netic markers. Nucleic Acids Res, 1990, 18: 6531~ 6535
9 Giovannoni J J, Wing R A, Ganal M W et al. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res, 1991, 19: 6553~ 6558
10 Michelmore R W, Paran I, Kesseli R V. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA, 1991, 88: 9828~ 9832
11 Striem M J, Ben-Hayyim G, Spiegel-Roy P. Developing molecular genetic markers for grape breeding, using polymerase chain reaction procedures. Vitis, 1994, 33: 53~ 54

葡萄无核基因的 RAPD遗传标记

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摘 要 报道了用随机引物和混合分离分析(BSA)方法检测葡萄无核基因连锁的遗传标记。DNA混合样来自 B72-216× B45-187的杂交后代。两个混合样分别由 9个表现型为无核和有核杂种组成,除有核与无核性状外,其他性状是随机的。用 10碱基随机序列的 100个引物筛选混合样以期获得多态型 DNA片段。结果在引物 UBC-269扩增后, 500对碱基的 DNA多态型片段出现在无核样,而有核样不出现该多态型片段。进一步用杂种、父本及其无核基因的供给者无核白(Thompson Seedless)和百乐葡萄(Perlette)作模板,用引物 UBC-269扩增,一些杂种、亲本及无核基因的供给者也拥有 500对碱基的多态型片段。据此分析,葡萄的无核性是由多基因控制的,这些基因有主效和微效之分;本研究获得的分子标记 $UBC\text{-}269_{500}$与葡萄无核基因之一有连锁关系,而且与主效基因之一有连锁。

关键词 BSA,遗传标记,葡萄无核基因, RAPD,葡萄
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Identification of Genetic Marker Linked to Seedless Genes in Grapes Using RAPD

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Abstract This paper reports the rapid detection of a genetic marker linked to seedless genes in segregant populations of grapes using random primers and BSA (bulked segregant analysis). Two bulked DNA samples were generated from cross combination of B72-216× B45-187. One bulk contains mixed DNA samples of 9 individuals that are identical for the seedlessness trait in phenotype, and another bulk are those of 9 individuals of the same family with the seeded trait, but arbitrary at all unlinked regions. The bulks were first screened for polymorphism using a set of 100 arbitrary 10-mers primers. Only one polymorphism in the seedless trait pool distinguished two bulks by primer UBC-269. The size of this polymorphism is 500 bp. The polymorphism was further featured in some hybrids and the pollen parent B45-187 as well as initial donors of seedless genes, Thompson seedless, and Perlette, when they were used as the templates for amplified by UBC-269. Based on the presence or absence of this polymorphism, segregation that can be mapped in the population occurs. Our results suggested that the seedlessness of grapes is controlled by multigenes, including major and minor genes; The marker, $UBC\text{-}269_{500}$ obtained in the study, was linked to one of the seedless genes in grapes. and it appears to be related to a major seedless gene.

Key words BSA, genetic marker, grape seedless genes, RAPD, *vitis*

Seedless grapes make up the majority of the production for table and raisin grapes. Thompson Seedless (Sym. Sultanina) is an old seedless cultivar known in many countries around the world. Thompson Seedless and its seedless progeny are now widely grown throughout the world. In California alone, Thompson Seedless acreage was estimated at 104,000 hm^2 in 1991[1]. At this time, Sultanina is important for both commercial production and for its seedless trait which has been used for breeding new seedless grape cultivars.

In the past, breeders produced new seedless cultivars only by making seeded by seedless crosses with the aid of conventional techniques. By using in ovulo embryo culture techniques[2,3], it is now possible to recover plants from seedless by seedless crosses and obtain a higher proportion of seedless progenies in the F_1 generation than conventional seeded by seedless crosses[4]. Even with these advanced breeding techniques, seed-

lessness in the hybrid can only be determined after fruit production. A technique capable of detecting seedlessness in young seedling still in the vegetative state, would greatly improve the efficiency of the selection process. The use of molecular markers offers a potentially unambiguous method for such determination.

It has been known that the unique traits or genes of crops can be tagged with genetic markers, such as isozyme and RFLP (Restiction Fragment Length Polymorphism) since the 1980s. Genetic analysis of isozyme polymorphism and RFLPs in *Vitis* have also been reported in recent years[5,6]. RAPD (Random Amplification Polymorphic DNA) can greatly facilitate identification and efficient mapping in plant breeding[7,8]. Michelmore et al (1991) have described the method called bulked segregant analysis (BSA) that permits an efficient screening for markers located in specific regions of the genome[10]. The possibles use of polymerase chain reaction procedure to develop genetic markers was recently suggested by Strime et al[11].

The objectives of this study was to use random primers and BSA for rapid identification and efficient mapping of RAPD marker linked to genes conditioning seedlessness in grapes.

1 Materials and Methods

1.1 Plant matetials

The parents (B72-216 and B45-187) and their 61 hybrids were obtained from the Horticultural Crop Research Laboratory, USDA, Fresno, California. DNA was extracted from leaves as described below. The pedigree of these plants is shown in Fig. 1. Similarly, DNA was also extracted from Thompson Seedless, Perlette, and Orlando Seedless maintained in the Center for Viticulture Science and Small Farm Development, Florida A& M University, Tallahassee, Florida, U. S. A.

1.2 DNA extraction

Young leaves (0.5g) from each plant were ground to a fine powder with liquid nitrogen, thawed and resuspended in a CTAB extracion buffer [1.4 mol/L NaCl, 20 mmol/L EDTA, 100 mmol/L Tris-HCl pH 8.0, and 0.4% β-mercaptoethanol]. The homogenate was incubated at 65℃ for 30 minutes. After the extraction was cooled to room temperature, 50 mg of PVPP (polyvinylpoly-pyrrolidone) and an equal volume of chloroform/octanol (24: 1) was added, and then centrifuged at 8000 rpm for 10 minutes. The upper (aqueous phase) was transferred to a 15 mL centrifuge tube. DNA was precipitated by two volumes of cold ethanol (- 20℃) and resuspended in 200μL TE buffer (10 mmol/L Tris, pH 8.0, 250 mmol/L EDTA).

1.3 Pooling of two BSA samples and selection of primers

Two bulks were made, with nine hybrids in each. The two BSA samples were pooled using equal volumes of DNA. Pool 1 contained the seedlessness trait and pool 2

the seeded trait (Fig. 2) The two BSA samples were screened using 100 arbitrary primers, UBC 201~ 300, purchased from University of British Columbia, Vancouver, BC, Canada.

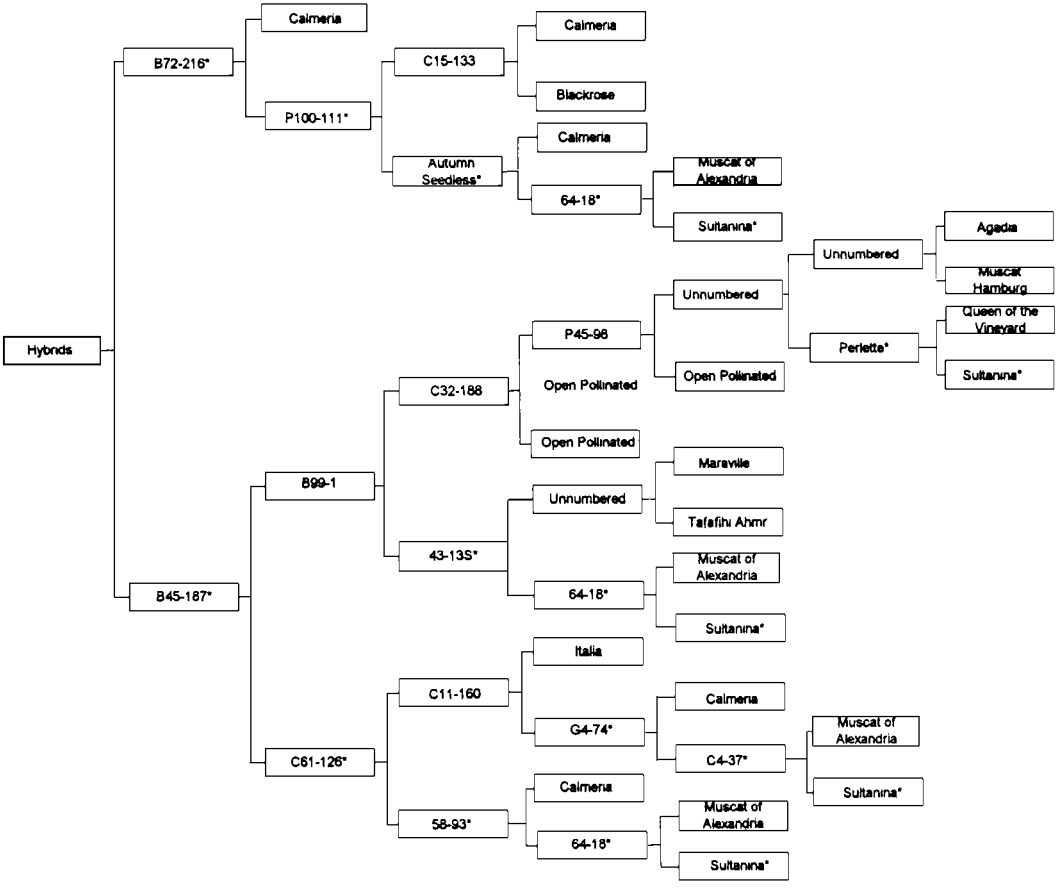

Fig. 1　The pedigree of parents and their hybrids

1.4 PCR conditions

For PCR amplification, the reaction mixture (25μL) contains 500 mmol/L KCl, 100 mmol/L Tris HCl (pH 9.0 at 25℃), 10% Triton X-100, 250μmol of each dNTP, 1 mmol/L magnesium, 0.15μmol/L of primer, 25~ 30 ng of genomic DNA, and 1 unit of Taq DNA polymerase (Promega Co. Madison, WI, U.S.A). After covering with 25 μL of mineral oil, DNA was amplified with an MJ Research thermal cycler (Model PTC-100) for 40 cycles. Each cycle consisted of: denaturation for 1 minute at 94℃, annealing for 2 minutes at 35℃, and elongation for 2 minutes at 72℃. The last cycle of elongation was extended to 8 minutes at 72℃ before the amplified products were stored at 4℃ for use. The amplified DNA was then subjected to 2% agarose gel (1% normal

molecular grade agarose plus 1% NuSieve GTG agarose, FMC) electrophoresis and visualized by ethidium bromide staining. In all cases, a 100 bp DNA ladder (GIBCO BRL) was used as the size marker.

1.5 Identification of RAPD Markers and Linkage Analysis

DNA samples isolated from parents and hybrids were amplified with the primers selected from the two BSA samples mentioned above. The amplification was repeated at least twice and only bands reproducible on several runs were considered for analysis. Some culitivars mentioned above, including the two progenitor parents of the segregation, were also amplified by the primer selected for analyzing the relationship between the marker and the pedigree of the segregation.

2 Results

Two pools BSA samples differing for the seedlessness trait were initially used as templates for amplification with each of 100 arbitrary oligonucleotide primers. The amplification patterns of the 100 RAPD primers generated different polymorphic DNA products (data not shown). Although 5 primers (UBC-234, UBC-239, UBC-266, UBC-269, and UBC-295) generated different DNA profiles, UBC-269 (5′-CCA GTT CGCC-3′) was the only primer that uniquely distinguished the two pools (Fig. 2). Pool 1 showed the band of 500 bp, whereas pool 2 did not. Further experiments showed that primer UBC-269 could generate the specific band of 500 bp with five individuals from pool 1 (accounted for 55.6%), but not at all with those from pool 2. UBC-269 also distinguishes the parents (Fig. 3), that is, pollen parent B45-187 and the five hybrids of pool 1 with the seedlessness trait displayed the 500 bp fragment.

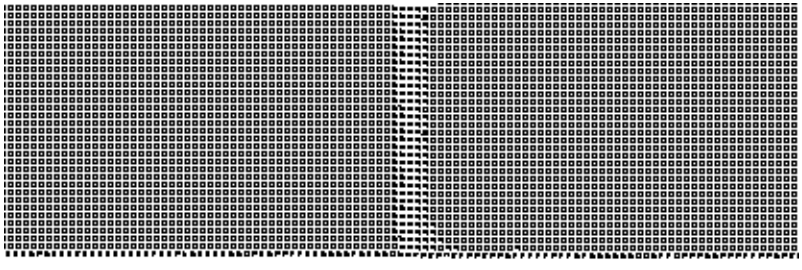

Fig. 2 Genetic basis of bulked segregant analysis

Lane 1— Marker; Lane 2, 4, 6,… 28— pool 2 with seeded trait; Lane 3, 5, 7,… 29— Pool 1 with seedless trait; Lane 24 and 25 show the polymorphism of pool 2 and pool 1 amplified by primer UBC-269, respectively. Lane 25 has specific band in 500 bp

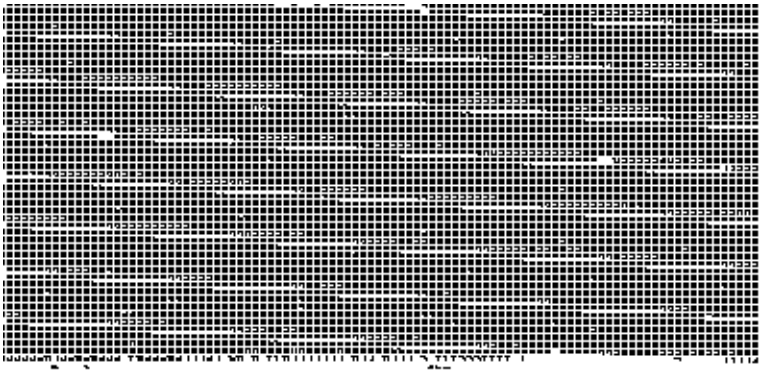

Fig. 3 Schematic showing the polymorphisms of individuals from pool 1, pool 2, and their parents Lane 1—— Marker; Lane 2—— B72-216; Lane 3—— B45-187; Lane 4, 5,… 12—— individuals of pool 2 (A56-9, A56-10, A56-11, A56-12, A56-14, A56-17, A56-37, A56-47, and A56-50); Lane 13, 14,… 21—— individuals of pool 1 (A56-16, A56-20, A56-25, A56-27, A56-31, A56-36, A56-38, A56-40, and A56-45)

To confirm that the specific RAPD products originated from the seedlessness region and select a molecular marker linked to this region, DNA from all hybrids in the segregant population were used as templates for amplification with primer UBC-269. The specific band of 500 bp was also found in eighteen offspring (Table 1). For the purpose of analysis, we converted our results (Table 1) into Table 2. As shown in Table 2, individuals of each grade were different in the population, and grade 0, 4 and 9 had more individuals than other grades. The 500 bp band was exhibited most frequently for grade 9. The 500 bp band was present more grade 4 than in grade 0. Overall, the frequency of the 500 bp fragment was greater in the higher grades. Additionally, we divided the population into three groups according to seed softness, group A(grade 0) with seeded trait, group B(grade 1~ 5) with medium to large soft seed, and group C(grade 6~ 9) with small soft seed to only the trace of seed (Table 2). The fact that the presence of the 500 bp band in group C was also the highest in the population, demonstrated that the band of 500 bp was linked to a gene or genes affecting seedlessness in grapes.

Our results showed that Thompson Seedless, Perlette, and Orlando Seedless also displayed the 500 bp band (Fig. 4). Thompson Seedless had another specific band of 450 bp; but of all the hybrids, only A56-13 showed the 450 bp band like its progenitor, Thompson Seedless (Fig. 4).

Table 1 The cosegregating pattern of seedlessnees and the RAPD marker

Parents or hybrids	Seedless status	UBC-269$_{500}$	Hybrids	Seedless status	UBC-269$_{500}$
B72-216	4[z]	-	A56-43	3	-
B45-187	7	+	A56-44	6	-
A56-8	3	-	A56-45	7	-
A56-9	0	-	A56-47	0	-
A56-10	0	-	A56-48	9	-
A56-11	0	-	A56-49	1	-
A56-12	0	-	A56-50	0	-
A56-14	0	-	A56-51	0	-
A56-15	8	+	A56-52	4	+
A56-16	9	+	A56-53	4	-
A56-17	0	-	A56-54	0	-
A56-18	4	-	A56-55	2	-
A56-19	6	+	A56-57	9	+
A56-20	8	-	A56-58	0	-
A56-21	4	+	A56-60	0	+
A56-22	4	-	A56-61	0	-
A56-23	3	-	A56-62	7	-
A56-24	6	+	A56-63	3	-
A56-25	9	+	A56-64	5	-
A56-26	4	-	A56-65	4	-
A56-27	9	-	A56-66	9	+
A56-30	3	+	A56-67	9	+
A56-31	9	-	A56-69	3	-
A56-32	4	-	A56-70	0	+
A56-33	5	-	A56-71	0	-
A56-34	3	-	A56-72	9	-
A56-35	5	-	A56-74	8	-
A56-36	9	+	A56-75	0	-
A56-37	0	-	A56-77	4	+
A56-38	9	+	A56-78	3	+
A56-40	8	+	A56-79	8	-

z Seed/aborted seed size rating. 0= seeded, 9= v. small like Thompson Seedless

Table 2 The analysis of cosegregating between seedlessness and the RAPD marker in the segregation

Seed Grade[z]	0	1	2	3	4	5	6	7	8	9
No. of grade	17	1	1	8	9	3	3	2	5	11
Presence of 500 bp	2	0	0	2	3	0	2	0	3	6
Absence of 500 bp	15	1	1	6	6	3	1	2	2	5
Accounted for each grade (%)	11.7	0	0	14.3	36.4	0	66.7	0	0	54.6
Accounted for population (%)	3.3	0	0	1.6	6.6	0	3.3	0	4.9	11.5
Group	A (grade 0)			B (grade 1~5)					C (grade 6~9)	
Frequency in each group (%)	3.3			21.7					55	
Frequency in the population (%)	3.3			8.3					18.3	

z Seed/aborted seed size rating. 0= seeded, 9= v. small like Thompson Seedless

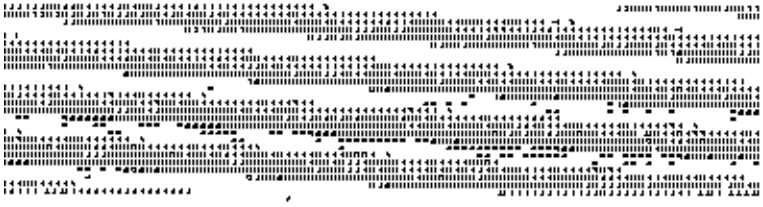

Fig. 4　Schematic showing the polymorphisms of some hybrids and pollen parents as well as Thompson Seedless, Perlette, Orlando Seedless, amplified with primer UBC-269

Lane 1— Thompson Seedless; Lane 2— perlette; Lane 3— Orlando Seedless; Lane 4— B45-187; Lane 5— A56-48; Lane 6— A56-21; Lane 7— A56-29; Lane 8— A56-29; Lane 9— A56-62; Lane 10— A56-67; Lane 11— A56-53; Lane 12— A56-68; lane 13— A56-66; Lane 14— A56-20; Lane 15— A56-51; Lane 16— A56-14; Lane 17— A56-13; Lane 18— A56-8; Lane 19— A56-23; Lane 20— Marker

3　Discussion

Although efforts to breed seedless grape varieties started over one hundred years ago[2,3], little is known about its mode of inheritance. Recently, Ledbetter and Burgos[1] advanced a hypothesis that three dominant complementary genes appear to be the simplest and most plausible as it explains the 9: 23 and 3: 13 seedless: seeded observed ratios that are possible only in a three-gene model, according to data from 70 years of grape breeding records at the Agricultural Research Service in Fresno, California. Loomis and Weinberger[3] reported that seedlessness appears to be recessive in nature, though not a simple recessive, based upon the evaluation of more than 10, 000 seedlings from different cross combinations.

All the previous results were obtained by phenotype rations of segregating in progeny population. Here, we pool two BSA samples based on the softness of seeds to form pool 1 (graed 7~ 9) and pool 2 (grade 0). Generally speaking, grades 6 or higher were considered to be used as seedless in commercial production. For example, Sultanina is 9, Perlette is 7, Flame Seedless is 6, and B45-187 is 7.

DNA molecular markers, which are direct products of PCR and unaffected by environment, may provide more objective descriptors for cultivar identification. In this study, presence: absence of the 500 bp band in the segregation is 18: 42. Analysis of the cosegregating pattern of the seedlessness trait and the RAPD marker ($UBC\text{-}269_{500}$) in the population (Table 2), suggests that the marker was linked to one of the genes con-

trolling the seedless trait in grapes, and a group of minor genes also has an effect of the seedlessness trait. Most hybrids containing the marker are to some extent seedless indicating that a gene or loci of genes affecting seed formation are colsely linked seedless marker. The variability in extent of seed formation in plant both with and without the marker suggests that other quantitive genes affect seed development. As for the two hybrids (grade 0) with the marker, although they are linked to one of seedless genes, the function of this gene could have been restrained by controlling seeded trait genes in lacking a group of minor seedless genes, thus showing seeded trait in phenotype. On the other hand, hybrids in grade 9 with no marker may have various minor genes contributing to seedlessness in phenotype. Therefore, according to the cosegregating pattern between the seedless trait and the RAPD marker in the population, we must conclude that the marker linked to the one of the major genes controlling the seedless trait, and the major seedless genes and those minor seedless genes are independently inherited in grapes. This is why obtaining the seedless hybrids are difficult during short breeding program, and little is yet known about the mode of seedlessness inheritance in grapes so far.

Based on the pedigree of hybrids and their parents, the genes controlling seedlessness or the seedless trait in B45–187 and its offspring are derived from the initial donor progenitor, Sultanina and Perlette. We have identified the RAPDs of Thompson Seedless and Perlette using primer UBC–269. The RAPD identification showed that they both had the same specific band of 500 bp (Fig. 4). In fact, Perlette is derived from Queen of the vineyard X Thompson Seedless (Sultanina), so Perlette also got seedless gene from Thompson Seedless. On the other hand, Thompson Seedless had another specific band of 450 bp (Fig. 4). In contrast to the two bands of Thompson Seedless, eighteen hybrids of the segregant population had the band of 500 bp (Table 1), and one hybrid, A56–13(gradeg 9), had the band of 450 bp (Fig. 4). Thus, we concluded that except for A56–13, the genes controlling seedlessness of the other hybrids and their pollen parent, B45–187, originated from Thompson Seedless and Perlette, and seedless genes in A56–13 were mainly derived from Thompson Seedless. As for the 450 bp band, because it was only displayed by one hybrid, A56–13, future work needs to be done in other segregating population related to Thompson Seedless to confirm its association with seedlessness.

In addition, when the seedless grape cultivar Orlando Seedless was used as a template for amplification by UBC–269, the same band of 500 bp was obtained (Fig. 4). However, further works need to be done among other seedless grape cultivars for generalizing the application of this marker for seedlessness in grapes.

Giovannoni et al[9] reproted seven to fourteen individual plants were used to generate each pool. Pooling larger numbers of individuals increases the probability that the

two pools will not differ for alleles other than those within and adjacent to the target interval. However, as the pool size increases, so does the probability that individuals will occur in the pool with a double crossover. Since the *V. vinifera* genome has a relatively high level of heterozyosity[6], and a segregating F_1 population is used for the linkage analysis, a relatively large number of individuals in each pool may be necessary. We used nine individual plants in each pool from the segregating F_1 population, and found that 4 out of 5 polymorphism were false linkage markers (Fig. 3). In contrast to the presence of the marker ($UBC\text{-}269_{500}$) between pool 1 and grade 6~9, the frequency accounted for 55.6% (Fig. 3) and 55% (Table 2), respectively. That is, the result obtained from the BSA sample (pool 1) is clearly related to that from a number of seedless hybrids in the segregant population. We therefore suggest that 9~15 individuals are an appropriarte number for making a pool of heterozygous grapes which segregate in the F_1 generation.

Our results suggested that the markert $UBC\text{-}269_{500}$, linked to seedless genes, is related to the one of major efficient genes of controlling seedless trait. This marker can be utilized as one anchor for mapping seedless genes in grapes for further research. Because the seedless trait of grapes is controlled by multiple genes, more markers linked to other seedless genes need to be detected in future. The results of this study show that RAPDs and BSA can be used as a rapid method of identification for progeny from segregant populations of seeded by seedless grape hybrids.

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References

1 Ledbetter C A, Burgos L. Inheritance of stenospermocarpic seedlessness in *Vitis vinifera* L. The Jounal of Heredity, 1994, 85: 157~160

2 Weinberger J H, Harmon F N. Seedlessness in *Vitis vinifera* grapes. Proc Amer Soc Hort Sci, 1964, 85: 270~274

3 Loomis N H, Weinberger J H. Inheritance studies of seedlessness in grapes. J Amer Soc Hort Sci, 1979, 104: 181~184

4 Barlass M, Ramming D W, Davis H P. In ovule emlbryo culture: a breeding technique to rescue seedless X seedless table grape crosses. Australian Grape Grower and Winemaker, 1988, 259: 123~125

5 Weeden N F, Reisch B I, Martens M H. Genetic analysis of isozyme polymorphism in grape. J Am Soc Hort Sci, 1988, 113: 765~769

6 Mauro M-C, Strefeler M, Weeden N F et al. Genetic analysis of restriction fragment length polymorphisms in *Vitis*. The Jorunal of Heredity, 1992, 83: 18~21

7 Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res, 1990, 18: 7213~7218

8 Williams J G K, Kubelik A R, Livak K J et al. DNA polymorphisms amplified as arbitrary primers are useful ge-