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Application of RAPDs to DNA Extracted from Apple Rootstocks

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Abstract. **Samples of 15 different rootstocks from mature apple trees (*Malus ×domestica* Borkh.) with 'Starkspur Supreme Delicious' as the scion were differentiated adequately using random amplified polymorphic DNAs (RAPDs). A procedure for extraction of DNA from root material is described. Patterns of DNA from leaf tissue of young trees of 10 of the rootstocks were compared with those from root tissue of mature trees grafted on the same rootstocks and at a different location to reaffirm identification of these rootstocks. Similar patterns were obtained for a) root vs. leaf tissue and b) tissue from two locations. Except for one apparent misidentification (within a single replication), patterns for root tissue of all rootstocks matched those of the corresponding leaf tissue.**

Apple scion cultivars often are identified easily by morphological characteristics exhibited in both their fruit and leaves (e.g., Thomas, 1906). Leaves also represent convenient material for analysis of DNA fingerprints (Mulcahy et al., 1993). Rootstocks, however, whether in the stoolbed or planted in the nursery row, provide few morphological characteristics for their identification, and even these few characteristics tend to be obscure and poorly standardized. Furthermore, in finished trees, the rootstock often does not produce any shoots and is, in fact, virtually impossible to identify. Only tree performance, such as canopy size and productivity, give a general indication of rootstock, and only those rootstocks resulting in dramatic differences can be separated this way. Both nurserymen and growers have long been interested in rootstock identification and would benefit from a simple and accurate identification procedure. This interest applies not only to rootstocks in the nursery field that are ready for budding, but also to rootstocks of finished trees in the orchard.

If nurserymen were confident that rootstocks were true to name, they would be able to provide better service to growers, and with the aid of testing laboratories, growers, in turn, would be able to identify plantings where rootstocks were labeled incorrectly. Random amplified polymorphic DNAs (RAPDs) are generated easily, require no prior knowledge of the molecular genetics of an organism, and are used widely in cultivar identification. The importance of rootstock identification has been emphasized by Lu et al. (1996), who employed leaves for RAPD identification of peach [*Prunus persica* (L.) Batsch] rootstocks. However, preparation of clean DNA from the tissues of rootstocks could be a problem. Therefore, a project was undertaken with the objective of assessing the viability of RAPDs for identifying rootstocks both from leaf tissue and from root tissue of mature, grafted apple trees.

Materials and Methods

Trees used in this study were a part of the 1984 NC-140 Cooperative Apple Rootstock Planting (NC-140, 1996), which included 'Starkspur Supreme Delicious' on 15 different rootstocks, including B.9, MAC.1, MAC.39, P.1, P.22, domestic seedling, M.4, M.7 EMLA, M.26 EMLA, B.490, P.2, P.16, P.18, C6, and A.313. Root samples were collected in Mar. 1994 from four replications of the 1984 planting at the Ohio Agricultural Research and Development Center, Wooster, Ohio. Samples were placed in plastic bags and sent immediately via overnight mail to the Univ. of Massachusetts, Amherst. Samples then were refrigerated at 8 °C, and DNA was extracted from each within 10 d. Liners of 10 of the clonally propagated rootstocks in the planting (B.9, MAC.39, P.22, M.4, M.7 EMLA, M.26 EMLA, P.2, P.16, P.18, and C6) were obtained from Treco (Woodburn, Ore.) in May 1993 and planted at the Univ. of Massachusetts Horticultural Research Center, Belchertown. Leaf samples were collected from these plants in Summer 1994 to compare with results from the Ohio root samples.

DNA extraction and purification. The procedures used to extract DNA from root and leaf samples were modified from those of Bernatzky and Tanksley (1986). For each leaf or root sample, 1.0 g fresh mass of the phloem/cambium layer (excluding the corky outer layer and any oxidized edges) and 4.0 g of sand were combined with liquid N in a mortar for grinding. Aliquots of ≈5 mL liquid N were added two to four times during the grinding. An extraction buffer was prepared, including sorbitol (63.75 $g \cdot L^{-1}$), Tris (12.10 $g \cdot L^{-1}$), and EDTA (1.68 $g \cdot L^{-1}$). The pH of the buffer was adjusted to 7.5 with HCl, and Na-bisulfite was added just before use (3.802 $g \cdot L^{-1}$). A lysis buffer was prepared, including 200 mL 1.0 M Tris (121.1 $g \cdot L^{-1}$), 200 mL 0.25 M EDTA (93.05 $g \cdot L^{-1}$), 400 mL 5.0 M NaCl (292.2 $g \cdot L^{-1}$), 20 g hexadecyltrimethylammonium bromide, and 200 mL deionized H_2O. The pH of the buffer was adjusted to 8.0 with HCl. After the samples were ground, the powder was added to 20 mL of a solution of 3 parts extraction buffer : 3 parts lysis buffer : 1 part sarcosine (5%, containing 0.50% Na-bisulfite and 2.0% PVP) (by volume) in a 50-mL polypropylene tube. Samples were vortexed and incubated at 65 °C for 20 to 30 min, vortexing again every 10 min during incubation.

To each sample, 10 mL of a solution of 25 phenol (pH 7.5) : 24 chloroform : 1 isoamyl alcohol (by volume) were added and samples were again vortexed for 2 min. Samples were centrifuged at 10,000 g_n for 10 s. The supernate from each sample was then transferred to a new tube, and an equal volume of a solution of 24 chloroform : 1 isoamyl alcohol (by volume) was added to each. Samples were then centrifuged at 10,000 g_n for 10 min. This last extraction was repeated two to three times, until the supernate was clear. The supernate was then removed, and two-thirds volume of 2-propanol was added to each sample. Each was mixed by inversion and centrifuged at 3,000 g_n for 5 min. Pellets were washed twice with 70% ethanol, once with 95% ethanol, and air-dried for 30 to 60 min.

DNA was purified by differential precipi-

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tation (Manning, 1991). Pellets were resuspended in 5.0 mL of 2-BE buffer (25 mM H_3BO_3, Tris pH 7.6, 1.25 mM Na_2EDTA, 100 mM NaCl), 2.0 mL of 2-butoxyethanol (2-BE) were added, and samples were incubated on ice for 30 min. Samples then were centrifuged at 15,000 g_n at 4 °C for 10 min. The supernate was removed, 4.5 mL of 2-BE were added, and the samples were incubated on ice for 30 min. The samples were centrifuged again at 15,000 g_n at 4 °C for 15 min. Pellets were washed consecutively with a) 0.2 M boric acid/Tris, b) a solution of equal parts (by volume) of 10 mM Na_2EDTA (pH 7.6) and 2-BE, c) 70% ethanol containing 0.1 M K acetate/acetic acid (pH 6.0), and d) absolute ethanol before drying under vacuum. Pellets were then dissolved in 400 µL TE [10 mM Tris/1mM Na_2EDTA (pH 8.0)]. Two subsamples of each sample were extracted and processed.

Polymerase chain reaction. For use in the polymerase chain reaction (PCR), the DNA was diluted with sterile distilled water to 5 to 10 ng·µL^{-1}, and 2.5 µL of this solution was added to 10.15 µL of reaction mixture for PCR. The PCR reaction mixture was 1.25 µL 10× buffer [100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Difco Bacto Gelatin (Difco Laboratories, Detroit)]; 1.0 µL of nucleotide solution (containing 2.5 mM of each of the following: dATP, dCTP, dGTP, and dTTP); 0.55 µL 10 mM $MgCl_2$; 0.5 µL 10 µM primer; 0.1 µL Taq (DNA polymerase, 5 units·µL^{-1}; one unit catalyzes the incorporation of 10 nmol of total nucleotide into DNA in 30 min at 70 °C); 2.0 µL DNA (5–10 ng·µL^{-1}); 6.75 µL H_2O. The total volume, 12.65 µL, was overlain with one drop of mineral oil. The PCR conditions were 94 °C for 1 min, followed by 40 cycles of the following: 94 °C for 1 min, 34 °C for 2 min, and 74 °C for 2 min. Some reactions were run on a Barnstead/Thermolyne Amplitron thermocycler (model DB 66935; Dubuque, Iowa) and others on a Perkin-Elmer thermocycler (model 480; Norwalk, Conn.).

Random 10 bp primers used were A02 (= Set A, primer 02 5´TGCCGAGCTG3´) and A09 (5´GGGTAACGCC3´), both from Operon Technologies (Alameda, Calif.). Several primers were tried, but these two allowed distinction of the genotypes tested. Nucleotides were from both Promega (Madison, Wis.) and Perkin-Elmer Cetus (Norwalk, Conn.). Taq polymerase was AmpliTaq (DNA polymerase AS) from Perkin-Elmer Cetus. The DNA size marker was the 100 bp ladder (catalog no. 27-4001-01; Pharmacia Biotech, Piscataway, N.J.).

Amplified DNA was separated in 2% agarose (1% NuSieve agarose; FMC Bioproducts, Rockland, Maine), 1% agarose (catalog no. A-6013; Sigma Chemical Co., St. Louis), and 1×TBE buffer [0.089 M Tris, 0.89 M boric acid, 0.002 M Na_2EDTA (pH 8.0)], and was stained with ethidium bromide. Each sample was run several times.

Results and Discussion

Rootstocks are not ideal material for DNA extraction since the corky outer layer, which is replete with biological contaminants, must be excluded for DNA extraction. In preliminary work, tissue was frozen immediately after sampling for convenience. We found, however, that attempts to remove the corky outer root layers from this frozen tissue usually resulted in tissue thawing and the release of oxidized phenolic compounds, indicated by tissue browning. These compounds severely

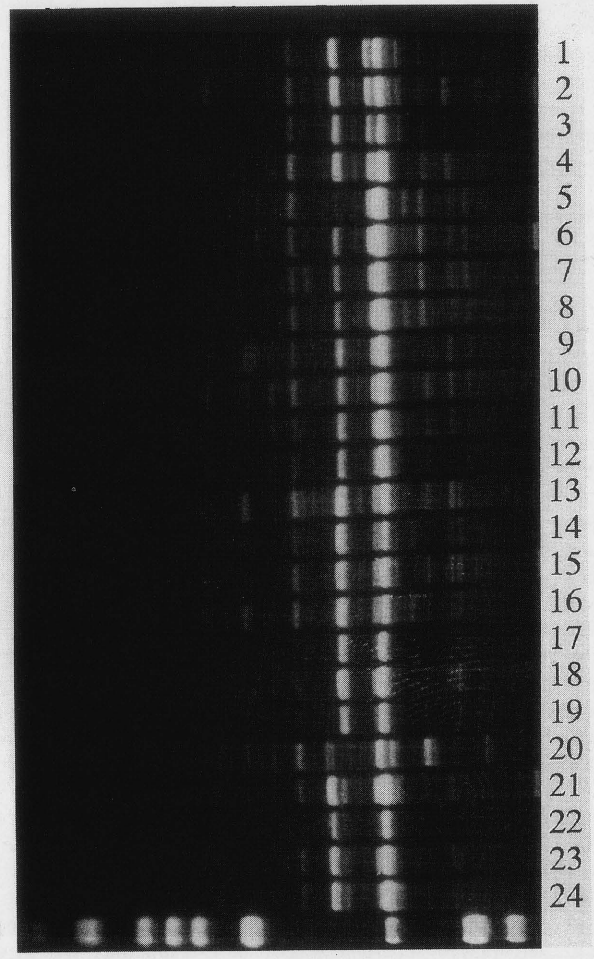

Fig. 1. RAPD fingerprints from four replicates of each of six apple rootstocks. Lanes 1 through 4 = B.9, lanes 5 through 8 = MAC.39, lanes 9 through 12 = P.22, lanes 13 through 16 = M.4, lanes 17 through 20 = M.7 EMLA, lanes 21 through 24 = M.26. EMLA. The last lane is a DNA size marker. Lambda DNA cut with EcoR I and Hind III.

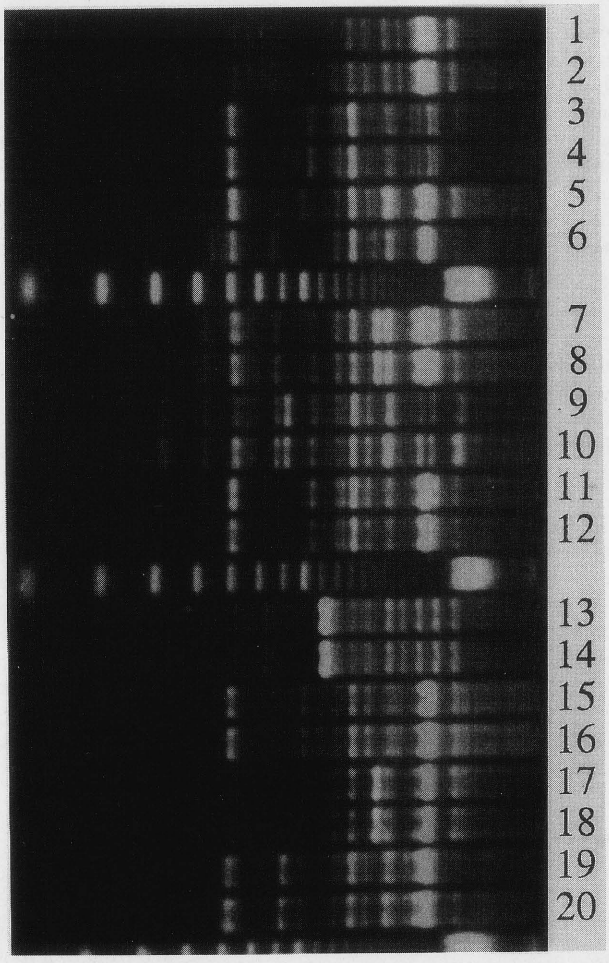

Fig. 2. RAPD fingerprints from paired root and leaf samples of 10 apple rootstocks. Lanes 1 and 2 are root and leaf samples, respectively, of B.9, lanes 3 and 4 = MAC.39, lanes 5 and 6 = P.22, lanes 7 and 8 = M.4, lanes 9 and 10 = M.7 EMLA, lanes 11 and 12, M.26 EMLA, lanes 13 and 14 = B.490, lanes 15 and 16 = P.2, lanes 17 and 18 = P.18, lanes 19 and 20 = C6. Lanes without numbers are a DNA size marker; each band is a 100 bp increment, with that at 800 bp brighter than other bands.

interfere with the polymerase chain reaction of RAPDs analysis. Therefore, we limited our study to fresh root material from rootstocks. Leaves were used successfully either fresh or frozen, since there was no lengthy dissection with associated risk of thawing. Note that, despite the extraordinary ability of PCR to reveal very small quantities of DNA, the most abundant DNA molecules have a correspondingly greater effect upon the outcome of the reaction. Thus, the ability to detect even a single molecule of DNA does not imply that the method will show rare DNA sequences within a mixture of abundant sequences. In fact, contaminants of 10% within a mixture of DNAs have gone undetected (Michelmore et al., 1991). This explains why separate accessions of field-grown leaf material will exhibit the same DNA fingerprints despite the presence of what must be profoundly different surface contaminants.

The four field replications of 13 of the 15 rootstock genotypes gave consistent patterns. As examples, six of the 15 are shown in Fig. 1. One group of four, shown in lanes 17 through 20 (Fig. 1), should exhibit a common fingerprint. Lane 20 was clearly different from 17 through 19, suggesting a misidentification of the rootstock from which the sample in lane 20 was taken. Rootstock 15 was domestic seedling and exhibited expected variation, since it was seed propagated, not clonally propagated.

In all 10 rootstocks of which we had leaf material, the rootstock fingerprints matched those of the leaves (Fig. 2). This result suggests that rootstock material can indeed be identified reliably with RAPDs, regardless of location, tissue used, or potential contamination. Because of variability from laboratory to laboratory and day to day, however, published standards cannot be used for identification. If samples from known tissue can be obtained and run at the same time as unknowns, then adequate comparisons can be made.

The DNA-extraction procedure used provided repeatable separations, but further work must be directed at simplifying the procedure for extraction of DNA from root samples.

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