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RFLP Report

Linkage of the *Fr2* locus controlling soybean root fluorescence and four loci detected by RFLP markers

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Source of probes

The probes used in this study were constructed and maintained as inserts in *E. coli* plasmid vectors. The probes pBLT6, pBLT42, and pBLT73 are in vector pIBI30 (International Biotechnologies Inc.) while pBLT68 is in vector pUC18 (Yanisch-Perron et al. 1985). Plasmid DNA was digested with a restriction endonuclease to release the insert. DNA fragments were purified on agarose gels. The DNA fragments used as probes were radiolabeled using the random oligonucleotide labeling procedure of Feinberg and Vogelstein (1983). Random hexamers [pd$(N)_6$] were purchased from Pharmacia (Piscataway, N.J.). Approximately 50 ng of DNA was used per 25 µl of reaction with 50 µCi of [α-^{32}P]-deoxycytidine 5′-triphosphate (3000 Ci/mmol) (NEN/DuPont, Wilmington, Del.). Reactions were incubated at room temperature for 2–3 h.

Plant analysis

A comprehensive genetic map of soybean would be very valuable to soybean breeders and geneticists in devising breeding strategies for soybean improvement. A rudimentary classical genetic map has been developed with great effort over many years. This map needs to be integrated with the molecular map now emerging from current research in order to use the molecular map as a bridge to obtain a comprehensive map of the organismically important genes. In the present study, we tested the as yet unmapped *Fr2* gene, controlling root fluorescence under UV irradiation, for linkage with numerous RFLP markers segregating in the cross of PI290136 X BARC-2(*Rj4*). This cross was made at Beltsville, Md. and F_2 and F_3 seeds were also produced in the field at Beltsville. PI290136, also referred to as 'Noir I', is an accession from the USDA germplasm collection carrying the alleles *fr2*, *rj4*, *w1*, and *i*. BARC-2(*Rj4*) is a near-isogenic line of the cultivar Clark 63 (Devine and O'Neill 1986) and carries the alleles *Fr2*, *Rj4*, *W1*, *i-i* (Table 1). Gene symbols were described by Palmer and Kilen (1987). The F_2 seeds were surface-sterilized by immersion in 50% ETOH for 25 s and rinsed in tap water. The seeds were then planted in sterilized vermiculite in growth trays (Devine and Reisinger 1978). At planting, the seeds were inoculated with a stationary-phase broth culture of bradyrhizobial strain USDA 61. Strain USDA 61 is definitive for distinguishing the ineffective vs effective nodulation responses conditioned by the allele *Rj4* vs *rj4*. The bacteria were grown in A1E medium (Kuykendall 1979). After 2 weeks of growth, plant roots were extracted from the vermiculite and scored for effective vs ineffective nodulation response (Devine and O'Neill 1986). Seedlings were then examined under UV light to classify for the root fluorescence phenotype controlled by the *Fr2* vs *fr2* allele. Seedlings were also classified for the *W1* vs *w1* allele based on stem color. Seedlings were transplanted to the field for further growth and seed production. Leaf tissue was harvested from individual F_2 plants for DNA extraction during the growing season. At maturity, F_3 seeds were harvested from individual F_2 plants. To obtain a more complete classification of genotypes, seeds of F_3 lines harvested from individual F_2 plants were planted in growth trays in a similar manner to that used to assay the F_2 plants, and the F_3 seedlings were scored, according to the same procedure, for

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Table 1. Genotypes of parental lines PI290136 and BARC-2(*Rj4*)

PI290136		BARC-2(*Rj4*)	
Gene symbol	Trait	Gene symbol	Trait
fr2	Non-fluorescent roots	*Fr2*	Fluorescent root
i	Self-colored seed coat	*i-i*	Seed coat color restricted to hilum
rj4	Effective nodulation with *R. japonicum* strain USDA 61	*Rj4*	Ineffective nodulation with *R. japonicum* strain USDA 61
w1	White flowers, green stems	*W1*	Purple flowers, purple stems
p(BLT6)1	Hybridizes with fragment at 3,800 bp derived from *Taq*I digest	*p(BLT6)1*	Hybridizes with fragment at 3,600 bp derived from *Taq*I digest
p(BLT42)3	Hybridizes with fragments at 4,400 and 5,000 bp derived from *Ava*II digest	*p(BLT42)3*	Does not hybridize with fragments at 4,400 and 5,000 bp derived from *Ava*II digest
p(BLT68)	Hybridized with fragments at 1,800 and 5,000 bp derived from *Hae*III digest	*P(BLT68)*	Hybridizes with fragments at 1,600 and 2,700 bp derived from *Hae*III digest
P(BLT73)	Hybridizes with fragment at 3,900 bp derived from *Hae*III digest	*p(BLT73)*	Hybridizes with fragment at 1,200 bp derived from *Hae*II digest

nodulation, root fluorescence and stem color. F_3 seed was also planted in a field and grown to maturity, when the seed coat color of the F_4 plants was classified to distinguish the genotype of the F_2 plants for the *I* locus.

Soybean leaves from field-grown or greenhouse-grown plants were harvested, freeze-dried and stored at −20 °C. Aliquots were finely ground in an analytical mill. DNA was extracted using a buffer containing 1% CTAB (hexadecyltrimethylammonium bromide), 50 mM EDTA, 50 mM Tris-HCl pH 8, 1 mM 1,10 o-phenanthroline, 0.7 M NaCl, and 0.1% β-mercapto-ethanol. This mixture was incubated at 60 °C for 1 h and then chloroform-extracted. DNA was precipitated with isopropanol, resuspended in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA), treated with RNAse, re-extracted with phenol and chloroform, ethanol-precipitated, and resuspended in TE (Keim et al. 1988).

Restriction endonuclease digestions of the DNA, with *Hae*III and *Ava*II, were performed according to instructions from the supplier (BRL, Life Technologies, Inc., Gaithersburg, Md.). Digests were typically incubated for 16 h using 2–5 units of enzyme per microgram of genomic DNA.

The digested DNA (10 µg per lane) was subjected to electrophoresis in 1% agrose gels for approximately 4 h at 90 mA (150 V) using TBE running buffer (90 mM Tris, 90 mM borate, 2 mM EDTA). Following electrophoresis, DNA was transferred to a nitrocellulose membrane according to the method of Maniatis et al. (1982).

Membranes were prehybridized at 42 °C in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA for 2–4 h. The ^{32}P-labeled probe was denatured by boiling and added to the prehybridization solution. Hybridization was carried out at 42 °C for 16–24 h. The membrane was washed several times at 42 °C with 2 × SSC, 0.1% SDS for a total of 1–2 h, followed by several washes at 60 °C with 0.1 × SSC, 0.1% SDS. The washed membranes were exposed to X-ray film (Kodak XOmatAR) for 2–4 days with intensifying screens.

Hybridization patterns for F_2 plants were compared to parental BARC-2(*Rj4*) and PI290136 patterns (Table 1) and scored.

The Mapmaker software program was used to screen the data for probable linkages (Lander et al. 1987), then a computer program devised in our laboratory was used for final analysis. Chi-square analysis was used to test the goodness-of-fit of observed to expected ratios. The total chi-square was partitioned to extract the chi-square deviation due to linkage. The method of maximum likelihood was used to estimate the recombination frequency (Mather 1951; Allard 1956). The bisection method was used to solve the maximum likelihood equations (Yakowitz and Szidarovszky 1989).

Location and order of the probes and gene loci

The four gene loci expressed phenotypically in the plants, i.e., *Fr2*, *I*, *Rj4*, and *W1*, were found to segregate independently of each other (Table 2).

pBLT68. DNA probe pBLT68 is a 1,750-bp cDNA clone encoding the entire coding region of a soybean gene for aspartate amino-transferase (AAT) (G. J. Wadsworth and B. F. Matthews, manuscript in preparation). The probe hybridizes to DNA fragments of 4,500, 2,300, 1,800 and 600 bp from *Hae*III-digested genomic DNA from PI290136 and with fragments of

Table 2. Results of genetic linkage tests and linkage analyses for four RFLP markers with the *Fr2* and other loci in soybean

Genes	Genotype[a]									Sum	χ^2L[b]	P(χ^2L)[c]	Rec[d]	SE[e]	Ph[f]	Ratio
p(BLT42)3, Fr2	e+f		g+h+i		j+k	l	m	n		117	94.043	<.001	6.0	2.3	R	3:6:3:1:2:1
p(BLT42)3, pBLT68	0		46		31	28	1	0		106	112.157	<.001	1.0	1.0	R	3:6:3:1:2:1
p(BLT42)3, pBLT73	6		42		29	29	1	1		108	97.944	<.001	8.2	2.7	R	3:6:3:1:2:1
p(BLT42)3, Rj4	19		34		32	5	19	8		117	3.581	.10–.20	48.6	5.7	R	3:6:3:1:2:1
p(BLT42)3, W1	15		46		24	9	17	6		117	1.849	.30–.50	42.2	5.5	R	3:6:3:1:2:1
p(BLT42)3, p(BLT6)1	13		17		19	12	3	1		65	16.754	<.001	27.4	6.3	R	3:6:3:1:2:1
p(BLT42)3, i-i	30		37		16	7	16	8		114	2.111	.30–.50	56.4	5.7	R	3:6:3:1:2:1
	e	f	g	h+i	j	k	l	m	n							
Fr2, p(BLT6)1	12	15	5	15	1	11	2	4	11	76	21.658	<.001	30.0	4.7	C	1:2:1:2:4:2:1:2:1
Fr2, pBLT68	28	5	5	52	0	4	0	2	30	126	177.460	<.001	6.6	1.6	C	1:2:1:2:4:2:1:2:1
Fr2, pBLT73	32	11	2	48	0	2	0	1	31	127	197.079	<.001	6.5	1.6	C	1:2:1:2:4:2:1:2:1
Fr2, Rj4	7	16	25	28	9	24	8	15	15	147	5.776	.20–.30	47.3	4.1	C	1:2:1:2:4:2:1:2:1
Fr2, W1	9	7	22	45	10	16	12	16	10	147	6.959	.10–.20	52.6	4.1	C	1:2:1:2:4:2:1:2:1
i-i, Fr2	8	19	20	31	11	17	12	16	8	142	1.775	.30–.50	54.3	4.1	C	1:2:1:2:4:2:1:2:1
i-i, pBLT68	7	17	15	30	13	12	8	14	7	123	3.878	.50–.70	55.5	4.4	C	1:2:1:2:4:2:1:2:1
i-i, pBLT73	10	22	16	22	12	13	10	12	7	124	1.887	.50–.70	53.1	4.5	C	1:2:1:2:4:2:1:2:1
i-i, Rj4	9	13	17	34	13	20	7	16	13	142	0.958	>.95	48.6	4.2	C	1:2:1:2:4:2:1:2:1
i-i, W1	4	15	28	36	7	16	8	16	12	142	7.197	.10–.20	49.3	4.2	C	1:2:1:2:4:2:1:2:1
i-i, p(BLT6)1	8	14	6	15	9	7	6	3	5	73	4.123	.30–.50	52.3	5.8	C	1:2:1:2:4:2:1:2:1
pBLT68, p(BLT6)1	14	14	5	14	1	7	1	4	15	75	43.693	<.001	24.4	4.2	C	1:2:1:2:4:2:1:2:1
pBLT73, p(BLT6)1	15	11	7	11	2	8	1	4	12	71	34.211	<.001	27.3	4.6	C	1:2:1:2:4:2:1:2:1
pBLT73, pBLT68	30	2	11	42	0	3	1	1	30	120	179.750	<.001	8.2	1.9	C	1:2:1:2:4:2:1:2:1
Rj4, p(BLT6)1	4	14	2	14	2	12	11	8	9	76	2.000	.70–.90	50.0	5.7	C	1:2:1:2:4:2:1:2:1
Rj4, pBLT68	4	19	8	28	8	13	10	23	13	126	2.968	.50–.70	50.7	4.5	C	1:2:1:2:4:2:1:2:1
Rj4, pBLT73	5	16	11	22	8	13	12	18	12	127	5.205	.20–.30	51.8	4.4	C	1:2:1:2:4:2:1:2:1
Rj4, W1	6	12	14	39	11	17	10	30	8	147	4.456	.30–.50	55.0	4.1	C	1:2:1:2:4:2:1:2:1
W1, p(BLT6)1	6	16	3	14	5	13	7	7	5	76	0.342	>.95	51.0	5.7	C	1:2:1:2:4:2:1:2:1
W1, pBLT68	10	20	6	39	10	16	3	14	8	126	8.222	.05–.10	44.9	4.4	C	1:2:1:2:4:2:1:2:1

[a] Class designations per Allard 1956
[b] Linkage chi-square tests for independence were calculated with one of two assumptions: a 3:6:3:1:2:1 F_2 ratio with 2 *df* or a 1:2:1:2:4:2:1:2:1 F_2 ratio with 4 *df*
[c] Chi-square probability
[d] Rec, estimate of the recombination frequency using the method of maximum likelihood
[e] SE, standard error of recombination estimate
[f] Phase: C, coupling; R, repulsion

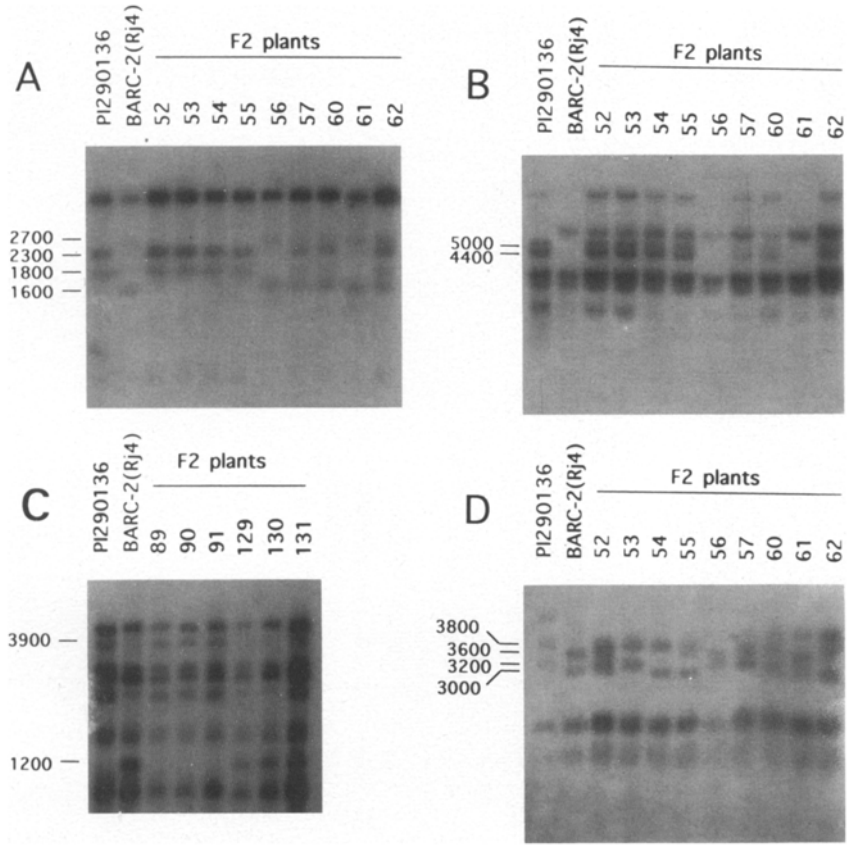

Fig. 1A–D. Hybridization of probes pBLT68, pBLT42, pBLT73 and pBLT6 to genomic DNA from the two parental lines PI290136 and BARC-2(*Rj4*) and F_2 progeny. Numbers on the left indicate the size (in bp) of bands scored in the F_2 analysis. **A** pBLT68 hybridized to *Hae*III-digested DNA. **B** pBLT42 hybridized to *Ava*II-di-gested DNA. **C** pBLT73 hybridized to *Hae*III-digested DNA. **D** pBLT6 hybridized to *Taq*I-digested DNA

4,500, 2,700, 1,600, and 600 bp from BARC-2(*Rj4*) DNA (Fig. 1A; Table 1). F_2 plants were screened for the presence of the cosegregating 2,300- and 1,800-bp fragments (PI290136 genotype), the co-segregating 2,700- and 1,600-bp fragments [BARC-2(*Rj4*) genotype], or all four fragments (heterozygote).

pBLT42. Probe pBLT42 is a 600-bp *Pst*I soybean genomic DNA fragment of undefined function. It hybridizes to *Ava*II-digested genomic DNA fragments from PI290136 of approximately 17,000, 5,000, 4,400, 3,400, 2,800, and 2,000 bp (Fig. 1B). With BARC-2(*Rj4*) it hybridizes with fragments of 5,600, 3,400, 2,800, and 2,000 bp. In the F_2 progeny the probe clearly distinguished fragments at 4,400 and 5,000 bp, defining the *p(BLT42)2* locus, and at 5,600bp, defining the *p(BLT42)3* locus. The F_2 plants were scored for the presence or absence of the fragments that define these two loci.

pBLT73. Probe pBLT73 is a 600-bp *Pst*I genomic DNA fragment of undefined function. It hybridized to more than ten fragments of *Hae*III-digested genomic DNA from either PI290136 or BARC-2(*Rj4*) (Fig. 1C). These fragments range in size from about 4,400bp to less than 650 bp. In addition to the common fragments, the probe hybridizes to a fragment of 3,900 bp in PI290136 and to a fragment of 1,200 bp in BARC-2 (*Rj4*). F_2 plants were scored for the presence of the 3,900-bp fragment (PI290136 genotype), the 1,200-bp fragment [BARC-2(*Rj4*) genotype], or both fragments (heterozygote), to distinguish the *p(BLT73)* locus.

pBLT6. Probe pBLT6 is a 600-bp soybean cDNA clone of undefined function. When hybridized to *Taq*I-digested genomic DNA, it distinguishes four polymorphic fragments in the comparison of PI290136 and BARC-2 (*Rj4*) (Fig. 1D). The gene locus *p(BLT6)1* is distinguished by the hybridization of this probe with a 3,800-bp fragment in PI290136 and with a 3,600-bp fragment in BARC-2(*Rj4*). The F_2 plants were classified for hybridization with the 3,800-bp fragment, the 3,600-bp fragment, or both fragments.

The heterozygous genotypes were distinguishable for the *Fr2* locus, the *p(BLT6)1* locus, the *p(BLT73)*, and the *p(BLT68)* locus (Table 2). The *p(BLT42)3* locus segregated 3:1. Linkage analysis indicated that the *p(BLT42)2* locus was not linked to the *Fr2* locus, but that the *p(BLT6)1*, *p(BLT42)3*, *p(BLT68)*, and *p(BLT73)* loci were linked to the *Fr2* locus and to

each other. Segregation data and linkage analysis are available from the senior author upon request. The linkage group and gene order computed with the Mapmaker program, and the estimated distances calculated from the recombination data without transformation and expressed in centimorgans (cM) indicated by our analysis are: *p(BLT73)* – 6.5+/–1.6 cM – *Fr2* – 6.0+/–2.3 cM – *p(BLT42)3* – 1.0+/–1.0 cM – *p(BLT68)* – 24.4+/–4.2 cM – *p(BLT6)1*.

Location of loci in the genome

Five distinct loci controlling root fluorescence have been reported (Delannay and Palmer 1982; Sawada and Palmer 1987). Only one, *Fr1*, has previously been located in the classical genetic map. *Fr1* is 41 map units distant from the *ep* locus in linkage group 12 (Griffin et al. 1989).

The *Fr2* locus has been determined to segregate independently of several other loci including: *Y12* in linkage group 1 (Devine 1992), *P1* in linkage group 2 (Devine and O'Neill 1989), *P2* in linkage group 4 (Devine 1991), *Y13* in linkage group 7 (Devine 1991), *I* in linkage group 7 (present study), *W1* in linkage group 8 (Devine and O'Neill 1989; Devine 1991), *Rj1* in linkage group 11 (Devine et al. 1983), *Rj2* in linkage group 19 (Devine and O'Neill 1989; Devine et al. 1991), and *Rj4* (Devine and O'Neill 1989). It is, therefore, unlikely, though possible, that the loci *p(BLT42)3*, *p(BLT68)*, *p(BLT73)* and *p(BLT6)1* would be linked to these genes. This is confirmed in the case of the *Rj4* locus, the W1 locus and the *I* locus segregating in the cross studied here by our test for linkage with *p(BLT6)1*, *p(BLT42)3*, *p(BLT68)*, and *p(BLT73)*. Thus, the *Fr2* locus has been located within an RFLP linkage series that is distinguished from elements of several classical linkage groups. The location of the *Fr2* locus in proximity to four RFLP detectable loci that are part of the grid map of RFLP markers is a contribution toward the construction of a comprehensive genetic map of soybean.

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