Molecular Markers Linked to Brown Stem Rot Resistance Genes, *Rbs$_1$* and *Rbs$_2$*, in Soybean

M. S. Bachman,* J. P. Tamulonis, C. D. Nickell, and A. F. Bent

ABSTRACT

Brown stem rot (BSR) of soybean [*Glycine max* (L.) Merr.] is caused by the fungal pathogen *Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams and occurs in soybean production areas around the world. Brown stem rot resistance genes *Rbs$_1$*, *Rbs$_2$*, and *Rbs$_3$* have been identified in soybean germplasm and plant introductions through traditional genetic analyses. Resistance to BSR has been shown to reduce yield losses in soybean, but selection for this trait is laborious and confounded by environmental variation. The objectives of this study were to identify molecular markers linked to BSR resistance genes *Rbs$_1$* and *Rbs$_2$*, and map these genes in the soybean genome. Genetic families of populations segregating for *Rbs$_1$* and *Rbs$_2$* were evaluated in the greenhouse for BSR phenotypic reaction and identified as resistant, segregating, or susceptible. Leaf tissue collected from members of $F_{2:3}$ families was bulked and DNA simple sequence repeat (SSR) marker analysis was used to identify markers that cosegregated with BSR reaction phenotypes. Five pairs of *Rbs$_2$* near-isogenic lines were subjected to a similar analysis to verify results obtained from marker analysis conducted on the population segregating for *Rbs$_2$*. Results of marker analyses indicated that SSR markers Satt215 and Satt431 were linked to *Rbs$_1$* and that Satt244 and Satt431 were linked to *Rbs$_2$*. Marker-assisted selection in the *Rbs$_1$* (using Satt431) and *Rbs$_2$* (using Satt244) populations would have correctly predicted 88 and 82%, respectively, of the BSR reaction phenotypes. The *Rbs$_1$* and *Rbs$_2$* loci map to Molecular Linkage Group J and lie in a region known to contain *Rbs$_3$*. This region also contains loci conditioning resistance to taxonomically diverse fungal pathogens and a locus affecting nodulation in response to a bacterial symbiont.

BROWN STEM ROT OF SOYBEAN, caused by the soilborne fungus *Phialophora gregata*, is an economically important disease in the north central USA. Yield losses as high as 38% have been recorded in fields with development of severe BSR symptoms (Bachman et al., 1997b; Dunleavy, 1966; Gray, 1972; Mengistu et al., 1986; Weber et al., 1966). Soybean yield loss to BSR in the USA in 1994 was 260 000 Mg (Wrather et al., 1997), and yield loss to BSR in 1996, 1997, and 1998 was estimated at approximately 837 500, 653 300, and 369 500 Mg, respectively (Wrather and Stienstra, 1999). Resistance to BSR has been identified and utilized in cultivar development and germplasm enhancement. Three loci designated *Rbs$_1$*, *Rbs$_2$*, and *Rbs$_3$* were identified in germplasm line L78-4094 (Hanson et al., 1988), PI 437833 (Hanson et al., 1988), and PI 437970 (Willmot and Nickell, 1989), respectively. Brown stem rot resistance at these loci is conditioned by dominant alleles, and the presence of a dominant allele at any one of these three loci has been associated with a resistant reaction to BSR (Hanson et al., 1988; Willmot and Nickell, 1989). Resistance thus appears to provide an efficacious, economical means of control of BSR.

Selection for BSR resistance is hampered by a high level of environmental variation in the field (Chamberlain and Bernard, 1968) and labor-intensive, time-consuming assays in the greenhouse (Sebastian and Nickell, 1985; Sebastian et al., 1985). However, the use of molecular markers linked to *Rbs* genes could accelerate selection and eliminate the effects of environmental variation. Molecular markers could also facilitate pyramiding of *Rbs* loci which could provide more temporally or geographically stable resistance to *P. gregata* in the future. Stable BSR resistance sources are desirable given the historical failure of many monogenic disease resistance mechanisms in plants and reports of physiological specialization in *P. gregata* (Gray, 1971; Willmot et al., 1989). There is also evidence that while single *Rbs* resistance alleles usually confer high-level resistance, they do not provide immunity from disease; there are reports of BSR symptom development on soybean lines containing *Rbs* resistance alleles (Bachman et al., 1997a; Hanson et al., 1988; Nelson et al., 1989).

Several different molecular marker systems have been used successfully in soybean, although these systems can be limited by expense, labor requirements, or a lack of repeatability if used for marker-assisted selection (Denny et al., 1996; Mudge et al., 1997). Restriction fragment length polymorphism (RFLP) markers were among the first molecular markers to be used in soybean. However, RFLP markers have several disadvantages for use in marker assisted selection, including relatively low levels of polymorphism (Keim et al., 1989, 1992) and time-consuming protocols (Mudge et al., 1997). SSR markers are DNA sequences consisting of short tandem repeats of two to five nucleotides (core sequences) flanked by conserved DNA sequences. These markers can vary in length, depending on the number of core sequences positioned in tandem arrangement. Sequence length polymorphism can be assayed by amplification of these regions with the conserved flanking sequences used as primer templates in the polymerase chain reaction (PCR) (Akkaya et al., 1992). SSR markers have been widely utilized in soy-

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Abbreviations: AFLP, amplified fragment length polymorphism; BSR, brown stem rot; MLG, molecular linkage group; PCR, polymerase chain reaction; PI, plant introduction; QTL, quantitative trait locus/loci; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat.

bean because they have high levels of sequence polymorphism, codominance, repeatability, and they are rapid and relatively inexpensive to use (Maughan et al., 1995; Mudge et al., 1997). Because many of these markers have been mapped in the soybean genome (Akkaya et al., 1995; Cregan et al., 1994), genes of interest can be placed relative to known markers.

Molecular markers have been utilized in soybean to identify a cluster of genes on Molecular Linkage Group J (MLG) involved in plant responses to fungal pathogens and a bacterial symbiont. Polzin et al. (1994) used RFLP markers to identify a cluster of three loci on MLG J involved in disease resistance and symbiosis. Comprising this cluster was a gene for resistance to Phytophthora rot (*Rps*$_2$), a gene for resistance to powdery mildew (*Rmd*), caused by *Microshaera diffusa* Che. & Pk. and a gene involved in nodulation (*Rj*$_2$). Webb (1997), using RFLP markers, mapped BSR resistance gene, *Rbs*$_3$, to the same region on MLG J. Lewers et al. (1999), using RFLP and amplified fragment length polymorphism (AFLP) markers on members of the same soybean population studied by Webb (1997), mapped a major quantitative trait locus (QTL) (likely *Rbs*$_3$) and a minor QTL for BSR resistance to the same region. More recently, the *Rcs*$_3$ locus, conferring resistance to frogeye leaf spot, was mapped by SSR markers to the same gene cluster (Mian et al., 1999).

The studies outlined above indicate that disease resistance genes can be successfully mapped in soybean by molecular marker systems. Given the operative and efficient SSR marker system, a reliable greenhouse assay for BSR resistance (Sebastian et al., 1983), and populations segregating for *Rbs*$_1$ and *Rbs*$_2$ BSR-resistance loci, our objectives were to (i) identify SSR markers linked to *Rbs*$_1$ and *Rbs*$_2$ to facilitate marker-assisted selection; and (ii) map *Rbs*$_1$ and *Rbs*$_2$ in the soybean genome.

MATERIALS AND METHODS

Sources of Populations and Near-Isogenic Lines

The plant populations used in this study were generated at the University of Illinois, Urbana.

Rbs$_1$ $F_{2:3}$ Families

In 1984, a cross was made between L78-4094, a BSR resistant germplasm line carrying *Rbs*$_1$, and susceptible cultivar Century (Wilcox et al., 1980). The F_1 plants were grown and harvested in 1985. The F_2 seed was kept in cold storage. In 1997, a portion of the F_2 seed from each of four F_1 plants was planted at the Crop Sciences Research and Education Center, Urbana, IL. Single plants were harvested and threshed individually to obtain $F_{2:3}$ families.

Rbs$_2$ $F_{2:3}$ Families

In 1983, a cross was made between PI 437833, a BSR resistant accession carrying *Rbs*$_2$, and susceptible cultivar Century. The F_1 plants were grown and harvested in 1984. The F_2 seed was kept in cold storage. In 1997, a portion of the F_2 seed from one F_1 plant was planted at Urbana. Single plants were harvested and threshed individually to obtain $F_{2:3}$ families. In the winter of 1996, a cross was made between PI 437833, a BSR resistant accession carrying *Rbs*$_2$, and susceptible cultivar Century 84 (Walker et al., 1986) in the greenhouse. The F_1 plants were grown in the summer of 1996 to produce F_2 seed. In 1998, a portion of the F_2 seed from one F_1 plant was planted at Urbana. Single plants were harvested and threshed individually to obtain $F_{2:3}$ families. All crosses were verified in subsequent generations using phenotypic markers, including flower color, pubescence color, and hilum color.

Rbs$_2$ Near-isogenic Lines

The *Rbs*$_2$ near-isogenic lines used in this study were developed at the University of Illinois as described in Bachman et. al. (1997b). Briefly, a cross was made between BSR-susceptible cultivar Century 84 and PI 437833 (*Rbs*$_2$). Segregating progeny were screened for BSR resistance and *Rbs*$_2$/*rbs*$_2$ heterozygotes were selected during inbreeding. Following four cycles of screening and selection for *Rbs*$_2$/*rbs*$_2$ heterozygotes, homozygous resistant (*Rbs*$_2$/*Rbs*$_2$) and susceptible (*rbs*$_2$/*rbs*$_2$) individuals were identified through progeny testing, and increased to generate F_6 derived *Rbs*$_2$ near-isogenic lines. The resulting five pairs of *Rbs*$_2$ near-isogenic lines, each pair initially derived from a different F_2 plant, were then increased for three generations and selected for uniform phenotype.

Inoculation with *Phialophora gregata* and Plant Culture

Soybean populations and lines were evaluated for BSR reaction after inoculation with a monoconidial isolate of *P. gregata*, designated PgOh2. This isolate was initially cultured from soybean tissue from Ohio (personal communication, 1994, L.E. Gray), and it was obtained from Dr. L.E. Gray, University of Illinois, USDA-ARS. (The isolate PgOh2 is currently maintained by Dr. Brian Diers, University of Illinois.) This isolate was chosen for this study on the basis of the following three criteria: (i) its ability to induce BSR foliar symptoms on susceptible soybean varieties, (ii) its relative avirulence against BSR resistance genes *Rbs*$_1$, and *Rbs*$_2$, and (iii) its stability over time in continuous culture (Bachman et al., 1997a; Bachman and Nickell, 1998; Bachman and Nickell, 2000a,b).

Inoculum of *P. gregata* was prepared as described by Bachman and Nickell (2000a). Briefly, cultures were initiated by transferring three agar plugs (each approximately 2.7 mm^3) containing hyphal tips of an active, 30- to 40-d culture from soybean stem agar minimal medium (Allington and Chamberlain, 1948) to 100 mL soybean seed broth (100 g of soybean seed/L water steamed, strained, and autoclaved). Both stem agar and seed broth were made from susceptible cultivar Century 84. Stationary liquid cultures were incubated at 24°C in the dark. After 4 wk, seed broth cultures of *P. gregata* were ground for 75 s in a blender at high speed. The concentration of propagative fragments (mycelial fragments and conidia) was determined with a hemocytometer. Blended cultures were then diluted with distilled water to a concentration of 1.2×10^6 propagules/mL. Carboxymethyl cellulose was added to the suspension at a rate of 7.5 g/L to act as a sticking agent.

Inoculation of plants for greenhouse evaluation was conducted by a root-dip technique developed by Sebastian et al. (1983) and modified in accordance with the following methods. Seed was germinated in commercial grade sand in 10-cm-diam plastic pots and grown to the V1 growth stage (Fehr et al., 1971) at temperatures ranging from 18 to 24°C. Sand was rinsed from the roots of the seedlings. Five healthy-appearing seedlings were selected, and the roots were blotted dry with paper towels and dipped into a beaker containing 50 mL of *P. gregata* inoculum for 2 to 3 s. The seedlings were removed from the inoculum and placed into a 6- to 8-cm depression in

steam-treated 1:1 sand:topsoil mixture filling a 15-cm-diam steam-sterilized clay pot. The remaining inoculum was then poured over the roots of the seedlings. The mixture of 1:1 sand:topsoil was used to cover the roots of seedlings to a level 1 to 2 cm below the cotyledons. Pots were placed on trough-shaped benches lined with 25 to 50 mm of commercial grade sand. Plants were maintained under a 14-h photoperiod at an average nighttime temperature of 18°C and an average daytime temperature of 24°C. All pots received approximately 300 mL of water daily. Pots were fertilized weekly with 150 mL of a nutrient solution containing 0.121 g N, 0.112 g P, 0.107 g K, 0.000044 g B, 0.00022 g chelated Fe, and 0.00011 g chelated Cu, Mn, and Zn.

Experimental Design for BSR Evaluation

Seventy-three $F_{2:3}$ families segregating for *Rbs*$_1$ and 77 $F_{2:3}$ families segregating for *Rbs*$_2$ (including 30 families derived from the cross of PI 437833 × Century and 47 families derived from the cross of PI 437833 × Century 84) were screened for BSR reaction in the greenhouse in the winters of 1997-1998 and 1998-1999 by the technique described above. The resistant and susceptible parents of each population, as well as each family segregating for *Rbs*$_1$ and *Rbs*$_2$, were represented by four pots (totaling approximately 20 plants). Families and parental controls for each population were arranged in a completely randomized design.

BSR Evaluation

Brown stem rot reactions of *Rbs*$_2$ near-isogenic lines were confirmed in field tests in 1994 and 1995 (Bachman et al., 1997b), and in a greenhouse evaluation in the winter of 1996. Resistant and susceptible near-isogenic lines were easily distinguished by the absence and presence, respectively, of foliar BSR symptoms to or above the node bearing the first trifoliolate leaf (Hanson et al., 1988) (data not included).

Brown stem rot reaction to inoculation is influenced by the inoculation efficiency, genetic backgrounds of the material under evaluation, and the environment. As a result, BSR reaction data can be quantitative in nature, and phenotypic classification of segregating populations can be difficult. In this study, restricted population size necessitated the use of a reliable method of phenotypic classification of genetic families for BSR reaction. As a means of verifying the results of our phenotypic classification, genetic families segregating for *Rbs*$_1$ and *Rbs*$_2$ were phenotypically classified by two methods.

To utilize the first method of phenotypic classification, families were initially evaluated for BSR mean foliar severity. Mean foliar severity of BSR was calculated by rating each plant for height of foliar symptom progression (the proportion of total nodes with an expanded leaf showing BSR foliar symptoms) and averaging these values over all plants in a family. Brown stem rot mean foliar severity ratings were expressed as a decimal value from 0 to 1. Mean foliar severity values were arbitrarily divided into phenotypic classes with low (homozygous resistant), intermediate (segregating), and high (homozygous susceptible) means (Hanson et al., 1988; Willmot and Nickell, 1989) (data not included).

The second classification method for families was based on the ratio of resistant to susceptible plants within a family. To utilize this method, individual F_3 plants within a family were phenotypically classified. Plants were classified as resistant if they had no disease or developed BSR foliar symptoms to a level below the first trifoliolate node, and susceptible if they developed BSR foliar symptoms to or above the first trifoliolate node (Hanson et al., 1988). Observed numbers of resistant and susceptible plants within a family were then compared, by Chi-square analysis, to expected numbers for segregation of a single dominant gene (by ratios of 1 resistant: 0 susceptible, 3 resistant: 1 susceptible, and 0 resistant: 1 susceptible)(data not included). A family was classified as resistant when the highest Chi-square probability was obtained from comparison to an expected ratio of 1 resistant: 0 susceptible. Likewise, a family was classified as segregating when the highest Chi-square probability was obtained from comparison to an expected ratio of 3 resistant: 1 susceptible. Finally, a family was classified as susceptible when the highest Chi-square probability was obtained from comparison to an expected ratio of 0 resistant: 1 susceptible. In the infrequent event of disagreement between the two classification methods, the family of interest was classified by the "Chi-square probability" method, because Chi-square analysis has been used in previous genetics studies involving BSR resistance (Hanson et al., 1988; Willmot and Nickell, 1989).

Tissue Sampling

Two weeks after inoculation (V2 growth stage), plants were tissue sampled. Approximately equal proportions of leaf tissue (young expanding trifoliolates) were harvested from 10 to 12 individual F_3 plants within each family segregating for *Rbs*$_1$ and *Rbs*$_2$. This tissue was bulked to reconstruct the F_2 plant from which those families were derived. Young trifoliolate leaves were collected from approximately 10 individual plants of each of the *Rbs*$_2$ near-isogenic lines and bulked. Leaf tissue collected from families and near-isogenic lines was placed in plastic heat-seal bags, and immediately placed on ice. Tissue samples were frozen in liquid nitrogen, lyophilized for approximately 48 h, sealed in bags, and stored at −20°C.

DNA Extraction

DNA was extracted by the method of Saghai-Maroof et al. (1984). Approximately 0.75 g of freeze-dried soybean leaf tissue was powdered with a modified paint shaker. Following addition of 10 mL extraction buffer [50 mM tris, pH 8.0, 0.7 *M* NaCl, 10 m*M* EDTA, 1% (w/v) hexadecyltrimethylammonium bromide, 0.1% (v/v) 2-mercaptoethanol], the slurry was incubated for 60 min at 60°C with occasional swirling. After incubation, 10 mL of chloroform-octanol (24:1, v/v) was added, and the solution was mixed by inversion and centrifuged at 5125 × *g* for 10 min at 4°C. The aqueous phase was removed and the DNA was precipitated by adding 2/3 volume of isopropanol. Precipitated DNA was spooled onto a glass rod and washed in 20 mL of a 76% (v/v) ethanol/ 10 m*M* ammonium acetate solution. The DNA was then dissolved in 1.5 mL of 10 m*M* ammonium acetate/0.25 m*M* EDTA.

SSR Amplification and Viewing

All SSR primers used in this study were described by Cregan et al. (1999) and are listed at http://129.186.26.94/SSR.html; verified November 17, 2000. The *Rbs*$_2$ near-isogenic lines were assayed for polymorphism with 154 SSR markers. Parents of progeny segregating for *Rbs*$_1$ and *Rbs*$_2$ were assayed for polymorphism with a set of 112 SSR markers from 20 linkage groups. Markers residing on Soybean Molecular Linkage Group J (Cregan et al., 1999) were tested initially because a cluster of disease resistance genes has been identified on that linkage group (Polzin et al., 1994; Webb, 1997). Subsequent analyses in parents of both *Rbs*$_1$ and *Rbs*$_2$ populations included SSR loci spanning the soybean genome. Polymorphic markers were then assayed on segregating progeny. Amplification of SSR loci was carried out as described by Akkaya et al. (1995).

Polymerase chain reaction mixtures included 30 ng of genomic DNA, 1.5 m*M* Mg^{2+}, 0.15 m*M* of 3′ and 5′ end primers, 200 m*M* each of dATP, dTTP, dCTP, and dGTP, 1× PCR buffer containing 50 m*M* KCl, 10 m*M* Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, and 1 unit *Taq* polymerase in a total volume of 10 mL. Thirty thermal cycles were run, each included a 25-s denaturation step at 94°C, a 25-s annealing step at 47°C, and a 25-s extension step at 68°C. PCR products were separated with 3.0% (w/v) Metaphor (FMC BioProducts, Rockland, ME) agarose gels, stained with ethidium bromide, and visualized under UV light.

Statistical Analyses

Broad sense heritability estimates for BSR mean foliar severity were calculated for each population under study. The variance among families in a population was used as an estimate of total phenotypic variance. The variance among four replicates of each of two homozygous parental checks was pooled and used as an estimate of error variance for each population. Total genetic variance was calculated as the difference between total phenotypic variance and error variance. The broad sense heritability was estimated as the proportion of total phenotypic variance attributed to the total genetic variance.

The observed BSR reaction phenotypes and SSR markers were tested for goodness-of-fit to expected ratios for segregation of a single gene using Chi-square analysis.

Simple sequence repeat markers were analyzed using single factor analyses in SAS (SAS Institute, 1985) to determine the proportion of variation in BSR reaction that was explained by individual markers. Markers significant at $P = 0.01$ probability level were included in linkage analysis using the Kosambi function of Mapmaker/EXP 3.0 (Lander et al., 1987; Lincoln et al., 1992a). The BSR phenotypic reaction was coded as a marker (resistant, heterozygous, or susceptible) and mapped as a qualitatively inherited trait with respect to SSR markers. In an additional analysis, the BSR mean foliar severity value of *Rbs*$_1$ and *Rbs*$_2$ $F_{2:3}$ families was treated as a quantitative trait, and putative quantitative trait loci (QTL) were identified and mapped by Mapmaker QTL 1.1 (Paterson et al., 1988; Lincoln et al., 1992b). Maps generated from *Rbs*$_1$, *Rbs*$_2$, and linked SSR markers were compared with a recently published molecular linkage map of soybean (Cregan et al., 1999) containing identical SSR markers.

RESULTS AND DISCUSSION

Broad sense heritability estimates of brown stem rot phenotypic reaction based on family means were 0.88 and 0.86, respectively, for the *Rbs*$_1$ and *Rbs*$_2$ populations.

Table 1. Results of Chi-square analysis on brown stem rot phenotypes and SSR markers in *Rbs*$_1$ and *Rbs*$_2$ populations.

Trait or marker	Observed ratio†	Expected ratio	Chi-square probability
***Rbs*$_1$ population**			
BSR phenotype	16 R:35 H:22 S	18.25 R:36 H:8.25 S	0.57
Satt215	19 R:32 H:18 S	17.25 R:34.5 H:17.25 S	0.82
Satt431	14 R:33 H:18 S	16.25 R:32.5 H:16.25 S	0.78
***Rbs*$_2$ population**			
BSR phenotype	16 R:42 H:19 S	19.25 R:38.5 H:19.25 S	0.65
Satt244	19 R:37 H:20 S	19 R:38 H:19 S	0.96
Satt431	20 R:37 H:12 S	17.25 R:34.5 H:17.25 S	0.33

† R = resistant phenotype or marker allele associated with resistant phenotype; H = segregating phenotype or marker alleles associated with segregating phenotype; S = susceptible phenotype or marker allele associated with susceptible phenotype.

These relatively high heritabilities provide additional evidence that the greenhouse screening procedure used in this study minimized environmental variation during BSR evaluation (Sebastian et al., 1985).

The two methods used to classify BSR phenotypic reactions were in agreement for 71 of 73 families segregating for *Rbs*$_1$ and 73 of 77 families segregating for *Rbs*$_2$. Agreement between these methods provided evidence that either classification scheme could distinguish phenotypic classes within the pool of segregating families.

Results of Chi-square analyses indicated that SSR markers and BSR phenotypic reactions for families in both the *Rbs*$_1$ and *Rbs*$_2$ populations fit expected ratios for segregation of a single gene (Table 1).

Rbs$_1$ $F_{2:3}$ Population

Of 112 SSR markers assayed, 14 were polymorphic between parents L78-4094 and Century 84. This low level of polymorphism can be attributed to the high degree of relatedness between these lines (Bernard et al., 1988; Sebastian et al., 1985). Single factor analyses of variance indicated that two of these markers, Satt215 and Satt431 were significant at $P = 0.001$ probability level. These markers explained 28 and 74%, respectively of the variation in BSR phenotypic reaction. Mapmaker EXP/3.0 placed Satt215 and Satt431 in flanking positions relative to *Rbs*$_1$, mapping this gene to a location 7.2 centimorgans (cM) from Satt431 and 25.7 cM from

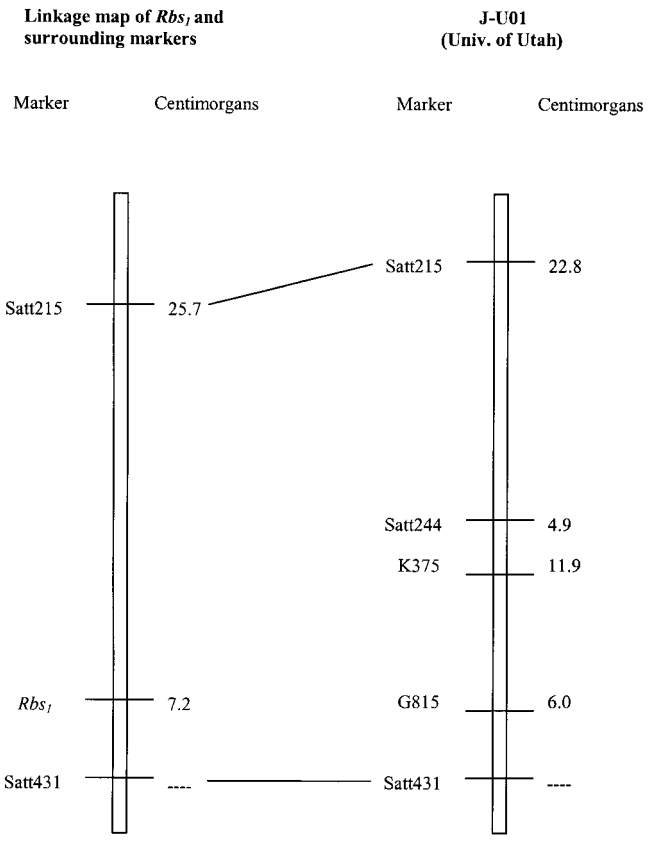

Fig. 1. Molecular linkage map of brown stem rot resistance gene, *Rbs*$_1$, and surrounding simple sequence repeat (SSR) markers, compared to a map of Soybean Molecular Linkage Group J from the University of Utah (Cregan et al., 1999).

Satt215 (Fig. 1). Although no RFLP markers were included in this analysis, the region indicated as the probable location of *Rbs1* appears close to RFLP marker G815 on MLG J of the integrated linkage map proposed by Cregan et al. (1999). These results were supported by analysis using Mapmaker QTL 1.1, which identified a peak explaining 89% of the variation in BSR phenotypic reaction, located 28.0 cM from Satt215 and 5.8 cM from Satt431. The proportion of variation explained in the latter analysis is approximately equal to the heritability of BSR reaction phenotype in the *Rbs1* population, indicating that this QTL is responsible for all of the genetic variation for BSR phenotype in this population.

Rbs2 $F_{2:3}$ Populations

Thirty-three SSR markers were polymorphic between parents PI 437833 and Century (or Century 84). Single factor analyses indicated that two of these markers, Satt244 and Satt431, were significant at the $P = 0.001$ probability level. Markers Satt244 and Satt431 explained 67 and 46%, respectively, of the variation in BSR reaction phenotype. Results of linkage analysis on the three loci [Satt244, Satt431, BSR phenotype (*Rbs2*)] indicated that the likelihood values of the two most likely map orders were not significantly different. The first linkage map placed *Rbs2* 10.0 cM from Satt244 and 23.8 cM from Satt431, whereas the second linkage map placed *Rbs2* between Satt244 and Satt431 at distances of 9.7 cM (from Satt244) and 17.1 cM (from Satt431). These results provide evidence that *Rbs2* lies within a 20-cM interval centered on Satt244 (Fig. 2).

The analysis for QTL affecting BSR mean foliar severity in the *Rbs2* population identified two peaks associated with 84 and 79%, respectively, of the variation in BSR mean foliar severity. These peaks mapped to locations 8.4 cM to one side of Satt244 and 6.0 cM to the other side of Satt244 (nearer Satt431). The two peaks identified by QTL analysis likely represent the same QTL because of the high level of variation explained at each peak. The presence of two peaks in likelihood value in this region, with a relatively small reduction in the vicinity of Satt244 (reduction in LOD score of approximately 5 vs. peaks of approximately 20), is indicative of some level of phenotypic misclassification (Lincoln et al., 1992b). This would be expected on the basis of historical difficulties associated with BSR phenotypic evaluation and the presence of several families in this study with ambiguous phenotypes. Overall, QTL analysis indicated that the region of MLG J spanning approximately 10 cM on either side of Satt244 explains nearly all of the heritable variation for BSR mean foliar severity among families segregating for *Rbs2* (Fig. 3).

Rbs2 Near-Isogenic Lines

Ten SSR markers were polymorphic between the resistant and susceptible members of the five pairs of

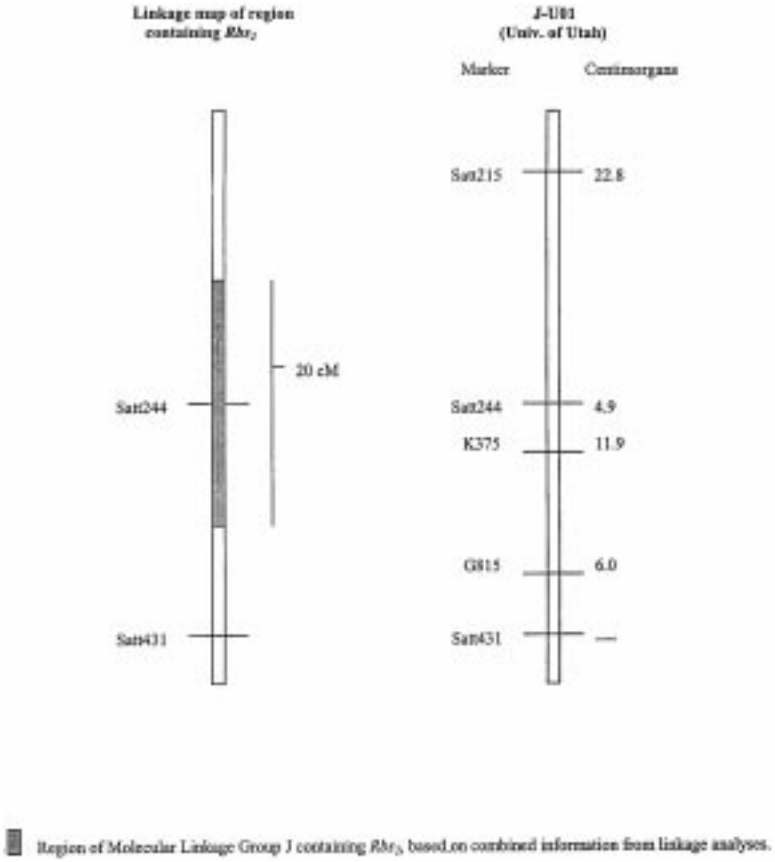

Fig. 2. Molecular linkage map of the region containing brown stem rot resistance gene, *Rbs2*, compared to a map of Soybean Molecular Linkage Group J from the University of Utah (Cregan et al., 1999).

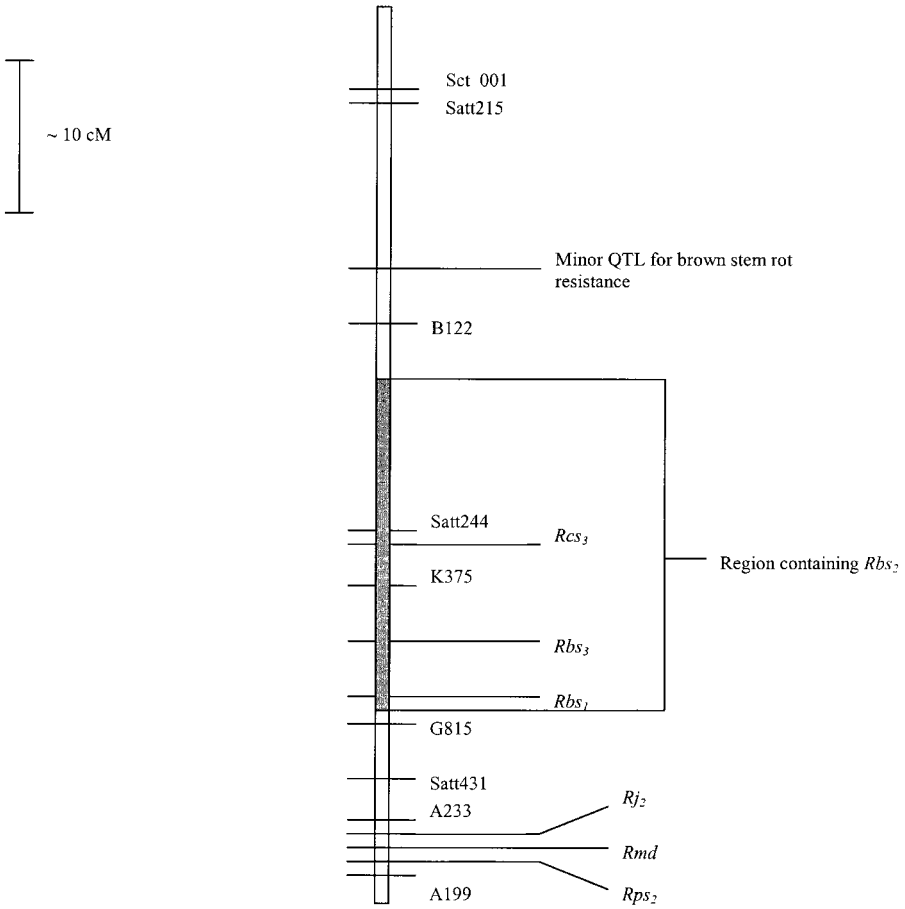

Fig. 3. Integrated map of Molecular Linkage Group J of soybean indicating approximate locations of molecular markers and disease resistance loci [based on present study and maps presented by Cregan et al. (1999), Kanazin et al. (1999), Mian et al. (1999), Polzin et al. (1994), and Webb (1997)].

Rbs$_2$ near-isogenic lines, and the number of polymorphic markers varied between one and five for each individual pair (Table 2). Simple sequence repeat marker Satt244 was the only marker polymorphic between resistant and susceptible members of all five pairs of *Rbs*$_2$ near-isogenic lines, and Satt431 was polymorphic between two pairs of *Rbs*$_2$ near-isogenic lines. Because Satt244 and Satt431 were polymorphic between the parents of the *Rbs*$_2$ near-isogenic lines, these markers would be expected to be polymorphic between resistant and susceptible near-isogenic lines if tight linkage prevented recombination between *Rbs*$_2$ and the marker loci. Polymorphism among near-isogenic lines was only observed for Satt244, indicating that *Rbs*$_2$ is likely closer to Satt244 than to Satt431. These results support map placement based on the analyses of BSR resistance as both a qualitative and quantitative trait, as described above.

Table 2. Simple sequence repeat (SSR) markers polymorphic between *Rbs*$_2$ near-isogenic lines.

Rbs$_2$ near-isogenic lines	Polymorphic SSR	Molecular Linkage Group‡
LN92-11976 (R†), LN92-11995 (S)	Satt244	J
	Satt263	E
	Satt290	D1b
	Satt431	J
LN92-12014 (R), LN92-12022 (S)	Satt244	J
LN92-12033 (R), LN92-12054 (S)	Sat_042	C1
	Satt244	J
	Satt442	H
	Satt472	G
	Satt530	N
LN92-12077 (R), LN92-12070 (S)	Satt244	J
	Satt260	K
LN92-12117 (R), LN92-12137 (S)	Satt008	D2
	Satt244	J
	Satt431	J

† R = resistant near-isogenic line, S = susceptible near-isogenic line.
‡ Soybean Molecular Linkage Groups assigned by Cregan et al. (1999).

Results of linkage analysis conducted on markers in each of the *Rbs*$_1$ and *Rbs*$_2$ populations indicate that *Rbs*$_1$ and *Rbs*$_2$ are linked on MLG J. On the basis of the data collected in this study, *Rbs*$_1$ and *Rbs*$_2$ are linked on MLG J. These results disagree with a genetic study conducted by Hanson et al. (1988) which indicated that *Rbs*$_1$ and *Rbs*$_2$ segregated independently. One explanation for the disagreement between the genetic study conducted by Hanson et al. (1988) and the present study could be an interaction between resistance loci and isolates of *P. gregata*. Different isolates were used for BSR evaluation in these studies, and the possibility exists that different isolates of the fungus triggered unique resistance genes in populations derived from L78-4094 (*Rbs*$_1$). Another possible explanation for the inconsistent results of these studies could be the existence of unlinked loci in the genome which interact with *Rbs* loci to confer a resis-

tance response. This type of model has been postulated to explain apparent redundancy of loci involved in defense responses (Glazebrook et al., 1997). If this were the case, *Rbs*$_1$ and *Rbs*$_2$ could be tightly linked or allelic and could be activated by independent loci that serve as resistance gene "regulators." In this scenario, BSR resistance in a population segregating for both *Rbs* genes (either linked or allelic) could appear to be conditioned by two independently segregating genes. This scenario was discussed in detail by Bachman and Nickell (2000b).

Marker Assisted Selection Using SSR Markers

On the basis of the data collected in this study, efficiency of selection for BSR reaction phenotype in populations segregating for the *Rbs*$_1$ allele would be highest if Satt431alone were used. The marker Satt431 predicted the BSR reaction phenotype in 88% of the $F_{2:3}$ families, whereas Satt215 predicted the correct reaction phenotype in only 62% of the families. Prediction of BSR reaction phenotype using flanking markers was less effective. When both Satt215 and Satt431 carried the marker alleles associated with a given BSR reaction, these marker loci predicted only 6% of the BSR reaction phenotypes of families. These trends in selection efficiency would be expected on the basis of the relatively large map distances between the SSR markers and *Rbs*$_1$.

Selection efficiency for *Rbs*$_2$-conferred resistance was not as high as that for resistance conferred by *Rbs*$_1$. When used alone, SSR markers Satt244 and Satt431 predicted the BSR reaction phenotype in 82 and 70%, respectively, of the families segregating for *Rbs*$_2$. Although marker assisted selection in this study was most efficient using Satt244, identification of markers with tighter linkage to *Rbs*$_2$ would be desirable to improve efficiency of selection.

Genes conferring resistance to a single pathogen or to taxonomically diverse pathogens have been mapped to discrete clusters in maize, *Zea mays* L., (Bennetzen et al., 1991; Hulbert and Bennetzen, 1991), rice, *Oryza sativa* L., (Kinoshita, 1993; Mackill and Bonman, 1992; Yoshimura et al., 1983), tomato, *Lycopersicon esculentum* Mill., (Dickinson et al., 1993), barley, *Hordeum vulgare* L., (Wise and Ellingboe, 1985), flax, *Linum usitatissimum* L., (Islam et al., 1989; Mayo and Shepherd, 1980; Shepherd and Mayo, 1972), lettuce, *Lactuca sativa* L, (Kesseli et al., 1990, 1992, 1993; Maisonneuve et al., 1994), and soybean (Diers et al., 1992; Polzin et al., 1994; Yu et al., 1994). Related structure and/or function among genes in these clusters has provided evidence that resistance genes occur in multigene families and have a common origin (Parniske et al., 1997; Ronald, 1998; Song et al., 1997).

In soybean, a cluster of disease resistance genes including *Rps*$_2$, *Rmd* (Polzin et al., 1994), *Rcs*$_3$ (Mian et al., 1999), *Rbs*$_1$, *Rbs*$_2$ (present study), and *Rbs*$_3$ (Webb, 1997; Lewers et al., 1999) has been mapped to Molecular Linkage Group J (Fig. 3). The loci *Rps*$_2$ and *Rmd* are involved in resistance responses to *Phytophthora sojae* M.J. Kaufmann & J.W. Gerdemann and *Microspaera diffusa* Cooke & Peck, respectively (Buzzell and Haas, 1978; Kilen et al., 1974; Lohnes and Bernard, 1992). The *Rcs*$_3$ gene conditions resistance to frogeye leaf spot in soybean, caused by *Cercospora sojina* K. Hara (Phillips and Boerma, 1982). The genes, *Rbs*$_1$, *Rbs*$_2$, and *Rbs*$_3$ provide resistance to brown stem rot of soybean (Hanson et al., 1988; Willmot and Nickell, 1989). The BSR resistance gene, *Rbs*$_3$, was mapped to this region of Molecular Linkage Group J using RFLP markers (Webb, 1997). In addition, Lewers et al. (1999) identified RFLP and AFLP markers in this region linked to two QTL associated with BSR resistance. Because the populations under study by Webb (1997) and Lewers et al. (1999) were the same, it is likely that the major QTL found in the latter study was *Rbs*$_3$. An important finding in the study by Lewers et al. (1999) was the association of a minor QTL for BSR resistance with a resistance gene analog. Multiple resistance gene analogs have been mapped to this region of Molecular Linkage Group J (Kanazin et al., 1996), and the possibility exists that these resistance gene analogues correspond to one or more of the BSR resistance genes identified in this region, or to unique BSR resistance loci.

Polzin et al. (1994) also mapped another locus, *Rj*$_2$, to the gene cluster discussed above (Fig. 3). Alleles at this locus have been implicated in nodulation response to specific strains of *Bradyrhizobium japonicum* (Caldwell, 1966). This region on MLG J is therefore involved in both defense responses to diverse fungal pathogens and symbiotic relationships with a bacterium.

In the initial search for SSR loci polymorphic between BSR resistant and susceptible parents, Molecular Linkage Group J was targeted because it contained a cluster of genes for resistance to several soybean pathogens. This approach proved successful in locating BSR resistance loci, *Rbs*$_1$ and *Rbs*$_2$, and the *Rcs*$_3$ locus conferring resistance to frogeye leaf spot (Mian et al., 1999). The presence of multiple resistance gene analogs in this region provides evidence for the existence of additional genes conferring resistance to soybean pathogens, and this region may be the focus of future attempts to map or clone resistance loci.

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QTL Analysis of Resistance to Fusarium Root Rot in Bean

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ABSTRACT

A major constraint to dry edible and snap bean (*Phaseolus vulgaris* L.) production worldwide is root rot, one form of which is caused by *Fusarium solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans (FSP). Sources of resistance to this pathogen exist in *P. vulgaris*, and, in the current paper, we studied the inheritance of one such source, FR266, using two recombinant inbred populations, MF and IF, derived from crosses of susceptible cultivars Montcalm (M) and Isles (I) with FR266 (F). Random amplified polymorphic DNA (RAPD) markers, associated with quantitative trait loci (QTL) controlling resistance to Fusarium root rot, also were identified. Genetic resistance to FSP, originally derived from PI 203958, was polygenically controlled and strongly influenced by environmental factors. Heritability estimates (h^2) were moderate and ranged from 0.48 to 0.71 for MF population. Several RAPD markers were identified that demonstrated significant associations with resistance to FSP determined from both greenhouse and field evaluations. Markers associated with field ratings tended not to be associated with greenhouse ratings and vice versa, except for the P_{700} marker which was significantly associated with both greenhouse and field data. Individual markers identified in this study did not explain more than 15% of the phenotypic variation for root rot resistance, whereas a combination of four markers explained 29% of the phenotypic variation for root rot ratings in the field. The two regions of the bean genome associated with root rot resistance corresponded to loci controlling the *Pv* pathogenesis-related proteins (*Pv*PR). Mechanisms associated with host defense responses may be involved in resistance to FSP and selection directed towards enhancing these traits may allow for rapid improvement of resistance to Fusarium root rot in bean.

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Root rot, caused by *Fusarium solani* f. sp. *phaseoli*, is considered among the most serious and widespread soil-borne diseases of bean with yield losses of up to 84% attributed to this pathogen (Park and Tu, 1994; O'Brien et al., 1991; Abawi and Pastor Corrales, 1990; Silbernagel, 1990; Dryden and Van Alfen, 1984; Miller and Burke, 1986; Beebe et al., 1981; Natti and Crosier, 1971). Fusarium root rot, characterized by reddish-brown lesions along the tap root and hypocotyl, is particularly severe on large-seeded Andean bean genotypes because of a lack of genetic resistance in these market classes (Abawi and Pastor Corrales, 1990; Dickson, 1973; Wallace and Wilkinson, 1973). 'Montcalm', a popular dark red kidney bean cultivar grown in Michigan, Minnesota, and Wisconsin exemplifies the high degree of susceptibility inherent to this market class (Estevez de Jensen et al., 1998; Schneider and Kelly, 2000). An overemphasis on quality traits in breeding red kidney and snap bean market classes, and the consequent reduction in genetic variability (Gepts, 1998), may have contributed to the lack of resistance in these seed and pod types. Small-seeded genotypes of Middle American origin, although not completely resistant to root rot, do not appear as susceptible as the large seeded types (Abawi and Pastor Corrales, 1990; Beebe et al., 1981). Genotypes from the large-seeded Andean gene pool are distinguished from the small-seeded, Middle American genotypes by morphological, biochemical, and molecular characteristics (Gepts, 1988; Haley et al., 1994). Like-

Abbreviations: BGMV, bean golden mosaic virus; bp, base pair; CBB, common bacterial blight; cM, centimorgan; DAP, days after planting; DTF, days to flower; FSP, *Fusarium solani* f. sp. *phaseoli*; IF, Isles/FR266; MAS, marker-assisted selection; MF, Montcalm/FR266; PI, Presque Isle county, MI; PCR, polymerase chain reaction; PM, Perham, MN; RAPD, random amplified polymorphic DNA; RCBD, randomized complete block design; RILs, recombinant inbred lines; QTL, quantitative trait loci.