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Identification of a codominant scar marker linked to the seedlessness character in grapevine

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Abstract The variety *Vitis vinifera* cv Sultanine presents a type of seedlessness in which fertilization occurs but seeds subsequently fail to develop. It has been suggested that this trait might be controlled by three complementary recessive genes regulated by a dominant gene named *I*. Bulk segregant analysis was used to search for random amplified polymorphic DNA (RAPD) markers linked to the *I* gene in progeny obtained by crossing two partially seedless genotypes. One hundred and forty decamer primers were screened using bulks obtained by pooling the DNA of extreme individuals from the phenotypic distribution. We identified two RAPD markers which appeared tightly linked to *I* (at 0.7 and 3.5 cM respectively). The closest marker was used to develop a codominant SCAR (sequence characterized amplified region), named *SCC8*. This latter marker appeared of great value either to exclude from the progeny potentially seeded individuals or to select for seedless individuals. Indeed, all the seeded individuals of the progeny were found to be homozygous $scc8^-/scc8^-$, and all the individuals homozygous $SCC8^+/SCC8^+$ were seedless. Moreover, this marker was successfully applied to other natural seedless varieties where codominance persisted. *SCC8* was also used to dissect more precisely the genetics of seedlessness. ANOVA analysis indicated that this SCAR marker accounted for at least 64.9% of the phenotypic variation of the seed's fresh weight and for at least 78.7% of the phenotypic variation of the seed's dry matter. These results confirmed the presence of a major gene, and also the existence of other complementary recessive genes, controlling the expression of seedlessness.

Key words *Vitis vinifera*. L · Seedlessness · RAPD · SCAR · BSA · Marker-assisted selection

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Introduction

Most of the grapes marketed in the New World for table consumption are now seedless. In the United States, they represent 80% of the total table grape production (California Table Grape Commission 1995). The most commonly grown seedless variety is *Vitis vinifera* cv Sultanine, also called Thompson Seedless. This variety presents a type of seedlessness named stenospermocarpy, where fertilization occurs but seeds fail to develop completely because of embryo and/or endosperm abortion (Stout 1936).

For the development of new seedless varieties, traditional breeding methods are based on crosses between seeded and seedless varieties, Sultanine being the main source of seedlessness. The proportion of seedless genotypes obtained in these progenies is low (10–30%) and depends on the parentage (Olmo and Baris 1973; Loomis and Weinberger 1979). Since seedlessness is only one of the important traits to consider for a new table grape variety, the selection process requires a great number of plants. By means of in vitro culture, viable embryos can be rescued from crosses between two seedless varieties (Cain et al. 1983; Emershad and Ramming 1984; Spiegel-Roy et al. 1985; Bouquet and Davis 1989). This technique allows the recovery of a higher proportion of seedless plants than can be obtained by traditional crosses (50–80% according to the degree of seedlessness of the parents). However, because the use of in vitro culture is labour intensive and time consuming, the breeding of seedless varieties is a tedious process. Moreover, as the grapevine does

not produce bunches before the age of 3 or 4 years, the seedlessness character can not be observed at an early stage of the breeding process. The identification of molecular markers linked to the seedless character would therefore be of great interest, creating the possibility to exclude seeded offspring at an early seedling stage. Consequently, there would be a considerable saving of time and space, and a reduction in overall costs.

Striem et al. (1996) reported the search for molecular markers associated with seedlessness. They identified 12 RAPD markers which were found to be correlated with several seedlessness subtraits. However, the usefulness of these markers in other crosses needs to be investigated, as well as the distance between the markers and the different components of seedlessness. Their study suggested that several genes might be involved in the expression of the character, which is in agreement with other published results (for a review see Bouquet and Danglot 1996). Recently, Bouquet and Danglot (1996) proposed that seedlessness might be controlled by three complementary recessive genes *a1, a2, a3,* independently inherited and regulated by a dominant gene *I*. Although seedlessness is a quantitative trait according to this hypothesis, molecular markers linked to this major gene could be identified using the "Bulk Segregant Analysis" technique (Michelmore et al. 1991) by pooling DNA from extreme individuals, as has been demonstrated for a major resistance gene in sugarbeet (Pelsy and Merdinoglu 1996).

In the present paper, we described the use of the RAPD technique in combination with BSA to identify markers linked to gene *I*. In addition, a codominant SCAR marker (Paran and Michelmore 1993) was developed from one of these markers, allowing a better evaluation of the genotype at the *I* locus and an estimation of the contribution of this locus to the phenotypic variation for several subtraits of seedlessness.

Materials and methods

Plant material

The progeny Mtp 3140 was obtained by crossing Mtp 2223-27 (Dattier de Beyrouth × 75 Pirovano) with Mtp 2121-30 (Alphonse Lavallée × Sultanine) as described by Bouquet and Danglot (1996). It was grown in a seedling block of the INRA viticultural experiment station "Chapitre – Vassal", near Montpellier, France. The 139 fertile plants were analysed over 3 years (from 1994 to 1996) and distributed into four phenotypic classes (Bouquet and Danglot 1996). Other seedless genotypes derived from different crosses with or without Sultanine, as well as several naturally occuring seedless varieties (Table 1), were analysed. Young leaves were harvested during the growing season, frozen in liquid nitrogen and kept at − 20°C until DNA extraction.

DNA extraction

In the case of the individuals used for the bulk segregant analysis, the extraction was made from 2 g of leaves according to the method described by This et al. (1997). For the other individuals, the procedure was adapted as follows for small quantities of leaves (0.2 g). Pieces of leaf were ground to a fine powder in liquid nitrogen and 0.2 g of the powder was transferred into 1 ml of extraction buffer (This et al. 1997). After homogenization, the tube was incubated for 45 min at 65°C. Then, 800 µl of chloroform/isoamyl alcohol (24 : 1) was added to the tube. After centrifugation at 12 000 rpm for 10 min, the supernatant was transferred into another tube. Subsequently 160 µl of 10% CTAB and 800 µl of chloroform/isoamyl alcohol (24:1) were added and a further centrifugation was performed under the same conditions. The supernatant was then transferred into another tube and nucleic acids were precipitated using 750 µl of cold isopropanol. The tube was kept on ice, gently agitated from time to time over a 30-min period and centrifuged at 12000 rpm for 10 min. Isopropanol was discarded and the pellet washed with 70% ethanol. The pellet was dried under vacuum and dissolved in 250 µl of TR (10 mM Tris HCl pH 7.5, 0.1 mM EDTA). Then, 2 µl of RNAse (10 mg/ml, Sigma) was added and the solution incubated for 60 min at 37°C. Similarly, 2 µl of Proteinase K (10 mg/ml, Sigma) was added and incubated for 30 min at 50°C. The DNA was purified by a phenol/chloroform and a chloroform extraction. Nucleic acids were then precipitated using 250 µl of 5 M ammonium acetate and 1.2 ml of cold absolute ethanol. After drying, the pellet was re-suspended in 100 µl of TR. The DNA was quantified on a 0.8% agarose gel by visual comparison with known quantities of lambda DNA (Boehringer Mannheim, Germany).

Table 1 Parentage of the seedless plants tested

Name	Parentage
Centennial Seedless	Complex hybrid derived from Sultanine
3046-20	Italia × Canner seedless[a]
3049-166	Italia × (Dattier de Beyrouth × 75 Pirovano[b])
3047-1	Italia × Sultanine
1992-9	Dattier de Beyrouth × 75 Pirovano[b]
2715-23	Bicane × Sultanine
2711-6	Madeleine angevine × Canner seedless[a]
2223-32	Dattier de Beyrouth × 75 Pirovano[b]
2212-30	Alphonse Lavallée × 75 Pirovano[b]
2121-61	Alphonse Lavallée × Sultanine
Canner seedless[a]	Hunisa × Sultanine
Attika seedless	Alphonse Lavalle × Black Monukka
Bayad	Natural origin[c]
Black Monukka	Presumably natural[c]

[a]Canner seedless = Hunisa × Sultanine
[b]75 Pirovano = Muscat d'Alexandrie × Sultanine
[c]From Branas and Truel (1965)

RAPD analysis

The RAPD reactions were carried out in a 25-µl volume containing 0.4 units of *Taq* DNA polymerase (Appligène-oncor, France), 1 × buffer [10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.02% (w/v) BSA or gelatin] provided with the polymerase, 120 µM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 30 ng of primer and approximately 50 ng of template DNA. The reaction mixture was overlaid with one drop of mineral oil. PCR was carried out in a Braun Bio-med Thermocycler (Germany) programmed as follows: one step of 3 min at 93°C, then 35 cycles of 1 min 15 s at 93°C, 1 min 15 s at 38°C, 1 min 15 s at 72°C, and a final step of 6 min at 72°C. The amplification products were electrophoresed in a 1.6% agarose gel for 4 h at 100 V and detected by staining with ethidium bromide.

Bulk segregant analysis

Equal quantities of DNA were pooled from eight individuals of the class-1 phenotype (berries without seed traces or containing small-sized seed traces with unsclerified integuments) and eight individuals of the class-4 phenotype (berries containing normally developed seeds with totally sclerified integuments), to produce two DNA bulks (BkA and BkP, respectively) which were used to screen 140 random, 10-mer primers (kits A–E and O–P, Bioprobe, France, as defined by Operon Technologies Inc.). Similarly, equal quantities of DNA of the two seeded grandparents (Dattier de Beyrouth and Alphonse Lavallée) were pooled. PCR was carried out simultaneously on the seeded grandparental bulk, BkP, BkA and Sultanine. Putative markers were validated by the analysis of the 16 individuals constituting the bulks.

Cloning and sequencing of the RAPD product

The RAPD band to be cloned was purified on a 2% low-melting agarose gel (Appligène-oncor, France) in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA). After ethidium-bromide staining, the band was excised and purified with the Wizard™ PCR Preps kit (Promega) according to the manufacturer's instructions. The fragment was then ligated into the pGEM-T vector (Promega). Fresh competent cells of *Escherichia coli* strain DH5 were prepared according to Hoisington et al. (1994) and were transformed with the ligated plasmids (Sambrook et al. 1989). Selection of the transformed clones was performed by PCR analysis directly on white colonies. A purification of the plasmids from the selected colonies was carried out using the alkaline-lysis method (Sambrook et al. 1989). The insert was then amplified by PCR using the T7 and SP6 primers and purified using the Wizard™ PCR Preps kit. The identity of the purified RAPD product was verified by hybridization to Southern blots of individuals that segregated for this marker. Sequencing of both ends of the insert was done by Génome Express SA (France).

SCAR design and analysis

Two oligonucleotides to be used as SCAR primers were designed using the PRIME command of the GCG package Version 8.1-UNIX (Wisconsin Package 1995) and synthesized by Oligo Express SA (France). The forward primer (SCC8-S) contains only the four 3′ end bases of the RAPD primer plus the 16 adjacent bases (5′GGTGTCAAGTTGGAAGATGG3′). The reverse primer (SCC8-AS) is located 16 bases upstream of the RAPD primer (5′TATGCCAAAAACATCCCC3′). The amplification was done in a standard PCR reaction using 10 pmol of each primer and the PCR conditions were as follows: 4 min at 94°C, 35 cycles of (1 min at 94°C, 1 min at 60°C, 1 min at 72°C), and 6 min at 72°C. Amplification products were resolved electrophoretically in a 2% agarose gel. Restriction of the amplified fragments was performed in a final volume of 10 µl using 7 µl of the PCR reaction and 10 units of the chosen restriction endonuclease, according to the manufacturer's instructions. Digestion products were resolved by electrophoresis in a 2% agarose gel.

Southern hybridization

Two micrograms of genomic DNA from two individuals of class-1 and class-4 phenotype respectively were digested with either *Eco*RI, *Eco*RV or *Hind*III restriction enzymes according to the manufacturer's instructions. Restriction products were separated on a 0.8% agarose gel in 0.5 × TBE and blotted by Southern transfer to a Biotrans™ nylon membrane following the manufacturer's instructions (ICN Biomedicals, Inc.). Similarly, RAPD amplification products obtained using DNA from individuals segregating for the cloned RAPD marker, as well as from other Sultanine-derived seedless varieties, separated on a 1.6% agarose gel, were blotted using the same process.

The membranes were pre-hybridized at 65°C for 1 h with 100 µg/ml of salmon sperm DNA in a solution of 5 × Denhardt's solution (2% Ficoll, 2% PVP, 2% BSA), 5 × SSC, 0.1% SDS. The probe was radio-labeled with α-[^{32}P]-dNTP using the Megaprime DNA labelling System Kit (Amersham) and added to the previous solution. The hybridization was then performed overnight under the same conditions. The membranes were washed successively with 2 × SSC – 0.1% SDS, 1 × SSC – 0.1% SDS and 0.1 × SSC – 0.1% SDS at 65°C for 15 min each and exposed to XAR-Kodak autoradiographic film for 3 to 4 h.

Statistical analysis

The putatively linked RAPD markers were analysed on all individuals of the progeny. They were scored as 1 for presence and 0 for absence. Similarly, the SCAR marker was analysed on 136 individuals of the progeny. They were scored as $SCC8^+/SCC8^+$, $SCC8^+/scc8^-$ or $scc8^-/scc8^-$, $SCC8^+$ being the allele linked to the seedless character. Goodness-of-fit between observed and expected segregation ratios at marker loci was tested by a chi-square analysis as too was the likelihood of independence between the absence of flanking markers and the seeded phenotype (class 4).

Marker order and map distances were estimated using the software Mapmaker 3.0 (Lander et al. 1987). A minimum LOD score of 3.0 and a maximum recombination fraction of 0.5 were used to form linkage groups. Recombination fractions were converted into centiMorgans (cM) by applying the Haldane function (Haldane 1919).

A one-way analysis of variance (GLM procedure, SAS Institute Inc. 1989) using the SCAR marker as the treatment was used to test for an association between this marker and subtraits of seedlessness [total fresh weight (TFW) and percentage of dry matter (DM) of seeds of 100 berries]. Certain individuals had seeds too small to be extracted and weighed, their TFW values were arbitrarily noted as 0 and their DM values as missing data, since no measure could be obtained. The proportion of the total phenotypic variance attributed to the SCAR marker was given by the R^2 value. As the residuals from the ANOVA were not normally distributed, the non-parametric Kruskal-Wallis test was used to verify the results obtained with the ANOVA. When a significant effect was detected, a Student-Newman-Keuls (SNK) test was performed using the SAS program in order to test for significant differences between the mean values of TFW and DM for each genotype at the SCAR locus. Similarly, a one-way analysis of variance and SNK tests were performed in order to test the validity of the phenotypic classification.

Results

Bulk segregant analysis

Among the 140 tested primers, only three did not initiate DNA amplification in both grandparents and bulks and four did not produce any discrete bands. Eight primers (opA11, opC08, opC12, opD15, opE05, opE08, opE13 and opP18) showed an amplification product present on Sultanine and bulk BkA profiles but absent on the seeded grandparental bulk and bulk

BkP profiles (Figs. 1 and 2 for opC08 and opP18 respectively). No fragment specific for the seeded bulks was revealed. These eight primers were then tested on each of the 16 individuals of bulks BkA and BkP. Only opC08 produced one phenotype-specific fragment, opC08–1020 (Fig. 1). Two other primers (opP18 and opC12) showed one fragment (opP18–530 and opC12–950, respectively) present in all BkA individuals but also in a few BkP individuals (Table 2, Fig. 2). The analysis of 14 other individuals (seven from phenotypic class 1 and seven from phenotypic class 4) confirmed these results, except that one seeded individual showed opC08-1020 (Table 2).

Linkage analysis

Only primers opC08 and opP18 were tested on all the progeny individuals (Table 3). According to the chi-square tests, the phenotype distributions at the marker loci did not deviate significantly from the 3 : 1 expected ratio (Table 3). When all the individuals of phenotypic classes 1 to 3 (i.e. never presenting fully developed seeds) were considered as seedless, the phenotypic distribution of the character conformed to a 3:1 segregation ratio, validating the single gene model. Both markers were found to be tightly linked to the seedless character (Table 3), in coupling with the I^+ allele.

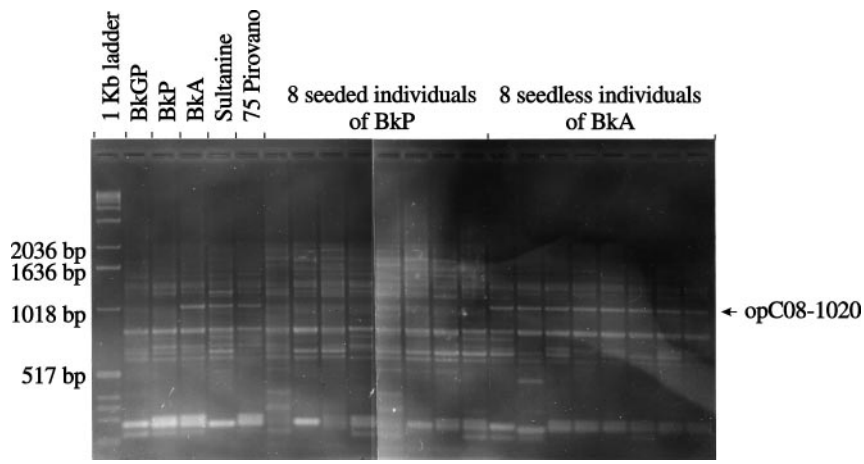

Fig. 1 RAPD amplification from genomic DNA of the parents, seeded and seedless bulks, and the individuals constituting these bulks, using primer opC08. The RAPD opC08–1020 is indicated by a *black arrow*.

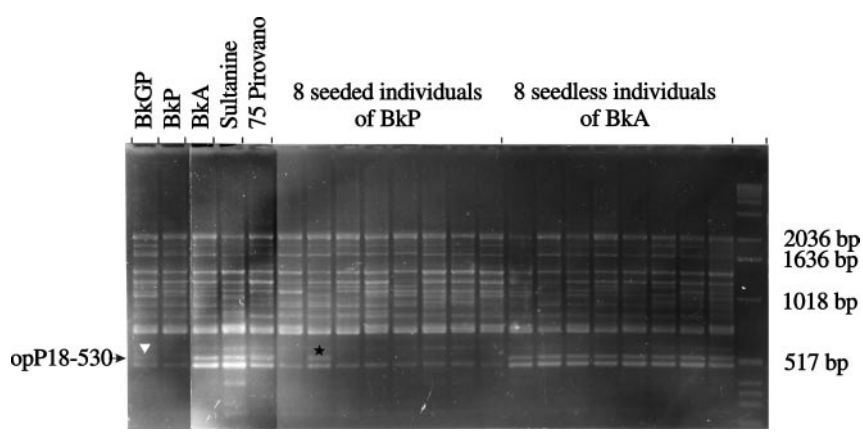

Fig. 2 RAPD amplifications from genomic DNA of the parents, seeded and seedless bulks, and the individuals constituting these bulks, using primer opP18. The RAPD opP18–530 is indicated by a *black arrow*. The *white arrow* shows a band which is slightly higher than opP18-530. ★ recombinant individual

Table 2 Number of individuals showing the markers in both seedless and seeded bulks and in 14 other individuals of the progeny

Markers	BkA	BkP	Seven other individuals class 1	Seven other individuals class 4
opC08-1020	8	0	7	1
opP18-530	8	1	7	0
opC12-950	8	2	7	2

Table 3 Phenotype occurence and χ^2 values for goodness of fit to 3 : 1 (χ^2A; χ^2B) and to 9 : 3 : 3 : 1 (χ^2AB) expected progeny segregation ratios

Locus		Phenotypes[a]				Chi-square value		
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB
I^+	opC08-1020	97	0	1	41	2.01	1.49	174.9**
I^+	opP18-530	94	3	2	40		2.61	158.7**
opP18-530	opC08-1020	94	2	4	39			149.9**

[a]A, B: Presence of I^+ (phenotypic class 1–3) or of the RAPD marker; a, b: Absence of I^+ (phenotypic class 4) or of the RAPD marker
**Significant at the 0.01 probability level

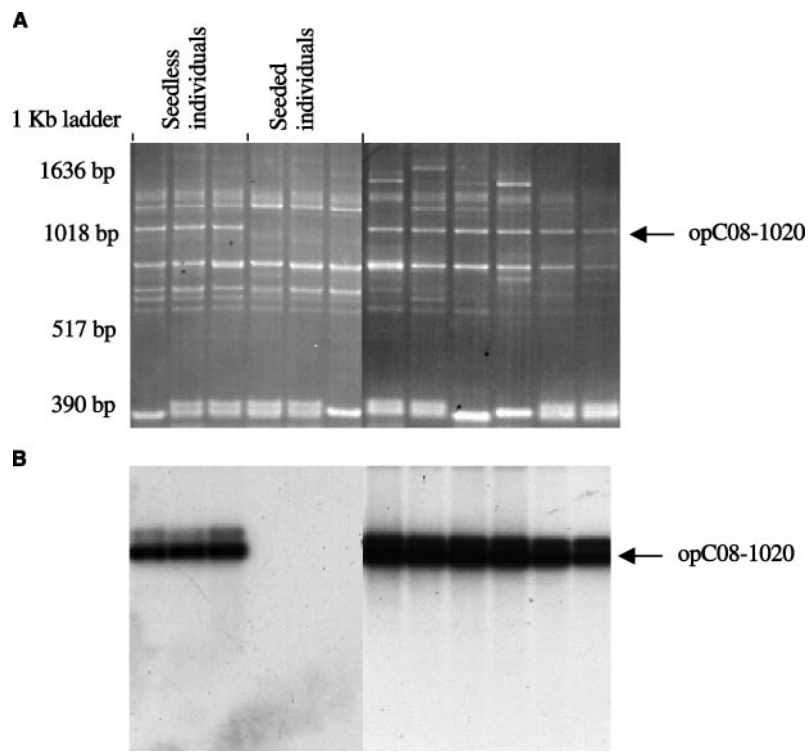

Fig. 3 A RAPD amplifications from genomic DNA of seedless and seeded progeny, and other seedless Sultanine-derived individuals, using primer opC08. The RAPD opC08-1020 is indicated by an *arrow*. **B** Verification of the homology of RAPD fragments between seedless progeny and other seedless Sultanine-derived individuals. The Southern blot of the RAPD profiles was hybridized using a ^{32}P-labelled opC08-1020 fragment as a probe

Marker opC08-1020, which revealed one recombinant, was located closer to the gene *I* than opP18-530 which showed five recombinants (Table 3). The genetic distances between the gene and opC08-1020 or opP18-530 were 0.7 and 3.5 cM respectively, the distance between the two RAPD markers being 4.2 cM.

Analysis of other Sultanine-derived seedless varieties

Ten other seedless plants derived from Sultanine (Table 1) were tested for the presence of fragments opC08-1020 and opP18-530, along with seeded and seedless individuals of the progeny. Both markers have been recovered from all the seedless individuals (Figs. 3 A and 4 A, respectively). The homology of these two RAPD bands among the varieties was tested by Southern hybridization. The two gels from Figs 3 A and 4 A were each blotted to a nylon membrane and hybridized with the corresponding cloned fragment. Both bands were cloned as described in Materials and methods. The two cloned fragments used as probes revealed the predicted fragments in the seedless individuals as well as in the ten other seedless Sultanine-derived individuals, but with no hybridization signal in the seeded individuals (Figs 3 B and 4 B). This confirmed that the same sequence was amplified in all the tested varieties.

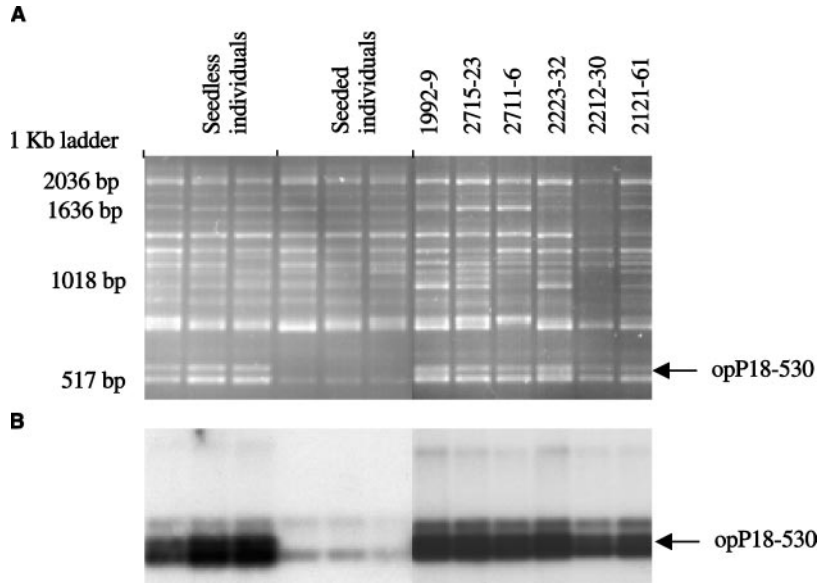

Fig. 4 A RAPD amplification from genomic DNA of seedless and seeded progeny, and other seedless Sultanine-derived individuals, using primer opP18. The RAPD opP18-530 is indicated by an *arrow*. **B** Verification of the homology of RAPD fragments between seedless progeny and other seedless Sultanine-derived individuals. The Southern blot of the RAPD profiles was hybridized using a ^{32}P-labelled opP18–530 fragment as a probe

Converting the opC08-1020 RAPD marker into a SCAR marker

In order to obtain a more reliable marker, a SCAR (*SCC8*) was derived from the cloned opC08-1020 fragment. Used as a probe against a Southern blot with genomic DNA from a seeded individual (class 4) and a seedless individual (class 1), this RAPD band revealed a unique fragment in both individuals (data not shown). This suggests that opC08-1020 corresponds to a single-copy sequence.

The GCG package allowed the design of primers specifically lacking palindromic regions and containing between 40 and 55% G + C taken from the sequences at the ends of fragment opC08-1020. As the chosen pair of primers (SCC8-S and SCC8-AS) were located upstream of the RAPD primers, the fragment produced with these SCAR primers was expected to be shorter (32 bp less) than the original RAPD product. Amplification with SCC8-S and SCC8-AS produced a single band of the expected size, both in the seedless and seeded individuals of the progeny (Fig. 5).

To reveal polymorphisms, restrictions were carried out directly on the amplification products using six restriction enzymes. Digestion with *Bgl*II produced from 1 to 3 bands (Fig. 5), identifying polymorphism between individuals. This marker was then tested on the grandparents and parents (Table 4), and on 136 individuals of the progeny (Table 5). Closer examination of these progeny revealed that there were two allelic forms at this locus, named $SCC8^+$ and $scc8^-$, one containing a restriction site for *Bgl*II. This produced the two smaller fragments (Fig. 5). The 1 : 2 : 1 segregation distribution ratio of *SCC8* (confirmed by the chi-square test; $\chi^2 = 2.42$, $P > 0.2$) is in keeping with the hypothesis that we are dealing with a codominant

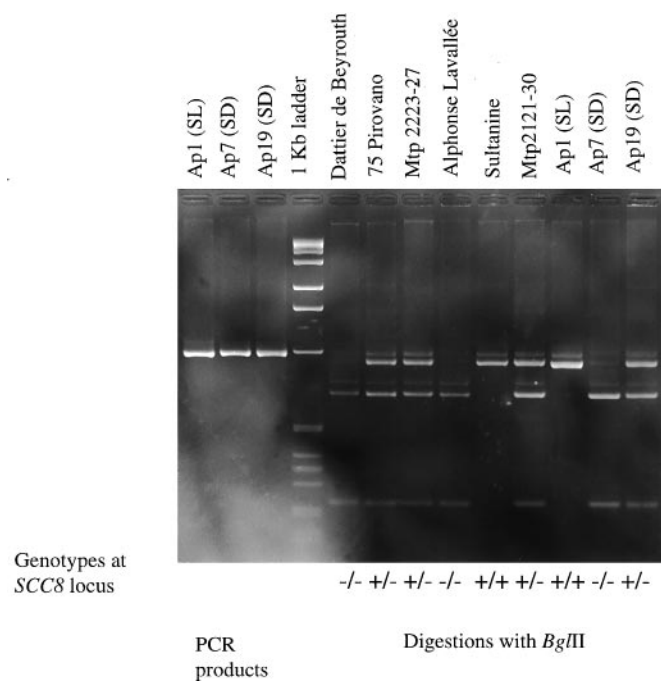

Fig. 5 Analysis of progeny using the *SCC8* marker. *Lanes 1 to 3* DNA fragments amplified from genomic DNA of seedless and seeded progeny using primers SCC8-S and SCC8-AS. *Lane 4* molecular-size marker. *Lane 5 to 13* polymorphism obtained after digestion of the amplification product with *Bgl*II: homozygous individuals $SCC8^+/SCC8^+(+/+)$ showed one band, homozygous individuals $scc8^-/scc8^-(-/-)$ showed two bands, whereas heterozygous $(+/-)$ individuals showed a three band pattern. *SL* seedless individual. *SD* seeded individual

marker, allowing one to distinguish homozygous from heterozygous individuals at this locus. Sultanine was found to be homozygous $SCC8^+/SCC8^+$, Dattier de Beyrouth and Alphonse Lavallée homozygous

Table 4 Genotypes at the *SCC8* locus for the grandparents and the parents of the progeny

Source	Genotype
Dattier de Beyrouth	$scc8^-/scc8^-$
75 Pirovano	$SCC8^+/scc8^-$
Sultanine	$SCC8^+/SCC8^+$
Alphonse Lavallée	$scc8^-/scc8^-$
Mtp 2223-27	$SCC8^+/scc8^-$
Mtp 2121-30	$SCC8^+/scc8^-$

Table 5 Distribution of progeny and phenotypic classes according to genotype at the *SCC8* locus

Genotype	$SCC8^+/SCC8^+$	$SCC8^+/scc8^-$	$scc8^-/scc8^-$
Total progeny	31	64	41
class-1 phenotype (no traces)[a]	31	10	0
Class-2 phenotype (intermediate)	0	25	0
Class-3 phenotype (intermediate)	0	28	0
Class-4 phenotype (normal seeds)	0	1[b]	41

[a]See Bouquet and Danglot (1996) for the definition of each phenotypic class
[b]Recombinant individual

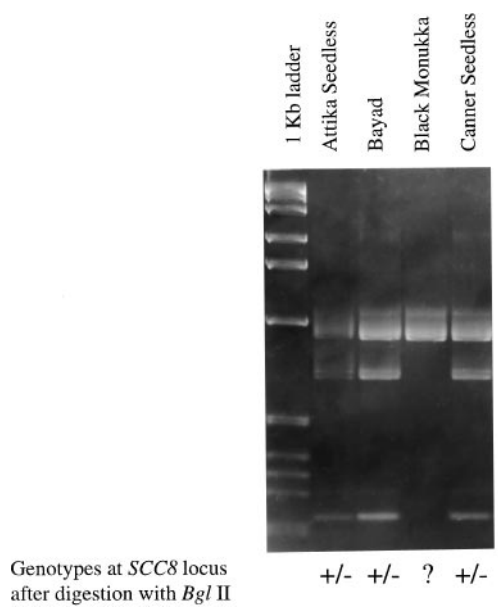

Fig. 6 Analysis of seedless varieties of natural origin or from crosses which do not contain Sultanine : polymorphism obtained after digestion with *Bgl*II of the amplification product at the *SCC8* locus. Individuals showing three bands are heterozygous but the homozygous status of Black Monukka needs to be tested

$scc8^-/scc8^-$, with both parents and 75 Pirovano being heterozygous.

This SCAR marker was also tested on several other naturally occuring seedless varieties as well as on seedless varieties obtained from crosses which do not contain Sultanine. Amplification and digestion with *Bgl*II (Fig. 6) revealed profiles showing three bands, characteristic of heterozygous individuals. This confirmed the codominant behavior of *SCC8*. However, the homozygous status of Black Monukka needs to be tested by progeny analysis.

Contribution of *SCC8* to seedlessness subtraits

According to the ANOVA test (Table 6), *SCC8* showed a highly significant correlation ($Pr < F = 0.0001$) with both seedless subtraits tested (TFW and DM). The association between *SCC8* and both quantitative subtraits was confirmed by the non-parametric Kruskal-Wallis test ($P < 0.001$). This marker accounted for 64.9–73.4% of TFW total phenotypic variation and for 78.7–89.1% of DM total phenotypic variation according to the year (Table 6). Mean values calculated for each genotypic class were significantly different from each other at $\alpha = 0.05$ according to the SNK test (Table 6). In order to control the validity of the phenotypic classification previously defined (Bouquet and Danglot 1996), individual progeny were sorted using a combination of the genotypes at *SCC8* and the seedless phenotypes. The SNK test revealed significant differences among the heterozygous individuals of the different phenotypic classes in 1995 (Table 7) and in 1996 (data not shown). However, for TFW, no significant differences were observed between the heterozygous and homozygous individuals of the class-1 phenotype. In the case of the heterozygous individual belonging to the class-4 phenotype, no significant differences with the other individuals of this class were observed for both subtraits.

Discussion

These results represent the first report of molecular markers genetically linked to seedlessness in grapevine. We used progeny, obtained from a cross between two partially seedless genotypes, in which phenotypic variation of seededness and seedlessness was equally distributed. Although the variation for this trait is continuous, the working hypothesis assumed the existence of a single major gene named *I*. Indeed, when classified into groups according to the total fresh weight of the seeds of 100 berries (TFW) and to the percentage of dry matter of the seeds of 100 berries (DM), as described by Bouquet and Danglot (1996), one-fourth of the individuals were coded as seeded, one-fourth as complete seedless, and the others as intermediates. By performing a BSA strategy using bulks derived from this population, we were able to identify two RAPD fragments which are linked in coupling with the allele I^+ present in the seedless individuals. The number of tested

Table 6 Results of the ANOVA, Kruskal-Wallis and Student-Newman-Keuls tests performed for the total fresh weight of seeds of 100 berries (TFW) and the dry matter of seeds (DM) using the SCAR marker *SCC8*

Seedless subtraits	Year	Mean values for each genotype			ANOVA		Kruskal-Wallis H value
		$SCC8^+/SCC8^+$	$SCC8^+/scc8^-$	$scc8^-/scc8^-$	Pr < F	R^2	
TFW	1994	0.00[c]	3.35[b]	10.04[a]	0.0001	0.65	87.56***
	1995	0.00[c]	3.92[b]	10.38[a]	0.0001	0.69	80.80***
	1996	0.00[c]	3.98[b]	12.48[a]	0.0001	0.73	86.49***
DM	1994	–	41.15[b]	63.84[a]	0.0001	0.79	69.56***
	1995	–	41.51[b]	66.83[a]	0.0001	0.89	54.05***
	1996	–	36.31[b]	60.78[a]	0.0001	0.86	60.77***

[a,b,c]Numbers followed by the same letter are not significantly different according to the SNK test at the 0.05% level
***Significant at the 0.001 probability level

Table 7 Results of the ANOVA, Kruskal-Wallis and Student-Newman-Keuls tests performed for the total fresh weight of seeds of 100 berries (TFW) and the dry matter of seeds (DM) in 1995 using as the treatment a combination of the genotypes at *SCC8* with the seedless phenotypes. N is the number of individuals. In parenthesis, the number of individuals for DM when different

Item	Mean values for each genotype-phenotype combination						ANOVA		Kruskal-Wallis H value
	Class 1 $SCC8^+/SCC8^+$	Class 1 $SCC8^+/scc8^-$	Class 2 $SCC8^+/scc8^-$	Class 3 $SCC8^+/scc8^-$	Class 4 $SCC8^+/scc8^-$	Class 4 $scc8^-/scc8^-$	Pr < F	R^2	
N	28 (0)	10 (3)	25	28	1	37			
TFW	0.00[c]	0.18[c]	3.87[b]	5.01[b]	11.93[a]	10.38[a]	0.0001	0.78	94.86***
DM	–	37.37[c]	38.08[c]	44.16[b]	65.2[a]	66.83[a]	0.0001	0.96	67.58***

[a,b,c]Numbers followed by the same letter are not significantly different according to the SNK test at the 0.05% level
***Significant at the 0.001 probability level

primers was relatively small compared to other studies using the BSA technique (Benet et al. 1995; Chagué et al. 1996; Cheng et al. 1996) probably due to the high level of polymorphism in grapevine (Grando et al. 1996). These markers did not show a distorted distribution, indicating that the embryo-rescue step did not introduce a significant bias in the number of seeded or seedless individuals recovered. The two markers, opC08-1020 and opP18-530, were close to the *I* gene (0.7 and 3.5 cM, respectively). Considering the small number of individuals constituting the bulks, we expected to find more distant markers. However, these genetic distances are likely to be underestimated because both parents have the same genotype at the *I* locus (I^+/i^-), and it is therefore possible that some recombinants were not detected. Nevertheless, the statistical analysis provided a way to confirm or identify some recombinants. The only seeded heterozygous individual was confirmed to be a recombinant since it did not significantly differ from the individuals of class-4 phenotype (Table 7). In any case, the markers bracket the *I* gene and are close enough to allow the development of a marker-assisted selection strategy (Tanksley 1983). Moreover, both markers proved to be population non-specific since they were recovered in all the Sultanine-derived seedless individuals tested. The homology of these similar-sized fragments was confirmed by DNA hybridization, using the RAPD fragments as a probe. However opC08–1020 and opP18–530 are dominant and both are in coupling phase with the I^+ allele. A RAPD marker linked in repulsion phase with I^+ could have improved the selection efficiency (Johnson et al. 1995).

In order to increase the specificity of our markers and simplify the PCR analysis, a SCAR (*SCC8*) was derived from opC08-1020. Amplification with the SCAR primers produced a single band both in the seedless and seeded individuals of the progeny, suggesting that the polymorphism observed with the opC08 primer was caused by a difference in one of the two RAPD primer-targeted sequences. A polymorphism was recovered after digestion of the amplification products with the restriction enzyme *Bgl*II. The resultant SCAR was codominant, eliminating the need for a marker in repulsion phase. Indeed, the transformation of the dominant RAPD marker opC08-1020 into the codominant marker *SCC8* allowed $SCC8^+/scc8^-$ individuals from $SCC8^+/SCC8^+$ individuals to be distinguished. This marker appeared to be very useful in a marker-assisted selection program aimed at seedlessness. The analysis of the progeny with *SCC8* indicated that the $scc8^-/scc8^-$ individuals were all seeded and that all the $SCC8^+/SCC8^+$ individuals were seedless (Table 5). This SCAR marker would thus have been of great value to exclude at the seedling stage the $scc8^-/scc8^-$ individuals that are probably seeded or to

select the $SCC8^+/SCC8^+$ seedless individuals. However, this selection process would exclude some seedless offspring, since we found that a quarter of the seedless individuals were $+/-$ at *SCC8* (Table 5). A selection process using this marker seems to be more efficient than that developed by Striem et al. (1996). Their system used a combination of seven markers, which allowed seeded offspring to be excluded and thus reduced the size of the population by 44%. As they had only a few seedless plants in their progeny, it is not possible to test if their selection process also leads to the exclusion of any seedless individuals. Moreover, due to the initial positive application of SCC8 to a few naturally occuring seedless varieties, we are currently testing other varieties and their progeny to extend the analysis of the codominant behavior of this marker. Nevertheless, SCC8 will be very usefull to select the parents for breeding and particularly to select homozygous seedless parents rather than heterozygous seedless parents.

The SCC8 marker also facilitates the more precise dissection of the genetics of seedlessness. Different hypotheses have been proposed for the number of genes involved in the control of this character (for a review see Bouquet and Danglot 1996). This present work was based on the assumption of a single dominant gene which regulates the expression of three other complementary, recessive genes. Our results confirmed the presence of a major gene whose expression is correlated with the incomplete development of the seeds. We propose to name it *sdI* (for seed development Inhibitor). The analysis of the genotypes at *SCC8* in relationship with the phenotypes for two subtraits of seedlessness (TFW and DM) allowed us to estimate the contribution of the *sdI* locus to the total phenotypic variation of these subtraits. The parametric (ANOVA) and non-parametric (Kruskal-Wallis) tests revealed a significant association between SCC8 and both subtraits. This marker accounted for a large proportion of the total phenotypic variation of both TFW and DM: more than 64.9% and 78.7% respectively according to the year (Table 6). The higher values obtained for DM may be due to the lack of data for one genotypic class ($SCC8^+/SCC8^+$). These values are higher than those obtained in other similar studies: a single RAPD marker accounted for 62.2% of the total phenotypic variation for rhizomania resistance in sugar beet (Pelsy and Merdinoglu 1996), while a SCAR marker accounted for 53% of the oleic acid content variance in spring turnip rape (Tanhuanpää et al. 1996). The determination of the broad-sense heritability of seedlessness would enable an estimation of the contribution of our marker to the genetic variation, as demonstrated by Pelsy and Merdinoglu (1996). When considering a combination of the genotypes at *SCC8* with the seedless phenotypes, the proportion of the phenotypic variance explained increased (Table 7). Indeed, there were significant differences among the heterozygous individuals of the different phenotypic classes for both subtraits. This suggests that, as proposed by Bouquet and Danglot (1996), other genes could be involved in seedlessness.

Moreover, our results also suggest that the *sdI* does not act as a completely dominant gene since all the intermediary individuals were heterozygous at *SCC8*. To confirm these results, the analyses of other progenies and other seeded varieties is in progress. However, they are not incompatible with the hypothesis of the production of a repressor by *sdI* (Bouquet and Danglot 1996). This gene could therefore repress other genes involved in the expression of different subtraits, like the weight or the degree of sclerification of the seeds. In order to identify possible targets for *sdI*, a histological analysis of seed development will be undertaken. The development of seeds for individuals from the different phenotypic classes will also be compared. In addition, a QTL analysis has to be devised to detect the minor genes involved in seedlessness. Since the only available *Vitis* map (Lodhi et al. 1995) was obtained with RAPD markers on a cross between two complex hybrids, another map has to be constructed using the progeny Mtp 3140.

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