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. 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 hardiness 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 seedless. 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 seedless 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 hardiness of the seedcoat (Striem et al., 1992). Furthermore, in progenies of crosses between seeded and seedless cultivars, we have noticed several levels of hardiness 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...
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,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-

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

| Marker | Primer sequence | Band size (bp) | Parental phenotype |
|--------|----------------|----------------|--------------------|
| 27.4.2 | TTCCCCCGCT     | 700            | Early Muscat      |
| 27.6.1 | TTCCCCCGCT     | 500            | Flame Seedless    |
| 33.7  | TCCGCTCTGG     | 400            |                    |
| 39.5  | CACGAGTTGA     | 900            |                    |
| 40.9  | CCCATTCACC     | 520            |                    |
| 46.1.1| CTGAAGCCGA     | 2200           |                    |
| 51.5  | ACACCGCACA     | 900            |                    |
| 60.1.3| CTCCATGGGG     | 480            |                    |
| 62.4  | TCCCCCGCT     | 1350           |                    |
| 87.8  | CGCCACCGT     | 700            |                    |
| 90.2  | GGGATTCGAC     | 1300           |                    |
| 90.7  | GGGATTCGAC     | 600            |                    |

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.

**Morphological trait**

| Mean one trace berry | All traces/ Seed size | Developmental degree |
|----------------------|-----------------------|----------------------|
|                      |                       | Seedcoat Endosperm   |
| Fresh wt             |                       |                      |
| Marker               |                       |                      |
| 27.4.2               | -0.48**               | ns                   |
| 27.6.1               | -0.32*                | ns                   |
| 33.7                 | -0.47***              | 0.38**              |
| 39.5                 | -0.41***              | 0.29*               |
| 40.9                 | ns                    | 0.36*               |
| 46.1.1               | -0.35*                | ns                   |
| 51.5                 | -0.40**               | 0.27                |
| 60.1.3               | ns                    | 0.36**              |
| 62.4                 | 0.28*                 | ns                   |
| 87.8                 | 0.38**                | -0.32*              |
| 90.2                 | 0.36*                 | ns                   |
| 90.7                 | -0.48**               | 0.27                |

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

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.
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.

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 unreliable 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

### Results and Discussion

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### 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 trace (mg) | All traces/berry (mg) | Seed size | Dev. degree |
|        | By perceptibility | Visually | Seedcoat | Endosperm | Embryo |
| 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   |
likely that a group of seeded offspring will share similar band
dividing the progeny into seeded vs. seedless offspring. It is more
mentioned above, one cannot expect to have one single marker
(Table 4).

perform multiple linear regression analysis, resulting in higher
that markers must be identified for each component. This led us to
more, the degree of recombination between a marker and one of the
(unless they are all linked, or a major gene is involved). Further-
more, it may be possible to find markers for each contributing locus
possible for a single marker to be closely linked to seedlessness but
and contribute to the evaluated phenotype. Thus, it was not
patterns for several markers. Furthermore, the opposite band
pattern should be found to characterize mainly seedless individu-
als. To locate such marker combinations, the data of the progeny
were sorted by the mean fresh weight per seed. Seedless individu-
als 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 addi-
tional markers: 39.5, 51.5, 87.8 (Table 5). The terms seeded and
seedless referred mainly to the size of the seeds and traces evalu-
atived 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 pheno-
type. It should also be noted that the development of additional
markers correlating to traits related to seedlessness should im-
prove 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 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.

| Fresh wt | Morphological trait |
|---|---|
| | Mean one trace | All traces/ berry | Seed size | Developmental degree |
| | (mg) | (mg) | By perceptibility | Visually | Seedcoat | Endosperm | Embryo |
| Marker | | | | | | | |
| 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 | 5 | 4 | 4 |
| Correlation | 0.675 | 0.779 | 0.684 | 0.570 | 0.526 | 0.424 | 0.492 |
Table 5. Partial list (last 41 individuals) of offspring analyzed, sorted by seed fresh weight.

| Vine no. | Seed fresh wt (mg) | Seedcoat$^a$ | Endosperm$^b$ | Embryo$^c$ | Visually$^d$ Perceptibility$^e$ | Molecular marker patterns |
|----------|-------------------|--------------|--------------|-----------|------------------|--------------------------|
| 188      | 13.6              | 1            | 2            | 1         | b                | 1–1–0–1                  |
| 130      | 14.3              | 1            | 3            | 2         | m                | 1–1–0–1                  |
| 119      | 15.2              | 3            | 3            | 3         | n                | 1–1–1–1                  |
| 173      | 16.3              | 3            | 3            | 3         | n                | 1–1–0–1                  |
| 136      | 16.5              | 1            | 2            | 1         | b                | 1–1–0–1                  |
| 145      | 17.9              | 1            | 2            | 1         | b                | 0–0–1–1                  |
| 95       | 19.8              | 1            | 3            | 3         | b                | 1–1–0–1                  |
| 73       | 20.5              | 3            | 3            | 3         | n                | 0–0–1–1                  |
| 114      | 21.1              | 1            | 2            | 2         | b                | 1–1–1–1                  |
| 185      | 23.4              | 3            | 3            | 3         | n                | 1–1–1–1                  |
| 187      | 23.5              | 1            | 2            | 2         | b                | 1–1–0–1                  |
| 135      | 23.7              | 3            | 3            | 3         | n                | 1–1–1–1                  |
| 131      | 24.0              | 1            | 2            | 2         | b                | 1–1–0–1                  |
| 190      | 25.7              | 1            | 2            | 1         | b                | 1–0–1–0                  |
| 167      | 26.7              | 2            | 2            | 1         | b                | 1–0–1–1                  |
| 118      | 27.8              | 1            | 2            | 1         | b                | 1–1–0–1                  |
| 81       | 29.1              | 3            | 3            | 3         | n                | 1–1–1–1                  |
| 121      | 29.3              | 3            | 3            | 3         | n                | 1–0–0–1                  |
| 107      | 29.7              | 3            | 3            | 3         | n                | 1–0–0–1                  |
| 102      | 29.8              | 3            | 3            | 3         | b                | 0–0–1–1                  |
| 180      | 30.2              | 2            | 2            | 2         | b                | 1–1–0–1                  |
| 183      | 31.1              | 3            | 2            | 2         | n                | 0–0–1–1                  |
| 172      | 32.5              | 2            | 3            | 2         | n                | 1–0–1–1                  |
| 189      | 32.8              | 3            | 3            | 3         | n                | 1–0–1–1                  |
| 86       | 33.7              | 3            | 3            | 3         | n                | 1–0–0–1                  |
| 198      | 34.5              | 3            | 3            | 3         | N                | 0–0–1–1                  |
| 76       | 34.2              | 3            | 3            | 3         | n                | 1–0–0–0                  |
| 78       | 35.2              | 2            | 3            | 3         | n                | 1–0–0–1                  |
| 77       | 39.1              | 2            | 3            | 3         | n                | 1–0–0–0                  |
| 84       | 39.3              | 3            | 3            | 3         | N                | 1–0–1–1                  |
| 196      | 39.8              | 3            | 2            | 3         | n                | 1–1–1–NA$^a$             |
| 144      | 40.1              | 3            | 3            | 3         | n                | 0–0–1–0                  |
| 149      | 42.7              | 3            | 3            | 3         | n                | 0–0–1–0                  |
| 88       | 43.4              | 3            | 3            | 3         | n                | 0–0–1–0                  |
| 137      | 46.1              | 3            | 3            | 3         | n                | 0–0–1–0                  |
| 193      | 46.9              | 3            | 3            | 3         | n                | 1–1–1–1                  |
| 158      | 47.0              | 3            | 3            | 3         | n                | 0–1–0–0                  |
| 115      | 50.6              | 3            | 3            | 3         | N                | 0–0–1–0                  |
| 116      | 54.2              | 3            | 3            | 3         | N                | 0–0–1–0                  |
| 160      | 60.3              | 3            | 3            | 3         | N                | 0–0–0–0                  |
| 103      | 65.4              | 3            | 3            | 3         | n                | NA–1–1–0                 |

$^a$1 = Soft, to 3 = hard.
$^b$1 = Partial, to 3 = full.
$^c$1 = Small, to 3 = full.
$^d$s = Small seed traces, m = medium seed traces, b = big seed traces, n = normal seeds.
$^e$N = Normal seeds, T = noticeable seed traces, S = seedless individual.
$^NA$ = 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 possibly 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. Bulked 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 progeny) 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 progeny |
|-------------------------|-------------|---------------|
| First selection         | Seeded      | Traces        | Seedless |
| Second selection        | (no.)       | (no.)         | (%)      |
| 27.4.2–33.7–62.4–90.7   | 6           | 2             | (9/59=) 15.3 % |
| 0–0–1–0                 | 0–1–0       | 0–0–0         | 0–1–1    |
|                         | 6           | 1             | 1        |
|                         | 4           | 1             | 1        |
|                         | 2           | 2             |          |
| Total excluded          | 21          | 4             | 1        |

*Progeny: 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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