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RESEARCH PAPER

Fruit ripening in *Vitis vinifera*: spatiotemporal relationships among turgor, sugar accumulation, and anthocyanin biosynthesis

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Abstract

This study reports the first observations indicating the spatiotemporal relationships among genetic and physiological aspects of ripening in the berry of *Vitis vinifera.* At the onset of ripening in the red flesh variety Alicante Bouschet, colour development began in the flesh at the stylar end of the fruit and progressed toward the pedicel end flesh and into the skin. Tissue solute potential and cell turgor also decreased first in the flesh. The decrease in flesh solute potential was due to accumulation of sugars, glucose and fructose, an accumulation that is integral to ripening. Expression of the anthocyanin biosynthesis-related genes *VvMybA* and *VvUFGT* was linearly related to the decrease in solute potential. Expression of *VvMybA*, and to a lesser extent *VvUFGT*, was correspondingly low in green tissue, higher in the red, stylar end flesh of berries beginning to ripen, and greatest in red berries. In contrast, expression of the abscisic acid biosynthesis-related genes *VvNCED1* and *VvNCED2* was not correlated with the other spatiotemporal aspects of the onset of ripening. These results, together with earlier work showing that sugar accumulation and acid loss also begin in the stylar flesh in other varieties, indicate that ripening in the grape berry originates in the stylar end flesh.

Key words: Capillary electrophoresis, cell pressure probe, flavonoid, gene expression, grape, non-climateric, tissue, veraison.

Introduction

Grapevine is economically the most important fruit crop in the world, but understanding of the control of berry ripening in this non-climacteric fruit lags behind that of several other, mostly climacteric, fruit crops (Giovannoni, 2004, 2007). Ripening in grape begins ~60 days after anthesis (DAA) and involves a coordinated shift in fruit development leading to softening, the accumulation of sugars and anthocyanins, and the resumption of growth, among other ripening processes. The onset of ripening, called veraison by viticulturists, is identified by the transition from green to red skin in berries of red grape varieties. The genetic control of ripening is not fully understood. Elucidating these mechanisms would be valuable for breeding improved varieties that achieve optimal ripening characteristics in different cultivation environments.

Research has been directed at the onset of ripening in what appears to be a coordinated process. Coombe and Phillips (1982) showed that softening (as measured by 'deformability'), sugar accumulation, and an increase in abscisic acid (ABA) were coincident at the onset of ripening. Findlay *et al.* (1987) also reported that softening, sugar accumulation, and colour change were coincident, and that these changes preceded the resumption of growth by 5–7 d. However, Matthews and Shackel (2005) found that several days before colour development, daily contraction decreased by 80% and nightly expansion increased by 50% compared with

Abbreviations: ABA, abscisic acid; DAA, days after anthesis; Ct, cycle threshold; P, cell turgor; *VvMybA*, *Vitis vinifera* MybA transcription factor; *VvNCED*, *Vitis vinifera* 9-cis-epoxycarotenoid dioxygenase; *VvUFGT*, *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase; Ψ_s, solute potential.

pre-veraison. They concluded that it was the resumption of growth and altered diurnal water relations in the berry that were early events in the transition from unripe to ripening. Subsequently, Thomas *et al.* (2008) and Matthews *et al.* (2009) showed that cell turgor (P) decreased and fruit softening (as measured by elasticity) began much earlier than previously recognized, ~10 d prior to rapid sugar accumulation and berry expansion. Thus, there is evidence of a separation in time of some components of the ripening process in grape.

In addition to identifying the timing of the onset of the many metabolic changes involved in ripening, it is important to know where to look for these changes. Because softening, sugar accumulation, and anthocyanin accumulation have traditionally been thought to be coincident at the onset of ripening, it has been implicit that ripening begins simultaneously in the skin, where colour accumulates, and in the flesh, where sugar accumulates. However, work has shown that decreases in P in the flesh precede sugar and anthocyanin accumulation (Thomas *et al.*, 2008; Matthews *et al.*, 2009). Still, the spatiotemporal relationship between these events remains unresolved.

Transcriptomic analysis of the grapevine genome has revealed that the onset of ripening involves the induction of genes related to several pathways such as the synthesis of secondary metabolites, sugar transport into the berry, and cell wall metabolism (Goes da Silva *et al.*, 2005; Terrier *et al.*, 2005; Deluc *et al.*, 2007; Pilati *et al.*, 2007; Lund *et al.*, 2008; Zenoni *et al.*, 2010). Nevertheless, what triggers this important transition in non-climacteric fruits is still unknown. In climacteric fruit, the onset of ripening can be controlled exogenously with ethylene and other effectors. Many studies have implicated sugar and ABA as endogenous signals regulating the onset of ripening in grape (Davies *et al.*, 1997; Atanassova *et al.*, 2003; Cakir *et al.*, 2003; Conde *et al.*, 2006; Lund *et al.*, 2008; Wheeler *et al.*, 2009; Gambetta *et al.*, 2010; Giribaldi *et al.*, 2010; Sun *et al.*, 2010). ABA increases at the onset of ripening (Davies *et al.*, 1997; Owen *et al.*, 2009), and there is evidence that this may arise through transient increases in the expression of ABA biosynthetic genes coding for the 9-*cis*-epoxycarotenoid dioxygenases (*NCED1* and *NCED2*) (Castellarin *et al.*, 2007; Lund *et al.*, 2008; Wheeler *et al.*, 2009; Sun *et al.*, 2010). Previous work demonstrated that exogenous sugar and ABA alone result in softening and anthocyanin accumulation in berry culture (Gambetta *et al.*, 2010). However, other hormones have been implicated in grape ripening processes such as auxins (Davies *et al.*, 1997; Bottcher *et al.*, 2010), brassinosteroids (Symons *et al.*, 2006), and ethylene (Chervin *et al.*, 2004; Sun *et al.*, 2010).

Ripening-related softening in fleshy fruit has been extensively studied (e.g. Harker *et al.*, 1997) and, while softening is typically attributed to changes in cell wall properties (e.g. Li *et al.*, 2010), recent work (Thomas *et al.*, 2008) has demonstrated that in grape, softening largely results from decreases in P. Direct measurements of P are rare, but similar results in tomato (Shackel *et al.*, 1991; Saladie *et al.*, 2007) and apple (Tong *et al.*, 1999) suggest that decreases in P may serve as a primary mechanism of softening in many fleshy fruits.

With respect to anthocyanin accumulation in grape, it is well documented that transcription factors of the *VvMybA* family regulate the expression of *VvUFGT*, which encodes an enzyme responsible for conversion of anthocyanidins to anthocyanins that accumulate in the vacuole (Ford *et al.*, 1998; Kobayashi *et al.*, 2004; Walker *et al.*, 2007). *VvUFGT* expression is strictly related to the activation of the anthocyanin pathway (Boss *et al.*, 1996*a*, *b*; Kobayashi *et al.*, 2001; Castellarin and Di Gaspero, 2007), hence it can be used as a molecular marker to discriminate the berry ripening stage with respect to colour development. In the berry skin of the red winegrape varieties, *VvMybA* is up-regulated at the onset of ripening (Kobayashi *et al.*, 2002; Castellarin and Di Gaspero, 2007). The focus on the role of *VvMybA* may oversimplify the nature of the regulation of anthocyanin biosynthesis in grape. In all other model systems studied, the regulation of anthocyanin accumulation, both spatially and developmentally, results from a cooperative, essential interaction between WD40 proteins, basic helix–loop–helix (bHLH) transcription factors, and Myb transcription factors (Ramsay and Glover, 2005). However, in a recent study, most of the natural diversity in the anthocyanin content observed across many grape cultivars was explained by the allelic variations at a single gene cluster that encompassed three *VvMybA* genes (Fournier-Level *et al.*, 2009).

In the vast majority of the cultivated grapevine varieties, anthocyanins are synthesized only in the skin. Alicante Bouschet is a Teinturier winegrape variety, varieties which are atypical in that they accumulate anthocyanins in the flesh as well (Ribéreau-Gayon *et al.*, 2004). Alicante Bouschet is used in winemaking to enhance the colour of red wines, in investigations of the genetic relationships between grape cultivars (Cabezas *et al.*, 2003), and in studies on the phenolic composition of the fruit (Castillo-Munoz *et al.*, 2010), but never to understand the ripening process itself. This study arose from an observation that colour began to accumulate in the flesh in the variety Alicante Bouschet before colour began to develop in the skin. Transcriptome, proteome analyses indicate that in common winegrape varieties, skin and flesh tissue differ greatly (Grimplet *et al.*, 2007, 2009). Older work also shows differences in solute accumulation between skin and flesh (Possner and Kliewer, 1985; Coombe, 1987; Iland and Coombe, 1988). Here the spatiotemporal relationships among sugar accumulation, turgor, and anthocyanin biosynthesis in skin and flesh of Alicante Bouschet were investigated.

Materials and methods

Plant material

Grape berries (*V. vinifera* L. 'Alicante Bouschet') were sampled from field-grown vines located in the Variety Collection Block of the Department of Viticulture and Enology facility at the University

of California, Davis, CA, USA (38°32' N latitude and 121°46' W longitude, elevation 18 m above sea level). The anthesis date was noted as the day on which 50% of the cluster was flowering, with time measured as days after anthesis (DAA). Samples of Green, Transition (just turning red), and Red berries were randomly collected from several clusters of different plants. Green and Transition berries were sampled at the beginning of fruit ripening as indicated by colour change, 52 DAA, and Red berries in the middle of the ripening process, 92 DAA. Berries were carefully trimmed off the cluster at the pedicel with a pair of scissors and placed into labelled plastic zip-top bags. Care was taken to avoid physical damage. For P measurements, berries were gently excised at the pedicel and immediately placed into small aluminized mylar zip-top bags to prevent water and P loss (Thomas *et al.*, 2008). Berries were immediately placed into a Styrofoam box at ambient temperature and transported to the laboratory.

Three berries for each class were longitudinally sectioned and analysed with a dissecting microscope. For Green and Transition berries, six replicates of five-berry samples were analysed for berry weight, solute potential, glucose, fructose, and total soluble solid concentration. Another three replicates of four-berry samples were stored at –80 °C for further RNA extraction and gene expression analysis. For Red berries, six replicates of one-berry samples were analysed for berry weight, solute potential, glucose, fructose, and total soluble solid concentration. Another three replicates of one-berry samples were stored at –80 °C for further RNA extraction and gene expression analysis. For solute potential, glucose and fructose concentration, total soluble solid concentration, and RNA extraction, berries were deseeded and peeled as described below and skin tissue was analysed separately from flesh tissue. Additionally, for the Transition berries, the green part of the flesh was separated from the red part of the flesh and analysed independently.

Tissue solute potential and sugars

Samples for solute potential measurements were processed under saturating humidity inside a box to avoid water loss from the tissues during the dissection (Boyer, 1995). Berries were deseeded and peeled with a scalpel. Skin and flesh tissues were placed in 2 ml tubes, immediately frozen in liquid nitrogen, and stored at –80 °C. In order to avoid contamination of the skin with flesh sap, skins were gently blotted with a Kimwipe before storing in the tube. For Transition berries, flesh was visually inspected and the green part was separated from the reddened part. Skin and flesh aliquots were immediately frozen under liquid nitrogen and stored at –80 °C until analysis. The tissue solute potentials from skin and flesh tissues were measured with the supernatant of fluid sap obtained by centrifuging at 2000 *g* for 10 min after thawing at 25 °C. An aliquot of 10 µl was used to determine tissue solute potentials (5500 Vapor Pressure Osmometer, Wescor Inc., Logan, UT, USA).

Total soluble solids were quantified on a separate aliquot of supernatant with a hand-held refractometer (Reichert A2R200, ReichertGmbH, Seefeld Germany) and reported as °Brix. The precise concentration of fructose, glucose, and sucrose was quantified using an Agilent capillary electrophoresis (CE) system (G1600AX, Agilent Technologies, Germany) according to Soga and Imaizumi (2001) and Wada *et al.* (2008). In short, separations were carried out on fused silica capillaries preconditioned for 5 min by flushing with Agilent basic anion buffer. The sample was injected with a pressure of 50 mbar for 6 s, the applied voltage was set at –30 kV, and the capillary temperature was thermostated to 15 °C. The detection wavelength was set at 350 nm for constant signal wavelength and at 230 nm for reference wavelength. Unknown peaks were identified by co-electropherogram with internal standard solutes.

Gene expression analysis

Berries for RNA extraction were peeled with a scalpel while still frozen and the obtained tissues, skin and flesh, were stored at –80 °C until RNA extraction. For Transition berries, flesh was visually inspected and the green part was separated from the reddened part. Total RNA was extracted from 0.1–0.3 g of tissue following the procedure described in Iandolino *et al.* (2004) and treated with 0.5 U μg^{-1} RQ1 DNase (Promega Corporation, Madison, WI, USA). First-strand cDNA was synthesized using ~0.5–2 µg of RNA, 0.5 µM (dT)18 primer, and 50 U of M-MLV reverse transcriptase (Promega Corporation). Quantitative real-time PCR was carried out in an ABI PRISM 7500 sequence detector (Applied Biosystems, Carlsbad, CA, USA). Each reaction (20 µl) contained 250 nM of each primer, 5 µl of 1:10 diluted cDNA, and 10 µl 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. Dissociation curves for each amplicon were then analysed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. No evidence for any primer dimer or other non-specific product formation was detected for any of the primer pairs used. Each PCR was run in duplicate within the same plate, and the cycle threshold (Ct) values obtained from the technical replicates were averaged. Gene transcripts were quantified by comparing the Ct of the target gene with that of *VvUbiquitin1* (Bogs *et al.*, 2005). Gene expression was expressed as mean and standard error calculated over the three biological replicates. Primer pairs for *UFGT* were retrieved from Goto-Yamamoto *et al.* (2002), and for *VvMybA*, *VvNCED1*, and *VvNCED2* from Castellarin *et al.* (2007).

In situ *cell turgor (P) measurements*

For P measurements, three one-berry samples of Transition berries were collected at 52 DAA. The cell pressure probe technique (Hüsken *et al.*, 1978) modified as described previously (Shackel *et al.*, 1987) was utilized to measure the P of individual cells in the berry mesocarp between depths of 100 µm and 2500 µm from the epidermis by using a Piezo-micro manipulator (PM-10, Stoelting Co., IL, USA). Microcapillary tips were prepared by a Koph 750 micropipette puller and were bevelled in a jet-stream of bevelling solution modified as described previously (Shackel *et al.*, 1987) to prepare 3–4 µm o.d. tips at the widest point of the bevelled portion. By looking at the flesh colour of Transition berries (see Fig. 1) from the outside, one or a few portions on the skin in each of berries, where the flesh colour either stayed green or was turning red, were gently marked with an ultra-fine black marker prior to the P measurements. Microcapillary tips were pointed at the mark to be inserted perpendicularly to the marked plane on the berry to determine P along a sequence of the cells. In some cases, in which a few marks were made, P measurements were conducted at each portion in the same fruit. After determining P at the first portion, the berry was immediately and precisely rotated one portion to another on the small apparatus as a berry holder (Thomas *et al.*, 2006) to point to the next portion, and immediately P in the next sequence of cells was determined. Preliminary experiments indicated that no significant difference in P are attributable to the order of the probing. All measurements were performed under laboratory conditions (diffuse fluorescent light, 25 °C air temperature, and localized 100% relative humididty obtained with a humidifier) and were generally completed within 3 h of detachment from the cluster depending on the sample size. Previous work has shown that the berry P does not significantly change for up to 48 h after being excised from the vine if water loss from the berry is prevented (Thomas *et al.*, 2006). The berries used for cell pressure probe experiments were cut in half along the direction in which the micropipette was introduced, identifying the marks on the berry skin. The berry sections were viewed with an

Olympus Vanox-AHBT (Olympus America, Melville, NY, USA) compound light microscope linked to a Pixera 600ES digital camera. The thickness of the berry skin was measured using image analysis software (NIH-Image Ver.1.61, National Institutes of Health, Bethesda, MD, USA).

Results

Initial observations

In the field most Alicante Bouschet berries were Green at the onset of ripening (Fig. 1A), but some had a slight red tint and red pedicel (Transition berries) (Fig. 1B). When examined under a dissecting microscope, it was clear that Green berries had green mesocarp and epicarp (Fig 1D), but in Transition berries incipient colour development was present, restricted to parts of the mesocarp, and had a predictable pattern. Red-coloured tissues originated in the stylar (distal) end flesh of Transition berries, near seeded ovules, transitioning to green tissues in the middle and pedicel (proximal) end flesh (Figs 1E, 5A). Later in development, after the onset of ripening, Red berries were dark red in both mesocarp and epicarp tissues (Fig. 1C, F). Because this observation might lead to insight into the geography and control of the onset of ripening, samples from meso- and epicarp tissues were collected and analysed for a variety of parameters associated with the onset of ripening including solute potential, P, glucose and fructose concentration, soluble solid concentration, and the expression levels of anthocyanin- and ABA-related genes.

At the first sampling, berries were harvested based on berry colour, leading to independent samples of fully Green and Transition berries (Table 1 and Fig. 1). At the second sampling, only Red berries were observed in the field and collected. Berry weight and soluble solids varied significantly among the three types of berry. Berry weight was lowest in Green berries and highest in Red berries (Table 1). Although still low in both Green and Transition berries, soluble solids in green berries were lower than in transition berries. Berry weight and soluble solids in Red berries were much greater than in the Transition berries.

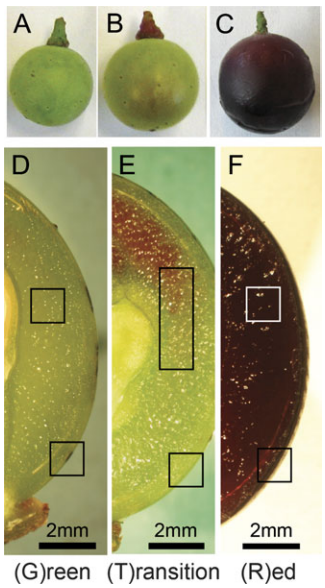

Fig. 1. Pictures of whole and dissected Alicante Bouschet berries at various ripening stages. Whole (A) Green, (B) Transition, and (C) Red berries. Dissected (D) Green, (E) Transition, and (F) Red berries with representative flesh and skin tissues boxed. Scale bars for D–F are 2 mm.

Table 1. Harvesting date, berry weight, and soluble solids of the Green, Transition and Red berries, Data are the mean ±SE (n=6).

Ripening stage	Sampling date	Berry weight (g)	Soluble solids (°Brix)
Green berry	7 July 2008 (52 DAA)	0.64±0.04	3.50±0.11
Transition berry	7 July 2008 (52 DAA)	0.98±0.03	4.20±0.11
Red berry	12 August 2008 (93 DAA)	2.09±0.17	18.50±0.76

Gene expression

VvMybA expression was detected at low levels in green tissues, and was always greater in red tissues (Fig. 2A). In flesh, *VvMybA* expression increased from Green to Transition green to Transition red to Red; and increases in flesh occurred before increases in skin. In Transition berries, *VvMybA* expression increased 2-fold from green to red flesh, and expression levels in the red flesh were ~5-fold higher than in the skins of the same berries. The expression pattern of *VvUFGT* was similar to that of *VvMybA* (Fig. 2B). Again *VvUFGT* expression was always higher in red than in green tissues. *VvUFGT* expression increased slightly in Transition red and greatly in Red flesh and in Red skin. Increases in expression were earlier in flesh than in skin.

VvNCED1 was expressed at extremely low levels during the first stages of ripening. In Red berries, *VvNCED1* was greatly up-regulated in the flesh but not in the skins (Fig. 2C). In contrast to *VvNCED1*, *VvNCED2* expression was greatest in Green berries and decreased through development in all tissues (Fig. 2D).

Tissue solute potential and sugar accumulation

Tissue solute potential decreased progressively in Green, Transition, and Red berries. These decreases were very slight between green and transition berries, and much greater from Transition to Red (Fig. 3A). In both Green and Transition berries, solute potential was higher in skins, approximately –0.6 MPa, than in flesh, (less than –0.80 MPa). This difference in skin and flesh solute potential was absent in Red berries. Glucose, fructose, and sucrose concentrations were quantified from the same samples used for solute potential measurements. Sucrose concentrations were zero, or negligible, for all samples measured. The sum of glucose and fructose concentrations reflected the pattern found for solute potentials, and were strongly correlated with tissue solute potential (Fig. 3B). Identical to decreases in tissue solute potential, sugar

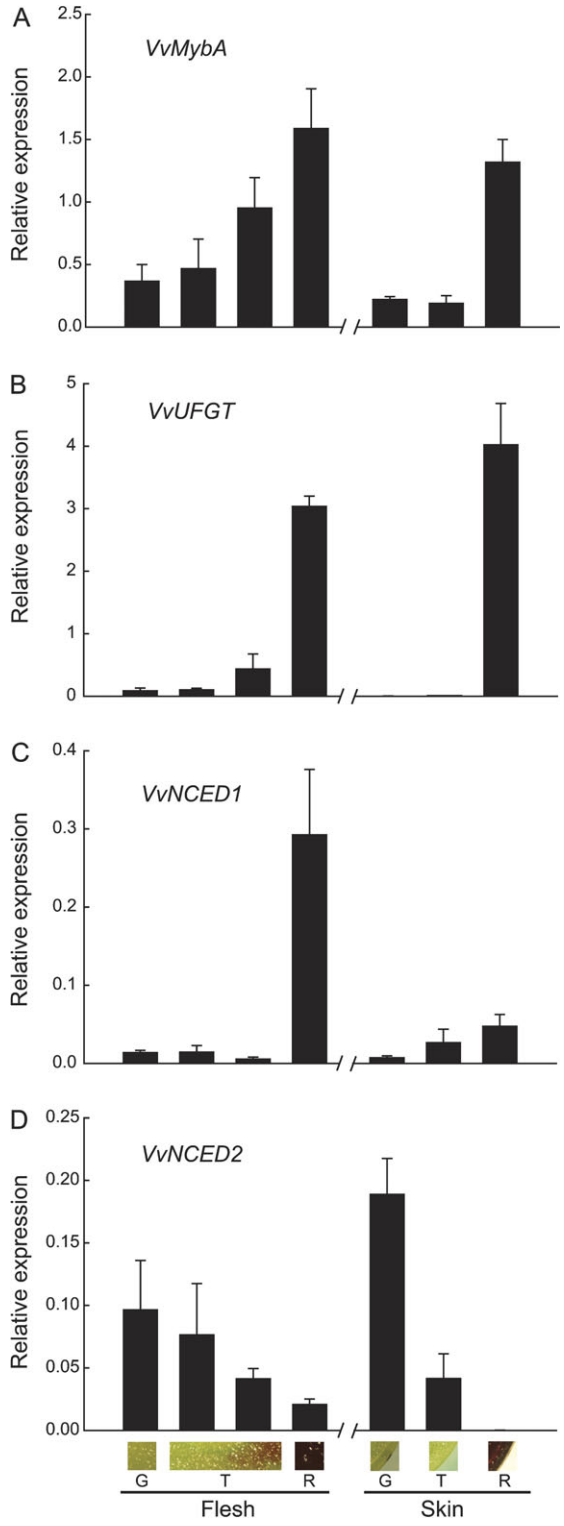

Fig. 2. Expression profiling of various ripening-related genes in flesh and skins of Alicante Bouschet berries around the onset of ripening. Expression profiles of the anthocyanin biosynthetic genes, (A) *VvMybA* and (B) *VvUFGT*, and of the ABA biosynthetic genes, (C) *VvNCED1* and (D) *VvNCED2*. Boxes on the *x*-axis report the analysed tissues with representative pictures from Fig. 1. Flesh and skins of G, Green; T, Transition; and R, Red berries. Data are the mean ±SE (n=3).

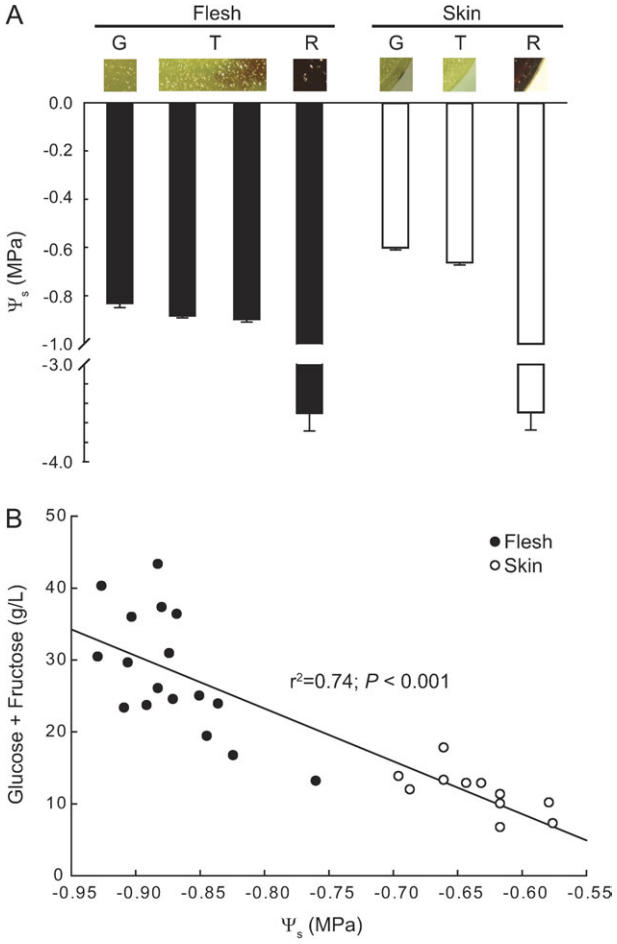

Fig. 3. Tissue solute potential and hexose concentration. (A) Skin and flesh tissue solute potential in G, Green; T, Transition; and R, Red berries. (B) Linear regression between glucose+fructose concentration and solute potential in flesh and skin tissues of Green and Transition berries. In Transition berries, green and red flesh were analysed separately. r^2 and one-way ANOVA significance value are presented.

concentrations were greater in flesh than skin tissues (data not shown).

VvMybA and *VvUFGT* expression levels were related to tissue solute potential (Fig. 4A, B). Across all data there is a strong relationship between the level of expression of *VvMybA* and *VvUFGT* and tissue solute potential. In green skin and flesh tissues, high tissue solute potentials are associated with low or undetectable levels of expression, while decreases in solute potential correlate with increases in expression levels. The same relationship was not found with *VvNCED1* and *VvNCED2* (data not shown).

Cell turgor (P)

P was measured in both green and red regions of Transition berries as shown in Fig. 5. In Transition berries the average cell P was ~0.37 MPa in the green region (Fig. 5A, B Loc1) and decreased from 0.35 MPa to 0.18 MPa as the probing locations approached the stylar end of the berry, where the flesh turned red (Fig. 5A, B). When P data are considered as

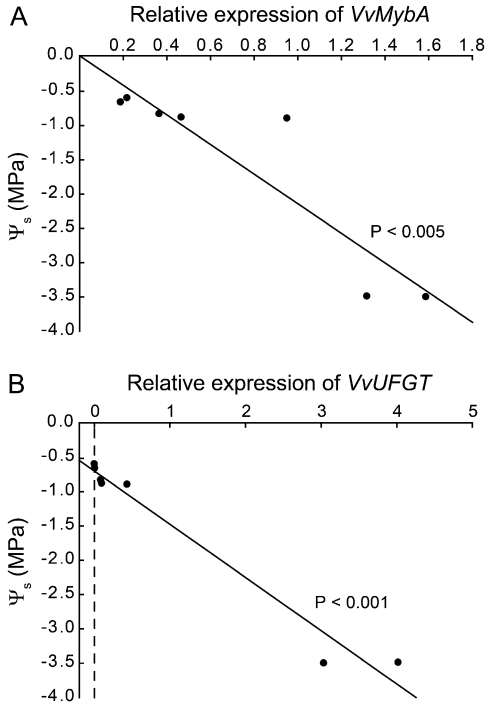

Fig. 4. Relationship between tissue solute potential (Ψ_s) and gene expression. Linear regression between Ψ_s and the magnitude of (A) *VvMybA* and (B) *VvUFGT* gene expression across all data. One-way ANOVA significance values are presented.

a function of probing depth, P was greater in the epicarp than in the mesocarp and tended to decrease with increased probing depth (Fig. 5C).

Discussion

Organ development in plants is typically initiated at a specific location and time, and the observations and data in this study show that in Alicante Bouschet, ripening originates in the flesh, more specifically in the flesh near the stylar end of the berry. The most important observation is that when fruit colour begins to develop in Transition berries, it begins in this tissue region and not in the skin. The spatiotemporal behaviour of several other genetic and physiological characteristics of the onset of ripening conformed to this interpretation. The decrease in P, increase in sugar concentration, and increase in anthocyanin-related gene expression begin in the flesh before being detected in the skin. These observations establish a geography of ripening for more refined studies of the control of ripening in the grape berry.

Expression patterns of *VvMybA* and *VvUFGT* were consistent with ripening beginning in the stylar flesh where colour originates. *VvMybA* expression in flesh increased from the lowest values in Green berries to Transition green to Transition red, to the highest values in Red berries, and these increases occurred before any increase in the skin. *VvUFGT* was not expressed in the skin of Green and Transition berries; expression increased slightly in Transition red, and greatly in Red flesh and in Red skin. As with *VvMybA*, the increase in expression was earlier in flesh than in skin. In previous work, *VvMybA* expression was always coupled with *VvUFGT* expression and anthocyanin biosynthesis (Kobayashi *et al.*, 2002; Castellarin and Di Gaspero, 2007). In this work, *VvMybA* was up-regulated in Transition green tissues prior to detectable *VvUFGT* expression and anthocyanin accumulation, representing an early molecular marker for colour development.

There was also a characteristic pattern of colour development in the rachis and berry pedicel, in which the

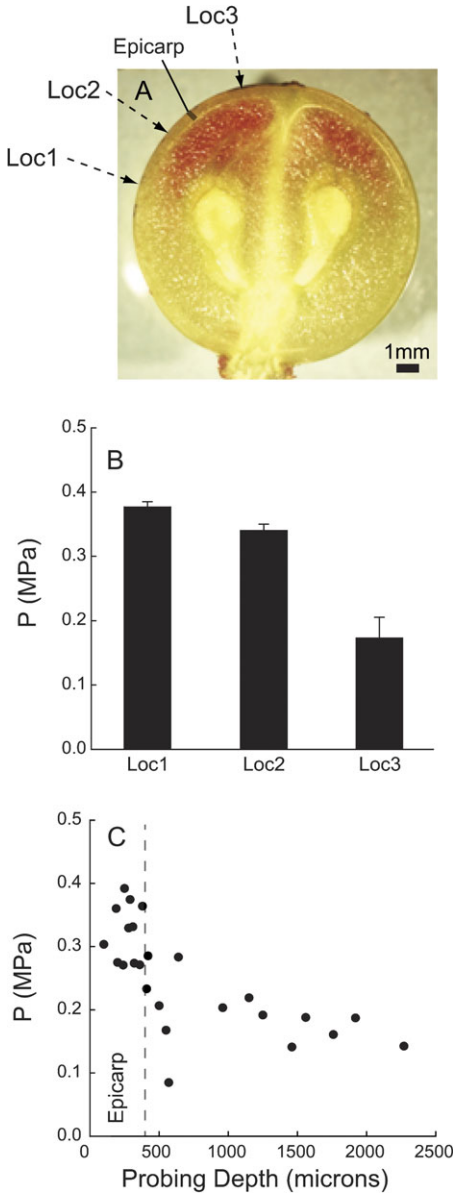

Fig. 5. *In situ* measurements of cell turgor pressure (P) in green and red sections of Transition berries. (A) Cross-section of a probed berry (taken after probing) showing the three locations which were probed: Loc1–Loc3. The scale bar is 1mm. (B) Average P ±SE across all depths in each location probed. (C) Relationship between probing depth and P across all data. Epicarp depth is denoted by the dotted line and is represented visually in A.

rachis began to change colour prior to the pedicel, which changed colour prior to the berry (data not shown). This pattern of colour change could represent an increase in exogenous stimulatory factors arriving via the phloem, whose influx increases ~10-fold at the onset of ripening (Greenspan *et al.*, 1994). Alternative explanations, such as long-distance translocation of anthocyanins, seem unlikely. It was once erroneously thought that in red flesh varieties anthocyanins leak from the skin into the flesh after the fruit become ripe (Winkler, 1973). In red flesh varieties all the molecular regulation of anthocyanin synthesis operates in the flesh as in the skin (Jeong *et al.*, 2006). Indeed, Jeong *et al.* (2006) showed that the mechanism that underlies anthocyanin accumulation in the flesh is the same as that in the skin, and is based on the up-regulation of anthocyanin biosynthetic genes via factors that include *VvMybA*.

Sugar has been shown to stimulate anthocyanin accumulation across numerous flowering plants such as *Arabidopsis* (Solfanelli *et al.*, 2006; Loreti *et al.*, 2008), corn (Straus, 1959), and grape (Pirie and Mullins, 1976; Larronde *et al.*, 1998; Hiratsuka *et al.*, 2001; Gollop *et al.*, 2002). In this study, expression of both *VvMybA* and *VvUFGT* exhibited a linear relationship with tissue solute potential. The decrease in solute potential as fruit developed was overwhelmingly due to accumulation of sugars in this and other studies (Thomas *et al.*, 2008; Wada *et al.*, 2009). Significant amounts of sucrose (10%), in addition to ABA, are necessary to bring about the onset of ripening in berry culture (Gambetta *et al.*, 2010). These levels of sucrose would correspond to –0.84 MPa of solute potential at 37 °C according to Michel (1972), a value strikingly similar to levels found in transition tissues in the current study (Fig. 3A). Further, many components of sugar signalling pathways function at the onset of ripening (Vitrac *et al.*, 2000; Conde *et al.*, 2006; Gambetta *et al.*, 2010). These results suggest that sugar accumulation is tantamount to decreasing solute potential and is integral in the regulation of colour development, and perhaps other processes of ripening.

Other early aspects of ripening include a decrease in P (Matthews *et al.*, 2009; Wada *et al.*, 2009) and a dramatic increase in ABA concentration (Davies *et al.*, 1997; Deluc *et al.*, 2009; Owen *et al.*, 2009; Wheeler *et al.*, 2009). In this study, cell P was highest in skin cells, intermediate in green flesh, and lowest in Transition red flesh, and *VvNCED2* expression decreased similarly. *VvNCED1* expression was low in all tissues except red flesh, in which there was a sharp increase. Thus, these analyses also showed that the transition to ripening originated in the stylar flesh. The decrease in P is implicated in the increase in ABA, via either activation of ABA synthesis or enhanced ABA influx via the phloem. Many studies have investigated the expression of the *VvNCED* genes, reasoning that if the increases in ABA result from its biosynthesis in the berry, this would be reflected in an up-regulation of the rate-limiting *VvNCED* genes. Indeed, as in the current study, one or both *VvNCED* genes are consistently up-regulated at the onset of ripening in grape (Castellarin *et al.*, 2007; Deluc *et al.*, 2007, 2009; Lund *et al.*, 2008; Wheeler *et al.*, 2009). However, there does not appear to be a specific pattern with respect to the regulation of individual isogenes (Castellarin *et al.*, 2007; Lund *et al.*, 2008; Deluc *et al.*, 2009), nor a strict correlation with measured ABA concentrations (Deluc *et al.*, 2009; Wheeler *et al.*, 2009). The interpretation that increases in *VvNCED* expression are causally connected with an increase in ABA is problematic because the *NCED* genes have been shown to be up-regulated in response to ABA itself (Wheeler *et al.*, 2009; Koyama *et al.*, 2010; Sun *et al.*, 2010). Therefore, it is still not clear what causes the initial increases in ABA in the berry tissues: *in situ* ABA biosynthesis, or exogenous ABA arriving from other plant organs. This question can only be resolved through intensive sampling at the onset of ripening.

The data suggest that the decrease in P leading up to veraison was greater in the mesocarp than in the epicarp, and this is further evidence that the epicarp and mesocarp follow different developmental trajectories (Schlosser *et al.*, 2008). In an earlier study, a decrease in P as a function of depth from the berry surface was observed pre-veraison but not at a transition stage in Chardonnay berries (Thomas *et al.*, 2006). However, Thomas *et al.* (2006) apparently sampled later in berry development than the Transition berries in this study. Although there may be fundamental differences among varieties, it is speculated that the Alicante Bouschet data reported in Fig. 5 represent a transient difference between tissues that had already passed in the berries investigated in Thomas *et al.* (2006). These data on Alicante Bouschet are supported by similar observations recently obtained in our laboratory in berries of wine and table grape varieties sampled at a similar developmental stage (data not shown). The suggested decrease in mesocarp P at the onset of veraison, as distinct from the epicarp cells, is consistent with the other data in this study showing that veraison originates in the mesocarp.

In this study it was shown that in the red flesh variety Alicante Bouschet, ripening originates in the stylar end flesh. Alicante Bouschet was a useful genotype in order to study the relationships between sugar accumulation, gene expression, and colour development because colour developed in both the flesh and the skin, but not simultaneously. Early work on berry ripening includes three studies in which berries were dissected and tissues analysed for sugars, acids, etc. Results in each of these studies were consistent with ripening beginning in stylar end flesh. Loss of malate began in flesh before skin (Iland and Coombe, 1988) and in stylar flesh before pedicel end flesh (Possner and Kliewer, 1985). In an intensive dissection of Muscat of Alexandria berries, Coombe (1987) reported large differences between skin and flesh in the accumulation of sugars. However, a re-examination of the data shows that the largest difference among his 18 berry sections was in the sugar concentration of flesh in the stylar end compared with that in flesh in the pedicel end. Hexose concentration was consistently different between skin and flesh only in the stylar end, where the data were similar to what is reported here for Alicante Bouschet. Thus, onset of sugar accumulation and acid loss apparently begins in stylar end flesh.

Unfortunately, contemporary 'omic'-level studies are not helpful in discerning the spatiotemporal nature of ripening in grape because all but two studies utilized whole berries, and in the two cases where skins and flesh were separated only a single sampling date was analysed (Ageorges *et al.*, 2006; Grimplet *et al.*, 2007).

The present data indicate that the ripening in *V. vinifera* starts in the stylar flesh and proceeds back towards the pedicel end and outwards to skin cells where colour development is normally observed. This basipetal pattern of ripening is not without its parallels in dicot plant development. *Arabidopsis* siliques senesce basipetally (reviewed in Roeder and Yanofsky, 2005), almost all aspects of simple dicot leaves develop in a basipetal fashion (e.g. Turgeon, 1989; Nelson and Dengler, 1997; Donnelly *et al.*, 1999), and several aspects of leaf senescence also proceed in a basipetal fashion (Thimann *et al.*, 1974; Paschalidis and Roubelakis-Angelakis, 2005). These parallels could be an interesting focus of future study given the evolutionary homology of the leaf, carpel, and fleshy fruit.

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