



Commentary

Cloning of a Specific Ripening-Related Gene From the Multiple of Ripening-Related Genes Identified From a Single Band Excised From a cDNA-AFLP Gel

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Abstract. High-throughput methods such as amplified fragment length polymorphism (AFLP) analysis of complementary DNA (cDNA) and cDNA microarrays are useful for identifying ripening-related cDNAs from grapevine. Here we describe the identification and cloning of a gene that is transcriptionally activated at the onset of grape berry ripening. In addition, we describe the presence of multiple ripening-related genes in a single band excised from a cDNA-AFLP gel and the additional steps implemented to identify a specific ripening-related gene. In total, 7 cDNAs were identified in the band excised from the cDNA-AFLP gel. All 7 cDNAs were shown to be ripening regulated during berry development, though most were characterised by low levels of expression during berry ripening. These results highlight the limitation placed on the isolation of a specific sequence from a cDNA-AFLP gel and the steps that can be taken to overcome this limitation.

Key words: affinity capturing, cDNA-AFLP analysis, differential amplification, fruit ripening, grapevine, ripening-related gene expression

Abbreviations: AFLP, amplified fragment length polymorphism; cDNA, complementary DNA; DDRT-PCR, differential display reverse transcription PCR; PCR, polymerase chain reaction.

Introduction

Analysis of differential gene expression is one of the cornerstones of modern molecular biology. It forms the basis for unraveling the control of plant growth and development, and it allows for the identification of specific control points of metabolism. In addition, it is an important prerequisite for the identification of specific promoter elements, which in turn is invaluable for gaining insight into the

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regulation of gene expression and the possibility to control gene expression in many areas.

Techniques for isolating differentially expressed genes include the now classic approaches of differential and subtractive hybridisation (Sargent, 1987; Gray et al., 1992; Woodhead et al., 1998; Davies and Robinson, 2000) and the polymerase chain reaction (PCR)-based approaches for selective amplification of complementary DNA (cDNA). The main advantage of PCR-based approaches is their ability to rapidly and simultaneously display mRNAs that are expressed in various eukaryotic cells or tissues, in different stages of development, or under altered conditions. Differential display reverse transcription PCR (DDRT-PCR), the first in vitro technique for the determination of transcript patterns, was developed in 1992 by Liang and Pardee and has since been widely applied to identify and clone a large number of genes that are differentially expressed (Liang et al., 1993; Aiello et al., 1994; Liu and Raghobama, 1995; Oh et al., 1995; Wilkinson et al., 1995; Baldwin et al., 1999). Unfortunately, the technique tends to give high rates of false positives (Debouck, 1995), which are primarily attributed to the presence of multiple DNA fragments in one particular band (Bauer et al., 1993; Li et al., 1994; McClelland et al., 1995; Men and Gresshoff, 1998).

To counteract this high rate of false positives, several improved PCR-based methods have been described (Kawamoto et al., 1999; Shimkets et al., 1999; Sutcliffe et al., 2000). The current method most widely used for expression analysis of multigene families is that of Fischer et al. (1995), who combined DDRT-PCR and amplified fragment length polymorphism (AFLP) (Vos et al., 1995). This technique, termed cDNA-AFLP analysis, has been used extensively in recent years to identify differentially regulated genes in plants and other organisms (Bachem et al., 1996; Breyne and Zabeau, 2001; Donson et al., 2002). It has proven itself highly reproducible and more reliable than DDRT-PCR.

Here, a putative ripening-related cDNA fragment, designated C2, was differentially amplified from ripening grape berries by means of cDNA-AFLP analysis. The single band excised from the cDNA-AFLP gel contained multiple ripening-related sequences. Steps for identifying a specific ripening-regulated gene are described.

Materials and Methods

Plant material

Berry and leaf material from *Vitis vinifera* L. cv. Clairette blanche were collected at the ARC-Nietvoorbij collection block in Stellenbosch, South Africa. The berries were collected 14-wk postflowering (wpf), which was 1-wk post-*véraison* and just prior to commercial harvest, 18-wpf. Ripening Merlot berries were collected at Grondves, an experimental farm of the KWV in Stellenbosch. These berries were collected 2-wpf and thereafter on a 2-weekly basis until berries reached full maturity, 16-wpf. The samples were designated M1-M8. Berries were deseeded, frozen in liquid nitrogen, and stored at -80°C until further use.

Isolation of the particular differentially-expressed cDNA fragment

The putative ripening-related cDNA fragment, designated C2, was identified from 14-wpf Clairette blanche berries by means of cDNA-AFLP analysis. Complementary DNA was synthesised from total RNA isolated from 14- and 18-wpf berries. The resulting cDNA was digested by using the restriction endonucleases *MseI* and *PstI* and subjected to cDNA-AFLP analysis as described in Venter et al. (2001). Amplification products were denatured, size-fractionated (5% [m/v] polyacrylamide, 80 W, 100 min), differentially amplified fragments excised from the dehydrated polyacrylamide gel, according to the procedures described in Venter et al. (2001).

Reamplification, cloning, and sequence analysis

Reamplification (30 cycles) was performed according to Habu et al. (1997) with the primers used for selective amplification, *MseI* (+CAT) and *PstI* (+C). For cloning procedures, 5 µL of this reamplified product was used for ligation into pGEM-T Easy Vectors (Promega Corporation, Madison, USA). Ligation and transformation of *Escherichia coli* strain DH10B cells were performed according to the supplier's protocol (Promega Corporation, Madison, USA). Nucleotide sequences were obtained by means of double-strand analysis using the Applied Biosystems PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, following the manufacturers instructions. Samples were run in an Applied Biosystems automatic sequencer model. Sequence analysis of the reamplified uncloned PCR product was performed by subjecting the agarose gel-purified reamplified PCR product to sequence analysis, using the selective amplification primers to prime the sequencing reaction. Sequences not corresponding to the selective nucleotides of the primers used for selective amplification were omitted from further analysis.

Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). All ambiguities and vector and primer sequences were removed before nucleotide sequences were submitted to search entries in the National Centre for Biotechnology Information (NCBI) nonredundant and expressed sequence tag genetic databases. The significance of the homology was based on the Score and E-value. A minimum Score of 50 was set as an indication of significant homology.

Total RNA and mRNA isolation

Total RNA was isolated from Merlot berry and Clairette blanche berry and leaf material according to Venter et al. (2001). Messenger RNA was isolated from total RNA by using PolyATtract mRNA isolation systems (Promega Corporation, Madison, WI, USA) and quantified fluorometrically (BIO-TEK Instruments Inc., Winooski, Vermont, USA).

Northern blot analysis

Messenger RNA (50 ng per lane) was transferred to positively charged nylon membrane (Roche Diagnostics Mannheim, Germany) by means of alkaline downward capillary blotting (Ingelbrecht et al., 1998). RNA cross-linking, hybridisa-

tion (using ULTRAhyb ultrasensitive hybridisation buffer), and washing procedures were carried out as described by the manufacturer (Ambion, Austin, USA). The fragments were radioactively labelled as described by Venter et al. (2001). Hybridisation was visualised by means of phosphoimaging with the Cyclone Storage Phospho System (Packard Instrument Co., Inc., Meriden, USA).

Cloning of differentially hybridised cDNAs

For cloning of the appropriate differentially hybridised cDNAs, the area of the nylon membrane displaying differential hybridisation was excised. The excised membrane was then subjected to the procedures described by Li et al. (1994) to strip cDNAs hybridised to the membrane. A 2.5- μ L aliquot of this supernatant was used for reamplification. The PCR reaction product was purified by using a QIAGEN PCR purification cleanup kit (QIAGEN Inc., Valencia, CA, USA) and ligated (3 ng) into the pGEMT easy vector.

Reverse Northern blot analysis

Plasmid DNA of each of the clones was dot blotted onto 4 duplicate positively charged nylon membranes (Roche Diagnostics Mannheim, Germany). The equivalent of 300 ng of insert plasmid DNA was applied to the 4 membranes. The membranes were air-dried. Plasmid DNA was denatured (1.5 M NaCl, 0.5 M NaOH), neutralised (1 M Tris [pH 7.4], 1.5 M NaCl), and rinsed in 2 \times SSC (0.3 M NaCl, 0.03 M trisodium citrate [pH 6.8], citric acid). Plasmid DNA was crosslinked to the membrane (2.5 min at 120 mJ/cm) with an ultraviolet crosslinker (ULTRA.LUM, Scientific Associates). The membranes were left to dry before used for hybridisation.

Four single-stranded cDNA probes, M1-M2 (2- to 4-wpf), M3-M4 (6- to 8-wpf), M5-M6 (10- to 12-wpf), and M7-M8 (14- to 16-wpf), were synthesized. Each of the 4 probes was synthesized from a total of 1 μ g mRNA, comprising 500 ng of mRNA from berries of each of the 2 ripening stages. Reverse transcription was performed by using Superscript II (Invitrogen life technologies, UK) and procedures according to the protocols supplied by the manufacturer. Modifications to the protocol included the replacement of the dNTP solution with dNTPs (-dCTP), final concentration 75 μ M, and the addition of 50 μ Ci [α - 32 P] dCTP (Amersham Pharmacia Biotech).

Hybridisation was performed by using Rapid-hyb buffer (Amersham Pharmacia Biotech). Hybridisation and washing procedures were according to the manufacturer's instructions. Equal counts (1×10^8 cpm/ μ g DNA) of cDNA probes were used to probe the 4 duplicate membranes. Hybridisation was visualised with phosphoimaging.

Results

Isolation and cloning of the cDNA corresponding to the ripening-related gene identified by cDNA-AFLP analysis

Approximately 63 discrete fragments ranging from 80-600 bp were amplified from the ripening Clairette blanche berries by cDNA-AFLP analysis using

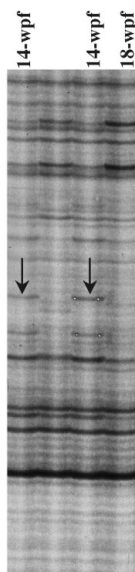


Figure 1. Portion of the complementary DNA–amplified fragment length polymorphism (cDNA-AFLP) gel autoradiogram showing the differential amplification of the putative ripening-related cDNA fragment, C2, from 14-wk postflowering (wfp) and 18-wpf Clairette blanche berries. The C2 fragment is indicated by the arrow. The first lane on the left represents the duplicate 14-wpf sample, which was included to verify the reproducibility of the analysis.

primers with selective nucleotides *Mse*I (+CAT) and *Pst*I (+C). The putative ripening-related cDNA fragment (C2), was differentially amplified from 14-wpf Clairette blanche berries (Figure 1). The putative ripening-related fragment was excised and reamplified. Northern blot analysis with the reamplified uncloned C2 product confirmed the ripening-related expression demonstrated by cDNA-AFLP analysis (Figure 2).

After cloning of the C2 fragment, 2 random colonies (C2-2 and C2-24) were picked for sequence analysis, which revealed that the nucleotide sequences of C2-2 and C2-24 were distinctly different. In addition, C2-24 was shown to be identical to the nucleotide sequence obtained from the reamplified uncloned C2. Homology searches revealed high sequence identity of both these sequences with mRNA sequences from grape berries (Table 1). However, ripening-related expression of the 2 sequences could not be confirmed by means of Northern blot analysis. In fact, no hybridisation of the 2 sequences to mRNA from ripening Clairette blanche berries or leaf material could be observed.

To clone the differentially hybridised cDNA(s) shown in Figure 2, the hybridised cDNA(s) were stripped from the membrane and cloned. Eighteen clones were picked for sequence analysis. From these, 8 additional sequences were identified (Table 1). Three of the sequences did not contain terminal sequences corresponding to the selective nucleotides of the primers used for amplification, and they were omitted from further analysis. Almost half of the remaining clones (44%) contained a sequence identical to that of clone C2-24. Abundance of the other clones was low: C2-5 and C2-23 (11% representation) and clones C2-6, C2-

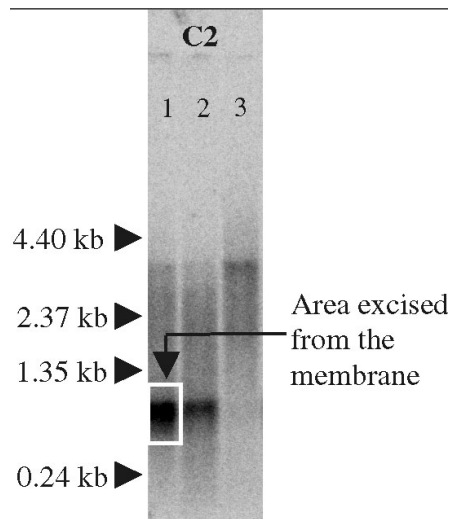


Figure 2. Visualisation of differentially-hybridised cDNAs by Northern blot analysis using the reamplified uncloned C2 product as probe. The higher level of hybridisation to mRNA from 14-wk postflowering (wpf) berries, (lane 1) confirms the ripening-related expression demonstrated by complementary DNA–amplified fragment length polymorphism (cDNA-AFLP) analysis. The area of membrane representing the differentially-hybridised cDNAs (indicated by the arrow) was excised and subjected to procedures to strip the hybridised cDNAs from the membrane. Position of the size standards are indicated by the arrowheads (kb). Lane 2, 18-wpf berries; lane 3, leaf tissue.

Table 1. Characterisation of the 7 cDNA sequences identified from the single band, C2, excised from the complementary DNA–amplified fragment length polymorphism (cDNA-AFLP) polyacrylamide gel.

Clone (size of the insert in base pairs, bp)	Homology (A minimum score of 50 was set as indication of significant homology)	Significance of Identity (Score and E-value)
C2-2 (123 bp)	mRNA sequence from stressed berries of grapevine var. Chardonnay, accession no. CB006924	Score, 223; E-value, 5e-56
C2-24 (123 bp)	mRNA from grapevine Cabernet Sauvignon berry Stage I, accession no. CB973539	Score, 236; E-value, 7e-60
C2-5 (120 bp)	No sequences with significant homology identified	-
C2-6 (122 bp)	mRNA sequence from grapevine-stressed leaves, accession no. CD721029 mRNA sequence from grapevine buds, accession no. CF514173	Score, 217; E-value, 7e-54
C2-17 (128 bp)	Grapevine ripening-induced protein <i>grip15</i> , accession no. AJ237984	Score, 234; E-value, 7e-60
C2-23 (127 bp)	No sequences with significant homology identified	-
C2-73 (126 bp)	mRNA sequences from young grape root, accession no. CF605780, and grape berries and petioles developmental stage <i>véraison</i> , accession nos. CF602951, CF519271.	Score, 206; E-value, 7e-51
	Grape ripening-induced protein (GASR) mRNA; Berries harvested at onset of ripening	Score, 200; E-value, 1e-49

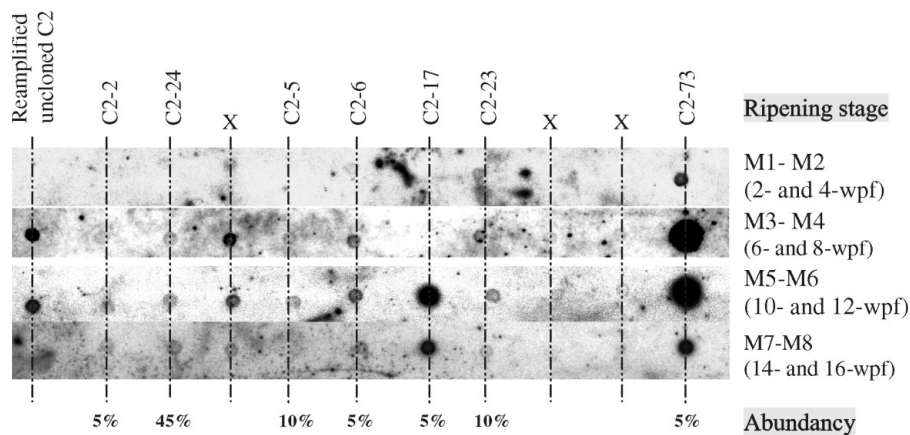


Figure 3. Reverse Northern blot analysis to display ripening-related expression and levels of expression of the reamplified uncloned fragment C2 and the 10 sequences identified from the reamplified uncloned fragment C2. Plasmid DNA, equivalent to 300 ng of the insert, was blotted onto duplicate membranes and probed with equal counts of the 4 cDNA probes M1-M2, M3-M4, M5-M6, M7-M8. Abundance refers to the number of clones containing the specific sequence, as a percentage of the 20 clones subjected to nucleotide sequence analysis. X refers to the 3 cDNAs that were omitted from further analysis because their terminal sequences did not respond to the selective nucleotides of the primers used for amplification.

17, and C2-73 (5.5% representation). The size of the sequences varied from 120-128 bp (Table 1). Homology searches revealed significant sequence identity of C2-6 with mRNA sequences from grapevine leaves and buds. Both C2-17 and C2-73 revealed high sequence identity with grapevine ripening-related mRNAs (Table 1). No sequences with significant homology to C2-5 and C2-23 could be identified. The 7 cDNA sequences were deposited in the NCBI genetic database, accession numbers CN069438 to CN069444.

Reverse Northern blot analysis

Expression analysis of the 7 cDNA sequences was done by using reverse Northern blot analysis. Contrary to the Northern blot analysis, which did not show any hybridisation of C2-2 and C2-24 to mRNA from ripening berries, the presence of the 2 sequences in ripening berries was confirmed by reverse Northern blot analysis (Figure 3). Like for most of the other cDNAs, reverse Northern blot analysis revealed that C2-2 and C2-24 were characterised by very low levels of the transcript in 6- to 12-wpf Merlot berries. All 7 sequences identified were shown to be ripening regulated during berry development, mimicking the differential amplification visualised by cDNA-AFLP analysis and the ripening-related expression visualised by the reamplified uncloned product (Figure 2). For all 7 sequences, immature berries (2- to 4-wpf) were characterised by no or low levels of the transcript, followed by a rapid accumulation of the transcripts in pre-*véraison* (6- to 8-wpf) or post-*véraison* (10- to 12-wpf) berries. Levels of the transcript declined toward the final stages of ripening, with no or much lower levels of expression visualised in 14- to 16-wpf berries. Only 2 cDNAs, clones C2-17 and C2-73,

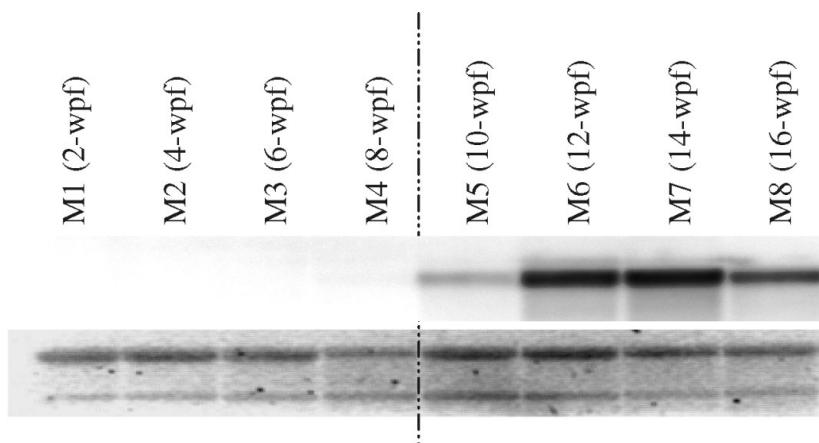


Figure 4. Northern blot analysis to illustrate ripening-related expression of clone C2-17 during Merlot berry ripening, 2- to 16-wk postflowering (wfp). *Véraison*, which is the onset of berry softening and sugar accumulation, is indicated by the dashed line. The bottom panel illustrates approximately equal amounts of intact total RNA used for the analyses.

showed to be abundantly expressed—possibly accounting for the high level of the transcript in 12- and 16-wpf berries, visualised by the reamplified uncloned product (Figure 2).

According to the ripening-related expression and significant homology of some of the sequences with entries in the NCBI genetic databases, clones C2-2, C2-5, C2-24, C2-17, C2-23, and C2-73 correspond to different genes.

Northern blot analysis

Clone C2-17 was identified for further characterisation, based on the relative high levels of the transcript and the initiation of gene transcription at the onset of *véraison* (10- to 12-wpf). Northern blot analysis confirmed the ripening-related accumulation of the transcript revealed by reverse Northern blot analysis (Figure 3).

Discussion

cDNA-AFLP analysis is a fast and reliable technique for the identification of differentially expressed genes (Habu et al., 1997; Baldwin et al., 1999; Breyne and Zabeau, 2001; Donson et al., 2002). In this study, a gene that is transcriptionally activated at the onset of grape berry ripening (*véraison*), was cloned following cDNA-AFLP analysis of immature (14-wpf) and mature (18-wpf) grape berry tissue. This gene is an ideal candidate for studying ripening-related gene expression during the post-*véraison* stages of grape berry ripening and for the isolation of a late-ripening-specific promoter to achieve transgene transcription in genetically modified grapevine.

This study shows that despite identification of multiple cDNAs in the single band excised from the cDNA-AFLP gel, the implementation of additional steps led to the identification of a specific ripening-related gene and 6 other genes

differentially expressed during grape berry ripening. The presence of multiple sequences in a single band excised from a DDRT-PCR gel is well documented (Callard et al., 1994; Wan et al., 1996; Zegzouti et al., 1997). Although the origin of these additional sequences remains unclear, the sequences are usually regarded as false positives. The 3 main sources of these false positives are considered to be (1) artifactual differences created in the original RNA populations by non-standardised extraction procedure, (2) false positives introduced through PCR reamplification of differential display cDNA, and (3) identical-sized cDNA fragments that comigrate with the band of interest on display gels (Miele et al., 1998).

In this study, all 7 sequences identified from the single excised band were shown to be ripening related. Although only 2 of these are considered to account for the ripening-related expression visualised by the reamplified uncloned product, none of the other 5 sequences were considered to be false positives. Considering the mRNA complexity of a cell (20,000-30,000 distinct mRNAs per cell) (Wan et al., 1996) and the high probability of *Mse*I restriction sites in the AT-rich 3' untranslated regions of the mRNAs, the 7 sequences identified from the single excised band are regarded as identical-sized cDNA fragments that comigrate with the band of interest on the cDNA-AFLP gel. Despite the high abundance of clone C2-24 among the total number of clones analysed in this study, reverse Northern blot analysis showed that the sequence is characterised by a low level of expression during grape berry ripening. This contradiction can possibly be attributed to phenomena such as preferential amplification or cloning efficiency (Liang and Pardee, 1992).

In this study, reverse Northern blot analysis was shown to be an effective tool for the expression analysis of the large number of different sequences and the identification of cDNAs accounting for the levels of expression visualised by the reamplified uncloned product. The procedure does require substantial quantities of poly(A)⁺ RNA, which might be a problem in situations in which large quantities of material are not available and where low-copy number transcripts are involved.

Unlike previously stated (Debouck, 1995), the reamplified uncloned product was found to be useful. Not only was it useful to confirm the presence of differentially-regulated cDNAs in the excised band, but the levels of expression visualised by the uncloned product were used to identify the cDNA corresponding to the differentially regulated gene identified by the analyses. In this study, the ripening-related expression visualised by the reamplified uncloned fragment was used as motivation for further investigation.

Contradictory to some reports (Linskens et al., 1995; Wang and Feuerstein, 1995; Martin et al., 1998), direct sequencing of the reamplified uncloned product was not of any help in the identification of the appropriate differentially expressed cDNA. Sequence analysis produced a clear sequence, creating the impression of homogeneity, not the presence of multiple different sequences. Since the nucleotide sequence of the uncloned product corresponded to that of clone C2-24, which represented 45% of the total number of clones sequenced, sequence analysis of the reamplified uncloned product was only helpful in the identification of the most abundant sequence.

With cDNA-AFLP analysis, it would be possible to eliminate most (possibly all) of the additional sequences from the PCR product by increasing the number of selective nucleotides of the primers used for selective amplification. Bachem et al. (1996) showed that simple and rapid verification of band identity could be achieved by using primers with 3 selective nucleotides for amplification. When the sequence of the target sequence is unknown, this requires an additional 4 reactions for each extra selective nucleotide. If extensive sets of primers are not available to accommodate this approach, time and costs involved in primer synthesis might impede on the usefulness of this approach.

The results of this study serve as confirmation of 2 aspects considered to be advantages of the cDNA-AFLPs approach. Firstly, except for the 3 cDNA sequences omitted from further analysis, all cDNA sequences identified contained terminal sequences corresponding to the primers and selective nucleotides used for amplification. No DNA sequences were identified, although the presence of genomic DNA in the total RNA used for cDNA synthesis is regarded to be one of the major sources of heterogeneous sequences (Liu and Raghothama, 1995). These results illustrate the stringency and fidelity of the analyses as performed in this study. Secondly, because most of the 7 genes identified are characterised by low levels of the transcript in the ripening grape berry, it confirms that sequence-based approaches are not biased toward abundant transcripts (Breyne and Zabeau, 2001).

Here, the capturing of differentially hybridised cDNAs from the reamplified uncloned product and the subsequent cloning and reverse Northern blot analysis was shown to be an effective approach for the identification of the cDNAs corresponding to the differentially-regulated genes visualised by cDNA-AFLP analysis. It is proposed that cDNA populations amplified by differential display is more complex than currently anticipated, and that this should be considered as a factor contributing to the incidence of the false positives that differential display is so often criticised for.

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