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Rpg1, a soybean gene effective against races of bacterial blight, maps to a cluster of previously identified disease resistance genes

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Abstract Alleles, or tightly linked genes, at the soybean (*Glycine max* L. Merr.) *Rpg1* locus confer resistance to races of *Pseudomonas syringae* pv. *glycinea* that express the avirulence genes *avrB* or *avrRpm1*. In this study we demonstrate that *Rpg1* maps to a cluster of previously identified resistance genes, including those effective against fungal, viral and nematode pathogens. *Rpg1* is in molecular linkage group (MLG) F, flanked by the markers K644 and B212. The RFLP markers R45, php2265 and php2385 cosegregated with *Rpg1*, as did the marker nbs61, which encodes a protein related to previously isolated resistance genes.

Key words Nucleotide binding site · Leucine-rich repeats · RFLP mapping · Recombinant-inbred lines

Introduction

Plant resistance to pathogens is often governed by the interaction of corresponding genes in the plant and pathogen; a specific resistance gene (R-gene) in the plant is matched by a specific avirulence gene (avr-gene) in the pathogen. Only if both genes are present is the pathogen rapidly detected and contained. The genetics of this type of interaction, often referred to as gene-for-gene resistance, were first described by Flor (1955) to account for the varietal resistance of flax to flax rust (*Melampsora lini*). Gene-for-gene resistance has since been described for interactions between plants and nematode, fungal, bacterial and viral pathogens (reviewed by Crute and Pink 1996).

R-genes in distantly related plant species occasionally share the same avirulence gene specificity. An example of two such functionally analogous R-genes are *RPM1* and *Rpg1* from Arabidopsis and soybean, respectively. Both respond to *Pseudomonas syringae* strains expressing *avrB*, an avirulence gene isolated from *P. syringae* pv. *glycinea* (Innes et al. 1993; Bisgrove et al. 1994; Mukherjee et al. 1966; Staskawicz et al. 1987; Keen and Buzzell 1991). The Arabidopsis *RPM1* gene is unusual in that it displays a dual specificity, also responding to *avrRpm1* (Bisgrove et al. 1994), an avirulence gene isolated from *P. syringae* pv. *maculicola* (Debener et al. 1991). It is not known whether functionally analogous R-genes result from the conservation of an ancestral gene through speciation or whether convergent evolution is responsible. The cloning and comparison of *RPM1* and *Rpg1* may help address this question.

Since 1993, a number of R-genes have been cloned from various plant species. Several of these R-genes, including *RPM1*, belong to a family of genes that contain a putative nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) (Grant et al. 1995 and reviewed by Bent 1996; Briggs 1995; Staskawicz et al. 1995). The NBS consists of a conserved P-loop motif (Saraste et al. 1990) and distal kinase 2 and kinase 3 domains (Traut 1994). LRRs are typically associated with protein-protein interactions (Kobe and Deisenhofer

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1994), and it has been suggested that R-genes of this type are receptors and that the LRRs interact directly with avirulence gene-derived ligands (discussed by Bent 1996). This remains to be demonstrated. The NBS-LRR containing family of R-genes includes those effective against bacterial, fungal or viral pathogens (reviewed by Bent 1996; Briggs 1995; Staskawicz et al. 1995).

To date, no R-genes have been cloned from soybean. However, primers corresponding to conserved regions of NBS-LRR-type R-genes from tobacco, Arabidopsis and flax have been used to polymerase chain reaction (PCR)-amplify related sequences from soybean (Kanazin et al. 1996; Yu et al. 1996). Several of the clones isolated mapped to the vicinity of known soybean R-genes. These data suggest that at least some soybean R-genes will fall into the NBS-LRR class.

Varietal resistance to bacterial blight (causative agent, *P. syringae* pv. *glycinea*, *Psg*) in soybean was noted as early as 1919 (Coerper 1919). A single, dominant gene conferring resistance to race 1 *Psg* was identified by Mukherjee et al. (1966) and named *Rpg1*. Subsequently, it was shown that the avirulence gene interacting with *Rpg1* was *avrB* (Staskawicz et al. 1987; Keen and Buzzell 1991). More recently, an allele, or tightly linked gene at the *Rpg1* locus, specific for *avrRpm1* was identified (Ashfield et al. 1995). For the sake of simplicity, the R-genes specific for *avrB* and *avrRpm1* will be provisionally referred to as alleles, designated *Rpg1-b* and *Rpg1-r*, respectively.

In the study presented here we report the genetic mapping of the *Rpg1* locus to a cluster of R-genes in soybean molecular-linkage group (MLG) F (Shoemaker and Olson 1993). We further demonstrate that a previously identified soybean clone displaying motifs characteristic of *RPM1* and other related R-genes cosegregates with *Rpg1-b* (0 ± 0.86 cM).

Materials and methods

Plant lines and growth

Seeds for the ‘PI 437654’ × ‘BSR 101’ recombinant inbred lines (RILs) were provided by Iowa State University, Ames, IA. The production of this population has been described previously (Baltazar and Mansur 1992; Keim et al. 1994). The placement of restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) markers in this population has been described by Webb et al. (1995) and Keim et al. (1997). The ‘Flambeau’ × ‘Merit’ RIL population was generated by crossing the parental cultivars followed by inbreeding to the F_8 generation by single-seed descent (Ashfield et al. 1995). The *Rps3-a* ‘Harosoy’ isoline and the two parental lines (‘PI 171442’ and ‘Harosoy’) were provided by R. Buzzell (Agriculture & Agri-food Canada, Harrow, Canada). The *Rps3-a* ‘Williams’ isoline L83-570 and the two parental lines (‘PI 86972-1’ and ‘Williams’) were obtained from the USDA soybean germplasm collection (Agricultural Research Service, Urbana, Ill.).

The soybean growth conditions used have been described previously (Ashfield et al. 1995).

Bacterial strains

*Psg*R4 (*avrB*) and *Psg*R4 (*avrRpm1*) have been described previously (Ashfield et al. 1995). Both strains are resistant to rifamycin and kanamycin. Strain *Psg*R4 (DSK600/*avrB*) was kindly provided by N. Keen (University of California, Riverside). This strain contains a 1.3-kb *avrB* fragment cloned in the vector pDSK600 at the *Kpn*I and *Xba*I sites. *Psg*R4 (DSK600/*avrB*) is resistant to streptomycin and spectinomycin.

Growth of bacteria and inoculum preparation

Bacterial lawns were grown on King’s medium B (King et al. 1954) supplemented with the appropriate antibiotics (from Sigma) at 30°C over-night. Rifamycin was included at 100 μg/mg, kanamycin at 50 μg/ml, streptomycin at 40 μg/ml and spectinomycin at 50 μg/ml. Bacterial suspensions were prepared from the lawns in 10 m*M* $MgCl_2$ and diluted to approximately 1×10^8 colony forming units (cfu)/ml (an OD_{600} of 0.1) for HR tests and approximately 5×10^8 cfu/ml (an OD_{600} of 0.5) for dip-inoculations (see below). The suspensions were used within 4 h of preparation.

Inoculation of plants with *P. syringae*

To test for a hypersensitive resistance (HR) response, we injected unifoliate leaves of 14-to 21-day-old soybean plants with bacterial suspensions as described by Ashfield et al. (1995). Strains *Psg*R4 (*avrB*) and *Psg*R4 (*avrRpm1*) were used to determine the responsiveness of soybean lines to *avrB* and *avrRpm1*, respectively.

To test for disease symptoms (susceptibility), we used a dip-inoculation assay (modified from protocols described by Salmeron et al. 1994 and Whalen et al. 1991). Plants were inoculated when the first set of trifoliate leaves were visible but only partially expanded (0.5–3 cm in length); typically 2–3 weeks after planting. *Psg*R4 (DSK600/*avrB*) bacterial inoculum was prepared as described above, diluted to an OD_{600} of 0.5 in 10 m*M* $MgCl_2$, and the surfactant Silwet L-77 (OSi Specialties) added to a final concentration of 0.025% (v/v). The plants were inverted and the foliage briefly submerged in the inoculation suspension before being transferred to a humidity-controlled growth chamber (16 h day, 22°C, 250 microeinsteins $m^{-2} s^{-1}$, 80% relative humidity). The symptoms were scored after 7 days as described in the Results section.

Plant DNA preparation

DNA was isolated from soybean tissue using a procedure modified from Doyle and Doyle (1990). Approximately 1.5 g frozen leaf tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen, and 0.15 g solid polyvinylpyrollidine (PVP-40) added. The material was resuspended in 15 ml pre-heated buffer [3% hexadecyltrimethylammonium bromide (CTAB), 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* TRIS-HCl pH 8, 0.2% mercaptoethanol] and incubated at 60°C for 1 h with occasional mixing. The DNA was purified by extracting once with an equal volume of chloroform : isoamyl alcohol (24 : 1; v : v) followed by precipitation with a two-thirds volume of isopropanol (2 h - overnight at −20°C). The DNA was pelleted, washed with 70% ethanol and resuspended in 1 ml TE (pH 7.5). RNA was removed by adding RNaseA to a final concentration of 10 μg/ml followed by incubation at 37°C for 1 h.

RFLP markers

The publicly available markers pA186, pB212, pK644 (Keim et al. 1990; Shoemaker and Specht 1995) and pR45 (Lark et al. 1993) were

kindly provided by R. Shoemaker (Iowa State University, Ames, Iowa). Markers php2265 and php2385 are Pioneer Hi-Bred International propriety markers. The publicly available markers were generated from a *Pst*I genomic soybean library (Keim and Shoemaker 1988).

RFLP analysis

Plant DNA was digested over-night with restriction enzymes (New England Biolabs) in a volume of 400 μl according to the manufacturer's instructions except that spermidine (Sigma) was included at a final concentration of 4 m*M*. The digested DNA was recovered by ethanol precipitation and separated through 0.9% agarose (0.8 V/cm), approximately 10 μg DNA per lane. Capillary transfer to Hybond-N membranes (Amersham) was conducted essentially as described by the manufacturer using 20 × SSC as the transfer buffer. [^{32}P](ICN)-labeled probes were generated by the random-primer method (Feinberg and Vogelstein 1983). Hybridizations (over-night) were in 1% BSA, 1 m*M* EDTA, 0.5 M Na_2HPO_4 pH 7.2, 7% SDS at 60°C. Membranes were washed twice with 1 m*M* EDTA, 40 m*M* Na_2HPO_4 pH 7.2, 5% SDS and twice with 1 m*M* EDTA, 40 m*M* Na_2HPO_4 pH 7.2, 1% SDS. All washes were at 55°C. Membranes were exposed over-night to Phosphor Screens (Molecular Dynamics) or to *RX* X-ray film (Fuji) for 1–7 days.

Microsatellite marker analysis

HSP176 primers were obtained from Research Genetics Inc., the Sct33 primers were kindly provided by K. G. Lark (University of Utah).

Markers were amplified by PCR in a 25-μl volume containing approximately 20 ng soybean DNA, 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8), 3 m*M* $MgCl_2$, 0.15 μM forward and reverse primers, 0.2 m*M* each dNTP, 0.05% (v/v) NP-40 and 0.15 units *Taq* polymerase. After a 3-min denaturation at 94°C, the reactions were cycled 32 times using the profile − 94°C for 25 s, 45°C for 25 s and 72°C for 25 s. After the last cycle the reactions were held at 72°C for 3 minutes to allow the completion of part-length products.

HSP176 PCR products were separated through 3% MetaPhor agarose (FMC) (10 cm long, 1 × TAE running buffer, 4–6 V/cm, room-temperature). Sct33 PCR products were resolved either through 3.5% MetaPhor agarose or 6% acrylamide. Separation through MetaPhor agarose was essentially as described by the manufacturer for optimum resolution (gels 20 cm long, 0.5 × TAE running buffer). Gels were run with pre-cooled buffer, in a 4°C room and the voltage was adjusted to between 10 and 14 V per centimeter so as to maintain the buffer temperature below 25°C.

Samples for loading on acrylamide gels were prepared by mixing the reaction products and a 0.5 volume of formamide loading buffer [89% (v/v) formamide, 10% (w/v) ficoll, 20 m*M* EDTA pH 8, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol], followed by a 10-min incubation at 95°C. Acrylamide gels (6% acrylamide, 5.6 *M* urea, 30% ultrapure-formamide, 1 × TAE), 1.5 mm thick/15 cm in length, were run at 30–50 W. After running, gels were immersed for 30 min in SYBR Green II stain (Molecular Probes) diluted in 1 × TBE buffer and the DNA visualized by exposure to UV light.

Linkage analysis

The initial placement of *Rpg1-b* to MLG-F was accomplished using the computer program MAPMAKER (Lander et al. 1987). Subsequently, genetic distances were calculated manually. Map distances in the recombinant-inbred populations were determined as described by Reiter et al. (1992) and Koornneef and Stam (1992). The frequency of recombinant gametes in a single meiosis (p) was calculated using the Haldane and Waddington equation; $p = R/(2\text{-}2R)$, where R is the proportion of recombinant lines. p was converted to a map distance using the Kosambi function; $D = 25\ln[(100 + 2r)/(100 - 2r)]$ where D is the map-distance (in cM) and r is the percentage of recombinant gametes in a single meiosis.

The standard error of p (s_p) was calculated using the equation; s_p = the square root of $p(1 - p)/n$, where n is the number of RI lines examined (Allard 1956). When the observed p was 0, the s_p presented is the value that would have been observed if a single recombinant had been detected.

Results

An efficient screen to score for *Rpg1*-mediated disease resistance

To facilitate the mapping of the soybean *Rpg1* locus we developed a dip-inoculation assay which permitted the efficient, and rapid, scoring of resistance to *P. syringae* expressing *avrB*. In this assay, infection is established by briefly submerging the aerial parts of the test plants in a suspension of *P. syringae* cells (expressing the appropriate avirulence gene), together with a low concentration of anionic surfactant (see Methods).

After dip-inoculation with a *P. syringae* strain expressing the avirulence gene *avrB*, the leaves of soybean cultivars lacking a functional *Rpg1-b* gene develop water-soaked pits surrounded by chlorotic halos (Fig. 1B, D), the symptoms first becoming visible 3–4 days after inoculation. In heavily infected leaves these lesions coalesce, especially around the margins of the leaf, to form areas of confluent watersoaking (data not shown). Only leaves partially expanded at the time of inoculation subsequently develop symptoms; fully expanded leaves remain macroscopically healthy. Cultivars expressing *Rpg1-b* either show no visible response or develop small brown flecks with no accompanying chlorosis (Fig. 1A, C).

Rpg1 maps to a cluster of previously identified disease resistance genes

Rpg1-b was initially mapped in a large RIL population derived from a cross between the *G. max* lines 'PI 437654' and 'BSR 101'. A large number of RFLP and AFLP markers have previously been scored in this population (Keim et al. 1997; Webb et al. 1995). Scoring of the parental lines for responsiveness to *avrB* and *avrRpm1* revealed that *Rpg1-b* is segregating in this population; 'BSR 101' and 'PI 437654' having the genotypes *Rpg1-b* and *rpg*1, respectively (Fig. 1C, D). The dip-inoculation procedure described above was used to score 157 of the RILs for *Rpg1-b* function. As expected for a single dominant gene, resistance/susceptibility segregated 1 : 1 ($\chi^2 = 0.16$, $P > 0.5$). Six of the scored lines were still segregating for *Rpg1-b*. The computer program MAPMAKER was used to detect linkage

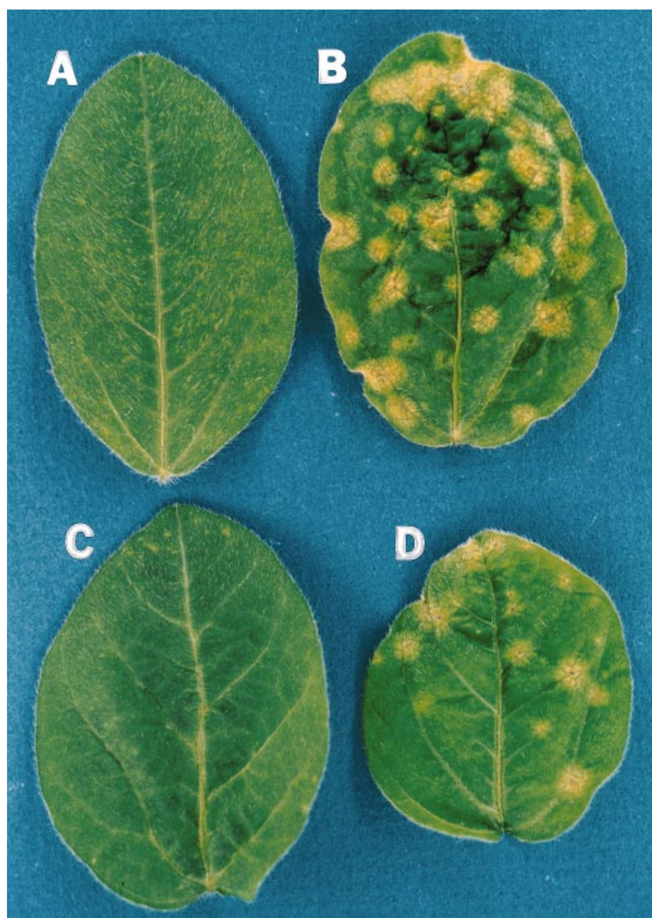

Fig. 1A–D Disease symptoms on leaves from resistant ('Merit' and 'BSR 101') and susceptible ('Flambeau' and 'PI 437654') soybean cultivars dip-inoculated with *Pseudomonas syringae* pv. *glycinea* race 4 expressing *avrB*. **A** cv. 'Merit' (*Rpg1-b*), **B** cv. 'Flambeau' (*Rpg1-r*), **C** cv. 'BSR 101' (*Rpg1-b*) and **D** line 'PI 437654' (*rpg*1). Leaves from the indicated cultivars were photographed 7 days after dip-inoculation with 5×10^8 cfu/ml *Psg* R4 (DSK600/*avrB*). *cfu* Colony forming units

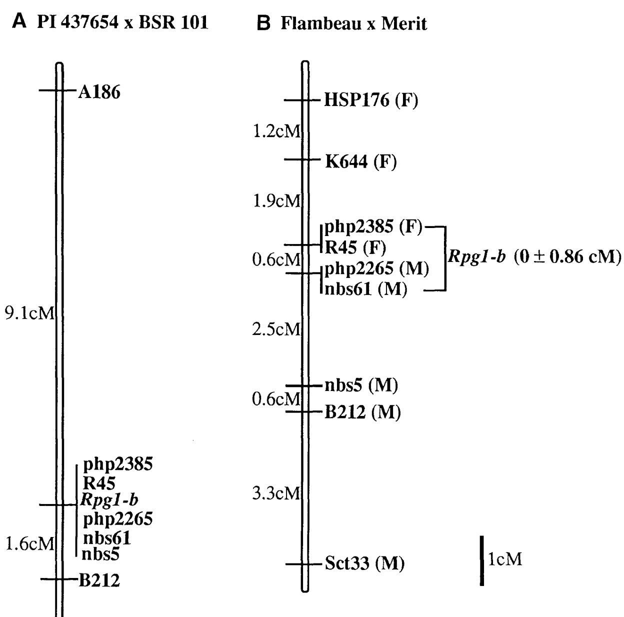

Fig. 2 Deduced order of molecular markers surrounding *Rpg1-b* in soybean molecular linkage group F (as defined by Shoemaker and Olson 1993). Genetic distances between the indicated markers were determined in two mapping populations: **A** 'PI 437654' × 'BSR 101' and **B** 'Flambeau' × 'Merit'. Genotypes of RFLP markers in line RI-61 are shown in *parentheses* (*M* 'Merit'-like, *F* 'Flambeau'-like). Number of lines scored varies for different markers (see Table 1 for details)

between *Rpg1-b* and the previously scored molecular markers. This analysis placed *Rpg1-b* in molecular linkage group F (MLG-F; as defined by Shoemaker and Olson 1993) flanked by the RFLP markers A186 and B212, and cosegregating with the markers R45, php2265 and php2385 (Fig. 2A, Table 1A). The HR hand-inoculation assay was used to confirm the *Rpg1-b* genotypes of RILs containing recombination events in the A186 - B212 interval (data not shown).

Additional markers were ordered relative to *Rpg1-b* in a second RIL population derived from a cross between *G. max* cultivars 'Flambeau' and 'Merit'. 'Merit' expresses *Rpg1-b*, 'Flambeau' expresses *Rpg1-r* (Ashfield et al. 1995; Fig. 1A, B). Two microsatellite markers, HSP176 and Sct33, and the RFLP marker K644 were scored. HSP176 and K644 mapped to one side of *Rpg1-b* (3.1 cM and 1.9 cM, respectively) and Sct33 to the other (6.4 cM) (Fig. 2B, Table 1B). In this population B212 mapped 3.1 cM from *Rpg1-b* (Fig. 2B, Table 1B).

Table 1 Linkage data for *Rpg1-b* and MLG-F molecular markers

Interval	r[a]	*n*[b]	centiMorgans[c]
A) PI 437654 × BSR 101 population			
A186 to *Rpg1-b*	23	151	9.1
Rpg1-b to php2385	0	154	–
Rpg1-b to R45	0	66	–
Rpg1-b to nbs5	0	56	–
Rpg1-b to nbs61	0	43	–
Rpg1-b to php2265	0	154	–
php2385 to php2265	0	330	–
Rpg1-b to B212	2	64	1.6
B) Flambeau × Merit population[d]			
HSP176 to K644	2	86	1.2
K644 to R45/php2385	3	84	1.9
R45/php2385 to *Rpg1-b*	0	83	–
Rpg1-b to php2265/nbs61	0	83	–
R45/php2385 to php2265/nbs61	1	84	0.6
php2265/nbs61 to nbs5	4	84	2.5
nbs5 to B212	1	84	0.6
B212 to Sct33	4	65	3.3

[a] Number of RIL families recombinant for indicated markers
[b] Total number of RIL families scored for both markers
[c] Deduced map distance separating markers (in cM)
[d] 'Flambeau' × 'Merit' population; markers php2385, php2265, R45, nbs5 and nbs61 were only scored in the 9 families preselected (from 84 lines) for recombination events between the *Rpg1-b* flanking markers K644 and B212

Nine lines with recombination breakpoints between the *Rpg1-b* flanking markers K644 and B212 were identified in the 84 lines scored from the 'Flambeau' × 'Merit' RIL population (Table 1B). These

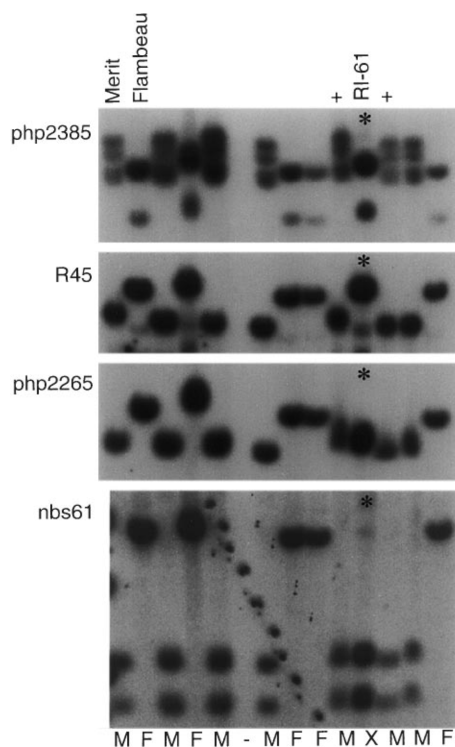

Fig. 3 Separation of *Rpg1-b*-cosegregating RFLP markers in line RI-61. Nine lines preselected for recombination events between the *Rpg1* flanking markers K644 and B212, plus 2 additional lines with recombination events between HSP176 and K644 (all from the 'Flambeau' × 'Merit' RIL population) were scored with the indicated RFLP markers. The *Rpg1-b* genotypes of the RILs are indicated at the *bottom* (*M* 'Merit'-like, *F* 'Flambeau'-like, *X* non-parental). The first 2 lanes contain DNA from the parental lines, 'Merit' and 'Flambeau', respectively. The *asterisk* indicates the lanes containing DNA from line RI-61, + symbols indicate lines with recombination breakpoints between the markers HSP176 and K644. DNA was isolated from the indicated soybean lines, digested with the appropriate restriction enzyme, separated through agarose, transferred to nylon membranes and hybridized with the indicated probes

informative recombinant lines were subsequently scored with the RFLP markers R45, php2265 and php2385. In 8 of the 9 informative recombinant lines, these markers cosegregated both with each other, and with *Rpg1-b* (Figs. 2B and 3, Table 1B). In the ninth line (RI-61) R45 and php2385 were separated from php2265 by recombination (Fig. 3). The deduced marker order is shown in Fig. 2B (marker genotypes in line RI-61 are shown in parentheses). It was impossible to order php2385, R45 and php2265 relative to *Rpg1-b* with certainty as line RI-61 had inherited neither parental resistance specificity (i.e. has the genotype *rpg*1) (Ashfield et al. 1995). However, the data is consistent with the double-susceptible phenotype of line RI-61 resulting from intragenic recombination or unequal exchange at the *Rpg1* locus. We therefore tentatively place markers php2385/R45 and php2265 on opposite sides of *Rpg1-b*.

These analyses clearly place *Rpg1-b* in MLG-F in the interval between the RFLP markers K644 and B212. The inclusion of microsatellite markers in the analysis precludes any possible confusion with homeologous linkage groups. Significantly, at least 5 other R-genes with specificities for a range of nematode, fungal and viral pathogens have previously been mapped to this region of the soybean genome (see Discussion).

Rpg1 and *Rps3* are linked but not allelic

As an initial step towards characterizing linkage between *Rpg1* and other R-genes in the A186/B212-linked cluster we focused on *Rps3*. Alleles at the *Rps3* locus confer resistance to races of *Phytophthora sojae* (the causative agent of root and stem rot of soybean) and have previously been shown to map between RFLP markers A186 and R45 (Diers et al. 1992). To obtain direct evidence for the predicted linkage between *Rpg1* and *Rps3* we determined whether both loci are present in the introgressed segments of near-isogenic lines (NILs) in which *Rps3* has been introgressed into established cultivars. Two independently generated NILs were used; L83-570 and HARO 3272 (*Rps3-a* introgressed into cvs 'Williams' and 'Harosoy', respectively). The cv 'Williams' NIL L83-570 still carries the *Rpg1* allele characteristic of the donor parent, despite the six rounds of backcrossing (Table 2), a result consistent with tight linkage between *Rpg1* and *Rps3*. Conversely, the HARO 3272 NIL carries the *Rpg1* allele characteristic of the recurrent parent, 'Harosoy', demonstrating that *Rpg1* and *Rps3* can be separated by recombination.

These data are consistent with *Rpg1* and *Rps3* being two closely linked genes. Analysis of segregating populations will be necessary to quantify this linkage.

Table 2 Analysis of *Rpg1* and *Rps3* linkage in near-isogenic lines

Line	Parentage	*Rps3-a*	*Rpg1-b*	*Rpg1-r*
PI 86972-1[a,b]	–	+	+	+
Wm	–	–	+	–
L83-570	Wm(6) × PI86972-1	+	+	+
PI171442[c]	–	+	–	–
Harosoy	–	–	+	–
HARO 3272	Harosoy (6) × PI 171442	+	+	–

The presence/absence of *Rpg1* alleles was determined by flooding small areas of the leaf with *P. syringae* py. *glycinea* race 4 expressing *avrB* or *avrRpm1* and scoring for the development of a hypersensitive response. Resistance reactions were scored after 24 h. + = presence of allele; – = absence of allele. Wm = cv 'Williams'. Numbers in parentheses represent number of backcrosses to recurrent parent

[a] Muller et al. (1978)

[b] Line 'PI 86972-1' expresses both the *Rpg1-b* and *Rpg1-r* specificities. It is not known whether the line contains a distict *Rpg1* allele with both specificities, or whether *Rpg1-b* and *Rpg1-r* have been recombined onto a single chromosome in this line.

[c] Kilen and Keeling (1981)

Clones encoding putative NBS-LRR-type R-genes map to the vicinity of *Rpg1*

The clones nbs5 and nbs61 have previously been shown to contain sequence motifs characteristic of known NBS-LRR-type R-genes and have been mapped to the vicinity of the A186/B212-linked cluster of resistance specificities (Yu et al. 1996). To determine if either clone could represent *Rpg1* we mapped these clones relative to *Rpg1-b* in both the 'PI 437654' × 'BSR 101' and 'Flambeau' × 'Merit' RIL populations. On genomic blots of soybean DNA, clone nbs5 hybridized to only a single fragment, while nbs61 hybridized strongly to a single fragment and weakly to two others (data not shown). Only the strongly hybridizing fragments were mapped relative to *Rpg1-b*.

Analysis in the 'PI 437654' × 'BSR 101' population demonstrated close linkage between the nbs clones and *Rpg1-b* with both nbs5 and nbs61 cosegregating with the resistance gene (in 56 and 43 lines, respectively; Table 1A).

The genetic resolution was further increased by characterizing 84 of the 'Flambeau' × 'Merit' RILs. This analysis was accomplished by scoring the 9 lines known to contain recombination break-points between the *Rpg1*-flanking markers K644 and B212. Probe nbs61 cosegregated with *Rpg1-b* in all 8 lines that had inherited a parental *Rpg1* allele (Fig. 3). As noted earlier, the ninth line (RI-61) had inherited neither parental *Rpg1* allele but contains the 'Merit' allele of nbs61. Probe nbs5 mapped 2.5 cM from *Rpg1-b* in the interval between *Rpg1-b* and B212 (Fig. 2B, Table 1B).

These data confirm the proximity of the two putative R-gene clones, nbs5 and nbs61, to the A186/B212-linked cluster. The failure to separate nbs61 from *Rpg1-b* in the 84 lines scored places this clone 0 ± 0.86 cM from the R-gene.

Discussion

In this study we show that the *Rpg1* locus is located in a cluster of previously mapped R-genes. Furthermore, we demonstrate that a previously isolated clone (nbs61), which shows striking similarity to cloned NBS-LRR-type R-genes (Yu et al. 1996), cosegregates with *Rpg1-b* (0 ± 0.86 cM).

Mapping *Rpg1* relative to molecular markers has resulted in its placement in molecular linkage group F (MLG-F) flanked by the RFLP markers K644 and B212. Three other major R-genes have previously been mapped to this region of the soybean genome; *Rps3* (Diers et al. 1992), *Rsv1* (Yu et al. 1994) and *Rpv1* (Roane et al. 1983). *Rps3* is effective against the fungal pathogen *Phytophthora soja*, while *Rsv1* and *Rpv1* act against strains of the viral pathogens soybean mosaic virus (SMV) and peanut mottle virus, respectively. Also present in this cluster of R-genes are two quantitative trait loci (QTLs) conferring resistance to different species of root-knot nematode (Tamulonis et al. 1997a, b). All these resistance specificities are tightly linked to RFLP markers A186 and B212 or to each other. Only *Rsv1* and *Rpv1* have been scored together in a single population and subsequently found to be separated by 3.7 cM (Roane et al. 1983). Similar analysis with the other resistance specificities will be necessary to determine more accurately the linkage relationships within the cluster.

The diversity of specificities in the A186/B212-linked cluster is further increased by the existence of allelic series at several of the R-gene loci. We have previously demonstrated the existence of alleles, or closely linked genes, at the *Rpg1* locus; one specific for *avrB*, the other for *avrRpm1* (Ashfield et al. 1995). Allelic series are known at other R-gene loci in the cluster. For example, three alleles of the *Rps3* locus have been identified; *Rps3-a* (Kilen and Keeling 1981; Laviolette et al. 1979; Mueller et al. 1978), *Rps3-b* (Ploper and Athow 1985) and *Rps3-c* (Athow et al. 1986). Each allele is effective against different races of *Phytophthora sojae*. Similarly, multiple alleles at the *Rsv1* locus are known (Chen et al. 1991; Chen et al. 1994; Ma et al. 1995). Alleles differ both in respect to the range of SMV strains to which they respond and in the strength of response they condition to particular strains. It should be noted that the soybean populations used to define the proposed alleles at the *Rpg1*, *Rps3* and *Rsv1* loci were not large enough to exclude the possibility of tightly linked genes.

The clustering of R-genes to distinct races of certain fungal pathogens is well-documented (reviewed by Pryor and Ellis 1993). For example, 14 resistance specificities to different races of the rust pathogen *Puccinia sorghi* map to the *Rp1 complex* in maize (Saxena and Hooker 1968). Molecular and genetic analysis indicate that at least some of these specificities are encoded by linked genes (as opposed to alleles) (Hulbert and Bennetzen 1991). Similarly, the flax *M* locus consists of several tightly linked genes conferring resistance to races of the rust *Melampsora lini* (Mayo and Shepherd 1980).

The clustering of R-genes effective against unrelated pathogens has also been described. For example, in lettuce the *Dm5/8* and *Dm10* loci (confer resistance to the downy mildew pathogen *Bremia lactucae*) are closely linked to *Tu* and *plr*, all present within a 6.4-cM genetic interval (Witsenboer et al. 1995). *Tu* and *plr* confer resistance to turnip mosaic virus and *Plasmopara lactucae-radicis* (root-infecting downy mildew), respectively. Similarly, the tomato *Cf-2*/*Cf-5* R-genes (to the leaf mold *Cladosporium fulvum*) are closely linked to *Mi*, a gene effective against the root-knot nematode *Meloidogyne* spp. (Dickinson et al. 1993). Soybean also contains a second such cluster – *Rps2*, *Rmd* and *Rj2*, all lying within a 3.8-cM region in MLG-J (Polzin et al. 1994). *Rps2* confers resistance to certain

races of *Phytophthora sojae* (causal agent of root and stem rot), *Rmd* confers resistance to *Microsphaera diffusa* (causal agent of powdery mildew), while *Rj2* modulates *Bradyrhizobia japonicum*-mediated nodulation.

It has been speculated that the clustering of related R-genes may facilitate the generation of new specificities. Unequal exchange between family members could theoretically increase or decrease the size of the cluster. Gene-conversion and/or intragenic recombination could combine existing genes to generate new specificities (discussed by Crute and Pink 1996). Evidence for such a process has been provided by studies of the *Rp1 complex* in maize where the generation of new specificities is associated with crossing over within the complex (Richter et al. 1995). Other studies suggest a role for both unequal exchange and gene conversion in the evolution of the *Rp1 complex* (Hu and Hulbert 1994; Sudupak et al. 1993).

Previously, we described the isolation of a soybean line (RI-61) which had inherited neither parental *Rpg1* allele from a *Rpg1-r Rpg1-r* × *Rpg1-b Rpg1-b* cross (Ashfield et al. 1995). Here we demonstrate that the new genotype correlates with recombination between *Rpg1*-flanking markers. These results strongly suggest that the line RI-61 genotype arose from a recombination-based event rather than a chance point-mutation or transposon insertion. It remains to be shown whether the line arose from recombination between two tightly linked genes or through the loss of an allele by unequal exchange or intragenic recombination. In either case, it is likely that this line will facilitate the positional cloning of *Rpg1* by providing a recombination break-point within, or close to, the R-gene. It should be noted that line RI-61 carries the nbs61 RFLP characteristic of the 'Merit' parental line, indicating this sequence has not been lost by unequal exchange in this genotype.

Yu et al. (1996) and Kanazin et al. (1996) have previously reported on the successful use of primers corresponding to conserved NBS domains in the *RPS2*, *N* and *L6* R-genes to PCR-amplify related sequences from soybean. As expected for R-gene sequences, many of these clones correspond to families of linked sequences in the soybean genome. Two of the clones isolated (nbs5 and nbs61) were found to map to the A186/B212 region of MLG-F (Yu et al. 1996). In this study we demonstrate that one of these clones (nbs61) cosegregates with *Rpg1-b* (0 ± 0.86 cM). It should be emphasized that R-gene sequences are typically clustered (reviewed by Bent 1996) and other, yet to be identified, NBS-LRR-type genes will almost certainly map to this location. Further analysis will be necessary to determine if nbs61, or a related NBS-LRR type gene, encodes *Rpg1*. It is intriguing that *RPM1*, a functionally analogous R-gene cloned from Arabidopsis (both *Rpg1* and *RPM1* respond to *avrB*), is also a member of the NBS-LRR family of R-genes (Grant et al. 1995). It seems plausible that the functional relatedness of *RPM1* and *Rpg1* could be reflected in sequence similarities.

The A186/B212-linked cluster of R-genes provides an excellent system for studying the evolution of new resistance specificities. In addition, comparisons of *Rpg1* with the functionally analogous *RPM1* gene from Arabidopsis may throw light on the evolutionary relationship of R-genes with the same specificity found in different species. Future work will concentrate on the cloning of the alleles or linked genes present at the *Rpg1* locus.

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