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Production of Reliable Randomly Amplified Polymorphic DNA (RAPD) Markers from DNA of Woody Plants

Amnon Levi, Lisa J. Rowland, and John S. Hartung
Fruit Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, Beltsville, MD 20705

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Abstract. **A procedure for identifying reproducible RAPD markers from woody plant DNA is presented. The procedure relies on using a PCR buffer that contains 1% Triton-X-100 and 0.1 % gelatin [previously described for successful polymerase chain reaction (PCR) amplification of 16S/23S rRNA intergenic spacer regions from eubacteria], and amplification conditions of 50 cycles: 30 sec at 94C, 70 sec at 48C, and 120 sec at 72C. The combination of this buffer and these conditions amplified consistent fragments in higher amounts, as compared to other standard PCR buffers and conditions generally used for RAPD analysis. This procedure resulted in reliable RAPD patterns for all organisms tested. Chemical name used: α-[4-(1,1,3,3,-tetramethylbutyl)phenyl]-co-hydroxypoly(oxy-l,2-ethanediyl) (Triton-X-l00).**

The polymerase chain reaction (PCR) method of DNA amplification (Saiki et al., 1988) has provided molecular biology with novel tools and applications. One of these applications is the use of short primers to amplify polymorphic DNA sequences randomly (RAPDs; for reviews see Hadrys et al., 1992; Tingey et al., 1992; Weeden et al., 1992). RAPDs have generated great interest among plant geneticists because these fragments can be useful markers for constructing genetic linkage maps (Williams et al., 1990), tagging chromosomes (Martin et al., 1991), studying populations and phylogenetics (Van Heusden and Bachmann, 1992; Vierling and Nguyen, 1992), and identifying varieties (Caetano-Anolles et al., 1991; Hu and Quiros, 1991). Additionally, RAPD methodology is technically simple, can be performed quickly, requires only small amounts of DNA, and involves no radioactivity (Munthali et al., 1992; Tingey et al., 1992). However, many investigators, including ourselves, have been confronted with the problem of a lack of reproducible results.

Our initial experiments revealed inconsistencies in RAPD fragment patterns even between aliquots of the same reaction cocktail that were incubated in adjacent wells of the same thermocycler. Also, we generally observed low amplification of DNA fragments. Since the DNAs used in our experiments had normal spectrophotometric absorbance (A260/A280 = 2) and were intact and free of RNA, the inconsistencies in our early experiments could not be attributed to poor DNA quality. Therefore, our efforts focused mainly on optimizing the experimental conditions for obtaining reproducible results, which is a prerequisite for our goal of constructing a genetic linkage map for blueberry *(Vaccinium,* section *Cyanococcus)* using RAPD markers. Here, a PCR buffer, previously used by Barry et al. (1991) for amplifying the 16S/23 S rRNA intergenic spacer regions from eubacteria, has been used for reliable RAPD analysis of blueberry DNA.

We describe a method that uses stringent conditions for annealing primer and template DNA while maintaining favorable conditions for DNA amplification. This method can be used for identifying reliable RAPD markers from DNA of various organisms.

Materials and Methods

Lyophilized dATP, dCTP, dGTP, and dTTP (Sigma Chemical Co., St. Louis) were used to prepare working solutions that contained 2.5 mM of each nucleotide and were stored at –20C. Triton-X-100 (electrophoresis grade) and gelatin (granular) were purchased from Fisher Scientific (Pittsburgh). Primers (single-stranded 10 base oligonucleotides), purified by elution with Tris/EDTA buffer through NAP-5 drip columns, were purchased from the Biotechnology Laboratory, Univ. of British Columbia, Vancouver. Working solutions of 15 μM primer in H_2O were prepared and stored at –20C. Blueberry, strawberry *(Fragaria* × *ananassa* Duch), cherry *(Prunus avium* L.), peach *(Prunus persica* L. Bastch.), apple *(Malus domestics* Borkh.), and pear *(Pyrus communis* L.) leaf DNAs were isolated using a hexadecyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987) primarily based on one from Murray and Thompson (1980) and further modified for isolating DNA from woody plants by adding more CTAB after the chloroform extraction step (as-described by Rowland and Nguyen, 1993). Genomic DNA was isolated from *Xanthomonas campestris* pv. citri. (Hasse) Dye. and *Xylella fastidiosa* Wells et al. as described previously (Hartung and Civerolo, 1987).

Our experiments to optimize reaction conditions employed five $MgCl_2$ concentrations; three sources of Taq-DNA-polymerase (Perkin Elmer, Norwalk, Corm.; Promega, Madison, Wis.; and Stratagene, La Jolla, Calif.) used at three concentrations; nine primer : template ratios; and nine annealing temperatures, three durations of the DNA denaturation temperature, and four numbers and patterns of thermal cycles (Table 1). Test amplification reactions for blueberry DNA were performed in 25μl volumes that contained one of the following buffers: 1) 10 mM Tris-HCl (pH 8.3) and 50 mM KC1; 2) 10 mM Tris-HCl (pH 9.0), 50 mM KC1, and 0.1 % Triton-X-100; or 3) 50 mM Tris-HCl (pH 9.0) and 20 mM NaC1. Various concentrations of Triton-X-100 (0% to 2%) and gelatin (0% to 0.5%); 100 to 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.12 to 0.36 μM primer; 0.2 to 2 ng genomic DNA/pi; 0.014 to 0.036 units Taq-DNA-polymerase/μ1; and 0.5 to 4 mM $MgCl_2$ also were used.

Table 1. Optimum concentrations and conditions for RAPD protocol.

Variable	Concentrations or conditions	
	Evaluated	Optimum
Buffers[z]	1,2, and 3	3
Triton-X-100[y]	0%, 0.01%, 0.1%, 1%, 2%	1%
Gelatin	0%, 0.01%, 0.1%, 0.5%	0.1%
dATP, dCTP, dGTP, and dTTP	100, 200 μM	200 μM of each
Primer	0.12, 0.2, 0.36 μM	0.2 μM
Template	0.2, 0.5, 1, 2 ng/μl	0.5–1 ng/μl
Taq-DNA-polymerase	0.014, 0.028, 0.036 units/μl	0.028 units/μl
$MgCl_2$	0.5, 1, 1.6, 2, 4 mM	1.6 mM
Annealing temp (°C)	36, 38, 40, 42, 45, 48, 49, 50, 51	48 and 49
Incubation time (sec)		
94C (denaturation)	30, 40, 45, 50, 60	30–45
48C (annealing)	50, 60, 70, 90	70
72C (elongation)	60, 90, 120	120
No. cycles	40, 45, 50, 56	50

[z]For content of buffers 1, 2, and 3, see Material and Methods section.
[y]All remaining variables were optimized using buffer 3.

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DNA was amplified using thermal cycle programs of 40 to 56 cycles of 30 to 60 sec at 94C, 50 to 70 sec at 36 to 48C, and 120 sec at 72C (Table 1). After reaction conditions were optimized, amplification was tested in four thermocyclers: the original Perkin Elmer thermal cycler, Hybaid thermal reactor (Hybaid, Teddington, Middlesex, U.K.), MJ programmable thermal controller (model PTC-100; MJ Research, Watertown, Mass.), and ISS Programmable Oven II (Integrated Separation System, Natick, Mass.).

Results

Our experiments indicated that the combination of buffer type, annealing temperature, and duration of incubation at 94C (for DNA denaturation) were most critical for obtaining a high amplification rate and reproducible phenotypes. Standard PCR buffers that contained 10 to 50 mM Tris-HCl, 10 to 50 mM KC1, 1 to 4 mM $MgCl_2$, and no stabilizing detergents, as well as annealing temperatures of 36 to 42C, all resulted in poor amplification and nonreproducible phenotypes. In contrast, adding 0.1% to 2% Triton-X-100 to a buffer containing 50 mM Tris-HCl (pH 9.0) and 20 mM NaCl, and incubating it at a 48C annealing temperature significantly improved DNA amplification and resulted in consistent phenotypes (Fig. 1). Furthermore, a 30- and 15-sec incubation at 94C (to denature blueberry and *Xanthomonas* DNAs, respectively) increased the amplification products by a few fold compared to a 60-sec incubation (data not shown). Yu and Pauls (1992) reported that a 30-sec incubation at 94C was optimal for amplifying RAPD fragments from alfalfa DNA using primers with 50% to 80% guanine and cytosine (GC) content, whereas a 5-sec incubation was sufficient for primers with 70% to 80% GC. The longer incubation time (60 see) at 94C likely resulted in inactivation of Taq-DNA-polymerase and, consequently, low amplification. The addition of gelatin was not as effective as Triton-X-100. However, the combination of 1% Triton-X-100 and 0.1% gelatin was optimal for consistent amplification of 0.1- to 3-kb fragments (Fig. 1), as were 0.5 to 1 ng template DNA/µl, 0.2 µM primer, and 1.6 mM $MgC1_2$ (Table 1).

From the results described above, the following RAPD protocol was developed for obtaining optimal amplification levels. A stock buffer (10×) containing 200mMNaCl, 500 mM Tris-HCl (pH 9.0), 10% Triton-X-100, and 1% gelatin was prepared and autoclave. After being autoclave, the warm solution was cloudy but became clear when cooled. A 25-µl reaction solution contained l× stock buffer; 1.6 mM $MgC1_2$; 0.2 µM primer; 1 ng template DNA/pi; 200 µM each of dATP, dCTP, dGTP, and dTTP; and 0.028 units Taq-DNA-polymerase (Promega)/µl. The reaction solution was mixed and centrifuged for 2 min at 2 × 10^3× *g*, and an equal volume of mineral oil was added. The samples were incubated through 50 thermocycles of 40 sec at 94C, 70 sec at 48C, and 120 sec at 72C while employing the fastest attainable transition between each temperature. The amplification products were analyzed by electrophoresis in 1.4% agarose gels containing 0.5 µg ethidium bromide/ml.

Discussion

Triton-X-100 (nonionic detergent, emulsifier) and gelatin (a heterogeneous mixture of water-soluble proteins of high molecular weight) possibly decreased the relative volume of the reaction cocktail and, thus, maximized template **x** primer interaction; while the relatively high annealing temperature (48C) may have increased template/primer specificity. The ability of template DNA and primer to anneal at 48C also suggests that the presence of Triton-X- 100 and gelatin stabilized DNA/DNA hybrids and, thus, raised their melting temperature. Our experiments with annealing at 36 to 42C and using standard buffers resulted in inconsistent products even among aliquots of the same reaction solution when incubated in different wells of the same thermocycler. Initially, we attributed this inconsistency to a flaw in thermocycler performance as shown by Linz (1990). However, the use of Barry's buffer (Barry et al., 1991), higher annealing temperature (48 C), and short denaturation time (30 see) nullified any differences between well performance and produced consistent results and higher amplification in all thermocyclers tested. The inconsistencies in our early experiments could not be attributed to DNA quality because, in contrast to our initial experiments, the same DNAs yielded consistent patterns using the RAPD protocol presented here. Compared with other RAPD protocols, where only 35 to 40 therrnocycles are required, this procedure requires≈50 cycles for optimal amplification. The stringent reaction conditions likely resulted in fewer amplification products in the first cycles. Therefore, more cycles are required to obtain sufficient amplification.

Using the RAPD protocol described here, we have been able to screen 180 primers and identify >80 RAPD markers that have been useful for mapping the blueberry genome. Reactions with primers with 70% to 80% GC content consistently produced more amplified DNA fragments (2–15) than reactions with 50% to 60% GC (0-8). Only a few (≈12 out of 35) primers with 50% GC content produced amplified fragments. For some plant species, the GC content of a primer is the best indicator of whether it will result in amplification (Fritsch et al., 1993). The higher the GC content, the higher the likelihood of amplification.

This procedure's reliability was confirmed using our test-cross population of diploid blueberry plants. Various primers revealed fragments common to all the plants in the population and fragments that segregated among the progeny (Fig. 2) at the expected 1:1 ratio. Using this procedure, we have been able to construct a genetic linkage map for blueberry that currently consists of 72 RAPD markers and 12 linkage groups corresponding to the basic blueberry chromosome number (Rowland and Levi, 1994). This procedure also was amenable for generating RAPDs from strawberry, cherry, peach, apple, and pear DNAs, as well as for standard PCR analysis of

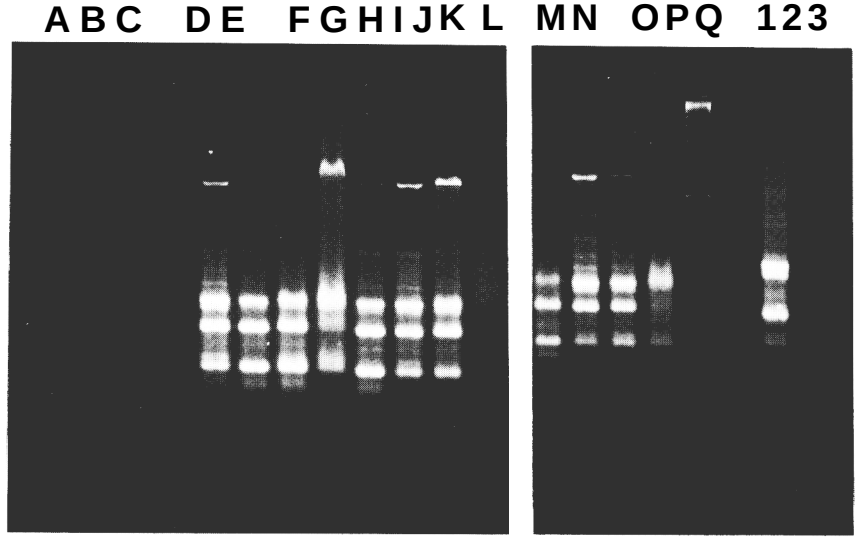

Fig.1.Amplificationofblueberry *(Vaccinium darrowi* Camp.,Florida-4B) DNA in solutions containing 50 mM Tris-HCl (pH 9.0) and 20 mM NaCl (buffer 3), and various concentrations of Triton-X- 100 and gelatin(lanes A-P).Tritonand gelatin concentrations, respectively, were: A (0, 0), B (0, 0.01%), C (0, 0.1%), D (0, 0.5%), E (0.1%, 0), F (0.1%, 0.01%), G (0.1%, 0.1%), H (0.1%, 0.5%), I (l%, 0), J (l%, 0.01%),K(l%, 0.l%), L(l%, 0.5%), M (2%, 0), N(2%, 0.01%), O (2%,0.1%), and P(2%, 0.5%). (lanes ; 1–3, respectively)Amplificationofblueberry *(V. elliotti* Champ.,Knight)DNA in solutions containing buffer 2,buffer3with1%Triton-X-100 and 0.1 % gelatin, and buffer 1. All reaction solutions had a final volume of 25 µl and contained 1 ng template DNA/µl; 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.2µM of primer; 0.028 units Taq-DNA-polymerase (Promega)/µl; and 1.6 mM $MgCl_2$. The DNA was amplifiedusingtheprimer5'-ATCTGGCAGC.Amplification was obtained through 50 cycles of 30 sec at 94C, 70 sec at 48C, and 120 sec at 72C. Lane Q is a l-kb ladder (GIBCO-BRL, Gaithersburg, Md.).

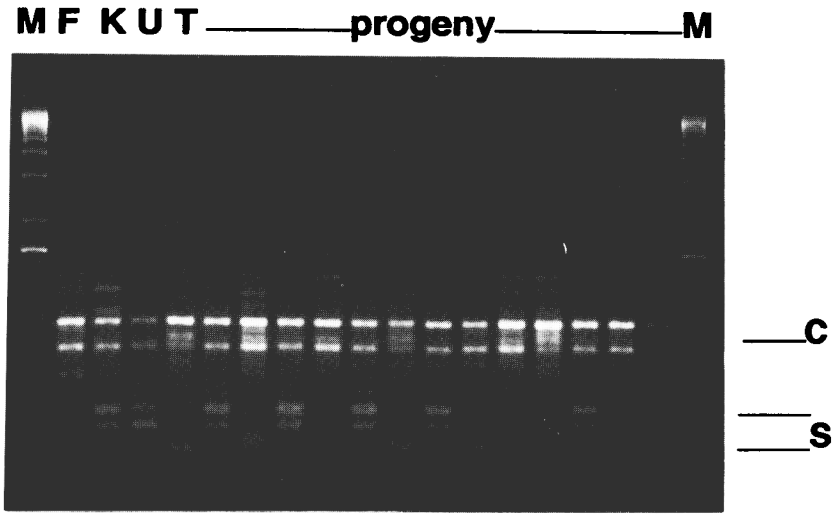

Fig. **2.** AmplificationofblueberryDNAs using the described protocol. RAPD fragments generated from DNAoforiginalparentplants *Vaccinum darrowi* Camp.[Florida-4B (F)] and *V. elliotti* Champ. [Knight (K)], their F_1 hybrid US-388 (U), test-cross parent *V. darrowi* Camp. [US-799 (T)], and progeny plants of the test-cross population (US-388 × US-799). Note common (C) and segregating fragments (S). The two segregating fragments shown here, in fact, cosegregate and, thus, were placed at the same location on the genetic map. M is a 1-kb DNA ladder (GIBCO-BRL, Gaithersburg, Md.). DNA was amplified using primer 5'-GAGAACTGGC.

theGramnegativephytopathogenicbacterium *Xanthomonas **campestris*** pv. citri (Hartung et al., 1993) and RAPD-PCR analysis of *Xylella fastidiosa*, responsible for citrus canker and variegated chlorosis, respectively (data not shown).

The procedure presented here is reliable and can be used for genome mapping and chromosome tagging in many plant and bacterial species.

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