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The *Avr1b* Locus of *Phytophthora sojae* Encodes an Elicitor and a Regulator Required for Avirulence on Soybean Plants Carrying Resistance Gene *Rps*1b

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We have used map-based approaches to clone a locus containing two genes, *Avr1b*-1 and *Avr1b*-2, required for avirulence of the oomycete pathogen *Phytophthora sojae* (Kaufmann & Gerdemann) on soybean plants carrying resistance gene *Rps*1b. *Avr1b*-1 was localized to a single 60-kb bacterial artificial chromosome (BAC) clone by fine-structure genetic mapping. *Avr1b*-1 was localized within the 60-kb region by identification of an mRNA that is expressed in a race-specific and infection-specific manner and that encodes a small secreted protein. When the *Avr1b*-1 protein was synthesized in the yeast *Pichia pastoris* and the secreted protein infiltrated into soybean leaves, it triggered a hypersensitive response specifically in host plants carrying the *Rps*1b resistance gene. This response eventually spread to the entire inoculated plant. In some isolates of *P. sojae* virulent on *Rps*1b-containing cultivars, such as P7081 (race 25) and P7076 (race 19), the *Avr1b*-1 gene had numerous substitution mutations indicative of strong divergent selection. In other isolates, such as P6497 (race 2) and P9073 (race 25), there were no substitutions in *Avr1b*-1, but *Avr1b*-1 mRNA did not accumulate. Genetic complementation experiments with P6497 revealed the presence of a second gene, *Avr1b*-2, required for the accumulation of *Avr1b*-1 mRNA. *Avr1b*-2 was genetically mapped to the same BAC contig as *Avr1b*-1, using a cross between P7064 (race 7) and P6497. The *Avr1k* gene, required for avirulence on soybean cultivars containing *Rps*1k, was mapped to the same interval as *Avr1b*-1.

Interactions between disease resistance genes (*R* genes) in plants and so-called "avirulence" genes (*Avr* genes) in pathogens determine the outcome of infection in many plant-pathogen and plant-pest interactions. Many *R* genes now have been cloned (Ji et al. 1998; Young 2000). A large majority of these encode proteins with nucleotide binding sites and leucine-rich repeats (NBS-LRR proteins) that have a likely or, in some cases, proven (Boyes et al. 1998) intracellular location (Young 2000). *Avr* genes initially were identified genetically, and later cloned, because their presence in the pathogen is required for a particular *R* gene to be effective (Day 1974; Staskawicz et al. 1984). Many *Avr* genes have been cloned from bacteria and a smaller number from fungi (Ji et al. 1998; Lauge and De Wit 1998; Luderer and Joosten 2001; White et al. 2000). Several of the proteins encoded by fungal *Avr* genes, especially those isolated from *Cladosporium fulvum,* trigger defense responses when applied directly to plant tissue (Lauge and De Wit 1998). None of the bacterial proteins encoded by *R* gene-specific *Avr* genes do so. However, several of the proteins have been demonstrated to trigger a defense response when delivered inside the plant cell by the pathogen's bacterial type III secretion system, and many more are inferred to do so (Ham et al. 1998; Van den Ackerveken et al. 1996; White et al. 2000). These Avr proteins are injected into the plant cells by the same mechanism by which some virulence factors are injected into the host cells of bacterial pathogens of animals and humans, such as *Yersinia pestis.* Therefore, it seems likely that the Avr proteins have a dual role as virulence factors (Ham et al. 1998; Van den Ackerveken et al. 1996; White et al. 2000). This has been demonstrated directly in more than 14 cases (White et al. 2000). Two fungal avirulence proteins, NIP1 from *Rhynchosporium secalis* (Rohe et al. 1995) and Ecp2 from *C. fulvum* (Lauge et al. 1997) also have been demonstrated to have a virulence function. These two proteins are secreted into the intercellular space and do not appear to enter the plant cell.

On the basis of the specificity of the so-called "gene-for-gene interaction" between *R* genes and *Avr* genes, it was proposed that the products of *R* genes and *Avr* genes interact physically. This has been demonstrated directly in two cases, namely interaction of the tomato *Pto R* gene product with the product of the *AvrPto* gene from the bacterium *Pseudomonas syringae* pv. *tomato* (Scofield et al. 1996; Tang et al. 1996), and interaction of the rice *Pi-ta R* gene product with the product of the *AvrPi-ta* gene from the fungus *Magnaporthe grisea* (Jia et al. 2000). However, despite much effort, direct interactions between other *R* gene and *Avr* gene products has not been detected (Luderer et al. 2001), leading to the hypothesis that *R* gene products recognize *Avr* gene products only when they are bound to other plant components, such as those targeted by the virulence function of the *Avr* gene products (Van der Biezen and Jones 1998).

Despite progress in understanding the role of avirulence genes in bacteria and true fungi, little is known about avirulence genes in oomycetes (Tyler 2001, 2002). Oomycetes are phylogenetically distant from true fungi, and are most closely related to golden-brown algae such as diatoms (Forster et al. 1990). Oomycete pathogens are responsible for a wide variety of destructive diseases and include over 60 species of *Phytophthora,* over 100 species of *Pythium,* and a variety of biotrophic downy mildews and white rusts.

Phytophthora sojae (Kaufmann & Gerdemann) causes root and stem rot of soybean and causes over $300 million in dam-

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age annually in North America. More than 14 resistance genes at seven loci conferring to individual races of *P. sojae* have been identified, including 6 at the *Rps*1 locus and 3 at the *Rps*3 locus (Anderson and Buzzell 1992; Buzzell and Anderson 1992). Near-isogenic lines containing individual *Rps* genes have been developed in two genetic backgrounds (Buzzell et al. 1987). Several *Rps* genes are used commercially to protect against *P. sojae,* particularly *Rps*1k. However, in all cases, races of the pathogen able to infect resistant cultivars have arisen, limiting the usefulness of *Rps* genes (Schmitthenner et al. 1994). To date, more than 45 races have been reported in the United States, and many other unnamed pathotypes have been identified (Abney et al. 1997; Kaitany et al. 2001; Schmitthenner et al. 1994). Genetic analysis of *P. sojae* has shown that single dominant avirulence genes matching the *Rps* genes are present in the pathogen (Gijzen et al. 1996; May et al. 2002; Tyler et al. 1995; Whisson et al. 1994, 1995). In contrast to the situation in true fungi, a number of *Avr* genes in *P. sojae* are clustered (Tyler 2002). *Avr1b* and *Avr1k* are tightly linked (Whisson et al. 1995), as are *Avr4* and *Avr6* (Gijzen et al. 1996; Whisson et al. 1995), whereas *Avr3a* and *Avr5* are 5 cM apart (Whisson et al. 1995). Clustering of *Avr* genes also occurs in *P. infestans* (van der Lee et al. 2001).

There are four major genotypes of *P. sojae,* based on restriction fragment length polymorphism (RFLP) analysis, with some geographical localization (Forster et al. 1994). Field isolates of *P. sojae* either match one of these four genotypes directly or are hybrids resulting from rare outcrosses between strains of different genotypes in the field. The most common genotype (genotype I) includes the most common race (race 1) (Forster et al. 1994). Race 1 isolates express avirulence genes for all known *Rps* resistance genes, except *Rps*7. Isolates typical of the other three genotypes lack different combinations of *Rps* genes. For example, isolates of genotype II typically lack avirulence against *Rps*1b and *Rps*1k. Outcrosses among strains of different genotypes have resulted in many new races of *P. sojae* as a result of reassortment of virulence alleles coming from genotypes II, III, and IV (Forster et al. 1994). In addition, new races have appeared within genotype I as a result of mutations (Forster et al. 1994).

P. sojae easily can be cultured in vitro, and viable sexual oospores and uninucleate asexual zoospores can be obtained readily. It is diploid and homothallic but can be outcrossed readily by using molecular markers to distinguish F_1 hybrids (Tyler et al. 1995; Whisson et al. 1994). A genetic map of over 240 markers has been developed (May et al. 2002; Whisson et al. 1995) and a 9× bacterial artificial chromosome (BAC) library has been constructed (F. Arredondo and B. M. Tyler, *unpublished data*).

In this article, we describe the map-based cloning of the *Avr1b* locus from *P. sojae* and the identification of two genes within the locus required for the Avr1b phenotype We show that one of the genes (*Avr1b*-1) encodes a secreted elicitor, while the other (*Avr1b*-2) controls the accumulation of *Avr1b*-1 mRNA. *Avr1b*-1 is the first genetically defined avirulence gene cloned from an oomycete.

RESULTS

Map-based cloning of the *Avr1b/Avr1k* locus.

Crosses with four isolates, P6497 (race 2, Avr1b$^-$, Avr1k$^+$), P7064 (race 7, Avr1b$^+$, Avr1k$^+$), P7076 (race 19, Avr1b$^-$, Avr1k$^-$), and P7081 (race 25, Avr1b$^-$, Avr1k$^-$) were used to map the *Avr1b* and *Avr1k* loci. We previously showed that the *Avr1b* locus mapped as a single dominant locus in a cross between P6497 and P7064 (Tyler et al. 1995). Whisson and associates (1995) showed that the *Avr1b* and *Avr1k* loci were tightly linked in a cross between a race 7 and a race 25 isolate. In this study, we confirmed that the two loci were tightly linked in a cross between P7064 and P7081; no recombinants were

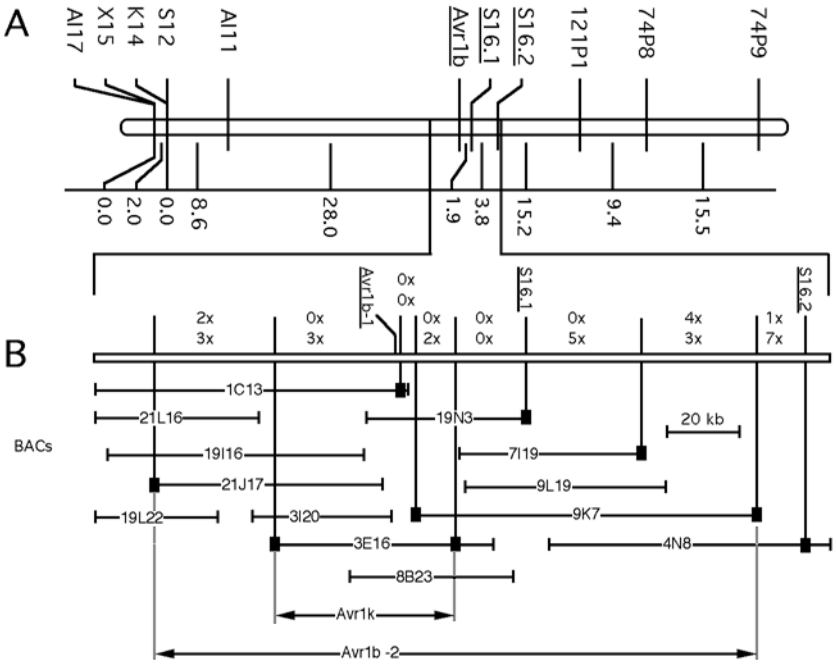

Fig. 1. Genetic and physical map of the *Avr1b/Avr1k* locus. **A,** Genetic map. The map was constructed by analysis of 100 F_2 progeny from a cross of P6497 × P7064, using Mapmaker 1.0. All molecular markers are random amplified polymorphic DNA markers except 121P1, 74P8, and 74P9 which are restriction fragment length polymorphisms (RFLPs). Distances are in centimorgans. Genetic markers common to A and B are underlined. **B,** Physical and genetic map of a 200-kb bacterial artificial chromosome (BAC) contig spanning the *Avr1b* locus. Black boxes and vertical lines indicate RFLP and cleaved amplified polymorphic sequence markers; 2×, 3×, and so on indicate the number of crossovers in an interval; the upper number is from P7064 × P6497 and the lower from P7064 × P7081. Dotted lines show the limits of *Avr1k* (mapped in P7064 × P7081) and *Avr1b*-2 (mapped in P7064 × P6497). The expansion lines show the position and orientation of the contig on the genetic map.

observed among 595 F_2 progeny scored for both *Avr1b* and *Avr1k*. Both loci behaved as single dominant genes in this cross.

We previously identified three RFLP markers linked to the *Avr1b* locus in P6497 × P7064 (Fig. 1A). We used 11 selected progeny from P6497 × P7064 that were either avirulent or virulent and were homozygous for the closest RFLP marker 121P1 to identify seven additional random amplified polymorphic DNA (RAPD) markers linked to the *Avr1b* locus by bulked segregant analysis (Fig. 1A). Two of these markers, S16.1 and S16.2, both amplified by primer OPS16, were identified 1.9 and 5.7 cM, respectively, from the *Avr1b* locus (Fig. 1A).

A bidirectional chromosome walk was initiated by using cloned S16.1 and S16.2 marker fragments to screen a BAC library from P6497 containing seven genome equivalents. S16.1 is single-copy DNA whereas S16.2 is moderately repetitive. Four BACs (19N3R, 7I19, 9L19, and 9K7) matching S16.1 were identified. A single BAC, 4N8, matching S16.2 was identified by polymerase chain reaction (PCR) screening of positively hybridizing clones with the RAPD primer OPS16. Restriction analysis of the BACs with *Eco*RI and *Hin*dIII, together with filter hybridizations, revealed that 4N8 overlapped 7I19, 9L19, and 9K7, placing the two RAPD markers approximately 90 kb apart. S16.1 and S16.2 mapped close to the right ends of 19N3 and 4N8. The right hand ends of 7I19 and 9K7 and the left end of 9K7 were mapped relative to the *Avr1b* locus, S16.1 and S16.2 among 98 progeny from P6497 × P7064 (19N3L was repetitive and could not be mapped.) The results placed the *Avr1b* locus to the left of 9K7R and S16.2 (Fig. 1B). 7I19R, S16.1, and 9K7L showed no recombination with each other, but three progeny appeared to show recombination between these markers and the *Avr1b* locus. A further eight BACs were isolated from the left end of the contig, extending the length of the contig to approximately 200 kb (Fig. 1B). Genetic mapping of 21J17L, near the left end of the contig, revealed three additional recombination events with the *Avr1b* locus and with 7I19R, S16.1, and 9K7L, suggesting that the *Avr1b* locus was located on the 200-kb BAC contig. However, using the molecular markers, no recombination events could

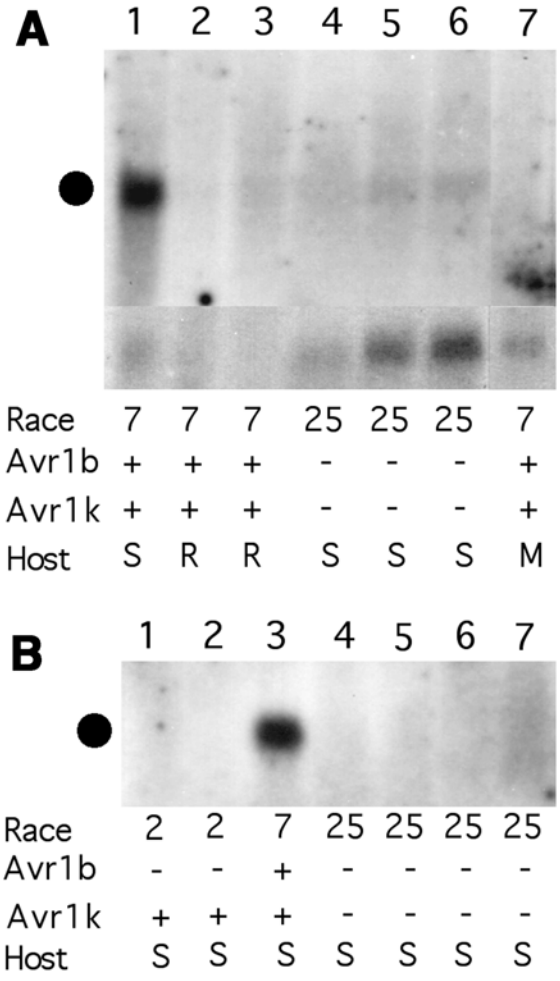

Fig. 2. Expression of *Avr1b*-1 mRNA in avirulent isolates. **A** and **B,** Hybridization of the 650-bp *Hin*dIII/*Nco*I fragment containing *Avr1b*-1 to total RNA extracted **A,** 48 h (upper panel) or **B,** 24 h after infection of resistant (R) or susceptible (S) soybean hypocotyls, or extracted from mycelia (M). Black circles indicate *Avr1b*-1 mRNA. **A,** RNA from hypocotyls of Williams (lanes 1 and 4), L77-1863 (lanes 2 and 5), or Williams 82 (lanes 3 and 6) infected with P7064 (lanes 1 through 3) or P7081 (lanes 4 through 6). Lane 7: P7064 mycelial RNA. Lower panel shows hybridization with a control gene (*His*-4). Variation in the *His*-4 signal in the compatible interactions reflects RNA loading variations, not the level of infection. **B,** RNA from hypocotyls of Williams (lanes 1, 3 through 5), L77-1863 (lanes 2, 5, and 6), or Williams 82 (lane 7) infected with P6497 (lanes 1 and 2), P7064 (lane 3), P7081 (lane 4), or P9073 (lanes 5 through 7). RNA loading was verified by ethidium bromide staining of the gel before transfer (not shown).

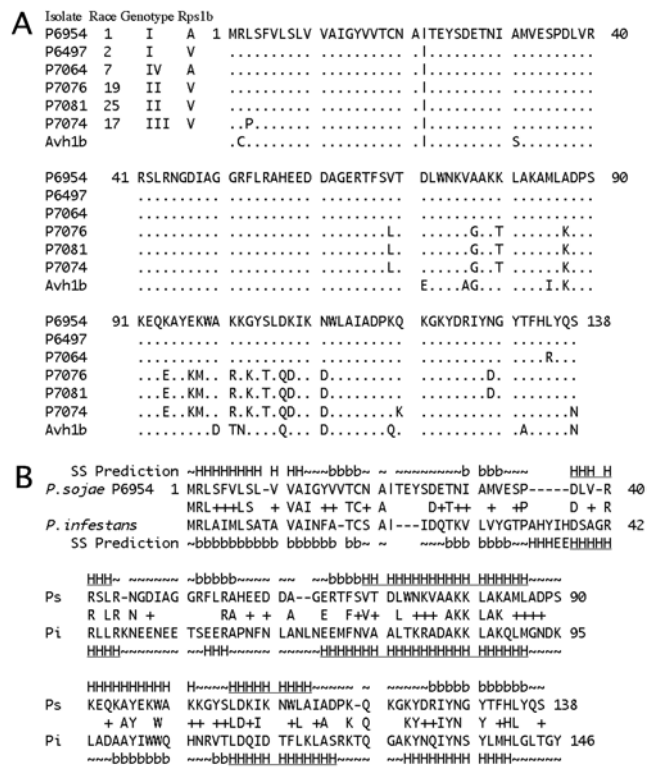

Fig. 3. Sequence comparison of *Avr1b*-1 alleles and homologs. **A,** Sequences of *Avr1b*-1 alleles and homolog (*Avh1b*) from *Phytophthora sojae.* Identities are indicated by dots, substitutions by the substituted residue. In both A and B, | indicates the cleavage site for the secretory leader predicted by the software SignalP V2.0 (Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark). The *Avh1b* sequence is identical in P6954, P6497 and P7081. Corresponding DNA sequences are available from GenBank. Accession numbers are: P6954 (AF449622), P6497 (AF449619), P7064 (gene: AF449620; cDNA: AF449621; 6.5-kb *Hin*dIII fragment: AY426744), P7081 (AF449623), P7076 (AF449624), P7074 (AF449625), and *Avh1b* (AF449626). **B,** Comparison of sequences and secondary structure predictions between *P. sojae Avr1b*-1 and *P. infestans* homolog. The *P. infestans* sequence is derived from expressed sequence tags MY-06-F-06 (BE775766), MY-15-C-09 (BE776395), and MY-36-F-02 (BE777445). Identities between the sequences are indicated by the particular residue, conservative substitutions by +, and gaps in the alignment by –. In the secondary structure predictions (3D-pssm; Imperial College of Science, Technology and Medicine, London), H = helix, b = beta strand, and ~ = unstructured. Underlined helical regions are common to both the *P. sojae* and *P. infestans* predictions.

be identified in the original three progeny that showed "recombination" between the *Avr1b* locus and S16.1, leading us to hypothesize that these three progeny resulted from unusual events such as gene conversion. Previously, we have shown that gene conversion events are extremely frequent in some F1 hybrids of P6497 and P7076, and occur at lower frequencies in other hybrids (Chamnanpunt et al. 2001). To resolve the position of the *Avr1b* locus more clearly and to place *Avr1k* on the molecular map, the *Avr1b* and *Avr1k* loci were mapped relative to the molecular markers in 324 F_2 and F_3 progeny from the cross P6497 × P7076, in which *Avr1k* segregated, and 595 F_2 progeny from the cross P7081 × P7064, in which both the *Avr1k* and *Avr1b* loci segregated. A total of 23 progeny showing recombination between the distal markers 4N8R and 21J17L were identified. Characterization of these recombinants placed both the *Avr1b* and *Avr1k* loci on a single 60-kb BAC, 3E16, as defined by five genetic recombinants between RFLP markers developed from the ends of BAC 3E16 (Fig. 1B). Two additional RFLP markers, 9K7L and 1C13R, were developed within 3E16. Three recombinants were identified between 3E16L and 1C13R, and two recombinants between 9K7L and 3E16R. No recombinants were identified among 1C13R, 9K7L, or the *Avr1b* or *Avr1k* loci (Fig. 1B).

Identification of an *Avr1b*-specific, infection-induced mRNA within BAC 3E16.

We attempted to localize the *Avr1b* and *Avr1k* genes in the 60-kb region using transformation. The protoplasting enzyme, Novozyme 234, previously used for *P. sojae* transformation (Judelson et al. 1993) was no longer available; therefore, we attempted to obtain transformation by electroporation of zoospores, but were unable to obtain any transformants useful for localizing *Avr1b* genes. As an alternative to transformation, we screened for the possible presence of mRNAs that hybridized to the 60-kb region differentially in Avr1b$^+$ versus Avr1b$^-$ or Avr1k$^+$ versus Avr1k$^-$ isolates. Total RNA was isolated from susceptible soybean tissues infected with isolates P7064 (Avr1b$^+$, Avr1k$^+$), P6497 (Avr1b$^-$, Avr1k$^+$), or P9073 (Avr1b$^-$, Avr1k$^-$). Radioactive cDNA then was synthesized from the RNA and hybridized to restriction digests of BACs spanning the region containing the avirulence genes. A 650-bp *Hin*dIII/*Nco*I fragment finally was identified to hybridize with cDNA from tissue infected with P7064 but not with cDNA from infections with either of the other two isolates (data not shown). Thus, the hybridization appeared to be specific to the Avr1b$^+$ phenotype. No hybridization was observed that was specific to the Avr1k$^+$ phenotype. Hybridization of the 650-bp *Hin*dIII/*Nco*I fragment to a gel blot of total RNA revealed a 0.5-kb mRNA specific to tissue infected with the Avr1b$^+$ isolate P7064 (Fig. 2A and B). The mRNA was absent or much reduced in soybean tissues infected with Avr1b$^-$ isolates, including P6497, which is Avr1k$^+$. As further documented below, the gene encoding this mRNA is, in fact, the first of two genes required for the Avr1b phenotype; therefore, for clarity, the gene will be referred to from here on as *Avr1b*-1.

Although *Avr1b*-1 is strongly expressed 24 and 48 h after infection (Fig. 2A and B), no *Avr1b*-1 mRNA could be detected in RNA from vegetatively grown mycelia. *Avr1b*-1 mRNA also was undetectable in infected tissue from resistant soybean cultivars, but this is likely due to the very small amount of pathogen biomass present in the resistant plant tissues.

***Avr1b*-1 is polymorphic and encodes a secreted protein.**

The BAC library was prepared using P6497, which is Avr1b$^-$; therefore, we amplified a 12-kb fragment from genomic DNA of the Avr1b$^+$ isolate P7064 using long-range PCR. A 6.5-kb *Hin*dIII fragment bearing *Avr1b*-1 was subcloned from the 12-kb fragment and sequenced (GenBank accession no. AY426744). The 650-bp *Hin*dIII/*Nco*I fragment hybridizing to the *Avr1b*-1 mRNA contained a 417-bp open reading frame that encoded a 138-amino acid (aa) protein, including a predicted 21-aa secretory leader signal (Fig. 3). To confirm the reading frame and the absence of introns, a full-length *Avr1b*-1 cDNA was isolated from P7064 by reverse transcriptase-PCR (RT-PCR) and sequenced (data not shown). The predicted amino acid sequence of *Avr1b*-1 showed no significant matches to the public DNA sequence databases, except for three expressed sequence tags from *P. infestans* that encoded a secreted protein with weak to moderate similarity to *Avr1b*-1 (Fig. 3B).

The *Avr1b*-1 gene was amplified, cloned, and sequenced from P6954 (race 1, Avr1b$^+$, Avr1k$^+$), P6497 (race 2, Avr1b$^-$, Avr1k$^+$), P7076 (race 19, Avr1b$^-$, Avr1k$^-$), P7081 (race 25, Avr1b$^-$, Avr1k$^-$), and P7074 (race 17, Avr1b$^-$, Avr1k$^+$). The P6954 and P6497 alleles were identical to one another, and differed from the P7064 allele at only a single position, resulting in an amino acid substitution (R135L). The P7081 and P7076 alleles were identical to one another, but showed 21 nucleotide substitutions from that of the P7064 allele, all but one of which created amino acid substitutions. The allele from P7074 resembled that from P7076 except at four positions. The alleles in each isolate are summarized in Table 1.

None of 40 Avr1b$^-$ fresh field isolates examined by Southern blot analysis or PCR showed a deletion of the *Avr1b*-1

Table 1. *Phytophthora sojae* strains used in this study

			Virulence[a]		*Avr1b*-1		
Isolate[b]	Genotype[c]	Race[d]	*Rps*1k	*Rps*1b	Allele[e]	mRNA[f]	*Avh1b*[g]
P6954	I	1	A	A	I	++	+
P6497	I	2	A	V	I	–	+
P7373	I	2	A	V	I	–	+
P9073	I	25	V	V	I	–	+
P7081	I/II	25	V	V	II	+	+
P7076	II	19	V	V	II	++	–
P7074	III	17	A	V	III	±	–
P7064	IV	7	A	A	IV	++	–

[a] A = avirulent (i.e., unable to infect plants containing the specified *Rps* resistance gene) and V = virulent (i.e., able to infect).
[b] Isolates are as described by Förster and associates (1994), except for P9073, which is identical to UQ1200 described by Whisson and associates (1995).
[c] Genotype is defined by genome-wide restriction fragment length polymorphism markers (Förster et al. 1994). P7081 is a naturally occurring hybrid between strains of genotypes I and II.
[d] Race is defined by the avirulence or virulence reactions on soybean cultivars containing 12 individual *Rps* resistance genes (Schmitthenner 1989).
[e] *Avr1b*-1 allele is defined by the sequence as shown in Figure 3A.
[f] *Avr1b*-1 mRNA levels 24 h postinfection.
[g] Presence of *Avh1b* was determined by Southern blot hybridization and by polymerase chain reaction.

gene. Two Avr1b$^-$, Avr1k$^+$ isolates, P7373 (race 2, genotype I) and P7074 (race 17, genotype III), appeared to have an insertion of approximately 3 kb within the region 2 kb upstream from *Avr1b*-1 (data not shown).

A second gene, similar to *Avr1b*-1 but differing at 20 positions, was isolated from P6954 and P6497 by PCR. Southern blot analysis of genetic progeny from P7064 × P6497 (data not shown) revealed that the second gene was located at a locus different from *Avr1b*-1, albeit genetically linked to it (approximately 10 cM distant). We have designated this gene *Avh1b* (Avirulence-homolog 1b). *Avh1b* does not cosegregate with any other known avirulence genes, and did not appear to be transcribed, even during infection (see below). *Avh1b* is present in all strains of genotype I, and in the genotype I/II hybrid P7081, but *Avh1b* was not detected in strains of any other genotype, such as P7076 (genotype II), P7074 (genotype III), or P7064 (genotype IV) (Table 1).

Avr1b-1 protein triggers a spreading hypersensitive response in soybean plants containing *Rps*1b.

Avr1b-1 appeared to encode a secreted protein; therefore, we hypothesized that the protein itself might act as a specific elicitor on soybean plants containing the *Rps*1b resistance gene. In order to obtain Avr1b-1 protein, we introduced the *Avr1b*-1 cDNA, including the native secretory leader, into the methylotrophic yeast *Pichia pastoris* using the protein expression vector PICZ. When expression of the cDNA was induced, a small amount of protein of the predicted size was secreted into the culture medium; the protein was absent from supernatants of a control transformant (Fig. 4).

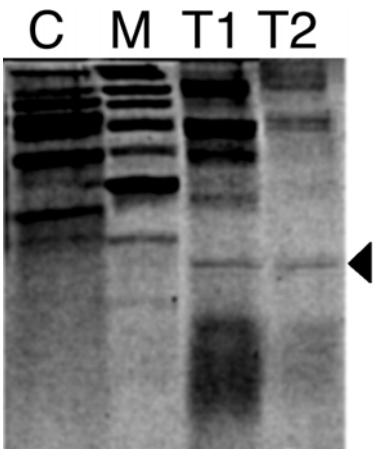

Fig. 4. Secretion of Avr1b-1 protein from *Pichia pastoris.* Crude culture supernatants are shown for two transformants (T1 and T2) containing the *Avr1b*-1 gene. C indicates a control transformant not expressing *Avr1b*-1. M indicates size markers. The gel was stained with Coomassie blue. The triangle indicates proteins of the size expected for mature *Avr1b*-1.

The culture supernatants from induced *Avr1b*-1 transformants and control transformants were filtered, diluted 10- to 500-fold, and infiltrated into leaves of soybean seedlings of isogenic cultivars containing the *Rps*1b gene, the *Rps*1k gene, or no *Rps* gene (*rps*). Supernatants from three independent *Avr1b*-1 transformants but not from two independent control transformants triggered a severe necrosis in the leaves of *Rps*1b cultivars 2 to 3 days after infiltration, even at a dilution of 500-fold; a representative experiment is shown in Figure 5. No necrosis was observed in leaves of the *rps* cultivars. Because the *rps* cultivars are isogenic to the *Rps*1b cultivars other than at the *Rps*1 locus, the response to the culture supernatants clearly is specific to the correct resistance gene, even though the Avr1b-1 protein was not purified from the culture supernatants. In the case of *Rps*1k cultivars, a small amount of localized necrosis was observed at the site of infiltration that was not observed in the controls.

Surprisingly, we observed that, when the elicitor preparation was infiltrated at higher concentrations (10× to 100× dilution) into *Rps*1b plants, the severe necrosis observed in the infiltrated leaves spread to the entire mid and upper regions of the plants from day 4 onward, beginning not with the nearest uninfiltrated leaves but with the uppermost leaves, then moving downward through the plant. An example of collapsed *Rps*1b plants (photographed at day 10 after infiltration) is shown in Figure 6. The control *rps* plants showed no necrosis or wilting. In the *Rps*1k plants, some infiltrated leaves eventually showed complete collapse, but the necrosis did not spread to the rest of the plant.

Identification of a second gene, *Avr1b*-2, required for accumulation of *Avr1b*-1 mRNA.

The sequence of the *Avr1b*-1 allele from P6497 (race 2), which has an *Avr1b*– phenotype, has no mutations in the coding region, compared with the allele from P6954 (race 1) which is *Avr1b*+ (Fig. 3A). However, in P6497, no *Avr1b*-1 mRNA could be detected (Fig. 2), likely explaining its

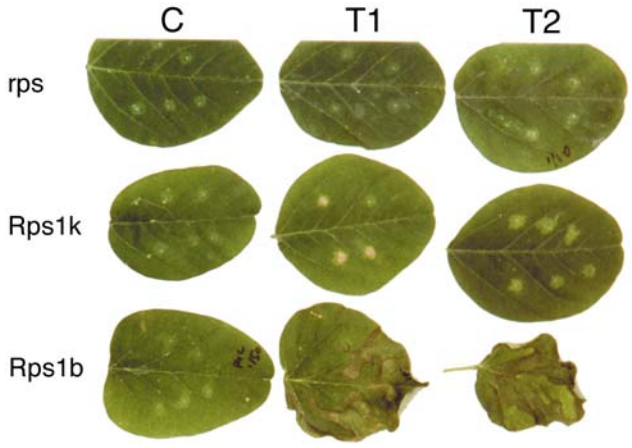

Fig. 5. Avr1b-1 protein triggers host genotype-specific hypersensitive responses in soybean leaves. Culture supernatants from *Pichia pastoris* transformants, diluted 100×, were infiltrated into nontrifoliate leaves of the indicated cultivars. T1 and T2 are independent *P. pastoris* transformants producing Avr1b-1 protein and C is a control with only the transformation vector.

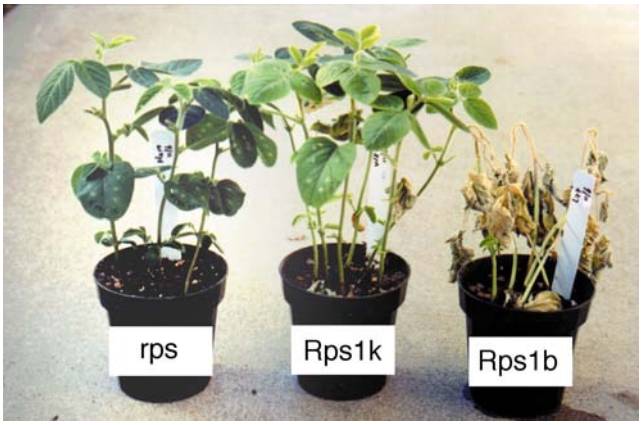

Fig. 6. Plant collapse caused by Avr1b-1 protein on *Rps1b* cultivars First nontrifoliate leaves of the indicated cultivars were infiltrated with Avr1b protein expressed by *Pichia pastoris* (10× and 100× dilutions of the culture supernatants). Collapse appeared 4 days after infiltration, beginning with the inoculated leaves, followed by the uppermost leaves. The picture was taken 10 days after infiltration.

Avr1b-1$^-$ phenotype. Further sequencing showed that there were no differences in P6497 compared with P6954 within a 4.9-kb region upstream of the gene nor within 0.4 kb downstream, raising a question as to the mechanism by which mRNA accumulation was suppressed. To test whether the lack of *Avr1b*-1 mRNA in P6497 was determined in *cis* or in trans, we assayed for mRNA from the P6497 allele of *Avr1b*-1 when it was heterozygous with the transcriptionally active P7064 allele. We carried out RT-PCR to amplify *Avr1b*-1 mRNA sequences from four independent heterozygous F_2 progeny from P6497 × P7064 and digested the amplification products with *Age*I, which detects the single polymorphism that distinguishes the two alleles. In all four heterozygotes, the P6497 allele is represented equally as well as the P7064 allele in *Phytophthora sojae* infection RNA (Fig. 7). The observation is not an artifact caused by DNA contamination of the RNA preparations, because no products were obtained without reverse transcriptase, and also because *Avh1b* sequences (that produce a more slowly migrating fragment) were not amplified. This result demonstrates that the lack of *Avr1b*-1 mRNA in P6497 is due to the lack of a trans-acting factor that can be provided by the genome of P7064. The result further implies that the genetic difference between P7064 and P6497 that is responsible for the *Rps*1b-specific virulence of P6497 is not located in the *Avr1b*-1 gene itself, but in a different cistron that we have designated *Avr1b*-2. Thus, the gene segregating in the cross of P6497 and P7064 that we originally used to map and clone the *Avr1b* locus is actually *Avr1b*-2 (Fig. 1). The recombination breakpoints within the 200-kb BAC contig in this cross place *Avr1b*-2 within a 125-kb region that spans *Avr1b*-1 and *Avr1k* (Fig. 1B). In isolate P7076 (race 19; Avr1b$^-$), *Avr1b*-1 is transcribed during infection (Table 1), but does not confer avirulence, presumably because of the many substitution mutations in the coding region. To test for formal complementation between *Avr1b*-1 and *Avr1b*-2, we tested eight F_1 and F_2 hybrids from a cross of P6497 and P7076 that were heterozygous for the *Avr1b* locus; these strains were previously constructed to map *Avr1k.* All eight heterozygotes were avirulent on HARO13, which contains *Rps*1b, but were fully virulent on Harosoy, which contains no *Rps* genes (data not shown). Both parental strains were fully virulent on HARO13. This experiment formally demonstrates complementation between Avr1b-1 and Avr1b-2.

DISCUSSION

Cloning of *Avr1b.*

The evidence that we have cloned the *Avr1b* locus is threefold. First, the locus lies within a 60-kb interval that cosegregates with the *Avr1b* phenotype in more than 1,000 progeny. Second, the protein encoded by the *Avr1b*-1 gene within the locus triggers a hypersensitive response in soybean plants containing the *Rps*1b gene, but not in near-isogenic cultivars lacking *Rps*1b. The genetic specificity in soybean of the response to the expressed protein provides the most compelling evidence that *Avr1b*-1 encodes the elicitor responsible for avirulence. Third, in virulent races of *P. sojae* that can infect soybean plants containing *Rps*1b, the *Avr1b*-1 gene contains large numbers of base substitutions that result in nonconservative amino acid substitutions, or else its mRNA does not accumulate due to an alteration in the *Avr1b*-2 gene located within the same locus. These three lines of evidence strongly support our conclusion that we have cloned the *Avr1b* locus and have identified one of the two genes within it responsible for avirulence on *Rps*1b-containing soybean cultivars. In the future, DNA transformation experiments will be required to add further confirmation of the identify of these genes and to identify if there are other genes in the locus or elsewhere that are involved in conferring the Avr1b phenotype.

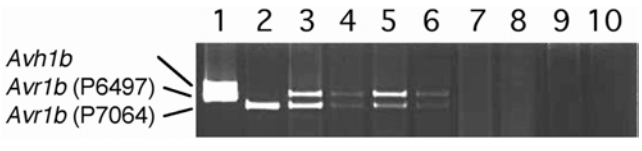

Fig. 7. Accumulation of mRNA from the P6497 allele of *Avr1b*-1 in heterozygotes. Lanes 1 and 2 were amplified from genomic DNA of P6497 and P7064, respectively, and digested with *Age*I. Lanes 3 through 6 are *Age*I-digested reverse- transcriptase polymerase chain reaction products from infection RNA from four heterozygous F_2 progeny from the P6497 × P7064 cross. Lanes 7–10 are the same as 3 through 6 but without reverse transcriptase.

Relationship between *Avr1b* and *Avr1k.*

We mapped *Avr1k* to the same 60-kb interval as *Avr1b*-1. However, the relationship between *Avr1b*-1 and *Avr1k* remains to be resolved. Avr1b-1 protein triggers a weak hypersensitive response on cultivars containing *Rps*1k. Furthermore, no isolates have been documented that are virulent against *Rps*1k but avirulent against *Rps*1b. This raises the possibility that, at least in some strains, *Avr1b*-1 is the same gene as *Avr1k.* This could account for the fact that *Avr1b*-1 and *Avr1k* map genetically to the same 60-kb region in several crosses. However, *Avr1b*-1 and *Avr1k* are unlikely to be the same gene in all isolates, because several *P. sojae* isolates exist which are virulent against *Rps*1b but avirulent against *Rps*1k; for example, P6497 and P7074. These strains likely express a distinct *Avr1k* gene. Alternatively, an Avr1k$^+$, Avr1b$^-$ phenotype possibly could be created through certain amino acid substitutions or changes in expression levels of *Avr1b*-1. It is unlikely that *Avh1b* is the distinct *Avr1k* gene because P7074 lacks *Avh1b* but has an Avr1k$^+$ phenotype, and P9073 and P7081 both contain *Avh1b* but are Avr1k$^-$. No other homologs of *Avr1b*-1 were detected by Southern blots to genomic DNA. Therefore, if there is a distinct *Avr1k* gene, its sequence must be quite dissimilar to *Avr1b*-1; *Rps*1k would thus recognize two distinct proteins, Avr1b-1 and Avr1k. A parallel exists in *Arabidopsis thaliana,* where the *Rpm*1 resistance gene recognizes two dissimilar avirulence genes from *Pseudomonas syringae, Avr*B and *AvrRpm1* (Bisgrove et al. 1994). A different possibility is that Avr1b-1 protein might not be recognized by *Rps*1k itself, but by the product of an *Rps* gene paralog closely linked to *Rps*1k. Future transformation experiments should resolve this issue.

Genetic mechanisms by which avirulence is lost.

The presence of *R* genes in a plant population creates selection pressure in favor of novel "races" of the pathogen that no longer express the relevant avirulence gene or express altered

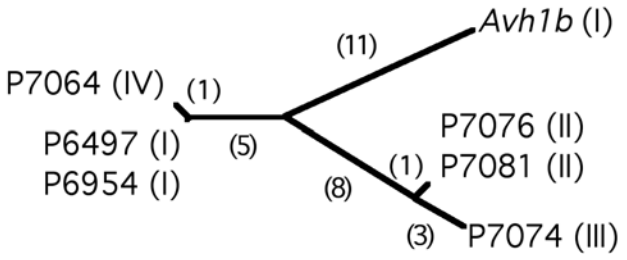

Fig. 8. Relationships of *Avr1b*-1 alleles and homologs. Arabic numerals in parentheses indicate the number of amino acid substitutions. The tree was constructed using the ProtPars function of the Phylip 3.5c package (Felsenstein 1989).

versions of the genes which encode proteins no longer recognized by the *R* gene product. Losses of avirulence genes in fungal pathogens have occurred by partial or complete deletions (Jia et al. 2000; Lauge and De Wit 1998; Orbach et al. 2000; Rohe et al. 1995) or point mutations (Joosten et al. 1994; Rohe et al. 1995). Bacterial avirulence genes have been lost by point mutations (Ji et al. 1998; Keen et al. 1990), deletions (Ji et al. 1998), transposon insertions (Kearney and Staskawicz 1990), or loss of the plasmid carrying the genes (Ji et al. 1998).

We have identified at least two different mechanisms by which the Avr1b phenotype was negated, determined by two different genes. In some virulent strains, such as P6497 and P9073, the sequence of the *Avr1b*-1 gene was identical to that of an avirulent strain, P6954 (race 1), but no mRNA from the *Avr1b*-1 gene could be detected. In other virulent strains, such as P7081, P7076, and P7074, a large number of amino acid substitutions were present in the gene, suggesting that loss of the Avr1b$^+$ phenotype was due to these mutations. In P7081 and P7074, mRNA levels of *Avr1b*-1 also were reduced but not abolished. Of 40 isolates examined by Southern blots or PCR, none showed deletion of the *Avr1b*-1 gene.

The pattern of sequence differences among the *Avr1b*-1 alleles indicates that *Avr1b*-1 is under strong divergent selection (Meyers et al. 1998). Of 15 codons containing nucleotide differences between P6954 and P7076, only one contains a silent substitution. Similarly, of 17 codons divergent between P6954 and P7074, only one contains a silent substitution. The *Avr1b*-1 alleles from P6954 (genotype I) and P7064 (genotype IV) which confer avirulence are most similar to one another, whereas the *Avr1b*-1 alleles from P7076 (genotype II) and P7074 (genotype III) that confer virulence closely resemble each other (Fig. 8). Genotype I and genotype IV isolates are found most commonly in the northern Midwest region of the United States (Forster et al. 1994), where *Rps*1a, *Rps*1c, and *Rps*1k often are included in commercially grown soybean cultivars (Schmitthenner 1989), whereas genotype II and genotype III isolates more commonly are recovered in the southern Mississippi valley (Forster et al. 1994), where a combination of *Rps*1b and *Rps*3a was used extensively for protection against *P. sojae* in the 1970s and 1980s (Dorrance and Schmitthenner 2000). Thus, the *Avr1b* alleles found in the southern isolates may reflect exposure to cultivars containing *Rps*1b. P6497 also was recovered from the South (Forster et al. 1994).

The mechanisms by which *Avr1b*-1 mRNA levels are reduced in some virulent isolates are not yet fully resolved. In the case of P6497, the loss of mRNA accumulation was shown to be due to a tightly linked trans-acting gene, *Avr1b*-2. *Avr1b*-2 has not yet been localized within the locus, and we do not yet know its mechanism of action. The ability of *Avr1b*-2 to elevate *Avr1b*-1 mRNA levels is dominant in heterozygotes. Therefore, the *Avr1b*-2 gene product must act positively, either to stimulate *Avr1b*-1 transcription initiation or to stabilize *Avr1b*-1 mRNA. In a genetic analysis of virulence in *P. infestans* to potato, Al Kherb and associates (1995) obtained avirulent progeny from parental strains virulent on a specific *R* gene, suggesting that two complementing genes may be responsible for avirulence in that case also.

In P9073, like P6497, no *Avr1b*-1 mRNA accumulates and DNA sequencing revealed no sequence alterations at all up to 7 kb upstream of the genes, or 1.5 kb downstream. However, P9073 has concomitantly lost the Avr1k phenotype, whereas P6497 has not, raising the possibility that a different gene than *Avr1b*-2 might be responsible for the virulence phenotype in P9073. In P7074 and in another *Avr1b*$^-$ isolate, P7373 (race 2), a 3-kb insertion was detected upstream of *Avr1b*-1, suggesting that the insertion may have been responsible for the loss of transcription.

Loss of transcription in virulent isolates also was observed in the case of another *Phytophthora* avirulence factor, namely elicitin (Kamoun et al. 1993a). Elicitins are proteins secreted by all *Phytophthora* spp. which act as avirulence factors in the interaction of *Nicotiana* spp. with *P. infestans* and *P. parasitica* (Kamoun et al. 1993b; Kamoun et al. 1998; Ricci et al. 1989). Loss of elicitin expression in virulent isolates of the tobacco pathogen *P. parasitica* results from the loss of transcription of the entire multigene family encoding the elicitins (Kamoun et al. 1993a). Thus, loss of transcription may be a common mechanism in oomycetes for inactivating genes that are temporarily disadvantageous, especially genes like avirulence genes that may be advantageous during infection of one host cultivar but disadvantageous during infection of a different cultivar. Epigenetic changes in transcription levels of avirulence genes could explain rapid reversible changes observed in the avirulence phenotypes of some *Phytophthora* strains. For example, changes from avirulence to virulence and vice versa were observed among two generations of single zoospore lines of *P. sojae* (Rutherford et al. 1985), and also among single zoospore lines in *P. infestans* (Abu-El Samen et al. 2003). During serial subculture of *P. infestans* on detached potato leaves, avirulence in the presence of host resistance gene *R4* was lost without selection as early as the third or fourth passage (Denward 1970). In the field, the rapid appearance of new races of *P. infestans* has made single major resistance genes useless in potato (Fry 1982).

Nature of the *Avr1b*-1 gene product.

Avr1b-1 is the first genetically defined oomycete avirulence gene to be cloned. The gene encodes a small, hydrophilic secreted protein. However, there are no sequence similarities to fungal avirulence proteins or elicitors (Lauge and De Wit 1998; Tyler 2002). *Avr1b*-1 is unusual for a secreted protein in that it lacks any disulfide bonds (Lauge and De Wit 1998; Templeton et al. 1994). The predicted secondary structure of *Avr1b*-1 (3D-pssm; Imperial College of Science, Technology and Medicine, London) is rich in alpha helices (Fig. 3B). Three of the helices predicted in the *P. sojae* P6954 allele of the Avr1b-1 protein match helices predicted for the *P. infestans* homolog (Fig. 3B), despite limited sequence identity between the two proteins, suggesting that the predictions may be biologically relevant. A structural similarity search (3D-pssm; Imperial College of Science, Technology and Medicine) suggests that the tertiary structure of *Avr1b*-1 is most similar to tri-helical bundle DNA and RNA binding proteins, and to cytochrome c. The significance of this similarity remains to be determined.

Role of *Avr1b*-1 in infection of soybean.

A number of bacterial and fungal avirulence gene products enter plant cells or have a demonstrated role in virulence; therefore, it seems plausible to imagine that *Avr1b*-1 also might have a positive role. This speculation is reinforced by the observation that the gene is induced specifically during infection. The observation that Avr1b-1 protein triggers a systemic hypersensitive response when infiltrated into soybean leaves also would fit a hypothesis that Avr1b-1 protein itself spreads systemically as part of its role in infection, though we cannot rule out the possibility that Avr1b-1 actually triggers the release of a plant-derived systemic signal. *Phytophthora* elicitins have been directly demonstrated to spread systemically throughout the plant from the site of infection or infiltration (Devergne et al. 1992). The fact that *P. infestans* contains an *Avr1b*-1 homolog, and that the alleles in the virulent races P7076 and P7074 appear not to be pseudogenes—they encode intact proteins and do not contain frameshift or stop mutations,

or mutations that create rarely used codons—also would be consistent with a positive role for *Avr1b*-1.

Resistance genes that protect against bacterial and viral pathogens typically have NBS-LRR structures consistent with an intracellular location, as expected for receptors required to detect intracellular pathogen molecules (Dangl and Jones 2001; Ellis et al. 2000). However, a number of resistance genes that protect against fungal and oomycete pathogens also have NBS-LRR structures; for example, the L, M, N, and P families of rust resistance genes of flax (Ellis et al. 2000); the *Pb* (Wang et al. 1999) and *Pi-ta* (Jia et al. 2000) rice blast resistance genes of rice; the *Rpp1*, *Rpp5*, *Rpp8*, and *Rpp13* resistance genes of A*rabidopsis* spp. that protect against the oomycete *Peronospora parasitica* (Dangl and Jones 2001; Ellis et al. 2000); the *R1* gene of potato for resistance against *Phytophthora infestans* (Ballvora et al. 2002); and the *Dm3* resistance gene of lettuce that protects against the oomycete *Bremia lactucae* (Chin et al. 2001). The predicted intracellular locations of these resistance gene products imply that fungal and oomycete pathogens have mechanisms for introducing proteins inside the cells of their hosts. However, presently there is no information as to what those mechanisms might be. We have shown here that Avr1b-1 protein secreted from *Pichia pastoris* can trigger a specific hypersensitive response in the absence of the pathogen. The *Rps*1b gene of soybean has not yet been cloned; therefore, the cellular location of its product is not yet known. However, *Rps*1k, which is allelic to *Rps*1b, has been cloned and encodes an intracellular coiled-coil class NBS-LRR protein (M. Bhattacharya, *personal communication*). Therefore, it is very likely that *Rps*1b also encodes an intracellular NBS-LRR protein and that Avr1b-1 protein has the ability to enter soybean cells in the absence of the pathogen.

MATERIALS AND METHODS

Genetic methods.

Phytophthora sojae strains used in this study are summarized in Table 1. *P. sojae* was cultured, selfed, and outcrossed as described (Tyler et al. 1995). *P. sojae* strains were tested for specific virulence by the seedling hypocotyl inoculation method (Tyler et al. 1995) using soybean cvs. Williams (*rps*), Harosoy (*Rps*7), HARO(1-7)1 (*rps,* Harosoy background), L77-1863 (*Rps*1b, Williams background), HARO13 (*Rps*1b, Harosoy background), Williams 82 (*Rps*1k), and HARO15 (*Rps*1k, Harosoy background) generously provided by T. Anderson and R. Buzzell (Harrow, Ontario, Canada).

Genetic markers.

Bulked segregant analysis (Michelmore et al. 1991) was used to identify RAPD markers tightly linked to the *Avr1b* locus using 11 F_2 progeny from P6497 × P7064 homozygous for RFLP marker 121P1 located 19 cM from *Avr1b.* Four individuals avirulent on *Rps*1b cultivars and seven virulent individuals formed the avirulent and virulent pools, respectively. Candidates identified by bulked segregant analysis then were screened against individual F_2 progeny. The two RAPD markers most closely linked to *Avr1b,* S16.1 and S16.2, both were generated by primer OPS16 (5′-AGGGGGTTCC-3′ [Operon Technologies, Alameda, CA, U.S.A.]). Unusually low primer concentrations (0.03 to 0.05 μM; normal concentration is 0.44 μM) were required to reliably amplify these two marker fragments, especially in the case of S16.2. The polymorphic fragments of S16.1 and S16.2 were cloned and sequenced from P6497, P7064, and P7076 in order to create codominant cleaved amplified polymorphic sequences (CAPS) markers designated 19N3R and 4N8R. 19N3R and 4N8R were used extensively for screening for hybrids from outcrosses, screening the BAC library, and screening for genetic recombinants.

BAC library and chromosome walking.

A genomic library of *P. sojae* strain P6497 (Avr1b$^-$, Avr1k$^+$), consisting of 14,592 clones, equivalent to nine genomes (F. Arredondo and B. M. Tyler, *unpublished data*), was constructed in BAC vector pBeloBACII. For library screening, sets of 1,536 BAC clones from four 384-well plates each were arrayed onto N+ nylon membranes (Amersham. Arlington Heights, IL, U.S.A.), then hybridized with radioactively labeled DNA probes. To confirm positive BAC clones, the DNA was digested by *Hin*dIII and *Eco*RI, transferred onto N+ nylon membranes, and subjected to hybridization. To identify fragments at the ends of the BAC inserts, the BAC DNAs were digested with *Sal*I or *Bgl*II, then used as templates to generate radiolabeled RNA probes using either T7 or SP6 RNA polymerase (Promega Corp., Madison, WI, U.S.A.) following the manufacturer's instructions. The labeled RNA probes then were hybridized to restriction digests of the BAC DNAs. For chromosome walking, cloned BAC ends or selected DNA fragments were radiolabeled by random priming and hybridized back to the BAC library.

At each step of the walk, the ends of the BACs were genetically mapped. If the endmost fragment of a BAC insert was repetitive, total *P. sojae* genomic DNA was radiolabeled and hybridized to restriction-digested BAC DNA. Potential low copy number DNA fragments close to the BAC end were identified as faintly hybridizing fragments; these then were cloned and retested by hybridization to restriction digests of genomic DNA. Fragments to be mapped were hybridized to genomic DNA from parental strains P6497, P7064, P7076, and P7081 to identify RFLPs. Probe-enzyme combinations that identified polymorphisms then were used to screen segregating populations.

DNA and RNA manipulations.

Total genomic DNA of *P. sojae* was isolated as described (Tyler et al. 1995). Alkali lysis was used for all plasmid isolation. pBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.) was used for routine cloning and sequencing. pCR-XL-TOPO (Invitrogen, San Diego, CA, U.S.A.) was used for cloning PCR products. *Escherichia coli* strains DH5α and DH10B were used for routine cloning experiments. For gel-blot ("Southern") hybridization analysis, *P. sojae* genomic DNA (2 μg) or plasmid DNA (0.5 μg) was digested with selected restriction enzymes, electrophoresed through 0.6 to 1% agarose gels, and blotted onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) using 0.4 M NaOH as transfer agent.

To isolate RNA from *P. sojae*-infected tissue, hypocotyls of 7- to 10-day-old seedlings were inoculated with small pieces of mycelia (Tyler et al. 1995). After 24 h (before the appearance of disease symptoms in most compatible isolate–cultivar combinations) or 48 h (disease symptoms apparent), the infected regions of the hypocotyls were excised, frozen in liquid nitrogen, and stored at –80°C. The hypocotyl fragments were ground to a fine powder in liquid nitrogen and total RNA was isolated using guanidinium isothiocyanate (Chomczynski and Sacchi 1987). mRNA was isolated using polyA Spin mRNA Isolation Kit (New England Biolabs, Beverly, MA, U.S.A.). For RNA gel blot ("Northern") analysis, approximately 20 μg of total RNA samples were denatured by heating at 70°C for 5 min in 1 M glyoxal, 50% dimethyl sulfoxide, 20 mM Hepes, 1 mM EDTA, pH 7.0, resolved by electrophoresis through a 1.2% agarose gel in 20 mM Hepes and 1 mM EDTA, then transferred to Hybond N nylon membranes (Amersham Phamarcia Biotech) overnight using 10× SSC (1× SSC = 0.15

M NaCl and 15 mM sodium citrate) as transfer agent. The membranes then were exposed to UV light for 2 min in a sterile transfer hood at a distance of 50 cm, followed by baking at 80°C for 2 h in a vacuum chamber.

PCR amplification conditions.

RAPD amplification conditions were as described (Whisson et al. 1994) except where indicated otherwise. All CAPS markers were amplified using the following conditions: step 1, 94°C for 2 min, 1 cycle; step 2, 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, 5 cycles; step 3, 94°C for 30 s, 55 to 60°C for 1 min, 72°C for 1 to 2 min, 30 cycles; step 4, 72°C for 10 min, followed by extended incubation at 4°C (the annealing temperature and extension time were varied depending on the specific pairs of primers). Reactions (10 to 50 µl) contained 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 2.0 mM $MgSO_4$; 100 µM each dNTP; 0.03 to 0.5 µM primers; 0.01% bovine serum albumin; 5 to 20 ng of template genomic DNA or 0.1 to 0.5 ng of plasmid DNA; and 0.4 to 2.0 U *Taq* DNA polymerase. Up to 5% (vol/vol) dimethyl sulfoxide was included for amplification of selected DNA fragments. Amplifications were performed using either a PE Thermocycler 480 or an MJ Research thermocycler. To amplify DNA fragments over 10 kb in length (long-range PCR [L-PCR]) the following amplification conditions were used: step 1, 95°C for 1 min, 1 cycle; step 2, 94°C for 1 min, 65°C for 1 min, 70°C for 10 min, 5 cycles; step 3, 94°C for 40 s, 60°C for 1 min, 70°C for 12 min, 10 cycles; step 4, 94°C for 30 s, 60°C for 15 min, 10 cycles; step 5, 70°C for 20 min, followed by extended incubation at 4ºC. Perkin-Elmer Thermocycler 480 was used. The amplification was done in 50 µl containing 50 ng of genomic *P. sojae* DNA, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1% Triton X-100, 500 µM each dNTP, and 0.5 µM primers. One unit of DyNAzymeTM EXT DNA polymerase (Finnzymes, Finland) was added after the reaction reached 80°C ("hot start").

RT-PCR was performed in two separate steps. The first-strand cDNA was synthesized following manufacturer's recommendation using mRNA (0.1 µg) as template, RTP1 (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTT-3′) as primer and M-MLV reverse-transcriptase. The RNA/DNA hybrids were hydrolyzed using NaOH, extracted with phenol: chloroform (1:1), precipitated with isopropanol, and finally dissolved in 20 µl of H_2O (Sambrook et al. 1989). The resulting cDNA solution (1 µl) was used as template for PCR using primer RTP2 (5′-GACTCGAGTCGACATCGA-3′) and primer 381P2 (5′-AAGTCGCCAAGTATCACGAACC-3′) specific to the putative 5′ untranslated region of the *Avr1b*-1 gene. PCR amplification conditions were as described for CAPS markers except for a lower annealing temperature (55°C). To amplify and clone the 5′ end of the *Avr1b*-1 mRNA, the first-strand cDNA was synthesized using a gene-specific primer, 1C13RP11 (5′-AGATTCCACCATGGCGATATTGG-3′), which corresponds to nucleotides 105 to 83 in the sequence of *Avr1b*-1 gene. A poly(dA) sequence was added to the first-strand cDNA following RNA hydrolysis using terminal transferase (New England Biolabs) according to the manufacturer's instructions. The tailed cDNA then was used as a template for amplification using primers RTP1 and 1C13RP11 following the conditions described above.

Heterologous expression of *Avr1b*-1 in *Pichia pastoris*.

To obtain expression of *Avr1b*-1 in *Pichia pastoris* using the native secretory signal sequence, *Avr1b*-1 cDNA, including 7 bp of 5′ noncoding sequence, the coding sequence, and 5 bp of 3′ noncoding sequence, were amplified using primers that introduced a 5′ *Sfu*I site (5′-TTCGAACCATGCGTCTATCTTTTGTGC-3′) and a 3′ *Eco*RI site (5′-GAATTCTCAGCTCTGATACAGGTGAAAGG-3′). The PCR product was cleaved with *Sfu*I and *Eco*RI and ligated into PICZ (Invitrogen). Transformation of *P. pastoris* strains GS115 *his*4 and KM71 *his*4 was done by electroporation following the manufacturer's protocol (Invitrogen). Plasmid DNA linearized with the restriction enzyme *Pme*I was used for transformation. Selection of transformants and expression of *Avr1b*-1 protein were carried out following the manufacturer's protocol (Invitrogen).

ACKNOWLEDGEMENTS

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark. SignalP V2.0.b2 website: www.cbs.dtu.dk/services/SignalP-2.0/

Imperial College of Science, Technology and Medicine, London, 3D-pssm website: www.sbg.bio.ic.ac.uk/~3dpssm/

Department of Genome Sciences, University of Washington, PHYLIP website: http://evolution.genetics.washington.edu/phylip.html