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Analysis of the Expression of Anthocyanin Pathway Genes in Developing *Vitis vinifera* L. cv Shiraz Grape Berries and the Implications for Pathway Regulation

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Anthocyanin synthesis in *Vitis vinifera* L. cv Shiraz grape berries began 10 weeks postflowering and continued throughout berry ripening. Expression of seven genes of the anthocyanin biosynthetic pathway (phenylalanine ammonia lyase [PAL], chalcone synthase [CHS], chalcone isomerase [CHI], flavanone-3-hydroxylase [F3H], dihydroflavonol 4-reductase [DFR], leucoanthocyanidin dioxygenase [LDOX], and UDP glucose-flavonoid 3-*o*-glucosyl transferase [UFGT]) was determined. In flowers and grape berry skins, expression of all of the genes, except UFGT, was detected up to 4 weeks postflowering, followed by a reduction in this expression 6 to 8 weeks postflowering. Expression of CHS, CHI, F3H, DFR, LDOX, and UFGT then increased 10 weeks postflowering, coinciding with the onset of anthocyanin synthesis. In grape berry flesh, no PAL or UFGT expression was detected at any stage of development, but CHS, CHI, F3H, DFR, and LDOX were expressed up to 4 weeks postflowering. These results indicate that the onset of anthocyanin synthesis in ripening grape berry skins coincides with a coordinated increase in expression of a number of genes in the anthocyanin biosynthetic pathway, suggesting the involvement of regulatory genes. UFGT is regulated independently of the other genes, suggesting that in grapes the major control point in this pathway is later than that observed in maize, petunia, and snapdragon.

Anthocyanin biosynthesis has been extensively studied in petunia, snapdragon, and maize, resulting in the elucidation of the biosynthetic pathway in which the various anthocyanin pigments are synthesized from Phe. This work has been aided by the availability of a large number of anthocyanin mutants that are nonlethal and have been selected over many years by both plant breeders and geneticists (for a review, see Martin and Gerats, 1993). More recently, it has become apparent that the mutants generally fall into two groups. The first group results from mutations in the structural genes coding for enzymes in the anthocyanin biosynthetic pathway, and many of these genes have been isolated or identified using these mutants. The second group displays altered expression of more than one structural gene, and these are usually the result of mutations in regulatory genes. It has been shown that these regulatory genes are homologous to members of the *myc* and *myb* families of transcription factors (Paz-Ares et al., 1987; Ludwig et al., 1989; Goodrich et al., 1992). It is interesting that the control of the anthocyanin pathway differs in the three plant species mentioned above. In maize, it appears that the regulation start point is CHS, whereas in snapdragon and petunia the control start points are further on in the pathway, at F3H and DFR, respectively (for a review, see Martin and Gerats, 1993). As yet, the control of the expression of the anthocyanin pathway genes in fruit tissues has not been studied.

The color of red and black grapes results from the accumulation of anthocyanins that are usually only located in the skin of the berry. The quantity and quality of color in grape berries at harvest are crucial factors that influence wine making. Each species or variety of grapes has a unique set of anthocyanins, and the anthocyanin profiles of many *Vitis* spp. and varieties have been described (for a review, see Mazza and Miniati, 1993). *Vitis vinifera* varieties usually produce 3-monoglucoside, 3-acetylglucoside, and 3-*p*-coumarylglucoside derivatives of the aglycones delphinidin, cyanidin, peonidin, petunidin, and malvidin, with malvidin derivatives often being the major forms present. However, there are exceptions. The cultivar Pinot Noir produces only nonacylated anthocyanins (Fong et al., 1971), and many muscat cultivars produce less malvidin derivatives than other anthocyanins (Cravero et al., 1994). There are no reports of any pelargonidin derivatives isolated from grape berry skins, and thus the general pathway for anthocyanin synthesis can be modified to account for this fact (Fig. 1).

Grapes are a nonclimacteric fruit, and berry growth follows a double-sigmoid pattern (Coombe and Bishop, 1980). Anthocyanin biosynthesis commences only when ripening of the berry begins (termed véraison by viticulturists) and normally continues throughout the ripening phase of growth. Factors such as variety, growing region, and growth conditions can influence the levels of anthocyanins

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Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone-3-hydroxylase; LDOX, leucoanthocyanidin dioxygenase; PAL, Phe ammonia lyase; UFGT, UDP Glc-flavonoid 3-*o*-glucosyl transferase.

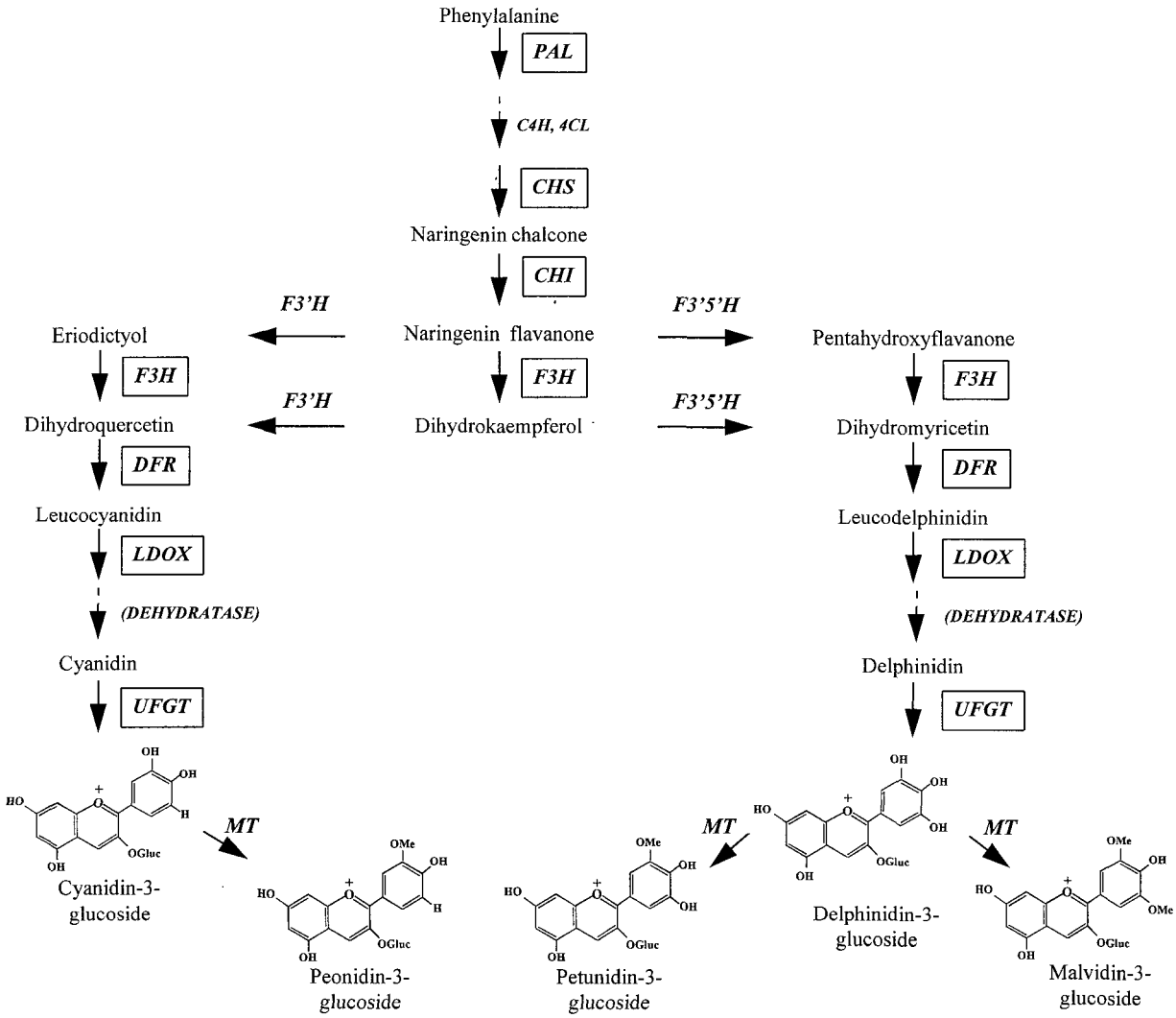

Figure 1. Simplified schematic of the anthocyanin biosynthetic pathway, modified to account for the major products found in grapes. The genes examined in this study are boxed. The dehydratase is putative and is thus written in brackets, and the substrates for flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) have not been determined for grapes. C4H, Cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; MT, methyltransferase.

produced and the profile of different pigments (for review, see Mazza and Miniati, 1993). Hrazdina et al. (1984) measured the activity of some anthocyanin biosynthetic enzymes during the development of De Chaunac berries. Anthocyanin accumulation began when these berries had just begun to accumulate sugar and increased rapidly until the concentration leveled off late in ripening, when the soluble solids were approximately 24 °Brix (a refractive index measure of the total dissolved solids). The increase in activity of the enzymes cinnamate-4-monooxygenase, *p*-coumarate:CoA-ligase (which catalyzes reactions before CHS in the pathway), and UFGT paralleled the increase in anthocyanin content. However, the activity of both PAL and CHI decreased as accumulation began and then subsequently increased. CHS activity was not detected until 3 weeks after anthocyanins were detected, even though it is the first committed step of the anthocyanin biosynthetic pathway. In cv Cardinal grapes, Roubelakis-Angelakis and Kliewer (1986) found that the increase in PAL activity paralleled anthocyanin accumulation as ripening progressed. Thus, the limited enzymatic studies of anthocyanin accumulation in grapes have not revealed a great deal about the control of this biosynthetic pathway and what causes anthocyanin biosynthesis to be switched on during ripening.

To further investigate the regulation of anthocyanin production in grape berries, we utilized grapevine cDNAs encoding enzymes of the anthocyanin biosynthetic pathway isolated by Sparvoli et al. (1994) from grape leaf tissue. Berries from cv Shiraz were sampled throughout development, and the tissue was used to study anthocyanin profiles and the expression of seven structural genes from the anthocyanin biosynthetic pathway. Our results suggest that the pattern of control for the anthocyanin pathway in grape berry skin tissue is different from that observed in petunia, snapdragon, and maize.

MATERIALS AND METHODS

Plant Tissue

Berries from *Vitis vinifera* L. cv Shiraz were sampled at 2-week intervals during the 1994 to 1995 growing season from vines grown at a commercial vineyard in Willunga, South Australia. To define the stage of berry development, a sample of 100 randomly selected berries from 30 bunches was individually labeled and scored each week for deformability, length, and width using a Harpenden (British Indicators, Burgess Hill, West Sussex, UK) skinfold calliper gauge as described by Coombe and Bishop (1980). The volumes of the Shiraz berries were calculated using the formula for an ellipsoid ($4/3\pi abc$, where a, b, and c are the semiaxes of the ellipsoid). Another random sample of 50 berries was measured for soluble solids (°Brix) with a refractometer (model 10430; Reichert, Vienna, Austria). Berries for RNA extraction and anthocyanin analysis were randomly sampled every 2 weeks postflowering from randomly selected bunches, deseeded, and immediately frozen in liquid nitrogen. These samples were stored at −80°C pending further analysis. Separate skin and flesh samples were obtained by peeling frozen berries.

Anthocyanin Extraction

To prepare samples for HPLC analysis, 10 to 20 frozen berries were removed from storage and peeled. The peel tissue was ground in liquid nitrogen using a mortar and pestle. A 0.5-g subsample of the tissue was then added to 1 mL of methanol, and the anthocyanins were extracted for 1 h at −20°C. The grape tissue was pelleted by centrifugation at 10,300*g* for 15 min at 4°C, and the supernatant was retained for HPLC analysis. A 5-μL aliquot of this sample was diluted to 1 mL in methanol and 1% (v/v) HCl, and total anthocyanins were measured by reading at A_{520}.

HPLC Analysis of Anthocyanin Extracts

A 5-μm Gold Pack C18 column (4.6 × 25 mm; Activon, Sydney, Australia) and Varian (Melbourne, Australia) equipment consisting of the Vista 5500 pumps and solvent programer, a Rheodyne (Cotati, CA) injector, and a UV-200 detector (Varian, Melbourne, Australia) operating at 520 nm were used. The signal was received and analyzed using a data acquisition, plotting, and analysis package from DAPA Scientific (Kalamunda, Australia), which measured retention times and peak areas. The weak solvent A was 1.4% (v/v) perchloric acid, the strong solvent B was 100% methanol, and solvent C was water. Solvent A was maintained at 30% throughout the analyses, and the flow rate was 1.5 mL/min. The initial condition of solvent B was 20%, increased to 35% in 5 min, and then increased to 55% in 35 min. In all cases 100 μL of extract (see above) were injected.

Isolation of Total RNA

Total RNA was isolated from grape berry skin and flesh tissue using the perchlorate method of Rezaian and Krake (1987) with modifications. A 4-g sample of tissue was removed from −80°C storage and ground to a powder using a coffee grinder. The powder was added to 16 mL of extraction buffer (0.3 M Tris-HCl [pH 8.3], 2% [w/v] PEG 4000, 5 M sodium perchlorate, 1% [w/v] SDS, 8.5% [w/v] PVP, and 1% [v/v] β-mercaptoethanol) and stirred rapidly for 30 min at room temperature. This slurry was then centrifuged at 200*g* for 15 min at 4°C through Centriflo cones (Amicon, Beverly, MA) packed with glass wool, and the "raft" was discarded. The eluate was collected, and nucleic acids were precipitated with 2.5 volumes of ethanol, incubated at −20°C for 20 min, and then pelleted by centrifugation at 7700*g* for 15 min at 4°C. This pellet was rinsed with 70% ethanol, dried under vacuum, and resuspended in 1 mL of 0.1 mM Tris/1 mM EDTA (pH 7.6) and 0.2% (v/v) β-mercaptoethanol. The suspension was then extracted three times with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1, v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol to the aqueous phase and incubating at −20°C for at least 20 min. Finally, the RNA was pelleted by centrifugation at 7700*g* for 15 min at 4°C, washed with 70% ethanol, dried under a vacuum, and resuspended in 300 μL of water.

Northern Blot Analysis

Total RNA was extracted from grape tissues as described above. Aliquots of 4 μg were denatured and fractionated on a 1.2% agarose gel containing 8% formaldehyde. RNA loadings were checked on ethidium bromide-stained gels to confirm that they were equal. The RNA was transferred to a ZetaProbe membrane (Bio-Rad) for at least 15 h and then prehybridized for 2 h at 65°C in 0.25 M sodium phosphate (pH 7.0), 1 mM EDTA (pH 8.0), and 7% (w/v) SDS. Membranes were hybridized for 15 h at 65°C in the same buffer with the addition of denatured ^{32}P-labeled probes of the anthocyanin genes. Probes were prepared by random primer labeling to approximately equal specific activities of at least 3×10^6 cpm ng^{-1} DNA. The membrane was then washed twice for 10 min in 2× SSC (150 mM NaCl and 15 mM tri-sodium citrate, pH 7.0) and 0.1% (w/v) SDS (65°C) and then for 15 min in 1× SSC and 0.1% (w/v) SDS (65°C). The membranes were exposed to Kodak XAE film with intensifying screens at −80°C.

RESULTS

Grape Berry Development

Data from the measurement of various ripening parameters throughout the development of the cv Shiraz berries sampled are presented in Figure 2 and show that berry growth followed the typical double-sigmoid curve. The volume of the berries (Fig. 2A) increased during the first 7 weeks of development to approximately 650 mm^3, followed by a cessation in the berry expansion until 9 weeks postflowering, after which time the volume began to increase again. Berry volume peaked at week 11 (1183 mm^3) and then decreased to a final value of 765 mm^3 at harvest. The onset of ripening is indicated by an increase in softness, sugar content, berry size,

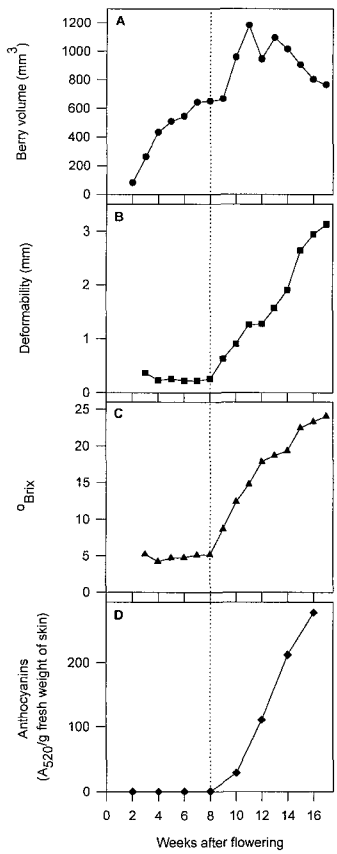

Figure 2. Changes in various parameters measured during the development and ripening of cv Shiraz grape berries. A, Berry volume; B, berry deformability; C, total soluble solids in the berry juice, measured as °Brix; D, total anthocyanins per gram fresh weight of berry skin. The vertical, dotted line represents véraison.

and, in red and black grapes, the development of skin color. Deformability (a measure of berry softness) began to increase 8 weeks postflowering (Fig. 2B). By 16 weeks postflowering the berries were very soft, deforming by 3.1 mm on average. Soluble solids (measured as °Brix) also began to increase 8 weeks postflowering and continued to increase, reaching a value of 24 °Brix 16 weeks postflowering (Fig. 2C). Anthocyanins were first detected in the sample taken 10 weeks postflowering (Fig. 2D), although field observations indicated that some pigmentation was present after 9 weeks (samples for RNA and anthocyanin extraction were only taken on even-numbered weeks). Thus, there seemed to be a slow accumulation of anthocyanins between 9 and 10 weeks postflowering, followed by a substantial increase in anthocyanin levels up to harvest, 16 weeks postflowering. From the data, véraison is considered to occur between 8 and 9 weeks postflowering, and this is indicated by the vertical, dotted line in Figure 2. Although no anthocyanins were detected in the flower sample or in the berry skin samples taken up to 8 weeks postflowering, hot acid extraction, which hydrolyzes unpigmented proanthocyanidins (condensed tannins) into colored anthocyanidin monomers (Harborne, 1989), revealed the presence of both procyanidin and prodelphinidin (data not shown). Hot acid extraction of the berry flesh samples suggested that the 4- and 8-week postflowering samples possessed proanthocyanidins, whereas the 12- and 16-week postflowering samples did not (data not shown).

HPLC Analysis of Anthocyanin Accumulation

HPLC techniques were used to follow the accumulation of the individual anthocyanins present in cv Shiraz berry skin tissue. Seventeen anthocyanins were present in all samples and 16 of these were identified by comparing their retention times and elution order with previous studies of grape and wine anthocyanins (Wulf and Nagel, 1978; Roggero et al., 1986; González-SanJosé et al., 1990). Figure 3 shows a typical elution pattern of cv Shiraz pigments under the HPLC conditions used. The most abundant anthocyanins present in all samples were malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvidin 3-*p*-coumaryl-

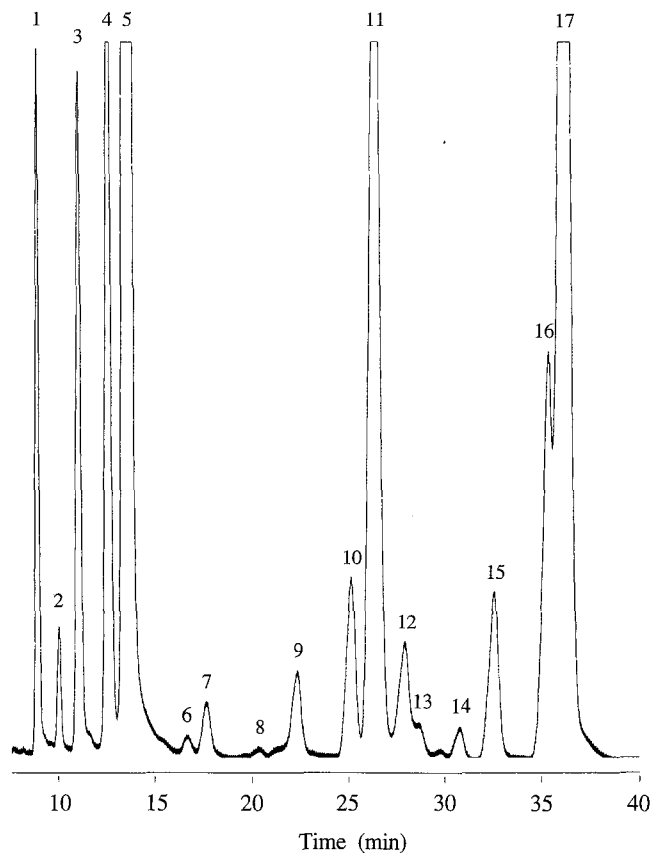

Figure 3. Typical separation of the anthocyanins of cv Shiraz grapes. Peak 1, Delphinidin-3-monoglucoside; peak 2, cyanidin-3-monoglucoside; peak 3, petunidin-3-monoglucoside; peak 4, peonidin-3-monoglucoside; peak 5, malvidin-3-monoglucoside; peak 6, unknown; peak 7, delphinidin-3-acetylglucoside; peak 8, cyanidin-3-acetylglucoside; peak 9, petunidin-3-acetylglucoside; peak 10, peonidin-3-acetylglucoside; peak 11, malvidin-3-acetylglucoside; peak 12, delphinidin-3-*p*-coumarylglucoside; peak 13, malvidin-3-caffeoylglucoside; peak 14, cyanidin-3-*p*-coumarylglucoside; peak 15, petunidin-3-*p*-coumarylglucoside; peak 16, peonidin-3-*p*-coumarylglucoside; peak 17, malvidin-3-*p*-coumarylglucoside.

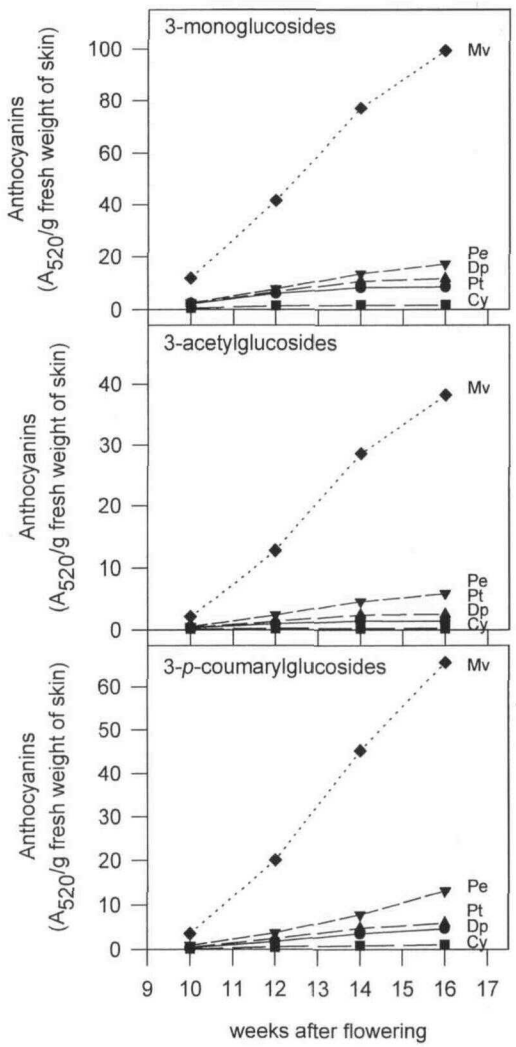

Figure 4. Changes in the total amounts of the major individual anthocyanin species found in grapes during development. The data are grouped as anthocyanin 3-monoglucosides, 3-acetylglucosides, and 3-*p*-coumarylglucosides, as indicated. Cy, Cyanidin; Dp, delphinidin; Mv, malvidin; Pe, peonidin; Pt, petunidin.

glucoside (Fig. 4). Of the peonidin, delphinidin, and petunidin anthocyanins, the 3-monoglucoside derivatives were the major contributors, and there was very little of any cyanidin-based anthocyanins. The malvidin derivatives increased as a percentage of the total anthocyanins from 62 to 73% over the ripening period, whereas the percentage of the other compounds decreased slightly during this time. Although the anthocyanin 3-monoglucosides increased in concentration, their percentage of the total anthocyanins decreased during ripening, probably because of the more rapid rate of accumulation of malvidin 3-acetylglucoside and malvidin 3-*p*-coumarylglucoside. During this time the percentage of total 3-acetylglucosides and 3-*p*-coumarylglucosides increased between 10 and 16 weeks postflowering. Thus, there was an increase in the concentration of anthocyanins throughout ripening of the berries but no major changes in the proportion of the 3′-substituted and 3′,5′-substituted anthocyanins.

Expression of Anthocyanin Biosynthetic Genes in cv Shiraz Berry Skin

The expression of seven anthocyanin biosynthetic genes (PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT; Fig. 1) was investigated in samples taken throughout the development of grape berry skin tissues (Fig. 5). Northern blot analysis indicated that anthocyanin pathway gene expression occurred in two phases. Most genes in the pathway were expressed briefly early in berry development and again after véraison, when color development occurred. All of the anthocyanin genes examined except UFGT were expressed in flowers and in the berry skin up to 4 weeks postflowering. In some cases (PAL, CHS, and LDOX), maximum expression occurred in the flowers, whereas CHI, F3H, and DFR showed maximum expression in the berry sample 2 weeks postflowering. There was then a reduction in expression of these genes 6 to 8 weeks postflowering,

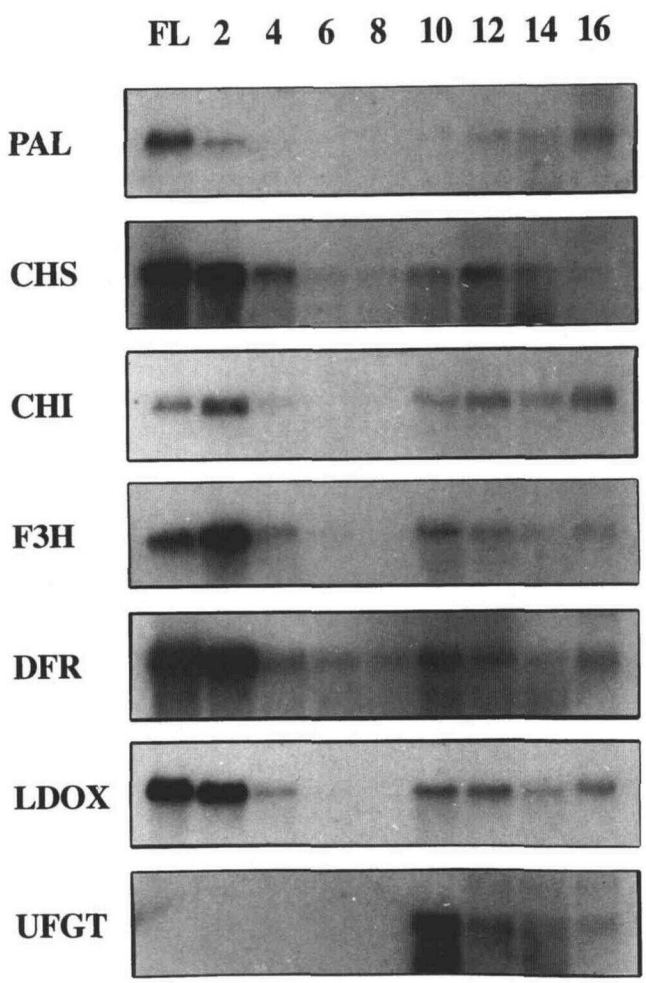

Figure 5. Temporal expression of anthocyanin biosynthetic genes in grape berry skin during berry development. Northern blots are of total RNA (4 μg) from grape flowers and grape berry skin samples taken at eight different stages during development, probed with grape cDNA clones for PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT. FL denotes flower RNA, and the numbers indicate weeks postflowering at which berry skin RNA was extracted. Véraison occurred between 8 and 10 weeks postflowering.

which coincided with the observed lag phase in berry volume increase (Fig. 2A). It should be noted that high-level expression of invertase genes was detected in the same RNA samples used in this study for this specific period of berry development (Davies and Robinson, 1996). This demonstrates, first, that the total RNA samples extracted at these times were intact and, second, that the decline in expression of anthocyanin genes does not reflect a general reduction in mRNA production in grape berry skin at this stage of development. Following this period of little or no expression, there was a coordinate increase in expression of all of the genes except PAL in the 10-week postflowering sample at approximately the time of véraison. Expression of these genes then continued throughout the remainder of berry development. The expression of PAL showed a similar increase following véraison but did not commence until 12 weeks postflowering. Thus, all of the genes of the anthocyanin biosynthetic pathway showed a similar pattern of expression except for the UFGT gene, which was found to be expressed only 10 to 16 weeks postflowering and this expression coincided precisely with the accumulation of anthocyanin pigments in the berry skin (Figs. 2D and 5).

Expression of Anthocyanin Biosynthetic Genes in Shiraz Berry Flesh

The expression of the seven anthocyanin biosynthetic genes was also studied in four cv Shiraz berry flesh samples taken 4, 8, 12, and 16 weeks postflowering (Fig. 6). These tissues contained no detectable anthocyanins (data not shown). No PAL or UFGT mRNA was detected in any of the samples tested. Both CHS and LDOX mRNA levels were at a maximum 4 weeks postflowering and subsequently decreased during development. The other genes tested (CHI, F3H, and DFR) showed maximum mRNA levels 4 weeks postflowering and a minimum expression 4 weeks later. Expression was again detected in the 12- and 16-week postflowering samples but at a lower level than that observed 4 weeks postflowering.

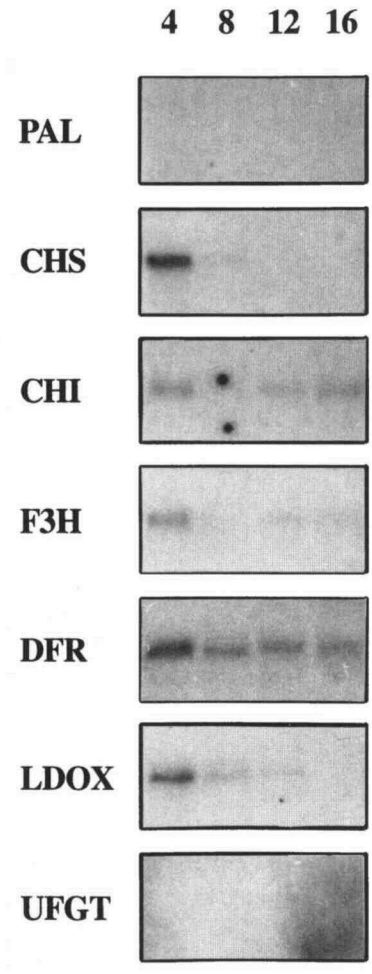

Figure 6. Temporal expression of anthocyanin biosynthetic genes in grape berry flesh tissue during berry development. Northern blots are of total RNA (4 μg) from grape berry flesh samples taken at four different stages during development, probed with grape cDNA clones for PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT. Numbers indicate weeks postflowering at which berry flesh RNA was extracted.

DISCUSSION

The increase in volume of the developing grape berries displayed a double-sigmoid growth curve. This is typified by two periods when berry volume increases, separated by a lag phase of little or no change (Fig. 2A). The véraison at the end of the lag phase, 8 weeks postflowering, is characterized by an increase in the deformability of the berries (Fig. 2B) and an accumulation of both soluble solids and anthocyanins (Fig. 2, C and D).

Predominantly, malvidin-based anthocyanin derivatives accumulated during the ripening period. The concentration of anthocyanins present in the berry skins did not decrease toward the end of ripening, and there was no evidence for the conversion of cyanidin-based anthocyanins to delphinidin derivatives as reported by Roggero et al. (1986). The percentage of delphinidin, petunidin, and malvidin derivatives remained relatively constant during ripening, as did the percentage of cyanidin- and peonidin-derived anthocyanins. So it seems that the rate of flux down either "branch" of the biosynthetic pathway (Fig. 1) is nearly constant throughout ripening.

Northern analysis of the expression of the anthocyanin biosynthetic genes in Shiraz berry skins supports the finding that anthocyanin accumulation continues throughout ripening. All of the genes studied were expressed in samples taken at the four different times after véraison. This suggests that the enzymes of the anthocyanin biosynthetic pathway encoded by these genes are being translated and catalyzing their respective reactions. Surprisingly, we also detected expression of all of these genes, except UFGT, in flowers and in berry skin up to at least 2 weeks postflowering. No anthocyanins could be detected in these samples, presumably because UFGT is missing. It is possible that these flavonoid genes are expressed in tissues not producing flavonoids. Nevertheless, the early committed steps of the pathway depicted in Figure 1 are common to other biosynthetic pathways, notably those involved in the biosynthesis of aurones, flavones, flavonols, isoflavonoids, and proanthocyanidins, and this may explain

expression of these genes in the absence of anthocyanin synthesis in the flowers and young berries. In the floral tissues, it is likely that flavonols are being produced, since they are known to accumulate in the pistils of flowers (Koes et al., 1990) and are essential for pollen viability in maize (Mo et al., 1992). It is also possible that any flavonols produced could be protecting the developing tissues from UV damage (Schmelzer et al., 1988). The young, developing berries could also be producing a number of flavonoid-derived compounds. For example, isoflavonoids (flavonoid derivatives) may play a role in protecting the young berries from various phytopathogens (Lamb et al., 1989) or as a feeding deterrent to insects (Caballero et al., 1986). Young, developing seeds (which may be present in the flower sample) may also accumulate leucoanthocyanidins, as occurs in petunia (Koes et al., 1990). Expression of the anthocyanin genes up to and including DFR would also be necessary for proanthocyanidin production, and these compounds were detected in young berries and flowers. Nevertheless, the observed expression of LDOX in these early samples is puzzling. LDOX is the putative leucoanthocyanidin dioxygenase required for the first of two enzymatic steps between leucoanthocyanidins and anthocyanidins (Martin and Gerats, 1993), the other step probably being catalyzed by a putative dehydratase (Heller and Forkmann, 1988). Any intermediates between these reactions are presumed to be unstable (Heller and Forkmann, 1988), and no colored anthocyanidins were detected.

In Shiraz grape berries anthocyanins accumulate in the skin but not in the flesh. The pattern of expression seen in the berry flesh samples was similar to that in the berry skin, except that neither PAL nor UFGT expression was detected, and CHS was not expressed late in development. Both PAL and CHS are encoded by multigene families in grapes (Sparvoli et al., 1994), and thus, other gene family members, not detected by northern analysis, may be expressed in this tissue. Only one UFGT gene seemed to be present in the grape genome (Sparvoli et al., 1994), and this was not expressed in the berry flesh tissue. The 4- and 8-week postflowering samples possessed proanthocyanidins, whereas the 12- and 16-week postflowering samples did not, and this may indicate that proanthocyanidin production in berry flesh is blocked by the lack of PAL and CHS gene expression in the 12- and 16-week postflowering samples.

Anthocyanins begin to accumulate at about véraison, and this coincides with the increase in expression of all seven genes tested from the anthocyanin biosynthetic pathway. This suggests that there is coordinate regulation of all of these genes at this time in the developing grape berry skin. Sparvoli et al. (1994) have also shown that, as anthocyanins accumulate in dark-grown grape seedlings subsequently exposed to light, there is a coordinate induction of the genes from the committed steps of the anthocyanin biosynthetic pathway (CHS, CHI, F3H, DFR, LDOX, and UFGT). This is similar to the control of the anthocyanin biosynthetic pathway in maize aleurone, which is regulated by the *R* and *C1* gene families (Martin and Gerats, 1993). Nevertheless, the pattern of expression seen in the flower and young berry skin samples prior to véraison suggests that UFGT expression is under a different regulatory regime. In these samples, all of which did not possess anthocyanins, UFGT was the only structural gene tested that was not induced.

The start points for the control of the anthocyanin pathway in the species most studied (maize, antirrhinum, and petunia) are different. In maize aleurone, the regulatory genes *C1* and *R*(S) regulate CHS, DFR, and UFGT gene expression (Dooner and Nelson, 1977; Dooner, 1983; Cone et al., 1986; Ludwig et al., 1989). The F3H activity in the aleurone is also influenced by the *R* gene (Larson, 1989). Thus, it appears that *R* and *C1* may regulate the transcription of all of the anthocyanin biosynthetic genes in the aleurone (Martin and Gerats, 1993). There are several homologs of *R* and *C1*, and these regulate pigmentation in other maize tissues (Ludwig and Wessler, 1990). In snapdragon flowers, mutations in the regulatory gene *delila* have little effect on the expression of CHS and CHI (Almeida et al., 1989), but there is repression of F3H, DFR, LDOX (called *candi*), and UFGT expression in the flower tube (Martin et al., 1991). Studies of mutants of the regulatory genes *eluta* and of two alleles of the *Rosea* locus in snapdragon have shown similar results (Martin and Gerats, 1993). This led Martin and Gerats (1993) to suggest that the "key regulatory point" of the production of anthocyanin compounds in this species is the activity of F3H. There also appears to be a link between genes that are induced during anthocyanin biosynthesis and those under the control of *myc*- and *myb*-like regulatory genes. For example, in snapdragon flowers, F3H, DFR, LDOX, and UFGT are induced in tissue-accumulating anthocyanins, whereas PAL, CHS, and CHI seem to be constitutively expressed (Jackson et al., 1992). The genes induced in these flowers are the same genes that are not expressed in snapdragon regulatory gene mutants (Martin et al., 1991). Quattrocchio et al. (1993) showed a similar link between developmental expression and regulatory gene mutants in petunia. The regulatory genes involved in anthocyanin biosynthesis in petunia flowers have been traced to the loci known as *an1*, *an2*, and *an11* (Beld et al., 1989). Another locus, known as *an4*, controls anthocyanin biosynthesis in petunia anthers (Quattrocchio et al., 1993). The expression of CHS, CHI, and F3H genes in petunia mutated at the *an* loci is not affected. However, there is a reduction in the expression of DFR and UFGT (Beld et al., 1989; Quattrocchio et al., 1993). Thus, in the case of petunia, the major control point seems to be one step further down the pathway than in snapdragon (compare DFR and F3H).

The structural genes from the grape anthocyanin biosynthetic pathway may also be controlled by *myc*- and *myb*-like regulatory genes. However, the way in which the structural genes are regulated in grape berry skins appears to be different from the patterns observed in snapdragon, petunia, and maize. The pattern of gene expression in grape berry skins could be explained in relation to regulatory genes in two ways. First, two types of regulatory genes may be active in the berry skin, one of which is expressed early and that induces expression of all of the structural genes except UFGT, and another that is expressed later and results in the induction of expression of all of the structural genes. Alternatively, two types of regulatory genes may be present, one that controls

expression of PAL, CHS, CHI, F3H, DFR and LDOX and another that induces UFGT gene expression. In this case the regulatory gene that controls expression of PAL, CHS, CHI, F3H, DFR, and LDOX is expressed early in berry development, whereas both the regulatory genes are expressed as the grape ripens, resulting in induction of all of the genes and thus in anthocyanin biosynthesis. In either case, it appears that the major control point to anthocyanin biosynthesis in grape berry skins is UFGT, and this control is later in the pathway than has been observed in the studies of maize, petunia, and snapdragon anthocyanin biosynthesis.

In summary, the appearance of anthocyanins in grape berry skins at the onset of ripening coincides with increased expression of each of the genes encoding biosynthetic enzymes in this pathway. This suggests that the induction of anthocyanin synthesis is triggered by regulatory genes. The isolation of these ripening-specific regulatory genes in grape berries is currently being undertaken to understand more fully the nature of this regulation.

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