Crop Breeding and Applied Biotechnology 6: 209-214, 2006
Brazilian Society of Plant Breeding. Printed in Brazil

Molecular analysis of the seedlessness character in grape using RAPD markers

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Received 12 June 2006

Accepted 28 July 2006

ABSTRACT - *Forty-eight polymorphic RAPD primers were used to study the genetic similarity of eight grapevine (*Vitis *spp.) accessions: Itália, Seyve Villard 12327, Seyve Villard 12375, Crimson Seedless, CG 87746, A 1976, Gota de Ouro and CNPUV 154-27. Gota de Ouro, the only* Vitis labrusca *cultivar, was the most distant cultivar from the others. The population CNPUV 154 (Seyve Villard 12327 x CG 87746) was evaluated for fresh and dry seed weight. Four phenotypic classes were considered for dry matter percentage (DM%): 1 (DM ≥ 55), 2 (45 ≤ DM < 55), 3 (35 ≤ DM < 45) and 4 (DM < 35). The observed frequencies for the classes 1, 2, 3, and 4 were 37, 27, 53, and 9 individuals, respectively. Of the 500 RAPD screened primers, 111 were selected as polymorphic. According to BSA, markers generated by OPK02, OPM16, OPS03, and OPU13 primers could be related to the region that controls seedlessness.*

Key words: *Vitis* spp., genetic diversity, RAPD, BSA, table grape breeding.

INTRODUCTION

The table grape industry is one of the most relevant in the Brazilian fruit business with a leading role in the social and economic situation of the Northeastern Region, mainly in the semi arid zone, as well as in other production areas in tropical and subtropical regions. There is a major world market for seedless grapes; however, the cultivars bearing this character introduced in Brazil have not adapted satisfactorily to date.

One of the most relevant traits in table grapes is the absence of palatable seeds, caused by the abortion of the embryo during the early steps of seed development (stenospermocarpy), leaving almost non-detectable traces or rudiments (Ledbetter and Ramming 1989). A consumer's idea of a seedless grape might be one in which the seed trace is undetectable. The phenotypic evaluation of seedlessness in not a simple task because berry size, flesh firmness and crispness, seed size and the degree of sclerification of seed integuments have a direct effect on whether a seed or seed trace is detectable or not (Ledbetter and Shonnard 1991).

This report describes the first attempt of the Embrapa Uva e Vinho (National Research Center for Grape and Wine) to perform a molecular marker-assisted selection for the character seedlessness in support of the Embrapa table grape (*Vitis* spp.) breeding program. The new approach will be included in the program as well as the promising results achieved earlier with the embryo rescue technique (Amaral et al. 2000). Above all for perennial crops, molecular marker-based tools can help breeders select any character at an early stage,

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allowing a reduction in time and experimental area (Ferreira and Grattapaglia 1998).

The identification of RAPD (Random Amplified Polymorphic DNA) markers as described by Williams et al. (1990) has been extensively applied to mapping because of its relatively low cost and ease of use. Striem et al. (1996) identified twelve RAPD markers that could be associated to the seedlessness character. Lahogue et al. (1998) developed a codominant SCAR (Sequence Characterized Amplified Region) marker, the SCC8, sequenced from a RAPD marker linked at 0.7 cM (opC08-1020) from the *sdI* gene. Adam-Blondon et al. (2001) sequenced the RAPD marker opP18-530 found by Lahogue et al. (1998) and a SCAR (SCP18) was developed. These two SCAR markers, SCC8 and SCP18, were mapped at a distance of 4.8 cM and 14.9 cM from the *sdI* gene, respectively.

The BSA (Bulked Segregant Analysis), proposed by Michelmore et al. (1991), has been widely used in the search for DNA markers linked to important agronomic characters and is very useful in the marker-assisted selection strategy.

The objectives of this study were: (1) to identify an adequate population for the BSA, and (2) to find RAPD markers associated to the region that controls the trait seedlessness in grape.

MATERIAL AND METHODS

Plant material and DNA extraction

Four cultivars with (Itália, Gota de Ouro, Seyve Villard 12327 and Seyve Villard 12375) and four without seeds (CNPUV 154-27, Crimson Seedless, A 1976 and CG 87746) obtained from the Grape Genepool of Embrapa Uva e Vinho (Brzailian Research Center for Grape and Wine) were used in this study. These accessions are currently used in the Embrapa table grape breeding program. Itália, Crimson Seedless and CG 87746 are *Vitis vinifera,* Gota de Ouro is *V. labrusca,* Seyve Villard 12327 and 12375, CNPUV 154-27 and A 1976 are complex hybrids.

Partially expanded young leaves were harvested for the DNA extraction (Lodhi et al. 1994). Total DNA was extracted using the CTAB method, according to Ferreira and Grattapaglia (1998), with some modifications. The samples were ground with liquid nitrogen (about 150 mg of fresh leaf tissue) in 1.5 mL microtubes. 700 mL of a CTAB 3X buffer solution heated to 65 ºC (3% CTAB; 1.4 M NaCl; 20 mM EDTA; 100 mM Trizma base pH 8.0; 1% PVP; 0.4% 2-mercaptoethanol) was added to each sample and 2-mercaptoethanol was added to the buffer solution right before its use. The samples were put in a water bath at 65 ºC for approximately 1 h. A total of 600 mL chloroform isoamyl alcohol (24:1) was mixed with the samples and centrifuged at 14000 rpm for 10 min. The supernatant was transferred to a new microtube; 500 mL of cold isopropanol was added to each sample right after the transfer. The samples were centrifuged again at 7000 rpm for 10 min at 4 ºC. The DNA pellet was washed with 70% alcohol and dried at room temperature. A volume of 50 - 100 mL TE pH 8.0 (1 M Trizma base pH 8.0; 0.5 M EDTA) was added to the samples, according to the amount of the pellet. 1 mL RNAse [10 mg mL^{-1}] per 50 mL TE was added. The microtubes were incubated at 37 ºC for at least 30 min. The DNA was quantified in 1% agarose gels under electrophoresis for about 1 h at 100 volts. To quantify the DNA the samples were compared with l DNA patterns of 50, 150 and 500 ng.

RAPD primer screening

A total of 500 RAPD primers (kits from A to Z of Operon Technologies) were tested. DNA samples were amplified by PCR and evaluated for the presence and absence of bands. Our criterion was to select primers which had at least two non-common bands of the seeded and seedless groups.

The PCR had a final volume of 13 mL: 1.3 mL 10X buffer (CBIOT/RS); 1.04 mL dNTPs [2.5 mM]; 5.2 mL BSA (Bovine Serum Albumin – 2.5 mg mL^{-1}); 1.5 mL primer [10 ng ml^{-1}]; 0.2 mL Taq DNA Polymerase [5U ml^{-1}] (CBIOT/RS), and 15 ng DNA and distilled water. The PCR program was slightly modified compared to Williams et al. (1990): 92 ºC for 1 min; 35 ºC for 1 min; 72 ºC for 2 min – 40 cycles and 72 ºC for 5 min as final extension temperature.

Genetic diversity trial

Genetic diversity of the above-mentioned cultivars was assessed in a trial using 48 polymorphic RAPD primers (OPB05/ 17/ 18; OPC18/ 19/ 20; OPE01/ 02/ 06/ 16; OPF10; OPG06; OPH04/ 06/ 12/ 14/ 19; OPI09/ 14/

16/ 17/ 18/ 20; OPJ04/ 06/ 07/ 10/ 16/ 17; OPK02/ 04/ 08/ 13/ 16/ 17/ 20; OPL04; OPO05/ 10/ 11/ 14/ 15/ 19; and OPP03/ 04/ 05/ 14/ 18). Clustering was performed by the UPGMA method and the similarity by Dice's coefficient; NTSYSpc 2.0 (Rohlf 1997) was used to generate a dendogram.

Phenotypic evaluation

Based on the methodology proposed by Bouquet and Danglot (1996), 126 individuals from the CNPUV 154 (Seyve Villard 12327 x CG 87746) segregating population were evaluated for the seedlessness character. The female parent (Seyve Villard 12327) is seeded and the male (CG 87746) has seedless berries. The clusters of the CNPUV 154 population were harvested from an experimental field of the National Research Center for Grape and Wine in Bento Gonçalves, RS.

Two clusters were collected from each individual of the segregating population. The 20 largest berries were chosen for the seedlessness character evaluation. The berries were frozen (-20 ºC) and later thawed in a microwave oven for 3min. Extracted seeds were weighed to obtain their fresh weight and then dried at 70 ºC for 72 h to determine their dry weight and dry matter percentage (DM%).

Four phenotypic classes were established according to the seed DM%: 1- totally sclerified normal seeds (DM $\geq$ 55); 2- partially sclerified hard seed traces (45 $\leq$ DM < 55); 3- non-sclerified soft seed traces (35 $\leq$ DM < 45); and 4- very small seed traces or no seeds (DM < 35).

Bulked Segregant Analysis

According to Michelmore et al. (1991) and Lahogue et al. (1998) and based on the dry matter percentage of seeds observed in the phenotypic evaluation, two DNA bulks were established for the molecular characterization of the seeded and seedless plants. Hybrids with the greatest dry matter percentage (class 1) were selected for the seeded bulk and hybrids with the smallest dry matter percentage (class 4) were selected for the seedless bulk. DNA samples of ten seeded (19, 48, 49, 69, 94, 112, 114, 134, 149, and 153) and ten seedless seedlings (20, 22, 24, 27, 34, 43, 72, 80, 145, and 160) of the CNPUV 154 population were mixed to represent the seeded and seedless bulks, respectively. Each sample had an equal DNA concentration of 5 ng μL^{-1}. The PCR was the same as used in the initial primer screening. A total of 111 primers with polymorphism in the initial screening were tested in the CNPUV 154 parental lines (Seyve Villard 12327 - seeded parent x CG 87746 - seedless parent) and in the contrasting bulks.

RESULTS AND DISCUSSION

RAPD primer screening

Of the 500 evaluated primers, 326 produced bands, and 111 showed polymorphism (OPB05/ 17/ 18; OPC18/ 19/ 20; OPD07; OPE01/ 02/ 06/ 10/ 16; OPF10; OPG06; OPH04/ 06/ 12/ 14/ 19; OPI09/ 14/ 16/ 17/ 18/ 20; OPJ04/ 06/ 07/ 10/ 16/ 17; OPK02/ 04/ 08/ 13/ 16/ 17/ 20; OPL04; OPM01/ 02/ 03/ 05/ 06/ 07/ 12/ 13/ 16/ 18; OPN02/ 03/ 04/ 11; OPO05/ 10/ 11/ 14/ 15/ 19; OPP03/ 04/ 05/ 14/ 18; OPQ01/ 04/ 05/ 06/ 09/ 20; OPR01/ 08/ 12/ 20; OPS03/ 16; OPT02/ 04/ 07; OPU06/ 09/ 10/ 11/ 13/ 14/ 18; OPV04/ 06/ 07/ 14/ 15/ 16/ 17/ 18/ 19; OPW04/ 06/ 07/ 09/ 11/ 17/ 19/ 20; OPX01/ 02/ 06/ 07; and OPZ06/ 07/ 10/ 19). This abundance of polymorphic RAPD primers confirms previous results obtained earlier with molecular-assisted selection (Striem et al. 1994, 1996) and grapevine characterization (Lodhi et al. 1997, Tessier et al. 1999, Luo and He 2001).

Genetic similarity

The genetic relationship achieved by applying RADP markers is shown in Figure 1. As expected, the cultivars

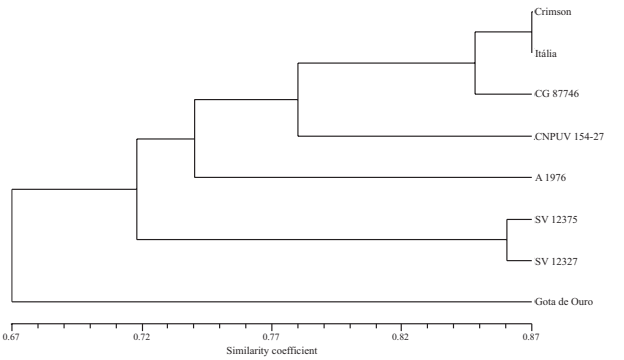

Figure 1. Dendogram of UPGMA

Crimson Seedless, Itália and CG 87746 were grouped together because they are *vinifera* types. The proximity between Itália and Crimson Seedless can be explained because Itália is one of the parents of Crimson (Ramming and Tarailo 1995). Another expected result was the observed position of CNPUV 154-27 compared to its parents Seyve Villard 12327 and CG 87746. Gota de Ouro was most distant from the others, possibly because it was the only *Vitis labrusca*.

Phenotypic evaluation and BSA

On the underlying results of the genetic distance trial, the CNPUV 154 population (Seyve Villard 12327 x CG 87746) was judged adequate for BSA. The frequency of segregation observed in this population is shown in Figure 2. A small number of seedless individuals (class 4) was obtained. However, if we would consider the sum of classes 1 and 2 as a seeded group (64 individuals) and the sum of the classes 3 and 4 (62 individuals) as a seedless group, since this is the usual criteria in the selection process, then the observed phenotypic ratio would be 1:1. Results reported in literature show a variable proportion of seedless offspring, depending on the parents. In crosses between seeded x seedless parents, segregation data showed a predominant 3:1 (seeded to seedless) ratio (Pospíšilová and Páleník 1988, Roytchev 1998, Spiegel-Roy et al. 1990), but the most accepted hypothesis to explain the inheritance of seedlessness in grape was proposed by Bouquet and Danglot (1996). Based on crosses between partially

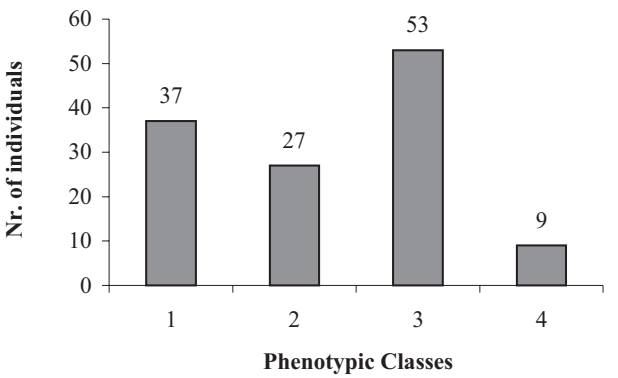

Figure 2. Frequency for the phenotypic classes 1 (DM ≥ 55), 2 (45 ≤ DM < 55), 3 (35 ≤ DM < 45) and 4 (DM < 35) according to the seed dry matter percentage (DM%) in the CNPUV 154 (Seyve Villard 12327 x CG 87746) population

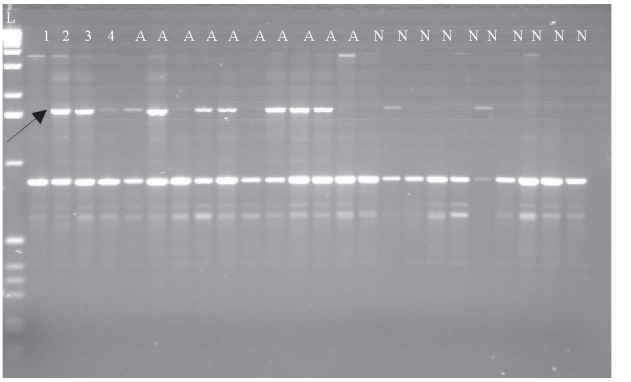

Figure 3. Electrophoretic analysis in agarose gel of the OPK02 primer with the open bulks. The arrow indicates the marker OPK02 (1700 bp). Columns 1, 2, 3 and 4 indicate seeded and seedless parents, seedless and seeded bulk, respectively. Columns A and N indicate seedless and seeded seedlings, respectively. Column L: ladder 1 kb

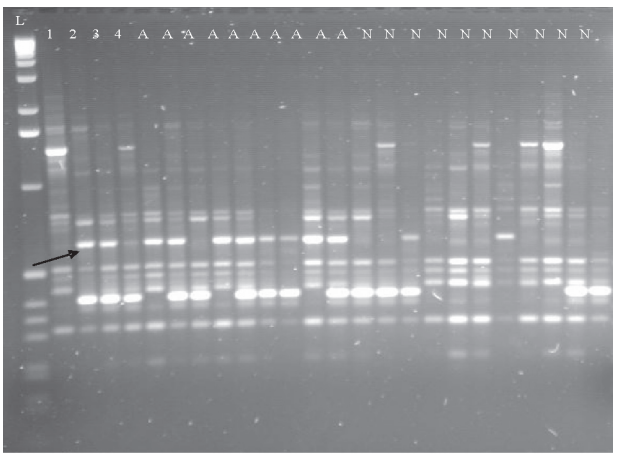

Figure 4. Electrophoretic analysis in agarose gel of the OPM16 primer with the open bulks. The arrow indicates the marker OPM16 (650 bp). Columns coded according to Figure 3

seedless selections, they suggested the existence of a complex system in which the expression of three independently inherited recessive genes is controlled by a dominant regulator gene.

The BSA seemed to be adequate for polymorphic RAPD marker identification in the seeded SV 12327 and seedless CG 87746 parents. Among the 111 primers screened for their polymorphism, the markers OPK02 (1700 bp), OPM16 (650 bp), OPS03 (900 bp) could be related to the chromosome region that controls seedlessness (Figures 3, 4 and 5). On the other hand, markers as the one generated by OPU13 (650 bp) can be

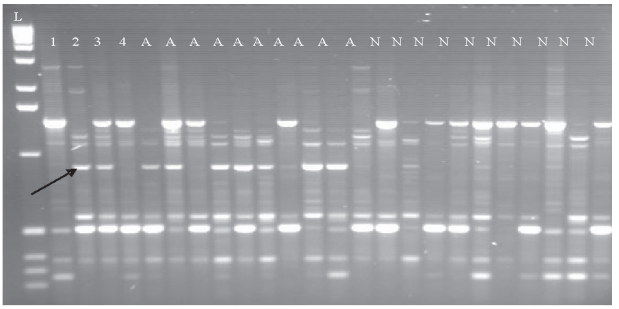

Figure 5. Electrophoretic analysis in agarose gel of the OPS03 primer with the open bulks. The arrow indicates the marker OPS03 (900 bp). Colunns coded according to Figure 3

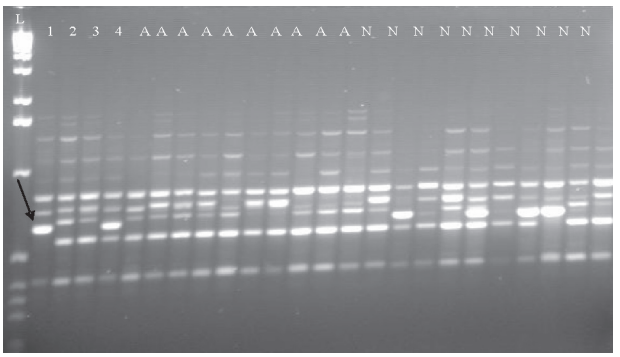

Figure 6. Electrophoretic analysis in agarose gel of the OPU13 primer with the open bulks. The arrow indicates the marker OPU13 (650 bp). Colunns coded according to Figure 3

related to the chromosome region that controls the seeded character since it is present in the seeded parent and in the seeded individuals (Figure 6). This can therefore be another method of marker-assisted selection that identifies markers that are present in the seeded and absent in the seedless plants. Each one presented similar DNA patterns for the parents and their respective bulks.

The RAPD markers that appeared in the seeded or seedless parent and in its corresponding bulk as well were also tested in the open bulks (Figures 3, 4, 5 and 6). The amplification of these selected primers in the open bulks showed unexpected patterns for some plants, which could mean the occurrence of recombination events. Another aspect, as pointed out by Striem et al. (1996), is related to the varied development degree of the seed components (seedcoat, endosperm and embryo); as they have influence on seedlessness, they might have caused misinterpretations in our phenotypic evaluation.

Since only a few, yet un-mapped RAPD markers were selected, more RAPD markers should be evaluated in a posterior screening that could be linked to seedlessness. Aiming to develop a reliable marker-assisted selection process for the seedlessness character, additional studies are necessary and should include both new populations with different genetic background and other powerful molecular markers such as SSR and AFLP. Besides, as pointed out by Striem et al. (1996), the criteria used in future phenotypic evaluations of the seedless trait should be chosen more carefully.

ACKNOWLEDGEMENTS

JC Lima and A Crippa were holders of scholarships granted by CNPq/ MCT (The National Council for Scientific and Technological Development/ Ministry of Science and Technology).

Análise molecular do caráter apirenia em uva usando marcadores RAPD

RESUMO – *Foram usados quarenta e oito iniciadores RAPD polimórficos para estudar a similaridade genética de oito acessos de videira (*Vitis *spp.): Itália, Seyve Villard 12327, Seyve Villard 12375, Crimson Seedless, CG 87746, A 1976, Gota de Ouro e CNPUV 154-27. Gota de Ouro, a única cultivar de* Vitis labrusca*, foi a mais distante. A população CNPUV 154 (Seyve Villard 12327 x CG 87746) foi avaliada para peso fresco e seco de semente. Foram consideradas quatro classes fenotípicas para porcentagem de matéria seca (%MS): 1 (MS ≥ 55), 2 (45 ≤ MS < 55), 3 (35 ≤ MS < 45) e 4 (MS < 35). As freqüências obtidas para as classes 1, 2, 3 e 4 foram de 37, 27, 53 e 9 indivíduos, respectivamente. Dos 500 iniciadores RAPD testados, foram selecionados 111 polimórficos. Pela BSA, os marcadores gerados pelos iniciadores OPK02, OPM16, OPS03 e OPU13 podem estar associados à região que controla a apirenia.*

Palavras-chave: *Vitis* spp., diversidade genética, RAPD, BSA, melhoramento de uva de mesa.

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