DNA Markers Associated with Resistance to Javanese Root-Knot Nematode in Soybean

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ABSTRACT

Javanese root-knot nematode [*Meloidogyne javanica* (Treub) Chitwood] (Mj) is a pathogen of soybean [*Glycine max* (L). Merr.] in the southern USA. Although breeding for root-knot nematode resistance is an important objective in many plant breeding programs, progress in selection for nematode resistant lines is hampered by laborious screening procedures. The inheritance of resistance to Mj is quantitative and has a moderate to high heritability (0.48–0.76). The objectives of this study were to use restriction fragment length polymorphism (RFLP) markers to identify quantitative trait loci (QTL) conditioning resistance to Mj and to determine the genomic location and the relative contribution to resistance of each QTL. Eighty-four F_2 progeny from a cross between "CNS" and a root-knot nematode resistant soybean plant introduction, PI230977, were used to map 86 RFLP markers and three morphological traits. The 89 markers converged on 18 linkage groups spanning a total of 1053 centimorgan (cM). Sixty-eight of the 84 $F_{2:3}$ families were assayed for Mj galling in the greenhouse. Analysis of variance and interval mapping were used to identify QTL associated with galling. Two QTL with alleles derived from PI230977 conditioning resistance to Mj were identified on two Linkage Groups (LG). Marker B212-1 on LG-F accounted for 46% of the variation in gall number, whereas A725-2 on LG-D1 accounted for 13%. The additive model best fit the data, and together the two markers accounted for 54% of the variation in gall number. Marker B212-1 is within a cluster of seven other disease resistance loci that span a 5- to 10-cM region on LG-F.

THE ROOT-KNOT NEMATODES, *Meloidogyne* spp., are distributed worldwide and have a broad host range that includes most crop plants (Sasser, 1977). Root-knot nematodes (RKN) cause serious damage through tissue abnormalities, direct feeding, wounding, and induction of disease complexes (Sasser, 1977). The severity of the disease is determined by the nematode population density, environmental conditions, and host genotype. The three main species of *Meloidogyne* that cause damage in soybean are *M. incognita* (Kofoid and White) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. javanica* (Riggs and Schmitt, 1987). In the southern USA, soybean growers annually lose an estimated $28 million because of plant damage caused by RKN (Sciumbato, 1993). Of the three main species of RKN, Mj occurs the least frequently in soybean fields in the southern USA (Garcia and Rich, 1985). However, Mj is potentially a serious threat to soybean producers since 60% of cultivars are susceptible (Hussey et al., 1991).

The development and use of RKN-resistant cultivars in combination with crop rotation, is the most effective control measures (Roberts, 1992). Considerable effort has been directed toward development of RKN-resistant soybean cultivars (Mai, 1985; Boerma and Hussey, 1992). The Southern Soybean Germplasm Collection was evaluated for resistance to Mj and PI230977 was identified as having the highest level of resistance to Mj for gall formation and nematode reproduction (Luzzi et al., 1987). PI230977 is a valuable source of resistance as it provides higher levels of resistance to Mj than currently exists in modern soybean cultivars. Heritability estimates for Mj range from 0.48 to 0.76 on a mean basis and soybean genotypes with different Mj resistance genes have been identified (Luzzi et al., 1995).

Many studies using DNA markers have concentrated on QTL that control important agronomic traits. In soybean, RFLPs were used to locate and determine effects of QTL associated with seed protein and oil content (Diers et al., 1992a; Lee et al., 1996b; Brummer et al., 1997); plant height, lodging, and maturity (Lee et al., 1996a,c; Mansur et al., 1993); pod dehiscence (Bailey et al., 1997); hard seededness (Keim et al., 1990b); and

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Abbreviations: cM, centimorgan; LG, linkage group; QTL, quantitative trait locus(i); Mj, *Meloidogyne javanica*; RFLP, restriction fragment length polymorphism; RKN, root-knot nematode.

various other traits (Keim et al., 1990a; Mansur et al., 1993).

Genetic markers have also been used for mapping disease resistance genes in soybean. DNA markers associated with *Phytophthora sojae* Kauf. and Genre. resistance (Diers et al., 1992b) and soybean mosaic virus resistance have been identified (Yu et al., 1994). In addition, studies have also used DNA markers to dissect quantitatively inherited resistance including soybean cyst nematode, *Heterodera glycines* Ichinohe, (Concibido et al., 1994; Webb et al., 1995) and soybean sudden death syndrome, *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. & Hans., type A FSA (Hnetkovsky et al., 1996).

The objectives of our study were two-fold: (i) use RFLP markers to identify QTL conditioning resistance to Mj and (ii) determine the relative contribution to resistance and genomic location of each QTL.

MATERIALS AND METHODS

An F_2 population consisting of 84 individuals was developed from the cross of CNS, susceptible to Mj, with a Mj-resistant plant introduction, PI230977 (Luzzi et al., 1987). The 84 F_2 plants and the parents were grown in the greenhouse in Athens, GA, and used as source of leaf tissue for DNA extraction and RFLP analysis. The F_2 plants were allowed to set seeds and data were collected on pod wall, hilum, and seed coat color. The genotypes of 11 F_2 plants were reconstituted by combining the leaves from 25-d-old plants from $F_{2:3}$ families, and a minimum of 14 plants was used to reconstitute the genotypes from these families.

Root-Knot Nematode Assay: Greenhouse Screening Procedure

Because of insufficient seed, only 68 of the 84 $F_{2:3}$ families were evaluated for resistance to Mj. Parental genotypes and $F_{2:3}$ families were evaluated for Mj in a randomized complete block design with three replications by means of greenhouse screening procedures (Luzzi et al., 1987). Seeds were planted in polystyrene Todd Planter Flats (Model 150-5, Speedling, Sun City, FL) and inoculated with 2000 eggs of greenhouse maintained Mj 7 d after planting (Hussey and Barker, 1973). Thirty days after inoculation, soil was washed from each root system, and the galls on each root system were counted.

DNA Clones, DNA Extraction, Restriction Digestion, Electrophoresis, and Blotting

Most of the RFLP markers mapped in this study were provided by R.C. Shoemaker, USDA-ARS and Iowa State Univ. A soybean heat shock protein gene (*Gm*HSP) (Nagao et al., 1985) provided by Dr. Ron Nagao (Univ. of Georgia, Athens, GA) was also mapped.

Soybean DNA was extracted from parental genotypes, individual F_2 plants, and bulked $F_{2:3}$ families according to previously published procedures (Keim et al., 1988). The DNA was quantified by spectrophotometric analysis and 10 μg were restriction enzyme-digested overnight. The five enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Taq*I, used to construct the USDA-ARS-ISU soybean map (Shoemaker and Specht, 1995) were also used in this study. Digested DNA was electrophoresed (22 V) for 16 h on 8 g L^{-1} agarose 10- by 20-cm gels and transferred onto GeneScreen+ membranes (Dupont, Wilmington, DE) (Southern, 1975).

DNA Hybridization and Map Construction

Lithium chloride mini-prep plasmid preparations from bacterial lysates were prepared (Kochert et al., 1991). Cloned DNA inserts were amplified from the mini-preps by the polymerase chain reaction. Hybridization conditions were essentially the same as in a previous study (Lee et al., 1996a). When a single probe detected several polymorphic fragments, individual fragments were designated by the same probe name but distinguished by a dash and a number (e.g., A757-1). The autoradiogram was compared with images downloaded from the soybean database to identify anchor markers on the USDA-ARS-ISU soybean map (SoyBase, 1995).

Multi-point linkage analysis was performed with MAPMAKER-EXP using data from the 84 F_2 individuals (Lander et al., 1987; Lincoln et al., 1992a). Linkage data were used to assign markers to linkage groups if the distance was ≤ 37 cM with Kosambi (1944) mapping function and the log likelihood of the odds (LOD) was ≥3.0.

Statistical Data Analysis

The gall number data were subjected to analysis of variance. Analysis of the raw data resulted in a correlation of $F_{2:3}$ line means and variances. To dissociate means and variances, a transformation [$\log_{10}(x + 1)$] was used on counts of galls per plant. The analysis was reconfirmed with untransformed data. All values presented for gall number are the antilog of the mean minus one. Heritability based on variance component estimates was calculated on a mean basis with the following formula: $h^2 = \hat{\sigma}^2_{F3} / [\hat{\sigma}^2_{F3} + \hat{\sigma}^2_e / r]$, where $\hat{\sigma}^2_{F3}$ is the genotypic variance for gall number per plant among F_3 families, $\hat{\sigma}^2_e$ is the error variance, and r is the number of replications.

Two methods of analysis were employed to identify markers associated with Mj resistance. With marker genotypic classes (e.g., A_1A_1, A_1A_2, A_2A_2) as the predictor variable and gall number as the response variable, data were analyzed with a general linear model (SAS, 1988). A significant association between a DNA marker and Mj response was declared if the probability was <0.001. The coefficient of determination (R^2) was used as a measure of the magnitude of the marker association. Significant differences between means were determined by LSD ($P = 0.001$) performed on $\log_{10}$ transformed data.

For the second method of analysis, interval mapping (Paterson et al., 1988; 1991) was used with MAPMAKER-QTL (Lincoln et al., 1992b) to identify putative QTL. A QTL was declared significant if the LOD score exceeded 2.2. Tests for each mode of inheritance (additive, dominant, or recessive) for QTL were performed as described in Paterson et al. (1991). The R^2 values presented are those obtained from unconstrained genetic analysis. Weights (the effect of allele substitution) were obtained from the map and scan commands. The scan command determines the maximally likely QTL likelihood at 2-cM intervals along the specified linkage group or entire map length. A QTL's position was fixed and additional QTL were searched for by determining the maximally likely QTL likelihood while controlling some of the variation with the first QTL. A LOD increase of 2.0 above the fixed QTL was used to declare any additional QTL significant. A LOD decrease of 1.0 for any constrained mode of inheritance was considered adequate to exclude specific modes of inheritance for the QTL.

RESULTS AND DISCUSSION

Molecular Markers and the RFLP Map

Four hundred fifty-one probes were tested against the restricted digested DNA of the two parental genotypes.

With at least one restriction enzyme, polymorphisms were detected with 178 (39%) of the probes. This level of polymorphism is higher than in a previously reported soybean study (Apuya et al., 1988), but was similar to polymorphism rates observed in other studies (Skorupska et al., 1993; Boutin et al., 1995; Lee et al., 1996a; Chen et al., 1996).

Eighty probes detecting 86 (six duplicate) informative markers, and three morphological traits were mapped in our study. The 89 markers converged into 18 linkage groups, spanning a total of 1053 cM, with an average of 13.5 cM between markers. Eleven markers remained unlinked. The unlinked markers contributed an additional 220 cM to the map length, estimated by adding 10 cM on each side of each unlinked marker (Danesh et al., 1994) for a total map length of 1273 cM. Twelve linkage groups contained three or more markers, and six linkage groups contained only two markers. For most linkage groups, the marker order was identical and distances were similar or longer than the USDA-ARS-ISU map distances (Shoemaker and Specht, 1995).

Mj Disease Response among $F_{2:3}$ Lines and Associated Markers

CNS averaged 39 galls per plant, compared with 6 galls per plant for PI230977. The $F_{2:3}$ families ranged from 4 to 51 galls per plant (data not shown). After transformation, the distribution of gall number in the F_3 was continuous. The variance component heritability estimate on a family mean basis was 0.55, which is consistent with an estimate from a previous study (Luzzi et al., 1995).

Resistance to Mj was mapped with gall number per plant from 68 of the 84 $F_{2:3}$ families. Eight RFLP markers ($P < 0.001$) were associated with Mj gall number (Table 1). Among the seven significant markers on LG-F, marker B212-1 accounted for the greatest variation in gall number (46%), and marker A725-2 on LG-D1accounted for 13%. For a given genotypic class, PI/PI (homozygous PI230977), PI/CNS (heterozygous), or C/C (homozygous CNS), the marker allelic means were similar, and markers from PI230977 were associated with fewer galls. Because of co-segregation of B212-1 and other significant markers on LG-F (a span of 13 cM), the significant marker alleles from PI230977 had a strong effect on reducing gall number (Table 1). Though the allelic means were similar, the R^2 values ranged from 19% (A806-1) to 46% (B212-1).

Together, markers B212-1 and A725-2 accounted for 54% of the variation in gall number (SAS, 1988) in a two QTL model. When lines were homozygous for markers derived from CNS (CC-CC), the highest number of galls was observed (33.6) (Fig. 1). Conversely, the lowest number of galls (7.4) was observed when lines were

Table 1. RFLP markers significantly ($P = 0.001$) associated with *Meloidogyne javanica* disease resistance based on analysis of variance.

RFLP marker†	Linkage group	R^2	Marker allelic means		
			PI/PI‡	PI/CNS	CNS/CNS
		%	galls plant^{-1}		
A806-1	F	19	10.6	18.8	17.9
*Gm*HSP	F	39	11.2	19.1	27.0
A186-1	F	30	11.5	18.5	26.0
A757-2	F	36	11.1	18.0	27.4
B212-1	F	46	10.3	18.9	27.1
R045-1	F	42	10.0	18.2	27.0
B174-1	F	28	10.3	17.7	22.5
A725-2§	D1	13	11.9	16.4	21.2

† Markers are shown in the order in which they map on Linkage Group F.
‡ PI = PI230977.
§ The probability value for marker A725-2 was 0.0081.

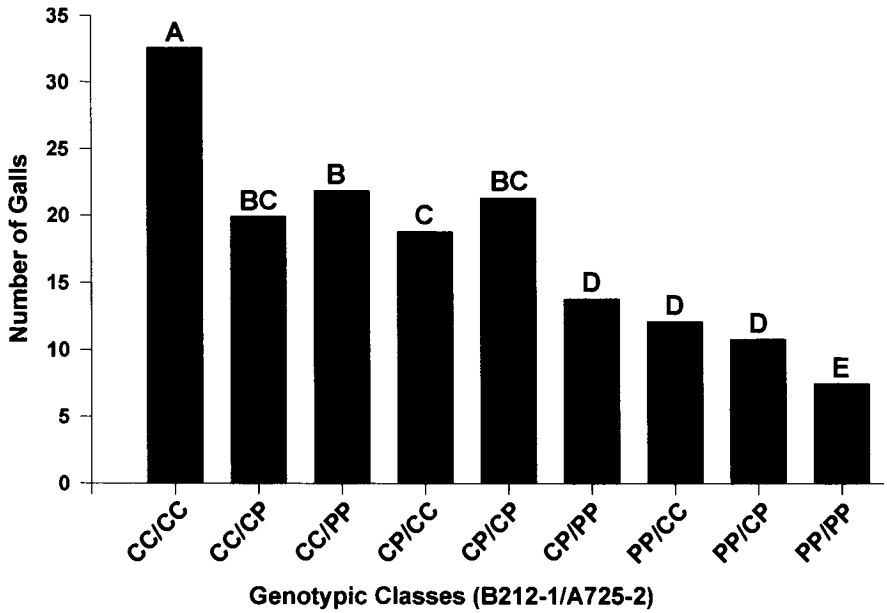

Fig. 1. Effects of the two RFLP markers, B212-1 and A725-2, for number of galls per plant (antilog of the mean minus one). CC-CC and PP-PP are homozygous for markers alleles at B212-1 and A725-2 for CNS (C) and PI230977 (P), respectively. Bars with the same letter did not differ significantly ($P = 0.001$).

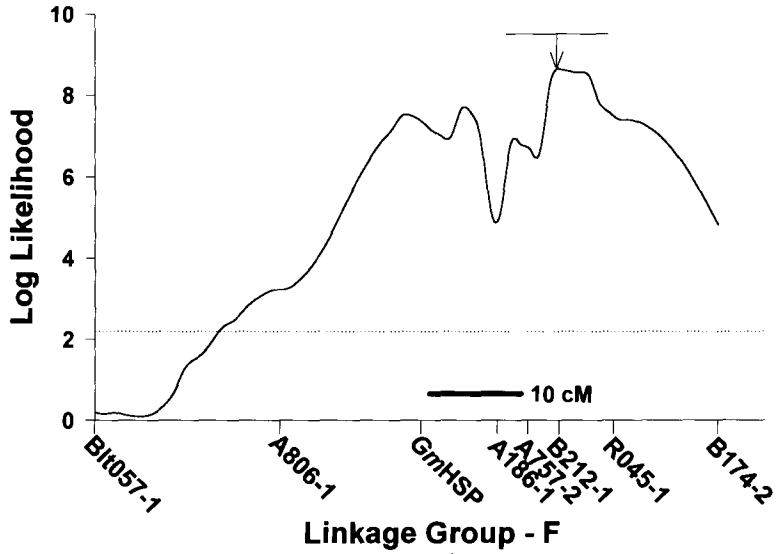

Fig. 2. QTL likelihood plot indicating LOD scores for *Meloidogyne javanica* gall per plant (transformed data) for $F_{2:3}$ lines on LG-F. The QTL peak is shown by the downward pointing arrow and its corresponding (10%) confidence interval (Lincoln and Lander, 1992b) is shown as horizontal line above the arrow. The horizontal dotted line at LOD of 2.2 represents the minimum LOD required for significance. The thick bar represents 10 cM.

homozygous for the two markers originating from PI230977 (PP-PP), and the mean gall number approached the level of galling observed for the resistant parent (6.0 galls per plant).

The data were also examined using MAPMAKER-QTL (Lincoln et al., 1992b). A LOD peak (LOD = 8.7) was detected at the marker B212-1 on LG-F (Fig. 2). The confidence interval for the peak was 11 cM (Lincoln and Lander, 1992b). The dominance to additive ratio (d/a) was −0.27 for B212-1, indicative of additive gene action (Stuber et al., 1987). The main effect (average effect of allele substitution) for B212-1 was −10.4, and the predicted mean was 6.3 galls per plant when families were homozygous for the PI230977 marker. Similarly, the main effect for A725-2 was −5.2, and the predicted mean was a slightly higher 10.7 galls per plant when families were homozygous for the PI230977 markers. With MAPMAKER-QTL, the additive model best fit the data, and no significant difference between the unconstrained and additive genetic models was found. Another LOD peak was detected on LG-F between marker *Gm*HSP and A186-1; however, the LOD did not increase significantly when B212-1 was fixed and the map rescanned in this area. The QTL position was fixed at B212-1 and the framework map rescanned in the search for other QTL; a significant LOD increase was detected at marker A725-2 on LG-D1 (LOD = 10.9, R^2 = 54%). Since both peaks were at the markers, interval mapping and analysis of variance yielded similar results. Additionally, the same QTL were found when data was analyzed by both ANOVA and interval mapping analysis with the non-transformed data.

The theoretical limit for the percent genotypic variation that can be accounted for in this population was 55% (the heritability estimate). The phenotypic variance explained by the markers B212-1 and A725-2 was 54%. Therefore, 98% of the genetic variation was explained by the two locus additive genetic model. Any additional QTL found in this population would likely account for only a small amount of the variation in gall number.

Clustering of Disease Resistance Genes on LG-F of the USDA/ARS-ISU RFLP Map

In addition to the Mj resistance QTL on LG-F reported in this study, seven other disease resistance QTL are known to reside in the same 10-cM region of LG-F (Table 2). The clustered QTL confer resistance to a diverse group of pathogens from three kingdoms (animal, monera, and fungi) and two viruses.

Table 2. Disease resistance genes and QTL tightly clustered on Linkage Group F of the USDA/ARS-ISU RFLP map (Shoemaker and Specht, 1995).

Pathogen/pest	Resistance gene or source	RFLP marker	Reference
Soybean mosaic virus	Rsv_1	A186-1	Yu et al., 1994
Peanut mottle virus	Rpv_1	A186-1	Palmer and Kiang, 1990
Heterodera glycinea	PI90763	A186-1†	N. Young, 1996, personal communication
Meloidogyne javanica	PI230977	B212-1	This study
Meloidogyne arenaria	PI200538	B212-1	Tamulonis et al., 1997a
Helicoverpa zea (corn ear worm)	'Cobb'	B212-1	B. Rector, 1996, personal communication
Pseudomonas syringae pv. *glycinea*	Rpg_1	K644-1	R. Innes, 1996, personal communication
Phytopthora megasperma	Rps_3	R045-1	Diers et al., 1992b

† A minor QTL was detected at marker A186-1.

The soybean genome has undergone one or possibly several rounds of duplication during its evolution (Zhu et al., 1995; Shoemaker et al., 1996). For example, on LG-F of the USDA-ARS-ISU RFLP map, marker K644-1, to which all resistance genes found on LG-F (Table 2) are closely linked, is duplicated four times in a *cis*-configuration. This suggests that the resistance genes may have been duplicated as well. Duplication followed by mutation, genetic divergence, and specificity could account for the clustered resistance genes. Alternatively, a gene may confer resistance to more than one pathogen. In addition, the genomic region on LG-F that encompasses the resistance loci is homoeologous with LG-O, and at least one other resistance QTL for *M. incognita*. Marker K644-5 is closely linked to marker G248-2, which is also duplicated on LG-O. A major QTL for resistance to the nematode *M. incognita*, (Tamulonis et al., 1997b) has been found on LG-O, 10 cM from G248-1. Supporting evidence for duplication of resistance QTL comes from comparative mapping (Boutin et al., 1995) that shows a region on LG-A of the common bean map conserved on LG-F and duplicated on LG-O of the USDA/ARS-ISU soybean RFLP map (Shoemaker and Specht, 1995).

Besides the cluster of resistance genes in soybean, studies in other crops such as lettuce, *Lactuca sativa* L., (Witsenboer et al., 1995), tomato, *Lycoperisicon esculentum* Miller, (van der Beek et al., 1992; Sarfatti et al., 1991), flax, *Linum usitatissimum* L., (Hammond-Kosack and Jones, 1996), and maize, *Zea mays* L., (Richter et al., 1995), have also shown clustering of resistance gene. Speculation regarding the mechanisms for resistance gene clustering in genomes includes unequal crossing and genetic specificity for the various pathogens (Staskawicz et al., 1995; Richter et al., 1995; Charlesworth et al., 1994; Hammond-Kosack and Jones, 1996).

SUMMARY

Using both analysis of variance and maximum likelihood methods of analysis, we report the identification and mapping of two QTL from PI230977 conditioning resistance to Mj. A LOD peak was obtained at marker B212-1 (LG-F) and explained the greatest amount of variation (46%) for gall number. Marker A725-2 on LG-D1 explained 13% of the variation. Together the markers acted additively and accounted for 54% of the variation in gall number, which was of the same magnitude as the heritability estimate ($h^2 = 0.55$) of this trait. From the amount of variation explained for each marker it could be surmised that one major gene, closely linked to B212-1, and a minor gene, linked to A725-2 at a less definitive distance, together control the resistance to Mj in soybean.

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