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Reproducibility of the differential amplification between leaf and root DNAs in soybean revealed by RAPD markers

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Abstract Random amplified polymorphic DNA (RAPD) was used to determine whether such markers can be employed for detecting genomic modification during plant development or under certain stress environments. Pairwise comparisons in RAPD patterns of leaf and root DNA amplifications were studied for 11 soybean accessions representing different origins. Hydroponic culture was used for the ease of harvesting roots. From a total of 40 primers screened, it was found that 16 can detect leaf DNA polymorphism, 19 for root DNA polymorphism, while 10 show a greater consistency for detecting polymorphism between leaf and root (L/R) DNAs. Nevertheless, problems were encountered when the newly synthesized oligo-primers and different thermal cyclers were used to check the data. Several factors were then tested for their reproducibility. The results indicated that the amplified differences between root and leaf DNAs are mostly not affected by template DNA concentrations. The addition of DMSO (dimethyl sulphoxide) or TMAC (tetramethyl-ammonium chloride) also did not mask the L/R differences. However, DNA polymerase and oligo-primers synthesized from different manufacturers, as well as the thermal cyclers, reacted differently sometimes. Regardless of the general problems of reproducibility in RAPD patterns, some amplified differences remain between the L/R DNAs. The most distinct patterns involve differences in the relative intensity of amplified bands. Differential amplification might have occurred during plant leaf and root development. Southern hybridization of the eluted polymorphic bands against restriction digestion of total genomic DNA confirms their being homologous to soybean DNA fragments. Polymorphism of these specific L/R differences also exists among varieties. RAPD should be a useful tool in detecting genomic alterations during plant development or under certain stress environments, as long as the factors affecting the reproducibility of RAPD patterns can be properly controlled. An additional cycle of selection would be possible if such a type of polymorphism is proved to be correlated with certain developmental characters.

Key words Root · Leaf · Random amplified polymorphic DNA · Polymerase chain reaction · *Glycine max* L. Merr.

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Introduction

Random amplified polymorphic DNA (RAPD) derived from AP-PCR (arbitrarily primed polymerase chain reaction) was first reported, almost simultaneously, by two research groups (Welsh and McClelland 1990; Williams et al. 1990). Since then, over 1000 articles have been published on its applications (McClelland et al. 1995). The popularity of RAPD studies is due to its rapidity, simplicity and automation in operation, coupled with the fact that DNA sequence information is not necessary for primer design, no radioisotope labelling is needed for sample detection, and only a small amount of template DNA is required (Williams et al. 1990). Various applications of RAPD have been summarized by Newbury and Ford-Lloyd (1993). Young (1993) proposed that DNA markers such as restriction fragment length polymorphisms (RFLPs) and RAPDs can be used in detecting changes in genomic organization during plant development or in response to environmental signals. Rapid genomic changes involving chromosome rearrangement,

chromosome imprinting, gene amplification, loss, and transposable elements within the lifetime of an organism have been addressed by Walbot and Cullis (1985). In plants, phenomena such as nuclear DNA variation, endoreplication, DNA amplification, DNA rearrangement and DNA methylation are known to occur during plant differentiation and development or under certain stress environments (D'Amato 1964; Nagl 1981; Van't Hof et al. 1983; Kraszewska et al. 1985; Murry et al. 1987; Vanyushin and Kirnos 1988; Kowles et al. 1990; Shang and Wang 1991). Nagl (1981) suggested that, during plant development, DNA replication in endocycles can follow different pathways: either the total genome is replicated (i.e. endoreplication or endomitosis), or a small portion of nuclear DNA is not replicated, or is not as often replicated, as the main part of the genome (i.e. DNA under-replication), while in a few cases a small portion of the genome is extra-replicated (i.e. DNA amplification). We are interested in determining whether the RAPD markers are capable of detecting a difference in such types of genomic modification during plant development. Our earlier observation on the comparison of RAPD patterns within genotypes of leaf and root DNAs in soybean have indicated that there were amplification differences between these two types of DNA (Chen et al. 1994). Nevertheless, this type of variation suffers from problems of reproducibility in successive studies.

Several reports have emphasized the problem of reproducibility in RAPD studies. For example, Devos and Gale (1992) reported that factors such as the concentrations of template DNA, Mg^{2+}, *Taq* DNA polymerase and the denaturing temperature all tended to affect the RAPD patterns. Penner et al. (1993) indicated that different laboratories amplified different size ranges of DNA fragments and that reproducibility among laboratories sometimes depended on the primer. Meunier and Grimont (1993) noted that, in addition to between-oligonucleotide and between-DNA variations, between-thermal cycler and between-DNA polymerase variations were also observed. Similar results on the effects of different DNA concentrations, primers and thermal cyclers have also been documented (MacPherson et al. 1993; Schierwater and Ender 1993). Other factors, such as RNA levels, the inclusion of dimethyl sulphoxide (DMSO) and tetramethyl-ammonium chloride (TMAC), are also known to affect PCR amplification (Shen and Hobn 1992; Vierling and Nguyen 1992; Pikaart and Villeponteau 1993). Studies of snap bean (*Phaeolus vulgaris*) genotypes, indicated that both qualitative and quantitative variations occurred in RAPD patterns (Skroch and Nieuhuis 1995 a). A 2% data-scoring error and 76% reproducibility were observed in their successive estimations (Skroch and Nieuhuis 1995 b).

In the present communication, we report the differential amplification in RAPD patterns of leaf and root DNAs in soybeans grown under hydroponic culture and reveal factors that might affect the reproducibility of these RAPD differences in soybean. The possible causes of this type of variation, and the advantages of using this method in detecting genetic modification during plant development or in stress environments, are also discussed.

Materials and methods

Plant material and DNA extraction

A total of 11 soybean lines, representing two local land varieties of Taiwan (FWCP and HCWT), two cultivated varieties (TN 15 and AGS-58), two pairs of isogenic lines derived from F_5 selfed progeny (BB120 W, BB120P, BB105 W and BB105 P), and three plant introductions (TARI-5, TARI-23 and TARI-26) were obtained from the Taiwan Agricultural Research Institute. Seeds of soybean were germinated and grown under hydroponic culture at a 28°C-day/24°C-night temperature in a growth chamber. Nutrient components in hydroponic cultures were 2.5 mM KNO_3 and $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 20 ppm FeNaEDTA and microelements (Barrentine et al. 1976). Trifoliolate leaves, at the V3 stage (Fehr and Caviness 1977), and roots were sampled individually from each accession for DNA isolation. The procedures for DNA extraction were similar to those of Murray and Thompson (1980), as described in Chen et al. (1993). A TKO 100 fluorometer (Hoeffer Science Instrument, Calif., USA) was used for DNA quantitation. DNAs were further diluted to 100 ng/μl with TE (10 : 0.1) and served as template DNA for the polymerase chain reaction (PCR).

Arbitrarily primed polymerase chain reaction (AP-PCR)

The primers used for this study included sequencing primers (M13, M13R, KS17, SK17 and T7), oligonucleotide-synthesized decamers, including four from Williams et al. (1990), and 20 OPF 10-mers from Operon Technologies Inc. (Alameda, Calif., USA), as well as partial sequences obtained from some known genes such as the soybean actin gene, soybean glycinin gene, pea rbcs gene, tobacco peroxidase gene and the rice salt-induced protein gene. The original sources and sequences of these primers are listed on Table 1.

Unless otherwise described, a Techne pHC-3 Dri-Block Cycler (Techne Ltd., UK) was used for temperature control and cycling. For those primers with 17–22-mers, three cycles at 94°C for 5 min, 40°C for 5 min and 72°C for 5 min, respectively, for denaturing, annealing and primer extension were initiated and then followed by 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. For all the decamers, 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min were programmed. The reaction components in the PCR reaction include 50 mM KCl, 10 mM Tris-HCl (pH 8.3) dNTP (dATP, dCTP, dGTP and dTTP each 200 μM), 1.0 μM of primer and 3.0 mM of $MgCl_2$. The amount of template DNA for each reaction is 100 ng and the concentration of Ampli-*Taq* DNA polymerase (Perkin Elmer Cetus) is 2.5 units/100 μl-reaction. Generally, a 50-μl reaction volume per tube was used.

After the cycles, 15 μl of amplified DNA for each sample were loaded in 2.0% agarose gels in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) and run at 60 V for about 7 h. The DNA molecular-weight-marker VI (pBR 328 DNA-*Bgl*I + pBR328 DNA-*Hinf*I, Boehringer Mannheim) was used as a molecular standard. The gel was stained with 10 ppm of ethidium bromide solution, de-stained with 1 × TBE buffer, and then photographed under UV light with Polaroid film 667. The band pattern classification is similar to that of Hu and Quiros (1991) with the primer name following the estimated base pairs. All reactions were repeated at least twice and the reproducible bands were recorded for each

Table 1 Summary of primer sources, number of bases and the sequences used for RAPD analysis

Primer name	Sequences 5′ to 3′	No. of base	Primer origin[a]
M13	GTAAAACGACGGCCAGT	17	Sequencing primer
M13R	AACAGCTATGACCATG	16	Sequencing primer
KS17	CGAGGTCGACGGTAT	17	Sequencing primer
SK17	TCTAGAACTAGTGGATC	17	Sequencing primer
T7	GTAATACGACTCACTATAG	19	Sequencing primer
Act-22	AAGCTGTTCTCTCCTTGTATGC	22	Soybean actin gene[1]
Act1R-22	GGTGTCCTTCACGAAGAT	22	Soybean actin gene[1]
SoyGy	TTTCAGTGGCTGCTGCTTCG	20	Soybean Gy3 glycinin gene[2]
SoyGy3	GACTTAATGCACTCTTCATGAA	22	Soybean Gy3 glycinin gene[2]
PRR1	TTCCAGTGCC	10	Pea rbcS-3A gene[3]
PRR2	TGAGGATTGT	10	Pea rbcS-3A gene[3]
RS1	AAGTTGGTGATGTAT	15	Rice salt induced protein[4]
RSR1	TAGGCGTGAC	10	Rice salt induced protein[4]
RSR2	AGCGGACCAG	10	Rice salt induced protein[4]
RSR3	GTCCGTACTA	10	Rice salt induced protein[4]
TPXR1	CATTTGGAAG	10	Tobacco peroxidase gene[5]
TPXR2	CTCAAGGTGG	10	Tobacco peroxidase gene[5]
AP11	ACCTCGAGCACTGTCT	16	Arbitrary primer [6]
APW1	TGGTCACTGA	10	Arbitrary primer[6]
APW2	CGGCCCCTGT	10	Arbitrary primer[6]
APG3	AGTCAGCCAC	10	Arbitrary primer[6]
OPFO1 to OPF20	Omitted	10	Arbitrary primer

[a] Numbers on the superscript indicates references: [1] Shen et al. 1982;[2] Chao et al. 1989; [3] Gilmartin et al. 1990; [4] Claes et al. 1990; [5] Lagrimini et al. 1987; [6] Williams et al. 1990

analysis. Polymorphic differences among accessions in leaf, root, and between leaf and root (L/R), were examined.

Factors affecting RAPD patterns

The observed leaf and root DNAs differences in RAPD patterns were further checked with the following factors. Two brands of thermal cycler, Techne PHC-3 (Techne Ltd, UK) and PTC-100 (MJ Research, Inc. USA), as well as *Taq* DNA polymerases of Ampli-*Taq* (Perkin Elmer Cetus, Norwalk, Conn., USA) and Super- *Taq* (HT Biotechnology LTD, UK). Oligo-primers denoted as AS, KS and NS sources were synthesized respectively by a ABI 380B DNA synthesizer (ABI, Applied Biosystems Inc, Calif., USA) in our institute, the Kwai-Shing Quality system Inc. (QSI, Taipei, Taiwan) and the National Biosciences Inc. (NBI, Plymouth, Minn., USA). Components of the PCR reaction were mostly the same except for the $10\times$ buffers recommended by each manufacturer, and the enzyme concentration was $4\times$ higher in Super-*Taq*. The addition of tetramethyl ammonium chloride (TMAC) at final concentration of 2×10^{-5} M, and 5% DMSO (dimethyl sulphoxide) were also investigated in PCR amplification. Furthermore, template DNA concentrations of 1 ng, 10 ng, 100 ng, 1000 ng per 100-µl reaction were evaluated to determine whether the observed differences were due to variation in template DNA concentration. Modifications in cycling conditions, such as in the decamer primers preceeding a cycle of 94°C (5 min)-36°C (1 min)-72°C (2 min) before a regular 94°C (1 min)-36°C (1 min)-72°C (2 min) for 45 cycles, then shortening the cycling times to 94°C (5 s)-36°C (30 s)-72°C (1 min) for 35 cycles, were also performed to see if there was any difference in the amplified patterns.

Elution of specific RAPD bands, labelling and Southern hybridization

Specific RAPD bands revealed root and leaf DNA differences that were separated in 2% Metaphor agarose (FMC Bioproducts, Me., USA) dissolved in $1\times$ TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Bands of interest were cut with a scalpel from the gel under UV illumination and extracted with a GeneClean II kit (Bio 101 Inc., La Jolla, Calif., USA) according to the instructions of manufacturer. The eluted fragments could be either directly used for labelling or cloned into a pGEM-T Vector system II using the (Promega Corp., Madison, Wis., USA) kit. For Southern hybridization, 10 µg of genomic DNA for each soybean leaf and root were digested with *Hin*dIII and *Taq* I (3–4 U/µg DNA) and were blotted onto the positively charged Nylon membrane by vacuum blotting (Bio-rad, Hercules, Calif., USA). The Dig high-prime DNA labelling and detection starter kit II was used for probe labelling and detection (Boehringer Mannheim, Germany). Pre-hybridization, hybridization and chemillumination detection followed the protocols suggested by the manufacturers (Boehringer Mannheim, Germany) with a slight modification in the CSPD concentration. A general $20\times$ to $50\times$ dilution of CSPD was adopted.

Results

Pairwise comparisons of leaf and root DNA amplifications in RAPD patterns were studied in 11 soybean accessions from various resources. From a total of 40 single primers with oligonucleotide lengths varying between 22 and 10 bases (Table 1), it is evident that, in addition to polymorphisms which occur among lines, there are some differences between leaf and root DNAs even within a line. In other words, differential amplification between root and leaf DNA has occurred. As listed in Table 2, primers such as M13, KS17, RS1, RSR1, RSR2, OPF2, OPF3, OPF4, OPF5, OPF6, OPF7, OPF8, OPF10, OPF12, OPF14, OPF16, OPF18, OPF19, and OPF20 show polymorphic

Table 2 Numbers of RAPD products, polymorphic bands and bands revealing differences in leaf and root DNAs of soybean for each primer

Primer	Leaf[a]	Root[a]	Polymorphic bands (L/R)[b]	Primer	Leaf[a]	Root[a]	Polymorphic bands (L/R)[b]
M13	7(5)	21(18)	17(3)	OPF-1	12(0)	12(0)	0
M13R	10(0)	10(0)	0	OPF-2	10(1)	11(2)	1
KS17	12(2)	17(11)	14(3)	OPF-3	10(3)	10(3)	0
SK17	3(0)	3(0)	0	OPF-4	10(3)	13(5)	2(1)
T7	6(0)	6(0)	0	OPF-5	11(5)	11(3)	0
Act-22	2(0)	2(0)	0	OPF-6	7(0)	10(2)	2
Act1R-22	2(0)	2(0)	0	OPF-7	7(2)	8(3)	1(1)
Soygy3	3(0)	3(0)	0	OPF-8	4(1)	8(4)	3(2)
PRR-1	4(0)	4(0)	0	OPF-9	9(0)	10(0)	0
PRR-2	5(0)	5(0)	0	OPF-10	11(1)	12(1)	0
RS-1	7(3)	9(3)	5(2)	OPF-11	4(0)	4(0)	0
RS-2	6(0)	6(0)	0	OPF-12	14(1)	14(1)	0
RSR-1	7(3)	10(3)	1(1)	OPF-13	14(0)	14(0)	0
RSR-2	9(2)	12(6)	7(4)	OPF-14	10(1)	11(2)	1(1)
TPXR-1	4(0)	4(0)	0	OPF-15	8(0)	8(0)	0
TPXR-2	5(0)	5(0)	0	OPF-16	15(2)	16(3)	1
AP-11	7(0)	7(0)	0	OPF-17	12(0)	12(0)	0
APW-1	3(0)	3(0)	0	OPF-18	3(2)	4(3)	1
APW-2	8(0)	8(0)	0	OPF-19	11(0)	12(1)	1
APG-3	6(0)	6(0)	0	OPF-20	6(0)	10(3)	4(1)

[a] Numbers within parentheses indicated polymorphic bands
[b] Numbers within parentheses indicate those polymorphic bands revealing leaf and root DNA differences and which are reproducible

differences either among soybean accessions or between L/R DNAs. The number of polymorphic bands explored were 0–21 (Table 2). A total of about 100 polymorphic bands were observed from either leaf or root DNA (Table 3). The two sequencing primers M13 and KS17 had the highest number of polymorphic bands, but most of these bands were not consistently found when newly synthesized primers were used (Fig. 2). Both qualitative (presence or absence, Type I, Fig. 1 arrowed) and quantitative (variation in relative band intensity, Type II, Fig. 1 double arrowed) differences were noted. However, even the latter bands indicate qualitative variation when resolved by the polyacrymide gel and silver-staining method (Pang et al. 1992); a weak band at the same migrated position can also be seen (data not shown). In general, root DNA tended to generate more extra bands, or show bands with higher intensity, in comparison with leaf DNA (Table 2). Nevertheless, occasionally some bands with a relatively higher intensity in the leaf than in the root were also observed (Figs. 1, 2 A, blank triangle). Bands that consistently occurred in leaf DNA were not necessary amplified in the root and *vice versa* . The frequencies of polymorphic band patterns among screened accessions are listed in Table 3. Among the 40 primers, 16 (40.0%) detect leaf DNA polymorphism, 19 (47.5%) indicate root DNA polymorphism, and 10 (25%) show more consistency in detecting polymorphism between leaf and root DNAs. Bands over 2 kb, or less than 400 bp, are generally less stable in repeated studies. Due to the inconsistencies found when the new synthesized oligo-primers and different thermal cyclers were used, several factors were tested to see if such a type of variation in revealing an L/R difference is reproducible.

Effects of synthesized oligo-primers, template DNA concentrations, *Taq* DNA polymerases and thermal cyclers

The same primer sequences synthesized from three sources (ABI, KS-QSI and NBI), as described in Materials and methods, were compared for their efficiency in revealing L/R differences. Among these, ABI and KS-QSI were purified by a oligonucleotide purification cartridge, while NBI was not purified. Primers synthesized from different sources sometimes varied in their sensitivity for detecting L/R differences and banding patterns. Figures 2 and 3 are actually the same M-13 sequences but synthesized by ABI, KS-QSI and NBI respectively. Although they retain the ability to distinguish the difference between L/R DNAs, band types were not completely identical. For example, no scorable bands were observed in the < 653-bp area of AS-M13 (Fig. 2 A) and in the > 1230-bp area of the KS-M13 primer (Fig. 2 B). A decrease in band number, intensity and resolution was frequently observed in NBI primers and it was generally difficult to clearly reveal the L/R difference (Fig. 3). Furthermore, it was likely that more bands, especially in the higher-molecular-weight area (above 2 kb), were generated with the

Table 3 Allele frequencies for each polymorphic RAPD marker in leaf and root DNA amplification of 11 soybean lines. Frequencies within parentheses indicate alleles with the same band but lighter in intensity

Marker Name	Leaf	Root	Marker name	Leaf	Root	Marker name	Leaf	Root
M13-2300	100.0	72.7	KS17-470	100.0	18.2	OPF4-1030	9.1	9.1
M13-2080	63.6	81.8	KS17-440	0.0	72.7	OPF4-300	0.0	90.9
M13-1910	9.1	54.5	KS17-380	0.0	9.1	OPF5-2100	63.6	90.9
M13-1840	0.0	45.5	KS17-280	9.1	72.7	OPF5-1640	54.5	81.8
M13-1560	18.2	100.0	RS1-1870	100.0	0.0	OPF5-1430	72.7	100.0
M13-1510	0.0	72.7	RS1-1440	100.0	(72.7)	OPF5-980	72.7	100.0
M13-1430	0.0	27.3	RS1-1340	100.0	(72.7)	OPF5-900	27.3	9.1
M13-1270	100.0	(100.0)	RS1-1230	100.0	(72.7)	OPF6-2180	0.0	18.2
M13-1200	0.0	45.5	RS1-1010	100.0	81.8	OPF6-980	0.0	9.1
M13-1060	72.7	54.5	RS1-920	(100.0)	100.0	OPF7-2180	36.4	72.7
M13-950	0.0	81.8	RS1-880	100.0	90.9	OPF7-1980	0.0	9.1
M13-890	0.0	100.0	RS1-600	0.0	100.0	OPF7-1120	27.3	72.7
M13-840	0.0	18.2	RS1-460	0.0	100.0	OPF8-1700	36.4	45.5
M13-810	18.2	90.0	RS1-400	0.0	(18.2)	OPF8-1650	0.0	54.6
M13-740	0.0	54.5	RSR1-2300	0.0	100.0	OPF8-1440	0.0	9.1
M13-670	0.0	45.5	RSR1-2060	45.5	36.4	OPF8-840	0.0	36.4
M13-650	0.0	9.1	RSR1-1230	45.5	45.5	OPF10-1030	0.0	9.1
M13-550	0.0	9.1	RSR1-990	54.5	54.5	OPF10-900	81.8	81.8
M13-470	0.0	9.1	RSR2-1000	100.0	(54.5)	OPF12-1910	81.8	81.8
M13-450	0.0	9.1	RSR2-940	0.0	45.5	OPF14-900	63.6	63.6
M13-430	0.0	9.1	RSR2-860	90.9	81.8	OPF14-600	0.0	(36.7)
KS17-1300	0.0	27.3	RSR2-780	100.0	(100.0)	OPF16-1840	0.0	9.1
KS17-1130	0.0	27.3	RSR2-700	(27.3)	100.0	OPF16-1500	27.3	27.3
KS17-1060	0.0	18.2	RSR2-320	100.0	45.5	OPF16-370	54.5	63.6
KS17-1000	0.0	72.7	RSR2-280	0.0	36.4	OPF18-2180	9.1	27.3
KS17-910	0.0	100.0	RSR2-120	0.0	72.7	OPF18-1310	0.0	9.1
KS17-840	27.3	27.3	OPF2-1630	18.2	36.4	OPF18-380	54.5	63.6
KS17-780	100.0	27.3	OPF2-610	0.0	9.1	OPF19-580	0.0	9.1
KS17-750	0.0	9.1	OPF3-1770	36.4	36.4	OPF20-2180	0.0	90.9
KS17-730	0.0	9.1	OPF3-1320	(90.9)	(90.9)	OPF20-1340	0.0	90.9
KS17-690	100.0	(100.0)	OPF3-1030	(9.1)	9.1	OPF20-1230	0.0	90.9
KS17-630	0.0	36.4	OPF4-1770	72.7	72.7	OPF20-420	0.0	100.0
KS17-550	0.0	27.3	OPF4-1500	0.0	81.8			
KS17-500	0.0	18.2	OPF4-1160	18.2	36.4			

ABI primers synthesized in our own institute (Fig. 2 A). Nevertheless, we cannot rule out the possibility of combining-effects on batch differences of the *Taq* enzymes or on the stability of the thermal cycler.

The concentrations of template DNA tested were 1 ng, 10 ng, 100 ng, 1000 ng/100-µl reaction. Figure 3 A is an indication of the effects of template DNA concentrations in two soybean accessions. Template DNA concentration had less effect on band patterns for the leaf than for the root. In general, higher concentrations tend to increase the number of bands generated. A DNA concentration over 100 ng has a tendency to decrease the ability to detect L/R differences, especially when the difference concerns the relative band intensity. Nevertheless, some polymorphic bands remain regardless of template DNA concentrations.

Both Ampli-*Taq* and Super-*Taq* DNA polymerases were examined in terms of their ability to reveal differences between L/R DNAs. From this study, it is evident that slight variation of the amplified RAPD patterns did exist between the two enzymes (Fig. 3 B). Ampli-*Taq* has a higher ability to differentiate L/R DNAs; however, batch-to-batch variation of the same enzyme brand was also sometimes observed. As for the response of the thermal cyclers, Techne PHC-3 seems more sensitive in revealing the L/R differences than MJ PTC-100. The two thermal cyclers react differently sometimes with different *Taq* polymerases (Fig. 3 B). Figure 4 indicates an inconsistent amplification when primers synthesized from different sources and different polymerases were employed. Most of the DNAs in our study were amplified by using Techne PHC-3 and Ampli-*Taq*.

A modification of cycling duration, such as a cycle of 94°C (5 min) for a complete denature before the routine 94°C (1 min)-36°C (1 min)-72°C (2 min) for decamers, tends to overwhelm the L/R differences when the variations are due to relative band intensity. For the 22–17-mers, it was also noted that slight variation in RAPD band patterns occurred when the periods of the cycling times were modified. Other factors, such as the addition of 5% (v/v) DMSO or 20 µM of TMAC, have effects on the number of band generated but no obvious effect on those bands revealing L/R differences.

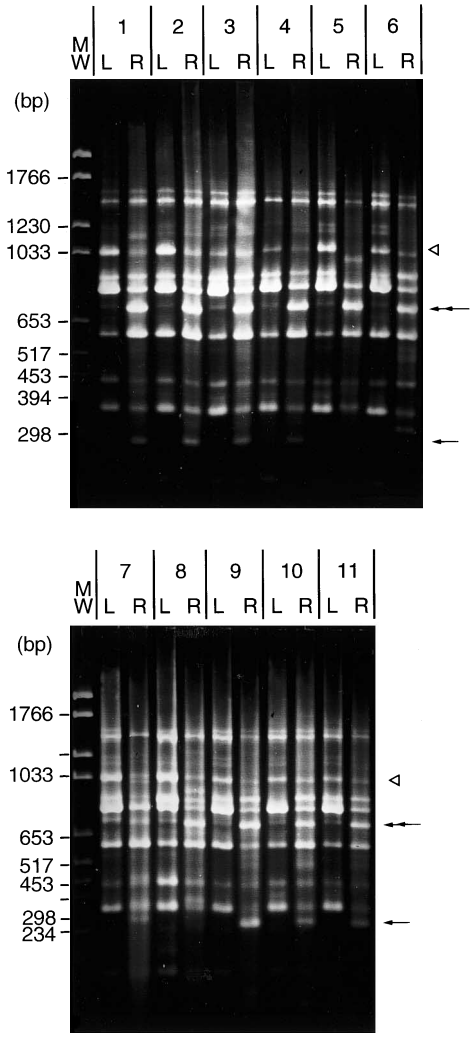

Fig. 1 Pairwise comparisons of RAPD patterns between leaf(L) and root(R) DNAs of 11 soybean accessions with RSR-2(5′-AGCGGAC-CAG-3′) primer. MW: molecular markers; 1–11: soybean accessions. 1: BB120 W, 2: BB120P, 3: BB105 W, 4: BB105P, 5: AGS-58, 6: HCWT, 7: TN15, 8: FWCP, 9: TARI-5, 10: TARI-23, 11: TARI-26. Single arrow indicates the Type I variation(presence or absence), double arrow indicates Type II variation(variation in relative band intensity) and blank triangle indicates Type II variation with band more distinct in leaf

Hybridizing confirmation of the eluted specific fragments

Both types of band which reveal L/R differences, as well as non-polymorphic band, were selected and eluted from the agarose gel for hybridization studies. Results from Southern hybridization demonstrated that both Type-I and Type-II polymorphic bands, as well as the non-polymorphic band, were capable of hybridizing with soybean DNA and are unlikely to be due to contamination. As shown in Fig. 5, two accessions (TARI-23 and HCWT) of soybean leaf and root DNAs were digested with *Hin*dIII and *Taq*I and equal amounts of DNA were loaded for electrophoresis (Fig. 5 A). Probes of M13-670 (Type I) , RSR2-700 (Type II) and M13-780 (non-polymorphic band) were then hybridized onto the membrane. Membranes were washed to high stringency (0.1 × SSC, 0.1% SDS, 68°C) and multiple discrete bands were produced (Fig. 5 B–D) which are apparently, involved in repeated sequences. There was some similarity in the hybridized patterns among these three probes; however, some relative differences in the signal intensity of leaf and root DNAs within and between accessions were noted (Fig. 5 B–D).

As previously mentioned, band M13-670 was classified as a Type-I polymorphic band for revealing an L/R DNA difference. Some notable differences are indicated in the Fig. 5 B. According to our original RAPD record, band M13-670 occurred in the root DNA of some soybean accessions (Table 3); TARI-23 did not have this band in its root DNA whereas HWCT did. This might explain, in part, why the hybridization pattern in TARI-23 was not as significant as in HCWT. Band RSR2-700 was classified as a Type-II polymorphic band. Both TARI-23 and HCWT had this band and generally the root DNA showed a relatively higher intensity. However, the most obvious difference between root and leaf was the occurrence of a high-molecular-weight band near the 23-kb area when root DNAs were digested with *Hin*dIII and hybridized either to M13-670 or RSR2-700 (Fig. 5B, C). Although the non-polymorphic probe M13-780 also shows the same band near 23 Kb in both root and leaf samples, the band intensity is relatively weaker in leaf samples. It is also worth noting that the counterpart leaves have a more distinct low-molecular-weight band which is not distinguishable in the root samples (Fig. 5 D, arrows). Some other minor differences can also be seen in the figures (Fig. 5B–D).

Discussion

RAPD is a rapid method for detecting genetic variation. In plants, genomic modifications, such as DNA amplification, endoreduplication and underreplication, are known to be commonly associated with cell differentiation and plant development (D'Amato 1964; Nagl 1981; Knowles and Phillips 1985; Shang and Wang 1991). Therefore, in the present study, we were interested to determin if RAPD markers could be used in monitoring genetic alternation during plant developments. Leaves and roots of soybean grown under hydroponic culture were used for comparison. Besides the general polymorphisms among lines, some extra band patterns were occasionally observed, especially in root DNA. Both qualitative and quantitative variations of band types were noted among lines in either leaf or root DNA and between L/R DNAs. At least 40 single primers have been screened for pairwise comparisons of RAPD patterns between L/R DNAs of 11 soybean

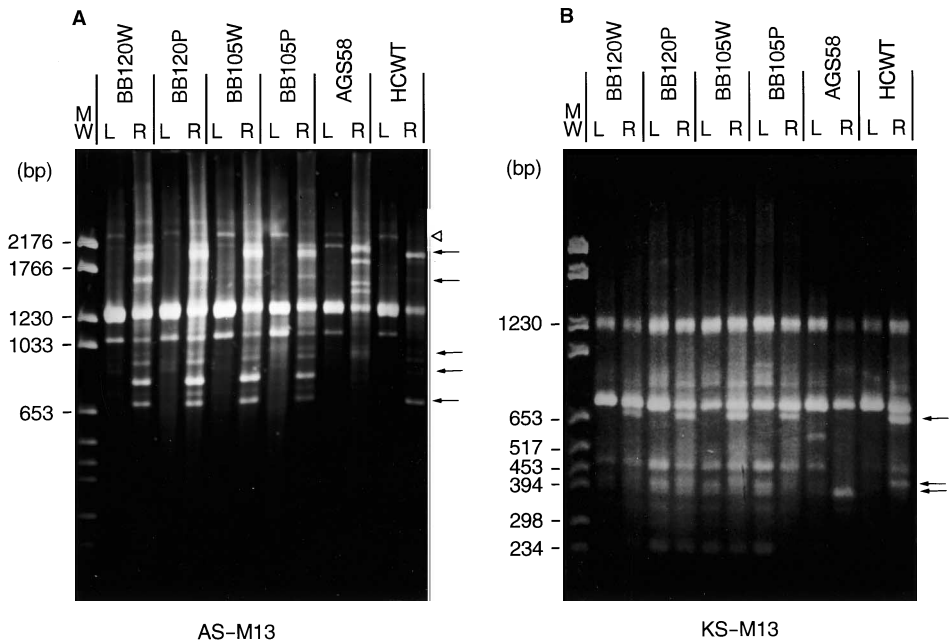

Fig. 2A,B Effects of synthesized primers on the RAPD patterns of leaf(L) and root(R) DNAs. AS-M13 was M13 primer(5′-GTAAAACGACGGCCAGT-3′) synthesized by the facility of this institute and KS-M13 was synthesized by a domestic commercial company. Arrows indicates the polymorphic bands between L/R

accessions. Among these, ten primers were found to be more consistent in detecting L/R differences. However, depending on the primer, some polymrophic bands between L/R tend to occur in most accessions whereas others occur only in a few accessions (Table 3). Nevertheless, some of the polymorphic bands revealing L/R differences did suffer from the problem of reproducibility in our repeated studies.

Problems regarding RAPD reproducibility have been addressed in several studies (Devos and Gale 1992; MacPherson et al. 1993; Meunier and Grimont 1993; Penner et al. 1993; Schierwater and Ender 1993). We, therefore, evaluated the reality of these L/R differences by a comparison of various parameters including template DNA concentrations, *Taq* enzyme variants, agents for improving PCR amplification [e.g. DMSO (dimethyl sulphoxide, TMAC (tetra-methylammonium chloride)], sources of synthesized oligo-primers, brands of thermal cyclers, and varying cycling conditions. As previously mentioned in the Results section, two types of polymorphic bands revealed L/R differences. Type-I variation was found to be more stable and less affected by testing parameters. Type-II bands, which were distinct in terms of relative band intensity, were more sensitive to the factors tested. Nevertheless, under certain conditions, the observed L/R differences were repeatable in our study. For example, when the template DNA concentration was no more than 100 ng/100-µl reaction, a RSR2-700 polymorphic band exhibited a relatively higher intensity in most root accessions (Fig. 1). However, when the template DNA concentration increased up to 200 ng/100 µl or more, the difference became insignificant. In contrast, as shown in Fig. 3 A, the polymorphic bands revealed by the M13 primer were even more clear as the template DNA content increased. Meunier and Grimont (1993) identified RAPD variations associated with the brand of *Taq* polymerase and the make of the thermal cycler. They also pointed out that it is difficult for one to guarantee the invariability of a given performance of a *Taq* DNA polymerase or a PCR apparatus over years. Our experience also shows that synthesized primers from different sources make a difference in some cases (Figs. 2 and 3). A similar comment was recently made by Virk et al. (1995).

Apparently, RAPD differences should result from structural or sequence variations of the target-site DNA and the relative binding efficiency of each particular primer. The finding of differential amplification in the RAPD study between L/R DNAs in soybean might suggest that a modification of the genetic constitution has occurred between two organs. The instability of some of these polymorphic bands between L/R DNAs might indicate that only a minor sequence alteration or a structural variation of a nearby target site has occurred. Thus, the detection of this type of variation requires a proper match of reaction conditions. The nucleus of differentiating root hairs contains amplified, extra-chromosomal, DNA sequences as reported by Murry et al. (1987). They noted that this amplification disappears under salt stress. In soybean, preferential replication of repeated DNA sequences is known to occur in nuclei isolated from suspension culture (Caboche and Lark 1981). In the present study,

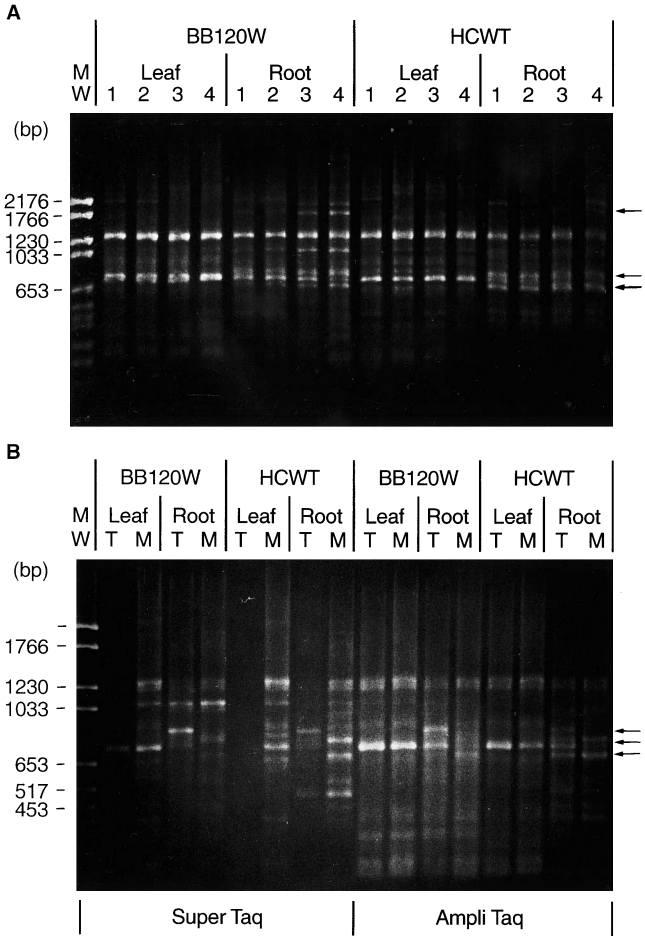

Fig. 3A, B Effects of template DNA concentrations (A), thermal cyclers and *Taq* DNA polymerases (**B**) on RAPD patterns in L/R DNAs of BB120 W and HCWT. Arrows indicates polymorphic bands between L/R DNAs appeared in the previous studies. 1, 2, 3, 4 in (A) were template DNAs 1 ng, 10 ng, 100 ng and 1000 ng respectively per 100 µl reaction; T, M in (B) stands for Thermal cyclers Techne PHC-3(T) and MJ PTC-100(M) respectively. The primer is M-13 sequencing primer synthesized by National Bioscience Inc.

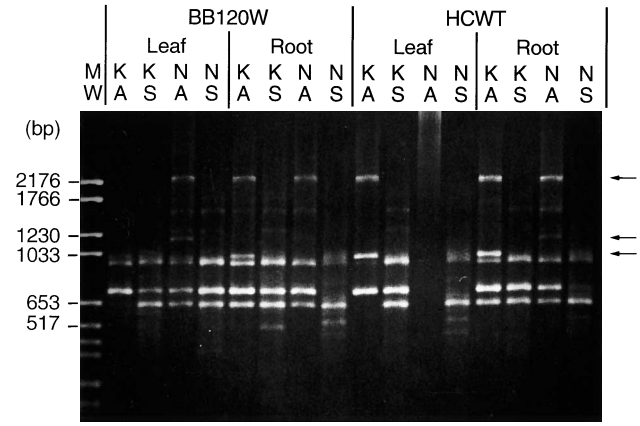

Fig. 4 Effects of primer sources and brands of *Taq* DNA polymerase on the reproducibility of polymorphic bands between leaf and root DNAs. Primer sequence was OPF-20 (5′-GGTCTAGAGG-3′), and were synthesized from Kwai-Shing Quality System Inc. (K) and National Bioscience Inc. (N) individually; Lines A: were amplified with Ampli- *Taq* and Lines S with Super- *Taq* as described in the Material and methods. Arrows indicates the polymorphic bands which occurred between L/R DNAs when primer OPF-20 was obtained originally from Operon Inc.

some amplified bands show a difference in relative band intensity between the root and leaf samples. Southern hybridization from this study also demonstrated that most of these bands were related to repeated sequences (Fig. 5 B–D). Genetic variation of tissue-culture materials derived from root tissue of soybean has been revealed by the restriction fragment length polymorphism study of Roth et al. (1989). These authors reported that tissue cultures prepared from root tissue of individual soybean plants develop RFLP allelic differences at various loci and that the alleles generated are almost always the same as ones previously found and characterized in other varieties of cultivated soybean. In our study, polymorphic loci between root and leaf DNAs are sometimes inconsistent among lines and some amplified bands which predominate in root DNA amplifications in most soybean lines are found sporadically in a few leaf DNA amplifications. Whether there is any correlation between the two studies is not known.

Brown et al. (1993), from an analysis of single protoplasts by RAPD technology, found some slight variation between them. They suggested that the observed difference is probably a reflection of the loss of DNA during isolation rather than of inherent differences between the protoplasts. Adams (1990) pointed out that although DNA is a very stable molecule whose structure is faithfuly maintained from generation to generation, yet, with each round of replication, this structure is modified by base methylation in nearly all cells and organisms. Striking differences in the DNA methylation of root cambium, secondary phloem and leaf petioles have been demonstrated in carrot (Arnholdt-Schmitt 1995). In Fig. 5 B and C, it is apparent that the polymorphic bands tend to hybridize near a 23-kb band, which might be due to incomplete *Hin*dIII digestion in the root samples. According to the BRL catalogue for restriction enzymes, *Hin*dIII does not cleave DNA when either the 5′ A or the C residue is N^6-methyladenine or 5-methylcytosine, respectively, while *Taq*I does not cleave DNA when the A residue is N^6-methyladenine, but cleaves DNA when the C-residue is 5-methylcytosine. Our recent study on neoschizomeric enzyme digestion also found that the methylation patterns between leaf and root DNA look different (data not shown). Whether DNA methylation has an effect on RAPD patterns requires further study.

Instability of the plant genome has been addressed by Marx (1984) who confirmed that DNA alteration could arise during mitosis when the original strains were growing in stress environments. Nevertheless, Wesising and Kaemmer (1992) pointed out that no

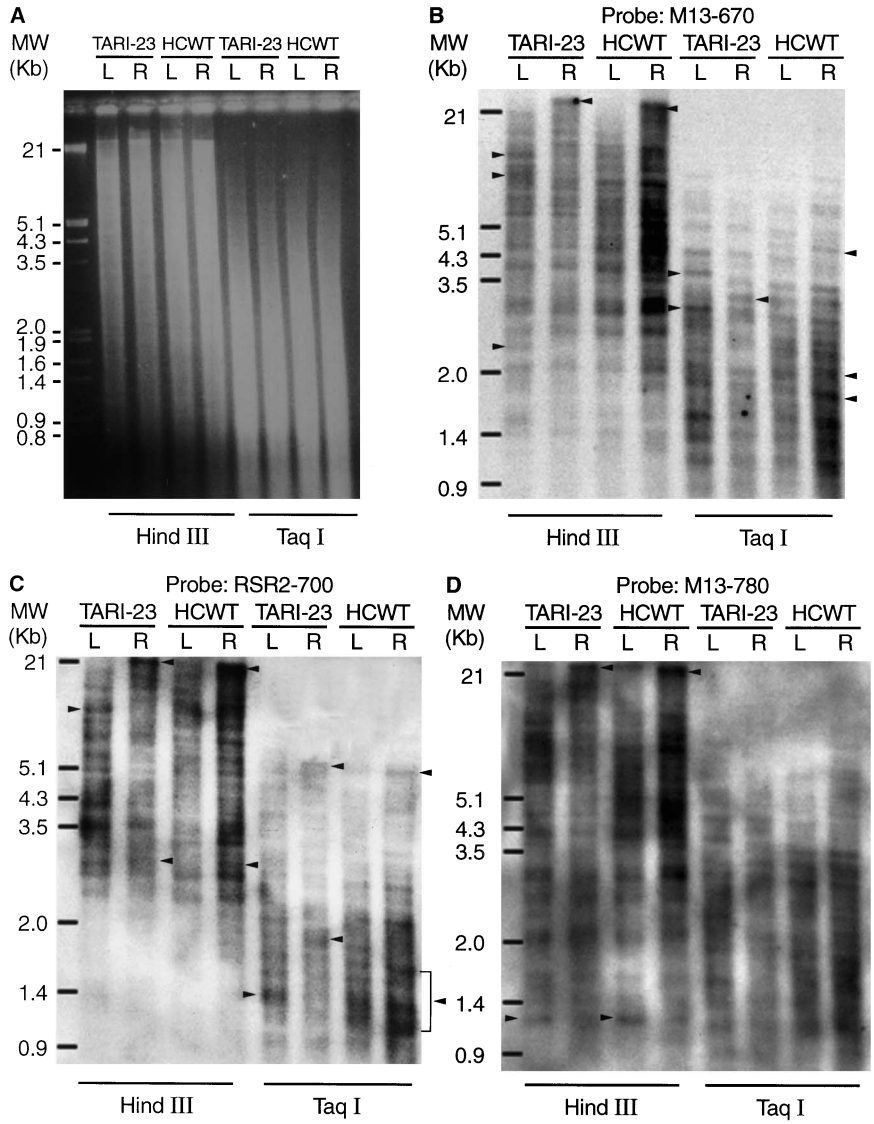

Fig. 5A–D Southern analysis for the Type I and II polymorphic and non-polymorphic bands. (A) Nearly equal amount of leaf and root genomic DNA of soybean TARI-23 and HCWT digested with *Hin*dIII and *Taq* I and separated in an 0.8% agarose gel stained with ethidium bromide. The gel was then blotted onto positive charged nylon membrane as described in the Materials and Methods. (B–D) Dig-chemillumination detection for the blotted membranes probing with M13-670 (Type I) (B), RSR2-700 (TypeII) (C) and M13-780 (non-polymorphic) (D) of eluted fragments. Arrows indicates some of the distinguishable variations

somatic variation was found when different tissue DNA digestions were probed by simple repetitive DNA sequences. However, our study did show differences between root and leaf DNA amplification with some primers. McClintock (1984) suggested that the genome may modify itself when confronted with unfamiliar conditions. In our case plant materials were grown in hydroponic culture for the ease of root harvesting, but whether hydroponic culture is a stress to soybean roots is not known. The differences between root and leaf DNA amplification might be an indication of a developmental modification in the soybean root genome or else be induced under hydroponic stress. We recently found it can also be used to differentiate rice shoot and root. We believe that RAPD analysis should be a very good tool for detecting genomic modification during developmental stages without the need of target gene sequences. Tissue bands or organ-specific amplification might then be further tagged for mapping, sequencing, and tracing the possible genes related to developmental changes. Nevertheless, a consistent control of factors related to RAPD stability should be essential to make the results more reproducible in this type of study. Differential display (Liang and Pardee 1992), a similar approach to the use of RAPD in RNA PCR, has been applied very successfully in gene isolation for different types of environmental stress or developmental stages (Gibson and Somerville 1993; McClelland et al. 1995). However, it is not known whether the observed difference results simply from RNA processing (transcription level) or from the involvement of genomic modification. The existence of RAPD differnces in

different organ or tissue DNAs, or under certain stresses, if proved to be correlated with specific developmental characters, will be valuable in serving as an additional selection cycle for regular marker-type selection.

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