

Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries

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Abstract Water deficits consistently promote higher concentrations of anthocyanins in red winegrapes and their wines. However, controversy remains as to whether there is any direct effect on berry metabolism other than inhibition of growth. Early (ED) and late (LD) season water deficits, applied before or after the onset of ripening (veraison), were imposed on field grown *Vitis vinifera* “Cabernet Sauvignon”, and the responses of gene expression in the flavonoid pathway and their corresponding metabolites were determined. ED accelerated sugar accumulation and the onset of anthocyanin synthesis. Both ED and LD increased anthocyanin accumulation after veraison. Expression profiling revealed that the increased anthocyanin accumulation resulted from earlier and greater expression of the genes controlling flux through the anthocyanin biosynthetic pathway, including *F3H*, *DFR*, *UFGT* and *GST*. Increases in total anthocyanins resulted predominantly from an increase of 3′4′5′-hydroxylated forms through the differential regulation of *F3′H* and *F3′5′H*. There were limited effects on proanthocyanidin, other flavonols, and on expression of genes committed to their synthesis. These results demonstrate that manipulation of abiotic stress through applied water deficits not only modulates compositional changes during berry ripening, but also alters the timing of particular aspects of the ripening process.

Keywords Anthocyanin · Berry · Flavonoid ·
Vitis vinifera · Water deficits · Wine

Abbreviations

C	Control(s)
ED	Early deficit
LD	Late deficit
DAA	Days after anthesis

Introduction

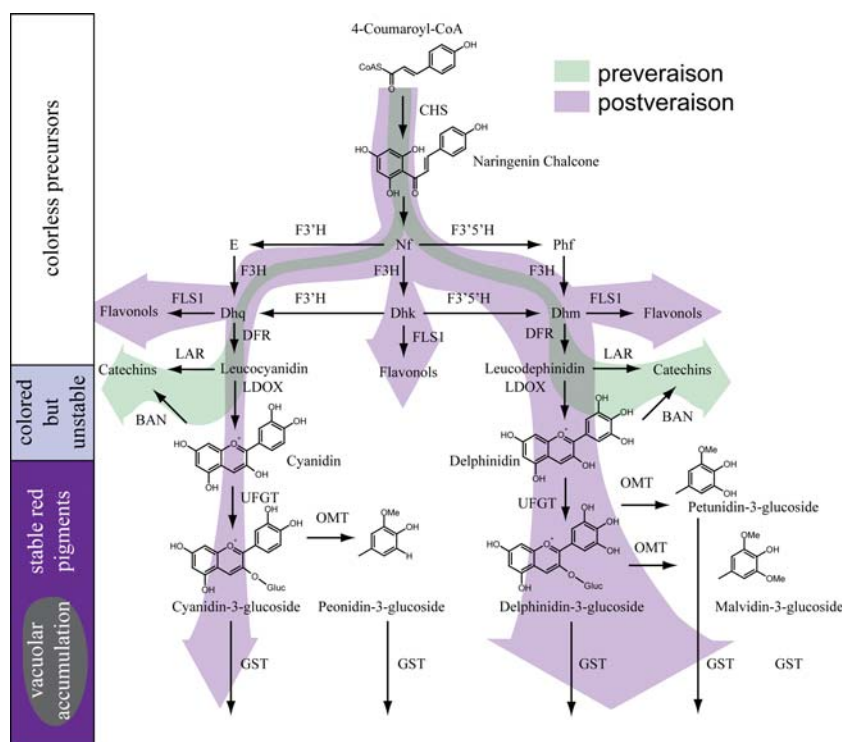
Flavonoids are regarded as one of the most important determinants of quality in red grapes and wines. Color and taste of red wines are strongly related to the amount of anthocyanins, flavonols, and proanthocyanidins. Moreover, in recent years some flavonoid compounds (anthocyanins, proanthocyanidins, and flavonols) have attracted additional attention for their potential health benefits (e.g., Cos et al. 2004; Thole et al. 2006). Vine water status affects the accumulation of flavonoids (Matthews and Anderson 1988; Kennedy et al. 2002), and regulating vine water deficits is considered a tool for increasing flavonoid content and improving quality in red winegrapes (Matthews et al. 1990; Kennedy et al. 2002).

Berry growth follows a double sigmoid habit that can be divided into two growth phases (Stage I and III) separated by a lag phase (Stage II) (Coombe 1976; Matthews et al. 1987b). The transition from Stage II to Stage III is named veraison and is considered to be the onset of ripening, as at this time sugars and anthocyanins begin to accumulate in red winegrapes. Tannins are synthesized before veraison, while anthocyanins are synthesized after veraison. Anthocyanins, proanthocyanidins, and flavonols are synthesized via the flavonoid pathway (Fig. 1) and share several early steps in the pathway. Other enzymes control the branch

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Fig. 1 Summary of changes in the flavonoid pathway during berry development. At veraison flux through the flavonoid pathway shifts from the production of proanthocyanidins preveraison (*green*), to flavonols and anthocyanins postveraison (*purple*)



points of the pathway. For example, flavonol synthases (FLS) lead to the production of flavonols. Downstream of this point, leucoanthocyanidin-reductase (LAR) and anthocyanidin-reductase (BAN) convert leucoanthocyanidins and anthocyanidins into catechin and epicatechin, which form the subunits of skin proanthocyanidins, also known as tannins. The first committed step in anthocyanin biosynthesis is catalyzed by UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT), which catalyzes the glycosylation of unstable anthocyanidin aglycones into pigmented anthocyanins. Two primary anthocyanins (cyanidin and delphinidin) are synthesized by UFGT. Cyanidin has a B-ring dihydroxylated at the 3' and 4' positions whereas delphinidin has a tri-hydroxylated B-ring due to an additional hydroxyl group at the 5' position. Cyanidin and delphinidin are precursors derived from parallel pathways that originate downstream of flavonoid 3'-hydroxylases and flavonoid 3'5'-hydroxylases (Bogs et al. 2006; Castellarin et al. 2006). One or more members of the glutathione *S*-transferase (GST) protein family are believed to participate in vacuolar trafficking and sequestration of anthocyanins in vacuoles (Marrs et al. 1995; Mueller et al. 2000; Kitamura et al. 2004; Terrier et al. 2005; Ageorges et al. 2006).

It remains unclear whether water deficits alter the pertinent biosynthetic pathways or high concentrations arise from the high sensitivity of berry growth to water deficits. Matthews and Anderson (1989) and Matthews et al. (1990) showed that the growth of berries was inhibited more and the concentration of flavonoids in fruit and resultant wine

increased more when water deficits were imposed before veraison than after veraison. Based on the observation of similar flavonoid content per berry between treatments, Kennedy et al. (2002) concluded that postveraison water deficits only inhibited fruit growth. Roby et al. (2004) dissected the effects of berry size and of low vine water status on flavonoid concentration to show that there are effects of vine water status on fruit composition that are independent of the inhibition of berry growth. However, this analysis also indicated that the fruits developed during water deficits had relatively more skin, the predominant tissue of flavonoid biosynthesis, than control fruit. Profiles of gene expression combined with metabolite data should clarify whether or not water deficits differentially promote the biosynthesis of flavonoid compounds. The Kennedy et al. (2002) and the Roby et al. (2004) studies involved a single water deficit treatment that developed after veraison. Therefore, in this study we report the effect of the timing of water deficits on: (1) berry maturation, (2) the accumulation of anthocyanins, proanthocyanidins and flavonols, and (3) the expression of flavonoid pathway genes in berry skin.

Materials and methods

Field plots and physiological measurements

Field experiments were conducted during 2006 in a commercial vineyard of R.H. Phillips Winery in the Dunnigan

Hills American Viticultural Area (Esparto, CA, USA) (38°45'N, 125°57'W). Grapevines of “Cabernet Sauvignon” clone 337 grafted onto rootstock 140R were planted at 2.43 m × 1.83 m spacing (2,242 vines per hectare) in east–west oriented rows and trained to bilateral cordons.

Three irrigation treatments were established. Irrigation was applied to control (C) vines in order to maintain midday leaf water potential (ψ_l) between -0.9 and -1.2 MPa throughout the season. Irrigation was cut off to ED vines from fruit set till two days before the end of veraison (77 days after anthesis, DAA); then vines were irrigated at the C rate till harvest (133 DAA). LD vines were irrigated at the same rate as C vines till 77 DAA when irrigation was cut off. Plant water status was monitored weekly by measuring midday leaf water potential (ψ_l) of fully mature, sun-exposed leaves, as described previously (Matthews et al. 1987a). Water was supplied by an underground drip irrigation system at approximately 50 l per vine per week. No rain occurred after fruit set.

Each treatment was replicated four times in 0.5 ha plots dispersed over a 36 ha vineyard according to a randomized complete block design. Each plot consisted of five rows separated by one buffer row. Samples were collected from ten vines located in the central row of the plots.

Berries were sampled ten times between 27th June (33 DAA) and 5th October (133 DAA), when the grapes were harvested. In order to minimize the effect of cluster exposure to direct sun, and to use the fruits exposed to a uniform diffuse light intensity, berries were collected from the north side of the row and only from the sides of clusters not directly exposed to the sun. Two sets of 20 berries were collected from each plot. The first set was used for measurements of berry weight and soluble solids, whilst the second was used for flavonoid analyses and studies of gene expression. During veraison, additional samples of green berries were collected separately from the same clusters and analyzed separately. Data were then expressed as weighted average.

Metabolite profiling

Samples for soluble solids measurements were weighed and pressed. Total soluble solids were quantified in juice with a hand held refractometer and expressed as °Brix. The corresponding sample of 20 berries was peeled and skins were immediately frozen in liquid nitrogen. After being finely powdered, one aliquot of 0.1 g was used for anthocyanin and flavonol analyses, one aliquot of 0.1 g was used for proanthocyanidin analysis, and one aliquot of 0.3 g was used for RNA extraction.

Anthocyanins and flavonols were extracted in 1/10 (w/v) skin/solvent suspension of 50% methanol in water for 20 min in an Ultrasonic Cleaner bath (Branson 2000). After

centrifugation at $13,000 \times g$ for 15 min, samples were injected into an Agilent 1100 Series HPLC system equipped with a SGE Wakosil II C18 RS column (3 μ m, 150 × 4.6 mm) and a guard SGE Wakosil II C18 RS cartridge. Gradient conditions were the same as reported by Bogs et al. (2005). Total anthocyanins were expressed as mg L^{-1} of malvidin 3-glucoside and included monoglucoside, acetyl-glucoside, and *p*-coumaroyl-glucoside fractions. The composition of monoglucoside anthocyanins was used for the calculation of the proportion of 3'4'5'-OH/3'4'-OH derivatives.

Two major flavonol peaks, corresponding to quercetin-3O-glucuronide and quercetin-3O-glucoside peaks, were identified by comparison of elution time and absorbance spectra with commercial standards (Extrasynthese). The concentration of flavonols was calculated from the integrated absorbance of the two peaks at 353 nm.

Proanthocyanidins were extracted in 1/10 (w/v) skin/solvent suspension of 70% acetone in water (v/v) for 20 min in an Ultrasonic Cleaner bath. After centrifugation at $13,000 \times g$ for 15 min, acetone was evaporated in a Speed Vac Concentrator (Savant) for 1 h. Water was added to obtain the initial volume. Proanthocyanidins were analyzed as reported in Harbertson et al. (2002). Absorbance was determined with a UV-160 spectrophotometer (Shimadzu). A standard curve was prepared using (+)-catechin. Proanthocyanidin values for skin extracts were obtained from the standard curve and reported in catechin equivalents.

Transcript profiling

Total RNA was extracted from 0.3 g of berry skin following the procedure described in Iandolino et al. (2004) and treated with 0.5 U/ μ g RQ1 DNase (Promega). First strand cDNA was synthesized using 2 μ g of RNA, 0.5 μ M (dT)₁₈ primer, and 50 U of M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was carried out in an ABI PRISM 7700 sequence detector (Applied Biosystems). Each reaction (20 μ L) contained 250 nM of each primer, 5 μ L of 1:400 diluted cDNA, and 10 μ L of Power SYBR Green Master Mix (Applied Biosystems). Thermal cycling conditions were 95°C for 10 min followed by 95°C for 30 s, 58°C for 30 s, and 65°C for 60 s for 40 cycles, followed by a melting cycle from 65 to 95°C. Each cDNA sample was run in duplicate. Gene transcripts were quantified upon normalization to *VvUbiquitin1* (TC32075, TIGR database) by comparing the cycle threshold (C_T) of the target gene with that of *VvUbiquitin1* (Bogs et al. 2005). Gene expression was expressed as mean and standard error calculated over all biological and technical replicates. Primers pairs for *DFR*, *LDOX*, and *UFGT* were retrieved from the literature (Goto-Yamamoto et al. 2002; *FLS1* from Downey et al. (2003); *LAR2* from Bogs et al. 2005; *GST* from Terrier

et al. 2005). *F3'H* was amplified using the primer pair coded F3-1 in Castellarin et al. (2006) which amplifies all grape *F3'H* expressed in fruit among those deposited in NCBI (Bogs et al. 2006; Jeong et al. 2006). *F3'5'H* was amplified using the primer pair coded F35-1 in Castellarin et al. (2006) which amplifies the *F3'5'H-1* isogenes of Castellarin et al. (2006) and the *F3'5'H* sequences of Bogs et al. (2006) and Jeong et al. (2006). *F3H* (Sparvoli et al. 1994) and *BAN* (Tanner et al. 2003) primers were newly designed by Castellarin et al. (2007).

Cumulative expression of genes throughout the ripening period was calculated as the area below the expression curves. Cumulative expression of genes of the core flavonoid pathway *F3H*, *DFR* and *LDOX* was calculated from 33 DAA to 133 DAA. The same was done for genes *FLS1*, *BAN* and *LAR2* since flavonols and proanthocyanidin accumulated in the berries prior to veraison. Cumulative transcript levels of genes specific to anthocyanin biosynthesis, *F3'H*, *F3'5'H*, *UFGT*, and *GST*, were analyzed from 63 DAA to 133 DAA; the window of time corresponding to anthocyanin biosynthesis.

Statistical analysis

Differences between the treatments were tested for significance by one way randomized blocks analysis of variance using the COSTAT statistical package (CoHort software, CA, USA) and means were separated using Student–Newman–Keuls test.

Results

Plant water status and berry growth

The irrigation treatments established significant differences in vine water status before and after veraison. Midday ψ_1 of control (C) and LD vines was similar and maintained between -0.9 and -1.1 MPa before veraison (Fig. 2a). Midday ψ_1 in ED vines was consistently around -1.4 MPa during the same period. At 77 DAA, water was supplied to ED vines and taken away from LD vines. As a consequence, ψ_1 in ED vines reached values close to C vines (approx. -1.2 MPa) at 85 DAA and maintained the same water status as C till harvest. After veraison, midday ψ_1 in the LD treatment decreased approximately 0.01 MPa/day, reaching -1.4 MPa by 112 DAA. Vine water status of C vines was maintained near -1.1 MPa after veraison, except for the low value of -1.26 MPa at 112 DAA. Thus, the low water potential of ED vines and LD vines was approximately 0.3 MPa more negative than C vines.

Berry growth in both C and LD vines before veraison was similar and significantly greater than in ED vines

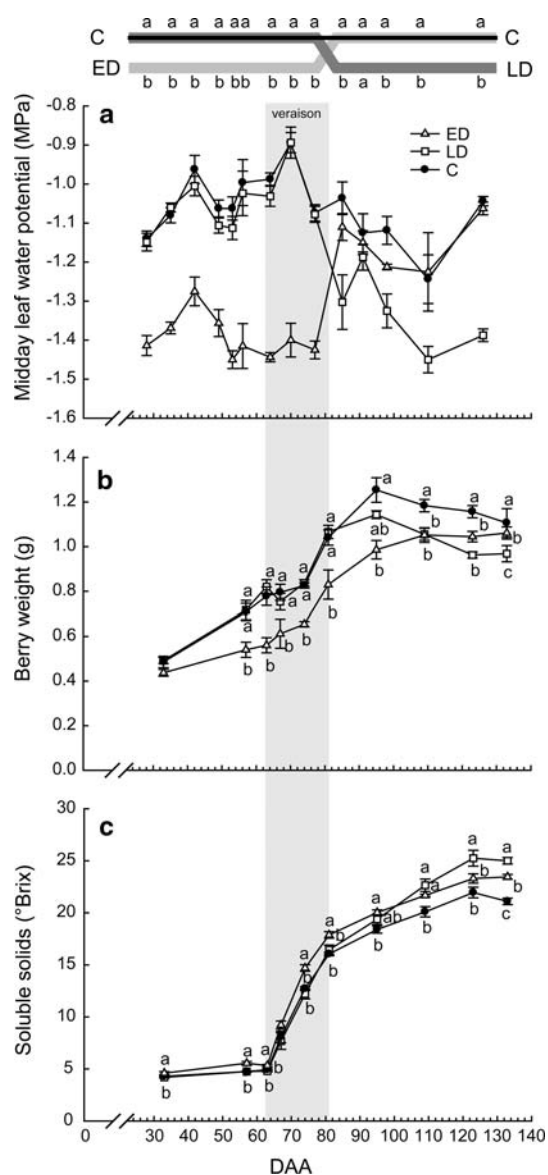


Fig. 2 Midday leaf water potential (a), berry weight (b), and berry soluble solids (c) in Cabernet Sauvignon vines subjected to three irrigation regimes: C control, ED early deficit (deficit imposed before veraison), and LD late deficit (deficit imposed after veraison). Vertical bars indicate \pm SE, Values with different letters indicate significance at $P \leq 0.05$. Grey boxes indicate the period of color transition from 1 to 100% colored berries

(Fig. 2b). Berries on C and LD vines reached maximum size at 96 DAA. At that time, C fruit were largest followed by LD fruit and then ED fruit. Water deficit applied before veraison strongly influenced berry weight throughout ripening. Nevertheless, ED vines resumed berry growth in the last 20 days before harvest and reached the same weight as the controls at harvest. LD caused the maximum reduction in berry size, particularly in the last period of ripening. The C and LD fruit lost about 6 to 8% of maximum fresh weight. Berries in ED vines did not reach maximum size

until 125 DAA, when ED fruit were intermediate in size to C and LD fruit. At harvest berry weight was 1.11, 1.06, 0.969 g in C, ED, LD respectively. Yield per vine was 6.73, 6.48, and 5.62 kg of grapes vine⁻¹, in C, ED, and LD respectively.

The transition from Stage II to Stage III based upon change in berry size was similar (77 DAA) for all treatments (Fig. 2b). The onset of sugar accumulation was also the same in all treatments (63 DAA) (Fig. 2c). The increase in Brix was rapid between 63 and 82 DAA and considerably slower after that. Water deficits influenced sugar concentration, and the influence depended upon the timing of the water deficit (Fig. 2c). Sugar accumulation was faster in ED vines than in C and LD vines between 63 and 82 DAA. Thereafter, increase in Brix was similar in C and ED vines but considerably faster in LD vines. At harvest, Brix values were 21.1, 23.4, and 25°Brix in C, ED, and LD vines, respectively.

However, the same transition based on color change was dependent on vine water status. Color change was accelerated in ED vines. At 67 DAA, 20% of berries on ED plants had already turned red, while no red berries were observed in C and LD treatments. At 74 DAA, 100% of ED berries were red, but only 70% were red in C and LD plants. By 81 DAA, all berries had turned red in all treatments.

Anthocyanin accumulation

Anthocyanin concentration (mg per g FW) and content (total mg per berry) were increased by water deficits. The concentration of anthocyanins was greater in ED vines than in controls at all sample dates except the last one (Fig. 3a). After veraison, the concentration of anthocyanins was also greater in LD vines than in controls at all sample dates except the last one. A similar pattern was exhibited in the anthocyanin content, except there were fewer sample dates on which the higher content in ED and LD vines was statistically significant (Fig. 3b).

ED vines started accumulating anthocyanins earlier than the other treatments; anthocyanins were easily detectable in ED at 67 DAA, while anthocyanin peaks just emerged from the background signal in C and LD berries (Figs. 3a, b). The maximum content of anthocyanins was attained 110 DAA in ED and LD vines, but not until 134 DAA in C vines.

Flavonoid pathway and berry development

The expression of nine structural genes of the flavonoid pathway (*F3H*, *F3'H*, *F3'5'H*, *FLS1*, *LDOX*, *DFR*, *LAR2*, *BAN*, *UFGT*) and of *GST*, thought to play a role in the transport of anthocyanins into the vacuole (Ageorges et al. 2006), were investigated throughout ripening by real-time

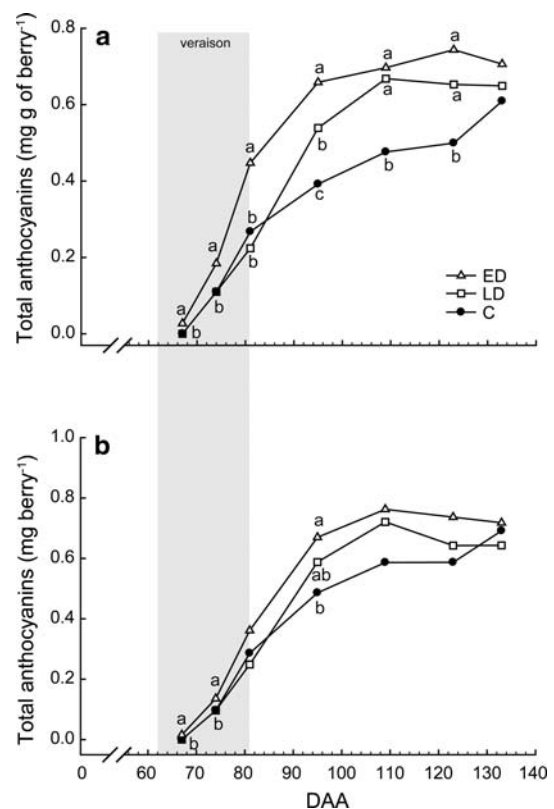
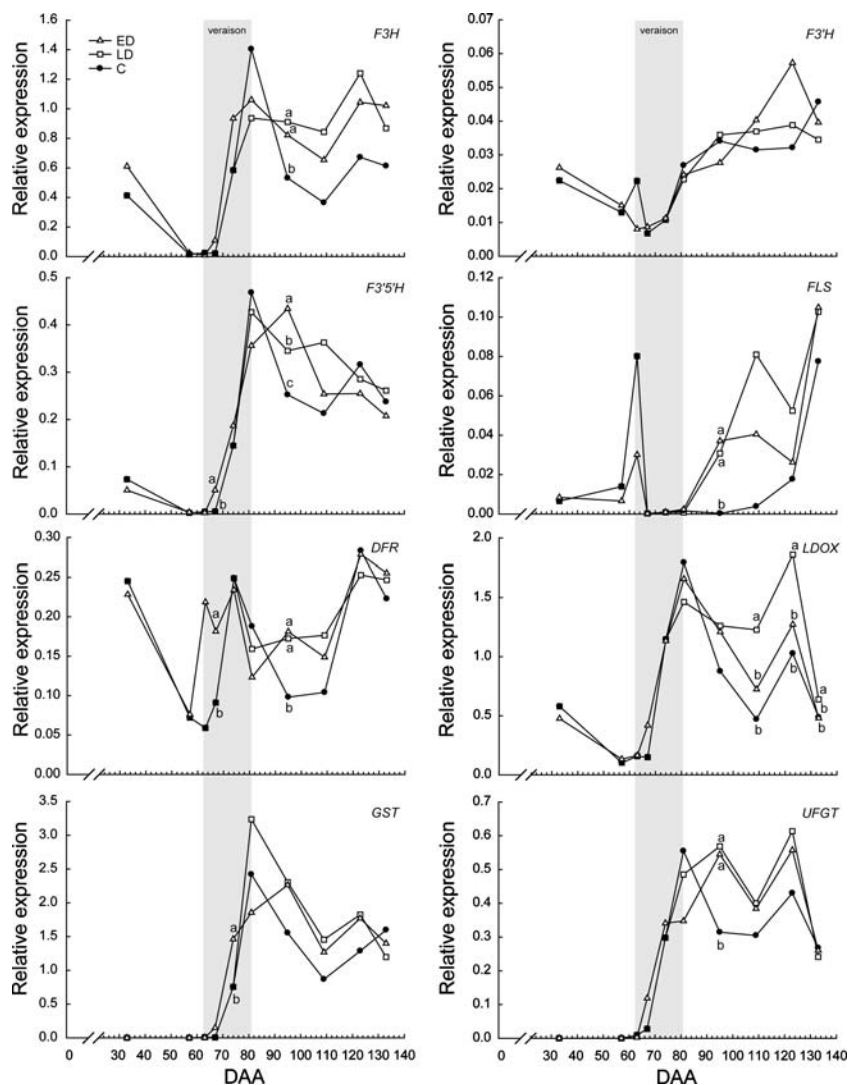


Fig. 3 Total anthocyanins expressed per g of berry (a) and per berry (b) in C, ED, and LD fruit. Values with different letters indicate significance at $P \leq 0.05$. Grey boxes indicate the period of bunch color transition from 1% colored berries to 100% colored berries

RT-PCR. All of these genes were expressed at some time of berry development (Figs. 4, 7). *F3H*, *F3'H*, *F3'5'H*, *DFR* and *LDOX* were all expressed early in berry development and were down-regulated just prior to veraison (Fig. 4). At the onset of veraison a strong increase of transcript level was observed for each of these genes. These increases in expression at veraison were coincident with changes in the color of individual berries. When compared to red skinned berries, expression of all of the above genes was substantially lower or absent in green skinned berries, even those collected at the same time on the same cluster (data not shown).

The expression profiles of most structural genes corresponded with the observed changes in anthocyanin accumulation. The expression of genes common to the biosynthesis of anthocyanins, proanthocyanidins, and flavonols, *F3H*, *F3'5'H*, *LDOX*, and *DFR*, were induced by water deficit. In ED berries their expression was triggered earlier at veraison, and in general, remained greater than C berries for some time after the removal of water deficits (Fig. 4). LD treatment strongly enhanced gene expression in the last 20–30 days before harvest. In most cases expression returned to levels similar to C by 133 DAA.

Fig. 4 Transcript profiling of genes of the flavonoid pathway in berry skins of *C*, *ED*, and *LD* vines. Cumulative expression during ripening, expressed as the area below the curve, can be found in Table 1. Values with different letters indicate significance at $P \leq 0.05$. Grey boxes indicate the period of bunch color transition from 1% colored berries to 100% colored berries



Core flavonoid pathway genes and water deficits

F3H transcripts were clearly enhanced by water deficit in both ED and LD treatments. The expression was triggered at veraison 7 days earlier in ED than in C (Fig. 4). From 67 DAA to harvest, the gene expression was greater in ED than in C berries, except at 81 DAA. A similar pattern of expression was observed for the LD treatment, although for both the treatments, differences with C were statistically significant only at 95 DAA. Transcripts of *F3H* were higher in LD than in ED, from 95 to 123 DAA. By observing the cumulative expression of *F3H* throughout the season, late season water deficits had the greatest impact on *F3H* transcription (Table 1).

Expression of *F3'5'H* encoding the hydroxylase responsible for the hydroxylation of the 5' position on the B ring of the flavonoid skeleton drastically increased at 67 DAA (Fig. 4). Again, expression increased 1 week earlier in ED than C berries. After veraison, up-regulation of *F3'5'H* in

ED persisted for approximately 1 month. Transcript levels were higher in LD in the middle of the ripening period, but the differences were statistically significant only at 95 DAA. Cumulative expression of *F3'5'H* was higher in both deficit treatments compared to C, and was greatest in LD berries (Table 1).

DFR was up-regulated in ED vines at 63 DAA, while in C berries the expression remained low until 74 DAA (Fig. 4). Differences were statistically significant at 67 DAA. Expression was greater in both ED and LD berries throughout the middle of the ripening period; 95, 109 and 133 DAA.

The expression profile of *LDOX* was similar between treatments until the completion of veraison, but from 95 DAA to harvest, *LDOX* expression was higher in LD than in C and ED with statistically significant differences at all sampling points except at 95 DAA (Fig. 4). Cumulative expression was 61.0, 71.5 and 84.2 in C, ED and LD, respectively (Table 1).

Table 1 The cumulative expression of anthocyanin pathway genes throughout development

Gene	Treatment		
	C	ED	LD
<i>F3H</i>	42.57	56.35	57.77
<i>F3'H</i>	0.62	0.55	0.59
<i>F3'5'H</i>	17.43	18.95	20.16
<i>DFR</i>	11.38	12.81	12.89
<i>LDOX</i>	60.96	71.54	84.23
<i>GST</i>	88.02	107.9	119.82
<i>UFGT</i>	23.16	27.45	29.4
<i>BAN</i>	2.44	1.92	2.46
<i>LAR2</i>	5.38	5.72	5.85
<i>FLS</i>	1.37	2.31	3.41

The value given is expressed as the area below each curve given in Figs. 4 and 7

Transcripts of *F3'H* constantly increased in all treatments throughout ripening. The maximum level of the transcripts was reached at harvest for C and ED, whereas in LD berries the peak of expression pointed to 10 days before. Expression patterns in different treatments overlapped, except for 123 DAA when expression in LD berries was greater, but not statistically significant, than in C and ED berries. Cumulative expression of *F3'H* was approximately equal across all treatments (Table 1).

Response of anthocyanin biosynthesis and storage genes to water deficits

Genes strictly related to anthocyanin biosynthesis and storage, *UFGT* and *GST*, had similar patterns of expression (Fig.4). Transcripts were undetectable in green berries, and expression was triggered at the onset of veraison, and as the ripening process progressed, transcript levels greatly increased and remained at high levels. Expression was higher in ED and LD berries, from 95 to 123 DAA, but differences were statistically significant only for *UFGT* at 95 DAA. At 133 DAA, expression of both the genes decreased to C levels. Cumulative expression throughout ripening confirmed a strong up-regulation of *UFGT* and *GST* under water deficit (Table 1).

Since ED berries changed color and began accumulating anthocyanin at 67 DAA, 7 days earlier than C berries, correspondingly these two genes were expressed at a much higher level in ED berries at this date (Fig. 5). Seven days later, C berries began undergoing the same changes, and accordingly *UFGT* and *GST* expression increased, yet ED berries contained higher concentrations of anthocyanins and higher levels of *GST* expression. At 81 DAA, expression of *UFGT* and *GST* in C berries surpassed that of ED

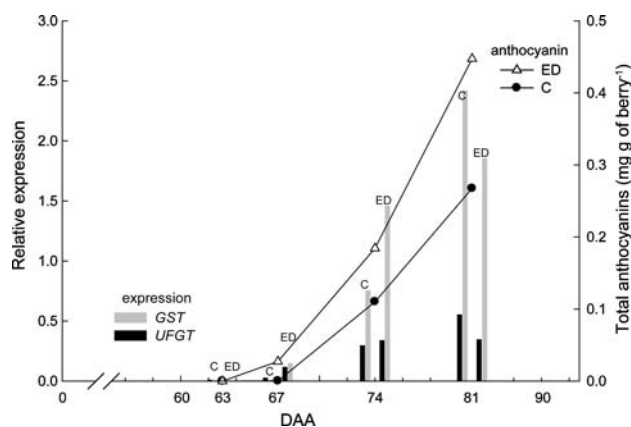


Fig. 5 Summary of anthocyanin accumulation, and *UFGT* and *GST* expression at veraison in berry skins of C and ED vines

berries, although ED berries still contained higher concentrations of anthocyanins. When examined across veraison ED berries accumulated anthocyanins earlier, more rapidly, and to higher concentrations, than C berries, and these increases were paralleled with changes in *UFGT* and *GST* expression.

Regulation of flavonoid hydroxylases and the anthocyanin profile

The relative expression of *F3'5'H* to *F3'H* increased dramatically at 67 DAA remaining high throughout veraison and then decreasing until 133 DAA (Fig. 6a). ED had a higher ratio than C at 67, 74 and 95 DAA, while later during ripening the ratio fell to levels similar to or less than C. In LD berries the *F3'5'H* to *F3'H* expression ratio was higher during the middle of the ripening, from 95 to 109 DAA.

The ratio between tri- and di-hydroxylated anthocyanins reflected the changes observed in the ratio of *F3'5'H* to *F3'H* expression, increasing from 67 to 123 DAA and finally decreasing at harvest (Fig. 6b). All treatments had the same trend but ED berries synthesized a higher percentage of tri-hydroxylated anthocyanins than C berries throughout ripening. These differences were statistically significant at all dates except 133 DAA. In LD berries this ratio was higher than in C berries only in the last phase of ripening, from 123 to 133 DAA. There were no differences between treatments in the concentration of di-hydroxylated anthocyanins; increases in the ratio resulted solely from increases in the tri-hydroxylated forms (data not shown).

Flavonol and proanthocyanidin accumulation

Total flavonols per berry increased in all treatments from the beginning of berry development till the end of ripening, but the concentration of flavonols, whether expressed per

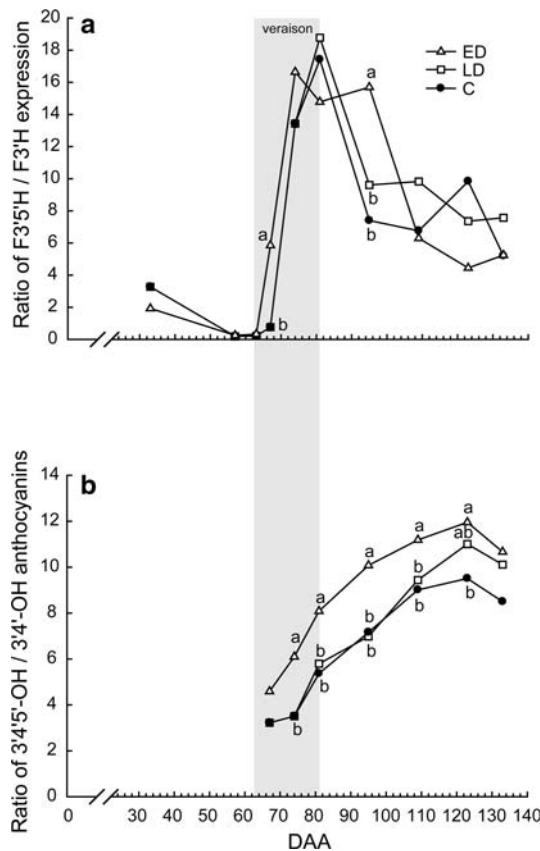


Fig. 6 The ratio of *F3'5'H/F3'H* expression (a) and of 3'4'5'-hydroxylated (3'4'5'-OH)/3'4'-hydroxylated (3'4'-OH) anthocyanins (b) in berry skins of C, ED, and LD vines. Values with different letters indicate significance at $P \leq 0.05$. Grey boxes indicate the period of bunch color transition from 1% colored berries to 100% colored berries

gram of skin or per g of berry, did not change except for a sharp increase during the last 10 days of ripening (data not shown). The flavonol concentration was 19% higher and 20% lower than controls at harvest in ED and LD fruit, respectively. These differences were not statistically significant. *FLS1* transcripts were low for all treatments till the end of veraison (81 DAA), except for a peak of expression that occurred at 63 DAA (Fig. 4). From 95 DAA, *FLS1* expression increased in both ED and LD treatments. In C berries expression did not increase significantly until 109 DAA and did not reach levels equal to those of the water deficit treatments until 133 DAA.

Few significant differences were found in proanthocyanidin concentration under water deficit (Fig. 7). The pattern of proanthocyanidin accumulation was similar in all treatments with a peak of accumulation before veraison. Proanthocyanidin concentrations were slightly greater in ED and LD treatments at most sampling times prior to 133 DAA. *LAR2* and *BAN* expressions were high prior to veraison, decreasing to almost zero by 67 DAA. For the remainder of ripening, transcript levels were extremely low for all

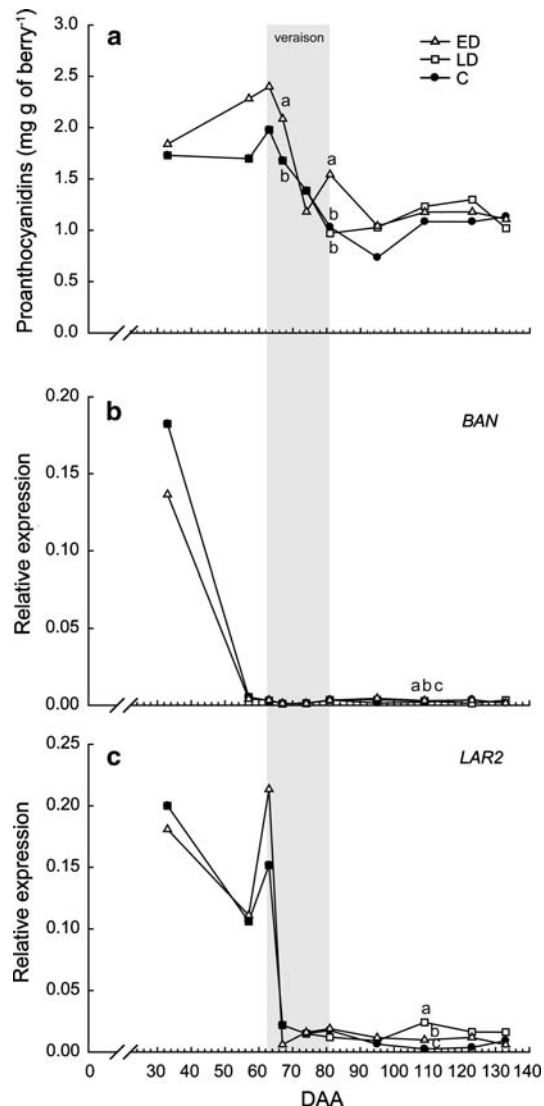


Fig. 7 Proanthocyanidins expressed per g of berry (a) and transcript profiling of *BAN* (b) and *LAR2* (c) expression in berry skins of C, ED, and LD vines. Cumulative expression of *BAN* and *LAR2* during ripening, expressed as the area below the curve, can be found in Table 1. Values with different letters indicate significance at $P \leq 0.05$. Grey boxes indicate the period of bunch color transition from 1% colored berries to 100% colored berries

treatments, although a higher level of *LAR2* and *BAN* was detected in ED and LD berries from 109 DAA onwards. The differences between the treatments were significant only for *BAN* at 109 DAA. The other leucoanthocyanidin-reductase isoform, *LAR1*, was not investigated because it is just barely expressed in skin tissue (Bogs et al. 2005).

Discussion

The results show that water deficits accelerated anthocyanin accumulation, particularly tri-hydroxylated anthocyanins,

and increased the expression of many genes responsible for the biosynthesis of anthocyanins, including *F3H*, *DFR*, *LDOX*, *F3'5'H*, *UFGT*, *GST*. The consequences of early and late deficits were similar in the rapid accumulation of anthocyanins, transient expression pattern that favors tri- versus di-substituted anthocyanins, and limited effects on other flavonoids. The consequences were different in that ED had greater effects than LD, and ED advanced the onset of anthocyanin biosynthesis. The higher amount of anthocyanin per berry in ED fruit and the up-regulation of pertinent biosynthetic genes make clear that biosynthesis was increased and that higher concentrations of anthocyanin in ED fruit was not simply the result of reduced berry growth.

Early water deficits accelerated the shift to anthocyanin biosynthesis and increased the initial rate of sugar accumulation, but water deficits did not accelerate all the changes associated with the onset of ripening. Color change is one of the most universal and easily recognizable aspects of the ripening process. The changes in the flavonoid pathway that bring about this are part of a larger coordinated change in berry development at the onset of ripening that includes growth, softening, and sugar accumulation. Genes strictly related to anthocyanin biosynthesis and storage, *UFGT* and *GST*, were activated earlier in ED berries than in controls along with the core flavonoid pathway genes needed for anthocyanin biosynthesis. The accelerated increase in anthocyanins and anthocyanin-related gene expression caused by ED suggests that water deficits accelerated the onset of ripening, i.e. veraison. The earlier appearance of red berries in the field is consistent with this suggestion.

However, several lines of evidence argue that ED affected anthocyanin synthesis per se and not the suite of changes that comprise ripening. In contrast to the accelerated accumulation of anthocyanins, there was no effect of ED on the onset of Stage III growth, which occurred almost 2 weeks later. Also, the onset of rapid sugar accumulation was coincident in all treatments, although the Brix was higher in ED berries early in development. These same responses to early water deficits, i.e., no change in timing of growth or sugar accumulation but an acceleration of anthocyanin accumulation, were observed in Cabernet Sauvignon (Acevedo et al. 2004) and in Merlot (Castellarin et al. 2007), but no effect on anthocyanins was observed in Shiraz (Ojeda et al. 2002). Also, if the ripening process was accelerated in ED, an earlier down-regulation of the genes responsible for proanthocyanidins would be expected. There was no evidence for acceleration of the loss of *BAN* and *LAR2* expression or of proanthocyanidin accumulation. Thus, in several studies the onset of growth and sugar accumulation aspects of ripening were not advanced by water deficits. Even within grapevine, water deficits reportedly accelerate (Keller 2005) and delay (Sipiora and Granda 1998) fruit maturation.

A more complex story is emerging where control of ripening results from the integration of a variety of signal transduction pathways including those mediated by sugar, abscisic acid (ABA), and brassinosteroids (Symons et al. 2006). Sugar accumulation may be required for anthocyanin biosynthesis in grape berries. When clusters are phloem girdled well before veraison to prevent sugar influx, berries remain firm and green for 8–10 weeks (data not shown). On the other hand, anthocyanin production was induced where it was otherwise not present in a sugar accumulating mutant of *Arabidopsis* (Solfanelli et al. 2006) and cultured grape berry cells when they were exposed to exogenous sugars (Gollop et al. 2002). In the present study, although ED berries accumulated anthocyanins and sugars more rapidly, the relationship between anthocyanin content and Brix was the same for Control and ED fruit early in ripening (Fig. 8). The onset of sugar accumulation preceded anthocyanin accumulation in both the treatments. These observations show that the onset of anthocyanin accumulation was closely coordinated with sugar accumulation, consistent with a sugar-dependent trigger as suggested by Solfanelli et al. (2006), and show sugar and anthocyanin accumulation cannot be uncoupled by water deficit, at least in the first weeks following veraison when most anthocyanin is synthesized.

ABA increases at veraison and is widely used to promote ripening in grapes. For example, exogenous ABA increased expression of *VvmybA1*, expression of related genes for anthocyanin synthesizing enzymes, and the concentration of anthocyanins (Jeong et al. 2004). ABA also activates invertases (Pan et al. 2005) and in grape berries monosaccharides accumulate, not sucrose. A monosaccharide transporter homologue (*VvHT1*, *Vitis vinifera* hexose transporter 1) has been isolated from grape berries at veraison, and its expression was shown to be regulated by sugars and

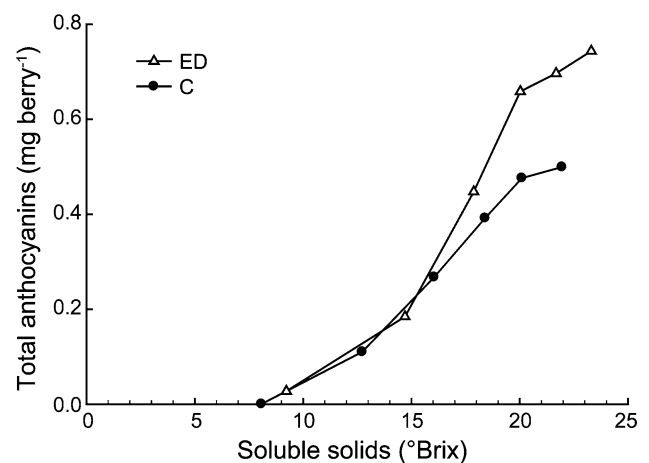


Fig. 8 Relationship of anthocyanin content to sugar concentration during berry development. Data taken from Figs. 2c and 3b

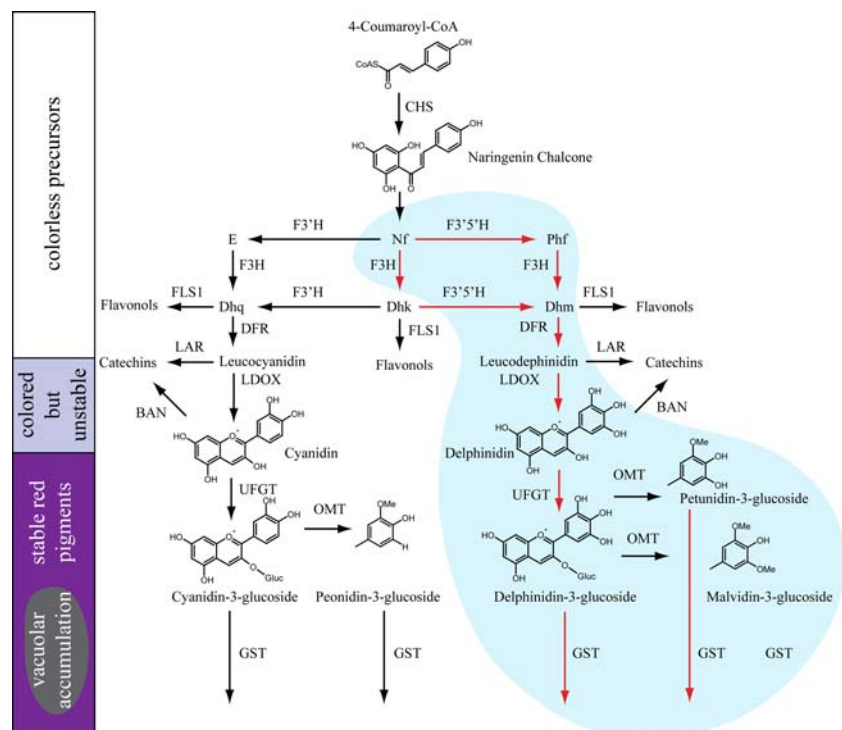
ABA (Cakir et al. 2003). Presumably the hexoses involved are imported as sucrose via the dramatic increase in phloem influx at veraison (Greenspan et al. 1994, 1996). The transition to phloem influx and anthocyanin accumulation is accompanied by a decrease in fruit cell turgor (Thomas et al. 2006) that may be required for sink accumulation of phloem sap (Patrick 1997). Thus, preveraison water deficits, which lower turgor even more (Thomas et al. 2006), may cause increases in sugar influx and ABA, which in turn activate the expression of sugar transporters and anthocyanin pathway genes. However, grape may be unusual in that when ABA-related genes commonly induced by water deficits (*NCED1*, *NCED2*, and *rd22*) were assayed in berry skin, water deficits only transiently increased their expression at veraison (Castellarin et al. 2007).

Water deficits had only a mild effect on proanthocyanidin biosynthesis and accumulation. ED enhanced the expression of *LAR2* before veraison, but these changes were minor. During ripening there were no statistically significant differences in mean proanthocyanidin concentrations, although they were greater in both early and late water deficits and similar in magnitude to differences reported by Ojeda et al. (2002). Kennedy et al. (2002) also showed that proanthocyanidin production is enhanced by water deficit. In this study, the difference in proanthocyanidin concentration essentially disappears when the data are expressed on a per berry basis (data not shown).

The greater effects of water deficits on anthocyanin concentrations than on flavonols and proanthocyanidins in this

study are consistent with the results of Roby et al. (2004) who reported that water deficits caused significant increases in anthocyanins but had less effect on skin tannins. The intermediate effects of LD on anthocyanin accumulation and expression of related genes in this study are similar to previous observations on growth, color, and soluble phenolics (Matthews and Anderson 1988, 1989). However, it is interesting that LD was effective at all given evidences for hydraulic isolation of the berry during ripening (Coombe 1992; Matthews and Shackel 2005). The efficacy of LD in altering expression patterns in the berry suggests that either a hydraulic connection remains present during ripening (Bondada et al. 2005), or a signal is translocated to the fruit via the phloem. It is also interesting that ED effects on gene expression and anthocyanin accumulation were maintained far after vines were released from water deficit. Indeed, ED resulted in changes in gene expression postveraison that were strikingly similar to those changes observed under LD. For example, during ripening, the relative composition of the anthocyanins was modified towards a higher percentage of tri-hydroxylated anthocyanins (Castellarin et al. 2006). Both the early and late water deficits promoted the accumulation of anthocyanins, in particular delphinidin, petunidin, and malvidin particularly the tri-hydroxylated malvidin-, petunidin-, and delphinidin-3-glucosides (Fig. 9). Water deficits increased the biosynthesis of 3'4'5'-hydroxylated anthocyanins in part through increases in *F3'5'H* expression, although mechanisms in addition to changes in gene expression have been shown to influence

Fig. 9 Summary of changes in the flavonoid pathway in response to water deficit. Many flavonoid pathway genes are up-regulated (red arrows) that enhanced the biosynthesis and accumulation of anthocyanins, as well as the change to predominantly tri-hydroxylated forms (blue shading)



anthocyanin composition (Jeong et al. 2006). In this study, the increase in the 3'4'5'-hydroxylated forms accounted for all of the overall increase of total anthocyanins. This demonstrates that even transient exposure to water deficits, and perhaps other environmental cues (Dokoozlian and Kliewer 1996), before the onset of ripening may have profound and prolonged effects on the ripening process.

These results have implications for winegrape production. Water deficits had direct effects on flavonoid gene expression and metabolism. Therefore, differences in fruit composition were not attributable simply to effects of water deficits on berry growth (Roby et al. 2004). Sugars and anthocyanins accumulated more rapidly in ED and LD in comparison to C vines. Maximum anthocyanin content was attained 24 days earlier in ED and LD. Maintaining these fruit on the vine beyond this peak of anthocyanin content resulted in some loss of color. Thus, although water deficits did not advance the onset of ripening, the data suggest that ED and LD accelerated fruit development to maturity, and both LD and ED fruit could be harvested before C fruit and have the same or higher concentrations of sugars, anthocyanins, and proanthocyanidins. The increase in 3'4'5'-hydroxylated anthocyanins constitutes an enrichment of purple/blue pigments, hence modifying grape and must color (Castellarin et al. 2006). This modification puts more of the pigments in moieties that resist oxidation and resultant color change (Sarni et al. 1995).

Finally, fewer significant differences were observed in the expression data than in other assays. There were significant treatment effects on plant water status, berry size, and concentrations of sugars and anthocyanins on essentially every sample date. In contrast, gene expression assays revealed far fewer statistically significant differences. Although mitigated by the observed differences in means maintained over the time course, this contrast cannot be resolved without further work, but has important implications for interpretation in similar studies. We can argue that analysis of gene expression results inherently in more noise than is produced in plant water status, sugar concentration, etc. by the vagaries of the field environment. This may be compounded by the reality that ripening is asynchronous among berries within clusters, especially at veraison, making samples rather heterogeneous. Therefore the variance between biological replicates may reflect this biological heterogeneity. More rather than less replication may be important for accurate insight into the responses of gene expression to environmental conditions in the field.

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