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Molecular markers located proximal to the soybean cyst nematode resistance gene, *Rhg4*

J. M. Weisemann, B. F. Matthews and T. E. Devine

Plant Molecular Biology Laboratory, Plant Sciences Institute, USDA-ARS, Beltsville Agricultural Research Center-West, Beltsville, MD 20705, USA

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Source of the probes

Probe pBLT24 is a cDNA encoding a 34 kDa soybean seed protein with sequence homology to thiol proteases of the papain family (Kalinski et al. 1990). Probe pBLT65 was isolated from a λgt11 cDNA library screened with a carrot cDNA encoding aspartokinase-homoserine dehydrogenase (Weisemann and Matthews, unpublished). The two probes used in this study were maintained as inserts in *E. coli* plasmid vectors. Plasmid DNA was digested with a restriction endonuclease to release the insert. DNA fragments were purified on agarose gels. The DNA fragments to be used as probes were radiolabeled using the random oligonucleotide labeling procedure of Feinberg and Vogelstein (1983).

Plant analysis

The cross PI290136 × BARC-2 (*Rj4*) and F_2 and F_3 seeds were produced at Beltsville, Maryland. The self-colored seed line PI290136 from the USDA germplasm collection carries the allele *i*, and BARC-2 (*Rj4*) carries *i-i* inhibiting the expression of seed coat color (Devine and O'Neill 1986). Gene symbols were described by Palmer and Kilen (1987). No direct assay for the nematode resistance gene *Rhg4* was performed during the course of these studies since the tight linkage of the *i* locus to *Rhg4* had already been established to be less (0.35%) than one percent (Matson and Williams 1965). The F_2 seeds were planted in vermiculite in the greenhouse. After 2-weeks growth, seedlings were transplanted to the field for further growth and seed production. At maturity, F_3 seeds were harvested from individual F_2 plants. F_3 seeds of each F_2 line were planted in a field at Beltsville and plants were grown to maturity when the seed coat color of the F_4 seed was classified to distinguish the genotype of the F_2 plants. The initial classification for seed coat color, controlled by the *I* locus, was done on the F_3 seed. Since the seed coat is maternal tissue, the superficial phenotype of the F_3 seeds is the phenotype of the F_2 and the superficial phenotype of the F_4 seeds reveals the genotype of the F_2.

Leaf tissue was harvested from individual F_2 plants for DNA extraction during the growing season (Keim et al. 1988). Restriction endonuclease digestions of the DNA 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% agarose 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.

The Mapmaker software program (Lander et al. 1987) was used to screen the data for probable linkages, 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 method of maximum likelihood was used to estimate

Correspondence to: T. E. Devine

Table 1. Results of genetic linkage tests and linkage analysis for the *I* locus with RFLP probes pBLT24 and pBLT65

Genes	Genotypic classes[a]										χ^2 L	$P(\chi^2 L)$[b]	R[c]	SE[d]	Ratio
	e	f	g	h+i	j	k	l	m	n	sum					
I, pBLT65	0	1	3	57	35	3	28	1	1	129	222.29	<0.001	4.0	1.2	121242121
	e+f		g+h+i		j+k		l	m	n						
pBLT24, pBLT65	4	57			41		24	1	0	127	79.59	<0.001	4.5	1.9	363121
pBLT24, I	39	59			5		0	0	25	128	82.97	<0.001	4.4	1.8	363121

[a] Class designations per Allard (1956) Linkage chi-square
[b] Chi-square probability
[c] Percent recombination
[d] Standard error for recombination 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

Probe pBLT24 is a 1,350 bp cDNA clone encoding a 34 Kda soybean seed thiol protease (Kalinski et al. 1990). On Southern blots of genomic DNA digested with the enzyme *TaqI* the probe hybridized mainly with two fragments for PI290136 of about 2,800 and 1,500 bp. Probe pBLT24 hybridized with three fragments for BARC-2 (*Rj4*) of about 2,800, 2,200, and 1,500 bp (Fig. 1A). F_2 progeny were scored on Southern blots for the presence [BARC-2 (*Rj4*) genotype] or absence (PI290136 genotype) of the 2,200 bp fragment. The heterozygote was not distinguishable and the presence of the fragment was dominant.

Probe pBLT65 is a 450 bp fragment of a soybean cDNA clone encoding the enzyme aspartokinase-homoserine dehydrogenase (J. M. Weisemann and B. F. Matthews, unpublished). For PI290136 this probe hybridized with *HaeIII* fragments of 7,000, 1,500 and 850 bp (Fig. 1B). For BARC-2 (*Rj4*) it hybridized with fragments of 5,500, 1,500, and 850 bp. The F_2 progeny were scored for the presence of the 7,000 bp fragment (PI290136 genotype), the 5,500 bp fragment [BARC-2 (*Rj4*) genotype], or both fragments (heterozygote).

The chi-square analyses for linkage strongly indicated linkage of the *I* locus with both pBLT24 and pBLT65, and linkage of pBLT24 with pBLT65 (Table 1). The maximum likelihood estimate of linkage indicated a very tight mutual linkage of these three loci. Due to the very close linkage, the probability of double crossovers would be very low. We calculated the gene order that would require the lowest frequency of double crossovers (0%) as pBLT24 – *I* – pBLT65 with 4.4% recombination between pBLT24 and the *i* locus and 4.0% recombination between *I* and pBLT65. This was substantiated by the Mapmaker estimate of the most likely gene order. Thus, the *I* gene is positioned between the two tightly linked RFLP markers.

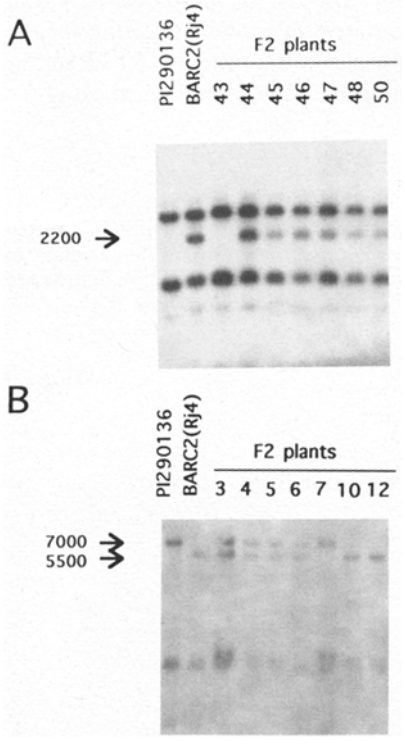

Fig. 1 A, B. Hybridization of probes pBLT24 and pBLT65 to genomic DNA from the two parental lines PI290136 and BARC-2 (*Rj4*) and seven F_2 progeny. **A** pBLT24 hybridized to *Taq*I-digested DNA. **B** pBLT65 hybridized to *Hae*III-digested DNA

Location of loci in the genome

Both the *I* gene and the *Rhg4* gene are located in linkage group 7 of the classical genetic map (Matson and

Williams 1965; Weiss 1970). The *I* gene locus also serves as a bridge to the RFLP map now being constructed, since the *I* locus is also known to be located in linkage group A of that map (Keim et al. 1989).

Since the *I* gene locus is very tightly linked to the *Rhg4* locus, both pBLT24 and pBLT65 must not only be linked to *I* but also to the *Rhg4* locus. Since the *Rhg4* locus was reported to be considerably closer to the *I* locus, 0.35 centimorgans (cm), than either pBLT24, 4.4 cM, or pBLT65, 4.0 cM, it is most probable that *Rhg4* is also located between these two RFLPs. Both the pBLT24 and pBLT65 probes have been sequenced. The close proximity of these loci and their position straddling the *I* locus greatly enhances the opportunity for chromosome walking to isolate and identify the *Rhg4* locus itself. The location of these two RFLPs provides soybean breeders with an alternative method of selecting for the *Rhg4* gene by allowing selection for the two RFLP markers rather than selection by phytopathological assay for disease resistance.

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Probe availability

Contact B. F. Matthews and E. M. Herman.

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