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Deletion of a Disease Resistance Nucleotide-Binding-Site Leucine-Rich-Repeat-like Sequence Is Associated With the Loss of the Phytophthora Resistance Gene *Rps4* in Soybean

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ABSTRACT

Resistance of soybean against the oomycete pathogen *Phytophthora sojae* is conferred by a series of *Rps* genes. We have characterized a disease resistance gene-like sequence *NBSRps4/6* that was introgressed into soybean lines along with *Rps4* or *Rps6*. High-resolution genetic mapping established that *NBSRps4/6* cosegregates with *Rps4*. Two mutants, M1 and M2, showing rearrangements in the *NBSRps4/6* region were identified from analyses of 82 F_1's and 201 selfed HARO4272 plants containing *Rps4*. Fingerprints of these mutants are identical to those of HARO4272 for 176 SSR markers representing the whole genome except the *NBSRps4/6* region. Both mutants showed a gain of race specificities, distinct from the one encoded by *Rps4*. To investigate the possible mechanism of gain of Phytophthora resistance in M1, the novel race specificity was mapped. Surprisingly, the gene encoding this resistance mapped to the *Rps3* region, indicating that this gene could be either allelic or linked to *Rps3*. Recombinant analyses have shown that deletion of *NBSRps4/6* in M1 is associated with the loss of *Rps4* function. The *NBSRps4/6* sequence is highly transcribed in etiolated hypocotyls expressing the Phytophthora resistance. It is most likely that a copy of the *NBSRps4/6* sequence is the *Rps4* gene. Possible mechanisms of the deletion in the *NBSRps4/6* region and introgression of two unlinked *Rps* genes into Harosoy are discussed.

OVER 40 disease-resistance genes (*R*) have been cloned in plants (Hulbert *et al.* 2001; Martin *et al.* 2003). It has been observed that the proteins encoded by most of the resistance genes are structurally very similar although they provide resistance against a wide range of phytopathogens and pests, such as viruses, bacteria, fungi, nematodes, and aphids. Broadly, cloned disease-resistance genes can be classified into eight classes (Hulbert *et al.* 2001). The majority of the resistance genes encode nucleotide-binding sites (NBS) and leucine-rich repeat regions (LRR). This class includes genes from dicotyledons as well as monocotyledons. Some members of this class contain an N-terminal coiled coil domain and others contain a toll-interleukin receptor-like domain (Dangl and Jones 2001; Mondragon-Palomino *et al.* 2002). Overall sequence homology among members of the class is low but small domains are well conserved (Dangl and Jones 2001). *R*-gene diversity is largely controlled by LRR domain, which goes through adaptive selection to generate new race-specificities (Dangl and Jones 2001). Resistance genes are evolved at a fast rate to protect plants against rapidly evolving new pathogenic races or isolates.

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Soybean [*Glycine max* L. (Merrill)] is an important oilseed crop and the United States is a major soybean producer. An average 71 million tons of soybean valued at up to ~16 billion dollars is produced in the United States annually (Wrather *et al.* 2001). Soybean yields per hectare are significantly reduced due to diseases. Soybean suffers annual yield losses worth nearly 2.7 billion dollars from all diseases together (Wrather *et al.* 2001). Phytophthora stem and root rot disease caused by *Phytophthora sojae* alone results in annual yield losses worth up to ~300 million dollars (Wrather *et al.* 2001). The disease can occur at any growth stages. Application of chemicals is expensive and sometimes ineffective in controlling the pathogen. Monogenic resistance encoded by *Rps* (**r**esistance to ***P**hytophthora* ***s**ojae*) genes has provided reasonable protection for soybean against this pathogen for the last 4 decades. Over 50 physiological races of this oomycete pathogen have been reported, and the number of races is increasing rapidly (Leitz *et al.* 2000). Mutation and rare outcrossing between races and isolates are the main mechanisms of evolution of novel races in this pathogen (Förster *et al.* 1994). Due to the highly variable nature of *P. sojae* it is difficult to control this pathogen by the use of most *Rps* genes (Schmitthenner *et al.* 1994). Identification of new useful *Rps* genes is a constant need for protecting soybean against newly evolved races of the pathogen. To date, 14 *Rps* genes have been mapped to eight genomic loci:

Rps1, *Rps2*, *Rps3*, *Rps4*, *Rps5*, *Rps6*, *Rps7*, and *Rps8*. *Rps1* carries five *Rps* genes, *viz*. *Rps1-a*, *-b*, *-c*, *-d*, and *-k*, and was mapped to the molecular linkage group (MLG) N. *Rps2*, *Rps3*, *Rps4*, *Rps5*, *Rps6*, *Rps7*, and *Rps8* were mapped to MLG J, F, G, G, G, N, and A2, respectively. Three functional *Rps* genes, *viz*. *Rps3-a*, *-b*, and *-c*, were mapped to the *Rps3* locus (Diers *et al.* 1992; Lohnes and Schmitthenner 1997; Demirbas *et al.* 2001; Weng *et al.* 2001; Burnham *et al.* 2003). Map location of *Rps6* has not been consistent in previous studies (Athow and Laviolette 1982; Demirbas *et al.* 2001). Demirbas *et al.* (2001) putatively mapped *Rps4* and *Rps6* to a similar region in the linkage group G. Furthermore, both genes encode resistance against almost the same set of *P. sojae* races (Buzzell *et al.* 1987).

We are interested in investigating the molecular differences that make the two genes distinct in their race specificities. Here we have shown that *Rps4* and *Rps6* are either allelic or tightly linked genes. We have isolated an NBS-LRR-type disease-resistance sequence from the *Rps4/6* region that was introgressed into soybean isolines. Deletion of a few copies of this sequence is associated with the loss of *Rps4* function. In this investigation we have also shown that additional *Rps* genes were introgressed into isolines during incorporation of *Rps4* through backcrossing. Possible mechanisms of the deletion in the *NBSRps4/6* region and introgression of two unlinked *Rps* genes into the cultivar Harosoy are discussed.

MATERIALS AND METHODS

Plant materials: The near-isogenic lines carrying different *Rps* genes [Harosoy lines: HARO14 (*Rps1*-c), HARO1472 (*Rps1*-c, *7*), HARO13 (*Rps1*-b), HARO15 (*Rps1*-k), HARO(1-7) (*rps1-7*), HARO1372 (*Rps1*-b, *7*), HARO16 (*Rps1*-d), HARO1572 (*Rps1*-k, *7*), L-70-6494 (*Rps2*, *7*), HARO3272 (*Rps3*, *7*), HARO 5272 (*Rps5*, *7*), HARO4272 (*Rps4*, *7*), HARO6272 (*Rps6*, *7*), HARO72 (*rps1*, *Rps7*); Williams lines: Williams (*rps*), L-75-6141 (*Rps1*-a), L-77-1863 (*Rps1*-b), L-75-3735 (*Rps1*-k), Williams 82 (*Rps1*-k), L-76-1988 (*Rps2*), L-83-570 (*Rps3*), L-85-2352 (*Rps4*), L-85-3059 (*Rps5*), L-81-4352 (*Rps1*-c, *2*), Williams 79 (*Rps1*-c), L-88-1479 (*Rps3*-b), L-89-1581 (*Rps6*)] were used to study the organization of the NBS sequence isolated in a previous study (M. K. Bhattacharyya, unpublished results). For mapping the *Rps4* gene, 17 F_1's were generated from crosses made between Williams (*rps4*) and HARO4272 (*Rps4*) at the Agronomy Research Center, Ames, Iowa, during 2000. Additional 65 F_1's were generated from crosses between 20 Williams and 20 HARO4272 plants at the Iowa State University soybean research site at the Isabela substation of the University of Puerto Rico during the winter of 2000–2001. The resulting F_1 plants were selfed to produce F_2 populations. Crosses were also made between HARO4272 (*Rps4*) and L89-1581 (*Rps6*) and an F_2 population was developed to determine the segregation ratio of *Rps4* and *Rps6* and also to map the *Rps6* gene.

Disease evaluation: The pathogen races were grown for 6 days in dark at 22° and zoospores were produced by following published methods (Ward *et al.* 1979). F_2 plants were screened by inoculating detached leaves (Bhattacharyya and Ward 1986). Two unifoliate leaves of 14-day-old F_2 plants were detached and placed in moist petri plates. Fifteen microliters of *P. sojae* race 1 zoospore droplets (10^5 spores/ml) were placed on each half of the leaf. Disease reactions were scored 3 and 5 days following inoculation. For wounded green hypocotyl inoculation, mycelia from a 6-day-old culture were used. Hypocotyls of 7-day-old light-grown seedlings were slit 1.0 cm open and fungal mycelia grown in 1/4 V8 medium were introduced into the slits (Schmitthenner *et al.* 1994). Resistant and susceptible seedlings were scored at a 24-hr interval up to 1 week following inoculation.

Pathogen used: *P. sojae* races 1 and 4 and isolates I-CC5A, I-CC5C, I-CC8, I-CC9A, I-CC10D, I-CE5, I-CE10, I-CE20, I-CW1, I-LEE1, I-MARIONCO1-2, I-MARSHALL1-1, I-MARSHALL2-1, I-NW1, I-NW5A, I-NW8A, I-NW8B, I-NW9B, I-NW10, I-POLK1-1, I-POLK3-3, I-SC1A, I-SC4A, I-SC10, I-SE10, I-SE10A, I-SE11A, I-SW1, I-SW11A, and I-SW11B were used. Isolates were identified in a previous investigation (S. Cianzio and X. B. Yang, unpublished results).

DNA analysis: Genomic DNA was prepared according to Anderson *et al.* (1992) for DNA gel blot and PCR analyses. Ten micrograms of genomic DNA was digested with appropriate restriction enzymes and electrophoretically separated on 0.8% agarose gels (Kasuga *et al.* 1997). DNA was blotted from the gel onto nylon membrane (Zeta Probe, Bio-Rad Laboratories, Hercules, CA) using capillary action of 0.4 M NaOH overnight at room temperature.

Probe preparation, hybridization, and autoradiography: About 80 ng DNA was labeled with 50 μCi of [α^{32}P]dATP (Feinberg and Vogelstein 1983). Hybridization was performed in 10 ml of hybridization buffer (50% formamide, 1% SDS, 1 M NaCl, 5× Denhardt's, 100 μg/ml herring sperm DNA), incubated at 42° for 16–18 hr in a hybridization rotisserie oven (Hybaid). Blots were washed with 2× SSC for 5 min at 42° followed by 65° in 0.2× SSC, 0.1% SDS solution for 45 min, and then once more at 65° in 0.1× SSC, 0.1% SDS for 45 min. Blots were exposed to X-ray films for 3–7 days.

Marker analysis: For SSR analysis 30 ng genomic DNA was used as template in 10-μl reaction mixtures containing 1× buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$, 0.25 μM of each primer, 200 μM dNTPs, and 0.5 units of Biolase DNA polymerase (Bioline). The PCR conditions were as follows: initial 2 min at 94° followed by 35 cycles consisting of denaturation at 94° for 30 sec, primer annealing at 58° for 30 sec, and extension at 72° for 1 min. A single 8-min period for extension was provided at the end of the amplification reactions. The amplification products were size separated on a 4% agarose gel.

Mapmaker 2.0 program was used for determining map distances and developing genetic maps (Lander *et al.* 1987). Marker orders were determined at a LOD score of 3.0.

Physical mapping: A Williams 82 soybean BAC library representing 10 soybean haploid genomes was screened with the *NBSRps4/6* probe (M. K. Bhattacharyya, unpublished results). BAC160N2 containing the *NBSRps4/6* region was identified, and both ends of this clone were sequenced. The primers for one end of the BAC clone were designed on the basis of the open reading frame sequence for the putative protein phosphatase 2 gene, and the PCR product for this end was named 160N2FEP. Similarly, primers designed on the basis of the putative importin gene sequence from the other BAC160N2 end were used to PCR amplify the 160N2REP probe. Both probes were used to screen the soybean BAC library and identify a clone that overlapped with the 160N2REP end. No clones were obtained for the 160N2FEP end. To identify clones for this end, the Faribault soybean BAC library constructed at the University of Minnesota was screened, and three clones that overlapped with the 160N2FEP end were identified (Danesh *et al.* 1998). Each end of the four new BAC clones was se-

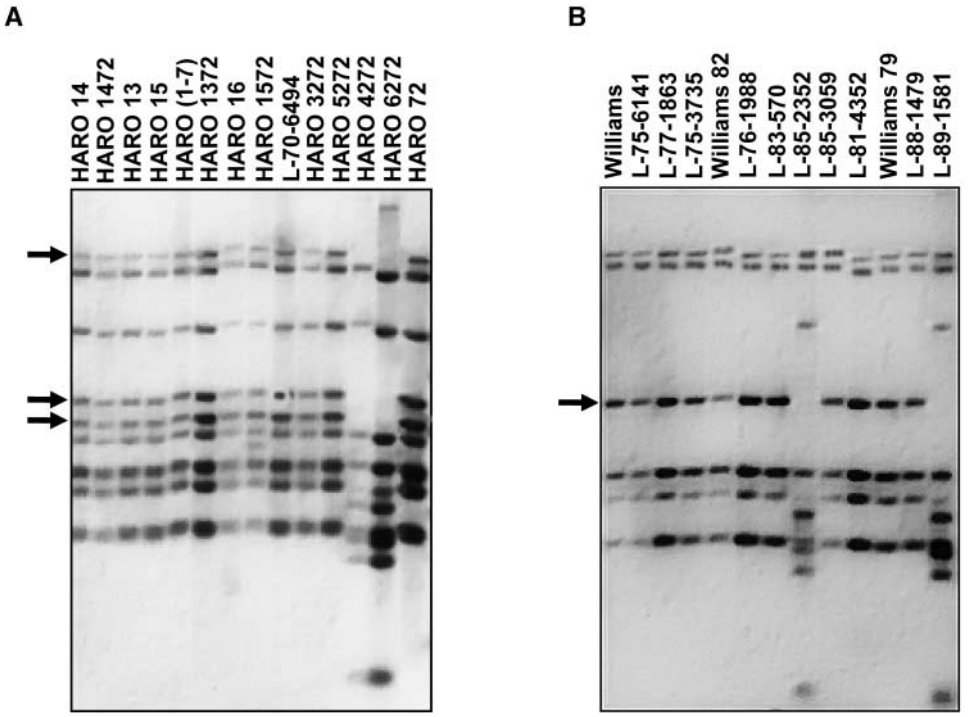

FIGURE 1.—Genomic organization of *NBSRps4/6* among near-isogenic soybean lines. (A) Harosoy isolines (details for isolines in MATERIALS AND METHODS) for *Rps* genes. (B) Williams isolines for *Rps* genes. Arrows indicate NBS-specific *Hin*dIII fragments of the recurrent parent that are linked to *Rps4* and *Rps6* in the repulsion phase.

quenced to develop PCR primers, which were subsequently used to generate the BAC contig of the *Rps4* region.

cDNA cloning: It has been shown that the upper one-third portion of the etiolated hypocotyls of 7-day-old dark-grown seedlings expresses gene-specific Phytophthora resistance (LAZAROVITS *et al.* 1981). We therefore used the upper one-third portion of the etiolated hypocotyls of 7-day-old dark-grown HARO4272 seedlings (*Rps4*) for generating a cDNA library in the Uni-ZAP XR λ-vector (Stratagene, La Jolla, CA; BHATTACHARYYA 2001). The unamplified library was screened using the *NBSRps4/6* probe. Positive clones were purified and excised. The cDNA clones that hybridized to all four *NBSRps4/6*-specific *Hin*dIII fragments of BAC160N2 were sequenced to confirm their identities. Sequencing was carried out in an ABI 3100 automated DNA sequencer at the DNA facility, Iowa State University. Sequence of a representative cDNA clone has been deposited in GenBank (accession no. AY258630).

RESULTS

Cosegregation of the disease-resistance-gene-like sequence *NBSRps4/6* with the Phytophthora resistance genes *Rps4* and *Rps6*: A pulsed-field gel-purified DNA fraction containing molecular markers that flank both sides of the *Rps1* locus, and primers synthesized for the conserved sequences of the NBS domain of cloned disease-resistance genes were used in PCR experiments (YU *et al.* 1996; BHATTACHARYYA *et al.* 2001). The amplified product showed high sequence identity to the NBS domain of cloned NBS-LRR-type disease-resistance genes and hybridized to several *Hin*dIII DNA fragments in DNA gel blot analyses (Figure 1, A and B). This sequence showed polymorphisms between near-isogenic lines differing at the *Rps4* and *Rps6* regions, but not among near-isogenic lines that differ for other *Rps* genes (Figure 1, A and B).

To determine the extent of linkage between *NBS Rps4/6* and *Rps4*, an F_2 population developed by crossing Williams with HARO4272 was investigated. Williams is susceptible to *P. sojae* and does not contain any known *Rps* genes. Detached leaves of F_2 plants were inoculated with *P. sojae* race 1 zoospores for determining the host responses (BHATTACHARYYA and WARD 1986). On the basis of previous information (DEMIRBAS *et al.* 2001) three SSR markers, Sat_064, Satt191, and Satt472, were selected for mapping *Rps4*. A total of 1295 F_2 plants were screened, and 254 of these plants showing susceptibility to *P. sojae* race 1 were selected for mapping the *Rps4* region using these three SSR markers and *NBSRps4/6*. Sat_064 and *NBSRps4/6* cosegregated with *Rps4* (Figure 2A).

NBSRps4/6 detected several similar introgressed *Hin*d III DNA fragments in near-isogenic lines containing either *Rps4* or *Rps6* (Figure 1, A and B). Introgression of these fragments was also accompanied by loss of *Hin*dIII fragments that were presumably linked tightly to the *Rps4* and *Rps6* loci in repulsion (Figure 1, A and B). These data strongly indicate that *Rps4* and *Rps6* are linked genes. However, earlier reports suggested that *Rps4* and *Rps6* segregated independently (ATHOW and LAVIOLETTE 1982). We therefore reinvestigated the possible linkage between these two *Rps* genes. An F_2 population developed from the cross between HARO4272 (*Rps4*) and L89-1581 (*Rps6*) was used to map these two *Rps* genes. A total of 120 plants were leaf inoculated with *P. sojae* race 1. Both genes confer resistance against race 1. All 120 F_2 plants were resistant to race 1. No double-reces-

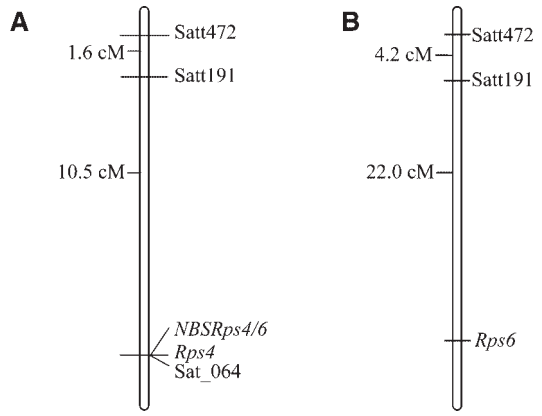

FIGURE 2.—Genetic linkage maps of the *Rps4* and *Rps6* regions. (A) High-resolution genetic linkage map of the *Rps4* region. (B) Genetic linkage map showing the location of *Rps6*.

sive susceptible recombinants were identified in this study. If these genes were to assort independently, ~8 susceptible plants (*rps4rps6*) should have been identified. These results suggest that two genes are linked with a χ^2 probability of 0.0046.

To distinguish the race specificity of *Rps4* and *Rps6*, we infected HARO4272 (*Rps4*) and L89-1581 (*Rps6*) with five selected *P. sojae* isolates, *viz.* I-CC5C, I-CC8, I-CE5A, I-NW8A, and POLK3-3 that were collected from soybean fields of Iowa. Isolate I-NW8a was found to be virulent to HARO4272 (*Rps4*) and avirulent to L89-1581 (*Rps6*). This isolate was used to screen 38 $F_{2:3}$ families generated from the cross between HARO4272 (*Rps4*) and L89-1581 (*Rps6*). The families segregated in a 1:2:1 ratio with a χ^2 probability of 0.08. Relative positions of polymorphic SSR markers Satt191 and Satt472 in relation to *Rps6* suggest that *Rps4* and *Rps6* are most likely allelic or tightly linked genes (Figure 2B).

It was observed that seven *NBSRps4/6*-specific *Hin*dIII fragments of the resistant parent HARO4272 were missing among all susceptible F_2 plants (Figure 3). These fragments were mapped to the *Rps4* region. One *Hin*dIII fragment specific to HARO4272 and one to Williams segregated independently of *Rps4*. Presumably, these sequences mapped to a second homeologous locus (shown by asterisks in Figure 3). Four fragments were not polymorphic between two parents, and therefore map positions of these fragments could not be predicted. These results suggest that the *NBSRps4/6* sequence mapped to at least two loci, one of which cosegregates with *Rps4*.

Identification of rearrangements at the *NBSRps4/6* region: Seventeen F_1's were generated by crossing Williams with HARO4272 for developing a mapping population that segregated for the *Rps4* region. In one F_1, all except one HARO4272-specific *NBSRps4/6 Hin*dIII fragment were lost (data not shown). One novel *Hin*dIII fragment was evolved in this F_1, indicating the presence of a deletion in the *NBSRps4/6* region. We were therefore interested in studying (i) if this locus is unstable and (ii) if deletion of this sequence resulted in the loss of *Rps4* function.

Identification of a loss-of-function mutant could establish the *NBSRps4/6* sequence as a potential candidate for the *Rps4* gene. This mutant will then facilitate the cloning of *Rps4*. We analyzed 65 additional F_1's and 201 selfed HARO4272 plants for detecting any possible rearrangements in the *NBSRps4/6* sequence and identified five additional mutants. These five mutant plants were classified into two classes on the basis of their *NBSRps4/6*-specific *Hin*dIII fingerprints (data not shown). In total we identified three classes of mutations. The mutant originally identified from analysis of 17 F_1's is termed M1, and the other two classes of mutants identified from selfed HARO4272 lines are M2 and M3, respectively. Of the four rearranged plants of the M2 class, only one was in homozygous condition; the *NBSRps4/6*-specific *Hin*dIII fingerprint in this mutant is identical to that of the recurrent parent Harosoy (data not shown). M3 was found in homozygous condition, and its *Rps4*-linked *NBSRps4/6*-specific *Hin*dIII fingerprint is comparable to that of M1 (Figure 4A).

We investigated if the observed rearrangements were due to methylation. It has been considered that *Hin*dIII is sensitive to cytosine methylation (KESSLER and MANTA 1990). To confirm that the differences in restriction patterns observed among mutants were not due to differences in DNA methylation, we compared *NBSRps4/6* fingerprints of HARO4272 and mutants using methylation-insensitive restriction enzyme *Dra*I (Figure 4B). *Dra*I-digested DNA samples of all three mutants resulted in *NBSRps4/6*-specific fingerprints that are distinct from the one for HARO4272. These results suggested that the rearrangements were not due to DNA methylation.

Rearrangements at the *Rps4* region were not generated from seed or pollen contamination: To investigate if the mutations originated from mechanical seed mixture, cross-fertilization, or through recombination, we genotyped all three mutants and HARO4272 using 180 SSR markers representing all 20 soybean chromosomes (see supplemental Table S1 at http://www.genetics.org/supplemental/). Mutants were identical to HARO4272 for all SSR markers except for a small region of MLG G that contains *Rps4* (Figure 5). Haplotypes for M1 and M3 are very comparable for the *Rps4* region. M1 and M3 differ only for Satt472. This SSR marker showed the HARO4272-type pattern in M1 and the Harosoy-type pattern in M3 haplotype (Figure 5B and Figure 6). These results indicate the occurrence of a recombination event between M1 and Harosoy haplotypes for generation of the M3 haplotype. M2 and Harosoy haplotypes are similar for the whole *Rps4* region except Satt191 (Figure 5B and Figure 6). On the basis of the variations in linked SSR markers and *NBSRps4/6* we concluded that there are at least four haplotypes for the *Rps4* region in the cultivar HARO4272 (Table 1 and Figure 6).

All four haplotypes are, however, identical for all 176

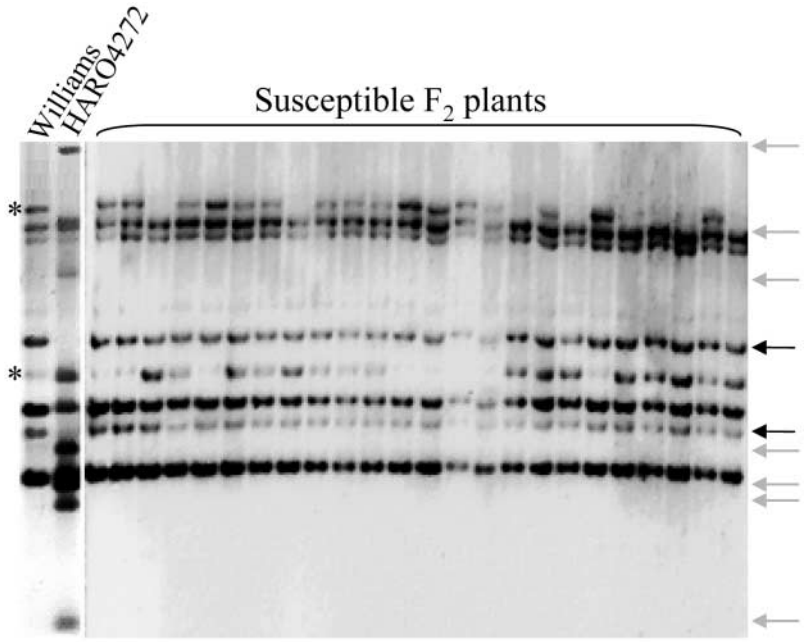

FIGURE 3.—Cosegregation of *NBSRps4/6* with the *Rps4* locus. Solid arrows represent *Hin*dIII DNA fragments that cosegregate with the *rps4* allele; shaded arrows represent *Hin*dIII fragments that cosegregate with *Rps4*, which are absent in all susceptible F_2 plants. (*) *Hin*dIII fragments that segregate independently of the *Rps4* locus.

SSR markers, unlinked to the *Rps4* region. PI86050 was the donor parent of HARO4272. As expected, HARO4272 and PI86050 are identical for Sat_064, Satt191, Satt472, and *NBSRps4/6*. Twenty individual PI86050 plants were evaluated for Sat_064 and Satt472. All 20 plants showed identical banding patterns for these two markers (data not shown). It is most unlikely that four novel haplotypes identified in HARO4272 were introgressed from the donor parent. Selection for Phytophthora resistance after each backcrossing should have allowed the introgression of a specific genomic region from the donor parent, because a single *Rps* gene should have been enough to confer resistance against race 1 in each backcross generation during development of HARO4272. Second, only a single resistant plant was selected for the next backcrossing. Therefore, it is very unlikely that the introgression of more than one haplotype was possible during introgression of *Rps4*. These results strongly suggest that the additional three haplotypes varying at the *Rps4* region evolved from rearrangements through recombination events rather than from mechanical seed mixture or pollen contamination.

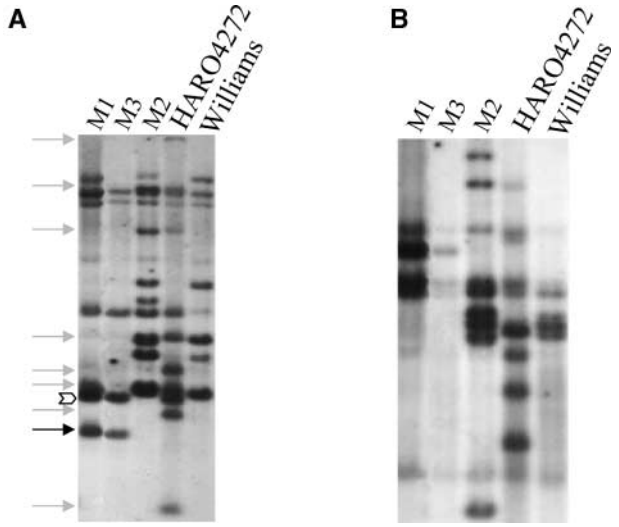

FIGURE 4.—Genomic organization of *NBSRps4/6* among the mutants. (A) DNA digested with *Hin*dIII. Loss of eight *Hin*dIII fragments (shaded arrows) and gain of a novel fragment (solid arrow) are shown. Both M1 and M3 retained a unique *NBSRps4/6*-specific *Hin*dIII fragment shown by an open arrowhead. (B) DNA digested with *Dra*I.

Loss of *NBSRps4/6* is associated with the loss of *Rps4* function in M1: We hypothesized that if a copy of *NBSRps4/6* is the *Rps4* gene sequence, then deletion of this sequence in M1 should have resulted in the loss of *Rps4* function. F_2 individuals of the cross between Williams and M1 were segregating for resistance and susceptibility when leaves were inoculated with race 1, suggesting the intactness of Phytophthora resistance function in this mutant.

Although leaves of HARO4272 are resistant to *P. sojae* race 1 (Figure 2A), the cultivar is highly susceptible to race 1 when wounded hypocotyls of light-grown plants are inoculated with the race. Responses of leaves and hypocotyls to the race were consistent throughout the investigation. On the other hand, wounded hypocotyls of mutants were highly resistant to this race (see supplemental Figure S1 at http://www.genetics.org/supplemental/). Thirty *P. sojae* isolates (see MATERIALS AND METHODS) collected from different soybean fields of Iowa were used to determine if there were any differences between Phytophthora resistance in mutants and the one encoded by *Rps4* in HARO4272. Four of these isolates and race 1 can distinguish mutants and HARO4272 (Table 2). M1 and M3 showed similar dis-

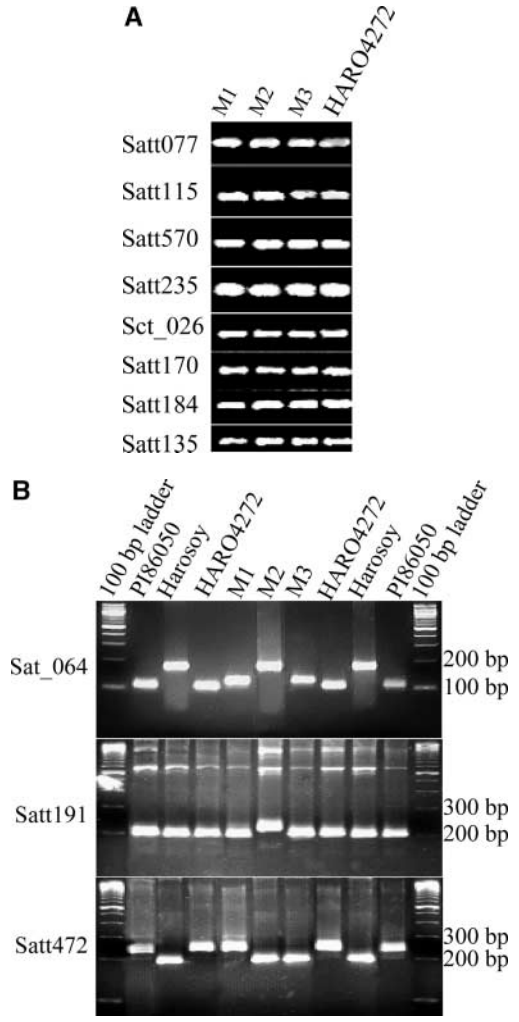

FIGURE 5.—Genome-wide simple sequence repeat analyses. (A) Identical simple sequence repeat phenotypes among mutants and HARO4272. Amplification products of a few selected SSR markers are shown. (B) Simple sequence repeat sequences depicting differences of haplotypes at the *Rps4* region. PI86050, donor parent; Harosoy, recurrent parent; HARO4272, the *Rps4* line; M1, M2, and M3 are three mutants.

ease reactions against all 30 isolates. Furthermore, except for the loosely linked SSR marker Satt472, both mutants showed the same allelic constitutions for all molecular loci of the *Rps4* region (Figure 6). Therefore, most likely M1 and M3 were evolved from the same original mutation. M2 showed the race specificity, which is distinct from those of HARO4272, M1, M3, and Harosoy. These results suggest that there are at least two distinct race specificities among three mutants.

P. sojae race 1 has been reported to be avirulent against soybean lines containing *Rps4* (BUZZELL *et al.* 1987). In our investigation, HAR04272 containing *Rps4* was susceptible to race 1 when wounded hypocotyls were inoculated, but resistant when leaves were inoculated. We investigated if race 1 used in this study still maintains its original race specificity against various *Rps* genes. Differential lines carrying all 14 *Rps* genes were evaluated for their responses to race 1. HARO4272 and PI91160 carrying *Rps4* and *Rps5*, respectively, were susceptible to this race (Table 3). *P. sojae* race 1 is expected to be avirulent to all differential lines carrying any *Rps* genes except *Rps7* (Table 3; BUZZELL *et al.* 1987). It appears that race 1 used in this study has lost its avirulence function against two known *Rps* genes.

We were interested in understanding the mechanism of gain of Phytophthora resistance function associated with deletion of the *NBSRps4/6* sequence. As a first step, we investigated the inheritance of resistance in M1. Wounded green hypocotyls of 96 $F_{2:3}$ families obtained from the cross between Williams and M1 were infected with race 1. As a control, 48 $F_{2:3}$ families derived from the cross between Williams and HARO4272 were similarly inoculated with race 1. Each family consisted of 20 progeny plants. All $F_{2:3}$ families obtained from the cross between Williams and HARO4272 were susceptible to race 1 (see supplemental Figure S2 at http://www.genetics.org/supplemental/). On the other hand, $F_{2:3}$ families of the cross between Williams and M1 segregated in a 1:2:1 ratio for a single dominant gene with a χ^2 probability of 0.573. These data suggest that a single major gene controls the Phytophthora resistance expressed in wounded hypocotyls of M1 against race 1.

During backcrossing usually only a single genomic region carrying a desirable trait is introgressed into recurrent parent. Therefore, except for the introgressed region, the rest of the genome in the near-isogenic line is expected to be identical to that of the recurrent parent. Gain of Phytophthora resistance in this mutant indicated that deletion in the *NBSRps4/6* region is responsible for expression of this novel race specificity. Loss of a genetic factor or element from the *NBSRps4/6* region could be responsible for gain of the novel race specificity in M1. To test this hypothesis, SSR markers were used to map the novel Phytophthora resistance in M1. Bulk segregant analysis was applied to map the new Phytophthora resistance gene (MICHELMORE *et al.* 1991). Resistant and susceptible bulks were prepared from 8 homozygous-resistant and 8 homozygous-susceptible F_3 families, respectively. These families were selected from 96 $F_{2:3}$ families obtained from the cross between Williams and M1. A total of 379 SSR markers representing the whole soybean genome were used to PCR amplify from both bulks. Satt334 showed polymorphism between resistant and susceptible bulks. Satt334 and polymorphic SSR markers close to Satt334 were used to map the unknown gene by using 31 selected $F_{2:3}$ families generated from the cross between Williams and M1. Map position of the unknown gene is shown in Figure 7. The *Rps* gene identified in this mutant mapped closely to the *Rps3* locus that contains three functional alleles. We conclude that the *Rps* gene identified in M1 could be either allelic or tightly linked to *Rps3*. We tentatively named this new *Rps* gene "*Rps3?*."

Analysis of the segregating $F_{2:3}$ families and identifica-

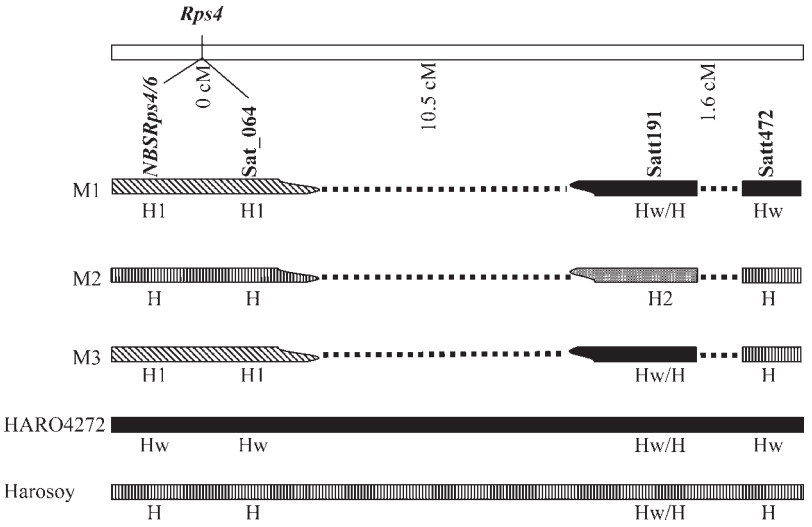

FIGURE 6.—Identification of four haplotypes at the *Rps4* region in HARO4272. Dotted lines represent genomic region for which no information is available. M1, M2, and M3 are mutants; H1, haplotype for M1; H2, haplotype for M2; H, Harosoy haplotype; Hw, HARO4272 haplotype. M1 and M3 are different only for Satt472.

tion of the map location of the gene *Rps3?* in M1 allowed us to investigate whether deletion in the *NBSRps4/6* (Δ*NBSRps4/6*) region played any role in the expression of *Rps3?*. If a negative regulator in the *NBSRps4/6* region were controlling the expression of the Phytophthora resistance encoded by *Rps3?*, then lines carrying Δ*NBSRps4/6* should always be resistant. Both *Rps3?* and Δ*NBSRps4/6* segregated independently, and we were able to identify susceptible genotypes homozygous for Δ*NBSRps4/6* and *rps3?* alleles (*e.g.*, families Ax17931-1-85, Ax17931-1-103). Therefore, the deletion unlikely resulted in the expression of *Rps3?*.

Generation of recombinant $F_{2:3}$ families homozygous for Δ*NBSRps4/6* and *rps3?* alleles also allowed us to investigate if deletion of any *NBSRps4/6* copies in M1 resulted in loss of the *Rps4* function. Earlier, leaf inoculation of F_2 plants of the cross between Williams and M1 showed segregation of resistance and susceptibility in a 3:1 ratio, suggesting that there was no loss of Phytophthora resistance (data not shown). Identification of families such as Ax17931-1-85 and Ax17931-1-103 carrying Δ*NBSRps4/6* and *rps3?* alleles in homozygous condition allowed us to investigate whether the leaf-specific *Rps4* function against race 1 is intact in the haplotype containing the Δ*NBSRps4/6* allele. Results of such a leaf-inoculation study are presented in Figure 8. It appears that progenies of the families homozygous for Δ*NBSRps4/6* and *rps3?* alleles were susceptible to race 1. This observation strongly supported that *Rps4,* which cosegregates with *NBSRps4/6* (Figure 2A), must have been deleted in M1. We hypothesize that a copy of *NBSRps4/6* is the *Rps4* gene.

Physical mapping of the *Rps4* region: To generate a physical map for the region, the *NBSRps4/6* probe was used to screen a Williams 82 BAC library (M. K. BHATTACHARYYA, unpublished results). Screening of the library resulted in identification of BAC160N2 containing all four *NBSRps4/6*-specific *Hin*dIII restriction fragments of the susceptible *rps4* haplotype of Williams 82 (Figure 1). Ends of this BAC were sequenced to develop end-specific primers. Open reading frames identified in each end were used to generate primers. Amplified sequences were used to isolate BAC44C18 that overlaps with the BAC 160N2R end. The BAC160N2F end was used to identify three clones, BAC50O5, 4N10, and 186O2, from a separate BAC library constructed in the Young Laboratory (DANESH *et al.* 1998). Ends of these BACs were sequenced

TABLE 1

Identification of multiple haplotypes at the *Rps4* region

Line	Proportion in population	*NBSRps4/6*	Sat_064	*Rps4/rps4*	Satt191	Satt472
M1	0.059	H1	H1	NK	Hw/H	Hw
M2	0.020	H	H	NK	H2	H
M3	0.005	H1	H1	NK	Hw/H	H
HARO4272	NA	Hw	Hw	*Rps4*	Hw/H	Hw
Harosoy	NA	H	H	*rps4*	Hw/H	H

M1, mutant 1; M2, mutant 2; M3, mutant 3; Hw, wild-type haplotype (HARO4272); H, Harosoy-type haplotype; Hw/H, not polymorphic between HARO4272 and Harosoy; H1, M1-type haplotype; H2, M2-type haplotype; NA, not applicable; NK, not known.

TABLE 2

Differential responses of mutants against *P. sojae* isolates

	P. sojae isolate				
Soybean line	I-CC5C	I-CC8	I-CE5	I-POLK3-3	Race 1
PI86050 (*Rps4*)	R	R	R	R	R
HARO4272 (*Rps4*)	R	S	S	I	S
M1	R	R	R	R	R
M2	R	S	S	R	I
M3	R	R	R	R	R
Harosoy (*Rps7*)	S	S	S	S	S

R, resistant, *i.e.*, >80% plants survived; I, intermediate, *i.e.*, 50–80% plants survived; S, susceptible, *i.e.*, <50% plants survived.

to develop BAC-end-specific probes for developing a BAC contig of the *Rps4* region (Figure 9). The contig most likely includes the *NBSRps4/6* region, because BAC160N2 contains all four *Hin*dIII *NBSRps4/6* fragments specific to the susceptible Williams haplotype. Marker 44C18R was mapped to the *Rps4* region by using an F_2 population of the cross between Williams and HARO4272 (Figure 9). BAC160N2 and BAC44C18 carry the Sat_064 marker that cosegregates with *Rps4* (Figure 9).

The candidate *NBSRps4/6* sequence is transcribed in tissues that confer *Rps4*-specific Phytophthora resistance: High-resolution genetic and physical mapping data and loss of *Rps4*-encoded Phytophthora resistance in M1 indicate physical association of *NBSRps4/6* with *Rps4*. Random sequencing of BAC160N2 indicated that *NBSRps4/6* may be the only class of disease-resistance-gene-like sequence in the *Rps4* region (H. Gao, unpublished results). We therefore investigated if the *NBSRps4/6* sequence is transcribed. The *NBSRps4/6* probe was used to screen a cDNA library generated from HARO4272 (*Rps4*) etiolated hypocotyls. The upper one-third portion of etiolated hypocotyls expressing *Rps4*-specific resistance was used to generate the cDNA library (Bhattacharyya 2001). About 1.5×10^6 plaque-forming units were screened for the *NBSRps4/6* sequence. Thirty-two positive clones were identified from this screening. Identity of individual clones was verified by gel blot and sequence analyses. Blast search of a partial cDNA sequence (GenBank accession no. AY258630) revealed 43% amino acid identity of *NBSRps4/6* with the *Arabidopsis thaliana RPP13* gene. Search for conserved domains using Reverse Position Specific BLAST (RPBLAST of NCBI) revealed an NB-ARC domain. No similarity with any known domains was found for the truncated N-terminal region.

TABLE 3

Responses of differentials against *P. sojae* race 1

Rps gene	Line	Expected reaction	Observed reaction
1a	Mukden/L75-6141	R	R
1b	Sanga	R	R
1c	Mack	R	R
1d	PI103091 Wu An	R	R
1k	Kingwa	R	R
2	CNS	R	I
3a	PI171442	R	R
3b	PI172901	R	R
3c	PI380046	R	R
4	PI86050 Rasuto San	R	R
5	T240/PI91160	R	S
6	Altona	R	R
7	Harosoy	S	S
8	PI399073	—	R
4, 7	HARO4272	R	S
—	Williams	S	S

R, resistant; I, intermediate; S, susceptible; —, data not available.

DISCUSSION

In this study, we have identified the disease-resistance-gene-like sequence *NBSRps4/6* that was introgressed into a soybean line along with Phytophthora resistance genes *Rps4* and *Rps6* from PI86050 and Altona, respectively. Earlier Athow and Laviolette (1982) reported independent assortment between *Rps4* and *Rps6* genes. However, Demirbas *et al.* (2001) identified a SSR marker that mapped closely to both *Rps4* and *Rps6*. Loose linkages of RFLP and SSR markers with *Rps4* have also been reported (Diers *et al.* 1992; Demirbas *et al.* 2001). High-resolution genetic mapping data showed that the SSR marker Sat_064 cosegregates with *Rps4* and is physically linked to *NBSRps4/6*. *Rps4* and *Rps6* are reported to encode resistance against very comparable sets of *P. sojae* races (Buzzell *et al.* 1987). Therefore, most likely these two genes are related. *Rps6* and *Rps4* isolines also exhibit similar DNA fingerprints for *NBSRps4/6* (Figure 1). Mapping data have shown that *Rps4* and *Rps6* are either allelic or clustered genes (Figure 2).

In this investigation we have discovered several rearrangements at the *NBSRps4/6* region in the cultivar HARO4272 (Table 1). On the basis of race specificities,

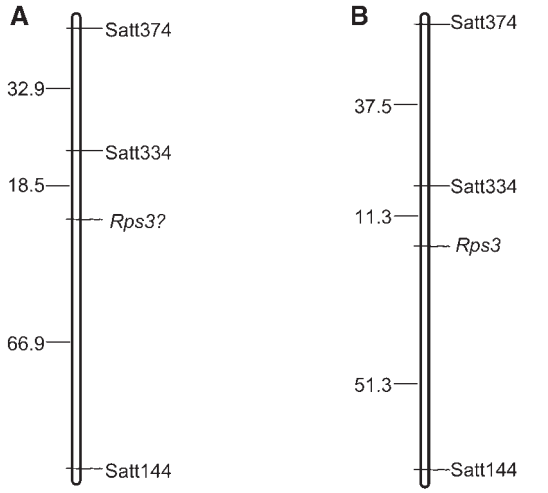

FIGURE 7.—The gene encoding Phytophthora resistance in M1 mapped to the *Rps3* region. (A) Because the gene encoding Phytophthora resistance in M1 mapped to the *Rps3* region, it could be allelic to *Rps3* and is termed as *Rps3?*. (B) Map shows the *Rps3* region in the soybean composite genetic linkage map for MLG F (http://soybase.ncgr.org/cgi-bin/ace/generic/pic/soybase?name=F-Composite&class=Map; SONG *et al.* 2004).

FIGURE 8.—Recombinants showing the association of deletion at the *NBSRps4/6* region with the loss of *Rps4* function. Disease reactions of mutants, recombinants, and control genotypes are shown. *Rps3?*, *Rps* gene mapped to the *Rps3* region; *Rps?*, *Rps* gene that has not been characterized; Δ*NBSRps4/6*, deletion in the *NBSRps4/6* region; *nbsrps4/6^{w}*, *NBSRps4/6* allele from Williams; *nbsrps4/6^{h}*, *NBSRps4/6* allele from Harosoy. In this experiment *P. sojae* race 1 zoospores were used. In leaves *Rps4* confers resistance against this race. (A) Williams (*nbsrps4/6^{w}*, *rps3?*). (B) 17931-1-85 (Δ*NBSRps4/6*, *rps3?*). (C) 17931-1-103 (Δ*NBSRps4/6*, *rps3?*). (D) Harosoy (*nbsrps4/6^{h}*, *rps3?*). (E) HARO4272 (*NBSRps4/6*, *rps3?*). (F) 17931-1-81 (Δ*NBSRps4/6*, *Rps3?*). (G) 17931-1-102 (*nbsrps4/6^{w}*, *Rps3?*). (H) 17931-1-113 (*nbsrps4/6^{w}*, *Rps3?*). (I) M2 (*nbsrps4/6^{h}*, *Rps?*). (J) M3 (Δ*NBSRps4/6*, *Rps3?*). (K) PI86050 (*NBSRps4/6*, *Rps3?*).

mutants can be classified into two classes: (i) M1 and M3 in class I and (ii) M2 in class II. However, molecular marker data showed that mutants M1 and M3, although they have the same race specificity, are different for Satt472, possibly because of a recombination event (Figure 5B and Figure 6).

A significant number of polymorphisms can be detected in soybean with the aid of SSR markers. A genetic diversity study of 131 Asian soybean accessions revealed that the average probability of detecting SSR-based polymorphisms between any two lines is 0.78. A minimum probability of 0.05 for detecting polymorphisms was observed between highly related Japanese soybean lines originating from the same cultivar group (ABE *et al.* 2003). In our study, even if we consider the lower probability value for detecting polymorphism by any SSR marker, the use of 176 SSR markers representing the whole genome, except the *Rps4* region, should have detected at least nine polymorphisms if mutants were to originate from mechanical seed mixture or cross-pollination rather than from recombination events. These data, therefore, suggest that genetic recombination at the *Rps4* region was the mechanism for generation of novel Phytophthora race specificities in HARO4272 (Table 2).

Investigation of the Phytophthora resistance encoded in M1 led to the identification of a novel gene *Rps3?*, which could be either allelic or linked to *Rps3* in MLG F. Eight loci have already been assigned to accommodate 14 *Rps* genes (DIERS *et al.* 1992; LOHNES and SCHMITTHENNER 1997; DEMIRBAS *et al.* 2001; WENG *et al.* 2001; BURNHAM *et al.* 2003). *Rps3?* was most likely introgressed along with *Rps4* into the Harosoy background. HARO 4272 was developed in Harrow, Ontario, during 1980s. The initial cross was made between L62-904 (white-flowered Harosoy) and PI86050 (*Rps4*). Selection for the Phytophthora resistance gene *Rps4* was accomplished by inoculating each backcrossing generation with *P. sojae* race 4 and race 5. *Rps4* encodes resistance against race 4 but not race 5 (BUZZELL *et al.* 1987). Nine BC_6F_1 plants were selfed to generate nine segregating F_2 populations that were evaluated for resistance against *P. sojae* race 1. Homozygous-resistant families were subsequently bulked and named HARO4272 (BUZZELL *et al.* 1987; D. BUZZELL, personal communication). Our data established that HARO4272 is composed of several haplotypes and carried multiple *Rps* genes. This can be explained if we assume that race 4 used for selection of *Rps4* was a mixture of isolates because a mixture of distinct isolates can provide selection pressure for two or more independent resistance genes. For example, one isolate

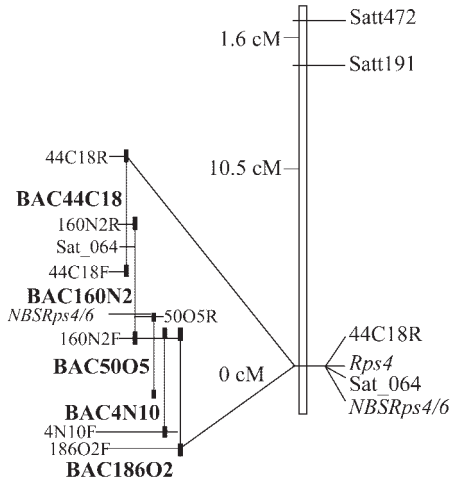

FIGURE 9.—Physical map of the *Rps4* region. A BAC contig is shown in parallel to the linkage map. BAC160N2, which is ~70 kb, contains all four *NBSRps4/6 Hin*dIII fragments specific to the Williams haplotype and the Sat_064 marker.

carried the corresponding *Avr* gene for *Rps4*, while the other one carried the corresponding *Avr* gene for *Rps3?*. As a result of the use of a mixture of two isolates, simultaneous selection for both *Rps4* and *Rps3?* was possible. M2 showed a distinct race specificity (Table 2) and contains an unknown *Rps* gene that confers resistance in leaves against race 1 (Figure 9). The presence of a third isolate in the race mixture can be considered for selection of this unknown *Rps?* gene detected in M2. Introgression of all three *Rps* genes was therefore accomplished in each backcross generation with the use of an isolate mixture. Use of single-spore-derived races was not in practice until the 1990s and consideration of an isolate mixture as a race was quite possible in the 1980s (BHAT *et al.* 1993; T. ANDERSON, personal communication).

Recombination most likely has taken place at the *NBSRps4/6* region between Harosoy and the donor parent PI86050 during selfing generations of nine BC_6F_1 plants. Occurrence of such a recombination process during backcrossing generations was very unlikely because only a single resistant plant was selected for crossing with the recurrent parent after each backcrossing generation, allowing introgression of only one haplotype into Harosoy. Instead, we have documented four haplotypes at the *Rps4* region in varying proportions in the cultivar HARO4272 (Table 1). We hypothesize that M1 was derived from a single unequal recombination event during selfing generations following the completion of backcrosses. This recombination event led to a deletion in the *NBSRps4/6* region. M3 is most likely derived from M1 through a subsequent recombination event between *NBSRps4/6* and Satt472 (Figure 6).

Unequal crossing over is considered as one of the most important mechanisms for evolution of disease-resistance genes. For example, in maize unequal crossing over is the main mechanism of meiotic instability at the *Rp1* region (SUDUPAK *et al.* 1993). In the Arabidopsis ecotype Columbia, the nonfunctional chimeric *rpp8* gene was most likely evolved from unequal crossing over between the functional oomycete resistance gene *RPP8* and its homolog *RPH8A* (MCDOWELL *et al.* 1998). It has been proposed that the *HRT* gene encoding viral resistance was evolved by unequal crossing over between progenitor genes related to *RPP8* and *RPH8A* (COOLEY *et al.* 2000). Unequal crossing over has also been considered to play an important role in the generation of alleles at the *Rsv1* locus in soybean (HAYES *et al.* 2004). The *NBSRps4/6 Hin*dIII fingerprints show that the sequence has many more paralogous copies in lines carrying either *Rps4* or *Rps6* than in those carrying the corresponding recessive alleles (Figure 1). Therefore, this region is highly diverse between the resistant donor and the susceptible recurrent parents, and a recombination at this region most likely will be an unequal one, which can cause loss of sequences. For example, nine deletions in the maize *Rp1-D* gene family have been considered to generate from unequal crossing over (COLLINS *et al.* 1999). The unequal crossing over between donor and recurrent parents at the *NBSRps4/6* region most likely led to deletion of *Rps4* in M1.

We have shown through analysis of segregants that leaves of recombinant lines homozygous for Δ*NBSRps4/6* and *rps3?* alleles are susceptible to race 1 (Figure 8, B and C). Thus, M1 carrying Δ*NBSRps4/6* does not contain *Rps4*. M2 also does not contain *Rps4* (Table 2; and slight spread in Figure 8I). Loss of *Rps4*, presumably through unequal crossing over, led to selection pressure for *Rps3?* in M1 or M3. Earlier studies have shown that most *Rps* genes confer resistance against race 1 (BUZZELL *et al.* 1987). Therefore, lines carrying any of these three genes should be selected during the selfing generations, because race 1 but not race 4 was used to inoculate the segregating population (D. BUZZELL, personal communication). If we assume that *Rps4* provided the strongest resistant response as compared to that of the other two genes against race 1, then use of race 1 instead of race 4 during selfing generations most likely relieved the selection pressure for *Rps3?* and *Rps?*. Lack of selection pressure for either *Rps3?* or *Rps?* among individuals carrying *Rps4* most likely led to partial loss of *Rps3?* and *Rps?* from the HARO4272 population (see supplementary Figure S1 at http://www.genetics.org/supplemental/).

High-resolution genetic mapping data have shown that *Rps4* and *NBSRps4/6* cosegregate. *NBSRps4/6* is also transcribed and most likely is the only candidate disease-resistance-gene-like sequence at the *NBSRps4/6* region (H. GAO, unpublished results). Loss of a few copies of the *NBSRps4/6* gene family is also associated with the loss of *Rps4*-specific resistance, suggesting that a copy of the *NBSRps4/6* gene family could be the *Rps4* gene.

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