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ORIGINAL PAPER

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Identification of a large cluster of coiled coil-nucleotide binding site–leucine rich repeat-type genes from the *Rps1* region containing Phytophthora resistance genes in soybean

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Abstract Fifteen *Rps* genes confer resistance against the oomycete pathogen *Phytophthora sojae*, which causes root and stem rot disease in soybean. We have isolated a disease resistance gene-like sequence from the genomic region containing *Rps1*-k. Four classes of cDNA of the sequence were isolated from etiolated hypocotyl tissues that express the *Rps1*-k-encoded Phytophthora resistance. Sequence analyses of a cDNA clone showed that the sequence is a member of the coiled coil-nucleotide binding site–leucine rich repeat (CC-NBS–LRR)-type of disease resistance genes. It showed 36% identity to the recently cloned soybean resistance gene *Rpg1-b*, which confers resistance against *Pseudomonas syringae* pv. *glycinea*, and 56% and 38% sequence identity to putative resistance gene sequences from lotus and *Medicago truncatula*, respectively. The soybean genome contains about 38 copies of the sequence. Most of these copies are clustered in approximately 600 kb of contiguous DNA of the *Rps1*-k region. We have identified a recombinant that carries both *rps1*-k- and *Rps1*-k-haplotype-specific allelomorphs of two *Rps1*-k-linked molecular markers. An unequal crossover event presumably led to duplication of alleles for these two physically linked molecular markers. We hypothesize that the unequal crossing over was one of the mechanisms involved in tandem duplication of CC-NBS–LRR sequences in the *Rps1*-k region.

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Introduction

Plants evolved with unique defense mechanisms to defeat invading pathogenic organisms. Active defense compounds are rapidly synthesized following infection to confer disease resistance. Accumulation of these molecules is regulated by single race-specific disease resistance (R) genes that encode receptors to recognize specific ligand molecules of invading pathogens (Dangl and Jones 2001). The genetic basis of this recognition phenomenon was first described by Flor for the flax and *Melampsora lini* interaction (Flor 1955). In recent years, over 40 R genes have been isolated (Martin et al. 2003). The majority of R genes encode proteins with nucleotide

binding (NB) and leucine rich repeat (LRR) domains. The NB–LRR group can be divided into two subfamilies based on their predicted N-terminal structures. One subfamily, CC-NBS-LRR, carries a coiled-coil or leucine zipper domain at the N-terminal region (Pan et al. 2000). The other subfamily, TIR-NB-LRR, has an N-terminal domain showing homology to a domain found in *Drosophila* Toll and mammalian interleukin-1 receptors, or TIRs (Whitham et al. 1994).

Soybean is a major oil seed crop grown across the world. The United States alone meets over half of the world's consumption. In North America, soybean suffers from a root and stem rot disease caused by the oomycete pathogen *Phytophthora sojae*. Extensive outbreaks of Phytophthora root rot in soybean fields were first documented in Indiana in 1948 and then in Ohio in 1951 (Schmitthenner 1989). The disease can occur at any stage of soybean development. In the United States, Phytophthora root and stem rot disease has been the second most destructive soybean disease after soybean cyst nematodes, and annual crop losses from this disease were valued to be about $273 million (Wrather et al. 2001). More than 50 races of *P. sojae* have been reported, and new races are rapidly evolving (Leitz et al. 2000). Monogenic resistance encoded by *Rps* genes has been providing reasonable protection against this disease for the last four decades. The soybean genome contains 15 *Rps* genes (Sandhu et al. 2004, and references there in). The *Rps1* locus carries five alleles; *Rps1*-a, *Rps1*-b, *Rps1*-c, *Rps1*-d, and *Rps1*-k. Among these Phytophthora resistance genes, *Rps1*-k confers resistance against a large number of the *P. sojae* races and has been widely used (Schmitthenner et al. 1994). Resistance encoded by this locus against *P. sojae* races 4 and 7, but not race 1, is significantly compromised in the ethylene mutant *etr1*. Therefore, the *Rps1*-k locus has been considered to carry more than one Phytophthora resistance gene, one of which requires a member of the ethylene-signal pathway for expression of Phytophthora resistance (Hoffman et al. 1999).

SSR markers linked to eight Phytophthora resistance loci except *Rps5* have been identified. *Rps1*, *Rps2*, *Rps3*, *Rps7*, and *Rps8* were mapped to the soybean molecular linkage groups N, J, F, N, and A2, respectively, whereas *Rps4*, *Rps5*, and *Rps6* are clustered in the linkage group G (Sandhu et al. 2004, and references therein). Single, dominant avirulence genes corresponding to 11 of the *Rps* genes have been mapped. Several *P. sojae Avr* genes have been targeted for positional cloning, and recently, *Avr1b* has been cloned (Tyler 2002; Shan et al. 2004).

In soybean, a major R gene cloning effort has been the identification and use of resistance gene analogues for the possible isolation of functional resistance genes (Graham et al. 2002; Hayes et al. 2004; Sandhu et al. 2004). Positional cloning experiments recently led to the cloning of *Rpg1*, which encodes the resistance of soybean against the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Ashfield et al. 2004). It is a CC-NBS–LRR-type resistance gene. Earlier, we reported the high-resolution genetic and physical maps of the *Rps1*-k region (Bhattacharyya et al. 1997; Kasuga et al. 1997). Here, we report the isolation and characterization of a family of CC-NBS–LRR-type sequences from the *Rps1*-k region. We also discuss the possible evolutionary mechanism of this large gene family.

Materials and methods

Growing of soybean and *P sojae*, inoculation, and disease scoring

Soybean seedlings were grown under light conditions for 2 weeks (Bhattacharyya and Ward 1986). *P. sojae* race 1 was grown in the dark at 22°C, and zoospores were obtained from 6-day-old cultures (Ward et al. 1979). Segregating materials from the cross between the pair of near-isogenic lines (NILs) Elgin (*rps1*-k) and Elgin 87 (*Rps1*-k) were tested for their responses to *P. sojae* race 1 by inoculating detached leaves with zoospore suspensions (Bhattacharyya and Ward 1986). Unifoliates of 2-week-old F_2 seedlings were detached and placed in 90-mm diameter petri plates carrying Whatman filter papers moistened in 10 ml water. Petioles of leaves were kept under a film of water, and leaf blades were inoculated with droplets of 10 µl zoospore suspensions carrying about 1,000 zoospores. The infected leaves were scored for disease development 3 days and 5 days following inoculation. Susceptible responses are characterized by rapidly spreading light-brown lesions. Resistant symptoms are normally dark-brown necrotic lesions about the size of inoculum droplets. Occasionally, a little spread was seen during the first 3 days, but the spreading lesions were dark brown.

Analysis of segregants and identification of recombinants

Three molecular markers, 120(1 + 2), CG1, and TC1, were used in isolation and characterization of recombinants. CG1 and TC1 were described earlier (Kasuga et al. 1997). 120(1 + 2) is a cleaved amplified polymorphic marker (Konieczny and Ausubel 1993). It was developed by *Bcl*I digestion of PCR products (primers: 120-1-F: 5′-GGT GAT GCT GGC CCA TCA TTT GGT ATT-3′ and 120-67-F: 5′-CCA CCC TCA ATG ATA CTT GGC TAC-3′) representing a 2-kb single-copy sequence identified from BAC120.

DNA preparation, PCR, and Southern analyses

Soybean genomic DNA for molecular mapping or Southern blot experiments was prepared from leaves according to White and Kaper (1989). A touchdown PCR program with initial denaturing temperature of 94°C for 2 min, and then a denaturing temperature of 94°C for 30 s at the beginning of each cycle, an

annealing temperature which dropped from 60°C to 55°C at −1°C/cycle, and extension temperature of 72°C for 1 min/cycle was used. A total of 35 cycles were carried out, with the final annealing temperature at 55°C or 48°C. Southern analysis was carried out according to the protocol described earlier (Kasuga et al. 1997).

Linkage analysis

To isolate the recombinant R910, a total of 480 chromosomes were evaluated for co-dominant molecular markers, whereas for the recombinant R213 1724 chromosomes were analyzed. Phytophthora resistance and susceptibility segregated at a 3:1 ratio. In calculating genetic distances between *Rps1*-k and TC1 and *Rps1*-k and CG1, information from Kasuga et al. (1997) was also considered. Map distances were calculated by using the MapMaker program, version 2.0 (Lander et al. 1987).

Preparation of high-molecular-weight DNA and construction of BAC libraries

Two BAC libraries were constructed for the cultivar Williams 82, which carries the *Rps1*-k gene. Two additional BAC libraries including Gm_ISb001, carrying Williams 82 DNA, were also screened in this investigation (Marek and Shoemaker 1997; Salimath and Bhattacharyya 1999). Williams 82 was a donor of the *Rps1*-k gene for many soybean cultivars including Elgin 87. Therefore, both Williams 82 and Elgin 87 carry the same gene. RFLP mapping data also suggested that the introgressed region in Elgin 87 is large and similar to the one in Williams 82 (Kasuga et al. 1997). Plants were grown in growth chambers under standard growing conditions (Bhattacharyya and Ward 1986; Kasuga et al. 1997). High-molecular-weight (HMW) DNA from young unifoliates of Williams 82 was prepared following the protocols described earlier (Salimath and Bhattacharyya 1999). Agarose plugs (76 µl) carrying approximately 8–10 µg of HMW DNA were prepared. About 150 plugs were prepared in a single batch and tested for quality by digesting the DNA with *Hin*dIII in the presence or absence of 10 mM $MgCl_2$. The plugs were used to construct a BAC library in the pBeloBAC11 vector at GenomicSystems (St. Louis, Mo., USA) (Kim et al. 1996). A copy of the library and two sets of DNA filters carrying DNA samples from individual BAC clones, kindly provided by GenomicSystems, were used in the present investigation. In addition to this large-insert BAC library, a small-insert BAC library was constructed from Williams 82 DNA. This library contains about five genome equivalent clones (S.S. Salimath and M.K. Bhattacharyya, unpublished results). Analyses of BAC clones of the *Rps1*-k region were carried out according to Salimath and Bhattacharyya (1999). BAC clones identified from the Gm_ISb001 and GenomicSystem libraries were referred to in the Fig. 1 with the prefixes IS_ and GS_, respectively. BAC clones identified from two pool BAC libraries were referred to in the Fig. 1 and text with serial numbers.

cDNA cloning

The upper one-third portion of etiolated hypocotyls of 7-day-old, dark-grown seedlings of Williams 82 expressing the *Rps1*-k gene were previously used to generate a cDNA library in the Uni-ZAP XR lambda vector (Stratagene, La Jolla, Calif., USA) (Ward et al. 1981; Bhattacharyya 2001). About 4.6×10^6 plaque-

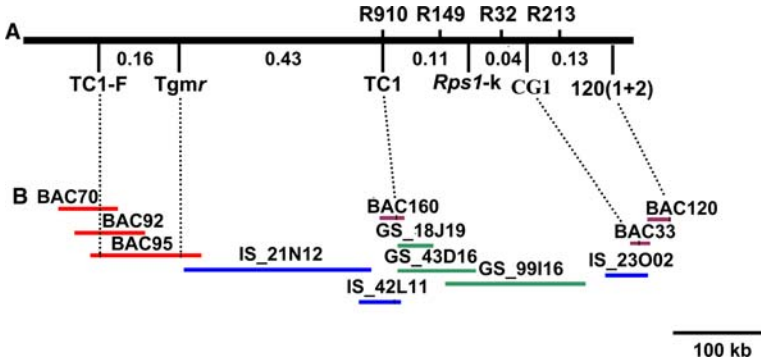

Fig. 1 High-resolution genetic and physical maps of the *Rps1* region. **a** High-resolution genetic map showing the locations of chromosomal breakpoints in four recombinants R910, R149, R32, and R213. The map is based on the results of this and earlier investigations (Kasuga et al. 1997). Two new recombinants, R910 and R213, and a new marker, 120(1 + 2), are added to this map. The location of the chromosomal breakpoint in R910 is based on the unequal crossover between TC1 allelomorphs of two haplotypes *rps1*-k (Elgin) and *Rps1*-k (Elgin 87) (additional information in Fig. 5). Map positions of Tgm *r* and TC1-F are based on previous data (Kasuga et al. 1997). 120(1 + 2) is a marker generated from BAC120 (Fig. 1b). **b** BAC contig containing the *Rps1*-k region. Four BAC libraries carrying 24 haploid genome equivalents of DNA were screened for isolating BAC clones of the *Rps1*-k region. BAC clones are *colored* to show the BAC libraries from which they originated. *Purple lines* represent BACs from the Gm_ISb001 library constructed by Marek and Shoemaker (1997); *black lines*, BACs from the library constructed by S.S. Salimath and M.K. Bhattacharyya, unpublished results; *green lines*, BACs from the library constructed by C. Baublite and M.K. Bhattacharyya, unpublished results; *red lines*, BACs from the library constructed by Salimath and Bhattacharyya (1999)

forming units (pfu) from this unamplified library were screened for the LRR160 sequence. Positive clones were purified and excised for sequencing.

Phosphorimage analysis and copy number estimation

Following Southern hybridization, DNA blots were exposed to storage phosphor screens for 48 h. The extent of hybridization of the LRR160 probe to a 2.3 kb *Hin*dIII fragment was determined using a PhosphorImager system (Variable mode imager Typhoon 8600, Molecular Dynamics, Sunnyvale, Calif., USA). To determine the intensity of each hybridizing signal, phosphorimages were analyzed by using ImageQuant software (tutorial, version 5.0).

Results

Isolation of recombinants for the *Rps1* region

In our previous study, we had developed a high-density and high-resolution map of molecular markers for the *Rps1*-k region and mapped the *Rps1*-k gene in between two AFLP markers CG1 and TC1, in a 0.13-cM interval (Kasuga et al. 1997). In that study, two recombinants containing crossover breakpoints in the TC1–CG1 interval were isolated from the F_2 populations developed from crosses between near-isogenic Elgin lines but not Williams lines. Therefore, to identify additional recombinants carrying crossover breakpoints at the *Rps1* region, 979 F_2 plants developed from the cross between near isogenic lines NILs Elgin (*rps1*-k) and Elgin 87 (*Rps1*-k) were infected with *P. sojae* race 1 and screened for segregation of resistant and susceptible phenotypes. Two hundred and forty susceptible plants identified from that screen were investigated further. A single recombinant 910 (R910) was identified from the screening of these 240 plants. R910 was susceptible to the *P. sojae* race 1 but contained both Elgin- and Elgin-87-specific TC1 alleles. This indicated that the crossover breakpoint in this recombinant was located in between *Rps1*-k and TC1. F_3 and F_4 progenies of R910 were investigated for segregation of TC1 alleles. A single individual containing only the Elgin-specific TC1 allele was detected among the ten F_3 progenies. Because this individual contained only the Elgin-specific TC1-allele, it was not taken to F_4. We failed to recover a single F_3 or F_4 individual containing only the Elgin-87-specific TC1 allele. Investigation of 14 additional F_3 progenies also did not reveal a single individual containing only the Elgin-87-specific TC1 allele. These data suggested that an unequal crossover between TC1 alleles led to the reconstitution of both Elgin- and Elgin-87-specific TC1 alleles in a single recombinant haplotype in R910. Therefore, we concluded that the recombination break point should be located in the TC1-locus (Fig. 1). This conclusion was supported by similar observations (described later) for a separate molecular marker physically linked to TC1.

Recombinant R213 was obtained from the screening of 862 F_2 plants. It was heterozygous for *Rps1*-k, CG1, and TC1 but contained only the Elgin-specific allele for 120(1 + 2). Therefore, it contained a breakpoint between CG1 and 120(1 + 2), and the genetic map distance between CG1 and 120(1 + 2) was calculated to be 0.13 cM. Progenies of R213 were segregating for resistance and susceptibility, whereas those of R910 were all susceptible. Recombinants R910 and R213 were reevaluated in the F_3 generation for the disease phenotype by inoculating leaves with *P. sojae* race 1 zoospore suspensions. Progenies of R213 segregated for resistance and susceptibility, whereas those of R910 were all susceptible. In a previous investigation, we identified a recombinant R149 containing a crossover breakpoint in the TC1–*Rps1*-k interval. This recombinant was obtained from the screening of 693 susceptible F_2 individuals for the codominant marker TC1 (Kasuga et al. 1997). Therefore, R149 and R910, showing crossover breakpoints in the TC1–*Rps1*-k interval, were obtained from 933 susceptible F_2 individuals, and the genetic map-distance between TC1 and *Rps1*-k was calculated to be 0.11 cM (Fig. 1). In the previous study of 1,770 chromosomes, we identified a single recombinant, R32, containing a crossover breakpoint in the *Rps1*-k–CG1 interval (Kasuga et al. 1997). In this investigation, no recombinants containing crossover breakpoints in this interval were identified from the screening of an additional 862 F_2 plants. The map distance between these two loci was therefore calculated to be 0.04 cM (Fig. 1a).

Isolation of BAC clones containing the *Rps1*-k region

In order to isolate the *Rps1*-k gene, we attempted to develop a contig for the *Rps1*-k region. A cosmid library carrying eight haploid genome equivalents of DNA and three BAC libraries carrying about 14 haploid genome equivalents of DNA were initially screened for developing a contig in the *Rps1*-k region (Bhattacharyya et al. 1997; Marek and Shoemaker 1997; Salimath and Bhattacharyya 1999; S.S. Salimath and M.K. Bhattacharyya, unpublished results). These libraries were constructed using HMW DNA prepared from the cultivar Williams 82. No cosmid clones containing either TC1 or CG1 were obtained. BAC33, BAC120, and BAC160 were obtained from a BAC library carrying five genome equivalents of DNA with an average insert size of ~50 kb (S.S. Salimath and M.K. Bhattacharyya, unpublished results). BAC160 and BAC33, carrying TC1 and CG1, respectively, were isolated by applying the RFLP-based screening method described earlier (Fig. 1b; Salimath and Bhattacharyya 1999). BACs IS_42L11, containing TC1, and IS_23O02, carrying one end of BAC33, were isolated from the Gm_ISb001 library containing five genome equivalents DNA (Marek and Shoemaker 1997). BAC IS_21N12 spans the dis-

tance between IS_42L11 and BAC95. BAC92 and BAC95 were isolated previously by Shalimath and Bhattacharyya (1999). Both BAC70 and BAC92 were isolated by using the TC1 probe, but based on their RFLP patterns, these two BACs were mapped to the TC1-F locus. Chromosomal walks from BAC160 and BAC IS_23O02 were unsuccessful due to repetitive sequences.

BAC clones GS_18J19, GS_43D16, and GS_99I16, abbreviated as BAC18, BAC43, and BAC99, respectively, were isolated as follows. All disease resistance genes except tomato *Pto* and barley *Rpg1* conferring race-specific resistance carry LRR sequences. Most LRR-type disease resistance genes occur in clusters, and usually, only a single type of LRR genes is predominant in a given genomic region. Therefore, we hypothesized that if *Rps1*-k were an LRR-type resistance gene, then paralogous *Rps1*-k sequences would be present in BAC160 and/or IS_23O02, closest to the *Rps1* locus. Identification of such a sequence would then allow us to identify BACs from the *Rps1*-k region located in between BAC160 and IS_23O02. We partially sequenced both BAC160 and IS_23O02 and identified an LRR sequence from BAC160. The sequence was named LRR160. DNA blot analysis of LRR160 revealed that the sequence is highly polymorphic between two NILs that differ for the *Rps1*-k alleles. Many copies of the sequence were found in the soybean genome (Fig. 2). We applied an RFLP mapping approach to identify the copy that co-segregated with the *Rps1*-k locus. $F_{3:4}$ progenies of R910 and $F_{2:3}$ progenies of R213 were used in conducting the RFLP analyses. Genetic make-ups of each of these two recombinants are shown in Fig. 3a. We reasoned that if a dimorphic copy of the sequence were present at the *Rps1*-k region, then it would segregate among progenies of R213 but not among those of R910, because R213 was heterozygous for *Rps1*-k and R910 was homozygous for *rps1*-k. Progenies of R910 and R213 were investigated for segregation of LRR160 sequences by using restriction endonucleases *Bcl*I, *Bgl*II, *Eco*RI, and *Taq*I. A single *Eco*RI 1.6-kb fragment was mapped to the TC1-120(1 + 2) interval containing the *Rps1*-k locus. The fragment was slightly bigger among progenies of R910 and Elgin (Fig. 3b). Elgin- and Elgin-87-specific alleles of the 1.6-kb *Eco*RI fragment segregated among progenies of R213. Identification of this molecular locus facilitated the identification of BAC clones containing the *Rps1*-k region.

LRR160 was used to screen a BAC library constructed in collaboration with GenomeSystems(C. Baublite and M.K. Bhattacharyya, unpublished results).

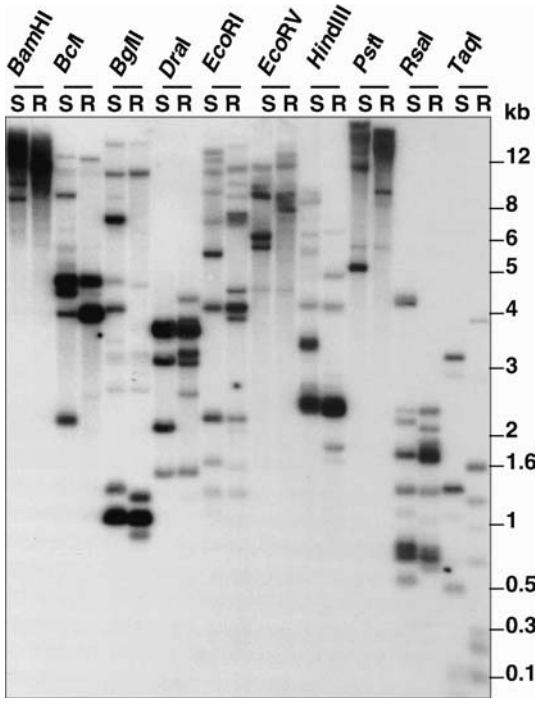

Fig. 2 LRR160 is a highly polymorphic sequence. DNA samples from NILs Elgin (*rps1*-k) and Elgin 87 (*Rps1*-k) were digested with ten restriction enzymes. *S* Elgin, *R* Elgin 87. Similar results were obtained when Williams (*rps1*-k) and Williams 82 (*Rps1*-k) were compared

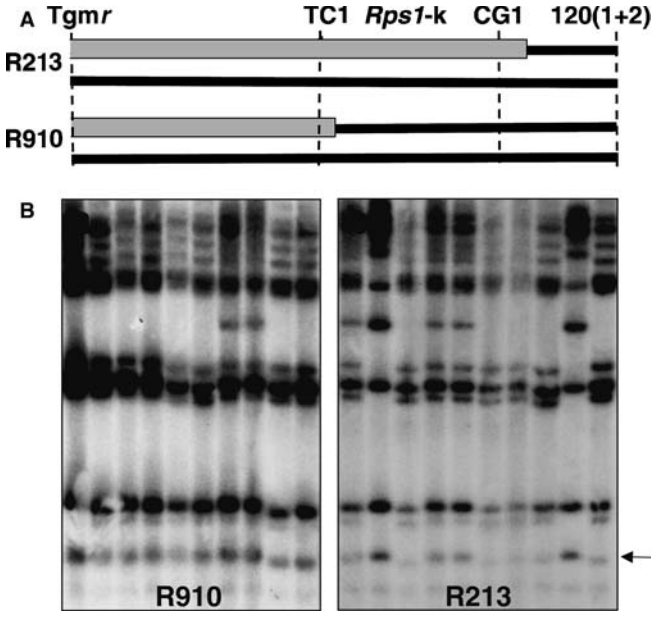

Fig. 3 Identification of the LRR160 copy that co-segregated with the *Rps1* locus. **a** Genetic make-ups of R910 and R213. *Black lines* represent DNA from Elgin (*rps1*-k) and *gray lines* DNA from Elgin 87 (*Rps1*-k). Progenies of R910 did not segregate for the genomic region containing the *Rps1*-k locus, because R910 contained the *rps1* allele in the homozygous condition. This *Rps1*-k region, in between TC1 and CG1 markers, was heterozygous in R213, and therefore, progenies of R213 segregated for DNA of the *Rps1*-k region located in between TC1 and 120(1 + 2) markers. **b** Segregation of LRR160 sequences among progenies of R910 and R213. Two *Eco*RI fragments, shown by an *arrow*, segregated among progenies of R213 but not among those of R910. The LRR160 probe hybridized more strongly to the Elgin-specific allele of the 1.6-kb *Eco*RI fragment than to the Elgin-87-specific allele. The Elgin-specific allele was slightly larger than the Elgin-87-specifc allele

The library consisted of 92,160 BAC clones representing ten soybean haploid genome equivalents of DNA. The average insert size of the BAC clones in this library was about 125 kb. Fifty-five BACs were isolated from screening of this library by using LRR160 as the probe. Of these BACs, only BAC43 and BAC99 contained the 1.6-kb *Eco*RI fragment. BAC43 and BAC99 were used for further investigation. In Southern analyses, one end of BAC43 hybridized to BAC99. The other end of BAC43 was used to isolate BAC18. BAC18 and BAC43 carry an identical end that overlaps with BAC160. BAC18 is much smaller than BAC43 and does not overlap with BAC99.

Unequal crossing over in the *Rps1*-k region

In an attempt to confirm the genetic map location of BAC18 and BAC43, recombinants R910 and R213 were used to map the BAC18-end 18R, which hybridized to three Elgin-specific and four Elgin-87-specific, polymorphic *Bcl*I fragments (Fig. 4a). Southern blot analysis revealed that Elgin- and Elgin-87-specific 18R alleles segregated normally among progenies of R213 (Fig. 4b). All three Elgin-specific or all four Elgin-87-specific 18R-*Bcl*I fragments segregated in groups among $F_{2:3}$s developed from R213. This suggested that Elgin- and Elgin-87-specific *Bcl*I fragments are clustered and most likely physically linked. Surprisingly, all $F_{3:4}$ progenies of R910 showed to contain two Elgin-specific *Bcl*I fragments in addition to all Elgin-87-specific fragments (Fig. 4a). We could not identify a single $F_{2:3}$ progeny that contained only all four Elgin-87-specific fragments. Based on the normal segregation pattern of clustered *Bcl*I fragments among progenies of R213 and the near-isogenic nature of the two parents of the cross, we concluded that: (1) R18-specific *Bcl*I fragments were mapped only to the *Rps1*-k region and (2) an aberrant segregation resulted in reconstitution of both Elgin- and Elgin-87-specific fragments into a single novel haplotype. A similar novel pattern was also observed for TC1 alleles among progenies of R910 but not R213 (Fig. 4c, d). The crossover breakpoint in R910 was in between TC1 and *Rps1*-k (Fig. 3a). If this breakpoint had originated from an equal crossover event, then 18R and TC1 alleles would have segregated normally among progenies of R910, as it did among progenies of R213 (Fig. 4). The novel rearrangement for 18R and TC1 in R910 suggested the occurrence of an unequal crossover in between *rps1*-k and *Rps1*-k haplotypes at the region

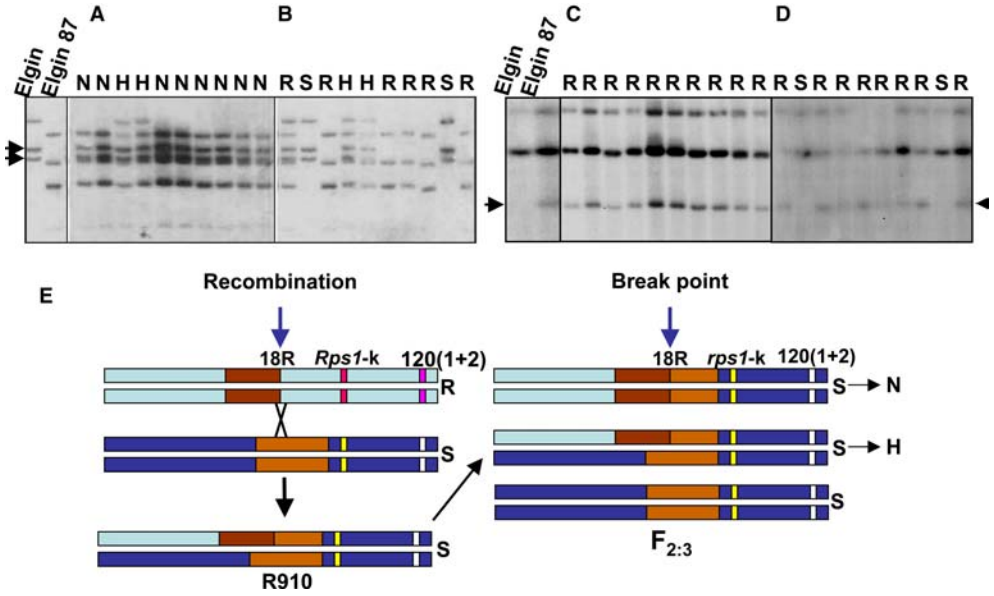

Fig. 4 Unequal crossing over in the *Rps1*-k region. The BAC18 end (18R) that overlaps with BAC160 and BAC43 was hybridized to: (**a**) DNA prepared from ten $F_{3:4}$ plants originating from the F_2 plant R910, which was susceptible to *Phytophthora sojae* race 1 (*lanes 1–10*); and (**b**) DNA isolated from ten $F_{2:3}$ progenies of R213 that segregated for resistance and susceptibility against *P. sojae* race 1 (*lanes 1–10*). The DNA samples shown in **a** and **b** were digested with *Bcl*I. *R* Resistant parent Elgin-87-specific genotype, *S* susceptible parent Elgin-specific genotype, *H* heterozygotes that contain novel and Elgin haplotypes, *N* novel genotype that carries all R-specific and two S-specific fragments (*shown by arrows*). **c** The blot shown in **a** was hybridized to TC1. *Arrowheads* show the DNA fragment that distinguished Elgin from Elgin 87. All ten $F_{3:4}$ genotypes of R910 showed the Elgin-87-specific TC1 allele, although they were all susceptible (*rps1*-k) to *P. sojae* race 1. **d** Blot of **b** was hybridized to TC1 to show the segregation of TC1 alleles (*arrowhead*) among ten $F_{2:3}$ progenies of R213. **e** Diagrammatic representation of the putative unequal crossing over event that resulted in duplication of 18R alleles in R910. *Left panel* Unequal crossing over between R (Elgin 87)-haplotype-specific dark brown allele (three *Bcl*I fragments) and part of the S (Elgin)-haplotype-specific, light-brown alleles (two of three *Bcl*I fragments) resulted in the generation of R910, progenies of which segregated as shown in the *right panel*. An *arrow* shows the breakpoint in R910 that caused the accumulation of alleles from both R and S haplotypes in the same N haplotype. *N* homozygous for the novel haplotype, *H* heterozygous for novel and Elgin haplotypes as in **a**

containing multiple copies of both TC1 and 18R (Fig. 4e).

LRR160 showed polymorphisms among NILs that differ only for *Rps1* alleles

Southern blot analysis of the NILs Elgin and Elgin 87 indicated that there are many copies of the LRR160 sequence in the soybean genome. Most copies were shown to be polymorphic between two NILs that differ for the *Rps1*-k region for all ten restriction endonucleases tested (Fig. 2). The highly polymorphic nature of this sequence between pairs of NILs differing for the *Rps1*-k region indicated that most copies, if not all, are located somewhere in the introgressed DNA fragment containing *Rps1*-k.

We investigated the organization of LRR160 among a few selected Williams lines that differ for *Rps* genes. Lines carrying all of the *Rps* genes except functional *Rps1* alleles showed the characteristic *Hin*dIII fingerprint of the cultivar Williams (*rps1*-k) (Fig. 5). Lines that differ for *Rps1* functional alleles showed polymorphisms for the sequence. Several Williams-specific DNA fragments were replaced by *Rps1*-allele-specific fragments during introgression of *Rps1* functional alleles.

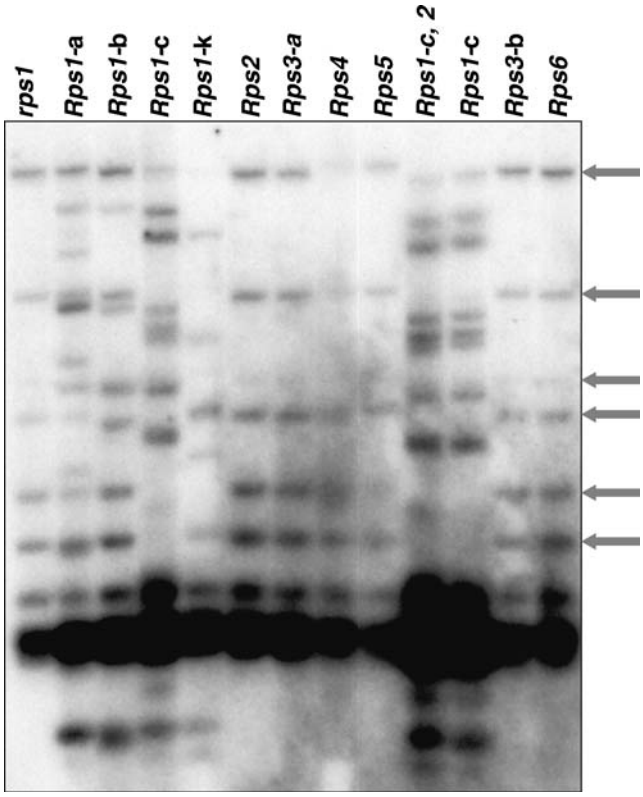

Fig. 5 Cultivar Williams isolines carrying *Rps1* alleles showed distinct polymorphisms for introgressed LRR160 sequences. Williams and near-isogenic Williams lines containing *Rps2, 3, 4, 5*, and *6* showed similar *Hin*dIII fingerprints for LRR160 (shown by *arrows*). However, distinct polymorphisms for LRR160 sequences were observed for lines containing functional *Rps1* alleles *Rps1*-a, -b, -c and -k. Introgression of *Rps1*-allele-specific fragments displaced Williams-specific fragments shown by *arrows*. *rps1* Williams, *Rps1-a* L75-6141, *Rps1-b* L77-1863, *Rps1-c* L75-3735, *Rps1-k* Williams 82, *Rps2* L76-1988, *Rps3-a* L83-570, *Rps4* L85-2352, *Rps5* L85-3059, *Rps1-c, 2* L81-4352 *Rps3-b* L88-1479, *Rps6* L89-1581

LRR160 is a member of a clustered large family of sequences

Southern blot analysis of NILs differing at the *Rps1*-k region showed that there were many copies of LRR160 in the soybean genome, the majority of which were polymorphic. This result suggested that LRR160 sequences were clustered in the introgressed region containing *Rps1*-k. We were, therefore, interested in estimating the copy number and genomic organization of paralogous LRR160 sequences. *Eco*RI and *Taq*I digestions released many polymorphic DNA fragments between the two NILs differing for the *Rps1*-k region. In contrast, the *Hin*dIII digestion produced a single, intensely hybridizing band and eight additional *Hin*dIII DNA fragments in the cultivar Williams 82 (Fig. 2). The 2.3-kb intense *Hin*dIII band represented a group of *Hin*dIII fragments that originated from a single progenitor copy through duplication. Two *Hin*dIII sites of the 2.3-kb fragment were conserved among most copies of the sequence. We carried out a Southern blot experiment in order to estimate the copy number of the sequence in the intensely hybridizing 2.3-kb *Hin*dIII fragment.

To determine the copy number of LRR160 sequences, Southern blot analysis was carried out for variable amounts of soybean genomic and the pG02 plasmid DNA (Fig. 6a). pG02 contained a single copy of the LRR160 sequence. Insert DNA in this plasmid, originating from BAC43, was 8.7 kb. The total size of pG02 was 17.8 kb. Salmon sperm DNA was digested with *Bam*HI and mixed with *Hin*dIII-digested soybean genomic or pG02 DNA to adjust the amounts of all samples to 5 μg DNA to avoid any variation in DNA blotting efficiencies. The DNA samples were run on a 0.8% agarose gel at 20 V for 24 h. The gel was blotted and hybridized to the [^{32}P]-labeled LRR160 copy isolated from the plasmid pG02. Two linear graphs, one for pG02 and the other for soybean DNA, were developed for DNA amounts and hybridization signals using the Microsoft Excel program (Fig. 6b, c). Based on the linear relationships between amounts of DNA and band intensities determined by a phosphorimager, the amounts of 8.7-kb insert DNA of the pG02 plasmid and soybean genomic DNA required for 500,000 units of band intensity were calculated to be 702 pg and 3.12 g, respectively. If the soybean genome (1150 Mb, Arumuganathan and Earle 1991) carries only a single copy of LRR160, then the amount of soybean DNA necessary for 500,000 units of band intensity is 92.84 μg [(1,150,000 kb/8.7 b) × (702 pg/1,000,000)]. However,

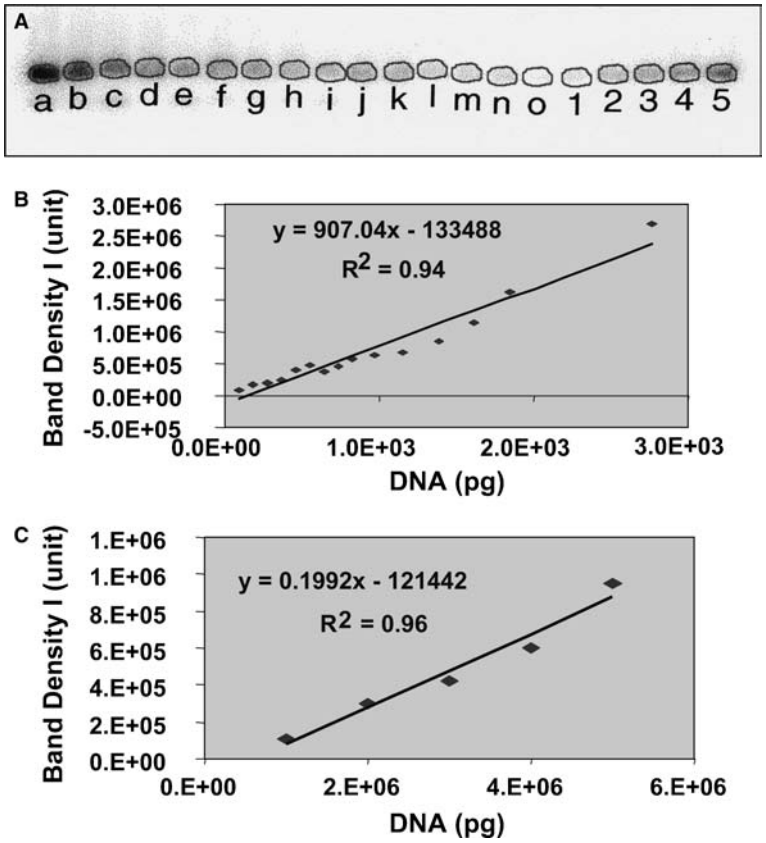

Fig. 6 LRR160 is a member of a large gene family. **a** Phosphorimage of a Southern blot carrying *Hin*dIII-digested plasmid pG02 DNA (*a* 5,700 pg, *b* 3,800 pg, *c* 3,325 pg, *d* 2,850 pg, *e* 2,375 pg, *f* 1,990 pg, *g* 1,710 pg, *h* 1,520 pg, *i* 1,330 pg, *j* 1,140 pg, *k* 950 pg, *l* 760 pg, *m* 570 pg *n* 380 pg, and *o* 190 pg) and soybean genomic DNA (*1* 1 μg, *2* 2 μg, *3* 3 μg, *4* 4 μg, and *5* 5 μg) was hybridized to the pG02-specific LRR160 sequence. **b** Linear relationship between intensity of hybridization signals and adjusted concentration of pG02 DNA content is shown. Adjusted concentrations of pG02 were obtained by subtracting the amount (in picograms) for the 9.1-kb pTF101.1 vector back-bond from each value shown in **a**. For example, adjusted values for *a*, *b*, and *o* are 2,770, 1,847, and 92 pg, respectively. **c** Linear relationship between intensity of hybridization signals and concentrations of soybean genomic DNA in picograms is shown

only 3.12 μg of genomic DNA was required to obtain the same level of intensity. Therefore, there are 30 copies of LRR160-like sequences in the 2.3-kb *Hin*dIII fragment (92.84/3.12). In addition to this strong *Hin*dIII fragment, eight additional *Hin*dIII fragments were hybridized to the LRR160 probe (Fig. 2). Therefore, there are at least 38 copies of the LRR160 sequence in the soybean genome.

In order to investigate the organization of LRR160 sequences in the *Rps1* region, Southern blot analysis was conducted using the contiguous BAC clones of the *Rps1*-k region shown in Fig. 1. No paralogous LRR160 sequences were detected in IS_23O02, BAC33, and BAC120, mapped to one side of the *Rps1*-k locus (Fig. 7). There are at least 15 unique *Eco*RI fragments in the soybean genome (Fig. 2). Of these 15 fragments, ten were mapped to the TC1-F–CG1 interval spanning about 600 kb of contiguous DNA and 0.74 cM (Fig. 1).

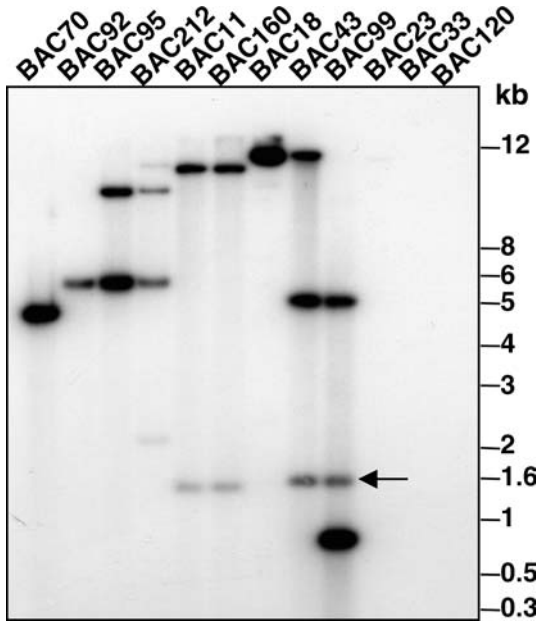

Fig. 7 Identification of a cluster of LRR160 sequences from the *Rps1*-k region. BAC clones that span the region from TC1-F to 120(1 + 2) (Fig. 1) were digested with *Eco*RI and hybridized to an LRR160 copy (pGO2). The probe did not hybridize to any of IS_23O02, BAC33, and BAC120. The 1.6 kb *Eco*RI fragment co-segregating with the *Rps1*-k locus is shown by an *arrow* (Fig. 3)

Members of the LRR160 gene family are transcribed and encode CC-NBS–LRR-type of disease resistance genes

A cDNA cloning approach was applied to investigate (1) if any members of the LRR160 gene family were transcribed, (2) the structure of the LRR160 gene family, and (3) the extent of transcription of the gene family members. The *Rps*-specific resistance is expressed in the upper one-third portion of etiolated soybean hypocotyls (Ward et al. 1981). The lower two-third portion of hypocotyls could be resistant to a virulent *P. sojae* race,

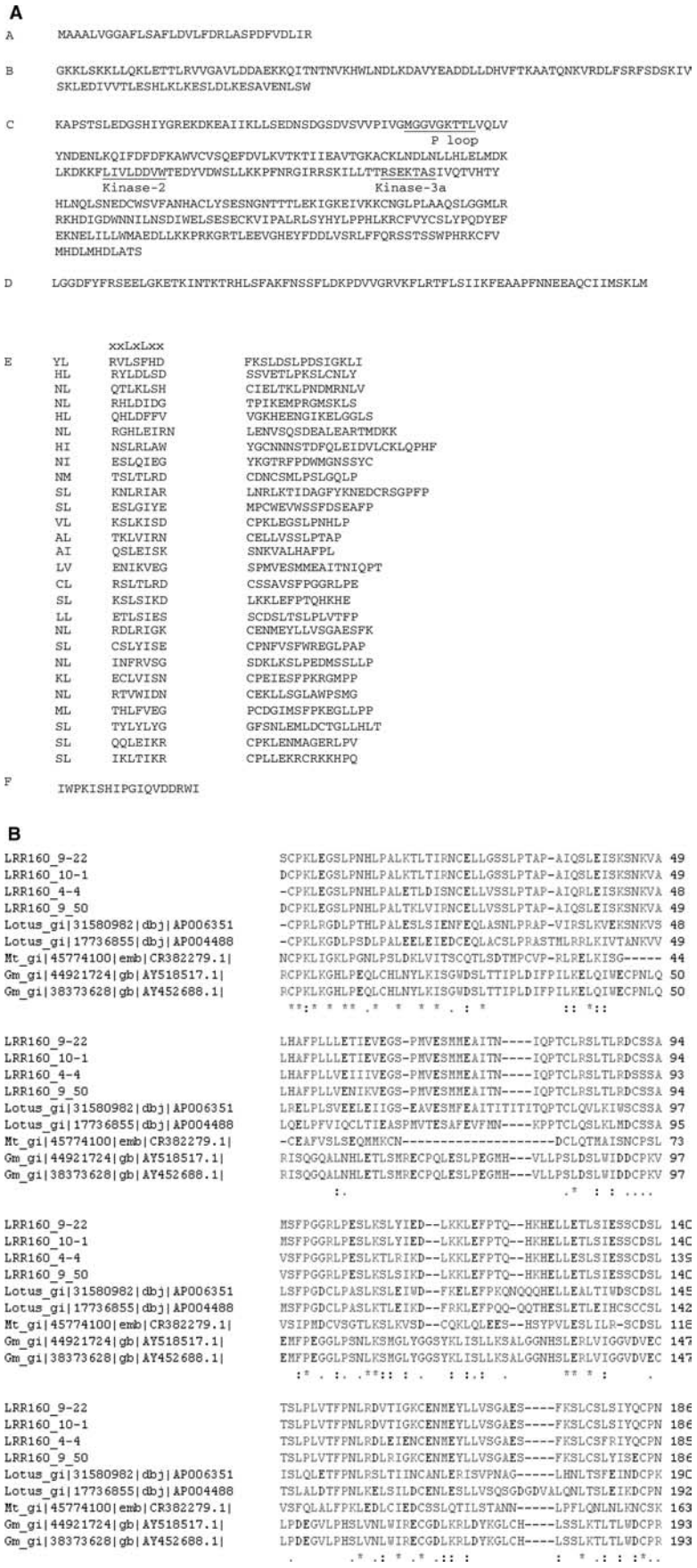

Fig. 8 A member of the LRR160 gene family encodes a coiled coil-nucleotide binding site–leucine rich repeat (CC-NBS–LRR)-type disease resistance gene. **a** Structure of the protein deduced from the cDNA LRR160_9_50. Six domains, A–F, were predicted from the deduced amino acid sequence of LRR160_9_50. *A* represents the region that does not contain any known motifs. *B* contains a CC domain. The CC structure was based on the analysis performed using the COILS algorithm (Lupas 1997). *C* shows the conserved P loop as well as kinase 2 and kinase 3a sequences in the NB domain. The LRR alignment is shown in domain *E*. The consensus sequence in LRRs is indicated by *xxLxLxx* in the *line above the alignment of LRRs*. Domain *F* is the C-terminus. **b** Comparison of deduced protein sequences of four representative LRR160 cDNAs with five most closely related LRR-type resistance gene sequences. The Clustal W program was applied to conduct the multiple alignments of the sequences (http://www.ebi.ac.uk/clustalw/index.html). *LRR160_9-22 through LRR160_9_50* are four cDNA that represent four classes of cDNA clones isolated in this investigation. *Mt Medicago truncatula*, *Gm Glycine max* (soybean cultivars)

presumably due to the expression of age-related resistance (Ward et al. 1981). Therefore, a cDNA library previously constructed from the upper one-third portion of etiolated hypocotyls of the cultivar Williams 82 was screened for possible LRR160 cDNA (Bhattacharyya 2001). A total of 4.6×10^6 pfu from the unamplified cDNA library were screened using the LRR160 sequence as the probe. Seven cDNA representing four gene members were isolated from this cDNA cloning experiment. One of the cDNA contained the whole open reading frame and encodes a CC-NBS–LRR protein (Fig. 8a). The cDNA showed 36% sequence identity to the recently cloned soybean resistance gene *Rpg1-b* that confers resistance against *Pseudomonas syringae* pv. *glycinea*, whereas it showed 56% and 38% sequence identities to the putative R gene sequences from lotus (GenBank no. AP00635) and *Medicago truncatula* (GenBank No. AC122163), respectively. A database search for a part of the LRR domain revealed that LRR160 cDNA sequences are more close to lotus and *M. truncatula* than to the cloned soybean R genes (Fig. 8b; Ashfield et al. 2004; Hayes et al. 2004). These results may suggest that the evolution of the gene cluster predates the divergence of soybean from lotus and *M. truncatula*.

Discussion

Five functional Phytophthora resistance genes, *Rps1*-a, -b, -c, -d, and -k, were genetically mapped to the *Rps1* locus in soybean (Schmitthenner 1989). Here, we have described the isolation and organization of a large CC-NBS–LRR-type disease resistance gene family from the *Rps1*-k region. This sequence is highly polymorphic among NILs that differ for the functional *Rps1* alleles.

Related members of LRR160 are organized as clustered loci

Four distinct members of the LRR160 gene family are transcribed at a low level in etiolated hypocotyls that confer race-specific Phytophthora resistance. The sequence of a cDNA showed that the LRR160 gene family is composed of CC-NBS–LRR-type disease resistance genes. Sequencing of BAC18, BAC43, and BAC99 revealed that this is the only class of disease resistance gene-like sequences found in this region (H. Gao and M.K. Bhattacharyya, unpublished results). Therefore, one member of the gene family is considered to be a candidate *Rps1*-k gene.

Southern blot analysis showed that one or two intensely hybridizing *Bcl*I, *Bgl*II, and *Hin*dIII DNA fragments represent most LRR160 copies (Fig. 2). This suggests that most copies of the gene family are highly similar and were probably descended from a single progenitor gene. For example, at least 30 copies of LRR160 sequences carry a unique 2.3-kb *Hin*dIII fragment in an *Rps1*-k line (Figs. 2, 6). We have shown that a majority of the copies are organized in a small introgressed region (Fig. 7), to which several duplicated molecular markers were also mapped (Kasuga et al. 1997). These results suggest recent expansion of the gene family along with other molecular loci in the *Rps1*-k region through a possible local duplication phenomenon.

Clustering of paralogous R-gene sequences appeared to be the rule rather than the exception. Genes within one cluster are mostly derived from a common ancestor (Richly et al. 2002). This clustering feature can facilitate the expansion of R-gene numbers and the generation of new R-gene specificities through recombination and positive selection (Michelmore and Meyers 1998). The rice *Xa21* locus contains seven family members of two distinct classes. Most members of the gene family are located within a 230-kb region (Song et al. 1997). Seven members of the *I2* family in tomato reside in a 90-kb region (Simons et al. 1998). The tomato resistance locus *Cf-2* contains two functional genes that are almost identical, with the exception of three amino acid residues (Dixon et al. 1996). Three haplotypes of the tomato *Cf* locus, *Cf-0*, *Cf-2*, and *Cf-5*, contain two, three, and four *Cf* homologues, respectively (Dixon et al. 1998). Both the *Cf-4* and the *Cf-9* loci are composed of four additional tandemly duplicated homologous genes within a 36-kb region (Parniske et al. 1997). The flax *M* locus has approximately 15 related genes, with only one conferring *M*-resistance specificity (Anderson et al. 1997). The *Rp1* rust resistance locus in maize contains nine paralogous R-gene sequences (Hulbert et al. 2001). Some R-gene families are often far apart. The *RGC2* (*Dm3*) family in lettuce spans at least 3.5 Mb. The estimated average distance between members of the *RGC2* family is at least 120 kb (Meyers et al. 1998).

Rarely, complex resistance gene loci carry distinct R-gene sequences. Three distinct CC-NBS–LRR gene families were identified within the 240-kb *Mla* region (Wei et al. 1999). Four related groups of CC-NBS–LRR genes were identified from the region containing the *Rpg1-b* gene in soybean (Ashfield et al. 2003). *Prf* required for *Pto*-mediated resistance in tomato is located within approximately 24 kb from the *Pto* gene (Salmeron et al. 1996). In addition to these results on the clustering of functional R genes, resistance gene analogues also show characteristic clustering (Kanazin et al. 1996; Leister et al. 1996; Graham et al. 2002).

Unequal crossing over for tandem duplication and creation of new race specificities

Local duplication is a characteristic of the *Rps1*-k region. Copies of several DNA markers have been mapped to multiple loci within the *Rps1*-k region (Kasuga et al. 1997). Duplication of CC-NBS–LRR sequences along with other molecular marker loci of the *Rps1*-k region

were most likely evolved through unequal crossing over, as shown for 18R in Fig. 4e. Unequal recombination could be either inter- or intra-genic. Intra-genic recombination has been shown to create new race specificity in flax (Luck et al. 2000). A similar mechanism may be responsible for creation of new race specificities in the *Rp1* and *Rp3* loci of maize. The *Rp1* locus is a good example of unequal recombination at a complex R locus (Hulbert et al. 2001). In *Arabidopsis* ecotype Columbia, the nonfunctional chimeric *rpp8* gene presumably evolved from unequal crossing over between the functional oomycete resistance gene *RPP8* and its homologue, *RPH8A* (McDowell et al. 1998). It has been proposed that *HRT*, encoding viral resistance, was evolved through unequal crossing over between progenitor genes related to *RPP8* and *RPH8A* (Cooley et al. 2000). Contrary to these observations on intra-genic recombination, sequence analyses indicated that ectopic interhomologous recombination was involved in the evolution of *Cf-9* gene clusters in tomato (Parniske and Jones 1999).

Rps1-k and *rps1*-k haplotypes are highly diverse, and therefore, any crossing over in this region will presumably be unequal as proposed for 18R and TC1 loci (Fig. 4). R910 recombinant carries *rps1*-k- and *Rps1*-k-haplotype specific alleles for both 18R and TC1 loci that are physically linked. Allelomorphs of these two loci from both haplotypes recombined in R910, through an unequal crossover event (Fig. 4e). Such an event between two diverse haplotypes at the *Rps1*-k region can easily lead to tandem duplication of LRR160 sequences. Duplicated functional *Rps1*-k genes resulting from such an event can then undergo diversifying selection for evolution of new race specificities. In this process, the frequency of mutants showing new race specificities will be much fewer than the number of loss of function mutants. The availability of many such duplicated copies of a functional R gene facilitates the generation of new genetic variation without compromising the resistance specificities against the prevalent pathogenic races.

Frequent unequal crossing over, however, tends to homogenize duplicated sequences that have undergone mutation and diversification. This slows down the process of evolution for new race specificities among duplicated genes (Hulbert et al. 2001). The sequence homogenization or concerted evolution is a major mechanism for the evolution of tandemly arranged repeat sequence families, such as rDNA in yeast (Gangloff et al. 1996). In R-gene clusters, concerted selection resulting from frequent unequal crossing over reduces the scope of diversifying selection necessary for the creation of new race specificities. Therefore, unequal crossing over has to be infrequent. Paralogous *Dm3* sequences in lettuce and *Pto* sequences in tomato are more diverse than their corresponding orthologous sequences, indicating the rareness of unequal crossing over in these two R-gene clusters (Michelmore and Meyers 1998). The presence of highly polymorphic intergenic regions has been thought to play a role in the suppression of unequal crossing over in R-gene clusters (Parniske et al. 1997). It is possible that the usual location of R-gene clusters in a recombination-suppressed region allows the newly duplicated paralogues to diversify freely for the generation of new race specificities.

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