TRANSCRIPTOMIC ANALYSIS OF GRAPE BERRY SOFTENING DURING RIPENING

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Abstract

Aims: The aim of this paper was to use recent transcriptomic tools available for grape in order to understand berry softening.

Methods and results: A microarray bearing specific 50 mer oligonucleotide for 3,200 genes was used to study gene expression along 8 stages of berry development in Chardonnay and Shiraz berries. Transcripts corresponding to aquaporin genes and to genes involved in cell wall metabolism were studied in detail and ranked according to their pattern of expression.

Conclusion: Several structural and regulatory genes whose expression pattern correlated with the late phases of ripening were identified.

Significance and impact of study: This study provides a preliminary molecular basis to identify molecular markers of berry ripening.

Keywords: cell wall modification, fruit softening, grape berry, fruit ripening, *Vitis*

Résumé

Objectif : Le but de cet article est d'utiliser des outils transcriptomiques récents pour comprendre le processus de ramollissement de la baie de raisin.

Méthodes et résultats : Des lames microarrays portant des oligonuclétides (50 mer) spécifiques de 3 200 gènes ont été utilisées pour étudier l'expression de gènes au cours de 8 stades de développement de baies de Chardonnay et de Shiraz. Les transcrits correspondant aux aquaporines et aux gènes codant des enzymes du métabolisme de la paroi cellulaire ont été étudiés en détail et classés en fonction de leur profil d'expression.

Conclusion : Plusieurs gènes de structure et gènes régulateurs dont l'expression est corrélée avec les phases tardives du développement ont été identifiés.

Signification et impact de l'étude : Ce travail fournit une étude moléculaire préliminaire pour identifier des gènes marqueurs de la maturation.

Mots-clés : modifications de paroi cellulaire, ramollissement du fruit, baie de raisin, maturation du fruit, *Vitis*

Taxonomic:
Vitis vinifera L. cv. Chardonnay, Ugni blanc, Cabernet-Sauvignon, Shiraz

Abbreviations (alphabetical order):
AGI, type-I arabinogalactan;
ARA-1, Arabinosidase; PIP, plasma membrane protein;
PG, polygalacturonases; PME, pectin methylesterase;
TIP, tonoplast intrinsic protein; XET, xyloglucan endotransglycosylase

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INTRODUCTION

The harvest date of grape berries is still more or less based on macroscopic parameters and is not always easy to determine. In addition to sugar content and phenolic maturity, it is important to take into account berry softening, both for table grape and wine grapes. Table grape berries must be soft enough, and softness and cell wall loosening are also important for the extractibility of secondary compounds during wine making. Grape berries exhibit a double sigmoid pattern of growth (COOMBE, 1992), and during ripening, softening occurs concomitantly with the second growth phase (NUNAN *et al.*, 2001). Softening may be due to changes in the composition of the cell walls (ROSLI *et al.*, 2004), and/or to changes in turgor. The paper presents a transcriptomic analysis of berry softening.

Grape berry softening is a complex process involving subtle changes in the cell wall, which would require only small amounts of enzyme activity (NUNAN *et al.*, 2001). Numerous studies have described changes in cell wall composition and cell wall associated enzymes along fruit ripening, but only a few have focused on grape.

Fruit ripening is accompanied by alterations of the cell wall composition that mainly concern pectins, the major compounds of the primary cell wall. In various species like strawberry, mango, tomato, avocado, fruit ripening is characterized by a decrease in the degree of pectin methylation, and in the pectin content accompanied by a rise in the content of soluble pectins (REDGWELL *et al.*, 1997; STEELE *et al.*, 1997; WAKABAYASHI *et al.*, 2000; PRASANNA *et al.*, 2003; ROSLI *et al.*; 2004). The analysis of cell wall polysaccharides during berry development and ripening did not show any major changes in the types of polysaccharides (NUNAN *et al.*, 1998). However, significant modifications of specific polysaccharides were observed, i.e. an increased solubility of the galacturonans and a decrease in the type-I arabinogalactan (AGI) of the pectic polysaccharide fraction. Grape berry ripening is associated with a decrease in the methylation level of pectins (BARNAVON *et al.*, 2001). Pectins may be degraded by esterases (pectin methylesterase = PME, and pectin acetylesterase), glucosylhydrolases (polygalacturonases, PG) and lyases (pectate lyase). In many species, PME activity increases during ripening, but in grape, this activity is low compared to other species (NUNAN *et al.*, 2001). Pectate lyase activity is difficult to detect in fruits, but it was shown to increase during the ripening of banana fruits (MARIN-RODRIGUEZ *et al.*, 2002). In peach, two pectate lyase genes are expressed early during fruit development, before PG and PME (TRAINOTTI *et al.*, 2003). In tomato, where PG transcripts represent about 1 % of total mRNA in the ripening fruit, a substantial cell wall pectinase activity develops with the induction of ripening and softening. PG was thought to play a major role in softening in many fruits (GIOVANNONI, 2004). However, down-regulation of PG does not abolish softening, indicating that no single enzyme plays a dominant role in the change of texture associated with ripening (MANNING, 1998).

Whereas the cellulose content is stable or slightly increases (AHMED and LABAVITCH, 1980), the xyloglucan fraction of hemicellulose is partially depolymerised during fruit ripening in some species like strawberry (ROSLI *et al.*, 2004).

Fruit ripening is also associated with an increase of soluble galactose resulting from rhamnogalacturonans degradation, and several (but not all) isoforms of galactosidases are increasingly expressed along ripening of tomato (SMITH AND GROSS , 2000), papaye (ALI *et al.*, 1998) and strawberry (TRAINOTTI *et al.*, 2001).

Water represents 90 % of the harvest weight in grape, a crop valued primarily on a fresh weight basis (LANG and THORPE, 1989). Measurements of water fluxes and transpiration in control and phloem-girdled plants indicate that most of the water coming into the berry after *véraison* is imported by the phloem, and thus has to cross biological membranes (LANG and THORPE, 1989). Using fluorescent dyes, BONDADA *et al.* (2005) confirmed that a decrease in xylem flow occurs in grape berries at *véraison* stage, but the tracheids of both the axial and the peripheral xylem remain intact and apparently functional throughout grape berry development. Moreover, significant changes in the pattern of solute partitioning between the grape berry symplast and apoplast occurs at the same stage (BONDADA *et al.*, 2005). KELLER *et al.* (2006) proposed that apoplastic phloem unloading coupled with solute accumulation in the berry apoplast may be responsible for the decline in xylem water influx into ripening grape berries. Instead, the xylem may serve to recycle excess phloem water back to the shoot. Water channels (aquaporins) located in the plasma membrane and in the tonoplast play a major role in the control of inter and ontracellular water fluxes. Several plasma membrane aquaporins (PIP) have been cloned from grape berries (BAIGES *et al.*, 2001; PICAUD *et al.*, 2003). For three cultivars (Chardonnay, Ugni blanc and Pinot meunier), PIP expression was lower in the younger stages of development than in the olders one. However, although PIP expression peaked after *véraison* in Chardonnay and Ugni blanc, the highest amounts of PIP transcripts were observed at the harvest time in Pinot meunier. Thus, VvPIP1s are mainly expressed in the berries after *véraison*, and may be involved in the water exchanges accompanying the ripening of the berries (PICAUD *et al.*, 2003).

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Although dramatic changes in mRNA population occurred in grapes berries during ripening (NUNAN *et al.*, 2001; TERRIER *et al.*, 2005; WATERS *et al.*, 2005) the understanding of softening process remains still partial. The present work investigates the expression of genes encoding enzymes involved in cell wall modification and of those encoding aquaporins along grape berry ripening. It also compares two cultivars, Chardonnay and Shiraz, and identifies some transcription factors as candidate genes that may be specifically involved in the control of some cell wall enzymes. The data obtained with the Shiraz cultivar were previously reported by TERRIER *et al*. (2005), nevertheless these data were exploited in another way.

MATERIAL AND METHODS

1. Grape berry used for microarray analysis

Grape berries from *Vitis vinifera* L. cv. Chardonnay were collected in the Domaine Fort Chabrol vineyard (Epernay, France). The climatic conditions and the sampling periods are presented in figure 1. The average temperature during grape development showed a minimum value of 12.5 °C in September (24/09) when the berries reached 11° alcohol and it was maximal (25 °C) at the three stages collected before *véraison* (30/07). Rainfalls occured regularly during the grape development period, avoiding water stress (figure 1, black bars). Chardonnay berries were collected at eight stages of development in 2002: pea-sized (26/06), half-green (9/07), before *véraison* (30/07), 50 % of *véraison* (19/08), after *véraison* (30/08), half-ripe berries (7 ° alcohol, 4/09), 11 ° alcohol (24/09) and over-ripening (4/10). The berries were selected according to their size during the green stage according to their softness, color and size at *véraison* and according to their size and sugar content, as estimated by density measurement after *véraison*. Berries were frozen in liquid nitrogen and stored at -80 °C until further use.

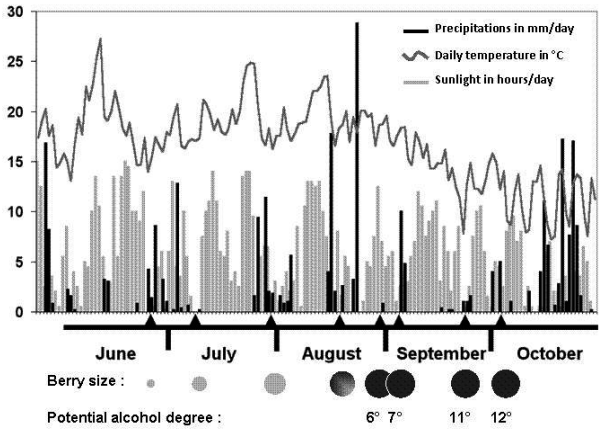


Figure 1 - Weather conditions during samplings (black arrows) of Chardonnay berries in Epernay (Champagne, France).
Temperature (°C) : curve, sunlight (h) : light bars and precipitations (mm) : dark bars. Relative berry size and potential alcohol degree of berries is shown below the graphic for each sampling.

2. Construction of cDNA libraries and sequence analysis

Grape berries from white cultivars Chardonnay and Ugni blanc and black cultivars Cabernet-Sauvignon and Shiraz were collected and used to generate cDNA libraries (TERRIER *et al.*, 2005).

EST generation was described in TERRIER *et al.* (2001). Sequence analysis, isogenes detection and functional annotation were described in TERRIER *et al.* (2005).

3. Microarray preparation and use

A set of 3,175 unigenes generated from grape berry cDNA libraries and a few miscellanous clones, either from our experiments or from public databases were used to prepare a microarray according to TERRIER *et al.* (2005). Fifty-mer oligonucleotides were designed preferentially in 3' non-coding regions, taking into account Tm compatibility and specificity. For control purpose, oligonucleotides corresponding to luciferase, desmin and Arabidopsis sequences were added on the slide. Each 5'amino-modified oligonucleotide was deposited in duplicate onto the arrays. The design, the synthesis, the spotting of the oligonucleotides were carried out by MWG Biotech (Ebersberg, Germany). The probe synthesis and labelling by Cy3- and Cy5-, hybridization and the scanning of oligo arrays with an Axon Genepix 4000B scanner were carried out as described in TERRIER *et al.* (2005).

4. Data analysis

The intensities of the spots were calculated as « the median intensity value of the spot - the median local background value ». After excluding 15 % of the highest and 15 % of the lowest values, the calculated mean ratio on the 70 % remaining values was used to normalise the intensity ratios to 1 (GIRKE *et al.*, 2000). For clustering, the total sum of the spot intensities was arbitrarily adjusted to 107 for the middle ripe stage (7° potential alcohol). Spot intensities at other stages were calculated using normalized ratios.

The time course profiles of expression of berries transcripts were finally obtained by comparing eight developmental stages using the half-green stage as a common reference.

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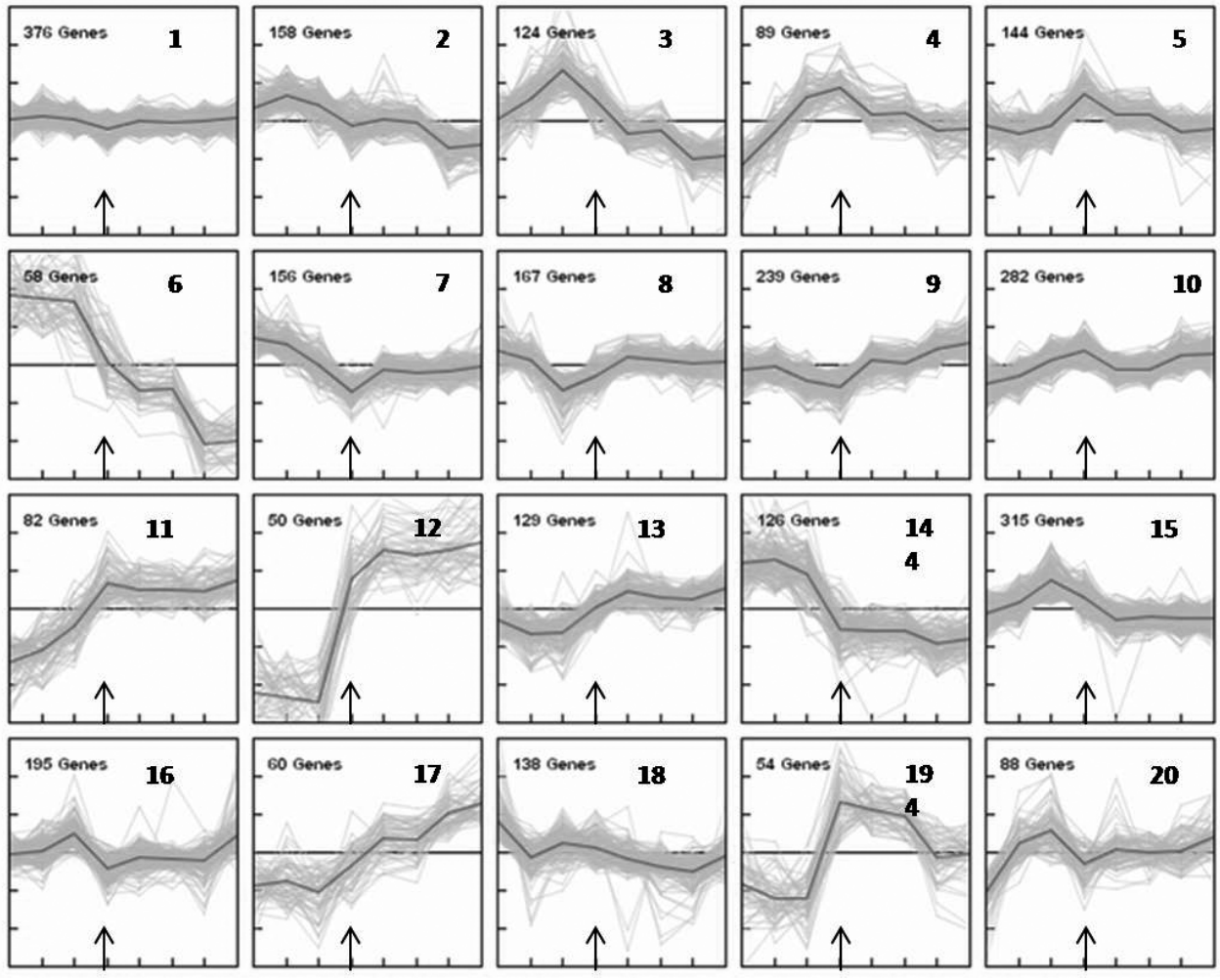


Figure 2 - Major expression profiles (1 to 20) during Chardonnay grape berry development by K-means (K=20) calculation from Genesis software, for centered and normalized means ratios.

The eight developmental stages studied are reported in abscissa, and differential expression (up to 6-fold) is represented in ordinate. The dark curve indicates the linear regression of each expression profile. Véraison stage, represented by a black arrow, is the fourth point of the abscissa.

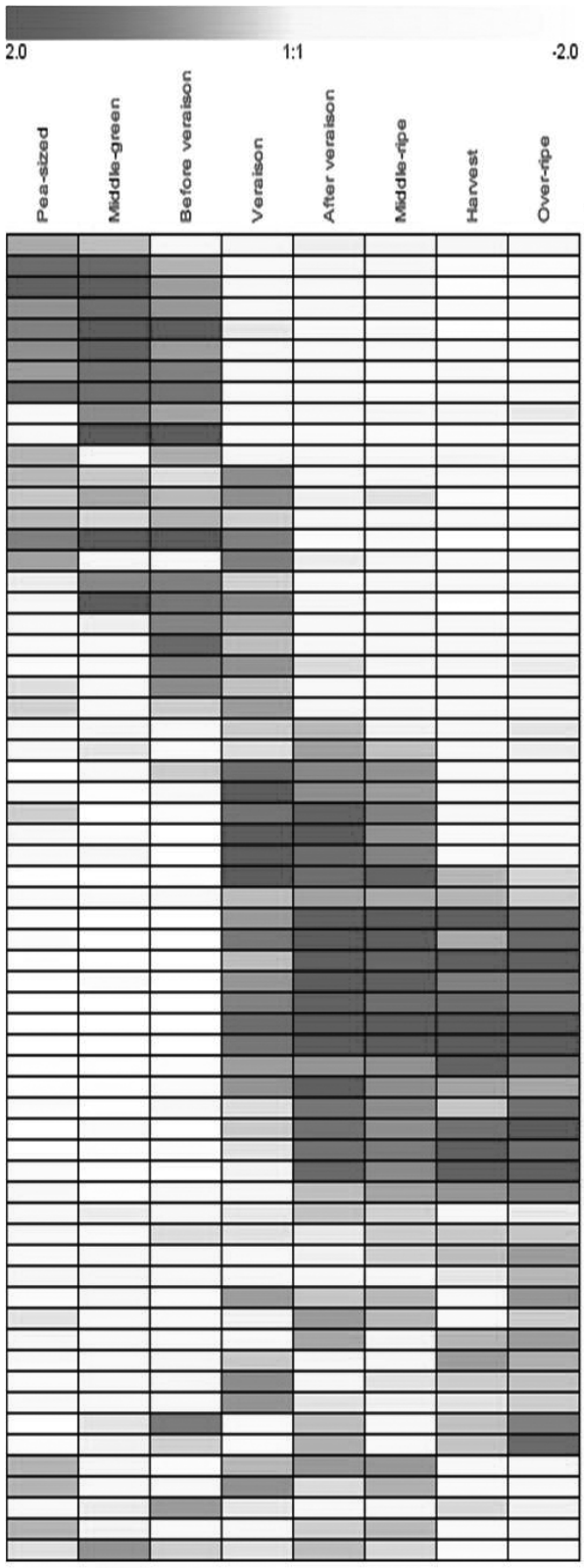
This common reference had the advantage of allowing an analysis and a direct interpretation of the results without the need of additional statistical tools (YANG and SPEED, 2002). Two developmental stages was technically replicated by reversing dye labeling (swapping). The comparisons of the half-green and half-ripe stages were reproduced four times. An analysis of variance of gene expression was performed using ANOVA from GENESIS (STURN *et al.*, 2002). Gene induction or repression was considered significant if the mean ratio for the four repetitions with a 95 % confidence interval was either greater than two or less than 0.5 respectively. For expression profile clustering, the data was pre-processed by dividing each expression value by the mean expression of the corresponding transcript. Ratios were log10 transformed and submitted to clustering in GENESIS software. Hierarchical clustering and k-means algorithm were used, with the Euclidean distance measure.

RESULTS

1. Time course studies

To study gene expression along ripening, total RNA samples from berries collected at eight developmental stages were isolated and retrotranscribed. After purification of the double stranded cDNA, an amplification with fluorescently labeled UTP was performed and the resulting cRNA was used for hybridization with microarrays bearing about 3200 independent clones. The expression profiles of 3030 clones could be resolved into 20 clusters (figure 2). Part of the transcripts (376, corresponding to 12.4 % of total transcripts analyzed) did not show any change in expression along ripening (cluster 1, figure 2). The expression level of 58 genes (1.9 %, gathered in cluster 6) and 126 other genes (4.2 %, cluster 14) decreased from pea-sized to over-ripening stages, and this decrease was

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EL	EST similar to :	Accession number :
7	contains similarity to alpha galactosidase	TT282G09
10	phosphomannomutase	PT005C11
4	beta-galactosidase	TB000A23
5	GDP-mannose pyrophosphorylase [Arabid.	GT174G08
3	putative glucanase [Arabidopsis ou cellulase	GT171A06
10	putative pectin methylesterase pectinesterase family	TB006F03
22	proline-rich protein, putative [Ar...	RT022E09
12	cellulose synthase	CS001A01C
1	extensin (class II)	PT002C02
5	xyloglucan endotransglycosylase 2	GB004G07
1	polygalacturonase-like protein	PT001B09
1	pectate lyase	G7547008
2	alpha-mannosidase	TT281B04
8	putative cellulose synthase catalytic subunit	PT013C05
9	beta-galactosidase	RB007C05
2	PECTINESTERASE 3 PRECURSOR	TB000A72
2	xyloglucan endotransglycosylase precursor	TT264D11
2	xyloglucan endotransglycosylase	RT042A03
6	cell wall-binding protein homolog	GB006D11
5	pectin methylesterase	RT043G01
11	Similar to beta-glucosidases [Arabid	RT021B05
5	putative pectinesterase [Arabidops... 301/717, pectin methylesterasi	GT172D10
4	4-alpha-glucanotransferase	PT012C01
2	putative alpha-mannosidase	TT251B04
4	alpha-mannosidase, putative [Arabi...	GT193H01
24	polygalacturonase inhibiting protein [Vitis vinifera]	AF305093
3	putative pectinesterase	RT062G12
4	polygalacturonase	CT003A08
10	pectate lyase	AY043234
11	pectate lyase	RT033H11
12	beta-glucosidase	RT081G10
5	endoxyloglucan transferase	RT073H01
133	GRIP28	RT061C10
23	alpha-expansin	RT083F08
132	GRIP15	RT052C11
63	xyloglucan endo-transglycosylase	TB001C02
121	alpha-expansin 3	RT094D07
133	GRIP13	RB005D10
209	GRIP4	RT093F08
10	xyloglucan endo-1,4-beta-D-glucanase Arabidopsis	CT006H11
42	mannan endo-1,4-beta-mannosidase	TT251A06
3	mannan endo-1,4-beta-mannosidase [Arabido	GT201H11
101	1.3 beta glucanase	TT281E02
177	GRIP 31	RT083B07
61	alcaline alpha-galactosidase II	RT043G05
1	GRIP15	CT002B04
2	pectin methylesterase	PT004D09
1	putative alpha-galactosidase [Arabidop	CM004F04
8	arabinosidase ARA-1	RT052C05
6	beta-glucosidase, putative [Arabid...	GT201C10
14	xylose isomerase	RT061F12
4	xylose isomerase	TT274E03
8	alpha galactosyltransferase	TT251E11
2	1.3 beta glucanase	AF239617
6	proline rich protein	RT032C11
1	beta-glucosidase	AY039034
44	pectin acetylesterase (EC 3.1.1.-) precursor	BM437268
10	pectin acetylesterase (EC 3.1.1.-) precursor	RB000A83
9	putative mannose-6-phosphate isomerase	RT023D02
4	putative beta-1,3-glucanase [Arabi..., endosperm cell wall weakening	GT171E03
7	putative glucan synthase [Arabidop...	GT191A02
5	1,4-alpha-glucan branching enzyme	GB000A76
3	putative polygalacturonase [Arabid...	GT181B04

Figure 3 - Expression profile cluster of transcripts potentially involved in cell wall modification during Chardonnay berry development.

After hierarchical clustering with average linkage (Genesis software), subclusters were reassembled manually in order to classify them in a time course of gene activation. Transcripts presenting different expression peaks along berry development were gathered at the bottom part of the cluster. EL: Expression Level.

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stronger for cluster 6 than for cluster 14. In contrast, 50 genes (1.6 %, cluster 12) and 60 genes (2.0 %, cluster 17) showed increased transcripts levels during ripening process. Some transcripts peaked at the *véraison* stage; in some cases, transcript levels started to increase from the beginning, at the pea-sized stage (cluster 4, 89 genes, 2.9 %) and cluster 11 (82 genes, 2.7 %), while for other genes, transcripts accumulate just before *véraison* stage (cluster 5, 144 genes, 4.8 % and cluster 19, 54 genes, 1.8 %).

2. Cell wall metabolism

Figure 3 focuses on 63 genes belonging to the major gene families potentially controlling cell wall metabolism. This includes protein families involved in reassembly and degradation of primary wall, as well as structural proteins and enzymes catalyzing polysaccharide synthesis, monosaccharide activation and interconversion.

a- Enzymes involved in synthesis, reassembly and degradation of cell wall polysaccharides

- Polysaccharide synthesis family

Cellulose synthase (CS001A01C, PT013C05) was expressed during the first stages of berry development (figure 3). Glucane synthase (GT191A02), which mediates callose synthesis was expressed at a low level throughout development, but a slight optimum was observed before *véraison*.

Arabinosidase (ARA-1, RT052C05) and GDP-mannose pyrophosphorylase (CYT1, GT174G08) are two enzymes involved in monosaccharide activation and interconversion (GRIKE *et al.*, 2004). GDP-mannose pyrophosphorylase transcripts were detected from pea-sized to before *véraison* stages. Arabinosidase (ARA-1) transcripts were present, but only in low amounts during the later stages of berry ripening.

- Hemicellulose fraction

α- and β-galactosidases belong to the glycoside hydrolases controlling the metabolism of the primary cell wall. One β-galactosidase isoform (TT282G09) showed a weak expression at pea-sized and half-green stages, whereas two others isoforms (RT043G05; CM004F04) were expressed from the pre-*véraison* stage until over-ripening. RT043G05 was much more strongly expressed than CM0004F04. ß-galactosidases (TB000A23, RB007C05) were expressed during the first four stages of development. RB007C05 was strongly expressed at half-green and pre-*véraison* stages, slightly later than TB000A23.

Among the four glucanase homologues spotted on the microarray slide (GT171A06, TT281E02, AF239617, GT171E03), three isoforms (corresponding to TT281E02, AF23917, GT171E03), have been involved in plant-pathogen interactions or in developmental processes in vegetative parts of number of plant species. Two isoforms (GT171A06, TT281E02) were strongly expressed, respectively during the early and late stages of berry development and ripening, whereas the two other ones were only weakly expressed throughout time. TT281E0 presents 98 % identity with VvGlu1/7 (accession number U68144) which corresponds to a *V. vinifera* ß-1,3-glucanase partial sequence detected only at the beginning of ripening and in the ripening stage (KRAEVA *et al.*, 1998). The exact role of glucanases in plant development is still unknown, but glucanase activity in young berries might be associated with the growth of tissues, since expansion of the cell wall requires the partial hydrolysis of glucan.

Three homologues of α-mannosidase were studied with the microarray. Two isoforms were expressed at a relatively low level, one before *véraison* (TT281B04), the other one after *véraison* (TT251B04). A third isoform (GT193H01) was more abundant and peaked at *véraison*.

β-glucosidase transcripts (AY039034, RT021B05, RT081G10, GT201C10) were absent before *véraison*, but they were detected from *véraison* (RT081G10) until the end of ripening (GT201C10, AY039034).

The transcripts of a xyloglucan endo-1,4 ß-D-glucanase (CT006H11) started to accumulate at 50 % of *véraison* stage and their amount was maximal at harvest stage.

The xyloglucan endotransglycosylase (XET) belongs to a family of enzymes mediating hemicellulose reassembly. Four isoforms of XET are spotted on the microarray, among which three (GB004G07, TT264D11, RT042A03) were mostly expressed at the beginning of the maturation process, half-green or before *véraison* stages. The fourth isoform (TB001C02) peaked after *véraison*.

- Pectin fraction

The release and depolymerization of polygalacturonans play a major role in fruit softening. Pectin methylesterases which catalyse the hydrolysis of methyl-ester groups from galacturonosyl residues, control the accessibility of polygalacturonans to polygalacturonases. PME and pectin esterases transcripts were detected until middle-ripe stage (figure 3). Among the six PME isoforms spotted on the microarray, one (PT004D09) was weakly expressed from the pea-sized until the middle ripe stage. TB000A72 and TB006F03 peaked at *véraison* and at the middle green stage respectively. One isoform (RT062G12) was most abundant at *véraison*, and then declined. For

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the last isoforms (RT043G01; GT172D10), two peaks were observed, at pea-sized and *véraison* stages.

Two sequences homologous to pectin acetylesterase (BM437268, RB000A83) are both abundant at the overripen stages (figure 3).

Three isoforms of grape polygalacturonase were identified in our EST and spotted on microarray slide. Two transcripts (PT001B09; GT181B04) were detected, at a relatively low level at the beginning of grape development. In contrast, another isoform (CT003A08) was more abundant and transiently expressed from *véraison* to middle-ripe stages. Maximal accumulation of polygalacturonase inhibiting protein (PGIP, AF305093) transcript occurs at *véraison*, approximately at the same time as transcripts corresponding to polygalacturonase (isoform CT003A08).

Among the 3,175 grape isogenes, three pectate lyase isoforms have been identified (G7547008; AY043234, RT033H11). Whereas G7547008 transcripts was detectable from pea-sized until *véraison* stages, the two other isoforms are mostly abundant at *véraison* and after *véraison*. Pectin degradation by pectate lyase occurs by a β-elimination reaction, in contrast to the hydrolytic mechanism of polygalacturonases. Pectate lyase catalysed the cleavage of de-esterified pectin. Thus, pectin methylesterase and acetylesterase activity must either precede the activity of pectate lyase or occur at the same time.

3- Cell wall proteins

Hyp-rich glycoproteins or extensins, pro-rich proteins, leu-rich repeat extensins, gly-rich proteins and arabinogalactan proteins are the major structural proteins of the cell wall. The expression of two pro-rich proteins and one extensin (class II) were studied with the microarray. One grape pro-rich protein (RT022E09) and one extensin (PT002C02) were abundant from pea-sized to before *véraison* stages (figure 3) whereas the second isoform of pro-rich protein (RT032C11) was poorly abundant, and was detected only at the *véraison* stage.

Several Grip proteins, such as Grip 4, 13, 15, and 28, are cell wall structural protein (DAVIES and ROBINSON, 2000). Deduced amino acid sequences of Grip 4, 13, 15, and 28 best matched with a putative pro-rich cell wall protein present in the database. Grip 4 (RT093F08), Grip 13 (RB005D10), Grip 28 (RT061C10) and one isoform of Grip 15 (RT052C11) were very abundant from *véraison* until over-ripening. Another isoform of Grip 15 (CT002B04) and Grip31 (RT083B07) were expressed at a lower level, and were mostly abundant at the harvest and overripe stages. Grip 31 best matches with a protein induced in nitrogen-fixing nodules involving the actinomycetous bacteria *Frankia* (DAVIES and ROBINSON, 2000).

Another protein involved in reassembly, degradation and expansion of cell wall is expansin. The two grape isoforms of α-expansin (RT083F08, RT094D07) present on the microarray showed increased transcripts amounts after *véraison*.

4- Comparison between Chardonnay and Shiraz

Expression profiles of several genes encoding enzymes involved in cell wall metabolism were compared throughout berry development for two cultivars, Chardonnay and Shiraz. Pectate lyase (RT033H11) and pectinesterase (TB000A72) profiles expression were identical for both grape cultivars (figure 4). However, maximal transcript levels were observed earlier in Shiraz than in Chardonnay. α-expansin (RT094D07) and 1,3-β glucanase (TT281E02) shared similar profiles in both cultivars: 1,3-β glucanase transcripts increased during the late stages of maturation, and α-expansin mRNA levels peaked after *véraison*. Both in Chardonnay and Shiraz, endoxyloglucan transferase (RB005B09) reached a maximum at veraison or shortly after *véraison*, and its level then remained stable. Also, in both varieties, the pectinase esterase precursor (TB000A72) and the mannan endo-1,4-beta-mannosidase (GT201H11) peaked around veraison (figure 4 E, F).

5- Aquaporins

Different EST encoding aquaporins were identified from grape berry cDNA libraries (TERRIER *et al.*, 2005; FOUQUET *et al.*, submitted). The expression pattern of various PIP and TIP during berry development is shown in figure 5 for Chardonnay and Shiraz berries.

One TIP3 isoform (RB003A08) exhibited two phases of strong expression (figures 5A and 5B), during early development, and at *véraison*, whereas the other one (TI3000A01C) presented a single peak at *véraison*. For both isoforms and in both varieties, transcript amounts decreased down to near zero at harvest and over-ripening stages (figures 5A and 5B). Another TIP3 transcript (AF271662) remained poorly abundant throughout all stages of development for both Chardonnay and Shiraz berries (figures 5C and 5D). The quantity of TIP1 (AF271661) transcript decreased during grape development in both cultivars (figures 5E and 5F). Nevertheless, the level of this transcript in Shiraz was constant from pea-sized to before *véraison* stages.

Transcripts amounts of plasma membrane proteins as PIP1-3 (RB000A23; AF141899) are given in figures 5E and 5F). In Chardonnay, one isoform of PIP1-3 (AF141899) was poorly abundant throughout berry development. Another PIP1-3 isoform (RB000A23) was

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abundant at the beginning of development, decreased before *véraison*, and peaked transiently at *véraison*. The expression pattern of this isoform was different in Shiraz since transcript was the most abundant at the *véraison* stage (figure 5F). The transcript amount of PIP2-1 (TT283E06; AF141642) reached a peak at *véraison* for both cultivars (Figures 5G and 5H). AF141642 transcripts were more abundant in Shiraz than in Chardonnay, and more abundant than the other isoform (TT283E06). In the case of PIP2-5 (GT204A12), the higher level of transcript was observed at the préveraison stage and at the mid veraison stage respectively in Chardonnay and in Shiraz (figures 5C and 5D).

In summary, PIP2-5 was thus expressed during the first stages of development while PIP2-1 and PIP2-2 were expressed later, during the second half of the development (figures 5G and 5H).

5- Co-expressed genes

Several remarkable examples of co-expression, suggesting potential functional relationships, were identified during our analysis. The expression profiles of the cellulose synthase catalytic subunit (PT013C05) and of a transcriptional activator protein (RT042F04) were parallel (figure 6). The expression pattern of a zinc finger protein (GT172A07) was parallel to that of a polygalacturonase like protein (PT001B09) (figure 7). Finally, the profiles of expression across the berry development of two pectin acetylesterase isoforms

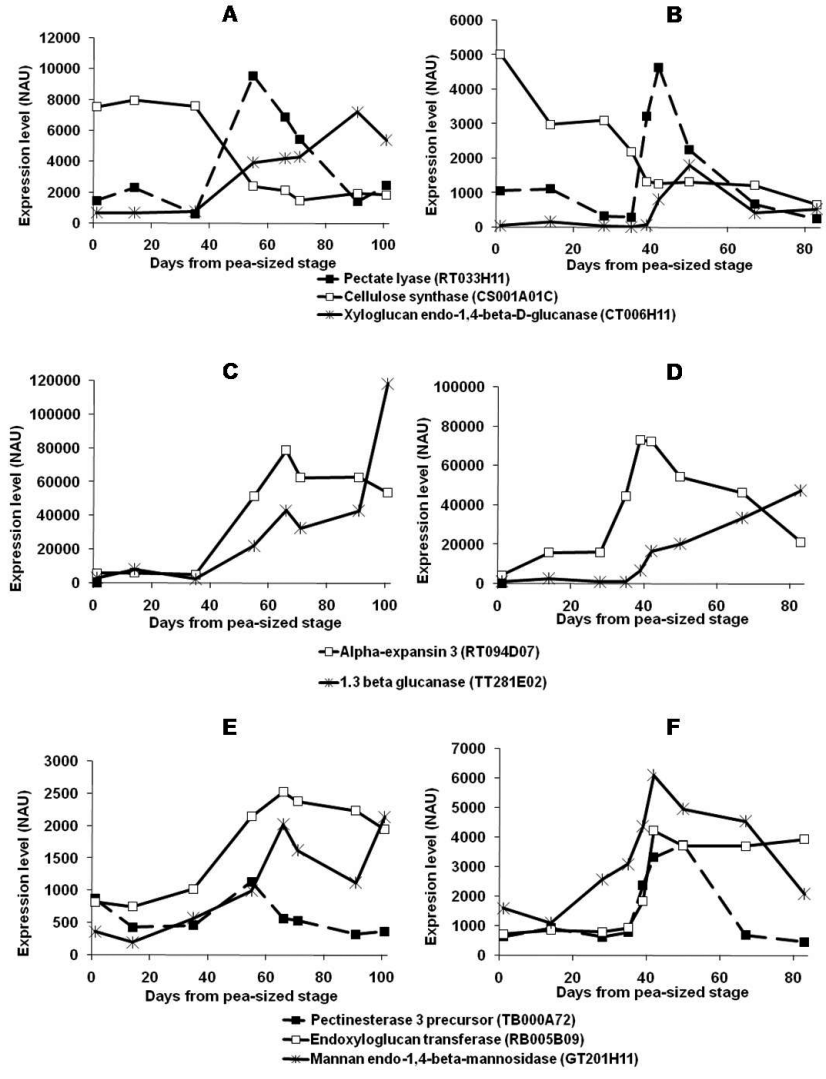


Figure 4 - Expression profiles of 8 transcripts involved in cell wall modification of Chardonnay grape berry development (A, C and E) and Shiraz grape berry development (B, D and F) which have similarity in their pattern of expression between the both cultivars.

Véraison is day 40 for Shiraz and day 55 for Chardonnay.

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(BM437268; RB000A83) were close to that of a zinc finger protein (B-box type, RT074A05) (figure 8).

DISCUSSION

Grape ripening is a complex process still poorly understood at the molecular level. Although the berries accumulate ABA during ripening, the hormonal and molecular control of berry development is still being investigated (KOUSSA *et al.*, 2004). Recent studies started to develop a molecular approach to this process (TERRIER *et al.*, 2005; DELUC *et al.*, 2007; GRIMPLET *et al.*, 2007; PILATI *et al.*, 2007). The present work uses the same approach and focuses on genes that may affect the firmness of grape berry and the metabolism of the cell wall.

During ripening, sugar accumulation in the berry is accompanied by concomitant water fluxes, in such a way that the dry weight increment to volume increment of the berry stays at a relatively stable ratio (PICAUD *et al.*, 2003). This indicates that a balance is kept within the berry between water and dry matter (COOMBE, 1989). The increase in berry weight in the second phase of berry development is due to the accumulation of water and sugar and the increase in berry size is due to cell expansion (COOMBE, 1992). Both in Chardonnay and Shiraz, PIP2-2 transcripts (TT283E06) and PIP 1-3 transcripts (RB000A23) present a large peak of expression at the

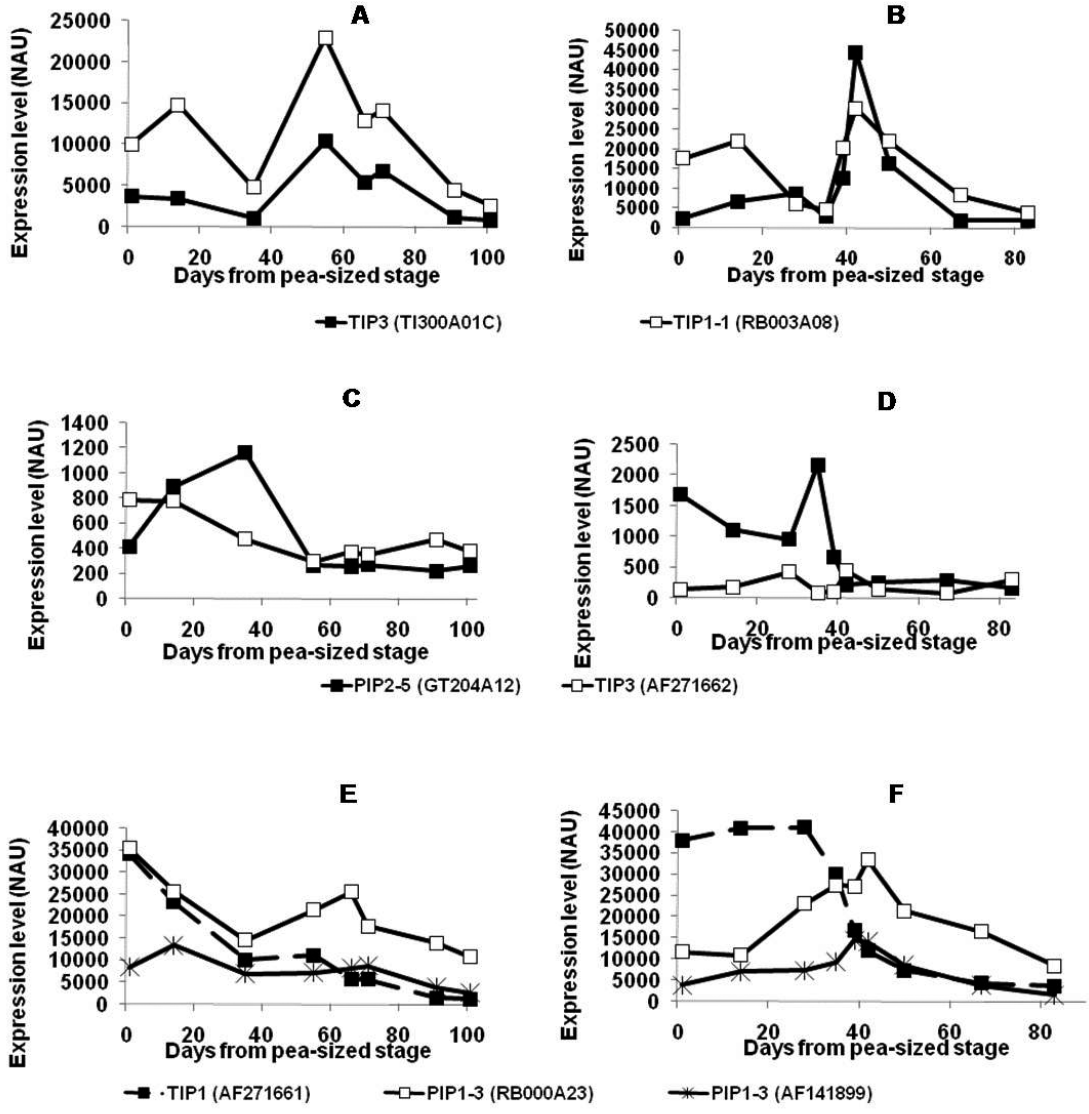


Figure 5 - Developmental expression profiles of 9 aquaporin genes in Chardonnay (A, C, E and G) and Shiraz (B, D, F and H) berries. Véraison is day 55 for Chardonnay and day 40 for Shiraz.

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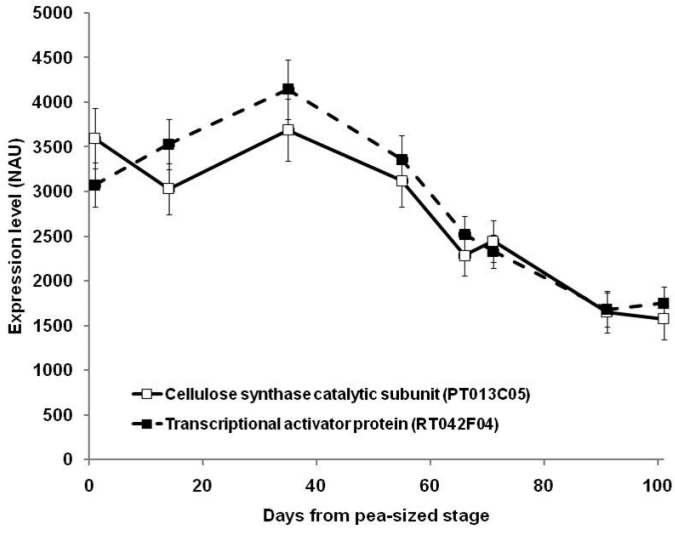


Figure 6 - Expression profiles of the transcripts of a cellulose synthase catalytic subunit (PT013C05, white squares) and of the transcriptional activator protein (RT042F04, black squares) along Chardonnay berry development.

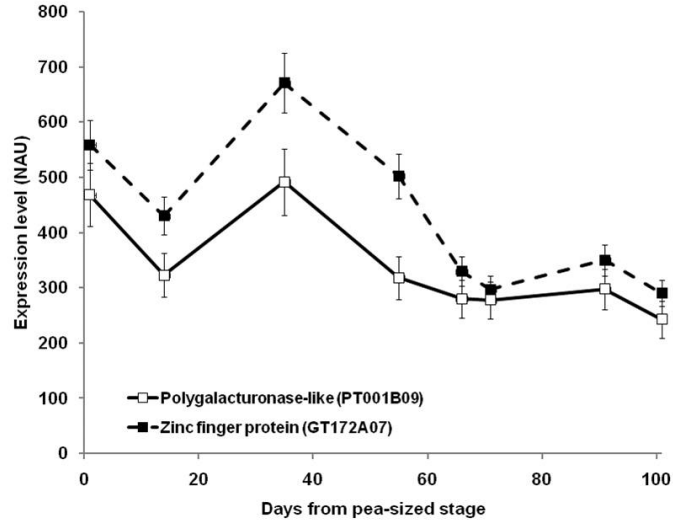


Figure 7 - Expression profiles of the transcripts of a polygalacturonase-like protein (PT001B09, white squares) and the zinc finger protein (GT172A07, black squares) along Chardonnay grape berry development.

veraison time, and may be involved in the second phase of berry swelling. The same is true for the tonoplast aquaporin TIP 3 (TI300A01C) and TIP1-1 (RB003A08). In contrast, TIP3 (AF271662) and PIP2-5 (GT204A12) are poorly expressed in the berries throughout development. TIP1 (AF271661) may be implicated in the first phase of berry swelling, as it is expressed mainly at the early stages of development. Although the general trends of aquaporin expression were similar in Chardonnay and Shiraz, some significant differences may be found, concerning for example AF271661 and TT283E06, whose expression patterns and/or levels significantly differs between both cultivars (figure 5). Likewise, PIP 1-3 transcripts (RB000A23) behave in the same way in Chardonnay and Shiraz (figure 5) and in Cabernet-Sauvignon berries (FOUQUET *et al.*, submitted), but this is not the case for the other aquaporins studied. It is not known whether these differences are due to a different genetic background and/or to different environmental conditions. In any case, it appears that aquaporin expression obeys a complex developmental pattern which probably adds to the diversity of their expression in different organs in response to water stress (GALMES *et al.*, 2007).

Softening of ripe fruits is essentially due to the degradation of the middle lamella of cortical parenchyma cells, mainly composed of pectins. Pectic polymers consists of linear polygalacturonate chains interspersed with highly branched rhamnogalacturonan chains. Some of the galacturonate residues are modified by methyl and/or acetyl esterification. The extent of methylation and acetylation varies greatly, depending on the source of pectin. Plant cell walls contain up to 7 % (on a dry weight basis) O-bound acetyl groups, mostly linked to two polysaccharides, xylan and pectin. Esterases are necessary to remove this modification, thus facilitating the further degradation of the polysaccharidic chain (SHEVCHIK and HUGOUVIEUX-COTTE-PATTAT, 2003).

In grape berries, PME mRNA accumulated from one week before *véraison* to the end of maturation period (BARNAVON *et al.*, 2001). Similar results were obtained for raspberry, another nonclimacteric fruit (IANNETTA *et al.*, 1999). Our results indicate that different isoforms of PME are expressed at different levels and at different times during grape berry development. Thus, TB006F03 is strongly expressed at the mid-green stage, whereas RT043G01 is strongly expressed at *véraison*, and PT004D09 slightly peaks after *véraison* (figure 3). *In situ* hybridization would be helpful to determine the precise localization of these transcripts in various tissues of the berries.

Polygalacturonases degrade de-esterified pectins, whereas pectate lyases may act on esterified pectins. In strawberries, a pectate lyase gene contributing to pectin degradation is expressed only in ripening fruits, and repressed by auxin treatment (JIMÉNEZ-BERMUDEZ *et al.*, 2002). In tomato, polygalacturonase is the most significant pectin-degrading enzyme. According to MANNING (1998), down-regulation of polygalacturo-

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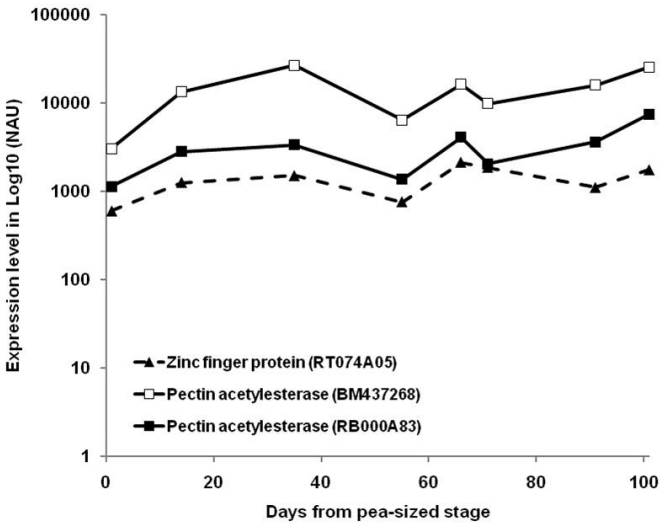


Figure 8 - Expression profiles of two pectin acetylesterase isoforms (BM437268 ; RB000A83, white and black squares) and of a zinc finger B-box type (RT074A05, black triangles) along Chardonnay berry development.

nase activity does not abolish softening in tomato, indicating that no single enzyme plays a dominant role in the texture changes associated with ripening fruits However, suppression of LePG, a tomato polygalacturonase results in altered softening (POWELL *et al.*, 2003). Our results show that polygalacturonase and pectate lyase form a group (figure 3, dark square) strongly and suddenly expressed at veraison. Surprisingly, a polygalacturonase inhibiting protein (AF305093) is expressed at the same time. This rises the limits of the transcriptomic data, because they do not give any information concerning protein amount, activity, and tissue/cell localization.

A second group of transcripts (shown in the grey rectangle, figure 3) presents a maximal amount after veraison and at the mid-ripe stage. This group includes alpha-expansins (RT083F08; RT094D07), GRIPs (RT061C10; RT052C11; RB0005D10; RT093F08; RT083B07), and several enzymes of the hemicellulose metabolism like xyloglucan endo-transglycosylase (TB001C02), xyloglucan endoglucanase (CT006H11) and mannan endomannosidases (TT251A06, GT201H11), as well as a beta-glucanase (TT281E02) and an alpha-galactosidase (RT043G05).

Endo-β-1,4-glucanases,xyloglucan endotransglycosidases and expansins have been proposed as allies cooperating in the modification of the hemicellulose network during fruit ripening, and this is confirmed by the data presented in figure 3. These enzymes contribute to the depolymerization of xyloglucan, the main component of hemicelluloses. However, contribution of each enzyme in fruit softening is not clear (LLOP-TOUS *et al.*, 1999).

The expansin superfamily of plant proteins contains four families, designated as α-expansin, β-expansin, expansin-like A and expansin-like B. α-expansin and β-expansin have cell-wall loosening activity and are involved in cell expansion (SAMPEDRO and COSGROVE, 2005). During growth and maturation of green tomato fruit, at least six expansin genes showed overlapping periods of mRNA accumulation (CATALA *et al.*, 2000). The LeExp1 expansin is abundant in the ripening tomato and was proposed to contribute to cell wall disassembly by nonhydrolytic activity, possibly by increasing substrate accessibility to other enzymes (POWELL *et al.*, 2003). ISHIMARU *et al.* (2006), reported the presence of three putative expansin cDNAs in grape, Vlexp1, Vlexp2 and Vlexp3 (AB104445) in V. labruscana cv.Kyoho berries. Their expression profiles suggest that the three expansin genes are associated with cell division or expansion and berry ripening and that Vlexp3 is most likely to play a role in berry softening. The fact that the α-expansin transcripts (RT083F08, RT094D07) amounts increased after *véraison* suggested that these isoforms may play a role in grape berry softening. The α-expansin sequence (RT083F08) presented 97 % of identity to Vlexp3.

The endo-cleavage of xyloglucans can be achieved not only irreversibly by endo- ß-1-4-glucanase, but also reversibly by xyloglucan endotransglycosylase (XET) (BRUMMELL and HARPSTER, 2001). In tomato, apple and kiwi, XET activity is high in young expanding fruit, declines during maturation and rise again slightly during ripening (PERCY *et al.*, 1996).

Two cDNAs clones, Cel1 and Cel2 encoding endo-β-1,4-glucanases were isolated from ripe strawberry (LLOP-TOUS *et al.*, 1999). Cel2 was expressed in green fruits, accumulating as the fruit turned from green to white and remaining at an elevated constant level throughout fruit ripening. The level of Cel1 transcripts increased gradually during ripening. Thus, the high levels of Cel1 and Cel2 mRNA in ripe fruit suggest that endo-β-1,4-glucanases play an important role in softening during maturation. Given the high expression of beta 1,3 glucanase (TT28102) at the overripe stage in grape berries, this enzyme and the pectin acetylesterase precursors (BM437268; RB000A83) might be considered as markers of over-ripening.

Correlations observed in microarray studies may also be useful to identify transcription factors potentially controlling the expression of one or several genes.

A detailed analysis of the data allowed to show strong expression correlations between a cellulase synthase

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catalytic subunit and a transcriptional activator (figure 6), as well as between a polygalacturonase-like transcript and a zinc finger protein (figure 7) or between two pectin acetylesterase isoform and a zinc finger protein (figure 8).

This type of correlation may indicate that both the structural gene(s) and the transcription factors showing a parallel expression pattern are controlled by the same regulatory mechanisms, or that the transcription factor does indeed control the expression of the structural gene(s). To discard the possibility that the observed parallels just result from chance, more detailed studies involving promoter analysis, reporter genes, and infiltration of recombinant transcription factors will be performed.

Although still partial, since the microarrays used contain only about one tenth of the grape genome, these data pave the way for a complete analysis of aquaporins and cell wall enzymes during berry softening. They will be helpful to identify, among the structural and the regulatory genes, the major players involved in the softening process. Some of the genes identified will now be confirmed by RT-PCR analysis on different parts of the berries (seeds, pulp, skins), and a similar approach will be developed with Combimatrix microarrays bearing almost the entire grape genome. This, together with other data (Terrier *et al.*, 2005; Deluc *et al.*, 2007; Grimplet *et al.*, 2007; Pilati *et al.*, 2007) should allow to identify the most relevant genes that may be considered as markers of ripening under different environments and for different varieties. In the long term, specific immumological probes raised against the encoded proteins may be used to monitor the ripening process.

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