High Resolution Genetic and Physical Mapping of Molecular Markers Linked to the *Phytophthora* Resistance Gene *Rps1-k* in Soybean

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The resistance of soybean to *Phytophthora* root and stem rot caused by *Phytophthora sojae* is conferred by a series of single-dominant *Rps* genes. We have applied random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analyses to isolate molecular markers linked to *Rps1-k*. Five RAPD markers were identified and mapped closely to one side of *Rps1-k*. AFLP analysis was carried out with near isogenic lines and bulks obtained from F₃ families. Twenty-seven markers were identified. Nineteen of these were specific to the resistant parent. Five AFLP markers were amplified from the susceptible parent. One of these markers, TC1, mapped at 0.07 centimorgans (cM) from the *Rps1* locus. Three AFLP markers were co-dominant, and one of these, CG1, mapped at a distance of 0.06 cM from the *Rps1* locus on the opposite side of the rest of the markers. Two RAPD, 17 AFLP, and 14 restriction fragment length polymorphism (RFLP) markers originating from duplicated sequences were mapped within a 3-cM map interval. These results suggest that *Rps1-k* is located at the end of an introgressed region. Physical mapping data indicate that the *Rps1-k*-flanking markers CG1 and TC1 may be located within a 125-kb chromosomal fragment.

Root and stem rot of soybean is caused by *Phytophthora sojae* M. J. Kaufmann & J. W. Gerdemann (*Phytophthora megasperma* Drechs. f. sp. *glycinea* T. Kuan & D. C. Erwin). *Phytophthora* rot is one of the most destructive diseases of soybean and occurs in most of the soybean-growing areas in the United States, Canada, Australia, Hungary, Japan, and New Zealand (Athow 1987). Minor genes of soybean confer different levels of resistance to *P. sojae*, referred to as field resistance, tolerance, or rate-reducing resistance, and contribute toward defending soybean against this pathogen (Olah et al. 1985; Schmitthenner 1989). The major source of resistance against this pathogen is, however, conferred by a series of single-dominant *Rps* genes that provide race-specific resistance

among soybean cultivars. At least 14 *Rps* genes at seven loci have been reported to provide resistance against 37 recorded *P. sojae* races (Schmitthenner 1989; Ward 1990; Anderson and Buzzell 1992; Förster et al. 1994; Polzin et al. 1994). Of these 14 *Rps* genes, six occur at the *Rps1* locus (Polzin et al. 1994). Recently, the genetics of virulence in *P. sojae* has been studied (Whisson et al. 1994; Gijzen et al. 1996). The genetics of the *Rps* genes in soybean and the corresponding avirulence genes in *P. sojae* suggest a classical gene-for-gene interaction (Flor 1956) for this host-pathogen system. In soybean, *Rps* gene-controlled resistance is expressed as a variety of defense responses, including the hypersensitive reaction and accumulation of the phytoalexin glyceollin. This occurs upon infection with a *P. sojae* race carrying a corresponding avirulence gene (Keen and Yoshikawa 1990).

Soybean chromosomes have been mapped in detail by restriction fragment length polymorphism (RFLP) (Lorenzen et al. 1995; Shoemaker and Specht 1995) and amplified fragment length polymorphism (AFLP) analyses (Keim et al. 1997). A few RFLP markers linked to the *Rps1* locus have previously been identified, and the closest marker mapped 2 centimorgans (cM) from the *Rps1-k* allele (Diers et al. 1992; Polzin et al. 1994). For map-based cloning of this gene, however, a much higher density genetic map is required (Tanksley et al. 1995). In this study, we constructed a high density genetic map in the *Rps1* region by means of the AFLP marker technique (Vos et al. 1995), as well as by random amplified polymorphic DNAs (RAPDs; Williams et al. 1990). AFLP analysis was carried out with a pair of near isogenic lines (NILs) with and without the *Rps1-k* allele, and F₃ bulks obtained from the cross between these NILs. We also carried out physical mapping of the markers linked tightly to either side of *Rps1-k*. High resolution genetic mapping of the markers indicates that *Rps1-k* is located at the end of an introgressed region. The feasibility of isolating this gene by map-based cloning is discussed in the light of the genetic and physical mapping data.

RESULTS

In this mapping project, we initially used the RAPD method to identify markers linked to the *Rps1* locus by comparing two pairs of NILs differing at the *Rps1-k* allele. Recombinants

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were isolated with a tightly linked RAPD marker, and subsequently the regions flanking the *Rps1* locus were nearly saturated with AFLP markers.

Identification and mapping of RAPD markers linked to the *Rps1* locus.

The screening of 400 decamer primers against the cultivar Williams (*rps1-k*) and its NIL Williams 82 (*Rps1-k*) resulted in the amplification of approximately 2,000 discrete DNA fragments ranging from 200 to 3,000 bp, with an average of 5 fragments per primer. Of the primers screened, six generated fragments that were present in resistant cv. Williams 82 but not in susceptible cv. Williams. Three hundred and fifty primers were screened against Elgin (*rps1-k*) and its NIL E420 (*Rps1-k*). Two hundred and fifty of these primers were the same as those used for Williams and Williams 82. The majority of the amplified fragments were identical between these two pairs of NILs. All six primers except the primer UBC123 that produced polymorphic fragments in resistant cv. Williams 82 also generated the same fragments in resistant E420 but not in susceptible cv. Elgin. Two primers (UBC330 and UBC348), which did not reveal polymorphisms between Williams and Williams 82, generated fragments that were present in resistant cv. E420 but not in susceptible cv. Elgin. The polymorphic fragment produced with primer UBC348 did not segregate in a Mendelian fashion in a segregating population obtained from the cross Elgin $\times$ E420. The polymorphic fragment generated with primer UBC330 showed a 3:1 segregation ratio but was not linked to the *Rps1* locus.

Linkage analysis of the five RAPD markers that produce E420-specific bands along with two RFLP markers (Diers et al. 1992) relative to the *Rps1* locus was conducted on 54 susceptible F_2 plants of the cross Elgin $\times$ E420. All RAPD markers along with two RFLP markers were positioned on one side of *Rps1* (Fig. 1; Table 1). RAPD271 and RAPD217 were converted to co-dominant and dominant RFLP markers, respectively, and their map positions were determined by RFLP analysis. Although markers RAPD271 and RAPD217 co-segregated with RFLP markers pA-71 and pA-280, respectively, they showed different hybridization patterns on DNA gel blots when these RAPD fragments were used as probes, indicating that they are different from these two previously reported RFLP markers (Diers et al. 1992).

Identification of AFLP markers linked to the *Rps1* locus.

A high density genetic map of the region carrying *Rps1-k* is required for map-based cloning of the gene. AFLP analysis has recently been applied to soybean with *EcoRI* and *MseI* restriction enzymes, with a total of six selective nucleotides (Lin et al. 1996). We chose *HindIII* instead of *EcoRI*, since *HindIII* gave complete digestion of soybean genomic DNA more efficiently for us.

To generate tightly linked *Rps1-k* markers efficiently, two steps of AFLP analysis were performed. In the first step, in addition to the cvs. Williams and Williams 82, two bulked segregant pools (Michelmore et al. 1991) derived from F_3 progeny of the cross Williams $\times$ Williams 82 were used in AFLP analysis. One bulk was composed of 20 F_3 families homozygous for the dominant allele *Rps1-k* (pool A), and the other was of 20 F_3 families homozygous for the recessive allele *rps1-k* (pool B). None of the F_3 families of these two

bulks were heterozygous for co-dominant RFLP marker RAPD271. The four DNA templates—Williams, Williams 82, and pools A and B—were screened with 1,240 out of the possible 4,096 randomly selected *HindIII* $\times$ *MseI* primer combinations (Fig. 2). On average, 80 DNA fragments were ampli-

Fig. 1. Random amplified polymorphic DNA (RAPD) map of the *Rps1* region. RAPD217 and RAPD271 were converted to dominant and co-dominant restriction fragment length polymorphism (RFLP) markers, respectively, and mapped by Southern analysis. The rest of the resistance-dominant RAPD markers were mapped by polymerase chain reaction (PCR) followed by Southern hybridization of PCR products with RAPD fragments as probes. RFLP markers pA-71 and pA-280 are those identified previously by Diers et al. (1992). Fifty-four susceptible F_2 plants of the cross Elgin (*rps1-k rps1-k*) $\times$ E420(*Rps1-k Rps1-k*) were used in mapping the molecular markers. Genetic distances in centimorgans are presented on the left side of the map.

Table 1. *Rps1*-linked markers isolated by random amplified polymorphic DNA (RAPD) analysis

Marker	RAPD phenotype ^a	RFLP phenotype ^{a,b}	Map distance (cM) ^c
RAPD217 ^d	RD ^b	RD	2.8
RAPD271	RD	CD	1.9
RAPD274	RD	Mono	8.5
RAPD206	RD	ND	2.8
RAPD304	RD	ND	2.8

^a RD, resistance-dominant; CD, co-dominant; Mono, monomorphic; ND, not determined.

^b RAPD markers were converted to restriction fragment length polymorphism (RFLP) markers by using RAPD fragments as probes in Southern blot analysis.

^c Map distances in centimorgans (cM) were determined by RFLP (RAPD217 and RAPD271) and RAPD (RAPD274, RAPD206 and RAPD304) analyses of 54 F_2 susceptible segregants of the cross Elgin (*rps1-k rps1-k*) $\times$ E420 (*Rps1-k Rps1-k*).

^d All five primers that produce RD phenotype in E420 not in Elgin were mapped.

fied per primer; therefore, approximately 100,000 loci were evaluated for polymorphism. Thirty-four primer combinations revealed polymorphisms between the parents, and between the two pools. These primer combinations were then reassessed in the second AFLP step to confirm reproducibility and also to help eliminate loosely linked markers.

In the second AFLP screening step, three additional DNA templates—pools C and D and donor parent Kingwa—were used. Pool C was composed of three F_3 families homozygous for the *Rps1-k* allele and heterozygous for RAPD271, while pool D was composed of five F_3 families homozygous for the *rps1-k* allele but heterozygous for RAPD271 (Fig. 2). The seven DNA templates, consisting of two NILs, four bulk pools, and the donor parent, were assayed with 34 informative primer combinations obtained in the first screening step. Recombinant plants have crossover points in the interval between the *Rps1* and RAPD271 loci (see Figure 2A). Markers present in pools A and C but absent from pool B and D, or present in pools B and D but absent from pools A and C, represent markers most closely linked to the *Rps1* locus.

Twenty-seven out of 34 primer combinations reproduced AFLP markers, and were classified into three different classes: resistance-dominant; susceptibility-dominant; and co-dominant (Table 2; Fig. 2B). Seven primer combinations,

which produced polymorphisms in the first screening, did not show reproducible results in the second screening.

Nineteen primer combinations amplified products only from resistant parent or pools carrying *Rps1-k*. These markers are termed resistance-dominant markers. For example, AFLP marker CT2 (Fig. 2B-a) is present in Williams 82, and pools A and C carrying the *Rps1-k* gene, but is absent from susceptible cv. Williams, and pools B and D.

Five primer combinations amplified fragments only from susceptible parent or pools B and D carrying the *rps1-k* allele. This class of AFLP markers is termed susceptibility-dominant. For example, TC1 (Fig. 2B-b) was amplified from the recurrent parent Williams, and pools B and D, but not from Williams 82, or pools A or C.

Three primer combinations generated co-dominant markers. An example of a co-dominant marker showing a length heterogeneity in the fragments from plants with genotype *Rps1-k/Rps1-k* and genotype *rps1-k/rps1-k* is CG1 (Fig. 2B-c). Sequence analysis of this AFLP marker has shown that the susceptibility-specific fragment and resistance-specific fragment are almost identical, though the susceptibility-specific fragment has a 3-bp deletion.

In this second step of AFLP analysis, inclusion of pools C and D aided the identification of markers that are linked

Fig. 2. Strategies used in isolating amplified fragment length polymorphism (AFLP) markers that are linked to *Rps1-k*. **A**, Schematic representation of genotypes in F_3 bulked segregant pools, and recombinant pools used for AFLP analysis. Chromosome fragments of cv. Williams 82 are derived from cv. Kingwa and are denoted as thick lines; chromosome fragments of cv. Williams are shown as thin lines. Pool A is homozygous for *Rps1-k* and RAPD271(+) alleles; pool B is homozygous for *rps1-k* and RAPD271(-). Pool C consists of recombinant homozygous resistant plants heterozygous for RAPD271. Pool D consists of recombinant homozygous susceptible plants heterozygous for RAPD271. **B**, Examples of AFLP marker phenotypes. Three classes of AFLP markers were found: a, resistance-dominant markers linked to the *Rps1-k* allele; b, susceptibility-dominant markers linked to the *rps1-k* allele; and c, co-dominant markers. d is an example of a loosely linked marker indicating recombination with *Rps1-k*. In each panel, from the left, lanes are S, susceptible cv. Williams; R, resistant cv. Williams 82; A, pool A; B, pool B; C, pool C; D, pool D and K, the *Rps1-k* source cv. Kingwa. Arrows depict polymorphic bands.

loosely to the *Rps1* locus. Figure 2B-d shows an example of a loosely linked marker. The marker AA1 is a resistance-dominant marker. Recombinant pool D shows this fragment, indicating that this marker is loosely linked to the *Rps1* locus.

Construction of a high density genetic map.

A high density genetic map of the AFLP markers was constructed as follows. The resistance-dominant and co-dominant markers were mapped by AFLP analysis of the susceptible segregants that are recombinants for RAPD271. Several AFLP markers, including resistance-dominant, co-dominant, and susceptibility-dominant markers, were mapped by RFLP analysis of the segregating population.

AFLP mapping. Sixteen recombinant individuals with genotypes *rps1-k/rps1-k* and RAPD271(+)/RAPD271(-) were subjected to AFLP analysis to place resistance-dominant and co-dominant marker loci in the RAPD271-*Rps1-k* interval (3.0 cM) (Figs. 3 and 4). Among the 16 recombinants, no recombination events were detected between the *Rps1-k* allele and markers CG1, AA3, and CC3.

RFLP mapping. Fragments of DNA corresponding to the polymorphic fragments identified by AFLP analysis were pu-

rified from the polyacrylamide gel and then re-amplified by polymerase chain reaction (PCR) with the same primer combinations that generated the original polymorphic fragments. Southern blot analysis of NILs Williams and Williams 82 DNA digested separately with *Bam*HI, *Bcl*II, *Bgl*II, *Dra*I,

Fig. 3. Amplified fragment length polymorphism (AFLP) mapping of the marker CC1. Homozygous susceptible recombinant individuals heterozygous for random amplified polymorphic DNA 271 (lanes 1 to 15) as well as the resistant cv. Williams 82 (lane 16) were subjected to AFLP analysis. CC1 is a resistance-dominant marker producing resistance-specific bands (arrow) in recombinant susceptible plants (lanes 9, 13, 14). In this figure, 15 out of 16 susceptible recombinants used in AFLP mapping are shown.

Table 2. *Rps1*-linked markers isolated by amplified fragment length polymorphism (AFLP) analysis

Marker	AFLP phenotype ^a	RFLP phenotype ^{a,b}	Map distance (cM) ^c
AA1	RD ^b	ND	3.0
AA3	RD	Mono	0
AA4	RD	Mono	1.59
AA5	RD	CD	0.80
AC1	RD	ND	1.27
AC2	RD	ND	2.23
AC3	RD	CD	0.80
AT1	RD	ND	ND
AT2	RD	ND	ND
AT3	RD	ND	ND
CA1	RD	Mono	0.80
CC1	RD	RD	0.48
CC3	RD	Mono	0
CG1	CD ^d	CD	0.06 ^c
CG2	CD ^d	ND	2.70
CT2	RD	RD and SD	0.80
CT3	SD	Mono	ND
GA3	CD ^d	ND	2.07
GT1	RD	SD	0.32
TA1	RD	ND	0.64
TC1	SD	CD	0.07 ^c
TC2	SD	SD	ND
TC3	SD	Mono	ND
TG1	RD	ND	ND
TG2	RD	ND	ND
TT3	RD	ND	ND
TT4	SD	ND	ND

^a RD, resistance-dominant; CD, co-dominant; SD, susceptibility-dominant; Mono, monomorphic; ND, not determined.

^b AFLP markers were converted to restriction fragment length polymorphism (RFLP) markers by using polymerase chain reaction amplified AFLP DNA fragments as probes in Southern blot analysis.

^c Map distances in centimorgans (cM) between CG1, TC1, and *Rps1* were determined by RFLP analysis. Other map distances were determined by AFLP analysis.

^d Resistance- and susceptible-specific bands are observed for CG1, CG2, and GA3 in AFLP gels. Sequence analysis confirmed that CG1 is a co-dominant marker. Sequence information for CG2 and GA3 is not available. Therefore, they are considered to be putative co-dominant markers.

Fig. 4. A genetic map of the *Rps1* region. Two random amplified polymorphic DNA (RAPD) markers (RAPD271 and *Tgmr*), 17 amplified fragment length polymorphism (AFLP) markers, and 14 restriction fragment length polymorphism (RFLP) markers originating from duplicated sequences are shown. Genetic distances in centimorgans are presented on the left side of the map. AFLP markers AA3 and CC3 are repetitive sequences, and are not polymorphic between resistant and susceptible parents. Therefore, the map positions of these two markers were determined only by the AFLP mapping of 16 recombinants (*rps1-k rps1-k*, RAPD271[+/-]) (see Linkage Analysis under Materials and Methods), whereas TC1 and CG1 were mapped by RFLP analysis of a large segregating population (see Results).

EcoRI, *EcoRV*, *HindIII*, *PstI*, *RsaI*, and *TaqI* with radiolabeled AFLP probes revealed that eight out of 14 AFLP markers produced polymorphisms between NILs with at least one restriction enzyme. The remaining six AFLP probes produced monomorphic patterns with all 10 tested restriction enzymes. Four resistance- or susceptibility-dominant AFLP markers behaved as co-dominant markers in RFLP analysis (Table 2). Total populations of F₂, F₃, and F₄ progeny, or only recombinant plants in the *RpsI*-k and RAPD271 interval, were then digested with suitable enzymes and the segregation of alleles at marker loci was tested by conventional RFLP analysis.

The susceptibility-dominant marker TC1 was converted to a co-dominant RFLP marker and mapped with segregating materials equivalent to 1,386 chromosomes. A single recombination event between the *RpsI* and TC1 loci was identified when *Bgl*III restriction enzyme was used in RFLP mapping, indicating a map position of 0.07 cM from the *RpsI* locus (Figs. 4 and 5). The co-dominant marker CG1 was converted to a co-dominant RFLP marker. An RFLP analysis of segregating materials representing 1,770 chromosomes revealed a single recombination event between CG1 and *RpsI*-k, resulting in a map position for CG1 of 0.06 cM from the *RpsI* locus on the opposite side of all the other molecular markers described in this study (Figs. 4 and 5).

The *RpsI*-k-linked chromosomal region comprises duplicated sequences that map to multiple loci.

Several *RpsI*-linked probes derived from AFLP markers or from characterization of adjacent regions of AFLP or RAPD markers revealed hypervariable polymorphic bands between

Fig. 5. Restriction fragment length polymorphism (RFLP) analysis of DNA markers linked to *RpsI*-k. A part of the F₂ population obtained from the cross Elgin × E420 showing recombinant plants 32 (*RpsI*-k *rpsI*-k) and 35 (*rpsI*-k *rpsI*-k) is presented. The molecular markers pA-71, RAPD271, Tgmr, TC1, and CG1 were used as probes in RFLP analysis. In plant 32 the crossover occurred between CG1 and *RpsI*-k, while in 35 the crossover occurred between Tgmr and RAPD271. Plant numbers 22 to 38 are shown at the top of the blot. The disease phenotype, either S, susceptible or R, resistant, is shown under each plant number. On the right hand side of the panels: R, resistance- and S, susceptible-specific bands are shown with arrow heads.

NILs Williams and Williams 82 in Southern blot analysis. For example, AFLP marker CT2 hybridized to 12 DNA fragments in a *TaqI* digestion that were designated CT2-A, -C, -D, -E, -G, -I, -M, -N, -O, -P, -Q, and -R (Fig. 6). RFLP analysis indicated that six of the hybridizing DNA fragments—CT2-C, -D, -E, -M, -N, and -O—are linked to the *RpsI* locus. The map distances for three markers (CT2-C, -D, and -O) were determined and are shown in Figure 4.

A soybean bacterial artificial chromosome (BAC) library (S. S. Salimath and M. K. Bhattacharyya, *unpublished*) was screened with the marker TC1. One BAC clone (TC1-BAC) carrying TC1 sequences was identified. One end of the insert of TC1-BAC hybridized to seven additional restriction fragments specific to Williams 82 in Southern blot analysis (data not shown). Of seven fragments, TC1-A, TC1-B, TC1-C,

Fig. 6. The polymorphic fragment of the amplified fragment length polymorphism (AFLP) marker CT2 reveals multiple, polymorphic bands when used as a probe in Southern analysis. Susceptible cv. Williams (S) and resistant cv. Williams 82 (R) were compared with four restriction enzymes: 1, *Bcl*I; 2, *Dra*I; 3, *Eco*RI; and 4, *Taq*I. Polymorphic bands CT2-C, -D, -E, -M, and -O in *TaqI* digestion were found to be linked to the *RpsI* locus. Map distances of CT2-C, -D and -O were presented in the AFLP map (Fig. 4).

TC1-D, TC1-E, and TC1-G were mapped to one locus at 0.8 cM distance from *Rps1*, while fragment TC1-F mapped at 0.64 cM from the *Rps1* locus.

The third probe that showed hypervariable polymorphisms was derived from the flanking sequences of RAPD marker OPRK15. Sequence analysis of this marker established that this marker is a part of *copia*-like retrotransposon Tgmr integrated in the linked chromosomal region of the *Rps1*-k allele, but not in that of four other functional *Rps1* alleles (Bhattacharyya et al. 1997b). Southern blot analysis of the Tgmr flanking sequences revealed four Williams 82-specific and two Williams-specific fragments. Of the four Williams 82-specific fragments, three co-segregated with the Tgmr locus. The fourth fragment, Tgmr-F, was mapped at a 2 cM distance from the *Rps1* locus (Fig. 4).

Determination of physical distance between markers flanking *Rps1*-k.

The AFLP markers TC1 and CG1 were mapped on either side of the *Rps1* locus at 0.07 and 0.06 cM distances, respectively. AA3 and CC3 co-segregated with *Rps1* in AFLP analysis (Fig. 4). These two markers hybridized to many monomorphic DNA fragments in Southern blot analysis. Therefore, they could not be mapped more precisely by carrying out RFLP analysis. An analysis of the physical distance between TC1 and CG1 was undertaken in order to find out the approximate size of the DNA fragment containing *Rps1*-k. High molecular weight DNA was digested separately with the fol-

lowing rare cutting enzymes: *Bsu*36I, *Bss*HI, *Bst*XI, *Nar*I, *Nru*I, *Mlu*I, *Sal*I, *Sma*I, *Sfi*I, *Xma*III, and *Xho*I. The enzyme *Mlu*I produced ca. 125-kb DNA fragments hybridizing to both TC1 and CG1 probes in cvs. Williams and Williams 82 (Fig. 7). In addition to *Mlu*I, *Sal*I digestion also resulted in common fragments slightly larger than *Mlu*I fragments for both probes and cultivars. These data indicate that the DNA fragment to be analyzed for the cloning of *Rps1*-k, with the TC1 and CG1 markers, may be less than 125 kb.

DISCUSSION

Several plant disease resistance genes that follow the classical gene-for-gene hypothesis (Flor 1956) have recently been cloned. Resistance genes isolated so far can be classified into four groups based on the structural analyses of their deduced amino acid sequences: (i) proteins with serine/threonine kinase activity, e.g., *Pto* (Martin et al. 1993); (ii) proteins with nucleotide binding sites and leucine-rich repeats, e.g., *RPS2*, *N*, *L6*, *RPM1*, *Prf*, *M*, and *I2* (Bent et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994; Grant et al. 1995; Lawrence et al. 1995; Salmeron et al. 1996; Anderson et al. 1997; Ori et al. 1997); (iii) proteins with leucine-rich repeats and transmembrane domains, e.g., *Cf2*, *Cf9*, and *Hs1^{pro-1}* (Jones et al. 1994; Dixon 1996; Cai et al. 1997); and (iv) proteins with leucine-rich repeats and transmembrane and serine/threonine kinase domains, e.g., *Xa21* (Song et al. 1995). Recently, a tomato gene, *Pti1*, encoding a serine/threonine kinase was cloned

Fig. 7. Southern blot analysis of pulse-field gel electrophoresed DNA with *Rps1*-k-flanking markers: **A**, cv. Williams; **B**, cv. Williams 82. Lane 1, *Mlu*I; lane 2, *Sal*I; and lane 3, *Sma*I. Common hybridizing bands are shown by arrow heads.

(Zhou et al. 1995). Pti1 is phosphorylated by Pto, and is involved in the hypersensitive response.

The *RpsI-k* gene was introgressed into cv. Williams from distantly related cv. Kingwa. An AFLP analysis revealed that about 10% of amplified fragments were polymorphic between Williams and Kingwa and 2% between Williams and Elgin (data not shown). Although genetic diversity among soybean cultivars is low (Apuya et al. 1988), we observed a relatively high level of polymorphism between cvs. Williams and Kingwa, thus allowing us to identify markers from the introgressed region carrying the *RpsI-k* allele.

In each AFLP assay 48 combinations of primers can be screened for two DNA templates. There are about 80 fragments amplified in each primer combination. Thus, about 400 polymorphic fragments between cvs. Kingwa and Williams can be obtained in each experiment. By contrast, previous studies indicate that only 20% of randomly chosen genomic DNA probes produced RFLPs, and 35% of RAPD primers identified polymorphic bands between different soybean cultivars (Apuya et al. 1988; Lin et al. 1996). Furthermore, each successful RAPD or RFLP analysis yields only one or two polymorphic bands. AFLP analysis is far superior to RFLP and RAPD as a method for identifying polymorphisms. Recently, Lin et al. (1996) compared these three methods in soybean, and came to the conclusion that AFLP is the most useful method in isolating molecular markers. AFLP analysis has been successfully applied to many different species, including the dicotyledonous plants potato (Ballvora et al. 1995; Meksem et al. 1995), *Arabidopsis thaliana* and tomato (Thomas et al. 1995; Vos et al. 1995), and soybean (Lin et al. 1996; Keim et al. 1997), the monocotyledonous plants maize (Vos et al. 1995) and barley (Becker et al. 1995), fungi (Mueller et al. 1996), and nematodes (Folkertsma et al. 1996).

The most recent soybean molecular map based primarily on AFLP markers has 28 linkage groups encompassing a genetic map distance of 3,441 cM (Keim et al. 1997). The linkage group N is 135 cM long and includes the *RpsI* locus. Substitution of these values to the formula summarized by Muehlbauer et al. (1988) shows that a soybean NIL developed through six backcrosses would have on average 1.6% (or 54 cM) of its genome introgressed from the donor parent. Of the contribution from the donor parent, an estimated 52% (28 cM) would be in a linkage block surrounding the desirable gene transferred into the NIL. This would account for 0.8% of the total genome. Between the recurrent parent Williams and the donor Kingwa, about 10% of fragments produced by AFLP analysis were polymorphic. Therefore, 0.08% of AFLP fragments, linked to *RpsI*, would be polymorphic between Williams and Williams 82. In this study, 1,240 combinations of primers were examined. Around 80 fragments were amplified in each primer combination. In the AFLP analysis of the present investigation, with a window of 3 cM, 27 *RpsI-k*-linked markers were identified. The 3-cM introgressed region representing 0.09% of the soybean genome should, however, result in only nine polymorphic bands among the 100,000 fragments analyzed in this study. This suggests a relatively higher level of DNA variability in the *RpsI-k* linked region than in the rest of the genome.

In Southern analysis, probes derived from CT2, from one end of a BAC clone carrying TC1, or from sequences flanking Tgm τ showed multiple, highly polymorphic fragments. These polymorphisms mapped to multiple loci, linked within a map

distance of 3 cM from the *RpsI* locus. Furthermore, TC1 was mapped to two independent loci. Hong et al. (1993) also observed RFLP markers that detected middle repetitive DNA sequences that co-segregated with the rust resistance phenotype determined by the *RpI* gene. The cloning of disease resistance (*R*) genes from other plant species has shown that in many cases *R* genes occur in multiple copies in physically linked clusters (Jones et al. 1994; Martin et al. 1993, 1994; Dixon 1996; Anderson et al. 1997). The *Cf-9* gene cluster has recently been sequenced. It is composed of four homologues of *Cf-9* that show a high level of sequence identity with *Cf-9* (Bhattacharyya et al. 1997a). These results may imply that, in disease resistance loci, unequal crossing over may generate duplicated sequences. Rearrangements following recombination in *R* loci may result in new specificities for *R* genes (Bennetzen and Hulbert 1992). Duplication of several sequences within a short genetic distance, and the occurrence of six *RpsI* functional alleles in a single locus, may indicate similar phenomena responsible for the evolution of *RpsI* alleles with distinct *P. sojae* race-specificity. The occurrence of threefold higher DNA variability in this region may also result from rearrangements following recombination. However, suppression of recombination may also cause apparent higher levels of polymorphism per map unit, as is observed for centromeric regions. It has been reported that the relationship between physical and genetic distance may vary widely within a chromosome (Tanksley et al. 1992).

In the present investigation we carried out AFLP analysis with 1,240 out of 4,096 possible primer combinations. We mapped 33 molecular markers (two RAPD, 17 AFLP, and 14 RFLP) originating from duplicated sequences within a 3-cM map interval. All markers except CG1 were mapped to the same side of the *RpsI* locus. Therefore, marker CG1 and *RpsI-k* are located most likely near the end of the introgressed region. Based on the soybean genome size and total recombination distance, 1 cM corresponds to approximately 320 kb DNA (Arumuganathan and Earle 1991; Keim et al. 1997). Physical mapping of *RpsI*-flanking markers CG1 and TC1 indicated that these AFLP loci may be located within a 125-kb DNA fragment. Identification and mapping of these two markers has provided the foundation for initiation of a chromosome landing (Tanksley et al. 1995) or walking experiment, thus enabling the isolation and identification of the agronomically important soybean disease resistance gene *RpsI-k*. The cloning of this *Rps* gene may contribute toward elucidation of the recognition process, and the signal transduction pathway involved in the expression of race-specific resistance in the soybean-*P. sojae* interaction. Furthermore, the cloned *RpsI-k* gene may also be introduced via direct transformation into soybean cultivars carrying alternative *RpsI* functional alleles to incorporate resistance to additional *P. sojae* races.

MATERIALS AND METHODS

Plant material and growth conditions.

Soybean cv. Williams and its NIL Williams 82, cv. Elgin and its NIL E420, cv. E300 and its NIL OX7171, and the *RpsI-k* source cv. Kingwa were used in this study. The NILs Williams 82, E420, and OX7171 contain the *RpsI-k* allele and are resistant to at least 21 races, including race 1 of *P. sojae*

(Schmitthenner et al. 1994). Williams 82 is a backcross-derived variety developed at the University of Illinois, Urbana. Williams was crossed with Kingwa as the source of *RpsI*-k, followed by six backcrosses and selection for resistance, with four BC6 F_{2,3} lines homozygous for *RpsI*-k being bulked for Williams 82. Elgin 87 is a backcross-derived variety developed at Iowa State University, Ames, by crossing Elgin with Williams 82. After four backcrosses and selection for resistance, 21 BC4 F₂ lines homozygous for *RpsI*-k were bulked for Elgin 87. OX717 was obtained at the Harrow Research Center, Ontario, Canada, by backcrossing Elgin 87 to Elgin and selecting BC5 F₂ lines homozygous for *RpsI*-k. E300 and E420 were obtained at the Harrow Research Center by mutagenizing Elgin and Elgin 87, respectively, with ethyl-methane sulfonate and selecting supernodulating lines; E300 is homozygous *rpsI* and E420 is homozygous *RpsI*-k. For linkage analysis three independent populations were analyzed. These consisted of the F₂ and F₃ progeny of the cross Elgin × E420, the F₂ and F₃ progeny of the cross E300 × OX717, and the F₃ and F₄ progeny of the cross Williams × Williams 82. The F₄ families were obtained from selected F₃ families that were heterozygous for both *RpsI* and RAPD 271 loci. Etiolated 7-day-old seedlings or 2-week-old green seedlings were grown under conditions previously described (Ward et al. 1979; Bhattacharyya and Ward 1986).

Disease evaluation.

Segregating materials were tested for their responses to *P. sojae* race 1 by inoculating unwounded, etiolated seedlings or detached leaves with *P. sojae* (Ward et al. 1979; Bhattacharyya and Ward 1986). *P. sojae* race 1 was grown in the dark at 20°C and zoospores were obtained from 6-day-old cultures. Seven-day-old etiolated seedlings were inoculated and maintained at 100% relative humidity in the dark at 25°C. Resistant and susceptible reactions were scored 24 h after inoculation. After disease evaluation, tissues free of fungal invasion were harvested from each individual seedling and frozen immediately in liquid nitrogen for DNA isolation. Twenty etiolated seedlings of each F₃ family were evaluated for disease development and DNA was prepared from cotyledons of these seedlings. Unifoliate leaves of 2-week-old F₂ seedlings were detached and placed in petri plates, inoculated with zoospores, and scored for disease development 3 and 5 days following inoculation. The leaf materials from the susceptible plants were collected for DNA preparation.

RAPD analysis.

Soybean genomic DNA was prepared by the method of White and Kaper (1989). Primers of 10-mer oligonucleotides were purchased from the Oligonucleotide Synthesis Laboratory, University of British Columbia, Vancouver, Canada, and Operon Technologies, Alameda, CA. The PCR procedure reported by Williams et al. (1990) was followed with minor modifications. Amplification reactions were in 25-μl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.25 mM MgCl₂, 150 μM each dNTP (Pharmacia Biotech, Alameda, CA), 0.2 μM primer, 25 ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Perkin Elmer, Roche, Branchburg, NJ). Amplification was carried out in a DNA Thermal Cycler (Ericomp, San Diego, CA) programmed for one cycle of 2 min at 96°C, and 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by 7 min

at 72°C. Reaction products were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide.

AFLP analysis.

A protocol of the AFLP technique reported by Zabeau and Vos (1993) was used after suitable modifications. A 0.5-μg sample of soybean genomic DNA was digested with *MseI* and *HindIII* enzymes for 1 h. *MseI* adapter and biotinylated *HindIII* adapter, ATP and T4 DNA ligase were then added to the restriction digests, and were incubated for 3 h (Zabeau and Vos 1993). The structure of the *MseI*-adapter is

5-GACGATGAGTCCTGAG

TACTCAGGACTCAT-5

The structure of the biotinylated *HindIII*-adapter is

Bio-5-CTCGTAGACTGCGTACC

CTGACGCATGGTCA-5

After ligation, biotinylated fragments were separated from non-biotinylated fragments by binding to streptavidin beads (Dyna, Oslo, Norway).

HindIII primers and *MseI* primers used for AFLP amplifications consisted of a core sequence and selective nucleotide (SN):

HindIII primer 5-AGACTGCGTACCAGCTT NNN-3

MseI primer 5-GACGATGAGTCCTGAGTAA NNN-3

A cascade amplification protocol of two consecutive amplifications was carried out with primers with one (+1) and three (+3) selective nucleotides, respectively (Zabeau and Vos 1993). In the first amplification, the total set of 16 (4 × 4) amplifications, each with two primers with one selective nucleotide, was performed. A reaction mixture (25 μl) contained 5.6 pmol of *HindIII* and *MseI* primers, 2.5 μl of template DNA, 0.5 U *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN), 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.2 mM of each dNTP. There were 20 cycles of amplification under the following conditions: a 30 s DNA denaturation step at 94°C, a 30 s annealing step at 60°C, and a 1 min extension step at 72°C. After this selective amplification, 10 μl of the reaction was diluted with 190 μl of Tris-EDTA (TE) and used for a second PCR.

In the second amplification, *HindIII*-primers with three selective nucleotides (+3) were end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase. Amplifications were performed in a total volume of 10 μl with 2.25 pmol *MseI*-primer (+3), 0.38 pmol labeled *HindIII*-primer (+3), and 1 μl of template DNA. These reactions were carried out for 36 cycles with the following profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step (see below), and a 1 min extension step at 72°C. The annealing temperature in the first cycle was 65°C. This annealing temperature was subsequently reduced at a rate of -0.7°C per cycle for the next 12 cycles and, thereafter, the PCR was continued at an annealing temperature of 56°C for the remaining 23 cycles. All AFLP reactions were performed in a PTC1-100 thermal cycler (MJ Research, Watertown, MA). Following amplification, products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, and bromophenol blue and xylene cyanol as tracking dyes). The resulting mixtures were heated for 3 min at 90°C, and quenched on ice. Each sample (2 μl) was then loaded on a 4.5% polyacrylamide sequencing gel. After electrophoresis, the gel was vacuum dried and exposed to Kodak BIOMAX MR X-ray film.

Southern blotting analysis.

Polymorphic RAPD or AFLP bands were isolated from agarose or sequencing gels, re-amplified, and radiolabeled with [α - 32 P]dATP by following the random-hexamer radiolabeling method (Feinberg and Vogelstein 1984). Genomic DNA was digested with one of 10 restriction enzymes (*Bam*HI, *Bcl*II, *Bgl*III, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Rsa*I, or *Taq*I) and resulting fragments were electrophoretically separated on 0.8% agarose gels, then capillary blotted onto Zeta Probe blotting membrane (Bio-Rad, Hercules, CA) with 0.4 M NaOH, 1.5 M NaCl as transfer solution. The filters were vacuum dried and prehybridized in 0.5 M Na₂HPO₄, 1% bovine serum albumin (BSA), 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), pH 7.2 at 65°C for 2 to 3 h. The hybridization was carried out in the same solution (fresh) with addition of 32 P-labeled probe at 65°C for over 16 h. Filters were then washed with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS twice at room temperature and once with 2 $\times$ SSC, 1% SDS for 30 min at 65°C. Finally, filters were rinsed briefly in 2 $\times$ SSC at room temperature and exposed to Kodak BIOMAX MR X-ray film for a period of 1 to 4 days.

Linkage analysis.

Segregation of *RpsI*-k, RAPD, and RFLP markers in the F₂, F₃, and F₄ populations was scored. From 1,289 segregating genotypes (either F₂ or F₃) 314 susceptible genotypes were identified. RFLP analysis of this subset population with RAPD271 revealed 19 recombinants between *RpsI* and RAPD271, of which 16 were used in the AFLP mapping of the resistance-dominant or co-dominant AFLP markers. Susceptible-dominant AFLP markers, or AFLP markers that co-segregated with *RpsI*-k in AFLP mapping, were converted to RFLPs by isolating the AFLP fragments by PCR followed by cloning in a T-vector (Bhattacharyya et al. 1997b) and use of these fragments as probes in Southern blot analysis. These cloned fragments were then used as probes in mapping these AFLP markers by using the whole or part of the segregating materials, including F₂, F₃, and F₄ populations. Duplicated sequences identified by AFLP or adjacent regions of RAPD or AFLP markers were mapped by gel blot analysis of recombinant plants. Genetic maps were constructed by the aid of the Map Manager program (Manly and Cudmore 1995).

Physical mapping

High molecular weight DNA of soybean NILs Williams and Williams 82 was isolated following the protocol of Liu and Whittier (1994), with suitable modifications (S. S. Salimath and M. K. Bhattacharyya, *unpublished*). The final nuclei suspension was mixed with an equal volume of 2% low melting point Sea Plaque GTG agarose (FMC, Rockland, ME), prepared in 50 mM EDTA and 10 mM Tris-HCl (pH 9.4), and poured into a mold to form plugs. The agarose plugs were treated with 0.5 M EDTA (pH 9.3), 1% Sarkosyl and 1 mg of proteinase K per ml. Finally, the plugs were washed with 1 mM PMSF, 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl and then dialyzed in 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl.

For CHEF (clamped homogeneous electric field) gel electrophoresis approximately 5 μ g of high molecular weight DNA was digested separately with the following rare cutting enzymes: *Bsu*36I, *Bss*HI, *Bst*XI, *Nar*I, *Nru*I, *MluI, *Sal*I, *Sma*I, *Sfi*I, *Xma*III, and *Xho*I (Stratagene, La Jolla, CA). For com-*

plete restriction digestion the plugs containing high molecular weight DNA were incubated overnight in a 200- μ l restriction cocktail (20 units of enzyme + 1 $\times$ buffer) and later incubated at the appropriate temperature for 6 to 8 h.

Restriction digested, high molecular weight DNA along with the Megabase I and II DNA standards (Gibco BRL, Grand Island, NY) were separated on 1% pulsed field certified agarose (Bio-Rad) in 0.5 $\times$ Tris-borate-EDTA, using a Bio-Rad CHEF mapper. CHEF electrophoresis was for 26.56 h with an auto algorithm program with switch time 2.98 s (initial) and 35.38 s (final), angle 120° and gradient 6 V/cm. After electrophoresis the gel was stained with ethidium bromide, visualized in UV light, and photographed. The DNA was then transferred to Zeta probe blotting membrane (Bio-Rad) and hybridized with radiolabeled AFLP probes.

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