Abundant DNA Markers Detected by PCR Using Simple Sequence Repeat Primers without Anchor Sequences in Soybean

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Simple sequence repeats (SSRs) or microsatellites have been widely used as DNA markers in the analysis of eukaryotic genomes. These markers are detected by PCR using SSR primers with or without anchored sequences. Anchored SSR primers have been used for detecting DNA markers and analyzing genome fingerprinting in a wide range of animal and plant species (Zietkiewicz *et al.* 1994).

On the other hand, Gupta *et al.* (1994) reported that the SSR primers without anchor sequences or non-anchored SSR primers gave a more polymorphic banding pattern by PCR in various animals and plants than RFLP. Among the primers used, those consisting of 16-mer oligonucleotides with 4 base-repeats and 50% GC contents were most effective in detecting the polymorphic bands.

We explored the polymorphisms among soybean cultivars using non-anchored SSR primers, which consisted of 16-mer oligonucleotides with 4 base-repeats. The GC contents of the primers were either 50% or 75%. The effectiveness of the detected polymorphic fragments in linkage analysis was also surveyed using recombinant inbred lines (RILs) of soybean.

Plant material, DNA extraction, primers, DNA amplification and linkage analysis

Three cultivars of soybean, Heihe 5, Akisengoku and No. 29, which have been used for genetic analysis of flowering time in our lab (Li and Niwa 1996, Yang and Niwa 2000), were used as plant materials. Total DNA was extracted from young-fresh leaves according to the method described by Tabei *et al.* (1998).

The primers used in this study are listed in Table 1. Sixteen-mer oligonucleotides with 4 base-repeats were used as primers for PCR. Those primers, which involved the

same core sequence, were grouped as one series, so that the maximum possible number of primers belonging to the same series was 4 by differing the first base at the 5' end. The possible number of the series for the primers with a 50% GC content was 22, of which 16 were tested here. For the primers with a 75% GC content, the possible number of the series was 16, all of which were tested. Some of the series tested covered all possible sequences, while the others included a part of them. Forty-nine and 50 primers were tested for 50% and 75% GC contents, respectively.

DNA was amplified according to the method described by Gupta *et al.* (1994) in a final volume of 20 µl with 500 mM KCl, 15 mM MgCl₂ and 100 mM Tris-HCl, pH 9.0, 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP), 1 µM primer, 5-100 ng template DNA, 1.5 U *Taq* DNA Polymerase (Pharmacia Biotech.) and overlaid with mineral oil. Amplification was performed in a Cyclogene or MJ Research Thermal Cycler, following an initial denaturation step at 95°C for 3 min. The amplification program using the primers with a 50% GC content was as follows: 35 cycles of 95°C for 1 min, 45°C for 1 min, 70°C for 5 min followed by final extension at 70°C for 5 min. Annealing temperature of 55°C was applied when the primers with a 75% GC content was used.

The amplified products were electrophoresed on 8% polyacrylamide gel circulating TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0), for about 1-1.5 h. The separated fragments were visualized by ethidium bromide staining and photographed under ultra violet light (Polaroid Fuji film FP-3000B). The banding patterns generated were highly reproducible through the twice trials of PCR.

Forty-eight F₅ RILs developed by the single-seed descent of F₂ plants derived from the cross Heihe 5 × No. 29 were used for linkage analysis of the markers detected by inter-SSR PCR. The linkage analysis was conducted by using the computer program MAPMAKER/EXP (v3.0b) (Lander *et al.* 1987) with a LOD score of 3.0 as the threshold value for declaring linkage in the pairwise loci analysis. Map distance (cM) was calculated according to the Kosambi function method (Kosambi 1994).

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Table 1. Sequences and GC contents of primers used

Series	Core sequence	Primers used ¹⁾	GC content (%)
SP1	5'(GGAT)	SP1-1 to 4	50
SP2	5'(ATCC)	SP2-1 to 4	50
SP3	5'(CCTA)	SP3-1 to 4	50
SP4	5'(TAGG)	SP4-1 to 4	50
SP5	5'(ACTC)	SP5-1 to 4	50
SP6	5'(GAGT)	SP6-1 to 4	50
SP7	5'(GACA)	SP7-1 to 4	50
SP8	5'(TGTC)	SP8-1	50
SP9	5'(AAGG)	SP9-1 to 4	50
SP10	5'(CCTT)	SP10-1	50
SP11	5'(ACTG)	SP11-1 to 4	50
SP12	5'(CAGT)	SP12-1	50
SP13	5'(AACC)	SP13-1	50
SP14	5'(GGTT)	SP14-1	50
SP15	5'(AAGC)	SP15-1 to 4	50
SP16	5'(CGAA)	SP16-1 to 4	50
SP17	5'(GACG)	SP17-1 to 4	75
SP18	5'(AGGG)	SP18-1	75
SP19	5'(ACCC)	SP19-1 to 2	75
SP20	5'(TGGG)	SP20-1	75
SP21	5'(TCCC)	SP21-1 to 2	75
SP22	5'(AGGC)	SP22-1 to 4	75
SP23	5'(AGCG)	SP23-1 to 2	75
SP24	5'(AGCC)	SP24-1 to 4	75
SP25	5'(ACGC)	SP25-1 to 4	75
SP26	5'(ACCG)	SP26-1 to 4	75
SP27	5'(TGGC)	SP27-1 to 4	75
SP28	5'(TGCG)	SP28-1 to 4	75
SP29	5'(TCGG)	SP29-1 to 4	75
SP30	5'(TGCC)	SP30-1 to 4	75
SP31	5'(TCGC)	SP31-1 to 4	75
SP32	5'(TCCG)	SP32-1 to 2	75

¹⁾ The primers grouped in the same series differed from each other by the first base at 5' end, e.g. SP1-1, (GGAT)₄; SP1-2, (GATG)₄; SP1-3, (ATGG)₄; SP1-4, (TGGA)₄.

Polymorphism of amplified DNA patterns

Sixty-three of the 99 primers tested, 36 and 27 for 50% and 75% GC contents, respectively, generated 283 poly-

Fig. 1. EtBr-stained 8% polyacrylamide gel showing PCR amplified band patterns by using primers SP16-1 and SP16-2. The arrowheads indicate polymorphic bands among cultivars. M, Molecular marker; H, Heihe 5; A, Akisengoku; N, No. 29.

morphic fragments between 2 pairs of cultivars (Table 2). Numbers of polymorphic fragments detected by each of those primers ranged from 1 to 7. The primer SP16-2, (GAAC)₄, generated the maximum number, 7, of polymorphic fragments among them. Thirty-five primers generated more than 2 polymorphic fragments.

The mean number of polymorphic fragments per primer was 1.16 and 0.75 for 50% and 75% GC contents, respectively. This suggests that the primers with a 50% GC content was more effective in detecting the inter-cultivar polymorphism of soybean than those with a 75% GC content. This result was consistent with that reported by Gupta *et al.* (1994).

Of the 32 series tested, the primers belonging to 22 series generated polymorphic bands. Twenty-one of the 22 series involved 4 primers differing in the base at the 5' end. Though the primers belonging to the same series harbored the same core sequences, they generated banding patterns different from each other (Fig. 1). This means that those

Table 2. Number of polymorphic fragments amplified by PCR using non-anchored SSR primers with 50 and 75% GC contents among soybean cultivars, Heihe 5, Akisengoku and No. 29

Between	Number of primers (A)	Number of primers detecting polymorphism	No. of polymorphic fragments of each parent type			Total No. polymorphic fragments (B)	Number of polymorphic fragments per primer (B/A)
			Heihe 5	Akisengoku	No. 29		
(50% GC content)							
Heihe 5 and Akisengoku	49	25	23	30	—	53	1.08
Heihe 5 and No. 29	49	25	31	—	27	58	1.18
Akisengoku and No. 29	49	29	—	35	24	59	1.20
Mean	49	26.3	27	32.5	25.5	56.7	1.16
(75% GC content)							
Heihe 5 and Akisengoku	50	17	15	9	—	24	0.48
Heihe 5 and No. 29	50	24	26	—	20	46	0.92
Akisengoku and No. 29	50	24	—	22	21	43	0.86
Mean	50	21.7	20.5	15.5	20.5	37.7	0.75

primers belonging to the same series could anneal to a different position of the genome due to the difference in their last base at the 3'-end, so that different DNA markers could be detected. This also implies the possibility that more polymorphic fragments can be detected with simple sequence repeat primers by differing the base at the 5' end.

Linkage map

Clear segregation was observed for 70 of the 104 polymorphic fragments among the 48 F₅ RILs. The chi-square test revealed that 5 of those markers showed a significant lack of fit to the expected ratio, 17:15, at the 1% level of probability in the RILs (Fig. 2). The small size of the tested population is considered to have brought about these discrepancies owing to a sampling error.

We conducted the linkage analysis using those 70 fragments as markers. Of them, 46 markers detected by 25 primers belonging to 13 series constructed 11 linkage groups involving 2 to 16 markers. These markers were located on 36 different positions covering 163.6 cM (Fig. 2).

More than 2 markers were located without any distance from each other on 6 positions. No map distance was detected in the linkage groups 10 and 11. Those 6 positions included different markers detected by not only the same primers but also those by the different ones (Fig. 2). It can be considered that those markers mapped without distance corresponded to the products of the amplification of the same or close intervals of the genome.

Except for one pair, SP4-2 and SP4-3a, those markers which were detected by the different primers but belonging to the same series were located at different positions. This proved that different loci could be identified using SSR primers with the same core sequences by changing the last base at the 3' end.

In conclusion, the current study revealed the high capability of non-anchored SSR primers to detect polymorphic DNA markers among soybean cultivars, although the linkage relationships of the markers detected here to those reported previously (Li and Niwa 1996) have not been researched yet.

Fig. 2. Linkage groups of DNA markers detected by inter-SSR PCR in F₅ recombinant inbred lines derived from the cultivars cross, Heihe 5 × No. 29. The asterisks indicate markers showing a significant difference from the expected segregation ratio (17:15) at the 1% level. The alphabetic letters were attached to the marker designations for distinguishing different fragments amplified by the same primer.

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