was observed at 9 WAF.

Table 1. Polymerase chain reaction (PCR) primers and reaction conditions for real-time quantitative PCR.

| Gene name and accession no. | Sequence of forward (F) and reverse (R) primer | Primer position* | Primer concn (µM)* | Annealing temp (°C) |
|----------------------------|-----------------------------------------------|------------------|-------------------|-------------------|
| VvMYBPA1 (TC54724) | F 5’TCCATGCGCTAGTTCGAG | +773 to +792 | 0.125 | 56 |
| | R 5’GAGTTGTCAGTGGTGGGAT | Complements of +900 to +919 | | |
| VvMYB5a (AY555190) | F 5’GTGACGACCCATCTAAATGTA | +844 to +865 | 0.375 | 58 |
| | R 5’GTGACCTAAAGGACGGATGTA | Complements of +954 to +975 | | |
| VvMYBA2 (AB252699) | F 5’GACCCATGGAGTGATGATT | +938 to +957 | 0.25 | 50 |
| | R 5’AACTAAAACATTAAGATAAC | Complements of +1055 to +1074 | | |

*Primer positions indicate the base from the start codon. The first nucleotide of the start codon was defined as position 1.

Table 2. Berry weight and juice composition at veraison and harvest in bunches of ‘Cabernet Sauvignon’ grapes shaded during different stages of development.

| Sampling time | Treatment* | Berry wt (g) | Soluble solids concn (%) | Titratable acidity (g L⁻¹) |
|---------------|------------|--------------|--------------------------|---------------------------|
| Veraison (9 WAF)$^a$ | L | 1.13 ± 0.07 | 6.9 ± 1.48 | 32.4 ± 1.43 |
| | S | 0.97 ± 0.02 | 5.3 ± 0.91 | 31.7 ± 2.29 |
| Significance$^b$ | * | NS | NS | |
| Harvest (18 WAF) | LL | 2.05 ± 0.09 | 17.8 ± 0.69 | 5.8 ± 0.43 |
| | LS | 1.99 ± 0.17 | 18.1 ± 0.50 | 6.3 ± 0.50 |
| | SL | 1.71 ± 0.13 | 18.9 ± 0.39 | 6.0 ± 0.42 |
| | SS | 1.72 ± 0.16 | 19.3 ± 0.21 | 7.1 ± 0.59 |
| Significance$^c$ | Shading during | * | * | NS |
| | Stages I and II | * | * | NS |
| | Shading during | NS | NS | * |
| | Stage III | | | |

$^a$Until veraison, two treatments were applied on bunches: L = light exposure, S = shading. After veraison, four treatments were applied: LL = light exposure during Stages I, II, and III; LS = light exposure during Stages I and II and then shading during Stage III; SL = shading during Stages I and II and then light exposure during Stage III; SS = shading during Stages I, II, and III.

$^b$t Test was used to evaluate significant differences between the treatments of the samples at veraison (*P < 0.05).

$^c$Two-factor analysis of variance was used to evaluate significant effects of shading during Stages I and II as well as shading during Stage III for the samples at harvest (*P < 0.05).

NS = nonsignificant.
The expression level of VvMYBPA1 at 4 WAF was lower in the shaded berries than the exposed berries, concomitant with the decrease of the PA concentrations. The expression level of VvMYBPA1 at 12 WAF was lower in the berry skins shaded during Stage III than that in the exposed berries, although the difference between the exposed (LL and SL) and shaded (LS and SS) berries was not high (16%).

The ectopic expression of VvMYB5a, another grapevine MYB transcription factor, induced the biosynthesis of anthocyanin, PA, flavonol, and lignin in tobacco (Deluc et al., 2006). The transcription levels of VvMYB5a were low and unaffected by shading during the study period (Fig. 2E). The mRNA levels were the highest at 4 WAF and gradually decreased through berry development. The profile did not change markedly on the onset of ripening, unlike the other MYB transcription factors examined in our study, in accordance with the report in which this gene was expressed in the berry skins before veraison.

A N A L Y S I S O F T H E A N T H O C Y A N I N PATHWAY AND OTHER MONOMERIC PHENOLIC ACCUMULATION IN BERRY SKINS. Based on color, the onset of ripening in the bunches shaded during Stages I and II appeared to be slightly delayed (Fig. 3A). Delayed onset of ripening and reduced berry size by bunch shading during Stages I and II were reported in a previous study in which the vines were grown in a sunlit phytotron to discriminate the effects of light and temperature (Dokoozlian and Kliewer, 1996). At harvest, the anthocyanin concentrations in the berries shaded during Stage III (LS and SS) were only 38% of the concentration in the exposed berries (LL and SL).

The VvUFGT expression profile corresponded to anthocyanin accumulation in accordance with its critical role in anthocyanin biosynthesis (Fig. 3B). A sharp increase in the transcription was observed at the onset of coloring (9 WAF). As a result of two-way ANOVA, both shading during Stages I and II and that during Stage III significantly reduced the level of expression at 12 WAF.

Two closely correlated MYB genes, VvMYBA1 and VvMYBA2, were cloned and characterized as transcriptional regulators of anthocyanin biosynthetic pathway (Kobayashi et al., 2004; Walker et al., 2007). Both genes (Fig. 3C–D) were expressed with only trace amounts during Stages I and II, and the...
expressions rapidly increased at the onset of veraison, corresponding to the profile of the anthocyanin concentrations and *VvMYBA1* expressions. The molar ratio of trihydroxylated PA subunits to dihydroxylated subunits in the berries shaded during Stages I and II (SL and SS) was lower than that in the exposed berries (LL and LS) through Stages I, II, and also III (Fig. 5A). At 4 WAF, a 13% reduction was observed. The degree of difference did not vary through Stages I, II, and also III (10% to 16%). At harvest, a slight increase in the ratio resulting from shading during Stage III from that in the exposed berries was observed. The effect of shading during Stage III was a minor factor compared with that during Stages I and II. The galloylation rates were higher than those of the exposed berries through Stages I, II, and also III by shading during Stages I and II (Fig. 5B). The galloylation rates gradually decreased during Stage III. The proportion of declining concentration did not differ between the shaded and exposed berries during Stages I and II. Similar to the change in the galloylation rate, the mDP of PAs gradually decreased during Stage III (Fig. 5C). Shading during Stages I and II tended to decrease the mDP through Stages I, II, and also III, although the effect was significant only at 12 and 18 WAF. During Stage III, mDP in the berries shaded during Stage III (LS and SS) was slightly lower than that in the exposed berries (LL and SL); however, the influence was minor compared with that during Stages I and II.

The relative abundance of trihydroxylated anthocyanins to dihydroxylated anthocyanins in the berry skins shaded during Stages I and II (SL and SS) was lower at 12 and 18 WAF than that in the exposed berries (LL and LS) (Fig. 6A). At 12 WAF, a 19% reduction was observed. Contrary, bunch shading during Stage III resulted in a slightly higher ratio than that in the exposed berries at harvest. The influence was minor compared with that during Stages I and II.

The proportion of acylated anthocyanins was higher in both the berry skins shaded during Stages I and II and those shaded during Stage III than in the exposed berry skins at 12 and 18 WAF (Fig. 6B). A higher percentage of acylated anthocyanins...
in the shaded berries was also reported in other studies (Gao and Cahoon, 1994; Haselgrove et al., 2000). At harvest, the effect of shading during Stage III was observed as the primary factor for the difference in the proportion of acylated anthocyanins in the berry skins.

The genes related to the hydroxylation pattern on the B ring in the flavonoids, \(VvF3'H3\), \(VvF3'H4\), and \(VvF3'5'H\), were transcribed in two phases. The mRNA levels of these genes were high at 4 WAF, decreased, and, after that, increased at the onset of ripening, concomitant with the active biosynthesis of PAs and anthocyanins at each phase (Figs. 2A and 3A). Such biphasic patterns were also observed in the profile of the other common genes on the flavonoid biosynthetic pathway (Boss et al., 1996). Shading during Stages I and II and also shading during Stage III showed lower induction of the transcriptions of \(VvF3'H4\) and \(VvF3'5'H\) in the berry skins at 12 WAF (Fig. 7B–7C). The expression level of \(VvF3'5'H\) was lowered to 50% at 4 WAF by shading. The relatively low ratio in the mRNA levels of \(VvF3'5'H\) to \(VvF3'Hs\) at 4 WAF was comparable with the low value of the relative abundance of trihydroxylated PA subunits to dihydroxylated ones at the same time (Figs. 5A and 7D). On the other hand, the mRNA ratio at 12 WAF was much higher than that at 4 WAF, reflecting the
higher ratio of trihydroxylated anthocyanins to dihydroxylated ones than that within PAs (Figs. 6A and 7D). The mRNA ratio in the berry skins at 4 WAF was lowered to 62% by shading. The ratio at 12 WAF was lower in the berry skins shaded during Stages I and II and also those shaded during Stage III than that in the exposed berry skins during the same period (Fig. 7D).

Similarly, the expression level of \( \text{VvCYTB5} \) tended to be lowered at 4 WAF by shading during Stages I and II, although no difference was observed (Fig. 7E). Shading during Stages I and II and also shading during Stage III showed lower induction of the transcriptions in the berry skins at 12 WAF.

**Discussion**

**Influence of bunch shading on the proanthocyanidin composition.** Bunch shading during Stages I and II did not affect the final PA concentrations; however, shading did affect the PA composition. Shading during Stages I and II decreased the ratio of trihydroxylated to dihydroxylated subunits within PAs. This result did not contradict the decreased ratio of the expression of \( \text{VvF3'5'H} \) to \( \text{VvF3'H} \) at 4 WAF, indicating that the modification of the transcription of \( \text{VvF3'5'H} \) and \( \text{VvF3'H} \) at the early developmental stage by shading contributes to the change in the composition of PAs (Figs. 5A and 7D).

With regard to the sequence of \( \text{VvF3'5'H} \), whole-genome sequencing of grapevine revealed that \( \text{VvF3'5'H} \) is multicopied, although complete sequences are not available at

During this period. The effects of increasing or decreasing light intensities on the contents of PAs have also been reported in other plant species. In *Lotus corniculatus* L. leaves, the accumulation of PAs was induced by the increasing light intensities (Paolocci et al., 2005). The late genes of the pathway (\( \text{DFR and ANS} \)) were upregulated by light and were considered to be the first rate-limiting steps in PA biosynthesis. In our study, shading from 1 week after anthesis resulted in the reductions in PA concentrations (23% of the sun-exposed berries) as well as in the transcription levels of \( \text{VvANR} \) and \( \text{VvMYBPA1} \) (45% and 36%, respectively) in the berry skin at 4 WAF (Figs. 2A, 2C, and 2D). Similar reduction in transcription of \( \text{VvANR} \) and \( \text{VvLAR2} \) with reduction in PA concentrations in shaded berries was previously reported by Fujita et al. (2007).

Bogs et al. (2007) reported that \( \text{VvMYBPA1} \) protein regulates the activity of the \( \text{VvANR} \), \( \text{VvLAR1} \), \( \text{VvF3'5'H} \), \( \text{VvLDOX} \), and \( \text{VvCHI} \) promoters, but not that of \( \text{VvUGFT} \), in a reporter assay using grape cells, suggesting that the gene specifically regulates the PA pathway. Thus, the reduction of the PA concentrations is explained by the reduced transcription of \( \text{VvMYBPA1} \) and \( \text{VvANR} \). In addition, it is possible that the decreases in the transcription of the upstream genes of \( \text{VvANR} \) in the PA pathway, or in that of the downstream genes related with the sequestration, condensation, and oxidation of PAs, contribute to the reduction of the PA concentrations by shading observed here. The transcription of \( \text{VvMYBPA1} \) was also observed during the early ripening stage (Fig. 2D) in agreement with the report of Bogs et al. (2007).

During Stage III, the PA concentrations in the bunches of all four different treatments gradually decreased (Fig. 2A). The different rates of the decrease of PAs during Stage III among these treatments indicate the changes in the proportion of the extractable portion of PAs during this period. Previous studies suggested that, as berry development progresses, the localization of some PAs changes from the vacuole to the apoplast in the plant cells and PAs are then oxidized and covalently attached to the cell wall, which makes them unextractable (Downey et al., 2003b; Gagne et al., 2006; Geny et al., 2003).

Interestingly, the exposed berries during Stages I and II (LL and LS) with a high PA concentration at veraison showed a larger decrease of extractable PAs during Stage III than shaded berries (SL and SS). As a result, no difference in the PA concentration was observed at harvest. In our study, it was clearly shown that whether the berries were shaded or exposed during Stage III did not influence the decline in the PAs (Fig. 2A). Further studies on this effect of shading during Stages I and II on the decrease of extractable PAs will be necessary, and the mechanism of the decline of extractable PAs during Stage III will need to be elucidated.

**The influence of bunch shading on proanthocyanidin composition.** Bunch shading during Stages I and II did not affect the final PA concentrations; however, shading did affect the PA composition. Shading during Stages I and II decreased the ratio of trihydroxylated to dihydroxylated subunits within PAs. This result did not contradict the decreased ratio of the expression of \( \text{VvF3'5'H} \) to \( \text{VvF3'H} \) at 4 WAF, indicating that the modification of the transcription of \( \text{VvF3'5'H} \) and \( \text{VvF3'H} \) at the early developmental stage by shading contributes to the change in the composition of PAs (Figs. 5A and 7D).

With regard to the sequence of \( \text{VvF3'5'H} \), whole-genome sequencing of grapevine revealed that \( \text{VvF3'5'H} \) is multicopied, although complete sequences are not available at
present (Velasco et al., 2007). On the other hand, the Unigene set of *V. vinifera* (National Center for Biotechnology Information, 2008) contains only one *F3'H*, Vvi.441, which consists of 57 expression sequence tags (ESTs). Among these ESTs, 22 ESTs were derived from grape berries and have sequences of their 3'-UTR region. These 22 ESTs were clustered into three groups by their sequences. The major group corresponding to TC51695 in DFCI consists of 18 highly homologous ESTs, 10 of which have the primer annealing sequence of *VvF3* '5'H used in this study. The other eight ESTs were all derived from the cDNA library made from *V. vinifera* cv. Muscat Hamburg pericarp tissue. These sequences contain a difference in a single nucleotide from our primer annealing sequence, indicating the single nucleotide polymorphism in this cultivar. Another group consists of three ESTs, including CF415436. This sequence was reported not to be significantly transcribed in the skin of 'Shiraz' (Bogs et al., 2006). The last group consists of only one EST and does not have our primer annealing sequences. Thus, our real-time Q-PCR primers of *VvF3'H* amplified the predominant *F3'H* in berry skins even if there are other *F3'H* sequences in the grape genome.

In addition, the expression of *VvCYTB5* was similarly reduced by shading to that of *VvF3'H*; thus, this gene possibly contributes to the change of PA composition observed here. The VvCYTB5 protein is regarded to modulate the *F3'H* and *F3'S'H* activity, affecting the hydroxylation pattern in the flavonoids. In *Petunia × hybrida* Vilm., cytochrome b5 affected only *F3'S'H* activity, although the function in grape still needs to be clarified (Bogs et al., 2006; Vetten et al., 1999).

An increase in the galloylation rate and a decrease in mDP within PAs by shading were also observed (Fig. 5B–C). These changes potentially influence the wine sensory properties: the overall astringency decreased as mDP decreased; increased galloylation was responsible for the rise in coarseness; and the decrease of trihydroxylation of the B ring increased the coarseness in a wine-like medium (Vidal et al., 2003). Wines made from shaded fruit were reported to be significantly less astringent overall (Cortell et al., 2008; Joscelyne et al., 2007; Ristic et al., 2007). Considering that shading had a much greater influence on the skin PAs than on the seed PAs (Cortell and Kennedy, 2006), these differences in sensory perceptions in wines are possibly

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**Fig. 7. Changes in the transcription levels of the flavonoid biosynthetic genes related with the hydroxylation profile of the metabolites in berry skins of 'Cabernet Sauvignon' grape shaded during different developmental stages.**

(A) Transcript profile of *VvF3'H* shown as the molar ratio of the mRNA level to that of *VvUbiquitin*. (B) Transcript profile of *VvF3'H4*. (C) Transcript profile of *VvF3'S'H*. (D) Ratio of *VvF3'S'H* transcription to that of *VvF3'H*s. (E) Transcript profile of *VvCYTB5*. Horizontal line represents weeks after flowering (WAF). The symbols indicate four treatments: LL = light exposure during Stages I, II, and III; LS = light exposure during Stages I and II and then shading during Stage III; SL = shading during Stages I and II and then light exposure during Stage III; SS = shading during Stages I, II, and III. Statistical analysis was carried out to test the significant effects of shading during Stages I and II (Shade I + II) as well as during Stage III (Shade III). The asterisks indicate significant differences between the treatments at *P* < 0.05 by the *t*-test before veraison (9 WAF) and by two-way analysis of variance after veraison. The vertical bars represent SD (*n* = 4).

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related to differences in skin PA contents and compositions between the treatments.

Bunch shading during Stage III did not affect these compositional changes, although a minor influence was observed compared with the influence of shading during Stages I and II (Figs. 5A and 5C).

**Influence of Bunch Shading on Anthocyanin Concentration and Composition.** Whether the berries were shaded or exposed during Stage III had a decisive impact on the concentrations of anthocyanin (Fig. 3A). The reduction rate of anthocyanin and flavonol by shading was much higher than that of PAs (Figs. 2A and 4A). It was shown that the shading during Stage III negatively influenced the transcription of VvMYBA2 as well as VvMYBA1. Similarly, the transcription of VvUFGT, which is the key enzyme for the anthocyanin biosynthetic pathway, as well as that of VvF3’H4, VvF3’S’H, and VvCYTB5, was markedly reduced by shading. The cumulative gene expression of VvUFGT, VvF3’S’H, VvCYTB5, VvF3’h4, VvF3’h3, VvMYBA1, VvMYBA2, and VvMYBPA1, but not VvMYB5a, was significantly correlated with anthocyanin accumulation through a time series during the early anthocyanin biosynthesis from 7 to 12 WAF (Table 3).

Bunch shading during Stages I and II retarded the onset of veraison. However, the treatment did not influence the final anthocyanin concentrations in the berries at harvest (Fig. 3A). Partially different from this result, Dokoozlian and Kliever (1996) reported that shading during Stages I and II delayed the onset of ripening and reduced the anthocyanin concentration at harvest from that of the exposed control.

Instead, bunch shading during Stages I and II affected the anthocyanin composition (Fig. 6). It decreased the relative abundance of 3′,4′,5′-hydroxylated anthocyanin to 3′,4′-hydroxylated anthocyanins throughout Stage III concomitant with the decreased mRNA ratio of VvF3’ S’H to VvF3’Hs at 12 WAF (Figs. 6A and 7D). Thus, this suggests that shading during Stages I and II has an effect on the activity of flavonoid hydroxylases, modifying the composition of anthocyanins during ripening. A similar proportional change was observed in another study in which the early deficit irrigation before veraison had an effect not only on the anthocyanin concentrations during ripening, but also on the composition through the modification of the transcription of the flavonoid hydroxylases (Castellarin et al., 2007b).

### Table 3. Correlation coefficients between the cumulative expression of the anthocyanin biosynthesis gene during a time series through ripening (from 7 to 12 weeks after flowering) and total anthocyanin content (mg g⁻¹ skin fresh weight) in berry skins of ‘Cabernet Sauvignon’ grapes.

| Gene       | Correlation coefficient (R²) |
|------------|-----------------------------|
| VvUFGT     | 0.88**                      |
| VvF3’ S’H  | 0.84**                      |
| VvCYTB5    | 0.88**                      |
| VvF3’ H4   | 0.84**                      |
| VvF3’ H3   | 0.41**                      |
| VvMYBA1    | 0.85**                      |
| VvMYBA2    | 0.79**                      |
| VvMYBPA1   | 0.66**                      |
| VvMYB5a    | 0.20                        |

**Significant at P < 0.01 (n = 24).**

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