Identifying Rose Cultivars Using Random Amplified Polymorphic DNA Markers

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Abstract. Five rose (*Rosa* spp.) cultivars were analyzed using random amplified polymorphic DNA (RAPD) markers. Using eight primers, all cultivars were distinguished by comparing differences in DNA banding patterns. The RAPD technique fingerprints rose cultivars rapidly and inexpensively for identification and patent protection purposes.

Developing highly reliable and discriminatory methods for identifying cultivars has become increasingly important to plant breeders and those in the nursery industry who need sensitive tools to differentiate among and identify cultivars for plant patent protection. In the past, cultivars were identified primarily based on horticultural, morphological, and physiological descriptions. In most cases, the descriptions and measurements varied considerably due to environmental fluctuation and human judgment. The development of new cultivars that lack distinguishing morphological characteristics has furthered the need for more positive identification methods. As an adjunct to morphological and physiological methods, identification tests based on isozyme patterns have been introduced to fingerprint ornamental cultivars of various species (Kuhns and Fretz, 1978; Messegueur and Arús, 1985; Wendel and Parks, 1983). This technique, however, is not powerful enough to distinguish among phenotypically similar cultivars. Tissue must be sampled at comparable physiological states to obtain uniform and repeatable banding patterns. Moreover, some genetic differences among cultivars may not be visualized by isozyme technique, since the number of loci that can be resolved is limited.

Methods developed over the past 20 years can detect differences in DNA sequence between individuals. Compared to isozyme techniques, analyzing DNA has many advantages: it is independent of environmental conditions; DNA sequence is identical, no matter what

plant tissue or tissue stage is analyzed; and the number of scorable loci is unlimited. The most widely used technique is restriction fragment length polymorphism (RFLP) analysis. Base substitutions in a restriction endonuclease site or insertions or deletions between sites result in detectable differences in the fragment length of restriction enzyme-digested DNA. These polymorphisms already have helped identify cultivars of some species (Apuya et al., 1988; Gebhardt et al., 1989), including rose (Hubbard et al., 1992). More recently, the random amplified polymorphic DNA (RAPD) technique (Williams et al., 1990) based on the polymerase chain reaction (PCR) has been used to detect polymorphism in some species (Welsh and McClelland, 1990; Welsh et al., 1991; Williams et al., 1990). The technique involves using a single primer ≈ 10 bases long to generate fingerprints of DNA segments that amplify in DNA preparations from one parent but not the other. Detected polymorphisms are inherited in a Mendelian fashion and can be produced from any species without any other DNA sequence information (Williams et al., 1990). The RAPD technique has similar uses as RFLP analysis—constructing linkage maps, identifying cultivars, and determining parentage are among the most valuable applications (Welsh et al., 1991). Advantages of the RAPD technique over RFLP analysis include the following: 1) the equipment and supplies necessary are inexpensive relative to those needed for RFLP analysis; 2) speed of analysis is <2 days, since Southern blotting and labeled probes are not necessary; 3) minimal quantities of DNA are required; and 4) a high degree of polymorphism is generated when the RAPD technique is used, a result indicating that selecting lines with diverse genetic backgrounds may be less critical than when using RFLP analysis (Weeden, 1991; Williams et al., 1990).

The objective of the present study was to assess the discriminating power of RAPD markers to identify and protect rose cultivar

patents. For this purpose, we surveyed variability patterns produced with eight primers and five rose cultivars. Samples of the following cultivars were provided by Universal Plantas S.A. of Sevilla, Spain: 'Cardinal' (A), 'Sonia' (B), 'Carta Blanca' (C), 'Laser' (D), and 'Carta de Oro' (E). DNA of each cultivar was extracted from ≈ 100 mg of young, unexpanded or partially expanded leaf tissue, because this tissue produced the highest DNA yield. Leaves were stored at -80°C if not used immediately. The extraction procedure was as described by Torres et al. (1993), a method based on (but considerably modified from) that of Lassner et al. (1989). Coprecipitated RNA was eliminated by adding 0.7 units of RNase A. The DNA was dissolved in tris-EDTA, and the final concentration was estimated by agarose gel electrophoresis and ethidium staining, using known concentrations of uncut λ DNA as a standard. To check the repeatability of the fingerprint pattern, more than one plant (in most cultivars) was included in the analysis. Thus, a total of 10 plants was analyzed.

Primers and amplification conditions. Eight 10-base-long arbitrary primers (Operon Technologies, Alameda, Calif.) were used for the experiments (Table 1). DNA amplification conditions were standardized for all primers. Amplification reactions were performed in 25 μl of 20 to 40 ng of plant genomic DNA, buffer [50 mM KCl, 10 mM tris-HCl (pH 8.3), 1.5 mM MgCl_2 , and 0.001% gelatin], and 100 μM each of dNTP, 0.2 μM primer, and 1 unit Taq DNA polymerase (Promega, Madison, Wis.). The reaction was overlaid with mineral oil. Amplification was performed in a Perkin Elmer (Norwalk, Conn.) Cetus DNA Thermal Cycler programmed for 40 cycles with the following temperature profile: 1 min at 94°C , 2 min at 35°C , and 2 min at 72°C , using the fastest available transitions between each temperature. Cycling ended with a final extension at 72°C for 8 min. Amplification products were electrophoresed in 1% agarose, 1% Nu-Sieve (FMC BioProducts, Rockland, Maine) agarose, and $1\times$ tris-borate-EDTA gels and visualized by ethidium staining. Controls lacking template DNA were included on each primer reaction mix used.

Cultivar comparisons. Identifying cultivars by the RAPD technique is possible because each cultivar yields a reproducible DNA band pattern. Since a limitless number of primers can be assayed and several DNA bands can be differentiated for each one, the number of

Table 1. Sequence of the eight oligonucleotide primers used [primer identification (ID) following OPERON's recommendations].

Primer ID	Sequence (5' to 3')
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG

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possible combinations is infinite. In the present work, differences among cultivars were obvious and expressed consistently with most primers. The most discriminatory primers were OPA-06 (Fig. 1) and OPA-08 (Fig. 2), which gave clear differences in banding patterns among cultivars. Discriminatory bands are indicated by arrows in the corresponding figures. For example, using OPA-06, 'Sonia' was discriminated from all other cultivars by the simultaneous presence of two intense bands of ≈ 950 and 1100 bp, while 'Carta Blanca' exhibited the 950 -bp band and a band of ≈ 800 bp (Fig. 1). The remaining cultivars were charac-

terized by 1) a combination of four bands ranging between ≈ 1250 and 950 bp ('Laser'); 2) three bands of ≈ 800 , 1100 , and 1150 bp ('Carta de Oro'); and 3) three bands of 950 , 1100 , and 1250 bp ('Cardinal') (Fig. 1). OPA-08 gave similar results (Fig. 2). With all primers, the DNA banding pattern for each cultivar was consistent. Occasional differences in the intensity of some nondiscriminatory bands between replicates of the same cultivar (e.g., 'Laser' in Figs. 1-3) probably were due to differences in the quality of DNA isolated from the respective samples. The remaining primers gave less conclusive results. OPA-05

(Fig. 3) clearly distinguished 'Sonia' (B), 'Carta Blanca' (C), 'Laser' (D), and 'Carta de Oro' (E), but 'Cardinal' (A) could not be distinguished from 'Laser'. OPA-04 was the only primer that failed to amplify rose genomic DNA and displayed faint, inconsistent bands; hence, OPA-04 was not considered in the analysis. The rest of the primers (Table 1) consistently identified 'Sonia', 'Carta Blanca', and 'Carta de Oro', but 'Cardinal' and 'Laser' were sometimes ambiguously classified (data not shown).

Although additional work is needed to confirm the results obtained in this initial study, our results indicate that the RAPD technique can characterize rose cultivars. This method offers a rapid and relatively inexpensive way to resolve many highly discriminatory bands, thus increasing the probability of correctly identifying cultivars to protect patent rights.

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Fig. 1. Amplifying rose cultivar DNA using the OPA-06 primer: (A) 'Cardinal' (lane 1), (B) 'Sonia' (lanes 2, 6, and 10), (C) 'Carta Blanca' (lanes 3 and 5), (D) 'Laser' (lanes 4 and 9), and (E) 'Carta de Oro' (lanes 7 and 8). Lane 0 = control sample lacking template DNA. Discriminatory bands among cultivars are indicated by arrows. Size marker was derived from Φ x174/Hind III digest.

Fig. 2. Amplifying rose cultivar DNA using the OPA-08 primer. Cultivar designations and corresponding lanes are the same as given in Fig. 1. Discriminatory bands among cultivars (or their absence) are indicated by arrows. Size marker was derived from Φ x174/Hind III digest.

Fig. 3. Amplifying rose cultivar DNA using the OPA-05 primer. Cultivar designations and corresponding lanes are the same as given in Fig. 1. Discriminatory bands among cultivars (or their absence) are indicated by arrows. Size marker was derived from Φ x174/Hind III digest.