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Ripening-related gene expression during fruit ripening in *Vitis vinifera* L. cv. Cabernet Sauvignon and Clairette blanche

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Summary

The gene expression patterns in ripening fruit of a high quality wine cultivar (Cabernet Sauvignon) and a poor quality wine cultivar (Clairette blanche) were studied using cDNA-AFLP fingerprinting. Total RNA from "immature" (14-weeks post flowering) and "mature" (18-weeks post flowering) berries were used to study ripening-related gene expression in post-véraison stages of berry development. A total of 1,276 fragments were analysed, of which 175 appeared to be ripening-related. Average pairwise differences of the fragments amplified from "immature" and "mature" Cabernet Sauvignon and Clairette blanche berries, revealed a high level of similarity between the two cultivars. 70 % of the ripening-related fragments were cultivar-specific. The number of cultivar-specific and/or ripening-related fragments amplified, depended on the selective nucleotides of the primers used in the cDNA-AFLP analysis. Reverse slot blot and northern blot analysis confirmed that the expression of the identified genes were ripening-related.

K e y w o r d s : Grapevine, ripening-related; gene expression; cDNA-AFLP, fruit ripening; fruit quality.

Introduction

Grape berry ripening is characterised by dramatic changes in characteristics determining the quality of the final product (Hawker 1969 a, b; Kanellis and Roubelakis-Angelakis 1993). In climacteric fruit - such as tomato - genes involved in these biochemical and physiological changes, which expression can possibly be manipulated to improve fruit quality, has been extensively studied (Aharoni *et al*. 2002; Moore *et al*. 2002; White 2002). On the contrary, little is known about the ripening-related expression of genes in non-climateric fruit - such as grapevine and strawberry. In grapevine, a number of ripening-related genes were identified by the characterisation of candidate genes (Boss *et al*. 1996; Tattersall *et al*. 1997; Davies *et al*. 1999; Nunan *et al*. 2001; Terrier *et al*. 2001 a), but often unexpected pathways were also activated during berry ripening (Tesnière and Verriès 2000). To date, however, most ripening-related genes reported in grapevine have been identified through sequence analysis and hybridisation-based differential screening of cDNA libraries (Davies and Robinson 2000; Ablet *et al*. 2000; Terrier *et al*. 2001 b) or the gel-based RNA fingerprinting technique DNA-AFLPs (Venter *et al*. 2001).

In an attempt to identify genes involved in the developmental processes related to berry quality, ripening-related gene expression in two cultivars with different wine qualities, was studied. Cabernet Sauvignon, the world's most renowned grape variety for the production of fine red wine, and Clairette blanche, a decidedly old-fashioned variety known for the production of flabby white wine, were selected for the analysis. Both cultivars were grown at the ARC-Nietvoorbij grapevine collection block in Stellenbosch. Date of bloom of both cultivars is late (3rd and 4th week of October) and maturation is late in the season (2nd and 3rd week of March), minimising the potential effect of various environmental factors on grape berry ripening and ripening-related gene expression. Given that the most dramatic changes in characteristics determining the quality of the final product, occur as the fruit enters into the ripening phase, this study focused on ripening-related gene expression in the post-véraison stage. The term post-véraison refers to the stages of ripening following the change in berry skin colour and the onset of sugar accumulation and berry softening.

Gene expression in ripening berries was studied using cDNA-AFLP fingerprinting. Amplified fragment length polymorphism (AFLP) is a powerful technique for fingerprinting of genomic (Vos *et al*. 1995) and complementary DNA (cDNA) (Money *et al*. 1996; Bachem *et al*. 1996; Habu *et al*. 1997). The technique represents an indigenous combination of restriction fragment length polymorphism (RFLP) analysis and polymerase chain reaction (PCR), resulting in highly informative fingerprints. cDNA-AFLP analysis consists of 4 steps: (1) synthesis of cDNA, (2) production of primary template by restriction digestion of cDNA with two restriction enzymes and ligation of adaptors to the termini of these cDNA fragments, (3) pre-amplification with primers corresponding to the adaptors ligated to the cDNA fragments, and (4) selective amplification of the cDNA fragments with primers extended with one or more specific bases. A fingerprint is produced by radioactive labeling of one of the primers used for selective amplification, polyacrylamide gel electrophoresis of the amplification product and visualisation of the amplification products by autoradiography (exposure to X-ray film). A step-by-step protocol is presented by Bachem *et al*. (1998).

Considerations of the cDNA-AFLP approach included (1) the extensive use of AFLPs to identify differentially-regu-

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lated genes in plants and other organisms (BREYNE and ZABEAU 2001; DONSON *et al*. 2002); (2) the observations that members of multigenic families often exhibit distinct developmental patterns during berry ripening (DAVIES *et al*. 1999; FILLION *et al*., 1999; TESNIÈRE and VERRIÈS 2000), which emphasise the utilisation of sequence-based analysis for the unambiguous characterisation of these isogenes and, (3) that contrary to hybridisation-based approaches, sequence-based approaches are not biased towards abundant transcripts (BREYNE and ZABEAU 2001). In sequence-based approaches identification of mRNAs is not limited by redundancy of highly expressed mRNAs or under-representation of rare mRNAs in a cDNA library. It is estimated that the 105,000 ESTs in the *Arabidopsis thaliana* collection are representative of only 60 % of all the genes, illustrating the extent to which cDNA libraries fail to represent all mRNAs (RICHMOND and SOMMERVILLE 2000). More recently, KUHN (2001) reported that only 1.4-5 % of the 1443 *Arabidopsis* genes analysed in cDNA microarrays represented highly expressed genes with abundance of more than 100-500 transcripts per cell. Most members in this class were well-characterised housekeeping or tissue-specific genes. The majority of the expressed genes were low abundance with levels of less than 10-50 transcripts per cell (RUAN *et al*. 1998). Many important regulatory genes can thus be overlooked by hybridisation-based approaches as abundant messages are over-expressed in cDNA libraries and rarely expressed genes are often missing.

Here we illustrate the usefulness of cDNA-AFLPs for the characterisation of ripening-related gene expression during grape berry ripening. In addition, the results suggest that gene expression in ripening berries of the cvs Cabernet Sauvignon and Clairette blanche, which differ largely phenotypically, is remarkably similar. Nevertheless, obvious differences in ripening-related gene expression of the two cultivars were identified.

Material and Methods

Cabernet Sauvignon and Clairette blanche berries were collected from the ARC-Nietvoorbij collection block in Stellenbosch. "Immature" berries were collected 14-weeks post flowering (14-wpf), which was one week post-véraison. The dark-red colour of the Cabernet Sauvigon berries and the yellowish-green colour of the 14-wpf Clairette blanche berries, were consistent with the post-véraison stage of berry development, which is characterised by the loss of chlorophyll from the skin and the accumulation of anthocyanins in berries of Cabernet Sauvignon. "Mature" berries were collected just prior to commercial harvest (sugar contents of Clairette blanche: 21.4 °Brix, Cabernet Sauvignon: 22.8 °Brix). Berries were deseeded, frozen in liquid N_2 and stored at –80 °C until further use.

Total RNA isolation and cDNA synthesis: Total RNA was isolated from ripening berries and leaf tissue using a modified sodium-perchlorate method (VENTER *et al*. 2001). Total RNA, 5 µg from each sample, was *Dnase*I-treated using the MessageClean kit (GenHunter Corporation) and subjected to first strand synthesis using an oligonucleotide 5'-AGTCTGCAGT_{12}V-3', where V denotes A, C or G (MONEY *et al*. 1996). Second strand synthesis was performed using the Universal Riboclone cDNA synthesis system (Promega Corporation, Madison, USA). Duplicate cDNA samples were prepared from RNA obtained from independent RNA isolations.

cDNA-AFLP analysis: cDNA-AFLP analysis was performed according to VENTER *et al*. (2001). Twenty-five combinations of *Pst*I and *Mse*I primers, containing up to three selective nucleotides each, were used for selective amplification (Tab. 1). To confirm that total RNA samples were free of genomic DNA, AFLP analysis was performed using an aliquot of the total RNA samples as template. To verify the reproducibility of the technique, duplicate cDNA

Table 1

Primer combinations used and the number of fragments visualised by cDNA-AFLP analysis of ripening Clairette blanche and Cabernet Sauvignon berries. The number of differentially amplified fragments identified by the analyses is indicated. *Mse*I primers used for selective amplification included *Mse*I (+CAA), (+CAC), (+CAG), (+CAT) and (+CTG)

	Number of cDNA fragments visualised (average number of fragments per primer combination)	Number of differentially amplified cDNA fragments (expressed as a percentage of the total number of differentially amplified fragments)
*Mse*I (+3) + *Pst*I (+ 0)	357 (71)	0 [1]
*Mse*I (+3) + *Pst*I (+ C)	315 (63)	24 (7.6 %)
*Mse*I (+3) + *Pst*I (+ G)	226 (45)	67 (29.6 %)
*Mse*I (+3) + *Pst*I (+ GT)	198 (40)	46 (23.2 %)
*Mse*I (+3) + *Pst*I (+ GTA)	180 (36)	38 (21.1 %)
Total	1,276	175

[1] The high number of fragments visualised per gel impeded the accurate scoring of the fragments, and these fragments were not included in further analysis.

samples were subjected to AFLP analysis using three primer combinations viz. *Mse*I (+CAA) and *Pst*I (+0); *Mse*I (+CAT) and *Pst*I (+C); *Mse*I (+CAC) and *Pst*I (+GTA).

Following visualisation of the amplification products, the amplified cDNA fragments were scored manually, "present" or "absent". Absolute pairwise differences of the cDNAs amplified were determined using PAUP version 4.0b10 for Macintosh. Differentially amplified fragments were excised from the dehydrated polyacrylamide gels and re-amplified as described in VENTER *et al*. (2001).

Reverse slot blot analysis: A 24 well slot blot manifold was used for this purpose (Sigma). Preparation and application of the cDNA samples (5 µl of each re-amplification product) to the positively charged nylon membrane (Roche Diagnostics Mannheim, Germany) were performed according to AUSUBEL *et al*. (1992). Filtered water (0.22 µm Cameo 25AS cellulose acetate syringe filter, Osmonics, USA) was applied to the membrane as negative control. Two identical membranes were prepared for each of the cultivars.

Single strand cDNA probes were synthesised by reverse transcription of 5 µg total RNA from each sample using Superscript™ II (Invitrogen life technologies, UK) according to the protocol supplied by the manufacturer. Modifications to the protocol included the replacement of the dNTP solution with dNTPs (-dCTP), final concentration 75 µM, and the addition of 50 µCi [α-^{32}P] dCTP (3,000 Ci·mmol^{-1}) (Amersham Pharmacia Biotech). The reaction volume was adjusted to 30 µl. Hybridisation was performed using Rapid-hyb buffer (Amersham Pharmacia Biotech) and equal counts (1 x 10^7 cpm·µg^{-1} DNA) of each of the cDNA probes. Procedures for hybridisation and stringency washes were according to the manufacturer's instructions. Hybridisation was visualised by autoradiography (Kodak BioMax MS film, intensifying screens, room temperature, overnight). Differences in hybridisation intensities were visually determined. Visual differences were considered significant if the hybridisation signal was (1) clearly stronger than the background signal produced by the negative control, and (2) clearly stronger than the hybridisation signal in the sample which it is compared to.

The membranes were stripped using boiling 0.1 % (w/v) SDS before being probed with cDNA from leaf material.

Messenger RNA isolation: Messenger RNA was isolated from Clairette blanche berries and leaf material from total RNA using PolyATtract mRNA isolation systems (Promega Corporation, Madison, WI, USA) and quantified fluorometrically (BIO-TEK Instruments Inc., Winooski, Vermont, USA).

Northern blot analysis: The re-amplified, uncloned fragments were radioactively labelled as described by VENTER *et al*. (2001). Messenger RNA (50 ng per lane) was denatured, size fractionated (1.2 % agarose, 3 V cm^{-1}; 2 h) and transferred to positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) by alkaline downward capillary blotting (INGELBRECHT *et al*. 1998). Duplicate membranes were prepared to accommodate analysis of all the selected cDNA fragments simultaneously. RNA was cross-linked to the membrane (2.5 min at 120 mJ·cm^{-1}) using an ultraviolet crosslinker (ULTRA.LUM, Scientific Associates). The membranes were dried before subjected to hybridisation procedures in a 50 % formamide-containing hybridisation buffer (ULTRAhyb™ Ultrasensitive Hybridisation buffer, Ambion, USA) at 42 °C. Stringency washes were performed according to the manufacturer's recommendations: 2 x 10 min in 2xSSC + 0.1 % (w/v) SDS at 42 °C, and 2 x 15 min in 0.1xSSC + 0.1 % (w/v) SDS at 42 °C. Hybridisation was visualised by phospho-imaging using the AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, USA). Differences in hybridisation intensities were visually determined, and regarded as significant, as stated for reverse slot blot analysis.

Results

Total RNA and mRNA isolation: The average total RNA yield from ripening Clairette Blanche and Cabernet Sauvignon berries was 25 ±5 and 8 ±2 µg·g^{-1} fresh weight for "immature" (14-wpf) and "mature" (18-wpf) berries respectively. In both "immature" and "mature" berries the poly(A)$^+$ mRNA comprised 0.85 % of the total RNA.

cDNA AFLP analysis: Approximately 50 discrete fragments, ranging from 80 to 600 base pairs, were visualised for each of the 25 *Mse*I and *Pst*I primer combinations used (Tab. 1). A total of 1,276 fragments was visualised. The high number of fragments for primer combinations *Mse*I (+3) and *Pst*I (+0) made accurate scoring of the fragments difficult and these were therefore not included in further analysis. cDNA-AFLP fingerprints generated from the duplicate cDNA samples were identical. No amplification products could be visualised in the reactions using total RNA as template.

Average pairwise differences revealed a 16 % difference in the fragments amplified from "mature" and "immature" Clairette blanche berries, and 19 % in the case of Cabernet Sauvignon, respectively (Fig. 1). The average pairwise difference between the two cultivars was 35 %.

100 and 75 ripening-related fragments were identified (Tabs 1 and 2). Only 52 of these (30 %) were similarly expressed in Clairette blanche and Cabernet Sauvignon, of

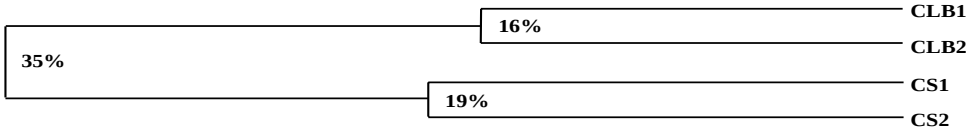

Fig.1: UPGMA tree of the average pairwise difference between cDNA fragments amplified from "immature" and "mature" Clairette blanche and Cabernet Sauvignon berries. CLB1 and CLB2: Clairette blanche "immature" and "mature" berries, respectively; CS1 and CS2: Cabernet Sauvignon "immature" and "mature" berries, respectively. The average pairwise difference is expressed as percentage of the total number of cDNA fragments scored.

Table 2

The number of differentially amplified fragments identified by cDNA-AFLP analysis and their expression in ripening Clairette blanche and Cabernet Sauvignon berries. CLB1 and CLB2: "immature" and "mature" Clairette berries: CS1 and CS2: "immature" and "mature" Cabernet berries. Presence or absence is indicated by “1” or “0”. Fragments similarly regulated in both cultivars are printed in *italics*. Arrowheads indicate fragments not present in the one cultivar, but ripening-related in the other. The number in parentheses was calculated by multiplying the score of “1” by the “number of fragments”. This was only performed in cultivars the fragment(s) of which were ripening-related

Number of fragments	Comment	Clairette blanche		Cabernet Sauvignon	
		CLB1	CLB2	CS1	CS2
21	Down-regulated in both cultivars	1 (*21*)	0	1 (*21*)	0
31	Up-regulated in both cultivars	0	1 (*31*)	0	1 (*31*)
▶ 20	Not present in Clairette, up-regulated in Cabernet	0	0	0	1 (20)
▶ 23	Not present in Clairette, down-regulated in Cabernet	0	0	1 (23)	0
6	Constitutive in Clairette, up-regulated in Cabernet	1	1	0	1 (6)
19	Constitutive in Clairette, down-regulated in Cabernet	1	1	1 (19)	0
▶ 16	Not present in Cabernet, up-regulated in Clairette	0	1 (16)	0	0
▶ 27	Not present in Cabernet, down-regulated in Clairette	1 (27)	0	0	0
10	Constitutive in Cabernet, up-regulated in Clairette	0	1 (10)	1	1
2	Up-regulated in Clairette; down-regulated in Cabernet	0	1 (2)	1 (2)	0
175 ◀	Totals ▶	(48)	(59)	(65)	(57)

which 60 % were more abundant in the "mature" berries (Tab. 2, printed in italics). Most of the remaining 123 fragments were cultivar-specific and up- or down-regulated during fruit ripening (Tab. 2, indicated by arrowheads). To determine whether ripening-related fragments were in general up- or down-regulated during berry ripening, the score of “1” was multiplied by the “number of fragments” (Tab. 2, indicated by the totals in parentheses). It was shown that in Clairette blanche, most of the ripening-related fragments were up-regulated during berry ripening. In Cabernet Sauvignon, however, most of the ripening-related fragments were down-regulated.

Primer combination affected the number of differentially amplified fragments that could be identified (Tab. 1). Most of the differentially amplified fragments were identified using the primer combinations *Mse*I (+3) and *Pst*I (+G), while the primer combinations *Mse*I (+3) and *Pst*I (+C) yielded the lowest number of differentially amplified fragments. The higher the number of selective nucleotides, the lower the number (and percentage) of differentially amplified fragments identified.

Some primer combinations resulted in the visualisation of a larger number of ripening-related fragments, while others amplified a large number of cultivar-specific fragments. For instance, the use of primer combinations *Pst*I (+C) and *Mse*I (+CAA; +CAC; +CAG; +CAT) did not result in the visualisation of a large number of ripening-related fragments, but instead a large number of cultivar-specific fragments. The use of primer combinations *Pst*I (+G and +GTA) and *Mse*I (+CAC; +CAG; +CAT) resulted in the visualisation of the highest number of ripening-related fragments in Clairette blanche and Cabernet Sauvignon (Fig. 2).

Expression analysis of ripening-related cDNA fragments: All fragments, except for fragments selectively amplified using the primer *Mse*I (+CAT), excised from dehydrated gels could be re-amplified. A subset of 23 fragments excised from the dehydrated gels was subjected to reverse slot blot analysis. To compensate for the background signal produced by the negative control, only sequences with stronger hybridisation signals were considered for further analysis. Of these, 15 of the Clairette blanche cDNA sequences were shown to be differentially expressed during berry ripening (Fig. 3 A). All 15 were characterised by high levels of expression in the "immature" berries. Five of the sequences appeared to be constitutively expressed (Fig. 3 A). None of these fragments were expressed in leaf tissue.

Differentially expressed cDNA fragments were subjected to northern blot analysis to verify their ripening-related expression. Five fragments (C2, D1, D3, D9.2 and G2) were characterised by higher levels of the transcript in "immature" berries, while no mRNA could be detected in leaf tissue (Fig. 3 B). The size of the 5 transcripts ranged from 1 to 1.5 kb.

Discussion

The method of cDNA-AFLP analysis allows the identification of differentially expressed transcripts. In this study, AFLP analysis of cDNA from 14- and 18-weeks post flowering Clairette blanche and Cabernet Sauvignon berries led to the identification of several transcripts differentially expressed during the post-véraison stages of berry development. Differences in the mRNA fingerprints produced from "immature" (14-wpf) and "mature" (18-wpf) berries, were referred to as “ripening-related gene expression”.

Complementary DNA was synthesised directly from total RNA, circumventing the isolation of mRNA as previously described (MONEY *et al*. 1996; BACHEM *et al*. 1996; HABU

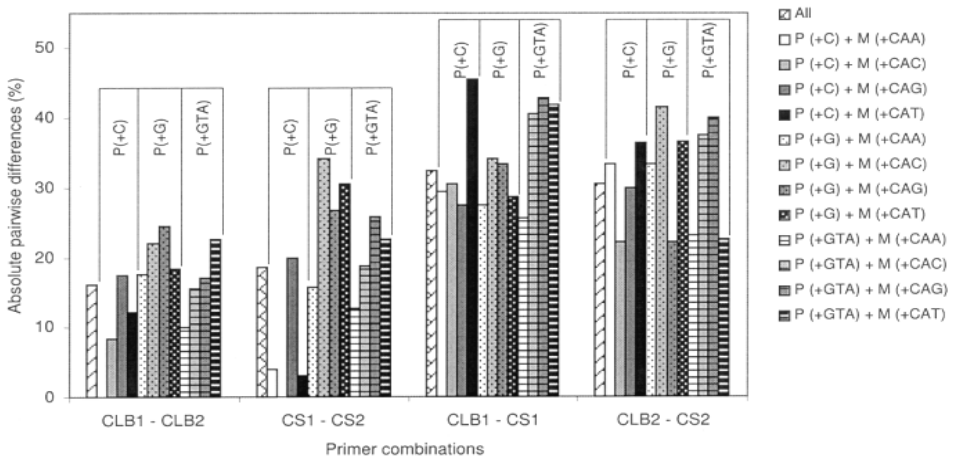

Fig. 2: Absolute pairwise difference of fragments amplified from cDNA isolated from ripening Clairette blanche (CLB1-CLB2) and Cabernet Sauvignon (CS1-CS2) berries. The 12 primer combinations used in the selective amplification procedure include *Pst*I (+C), (+G), and (+GTA) and *Mse*I with three selective nucleotides (+CAA), (+CAC), (+CAG) and (+CAT). Differences between fragments amplified from cDNA isolated from "immature" and "mature" berries are indicated by CLB1-CLB2 (Clairette blanche) and CS1-CS2 (Cabernet Sauvignon), while differences between the two cultivars are indicated by CLB1-CS1 and CLB2-CS2. Absolute pairwise difference is expressed as percentage of the total number of cDNA fragments scored.

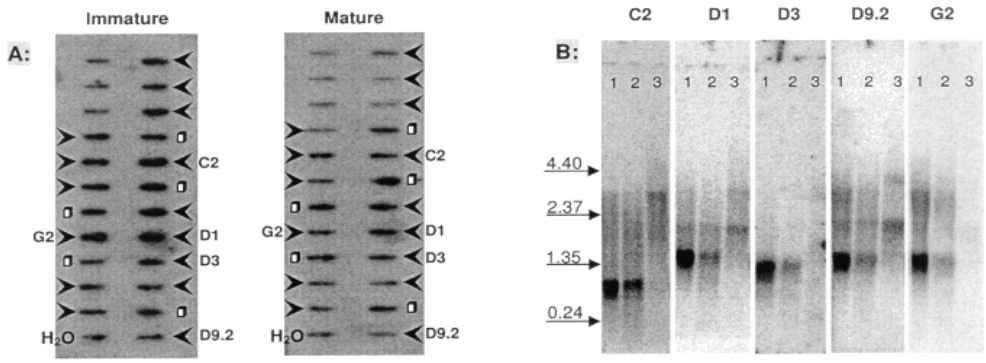

Fig. 3: Expression analysis of fragments differentially amplified from "immature" and "mature" Clairette blanche berries by cDNA-AFLP analysis. **A**: Reverse slot blot analyses were performed by probing duplicate membranes with cDNA probes synthesised from total RNA from "immature" and "mature" berries. The negative control (filtered water) is shown on the left bottom. Fragments differentially expressed during berry ripening are indicated by arrowheads, while fragments which appear to be constitutively expressed, are indicated by blocks. The 5 fragments identified for further analysis (C2; D1; D3; D9.2 and G2) are indicated. **B**: Ripening-related expression of the 5 fragments C2; D1; D3; D9.2 and G2. Lanes 1-3: mRNA from "immature" (lane 1) and "mature" berries (lane 2) and leaf tissue (lane 3). Molecular size (kb) is indicated by arrows.

et al. 1997). Considering that mRNA comprised 0.85 % of the total RNA isolated from ripening berries, and that first- and second-strand cDNA synthesis have been shown respectively about 12-50 %, and 80 % effective (Universal Riboclone cDNA synthesis system Technical Manual), it implies that cDNA-AFLP synthesis was performed using 5.1-17 ng of double-stranded cDNA as template.

Amplification using the primer combinations *Mse*I (+3) and *Pst*I (+0) resulted in the visualisation of a number of fragments, which was much higher than reported for wheat (Money *et al*. 1996). In fact, in this study it was shown that amplification using primer combinations with *Pst*I (+GTA) resulted in the visualisation of an average of 36 fragments per gel. If considering that first-strand cDNA synthesis was primed using the anchored primer VT_{12}GACGTCTGA, amplification using a *Pst*I primer with selective nucleotides other than a "T" would discriminate against cDNA fragments only containing the incorporated *Pst*I recognition site. It thus seems as if grapevine has a relative high proportion of cDNAs with *Pst*I sites and that dependence upon the presence of this 6-bp recognition site, is not a limitation in cDNA-AFLP analysis of grapevine.

This study confirmed the usefulness of cDNA-AFLPs to study grapevine ripening-related gene expression, and the identification of cDNA fragments differentially expressed during grape berry ripening. Considering that the two cultivars, Cabernet Sauvignon and Clairette blanche differ in many other aspects besides berry and wine qualities, it was surprising to find that ripening-related gene expression in the two cultivars is remarkably similar. Nevertheless, obvious differences in the ripening-related gene expression of the two cultivars were identified. These differences should be targeted to identify genes related to the phenotypical differences between the two cultivars, and to identify genes possibly involved in berry quality. cDNA-AFLPs are a sequence-based approach; therefore the differences in ripening-related gene expression of the two cultivars will also reflect single nucleotide polymorphisms (SNPs). Although

some SNPs may only be related to the genetic distance between the two cultivars, it may also be indicative of different members of a multigene family. In grapevine, members of multigenic families often exhibit distinct patterns of regulation during berry ripening (DAVIES *et al.* 1999; FILLION *et al.* 1999; TESNIÈRE and VIERRÈS 2000), which emphasise the utilisation of a sequence-based approach for the unambiguous characterisation of these isogenes. Further characterisation of differentially-amplified sequences by sequence- and hybridisation-based analysis in both cultivars, will clarify the issue.

Cloning and sequence analysis of differentially expressed fragments were not within the objectives of the study. The 5 ripening-related cDNA fragments shown in Fig. 3 B are however considered ideal candidates for further characterisation and possibly the isolation of fruit-specific, ripening-related genes. Cloning and sequence analysis of these five fragments will possibly lead to the identification of the corresponding genes and their function during grape berry ripening.

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