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Use of RAPD markers to detect chimerism in synthetic grape chimeras (*Vitis vinifera* L.)

by

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Summary: Shoot organogenesis was previously observed *in vitro* on a callus structure originating from an assemblage of two grape cultivars (*Vitis vinifera* L.): Chardonnay 7535 and Pinot noir 7613. Adventitious buds were assumed to be candidates of chimeras. RAPD analysis was used to distinguish between the two grapevine cultivars at a molecular level and to verify the hypothetical chimerical character of adventitious shoots.

Key words: Vitis vinifera L., Chardonnay, Pinot noir, RAPD, chimerism.

Introduction

Different grape sensitivity to the fungus *Botrytis cinerea* has been ascribed to differences in the epidermal tissue of the fruit. Therefore, in a previous *in vitro* investigation we tried to obtain a periclinal chimera whose fruits would combine the skin of the cv. Pinot noir (PN) 7613 (*Botrytis* tolerant) and the pulp of cv. Chardonnay (Ch) 7535 (sensitive). *In vitro* shoot organogenesis was only observed on a mixed callus structure originating from a grafting of the two cultivars (VERDISSON *et al.* 1998). The regenerated adventitious shoots were assumed to be good candidates of chimeras. However, in young *Vitis* plantlets, no difference between these two cultivars became visible (TORREGROSA 1995). An analysis by biochemical methods, *e.g.* isoenzymes, is not reproducible because of the strong dependence on environmental factors (COLLINS and SYMONS 1993). Molecular methods seem to be more appropriate. Compared to restriction fragment length polymorphism (RFLP) analysis which is extensive and time consuming (MORENO *et al.* 1995), random amplified polymorphic DNA (RAPD) provides a faster and easier approach to distinguish between Ch and PN (GRANDO *et al.* 1996). In RAPD single, short (9-10 bp), arbitrary primers are used to amplify unspecified regions of the genome. The amplification products frequently vary between genotypes and can also be used as genetic markers. In the present study we determined RAPD markers to distinguish between the two grapevine cultivars. These selected markers were used to verify the hypothetical chimerical character of the adventitious shoots previously obtained.

Material and Methods

DNA extraction: Genomic DNA was isolated according to a modified protocol of KIM *et al.* (1997). Plant material (2 leaves of a vitroplant) was ground by hand with a micropestle in a sterile 1.5 ml microtube containing 5 µl of 1 % β-mercaptoethanol, 100 µl of 20 % sarkosyl (w/v) and 300 µl of extraction buffer (250 mM NaCl; 25 mM EDTA; 0.5 % SDS w/v; 200 mM Tris-HCl pH 8). This homogenate was heated for 10 min at 65 °C. Polyvinylpyrrolidone (6 % w/v) and 200 µl of ammonium acetate (7.5 M) were added separately. This mixture was incubated on ice for 30 min and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was added to 1 volume of isopropanol and incubated at -20 °C for 30 min. After centrifugation at 10,000 *g* for 10 min, the pellet was resuspended in 500 µl TE buffer (10 mM Tris; 1 mM EDTA; pH 8), 2 µl of RNase were added and the solution was incubated for 15 min at 37 °C. RNase and plant pigments were removed by mixing with an equal volume of chloroform-isoamyl alcohol (24:1) followed by a centrifugation (10,000 *g*). The upper aqueous phase was transferred into a new tube, 50 µl of ammonium acetate (7.5 M) and 550 µl of isopropanol were added and the solution was mixed gently to precipitate the DNA. After centrifugation at 10,000 *g* for 10 min, the DNA pellet was washed with 70 % ethanol, air-dried and redissolved in 30 µl of TE. DNA concentration was measured by spectrophotometry.

Amplification conditions: All polymerase chain reactions (PCR) were performed in a total volume of 25 µl containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween 20, 1.5 mM of $MgCl_2$, 120 µM of each dNTPs, 0.3 µM of each primer (Bioprobe Systems), 1 ng·µl^{-1} of genomic DNA and 0.06 U·ml^{-1} of Taq polymerase (Eurobio). Negative controls (reaction mixture without genomic DNA) were run with each amplification. All mixtures were covered with mineral oil. Amplifications were performed in a Crocodile III Appligene Oncor thermocycler. The PCR program consisted of an initial denaturation at 94 °C for 2 min followed by 45 cycles of PCR at 94 °C, 40 °C and 72 °C for 1, 1 and 2 min, respectively. Amplification products were analysed by 1.5 % agarose gel electrophoresis and then visualised under ultraviolet radiation after staining with ethidium bromide. A total of 16 primers (10 nucleotides long) were investigated for RAPD analysis (Table).

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Table

Primer sequences used for RAPD analysis

Primer	Nucleotide sequence 5' to 3'
A01	CAGGCCCTTC
A02	TGCCGAGCTG
A11	CAATCGCCGT
A12	TCGGCGATAG
B01	GTTTCGCTCC
B02	TGATCCCTGG
B11	GTAGACCCGT
B12	CCTTGACGCA
C01	TTCGAGCCAG
C02	GTGAGGCGTC
C06	GAACGACTC
C07	GTCCCGACGA
H04	GGAAGTCGCC
H14	ACCAGGTTGG
H15	AATGGCGCAG
H18	GAATCGGCCA

Results and Discussion

RAPD analysis was conducted first to discriminate two *V. vinifera* cultivars: Chardonnay (Ch) and Pinot noir (PN). Then, a preliminary screening of primers has been carried out to distinguish between Ch and PN. A total of 16 primers were tested (Fig. 1). All primers used for DNA amplification reactions produced fragments of different size with different intensities. Repetitions showed the existence of some bands that were more or less erratic (not shown). Furthermore, we observed in the negative control several bands (Figs. 1 and 2, lanes C) corresponding to primer multimers (YU *et al.* 1993). So, faint bands and bands present in the control and template containing reaction have not been taken into account according to ORTIZ *et al.* (1997) and MORENO *et al.* (1995).

Each primer shows a specific profile, but most primers (A02, A11, B01, B02, B11, C01, C02, C07, H04) yielded similar amplified fragments between Ch and PN (Fig. 1). They showed no polymorphism and then were inadequate to reach our objective. A01, B12, H14, H15 and H18 primers induced the amplification of one band in one cultivar not observed in the other cultivar. But this band was insufficient to confirm a chimerical character. Only A12 and C06 primers induced the formation of constant (not erratic) polymorphic bands between the two cultivars (indicated by arrows in Figs. 1 and 2). These results indicate the aptitude of these two RAPD markers to distinguish between the two cultivars. A12 and C06 were then selected to detect the putative chimerism of adventitious shoots.

RAPD analysis of adventitious shoots showed all bands specific to both cultivars (Fig. 2, bands indicated by a white arrow) indicating a chimerical character. But, some new constant bands distinct from the two original cultivars were

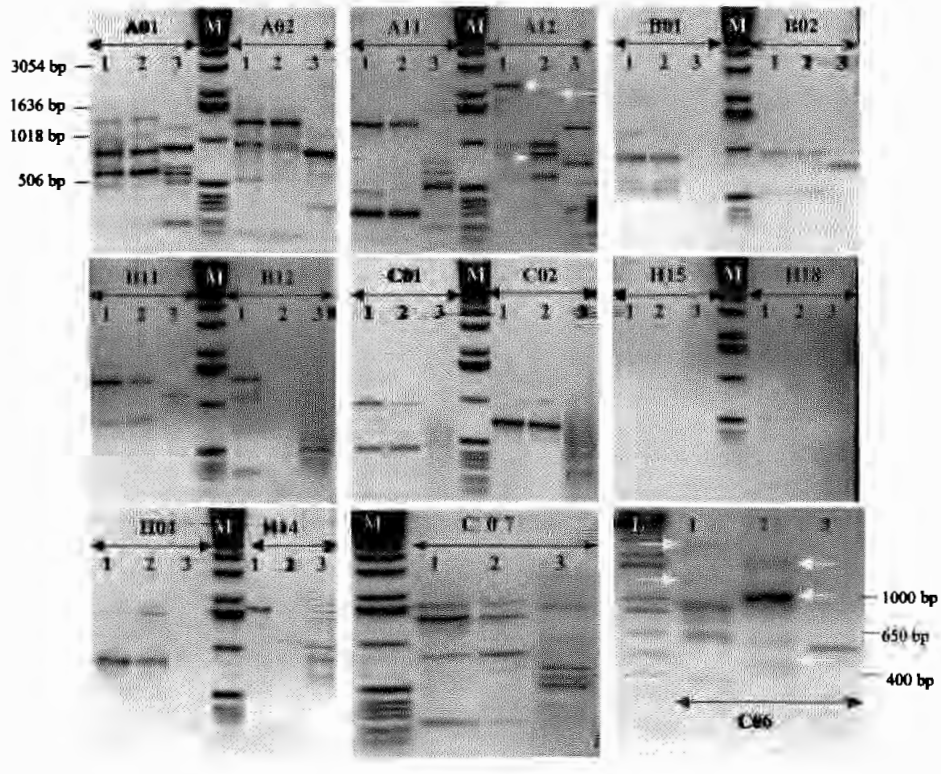

Fig. 1: RAPD profiles generated by tested primers. The RAPD products were fractionated on a 1.5 % agarose gel and stained with ethidium bromide. M: 1 kb DNA Ladder (GibcoBRL); L^+: 1 kb plus DNA Ladder (GibcoBRL) for the primer CO6 only; 1: Chardonnay 7535; 2: Pinot noir 7613; 3: control.

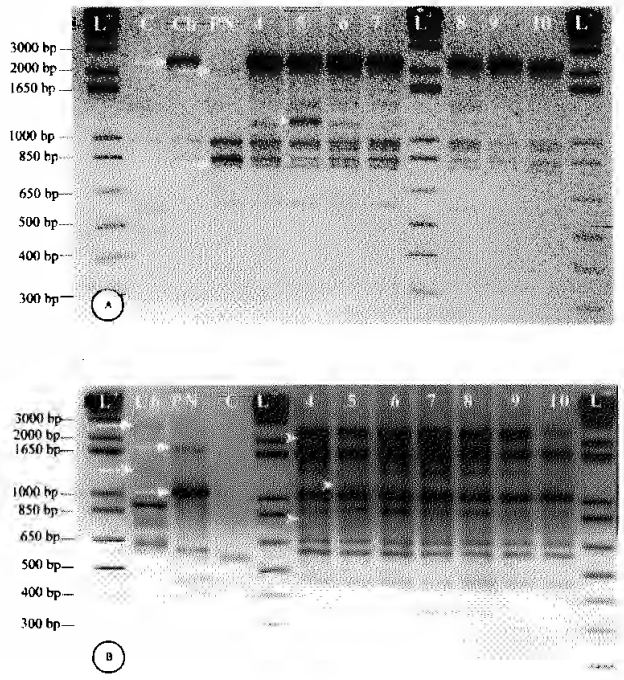

Fig. 2: RAPD profiles generated by A12 (**A**) and C06 (**B**) primers. The RAPD products were fractionated on a 1.5 % agarose gel and stained with ethidium bromide. L+: DNA size marker (1 kb plus DNA Ladder, GibcoBRL); Ch: Chardonnay 7535; PN: Pinot noir 7613; C: control; 4, 5, 6, 7, 8, 9, 10: adventitious shoots.

also amplified (Fig. 2, bands indicated by the head of an arrow). This may be due to somaclonal variation. Actually, RAPD can successfully detect somaclonal variation as has been shown in *Vitis* species (SCHNEIDER *et al.* 1996). Plant growth regulators, the process of dedifferentiation during the callus phase and redifferentiation during shoot organogenesis are known to enhance somaclonal variations (CLOG *et al.* 1990; STAMP *et al.* 1990; KARP 1994; VERDISSON *et al.* 1999) which can induce qualitative and quantitative changes in the genome. As mentioned by KARP (1994) different DNA sequences may be amplified or deleted during indirect shoot organogenesis. Furthermore we observed that these chimeras had nearly the same RAPD profile even if some of their bands are different. It can be assumed that the same bands appear during the callus common phase while the other should appear during the independent organogenesis process (VERDISSON *et al.* 1999).

Conclusion

As previously reported by different studies, RAPD has produced useful markers to characterise cultivars in herbaceous (RASMUSSEN and RASMUSSEN 1995) or woody species (SUGAWARA and OOWADA 1995; ORTIZ *et al.* 1997) such as grapevine (MORENO *et al.* 1995; STAVRAKAKIS *et al.* 1997; STAVRAKAKIS and BINIARI 1998). An easy and reliable distinction between Ch and PN can be carried out by this method. This method has already been used to detect chimerism in plants like *Citrus* species (SUGAWARA and OOWAD 1995), *Rubus* species (CHEN *et al.* 1996) or *Chrysanthemum* species (WOLFF 1996). But to our knowledge, this is the first report indicating that RAPD can be used to detect chimerism in grape.

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