

METHODOLOGY

RAPD-PCR amplification of soybean DNA using pairwise combinations of primers

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ABSTRACT

In order to increase the number of detectable polymorphisms for the construction of a RAPD-PCR based intraspecific soybean genetic map, two primers were used at a time, instead of only one. The DNA patterns obtained with each combination of primers were analyzed for intensity and number of bands, as well as for reproducibility. DNA samples of two soybean progenitors were tested initially with 72 random primers and 36 random pairwise combinations of these primers. Polymorphic fragments generated with the combinations of primers segregated 3 to 1 in an F₂ population of 68 individuals derived from these progenitors. Linkage analysis of some of the polymorphic bands revealed that they did not co-segregate with bands generated with the isolated primers. Our data show that pairwise combinations of primers can facilitate the detection of RAPDs.

INTRODUCTION

The RAPD technique uses single primers with random sequences to amplify discrete DNA fragments, which makes it appropriate to reveal polymorphisms among individuals (Williams *et al.*, 1990). It has been used with success on several species of agronomic interest, such as the common bean (Haley *et al.*, 1994), soybean (Vilarinhos *et al.*, 1994), tomato (Martin *et al.*, 1991), maize (Heun and Helentjaris, 1993), peach (Chaparro *et al.*, 1994), alfalfa (Yu and Pauls, 1993), *Eucalyptus* (Grattapaglia and Sederoff, 1994) and lettuce (Kesseli *et al.*, 1994).

We started to build a RAPD-PCR-based intra-specific genetic map for soybean (*Glycine max* L. Merrill), however, the number of polymorphisms available was relatively small due to the narrow genetic base of cultivated soybean (Abdelnoor *et al.*, 1995). In an effort to increase the number of detectable polymorphisms, we tested pairwise combinations of different primers. Although this procedure has been tried before (Williams *et al.*, 1993), it has not been tested in depth, possibly due to the extensive polymorphism normally detected with single primers in most species.

MATERIAL AND METHODS

The genetic material consisted of soybean lines UFV 91-717 and Ichigowase, an F₁ plant derived from a cross between these lines, and an F₂ population of 68

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individuals. Leaf DNA samples were extracted from young leaves according to Doyle and Doyle (1990).

Amplification reactions of 25 μ l contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.8 mM MgCl₂, 0.1 mM of each deoxynucleoside triphosphate, 0.4 μ M of a single primer or a pair of primers (Operon Technologies, Alameda, CA, USA), 1 unit Taq DNA polymerase, and 25 ng of DNA. The thermocycler model 9600 (Perkin Elmer-Cetus, Norwalk, CT, USA) was programmed for two cycles at 94°C for 2 min, 35°C for 1 min, and 72°C for 1 min; two cycles at 94°C for 1 min, 35°C for 30 s and 72°C for 1 min, and finally 40 cycles at 94°C for 15 s, 35°C for 30 s and 72°C for 1 min, and an additional elongation step at 72°C for 5 min.

Seventy-two arbitrary primers were tested individually and in 36 random pairwise combinations to amplify the DNA samples extracted from the soybean progenitors. The final concentration of primer(s) in all amplification reactions was 0.4 μ M. For the reactions containing two primers at a time, three proportions of primers were tested, 3:1, 1:1, and 1:3. Five of the primer combinations were used to amplify the DNA extracted from the F₂ population. The chi-square test was used to check the segregation ratios obtained. The software MAPMAKER.EXP 3.0b (Lander *et al.*, 1987) was used to analyze the segregation of the DNA bands. A LOD score minimum of 3.0 and a maximum percentage of recombination of 35% were used to establish linkage groups.

The DNA amplification products were separated electrophoretically on 2.8% agarose gels immersed in TBE (90 mM Tris-borate, 1 mM EDTA), stained with ethidium bromide and photographed under UV light.

RESULTS AND DISCUSSION

Among the 36 random pairwise combinations of primers tested with DNA samples of the two soybean progenitors, 15 generated a total of 20 polymorphic bands which were different from those obtained with the respective isolated primers (Figure 1). On average, each individual primer generated 5.3 bands, whereas 6.2 bands were produced for each combination of primers. Williams *et al.* (1993) obtained only 4.4 bands per combination of primers, while testing 21 pairs of primers to differentiate soybean cultivars. About one third of the DNA fragments generated with associations of primers were smaller than those produced with the individual primers. Additionally, most of the combinations of primers tested produced a sharper band pattern

when the ratio between the primers was 1:1 (Figure 1). When five of the combinations of primers were tested by another individual, 100% of the medium to intense bands were reproduced. However, about 8% of the fainter bands could not be reproduced (data not shown). Figure 1 illustrates what is meant by faint, medium and intense DNA bands.

To determine if the polymorphic bands generated with combinations of primers could be used as genetic markers, five of the associations that showed polymorphism between the progenitors were tested in an F₂ population of 68 individuals. All seven bands generated with the combinations of primers, and the eight bands produced with the respective isolated primers segregated at a ratio of 3:1 (Table I). Among these 15 bands, five segregated independently, whereas 10 formed five linkage groups (Table II). None of the markers generated with combinations of primers co-segregated with bands produced with the respective isolated primers, confirming that they map in different regions of the genome.

These data confirm that combinations of primers in RAPD reactions can generate reliable markers and can be used to increase the number of polymorphisms detected.

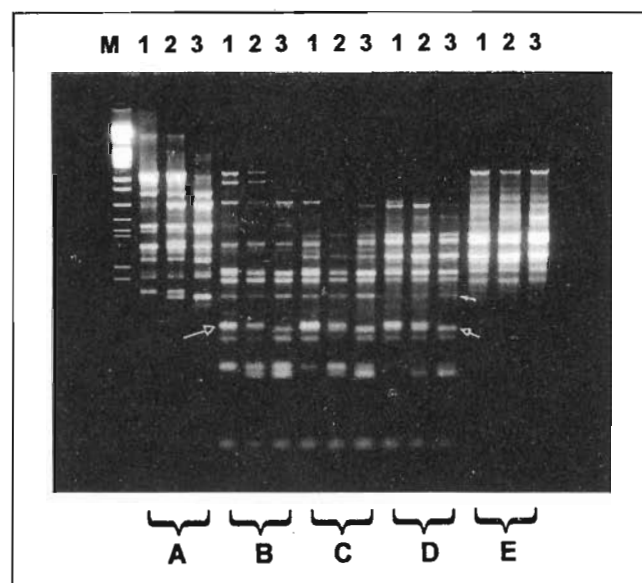


Figure 1 - Electrophoretic analysis of DNA amplification products obtained with two isolated primers and with combinations between them. Amplification reactions were done with: A, 0.4 μ M of primer OPAX09; B, 0.3 μ M of OPAX09 plus 0.1 μ M of OPAQ04; C, 0.2 μ M of OPAX09 plus 0.2 μ M of OPAQ04; D, 0.1 μ M of OPAX09 plus 0.3 μ M of OPAQ04; E, 0.4 μ M OPAQ04. Lane M was loaded with DNA from lambda phage digested with *Eco*RI, *Hind*III and *Bam*HI (size markers). DNA samples were extracted from 1- soybean line UFV 91-717, 2- F₁ (UFV 91-717 X Ichigowase), and 3- line Ichigowase. Arrows indicate a typical faint DNA band (small arrow), a typical medium band (medium arrow), and a typical intense band (large arrow).

Table I - Segregation analysis of DNA amplification products generated by five pairwise combinations of primers and by their respective isolated primers.

Primers	P	Band size (bp)	F ₂		χ^2 ^a
			+	-	
OPAK05	a	435	49	19	0.314
OPAK06	b	2,055	50	18	0.078
OPAK05 + OPAK06	b	120	57	11	2.824
OPAK05 + OPAK06	b	40	50	18	0.078
OPAK19	b	1,185	57	11	2.824
OPAK19	a	470	50	18	0.078
OPAN01	*				
OPAK19 + OPAN01	b	715	54	14	0.706
OPAM09	*				
OPAM14	*				
OPAM09 + OPAM14	b	330	56	12	1.961 ^{ns}
OPAQ04	*				
OPAX09	a	1,590	50	18	0.078
OPAQ04 + OPAX09	a	290	57	11	2.824
OPAQ04 + OPAX09	b	170	55	13	1.255
OPAT14	a	865	55	13	1.255
OPAT14	a	795	47	21	1.255
OPAU15	a	710	52	16	0.078
OPAT14 + OPAU15	a	1,215	51	17	0.000

P, Origin of polymorphic band: a, from progenitor UFV 91-717; b, from progenitor Ichigowase; (*), no polymorphic band; F₂, number of F₂ individuals with (+) or without (-) the polymorphic band.

a, The expected phenotypic ratio is 3:1. No significant differences were found between observed and expected ratios with one degree of freedom and 0.05 probability.

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RESUMO

Para aumentar o número de polimorfismos que pudessem ser detectados durante a construção de um mapa intra-específico de soja, baseado em marcadores moleculares do tipo RAPD-PCR, essa técnica foi modificada pelo uso de dois oligonucleotídeos iniciadores de cada vez. Os padrões eletroforéticos de fragmentos de DNA gerados com cada combinação de iniciadores foram analisados quanto à intensidade, número de bandas e reprodutibilidade. Amostras de DNA de dois progenitores de soja foram testadas com 72 iniciadores aleatórios e 36 pares desses iniciadores. Fragmentos polimórficos gerados pelas combinações de iniciadores

Table II - Linkage analysis of 15 RAPD markers in an F₂ soybean population.

Linkage group	Markers	Genetic distance (cM)
A	(OPAK05 + OPAK06) ₁₁₇ (OPAM09 + OPAM14) ₃₂₈	1.7
B	(OPAM09 + OPAM14) ₃₂₈ (OPAQ04 + OPAX09) ₂₈₈	4
C	OPAK19 _{1,185} (OPAX09+OPAQ04) ₂₈₈	12
D	OPAT14 ₇₉₅ OPAK05 ₄₃₅	18
E	OPAK19 ₄₇₃ OPAK06 _{2,054}	35
Unlinked markers		
(OPAK19+OPAN01) ₇₁₄ ; (OPAT14+OPAU15) _{1,215} ; OPAX09 _{1,592} ; OPAT14 ₈₆₆ ; OPAU15 ₇₁₂		

segregaram na proporção de 3:1 em 68 indivíduos de uma população F₂ derivada dos progenitores mencionados. A análise de ligação genética de algumas dessas bandas polimórficas revelou que elas não co-segregaram com as bandas geradas pelos iniciadores isolados. Os dados obtidos mostram que o uso de combinações de iniciadores representa uma ferramenta adicional para a detecção de polimorfismos do tipo RAPD.

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