Two RxLR Avirulence Genes in *Phytophthora sojae* Determine Soybean *Rps1k*-Mediated Disease Resistance

Tianqiao Song,¹ Shiv D. Kale,² Felipe D. Arredondo,² Danyu Shen,¹ Liming Su,¹ Li Liu,¹ Yuren Wu,¹ Yuanchao Wang,¹ Daolong Dou,^{1,2} and Brett M. Tyler²

¹College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China; ²Virginia Bioinformatics Institute, Virginia Tech, Blacksburg 24061, U.S.A.

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Resistance to *Phytophthora sojae* (*Rps*) genes have been widely used in soybean against root and stem rot diseases caused by this oomycete. Among 15 known soybean *Rps* genes, *Rps1k* has been the most widely used in the past four decades. Here, we show that the products of two distinct but closely linked RxLR effector genes are detected by *Rps1k*-containing plants, resulting in disease resistance. One of the genes is *Avr1b-1*, that confers avirulence in the presence of *Rps1b*. Three lines of evidence, including overexpression and gene silencing of *Avr1b-1* in stable *P. sojae* transformants, as well as transient expression of this gene in soybean, indicated that *Avr1b* could trigger an *Rps1k*-mediated defense response. Some isolates of *P. sojae* that do not express *Avr1b* are nevertheless unable to infect *Rps1k* plants. In those isolates, we identified a second RxLR effector gene (designated *Avr1k*), located 5 kb away from *Avr1b-1*. Silencing or overexpression of *Avr1k* in *P. sojae* stable transformants resulted in the loss or gain, respectively, of the avirulence phenotype in the presence of *Rps1k*. Only isolates of *P. sojae* with mutant alleles of both *Avr1b-1* and *Avr1k* could evade perception by the soybean plants carrying *Rps1k*.

The oomycete genus *Phytophthora* contains over 120 species, most of which are destructive plant pathogens (Erwin and Ribiero 1996; Kroon et al. 2012). Oomycetes form a distinct phylogenetic lineage of fungus-like eukaryotic microorganisms (Kamoun 2003; Tyler et al. 2006), related to diatoms. *Phytophthora sojae* (Kaufmann and Gerdeman 1958) was identified as a fast-spreading and devastating root rot pathogen of soybean (*Glycine max* L. Merr.) in the United States and Canada in the early 1950s (Kaufmann and Gerdemann 1958). Since then, the disease has become endemic in almost all soybean production regions around the globe. In the United States, losses due to

P. sojae have risen steadily and, in 2008 to 2010, averaged approximately \$400 million annually (Wrather and Koenning 2010). In China, the pathogen is a new and invasive problem in large-scale soybean-growing regions such as Heilongjiang province (Cui et al. 2010).

Resistance conferred by single dominant resistance to *Phytophthora sojae* (*Rps*) genes has been providing generally effective protection of soybean against this pathogen (Tyler 2008). To date, 15 *Rps* genes have been mapped to nine genetic locations (Bhattacharyya et al. 2005; Gordon et al. 2006; Lohnes and Schmitthenner 1997; Polzin et al. 1994; Sandhu et al. 2005; Wu et al. 2011), including five alleles of *Rps1* (*Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, and *Rps1k*) and three alleles of *Rps3* (*Rps3a*, *Rps3b*, and *Rps3c*). *Rps1k* confers stable resistance against most races of *P. sojae* and has been widely used for the past four decades in North America, indicating that its corresponding avirulence (*Avr*) gene or genes in the pathogen were not easily lost, even under high selection pressure.

The *Rps1* locus contains numerous genes encoding coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) proteins, and these genes show considerable variation in numbers and arrangement among different *Rps1* alleles (Bhattacharyya et al. 2005; Gao and Bhattacharyya 2008). Several of these genes have been cloned from the *Rps1k* allele of the *Rps1* locus and two of them, *Rps1k-1* and *Rps1k-2*, were confirmed to confer *P. sojae* resistance by soybean transformation (Gao et al. 2005).

Genetic analysis of *P. sojae* has revealed at least 12 single *Avr* genes matching *Rps* genes (Gijzen et al. 1996; May et al. 2002; Tyler et al. 1995; Whisson et al. 1994, 1995). Several pairs of *Avr* genes such as *Avr1b* and *Avr1k* (Shan et al. 2004; Whisson et al. 1995), *Avr3a* and *Avr5* (Whisson et al. 1995), and *Avr4* and *Avr6* (Gijzen et al. 1996; Whisson et al. 1995; 2004), appeared closely linked. Two of these pairs have proven to correspond to a single molecular gene, namely *Avr3a* and *Avr5* (Dong et al. 2011b; Qutob et al. 2009) and *Avr4* and *Avr6* (Dou et al. 2010). In addition to *Avr3a/5* and *Avr4/6*, *Avr1a* (Qutob et al. 2009), *Avr1b-1* (Shan et al. 2004), *Avr3b* (Dong et al. 2011a), and *Avr3c* (Dong et al. 2009) have been cloned and characterized. The first cloned *Avr* gene, *Avr1b-1* (Shan et al. 2004), encodes an effector protein that has an N-terminal host-targeting domain with motifs (known as RxLR and dEER) found in all cloned *P. sojae* avirulence effectors. The RxLR-dEER domain mediates effector translocation into host cells in the absence of pathogen machinery, and entry involves binding to phosphatidylinositol-3-phosphate (PI3P) on the surface of the plant plasma cell membrane (Dou et al. 2008b; Kale et al. 2010; Sun et al. 2013). The C-terminal sequences of *Avr1b* are moderately conserved and have the ability to suppress host defense (Dou et al. 2008a).

T. Song and S. D. Kale made equal contributions to the work.

Nucleotide sequence data are available in the GenBank database under accession numbers KC312950 to KC312957.

Current address of F. D. Arredondo, D. Shen, and B. M. Tyler: Center for Genome Research and Biocomputing and Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331, U.S.A.

Corresponding author: B. M. Tyler; Telephone: 541-737-3347; Fax: 541-737-3045; E-mail: brett.tyler@oregonstate.edu

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Oomycetes, including *P. sojae*, *P. infestans*, and *Hyaloperonospora arabidopsidis*, evade recognition by matching host resistance (*R*) genes through mutations in the responsible *Avr* genes (substitutions and deletions) or through transcriptional silencing of those genes (Qutob et al. 2013; Tyler 2009). Examples in *P. sojae* include *Avr1a*, *Avr1b*, and *Avr3a/5* (Dong et al. 2011b; Shan et al. 2004; Qutob et al. 2009). Although eight of the 12 genetically identified *P. sojae* *Avr* genes have been cloned thus far, the *Avr* gene or genes corresponding to *Rps1k* have still not been clearly elucidated. Here, we demonstrate that two genetically closely linked but distinct RxLR effector genes, including *Avr1b-1* and a new gene, *Avr1k*, are responsible for *Rps1k*-mediated gene-for-gene resistance. The recognition of two distinct effectors by plants carrying the *Rps1k* allele suggests a possible molecular basis for the durability of this resistance gene.

RESULTS

Genetic characterization of the *Avr1b/Avr1k* locus.

Previously, *Avr1b-1* and *Avr1k* were genetically defined as single dominant genes that co-segregated in all F2 progeny of the cross between isolates of race 7 (*Avr1b*⁺, *Avr1k*⁺) and race 25 (*Avr1b*⁻, *Avr1k*⁻) (Shan et al. 2004; Tyler et al. 1995; Whisson et al. 1995). *Avr1b-1* was genetically mapped to a single 60-kb bacterial artificial chromosome (BAC), 3E16, leading to its cloning (Shan et al. 2004). *Avr1k* was also localized to the same BAC by characterization of F2 and F3 progeny from the cross P6497 (race 2, *Avr1b*⁻, *Avr1k*⁺) × P7076 (race 19, *Avr1b*⁻, *Avr1k*⁻) and F2 progeny of the cross P7064 (race 7, *Avr1b*⁺, *Avr1k*⁺) × P7081 (race 25, *Avr1b*⁻, *Avr1k*⁻) (Shan et al. 2004). As part of that study, the region surrounding *Avr1b-1* was sequenced from P6497, P7064, and P7076. When the complete superfamily of RxLR genes was identified through use of a hidden Markov model (Jiang et al. 2008), a second RxLR effector gene, *Avh331*, was discovered 5 kb from *Avr1b-1* (Fig. 1A and B). Inspection of the sequence of *Avh331* in virulent strains such as P7076 revealed a frameshift

mutation in the gene in each strain, suggesting that it might be recognized by *Rps1k* (Fig. 1B and C). On the other hand, some avirulent strains such as P7063 (race 6) and P7064 (race 7) also carried the frameshift mutation in *Avh331*. Because P7063 and P7064 carried functional *Avr1b-1* genes, and no strains could ever be isolated that were virulent on *Rps1k* but avirulent on *Rps1b* (Shan et al. 2004; Whisson et al. 1995), we hypothesized that *Avr1b*, as well as *Avh331*, might be recognized by *Rps1k*. This hypothesis was encouraged by the observation that purified recombinant *Avr1b* protein from *Pichia pastoris* could trigger a small amount of localized necrosis at the site of infiltration of soybean leaves carrying *Rps1k* (Shan et al. 2004) and that *Avh331* protein, purified after expression in *Escherichia coli*, could trigger cell death on soybean plants containing *Rps1k* (Kale et al. 2010). For simplicity, we refer to *Avh331* as *Avr1k* in the remainder of this article.

Avr1b confers avirulence in the presence of *Rps1k*.

To test the hypothesis that plants carrying *Rps1k* recognize *Avr1b*, we used particle bombardment experiments with the *Avr1b-1* gene together with a gene encoding β-glucuronidase (GUS) in soybean leaves to measure cell death triggered by *Avr1b* in the presence of *Rps1k*. We used a double-barreled attachment for the Bio-Rad Gene Gun that enables simultaneous bombardment of a DNA test sample and a control sample into two sides of a soybean leaf with the same shot, which greatly reduces the variance of the results (Dou et al. 2008a and b; Gu et al. 2011; Kale et al. 2010; Liu et al. 2011). The number of tissue patches staining blue as a result of GUS expression was reduced approximately 76% by *Avr1b-1* expression compared with the empty vector (EV) in leaves containing *Rps1k* (Haro15) but not in leaves lacking *Rps1k* (*rps*) ($P < 0.001$) (Fig. 2). A similar reduction was observed in the presence of *Rps1b* (Fig. 2) (Dou et al. 2008b). These results indicate that *Avr1b-1* expression triggers *Rps1k*-dependent cell death in soybean leaves, as well as *Rps1b*-dependent cell death.

Fig. 1. Identification of the RxLR effector gene *Avr1k*. **A**, Region of *Phytophthora sojae* scaffold_6 (3070060-3070120; version 5 of the genome sequence) containing *Avr1b-1* and *Avr1k*. Black arrows indicate genes with syntenic orthologs in other *Phytophthora* spp. and white arrows show genes with no orthologs. Further details regarding the genes in this region are given in Supplementary Figure S1 and Supplementary Table S1. **B**, Predicted amino acid sequence encoded by *Avr1k* in the genome sequence of P6497, with key sequence features underlined. The triangle indicates the 8-bp frameshift insertion in some alleles. **C**, Alleles of *Avr1k* in other isolates; unlabeled black bars indicate the positions of nucleotide substitutions; the 8-bp frameshift insertion and the TGA stop codon it leads to are labeled.

In order to test *in vivo* whether *Avr1b-1* confers specific avirulence on *Phytophthora sojae* in the presence of *Rps1b* or *Rps1k*, we examined the phenotypes of two stably transformed *P. sojae* lines (T17 and T20) that produce high levels of *Avr1b-1* mRNA (Dou et al. 2008b). The transformants were avirulent on soybean cultivars containing *Rps1b* ('Haro13') or *Rps1k* ('Haro15') in a hypocotyl inoculation assay compared with plants lacking either *Rps* gene (Fig. 3B; Table 1), whereas the untransformed recipient strain (P7076) was virulent on all the cultivars. We also measured their virulence phenotypes quantitatively using a root lesion progression assay. The lesion lengths were significantly ($P < 0.001$) reduced by two- to threefold on soybean roots carrying *Rps1k* or *Rps1b*, compared with roots lacking the *Rps* genes (Fig. 3A).

Together, these results indicate that the *Avr1b* gene product is recognized by plant tissue containing either *Rps1k* or *Rps1b* when *Avr1b-1* is overexpressed in either the plant or the pathogen.

Silencing of *Avr1b-1* results in loss of the avirulence phenotype on soybean cultivars containing *Rps1k* or *Rps1b*.

In order to confirm that the endogenous *Avr1b-1* gene was responsible for avirulence on *Rps1k* cultivars as well as *Rps1b* cultivars, and to rule out the possibility that recognition by *Rps1k* plants was caused by overexpression of *Avr1b-1*, we silenced *Avr1b-1* in isolate P7063 (race 6) that is avirulent on soybean plants containing *Rps1b* or *Rps1k*. P7063 contains a frameshift mutation in *Avr1k*; therefore, avirulence on *Rps1b* or

Fig. 2. Cell death responses triggered by *Avr1b-1* and *Avr1k* in the presence of resistance to *Phytophthora sojae* (*Rps*)1b and *Rps1k*. Responses were measured by double-barreled particle bombardment of soybean leaves (Dou et al. 2008a; Kale et al. 2010), in which a mixture of β -glucuronidase (GUS) plus avirulence (*Avr*) gene DNA was bombarded through one barrel and GUS plus empty vector (EV) DNA was bombarded through the second barrel. Cell death resulted in a lowered ratio of GUS-positive tissue patches between *Avr* gene and control DNA bombardments. **A**, Average cell death ratios from 14 to 16 paired bombardments each. Error bars indicate standard errors. Asterisks indicate a significant difference ($P < 0.001$; Wilcoxon rank sum test) between bombardment of *Rps* gene-containing leaves (*Rps1b*: 'L77-1879' or 'Haro13'; *Rps1k*: 'Williams 82' or 'Haro15') compared with non-*Rps*-containing (*rps*: 'Haro(1-7)1' or 'Williams') leaves. **B**, Illustrative examples of double-barrel bombardments. Left barrels contained GUS plasmid plus EV plasmid. Right barrels contained GUS plasmid plus *Avr1b-1* driven by 35S promoter.

Fig. 3. Overexpression of *Avr1b-1* in *Phytophthora sojae* produces an avirulence phenotype on soybean cultivars containing resistance to *P. sojae* (*Rps*)1k or *Rps1b*. **A**, Root lesion lengths 5 days after inoculation. Lesions on the roots of 30 seedlings inoculated with wild-type or *Avr1b*-overexpressing *P. sojae* lines were measured in each of five independent experiments. Error bars show standard error. P values were determined with a t test. **B**, Representative phenotypes in hypocotyl inoculation assays of wild-type and transgenic lines on soybean cultivars: 'Haro1' (*rps*, Harosoy background), 'Haro13' (*Rps1b*, Harosoy background), and 'Haro15' (*Rps1k*, Harosoy background). Photographs were taken 4 days after inoculation. T20 produced results similar to those of T17.

Rps1k plants should be due to the *Avr1b-1* gene alone in this isolate. Three plasmids with sense, antisense, and hairpin *Avr1b-1* constructs (Supplementary Fig. S2) driven by the constitutive *Ham34* promoter (Judelson et al. 1991) were used to produce stable transformants of P7063 (Dou et al. 2008b; McLeod et al. 2008); all three strategies for silencing *Phytophthora* genes have been successfully used previously (Ah-Fong et al. 2008). Three independent transformants (T5-15 from hairpin, T7-1 from antisense, and T8-5 from sense constructs) with reduced *Avr1b-1* expression were obtained. Integration of the constructs into the genomes of the transformants was confirmed by Southern blot analysis (Fig. 4A). The transcript of *Avr1b-1* was almost undetectable in Northern blots whereas it was readily detectable in the wild type (Fig. 4B). To determine whether avirulence in the presence of *Rps1b* or *Rps1k* correlated with *Avr1b-1* mRNA levels, we inoculated the three silenced transformants onto hypocotyls of soybean cultivars containing *Rps1b* (Haro13) and *Rps1k* (Haro15). All three silenced transformants could kill soybean seedlings containing *Rps1b* or *Rps1k* (Fig. 4C; Table 1). In contrast, the control GUS transformant T9-1 remained avirulent on the cultivars containing *Rps1b* or *Rps1k*. These results indicate that the *Avr1b* gene product is responsible for avirulence in the presence of *Rps1k* as well as *Rps1b* in isolates such as P7063 that lack a functional *Avr1k* gene.

Characterization of *Avr1k*.

We used real-time reverse-transcriptase polymerase chain reaction (RT-PCR) to measure the transcript levels of *Avr1k* in P6497 in different infection stages. The actin gene was used as a reference. The transcript levels of *Avr1k* in the cultured mycelium were low but detectable. *Avr1k* transcript levels peaked at 12 h postinoculation (hpi) at levels 20-fold above the mycelial level (Fig. 5), consistent with the recognition of the *Avr1k* gene product during infection.

Kale and associates (2010) previously showed that particle-bombardment-mediated transient expression of *Avr1k* (called *Avh331* in that article) in soybean leaves conferred *Rps1k*-dependent cell death (Fig. 2). However, transient expression of *Avr1k* in *Rps1b*-containing leaves did not trigger cell death

(Fig. 2), indicating that *Rps1b*-containing tissue can recognize *Avr1b* but not *Avr1k*. This result is consistent with the observation that isolate P6497, that expresses *Avr1k* but not *Avr1b-1*, is virulent on *Rps1b*-containing cultivars, but is avirulent on *Rps1k*-containing cultivars.

Avr1k confers avirulence in the presence of *Rps1k*.

To determine directly whether *Avr1k* could confer avirulence during infection, the *Avr1k* gene was fused with a C-terminal His tag and placed under control of a strong constitutive promoter, *rpL41* (Dou et al. 2008b), then introduced into a strain, P7076, that is virulent on cultivars carrying the *Rps1k* gene. Only one *Avr1k*-expressing stable transformant (T12) was obtained from more than 100 putative transgenic lines that could grow on the G418-selection medium (Fig. 6A and B). Overproduction of *Avr1k* transcripts and of the *Avr1k* protein product were validated by RT-PCR and Western blot analyses, respectively (Fig. 6B and C). Neither the transcript nor protein product could be detected in the recipient P7076 (Fig. 6B and C). We inoculated T12 and the wild type onto hypocotyls of a cultivar containing *Rps1k* ('Williams 82') and a control isolate, 'Williams' (*rps*). When *Rps1k* was present, T12 could not kill the soybean seedlings whereas the wild type could (Fig. 6D; Table 1). Both T12 and the wild type were virulent on 'Williams' (Fig. 6D; Table 1.) These results indicated that *Avr1k* could confer avirulence on *Rps1k*-containing soybean cultivars, supporting the hypothesis that *Avr1k* can be recognized by *Rps1k* gene products.

Silencing of *Avr1k* results in loss of the avirulence phenotype on soybean cultivar containing *Rps1k*.

To directly test whether *Avr1k* was responsible for the avirulence of *P. sojae* isolates in the presence of *Rps1k*, and to rule out the possibility that recognition of *Avr1k* by *Rps1k* plants is an artifact of overexpression, we silenced *Avr1k* in P6497. The same construct used for *Avr1k* overexpression was used for co-transformation together with a plasmid containing the G418-resistance selectable marker; the sense construct can be as effective in silencing target genes as antisense and hairpin con-

Table 1. Characterization of *Phytophthora sojae* stable transformants

Strains ^b	Transgene ^c	<i>Avr</i> transcripts ^d	Virulence ^a		<i>P</i> value ^e
			<i>rps</i>	<i>Rps1k</i>	
P7076 (<i>Avr1b</i> ⁺ , <i>Avr1k</i> ⁻)	None	None	42/44 V	17/19 V	n.s.
T17 ^f	<i>Avr1b</i> -OX	<i>Avr1b</i> ++	34/35 V	1/22 A	<0.001
T20 ^f	<i>Avr1b</i> -OX	<i>Avr1b</i> ++	26/28 V	1/16 A	<0.001
T12	<i>Avr1k</i> -OX	<i>Avr1k</i> ++	27/33 V	0/33 A	<0.001
P7063 (<i>Avr1b</i> ⁺ , <i>Avr1k</i> ⁻)	None	<i>Avr1b</i> +	25/26 V	2/25 A	<0.001
T5-17	<i>Avr1b</i> -SIL	<i>Avr1b</i> -	27/30 V	20/26 V	n.s.
T7-1	<i>Avr1b</i> -SIL	<i>Avr1b</i> -	24/26 V	21/22 V	n.s.
T8-5	<i>Avr1b</i> -SIL	<i>Avr1b</i> -	23/25 V	17/21 V	n.s.
T9-1	GUS	<i>Avr1b</i> +	26/32 V	1/17 A	<0.001
P6497 (<i>Avr1b</i> ⁺ , <i>Avr1k</i> ⁺)	None	<i>Avr1k</i> +	32/32 V	1/50 A	<0.001
T30	<i>Avr1k</i> -SIL	<i>Avr1k</i> -	38/38 V	39/48 V	n.s.
T51	<i>Avr1k</i> -SIL	<i>Avr1k</i> -	45/45 V	42/53 V	n.s.
T131	<i>Avr1k</i> -SIL	<i>Avr1k</i> -	36/36 V	26/30 V	n.s.
T32	<i>NptII</i> only	<i>Avr1k</i> +	15/15 V	1/36 A	<0.001

^a The virulence of each strain was tested by inoculation of seedlings containing resistance to *Phytophthora sojae* (*Rps1k* ('Williams 82' or 'Haro15') or no *rps* gene ('Williams' or 'Haro(1-7)1'). The number of dead seedlings/total inoculated seedlings is shown, summed from all replicates. A = avirulent and V = virulent.

^b Recipient strains and their genotypes are listed above the transformants derived from them.

^c The presence of transgenes in the transformants was verified by polymerase chain reaction (PCR), Southern blots, or both. OX = overexpression transgene present and SIL = silencing transgene present. Transgene in control transformant T9 consisted of *nptII* plus a β -glucuronidase gene; transgene in T32 consisted of *nptII* plus a promoter-terminator construct lacking any coding region.

^d Avirulence (*Avr*) transcript levels of the indicated genes, as determined by Northern blots, qualitative reverse-transcriptase (RT)-PCR, or quantitative RT-PCR. - = reduced or undetectable transcript levels; + = native transcript levels; ++ = elevated transcript levels.

^e Fisher's exact test (one tailed) was used to compare the frequency of seedling survival between *rps* and *Rps1k* plants. A significant *P* value (<0.001) indicates that the transformant's phenotype is avirulent; n.s. = not significant.

^f T17 and T20 were described by Dou and associates (2008b), including transgene verification, *Avr1b-1* transcript levels, and *Avr1b* phenotype.

structs (Fig. 4) (Ah-Fong et al. 2008; van West et al. 1999). We obtained more than 100 putative transformants that could grow on the G418-selection medium. The transformed lines were screened at 12 hpi for loss of *Avr1k* transcript accumulation. In total, three independent transformants (T30, T51, and T131) were obtained with reduced *Avr1k* transcript levels, less than 20% relative to the wild type as measured by quantitative RT-PCR (Fig. 7A and B). We then examined the virulence phenotype of the silenced transformants on soybean by hypocotyl inoculation. All three silenced transformants could kill *Rps1k*-containing soybean seedlings (Fig. 7C; Table 1). In contrast, the recipient *P. sojae* isolate P6497 and a transformed line, T32, in which *Avr1k* was not silenced, remained avirulent on cultivars containing *Rps1k* (Fig. 7C; Table 1). These results confirm that the endogenous *Avr1k* gene is responsible for *Rps1k*-mediated gene-for-gene resistance in *P. sojae* isolates lacking *Avr1b-1* expression.

Sequence polymorphisms in *Avr1k*.

To more broadly survey polymorphisms in *Avr1k* that may be associated with the loss of the avirulence phenotype in virulent races, we cloned this gene from eight *P. sojae* isolates: P6954 (race 1, phenotype *Avr1b*⁺, *Avr1k*⁺), P6497 (race 2, *Avr1b*⁺, *Avr1k*⁺), P7063 (race 6, *Avr1b*⁺, *Avr1k*⁺), P7064 (race 7, *Avr1b*⁺, *Avr1k*⁺), P7360 (race 11, *Avr1b*⁺, *Avr1k*⁺), P7076 (race 19, *Avr1b*⁺, *Avr1k*⁺), P7081 (race 25, *Avr1b*⁺, *Avr1k*⁺), and PT2004C2.S1 (race 30, *Avr1b*⁺, *Avr1k*⁺) (Forster et al. 1994; Zhou et al. 2009). Three isolates (P6954, P6497, and P7360) contained identical *Avr1k* gene sequences with a full open reading frame, and all were avirulent on cultivars carrying *Rps1k* (Table 2). The other five isolates exhibited a variety of polymorphisms relative to *Avr1k* from the three avirulent isolates but shared a common insertion of eight nucleotides of TGCT ACTT between the regions encoding for the signal peptide and RxLR motif of *Avr1k*, leading to a frameshift and early stop in

the open reading frame (Fig. 1; Supplementary Fig. S3). These isolates were all virulent on cultivars containing *Rps1k*, except for isolates expressing a functional *Avr1b-1* gene; namely, P7063 and P7064 (Table 2). Overall, the relationship between avirulence on *Rps1k* plants and polymorphisms in *Avr1b-1* and *Avr1k* supports the conclusion that *Rps1k* gene products can recognize both *Avr1b* and *Avr1k* proteins.

DISCUSSION

We have demonstrated that two closely linked RxLR effector genes, *Avr1b-1* and *Avr1k*, confer avirulence in *P. sojae* in-

Fig. 5. *Avr1k* transcript levels during infection measured by quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from mycelia (0 h) and P6497-infected soybean leaves at 2 to 36 h postinoculation. Susceptible soybean ‘Williams’ was used. Quantitative real-time RT-PCR analysis employed primers specific for *Avr1k* and the *Phytophthora sojae actin* gene. Relative expression represents the ratio of *Avr1k* transcript levels compared with that in mycelia, standardized to *actin* transcript levels. Bars represent standard errors from two independent biological replicates each.

Fig. 4. Gene silencing of *Avr1b-1* produces a virulence phenotype on soybean lines containing resistance to *Phytophthora sojae* (*Rps1b* or *Rps1k*). **A**, DNA gel blots of transgenic *P. sojae* hybridized with an *Avr1b* probe. T5-17, T7-1, and T8-5 are three transformants with different constructs. Control line T9-1 was transformed with a β -glucuronidase (GUS) construct. H/E = *Hind*III + *Eco*R1 double digest; K = *Kpn*I digest. Arrows indicate fragments corresponding to the endogenous *Avr1b-1* gene. **B**, RNA gel blots of RNA extracted from *P. sojae* hyphae grown in vitro. Probe was *Avr1b-1*. The bottom panel shows ethidium bromide staining of RNA prior to transfer. **C**, Representative phenotypes in hypocotyl inoculation assays of wild-type and transgenic lines on soybean cultivars: ‘Haro52’ (*Rps5*, Harosoy background), ‘Haro13’ (*Rps1b*, Harosoy background), and ‘Haro15’ (*Rps1k*, Harosoy background). Photographs were taken 4 days after inoculation. T8-5 produced results similar to those of T5-15 and T7-1.

fecting soybean cultivars containing *Rps1k*. Overexpression of either gene in *P. sojae* transformants conferred specific avirulence, while silencing of either gene in appropriate *P. sojae* strains eliminated avirulence. Only *P. sojae* isolates carrying mutations in both *Avr1b-1* and *Avr1k* could infect cultivars carrying *Rps1k*. *Rps1k* has provided durable resistance against *P. sojae* in the northern Midwest of the United States since the 1970s. Only in recent years has *Rps1k*-mediated resistance become less reliable as *P. sojae* strains lacking functional *Avr1k* and *Avr1b-1* genes have spread. It seems likely that the durability of *Rps1k* has resulted from its ability to confer recognition of two different RxLR effectors. The very small physical distance of 5 kb between *Avr1b-1* and *Avr1k* would have greatly reduced genetic recombination events that might have enabled mutations in *Avr1b-1* and *Avr1k* to be combined through sexual outcrossing (Forster et al. 1994).

P. sojae has evolved several distinct mechanisms to avoid the recognition of avirulence effectors by soybean R proteins. Alterations of the *Avr1b-1* gene that result in loss of recognition by *Rps1b* cultivars include amino acid substitutions, complete deletions of the gene, or loss of transcript accumulation (Cui et al. 2012; Shan et al. 2004). In the case of *Avr1k*, all alleles found in isolates not recognized by *Rps1k* cultivars contained the same insertion of eight nucleotides, TGCTACTT, in the coding region, producing a pseudogene. The origin of this sequence is unclear; it is not a duplication of a nearby sequence, for example.

Avr1b represents the third example in which a *P. sojae* effector is recognized by products of two distinct soybean resistance genes (*Rps1b* and *Rps1k*). In the case of *Avr4/6*, distinct domains of the effector protein were recognized by plants con-

taining the two relevant *R* genes, *Rps4* and *Rps6* (Dou et al. 2010). In the case of *Avr3a/5*, distinct sets of *Avr3a/5* alleles were recognized by plants containing the two relevant *R* genes, *Rps3a* and *Rps5* (Dong et al. 2011b). In the case of *Avr1b*, however, we were unable to identify any mutants or alleles of the effector that were differentially recognized by plants containing *Rps1k* and *Rps1b*. The two *R* genes are distinguished only by their ability to confer recognition of *Avr1k*. *Rps1b* and *Rps1k* represent two of six alleles of the *Rps1* locus. The *Rps1* locus is highly polymorphic and contains a large number of similar genes encoding CC-NB-LRR proteins (Bhattacharyya et al. 2005; Gao and Bhattacharyya 2008). Two near-identical genes conferring *P. sojae* resistance were cloned from the *Rps1k* locus, *Rps1k-1* and *Rps1k-2* (Bhattacharyya et al. 2005; Gao and Bhattacharyya 2008; Gao et al. 2005). However it is currently unclear whether each individual cloned gene confers recognition of both *Avr1b* and *Avr1k*, which have very different amino acid sequences. It is possible that different CC-NBS-LRR proteins encoded by genes within the broader *Rps1k* region recognize the two different effectors. Furthermore, it is possible that the *Rps1b* and *Rps1k* loci share one or more identical genes that encode R proteins able to recognize *Avr1b*. In *Arabidopsis thaliana*, the R protein Rpm1 can recognize two dissimilar *Avr* genes from *Pseudomonas syringae*, *AvrB* and *AvrRpm1* (Bisgrove et al. 1994). However, in that case, Rpm1 guards a plant protein, RIN4, that is targeted by the two bacterial effectors (Mackey et al. 2002). Similarly, in tomato, the *Cf2* resistance gene can recognize the distinct effectors *Avr2* from the fungus *Cladosporium fulvum* and Gr-VAP1 from the potato cyst nematode *Globodera rostochiensis* because both effectors target a common plant protein, Rcr3, guarded by

Fig. 6. Overexpression of *Avr1k* in *Phytophthora sojae* results in an avirulence phenotype on soybean containing resistance to *P. sojae* (*Rps*)1k. **A**, Verification of the transgene integration in transformant T-12 by amplification of genomic DNA with oligonucleotides from the 5' end of *Avr1k* and from the pHam34 terminator. WT = wild-type recipient P7076, + = DNA template from the transformation plasmid, - = no DNA template, M = size markers. **B**, *Avr1k* transcripts in mycelia of T-12 grown in vitro compared with P7076, detected by reverse-transcriptase polymerase chain reaction analysis. Amplification of *actin* transcripts was used as a positive control; + = positive control consisting of plasmid DNA template and - = no DNA template. **C**, Western blot assay of *Avr1k* protein in mycelia of T-12 grown in vitro. Anti-His-tag monoclonal antibody was used to detect His-tagged *Avr1k* protein in T12. In P7076, *Avr1k* is not His-tagged. Total protein was extracted from in vitro-grown mycelia. Ponceau S staining verifies loading levels. **D**, Phenotypes in hypocotyl inoculation assays of P7076 and T-12 on 'Williams' (*rps*) and 'Williams 82' (*Rps1k*, 'Williams' background) soybean. Photographs were taken 4 days after inoculation.

Cf2 (Lozano-Torres et al. 2012). In flax rust *Melampsora lini*, the AvrL567-A gene product is targeted by three allelic *R* gene products: L5, L6, and L7 (Catanzariti et al. 2006). In that case, the *R* proteins interact directly with the effector (Dodds et al. 2006).

Like most of the *Avr* genes cloned from oomycetes, including *Avr1b-1*, *Avr1k* encodes a protein with the cell entry motifs RxLR and dEER. Accordingly, synthesis of *Avr1k* lacking its secretory leader inside soybean cells following bombardment of leaves with an *Avr1k* gene produced cell death specifically in leaves carrying *Rps1k* (Kale et al. 2010), indicating that *Avr1k* interacts with *Rps1k* gene products inside soybean cells. Kale and associates (2010) showed that purified *Avr1k* protein could enter both root and leaf cells of soybean autonomously; in both cases, the RxLR and dEER motifs of *Avr1k* were required. Kale and associates (2010) further showed that *Avr1k* could bind PI3P and that binding, which required the RxLR and dEER motifs, was required for cell entry.

The C-terminal domain of *Avr1k* contains two modules consisting of W-Y motifs, one of which also includes an L motif (Dou et al. 2008b; Jiang et al. 2008) (Fig. 1B). These motifs occur in almost half of the identified *Phytophthora sojae* RxLR

effectors (Dou et al. 2008b; Jiang et al. 2008) and structural studies have revealed that W-Y modules form a structural unit that supports a diversity of solvent-exposed surfaces (Boutemy et al. 2011).

Both *Avr1b-1* and *Avr1k* are upregulated during infection (Shan et al. 2004; Wang et al. 2011; this study), and both can suppress cell death triggered by the pathogen-associated molecular pattern INF1, by other *P. sojae* effectors, or even by the mouse pro-apoptotic protein Bax (Dou et al. 2008a; Wang et al. 2011). *Avr1k* could also suppress mitogen-activated protein kinase-based plant defense (Cheng et al. 2012). The plant proteins targeted by *Avr1b* and *Avr1k* are currently unknown. *P. infestans* effector Avr3a, which is closely related to *Avr1b* (Armstrong et al. 2005), suppresses cell death by targeting and then stabilizing host U-box E3 ligase CMPG1 (Bos et al. 2010). Because both *Avr1b* and *Avr1k* can suppress cell death triggered by Bax, it is possible that the two effectors interact with fundamental components of the plant cell death machinery. Identification of the plant targets of *Avr1b* and *Avr1k* should reveal how these two proteins can suppress a diversity of plant immune functions, and may shed light on their interactions with the products of *Rps1k* and *Rps1b* genes.

Fig. 7. Gene silencing of *Avr1k* produces a virulence phenotype on soybean containing resistance to *Phytophthora sojae* (*Rps1k*). **A**, Verification of the transgene integration in silencing transformants by amplification of genomic DNA with oligonucleotides from the 5' end of *Avr1k* and from the pHam34 terminator. WT = wild-type recipient P6497, + = DNA template from transformation plasmid, - = no DNA template, M = size markers. **B**, *Avr1k* transcript levels in transformants compared with P6497, measured by quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from infected soybean leaves at 12 h postinoculation. Levels of *actin* transcripts were used as a reference. Transcript levels relative to *actin* were normalized to that of P6497 using the 7500 System Sequence Detection Software. PCR reactions were replicated three times. Bars indicate standard errors. **C**, Phenotypes in representative hypocotyl inoculation assays of P6497 and transformants on 'Williams' (*rps*), and 'Williams 82' (*Rps1k*, 'Williams' background) soybean. Photographs were taken 4 days after inoculation. T30 and T131 produced results similar to those of T51 while the nonsilenced transformant T32 produced results similar to those of P6497.

Table 2. *Avr1b* and *Avr1k* genotypes and phenotypes

Race	Isolate	Phenotype ^a		Avirulence gene allele ^b		Avr1k phenotype contribution ^c
		<i>Rps1b</i>	<i>Rps1k</i>	<i>Avr1b</i>	<i>Avr1k</i>	
Race 1	P6954	A	A	Avr	Avr	<i>Avr1b</i> ⁺ <i>Avr1k</i> ⁺
Race 2	P6497	V	A	Vir(nt)	Avr	<i>Avr1b</i> ⁻ <i>Avr1k</i> ⁺
Race 6	P7063	A	A	Avr	Vir (fs)	<i>Avr1b</i> ⁺ <i>Avr1k</i> ⁻
Race 7	P7064	A	A	Avr	Vir (fs)	<i>Avr1b</i> ⁺ <i>Avr1k</i> ⁻
Race 11	P7360	V	A	ND	Avr	<i>Avr1b</i> (ND) <i>Avr1k</i> ⁺
Race 19	P7076	V	V	Vir	Vir (fs)	<i>Avr1b</i> ⁻ <i>Avr1k</i> ⁻
Race 25	P7081	V	V	Vir	Vir (fs)	<i>Avr1b</i> ⁻ <i>Avr1k</i> ⁻
Race 30	PT2004C2.S1	V	V	ND	Vir (fs)	<i>Avr1b</i> ⁻ <i>Avr1k</i> ⁻

^a Avirulence (A) or specific virulence (V) in the presence of the indicated resistance gene. *Rps* = resistance to *Phytophthora sojae*.

^b Avr = avirulence allele, Vir = virulence allele, nt = no transcript, fs = frameshift, and ND = not determined.

^c The contribution of each gene, *Avr1b-1* or *Avr1k*, to recognition by *Rps1k* plants: + = positive contribution and - = no contribution.

MATERIALS AND METHODS

Bioinformatic analyses.

The *P. sojae* and *P. ramorum* genome sequences and predicted gene models were accessed at the Eukaryotic Microbe database. Functional analyses of predicted genes were based on BLAST and FASTA searches against public databases. Sequence similarities were determined using the BLAST website. Genes sharing best bidirectional BLAST matches were considered to be candidate ortholog pairs. Synteny maps among the genomes of *Phytophthora* spp. were generated by using PHRINGE (Dehal and Boore 2006). Identification of transposable elements in syntenic regions were carried out with the program CRNSOR (Kohany et al. 2006) by searching Repbase (Jurka et al. 2005).

P. sojae strains and manipulation.

P. sojae isolates P6954 (race 1), P6497 (race 2), P7063 (race 6), P7064 (race 7), P7360 (race 11), P7074 (race 17), P7076 (race 19), P7081 (race 25), P9073 (race 25), and PT2004C2.S1 (race 30) (Forster et al. 1994; Whisson et al. 1995; Zhou et al. 2009) were routinely grown and maintained on V8 agar at 25°C in the dark. Procedures for *P. sojae* transformation, screening of *P. sojae* transformants and identification of *Avr1k* polymorphisms were as described (Dou et al. 2008a and b).

Transcriptional profiling.

To characterize *Avr1k* expression during infection, total RNA of *P. sojae* P6497 was collected from mycelium sandwiched between pairs of soybean leaves (Chen et al. 2007). The mycelium was removed at 2, 4, and 8 hpi or the infected soybean leaves were collected at 12, 24, and 36 hpi. All of the collected samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNAsimple total RNA kit (Tiangen, Beijing) was used for total RNA extraction. SYBR green real-time RT-PCR assays (TaKaRa, Tokyo) were carried out for *Avr1k* transcript profiling analysis following the manufacturer's described methods. We used *Avr1k*-RT-F and *Avr1k*-RT-R primers for *Avr1k*, and *actinAF2* and *actinAR2* primers for the reference gene *P. sojae actin* (all primers are listed in Supplementary Table S2). The reaction conditions were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 34 s; followed by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain melt curves. The cycle threshold (Ct) of each gene relative to average Ct values of the housekeeping gene ($Ct = Ct_{\text{gene}} - Ct_{\text{HKaverage}}$) were determined and analyzed using ABI 7500 System Sequence Detection Software, version 1.4 (Schuhmacher et al. 2010).

Construction of plasmids.

P. sojae transformation plasmids pUN (*NptII* gene driven by *P. sojae* *rpL41* promoter) and pHam*Avr1b*, and plant transient expression plasmids pUCA*Avr1b* and pUCGUS were described by Dou and associates (2008b). To make the antisense construct of *Avr1b-1*, the amplicons of *Avr1b-1* produced with primers of *Avr1bF* and *Avr1bR* were digested with *KpnI* and inserted into *KpnI*-digested pHamT35 (Judelson et al. 1991). The antisense construct of *Avr1b-1* was identified by restriction enzyme digestion. The hairpin construct of *Avr1b-1* (pHam: *Avr1b*[antisense]:*rpL41* intron: *Avr1b* [sense]) was obtained by two-step PCR with the primers *UblIF*, *UblIR*, *Avr1bHPF*, and *Avr1bHPR* and ligation of the three resultant fragments. To make the *Avr1k* construct for *P. sojae* transformation (pHam34-*Avr1k*), the *Avr1k* gene was amplified with the primers *Avr1k-Sma* I-F, *Avr1K*-His-F, and *Avr1K*-His-R, digested with *SmaI* and *KpnI*, and then inserted into the vector pUN digested with the same enzymes. All of the above constructs were confirmed

by sequencing. All constructs for overexpressing *Avr1b-1* and *Avr1k* utilized the full-length coding region, including the secretory leader.

Characterization of *P. sojae* transformants.

The two transformants (T17 and T20) that overexpress *Avr1b-1* were previously described by Dou and associates (2008b). Transformants with reduced *Avr1b-1* transcript levels were first screened following the methods of Dou and associates (2008b). For DNA gel blot hybridization, we purified genomic DNA from mycelium as described (Judelson et al. 1991), then digested 10 µg of genomic DNA with *KpnI* or *EcoRI* + *HindIII*. The digested DNA was size fractionated on a 0.7% agarose gel followed by transfer to Hybond Nt nylon membrane (Amersham, Tokyo). For RNA gel blot analysis, total RNA from the transformants was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. We electrophoresed samples of 5 to 10 µg of total RNA in a 1% agarose gel in morpholine-propanesulfonic acid running buffer at 45 mV for 50 min. The gel was then stained with an RNA staining buffer for 15 min. The gel was transferred overnight with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer. DNA probes for *Avr1b-1* were synthesized using a PCR digoxigenin probe synthesis kit according to the manufacturer's manual (Roche Diagnostics, Mannheim, Germany). The membrane was hybridized, and chemiluminescent detection was performed following the kit instructions.

Putative *P. sojae* transformants containing *Avr1k* were screened in three steps. First, DNA from each line was amplified with oligonucleotides *Avr1k*-His-F and *Ham34-R*, which are primers for the 5' end of *Avr1k* and for the *Ham34* terminator, respectively. Next, RT-PCR was performed on RNA extracted from each line using oligonucleotides *Avr1k*-harf-F and *Avr1k*-harf-R to confirm *Avr1k* expression. Finally, the transcript levels derived from the *Avr1k* transgenes were accurately quantitated by real-time PCR, as described above. Because the transcript levels of *Avr1k* are almost undetectable in mycelia and highly induced at 12 hpi, *Avr1k* silencing transformants were screened at 12 hpi.

For overexpression transformants of *Avr1k*, Western blots were used to confirm the protein levels. Total mycelial protein was extracted using Plant protein miniprep kit (Tianz, Beijing) for Western blot assays. A standard sodium dodecyl sulfate polyacrylamide gel electrophoresis protocol was used for protein separation. Proteins were transferred onto polyvinylidene difluoride membranes using a semi-wet apparatus (Bio-Rad, Hercules, CA, U.S.A.) according to the product instructions. Western blotting was performed as described (Yu et al. 2012). Anti-His-tag monoclonal antibody (Sigma-Aldrich, St. Louis) and anti-mouse immunoglobulin G-peroxidase conjugate (Sigma-Aldrich) were used as the primary and secondary antibodies, respectively. The membrane was treated with chemiluminescent peroxidase substrate-1 (Thermo Scientific, Sugarland, TX, U.S.A.) for 5 min. The membrane was briefly drained and exposed to BioMax (Kodak, Rochester, NY, U.S.A.) light film for several different time periods (depending on exposure).

The avirulence phenotypes of selected transformants were evaluated by hypocotyl inoculation (Tyler et al. 1995) using soybean 'Williams' (*rps*), 'Williams 82' ('Williams' background, *Rps1k*), 'Haro(1-7)' (*rps*), 'Haro13' ('Harosoy' background, *Rps1b*), and 'Haro15' ('Harosoy' background, *Rps1k*). The plants were grown in the greenhouse with a 16-h photoperiod at 25°C. One to two days after the first primary leaf appeared, the hypocotyl of the soybean was wounded with a short incision and the incision was inoculated with a small piece of V8 agar cut from the edge of a 3-day-old colony. Thereafter, the

plants were incubated in the greenhouse under the conditions described above. The numbers of dead and surviving plants were counted 4 days after inoculation. The numbers of surviving plants from *rps* and *Rps1k* or *Rps1b* cultivars were compared using Fisher's exact test. Only the transformants producing significant differences between *rps* and *Rps1k* or *Rps1b* cultivars were classified as avirulent. Each avirulence or virulence determination was repeated at least three times.

Particle bombardment assays for avirulence and virulence phenotypes.

Particle bombardment assays were carried out using a double-barreled extension of the Bio-Rad He/1000 Particle Delivery System (Dou et al. 2008a). To quantitate the avirulence (*R*-gene-dependent cell death) conferred by *Avr1b-1* constructs, DNA carrying the constructs (1.7 µg/shot) was co-bombarded into soybean leaves along with DNA carrying a GUS reporter gene (1.7 µg/shot). Avirulence activity was measured as the reduction in the number of blue-staining GUS-positive spots in leaves carrying *Rps1k* compared with leaves lacking either resistance gene. The double-barreled device was used to deliver a parallel control shot in every case that contained GUS DNA plus EV DNA. For each pair of shots, the logarithm of the ratio of the number of blue spots with various *Avr1b-1* constructs to the number with the EV control was calculated. Each assay consisted of eight pairs of shots and was conducted at least twice. The log ratios from all of the *Rps1k* leaves were then compared with those from the non-*Rps1k* leaves using the Wilcoxon rank sum test.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLAST Nucleotide Blast database:
www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
 The Eukaryotic Microbe database: eumicrobedb.org
 Genetic Information Research Institute (GIRI) CENSOR database:
www.girinst.org/censor/index.php
 GIRI Repetitive DNA database: www.girinst.org/repbase/index.html
 Phylogenetic Resources for the Interpretation of Genomes (PHRINGER)
 database: www.genomeprojectsolutions.com/PHRINGER_project.html