Cellular expansion and gene expression in the developing grape (Vitis vinifera L.)

J. Schlosser 2, N. Olsson 2, M. Weis 1, K. Reid 2, F. Peng 2, S. Lund 2, P. Bowen 1

1 Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, British Columbia
2 Faculty of Land and Food Systems, University of British Columbia, Vancouver, British Columbia

Received 26 January 2005; Accepted 2 April 2007; Published online 18 April 2008
© Springer-Verlag 2008

Summary. Expression profiles of genes involved in cell wall metabolism and water transport were compared with changes in grape (Vitis vinifera L.) berry growth, basic chemical composition, and the shape, size, and wall thickness of cells within tissues of the berry pericarp. Expression of cell wall-modifying and aquaporin genes in berry pericarp tissues generally followed a bimodal expression profile with high levels of expression coinciding with the two periods of rapid berry growth, stages I and III, and low levels of expression corresponding to the slow-growth period, stage II. Cellular expansion was observed throughout all tissues during stage I, and only mesocarp cellular expansion was observed during stage III. Expansion of only exocarp cells was evident during transition between stages II and III. Cell wall-modifying and aquaporin gene expression profiles followed similar trends in exocarp and mesocarp tissues throughout berry development, with the exception of the up-regulation of pectin methylesterase, pectate lyase, two aquaporin genes (AQ1 and AQ2), and two expansin genes (EXP3 and EXPL) during stage II, which was delayed in the exocarp tissue compared with mesocarp tissue. Exocarp endo-(1→3)-β-glucanase and expansin-like gene expression was concurrent with increases in epidermal and hypodermal cell wall thickness. These results indicate a potential role of the grape berry skin in modulating grape berry growth.

Keywords: Veraison; Aquaporin; Expansin; Pectate lyase; Pericarp; Mesocarp; Vitis vinifera.

Abbreviations: AQ aquaporin; BG endo-(1→3)-β-glucanase; CEL cellulase, endo-(1→4)-β-glucanase; dpa days post-anthesis; EXP expansin genes; EXPL expansin-like gene; PL pectate lyase; PME pectin methylesterase; XET xyloglucan endotransglycosylase transferase.

Introduction

During grape berry development from fertilization to maturity, there are two distinct periods of rapid growth divided by a period of substantially slower growth, resulting in a double sigmoid growth pattern. Although transition from one period to another is continuous, the distinctiveness of the double sigmoid pattern allows viticulturalists to conveniently delineate berry growth into three stages: stages I and III demarking the two periods of rapid growth and stage II the intervening slow period (Coombe 1973, 1976).

During stage I, cells of the ovary undergo a series of mitotic divisions and cell expansion resulting in the formation of the berry skin and flesh, known collectively as the berry pericarp. In seeded varieties, stage I is also the period during which cell division and expansion occurs in the integument, nucellus, endosperm, embryo, and various other tissues of the seed (Pratt 1971). At approximately four to six weeks postanthesis, cell division in the berry pericarp ceases and cell expansion subsides as the berry enters stage II (Harris et al. 1968). The berry pericarp is hard, green, and highly acidic during this period, and the seed has reached its full size (Nitsch et al. 1960). The inception of ripening, known as veraison, occurs during the latter portions of stage II. At this time, berry acidity decreases, soluble-solid content increases, and the skin of the berry softens and in red varieties changes color. Veraison signals the transition of the berry into stage III, during which berry growth is entirely due to cell expansion (Coombe 1992).

Fruit growth is dependent upon both cell division and expansion, and fruit development at the cellular level has been documented in several species, including apple (Blampied and Wilde 1968, Steudle and Wieneke 1985, Goffinet et al. 1995), peach (Scorza et al. 1991, Zanchin et al. 1994), cucumber (Marcelis and Hofmaneijer 1993),
kiwifruit (Hallett et al. 1992), melon (Higashi et al. 1999), and blueberry (CanoMedrano and Darnell 1997). Previous histological examinations of *Vitis vinifera* berry anatomy have used light, scanning, and electron microscopy on several cultivars, including Muscat Gordo Blanco (Consideine and Knox 1979), Traminer and Sauvignon blanc (Hardie et al. 1996), Delaware (Shiozaki et al. 1997), Sultanana (Harris et al. 1968), Granache (Nii and Coombe 1983), Pinot noir (Raijae 1987), Cabernet Sauvignon (Diakou and Carde 2001), Cabernet Franc (Chevalier et al. 2003), and Bacchus and Madeleine (Staudt et al. 1986). These investigations have shown that the pericarp of the grape berry consists of different tissue types that exhibit exclusive structural and cytological features throughout berry development. Hardie et al. (1996), for instance, described the skins of Traminer and Sauvignon blanc berries as consisting of thick-walled epidermal and hypodermal cells containing plastids, polyphenols, anthocyanins, and starch. Mesocarp cells of the flesh, on the other hand, have thin cell walls and become devoid of polyphenols with the progression of berry development. Duration of cell division has been observed to cease earlier amongst tissues of the flesh than amongst those of the skin (Pratt 1971). Variation in cell expansion rate and direction has been found to be tissue specific (Huang and Huang 2001).

The primary cell wall of plants is a complex structure of cellulose microfibrils cross-linked by hemicelluloses and embedded in a matrix of pectin polysaccharides. In grapes, although compounds from each of these groups of polysaccharides can be found throughout the berry pericarp, compositional differences have been observed between skin and flesh tissues. Doco et al. (2003), for instance, observed that skin and flesh tissues vary in xyloglucan composition, whereas Vidal et al. (2001) found a threefold higher concentration of rhamnogalacturonan II pectic polysaccharides in skin than in flesh tissues.

Expansion of cells requires a loosening of the primary cell wall and the synthesis and integration of new cell wall material. Loosening results from the disruption of chemical bonds between the structural components of the cell wall through either acidification or the action of hydrolyzing enzymes. Nunan et al. (2001) characterized the activity and mRNA expression of a number of cell wall-modifying enzymes and observed that β-galactosidase, α-galactosidase, pectin methylesterase, and pectate lyase expression and activity corresponded with changes in pectin polysaccharide composition of mesocarp cell walls during grape berry ripening. An increase in the expression of xyloglucan endotransglycosylase during ripening of Kyoho grape was similarly observed by Ishimaru and Kobayashi (2002). This increase in expression would coincide with depolymerization of xyloglucan, the major hemicellulose polysaccharide in dicotyledons, as observed before and during veraison by Yakushiji et al. (2001), indicating a possible role of xyloglucan endotransglycosylase in grape berry softening. Given their ability to hydrolyze (1→4)-β-D-linked glucan chains, members of the endo-(1→4)-β-D-glucanase family of proteins (EGase) can also hydrolyze xyloglucan as well as noncrystalline cellulose (Brummell and Harpster 2001). Although activity and expression data from studies of tomato and strawberry support a role of these enzymes in fruit growth (Brummell et al. 1997, Brummell and Harpster 2001), Nunan et al. (2001) failed to observe any expression and activity during grape berry ripening. Discussion of enzymes that elicit endo-(1→3)-β-glucanase activity generally focuses on antimicrobial activity, but recently an endo-(1→3)-β-glucanase was isolated and characterized from the pulp tissue of banana that may be involved in ripening and tissue softening (Peumans et al. 2000). This work may spur functional characterization of other enzymes, such as thaumatin-like proteins that are highly expressed in fruits such as grape (Tattersall et al. 1997) and can also exhibit endo-(1→3)-β-glucanase activity (Peumans et al. 2000).

Through its role in creation of turgor pressure, water is a key regulator of stage I and III growth. Considering that movement of water molecules into the vacuoles of cells requires passage through both the plasma and tonoplast membranes (Ollat et al. 2002), it is not surprising that interest has been growing in aquaporins found in plasma (PIP) and tonoplast (TIP) membranes, which facilitate water movement. Recently, the expression of two plasma intrinsic proteins was found to correlate with volume increases in apple fruit (Hu et al. 2003). In grapes, two PIP aquaporins have been identified, one of which was found to moderately increase water permeation.

With a few exceptions (Ishimaru and Kobayashi 2002, Picaud et al. 2003), previous studies of gene expression for grape cell wall-modifying enzymes have concentrated on the ripening period and the elucidation of genes responsible for berry ripening and softening. In this work, we investigated tissue-specific anatomical changes in grape pericarp cells, including cell dimensions and wall thickness, relative to changes in berry size, juice acidity, and soluble-solids content, beginning two weeks after anthesis and ending at commercial maturity. We then compared these developmental changes with expression of aquaporin, expansin, xyloglucan endotransglycosylase, cellulase/endo-(1→4)-β-glucanase, pectate lyase, and pectin methylesterase enzymes in the tissues.
Material and methods

Fruit sampling

Fruit was collected from four planted rows in a five-year-old block of Cabernet Sauvignon (clone 15) grafted on rootstock 101-14 at Vincor Canada’s Bull Pine Vineyard in Oliver, British Columbia, Canada. The vines were bilateral-cordon trained, spur pruned, and vertically shoot-positioned. In-row vine spacing was approximately 1 m. The rows were oriented north to south on a 2% slope with a 65° southern aspect. In each of the four rows, six grapevines were selected which had relatively straight trunks and at least six spurs on each of their two coronets.

In order obtain fruit from clusters that were nearly synchronous in development, clusters were selected according to the following criteria: the bearing shoots had similar leaf numbers and nutritional health and bore two clusters; and the selected clusters were in the distal position, east-facing, and at fifty percent anthesis when selected. Fruit was sampled eight times during the growing season, approximately every two weeks, on 2, 15, and 29 July, 11 and 22 August, and 4 October, corresponding to 15, 28, 42, 55, 71, 85, 99, and 112 days post-anthesis (dpa), respectively. To minimize the effect on fruit maturation of crop load reduction due to sampling, vines in each row were randomly spaced, divided into two groups sampled alternately, so that each vine was sampled monthly.

Metabolite analysis

On each sampling date, three clusters were removed from the preselected vines in each row, placed in plastic containers, and transported to the laboratory. Upon arrival, 50 berries were removed from each cluster, weighed, encased in cheesecloth, and crushed by mortar and pestle. The expressed juice was analyzed for titratable acidity by titrating a known volume of juice with 0.1 N NaOH with an autotitrator (655 Dosimat; Metrohm, Herisau, Switzerland). Percent soluble solids (°Brix) was measured with a digital refractometer (PR-101; Atago, Tokyo, Japan). Juice pH was measured with a standard pH meter (PHM82; Bachem Simpson Ltd., London, Ontario, Canada) and osmolality was measured with a freeze point depression osmometer (Advanced Micro-Osmometer Model 3300; Advanced Instruments, Norwood, Mass., U.S.A.).

Histology

For histological characterization, one berry from a cluster in each row was sampled, providing four berries per sampling time. A 1 mm thick section of tissue was cut from along the equatorial plane of each berry with two single-sided razor blades separated by a glass slide (Fig. 1). This section was placed immediately in glutaraldehyde (Ted Pella Inc., Redding, Calif., U.S.A.) diluted with 0.2 M phosphate buffer solution at pH 7.2 (Fig. 1). The glutaraldehyde concentration was adjusted to the freeze point depression osmometer (Advanced Micro-Osmometer Model 3300; Advanced Instruments, Norwood, Mass., U.S.A.).

Total RNA isolation and cDNA synthesis

Seeds were removed and exocarp and mesocarp tissues were separately sampled from partially thawed berries with forceps. The separated tissues were then quickly refrozen and the tissue was prepared for RNA isolation as follows: 2 g of tissue was ground to a fine powder in liquid nitrogen and placed in a 20 mL RNA later tube (Ambion, Austin, Texas). Three 1 mL aliquots of lysis buffer (RNeasy Plant Mini Kit; QIAGEN, Valencia, Calif., U.S.A.) were added, and the tissue was ground with a micropestle. The resulting suspension was incubated on ice for 5 min and then centrifuged at 18,000 x g for 5 min at 4°C. The supernatant was transferred to a fresh tube and 70% ethanol was added dropwise to bring the final volume to 1 mL. The suspension was then centrifuged at 13,000 x g for 5 min at 4°C. The supernatant was discarded and the remaining pellet was air-dried for 10 min. The RNA pellet was suspended in 100 μL DEPC-H2O and stored at -80°C.

Total numbers of cells used for calculation of cell area, cell width, and cell length are shown in Table 1; 40 measurements were used for the calculation of cell wall thickness for each time point.

<table>
<thead>
<tr>
<th>Days post-anthesis</th>
<th>Nr. of cells of tissue:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocarp</td>
<td>Hypodermis</td>
</tr>
<tr>
<td>15</td>
<td>149</td>
</tr>
<tr>
<td>28</td>
<td>127</td>
</tr>
<tr>
<td>42</td>
<td>105</td>
</tr>
<tr>
<td>55</td>
<td>99</td>
</tr>
<tr>
<td>71</td>
<td>84</td>
</tr>
<tr>
<td>85</td>
<td>96</td>
</tr>
<tr>
<td>99</td>
<td>105</td>
</tr>
<tr>
<td>112</td>
<td>74</td>
</tr>
</tbody>
</table>

Fig. 1. Diagrammatic representation of the grape berry sectioning procedure

Spurr resin, transferred to aluminum weighing dishes with fresh Spurr resin, and baked overnight at 65°C.

Sections, 1 μm thick, were taken from the embedded samples with a microtome (Ultracut E; Reichert-Jung, Vienna, Austria). The sections were stained with toluidine blue (Ted Pella Inc.) and mounted onto slides with a drop of Spurr resin. Images of sections from all samples were captured with a light microscope (Axioscop; Carl Zeiss International, Oberkochen, Federal Republic of Germany) equipped with a cooled charge-coupled-device camera (Retiga; Q Imaging, Surrey, British Columbia, Canada). For images of the outer mesocarp, bright-field optics and a 10× objective were used. Images of the hypodermis and epidermis were captured with bright-field optics and a 40× objective. Measurements of wall thickness and the area, radial width, and tangential length of cells were made by imaging software (Image Pro Plus; Media Cybernetics Inc., Bethesda, Md., U.S.A.) calibrated with a 10 μm stage micrometer. The outermost two cell layers were considered to be the epidermis, while the seven to eight cell layers immediately below the epidermis were considered to be the hypodermis (Hardie et al. 1996). Cells that fitted within the field of view of the 10× objective immediately below the dorsal vasculature were considered to be outer mesocarp.

Table 1. Number of cells used in the calculation of cell area, width, and length in each tissue type at each time point
and then added to 20 ml of extraction buffer prewarmed to 65 °C and containing 300 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% hexadecyltrimethylammonium bromide, 2% polyvinylpolypyrrolidone, 0.05% spermidine trihydrochloride, and 2% β-mercaptoethanol. Samples were incubated in a 65 °C water bath for 10 min with shaking. The mixtures were extracted twice with equal volumes of chloroform-isomylalcohol (24:1) followed by centrifugation at 3500 g for 15 min at 4 °C. The aqueous layer was transferred and further centrifuged at 30,000 g for 20 min at 4 °C. The supernatant was transferred to a new tube and RNA was precipitated at −80 °C for 30 min following the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol. The pellet was collected by centrifugation at 3500 g for 30 min at 4 °C and dissolved in Tris-HCl–EDTA. After addition of 0.3 volumes of 8 M LiCl, the mixture was incubated overnight at 4 °C followed by centrifugation at 20,000 g for 30 min at 4 °C. The next day, samples were centrifuged at 20,000 g for 30 min at 4 °C, and pellets were washed with ice-cold 70% ethyl alcohol, air dried, and dissolved in diethyl pyrocarbonate-treated water. RNA concentration and 260/280 nm ratios were determined before and after Dnase I digestion with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del., U.S.A.), and 1% agarose gels were run to visualize the integrity of the RNA. Total RNA was purified by an RNasey kit (Qiagen, Valencia, Calif., U.S.A.) with the addition of an on-column DNase digestion. cDNAs were synthesized from 2 μg of total RNA by the Superscript III First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, Calif., U.S.A.) with oligo(dT)15 primers, according to the manufacturer’s instructions. After cDNA synthesis, 1 μl of RNase H was added and the samples were incubated for 20 min at 37 °C. cDNA samples were stored at −20 °C until further analysis.

**PCR primer design and real-time PCR analysis**

Several common cell wall- and growth-associated genes were selected for expression analysis. Gene-specific primers were designed by Primer Express 2.0 software (Applied Biosystems, Foster City, Calif., U.S.A.) and had melting temperatures (Tm) of 56–60 °C, lengths of 20–24 nucleotides, and amplicon lengths of 67–141 base pairs (Table 2). Polymerase chain reactions were performed in optical 96-well plates with an ABI Prism 7500 sequence detection system (Applied Biosystems).

### Table 2. Selected genes and primers used for real-time PCR experiments with expected size for amplified fragments and the efficiency of the PCR reaction

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>GenBank accession number</th>
<th>A. thaliana ortholog locus</th>
<th>A. thaliana locus description</th>
<th>Forward/reverse primers</th>
<th>Product (bp)/efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXP1</td>
<td>EC969647</td>
<td>AT1G20190.1</td>
<td>expansin, putative (EXP1); alpha-expansin gene family expansin, putative (EXP8); alpha-expansin gene family expansin family protein (EXPL2); expansin-like gene expansin, putative (EXP4); alpha-expansin gene family glycosyl hydrolase family 17 protein, similar to beta-(1,3)-glucanase pectinesterase family protein</td>
<td>AAATGGGCACAAGGAGTTGATAG/ CTCAAATGGTGGCACAATGGA</td>
<td>73/1.98</td>
</tr>
<tr>
<td>EXP2</td>
<td>EC958403</td>
<td>AT2G40610.1</td>
<td>beta-(1,4)-glucanase 17 protein, similar to pectate lyase</td>
<td>AGGTTCTCGATGCTGATCACTACA/ AAACGAGGCGGCAGGTTGATG</td>
<td>73/1.95</td>
</tr>
<tr>
<td>EXP3</td>
<td>CB970308</td>
<td>AT2G39700.1</td>
<td>cellulose, similar to endo-beta-(1,4)-glucanase</td>
<td>TGCTGTTGCTGCTGCTAATG</td>
<td>70/1.83</td>
</tr>
<tr>
<td>PME</td>
<td>EC921503</td>
<td>AT3G43270.1</td>
<td>xyloglucan endotransglycosylase, putative</td>
<td>TATCCCTCTATGCCTGACTGGA/ AAGCCACCAAGGCTTGACATT</td>
<td>71/1.91</td>
</tr>
<tr>
<td>PL</td>
<td>EC929070</td>
<td>AT1G67750.1</td>
<td>cellulose, similar to endo-beta-(1,4)-glucanase</td>
<td>TGGTGTGGCTGCTGCTAATG</td>
<td>116/1.88</td>
</tr>
<tr>
<td>CEL</td>
<td>EC922445</td>
<td>AT1G64390.1</td>
<td>xyloglucan: xyloglucosyl transferase, putative; xyloglucan endotransglycosylase, putative</td>
<td>TGCCCTGCTGCTGCTAATG</td>
<td>69/1.79</td>
</tr>
<tr>
<td>XET</td>
<td>EC970173</td>
<td>AT2G36870.1</td>
<td>major intrinsic protein; MIP family membrane intrinsic protein 2B (PPIP2B); aquaporin PIP2.2 (PPIP2.2)</td>
<td>TGCCCTGCTGCTGCTAATG</td>
<td>74/1.81</td>
</tr>
<tr>
<td>AQ1</td>
<td>EC969647</td>
<td>AT2G36830.1</td>
<td>major intrinsic protein; MIP family membrane intrinsic protein 1A (PPIP1A); aquaporin PIP1.1 (PPIP1.1)</td>
<td>TGGACCCACCATGCTGACTGGA/ CATAGACCAAGGCTGACCATG</td>
<td>67/1.89</td>
</tr>
<tr>
<td>AQ2</td>
<td>EC969993</td>
<td>AT2G37170.1</td>
<td>major intrinsic protein; MIP family membrane intrinsic protein 1A (PPIP1A); aquaporin PIP1.1 (PPIP1.1)</td>
<td>TGGACCCACCATGCTGACTGGA/ CATAGACCAAGGCTGACCATG</td>
<td>90/1.93</td>
</tr>
<tr>
<td>AQ3</td>
<td>EC932586</td>
<td>AT3G61430.1</td>
<td>major intrinsic protein; MIP family membrane intrinsic protein 1A (PPIP1A); aquaporin PIP1.1 (PPIP1.1)</td>
<td>TGGACCCACCATGCTGACTGGA/ CATAGACCAAGGCTGACCATG</td>
<td>141/1.88</td>
</tr>
<tr>
<td>UBC</td>
<td>EC922622</td>
<td>AT1G64230.1</td>
<td>ubiquitin-conjugating enzyme</td>
<td>GAGGGTGCATGAGTGTGGA/ GCCCTGCATGCTGCTAATG</td>
<td>75/1.94</td>
</tr>
</tbody>
</table>

1 All grape sequences were named on the basis of their similarity to Arabidopsis thaliana proteins determined via BLASTX. In most cases, the name indicates only a gene family or subfamily rather than a specific member of a gene family, because partial grape sequences and BLAST will not necessarily identify the putative A. thaliana ortholog. Closest A. thaliana homolog was identified by TAIR BLAST 2.2.8 (http://www.arabidopsis.org/Blast/)
2 Database designations of matching TIGR gene index sequences and ortholog sequences from A. thaliana
using SYBR Green to detect double-stranded DNA synthesis. Reactions were done in a 20 μl total volume containing 200 nM (each) primer, 5 μl of cDNA (150-fold dilutions, corresponding to about 3 ng/sample) and 10 μl of 2X SYBR Green Master Mix reagent (Applied Biosystems). Aliquots of the same cDNA sample were used with all primer sets. The manufacturer’s recommended protocol and parameters were followed. PCR was carried out at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each PCR reaction was performed in triplicate and no-template controls were included. Dissociation curves for each amplicon were then analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. The PCR dissociation curve did not indicate amplification of multiple genes. Considering that primers were designed on limited expressed sequence tag data which may not have included all gene family members, amplification of similar family members that have yet to be sequenced and databased is still possible.

Real-time PCR data were analyzed using the SDS version 1.2.2 software (Applied Biosystems). Baseline data were collected between cycles 3 and 15, and all amplification plots were analyzed with an Rn threshold of 0.2 to obtain Ct (threshold cycle) values. LinReg software was used to calculate real-time PCR efficiency for each gene. This software uses absolute fluorescence data captured during the exponential phase of amplification of each real-time PCR reaction (Ramakers et al. 2003). This method, used by Czechowski et al. (2004), reduces the need for dilution curves. All primer pairs had amplification efficiencies higher than 1.80, except TU-CELa (cellulase), which had an efficiency of 1.79 (Table 2). Results from the SDS and LinReg analyses were imported into Microsoft Excel for correction of different PCR efficiencies and further analysis, as described by Pfaffl (2001). All Ct values were normalized to those of the reference gene UBC (ubiquitin conjugating enzyme).

Results

Berry growth and composition and changes in cellular anatomy

Accumulation of berry weight followed a double sigmoid curve with two periods of high growth rate indicative of developmental stages I (15–28 dpa) and III (71–99 dpa), separated by a slower growth period characteristic of stage II (28–55 dpa) (Fig. 2A). Titratable acidity of the juice increased during stage I and then decreased during stages II and III when soluble-solid accumulation and pH increased. These data are consistent with a typical growth pattern and
metabolite profile for grape berries (Coombe 1992) and indicate that normal berry development had occurred.

During stage I, when berry weight increased by over 250%, cells of the epidermis, hypodermis, and mesocarp increased in area by 15%, 33%, and 159%, respectively (Figs. 2B, 3, and 4). Cells of the mesocarp expanded in both the tangential and radial directions, while those of the epidermis and hypodermis expanded only in the tangential direction (Fig. 2C, D). From the end of stage I through to the end of stage II, there was little change in hypodermal and mesocarp cell areas, but there were reductions of about 20% in epidermal cell area and radial width (Fig. 2B, C). Concurrent with the increase in soluble solids during the latter portion of stage II, mesocarp cells decreased slightly in area and radial width, while large increases were observed in epidermal (34%) and hypodermal (44%) cell areas. The increase in epidermal cell area at this time was due to both tangential and radial expansion, while hypodermal cells expanded only radially. Through stage III, berry weight increased by more than 40% (Fig. 2A) and mesocarp cells expanded by approximately 30% both tangentially and radially, and 63% in area (Fig. 2B–D). Hypodermal and epidermal cells were similar in their growth pattern and gained and lost size inversely in comparison with mesocarp cells.

The thickness of epidermal cell walls increased by 82% through stage I to the beginning of stage II (Fig. 2E). A further 15% increase was observed at the beginning of stage III, which was followed by a decrease then increase in wall thickness before harvest maturity. From stage I through to the beginning of stage III, hypodermal cell walls thickened steadily and by 42%. There was an increase in the rate of thickening during stage III, adding 37% to the wall thickness, which was then followed by a 14% decrease.

Gene expression

Expression of all genes was compared to transcript concentrations at 15 dpa, a period of active cell division and
expansion. Expression of the three EXP genes and the EXPL gene investigated was down-regulated from stage I to stage II (Fig. 5). EXP1, EXP2, and EXPL were, however, subsequently up-regulated during the latter periods of stage II and into stage III, corresponding to the second period of active berry growth. EXP3 also showed a period of up-regulation during transition from stage II to III, but transcript levels were not observed to be as high as at 15 dpa. Generally, there was a trend of down-regulation amongst all the expansin genes two or three weeks before the attainment of maximum berry size and through to commercial maturity. With the exception of an earlier up-regulation of EXP3 and EXPL during stage II in the mesocarp tissue, very few differences were observed between the mesocarp and exocarp in expansin gene expression.

Xyloglucan endotransglycosylase transferase (XET) expression in exocarp and mesocarp tissues was severely down-regulated from 15 to 28 dpa but was up-regulated again during stage II and into stage III (Fig. 5). Pectin methylesterase (PME) expression levels were drastically up-regulated during stage II and continued at this level for the remainder of berry development. There was, however, a transient expression of PME in mesocarp tissue during the latter portions of stage III, which was not similarly evident in exocarp tissue. Endo-(1→4)-β-glucanase (CEL) expression in both the exocarp and mesocarp tissues was higher during the rapid period of berry growth during stages I and III compared with stage II and the latter portions of stage III. For pectate lyase (PL) and endo-(1→3)-β-glucanase (BG), different trends of expression could be seen in the exocarp and mesocarp. Most evident was a decrease in expression of both of these genes within the exocarp tissue between 42 and 55 dpa, while an increase in expression of PL occurred within the mesocarp.

Aquaporin (AQ) transcript levels generally decreased from 15 dpa through to commercial harvest (Fig. 5). Expression of AQ1 and AQ2, however, did exhibit a period of up-regulation coinciding with the transition of the berry from stage II to III. Expression profiles for AQ2 were consistent across both exocarp and mesocarp tissues, while the up-regulation of AQ1 during stage III appeared to be confined to the exocarp tissue.

Red berries sampled at veraison generally exhibited higher transcript levels than green berries sampled on the same day (Fig. 5). In the case of some genes, the higher transcript numbers in red berries were more pronounced in either exocarp or mesocarp tissues. For instance, exocarp tissue exhibited a larger increase in transcript levels for PME, AQ1, and CEL. In contrast, there was a larger increase in relative transcript levels of the EXPL gene in mesocarp than in exocarp tissue.
Discussion

Over the past few decades, our understanding of grape berry growth at the cellular level has been greatly improved through studies using histological and enzyme expression techniques. Here we have utilized both techniques simultaneously in order to correlate tissue-specific changes in berry cellular anatomy with changes in the expression of cell wall-modifying enzymes and enzymes involved in water transport. Of specific interest was the variability in cellular anatomy and gene expression during transition between the stages of berry development and within different tissue types (i.e., skin versus flesh).

Berry growth from cell expansion differed at the tissue level during stages I and III. Of primary difference was the lack of cellular expansion in epidermal and hypodermal tissues concurrent with the large increase in berry weight during stage III (71–99 dpa). Grape berry growth after ripening is therefore entirely dependent on mesocarp cellular expansion. The large increases in transcript numbers from stage II to III indicate that a number of the genes investigated here play a role in the second period of grape berry growth. Considering that trends were generally conserved between exocarp and mesocarp tissues, the individual activity of any of these enzymes is not likely to be responsible for the tissue-specific expansion of mesocarp cells during stage III. These results indicate that, despite large differences in the anatomy of cells walls between exocarp and mesocarp tissues, enzymes such as XET, PME, PL, and CEL carry out similar functions in skin and flesh tissues.

BG, EXP3, and EXPL showed an interesting expression trend in the mesocarp versus the exocarp between 71(r) and 85 dpa. During this rapid period of berry growth, the expression in the mesocarp decreased between the two sampling points, while it increased in the exocarp.
At present, there is little information pertaining to possible BG substrates within fruit, but it has been speculated that they are involved in fruit ripening (Peumans et al. 2000). Expansins, on the other hand, have exhibited the ability to extend cell walls rapidly through the disruption of noncovalent bonds that hold microcellulose fibrils together (Sampedro and Cosgrove 2005). The up- and subsequent down-regulation of the expansins EXP3 and EXPL in the exocarp at 85 and 99 dpa, respectively, corresponded to changes in cell wall thickness within epidermal and hypodermal tissue layers, indicating a role in cell wall loosening to accommodate expansion of the mesocarp tissues below.

The ability of epidermal tissues to control the expansion of underlying tissues has been previously documented in tomato (Thompson et al. 1998). Evidence that the berry skin can control post-veraison growth in grape has similarly been found (Matthews et al. 1987, Huang and Huang 2001, Huang et al. 2005). The expression results obtained here agree with the conclusions of these previous authors. There was evidence not only of specific expansin and BG expression trends in the mesocarp versus exocarp during stage III, but also of a lag in the up-regulated expression of exocarp PL, PME, and EXPL enzyme during stage II (42–55 dpa). Moreover, when these enzymes were up-regulated in the exocarp tissue during transition between stages II and III (between 55 and 71 dpa), a tissue-specific expansion of exocarp cells (both epidermal and hypodermal) was observed. Microbial PL enzymes have been shown to degrade pectins through a β-elimination reaction. In strawberry transgenic lines with repressed PL activity, there was a greater retention of fruit firmness indicating a role of this gene in fruit softening (Jimenez-Bermudez et al. 2002). PME enzymes de-esterify polygalacturonans after they are secreted from the Golgi in a highly methylesterified form. Such de-esterification can result in either increased wall stiffness through the promotion of calcium cross-linking of adjacent pectic chains or cell wall loosening by increasing the accessibility of hydrolases, such as polygalacturonases and pectate lyases, to their substrates (Bosch et al. 2005). Since PME was observed to increase in transcript number prior to PL, it is possible that activity of this enzyme may function in both cell wall stiffening and loosening within the grape berry.

Similar to stage III, stage I exhibited little variation in enzyme expression during cell expansion that resulted in substantial berry growth. Although all enzymes were down-regulated during this period, it should be kept in mind that transcript levels at 15 dpa were used to normal-ize the data acquired for each subsequent point of development. An appropriate reference point in studies such as these is difficult to choose given the constant changes in cell number and expansion that occur during stage I. The unfertilized ovary would possibly have been a better reference for characterization of stage I, considering that cell division and expansion took place at 15 dpa, and up-regulation of a number of the cell wall-modifying genes preceded berry expansion during stage III. However, the relative expression trends observed between the sampling stages would still be the same even if another reference point or tissue was used. It is likely that genes such as those encoding XET, PL, BG, CEL, and all of the expansins investigated in this study are involved in the first period of berry growth. Furthermore, exocarp cells lost volume by shrinking radially, signifying the development of pressure between exocarp and mesocarp tissues. It appears that grape berry skin is an influential factor in berry growth during this period.

With the discovery of aquaporins in plants, there has been a growing interest in their ability to affect water flow across plant membranes during fruit growth (Tyerman et al. 2002). Although previous research on pear (Shiratake et al. 2001) and peach (Sugaya et al. 2001) showed no correlation between tonoplast AQ expression and fruit growth, a good correlation was recently found between cell expansion and expression of a plasma membrane aquaporin in apple tissues (Hu et al. 2003). The aquaporins characterized in grape berries thus far are from a subgroup exhibiting low to moderate water transport activity (Picaud et al. 2003). All three V. vinifera plasma membrane aquaporin paralogs that we tested followed a bi-modal trend in transcript accumulation with the greatest expression found during stage I and at the onset of stage III, and the lowest during stage II and later stages of berry maturation. These results indicate, as would be expected, that active berry growth during stages I and III involves a large influx of water into the tissues of the grape berry, possibly dependent on the presence of aquaporin proteins. The large reduction in aquaporin expression in the latter stages of stage III may further explain the loss of berry size, which has previously been suggested to be due to the loss of aquaporin activity in xylem and phloem cells (Tyerman et al. 2004).

A similar lag to that observed in the expression of exocarp PME, PL, and the EXPL enzyme during stage II was also evident for the aquaporin gene AQ2. This aquaporin and AQ1 were up-regulated during transition from stage II to stage III (55–71[d] dpa), concurrent with a specific increase in exocarp cell expansion, indicating a further
difference between mesocarp and exocarp in gene expression prior to and during stage III.

During veraison, green and red berries can be observed simultaneously in clusters. In this study, there were substantially higher levels of expression of most of the cell wall-modifying and aquaporin genes in red than in green berries sampled at veraison. These results support the finding of Terrier et al. (2005) that there is an activation of hundreds of genes during grape berry softening prior to changes in color and sugar and water content. The results, however, also indicate that, at least for the genes examined here, changes in gene expression can precede grape berry softening.

In conclusion, cell wall-modifying and aquaporin enzymes generally followed a bimodal trend in which high levels of expression coincided with periods of rapid berry growth and cellular expansion during stages I and III, and low expression levels coincided with slow growth during stage II. These results indicate that, despite major differences in cell morphology between exocarp and mesocarp tissues, similar cell wall metabolic processes occur in these tissues. Some differences for the genes encoding BG, pectic methylesterase, PL, the expansins EXP3 and EXP1, and the aquaporin genes AQ1 and AQ2 were found prior to stage III when the expression trends for the mesocarp and exocarp were compared. These results, in combination with tissue-specific expansion of the exocarp cells, indicate a role of berry skin in moderating growth during stage III. Although no differences in gene expression were evident between tissues during stage I, signs of tissue compression in the radial direction also signified a similar role of the skin during the unripe green stage. Immunolocalization studies should be performed for those genes that exhibited delayed expression trends in the exocarp to seek further evidence of a potential growth-modulating effect.

Acknowledgments

We thank Vincor Canada for use of their vineyard. We acknowledge the critical reading of and helpful comments on this manuscript by Joerg Bohlmann, Peter Toivonen, and Kevin Usher.

References


Tattersall DB, van Heeswijck R, Hoj PB (1997) Identification and characterization of a fruit-specific, thiamin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol 114: 759–769


