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Clonal Stability of RAPD Markers in Three *Rhododendron* Species[1]

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Abstract

Amplification profiles produced by polymerase chain reaction (PCR) using randomly amplified polymorphic DNA sequences (RAPD) have the potential for species and cultivar identification. Since most rhododendron plants are vegetatively propagated, it is imperative that RAPD profiles be stable during this propagation. Three species of rhododendron, *Rhododendron arborescens*, *R. atlanticum* and *R. yedoense* var. poukhanense were used to produce species specific amplification profiles. Stability of amplification profiles among individually cloned plants of each species were studied. Ten plants of *R. atlanticum*, 9 of *R. arborescens*, and 10 of *R. yedoense* var. poukhanense were studied with 10 random primers. No polymorphism was observed among individual plants of *R. atlanticum* and *R. arborescens* with all the primers. The amplification product of one plant of *R. yedoense* var. poukhanense showed a difference of one band with one primer. The rest of the profiles with 9 primers were identical in all plants of this species. In order to ascertain that RAPD markers can indeed reveal real genetic differences among plants, F2 plants of two hybrids were analyzed. In contrast to the clonally propagated plants, extensive polymorphisms were observed among the individual F2 plants. Thus, RAPD analysis can be used to detect genetic variability. This stability of RAPD profiles in clonally propagated rhododendron indicates the usefulness of these markers in plant identification.

Index words: DNA fingerprints, vegetative propagation, plant identification.

Species used in this study: Coastal azalea (*R. atlanticum* (Ashe) Rehd.); Sweet azalea (*R. arborescens* (Pursh) Torr.); Korean azalea (*R. yedoense* var. poukhanense); Adele's Yellow azalea (*R. maximum* x *R. wardii* var. wardii); Yak x Max (*R. yakushimanum* x *R. maximum*).

Significance to the Nursery Industry

Recent reports in the nursery industry have indicated a serious problem with plant identification in rhododendrons. Several nurseries have suffered significant economic loss due to the clonal propagation of mislabeled plants. The results presented in this study demonstrate the potential use of DNA fingerprinting to identify plants at early stages of micropropagation. RAPD analysis was found to be a rapid, reliable and cost effective means of plant identification. The development of this biotechnology will benefit the nursery industry by reducing the economic impact of misidentification of plants during propagation.

Introduction

Wild species, breeding lines and cultivars are important sources of genetic material for use by plant breeders to develop new cultivars or hybrids of economically important plants. This material is also useful to the nursery industry for mass propagation of superior and desirable phenotypes. *Rhododendron* species and cultivars are widely grown throughout the U.S. and are vegetatively propagated by cuttings or via tissue culture. Misidentification of nursery stocks has resulted in major economic losses to nurseries (6). Morphological characters such as leaf and flower shape and color and growth habit, among others, are commonly used to confirm trueness to type of clonally propagated material. These characters are easily influenced by environmental factors such as light and temperature, as well as by growing conditions such as soil type and mineral nutrients, among many others. As such these phenotypic markers are not 100% reliable in plant identification. In addition, many of the morphological markers are observed in plants three to five years after cloning. Thus the nurseries have invested time, money, and labor before plant identification can be confirmed.

Recently molecular markers have been used to identify species, cultivars and/or varieties and are found to be highly reliable. Markers such as isozymes, seed storage proteins, restriction fragment length polymorphisms (RFLPs) and most recently randomly amplified polymorphic DNA (RAPD) profiles have been used. The use of isozymes and seed storage proteins is limited; although they are a product of genetic material, they can be affected by environmental factors. Moreover, polymorphisms of isozymes and seed storage proteins are rather restricted. RFLPs while useful have their own disadvantages. The polymerase chain reaction (PCR) has provided an alternative technique for genetic markers (3, 7, 11). In this technique, two primers of known sequence are usually required to amplify a specific fragment of DNA. However, with the RAPD analysis of Williams et al. (14) and arbitrary primed-PCR technique (AP-PCR) of Welsh and McClelland (13), only one random primer is required, and produces ample polymorphism.

RAPD amplification products reveal a large number of polymorphic patterns among different species and among cultivars (2, 8, 15). RAPD analyses have been recently used

[1]Received for publication October, 1994; in revised form December 5, 1994. This research was funded in part by a grant from the Research Foundation of the American Rhododendron Society and in part by the nursery industry through contributions to the **Horticulture Research Institute, 1250 I Street, N.W., Suite 500, Washington, DC 20005**. We thank Lisa M. McMurphy for editing the manuscript

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to develop species-specific markers for rhododendrons (4). Torres et al. (12) observed different banding patterns among four rose cultivars by RAPD analysis. RAPDs have the potential to reveal unlimited polymorphism due to the availability of a large number of primers or loci for analysis. The presence of a potentially large number of intervarietal and interspecific polymorphisms inevitably raises the issue of variability of individual plants within a species. In this paper, we report on intraspecific stability of amplification profiles of clonally propagated plants of three rhododendron species and individual F2 plants of two hybrids.

Materials and Methods

Plant Material. Ten plants of *R. atlanticum* (Ashe) Rehd., and and nine of *R. arborescens* (Pursh) Torr. were obtained from the Rhododendron Species Foundation (Federal Way, WA). The ten and nine plants were respectively cloned from one parental plant. Ten plants of *R. yedoense* var. poukhanense was obtained from Bright Star Nursery (Hawesville, KY) and also represented clonally propagated plants (the number of original parent plants were unknown). Six F2 plants from the hybrid *R. maximum* x *R. wardii* var. wardii (Adele's Yellow) and seven F2 plants from the hybrid *R. yakushimanum* x *R. maximum* (Yak x Max) were provided by Weldon Delp (Harrisville, PA).

DNA Isolation. Newly unfolding leaves were washed with distilled water and quickly frozen in liquid nitrogen. DNA was isolated following the procedure of Rogers and Bendich (10). To isolate DNA from Adele's Yellow and Yak x Max, three grams of leaf material was washed and ground into a very fine powder in liquid nitrogen. After transferring the plant material into a 50 ml centrifuge tube, 15 ml of hot (65C (149F)) 2X CTAB {2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH 8.0), 1% PVP, 1% 2-merceptoethanol} was added and incubated at 65C (149F) for 30 min with occasional swirling. The mixture was emulsified with an equal volume of chloroform:isoamyl alcohol (24:1) and spun at 4000 rpm for 10 min. The top phase was again emulsified with an equal volume of chloroform:isoamyl alcohol and spun for 10 min at 4000 rpm. The aqueous phase was removed and DNA was precipitated with 0.6 volume of isopropanol. DNA was pelleted at 4000 rpm for 2 min and supernatant was discarded. After adding 10 ml of 70% ethanol to the DNA pellet, it was kept at slow shaker for 20 min and then spun at 4000 rpm for 2 min. The above wash step was repeated and then it was spun for 10 min at 4000 rpm. The pellet was air dried (20 min) and resuspended in 0.5 ml of 0.1X TE. After treatment with RNase, the DNA concentration was measured using a UV-VIS spectrophotometer. DNA was diluted in sterile distilled water at a concentration of 12.5 ng/ul for use in PCR analysis.

PCR and primers. Decamer primers (Operon Technologies Inc., Alameda, CA) were dissolved in distilled water at a concentration of 15 ng/ul. A list of primers used, along with their sequence, is shown in Table 1. A 50 ul amplification reaction contained 10 mM Tris-HCl (pH 8.3 at 25C (77F)), 50 mM KCl, 3.5 mM $MgCl_2$ (Perkin Elmer, Norwalk, CT), 0.1 mM each of dATP, dGTP, dCTP and dTTP (Perkin Elmer, Norwalk, CT), 0.001% gelatin (Sigma, St. Louis, MO), 1 unit of Taq DNA polymerase (BRL, Bethesda, MD), 50 ng of template DNA, and 30 ng of primer. In order to avoid evaporation, the reaction mixture was overlaid with two drops of mineral oil (Sigma, St. Louis, MO). Amplification was conducted in a Hybaid Thermocycler (Hybaid Ltd. Middlesex, U.K.) programmed for first denaturation step at 94C (201F) for 3 min followed by 40 cycles of 94C (201F) for 1 min, 36C (96.8F) for 1 min, 72C (161F) for 2 min, with a ramp of 3 sec/1C (33.8F) between annealing and extension steps. After completion of 40 cycles, reactions were kept at 72C (161F) for 4 min and then held at 25C (77F) for at least 5 min. PCR products were separated on a 1.2% agarose gel and stained with Ethidium bromide which was added to the gel as well as to the 1X Tris Borate EDTA (TBE) buffer.

Table 1. List of primers and their base sequence (primer numbers are standard OPERON's recommended identification numbers.)

Primer number	Sequence (5' to 3')[z]
OPL04	GACTGCACAC
OPL05	ACGCAGGCAC
OPL07	AGGCGGGAAC
OPL11	ACGATGAGCC
OPL12	GGGCGGTACT
OPL14	GTGACAGGCT
OPL15	AAGAGAGGGG
OPL16	AGGTTGCAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG

[z]A = adenine; G = guanine; C = cytosine and T = thymine.

Results and Discussion

As expected from previous experiments, the DNA profiles revealed by RAPD analysis were different for each of the three species examined. These results are a further indication that DNA profiles can be used to distinguish between species in rhododendron. It should be noted that all of the plants from each species were cloned from one original parent plant. Thus, the true interspecific variability was not determined.

Fig. 1. Amplification profiles of 9 *R. arborescens* plants with OPL07 primer. Each lane represents different plants. M = Size marker (1 Kb DNA ladder).

Fig. 2. **a) Amplification profile of 10 *R. yedoense* var. poukhanense plants with OPL05 primer. M = Size marker (1 Kb DNA ladder). b) Amplification profile of ten *R. yedoense* var. poukhanense plants with OPA10 primer. Plant # 8 (lane 8) has a different profile as compared to the other plants. M = 1 Kb DNA ladder (BRL).**

To investigate intraspecific stability of amplification profiles, DNA from individual plants was isolated separately. All amplifications were performed in duplicates. For *R. atlanticum*, all plants gave the same banding pattern with the 10 primers used. The size of amplified fragments varied between 2 Kb to 550 bp and the number of amplification products varied between 2 with OPL05 to 6 with OPL04, OPL12, and OPL16.

The banding patterns generated among the nine *R. arborescens* plants with 10 primers were also consistent (Fig. 1). The number of bands varied from 2 with OPL05 to 6 with OPL04, OPL14 and OPL16 and the size of fragments range from 500 bp to < 2 Kb.

Similarly, a consistent DNA profile of 10 individual *R. yedoense* var. poukhanense plants was observed (Fig. 2a). All amplified bands were present in all 10 plants with nine primers. The number of amplified fragments varied between

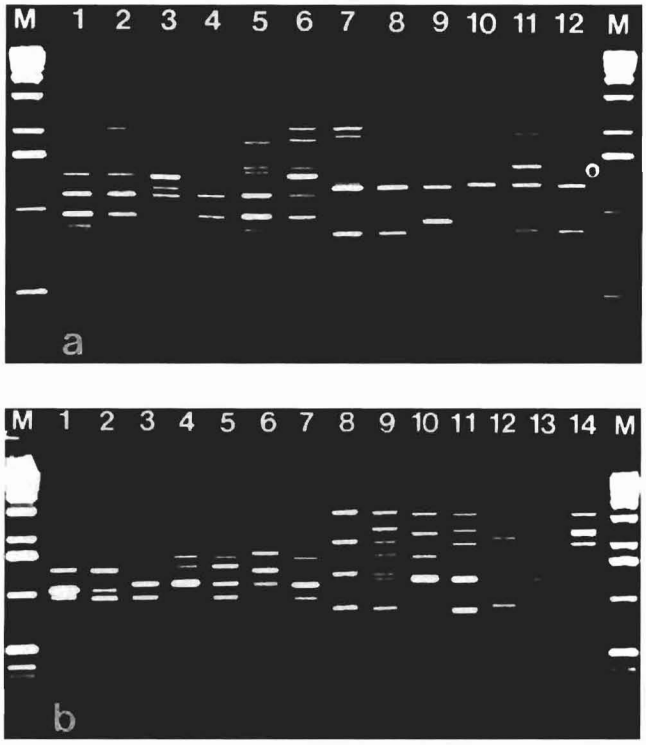

Fig. 3. **a) Amplification profile of six F2 plants of the Adele's Yellow hybrid amplified with OPA09 (lanes 1–6) and OPA10 (lanes 7–12). b) Amplification profiles of seven *R. yakushimanum* x *R. maximum* F2 plants with OPA09 (lanes 1–7) and OPA10 (lanes 8–14) primers.**

2 with OPA10 to 6 with OPL16 and the size of fragments ranged between 500 bp to < 2 Kb. With one primer OPA10, one plant gave a different pattern (Fig. 2b, lane 8). The amplification pattern of this plant did not have an extra band, but a 1.7 Kb fragment was missing. Whether this difference was due to a somatic mutation or an error in laboratory techniques, we do not know.

As compared to the clonally propagated rhododendrons, RAPD profiles revealed extensive polymorphisms among the individual F2 plants. All the six plants of *R.* Adele's Yellow showed polymorphisms with all the ten primers studied (Fig. 3a). Similarly, the seven plants of Yak x Max showed polymorphisms with all the primers (Fig. 3b). These F2 plants represent a genetically segregating population. RAPD analysis was successfully used to identify the large amount of genetic polymorphisms among these plants.

Variations in length or sequence of DNA can be used to measure the degree of relatedness between individual organisms, or inheritance in progeny populations (1). In clonally propagated rhododendrons, variation of a single band with one out of ten primers which amplified 42 DNA fragments is not significant as compared to the polymorphisms observed among F2 plants. These results are similar to the our previous results observed in rye which indicated a relationship between RAPD polymorphism and genetic diversity (5).

The insignificant variability among vegetatively propagated individual rhododendron plants signifies their use as a tool for varietal or clonal identification in nurseries. The polymorphisms among individual plants of the two rhododendron hybrids documents the ability of RAPD markers to

identify true genetic diversity. RAPDs, therefore should provide excellent markers for the identification of genetically distinct plants. Such markers would be useful in varietal identification by nurseries and arboreta and minimize the propagation of unwanted or mislabeled plants.

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