Sequence-specific Polymerase Chain-reaction Markers Derived from Randomly Amplified Polymorphic DNA Markers for Fingerprinting Grape (Vitis) Rootstocks

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Additional index words. sequence characterized amplified region, SCAR

Abstract. Randomly amplified polymorphic DNA (RAPD) markers were generated for identifying grape (Vitis) rootstocks. Seventy-seven primers (10 bases long) were screened using CsCl-purified leaf DNA derived from several field samples of nine rootstocks sampled in successive years. Nine RAPD markers were detected from six primers and, in combination, distinguished all nine rootstocks tested. Because inconsistencies were encountered in performing the RAPD assay, sequence-specific primers were derived from cloned RAPD bands for use under more stringent amplification conditions. Southern hybridization analysis of the RAPD gels with cloned RAPD bands as probes revealed deficiencies of scoring RAPD bands based solely on ethidium bromide staining. In some cases, bands of the same size generated by the same primer in different rootstocks-normally scored as the same marker-failed to cross-hybridize, implying lack of homology between the bands. More commonly, bands scored as absent based on ethidium bromide staining were detected by hybridization. Six of the nine cloned RAPD bands were partially sequenced, and sequence-specific primer pairs were synthesized. Two primer pairs amplified a product the same size as the original RAPD band in all rootstocks, resulting in loss of polymorphism. Two other pairs of sequence-specific primers derived from the same marker failed to amplify the expected band consistently. Three of the most useful primer pairs amplified apparent length variants in some accessions and will have value as polymerase chain-reaction markers for fingerprinting.

Since the first published description of randomly amplified polymorphic DNA (RAPD) markers, this polymerase chain-reaction (PCR)-based technique has been adopted as a convenient and powerful means of detecting genetic differences among closely related organisms (Welsh and McClelland, 1990; Williams et al., 1990). RAPD markers are generated from genomic DNA under conditions similar to those used in standard PCR, except that a single, short (usually 10 bases long) primer of arbitrary sequence is used at a low annealing temperature. Several loci may be detected per primer and no prior knowledge of target DNA sequence is required. Experimental protocols and genetic applications (Williams et al., 1993) and precautions (Clark and Lanigan, 1993) for RAPD analysis have recently been reviewed.

The RAPD reaction is competitive because the low annealing temperature and short primer presumably permit some mismatches between primer and template (Heun and Helentjaris, 1993). If perfect or near-perfect templates are not present in proper orientation or proximity to permit amplification, less-perfect templates may substitute. This flexibility in priming sequence would account for the observation that prokaryotic genomes give rise to more RAPD products than would be predicted on the basis of genome size (Williams et al., 1990). Events favoring one or another product in early cycles of PCR may have a far greater effect on the nature and quantity of the final products than under standard conditions, where only a perfect or near-perfect match will be productive.

The competitive aspect of RAPD analysis may explain in part why minor changes in almost any aspect of the amplification reaction have been reported to affect the outcome: DNA quality and quantity (Williams et al., 1993), choice of DNA polymerase (Schierwater and Ender, 1993), Mg concentration (Park and Kohel, 1994; Williams et al., 1993), choice of thermal cycler (Penner et al., 1993), primer concentration (Williams et al., 1993), use of ethidium bromide vs. silver for detection of products (Caetano-Anolles et al., 1992), and presence of RNA (Yoon and Glawe, 1993).

Although some workers have described potential difficulties in using RAPD markers (Ellsworth et al., 1993; Heun and Helentjaris, 1993; Penner et al., 1993; Riedy et al., 1992; Weeden et al., 1992), the present study was undertaken to evaluate their utility for fingerprinting grape (Vitis) rootstocks. Restriction fragment-length polymorphism (RFLP) analyses have revealed genetic differences among grape rootstocks (Bourquin et al., 1991) and cultivars (Bowers et al., 1993; Mauro et al., 1992; Sivolap et al., 1992; Thomas et al., 1993; Yamamoto et al., 1991), but the differences are not yet detectable in a PCR-based assay. In contrast, sequences flanking grape microsatellites have been developed for use as PCR markers (Thomas and Scott, 1993; Thomas et al., 1994). Although the potential of RAPD markers for fingerprinting grape cultivars has been reported (Collins and Symons, 1993; Jean-Jacques et al.,
1993), Büscher et al. (1993) have expressed reservations. We report here on the generation of RAPD markers to identify grape rootstocks and on the derivation of more reliable PCR markers from them.

Materials and Methods

Plant material. Rootstocks were sampled from late August through the first week of October 1991, 1992, and 1993 from vineyards at Oregon State Univ. (Woodhall III and the Lewis Brown Farm Mother block) and at Mahonia Nursery, Salem, Ore. Rootstock origins (Galet, 1979) are as follows: MG 420A (Vitis berlandieri x V. riparia), Richter 99 (V. berlandieri x V. rupestris), 5C (V. berlandieri x V. riparia), SO4 (V. berlandieri x V. riparia), Couderc 3309 (V. riparia tomentose x V. rupestris), MG 101-14 (V. riparia x V. rupestris), Couderc 1616 (V. solonis [riparia-rupestris-candicans] x V. riparia), Kober 5BB (V. berlandieri selection), and Riparia Gloire (V. riparia selection). Several independent sources of the rootstock SO4 were sampled. Based on isozyme analysis for asparagine amnontransferase (AAT) in our laboratory, two putative AAT samples (indicated as #3 or #3b, and #14 in the figures) are likely to be 5C (Walker and Boursiquot, 1992).

Grape DNA isolation. Young leaves were held on ice immediately after picking and generally were processed the same day. Leaves were never held for more than 2 days before DNA extraction. Fresh leaves (about 10 g) were rinsed in distilled water, frozen, and ground in liquid N. The powder was suspended in 80 ml of ice cold extraction buffer [0.35 m sorbitol, 0.1 m Tris, pH 8.0, 5.0 mM MgCl, 50 mM KCl, 10 mM EDTA, 10 mM sodium sulfite, 10 mM 2-mercaptoethanol, 1% polyvinylpyrrolidone-40 (PVP-40, Sigma Chemical Co., St. Louis)] and blended at high speed in a prechilled blender. The blended leaf extract was poured through a cheesecloth and centrifuged at 10,000× g for 15 min at 4C. The supernatant was discarded, and the pellet gently suspended in 8 ml of cold extraction buffer (Walker and Boursiquot, 1992).

Table 1. Sequence-specific primer pairs derived from cloned RAPD bands.

| Primer pair | Annealing temp (°C) |
|-------------|---------------------|
| OPG02U590 | 5'-GGC ACT GAG GCA ACA CAT AA-3' |
| OPG02I590 | 5'-CTG AGG AAT ATC TGG TGC AA-3' |
| OPG05U590 | 5'-CTG AGA CGG AGA GCT AAA AAA TAA-3' |
| OPG05I590 | 5'-CTG AGA CGG ACA TTA TCC ACA-3' |
| UBC204U1| 5'-TTC GGG CCG TGT CAC ATG AAT TCC-3' |
| UBC204I1| 5'-TTC GGG CCG TGT ACT TGG AGG AAA-3' |
| UBC231U1| 5'-AGG GAG TCC CTT GCA AGT CCT-3' |
| UBC231I1| 5'-AGG GAG TCC CAA CAA GAT TTA AGT-3' |
| UBC231U2| 5'-ACC GGT ATG GTA CAG ATG CT-3' |
| UBC231I2| 5'-GGG ATG TCC AAC AAG ATT TA-3' |
| UBC231U3| 5'-TTC ACG GAC GGG TGG GGA 'TAG ATT'-3' |
| UBC231I3| 5'-TTC ACG GAC GGA TTC CAG GGG GTC-3' |
| UBC251U| 5'-CTT GAC GGG GTA TGG GGT GCT GAG-3' |
| UBC251I| 5'-CTT GAC GGG GTA TGG GGT GCT GAG-3' |

Underlined sequences are derived from the original RAPD primers. The number and letters preceding the U (upper) and L (lower) and subscript (size of marker in bp) refer to the primer used to generate the marker. OP = Operon, UBC = Univ. of British Columbia.
Table 2. Rootstock-RAPD marker matrix.

| Rootstock       | OPG02<sub>400</sub> | OPG02<sub>120</sub> | OPG05<sub>550</sub> | OPG05<sub>1420</sub> | UBC204<sub>140</sub> | UBC231<sub>140</sub> | UBC231<sub>190</sub> | UBC251<sub>140</sub> | OPG06<sub>140</sub> |
|-----------------|---------------------|---------------------|---------------------|----------------------|----------------------|----------------------|----------------------|----------------------|---------------------|
| MG 420A         | +                   | +                   | +                   | +                    | +                    | +                    |                     |                      |                     |
| Richter 99      |                     | +                   | +                   | +                    | +                    | +                    |                     |                      |                     |
| 5C              | +                   |                      |                      |                      | +                    |                      | +                    |                      | +                   |
| SO4 #4          | +                   | +                   |                      |                      | +                    | +                    | +                    |                      | +                   |
| SO4 #12         | +                   |                      |                      |                      | +                    |                      | +                    |                      | +                   |
| Rtpartia Giroire| ?                   | ?                   |                      |                      |                      |                      | +                    |                      | +                   |
| Coudere 3309    |                     | +                   |                      |                      | +                    |                      | +                    |                      | +                   |
| MG 101-14       |                     | +                   |                      |                      | +                    |                      | +                    |                      | +                   |
| Kober 5BB       | ?                   | ?                   |                      |                      |                      |                      |                      | +                    | +                   |
| Coudere 1616    |                     |                      | +                   |                      | +                    |                      |                      | +                    | +                   |

<sup>1</sup>(+) The presence of the indicated marker when rootstock DNA was used in an appropriate DNA amplification reaction. (?) An ambiguous result. The number and letters preceding the subscript (size of RAPD marker in bp) refer to the primer used to generate the marker. OP = Operon; UBC = Univ. of British Columbia.

The pattern for 5C was the same obtained for two other putative SO4 isolates (SO4 #3 and SO4 #14).
Table 3. Hybridization analysis of cloned RAPD bands.

| Marker       | False negatives | False positives | Other hybridizing bands |
|--------------|-----------------|-----------------|-------------------------|
| OPG02<sub>860</sub> | No              | No              | Yes                     |
| OPG01<sub>1320</sub> | Yes             | No              | Yes                     |
| OPG01<sub>850</sub> | No              | No              | Yes                     |
| UBC204<sub>1,40</sub> | No              | No              | Yes                     |
| UBC231<sub>1,40</sub> | No              | No              | Yes                     |
| UBC234<sub>922</sub> | Yes             | No              | Yes                     |
| UBC231<sub>1,60</sub> | No              | No              | Yes                     |
| UBC251<sub>1,440</sub> | No              | No              | Yes                     |
| OPG06<sub>1,440</sub> | No              | Yes             | Yes                     |

RAPD gels were blotted and probed with individual cloned RAPD bands. Hybridizations were interpreted relative to expectations based on positive or negative scoring of the RAPD bands on the original ethidium bromide-stained gels. False negative = a hybridization signal was observed when the band was not seen on the RAPD gel; false positive = no hybridization signal or a substantially weaker one was observed when the putative band was seen on the RAPD gel. Other hybridizing bands = presence of hybridizing bands other than the expected RAPD band.

duced 1) no products, 2) smears instead of discrete bands, and 3) faint discrete bands. Generally, for every successful amplification, one to two attempts were made. Using fresh DNA dilutions, new mineral oil, and new buffer did not noticeably improve these amplifications. However, a systematic effort to determine the basis for the failed reactions was not undertaken. More often than not, the expected amplification was restored on repeating the assay without deliberate alteration in protocol.

Fig. 1. Hybridization of cloned RAPD band UBC234<sub>1,40</sub>(arrow) to a Southern blot of the RAPD gel. Rootstocks marked with asterisks had been scored positive for the marker. Because the intact recombinant vector was labeled and used as a probe, the hybridization signal in the unmarked first lane, containing the 123-bp molecular weight ladder, reflects homology between vector sequences and the 123-mer. Based on their isozyme (AAT) profiles, the rootstocks designated SO4 #3 and #14 are likely 5C.

Fig. 2. Hybridization of cloned RAPD band UBC204<sub>1,40</sub>(arrow) to a Southern blot of the RAPD gel. Rootstocks marked with asterisks had been scored positive for the marker. On this particular gel, the amplification with MG 420A produced no discrete bands. Because the intact recombinant vector was labeled and used as a probe, the hybridization signal in the unmarked outer lanes, containing the 123-bp molecular weight ladder, reflects homology between vector sequences and the 123-mer. SO4 #3 and #14 are likely 5C; refer to Fig. 1 legend.
An experiment was performed to determine if AmpliTaq used in a Perkin-Elmer thermal cycler would produce the same RAPD products obtained with Tfl DNA polymerase in an Ericomp thermal cycler. The three Operon primers-OPG02, OPG05, and OPG06-used with AmpliTaq produced banding patterns that contained new polymorphic bands but that-bore no resemblance to those observed using Tfl (data not shown). The minor differences in cycling parameters were in the total number of cycles (40 vs. 45), the lengths of the initial denaturation and final extension steps (5 vs. of 2 min at 94°C and 5 vs. of 1 min at 72°C), and in the temperature and time used for the repeated denaturation step (30 set at 94°C vs. 1 min at 92°C). While it is possible that these differences may account for the different products, dependence of RAPD bands on the DNA polymerase used has been noted in grapes (Büscher et al., 1993), corn (Williams et al., 1993), and in a crustacean (Schierwater and Ender, 1993). A third heat-stable DNA polymerase (Replitherm, Epicentre Technologies) used in buffer supplied by the manufacturer failed to generate RAPD products under the PCR conditions used for Tfl (data not shown).

Because we did not determine the causes of the failed reactions and wanted an assay that did not depend on a particular heat-stable DNA polymerase, we decided to design sequence-specific PCR primers from the RAPDs that could be used under more-stringent amplification conditions (Paran and Michelmore, 1993). Thus, we cloned the nine informative RAPD bands and designed primers for the six that were sequenced.

**Southern hybridization analysis of cloned RAPD markers.**

Confirmation that the correct bands had been cloned was based on probing blotted RAPD gels with the cloned markers and comparing the hybridization patterns with the banding patterns based on ethidium bromide staining (Table 3). In all cases, hybridizing bands in addition to the expected RAPD bands were observed. When hybridization signals corresponding to the RAPD markers were observed in rootstocks that had been scored as lacking the markers, they are indicated as false negatives because the bands are present, but at concentrations too low to be detected by ethidium bromide staining (Table 3). In all cases, hybridizing signals were not observed in rootstocks Riparia Gloire, Coudre 3309, MG 101-14, and Kober 5BB (Table 2), hybridizing bands of the correct sizes were observed (data not shown).

In the two cases indicated as false positives (Table 3), hybridizations were not observed in rootstocks that had been scored positive for the RAPD bands. In the example shown in Fig. 2, neither MG 420A nor Richter 99 produced a hybridization signal, although both were scored positive for marker UBC204L. The failure of MG 420A to hybridize can be explained by the fact that, in this particular amplification, MG 420A produced no discrete bands. The absence of a hybridization signal in Richter 99 cannot be accounted for by a failed amplification because the expected band was present on the ethidium bromide stained gel. However, a longer exposure of the X-ray film revealed a very weak hybridization signal of about the correct size, suggesting that the band identified as marker UBC204L in Richter 99 shares poor homology to UBC204L in the other rootstocks. These results demonstrate that bands of the same size generated in different samples using the same primer may not necessarily represent the same sequence, although such bands are typically scored as the same RAPD marker.

Figure 3 shows an example of specific detection of the expected marker OPG05L in the only rootstock that produced it, Kober 5BB. A slightly larger cross-hybridizing band is also seen. Interestingly, because OPG05L could no longer be detected by ethidium bromide staining in the third year of the study, an uncertain score for Kober 5BB is indicated in the rootstock-RAPD marker matrix (Table 2).

**Amplifications using sequence-specific primers.**

Six of the RAPD markers were partially sequenced and seven new sequence-specific primers were synthesized for use under more stringent amplification conditions. The most useful primer pairs-OPG02U and OPG02L (Fig. 4), OPG05U and OPG05L, and UBC231U and UBC231L—generated additional bands that may represent length variants. Southern blots of gels based on amplifications with the first two primer pairs with cloned markers OPG02 and OPG05, respectively, revealed that the additional bands were homologous to the original markers (data not shown). Southern analysis was not performed on the amplifications with primer pair UBC231U and UBC231L. Amplifications involving sequence-specific primer pairs UBC204U and UBC204L, and UBC251U and UBC251L, resulted in a loss of polymorphism because all rootstocks gave rise to the formerly polymorphic RAPD bands. Although rootstocks 5C and SO4 are polymorphic for RAPD marker UBC204L (Table 2), postamplification digestions of the product generated by primer pair UBC204U and UBC204L from these rootstocks with Bam HI, Eco RI, Hin dIII, Pst I, Xba I, Hae III, and Sau IIIA I failed to reveal restriction site differences. Primer pair UBC251U and UBC251L amplified other products in addition to the monomorphic band. Using higher- and lower-than-optimal annealing temperatures with this primer pair, or lower-than-optimal tem-
temperatures with UBC204U\textsubscript{100} and UBC204L\textsubscript{100} did not result in amplification of length variants (restoration of polymorphism). For unknown reasons, neither of the two pairs of sequence-specific primers designed from cloned marker UBC231\textsubscript{100} amplified the expected product consistently. Certain primers have been observed to amplify poorly, or not at all from sequences that lack obvious potential secondary structure (Innis, 1990). Using higher- and lower-than-optimal annealing temperatures with primer pair UBC231U\textsubscript{100} and UBC231L\textsubscript{100} did not restore polymorphism.

Discussion

Inconsistencies in results of RAPD reactions made the RAPD markers less useful than desired. To improve their utility, six of the nine cloned markers were partially sequenced, and sequence-specific primer pairs were synthesized. Two amplified a product the same size as the original RAPD band in all rootstocks, resulting in loss of polymorphism. Two other pairs of sequence-specific primers designed from the same cloned RAPD band failed to amplify the expected bands consistently. However, three of the primer pairs amplified apparent length variants from some accesses and will have value as PCR markers for fingerprinting.

Although RAPD markers were generated for fingerprinting grape rootstocks, the inconsistencies we encountered in performing the amplifications made the procedure impractical. For markers to have value in fingerprinting, they must be reliable, practical, and validated in independent laboratories (Smith and Smith, 1992), especially if they are to be used in cultivar patent disclosures and in legal cases of varietal infringement. The sensitivity of the RAPD reaction to various reaction parameters may demand a degree of standardization that is difficult to sustain even within a laboratory, let alone between laboratories.

Three of seven sequence-specific primer pairs derived from the cloned informative RAPD bands were reliable and useful because they amplified apparent length variants in addition to the expected products. Although Southern analysis established homology among the variants in the two cases tested, it is not possible to establish allelism in the absence of a pedigree. Paran and Michelmore (1993) reported that eight of nine RAPD-derived sequence-specific primers amplified the original polymorphic product or new alleles among segregating populations of lettuce.

Results from the Southern analysis showed lack of homology among some amplified bands of the same size generated with the same 10-mer primers in different rootstocks. It is important to point out that various Vitis species are often used as rootstocks for grape scion cultivars and that most of them are of interspecific hybrid origin. Weeden et al. (1992) correctly noted that, without detailed genetic, DNA hybridization, or DNA sequence analysis, it is extremely difficult to establish that RAPD bands amplified in individuals of undefined relationship are homologous. This is a major impediment to using RAPD bands to fingerprint distantly related cultivars.

Without knowing the source of the polymorphisms that produced the RAPD bands, one cannot predict whether simple extension of the 10-mers to make longer sequence-specific primers will retain them. In the present study, half of the attempts were successful. One interpretation of why two of the primer pairs amplified the same product in all rootstocks is that the original polymorphism is in the sequences to which the RAPD primer anneals. The rootstocks that failed to amplify the RAPD band may have had poor matches at one or both termini. Adding 10 to 14 bases to the 3’ terminus of the 10-mer primers in anpolyorphic region may have provided enough homology to overcome the original mismatch and to permit annealing and amplification. Alternatively, an insertion between primer binding sites might have increased the size of the product beyond the range of the PCR conditions applied. This latter possibility would account for lack of a product when the original 10-mer was used. However, in this case, extension of the 10-mers would not be expected to result in the observed amplification.

Our ongoing efforts to sequence regions in the grape genome to which the RAPD primers anneal will provide the evidence needed to assess the importance of primer-template mismatches and may also permit more rational design of RAPD-derived primers such that the original or newly discovered polymorphisms are retained.

![Fig. 4. Amplification products obtained with sequence-specific primers OPG02U\textsubscript{100} and OPG02L\textsubscript{100}. The lower of the two bands was expected, the upper is an apparent length variant. The outer lanes consist of a 123-bp molecular weight ladder. SO4 #3b is likely 5C; refer to Fig. 1 legend.](image)

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