Effect of Shading on Proanthocyanidin Biosynthesis in the Grape Berry

Akiko Fujita*, Noriko Soma, Nami Goto-Yamamoto, Akihiro Mizuno, Kuniaki Kiso and Katsumi Hashizume

National Research Institute of Brewing, Kagamiyama, Higashi-Hiroshima 739-0046, Japan

Proanthocyanidins, which are oligomers and polymers of flavan-3-ol units (e.g., (+)-catechin and (−)-epicatechin), are important components of grapes for red winemaking. Flavan-3-ols are biosynthesized by the catalysis of anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR). In this study, we investigated the effect of shading on proanthocyanidin biosynthesis in berries of Vitis vinifera ‘Cabernet Sauvignon’. Shading of the berries reduced the accumulation of proanthocyanidins and the transcription of ANR and LAR genes in the skins during berry development, while no significant effect was observed in the seeds. Because the proanthocyanidins significantly decreased in the skins and seeds of the control berries during ripening, the levels of proanthocyanidins were similar in the shaded and control berries at the harvest stage.

Key Words: anthocyanidin reductase, flavan-3-ols, grape, leucoanthocyanidin reductase, proanthocyanidins.

Introduction

Proanthocyanidins (PAs), also known as condensed tannins, contribute to the astringency and bitterness of grapes and wine. PAs, which accumulate in grape skins and seeds, are important flavonoid compounds, especially for red-wine grapes. PAs are oligomers and polymers of flavan-3-ol units (e.g., (+)-catechin and (−)-epicatechin) (Fig. 1). The key enzymes for flavan-3-ol biosynthesis are anthocyanidin reductase (ANR), which catalyzes the reaction from anthocyanidin to 2,3-cis-flavan-3-ols (e.g., (−)-epicatechin), and leucoanthocyanidin reductase (LAR), which catalyzes the reaction from leucoanthocyanidin to 2,3-trans-flavan-3-ols (e.g., (+)-catechin) (Tanner et al., 2003; Xie et al., 2003). The starter units of PAs are believed to be flavan-3-ols, while the origin of the extension units and the mechanism of PA polymerization have not been proven yet. Although quinone methides or carbocations derived from leucoanthocyanidins have been generally accepted as the precursors of the extension units, the fact that leucoanthocyanidin possesses 2,3-trans stereochemistry does not explain why the predominant extension units in PAs are in the 2,3-cis configuration (Xie and Dixon, 2005). Anthocyanidins have also been considered as potential substrates for PA polymerization. Recently, a model according to which 2,3-cis-flavan-3-ols compose not only the starter units but also the extension units has been presented because the ectopic expression of ANR in tobacco flower petals resulted in the accumulation of PAs (Xie et al., 2003, 2006). In grape seed PAs, 60% of the terminal units and 90% of the extension units were reported to be 2,3-cis-flavan-3-ols (Kennedy et al., 2000a,b).

With regard to grapes, Bogs et al. (2005) reported one ANR cDNA (VvANR) and two LAR cDNAs (VvLAR1 and VvLAR2) of Vitis vinifera ‘Shiraz’. They showed that both ANR and LAR contribute to PA synthesis in grape berries and that PA accumulation occurs early in berry development and is completed when ripening starts. At almost the same time, we also reported that the mRNA of VvANR accumulated in the berry skins and seeds of ‘Cabernet Sauvignon’ at the early stage of development, concomitantly with PA accumulation, and then decreased toward the ripening stage (Fujita et al., 2005).

Flavan-3-ols and PAs share the same upstream biosynthetic pathway with anthocyanins, which are red grape pigments, and with flavonols, which contribute to the bitter taste of red wine and stabilize or increase the color of red wine by copigmentation (Baranac et al., 1997). With regard to the effects of light on flavonoid accumulation in grape berries, many studies have been carried out. Increased exposure to light generally stimulates anthocyanin accumulation in grape berries (Jeong et al., 2004; Kataoka et al., 2003), while Bergqvist...
et al. (2001) reported that excessive sunlight exposure resulted in decreased anthocyanin concentrations, likely to be a result of the elevated temperature. Shading of the berries significantly reduces the flavonol content and mRNA accumulation of one of the flavonol synthase genes in berry skins (Downey et al., 2004; Fujita et al., 2006). Downey et al. (2004) reported that shading had no significant effect on the levels of anthocyanins or condensed tannins in ripe ‘Shiraz’ berries. However, the effects of shading on the enzymes or genes involved in the flavan-3-ol biosynthesis of grapes have not yet been studied.

In this study, we determined the concentrations of flavan-3-ol monomers and PAs and the mRNA levels of \( VvANR \), \( VvLAR1 \), and \( VvLAR2 \) in several organs of a major red-wine cultivar, ‘Cabernet Sauvignon’. In order to investigate whether PA biosynthesis in grape berries is regulated by light, we also examined the effect of shading on the mRNA accumulation of these genes, as well as on the accumulation of flavan-3-ol monomers and PAs, in the berry skins and seeds during development and ripening.

**Materials and Methods**

**Plant materials**

Four vines of *Vitis vinifera* ‘Cabernet Sauvignon’ (a red-wine cultivar) grown in an experimental vineyard in Higashi-Hiroshima, Japan, were used.

In 2004, 40 bunches each were used for an experiment under two conditions: shading and control. Shading of the bunches was started on June 7 (flowering) using four layers of Victoria lawn, which reduced the light intensity to 9% of that of control bunches during the daytime.

Two hundred berries each at different stages of development and ripening were sampled at random. The average berry weight was obtained by dividing the whole berry weight by the berry number. After peeling and de-seeding, the berry skins and seeds were immediately frozen in liquid nitrogen and kept at −80°C until use.

Leaves, flower buds, and flowers were also sampled, frozen, and kept as described above.

**Extraction, fractionation, and quantification of flavan-3-ol monomers and PAs**

The frozen samples were ground using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). Phenolic compounds were extracted from the ground samples with methanol, methanol/water (80/20, v/v), methanol/water (50/50, v/v), distilled water, and acetone/water (75/25, v/v) successively, as described by Bourzeix et al. (1986). Finally, the extracts were combined. Using \( tC_{18} \) and \( tC_{18} \) Sep-Pak Plus cartridges (Waters, Milford, MA, USA), the extracts were fractionated into three fractions: FI, flavan-3-ol monomers; FII, oligomeric PAs; and FIII, polymeric PAs, following the method described by Sun et al. (1998a).

The concentrations of flavan-3-ol monomers and PAs...
in each fraction were determined by the modified vanillin assay (Sun et al., 1998b) using a standard curve prepared with (+)-catechin (Sigma Chemical, St. Louis, MO, USA). Sample fractionation and analysis were carried out in triplicate. The concentration of each fraction was shown as the (+)-catechin equivalents per gram fresh weight. The total amount in the skins and seeds was also shown per berry.

RNA extraction and quantification of mRNA

Total RNA was extracted from the leaves, flower buds, flowers, berry skins, and seeds according to the procedure described by Loulakakis et al. (1996). The RNA concentration was determined by its absorbance at 260 nm.

For the determination of the mRNA levels of VvANR, VvLAR1, and VvLAR2, a real-time quantitative polymerase chain reaction (Q-PCR) was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA) and a QuantiTec SYBR Green PCR Kit (Qiagen, Hilden, Germany), as described in the manufacturers’ manuals. cDNAs of all the samples were prepared by reverse transcription of the samples were prepared by reverse transcription of mRNA using AMV (Avian Myeloblastosis Virus) reverse transcriptase XL (Takara Bio, Otsu, Japan). The real-time Q-PCR mixture (20 µL) contained 0.1 µL of the cDNA template and 0.3 µM each of the forward and reverse primers. Real-time Q-PCR was performed under the following conditions: at 95°C for 15 min followed by 45 cycles at 95°C for 15 s, at the annealing temperature for 30 s, and at 72°C for 30 s. The mRNA level of a ubiquitin gene (VvUbiquitin) was used as an internal control for Q-PCR, as described by Fujita et al. (2005). The primer sequences and annealing temperature for each primer set are shown in Table 1. Standard DNAs for the calibration curves were prepared as described by Jeong et al. (2004). Real-time Q-PCR was carried out in triplicate per total RNA sample. The average data are presented as the molar ratio of the mRNA level of VvANR, VvLAR1, and VvLAR2 to that of VvUbiquitin and, hence, have no units.

Genomic sequencing of the promoter regions of grape LAR genes

The promoter sequences of grape LAR genes were obtained by DNA walking using a Universal Genome-Walker Kit (BD Biosciences Clontech, Palo Alto, CA, USA). For the first PCR, the gene specific primers were designed in the 5’ region of the grape cDNA clones, VvLAR1 (GenBank accession no. AJ865336) and VvLAR2 (AJ865334). The genomic fragments amplified from the total DNA of ‘Cabernet Sauvignon’ using Takara Ex Taq polymerase (Takara Bio) were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) using a TA cloning system and sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed using a database of plant cis-acting regulatory DNA elements (PLACE, http://www.dna.affrc.go.jp/PLACE/signalscan.html, May 19, 2006).

Results and Discussion

Berry development and ripening

The increase in berry weight showed a double sigmoid curve during berry development and ripening (Fig. 2A). Both the control and shaded berries grew similarly during berry development, while the shaded berry weight was lower than the control berry weight after the veraison stage (August 16). At the harvest stage (October 12), the shaded berries weighed 10% less than the control berries.

The total soluble solids under both conditions increased markedly at around the veraison stage (Fig. 2B). Although the level of the total soluble solids in the shaded berries was lower than that in the control berries during ripening, the level under both conditions was similar (16.5°Brix) at the harvest stage.

On the whole, shading appeared to retard ripening of the berries in this study.

Accumulation of flavan-3-ol monomers and PAs

The concentrations of flavan-3-ol monomers and oligomeric and polymeric PAs in the leaves, flower buds, flowers, berry skins, and seeds of ‘Cabernet Sauvignon’

Table 1. PCR primers and annealing temperature for real-time Q-PCR.

| Genename | GenBank accessionno. | Sequence of forward(F) and reverse(R) primers | Primerposition ¤ | Annealing temperature(°C) |
|----------|----------------------|-----------------------------------------------|------------------|--------------------------|
| VvANR    | AB199315             | F 5’GCTGCTGTATACCATCAATCA 1774 to 1793 57   | 1774to1793       | 57                       |
| VvLAR1   | AJ865336             | R 5’GCAGGATACCCCGAATGAGG 690 to 709 57      | Compliments of 1867 to 1993 | 57                       |
| VvLAR2   | AJ865334             | F 5’GAATGAAATTCACTTCGAT 592 to 611 59       | Compliments of 688 to 707 59 |                       |
| VvUbiquitin* | F 5’TCTGAGGCTTCGTTGGAATAGG 210 to 229 60 | Compliments of 289 to 308 60     |                       |
|          | R 5’AGGCTGTGATACATTTGCG 289 to 308 60 |                       |                       |

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

* Ubiquitin primers were designed in the sequence (TCC38636) in an EST database of grape (TIGR, http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=grape, Release 4.0, September 21, 2004).
are shown in Figure 3. In all samples, the oligomeric and polymeric PAs accumulated more than the flavan-3-ol monomers. The flavan-3-ol monomers and PAs had already accumulated in small leaves, flower buds, flowers (Fig. 3A), and small-berry skins and seeds (June 21, Fig. 3B, D, F, H). From the small-berry stage until the veraison stage (August 16), the total amount of flavan-3-ol monomers and PAs per berry increased in the skins and seeds (Fig. 3B, D). The levels of flavan-3-ol monomers and PAs reached their maximum at around the veraison stage. Toward the harvest stage (October 12), the polymeric PAs decreased in the skins (Fig. 3B), and the flavan-3-ol monomers and PAs decreased in the seeds (Fig. 3D). These results showed a similar tendency to the data obtained from 'Cabernet Sauvignon' berries grown in the previous year (Fujita et al., 2005).

In the skins, the accumulation of flavan-3-ol monomers and PAs was suppressed by shading during berry development (Fig. 3B, C). However, the polymeric PAs significantly decreased in the control berries from September 13 to October 12. Thus, the differences between the control and shaded berries became small at the harvest stage. In the seeds, the total amount of flavan-

Fig. 2. Effect of shading on berry development and ripening of Vitis vinifera 'Cabernet Sauvignon'. Berry weight (A) and total soluble solids expressed as °Brix (B) are shown.

Fig. 3. Accumulation of flavan-3-ol monomers and proanthocyanidins (PAs) in the leaves, flower buds, and flowers (A); berry skins (B, C, F, G); and seeds (D, E, H, I) of 'Cabernet Sauvignon'. The concentrations of flavan-3-ols and PAs in the monomeric, oligomeric, and polymeric fractions were quantified by a vanillin assay. The results are expressed in (±)-catechin equivalents per gram fresh weight (A, F–I) or per berry skin or seeds (B–E). The vertical bars represent SD (n = 3).
3-ol monomers and PAs per berry reached its maximum at the veraison stage (Fig. 3D, E). The maximum level was slightly lower in the shaded than in the control berries. The levels of PAs significantly decreased in the control berries from September 13 to October 12. Thus, as observed in the skins, the levels of flavan-3-ol monomers and PAs in the shaded berry seeds were similar to those in the control berry seeds at the harvest stage. In addition, the concentrations per gram fresh weight in the shaded berries were also similar to those in the control berries at the harvest stage (Fig. 3F–I). This result that extractable PAs decreased after veraison agreed with reports from other groups (Kennedy et al., 2000a,b; Mateus et al., 2001). Although the mechanism whereby PAs become unextractable, as well as the mechanism of PA polymerization, has not yet been revealed, it is expected that PA oxidization and interaction with cell wall polysaccharides and other phenolics make PAs unextractable (Kennedy et al., 2000b; Pourcel et al., 2005).

Downey et al. (2004) investigated the effect of shading on flavonoid accumulation in ‘Shiraz’ berries and reported that there were significant differences in the condensed tannin content of the skins at around the veraison stage, but, in the course of commercial harvest, similar levels were observed between the shaded and exposed berries. They also reported that there was no effect of shading on the seed tannin content and composition. Therefore, the accumulation pattern of flavan-3-ol monomers and PAs in ‘Cabernet Sauvignon’ closely agreed with that in ‘Shiraz’.

Transcription of the ANR and LAR genes

The mRNA levels of VvANR, VvLAR1, and VvLAR2 were determined by real-time Q-PCR (Fig. 4). VvANR mRNA accumulated in the leaves throughout development and in the flower buds and flowers (Fig. 4A). The mRNA level of VvLAR1 was high in the flower buds and flowers (Fig. 4D), while that of VvLAR2 was high in the leaves (Fig. 4G). The mRNA level of VvANR was high in the small-berry skins and seeds at the early stage of development and decreased thereafter (Fig. 4B, C). The transcription of VvLAR1 showed similar patterns to that of VvANR in the skins and seeds (Fig. 4E, F), although the mRNA level of VvLAR1 differed from that of VvANR in the absolute value and moderately increased again in the skins at the veraison stage. The transcription of VvLAR2 showed a similar pattern to that of VvLAR1 in the skins (Fig. 4H) but differed from that of the other two genes in the seeds; in other words, the mRNA level of VvLAR2 increased toward the veraison stage and then decreased (Fig. 4I). Thus, the two LAR genes showed

![Fig. 4](image-url)
different transcription patterns depending on the organ and stage. The change in mRNA accumulation of VvANR, VvLAR1, and VvLAR2 does not contradict the accumulation of flavan-3-ol monomers and PAs in the berry skins and seeds. The transcription pattern of VvANR, VvLAR1, and VvLAR2 in ‘Cabernet Sauvignon’ berries agreed with that in ‘Shiraz’ berries (Bogs et al., 2005), except that VvLAR1 was transcribed in the small-berry skins.

In the skins, VvANR, VvLAR1, and VvLAR2 were transcribed mainly at the early stage of berry development (Fig. 4B, E, H). At this stage, the mRNA levels of these genes were lower in the shaded than in the control skins, although the effect of shading on the transcription of VvLAR1 was relatively weak. In the seeds, VvANR and VvLAR1 were transcribed mainly at the early stage of development (Fig. 4C, F). During development, the mRNA level of VvANR was lower in the shaded berry seeds, but the transcription of VvLAR1 appeared not to be affected by shading. The results suggest that, in the skins at the early stage of berry development, the transcription of ANR and LAR genes is suppressed by shading to a greater or lesser degree, which resulted in the limited accumulation of flavan-3-ol monomers and PAs. The transcription of these genes in the skins and that in the seeds were differently affected by shading. The genes in the skins are probably more sensitive to shading than those in the seeds because of direct exposure to light.

During ripening, the mRNA levels of the ANR and LAR genes were relatively low, but they remained higher in the shaded than in the control berries (Fig. 4B, C, E, H, I). The mRNA level of VvLAR2 was especially higher in the seeds of the shaded berries after the veraison stage. In the shaded berries, the decrease in the transcription of these genes appeared to lag because of the slow ripening.

**Promoter sequences of the ANR and LAR genes and transcriptional regulation of these genes by light**

In order to investigate the transcriptional regulation of LAR genes, we obtained the upstream genomic

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**Fig. 5.** Nucleotide sequences of VvLAR1 between −910 and −1 and VvLAR2 between −744 and −1 relative to the transcription start sites. Several putative cis-acting regulatory elements conserved in light-regulated genes are indicated in the VvLAR1 and VvLAR2 promoter regions. The VvLAR1 and VvLAR2 sequences were obtained from the genomic DNA of ‘Cabernet Sauvignon’ (GenBank accession nos. AB262457 and AB262458), except that a sequence between −66 and −1 in VvLAR1 is a cDNA sequence of ‘Cabernet Sauvignon’ (CF514437).
sequences of \textit{VvLAR1} and \textit{VvLAR2} of ‘Cabernet Sauvignon’ and have submitted them to the GenBank/EMBL/DBJ under accession nos. AB262457 and AB262458, respectively. The nucleotide sequences of the two promoter regions had no similar region to each other, while Bogs et al. (2005) reported that the coding regions of \textit{VvLAR1} and \textit{VvLAR2} of ‘Shiraz’ share 60% identity (77% similarity) at the protein level. A homology search using PLACE showed that some types of putative cis-acting regulatory elements conserved in light-regulated genes are present in the \textit{VvLAR1} and \textit{VvLAR2} promoter regions (Fig. 5): GT-1 sites (GRWAAW), I-box (GATAA), TGACG motif (Terzaghi and Cashmore, 1995), and GATA boxes (Gilmartin et al., 1990). The promoter region of \textit{VvANR} was previously reported to have one G-box (CACGTG) (Terzaghi and Cashmore, 1995), four GT-1 sites, one I-box, and four GATA boxes (Fujita et al., 2005). The presence of elements related to light does not contradict the fact that the transcription of these genes was affected by shading.

However, the effect of shading on the transcription of the flavan-3-ol biosynthetic genes was weaker than that of a biosynthetic gene of flavonols (Fujita et al., 2006), which act as a UV protectant (Flint et al., 1985), and the anthocyanin biosynthetic genes (Jeong et al., 2004).

In berry skins, flavonols accumulate at the very early stage of development and during ripening, while anthocyanins accumulate only during ripening. Although PAs, flavonols, and anthocyanins share a large part of the biosynthetic pathway, each biosynthesis appears to be under a different control system, even in the berry skins.

In this study, shading of the ‘Cabernet Sauvignon’ berries reduced the accumulation of flavan-3-ol monomers and PAs and the transcription of ANR and LAR genes in the skins during berry development; however, the levels of PAs at the harvest stage were similar in the shaded and control berries because extractable PAs decreased more markedly in the control than in the shaded berry skins and seeds during ripening. Downey et al. (2004) reported that shading had no significant effect on the levels of condensed tannins in ripe ‘Shiraz’ berries, but condensed tannins in the shaded berry skins were lower in terms of the mean degree of polymerization. The results obtained here support their report and suggest that light probably influences the qualitative change of PAs.

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Literature Cited

Baranac, J. M., N. A. Petranović and J. M. Dimitrić-Marković. 1997. Spectrophotometric study of anthocyan coipigmentation reactions. 2. Malvin and the nonglycosidized flavone quercetin. J. Agric. Food Chem. 45: 1694–1697.

Bergqvist, J., N. Dokoozlian and N. Ebisuda. 2001. Sunlight exposure and temperature effects on berry growth and composition of Cabernet Sauvignon and Grenache in the central San Joaquin Valley of California. Amer. J. Enol. Vitic. 52: 1–7.

Bogs, J., M. O. Downey, J. S. Harvey, A. R. Ashton, G. J. Tanner and S. P. Robinson. 2005. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. Plant Physiol. 139: 652–663.

Bourzeix, M., D. Weyland and N. Heredia. 1986. Étude des catéchines et des procyanidols de la grappe de raisin, du vin et d’autres dérivés de la vigne. Bull. OIV 669–670: 1171–1254.

Downey, M. O., J. S. Harvey and S. P. Robinson. 2004. The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. Aust. J. Grape Wine Res. 10: 55–73.

Flint, S. D., P. W. Jordan and M. M. Caldwell. 1985. Plant protective response to enhanced UV-B radiation under field conditions: leaf optical properties and photosynthesis. Photochim. Photobiol. 41: 95–99.

Fujita, A., N. Soma, N. Goto-Yamamoto, H. Shindo, T. Kakuta, T. Koizumi and K. Hashizume. 2005. Anthocyanidin reductase gene expression and accumulation of flavan-3-ols in grape berry. Amer. J. Enol. Vitic. 56: 336–342.

Fujita, A., N. Goto-Yamamoto, I. Aramaki and K. Hashizume. 2006. Organ-specific transcription of putative flavonol synthase genes of grapevine and effects of plant hormones and shading on flavonol biosynthesis in grape berry skins. Biosci. Biotechnol. Biochem. 70: 632–638.

Gilmartin, P. M., L. Sarokin, J. Memelink and N.-H. Chua. 1990. Molecular light switches for plant genes. Plant Cell 2: 369–378.

Jeong, S. T., N. Goto-Yamamoto, S. Kobayashi and M. Esaka. 2004. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. Plant Sci. 167: 247–252.

Kataoka, I., A. Sugiyama and K. Beppu. 2003. Role of ultraviolet radiation in accumulation of anthocyanin in berries of ‘Gros Colman’ grapes (\textit{Vitis vinifera} L.). J. Japan. Soc. Hort. Sci. 72: 1–6.

Kennedy, J. A., M. A. Matthews and A. L. Waterhouse. 2000a. Changes in grape seed polyphenols during fruit ripening. Phytochemistry 55: 77–85.

Kennedy, J. A., G. J. Troup, J. R. Pilbrow, D. R. Hutton, D. Hewitt, C. R. Hunter, R. Ristic, P. G. Iland and G. P. Jones. 2000b. Development of seed polyphenols in berries from \textit{Vitis vinifera} L. cv. Shiraz. Aust. J. Grape Wine Res. 6: 244–254.

Loulakakis, K. A., K. A. Roubelakis-Angelakis and A. K. Kanellis. 1996. Isolation of functional RNA from grapevine tissues poor in nucleic acid content. Amer. J. Enol. Vitic. 47: 181–185.

Mateus, N., S. Marques, A. C. Gonçalves, J. M. Machado and V. De Freitas. 2001. Proanthocyanidin composition of red \textit{Vitis vinifera} varieties from the Douro valley during ripening: influence of cultivation altitude. Amer. J. Enol. Vitic. 52: 115–121.
Pourcel, L., J.-M. Routaboul, L. Kerhoas, M. Caboche, L. Lepiniec and I. Debeaujon. 2005. *TRANSPARENT TESTA10* encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. Plant Cell 17: 2966–2980.

Sun, B. S., M. C. Leandro, J. M. Ricardo-da-Silva and M. I. Spranger. 1998a. Separation of grape and wine proanthocyanidins according to their degree of polymerization. J. Agric. Food Chem. 46: 1390–1396.

Sun, B. S., J. M. Ricardo-da-Silva and M. I. Spranger. 1998b. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46: 4267–4274.

Tanner, G. J., K. T. Francki, S. Abrahams, J. M. Watson, P. J. Larkin and A. R. Ashton. 2003. Proanthocyanidin biosynthesis in plants. J. Biol. Chem. 278: 31647–31656.

Terzaghi, W. B. and A. R. Cashmore. 1995. Light-regulated transcription. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46: 445–474.

Xie, D.-Y. and R. A. Dixon. 2005. Proanthocyanidin biosynthesis—still more questions than answers? Phytochemistry 66: 2127–2144.

Xie, D.-Y., S. B. Sharma, N. L. Paiva, D. Ferreira and R. A. Dixon. 2003. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. Science 299: 396–399.

Xie, D.-Y., S. B. Sharma, E. Wright, Z.-Y. Wang and R. A. Dixon. 2006. Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J. 45: 895–907.