Identifying Molecular Genetic Markers Associated with Seedlessness in Grape

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Abstract. Excluding seeded offspring at an early stage could be of great value to the breeder concerned with the development of seedless grapes (*Vitis vinifera* L.). We used the random amplified polymorphic DNA (RAPD) technique to identify molecular genetic markers, analyzing 82 individuals of a progeny resulting from a cross between 'Early Muscat' (seeded) and 'Flame Seedless'. Seven variables representing the traits of seedlessness were analyzed: mean fresh weight of one seed, total fresh weight of seeds per berry, perception of seed content, seed size categories evaluated visually, degree of hardness of the seed coat, degree of development of the endosperm, and degree of development of the embryo. Among 160 10mer primers, 110 gave distinct band patterns. Twelve markers yielded significant correlations with several subtraits of seedlessness, mainly with the mean fresh weight of one seed and the total fresh weight of seeds per berry. Multiple linear regression analysis resulted in high coefficients, such as $R = 0.779$ for fresh weight of seeds per berry, when the seven markers were included as independent variables in the model. Most of the seeded individuals, about 44% of the progeny, could be excluded using a two-step process of marker assisted selection.

In breeding table grapes, seedlessness is considered a trait of increasing importance to the consumer (Ledbetter and Ramming, 1989). Stenospermocarpic seedlessness in grapes has been discussed in detail since Pearson (1932), Stout (1936) and lately by Ramming et al. (1990), Spiegel-Roy et al. (1990a, 1990b), and Ledbetter and Burgos (1994). Aborted seeds in grapes after fertilization were termed stenospermic (Stout, 1936), and this term was applied to table grapes that were neither parthenocarpic nor seeded. A whole range of sizes of seeds and seed traces is found in progenies from crosses between seeded and seedless cultivars, as well as in crosses between two seedless cultivars. Clear classification of seeded vs. seedless individuals is influenced and biased by several factors. Perceptibility of seed traces may be affected by factors such as the firmness and crispness of the berry flesh (Ledbetter and Shonnard, 1991), the degree of development of the seed, its size and the sclerification of integuments (Ledbetter and Ramming, 1989). On the whole, organoleptic determination cannot be considered a sufficiently valid classification for the degree of seed development.

The need to divide grape progenies resulting from such crosses into seeded and seedless offspring led investigators to look for parameters correlated to the seed content of the berries (Perl et al., 1989). The continuous nature of the parameters used for quantifying the seed content of grape berries (e.g., fresh weight of seeds per berry) led to the need to devise a differentiating tool: 25 mg per aborted seed was used as threshold for seedlessness by Ramming et al. (1990). Our early investigations of this trait resulted in the identification and definition of seven traits related to seedlessness (Striem et al., 1992).

Numerous theories for the inheritance of the stenospermocarpic character have been proposed, analyzing the seeded versus seed-

less ratio in progenies. No wholly adequate inheritance scheme, taking into account the variable degrees of development of the seed traces, has yet been proposed. Although few genetic factors are assumed to be involved, it is hard to overlook the quantitative nature of seed development and its response to environmental effects (Kender and Remaily, 1970; Sandhu et al., 1984). We have noted that perceptibility of seed traces is not necessarily correlated to the size of the seed, but rather to the hardness of the seedcoat (Striem et al., 1992). Furthermore, in progenies of crosses between seeded and seedless cultivars, we have noticed several levels of hardness of the seedcoats occurring with different degrees of development of the endosperm. (These observations were considered also in the present study). The degrees of development of the seed components (i.e., seedcoat, endosperm and, possibly, embryo) were therefore evaluated as separate traits, rather than categorizing vines as either seeded or seedless.

The use of multi-loci DNA probes in order to uncover polymorphism between grape cultivars has been demonstrated (Striem et al., 1990). The genetic assay to detect nucleotide sequence polymorphism by polymerase chain reaction (PCR) procedures has been used in grapes. It was mainly used in cultivar identification (Büscher et al., 1993; Gogorcena et al., 1993; Jean-Jaques et al., 1993). We have reported preliminary results on the possibility to use PCR procedures in breeding for seedlessness (Striem et al., 1994). This approach contributed to the identification of several molecular markers correlating with the seedless character in stenospermocarpic grapes. Such markers could be useful as tools for preselection of seedless offspring by excluding the seeded seedlings prior to fruiting as suggested by Gray and Meredith (1992).

The objective of the present investigation was to develop markers associated with seedlessness traits, and to demonstrate their use for marker assisted selection.

Materials and Methods

Plant material. Progeny from a cross between 'Early Muscat' (E) and 'Flame Seedless' (F) was chosen for the analysis. This progeny was previously analyzed for the seed components of the stenospermocarpic seedlessness trait (Striem et al., 1992). Seven

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Table 1. Marker identification, primer sequence, and parental phenotype of the 12 markers discussed.

Marker	Primer sequence 5' —> 3'	Band size (bp)	Parental phenotype ²	
			Early Muscat	Flame Seedless
27.4.2	TTCCCCCGCT	700	1	1
27.6.1	TTCCCCCGCT	500	1	0
33.7	TCCGCTCTGG	400	0	1
39.5	CACCAGGTGA	900	0	1
40.9	CCCCATTCCC	520	1	1
46.1.1	CTGAGACGGA	2200	1	1
51.5	ACGACCGACA	900	0	1
60.1.3	CTCCATGGGG	480	0	1
62.4	TGCCCCGTCGT	1350	1	0
87.8	CGGCCCCACGT	700	1	0
90.2	GGGATTTCGAC	1300	1	0
90.7	GGGATTTCGAC	600	1	0

²1 = Band present; 0 = band absent.

seed traits related to seedlessness were defined: mean fresh weight per seed (mg), total seed fresh weight per berry (mg), seed contents evaluated by perceptibility (Spiegel-Roy, 1979), visual evaluation of four seed size categories, degree of hardness of the seedcoat (1 = soft to 3 = hard), and degree of development of the endosperm and the embryo (1 = partially developed to 3 = fully developed).

DNA was extracted from young grape leaves following the procedure given by Lodhi et al. (1994). Eighty-two individuals were included in the analysis (39 seedless, and 43 seeded, differentiated visually and by organoleptic perceptibility)

Development of molecular markers. Using the RAPD technique for producing molecular genetic markers, more than 160 10mer primers (Operon kits A,B,C,E,F,G,H,J and other primers) were tested.

Conditions for amplification. The PCR mixture contained 10-

to 20-ng template DNA in a 25-μL reaction volume with 2.5 μL 10× buffer (Promega Corp., Madison Wis.), 2.0 mM MgCl₂, 100 μM each dNTP and 1 unit Taq polymerase (Promega), and 0.2 μM primer, covered with a drop of mineral oil. Amplification was performed in a MJ-PTC-60 (MJ re-

Fig. 1. Electrophoretic separation of RAPD amplification products, using primers 33 and 39. Lanes 1–58: different individuals of the progeny from the cross of 'Early Muscat' (E) with 'Flame Seedless' (F). Marker (M): 100-bp DNA ladder.

Table 2. Correlation coefficients and significance between subtraits of seedlessness and the most significant randomly amplified polymorphic DNA markers, in an 'Early Muscat' x 'Flame Seedless' progeny.

Marker	Morphological trait						
	Fresh wt		Seed size		Developmental degree		
	Mean one	All traces/	By	Visually	Seedcoat	Endosperm	Embryo
	trace	berry					
	(mg)	(mg)	perceptibility				
27.4.2	–0.48**	–0.42**	NS	NS	–0.37*	NS	–0.27*
27.6.1	–0.32*	–0.34*	NS	NS	NS	NS	NS
33.7	–0.47**	–0.52**	0.38**	–0.32*	–0.42**	–0.34*	–0.33*
39.5	–0.41**	–0.44**	0.29*	–0.41**	NS	–0.38*	–0.40*
40.9	NS	0.28*	–0.36*	NS	NS	NS	0.27*
46.1.1	–0.35*	NS	NS	–0.30*	NS	NS	–0.30*
51.5	–0.40**	–0.36*	0.27*	–0.40**	–0.33*	NS	–0.30*
60.1.3	NS	–0.33*	NS	NS	–0.37*	NS	NS
62.4	0.28*	0.26*	NS	0.29*	0.40**	NS	0.30*
87.8	0.38**	0.38**	–0.32*	0.32*	NS	NS	NS
90.2	0.36*	0.31*	NS	NS	0.29*	0.35*	0.30*
90.7	–0.48**	–0.47**	0.27*	–0.29*	–0.35*	–0.27*	–0.34*

NS,*,** Nonsignificant or significant at $P = 0.01$ or 0.001 , respectively.

search, Watertown, Mass.) thermal cycler for 37 cycles, after initial denaturation for 30 s at 95 °C, one cycle of 1 min at 35 °C, and 2 min at 72 °C, was followed by 36 cycles consisting of 30 s at 94 °C, 1 min at 35 °C, and 1 min 45 s at 72 °C. Amplification products were resolved by electrophoresis at 10 V/cm for 3 h in a 2% NuSieve agarose gel, in TAE buffer. Gels were stained with ethidium bromide and visualized on UV light. Results were recorded from Polaroid pictures manually. Data on distinct, segregating bands were analyzed as

1/0 for presence/absence, respectively. Few markers were found to segregate in the progeny but the parents were not polymorphic. This is due to the dominant nature of RAPD markers when genotypes are heterozygous. Primer sequences and parental phenotypes for the 12 markers discussed are listed in Table 1. Marker identification which is used in this manuscript was compiled from a sequential number of the primer and the band number counted from top (large fragments) to bottom (small fragments) in each lane. All statistical analyses (data sorting, means, correlations, multiple stepwise regressions, analysis of variance and mean separation via Duncan multiple range tests) were performed using SAS (SAS Institute, Cary, N.C.) procedures.

Fig. 2. Schematic example of the definition of the associated effect of a marker on a morphological trait. Trait values were defined under Materials and Methods. Y_0 is the average value of the population missing a specific band while Y_1 if the average of the population having the band. Numbers in bars represent number of individuals rated at each trait value.

Results and Discussion

Some of the primers tested revealed a high degree of polymorphism between 'Early Muscat' (E) and 'Flame Seedless' (F). More than 70 primers producing polymorphic bands were all tested with 82 individuals of the progeny resulting from the cross between these cultivars (part of the progeny produced nonreliable band patterns with certain primers). More than 400 polymorphic markers were identified. Segregation of two markers is shown in Fig. 1.

About 10% (42) of the markers correlated with just one of the seven traits related to stenospermocarpic seedlessness trait examined. Twelve markers were significantly correlated with more than one component of seedlessness, mainly with the quantitative ones: the mean fresh weight per seed (mg) and the seed fresh weight per berry (mg) (Table 2).

For each marker, the progeny was divided into two groups according to the band pattern: 1) band present, and 2) band absent. The average value of the morphological traits in each group is associated and dependent on the number of individuals with the similar phenotype. Thus, the associated effect of each marker for each morphological trait was defined as the regression coefficient

Table 3. Marker effect, significance ($P > F$), and the ratio of the effect vs. the mean of the progeny (in percent), for the seven subtraits of seedlessness, for three markers: 33.7, 39.5, 90.7.

Marker	Morphological trait						
	Fresh wt		Seed size		Developmental degree		
	Mean one	All traces/	By	Visually	Seedcoat	Endosperm	Embryo
	trace	berry					
	(mg)	(mg)	perceptibility				
33.7							
Effect	-17.88	-54.17	0.84	-0.66	-0.95	-0.65	-0.76
Significance	0.0002	0.0001	0.0034	0.0174	0.001	0.0087	0.0122
Percent of mean	71.2	81.8	44.0	20.5	47.5	28.3	35.7
39.5							
Effect	-9.46	-28.30	0.52	-0.73	-0.52	-0.62	-0.75
Significance	0.0037	0.0017	0.0491	0.0045	0.0524	0.0087	0.0059
Percent of mean	46.5	51.4	25.5	23.8	29.0	28.3	40.1
90.7							
Effect	-16.21	-43.44	0.52	-0.54	-0.70	-0.46	-0.68
Significance	0.0001	0.0002	0.0447	0.0320	0.0078	0.0402	0.0112
Percent of mean	65.8	65.4	27.5	16.8	35.3	20.2	33.2

Table 4. Multiple regression analysis of molecular markers with the seven subtraits of seedlessness. Markers included in the linear model (+), number of markers included, and their correlation coefficients for the seven subtraits of seedlessness.

Marker	Morphological trait						
	Fresh wt						
	Mean one	All traces/	Seed size		Developmental		
	trace	berry	By	Visually	Seedcoat	Endosperm	Embryo
	(mg)	(mg)	perceptibility				
27.4.2		+			+		
27.6.1	+	+					
33.7	+	+	+	+	+	+	+
39.5	+	+	+	+		+	+
40.9		+	+				+
46.1.1	+			+			
51.5	+	+	+	+	+		+
60.1.3					+		
62.4	+				+		
87.8	+	+	+	+			
90.2						+	
90.7						+	
Markers	7	7	5	5	5	4	4
Correlation	0.675	0.779	0.684	0.570	0.526	0.424	0.492

(for the trait as the dependent variable) (Fig. 2). Since there are only two points of the independent variable (0 for band missing and 1 for band present) the difference between the two means ($Y_1 - Y_0$) is also the slope of the regression line. The larger this difference is, compared to the overall mean of the whole progeny, the higher the effect of the marker is on that specific trait (Table 3). For example, the effect of marker 33.7 on the mean fresh weight of one seed is -17.88 mg. This is the difference between the two groups classified according to band presence and absence. This difference is 71.2% of the overall mean of the whole progeny for this trait, which indicates a relatively high effect of the marker upon this trait. This marker was found to have significant correlations and effects upon most of the seven components of seedlessness investigated.

It should also be noted that the identification of several markers correlating to individual components of seedlessness supports, on one hand, our suggestion of dividing the trait into components (Striem et al., 1992), and on the other hand it gives more validity to the correlation of each marker by itself with the trait of seedlessness. Several of these markers were also significantly correlated among themselves, with recombination ratios of 17% to 30% (unpublished data).

The quantitative nature of the investigated traits suggests that several genes (probably at several loci) are involved in their control and contribute to the evaluated phenotype. Thus, it was not possible for a single marker to be closely linked to seedlessness but it may be possible to find markers for each contributing locus (unless they are all linked, or a major gene is involved). Furthermore, the degree of recombination between a marker and one of the loci involved in seedlessness cannot yet be estimated. We suggest that markers must be identified for each component. This led us to perform multiple linear regression analysis, resulting in higher coefficients, mainly with the quantitative traits, when four to seven markers as independent variables were included in the model (Table 4).

The main use of such markers is as a tool for preselection. As mentioned above, one cannot expect to have one single marker dividing the progeny into seeded vs. seedless offspring. It is more likely that a group of seeded offspring will share similar band

patterns for several markers. Furthermore, the opposite band pattern should be found to characterize mainly seedless individuals. To locate such marker combinations, the data of the progeny were sorted by the mean fresh weight per seed. Seedless individuals are located at the top of the list, and seeded ones, at the bottom (Table 5). It was found that the combination of the four markers: 27.4.2, 33.7, 62.4, 90.7, which gave a band pattern of 0-0-1-0 respectively, were found in six seeded individuals. Three different recombinant seeded individuals were found to have 0-1-0-0 and 0-0-0-0 band pattern combinations (Table 6). Thus, nine seeded individuals (which are 15.3% of the whole progeny) could be excluded based on their RAPD marker pattern. In order to exclude more seeded individuals this process was repeated. Elimination of 17 additional offspring (12 seeded, 4 with large traces, and 1 seedless) from the remaining progeny was obtained using additional markers: 39.5, 51.5, 87.8 (Table 5). The terms seeded and seedless referred mainly to the size of the seeds and traces evaluated visually, and to perceptibility. The second step of selection included some individuals which could not be analyzed in the first selection due to lack of data with some primers. Furthermore, in the first step, only seeded individuals were excluded, while in the second step some individuals bearing seed traces were excluded as well.

However, the existence of recombinant individuals bearing small seeds, but having rather well developed seedcoats and endosperm, might affect this classification. This could be one of the reasons for individuals not fitting into the described procedure. Thus, seven markers in a two-step selection process successfully excluded 21 out of 29 (72%) seeded individuals, and 4 out of 11 (36%) individuals with noticeable seed traces. Thus, 44.1% of the progeny analyzed could be excluded based on their marker phenotype. It should also be noted that the development of additional markers correlating to traits related to seedlessness should improve this analysis. Closer linkage with the genes involved with seedlessness will permit fewer crossing-over events, thus fewer recombinant individuals.

To conclude, 12 molecular genetic markers were identified, correlating to seven subtraits for seedlessness. Highly significant

Table 5. Partial list (last 41 individuals) of offspring analyzed, sorted by seed fresh weight.

Vine no.	Seed fresh wt (mg)	Development of seed components			Evaluation of seed size		Molecular marker patterns			
		Seedcoat ^z	Endosperm ^y	Embryo ^x	Visually ^w	Perceptibility ^v	First selection		Second selection	
							27.4.2	33.7 62.4 90.7	39.5 51.5 87.8	
							To stay	To exclude	To stay	To exclude
188	13.6	1	2	1	b	S	1-1-0-1			0-0-1
130	14.3	1	3	2	m	S	1-1-0-1		1-1-1	
119	15.2	3	3	3	n	T	1-1-1-1			0-1-1
173	16.3	3	3	3	n	N	1-1-0-1		1-0-1	
136	16.5	1	2	1	b	S	1-1-0-1		1-1-1	
145	17.9	1	2	1	b	T	0-0-1-1			0-0-1
95	19.8	1	3	3	b	T	1-1-0-1			0-0-0
73	20.5	3	3	3	n	N	0-0-1-1		1-1-0	
114	21.1	1	2	2	b	T	1-1-1-1			0-1-1
185	23.4	3	3	3	n	N	1-1-1-1			0-0-0
187	23.5	1	2	2	b	S	1-1-0-1		1-0-1	
135	23.7	3	3	3	n	N	1-1-1-1			0-0-1
131	24.0	1	2	2	b	T	1-1-0-1		0-1-0	
190	25.7	1	2	1	b	T	1-1-0-1		1-0-1	
167	26.7	2	2	1	b	T	1-0-0-1			0-1-1
118	27.8	1	2	1	b	T	1-1-0-1		1-1-1	
81	29.1	3	3	3	n	S?	1-1-1-1			0-0-0
121	29.3	3	3	3	n	N	1-0-0-1			0-0-1
107	29.7	3	3	3	n	N	1-0-0-1		1-1-0	
102	29.8	3	3	3	b	N	0-0-1-1		1-0-0	
180	30.2	2	2	2	b	N	1-1-0-1		1-1-1	
183	31.1	3	2	2	n	N	0-1-1-1			0-0-1
172	32.5	2	3	2	n	T	1-1-0-1		1-0-1	
189	32.8	2	3	3	n	N	1-1-0-1			0-0-1
86	33.7	3	3	3	n	N	1-0-1-1		1-0-1	
198	34.5	3	3	3	n	N	1-1-0-0		1-1-1	
76	34.2	3	3	3	n	N	1-1-0-0			0-0-1
78	35.2	2	3	3	n	T	1-1-0-1			0-1-1
77	39.1	2	3	3	n	N		0-1-0-0		0-0-1
84	39.3	3	3	3	n	N	1-1-0-1		1-1-1	
196	39.8	3	2	3	n	S?	1-1-1-NA ^u			0-0-0
144	40.1	3	3	3	n	N		0-0-1-0		NA-0-1
149	42.7	3	3	3	n	N		0-0-1-0		NA-0-NA
88	43.4	3	3	3	n	N		0-0-1-0		NA-NA-1
137	46.1	3	3	3	n	N		0-0-1-0		NA-0-1
193	46.9	3	3	3	n	N	1-1-1-1			0-0-0
158	47.0	3	3	3	n	N		0-1-0-0		NA-1-1
115	50.6	3	3	3	n	N		0-0-1-0		NA-0-1
116	54.2	3	3	3	n	N		0-0-1-0		NA-0-1
160	60.3	3	3	3	n	N		0-0-0-0		NA-0-0
103	65.4	3	3	3	n	N	NA-1-1-0			0-0-1

^z1 = Soft, to 3 = hard.^y1 = Partial, to 3 = full.^x1 = Small, to 3 = full.^ws = Small seed traces, m = medium seed traces, b = big seed traces, n = normal seeds.^vN = Normal seeds, T = noticeable seed traces, S = seedless individual.^uNA = not available.

correlation coefficients and associated effects of the markers upon the traits analyzed were found mainly with the quantitative subtraits. A combination of 4 markers identified 9 seeded offspring in the progeny investigated. Three additional markers identified 21 additional seeded individuals and four individuals with noticeable seed traces. The size of the population could be reduced by 44% using this marker-assisted-selection process. These results could possi-

bly be applied in breeding programs and be used as a tool for preselection. It should first be determined whether these chosen markers are polymorphic among parents. However, markers can be of value even though they are monomorphic among parents when segregating in the progeny. Bulk DNA analysis of the extreme groups can be used in order to estimate the biological effect of the loci involved.

Table 6. Number of individuals from a population of 82 individuals (and the percentage out of the whole progeny²) which were excluded by their band pattern combinations of the markers (27.4.2–33.7–62.4–90.7 and 39.5–51.5–87.8).

Band pattern of markers		Individuals			Whole
First selection	Second selection	(no.)			progeny
27.4.2–33.7–62.4–90.7	39.5–51.5–87.7	Seeded	Traces	Seedless	(%)
0–0–1–0		6			
0–1–0–0		2			(9/59=) 15.3 %
0–0–0–0		1			
	0–0–1	6	1	1	
	0–0–0	4	1		(12/59=) 28.8 %
	0–1–1	2	2		
Total excluded		21	4	1	(21/59=) 44.1 %

²Progeny: 19 seedless, 11 noticeable traces, 29 seeded individuals analyzed; 23 individuals could not be analyzed due to nonreliable bandpatterns with these primers.

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