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Molecular Mapping of the Soybean Nodulation Gene, *Rj4*

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Abstract

The *Rj4* allele in soybean protects the soybean plant from nodulation by many strains of *Bradyrhizobium elkanii*, a rhizobitoxine chlorosis-inducing species with generally less efficient symbiosis with soybean. However, the frequency of the *Rj4* phenotype shows progressive diminution with domestication in Asia and breeding for agronomic type in North America. This decrease in frequency might be due to linkage of the *Rj4* gene with unknown genes selected against during domestication and breeding for agronomic type. For this reason it is of interest to determine the location of the *Rj4* gene in the soybean genome. In this study, we tested the *Rj4* gene for linkage with Amplified Fragment Length Polymorphism (AFLP) markers segregating in recombinant inbred lines of the cross of PI290136 X BARC-2 (*Rj4*). The *Rj4* gene was mapped to a catena of AFLP markers.

Keywords: Genome mapping, chromosome, molecular markers

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1. Introduction

Several of the genes controlling nodulation in soybeans have been mapped in the classical genetic map. The *rj1* gene, conditioning restricted nodulation, was located 40 recombination units from the *f* locus controlling fasciated stem (Devine et al., 1983). Later, the *Idh1* locus, conditioning an electrophoretic variant of isocitrate dehydrogenase, was located between the *Rj1* locus and the *f* locus 26.9 recombination units from *Rj1* (Hedges et al., 1990). The *Rj2* gene, conditioning ineffective nodulation with certain bradyrhizobial strains, was mapped to linkage group 19 at a position 2.7 recombination units from the *Rps2* locus, conditioning resistance to phytophthora root rot disease (Devine et al., 1991). However, the *Rj4* gene, despite extensive efforts (Devine, 1992) has not been mapped until the present study.

The dominant allele *Rj4* in soybean (*Glycine max* (L.) Merr.) restricts nodulation principally, though not exclusively, with strains of *Bradyrhizobium elkanii* (Devine et al., 1990; Kuykendall et al., 1992; Devine and Kuykendall, 1996). *B. elkanii* strains occur with high frequency in soils of the southeastern U.S. and readily nodulate with most soybean cultivars (Weber et al., 1989). However, *B. elkanii* strains frequently produce rhizobitoxine-induced chlorosis symptoms on soybeans in Leonard jar tests and are generally poor microsymbionts of soybeans (Fuhrmann, 1990). The *Rj4* gene is considered a desirable allele for soybean cultivars where *B. elkanii* strains are common because it provides a degree of protection against nodulation by these strains which lack full competence as soybean microsymbionts (Qian et al., 1996). *B. elkanii* strains, however, serve as competent microsymbionts of other host legumes such as cowpea (*Vigna unguiculata* (L.) Walp) (Devine and Kuykendall, 1996). The *Rj4* phenotype is the most common phenotype (63.4%) in *Glycine soja*, the putative wild progenitor of the domesticated soybean, *Glycine max*. In a survey of over 800 plant introductions from 12 Asian countries, the *Rj4* phenotype was most frequent in the populations from Southeast Asia, i.e., Myanmar, Indonesia, Malaysia, Thailand, and Vietnam (all over 60%). The frequency of the *Rj4* phenotype in cultivars developed for mechanized agriculture in North America is much lower. The frequency of the *Rj4* phenotype was 13.7% among the entries in the preliminary test lines and 8.55% in uniform test lines (Devine and Breithaupt, 1981). The reduction in the frequency of the *Rj4* allele that has occurred with domestication in Asia and selection for agronomic characteristics in North America might be attributed to genetic linkage with traits selected against during domestication and agronomic improvement, e.g. seed shattering, viney growth habit, small seed. For this reason it is of interest to determine the location of the *Rj4* gene in the soybean genome and any possible linkages to characteristics disfavored during domestication and selection for agronomic traits.

2. Materials and Methods

The cross PI290136 X BARC-2 (*Rj4*) was made in a field nursery at Beltsville, MD (Devine, 1992). PI290136, also referred to as 'Noir 1', is an accession from the USDA germplasm collection carrying the recessive allele *rj4*. BARC-2 (*Rj4*) is a near isogenic line of the cultivar Clark 63 (Devine and O'Neill, 1986) and carries the dominant allele *Rj4*, conditioning ineffective nodulation with strains of bacteria principally, but not exclusively, classified as *B. elkanii* (Vest and Caldwell, 1972; Devine et al., 1990; Kuykendall et al., 1992). To develop the Recombinant Inbred Lines (RIL's) for genetic mapping, the F_1 generation was grown in the greenhouse at Beltsville and F_2 seed harvested. The F_2 and succeeding generations were grown either in the field or in the greenhouse for pedigree generation advance without selection. Seeds of the F_8 RIL's were surface sterilized by immersion in 50% v/v ETOH for 25 seconds and rinsed in tap water. The seeds were then planted in the glasshouse in sterilized vermiculite in growth trays (Devine and Reisinger, 1978). At planting, the seeds were inoculated with a stationary phase AIE broth (Kuykendall, 1979) culture of *B. elkanii* strain USDA 61. Strain USDA 61 is definitive for distinguishing the ineffective vs. effective nodulation responses conditioned by the alleles *Rj4* vs. *rj4*. After two weeks of growth, plant roots were extracted from the vermiculite and scored for effective vs. ineffective nodulation response (Devine and O'Neill, 1986).

DNA extraction

Leaf tissue of the soybean inbreds (103 F_8 progenies from the cross PI290136 X BARC-2 (*Rj4*)) planted in the greenhouse was harvested for DNA extraction and stored in a –80°C freezer until use. Genomic DNA was extracted as described previously (Michaels et al., 1994).

AFLP procedure

Amplified Fragment Length Polymorphism (AFLP) procedure was performed following the protocol of Life Technologies Inc., using the AFLP Primer Starter Kit and the Core Reagent Kit (Lin et al., 1996). Primary template DNA was prepared by completing a restriction enzyme digest and then an adapter ligation. 300 ng of DNA from each line was digested with 2 µl of *Eco*RI/*Mse*I (1.25 units of each enzyme/µl) at 37°C for 2 h, then heated to 70°C for 10 minutes to inactivate the enzymes. In addition to the DNA and enzymes the following were added to a 1.5 ml microcentrifuge tube: 5 µl of 5X reaction buffer and AFLP-grade water to a final volume of 25 µl. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters provided in the kit. The ligation mixture

Table 1. The primer selective nucleotides, marker names and their sizes for the 9 primer pairs used in the study

Primer pairs	AFLP Markers and their sizes in base pairs (bp)									
E-AAC/M-CAC	aBLT1 462bp	aBLT2 418bp	aBLT3 317bp	aBLT4 200bp	aBLT5 191bp	aBLT6 185bp	aBLT7 112bp			
E-ACA/M-CAC	aBLT8 467bp	aBLT9 450bp	aBLT10 425bp	aBLT11 263bp	aBLT12 258bp	aBLT13 250bp	aBLT14 119bp	aBLT15 <92bp	aBLT16 <92bp	
E-ACC/M-CAA	aBLT17 300bp	aBLT18 291bp	aBLT19 280bp	aBLT20 275bp	aBLT21 220bp	aBLT22 215bp	aBLT23 169bp	aBLT24 132bp	aBLT25 100bp	aBLT26 98bp
E-AGC/M-CTG	aBLT27 600bp	aBLT28 220bp	aBLT29 100bp	aBLT30 96bp	aBLT31 92bp	aBLT32 80bp	aBLT33 60bp	aBLT34 52bp		
E-AAC/M-CAT	aBLT35 300bp	aBLT36 209bp	aBLT37 165bp	aBLT38 159bp	aBLT39 110bp					
E-AAC/M-CTT	aBLT101 300bp	aBLT102 285bp	aBLT103 260bp	aBLT104 223bp	aBLT105 180bp					
E-AAG/M-CTC	aBLT106 550bp	aBLT107 490bp	aBLT108 325bp	aBLT109 60bp	aBLT110 55bp					
E-ACT/M-CTA	aBLT111 510bp	aBLT112 400bp	aBLT113 115bp	aBLT114 87bp						
E-AGG/M-CAC	aBLT115 320bp	aBLT116 275bp	aBLT117 236bp	aBLT118 230bp						

(containing fragments with adapters at both ends) was diluted 10-fold with sterile distilled water and the fragments were preamplified by 20 PCR cycles. The PCR reaction was performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) with the following temperature profile: 94°C for 30 s, 56°C for 60 s and 72°C for 60 s using *Eco*RI+A (5'-GACTGCGTACCAATTC+A3') and *Mse*I+C (5'-GATGAGTCCTGAGTAA+C3') primers (provided in the kit) described by Vos et al. (1995).

For selective amplification of restriction fragments, 9 sets of *Eco*RI (E) and *Mse*I (M) primer pairs (provided in the kit) were used (Table 1). Selective amplification was conducted by PCR using 5 µl aliquots of the preamplified fragments diluted 50-fold, using ^{32}P-ATP *Eco*RI+3 labeled primer with an unlabeled *Mse*I+3 primer and amplified by PCR using one cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, followed by lowering the annealing temperature each cycle 0.7°C for twelve cycles. The reaction amplified for 23 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 60 s. The reaction products were loaded on a 5% w/v polyacrylamide DNA sequencing gel containing 7.5M urea. Autoradiography was performed by exposing Kodak BioMax MR-2 film (Eastman Kodak Co., Rochester, NY, USA) to the dried gel overnight with intensifying screens in a –80°C freezer.

The following morphological traits were tested for linkage to *Rj4*: root fluorescence under UV light (Fr2), self colored seed coat (I), and purple flower with purple stem (W1). The Mapmaker/EXP version 3.0b computer software program was used to screen the data for linkage relationships and gene order using the criteria of a LOD of 4.0 and maximum distance of 35 centimorgans. The linkage map was constructed using the Kosambi Function (Lander et al., 1987).

3. Results

AFLP patterns were produced from the DNA extracted from the parents in the cross PI290136 X BARC-2 (*Rj4*) and the 103 F_8 recombinant inbred line progenies using 9 primer combinations. These 9 primer combinations resulted from seven *Eco*RI+3 and seven *Mse*I+3 primers. *Eco*RI and *Mse*I primers were used here because of their insensitivity to methylation patterns observed in plants (Keim et al., 1997). On the average 60 AFLP fragments in a size range of 50 to 500 base pairs were generated per primer combination. A total of 500–600 AFLP fragments were screened for polymorphism and 57 were found to be polymorphic (Table 1). This number (57/9 primer pairs) is similar to the 5.6 polymorphic bands per AFLP primer pair reported by Lin et al. (1996).

Four genetic markers scored in the studied population of inbred lines including the *Rj4* gene (Devine, 1992) were analyzed with the AFLP markers for linkage relationships. Ten of the 57 polymorphic AFLP markers were linked to

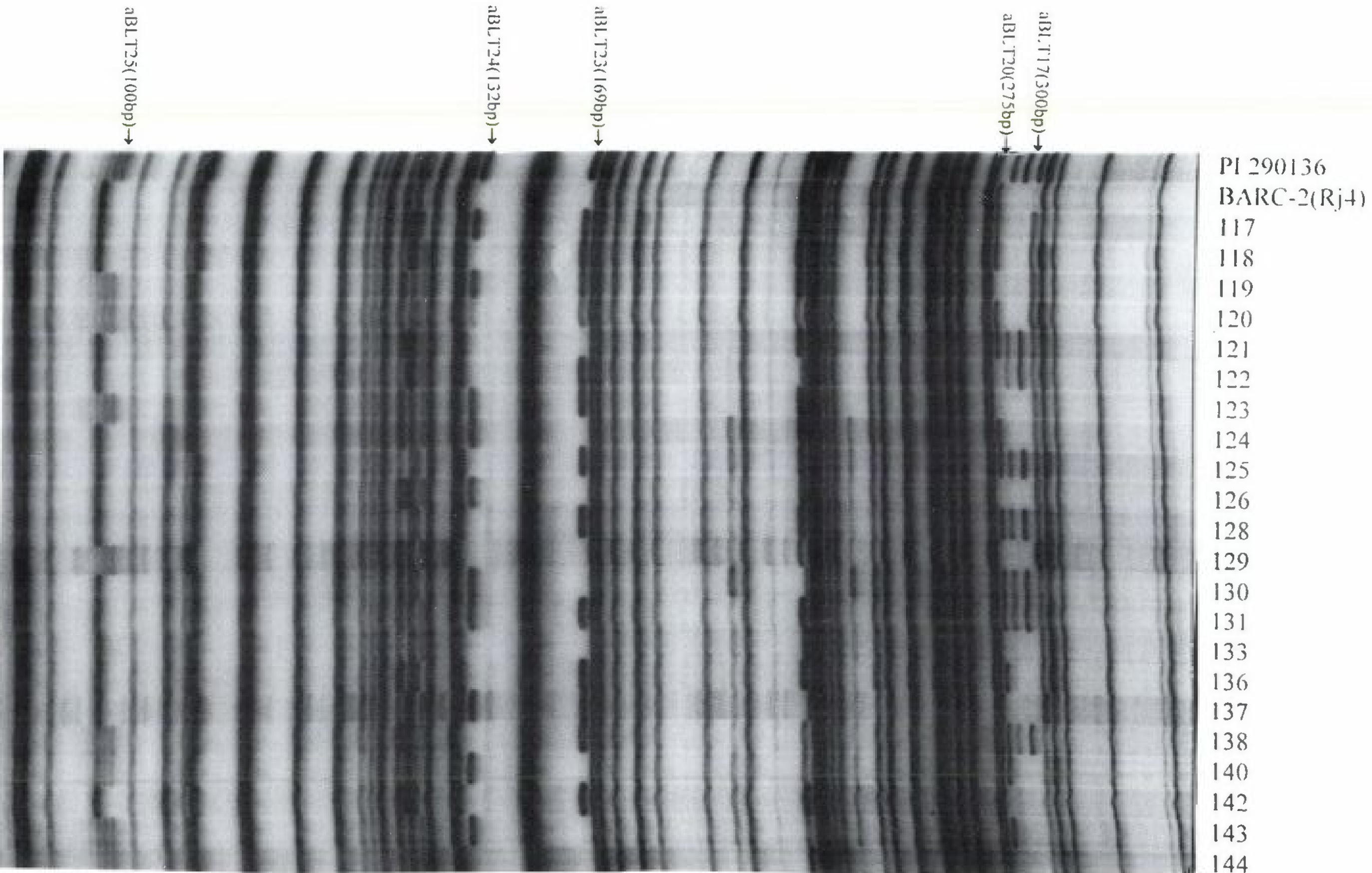

Figure 1: Autoradiogram obtained with *Eco*-ACC and *Mse*-CAA primer combinations showing segregation of alleles revealed by AFLP markers. The first two lanes contain the parents PI 290136 and BARC-2 (*Rj4*) followed by 22 of the 103 F_8 recombinant inbred lines used in this study.

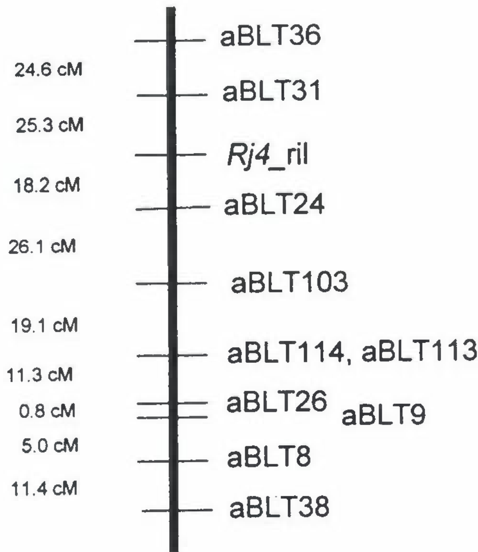

Figure 2: *Rj4* linkage group using LOD 4.0, maximum distance 35 cM.

the *Rj4* locus. This is a higher frequency of linked markers than would be expected based upon completely random distribution of AFLP markers and may represent an example of clustering of AFLP markers (Keim et al., 1997). The linkage map of the *Rj4* region (Fig. 2) is 141.8 cM long with an average distance of 12.7 cM between loci. The markers, aBLT24 (ACCCAA132) and aBLT31(AGCCTG92), are the more closely linked to the *Rj4* locus and are 18.2 and 25.3 cM from it, respectively. The morphological traits (*Fr2*, *I* and *W1*) segregated independently of each other and showed no linkage with the *Rj4* locus.

4. Discussion

The *Rj4* gene restricts nodulation by *Bradyrhizobium elkanii* (Devine et al., 1990; Kuykendall et al., 1992) which produce rhizobitoxine in plants and induces foliar chlorosis on soybean. *B. elkanii* strains are a prominent portion of the bradyrhizobial population in the soybean producing areas of the U.S. They are particularly common in soils subject to hot, dry conditions. The *Rj4* phenotype is the most commonly occurring type in *G. soja*, the wild progenitor of the domesticated soybean, and among the plant introductions of the domesticated soybean from Southeast Asia (Devine, 1987). The frequency of

the allele appears to have decreased progressively with domestication from *Glycine soja* in Asia and subsequent selection for agronomic type in North America. The diminishing frequency of the *Rj4* allele with selection raises the question of possible genetic linkage of the *Rj4* allele with other alleles conditioning qualities undesirable for agronomic production (Devine, 1992). The *Rj4* gene segregates independently of loci in nine of the linkage groups in the classical genetic map of soybeans, suggesting that it is less likely that the AFLP markers described here as linked to the *Rj4* locus, are associated with these linkage groups (Devine, 1992).

Molecular markers provide a quick and easy way of constructing high density molecular maps and saturating the existing gaps in existing genetic maps. High density maps have been found to be useful in molecular-marker-assisted selection of both qualitative and quantitative (QTLs) traits of agronomic importance and in the subsequent attempts to identify and clone the genes (Cai et al., 1997; Martin et al., 1993; Zhang et al., 1994). The development of a comprehensive map of the soybean genome, combining information on molecular markers and traits of agronomic significance, is an ongoing long term effort of several research groups (Keim et al., 1997).

The morphological traits (Fr2, I and W1) segregated independently of each other and were not linked with the *Rj4* locus. However, the *Rj4* gene showed close linkage to a catena of molecular markers (Fig. 2) with a total linkage distance of 141.8 cM. It is anticipated that the molecular linkage map published here will enhance further understanding of the linkage relationships of the *Rj4* gene with genes controlling other traits of significance in the domestication of soybean and its genetic adaptation to use in North American agriculture. Such an understanding may eventually provide an explanation for the shifts in the allelic frequencies at the *Rj4* locus concomitant with soybean domestication and agronomic adaptation. Secondly, to clone *Rj4*, a high-resolution genetic map can be constructed for the segment of chromosome that is bordered by the AFLP loci aBLT24 and aBLT31 and includes the *Rj4* locus (Meksem et al., 1995).

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