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Soybean *Phytophthora* Resistance Gene *Rps8* Maps Closely to the *Rps3* Region

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Abstract

Root and stem rot is one of the major diseases of soybean. It is caused by the oomycete pathogen *Phytophthora sojae*. A series of resistance genes (*Rps*) have been providing soybean with reasonable protection against this pathogen. Among these genes, *Rps8*, which confers resistance to most *P. sojae* isolates, recently has been mapped. However, the most closely linked molecular marker was mapped at about 10 cM from *Rps8*. In this investigation, we attempted to develop a high-density genetic map of the *Rps8* region and identify closely linked SSR markers for marker-assisted selection of this invaluable gene. Bulk segregant analysis was conducted for the identification of SSR markers that are tightly linked to *Rps8*. Polymorphic SSR markers selected from the *Rps8* region failed to show cosegregation with *Phytophthora* resistance. Subsequently, bulk segregant analysis of the whole soybean genome and mapping experiments revealed that the *Rps8* gene maps closely to the disease resistance gene-rich *Rps3* region.

Soybean root and stem rot, caused by the oomycete pathogen *Phytophthora sojae*, is one of the most important diseases of soybean. It causes nearly $200 million in annual yield losses (Wrather et al. 2001). A series of *Rps* genes have been extensively used in protecting soybean from this pathogen for several decades. To date, 15 *Rps* genes have been identified and mapped, and there are probably many unknown *Rps* genes in the germplasms (Anderson and Buzzell 1992; Burnham et al. 2003; Polzin et al. 1994; Sandhu et al. 2004; Schmitthenner 1989). Molecular markers linked to all of the known *Phytophthora* resistance genes have been reported. *Rps1*, *Rps2*, *Rps3*, *Rps4*, *Rps5*, *Rps6*, *Rps7*, and *Rps8* were mapped to molecular linkage groups (MLG) N, J, F, G, G, G, N, and A2, respectively (Burnham et al. 2003; Demirbas et al. 2001; Diers et al. 1992; Lohnes and Schmitthenner 1997; Sandhu et al. 2004; Weng et al. 2001).

The *Rps1* locus is complex. It contains five functional alleles including *Rps1*-k and is linked to *Rps7* (Bernard and Cremeens 1981; Lohnes and Schmitthenner 1997; Weng et al. 2001). *Rps1*-k has been the most extensively used gene in breeding *Phytophthora*-resistant soybean cultivars for about two decades, and it is still in use among cultivars of many soybean-growing areas (Schmitthenner et al. 1994). High-resolution genetic and physical maps have been constructed for the *Rps1*-k region, and the gene has recently been isolated through positional cloning and transformation experiments (Bhattacharyya et al. 1997; Kasuga et al. 1997; Gao et al. in press). Although previously *Rps1*-k was considered to be a single gene, two functional *Rps* genes were cloned from the *Rps1*-k locus. Analyses of recombinants strongly indicated that at least one additional functional *Rps* gene maps next to the *Rps1* locus (Seibel and Bhattacharyya unpublished data).

The *Rps2* region has been cloned and sequenced. It is comprised of three functional genes: (1) powdery mildew resistance gene *Rmd-c*, (2) an ineffective nodulation gene *Rj2*, and (3) *Rps2* (Graham et al. 2000, 2002). The *Rps4* region also has been recently characterized, and it has been shown that deletion of a disease resistance gene-like sequence leads to a loss of *Rps4* function (Sandhu et al. 2004). Although earlier results suggested no linkages between *Rps4* and *Rps6* (Athow and Laviolette 1982), two recent studies indicated that the two genes are either allelic or clustered genes (Demirbas et al. 2001; Sandhu et al. 2004).

The *Rps3* locus has been mapped to a disease resistance gene–rich region containing bacterial and viral disease resistance genes. Three functional *Phytophthora* resistance genes were mapped to the *Rps3* locus (Schmitthenner 1989). Recently, a novel *Phytophthora* resistance gene *Rps3?* has been mapped closely to the *Rps3* locus (Sandhu et al. 2004).

Continuous and enhanced uses of stable *Rps* genes in soybean cultivars against *P. sojae* races has created selection

pressures for the evolution of new pathogenic *P. sojae* races that can overcome resistance conferred by these genes. *P. sojae* evolves constantly, and the number of physiological races of this oomycete pathogen is rapidly increasing (Leitz et al. 2000). As a result, incorporation of new *Rps* genes becomes a priority to fight the newly evolved races. There were severe *Phytophthora* disease outbreaks in the Ohio valley for several years until *Rps8* was identified and introduced (Burnham et al. 2003). The new *Phytophthora* resistance gene *Rps8*, identified from a South Korean line (accession number PI 399073), confers resistance against most *P. sojae* isolates in Ohio (Schmitthenner personal communication). The gene can provide resistance against 26 out of 30 *P. sojae* isolates collected from Iowa soils (Sandhu et al. unpublished data). The *Rps8* gene has been mapped to MLG A2. The closest SSR marker was shown to map at about 10 cM from the locus (Burnham et al. 2003). Currently, tightly linked molecular markers for selection of the gene are unavailable. The objectives of our investigation were to (1) develop a high density molecular map of the *Rps8* region and (2) identify SSR markers linked to the *Rps8* gene.

Materials and Methods

Development of Genetic Materials

Two populations, AX18849 and AX18855, segregating for the *Phytophthora* resistance gene *Rps8* were developed by crossing a South Korean line (PI 399073) containing the *Rps8* gene with either (1) A95-684043 (*rps8*) or (2) IA2008R (*rps8*), respectively. The populations were developed at the ISU soybean research site of the Isabela Substation of the University of Puerto Rico, during the winter of 2002–2003.

Evaluation of Genetic Materials for *Phytophthora* Resistance

Plates of oatmeal agar were inoculated with *P. sojae* race 25 2 days before and on the day of sowing the $F_{2:3}$ seeds in the greenhouse. Plates were maintained at room temperature in the dark. A total of 208 and 202 $F_{2:3}$ families of the AX18849 and AX18855 population, respectively, were inoculated with *P. sojae* race 25 as follows. Fifteen seeds of an individual family were planted in soil around the edge of a 6-inch-diameter pot in the greenhouse. The cultivar Sloan with no known *Rps* gene was planted as a susceptible control. Seven days after sowing, each pot was thinned to 11 plants. A 3/4-inch vertical slit was cut below the cotyledons of each plant, and mycellia were inserted into the slits. Inoculated seedlings were misted every 20 s for 2 days in a mist chamber and then transferred to the greenhouse. Dead plants, plants with severe disease symptoms, and healthy plants in each pot were counted 3 and 5 days after inoculation. One unifoliate leaf was harvested from each plant on the day of inoculation, and 11 leaves from each family were bulked, frozen in liquid nitrogen, and stored at −80°C. Forty-seven homozygous resistant and 47 homozygous susceptible $F_{2:3}$ families from the AX18849 population and 45 homozygous resistant and 49 homozygous susceptible $F_{2:3}$ families from the AX18855 population were selected for mapping experiments. Homozygous resistant families were regrown, and an additional 11 progenies/family were infected with *P. sojae* race 25 to confirm their genotype. A total of 22 F_3 plants of each homozygous resistant family were evaluated for race reactions.

DNA Isolation and Pooling for Bulk Segregant Analyses

Genomic DNA of the selected homozygous resistant or susceptible families was isolated according to Anderson et al. (1992). Resistant and susceptible bulks for the bulk segregant analyses were prepared from DNA samples of either (1) 10 homozygous resistant (Resistant Bulk) or (2) 10 susceptible (Susceptible Bulk) $F_{2:3}$ families randomly selected from each of the two populations. DNA bulks were prepared by pooling 1 μg DNA from each selected family. Each bulk then was diluted to a final concentration of 50 ng DNA/μl (Michelmore et al. 1991).

Molecular Marker Analysis

Sequence information for developing SSR markers was obtained from a published report (Song et al. 2004; http://soybase.agron.iastate.edu/ssr.html). For SSR analysis, 30 ng DNA was used as the template in a 10 μl reaction containing 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$; 0.25 μM of each primer; 200 μM of each dNTP, and 0.25 U *Biolase* DNA polymerase (Bioline USA). The polymerase chain reaction (PCR) conditions were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C; followed by 8 min at 72°C. The amplification products were separated on a 4% agarose gel.

The Mapmaker 2.0 program was used to determine genetic linkages and genetic distances (Lander et al. 1987). Marker order was determined at a LOD threshold of 3.0.

Results and Discussion

Bulk Segregant Analyses of the *Rps8* Region

We investigated two segregating populations, AX18849 and AX18855, in identification of SSR markers linked to *Rps8*. Among the 208 $F_{2:3}$ families of the AX18849 population, 49 were homozygous resistant and 53 were homozygous susceptible. Similarly, among the randomly selected 202 $F_{2:3}$ families of the AX18855 population, 45 were homozygous resistant and 55 were homozygous susceptible. Twenty-two plants of each resistant homozygous family were scored. Therefore, the probability of mis-scoring a heterozygous family as resistant homozygous is only 0.0012 (Fairbanks and Rytting 2001). In both populations *Rps8* segregated as a single dominant gene, and the segregating ratios fit well with the genotypic Mendelian 1:2:1 ratio. χ^2 values for a single gene model were insignificant at p values of .89 and .60 for the AX18849 and AX18855 populations, respectively.

Table 1. Microsatellite markers from the *Rps8* region

S. no.	SSR markers	cM position on composite MLG A2	Population AX18849	Population AX18855
1	*Sat_232	112.88	−	+
2	Sct_194	113.57	−	−
3	Satt158	115.25	−	+
4	Satt421	115.93	−	−
5	Sat_382	116.41	+	−
6	Satt707	116.62	−	−
7	Sat_378	116.62	−	−
8	Sat_377	116.64	−	−
9	Satt470	116.73	−	−
10	Sat_040	118.64	+	+
11	Satt333	119.59	−	−
12	Sat_097	122.05	+	+
13	Sat_138	123.26	−	+
14	Satt209	128.44	−	−
15	Satt455	129.86	−	−
16	Sat_294	131.97	+	+
17	Satt409	145.57	−	−
18	Satt228	154.11	+	+
19	Sat_347	158.39	+	+
20	Satt538	159.63	+	+

+ Polymorphic.

− Non polymorphic.

* http://soybase.agron.iastate.edu/ssr.html.

Table 2. Microsatellite markers from the *Rps3* region

S. no.	SSR markers	cM position on composite MLG F	Population AX18849	Population AX18855
1	Satt374	43.01	+	+
2	Satt425	43.44	−	+
3	Satt516	44.42	+	−
4	Satt595	50.24	−	+
5	Sat_133	50.78	−	−
6	Satt663	56.17	−	−
7	Sat_103	57.77	−	−
8	Sat_297	59.60	−	−
9	Sat_229	62.79	−	−
10	Satt114	63.69	−	−
11	Sat_234	66.55	−	−
12	SOYHSP176	68.44	−	−
13	Sat_154	68.91	+	+
14	Satt510	71.41	+	−
15	Sat_317	72.97	−	−
16	Sct_033	74.13	−	−
17	Sat_120	75.97	+	+
18	Satt335	77.70	+	+
19	Satt334	78.06	−	−
20	Satt362	82.83	−	−
21	Sct_188	85.33	−	−
22	Satt072	87.01	−	−
23	Sat_375	88.09	−	−
24	Sat_313	91.87	−	−
25	Satt490	97.97	−	−
26	Satt144	102.08	−	−
27	Sat_197	103.51	+	+
28	Satt554	111.89	−	−

+ Polymorphic.

− Non polymorphic.

In a previous study, *Rps8* was shown to map in MLG A2 in two independent genetic maps (Burnham et al. 2003). The gene was mapped between SSR marker Sat_040 and the isozyme marker *Lap*. The genetic distances between these two markers ranged from 32 to 35 cM, and *Rps8* mapped closely to the marker Sat_040 at about 10–12 cM. To identify user-friendly, closely linked SSR markers, we evaluated 20 SSR markers spanning a 48-cM region, which includes the *Rps8* locus (Song et al. 2004; Table 1). Seven of the markers segregated in the AX18849 population, whereas nine did in AX18855. These markers were then evaluated for their possible linkages with *Rps8* using the resistant and susceptible bulks developed from each of the two populations. Resistant and susceptible bulks developed from either population showed similar patterns for these markers suggesting independent segregation between *Rps8* and the marker loci (Figure 1).

Burnham et al. (2003) studied two small $F_{2:3}$ populations (n_1, 39; n_2, 54) for mapping the *Rps8* gene and constructed two very comparable genetic maps for the *Rps8* region with complete collinearity of marker loci with that of a previously published map (Cregan et al. 1999). The probability of making exactly the same mistake in both maps by Burnham et al. (2003) should be nearly zero. Therefore, one possible explanation for our failure to map *Rps8* in MLG A2 is that race 25 used in our study did not recognize *Rps8*. Instead, it recognized a different *Rps* gene.

The race 25 used in our study was isolated from Iowa soil and was not the one (OH25) used by Burnham et al. (2003). The two races most likely differ for the *Avr8* gene that corresponds to *Rps8*. Our Iowa race 25 failed to recognize the *Rps8* gene and therefore should not contain *Avr8*, which is present in OH25. On the contrary, Iowa race 25 carries an avirulence gene, which is absent in OH25; therefore, the Ohio race failed to recognize the gene detected by Iowa race 25.

The presence of multiple *Rps* genes may also explain why the South Korean line has exhibited broad-spectrum resistance against most *P. sojae* races. We have observed that the line can resist infection in 26 out of 30 *P. sojae* isolates collected from Iowa soils. The presence of multiple *Rps* genes in soybean lines is not unusual. For example, we have recently documented the introgression of *Rps4* and a novel *Phytophthora* resistance gene *Rps3?* into a soybean cultivar from PI 86050 (Sandhu et al. 2004).

Phytophthora Resistance in the South Korean Line Putatively Mapped to the *Rps3* Region

To determine the map location of the unknown *Rps* gene, we applied bulk segregant analyses (Michelmore et al. 1991). A total of 96 SSR markers representing the whole soybean genome were evaluated using resistant and susceptible bulks developed from the AX18855 population (see supplemental

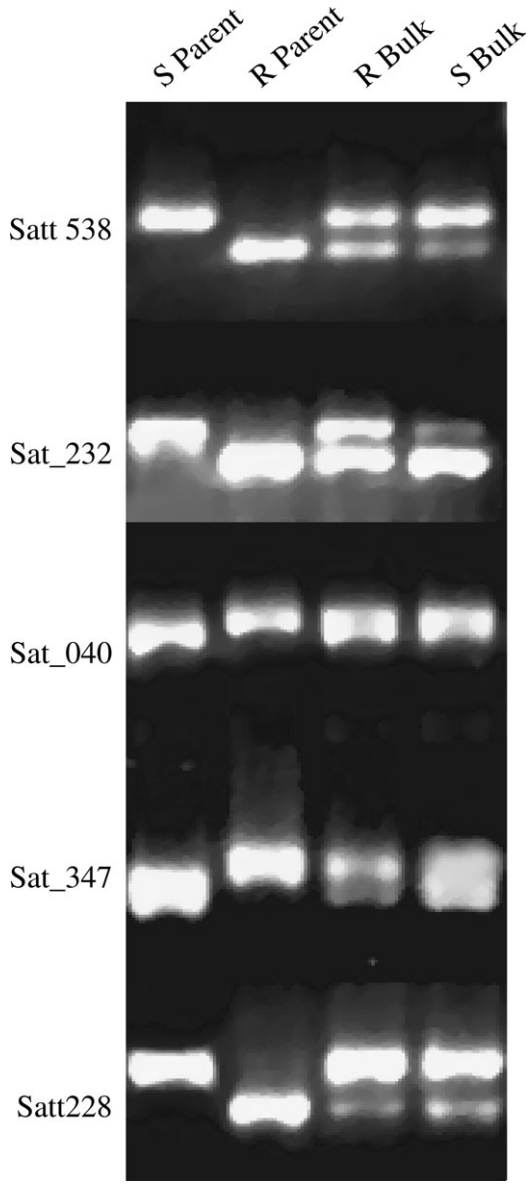

Figure 1. Bulk segregant analysis of the *Rps8* region. S Parent, susceptible parent A95–684043; R Parent, South Korean line (PI 399073) containing the *Rps8* gene; R Bulk, bulk of 10 resistant homozygous $F_{2:3}$ families; S Bulk, bulk of 10 susceptible $F_{2:3}$ families.

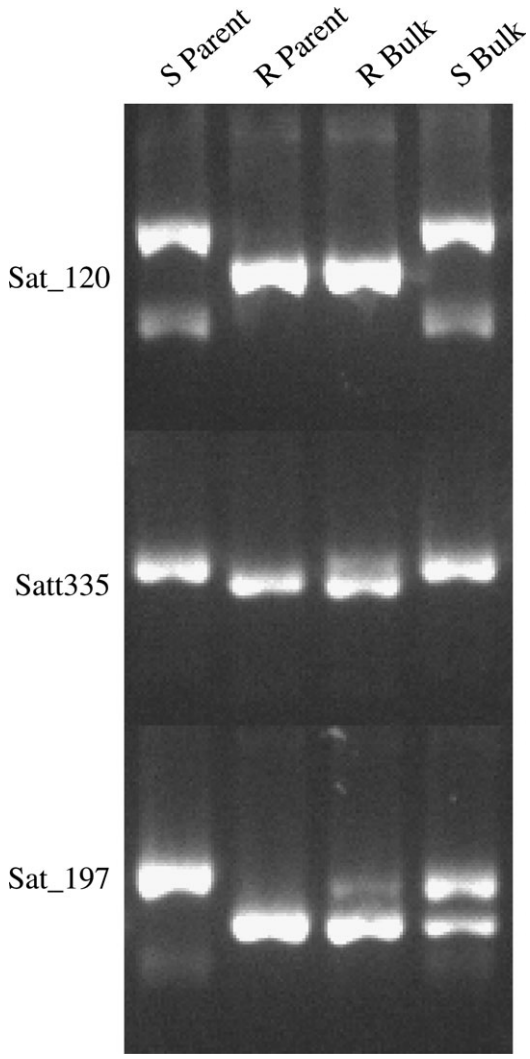

Figure 2. Identification of SSR markers linked to *Rps8*. S Parent, susceptible parent A95–684043; R Parent, South Korean line (PI 399073) containing the *Rps8* gene; R Bulk, bulk of 10 resistant homozygous $F_{2:3}$ families; S Bulk, bulk of 10 susceptible $F_{2:3}$ families.

Table 1). Sat_120 showed polymorphism between the resistant and susceptible bulks (Figure 2). Sat_120 also showed polymorphisms between the resistant and susceptible bulks developed from the AX18849 population. Additional SSR markers linked to Sat_120 also showed polymorphisms between the resistant and susceptible bulks (Figure 2). Based on data obtained from the bulk segregant analyses, we conclude that most likely the novel *Rps* gene is from the *Rps3* region because Sat_120 was shown to be linked to *Rps3* (Cregan et al. 1999).

To confirm the putative map location of the *Rps* gene, we conducted genetic mapping experiments by using 27 SSR markers linked to Sat_120 and segregating populations developed in this study (Table 2). Seven markers segregated in both the AX18849 and AX18855 populations. The AX18849 mapping population had 47 resistant homozygous and 47 susceptible $F_{2:3}$ families, and the AX18855 population had 45 resistant homozygous and 49 susceptible families. These homozygous families were used to map the selected SSR markers. Because OH25 was not available to us, we could not determine if we were studying the same gene *Rps8* as the Dorrance group did. However, a literature search led us to a recent meeting report from the Dorrance lab (Gordon et al. 2004). In that report the group has shown that

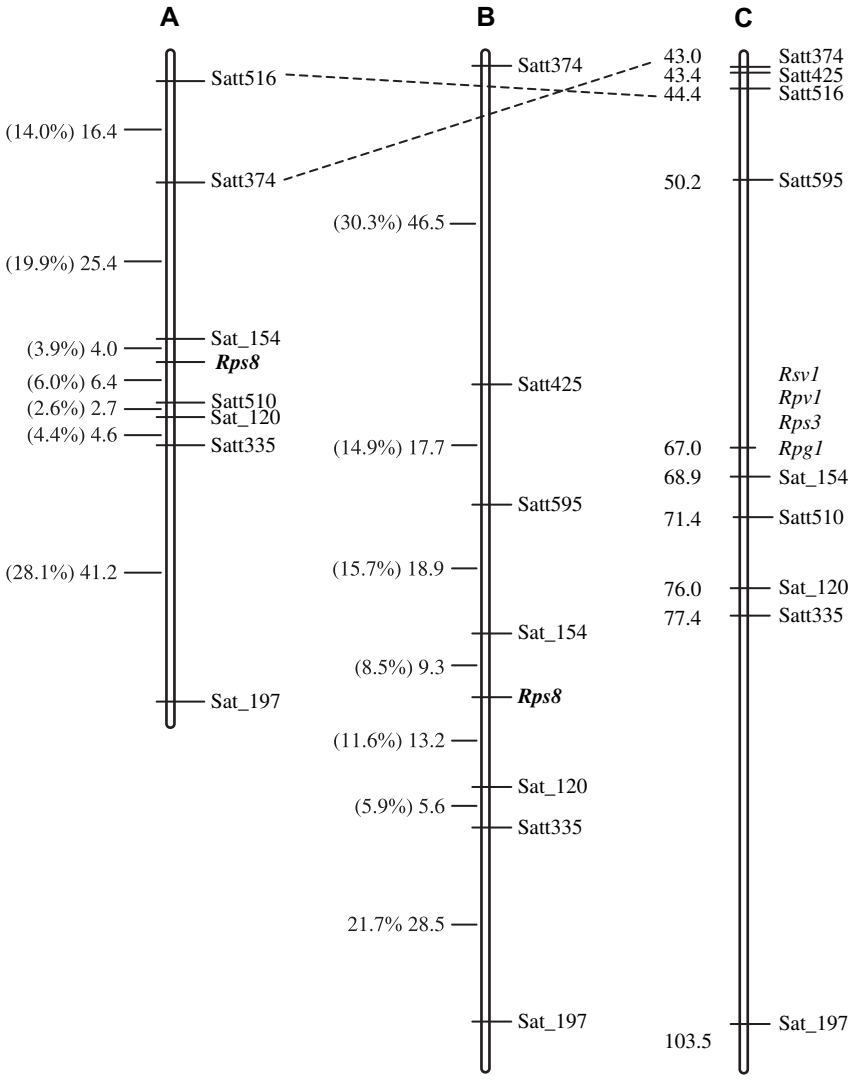

Figure 3. *Rps8* maps closely to the *Rps3* region. (**A**) Genetic map developed based on the AX18849 population; (**B**) genetic map developed based on the AX18855 population; (**C**) part of the composite MLG F (Song et al. 2004; http://soybase.ncgr.org/cgi-bin/ace/generic/pic/soybase?name=f-composite&class=map). Markers showing inversion are connected by dotted lines.

Rps8 mapped closely to the *Rps3* region in MLG F instead of its original location in MLG A2. Therefore, we conclude that the gene we have mapped to the *Rps3* region is in fact the *Rps8* gene. Analyses of the data obtained from both populations showed that the *Rps8* gene is located between Sat_154 and Sat_120 (Figure 3). A complete collinearity of marker loci was observed between the two maps. Apart from a single inversion, a good fit in collinearity of loci on our maps with that of Song et al. (2004) was observed. The map reported by Song et al. (2004) is a composite map; therefore, the observed inversion between this and one of our maps (Figure 3A) could have resulted from possible artifacts generated during composite map construction. The *Rps8* gene mapped closely to the recently cloned bacterial resistance gene *Rpg1*. The *Rps8* locus is located south of the *Rps3* locus (Cregan et al. 1999; Figure 3). Whether the *Rps8* gene is an allele of *Rps3* or tightly linked to *Rps3* is yet to be addressed.

Rps8 Maps to a Region Rich in Disease Resistance Genes

The *Rps3* locus is located in a disease resistance gene–rich region. The region also contains three additional disease resistance loci carrying bacterial disease resistance gene *Rpg1* and two viral resistance genes, *Rsv1* and *Rpv1*. To date, three functional *Rps3* alleles and four functional alleles of *Rsv1*, *Rpv1*, and *Rpg1* have been mapped to this region (Ashfield et al. 2004; Hayes et al. 2004; Schmitthener 1989;

Cregan et al. 1999; http://soybase.ncgr.org/cgi-bin/ace/generic/pic/soybase?name=fcomposite&class=map). *Rps3?* also has been recently mapped to this region (Sandhu et al. 2004). Including *Rps8*, at least 11 disease-resistance genes have been mapped to this small genomic region. It will be interesting to clone and characterize an *Rps* gene from this region and then investigate its evolutionary relationship with recently cloned bacterial resistance gene *Rpg1* and the candidate for *Rsv1* (Ashfield et al. 2004; Hayes et al. 2004).

Supplementary Data

A supplementary table is available at *Journal of Heredity* online (www.jhered.oxfordjournals.org).

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