Grapes on Steroids. Brassinosteroids Are Involved in Grape Berry Ripening[1]

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Fruit ripening is a unique plant developmental process with direct implications for our food supply, nutrition, and health. In contrast to climacteric fruit, where ethylene is pivotal, the hormonal control of ripening in nonclimacteric fruit, such as grape (*Vitis vinifera*), is poorly understood. Brassinosteroids (BRs) are steroidal hormones, essential for normal plant growth and development but not previously implicated in the ripening of nonclimacteric fruit. Here we show that increases in endogenous BR levels, but not indole-3-acetic acid (IAA) or GA levels, are associated with ripening in grapes. Putative grape homologs of genes encoding BR biosynthesis enzymes (*BRASSINOSTEROID-6-OXIDASE* and *DWARF1*) and the BR receptor (*BRASSINOSTEROID INSENSITIVE 1*) were isolated, and the function of the grape *BRASSINOSTEROID-6-OXIDASE* gene was confirmed by transgenic complementation of the tomato (*Lycopersicon esculentum*) *extreme dwarf* (d^x/d^x) mutant. Expression analysis of these genes during berry development revealed transcript accumulation patterns that were consistent with a dramatic increase in endogenous BR levels observed at the onset of fruit ripening. Furthermore, we show that application of BRs to grape berries significantly promoted ripening, while brassinazole, an inhibitor of BR biosynthesis, significantly delayed fruit ripening. These results provide evidence that changes in endogenous BR levels influence this key developmental process. This may provide a significant insight into the mechanism controlling ripening in grapes, which has direct implications for the logistics of grape production and down-stream processing.

Grapes (*Vitis vinifera*) and their processed products, wine, grape juice, and dried fruit, are economically and culturally important on a global scale. Of crucial importance to these industries is the timing and extent of grape berry ripening. The timing of commercial ripeness impacts on marketing of fresh fruit, drying of dried fruit, and on winery logistics. The extent of ripening can also greatly modify grape quality and wine style.

Grape berry development follows a typical double-sigmoid pattern, characterized by two phases of rapid growth separated by a lag phase, during which little or no growth occurs (Coombe and Hale, 1973). The onset of ripening (named véraison by viticulturists) occurs at the end of this lag phase and involves the accumulation of hexoses in berry vacuoles and colored anthocyanins in the berry skin, catabolism of organic acids, an increase in berry softness, and the development of compounds involved in flavor and aroma (Seymour et al., 1993). Despite the obvious economic implications of controlling these processes and a considerable research effort aimed at achieving this goal, the mechanisms that regulate grape berry ripening remain unclear.

Fruit species are categorized as either climacteric or nonclimacteric, based on physiological differences in their ripening patterns. While climacteric fruit such as tomato (*Lycopersicon esculentum*), apple (*Malus domestica*), and banana (*Musa* spp.) undergo a well-characterized peak in ethylene production and respiratory activity at the onset of ripening, little is known about the hormonal control of ripening in nonclimacteric fruits such as citrus and grape (Seymour et al., 1993; Adams-Phillips et al., 2004). By definition there is no major peak in ethylene levels or respiration during ripening of nonclimacteric fruit (Seymour et al., 1993), and early data showing only modest changes in ethylene levels at the onset of ripening in grapes formed the basis for its classification as a nonclimacteric species (Coombe and Hale, 1973). However, recent evidence of a transient increase in endogenous ethylene levels prior to véraison suggests that ethylene may play some role during grape berry development (Chervin et al., 2004).

Other classical plant hormones, such as auxin (indole-3-acetic acid [IAA]) and abscisic acid (ABA), have also been implicated in the control of ripening of grape berries (Seymour et al., 1993; Davies et al., 1997).

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Endogenous ABA levels have been shown to increase after véraison, and treatments that delay this increase delay the onset of ripening (Davies et al., 1997). For instance, application of the synthetic auxin-like compound benzothiazole-2-oxyacetic acid delays both the increase in endogenous ABA levels and ripening (Davies et al., 1997), suggesting that both endogenous auxins and ABA may play a role in the control of ripening in grapes. However, very little is known about the possible role of other key plant growth regulators.

Brassinosteroids (BRs) are a group of steroidal plant hormones that are essential for normal plant development (Clouse and Sasse, 1998). Extensive research over the past two decades has revealed the importance of BRs in numerous processes, including cell elongation, cell division, vascular differentiation, reproductive development, and pathogen and abiotic tolerance (Clouse, 2002). Although some data suggest that exogenous BRs may promote ripening (via increases in ethylene levels) in tomato (a climacteric fruit; Vardhini and Rao, 2002), BRs have not previously been implicated in the control of ripening in any nonclimacteric fruit.

We have isolated several key grape genes encoding BR biosynthesis enzymes *BRASSINOSTEROID-6-OXIDASE* (*VvBR6OX1*) and *DWARF1* (*VvDWF1*) and the BR receptor *BRASSINOSTEROID INSENSITIVE 1* (*VvBRI1*), and confirmed the function of the grape *VvBR6OX1* gene by transgenic complementation of the tomato *extreme dwarf* (d^x/d^x) mutant. Our survey of gene expression and plant hormone levels throughout grape (cv Cabernet Sauvignon) berry development indicates that BR levels may influence the process of berry ripening. We demonstrate a clear pattern of changes in the expression of genes controlling BR synthesis and endogenous BR levels, which coincide with the onset of ripening. Furthermore we show that the manipulation of BR levels via the application of exogenous BR and a BR biosynthesis inhibitor can significantly promote or delay berry ripening. The importance of these results for the control of ripening in grapes, and nonclimacteric fruit in general, is discussed.

RESULTS

Physical Changes during Grape Berry Development

Development of the Cabernet Sauvignon berries throughout the growing season followed a typical double-sigmoid growth pattern (Coombe and Hale, 1973) with a phase of rapid growth during the 4 weeks after flowering, followed by a lag phase between 4 and 8 weeks post flowering (wpf; Fig. 1A). The onset of ripening (véraison) is marked by a rapid increase in berry weight from 8 wpf onwards. This change also coincided with a sharp increase in the amount of soluble solids (°Brix) that occurred between 8 and 10 wpf (Fig. 1A).

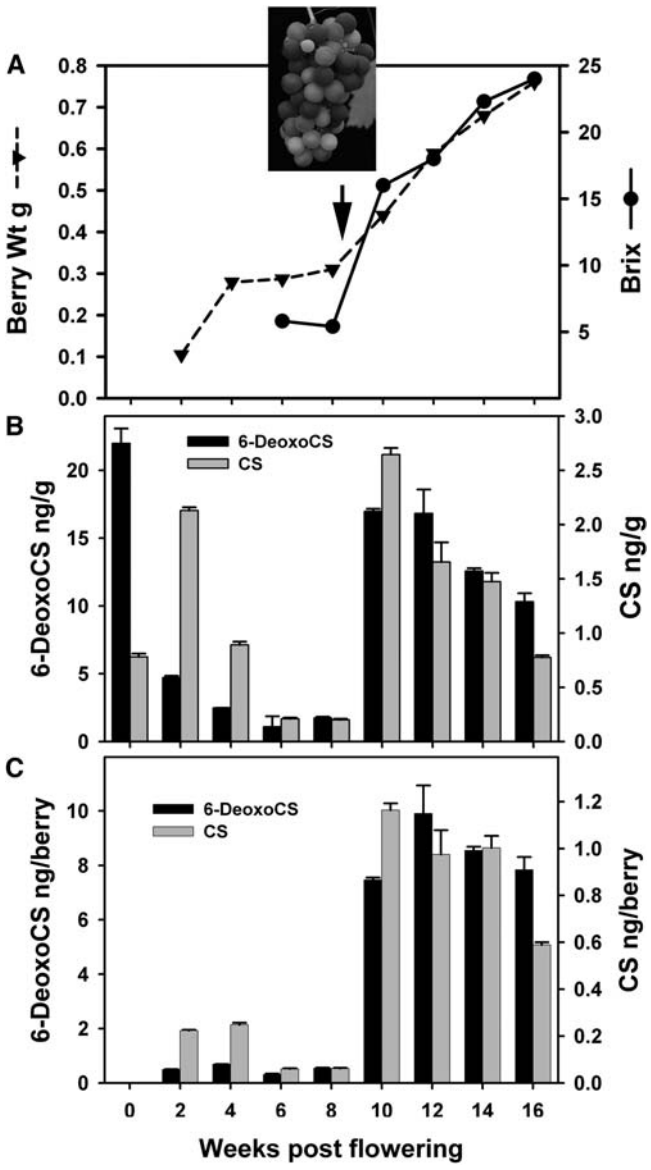

Figure 1. Changes in BR levels during grape berry development. A, The pattern of grape berry development as described by berry weight and total soluble solids (°Brix). Values presented are averages calculated by dividing the total berry weight and total soluble solids by the number of berries (between 50 and 100) sampled at each time point. The inset photograph shows a grape bunch in the early stages of ripening. B, Levels (ng g^{-1}) of 6-DeoxoCS and CS in developing grape berries. C, Levels (ng $berry^{-1}$) of 6-DeoxoCS and CS in developing grape berries. Values presented in B and C represent the mean ± SE derived from two individual replicates.

Endogenous BR Levels during Grape Berry Development

A clear pattern of changes in the endogenous BR levels was evident throughout grape berry development (Fig. 1). Levels (ng g^{-1} fresh weight) of the bioactive BR castasterone (CS) were elevated in flowers and during early berry development (2 wpf) but decreased markedly after this stage, reaching a minimum (10-fold lower) by 6 wpf (Figs. 1B and 2). CS levels remained low between 6 and 8 wpf (Fig. 1B). However, we observed a dramatic increase in the levels of CS and

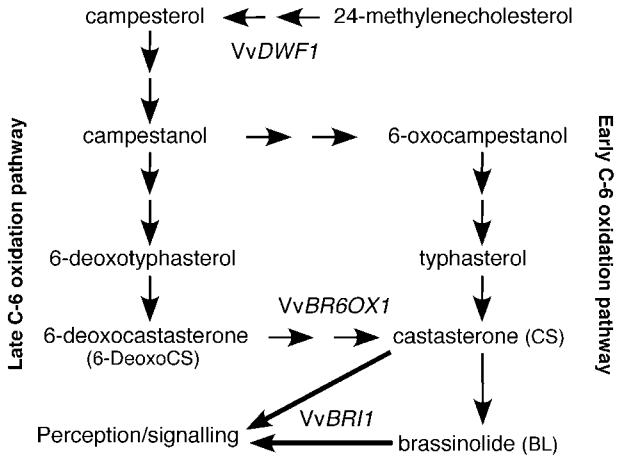

Figure 2. Stylized diagram of the BR biosynthesis pathway (after Bishop and Yokota, 2001), showing the alternative early and late C-6 oxidation pathways and the grape genes whose expression is described in Figure 4.

its direct precursor 6-deoxocastasterone (6-DeoxoCS; Fig. 2; 13- and 9-fold, respectively) between 8 and 10 wpf (Fig. 1B). This change coincided with the onset of ripening, as indicated by the increase in berry weight and soluble solids (°Brix), which also occurred at this time (Fig. 1A).

Typhasterol (an alternative precursor via CS in the early C-6-oxidation pathway; Fig. 2) levels were measured at 0.21, 0.29, and 0.42 ng g^{-1} at 0, 2, and 4 wpf, respectively, but remained below detection limits after 4 wpf. The most bioactive BR, brassinolide (BL; Wang et al., 2001), was not detected at any stage of grape berry development. The profile of endogenous BR levels observed in grape berries was similar to that found in grape internode tissues, where CS, 6-DeoxoCS, and typhasterol levels were 1.07, 5.96, and 0.28 ng g^{-1} (fresh weight), respectively. Once again, BL was not detected in grape internode tissues.

The reason for the observed decrease in concentration of CS (and 6-DeoxoCS) that occurs in the early stages of berry development (Fig. 1B) may be, at least in part, due to the concomitant increase in berry size (Fig. 1A) rather than to net catabolism. When expressed on a per berry basis there is little change in the amount of CS and 6-DeoxoCS between 2 and 8 wpf (Fig. 1C).

Endogenous IAA and GA Levels during Grape Berry Development

In contrast to the changes in BR levels (Fig. 1, B and C), there appeared to be no dramatic change in IAA and GA_1 levels that coincided with the various stages of berry development (Fig. 1A; Table I). Indeed, endogenous IAA levels remained relatively unchanged throughout berry development (Table I). Similarly, while the level of the bioactive GA, GA_1, was relatively high in the flowers (0 wpf), it had decreased dramatically by 2 wpf and remained low and unchanged throughout subsequent berry development (Table I). Another bioactive GA in grape, GA_4 (Boss and Thomas, 2002; Boss et al., 2003), was not detected in grape berry tissues.

Effects of Exogenous BR and Brassinazole on Grape Berry Ripening

The relationship between the endogenous increase in bioactive BR levels and the onset of ripening was further examined by studying the effects of exogenous BR and a BR biosynthesis inhibitor on this process in field-grown grape berries. Using the first appearance of coloring (anthocyanin production) in the berry skin as an indicator for the onset of ripening, we showed that the application of exogenous epi-BL significantly promoted véraison, while the application of the BR synthesis inhibitor, brassinazole (Brz), significantly delayed véraison (Fig. 3; overall $P < 0.001$ at 19 d post first treatment). This effect was observed even though treatments occurred at only four time points over an 18-d period, and involved a noninvasive application of relatively low levels of these compounds. Total soluble solids (°Brix), measured in berries 28 d after the first treatment (* in Fig. 3), were 12.7, 13.4, and 11.7 for control, epi-BL-treated, and Brz-treated fruit, respectively, indicating that BRs also influence sugar accumulation.

Isolation and Functional Analysis of *VvBR6OX1*

In contrast to the situation in tomato and Arabidopsis (*Arabidopsis thaliana*; Kim et al., 2005; Nomura et al., 2005), there was no evidence for the presence of more than one *BR6OX* gene in grape. Seven grape expressed sequence tag sequences (from the National Center for

Table I. *Endogenous IAA and GA_1 levels (ng g^{-1} fresh weight) in deseeded grape berries during berry development*

Values represent the mean ± SE of hormone levels obtained from two individual replicates (tissue samples were the same as those used for BR analysis).

Hormone Assayed	Weeks Post Flowering					
	0	2	4	8	12	16
IAA level	**5.3**[a] ± 0.6	**3.6** ± 0.8	**4.2** ± 0.5	**5.1** ± 0.1	**2.5** ± 0.07	**5.2** ± 0.3
GA_1 level	**8.67** ± 0.51	**0.08** ± 0.02	**0.05**[b]	**0.05**[b]	**0.04** ± 0	n.d.[c]

[a]Bold indicates mean throughout. [b]Indicates the value was obtained from one replicate only. [c]Not detected.

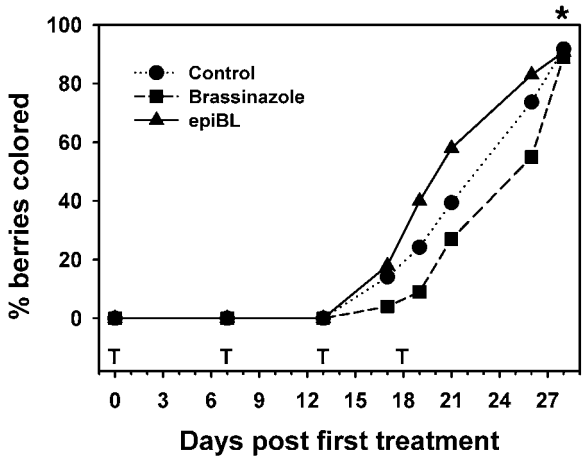

Figure 3. The effects of exogenous epi-BL and Brz (an inhibitor of BR biosynthesis) on grape berry ripening. Berries were treated with these exogenous compounds at 0, 7, 13, and 18 d post the first treatment (indicated by the T symbol). Percent colored berries were calculated for each observation time by counting the numbers of berries showing any degree of color development. The asterisk indicates the time of harvest (28 d post the initial treatment) when total soluble solids were measured.

Biotechnology Information database) were identified as homologs of the tomato *DWARF* gene. All seven grape expressed sequence tags (two from prevéraison berries, four from pedicles, and one from leaves) had identical sequences, which is suggestive of a single gene.

A full-length *VvBR6OX1* clone was isolated from a Cabernet Sauvignon prevéraison berry cDNA library. The sequence of the clone was 1,558 bp in length and contains a putative open reading frame of 460 amino acid residues. The putative protein was 81.3% identical (at the amino acid level) to that of the tomato *DWARF* gene, indicating that it is likely to be a grape *DWARF* homolog (*VvBR6OX1*). Analysis of the phylogenetic relationship of VvBR6OX1 to other plant P450 proteins shows that it is most closely related to the BR 6-oxidases from Arabidopsis, tomato, and rice (*Oryza sativa*) and distinct from more distantly related proteins involved in other reactions in the BR biosynthesis pathway (Fig. 4). This suggests that VvBR6OX1 has BR 6-oxidase activity and most likely converts 6-DeoxoCS to CS.

The function of the grape *VvBR6OX1* gene was confirmed by transgenic complementation of the tomato d^x/d^x mutant that lacks a functional endogenous *DWARF* (*CYP85A1*) gene (Fig. 5). This indicates that *VvBR6OX1* encodes a functional BR 6-oxidase that catalyses the conversion of 6-DeoxoCS to CS (Kim et al., 2005; Nomura et al., 2005). While the *35S::VvBR6OX1* transgene clearly restored the phenotype of the tomato dwarf mutant, minor leaf phenotypic differences were sometimes observed between the transgenic and wild-type tomato plants (Fig. 5). This is likely to be a consequence of the constitutive expression of the *VvBR6OX1* gene by the 35S promoter at higher levels or at different sites compared to the normal expression pattern of the endogenous *LeDWARF* gene in wild-type tomato plants.

Cloning of Putative Grape *DWF1* and *BRI1* Homologs

Two cDNA clones with similarity to *DWF1* homologs from other species were sequenced in full, and, although neither contained the entire open reading frame, the longest clone contained the 3′ end of the transcript and an open reading frame of 152 amino acids, which had an 84.9% identity, at the amino acid level, to the *DWF1* homolog from pea (*Pisum sativum*; gi 13194620; Schultz et al., 2001). This gene was therefore assigned as a putative grape *DWF1* homolog (*VvDWF1*).

A fragment of the putative *VvBRI1* homolog was cloned from Cabernet Sauvignon berries by PCR. This fragment, from the 3′ part of the gene, contained an open reading frame of 401 amino acids and was 91.3% identical (at the amino acid level) to the *BRI* homolog from pea (accession no. AB104529; Nomura et al., 2003). This gene was therefore assigned as a putative grape *BRI1* homolog (*VvBRI1*).

Expression Analysis of *VvBR6OX1*, *VvDWF1*, and *VvBRI1* during Grape Berry Development

We examined the expression of the grape *DWARF* homolog (*VvBR6OX1*) and the putative grape *DWF1* and *BRI1* homologs (*VvDWF1* and *VvBRI1*, respectively), during grape berry development. The expression

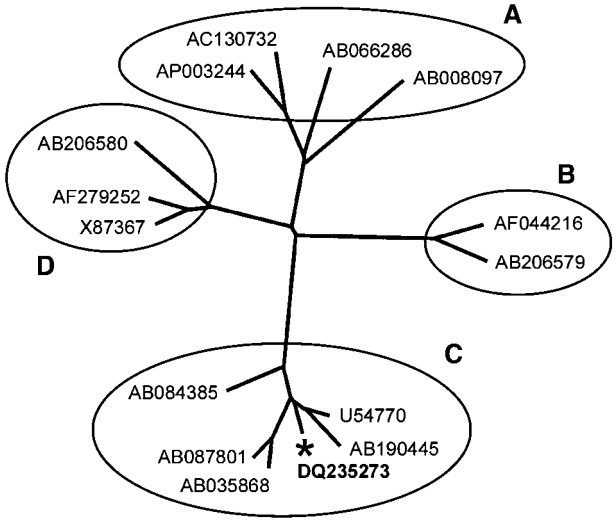

Figure 4. Phylogenetic relationships between VvBR6OX1 and related P450s. The unrooted phylogenetic tree is based on multiple sequence alignment of the full-length, deduced protein sequences. Asterisk (*) denotes the location of the grape VvBR6OX1 protein (DQ235273). Putative functional groupings are indicated on the diagram. A, Late-BR synthesis enzymes (including BR 3-oxidases and BR 2-hydroxylases): AB008097 (Arabidopsis, CYP90C1), AB066286 (Arabidopsis, CYP90D1), AP003244 (rice, CYP90D2), and AC130732 (rice, CYP90D3). B, BR 22α-hydroxylases: AB206579 (rice, CYP90B1) and AF044216 (Arabidopsis, CYP90B1). C, BR 6-oxidases: DQ235273 (VvBR6OX1), U54770 (tomato, CYP85A1), AB190445 (tomato, CYP85A3), AB035868 (Arabidopsis, CYP85A1), AB087801 (Arabidopsis, CYP85A2), and AB084385 (rice, CYP85A1). D, BR 23α-hydroxylases: X87367 (Arabidopsis, CYP90A1), AF279252 (mung bean [*Vigna radiata*], CYP90A2), and AB206580 (rice, CYP90A1).

Figure 5. Transgenic complementation of tomato d^x mutant by the grape *VvBR6OX1* gene. A, Ailsa Craig (wild type); B, Dwarf mutant (d^x/d^x); C, Dwarf tomato plant (T_0) transformed with *35S::VvBR6OX1* transgene.

patterns observed (Fig. 6) can be related to the observed changes in endogenous BR levels (Fig. 1B). For instance, increases in endogenous BR levels coincided with an increase in the transcript levels of *VvDWF1* and *VvBRI1*, between 8 and 10 wpf (Figs. 1, B and C, and 6, A and C). In contrast, *VvBR6OX1* expression, which increased steadily from flowering, was dramatically down-regulated during this period and was negatively correlated ($r = -0.79$, $P < 0.01$) with endogenous 6-DeoxoCS levels (Figs. 1, B and C, and 6B).

DISCUSSION

Endogenous BR Levels Influence the Onset of Grape Berry Ripening

While ethylene is known to be pivotal in the regulation of ripening in climacteric fruits (Seymour et al., 1993), our understanding of the hormonal regulation of ripening in nonclimacteric fruits (such as grape) is less clear. In this study we provide evidence that steroidal plant hormones (BRs) may play an important role in the ripening of grape berries. In particular, we observed a dramatic 13-fold increase in the level of a bioactive BR, CS, which coincided with the onset of berry ripening (Fig. 1). Expression analysis of grape genes involved in BR biosynthesis (*VvBR6OX1*, *VvDWF1*) and BR response (*VvBRI1*) during berry development revealed transcript accumulation patterns that were consistent with this increase in endogenous BR levels (Figs. 1, 2, and 6). In contrast, the endogenous levels of two other plant hormones, IAA and GA_1, remained relatively unchanged throughout berry development (Table I). IAA had previously been reported to be elevated early in berry development (in Concord, a *Vitis labrusca* cultivar) and to then decline to low levels as development progressed (Cawthon and Morris, 1982). The difference between these data and those presented here may be due to either the method of measurement or to species differences. As the levels of IAA in grape cultivar Cabernet Sauvignon did not appear to change throughout berry development, it may suggest that the inhibition of ripening initiation, previously shown to occur with the exogenous application of a synthetic auxin-like substance (Davies et al., 1997), may not accurately reflect the role of endogenous auxin in normal berry development. However, both the magnitude and timing of the increase in BR levels is suggestive of a role for BRs in the regulation of ripening in grape berries.

The link between BRs and the onset of ripening was further investigated by examining the effects of altered BR levels on this process. We showed that in field-grown grapes, application of exogenous epi-BL to individual berries significantly promoted ripening, while application of Brz, an inhibitor of BR biosynthesis, significantly delayed fruit ripening (Fig. 3). While the magnitude of these changes was not large, they represent a substantial change given the inherent

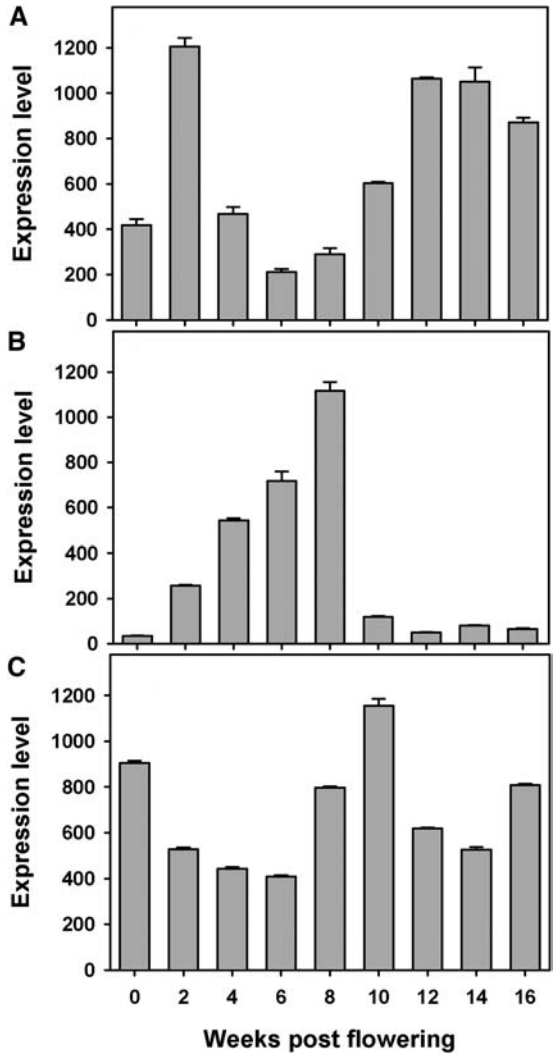

Figure 6. Relative transcript levels of three genes involved in BR biosynthesis and perception during grape berry development. Expression levels for *VvDWF1* (A), *VvBR6OX1* (B), and *VvBRI1* (C) were determined by real-time PCR analysis.

problems associated with hormone application studies, such as unpredictable uptake of the compound by the target tissues, and the small treatment exposure involving only four-point applications of epi-BL and Brz, spaced over 18 d (Fig. 3). The effects of point applications of both Brz and BRs to intact plant systems are known to be localized and/or relatively short lived (Symons and Reid, 2004). For instance, when Brz was applied directly to pea seeds (Symons et al., 2002), it only affected the growth of the first few internodes, while paclobutrazol (a GA synthesis inhibitor) applied in the same way inhibited the growth of at least eight successive internodes (Potts et al., 1985). Similarly, when BL is applied to the BR-deficient *lkb* mutant, it restores the expansion of only one to two internodes, while GA applied to a GA-deficient mutant promotes the growth of at least nine successive internodes (Potts et al., 1985). Unfortunately, a grape mutant with altered BR biosynthesis is currently not available to investigate the effects of a permanent change in endogenous BR levels on grape berry ripening.

The effects of the epi-BL and Brz treatments were evident in both the appearance of skin coloration and the final sugar levels in ripe berries. Thus, BRs affected indicators of ripening in different tissues of the berry; anthocyanin color accumulation is restricted to skin cells while berry flesh cells accumulate sugar. These results are consistent with the observed increase in endogenous BR at the onset of ripening (Fig. 1, A and B) and therefore provide further weight to the suggestion that changes in endogenous BR levels influence (either directly or indirectly) this key developmental process.

It is universally accepted that BRs are necessary for normal plant development (Clouse and Sasse, 1998). However, clear examples where plant developmental processes have been shown to be regulated by changes in BR levels (i.e. endogenous increases and decreases are associated with a phenotypic change during development) are rare (Yamamoto et al., 2001). Even in cases where circumstantial or indirect evidence suggests a regulatory role for BR levels in a given process, there exists little direct evidence of actual changes in endogenous BR levels to support these claims. For instance, despite the widely cited claim that BRs act as negative regulators of deetiolation in Arabidopsis, there exist no data that demonstrate the predicted changes in endogenous BR levels during this process (Symons and Reid, 2003b). However, results from this study are a clear example of changes in endogenous BR levels that correspond to an important developmental process. Such results provide further confirmation that BRs do not act merely as permissive factors that are simply necessary for plant development, but actually regulate these processes.

Insights into BR Biosynthesis in Grape

The profile of endogenous BR levels in grape berries provides an important insight into BR biosynthesis in this species. For instance, the relatively high level of 6-DeoxoCS compared with typhasterol is similar to the situation in tomato, pea, and Arabidopsis, where the late C-6 oxidation pathway (Fig. 2) is thought to be the predominant biosynthesis pathway for BRs (Nomura et al., 2001). The current data therefore suggest that a similar situation may occur in grapes. Furthermore, in grape berries CS was the only bioactive BR detected, while BL (the BR with the highest bioactivity; Wang et al., 2001) was not detected in any sample. This suggests that in grape berries the main bioactive BR is likely to be CS, and the conversion of 6-DeoxoCS to CS (catalyzed by the *VvBR6OX1* gene product) may be an important BR activation step in this species. This scenario is similar to the reported situation in vegetative tissues of tomato (Yokota, 1997; Bishop et al., 1999) and rice (Hong et al., 2002), where CS is considered to be the main bioactive BR. However, in many circumstances BL is thought to be the major bioactive BR. For instance, an examination of the BR profile in tomato fruits revealed that BL was detected at levels that were up to 84 times higher than CS (Montoya et al., 2005). Therefore, while CS is the major bioactive BR in vegetative tissues of tomato (Nomura et al., 2005), in the fruit the predominant bioactive BR is BL (Montoya et al., 2005). This observation was explained by recent results showing that a second *CYP85A* gene (*CYP85A3*), which encodes a BR 6-oxidase that converts 6-DeoxoCS to BL, is preferentially expressed in tomato fruits (Nomura et al., 2005). Importantly, there is no evidence to suggest a second *VvBR6OX1* gene in grape berries, which is consistent with the apparent absence of BL in these tissues. This contrast between grape and tomato provides a clear example of both tissue- and species-specific differences in the identity of the bioactive BR molecule and its synthesis.

It has previously been suggested that transcriptional regulation plays an important role in controlling BR biosynthesis (Bancos et al., 2002). This is based on a negative correlation between *CYP90A1/CPD* transcript levels and the amounts of the CYP90A1 substrate, 6-deoxocathasterone, in Arabidopsis shoots and roots (Bancos et al., 2002). Similarly, our results show a tightly linked, negative correlation between *VvBR6OX1* gene transcript levels and VvBR6OX1 substrate (6-DeoxoCS) accumulation (Figs. 1, B and C, and 6B). Furthermore, the down-regulation of *VvBR6OX1* transcript levels between 8 and 10 wpf coincides with a dramatic increase in the endogenous levels of the bioactive product, CS (Figs. 1, B and C, and 6B). This pattern is entirely consistent with the well-characterized feedback regulation of this step in several different species (Nomura et al., 2001; Bancos et al., 2002; Goda et al., 2002) and indicates that *VvBR6OX1* transcript levels may also be negatively regulated by bioactive BR levels in grape berries. Indeed, this is a clear demonstration of the feedback regulation of this step in response to changes in endogenous BR levels. In contrast, our results suggest that another grape BR biosynthesis gene, *VvDWF1*, is not negatively regulated

by bioactive BR levels, consistent with evidence from other species (Schultz et al., 2001). The *VvBRI1* gene, which encodes the putative BR receptor, was expressed in all the samples tested, indicating that it is most likely that berries can perceive BRs at any stage of their development.

It is also interesting that prior to the 10-wpf time point, high levels of *VvBR6OX1* transcript accumulation resulted in relatively low substrate (6-DeoxoCS) levels but was not accompanied by a resultant increase in the levels of the product (CS; Figs. 1, B and C, and 6B). This suggests that alternative biosynthetic pathways, BR metabolism (i.e. CS deactivation and/or conjugation), and/or posttranslational regulation also play an important role in the regulation of bioactive BR levels in grape berries.

BRs Influence the Onset of Ripening in Grape Berries

Together, the increase in endogenous BR levels at the onset of ripening and the effects of exogenous BR and Brz on this process indicate that BR levels may play a role in the regulation of ripening in nonclimacteric grape berries. The obvious question therefore is the following: By what mechanism(s) might BRs influence this process? Grape berry ripening is the result of the combination of a number of processes, such as berry expansion and softening, sugar and color accumulation, and flavor and aroma development. Much of the increase in berry size and weight after véraison is caused by an accumulation of water and sugar and an increase in cell expansion, facilitated by significant cell wall modification (Nunan et al., 1998). Expression patterns of genes encoding cell wall-modifying enzymes during grape berry development show increases in the expression of *VvXET* genes, which encode xyloglucan endotransglycosylases (XETs), post véraison (Nunan et al., 2001). In soybean (*Glycine max*), the *BRU1* gene, which encodes a functional XET involved in expansion growth, has been shown to be induced by BRs (Zurek and Clouse, 1994; Oh et al., 1998). Thus, in grape berries, the high BR levels during véraison (Fig. 1) may promote an increase in berry size, at least in part, by regulating *VvXET* gene expression. However, our understanding of ripening is far from complete, and the mechanism(s) by which BRs might influence other ripening processes are as yet unknown.

To fully understand the influence of BRs on the ripening of grape berries, it will also be necessary to understand any possible interactions between the BRs and other plant growth regulators that have also been implicated in the control of this process (Davies et al., 1997). While there appeared to be no relationship between changes in BR levels and endogenous GA, GA_1, or IAA levels during berry development (Table I; Fig. 1), it will be interesting to determine if BRs interact with ABA, which has also been shown to increase in concentration after véraison (Davies et al., 1997). Similarly, BRs are known to affect ethylene synthesis in some plant systems (Yi et al., 1999), and as such it will be important to investigate the relationship between the observed increase in BRs at the onset of ripening and the recently reported transient increase in endogenous ethylene levels prior to véraison (Chervin et al., 2004). Both ethylene and sugars, which increase during ripening, may play roles in the control of gene expression and, hence, grape berry development (Cakir et al., 2003; Chervin et al., 2004).

CONCLUSION

Our research indicates that endogenous BR levels influence the onset of ripening in grape berries, and further investigation may lead to a better understanding of the potential application of this finding to the grape and wine industries. Also worthy of investigation is the possibility that changes in BR levels may be a common regulatory mechanism for the control of ripening in other important nonclimacteric-fruit species, such as citrus and strawberry (*Fragaria* spp.).

Results from this study may also have wider ramifications for understanding the control of fruit ripening generally, as it has been suggested that common regulatory mechanisms may be operating early in the ripening processes of both climacteric and nonclimacteric species (Vrebalov et al., 2002). Indeed, exogenous BRs have been shown to promote ripening (albeit via an increase in ethylene levels) in tomato, a climacteric fruit (Vardhini and Rao, 2002). While Montoya et al. (2005) reported high endogenous BR levels in during early fruit development in tomato, further studies are required to investigate a possible role for BRs in ripening in this species.

MATERIALS AND METHODS

Berry Sampling for BR and Gene Expression Analysis

Flowers (at the 50% cap fall stage: anthesis) and berries (from 2–16 wpf, at fortnightly intervals), of grapes (*Vitis vinifera* L. cv Cabernet Sauvignon) were collected from a commercial vineyard (Slate Creek, Willunga, South Australia) in the 2002/2003 season. Berries were immediately deseeded, flowers and berries frozen in liquid nitrogen, and stored at −80°C until required. Soluble solids (°Brix) were measured with a refractometer (model 10430; Reichert).

Analysis of Endogenous Hormone Levels

Endogenous BR, IAA, and GA levels were quantified by gas chromatography-mass spectrometry-selected ion monitoring, using labeled internal standards as described previously (Symons and Reid, 2003a). Positive identification of CS and 6-DeoxoCS from grape berry tissue was obtained by full-scan mass spectrometry.

Epi-BL and Brz Treatment of Grape Berries

Cabernet Sauvignon grapevines growing in the field at the Waite Campus, Urrbrae, South Australia were tagged at flowering to allow inflorescences of the same stage to be used for experimentation. Twenty inflorescences of approximately the same size were chosen. On each of 10 bunches, 10 berries were tagged for treatment with epi-BL, and on each of these bunches five berries were tagged as controls (treated with 100% ethanol). On each of the other 10 bunches, 10 berries were tagged with the BR synthesis inhibitor Brz, while five berries per bunch were treated as controls as above. Epi-BL (Sigma catalog no. E 1641) was dissolved in 100% ethanol, and 5 μL (containing 200 ng) was applied to each berry at each time point. Brz was dissolved in

100% ethanol, and 5 μL (containing 10 μg) of solution was applied to each berry at each treatment time. This application technique resulted in an even coverage of ethanol over the majority of the surface of each berry. The three treatments were done late in the afternoon at four time points beginning on 02/01/04, which was approximately 5 wpf. The treated berries were observed daily and the first indication of anthocyanin production coloring the berry skin was recorded. Significance was determined by a χ^2 test. At the end of the experiment the °Brix of the three samples was measured as described above.

Cloning and Sequencing of a Putative *DWF1* (*DIM*) Homolog

Two cDNA clones with similarity to *DWF1* (*DIM*) homologs from other species were sequenced in full using synthetic oligonucleotide primers as appropriate with BigDye technology (Applied Biosystems).

Cloning and Sequencing the Grape *DWARF* Homolog

A full-length *VvBR6OX1* clone was isolated from a Cabernet Sauvignon prevéraison berry cDNA library and was sequenced in full as described above. A detailed sequence anaylsis of the *VvBR6OX1* gene and P450 genes from other species was conducted by multiple sequence alignment of the full-length, deduced protein sequences using ClustalW (Thompson et al., 1994). A distance matrix was calculated by Prodist (Felsenstein, 1989), and the unrooted phylogenetic tree was produced using Neighbor (Felsenstein, 1989).

Cloning and Sequencing the Putative *BRI1* Homolog

A fragment of the putative grapevine *BRI1* homolog was cloned from the 3′-terminal region of the *BRI1* cDNA by 3′-RACE PCR as described by Frohman et al. (1988). The first round of PCR employed the forward primer BRF1 (TCGATTTCAAGTACCCAGCA) and the $(dT)_{17}$-adaptor of Frohman et al. (1988) with first-strand cDNA made from Cabernet Sauvignon berries 4 (preripening) and 12 (post ripening) wpf. For the second round of PCR the forward primer BRF2 (TCATCGGAGACAAGCATCCC) was used with the $(dT)_{17}$-adaptor. The reactions were done using GIBCOBRL Platinum Taq High Fidelity polymerase in accordance with the manufacturer's instructions. PCR cycles were ×1 94°C, 2 min; ×25 94°C, 30 s; 55°C, 30 s; and 68°C, 2 min followed by 7 min at 68°C. After the first round of PCR 2 μL of a 1-in-20 dilution were used as template in the second round reaction of 25 μL. PCR products were run on an agarose gel, the single band was removed, and DNA isolated using a Qiaquick column (Qiagen) as described by the manufacturer. The resulting fragment was A-tailed using Taq polymerase and ligated into pGEM-T Easy vector (Promega) as described by the manufacturer. DNA was purified from selected clones and the inserts sequenced as described above.

Tomato Accessions

The dwarf tomato (*Lycopersicon esculentum*) mutant line GCR 567 with the d^x allele was obtained from G.J. Bishop (Institute of Biological Sciences, University of Wales Aberystwyth, Aberystwyth, UK). The Ailsa Craig cultivar having the wild-type *D* allele was obtained from C.M. Rick (Tomato Genetics Research Center, University of California, Davis, CA; accession no. LA2838A).

Generation of *VvBR6OX1* Expression Construct

A full-length *VvBR6OX* cDNA fragment was ligated into the pART7 vector (Gleave, 1992) between the cauliflower mosaic virus 35S promoter and octapine synthase terminator, and transformed into *Escherichia coli* (strain XL-1 blue) by electroporation. The expression cassette was released from the resultant vector by *Not*I digestion and ligated into the binary vector pART27 (Gleave, 1992) to produce pART27-VvBR6OX1. Positive clones were checked by DNA sequencing and selected clones were transformed into *Agrobacterium tumefaciens*, strain EHA105.

Tomato Transformation

Tomato plants were transformed with *A. tumefaciens* using the cotyledon method (Fillatti et al., 1987). Seeds of tomato dwarf mutant line GCR 567 and wild-type cultivar Ailsa Craig were surface sterilized, washed, and sown on seed germination Murashige and Skoog media (Murashige and Skoog, 1962) containing 1% Glc and 0.6% Phytagel (Sigma), pH 5.8. The seeds were incubated at 25°C with a 16-h photoperiod for 7 d. Epicotyl and stem pieces (5–10 mm) of seedlings (excluding the nodes) were excised and kept in sterile water prior to inoculation. *A. tumefaciens* cells containing the binary vector pART27-VvBR6OX1 were grown for 2 d at 30°C and then diluted in Murashige and Skoog media approximately 10-fold to give an OD_{600} of between 0.8 and 1.0. Prepared tomato explants were incubated with the *A. tumefaciens* suspension for 5 min with gentle inverting. Inoculated segments were placed on fresh seed germination media and incubated in the dark at 25°C for 2 d. The inoculated segments were then transferred to new selection media comprising the previous medium with additional supplements, i.e. zeatin riboside (1 μg mL^{-1}), kanamycin (100 μg mL^{-1}), and timentin (1 mg mL^{-1}). Explants were incubated at 25°C (16 h day) and subcultured every 2 weeks until calli were produced. The calli were cut from the explants and subcultured onto fresh media. Shoots generated from the calli were transferred into sterile tissue culture vials containing rooting media, where zeatin riboside was replaced with IAA (1.75 μg mL^{-1}) and the kanamycin concentration was reduced to 50 μg mL^{-1}. Two to three weeks later the rooted regenerated plantlets were potted into soil. Regenerated plants were allowed to self-pollinate and the resultant fruits were harvested. Southern-blot hybridization was used to confirm the insertion of the grape *VvBR6OX1* into the genome of the tomato d^x mutant T_0 regenerants and real-time PCR was used to confirm expression of the gene as describe below (data not shown).

Grapevine RNA Extraction, cDNA Production

Total RNA was extracted from grape floral and berry tissues as described by Davies and Robinson (1996). Total RNA was further purified using a Qiagen RNeasy column including a DNase digestion step as described by the manufacturer. First-strand cDNA for real-time PCR was made with Superscript III enzyme (Invitrogen) by using 2 μg of total RNA in a reaction volume of 40 μL as described by the manufacturer, with the $(dT)_{17}$-adaptor of Frohman et al. (1988) as primer.

Analysis of Gene Expression by Real-Time PCR

A template for a standard curve for the *VvBRI1* gene was created by PCR by producing a 207-bp fragment from the 3′ region of the cloned and sequenced cDNA from the putative grapevine *BRI1* homolog using the following primers: *BRI1*-fwd, AAGGTAGCGTGTGCCTGTTT; and *BRI1*-rev, GTTTCCCTGCTACTGCTTGC.

The melt curves for these assays produced a single peak indicating that a single species had been amplified; the products were also checked on an agarose gel. A 10-fold dilution series of the purified fragment was used to create a standard curve for the estimation of gene expression in the test samples. To normalize the level of cDNA in each real-time PCR reaction, each cDNA sample was assayed under the same conditions using primers designed to the grape *Ubiquitin1* (*VvUbi*) homolog (GenBank accession no. CA808925). The primers used were *Ubi*-fwd, AGTAGATGACTGGATTGGAGGT; and *Ubi*-rev, GAGTATCAAAACAAAAGCATCG. The reactions (done in triplicate) contained cDNA or purified fragment at the appropriate level, ×1 SYBR GREEN PCR Master Mix (Applied Biosystems), and forward and reverse primers at a final concentration of 0.28 μM. The cycles were as follows: 95°C, 8 min; 40 cycles of Step 1, 95°C, 30 s; Step 2, 58°C, 30 s; and Step 3, 72°C, 30 s. Melt (50°C–96°C) was hold 5 s on each step, one degree per step. The reactions were incubated in a Corbett RotorGene RG-3000 cycler and the data analyzed with RotorGene V6 software. The putative grapevine *DWF1* homolog was analyzed as described above but using the following primers, which produced a 206-bp fragment: *DWF1-fwd*, ACCGAGAAGGAAGTGCAGGAG; and *DWF1 -rev*, ACCATCACATTCGTTGAGCAGG. The grape *VvBR6OX1* was also analyzed as described above and yielded a 281-bp fragment when amplified with the following primers: *VvBR6OX1*-fwd, GACAAGAGCTTAGAGTCCCAAAAC; and *VvBR6OX1*-rev, GAAAATTATTGTACATCCATATTGCTT.

Sequence data from this article have been deposited with the GenBank data library under accession numbers CF372599, DQ235273, and CB975975 for *VvDWF1*, *VvBR6OX1*, and *VvBRI1*, respectively.

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