

Sugar and abscisic acid signaling orthologs are activated at the onset of ripening in grape

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Abstract The onset of ripening involves changes in sugar metabolism, softening, and color development. Most understanding of this process arises from work in climacteric fruits where the control of ripening is predominately by ethylene. However, many fruits such as grape are nonclimacteric, where the onset of ripening results from the integration of multiple hormone signals including sugars and abscisic acid (ABA). In this study, we identified ten orthologous gene families in *Vitis vinifera* containing components of sugar and ABA-signaling pathways elucidated in model systems, including PP2C protein phosphatases, and WRKY and homeobox transcription factors. Gene expression was characterized in control- and deficit-irrigated, field-grown Cabernet Sauvignon. Sixty-seven orthologous genes were identified, and 38 of these were expressed in berries. Of the genes expressed in berries, 68% were differentially expressed across development and/or in response to water deficit. Orthologs of several families were induced at the onset of ripening, and induced earlier and to higher levels in response to water deficit;

patterns of expression that correlate with sugar and ABA accumulation during ripening. Similar to field-grown berries, ripening phenomena were induced in immature berries when cultured with sucrose and ABA, as evidenced by changes in color, softening, and gene expression. Finally, exogenous sucrose and ABA regulated key orthologs in culture, similar to their regulation in the field. This study identifies novel candidates in the control of nonclimacteric fruit ripening and demonstrates that grape orthologs of key sugar and ABA-signaling components are regulated by sugar and ABA in fleshy fruit.

Keywords *Vitis vinifera* · Nonclimacteric fruit · Hormone action · Water deficit

Introduction

It is often asserted and then assumed that fundamental research from model systems, such as *Arabidopsis*, is valuable because this knowledge would be utilized later to elucidate practical aspects of plant function in species of agronomic importance. This has been successful in like organs and developmental processes. For example, a regulatory module involved in photoperiod control of flowering has been elucidated through extensive research in *Arabidopsis* (reviewed in Imaizumi and Kay 2006), and more recently this same mechanism has been shown to control flower time in *Populus trichocarpa* (Bohlenius et al. 2006). Unexpectedly, Bohlenius et al. (2006) revealed that the same regulatory module is involved in the control of bud dormancy. This illustrates that components of regulatory mechanisms can be conserved across disparate developmental processes that share commonalities; a dependence on daylength and action in the shoot apex in

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the case of flowering and bud dormancy. Less is known about the utility of extending knowledge to a class of organ not even present in a given model species.

The onset of ripening marks a transition in fruit development leading to changes in physiology involving sugar metabolism, softening, and color development. Most understanding of this process arises from work with climacteric fruits in which the control of ripening is predominately by ethylene. However, there are many fruits of economic importance that are nonclimacteric, such as grape, strawberry, and citrus, where the processes controlling the onset of ripening are still poorly understood. Study has traditionally focused on ethylene in climacteric fruits and on other hormones such as sugar and abscisic acid (ABA) in nonclimacteric fruits. However, there are numerous studies implicating ethylene in the ripening of nonclimacteric fruits (Chervin et al. 2004; Trainotti et al. 2005), and conversely, sugar and ABA in the ripening of climacteric fruits (Chernys and Zeevaart 2000; Leclercq et al. 2002; Lieberman et al. 1977; Telef et al. 2006). In addition, other hormones have been shown to influence the ripening processes such as auxin (Davies et al. 1997; Trainotti et al. 2007) and brassinosteroids (Fu et al. 2008; Lisso et al. 2006; Symons et al. 2006). Fruit ripening in general involves the integration of multiple hormone signals. Climacteric and nonclimacteric fruits are not delimited by phylogeny and both exist even among closely related species (reviewed in Giovannoni 2004). This suggests that ripening in all fleshy fruit may have a common foundation.

Sugar and ABA function as signals in many aspects of plant development (reviewed in Gibson 2004; Rook et al. 2006) and there are numerous lines of evidence correlating increases in sugar and ABA with the onset of ripening in grape (Davies et al. 1997; Deluc et al. 2009; Mei et al. 2009; Wheeler et al. 2009). In field-grown berries, exogenous ABA increases anthocyanin accumulation (Peppi et al. 2006, 2008; Wheeler et al. 2009), and in our own work berries fail to synthesize anthocyanins when sugar import into the berry is disrupted via phloem girdling prior to the onset of ripening (unpublished data). Exogenous applications of both sugars and ABA increase anthocyanin accumulation in cell and tissue culture (Hiratsuka et al. 2001; Larronde et al. 1998; Matsushima et al. 1989; Pirie and Mullins 1976). Finally, water deficits also result in increases in anthocyanin accumulation (Castellarin et al. 2007a, b; Deluc et al. 2009; Matthews and Anderson 1988), and have been shown to increase ABA accumulation at the onset of ripening (Okamoto et al. 2004; Antolin et al. 2008; Deluc et al. 2009).

Many studies investigating the role of sugar and ABA in ripening have focused on color development. In viticulture,

the onset of ripening is traditionally referred to as veraison and is defined by the initiation of color development. More contemporary genomic studies demonstrate that color can act as a proxy for developmental stage in that individual grape berries of like color have similar global gene expression patterns (Lund et al. 2008). A previous study in our laboratory found that water deficits advanced the onset of color development, but sugar and anthocyanin accumulation remained closely linked (Castellarin et al. 2007a). Furthermore, the induction of core flavonoid and anthocyanin-specific genes reflected this advancement as well (Suppl. Fig. 1). Therefore, in the current study, water deficit is used as a means to manipulate the timing of the onset of ripening in the field.

There are few examples of studies focused on identifying the molecular mechanisms responsible for sugar and ABA action in grape, although several recent studies have demonstrated roles for sugar and ABA in controlling sugar metabolism. ABA stimulates acid invertase activity (Pan et al. 2005), and expression of the grape hexose transporter *VvHT1* is regulated by both ABA and hexose (Cakir et al. 2003; Conde et al. 2006). Two other recent studies have focused on core sugar and ABA-signaling components. A calcium-dependent protein kinase is regulated by ABA during ripening (Yu et al. 2006), and a study in grape cell culture provides evidence that sugar induced increases in anthocyanin accumulation are dependent on calcium, calmodulin, and kinase activity (Vitrac et al. 2000).

In this study, we begin to examine the hypothesis that orthologous sugar and ABA-signaling components, characterized across such diverse processes as seed dormancy and stomatal control, function similarly in the control of the onset of ripening in fleshy fruit. This study employs a comprehensive approach in identifying novel sugar and ABA-signaling candidates in grape. Ten gene families, containing sugar and ABA-signaling components elucidated in model systems, were considered including; the putative sucrose sensor *SUT2*, core G-protein signaling components *GPA1* and *RGS1*, hexokinases (*Hxk*), *PP2C* protein phosphatases, *Snf1*-related kinases (*SnRK*), and the sugar-related *WRKY*, and ABA-related homeodomain–leucine zipper, or homeobox (*HB*), *ABRE*-binding factor (*ABF*), and *AP2* transcription factors (reviewed in Hirayama and Shinozaki 2007; Koch 2004; Leung and Giraudat 1998; Rolland et al. 2006; Rook et al. 2006). Their orthologous families in grape were identified, and the expression of individual genes were characterized in control- and deficit-irrigated, field-grown Cabernet Sauvignon. The onset of ripening was induced in cultured immature berries with sugar and ABA treatments. Finally, the effect of exogenous sugar and ABA on the expression of several key orthologs was investigated in berry culture.

Materials and methods

Field experiment and physiological measurements

The field experiment was the same as presented in Castellarin et al. (2007a). In short, experiments were conducted during 2006 in a commercial vineyard of R.H. Phillips Winery (Esparto, CA, USA) using *Vitis vinifera* “Cabernet Sauvignon” clone 337 grafted onto rootstock 140R. Two irrigation treatments were established. Irrigation was applied to control (C) vines in order to maintain midday leaf water potential (Ψ_{leaf}) between -0.9 and -1.2 MPa. Irrigation was cut off to early deficit (ED) vines at fruit set until 77 days after anthesis (DAA); then vines were irrigated at the C rate till 133 DAA. Plant water status was monitored weekly by measuring midday leaf water potential according to Matthews et al. (1987). Each treatment was replicated four times in 0.5-ha plots dispersed over a 36-ha vineyard according to a randomized complete block design. Samples were collected randomly from ten vines located in the central row of the plots. Berries were sampled six times between 27 June (33 DAA) and 5 October (133 DAA), when the grapes were harvested. Twenty berries were collected from each plot for use in studies of gene expression. During the onset of ripening, samples of green and red berries were collected separately from the same clusters and analyzed separately.

Bioinformatics and ortholog identification

Amino acid sequences of each ABA and sugar signaling gene of interest were obtained from NCBI. These sequences were then BLASTed against the predicted grape proteome via the Genoscope *Vitis* BLAST server (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) using a BLOSUM62 matrix. In cases with reasonable numbers (<10) of predicted orthologs, all significant hits were recorded, and their corresponding genomic and predicted cDNA sequences obtained through the Genoscope *Vitis* genome browser (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>). In cases with numerous (>10) predicted orthologs, protein sequences were obtained and nested within the Arabidopsis family (CLUSTALW, BLOSUM matrix with gap open penalty of 10, gap extension penalty of 1) producing rooted dendrograms from which predicted orthologs grouping within a particular subgroup could be determined (Figs. 1, 2, 3, Suppl. Figs. 3, 4). Then genomic and predicted cDNA sequences were obtained for these predicted orthologs as above. An identical methodology was used in incorporating the *P. trichocarpa* orthologs and producing the unrooted dendrograms.

Primer design and validation

Within a family, predicted cDNA sequences were aligned (CLUSTALW, IUB matrix with gap open penalty of 15, gap extension penalty of 6). All compatible primer pairs were determined using the FastPCR software’s unique primer design. The actual primer pair was chosen manually based on the following criteria making it most suitable for Quantitative PCR (QPCR): the product is approximately 150 bp in length, and the most 3’ sequence. Primers pairs for all the genes can be found in Suppl. File 1.

Every primer pair was validated in order to confirm that they were functioning and only yielding one specific product. This was performed by traditional PCR amplification of the product from a genomic DNA template. Total genomic DNA was obtained from *Vitis vinifera* “Cabernet Sauvignon” using a DNeasy Plant Kit (Qiagen, Inc., <http://www1.qiagen.com>) following the manufacturer’s protocol. Gene fragments were amplified in a 20- μ L volume PCR reaction containing 0.25 μ M of each primer, 1.5 mM MgCl_2 , 0.4 mM dNTPs, 0.4 U of Taq polymerase and 20 ng of template. The PCR profile was 30 cycles of 92°C for 30 s, 58°C for 30 s, and 65°C for 1 min. Gene amplicons were separated with a 2% agarose gel stained with ethidium bromide. PCR products were purified via the Qiaquick PCR Purification Kit (Qiagen, Inc., <http://www1.qiagen.com>) and directly sequenced to confirm their identity.

Semi-quantitative PCR analyses

In order to test the expression of all the orthologs in the grapevine berry, semi-quantitative PCR was performed on skin cDNA of control berries at three different stages of ripening, before, at and after the onset of ripening. The cDNA normalization among samples was based on the expression of the VvUbiquitin1 gene. PCR conditions were the same described above, and amplified fragments were separated with a 2% agarose gel stained with ethidium bromide.

Expression 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, <http://www.promega.com>). RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, <http://www.thermo.com>). Each sample was quantified twice and the mean of the two technical replicates calculated. 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, <http://www.promega.com>). QPCR was carried

out in an ABI PRISM 7500 sequence detector (Applied Biosystems, <http://www3.appliedbiosystems.com>). 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, <http://www3.appliedbiosystems.com>). Thermal cycling conditions were 95°C for 10 min followed by 95°C for 30 s, 60°C for 60 s, for 40 cycles, followed by T_m determination. Each sample was run in duplicate. In addition, VvUbiquitin1 (TC32075, TIGR database) was also quantified for every sample. For the field experiment, absolute expression level of VvUbiquitin1 was determined from standard curves established from genomic DNA standards and absolute levels of expression for each gene determined (Yun et al. 2006). Gene expression is expressed as \log_2 copy number/ng RNA. For berry culture experiments expression was not quantified absolutely due to limited total mRNA sample material, but instead expressed relative to VvUbiquitin1 expression using the comparative C_T methodology. Gene expression was expressed as mean and standard error calculated across three biological replicates.

Berry culture

Cabernet Sauvignon clusters were collected from field-grown vines (UC Davis experimental vineyard) at 61 DAA. Clusters were surfaced sterilized for 10 s in a 70% ethanol solution, 10 min in a 1% bleach solution, and then rinsed three times with sterilized water. Berries were removed from the clusters by cutting the stem at the peduncle level, and the peduncle was removed from the rest of the fruit with a scalpel. Berries were weighed and individually cultured for 23 days in 20 mL glass vials filled with 7 mL of six different 1% agarose media 2% sucrose, 10% sucrose, 200 μ M ABA ([+] *cis*, *trans*-ABA, A.G. Scientific, Inc., <http://www.agscientific.com>) alone, 2% sucrose 200 μ M ABA, 10% sucrose 10 μ M ABA, 10% sucrose 200 μ M ABA. The 2 and 10% sucrose concentrations used in this study approximate sugar concentrations of berries prior to (3–4°Brix) and at (>6–7°Brix), the onset of ripening in the field (Matthews et al. 1987; Matthews and Anderson 1988). Each treatment was replicated ten times. Berry color changes were visually analyzed twice, 10 days after T_0 and at the end of the experiment. Elasticity (E) was measured at T_0 and at the end of the experiment. In short, berry elasticity was determined non-destructively using a custom-fabricated instrument, which measured force and displacement during berry compression. As described in Thomas et al. (2008), the observed force/displacement relation was fit using SAS PROC NLIN (version 8.2, SAS Institute Inc., <http://www.sas.com>) to that expected for compression of a perfectly elastic sphere (Hertz equation, rearranged from Eq. 2 of Ravi et al. 2006). 23 days after

T_0 , berries were collected from the vials, weighed, and stored at -80°C till the tissues were processed for the RNA extractions.

Three berries from the 2% sucrose, 10% sucrose, 10% sucrose 10 μ M ABA, 10% sucrose 200 μ M ABA treatments were peeled and total RNA was extracted from each individual skin following the procedure described above.

Statistical analysis

All expression data was log transformed according to Rieu and Powers (2009) prior to statistical analysis. For the field experiment, ANOVAs were carried out comparing expression across time by each treatment. For treatment effects, means at each sample date were compared by Tukey's HSD. For berry culture experiments, differences between treatments were compared by Tukey's HSD. All analyses were carried out using JMP-SAS software (SAS Institute Inc., <http://www.sas.com>).

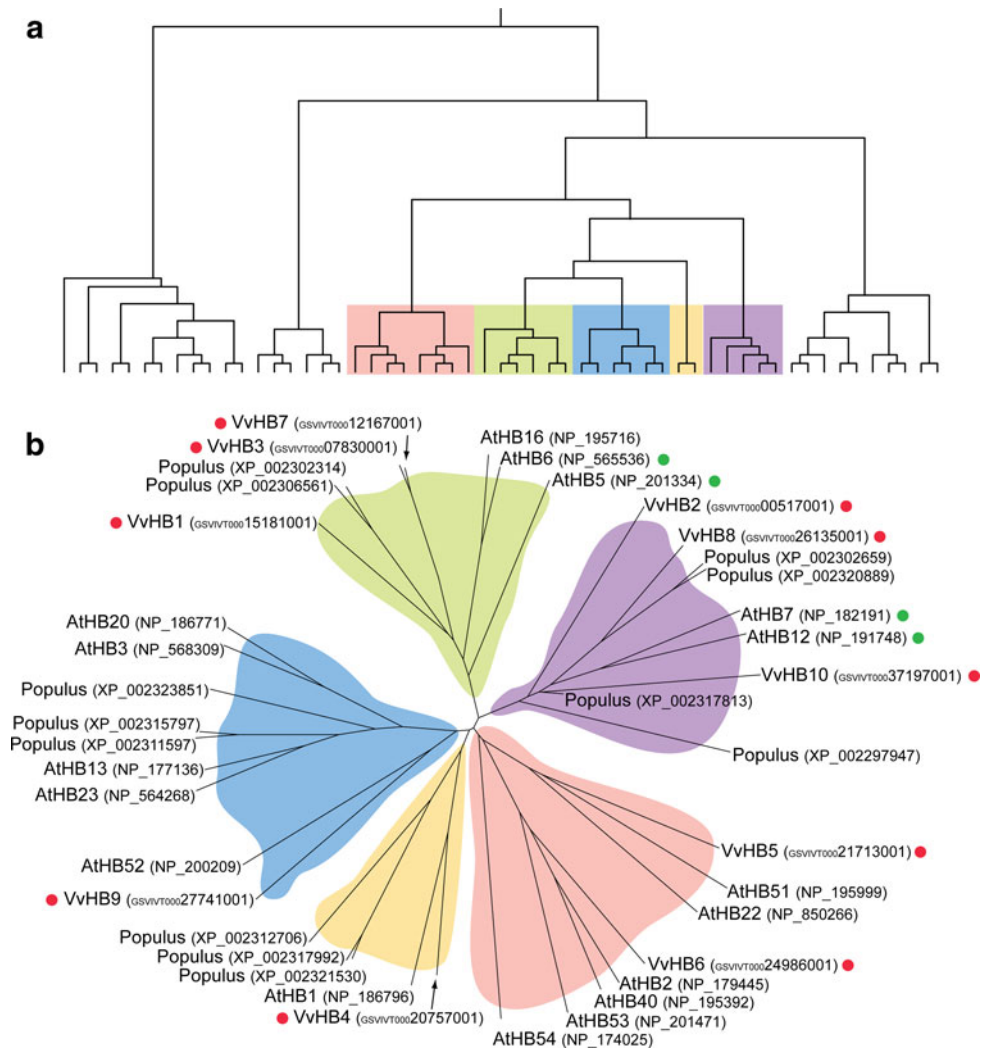
Results

Orthologous families

Utilizing the sequenced grape genome (Jaillon et al. 2007), 67 genes were identified representing orthologs of sugar and ABA-related genes in 10 gene families (Figs. 1–3, Suppl. Figs. 2–4). The genomic complexity of these families varied from small families with just one or few genes (e.g. the RGS1 and GPA1 families; see Suppl. Figs. 2b, c) to large families made up of nearly 100 genes (Figs. 1–3). Small gene families were comprehensively characterized for all grape orthologs, while only those subgroups containing genes specifically shown to be involved in sugar and ABA signaling were included for large gene families. Given the scope of this work, we focused primarily on those gene families encoding the following well characterized orthologs (1) the Arabidopsis Class I HB transcription factors, (2) the Arabidopsis ABA-insensitive mutants, *abi1* and *abi2*, encoding PP2C protein phosphatases, and (3) the WRKY transcription factors SUSIBA2, AtWRKY4, and AtWRKY34. Other gene families are summarized and referenced as supplemental material when pertinent.

The homeodomain–leucine zipper, or homeobox (HB) transcription factors represent a medium-sized gene family with approximately 47 members in Arabidopsis. This family has been delimited into four “Classes”, and 4 of the 17 members of the Class I AtHBs have been shown to be involved in ABA responses across diverse tissues (Henriksson et al. 2005). In addition, the expression of three *AtHB6*, 7, and 12, have been shown to be up-regulated by ABA (Johannesson et al. 2003; Lee and Chun 1998;

Fig. 1 *Vitis vinifera* orthologs of the Class I HB transcription factors. **a** Rooted dendrogram of the entire Arabidopsis HB family with nested grape orthologs. Class I, and various subgroups are colored. **b** Unrooted dendrogram of the Class I HBs clustering AtHB5, AtHB6, AtHB7, AtHB12 (green dots) and other *Vitis vinifera* (red dots), Arabidopsis, and *Populus trichocarpa* orthologs. NCBI and *Vitis* Genoscope accessions are given



Soderman et al. 1996, 1999). An examination of the grape genome identified 10 orthologs that cluster with the Class I HBs (Fig. 1).

The PP2C protein phosphatases represent another large gene family being made up of 80 genes in Arabidopsis (Xue et al. 2008). Within this family group a cluster of genes (referred to as Group A) containing many genes that have been characterized to function in the ABA-signaling pathway; most notably, the ABA-insensitive mutants, *abi1* and *abi2* (Leung et al. 1994, 1997; Meyer et al. 1994). In addition, AtPP2C-A, AtHAB1, AtHAB2, and AtAHG1, also members of Group A, function in ABA signaling across diverse tissues (Kuhn et al. 2006; Nishimura et al. 2007; Rubio et al. 2009; Saez et al. 2004). In Arabidopsis, all the members of this group are induced by ABA treatment. In grape, nine VvPP2Cs clustered in group A (Fig. 2).

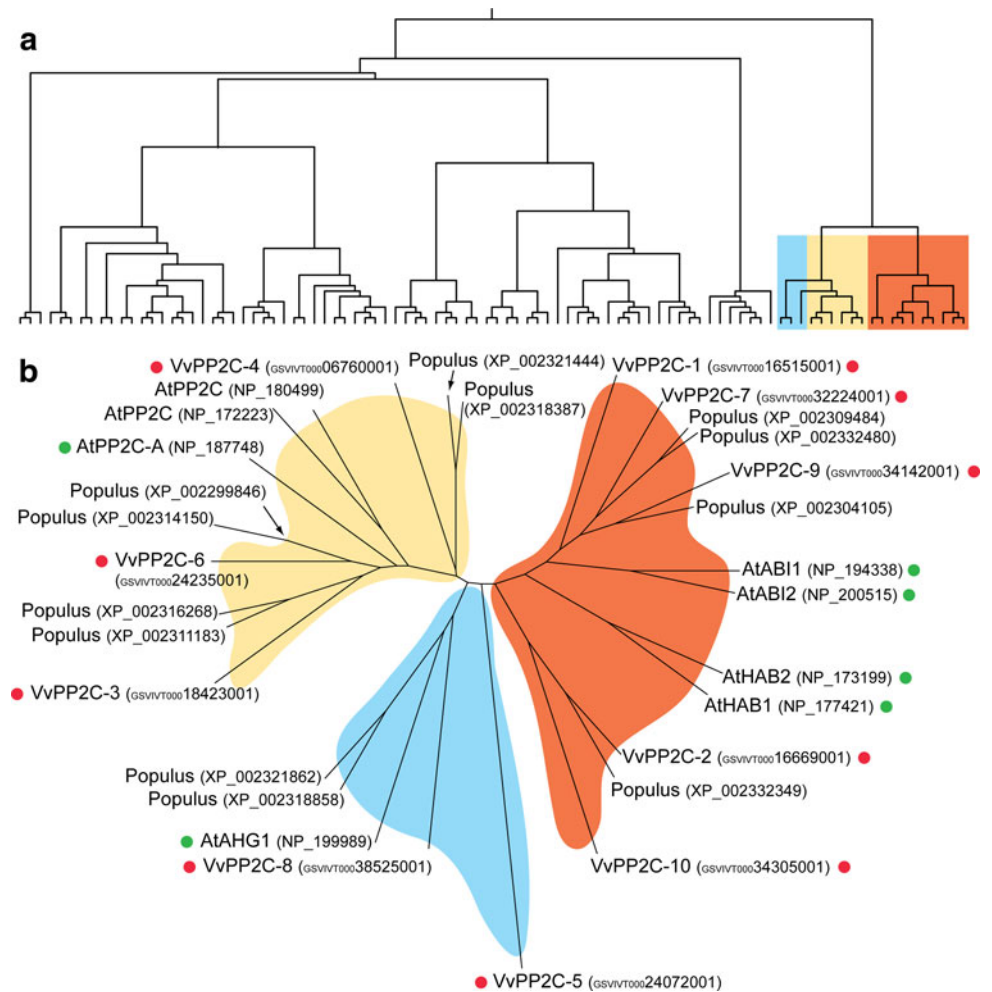
The WRKY transcription factors are a large gene family, consisting of approximately 70 members making up three groups in Arabidopsis (Eulgem et al. 2000; Wu et al. 2005). The barley (*Hordeum vulgare*) HvSUSIBA2, and

AtWRKY2, and AtWRKY34, all fall within the same group consisting of 14 members in Arabidopsis (referred to as Group I or Ia by Eulgem et al. 2000 and Wu et al. 2005, respectively). HvSUSIBA2 modulates the expression of a barley isoamylase gene during seed development via the binding of SURE (SUGAR RESPONSIVE) elements (Sun et al. 2003). In addition, Sun et al. (2003) demonstrated that expression of *HvSUSIBA2* is induced by exogenous sugar and its native expression profile during seed development correlates strongly with endogenous sucrose levels. Ham-margren et al. (2008) found that the sugar responsiveness of a nucleoside diphosphate kinase is altered in *Atwrky2* and *Atwrky34* mutant backgrounds. Grape contained 13 putative orthologs that fall within this group (Fig. 3).

Expression profiling

Expression profiling was carried out in berry skins of field-grown Cabernet Sauvignon in order to identify those orthologs expressed during ripening. In addition,

Fig. 2 *Vitis vinifera* orthologs of the ABA-related subfamily A PP2Cs. **a** Rooted dendrogram of the entire Arabidopsis PP2C family with nested grape orthologs. Group A, containing the ABA-insensitive mutants, *abi1* and *abi2*, are colored. **b** Unrooted dendrogram of Group A clustering *AtABI1*, *AtABI2*, *AtPP2C-A*, *AtHAB1*, *AtHAB2*, and *AtAHG1* (green dots) and other *Vitis vinifera* (red dots), *Arabidopsis*, and *Populus trichocarpa* orthologs. NCBI and *Vitis* Genoscope accessions are given



expression profiles under both control- and deficit-irrigated (denoted ED, Early water Deficit) conditions were compared in order to identify orthologs whose expression pattern reflected the advancement of ripening under ED. Water deficits were applied continuously from fruit set until to the onset of ripening, resulting in an average difference in midday leaf water potential of 0.36 MPa before the onset of ripening and no difference during ripening (Table 1). Of the 67 orthologous genes identified, 38 (57%) were expressed in grape berries. A summary of the expression profiles of all the genes examined in this study can be found in Figs. 4 and 5 while more detailed expression data is contained in Suppl. File 1. The majority of these genes were differentially regulated during berry development, with 26 (68%) exhibiting statistically significant changes with time in control and/or ED (Figs. 4, 5). There were few statistically significant differences in the magnitude of expression between control and ED (Figs. 4, 5, Suppl. File 1). Six genes exhibited statistically significant differences between control and ED. Four of these instances, *VvHB8*, *VvSnRK5*, *VvPP2C-3*, and *VvPP2C-7*, all exhibit elevated levels of expression in ED at or just prior

to the onset of ripening (See Suppl. File 1 for exact expression values).

Eight of the *VvWRKYs* selected for analysis were expressed in ripening grape. *VvWRKY3*, 5, and 6, were all differentially expressed during ripening and exhibited similar patterns of expression (Fig. 4, Suppl. File 1). They were up-regulated ranging from 4- to 16-fold at the onset of ripening. There were no significant differences in the expression of *VvWRKY1*, 2, 16, 18, and 19 across development or during water deficit (Fig. 4).

Of the 10 Class I, *VvHB* orthologs only four (*VvHB2*, 3, 4, and 8) are expressed in fruit during ripening (Fig. 5, Suppl. File 1). Both *VvHB4* and 8 were strongly up-regulated at the onset of ripening, exhibiting increases of >16-fold. *VvHB8* is up-regulated much earlier under water deficit and high levels persist until late in ripening. *VvHB4* is down-regulated early in development under ED. *VvHB2* expression in controls generally decreased during development with a small up-regulation at the onset of ripening, although these changes were not statistically significant. Under ED, however, this pattern of expression is more pronounced with a sharp eightfold decrease in

Fig. 3 *Vitis vinifera* orthologs of the sugar-related WRKY transcription factors. **a** Rooted dendrogram of the entire Arabidopsis WRKY family with nested grape orthologs. Group I, and various subgroups containing the sugar-related AtWRKY2 and AtWRKY34 are colored. **b** Unrooted dendrogram of Group I clustering *HvSUSIBA2*, AtWRKY2 and AtWRKY34 (green dots) and other *Vitis vinifera* (red dots), Arabidopsis, and *Populus trichocarpa* orthologs. NCBI and *Vitis* Genoscope accessions are given

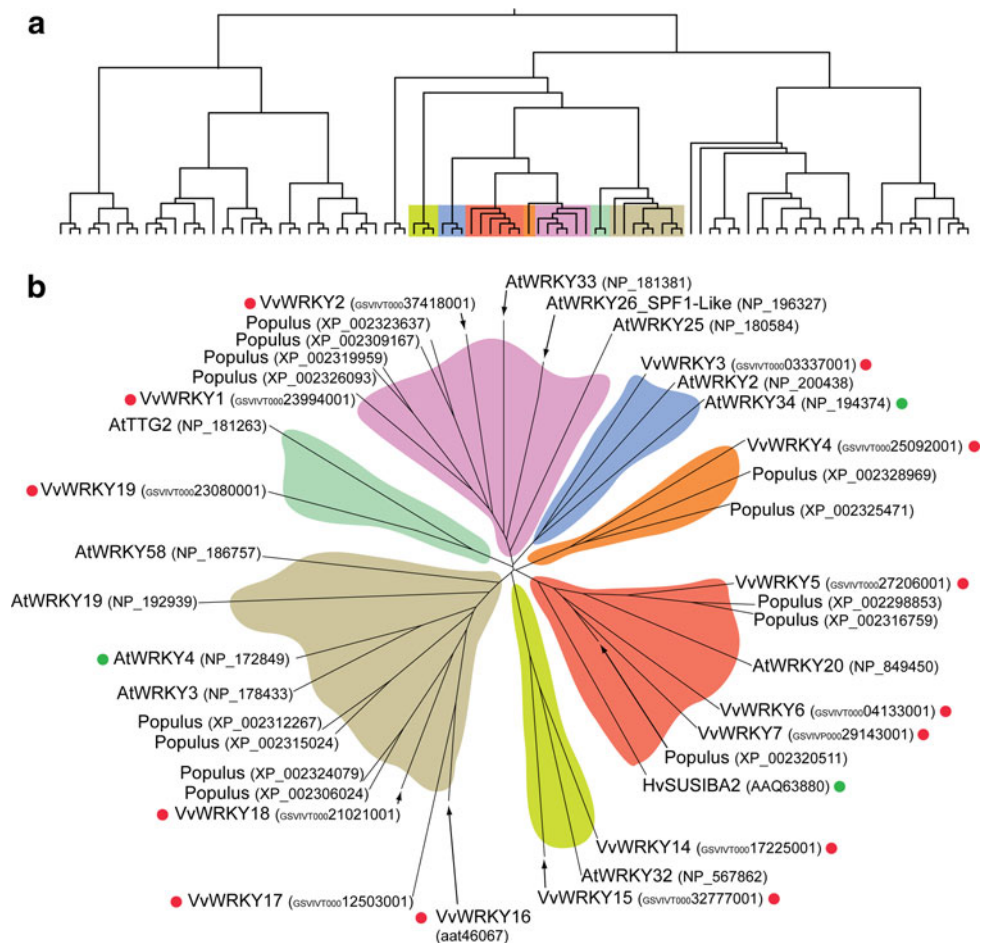


Table 1 Average midday leaf water potential (Ψ_{leaf}) in control (C) and early deficit-irrigated field-grown Cabernet Sauvignon grapevines during the periods prior to and after the onset of ripening (approx. 77 DAA)

DAA	Average Midday Ψ_{leaf} (MPa)	
	Control	Early deficit
33–77	-1.03 ± 0.07^a	-1.39 ± 0.05^b
77–133	-1.11 ± 0.08^a	-1.15 ± 0.07^a

Different letters denote significant differences ($P < 0.01$, Student's *t* test)

expression at 81 DAA (Fig. 5). *VvHB3* was constitutively expressed during ripening with no significant changes over time or under ED.

Six of the *VvPP2Cs* were expressed in grape berries. *VvPP2C-3*, 6, 7, and 9 were all differentially expressed during ripening, while *VvPP2C-1* and 5 expression did not change significantly (Fig. 5, Suppl. File 1). *VvPP2C-3*, 6, 7, and 9 were all up-regulated strongly at the onset of ripening increasing as much as 16-fold. *VvPP2C-3* and *VvPP2C-7* expression were clearly induced earlier and to

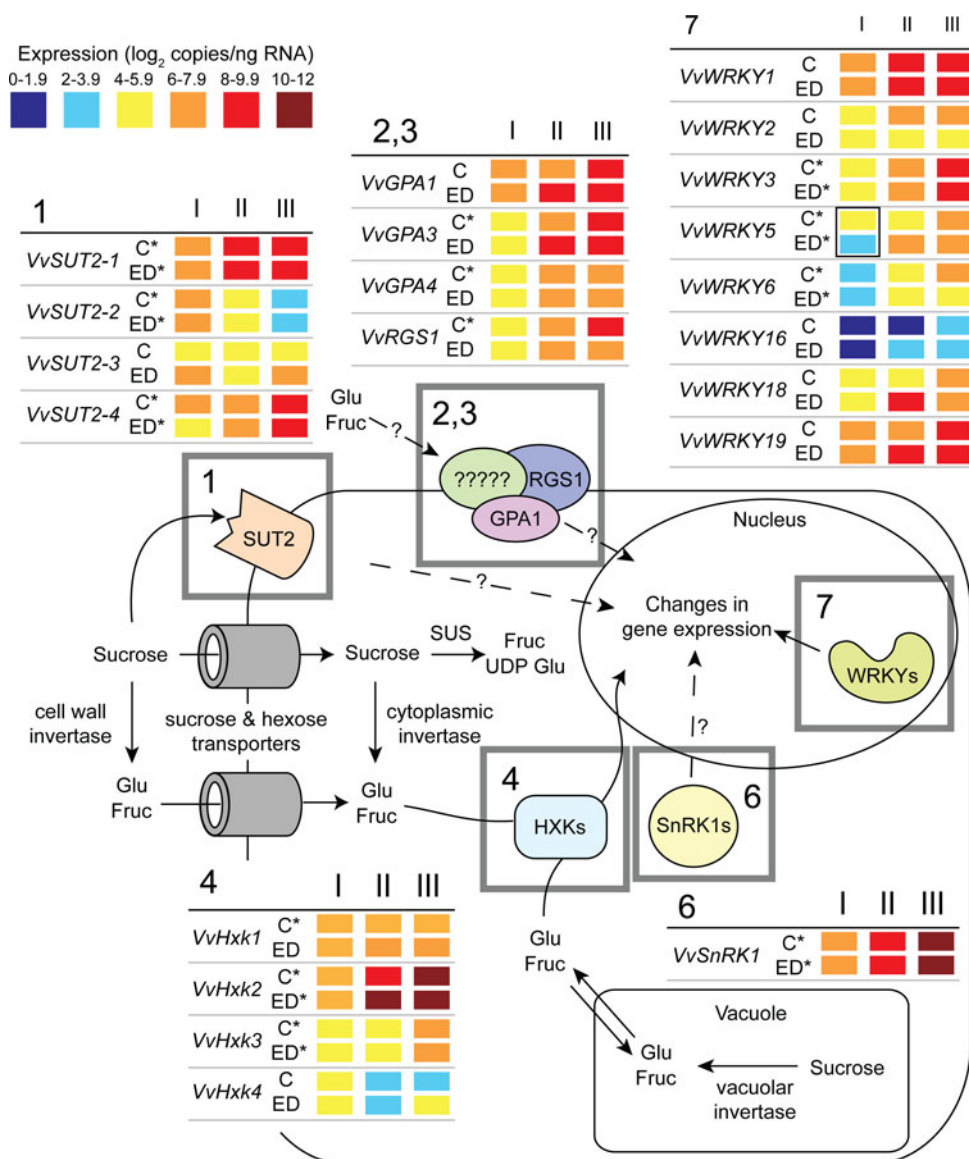
higher levels in ED. Both exhibited statistically significant two to fourfold greater levels of expression in green berries at the onset of ripening.

Responses of immature berries to sugar and ABA in berry culture

In order to more directly test the effects of sugar and ABA on the onset of ripening, immature Cabernet Sauvignon berries were harvested from the field at 61 DAA (T_0) and cultured in the presence of various combinations of sucrose and ABA until 84 DAA, a period of 23 days. The onset of ripening in the clusters from which these berries were collected in the field occurred at approximately 73 DAA, therefore the cultured berries were collected approximately 12 days prior to the onset of ripening.

Ripening phenomena were induced in berry culture when treated with sucrose and ABA as evidenced by changes in color, softening, and gene expression. Berries treated with 10% sucrose and various ABA concentrations changed color while those treated with 2 or 10% sucrose alone remained green (Fig. 6a). 200 μ M ABA and 2% sucrose + 200 μ M ABA treatments were included in our

Fig. 4 Summary of the gene families involved in sugar signaling examined in this work. Components are presented in their proposed positions in the signaling network. Gene families are 1 the putative SUT2 sucrose sensor, 2, 3 core G-protein signaling components GPA1 and RGS1, 4 hexokinases (*Hxk*), 6. Snf1-related kinases (*SnRK1s*), 7 WRKY transcription factors. The expression patterns of each gene in both control (C) and water deficit (ED) are summarized across three developmental stages I prior to the onset of ripening, II at the onset of ripening, and III late in ripening (See Supplemental File 1 for more detailed data). Asterisks denote significant changes in expression with time ($P < 0.05$, ANOVA), and boxes denote significant differences between treatments ($P < 0.05$, Tukey's HSD). Mean level of expression in \log_2 copies/ng RNA are expressed categorically as false color according to the legend above ($n = 3$). Signaling network figure adapted from Rolland et al. (2006)

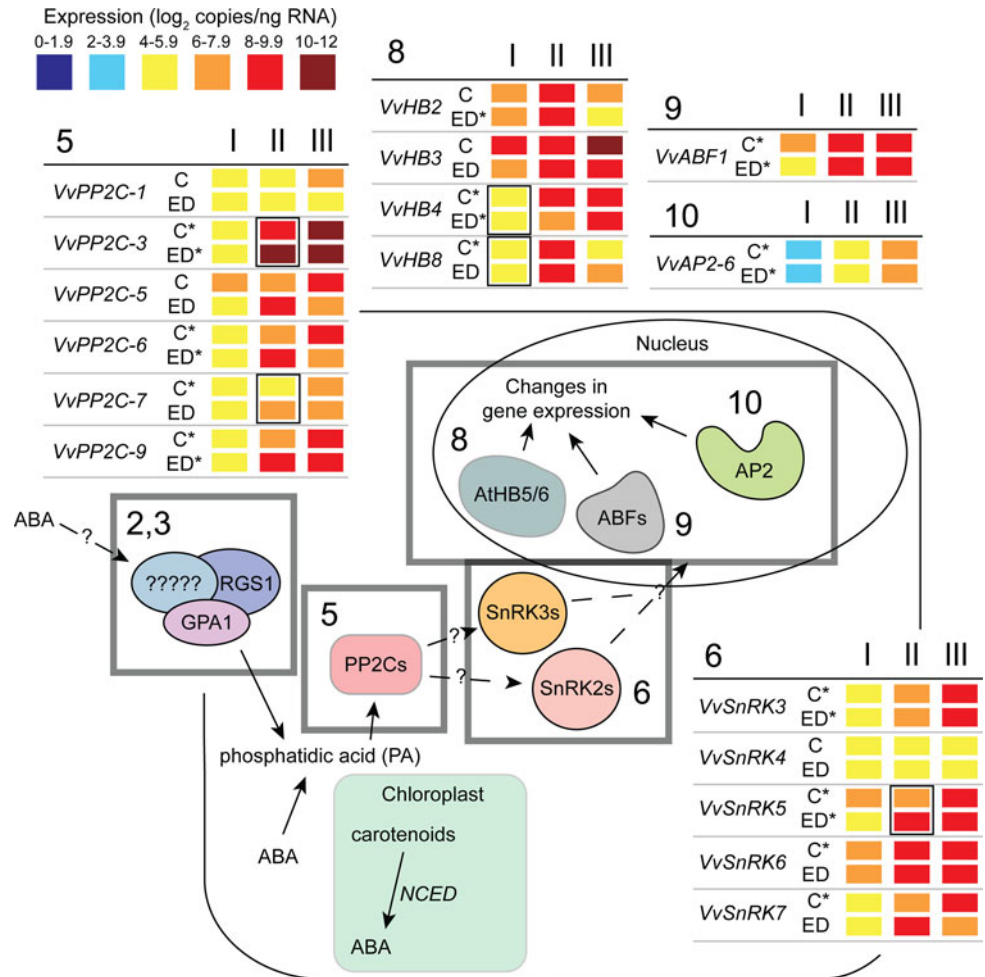


analyses but yielded no results because of a phenomenon where the berries exploded reproducibly (Suppl. Fig. 5). On average, cultured berries gained weight over the culturing period (Fig. 6a). Sucrose treatments of 2 and 10% showed the greatest weight gains corresponding to gains of 21 and 8%, respectively. Berries cultured in the presence of 10% sucrose with the addition of various ABA concentrations showed average weight gains of approximately 4%. Previous studies have found that a precipitous drop in grape berry elasticity (a measure of fruit softening) occurs just prior to the onset of ripening in grape (Thomas et al. 2008; Wada et al. 2009). In our current culture experiments, berry elasticity remained equal to that at T_0 in the 2 and 10% sucrose treatments, while sharply decreasing (95% of elasticity at T_0) with ABA treatment (Fig. 6b). Finally, the grape Myb transcription factor *VvMybA1* was

utilized as a molecular marker for the onset of ripening. *VvMybA1* is responsible for activating anthocyanin biosynthesis and has a distinct pattern of expression; being absent prior to the onset of ripening at which time it is strongly up-regulated (Kobayashi et al. 2002). In berry skins, *VvMybA1* expression was completely absent from the 2 and 10% sucrose treatments and was strongly up-regulated in the 10% sucrose + ABA treatments as expected (Fig. 6c).

We hypothesized that orthologs of gene families regulated by sugar and ABA, whose expression was strongly up-regulated at the onset of ripening and advanced under ED, would be regulated similarly by sugar and/or ABA in cultured berries. To test this, changes in the expression of *VvHB4*, *VvHB8*, *VvPP2C-3*, and *VvPP2C-6* were investigated in skins of cultured berries. In the field, all genes

Fig. 5 Summary of the gene families involved in ABA signaling examined in this work. Components are presented in their proposed positions in the signaling network. Gene families are 2, 3 core G-protein signaling components GPA1 and RGS1, 5 PP2C protein phosphatases, 6 Snf1-related kinases (*SnRK2s* and 3s), 8 homeobox (*HB*) transcription factors, 9 ABF transcription factors, 10 AP2 transcription factors. The expression patterns of each gene in both control (C) and water deficit (ED) are summarized across three developmental stages I prior to the onset of ripening, II at the onset of ripening, and III late in ripening (See Supplemental File 1 for more detailed data). Asterisks denote significant changes in expression with time ($P < 0.05$, ANOVA), and boxes denote significant differences between treatments ($P < 0.05$, Tukey's HSD). Mean level of expression in \log_2 copies/ng RNA are expressed categorically as false color according to the legend above ($n = 3$). Signaling network figure adapted from Hirayama and Shinozaki (2007)



were strongly up-regulated at the onset of ripening and advanced under ED (Fig. 7, left column) and in berry culture, expression was strongly induced in the presence of 10% sucrose + ABA when compared with treatments of 2 and 10% sucrose alone (Fig. 7, right column). Among those genes analyzed, the magnitude of induction in the field versus in berry culture was variable. For example, when data from Fig. 7 was expressed as fold change both *VvHB4* and *VvPP2C-3* were induced 10-fold from 57 to 74 DAA in the field compared to 6- and 40-fold in culture, respectively.

Discussion

The transcriptional data in this study demonstrate that numerous sugar and ABA-signaling orthologs are expressed during ripening in grape, and identify novel candidates in the control of nonclimacteric fruit ripening. Several genes exhibited patterns of expression correlating with sugar and ABA accumulation at the onset of ripening in field-grown fruit. Changes in color, softening, and gene

expression analogous to the onset of ripening in the field were induced in berry culture when treated with sucrose and ABA, demonstrating their role in controlling the onset of ripening. This study shows that many orthologous sugar and ABA-signaling components are regulated in fleshy fruit similar to their regulation originally characterized in model systems across diverse processes.

Sugar and ABA-signaling orthologs in fruit

These genes are easily delimited through nesting the currently available grape sequences comprising a gene family within their corresponding family in *Arabidopsis*. However, the current grape genome assembly, and its annotated proteome, certainly does not identify all the genes present in the grape genome so our analyses most likely failed to identify some orthologs.

In the current study, we chose to use QPCR in our expression analyses instead of the current grape microarray for several reasons. First, the majority of the genes analyzed here are not present on the current Affymetrix *Vitis vinifera* gene chip since the chip was derived from ESTs

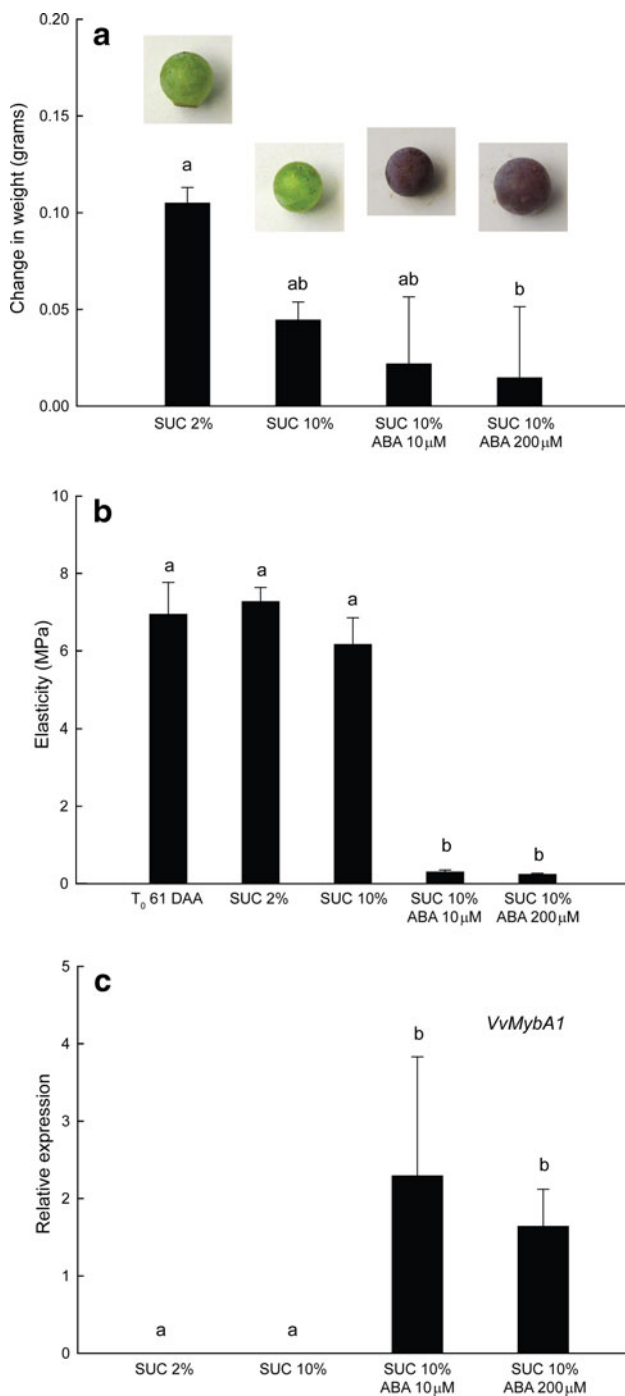


Fig. 6 Changes in immature berries cultured with various sugar and ABA treatments. **a** Change in berry weight over the course of treatment from T_0 (61 DAA) until 84 DAA. Photos of a representative berry from each treatment shown above. **b** Berry elasticity at T_0 and at the end of treatments 84 DAA. **c** *VvMybA1* gene expression at 84 DAA. Values are means with bars representing SE ($n = 3$). Different letters signify significant differences (**a** $P < 0.05$, **b**, **c** $P < 0.01$; Tukey's HSD)

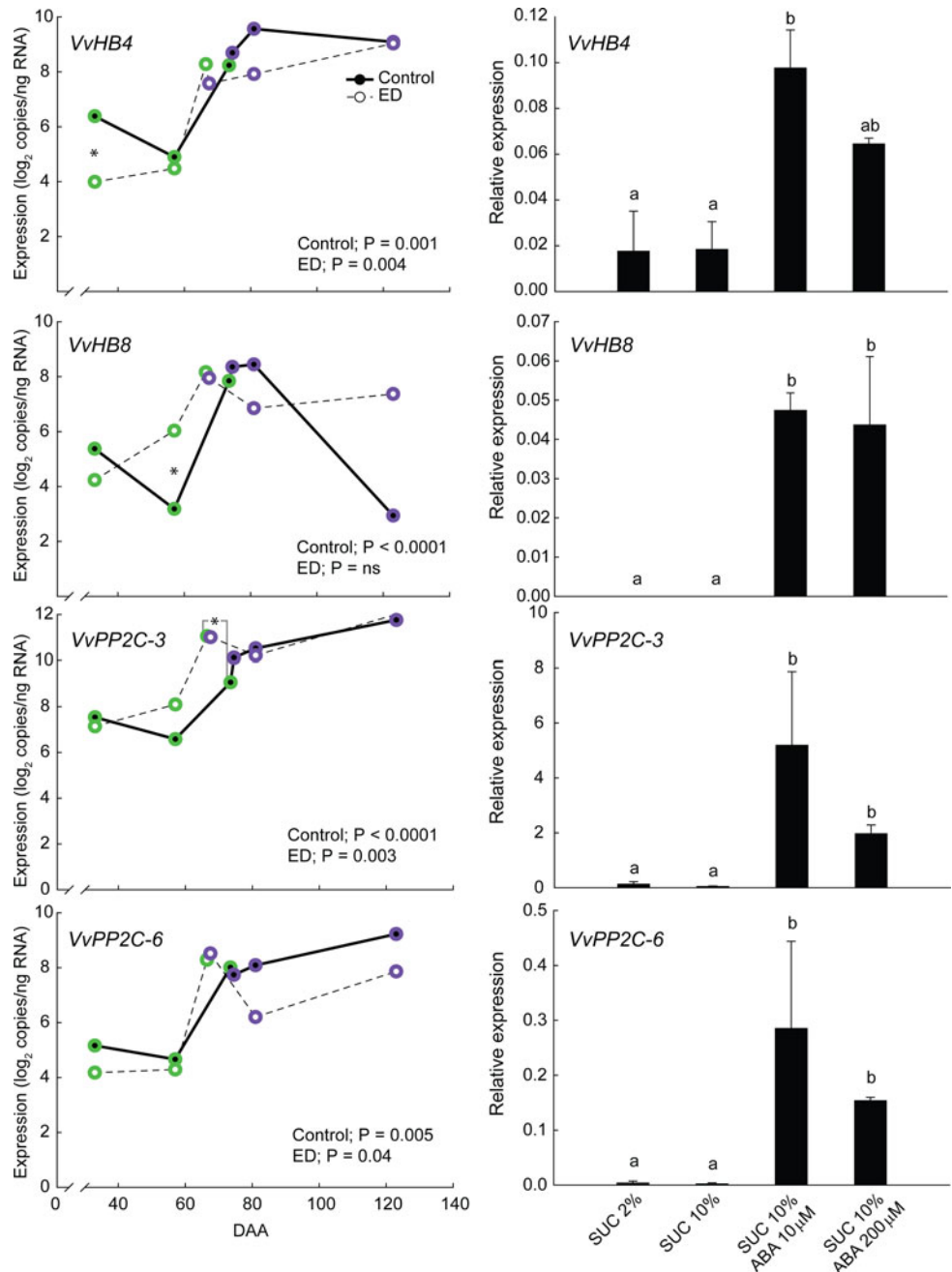
and the present study utilizes the complete genome. Second, microarrays suffer from several limitations one of which is that they are particularly insensitive in quantifying

low abundant transcripts (reviewed in Draghici et al. 2006). Transcription factors are a largely low abundance transcripts, and a study in *Arabidopsis* comparing QPCR and Affymetrix microarray approaches found that the microarray could detect <55% of 1,400 transcription factors tested, compared to >85% via QPCR (Czechowski et al. 2004). Furthermore, cross-hybridization is common on microarrays, which is especially problematic when considering conserved gene families like those examined here. However, those genes represented on the chip were identified and expression profiles were compared with those determined via microarray analyses in two recent studies. Notably, Koyama et al. (2009) demonstrated via microarray that a *VvHB* transcription factor (Probeset ID 1613946_s_at; Affymetrix *Vitis vinifera* gene chip), identical to *VvHB8* in this study, is also up-regulated at the onset of ripening and in response to exogenous sugar and ABA. For several other genes, expression profiles in the current study are nearly identical to those found by Deluc et al. (2007).

Many grape orthologs of genes shown previously to be modulated by sugar and/or ABA in model systems exhibited expression patterns during ripening, and in response to water deficit, consistent with their modulation by sugar and/or ABA in grape. More specifically, these genes are induced at the onset of ripening and induced earlier, and to higher levels, under water deficit. This characteristic pattern of expression is shared with many flavonoid pathway genes (Fig. 8) (Castellarin et al. 2007a), and these correlations in expression throughout ripening suggest common regulatory mechanisms. Experiments in berry culture demonstrated that several sugar and ABA-signaling orthologs, and *VvMybA1*, a transcriptional activator of anthocyanin biosynthesis, are up-regulated by exogenous ABA in the presence of high sucrose. These data suggest that sugar and ABA play a predominant role in regulating the expression of a suite of genes at the onset of ripening, including those responsible for anthocyanin biosynthesis and components of their own signaling pathways.

These results have interesting evolutionary implications demonstrating that some orthologs are consistently regulated by sugar and ABA across diverse developmental processes. Land plants, in general, have undergone abundant gene duplication through their evolutionary history (Doyle et al. 2008), although there is debate on the exact nature and timing of these events across angiosperms and in grape specifically (Jaillon et al. 2007; Velasco et al. 2007). Gene duplication is considered cornerstone to providing the raw material for evolution. A duplicate gene, now no longer essential, can undergo changes in its structure and/or regulation allowing for it to take up a novel role. The results of this study show that some of the Group A PP2Cs and Class I HBs have maintained their ABA

Fig. 7 Expression of select *VvHBs* and *VvPP2Cs* in the field (left column) and in berry culture (right column). Left column field-grown berries under control (solid lines, closed circles), and deficit irrigation (dotted line, open circles). Points are colored to denote berry color. Samples collected at the onset of ripening berries were pooled according to color and points offset slightly in graph for clarity. *P* values are given for ANOVAs of expression with DAA (by treatment) and points at which there are significant differences between treatments are noted with an asterisk ($P < 0.05$; Tukey's HSD, $n = 3$). Right column expression in berry culture under various treatments. Values are means with bars representing SE. Different letters denote significant differences ($P < 0.05$; Tukey's HSD, $n = 3$)



responsiveness during fruit ripening in grape. Fleshy fruit are a relatively recent evolutionary innovation (Doyle 1998; Eriksson et al. 2000). At least with regard to ABA responsiveness, the nature of regulation has been conserved, but co-opted into a completely different developmental context. This may provide for the discovery of novel *cis*-regulatory elements through promoter sequence comparisons across species.

In grape, advances in elucidating molecular mechanisms suffer from a lack of transgenic and related technologies on which most reverse genetic studies are based. This study demonstrates that model systems can provide fundamental

knowledge and insight into function of other agronomically important plant species even in extremely divergent developmental processes. Equally, this suggests that *Arabidopsis*, with its wealth of tools available for facilitating reverse genetic studies, may provide a valuable system to characterize genes of interest from grape or other crop species. Already there are several examples of the successful characterization of grape genes in *Arabidopsis* and tobacco (Bogs et al. 2007; Huguency et al. 2009; Poupin et al. 2007). This could prove especially useful considering the limitations of functional genetic analyses in perennial fruit crops where long propagation times are

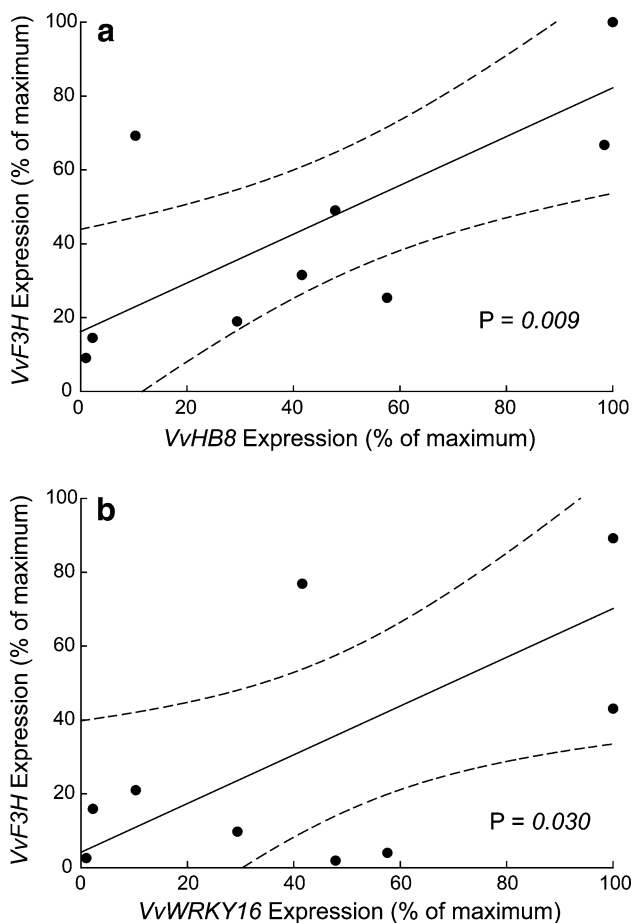


Fig. 8 Similarities in expression patterns in response to water deficit as represented by *F3H*, and *VvHB8* and *VvWRKY16*. Correlations between the expression of Flavanone 3-hydroxylase (*F3H*) from Castellarin et al. (2007a) and **a** *VvHB8* and **b** *VvWRKY16* from this study across all times and treatments, expressed as percent of maximum to equate scaling. Dotted lines represent 95% confidence intervals and regression *P* values are given

prohibitive. Future studies should include attempts to complement ABA and sugar signaling mutants in *Arabidopsis* and other model species with their orthologs implicated in ripening.

Water deficit, ABA, and acceleration of ripening

Changes in expression at the onset of ripening occurred earlier in ED than in controls. Previous work in our laboratory also demonstrated that ED advanced the onset of ripening, apparent from an earlier onset of color development and corresponding induction of flavonoid genes, and accelerated accumulation of anthocyanins and sugars after the onset of ripening (Castellarin et al. 2007a). Other studies examining the effects of water deficit have not found a temporal advancement in the onset of ripening, but did find that water deficits accelerated anthocyanin

accumulation after the onset of ripening (Castellarin et al. 2007b; Deluc et al. 2009). That some studies have not found a temporal advancement in the onset of ripening may reflect differences in the nature of the applied water deficits. ED fruit in the current study was subject to water deficit for more than a month prior to the onset of ripening, while fruit in Castellarin et al. (2007b) and Deluc et al. (2009) was subject to water deficit for only 1–2 weeks prior to the onset of ripening. Alternatively, the weekly sampling interval used in Castellarin et al. (2007b) and Deluc et al. (2009) may have missed the acceleration as the onset of ripening occurs over ~7 days. Regardless, these studies show that differences in fruit composition brought about by water deficit are established in a very short period of time surrounding the onset of ripening whether through advancement in the onset of ripening, accelerated accumulation, or both.

At this time, there is no direct evidence demonstrating that changes in ripening brought about by water deficit are indeed mediated by ABA. However, several studies demonstrate a strong correlation between the timing of the onset of ripening and ABA. In this study, berry culture treatments demonstrate that ABA, in the presence of high sugar, can bring about many of the changes associated with onset of ripening in grape. Wheeler et al. (2009) brought about an advancement in the onset of ripening through the repeated application of ABA to immature field-grown Cabernet Sauvignon, and Deluc et al. (2009) demonstrated that the acceleration in ripening in response to water deficit was correlated with increases in ABA. Without more sophisticated genetic studies, the evidence for ABA's role in mediating the effects of water deficit on fruit development remains corollary.

Berry culture and onset of ripening

Several lines of evidence suggest that changes in berries cultured with exogenous sucrose and ABA parallel changes in field-grown fruit at the onset of ripening. Softening, color development, and changes in gene expression (e.g. *VvMybA1*) associated with onset of ripening in were brought about by culture on high sucrose (10%) + ABA, but not with low or high sucrose treatments alone. The 2 and 10% sucrose concentrations used in this study approximate sugar concentrations of berries prior to (3–4°Brix) and at (>6–7°Brix), the onset of ripening in the field (Matthews et al. 1987; Matthews and Anderson 1988). ABA concentrations in the culture media match (10 μ M) or exceed (200 μ M) peak ABA concentrations (approximately 4–6 μ M) at the onset of ripening in the field (Owen et al. 2009; Wheeler et al. 2009). The induction of color development in the high sucrose + ABA treatments was not surprising given the strong evidence for sugar and ABA induction of

anthocyanin biosynthesis both in model systems (Loreti et al. 2008; Solfanelli et al. 2006) and in grape cell culture (Hiratsuka et al. 2001; Larronde et al. 1998; Matsushima et al. 1989; Pirie and Mullins 1976). Cultured berries grew and increased Brix at a rate similar to field-grown berries for the range of Brix represented by 2–10% sucrose. Similarly, the elasticity of both field-grown and cultured berries decreased from approximately 6 to 1 MPa or less from 61 to 84 DAA (Wada et al. 2009). The parallels in patterns of gene expression are striking. Those genes strongly up-regulated at the onset of ripening in control and ED field-grown fruit were similarly regulated between high sucrose, and high sucrose + ABA treatments (Fig. 7 and Suppl. Fig. 6). Other genes, such as *VvSUT2-1* were up-regulated at the onset of ripening in the field, while in culture they were up-regulated in response to sugar only (data not shown). Furthermore, other genes constitutively expressed during ripening in field experiments exhibit the same pattern of expression in culture (data not shown). Koyama et al. (2009) also demonstrated that, in culture, exogenous ABA brings about changes in gene expression analogous to the onset in ripening in the field.

Decrease in elasticity in response to exogenous ABA suggests that ABA treatment resulted in decreases in mesocarp cell turgor, although turgor was not measured directly in this study. Berry elasticity is tightly correlated with mesocarp cell turgor (Thomas et al. 2008). The decrease in elasticity in culture is attributable to a decrease in the $\Delta\Psi_s$ between the symplast and apoplast (Wada et al. 2008, 2009), and/or a decrease in cell wall rigidity through loosening of the cell wall, and not to desiccation because the berries grew during culture. In fruit, there is evidence that ABA plays a role in both of these processes through the stimulation of various invertases (Koyama et al. 2009; Pan et al. 2005; Pan et al. 2006) and cell wall-modifying enzymes (Koyama et al. 2009; Lohani et al. 2004; Parikh et al. 1990). The large weight gain, swelling of the mesocarp tissue, and berry cracking in low sucrose + ABA treatments, supports a role for ABA in stimulating cell wall loosening. This could be an interesting focus of future study.

In the introduction, we discussed the idea that components of regulatory networks can be conserved across disparate developmental processes that share commonalities. Sugar and ABA signals act across diverse processes (seed dormancy and germination, cessation of growth, stomatal control, the onset of ripening). Assuming that sugar and ABA signals work to control similar physiological processes in these different contexts what common physiology underlies their action? One strong candidate is the control of turgor, which is intimately connected to germination (Bradford 1990; Welbaum et al. 1990), stomatal control,

the onset of ripening (Thomas et al. 2006; Wada et al. 2008, 2009), and the control of growth.

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