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Grape is considered as a non-climacteric fruit, the maturation of which is independent of ethylene. However, previous work had shown that ethylene is capable of affecting the physiological processes during maturation of grape berries. Experiments were designed to screen the gene pool affected by ethylene at the ripening inception in Cabernet Sauvignon berries. The results showed that only 73 of 14 562 genes of microarray slides were significantly modulated by a 24-h ethylene treatment (4 $\mu l\ l^{-1}$), performed 8 weeks after flowering. The study then focused on accumulation of several mRNAs affected by ethylene in relation to the berry size. Indeed, we observed that ethylene application at véraison led to a berry diameter increase. This increase is mainly because of sap intake and cell wall modifications, enabling cell elongation. This was related to changes in the expression pattern of many genes, classified in two groups: (1) 'water exchange' genes: various aquaporins (AQUA) and (2) 'cell wall structure' genes: polygalacturonases, xyloglucan endotransglucosylases (XTH), pectin methyl esterases, cellulose synthases and expansins. The expression patterns were followed either along berry development or in three berry tissues (peel, pulp and seeds). Ethylene stimulates the accumulation of most gene transcripts in 1 h, and in several parts of the berry, this stimulation may last for 24 h in some cases. One XTH and one AQUA seem to be good candidates to explain the ethylene-induced berry expansion. This work brings more clues about the ethylene involvement in the development and ripening of grape berries.

Introduction

Grape (*Vitis vinifera* L.) has been classified as non-climacteric (Coombe and Hale 1973), but evidence suggests that climacteric and non-climacteric fruit may in fact share similar pathways of ripening (Barry and Giovannoni 2007). Indeed, recent studies have shown that grape berry tissues have a fully functional pathway for ethylene synthesis and that this pathway is activated just before véraison (Chervin et al. 2004) when berries start to accumulate sugars and, in red cultivars, accumulate anthocyanins. Furthermore, this study showed that ethylene perception is critical for some berry changes associated with ripening, including anthocyanin accumulation and berry expansion (Chervin et al. 2004).

Abbreviations – AQUA, aquaporin; CS, cellulose synthase; EX, expansin; 1-MCP, 1-methylcyclopropene; PG, polygalacturonase; qPCR, quantitative polymerase chain reaction; PME, pectin methyl esterase; TAI, transcript accumulation index; XTH, xyloglucan endotransglucosylase.

Besides, Tesniere et al. (2004) reported the involvement of ethylene signalling in the regulation of ADH expression in grapevine. There were other reports about the role of ethylene in non-climacteric fruit such as strawberries (Trainotti et al. 2005) and citrus (Katz et al. 2004).

These reports lead us to check more globally the effects of ethylene on gene expression during berry ripening in grape, and many new tools and technologies for dissecting the gene expression profiling were available, e.g. microarray technology. Recent experiments using this technology showed the variation of expression of many genes during grape berry development (Deluc et al. 2007, Pilati et al. 2007, Terrier et al. 2005, Waters et al. 2005).

In most fruit, an important part of the ripening process is berry expansion and berry softening and it is widely recognized that changes in cell walls accompany fruit ripening. Gross changes in wall composition may not always occur, and indeed more subtle structural modifications of constituent polysaccharides are often observed during softening (Brady 1987, Brummell and Harpster 2001).

The softening process is complex as breakdown or modifications of different components are usually accompanied by the incorporation of newly synthesized components into the wall (Gibeaut and Carpita 1994, Seymour and Gross 1996). The synthesis of cell wall polymers is probably continuous throughout ripening, and a change in the turnover rate of particular component will affect the overall wall composition (Brummell and Harpster 2001). Modifications of wall components might also be expected in ripening grape berries, and indeed several researchers investigated the modification of cell wall components and the expression of the gene for cell wall degradation-related enzymes during softening of the grape berries (Ishimaru and Kobayashi 2002, Nunan et al. 2001, Terrier et al. 2005, Waters et al. 2005), but the signals leading to berry expansion and softening are not fully understood.

Materials and methods

Plant material

Grapevines (*V. vinifera* L.) cv. Cabernet Sauvignon, grafted on 110 Richter rootstock, are grown in Toulouse, south-west of France (Domaine de Candie), in a non-irrigated vineyard. For the two seasons during which the experimentations have been performed, 2005 and 2006, full bloom occurred around mid-June and véraison occurred on weeks 8–9 after full bloom. The cluster harvest was recorded 14 or 15 weeks after full bloom. The equivalence between the time in weeks after full bloom and E-L growth scale (Coombe 1995) was the same for both years, and is given in Figs 1 and 2.

Treatments of grape clusters with ethylene, berry diameter and deformability measurements

Ethylene was applied for 24 h, once a week, at various times following full bloom. Ethylene gas was injected once at time 0; it was applied using a polyethylene bag wrapped around the cluster, at an initial concentration of 4 $\mu l\ l^{-1}$ and the clusters were kept inside the bag for 24 h or 1 h, depending on the experiment. Control clusters were also wrapped in plastic bags for 24 h or 1 h. For these experiments, clusters growing in a shaded area of the vines were chosen to avoid direct exposure to sunlight and overheating associated with such a treatment. Five replicates were performed using five clusters (each one from a different vine) at a similar stage of development. After treatments, the clusters were sampled and stored at −80°C pending further analyses. Every week, the berry diameter and the deformability were measured on 50 berries randomly chosen on the five clusters treated by ethylene and untreated controls using skinfold callipers (British Indicators, Burgess Hill, West Sussex, UK) according to the method described by Coombe (1992).

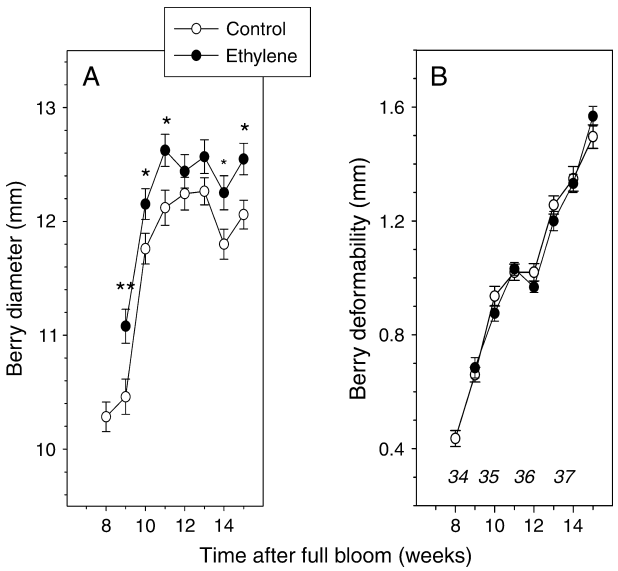

Fig. 1. Variations of diameter (A) and deformability (B) of Cabernet Sauvignon berries affected by an ethylene treatment (4 $\mu l\ l^{-1}$ for 24 h) at week 8, as a function of the time after full bloom. The first assessments in the 'ethylene' samples were made 1 week after the treatment; n = 50, error bars represent SE; * and ** show significant differences at 5 and 1%, respectively. Véraison happened between weeks 9 and 10. The numbers in italics in the right graph show E-L growth stages (Coombe 1995).

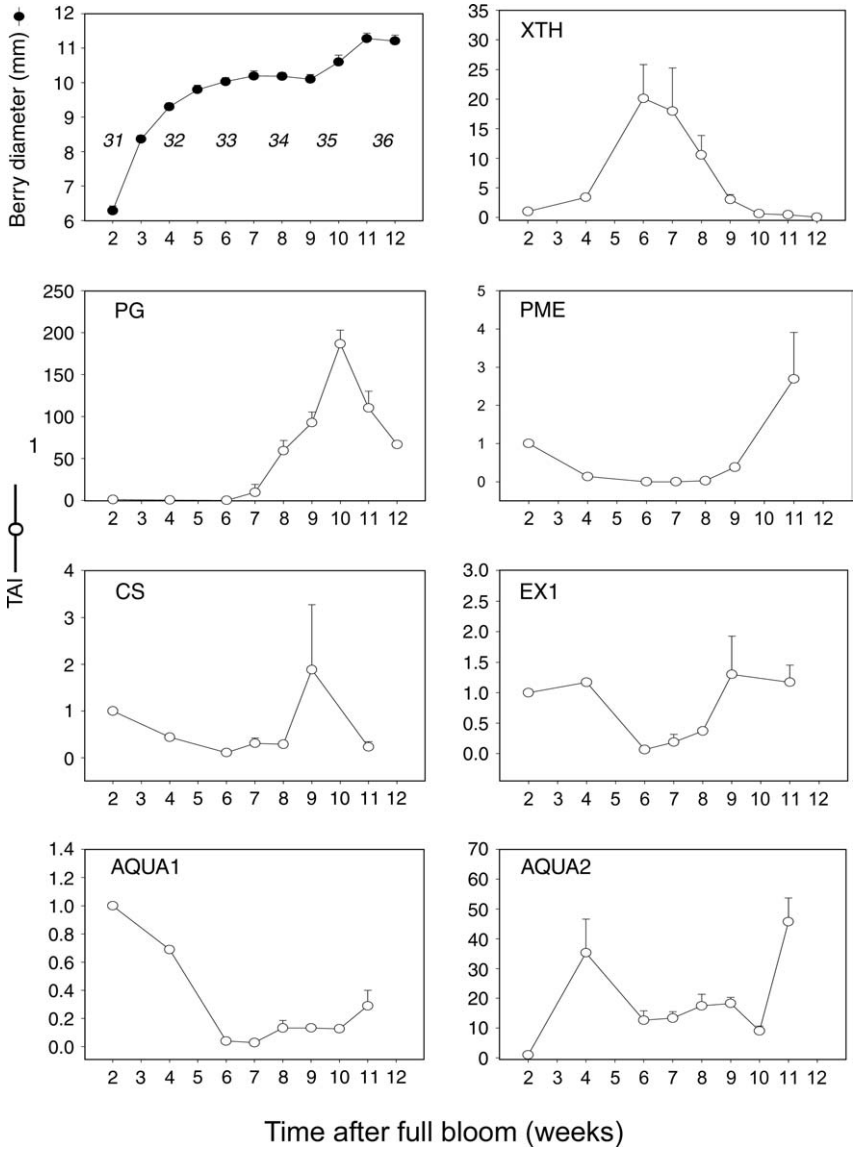

Fig. 2. Comparison of the diameter changes (n = 50 berries) and variations of gene TAI in Cabernet Sauvignon berries, at various times after full bloom. The transcript accumulation index was set at 1 for week 2 in each gene set; n = 6 replications in qPCR experiments, error bars represent SE. Véraison happened between weeks 9 and 10. The numbers in italics in the top left graph show E-L growth stages (Coombe 1995).

RNA extraction for microarray and quantitative polymerase chain reaction

Total RNA extraction was performed as previously described (Boss et al. 1996) with some modifications for microarray and quantitative polymerase chain reaction (qPCR). For the microarray, the RNAs were extracted from whole berries, whereas for qPCR they were extracted from isolated skin, pulp or seed tissues. To obtain pure RNA without DNA contamination, we used RNeasy mini kit (Qiagen, Courtaboeuf, France) and applied 10 μl of RNase-Free DNase I (Qiagen) onto the Rneasy column before elution; all other steps were performed according to Rneasy kit instructions. The absence of DNA was checked using UFGT intron primers (*UFGTi*-F 5′-CTGCAGGGCCTAACTCACTC-3′ and *UFGTi*-R 5′-TAGGTAGCACTTGGCCCATC-3′). DNase-treated RNA (4 μg) was reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen). Checking the quality of cDNA was performed by PCR with *EF1-α* primers (Supporting information Table S1).

Microarray experiments

The microarray experiment reported here was performed with three biological replications (year 2005) and two

treatments: applying ethylene at 4 μl l^{-1} or nothing (controls) and leaving the polyethylene bags for 24 h around the clusters, before sampling, freezing the berries in liquid nitrogen and storing them at −80°C until further analysis. This was performed 8 weeks after full bloom in triplicate (three separate clusters, each one on a different vine). Then three berries of each sample were regrouped in a nine berry biological replicate to extract RNAs. We processed three separate RNA extractions for each treatment.

We used the Array-Ready Oligo Set™ for the Grape (*Vitis vinifera*) Genome Version 1.0 containing 14 562 70mer probes representing 14 562 transcripts from The Institute for Genomic Research (TIGR) Grape Gene Index (VvGI), release 3. Oligonucleotides were re-annotated with the most recent release of the DFCI Grape Gene Index Version 5.0, June 2006 (Supporting information Table S2). Oligonucleotides were spotted on mirror slides and the probes were labelled with Cy3 and Cy5 dyes as described in Terrier et al. (2005). The experiment was performed with six slides (three biological replicates and a dye-swap, detailed in Supporting information Table S2). Hybridized microarrays were scanned simultaneously for Cy3- and Cy5-labelled probes with an Axon Genepix 4000B scanner (MDS Inc., Toronto, Canada).

qPCR experiments

The qPCR was performed using 100 ng of total RNA in a 10 μl reaction volume using SYBR GREEN PCR Master Mix (Applied Biosystems, Courtaboeuf, France) on an ABI PRISM 7900HT sequence-detection system (RNA samples obtained in 2006). The primers used are described in Supporting information (Table S1). All qPCR experiments were run using three biological replicates, in addition to three technical replicates in 384-well plates.

Relative fold differences were calculated based on the comparative Ct method using the *EF1-α* as an internal standard. To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount vs ΔCt was calculated for all the study gene and the referent gene (*EF-α*) as previously described (Terrier et al. 2005). To determine relative fold differences for each sample in each experiment, the Ct value for all the genes was normalized to the Ct value for *EF1-α* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

As EF1-α RNAs were used as internal standards, we checked that ethylene was not influencing significantly the EF1-α RNA accumulation (data not shown).

Statistical analyses and similarity search

For berry diameter and deformability, the means of the control and ethylene treatments were compared using *t*-tests. Regarding the microarray experiment, for each slide, data from both channels corresponding to Cy3- and Cy5-labelled probes were normalized using the LOWESS algorithm in TIGR Microarray Data Analysis System. No threshold value was applied for signal intensities and all the data were used for statistical analysis. Data from all the slides were log-transformed and normalized (centred on 0, variance equalized to 1), those data are available in Supporting information (Table S2). The significance was calculated at the 0.01 level by paired *t*-test in Multiexperiment Viewer from TIGR.

All genes that were affected by ethylene were tentatively identified by BLASTN similarity search with mRNA sequences from the whole genome sequencing project (http://www.genoscope.cns.fr/externe/English/Projets/Projet_ML/projet.htm). The complete mRNA sequences corresponding to these genes were used to identify the corresponding *Arabidopsis thaliana* proteins homologues (http://www.arabidopsis.org/) using BLASTX tool and an e-value <1e−5. These grape genes were then assigned the functions corresponding to the *Arabidopsis* homologues according to gene ontology annotations for (GOSLIM) in TAIR.

Results

Ethylene effects on transcript accumulation at the beginning of véraison: microarray experiments

Sampling for the microarray experiments has been performed after a rather long time following ethylene treatment (24 h after ethylene application), expecting to find pathways that are involved in the physiology of berry ripening, pathways for which the induction because of ethylene is sustained over many hours, more than the related signalling pathways. Experiments were performed on whole berries to detect global responses to ethylene, and in a second part, more detail expression patterns were performed on different tissues of the berry in to discriminate the response.

Table 1 show that ethylene modulate the expression of 73 genes of 14 562 genes analysed by microarray (three biological replications with a dye swap, $P < 0.01$). Among these genes, the transcription of 43 genes was stimulated (ratio >1), whereas it was reduced (ratio < 1) for 31 genes. Functional classification and the biological process were assigned for these genes as described in Materials and methods. This functional categorization indicate that ethylene response involves many loci encoding proteins implicated in response to abiotic or biotic stimuli, transport, transcription factors, cell organization and biogenesis and other process. The fact that

Table 1. Genes for which transcript accumulation is significantly affected by a 24-h treatment with 4 μl l^{-1} ethylene, in Cabernet Sauvignon berries, 8 weeks after full bloom. [a]List of genes validated with *t*-test statistics and a $P < 0.01$. [b]Transcripts deriving from the genome sequence identified using BLASTN as containing the sequence of the oligonucleotides on the grape arrays. [c]Description has been obtained by BLASTX of mRNA sequences from the whole genome sequencing project to *Arabidopsis thaliana* homologues, as described in Materials and methods. [d]Proteins with a function involved in cell elongation (and possibly berry expansion). The complete table with gene ontology groups and more details is available as supporting information (Table S3).

Oligo ID[a]	Transcript ID[b]	Description[c]	Ratio (ethylene/control)
Vv10010857	GSVIVT00026503001	Cupin family protein	2.26
Vv10009536	GSVIVT00034564001	Late embryogenesis abundant protein	2.12
Vv10003986	GSVIVT00019700001	Peroxiredoxin (PER1)	2.08
Vv10008997	GSVIVT00024981001	Cupin family protein	1.90
Vv10004533	GSVIVT00013854001	Major intrinsic family protein[d]	1.86
Vv10001169	GSVIVT00033512001	Expressed protein	1.86
Vv10006755	GSVIVT00028844001	Aldehyde dehydrogenase (ALDH2)	1.79
Vv10007437	GSVIVT00010950001	Cellulose synthase family protein[d]	1.73
Vv10011262	GSVIVT00019627001	DNAJ heat shock N-terminal domain-containing protein	1.71
Vv10003792	GSVIVT00025571001	Endopolygalacturonase, putative[d]	1.70
Vv10006756	GSVIVT00030581001	Nudix hydrolase homologue 18	1.62
Vv10010965	GSVIVT00011591001	Basic endochitinase	1.61
Vv10000294	GSVIVT00011782001	Unknown	1.61
Vv10000366	GSVIVT00020394001	Beta amylase, putative	1.56
Vv10003783	GSVIVT00025569001	Alkaline alpha galactosidase, putative	1.52
Vv10006086	GSVIVT00015029001	Amino acid permease 6	1.50
Vv10004510	GSVIVT00035855001	Leucine-rich repeat transmembrane protein kinase, putative	1.50
Vv10004205	GSVIVT00027622001	No apical meristem family protein (RD26)	1.47
Vv10007239	GSVIVT00029908001	Trehalose-6-phosphate phosphatase	1.43
Vv10005690	GSVIVT00023225001	RNA polymerase sigma subunit SigF (sigF)	1.38
Vv10008292	GSVIVT00000656001	Glutamate synthase (GLU1)	1.38
Vv10004211	GSVIVT00006236001	Xyloglucan:xyloglucosyl transferase[d]	1.37
Vv10003953	GSVIVT00014680001	Pathogenesis-related thaumatin family protein	1.34
Vv10005506	GSVIVT00031954001	ATP-dependent Clp protease putative	1.30
Vv10007442	GSVIVT00032365001	GHMP kinase family protein	1.29
Vv10000394	GSVIVT00033629001	Calcium-binding protein, putative	1.25
Vv10004402	GSVIVT00035972001	Arginine decarboxylase 2 (SPE2)	1.24
Vv10011468	GSVIVT00026451001	T-complex protein 11	1.23
Vv10013206	GSVIVT00008470001	Expressed protein	1.20
Vv10010716	GSVIVT00021505001	Cation exchanger, putative	1.19
Vv10008088	GSVIVT00023445001	Auxin-responsive factor (ARF8)	1.19
Vv10011080	GSVIVT00021669001	Phytosulphokines related	1.19
Vv10013102	GSVIVT00027080001	Ankyrin-repeat family protein	1.19
Vv10000779	GSVIVT00019025001	MIF4G domain-containing protein	1.19
Vv10002216	GSVIVT00037931001	Serine C-palmitoyltransferase (LCB2)	1.18
Vv10011103	GSVIVT00027238001	Unknown	1.17
Vv10008745	GSVIVT00031723001	Peroxidase 12 (PER12) (P12) (PRXR6)	1.15
Vv10003852	GSVIVT00024941001	Unknown	1.15
Vv10009962	GSVIVT00000758001	Structural maintenance chromosome (SMC) family protein	1.14
Vv10004009	GSVIVT00037443001	VHS domain-containing protein	1.10
Vv10012808	GSVIVT00016472001	Expressed protein	1.1
Vv10000894	GSVIVT00034747001	Ankyrin-repeat family protein	1.09
Vv10008032	GSVIVT00021497001	Yippee family protein	0.95
Vv10009881	GSVIVT00001121001	Zinc finger (C3HC4-type RING finger) family protein	0.92
Vv10011766	GSVIVT00030453001	Auxin efflux carrier family protein	0.91
Vv10002604	GSVIVT00036531001	Myb family transcription factor	0.89
Vv10009788	GSVIVT00011638001	Mannitol dehydrogenase, putative	0.88
Vv10001998	GSVIVT00023547001	Potassium transporter (HAK5)	0.86
Vv10003910	GSVIVT00014415001	Tubulin beta-1 chain (TUB1)	0.86
Vv10004817	GSVIVT00026731001	Heat shock protein 70, putative	0.86
Vv10004103	GSVIVT00028932001	DNA-binding family protein	0.85
Vv10001035	GSVIVT00005651001	Expressed protein	0.82

Table 1. Continued

Oligo ID[a]	Transcript ID[b]	Description[c]	Ratio (ethylene/control)
Vv10008728	GSVIVT00008725001	Homeobox-leucine zipper protein (HB-2)	0.81
Vv10002941	GSVIVT00014920001	Cytochrome P450 family protein	0.81
Vv10001518	GSVIVT00026288001	Cytochrome P450 76C2, putative	0.80
Vv10014094	GSVIVT00035958001	Zinc knuckle (CCHC-type) family protein	0.80
Vv10002056	GSVIVT00034638001	Class IV chitinase (CHIV)	0.80
Vv10011620	GSVIVT00024749001	E3 ubiquitin ligases	0.77
Vv10001666	GSVIVT00033153001	Ubiquitin-conjugating enzyme family protein	0.76
Vv10007063	GSVIVT00025111001	ABC transporter family protein	0.75
Vv10000906	GSVIVT00007730001	SWIB complex BAF60b domain-containing protein	0.75
Vv10000393	GSVIVT00025379001	Omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2)	0.75
Vv10008566	GSVIVT00014406001	Ferredoxin-NADP(+) reductase, putative	0.75
Vv10000324	GSVIVT00023677001	Dienelactone hydrolase family protein	0.72
Vv10007508	GSVIVT00028977001	5′-AMP-activated protein kinase related	0.72
Vv10003885	GSVIVT00015518001	Plastocyanin	0.71
Vv10000425	GSVIVT00000851001	Expansin, putative[d]	0.70
Vv10002996	GSVIVT00008438001	Expressed protein	0.69
Vv10001255	GSVIVT00006912001	Thiazole biosynthetic enzyme, chloroplast (ARA6)	0.69
Vv10011114	GSVIVT00030529001	Bet v I allergen family protein	0.64
Vv10003860	GSVIVT00029136001	Chlorophyll A–B-binding protein	0.64
Vv10001209	GSVIVT00032674001	Protease inhibitor/seed storage/lipid transfer proteins	0.63
Vv10003711	GSVIVT00022146001	Major intrinsic family protein[d]	0.56

about 10% of these loci share homology with transcription factors indicate that this mechanism requires complex regulatory control.

Interestingly, 12% of these genes are similar to proteins involved in cell transport and it is known that the berry ripening phase is partly dependent to the phloem downloading activity and associated metabolisms (Coombe 1992).

Among many functions, we noticed that ethylene regulates the expression of genes involved in the grape berry cellular expansion or softening such as xyloglucan endotransglucosylase (XTH) (ratio 1.37), endopolygalacturonase (ratio 1.70), cellulose synthase (CS) (ratio 1.73), expansin (EX) (ratio 0.70) or aquaporins (AQUA) (ratio 0.56 or 1.86), the latter being probably involved in water and sap transport within cells.

Some genes showed significant repression or induction by ethylene despite a ratio very close to 1 (more than 0.75 or less than 1.25), this means that there were very small variations in the hybridization signals of the three replicates, but the impact of such results on berry physiology have to be considered with care.

Because in a previous work (Chervin et al. 2004), we found that application of an inhibitor of ethylene perception led to berries with a smaller diameter than controls, we decided to check the effect of ethylene on berry diameter and deformability. We decided to further check by qPCR the ethylene effect on the transcript accumulation of a few genes that were shown to be upregulated by ethylene in the microarray experiments, but first we verified the ethylene effect on berry size.

Ethylene effect on berry diameter and transcript accumulation of genes related to cell expansion: physiological measurements and qPCR experiments

The results of Fig. 1A show that ethylene, when applied at a low dose (4 μl l^{-1}) at week 8 post-flowering, induced an increase in the berry diameter over the following weeks. This diameter increase was acquired within the week following the treatment and the difference with controls was maintained over the remaining ripening period. The results of Fig. 1B show that the difference in diameter was not paralleled by a difference in berry deformability, suggesting that the ethylene may affect cell wall changes and sap influx, leading to greater cell expansion rates for a few days following the application, but that turgor is not affected by this hormonal treatment.

When ethylene treatment was performed on grape berry at week 2 to week 7 after full bloom, and from week 10 to week 12, the diameter and the deformability of grape berries were not affected by ethylene (data not shown). Thus, the cells were sensitive to ethylene mostly at the beginning of véraison.

Then, we decided to check the variation in transcript accumulation of the genes detected in the 24-h-induction

microarray experiment and related to berry expansion. We ran a first set of qPCR with transcripts extracted at various stages of berry development (Fig. 2). These genes can be classified in two groups: cell wall structure genes and water exchange genes. In the first group, there are the *XTH*, polygalacturonase (*PG*), pectin methylesterase (*PME*), *CS* and *EX*. The second group (water exchange) includes several members of the AQUA gene family (*AQUA*). Most genes were known to be expressed in fruit during ripening, in tomato (Cho and Cosgrove 2004), mango (Sane et al. 2005), peach (Hayama et al. 2006), banana (Trivedi and Nath 2004, Wang et al. 2006) and strawberry (Tian et al. 2000).

In Fig. 2, we show some potential correlation between the variation of the berry diameter and the related gene expression pattern during the development of berry, between week 2 and week 11 after full bloom. The transcript accumulation index (TAI) value was set at 1 for week 2 in each gene set.

The time–course expansion of grape berries is characterized by two phases of growth, very characteristic in grape (Coombe 1992): a first phase (phase 1) of growth between week 2 and week 9, which is stopped around véraison and a second phase (phase 2) of growth from week 9 to week 14. The first phase of berry growth is in relation to the increase in the number of cells and their volume, and the second phase of the increase in the diameter is only because of the increase in cellular volume. The second phase of berry growth is also characterized by an increase in the softening of berry (Fig. 1).

The analysis of the transcript accumulation (Fig. 2) allowed classifying these genes into three categories. In the first category, for which the amount transcript was high during the first phase (phase 1) of diameter growth: it is the case for *AQUA1*. In the second category, the accumulation of transcripts was maximum at the beginning of the second phase (phase 2) of diameter growth: it is the case of *PG*, *PME*, *CS*. Variations of *XTH* transcripts are a particular case as they accumulate at the end of phase 1, when the berry diameter was not increasing as steadily as in the first weeks. The third category gathers the genes where expression was high over both expansion phases of the berries: it is the case of *EX1* and *AQUA2*. However, *EX1* transcripts seem to reach high levels before those of *AQUA2*.

These results suggest that some cell wall-modifying enzymes (EX1) and proteins involved in water exchange (AQUA1 and AQUA2) could be involved in both expansion phases of grape berries, as their transcripts' accumulation show a rather good correlation with both berry expansion phases. This is in agreement with previously published results (Nunan et al. 2001). However, transcripts of AQUA1 were not increasing as much as AQUA2 in the second phase.

Then we decided to check the effect of ethylene application on the accumulation of these transcripts. As we showed that ethylene affected berry diameter mainly when applied just before véraison, we decided to test the effect of ethylene application at week 8. After a preliminary set of RNA extraction of whole berry tissues, showing lots of variability in responses to ethylene, we decided to separate the berry tissue before RNA extraction: skin, pulp and seeds. This second season, we also ran an experiment with a short time induction in presence of ethylene: 1 h, to check the early responses to this hormone application.

For each set of results, we have chosen to give the relative value of '1' for the TAI of untreated controls (TAI = 1). In other words, the TAI of each gene after ethylene treatment was determined relatively to the control value.

We report here the ethylene effects on transcript accumulation over two durations: 1 h for the short time ethylene effect (Fig. 3) or 24 h for the long-time ethylene effect (Fig. 4).

The results presented in Fig. 3 show that ethylene has induced rapidly (1 h), a strong accumulation in the skin of most selected gene transcripts, except for *PME* gene. In the skin tissues, TAI was strongly increased for *AQUA2*, *EX2* or *PG* genes 1 h after ethylene treatment. Similarly, in the seeds, ethylene provokes a significant increase in the TAIs for *PME* and *AQUA1* genes but these bursts were less important than in the skin. In pulp, only the *XTH* gene expression seems to be clearly increased by ethylene, whereas the expression of the other studied genes is not affected by ethylene. The strongest impact being observed in the peripheral tissues like skin is also logical with the fact that skin is the first tissue in contact with ethylene, in the case of exogenous supply.

Indeed, after a 24-h induction, no transcript accumulation was boosted by ethylene in skin tissues any more (Fig. 4). Only *AQUA2* gene was still highly expressed in the pulp of berries treated by ethylene. In the seeds, only *PG* gene was strongly induced by ethylene; the stimulation of the other genes by ethylene, *XTH*, *CS*, *AQUA2*, *PME* and *EX1* was less marked.

These results show that the expression of these genes involved in the expansion of grape berry cells is strongly tissue and time specific. In the skin, the gene expression is rapidly stimulated and then comes back to a value similar to the control 24 h after the ethylene treatment. In contrary, in the seeds, a great number of genes were induced 24 h after ethylene treatment. In the pulp, ethylene does not seem to have marked effects on the gene expression 1 h or 24 h except for *XTH* and *AQUA2*.

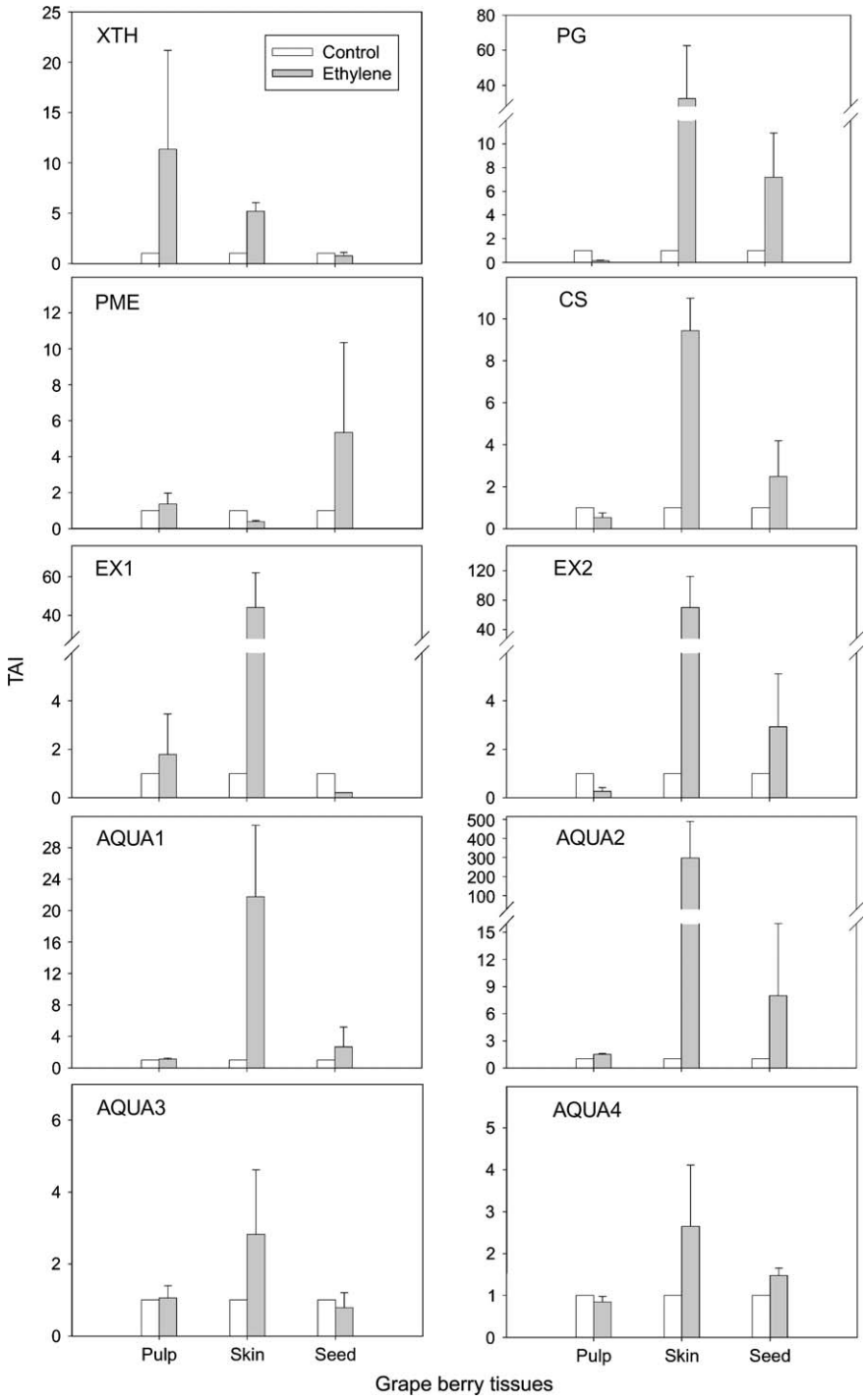

Fig. 3. The mRNA level (transcript accumulation index) of various genes in three tissue types after 1 h of ethylene treatment in Cabernet Sauvignon berries at week 8 after full bloom, was expressed relatively to controls (set at 1), reference gene *EF1-α*. n = 3 replications, error bars show SE.

These could be of great importance in the stimulation of berry expansion by ethylene observed in Fig. 1.

Independent of cell elongation, the influence of ethylene on transcript accumulation was checked by qPCR for some additional genes, that were known to respond to ethylene either in the present microarray experiment or in recent papers about ethylene effects on plant metabolism (De Paepe et al. 2004, Van Zhong

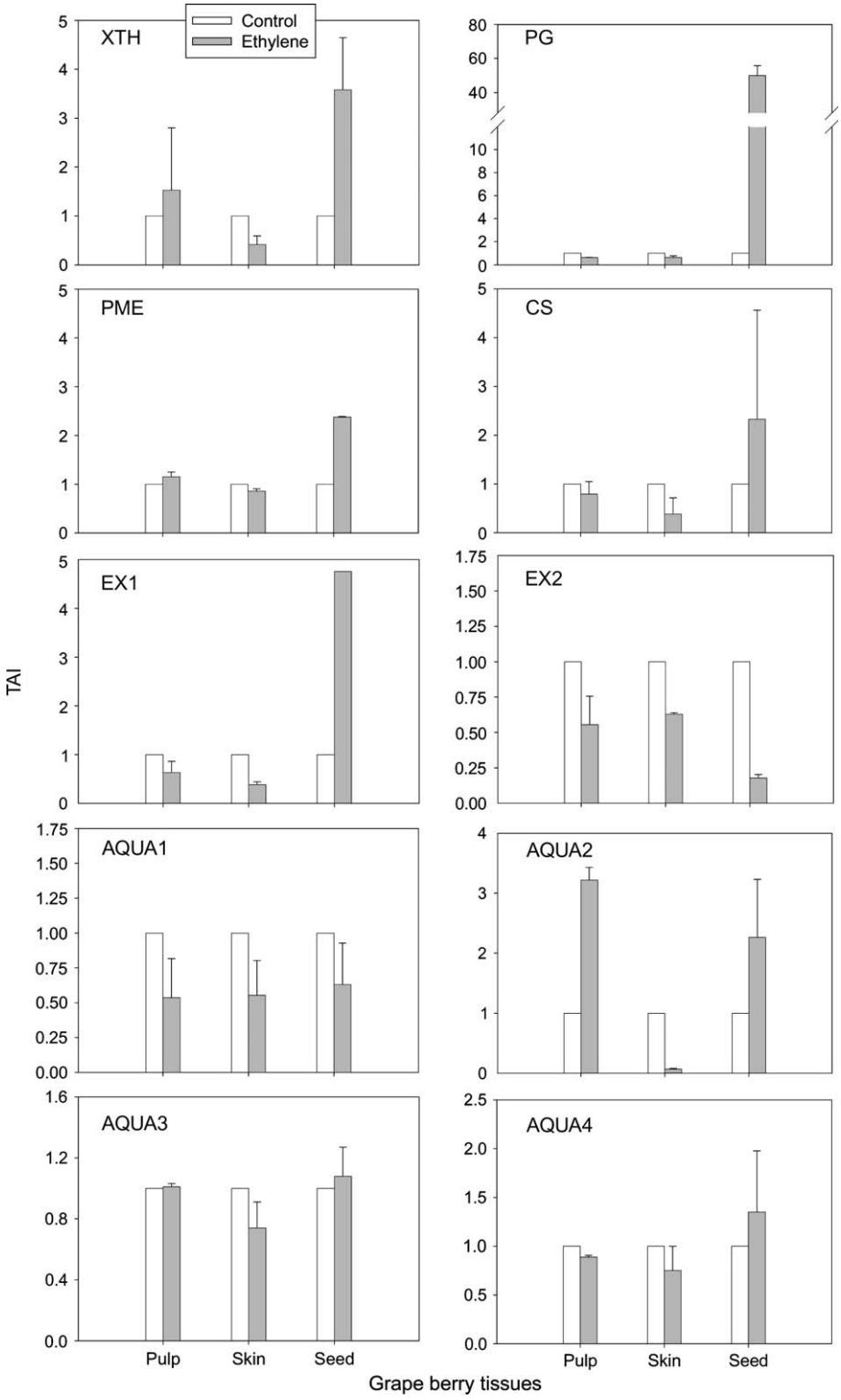

Fig. 4. The mRNA level (TAI) of various genes in three tissue types after 24 h of ethylene treatment in Cabernet Sauvignon berries at week 8 after full bloom was expressed relatively to controls (set at 1), reference gene *EF1-α*. n = 3 replications, error bars show SE.

and Burns 2003). The primer list and gene accession numbers are given as Supporting information (Table S1). After 1-h incubation with ethylene, we obtained the following 'ethylene/control' ratios for the TAI: 29, 5.6, 4.9, 3 and 1.5 for the cupin2, peroxiredoxin, ethylene responsive element (ERE)-binding protein, cupin1 and flavonone-3-hydroxylase, respectively, in the skin tissues. After 24-h incubation with ethylene, we obtained similarly the following ratios: 2.2, 1.8, 1.8, 1.7 and 1.6 for the chalcone synthase, peroxiredoxin, cupin2, ACC oxydase and alcohol dehydrogenase, respectively.

Discussion

According to our knowledge, this is the most extensive set of data targeting the role of ethylene in the grape berry expansion and softening processes. Fruit softening in grapes is clearly a complex process that involves subtle changes in different components of cell wall and in many cases would require only small amounts of enzyme activity indicating that a low change in gene expressions related to the control of cell wall composition could allow the beginning of softening. The fact that low doses of ethylene stimulated the grape berry expansion (Fig. 1) are in accordance with the fact that low doses of 1-methylcyclopropene (1-MCP), an ethylene inhibitor, limited the berry expansion (Chervin et al. 2004). Because at this stage of development, the berry growth relies mainly on cell elongation, these results are in accordance with previous results showing that ethylene stimulates plant cell elongation (Smalle et al. 1997).

In grape berries, it is noticeable that the applied ethylene treatment had a discrete impact on the global transcriptome on a quantitative and qualitative point of view: only 73 genes are significantly differentially expressed and the magnitude of the variation is rather low. But real-time PCR experiments presented in this paper revealed that this could be explained by the long lag time (24 h) between ethylene treatment and berry sampling and mixing of tissues because RNA was extracted from whole berry. Extended impact on transcription was measured when berries were sampled only 1 h after treatment and that the different tissues of the berries were separated before RNA extraction. This confirms that the transcriptional response of the plant to ethylene is quite rapid and tissue specific (Barry and Giovannoni 2007).

In the pulp, the changes induced by ethylene are not as remarkable as in the skin (Fig. 3), except for *XTH* (over 1-h induction; Fig. 3) and *AQUA2* (over 24-h induction; Fig. 4) that may be part of the expansion response to ethylene. Indeed, the expansion of pulp tissues is probably critical in the berry expansion phase, as they represent the major berry tissue. *AQUA2* is probably a good candidate to further study the role of AQUA in expansion and water exchange of grape berries. Aquaporin expression has already been observed in grape berries following véraison (Picaud et al. 2003, Reid et al. 2006), but these studies focussed on different transcripts than those described in this paper. The stimulation of *AQUA2* by ethylene in skin tissues reached 300% and was the biggest stimulation observed for the whole gene set.

XTH mRNAs are present mainly in the skin and ethylene increases their accumulation. Nunan et al. (2001) have also observed the presence of other *XTH* mRNAs in ripening grape berries and suggested a possible role for this enzyme in wall modification. In the time–course of developing berries (Fig. 2), the occurrence of *XTH* transcripts before others like *PME* or *PG*, suggest that XTH action may prepare the cell wall to further modifications by PME, PG and others.

PME and *PG* might contribute to increase the solubility of pectic polysaccharides of cell walls observed during ripening of grape berries (Nunan et al. 1998). *PG* transcript accumulations increase at véraison when berry expansion resumes (Fig. 2). Ethylene stimulates the accumulation of the *PG* transcripts, indicating that ethylene could be one of the signal triggering the berry expansion. *PME* transcripts also increased after véraison, but later than *PG* (Fig. 2), and in a previous study, the authors did observe a *PME* transcript accumulation but no related increase in PME activity over the second phase of ripening of grape berries, cv. Muscat Gordo Blanco (Nunan et al. 2001). In addition, the stimulation of PME by ethylene was less marked, and only in seeds (Figs 3, 4). Thus, *PME* seems less critical than *PG* to the berry expansion in response to an ethylene stimulus.

CS was marginally induced as the berry expansion resumed (Fig. 2) and was also stimulated by ethylene in the skin tissues (Fig. 3). This enzyme is known to be critical in cell wall expansion, as a sustained cellulose synthesis is then necessary. There is not a lot of data regarding ethylene and cellulose synthesis. Luo et al. (2007) reported recently that inhibition of ethylene perception by 1-MCP retarded the cellulose accumulation in bamboo shoots.

Nunan et al. (1998) have shown that the first major change of the cell wall composition during grape berry ripening was a decrease in galactose/galactan content. This decrease was related to β-galactosidase activities, which increased dramatically as the berry began to soften. Nunan et al. (2001) showed that accumulation of β-galactosidase mRNA was detected in pre-véraison and early post-véraison grapes. Similarly, Barnavon et al. (2000) showed that the pattern of a *β-gal* transcript accumulation was only detectable in the early stages of development. In our

microarray experiment, no transcript of β-galactosidase was detected among the strong changes induced by ethylene (Table 1), but we found an α-galactosidase in this category. This was not further developed.

Similarly, in the EX family, the accumulation of *EX1* transcripts was high in each expansion phase of the berry diameter (Fig. 2). The accumulation of *EX1* and *EX2* transcripts was strongly stimulated by ethylene in the skin 1 h after treatment (Fig. 3). The EX involvement in berry expansion may be linked to depolymerization of structural hemicelluloses and polyuronides (Brummell et al. 1999). In the seeds, *EX2* was inhibited by ethylene, but the expression of *EX1* was strongly increased. Whether the EX produced in the seeds can migrate to the pulp remains undetermined.

It seems logical that most stimulating effects by ethylene were observed in the skin tissues after 1 h (Fig. 3) as they were the first tissues in contact with this exogenous treatment, even if we used a very low dose of ethylene. It is worth noting that the recommended rates for commercial for applications of ethephon (ethylene precursor) to induce chemical thinning or promote ripening led to spraying very high doses of ethylene equivalent to more than 1000 $\mu l\ l^{-1}$, more than 200-fold the dose we used here, and at such high doses a diminution of berry diameter has often been observed. The genes whose transcript accumulated markedly in the skin were *PG*, both *EXs*, and *AQUA1* & *AQUA2*, and to some extent *CS*. This suggests that the skin loosening necessary to berry expansion may be controlled at véraison by the corresponding proteins.

Additionally, we verified that the accumulation of gene transcripts that was shown to be enhanced by ethylene in our microarray experiment was also observable by qPCR. It was the case for the genes we tested, two cupins and one peroxiredoxin, particularly for one cupin with an ethylene/control ratio as high as 29. This cupin superfamily encompasses a great number of functions (Dunwell et al. 2001), and we were not sure about their involvement in cell elongation. Thus, the study was not further extended with this gene family; however, we now think it would be worth more experiments. We also checked the induction of some gene transcripts well known to respond to ethylene (De Paepe et al. 2004, Tesniere et al. 2004, Van Zhong and Burns 2003). The qPCR results showed that it was the case for an ERE-binding protein and other genes like an ACC oxidase, an alcohol dehydrogenase or a chalcone synthase.

Ethylene has many roles in regulating the plant phenotype, and its particular role about cell elongation has been reviewed recently with other hormone interplay, such as auxins, giberrellins and brassinosteroids (Vandenbussche and Van Der Straeten 2007).

Conclusions

Grape berry size and softening are important characters in the grape production. We observed that low doses of ethylene application increased the berry diameter at the inception of the ripening stage: véraison. Ethylene did not affect the berry deformability.

This ethylene effect could be related to changes in transcript accumulation of XTHs and AQUAs that were found to accumulate in the pulp of ethylene-treated berries at higher rates than in controls. The physiological and biochemical changes in pulp tissues are probably critical to determine the berry diameter as the pulp represents a large part of the berry tissues. Our results also confirmed previous studies regarding the good correlations between berry ripening and the accumulation of various transcripts of PG, PME, CS and EX. Some of these enzymes may be involved in the increase of berry expansion by ethylene, even if their stimulation by ethylene was mainly observed in the skins, that may be regarded as less important than pulp tissues to explain variations in the berry diameter. However, the skin rigidity could restrict the berry expansion, thus enzyme actions leading to a higher plasticity of the skin may also be important in the increase of the berry diameter.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of primers used to control the induction by ethylene of a series of genes known to respond to this hormone.

Table S2. Experimental design and hybridization result log2 transformed and normalized.

Table S3. List of genes for which transcript accumulation is significantly affected by ethylene, analysis performed using microarrays, with gene ontology annotation.