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Molecular analysis of fruit ripening: The identification of differentially expressed sequences in *Vitis vinifera* using cDNA-AFLP technology

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Summary

Differential gene expression patterns were studied during the ripening process of grape berries (*Vitis vinifera* cv. Chardonnay). Thirty *Pst*I + *Mse*I primer combinations were used to generate 213 fragments that appeared to be differentially expressed of which 94 % were successfully re-amplified. Reverse northern dot-blot analysis indicated that 35 % of the fragments had similar gene expression profiles to cDNA-AFLPs regarding developmental-stage specificity. Northern blot analyses confirmed the tissue and/or developmental stage specific expression of three of these cDNA fragments. This work illustrates that developmentally regulated sequences can be identified from grape berry tissue using cDNA-AFLP technology.

K e y w o r d s : differential expression, cDNA-AFLP, berry ripening, *Vitis vinifera*.

Introduction

The understanding of the regulation of gene expression during fruit development has important agricultural implications. Fruit-specific genes can be used as molecular tools to modify the fruit ripening process (Edwards and Coruzzi 1990). Methods such as differential screening have been used to identify and isolate differentially expressed sequences/genes in grape berries during ripening (Davies and Robinson 2000). Other methods used for the identification of differentially expressed sequences include random sampling, subtraction cloning and differential display. All these methods are invaluable tools to select differentially expressed sequences but some of them suffer several drawbacks including the fact that these methods are labor intensive and time consuming (Sagerström *et al*. 1997). cDNA-AFLP technology largely overcomes these limitations, produces more reliable results than differential display (Habu *et al*. 1997) and is a broadly applicable technique for the identification of developmentally regulated genes (Bachem *et al.* 1996). However, the suitability of this methodology has been evaluated in a very limited number of plant species.

With respect to grape, a non-climacteric fruit, extensive research on fruit ripening has been conducted to identify certain biochemical and physiological changes during development (Coombe 1992). Major changes in several characteristics (shape, size, colour and metabolic changes) occur during fruit development and ripening which eventually have an effect on taste and quality (Archer 1981). However, success in biotechnological applications will only be possible if a better understanding is gained in the biochemical control and gene expression patterns in grape berries.

The implementation of an effective gene manipulation strategy is dependent on the isolation and characterization of genes that are specifically expressed in grape berry tissue. In this article we report on the isolation of differentially expressed fragments. It will be shown that the cDNA-AFLP technique allows the rapid identification of differentially expressed genes during grape berry ripening.

Material and Methods

P l a n t M a t e r i a l : Grape berries (*Vitis vinifera* L. cv. Chardonnay) were collected at 6 dates during development. The first date was 26 d after anthesis, the last when berries reached maturity (105 d after anthesis). Berries were deseeded, crushed in liquid nitrogen and stored at -80 °C until use.

S u g a r a n d o r g a n i c a c i d e x t r a c t i o n : 50 mg of frozen material was transferred to 2 ml Eppendorf tubes and suspended in 1.5 ml 80 % (v/v) EtOH containing 100 mM Hepes (pH 7.5) and 20 mM $MgCl_2$. The suspension was incubated at 70 °C for 14 h and the insoluble material removed through centrifugation.

H P L C a n a l y s i s : Sugars and organic acids were prepared for HPLC as previously described (Whittaker and Botha 1997). All analyses were conducted on a Shimadzu SCL-10AVP HPLC system. Sugars were separated for 20 min on a Supelco^{TM} LC-NH_2 column using with 80 % (v/v) acetonitril as the mobile phase and a flow rate of 1.2 ml·min^{-1}. Sugars were quantified by differential refractometry (Shimadzo RID-10A). Organic acids were separated over a 15 min period on a Aminex ion exclusion HPX-87H column with 0.02 M H_2SO_4 as the mobile phase, flow rate 0.6 ml·min^{-1}. Organic acids were quantified by UV spectrometry at 210 nm (Shimadzo SPD-10AVP UV/Vis).

R N A i s o l a t i o n a n d c D N A s y n t h e s i s : Total RNA was extracted from 4 g of ground, frozen berry material by a modified Na-perchlorate method (Rezaian and Krake 1987). The extraction buffer contained 5 M sodium perchlorate; 1M Tris-HCl (pH 8.3); 10 % (m/v) SDS; 20 %

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(m/v) PEG 6000; 10 % (m/v) PVPP and 1 % (v/v) β-mercaptoethanol. RNA was quantified fluorometrically (BIO-TEK® Instruments Inc., Winooski, Vermont, USA) and quality was visualised in ethidium bromide-stained 2 % (m/v) agarose gels.

5 µg total RNA, from each tissue sample, were used for first strand cDNA synthesis (Superscript™II, GibcoBRL Life Technologies Inc., Gaithersberg, MD, USA) followed by second strand cDNA synthesis (Universal Riboclone® cDNA synthesis system, Promega Corporation, Madison, USA).

cDNA-AFLP analysis: All AFLP-associated procedures were carried out according to a modified method (VOS *et al*. 1995).

Double strand cDNA templates were digested with 2.5 U of both *Mse*I and *Pst*I restriction enzymes at 37 °C overnight. Non-phosphorylated adaptor sequences were ligated to the restriction fragments at 20 °C overnight. The restriction-ligation products were subjected to 30 cycles of pre-amplification (94 °C denaturation, 30 s; 56 °C annealing, 1 min; 72 °C polymerization, 1 min) using primers with no selective nucleotides to obtain a sufficient amount of template. The pre-amplification products were diluted 1:10 with 1 x TE (10 mM Tris pH 8.0; 0.1 mM EDTA) and visualized in ethidium bromide-stained 1.5 % (m/v) agarose gels with expected sizes ranging from 100 bp to 1000 bp. The *Pst*I forward primer was radioactively labelled using 0.5 µCi γ^{33}P-ATP. Selective amplification was performed with 30 combinations of *Pst*I primer (5'-GACTGCGTACATGCAG+N-3') and *Mse*I primer (5'-GATGAGTCCTGAGTAA+N-3') extensions where 'N' represent two or three selective nucleotides (Tab. 1). Thirty-five cycles of amplification (12 cycles: 94 °C denaturation, 30 s; 65 °C annealing, 30 s; 72 °C polymerization; 1 min then 23 cycles: 94 °C denaturation, 30 s; 56 °C annealing, 30 s; 72 °C polymerization, 1 min) were carried out where the annealing temperature was lowered gradually from 65 °C to 56 °C at which efficient primer binding occurs. Thermocycling was started at 65 °C annealing temperature for optimal primer selectivity.

Table 1

Total *Pst*I and *Mse*I primers used in combinations with two or three selective nucleotides

	*Pst*I primer extension/s	*Mse*I primer extension/s
1)	GT*	CAA, CAC, CAG
2)	CT*	CAA, CAC, CAG
3)	GTA	TG, CAT, CTG
4)	TTT	TG, CAT, CTG
5)	TTT, GTA, GT	CA
6)	GTA*	CAA, CAC, CAG
7)	GA	CAA, CAC, CAG
8)	GC	CAA, CAC, CAG
9)	GT	TG, CAT, CTG
10)	TTT	CAA, CAC, CAG

**Pst*I-primer extensions +GT, +CT and +GTA in combinations with *Mse*I-primer extensions +CAA, +CAC and +CAG which generated the highest amount of polymorphic fragments.

Amplified products were heated at 95 °C for 5 min after addition of an equal amount of formamide dye (98 % (v/v) formamide, 10 mM EDTA pH 8.0 and 1 mg·ml^{-1} each of bromophenol blue and xylene cyanol) and immediately chilled on ice. Fragments were separated in 5 % (m/v) denaturing polyacrylamide gels and all gels were developed at 80 W for about 100 min. Gels were dried on to Whatman 3M paper on a slab gel dryer (Biorad Laboratories Inc., Hercules, CA, USA).

Radioactively labelled cDNA fragments were visualized on BioMAX MR film (Eastman Kodak Company, Rochester, New York) after exposure times ranging between 18 h and 72 h. Fragments that appeared to be selectively expressed were excised from the dried gels. cDNA was recovered from each band after heat treatment of 95 °C in 30 µl distilled water for 10 min. Fragments were re-amplified using the same selective primers and PCR conditions as used in the initial pre-amplification procedures and all re-amplified cDNA fragments were visualized in ethidium bromide-stained 2 % (m/v) agarose gels.

Reverse northern dot-blot analysis: A total of 192 fragments were selected and 2 µl of each re-amplified cDNA product was dot blotted on a nylon membrane (Boehringer Mannheim, Mannheim, Germany) in a 2 x 96 well PCR-plate configuration. Seven identical membranes were prepared. cDNA were denatured (1.5 M NaCl; 0.5 M NaOH), neutralized (1 M Tris pH 7.4; 1.5 M NaCl), rinsed in 2 x SSC (0.15 M NaCl; 0.015 Tri-sodium citrate pH 6.8; citric acid) and UV cross-linked before hybridization began.

Single strand cDNA probes were prepared from 5 µg total RNA at each stage of berry ripening and leaf samples using reverse transcriptase and an equimolar mix of primer 5'-AGTCTGCAGT$_{12}$-N-3', with 'N' representing A, C or G respectively (Superscript™II, Gibco BRL Life Technologies Inc., Gaithersberg, MD, USA). Modifications regarding 10 µCi ^{32}P-dCTP incorporation were made in our laboratory. Equal counts (1.5 x 10^7 cpm·ml^{-1}) of cDNA probes were used to probe the membranes. Hybridization was visualized by autoradiography. Dot intensities of sequences were analyzed using the AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, USA). Fragments were selected according to fruit-specificity and/or abundant expression. Promising fragments based on differential expression were cloned using pGEM®-T Easy Vectors (Promega Corporation, Madison, USA) to be used for Northern analysis.

Sequence analysis: Selected cDNA clones were sequenced (ABI PRISM™ dye terminator cycle sequencing) using a reaction kit with AmpliTaq® DNA polymerase (The Perkin Elmer Corporation, Norwalk, USA). The cDNA sequences were edited to discard the vector/linker and primer sequences.

Northern blot analysis: Northern blot membranes were prepared using total RNA (visualised in ethidium bromide-stained 1 % (m/v) agarose gels) of berries at grape ripening stages 1 to 6 and young grapevine leaves

and roots (10 μg per track). RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by upward capillary blotting (SAMBROOK *et al.* 1989) using 10 x SSC (standard saline citrate). The RNA was UV cross-linked and all hybridization (using ULTRAhyb™ ultrasensitive hybridization buffer) and washing procedures were carried out as described by the manufacturer (Ambion, Austin, USA). For preparation of probes, the re-amplified fragment of interest was radioactively labelled using 25 μCi [α-^{32}P] dCTP by 4 cycles re-amplification PCR using the same conditions as used in the initial pre-amplification procedures of this study. Hybridization was visualized using the Cyclone™ Storage Phosphor System (Packard Instrument Co., Inc., Meriden, USA).

Results and Discussion

Stages of fruit development: It is evident that the berries collected at stages 1 and 2 are typical berries prior to veraison, *i.e.* they contain similar levels of reducing sugars and sucrose and high levels of acid (Tab. 2). Stage 3 is characterised by a very rapid increase in both, glucose and fructose and a decrease in malate levels. Stage 6 represents fully ripened fruit where total sugars are in excess of 15 % of the total fresh mass and the acid levels are low.

cDNA-AFLP analysis: Total RNA was extracted from grape berry tissue and a decline of RNA yield (24 μg·g^{-1} to 2.75 μg·g^{-1} fresh weight) could be observed over the period from early to late berry ripening stages. cDNA pre-amplification products ranged in size from 200 bp to 1 kbp. For cDNA-AFLP *Pst*I and *Mse*I in combinations according to different selective nucleotide extensions on the primers were used. Although 6-bp restriction enzyme recognition sites would be present in only a minimal fraction of cDNA species (HABU *et al*. 1997), we retrieved 213 putative polymorphic bands with the primer combinations used for this study.

cDNA-AFLP reproducibility was examined by comparing reaction products that were derived from two sets of independent samples of total RNA, prepared from early and late developmental stages. Two different primer combinations, *Pst*I +CT with *Mse*I +CAT and +CTG gave identical band patterns (Fig. 1). A total of 213 polymorphic fragments

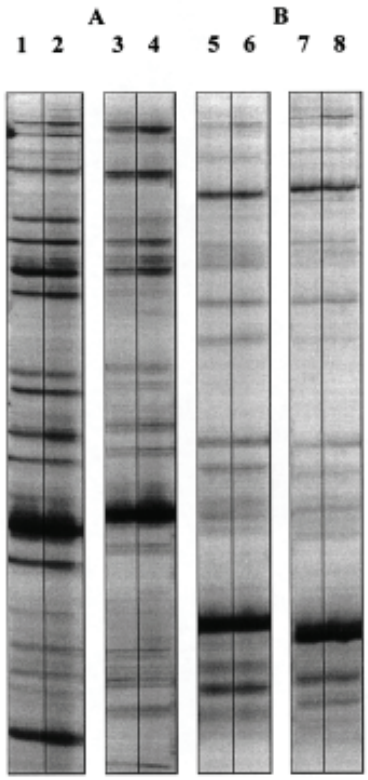

Fig. 1: Four sections of an autoradiograph with primer combination *Pst*I +CT with *Mse*I +CAT (A) and *Pst*I +CT with *Mse*I +CTG (B). Reproducibility examined with two independent total RNA samples of stage 1 (lanes 1 and 2 and lanes 5 and 6) and two independent total RNA samples of stage 5 (lanes 3 and 4 and lanes 7 and 8).

were isolated after visual analysis of cDNA-AFLP profiles using 30 *Pst*I + *Mse*I primer combinations. *Pst*I-primer extensions +GT, +CT and +GTA in combinations with *Mse*I-primer extensions +CAA, +CAC and +CAG generated the highest amount of polymorphic fragments. Stage-dependent expression as well as the gradual increase or decrease of gene expression intensities were observed (Fig. 2). cDNA-AFLP analysis conducted over the 6 stages of berry development, verified the presence or absence of bands at different ripening stages. This analysis was repeated in leaf material from the same cultivar. Ninety-four percent of all the fragments excised, could successfully be re-amplified (Fig.3).

Table 2

Sucrose, glucose, fructose and malate levels in the grape berries isolated at different stages during development and ripening. Each value is the average (± SD) of three extractions

Stage	Sucrose	Glucose	Fructose	Malate
	mol g^{-1} fresh weight			
1	21.0 ± 2.65	21.3 ± 2.08	23.3 ± 2.08	286.7 ± 30.55
2	19.3 ± 1.53	25.7 ± 1.53	24.0 ± 2.65	328.3 ± 16.07
3	17.3 ± 1.53	214.0 ± 12.29	211.3 ± 8.74	195.0 ± 13.23
4	19.0 ± 3.61	300.7 ± 11.02	330.0 ± 26.46	158.3 ± 18.93
5	18.7 ± 3.51	352.0 ± 14.11	355.0 ± 21.79	108.3 ± 10.41
6	25.3 ± 1.53	366.3 ± 14.57	392.7 ± 22.48	63.3 ± 18.93

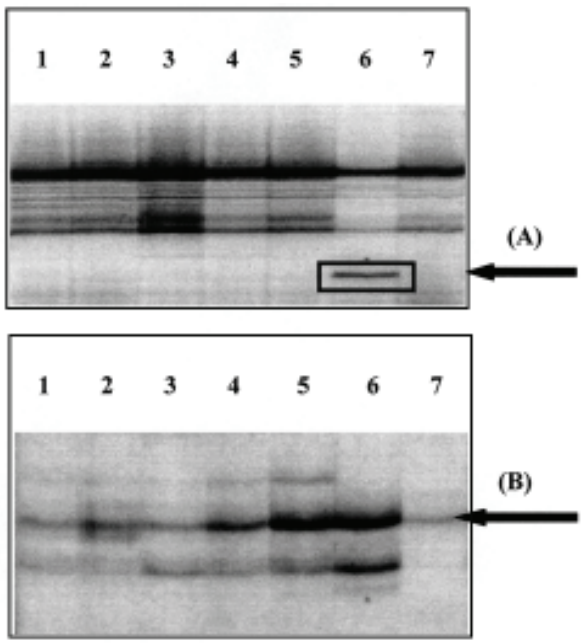

Fig. 2: Autoradiograph sections with primer combination *Pst*I +GT and *Mse*I +CAC showing (**A**) stage-specific expression and primer combination *Pst*I +CT and *Mse*I +CAA showing (**B**) gradual change of expression levels from early to late stages of grape development. Lanes 1 to 6 represent cDNA from ripening stages 1 to 6 and lane 7 is cDNA from young, field-grown leaf material.

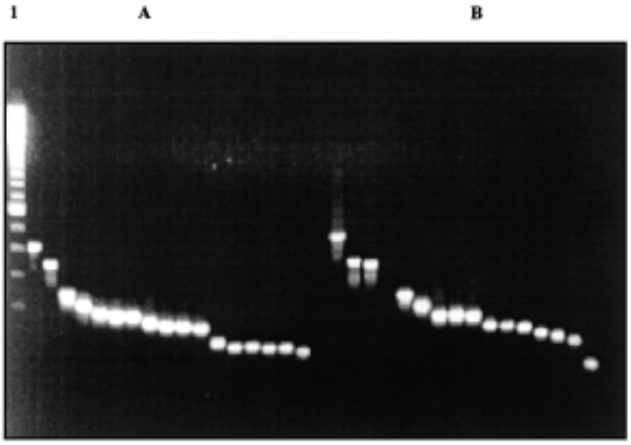

Fig. 3: Fragments excised and re-amplified with primer combination (A) *Pst*I +GTA and *Mse*I +CAC and (B) *Pst*I +GTA and *Mse*I +CAA. CDNA fragments range in sizes from 100 bp to 500 bp. Lane 1 is the 100 bp DNA ladder (Promega Corporation, Madison, USA).

Reverse northern and differential expression analysis: The reverse northern dot-blot technique is an effective method to test the feasibility of differential screening (ZHANG *et al*. 1996). Dot-blot results confirmed changes in gene expression patterns from early to late stages of berry development (Fig.4).

Reverse northern dot-blot analysis revealed the presence of the 98 most abundantly expressed fragments of which 58 were only expressed in the berry and not in the leaf, and designated as 'fruit-specific' during this study. Expression levels of 10 randomly chosen fragments were examined (Fig. 5). Analysis over a period of early to late stages of berry development revealed that 60 % (Fig. 5, fragments A, B, C, E, H, and I) of these fragments showed differential gene expression patterns similar to cDNA-AFLP profiles. Collectively these data indicate that approximately 35 % of the fragments identified during the initial cDNA-AFLP analysis are truly differentially expressed. Most of the fragments analyzed (Fig.5, fragments A, B, C, D, E, F, H and I) showed an increase of expression levels from early to late development with the highest expression occurring in the late stages of ripening. Some fragments (Fig.5, fragments G and J) revealed high expression levels in both early and late stages of berry development with lower expression between stages 1 and 6.

Northern blot and sequence analysis: Ten PCR fragments, abundantly differentially expressed as visualized with reverse northern analysis, and irrespective of their tissue and/or stage specificity, were selected and successfully cloned (Fig.6). Fragments excised from dried polyacrylamide gels usually contain more templates than the desired one, therefore two clones of each PCR product were isolated and successfully re-amplified. A total of 20 cloned fragments, designated as M1.A/M1.B to M10.A/M10.B (Fig. 6), were sequenced and analyzed (Tab. 3). Sequence-search (ALTSCHUL *et al.* 1990) results revealed two of the clones, M2.B and M8.A, to be homologues to known grape ripening related "Grip" genes (DAVIES and ROBINSON 2000) and fragment M10.A had homology to a H+-pyrophosphatase gene from *Vitis vinifera* (AF 192308.1). These three cDNA clones, M2.B, M8.A and M10.A, which had sequence similarity to grape genes were used as probes for northern blot analysis (Fig. 7). Fragment M10.A showed constitutive expression during berry ripening but was not

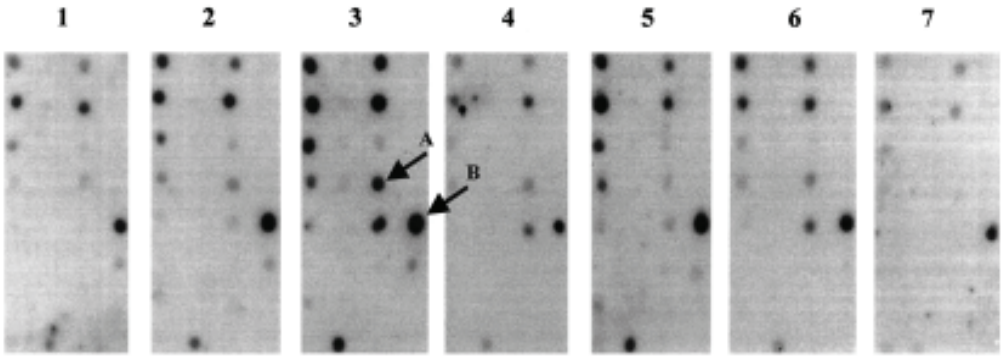

Fig. 4: Identical sections of original reverse northern blot membranes with examples of (A) stage-specific and (B) constitutive gene expression during berry ripening. Sections 1 to 6 represent membranes probed with cDNA from ripening stages 1 to 6 and section 7 probed with cDNA from young leaf material.

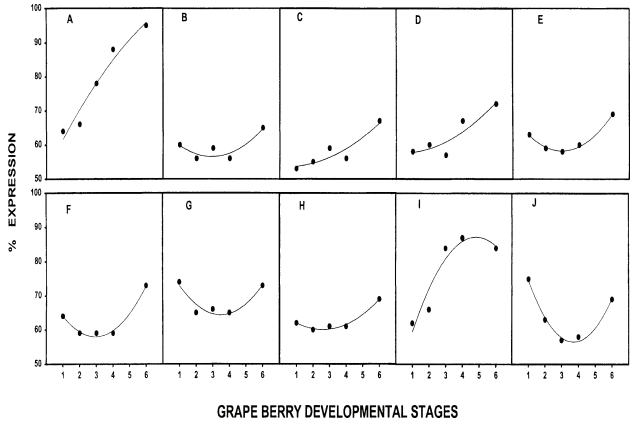

Fig. 5: Gene expression patterns, only visible in the berry, of 10 randomly selected sequences (**A** to **J**) from early to late berry development (stage 1 to stage 6).

present in leaf RNA. Fragments M8.A and M2.B were fruit-specific and showed gradual increase and stage specific expression patterns during ripening.

It has been shown in another study (Singh and Cheah 2000), using the differential display technique (Liang and Pardee 1992), that the lack of homology to known sequences in GenBank could be due to the fact that the cloned cDNAs were only fragments (200-600 bp). However, in this study we obtained partial cDNAs (62-315 bp) of which only 4 sequences showed no homology to known plant sequences. Other cDNAs had homology to genes from *Vitis vinifera* and to known DNA sequences, mostly from *Arabidopsis thaliana.* Sequences M1 and M7.B (Tab. 3) had similarity to expressed but as yet unidentified proteins. Analyses have also revealed sequence similarity to a putative epoxide hydrolase EphB gene from *Bradyrhizobium japonicum* which could be stress-induced and to a 19S ribosomal RNA gene from *Rafflesa pricei* (Tab. 3).

Table 3

Putative sequence identities of clones M1A/B to M10.A/B. The clones of 5 selected PCR products M1, M4, M5, M6 and M9 were identical

Clone no.	Length (bp)	Sequence similarity (accession no.)
M1.A=M1.B	296	*Arabidopsis thaliana* unknown protein (MBK23.13/AT5g41600) mRNA (AY035169.1)
M2.A	62	*Arabidopsis thaliana* chromosomeII section 208 of 255 of the complete sequence (AC005499.2)
M2.B*	237	mRNA for putative proline-rich cell wall from *Vitis vinifera* (AJ237982.1) Similarity to genes 'Grip' 3, 4, 13 and 15
M3.A	291	No significant similarity to plant sequences
M3.B	308	No significant similarity to plant sequences
M4.A=M4.B	179	*Arabidopsis thaliana* DNA, chromosome 5, BAC clone F21E1 (AL391716.1)
M5.A=M5.B	315	*Bradyrhizobium japonicum* putative epoxide hydrolase EphB (ephB), putative stress-induced protein Ohr (U33833.2)
M6.A=M6.B	193	*Arabidopsis thaliana* genomic DNA, chromosome 5, P1 clone:MNB8 (AB018116.1)
M7.A	173	*Arabidopsis thaliana* chromosome 1 BAC T22A15 genomic sequence (AC021666.5)
M7.B	170	*Arabidopsis thaliana* unknown protein (F12M12_190/AT3g46220) mRNA (AY034937.1)
M8.A*	292	mRNA for putative ripening-related protein from *Vitis vinifera* (AJ237986.1) Similarity to gene 'Grip' 31
M8.B	272	No significant similarity to plant sequences
M9.A=M9.B	128	No significant similarity to plant sequences
M10.A*	289	mRNA for H+-pyrophosphatase from *Vitis vinifera* (AF192308.1)
M10.B	289	*Rafflesia pricei* 19S ribosomal RNA gene, mitochondrial gene for mitochondrial rRNA (U96694.1)

*Fragments of which gene expression profiles were evaluated during berry maturation (Fig.7).

Conclusion

The reverse northern dot-blot technique confirmed differential expression similarity of isolated fragments to cDNA-AFLPs. Both methods used in conjunction, proved to be powerful and effective tools to identify and screen large quantities of polymorphic bands in grapevine. Northern blot results confirmed tissue and/or stage specific expression verifying the authenticity of the selected differentially expressed sequences. As mentioned before, a putative, poly-

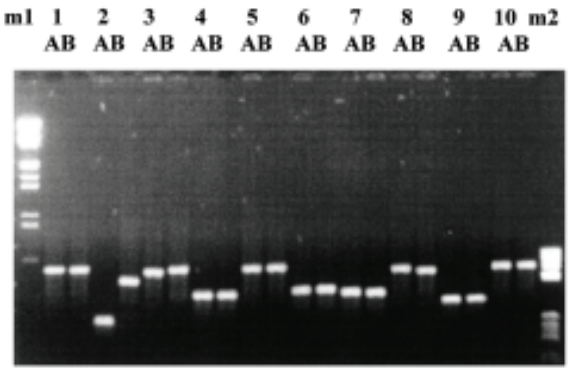

Fig. 6: Numbers 1 to 10 represent the 10 most abundantly expressed PCR fragments isolated after dot-blot analysis. Two amplified clones (A and B) of each PCR product can be visualized. Lanes m1 and m2 are the molecular weight markers III and V respectively (Boehringer Mannheim, Mannheim, Germany).

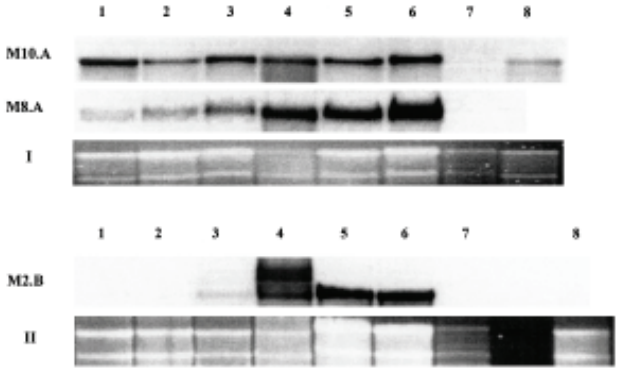

Fig. 7: Northern blot results of selected, tissue-specific fragments (marked by asterisks in Tab. 3) expressed constitutively M10.A and differentially M2.B and M8.A. Panels I and II show approximately equal amounts of intact total RNA with lanes 1 to 6 representing RNA from ripening stages 1 to 6 and lanes 7 to 8 representing RNA from young leaf and root material respectively.

morphic fragment initially excised from the dried gel, could be a mixed template and this can be considered as a major drawback. Therefore, cloning of excised fragments is a prerequisite for final evaluation and analysis. Nevertheless, results obtained during northern blot and sequence analysis suggest that the cDNA-AFLP method is a fast and reliable technique for identifying differentially expressed genes of grapevine.

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