Different Domains of *Phytophthora sojae* Effector Avr4/6 Are Recognized by Soybean Resistance Genes *Rps4* and *Rps6*

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Submitted 20 October 2009. Accepted 11 December 2009.

At least 12 avirulence genes have been genetically identified and mapped in *Phytophthora sojae*, an oomycete pathogen causing root and stem rot of soybean. Previously, the *Avr4* and *Avr6* genes of *P. sojae* were genetically mapped within a 24 kb interval of the genome. Here, we identify *Avr4* and *Avr6* and show that they are actually a single gene, *Avr4/6*, located near the 24-kb region. *Avr4/6* encodes a secreted protein of 123 amino acids with an RXLR-dEER protein translocation motif. Transient expression of *Avr4/6* in soybean leaves revealed that its gene product could trigger a hypersensitive response (HR) in the presence of either *Rps4* or *Rps6*. Silencing *Avr4/6* in *P. sojae* stable transformants abolished the avirulence phenotype exhibited on both *Rps4* and *Rps6* soybean cultivars. The N terminus of *Avr4/6*, including the dEER motif, is sufficient to trigger *Rps4*-dependent HR while its C terminus is sufficient to trigger *Rps6*-mediated HR. Compared with alleles from avirulent races, alleles of *Avr4/6* from virulent races possess nucleotide substitutions in the 5' untranslated region of the gene but not in the protein-coding region.

The genus *Phytophthora* belongs to the phylum Oomycota, within the Kingdom Stramenopila, which includes brown algae and diatoms (Förster et al. 1990; Harper et al. 2005). In addition to *P. sojae*, many oomycetes are destructive plant pathogens; for example, *P. infestans* is the potato and tomato late blight pathogen that caused the Irish potato famine in the nineteenth century. Well-advanced genetics, molecular biology, and genomics of *P. sojae* (Tyler 2007) and soybean (Stacey 2008) make this pathosystem a good model for studying the interaction of oomycete pathogens with their hosts.

Monogenic resistance encoded by resistance to *P. sojae* (*Rps*) genes has provided substantial protection for soybean against this pathogen for the last four decades. To date, 15 *Rps* genes have been mapped to eight genomic loci, including five at the *Rps1* locus and three at the *Rps3* locus (Tyler 2008). Cloning and characterization of the *Rps1k* locus identified two similar genes encoding coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) proteins (Gao et al. 2005). The *Rps4* region also has been characterized, and it was shown that deletion of a disease resistance gene-like sequence led to a loss of *Rps4* function. Furthermore, *Rps4* and *Rps6* were shown to be either allelic or clustered genes (Sandhu et al. 2004).

Over 50 physiological races of *P. sojae* have been reported (Leitz 2000), and the number of races is increasing rapidly because of mutation and sexual outcrossing (Förster et al. 1994; Ryley et al. 1998; Tyler 2007). Genetic analysis of *P. sojae* has shown that at least 12 single avirulence genes (Schneider 2002) matching the *Rps* genes are present in the pathogen (Gijzen et al. 1996a; May et al. 2002; Tyler 2007; Tyler et al. 1995; Whisson et al. 1994, 1995). These studies also reported close linkage of some pairs of avirulence genes such as *Avr1b* and *Avr1k*, *Avr3a* and *Avr5*, and complete cosegregation of *Avr4* and *Avr6*. Polymorphic DNA markers linked to many of these avirulence genes have been identified (Chamnanpant et al. 2001; MacGregor et al. 2002; May et al. 2002; Shan et al. 2004; Whisson et al. 2004), including *Avr4* and *Avr6* (Whisson et al. 2004). *Avr1b* has been cloned by a map-based strategy (Shan et al. 2004). Other cloned oomycete avirulence genes include *P. sojae* *Avr1a*, *Avr3a* (Qutob et al. 2009), and *Avr3c* (Dong et al. 2009); *P. infestans* *Avr2* (Lokossou et al. 2009), *Avr3a* (Armstrong et al. 2005), *Avr4* (van Poppel et al. 2008), *Avr-blb1* (Vleeshouwers et al. 2008), and *Avr-blb2* (Oh et al. 2009); and *Hyaloperonospora parasitica* *Atr1* (Rehmany et al. 2005) and *Atr13* (Allen et al. 2004).

Worldwide, soybean (*Glycine max* L. (Merrill)) is an important food, industrial, and fuel crop. *Phytophthora* stem and root rot disease, caused by *Phytophthora sojae* Kauf. & Gerd. (syn. *P. megasperma* f. sp. *glycinea*), is one of the most destructive soybean diseases and results in annual yield losses of up to 300 million dollars in North America (Wrather and Koenning 2006) and over 1 billion dollars worldwide (Nicholls 2004).

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Sequence data for *Avr4/6* is available from the GenBank database under accession number ABS50087 and accession numbers for all other sequences are listed in the captions for Figures 2 and 7.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables and one supplementary figure are available online. Also, Figure 5 appears in color online.

The genome sequence of *P. sojae* and *P. ramorum* (Tyler et al. 2006) revealed over 700 genes encoding proteins with sequence similarity to Avr1b (Tyler et al. 2006; Jiang et al. 2008). The protein family shares a common N-terminal motif RXLR-dEER (Rehmany et al. 2005; Tyler et al. 2006; Jiang et al. 2008) that enables entry of these proteins into plant cells (Whisson et al. 2007; Dou et al. 2008a) in the absence of the pathogen (Dou et al. 2008a). The proteins also contain C-terminal motifs (Jiang et al. 2008) that are involved in interactions with cognate resistance (R) genes and in suppression of host programmed cell death (Dou et al. 2008b).

The *P. sojae* Avr4 and Avr6 genes were previously mapped to a 24-kb interval in the genome (Whisson et al. 2004). In this article, we describe the identification of a single gene, Avr4/6, that interacts genetically with both *Rps4* and *Rps6*. Avr4/6 encodes a small effector protein with an RXLR-dEER host targeting signal (HTS). Surprisingly, the HTS domain in the N terminus is sufficient to trigger an *Rps4*-dependent hypersensitive response (HR) while its C terminus is recognized by the *Rps6* gene product.

RESULTS

Molecular characterization

of the *P. sojae* locus conferring Avr4 and Avr6 phenotypes.

Whisson and associates (2004) previously reported that Avr4 and Avr6 completely cosegregated in an F2 population of 99 individuals, and identified a randomly amplified polymorphic DNA marker, OPE7.1C, 2.0 centimorgans distant from the two

genes. Further mapping appeared to localize the genes to a physical region of approximately 24 kb, though some recombinants used for mapping displayed complex recombination events. Annotation of a 60-kb region encompassing this locus (Tyler et al. 2006) revealed 17 putative genes, including six corresponding to cDNA clones identified by Whisson and associates (2004) and 5 transposon-like sequences (Fig. 1) (Supplementary Table S1). Two of these transposon-like sequences shown in the annotated genome (Tyler et al. 2006) are incomplete and actually represent the 5' and 3' ends of a single Copia-like retrotransposon at this locus, as reported by Basnayake and associates (2009) (Fig. 1). Seven of the nontransposon protein-coding genes had syntenic locations in the *P. ramorum* genome sequence (Fig. 1). Two small genes, encoding a 123-amino-acid (aa) protein (Ps6) and a 127-aa protein (Ps7), had no syntenic orthologs in the *P. ramorum* genome and showed no match to any transposon. Many effector genes in the *P. sojae* and *P. ramorum* genomes are not syntenic (Jiang et al. 2006; Tyler et al. 2006), making Ps6 and Ps7 possible candidate effector genes. Ps6 and Ps7 were 1.56 and 0.6 kb, respectively, from cDNA71, which marks the left end of the genetic interval identified by Whisson and associates (2004) to contain the Avr4 and Avr6 genes, placing them just outside this interval.

Identification of two genes encoding RXLR-dEER-containing proteins in the region of Avr4 and Avr6.

The 10 cloned oomycete avirulence genes all encode small secreted proteins with N-terminal RXLR and dEER motifs. To search for candidates for Avr4 and Avr6, the proteins encoded

Fig. 1. Conservation of synteny between *Phytophthora sojae* and *P. ramorum* Avr4/6 regions. Scaffold_12: 600000-660000 from *P. sojae* sequence version 1.1 and scaffold_4: 524000-505000 from *P. ramorum* sequence version 1.0 are shown. Arrows indicate positions, lengths, and transcriptional orientations of the predicted genes. The dashed line indicates genes with high sequence similarity between the two species. Unk = unknown function; ARP3, adapter-related protein complex 3; TPR, TPR domain containing protein; BRAP, similar to B-cell receptor-associated protein 29; myosin heavy chain; PLP1, polyprotein: retrotransposable element; Copia, copia-like retrotransposable element; PLP2, polyprotein: reverse transcriptase; WD40, WD40 repeat-containing protein; and MVD, mevalonate pyrophosphate decarboxylase. cDNAs are from Whisson and associates (2004). The genetic interval defined by Whisson and associates (2004) lies between cDNA71 and cDNA100. Note that the two Copia-like retrotransposable elements identified in the published draft *P. sojae* genome sequence are fragments that represent the 5' and 3' ends of the single, full-length Copia-like retrotransposable element of 4,980 bp reported by Basnayake and associates (2009), and the intervening sequence between them includes various fragments of this retroelement that were linked incorrectly during the assembly of Scaffold 12. Avh171 and Avh432 correspond to Ps6 and Ps7, respectively.

Table 1. Secreted and RXLR-dEER motif proteins predicted in the vicinity of the *Phytophthora sojae* Avr4/Avr6 locus

ORF ^a	Gene name or ID ^b	Annotation	SignalP 3.0 ^c	iPSORT prediction ^c	PSORT II prediction ^c	RXLR-dEER ^d
Ps3	130722	Unknown	0.963	—	—	—
Ps4	cDNA57	BRAP29	0.792	—	—	—
Ps6	Avh171 = Avr4/6	Unknown	0.998	+	+	+
Ps7	Avh432	Unknown	0.999	+	+	+
Ps16	cDNA100	Unknown	0.697	+	—	—

^a Open reading frame in the region.

^b Six-digit gene identification numbers match those at the VBI Microbial Database and DOE Joint Genome Institute. cDNA names match those of Whisson and associates (2004). Avh designations are those of Jiang and associates (2008).

^c Full-length protein sequences were screened for signal peptides by SignalP 3.0, iPSORT, and PSORT II.

^d Oomycete host translocation RXLR-dEER motifs (Jiang et al. 2008).

by the 17 genes in the region were tested for the presence of a predicted signal peptide using the SignalP 3.0 algorithm (Bendtsen et al. 2004). Of the 17 encoded proteins, five contained a potential signal peptide and, for 2 of them (Ps6 and Ps7), the prediction was supported by two other signal peptide prediction algorithms, iPSORT (Bannai et al. 2002) and PSORTII (Nakai and Horton 1999) (Table 1). Ps6 and Ps7 also contained RXLR and dEER motifs, and were designated Avh171 and Avh432 by Jiang and associates (2008). Ps6 (Avh171) and Ps7 (Avh432) share 31% similarity at the protein level. The RXLR and dEER motifs in Avh171 were spaced more widely apart (26 residues) (Fig. 2) than usual (three to 15 residues) (Jiang et al. 2008). To test the function of the RXLR-dEER region and to determine if the Avh171 protein could enter soybean cells, we created a fusion of the entire Avh171 coding region with the region of *Avr1b-1* encoding Avr1b's C terminus (Dou et al. 2008a). The fusion was introduced into *P. sojae* strain P7076 (race 19; virulent on *Rps1b* plants) by transformation. Two transformants strongly expressing the fusion gene were avirulent in the presence of *Rps1b*, indicating that the Avh171 protein could carry the C terminus of Avr1b into soybean cells (Dou et al. 2008a), and that the Avh171 RXLR-dEER domain was functional. Avh171 is expressed and is functional in P7076 (described below) and, therefore, does not, by itself, confer avirulence in the presence of *Rps1b*.

Transient expression of Avh171 but not Avh432 in soybean leaves triggers *Rps4*- and *Rps6*-dependent cell death.

Many avirulence gene products trigger cell death associated with the HR in the presence of their cognate resistance gene product. This cell death can be monitored by co-expression of a second reporter gene; usually a gene encoding β -glucuronidase (GUS) or green fluorescent protein (GFP) is used for this purpose (Orbach et al. 2000; Allen et al. 2004; Dodds et al. 2004; Rehmany et al. 2005; Catanzariti et al. 2006). We previously described a double-barreled attachment for the Bio-Rad Gene Gun that enables us to simultaneously bombard a DNA test sample and a control sample into two sides of a leaf with the same shot (Dou et al. 2008a and b); this innovation greatly improves the reproducibility of the results. To test whether Avh171, Avh432, or both were responsible for the avirulence of *P. sojae* isolates in the presence of *Rps4* or *Rps6*, we used the attachment to introduce DNA encoding Avh171 or Avh432 proteins lacking their secretory leaders (pUCAvr46 and pUCAvr432, respectively) into soybean leaves containing *Rps4* or *Rps6*, together with DNA encoding GUS. The number of tissue patches staining blue as a result of GUS expression was reduced approximately twofold by Avh171 expression compared with the empty vector (EV) in leaves containing the *Rps4* (L85-2352 or HARO4272) or *Rps6* (L89-1581 or HARO6272) genes (Fig. 3). In contrast, equal numbers of blue tissue patches were observed in leaves from cultivars lacking *Rps4* and *Rps6*, namely Williams (W, *rps*) and HARO1(1-7) (H, *rps*). The differences in ratios between the *Rps4* and *Rps6* cultivars compared with the *rps* cultivars were statistically significant, with a *P* value < 0.05 comparing HARO6272 and HARO1(1-7), and < 0.001 for the other three comparisons. Expression of Avh432 significantly reduced blue tissue patches relative to the side-by-side EV control bombardment not only in the leaves containing the *Rps4* (L85-2352) or *Rps6* genes (L89-1581) but also in the leaves lacking *Rps4* and *Rps6* (Williams, *rps*) (Fig. 3B). These results show that Avh171 induced significant HR-related cell death in the presence of both *Rps4* and *Rps6* whereas Avh432 triggered cell death unrelated to *Rps4* or *Rps6*. Based on this evidence, we designated Avh171 as the *Avr4/6* gene.

Transcription of *Avr4/6* is induced during infection.

A small, 37-bp intron was predicted in the transcript of *Avr4/6* as a result of the computational annotation of the *P. sojae* genome sequence (Tyler et al. 2006). To test this prediction and determine the structure of the transcript, we carried out 5' rapid amplification of cDNA ends (RACE) and 3' RACE analysis of RNA from *P. sojae* isolate P6497 (race 2, genotype I). The sequences from 5' RACE and 3' RACE products indicated that *Avr4/6* does not contain the predicted intron and the full deduced open reading frame was transcribed. A transcription start point 53 bp upstream from the ATG initiator codon was identified, and diverse 3' ends were identified, ranging from 137 to 185 bp downstream of the TAA stop codon (Supplementary Fig. S1).

To test whether *Avr4/6* transcription was induced during infection, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was carried out on RNA from a plant challenge experiment in which mycelial preparations were sandwiched between leaves of susceptible soybean plants (Williams, no *Rps* genes) for 6 h, after which the mycelia were removed for analysis; this method produces physiological responses in the pathogen very similar to natural infection (Chen et al. 2007). Mycelia from each of four isolates representing the major genotypes of *P. sojae* (Förster et al. 1994)—P6497 (genotype I), P7076 (genotype II), P7074 (genotype III), and P7063 (genotype IV)—were tested. P6497 and P7076 are avirulent on *Rps4* and *Rps6* plants while P7063 and P7074 are virulent. In all four isolates, *Avr4/6* messenger (m)RNA levels were induced (relative to *Actin*) after 6 h of contact with host tissue compared with control mycelia taken directly from axenic culture (Fig. 4) but the magnitude of induction varied greatly from twofold in P7063 to 31-fold in P6497. However, neither the induction nor the final level correlated with virulence, because P7074 and P7076, which differ in virulence, had near identical levels of *Avr4/6* mRNA before and after contact with host tissue. Induction of *Avr4/6* mRNA levels during *P. sojae* infection soybean hypocotyls was also observed using microarray (Dong et al. 2009) and Tagman PCR (S. Basnayake and D. Maclean, data not shown) assays.

Silencing of *Avr4/6* results in loss of the avirulence phenotype on soybean cultivars containing *Rps4* or *Rps6*.

In order to test whether *Avr4/6* was necessary for the avirulence of *P. sojae* isolates in the presence of *Rps4* or *Rps6*, we examined the *P. sojae* transformants of isolate P7076 (*Avr4/6*+) containing the Avh171-Avr1b (i.e., *Avr4/6*-Avr1bCt) fusion to determine if *Avr4/6* had been silenced in any of the transfor-

Fig. 2. Alignment of Avh171(Avr4/6) and Avh432 predicted proteins. The predicted signal peptides and RXLR and dEER motifs are underlined. Identical residues are shown and conservative amino acid differences are indicated with a +. The separate functional regions in Avh171(Avr4/6) triggering *Rps4*- or *Rps6*-dependent responses are indicated with white or shaded boxes, respectively. Avh171 (GenBank accession no. GU214061) and Avh432 (GenBank accession no. GU214065) correspond to Ps6 and Ps7, respectively.

ants; in *P. infestans*, sense constructs can be as effective in silencing target genes as antisense constructs (van West et al. 1999). Of five independent transformants containing the fusion gene (based on genomic PCR analysis) (Fig. 5A), two (A46-7 and A46-10) contained reduced levels of *Avr4/6* mRNA as judged by semiquantitative RT-PCR (Fig. 5B) and quantitative real-time RT-PCR (Fig. 5C). Two further *P. sojae* transformants of P7076 were obtained using a sense construct of *Avr4/6* which was not fused to *Avr1b-1* (pHamAvr4/6). One of these (P7076-2) also had reduced levels of *Avr4/6* mRNA (Fig. 5B and C).

To determine whether avirulence in the presence of *Rps4* or *Rps6* correlated with *Avr4/6* mRNA levels, we inoculated the three silenced transformants onto hypocotyls of soybean isolines containing *Rps4* (L85-2352) and *Rps6* (L89-1581) in a Williams background, as well as isolines containing *Rps4* (HARO4272) and *Rps6* (HARO6272) in a Harosoy background. All three silenced transformants could efficiently kill *Rps4*- or *Rps6*-containing soybean seedlings in both Williams and Harosoy backgrounds (Fig. 5D; Table 2). In contrast, the untransformed recipient *P. sojae* isolate, P7076, remained avirulent on the tested soybean cultivars containing *Rps4* or *Rps6* genes (Fig. 5D; Table 2). These results indicated that *Avr4/6* was necessary for avirulence on both *Rps4*- and *Rps6*-containing cultivars, supporting the hypothesis that the *Avr4/6* gene product was recognized by both the *Rps4* and *Rps6* gene products. The silenced lines did not display any other obvious phenotypes such as loss or gain of aggressiveness.

Rps4 and *Rps6* recognition require different regions of the *Avr4/6* protein.

Avr4/6 lacks the conserved C-terminal motifs found in half the predicted RXLR effector proteins of *P. sojae* and *P. ra-*

morum and also in *P. infestans Avr3a* (Jiang et al. 2008); those motifs are required for the functional interaction of *Avr1b* with *Rps1b* (Dou et al. 2008b). To identify the functional region of *Avr4/6*, we created four N-terminal and two C-terminal deletion mutants and tested the ability of the deletion mutants to trigger *Rps4*- and *Rps6*-dependent cell death in the bombardment assay.

Fig. 4. *Avr4/6* is induced after challenge of host tissue in both avirulent (*Avr4/6*+) and virulent (*Avr4/6*-) strains. Real time reverse-transcription quantitative polymerase chain reaction (PCR) analysis of *Avr4/6* transcript levels from the indicated four *Phytophthora sojae* isolates in mycelia used to challenge soybean leaves of cv. Williams (*rps*) 6 h postinoculation compared with inoculum (0 h) controls. Expression levels relative to actin were calculated after 6 h of contact with host tissue compared with control mycelia taken directly from axenic culture. The PCR reactions were replicated three times (i.e., technical replicates). Bars indicate standard errors.

Fig. 3. *Avr4/6* can trigger cell death in the presence of soybean *Rps4* and *Rps6*. **A** and **B**, Ratio of β -glucuronidase (GUS)-positive blue spots following bombardment with **A**, *Avr4/6* DNA (pUCAvr4/6) or **B**, *Avr432* DNA (pUCAvr432) compared with the empty vector DNA. Soybean leaves were bombarded using the double-barreled device; *Avr4/6* or *Avr432* DNA-bearing particles were delivered to one side of the leaf and control (empty vector, EV) DNA to the other; both sides received GUS DNA. Two sets of soybean near-isogenic lines with Williams (W) or Harosoy (H) genetic backgrounds were used, namely Williams (W, *rps*), L85-2352 (W, *Rps4*), L89-1581 (W, *Rps6*), HARO(1-7) (H, *rps*), HARO4272 (H, *Rps4*), and HARO6272 (H, *Rps6*). The number of pairs of shots used to calculate the averages and standard errors are given in parentheses. *P* values comparing results from cultivars with *rps* to *Rps4* or *Rps6* were calculated from the log ratios using the Wilcoxon rank sum test. **C**, Direct comparison of bombardment with *Avr4/6* and EV onto the same leaf of soybean cv. L85-2352 (*Rps4*). The line indicates the position of a divider that prevents particles from the two shots from overlapping.

The results demonstrated that only deletion proteins retaining the 32-aa region from positions 53 to 84 could trigger *Rps4*-mediated cell death. This region corresponds to the residues between the RXLR and dEER motifs, together with the dEER motif. Three deletion mutants (*Avr4/6-4*, *Avr4/6-5*, and *Avr4/6-6*) lacking this region did not produce a cell death response in *Rps4* plants (Figs. 2 and 6; Table 3). However, the same three deletion mutants could trigger *Rps6*-dependent cell death. On the other hand, a C-terminal deletion (*Avr4/6-7*) abolished the recognition by *Rps6* (Figs. 2 and 6; Table 3). This indicates that the 30-aa region from residue 84 to residue 113 located in the C terminus is required for a *Rps6*-mediated response.

Avr4/6 alleles from virulent isolates contain polymorphisms only in the 5' upstream region.

To identify polymorphisms in *Avr4/6* that may be associated with the loss of the avirulence phenotype in virulent races, we

cloned this gene, including 576 nucleotides (nt) upstream of the translational start codon and 491 nt downstream from the stop codon from seven *P. sojae* isolates: P6954 (race 1, *Avr4/6+*), P6497 (race 2, *Avr4/6+*), P7076 (race 19, *Avr4/6+*), P9073 (race 25, *Avr4/6+*; same isolate as UQ1200), P7063 (race 6, *Avr4/6-*), P7074 (race 17, *Avr4/6-*) (Förster et al. 1994), and PT2004C2.S1 (race 30, *Avr4/6-*) (Zhou et al. 2009). All four avirulent isolates have an identical sequence within the sequenced region, designated the *Avr4/6⁺* allele (Fig. 7). The alleles from virulent isolates P7063 (race 6) and PT2004C2.S1 (race 30) share an identical sequence that we designated *Avr4/6^{vir2}*, whereas P7074 (race 17) had a different sequence, designated *Avr4/6^{vir1}*. *Avr4/6^{vir2}* shows two nucleotide substitutions (–2C-to-T, and –33A-to-G) relative to that of *Avr4/6⁺* in the 5'-untranslated region (UTR) but no substitutions in the coding region. *Avr4/6^{vir1}* also shows the substitution of A to G at –33 but does not have the substitution of C to T at –2

Fig. 5. Silencing of *Avr4/6* in *Phytophthora sojae* transformants abolishes avirulence on soybean cultivars containing *Rps4* or *Rps6*. **A**, Verification of the transgene integration by polymerase chain reaction (PCR) of genomic DNA with oligonucleotides from Ham34 promoter and terminator regions; the transgene should produce an amplified fragment of 747 bp for transformants A46-7 and A46-10 or 960 bp for 7076-2. P7076 (race19, *Avr4+*, *Avr6+*) is the recipient *P. sojae* isolate; A46-7 and A46-10 indicate two independent transformants which received plasmid pHamAvr4/6-*Avr1bCt*; 7076-2 indicates a transformant which received plasmid pHamAvr4/6. Ck- indicates a no template control and L indicates a 100-bp ladder. **B**, *Avr4/6* RNA levels in the transformed strains. *Avr4/6* (upper panel, 35 cycles) and *P. sojae* actin (lower panel, 30 cycles) genes were assayed by semiquantitative reverse-transcription (RT)-PCR. **C**, Relative expression of silenced transformants compared with P7076 mycelia, measured by real-time RT-PCR analysis. Levels of *actin* messenger RNA were used as a reference. Relative expression level was calculated using the 7300 System Sequence Detection Software (version 1.4). PCR reactions were replicated three times. Bars indicate standard errors. **D**, Soybean seedlings inoculated on the hypocotyls with P7076 wild-type (WT) and *Avr4/6*-silenced transformant A46-7. L85-2352 and HARO4272 contain *Rps4* while L89-1581 and HARO6272 contain *Rps6*. Photographs were taken 5 days postinoculation.

Table 2. Characterization of *Phytophthora sojae* stable transformants silenced for *Avr4/6*

Strains ^b	Transgene present ^c	<i>Avr4/6</i> expression ^d	Avirulence (surviving seedlings) ^a		
			<i>rps</i>	<i>Rps4</i>	<i>Rps6</i>
P7076	N	Y	7/49 V	96/113 A	58/101 A
A46-7	Y	N	0/28 V	12/82 V	11/82 V
A46-10	Y	N	0/16 V	16/50 V	5/64 V
7076-2	Y	N	0/41 V	13/130 V	6/126 V

^a Avirulence of each strain was tested by inoculation of seedlings containing *Rps4* (L85-2352 and HARO4272), *Rps6* (L89-1581 and HARO6272), or no *rps* gene (Williams and HARO1(1-7)). Number of surviving seedlings/total inoculated seedlings is shown, summed from all replicates. A = avirulent and V = virulent.

^b P7076 is the untransformed recipient used to create transformants A46-7, A46-10, and 7076-2. A46-7 and A46-10 contain an *Avr4/6-Avr1b* fusion transgene while 7076-2 contains a wild-type *Avr4/6* sense transgene.

^c Presence of transgenes was verified by polymerase chain reaction (PCR). Y = transgene present and N = transgene not detected.

^d Transgene expression was determined by qualitative reverse-transcription (RT)-PCR and by quantitative RT-PCR. Y = transgene transcripts present and N = transgene transcripts not detected or level significantly reduced.

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Avr4/6 region is a recombination hotspot and there was also evidence suggestive of substantial gene conversion in the region in some of the F2 individuals used to map the order of SNP and phenotypic markers (Whisson et al. 2004). The physical distance that separates *Avr4/6* from the 24-kb region probably corresponds to 1 or 2 mis-scored SNP. cDNA 71 has an unlinked, near-identical paralog in the *P. sojae* genome sequence (Tyler et al. 2006) undetected by Whisson and associates (2004), and this could also have affected accurate scoring of the cDNA 71 SNP. Alternatively, we cannot rule out the possibility that there is another locus within the 24-kb region that controls expression of the *Avr4/6* phenotype in the presence of a functional *Avr4/6* gene, although there are no obvious candidates.

***P. sojae* Avr4/6 gene contains a functional RXLR-dEER motif for translocation into host cells.**

Oomycete RXLR effector proteins *Avr1b* from *P. sojae* (Dou et al. 2008a) and *Avr3a* from *P. infestans* (Whisson et al. 2007) have been demonstrated to enter host cells across the plasma cell membrane by means of their RXLR-dEER motifs, and *ATR1* (Rehmany et al. 2005) and *ATR13* (Allen et al. 2004) from *H. arabidopsidis* have been inferred to do so also. More recently, the avirulence gene products *Avr1a* (Qutob et al. 2009), *Avr3a* (Qutob et al. 2009), and *Avr3c* (Dong et al. 2009) from *P. sojae* and *Avr2* (Lokossou et al. 2009), *Avr4* (van Poppel et al. 2008), *Avr-blb1* (Pieterse et al. 1994; Vleeshouwers et al. 2008), and *Avr-blb2* (Oh et al. 2009) from *P. infestans* have also been identified as small secreted RXLR-dEER proteins. *Avr4/6* can functionally replace *Avr1b* RXLR-dEER motifs when a fusion of the two genes is expressed in *P. sojae* transformants (Dou et al. 2008a), indicating that *Avr4/6* also has the capability to enter into host cells during infection. Our results confirm that *Avr4/6* acts inside host cells, because deletions of the protein that remove the signal peptide still allow interaction with the *Rps4* or *Rps6* gene products when expressed inside host cells as a result of biolistic transformation (Figs. 3 and 6). This is consistent with the likelihood that *Rps4* and *Rps6* encode intracellular NBS-LRR proteins (Sandhu et al. 2004) (next section). The precise mechanism by which RXLR-dEER effectors enter plant cells is currently under active study. We have demonstrated that translocation of *Avr1b* can occur in the absence of any pathogen-encoded machinery (Dou et al. 2008a). One possibility is that RXLR-dEER proteins can take advantage of host processes, such as endocytosis, to enter host cells.

The *P. sojae* Avr4/6 gene product is recognized by two *R* gene products.

Avr4 and *Avr6* have been genetically analyzed in several individual studies and, in all cases, the two genes were genetically inseparable. Gijzen and associates (1996a) obtained 55 F2 progeny from a cross of race 2 (*Avr4/6+*) and race 7 (*Avr4/6-*). Of these, 41 individuals were avirulent in the presence of both *Rps4* and *Rps6* genes and 14 virulent on both, with no recombinants. May and associates (2002) analyzed two groups of 53 F2 individuals from two crosses (race 7 × race 16 and race 17 × race 25) for avirulence on *Rps4* and *Rps6*, and for molecular markers. No evidence for recombination was observed between *Avr4* and *Avr6* in any of those progeny. In an enlarged population of 99 F2 from the cross of race 7 × race 25 isolates, both genes co-segregated completely in the ratio 82:17 (avirulent/virulent) (Whisson et al. 2004). All those results indicated a single locus controlling both phenotypes. Our finding that a single *P. sojae* gene accounts for both the *Avr4* and *Avr6* phenotypes provides the simplest explanation of the apparent cosegregation of the two genetic loci. Two other closely linked pairs of avirulence gene loci have

been identified on the *P. sojae* genetic map (*Avr1b* and *Avr1k*, and *Avr3a* and *Avr5*) (May et al. 2002; Shan et al. 2004; Qutob et al. 2009), and it seems plausible that single *P. sojae* genes might underlie each of those two pairs of specificities.

Interestingly, *Rps4* and *Rps6* recognize different sequences within the *Avr4/6* coding sequence, despite the fact that it is a very small protein (102 aa in the mature protein), suggesting that their recognition capabilities evolved independently. Based on the bombardment data, *Rps6* appears to recognize the C terminus of *Avr4/6*, similar to the recognition of the C terminus of *Avr1b* by *Rps1b* (Dou et al. 2008b), of the C terminus of *P. infestans* *Avr3a* by potato *R3a* (Bos et al. 2006), and of the C terminus of *Avr-blb1* by *Rpi-Blb1* (Champouret et al. 2009). On the other hand, *Rps4* appears to recognize the unusually long region of *Avr4/6* that lies between its RXLR and dEER motifs. We do not yet know whether recognition of the *Avr4/6* protein by *Rps4* and *Rps6* occurs by direct protein-protein interactions or by an indirect “guard” mechanism.

Overlapping recognition specificities have been reported in the interaction between flax and flax rust (*Melampsora lini*). The *M. lini* *AvrL567-A* gene product is recognized by three flax resistance genes: *L5*, *L6*, and *L7*. The product of the paralogous *AvrL567-B* is recognized most strongly by *L5* but only weakly by *L6* and not all by *L7*. The product of a third paralogue, *AvrL567-C*, is not recognized at all by *L5*, *L6*, and *L7*, despite the fact that *AvrL567-C* is more similar to *AvrL567-B* than *AvrL567-A* is to *AvrL567-B* (Catanzariti et al. 2006). In soybean, *Rps4* and *Rps6* also map to the same genetic locus and, thus, may be allelic. Sandhu and associates (2004) showed that isolines containing *Rps4* and *Rps6* contained a similar distinct cluster of NBS-LRR resistance gene analogs (RGA), and mapped the cluster to *Rps4*. During mapping, three distinct lines were identified in which most of the RGA had been lost, along with the *Rps4* function. Thus, *Rps4* and *Rps6* were inferred to be either allelic or clustered NBS-LRR genes. Considering that *Rps4* and *Rps6* recognize different regions of the *Avr4/6* protein, the recognition specificities of *Rps4* and *Rps6* may have arisen in paralogous (or the same) ancestral genes by convergent evolution. Alternatively, an *R* gene product ancestral to *Rps4* and *Rps6* might have recognized *Avr4/6* via contacts at both the N terminus and C terminus of the effector; subsequently, as mutations accumulated in *Avr4/6*, weakening recognition, independent variants of the ancestral *R* gene might have arisen with strengthened contacts in either the N terminus (i.e., *Rps4*) or C terminus (i.e., *Rps6*). Previously, differences in temperature responses between *Rps4*- and *Rps6*-mediated defenses were found, supporting our inference that *Rps4* and *Rps6* are different genes; Harosoy isolines containing *Rps6* were fully susceptible at 33°C while *Rps4* lines were only partially susceptible (Gijzen et al. 1996b). The future cloning of the *Rps4* and *Rps6* genes will help us to understand the specificity of the perception and uncover the mechanisms by which the genes trigger disease resistance.

Polymorphism and evolution.

Pathogens have evolved several distinct mechanisms to escape the recognition of their effectors by host defense systems. For instance, in fungal pathogens, loss of avirulence genes has occurred by partial or complete deletions (Van den Ackerveken et al. 1992; Orbach et al. 2000) or point mutations (Joosten et al. 1994; Dodds et al. 2004; Catanzariti et al. 2006). In oomycetes, amino acid substitutions have been observed in alleles of *Avr1b-1* (Shan et al. 2004), *Avr3a* (Armstrong et al. 2005), *Atr1* (Rehmany et al. 2005), and *Atr13* (Allen et al. 2004) from virulent races. In addition, *Avr1b-1* exhibits transcriptional polymorphism, in which *Avr1b-1* mRNA fails to accumulate although there are no substitutions in *Avr1b-1* coding or pro-

motor regions (Shan et al. 2004). In *P. infestans*, *Avr4* exhibits frame-shift mutations in the virulent alleles (van Poppel et al. 2008). *Avr4/6* is unusual in that there is no amino acid sequence variation among the alleles or transcriptional variation that correlates with the avirulence phenotype. Instead, we identified deletion and substitution mutations in the region upstream of the *Avr4/6* translational start site, including one, –33A-to-G, that was present in the 5'-UTR of all the virulence alleles. One possible explanation is that –33A-to-G may cause an alteration in translational regulation or efficiency of *Avr4/6* mRNA. Polymorphisms in the 5'-UTR regions cause reduction of protein expression in plant (Smith et al. 2004; Lee et al. 2006) and human (Ludwig et al. 2002) genes. This possibility will need to be tested experimentally in the case of *Avr4/6*. An alternative possibility, mentioned previously, is that another closely linked locus within the 24-kb region controls expression of the *Avr4/6* phenotype even when an intact *Avr4/6* gene is present and is transcribed. Shan and associates (2004) found that expression of *Avr1b-1* was controlled by a nearby locus, *Avr1b-2*, in some isolates that failed to express the *Avr1b* phenotype; in that case, expression was controlled at the level of transcript accumulation. In principle, coding region mutations might occur that differentially affect recognition by *Rps4* or *Rps6* plants. However, the only isolate reported to be recognized by *Rps4* but not by *Rps6*, P7361 (race 13), had no coding region mutations and did not consistently display differential recognition by *Rps4* and *Rps6*.

Function of *Avr4/6* effector protein in pathogenesis.

Bacterial effectors modulate diverse host processes to enhance virulence, including suppression of pathways of innate immunity and alteration of plant hormone signaling. Similar information is beginning to accumulate for oomycete effector proteins, including ATR1 and ATR13 (Sohn et al. 2007), *Avr3a* (Bos et al. 2006), and *Avr1b* (Dou et al. 2008b). In particular, several of the effectors that can suppress programmed cell death contain conserved C-terminal motifs found in more than half of the candidate *P. sojae* and *P. ramorum* RXLR effectors proteins (Dou et al. 2008b; Jiang et al. 2008). However, *Avr4/6* does not contain the motifs and, unlike effectors with the conserved C-terminal motifs, cannot suppress programmed cell death triggered by the mouse BAX protein (Dou et al. 2008b). Therefore, *Avr4/6* is likely to contribute to pathogenesis via a different mechanism. If *Avr4/6* does contribute to *P. sojae* pathogenicity, it is likely to be functionally redundant, because transformants silenced for *Avr4/6* remained fully pathogenic on soybean.

MATERIALS AND METHODS

Bioinformatic analyses.

The *P. sojae* and *P. ramorum* genome sequences and predicted gene models were accessed at the Virginia Bioinformatics Institute (VBI) Microbial Database website. Functional analyses of each predicted gene were based on assessment of BLAST and FASTA searches against public databases. The similarity of any two genes was determined by comparison of predicted protein sequences using Align. Signal peptides were identified by the SignalP 3.0 algorithm (Bendtsen et al. 2004) and validated by iPSORT (Bannai et al. 2002) and PSORTII (Nakai and Horton 1999).

Identification of polymorphisms.

The DNA sequences of the *Avr4/6* (= *Avh171*) and *Avh432* genes were determined by the VBI Core Laboratory Facility in the following *P. sojae* isolates: P6954 (race 1, *Avr4/6+*), P6497 (race 2, *Avr4/6+*), P7063 (race 6, *Avr4/6–*), P7076 (race 19,

Avr4/6+), P7074 (race 17, *Avr4/6–*), P7361 (race 13, *Avr4/6+*) (Förster et al. 1994), P9073 (UQ1200, race 25, *Avr4/6+*) (Whisson et al. 1995), and PT2004C2.S1 (race 30, *Avr4/6–*) (Zhou et al. 2009), where *Avr4/6+* indicates the isolate was avirulent on both *Rps4* and *Rps6* plants and *Avr4/6–* indicates virulence on plants lacking both *Rps* genes. DNA fragments amplified from each isolate with the oligonucleotides *Avr4PolyF* (all oligonucleotides used are listed in Supplementary Table S2) and *Avr4PolyR* were cloned into the sequence vector pCR2.1-TOPO (TOPO TA cloning vector; Invitrogen Inc., Shanghai, China) and sequenced by the VBI Core Laboratory Facility using the Sanger method. *Avh432* alleles were cloned using the combination of *Ps7PolyF* and *Ps7R* primers and sequenced as described above. To investigate polymorphisms in an additional 55 *P. sojae* isolates from the United States ($n = 3$) and China ($n = 52$), PCR products amplified from genomic DNA using *Avr4PolyF* and *Avr4PolyR* were cloned into pMD19 vector and sequenced by Invitrogen Inc. The sequences of the four alleles identified by this screen and their GenBank accession numbers are listed in Figure 7.

Plasmids and strain construction.

P. sojae transformation plasmids, described by Dou and associates (2008a), were as follows: pUN (*NptII* gene driven by *P. sojae* rpL41 promoter); pHamAvh171 (full sequence of *Avr4/6* gene [ABS50087, originally called *Avh171*] fused with the C-terminal domain of *Avr1b-1*, driven by Ham34 promoter and terminator); plant transient expression plasmids pUCAvr1b and pUCAvr46 (mature *Avr4/6* gene driven by *Cauliflower mosaic virus* [CaMV] 35S promoter); and pUCGUS (GUS plus gene driven by CaMV 35S promoter). Candidate gene *Ps7* (*Avh432*) was amplified with primers *Ps7F* and *Ps7R*. The *Avh432* amplicon was digested with *XmaI/KpnI* and inserted into plant transient expression plasmid pUCAvr1b (replacing *Avr1b-1* with *Avh432*, under control of a 35S CaMV promoter, creating pUCAvr432). pHamAvr4/6 (full sequence of *Avr4/6* gene driven by Ham34 promoter and terminator) was created by inserting *KpnI*-digested PCR products amplified with the combinations of the primers *avr4F3* and *avr4R2* into *KpnI*-digested pHamT35 (Judelson et al. 1991). To construct *Avr4/6* gene deletion mutants for plant transient expression assays, PCR products amplified using *Avr4-50F* and *Avr4R* were digested with *XmaI/KpnI* and inserted into *XmaI/KpnI*-digested pUCAvr1b (Dou et al. 2008b) to obtain pUCAvr4/6-2. Similarly, we obtained pUCAvr4/6-3 using *Avr4-54F* and *Avr4R*, pUCAvr4/6-4 using *Avr4-78F* and *Avr4R*, pUCAvr4/6-5 using *Avr4-85F* and *Avr4R*, pUCAvr4/6-6 using *Avr4-85F* and *Avr4-113R*, and pUCAvr4/6-7 using *Avr4F* and *Avr4-85R*. All the above plasmid constructs were validated by sequencing in the VBI Core Laboratory Facility.

Particle bombardment assays

of *Rps4*- and *Rps6*-mediated cell death.

Particle bombardment assays were carried out using a double-barreled extension of the Bio-Rad He/1000 Particle Delivery System (Dou et al. 2008b). To quantitate the activity of *Avr4/6* candidates and constructs in triggering *Rps4*- and *Rps6*-dependent cell death, DNA carrying the constructs (1.7 µg per shot) was co-bombarded into soybean leaves along with DNA carrying a GUS reporter gene (1.7 µg per shot). Specific cell death activity was measured as the reduction in the number of blue-staining GUS-positive spots in leaves carrying *Rps4* or *Rps6* 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 *Avr4/6* 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 the *Rps4* or *Rps6* leaves were then compared with those from the non-*Rps4/6* leaves using the Wilcoxon rank sum test (Dou et al. 2008b).

***P. sojae* strains, manipulation, and inoculation assay.**

P. sojae isolates were grown on V8 agar (Erwin and Ribiero 1996). *P. sojae* transformation was as described (Dou et al. 2008b). Putative *P. sojae* transformants were screened for *Avr4/6* transgenes by amplifying genomic DNA from each line (Dou et al. 2008b).

The avirulence phenotypes of selected transformants were evaluated by hypocotyl inoculation (Tyler et al. 1995) using soybean cvs. HARO(1-7) (*rps*), HARO4272 (Harosoy background, *Rps4*), HARO6272 (Harosoy background, *Rps6*), Williams (*rps*), L85-2352 (Williams background, *Rps4*), and L89-1581 (Williams background, *Rps6*). Each avirulence determination was repeated at least three times. A strain was considered avirulent if more than 50% of the inoculated seedlings survived, and the number surviving was significantly higher than among the seedlings lacking the *Rps* gene, as determined by Fisher's exact test (Sokal and Rohlf 1995).

Assay of *Avr4/6* mRNA levels.

For 5' and 3' RACE, RNA samples, prepared from V8-grown mycelia using the Qiagen RNeasy plant mini kit, were treated twice with RNase-free DNase (Qiagen), once in a microfuge tube and once on an RNeasy mini column, to remove contaminating DNA. The quality and quantity of the RNA was checked in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). The RACE reactions were conducted using the 5'-Full RACE kit (D315; Takara, China) and 3'-Full RACE Core Set (D314; Takara) with the combinations of the provided primers and two inner primers, respectively. Then, the PCR products from both reactions were cloned into pMD19 vector and sequenced by Invitrogen Inc.

To measure *Avr4/6* mRNA levels during infection using qRT-PCR, four *P. sojae* isolates—P6497 (genotype I), P7076 (genotype II), P7074 (genotype III), and P7063 (genotype IV) (Förster et al. 1994)—were cultured in 10% V8 broth in 90-mm petri dishes for 3 days. Seedlings of the soybean cv. Williams were grown in the greenhouse for 7 days (Dou et al. 2008a). The primary leaves were harvested and inoculated using the "leaf-sandwich" method (Chen et al. 2007) as follows: the leaves were washed once with 0.05% Tween 20, then with distilled water. Mycelia were carefully placed on the leaves with forceps and any liquid was removed with pipette. Then, the mycelia were covered with another leaf, placed in a petri dish sealed with parafilm, and incubated at 25°C in the darkness. After 6 h, the mycelia were harvested using forceps, discarding the leaves. RNA samples were prepared from the 6-h-harvested mycelia and from control (0 h) inoculum, and transcribed to cDNA using the PrimeScript RT-PCR kit (Takara). qRT-PCR was performed in 20-μl reactions including 20 ng of cDNA, 0.2 μM gene-specific primer for *Avr4/6* or reference *Actin* gene, 10 μl of SYBR Premix ExTaq (TaKaRa), and 6.8 μl of distilled H₂O. PCR reactions were performed on a ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) under the following conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 31 s to calculate cycle threshold values; followed by a dissociation program of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain melt curves. The 7300 System Sequence Detection Software (version 1.4) was used to obtain the expression level of each sample relative to the *Actin* control.

For screening transformants for silencing of *Avr4/6*, total RNA was extracted from mycelia from wild-type and putative transformants using the RNeasy plant mini kit (Qiagen) with β-mercaptoethanol added to buffer RLT, and genomic DNA was removed using RNase-Free DNase (Qiagen). RNA was quantified using a Nanodrop ND-1000 spectrophotometer. *Avr4/6* mRNA levels were initially evaluated by semiquantitative RT-PCR using the internal primers *Avr4ReF* and *Avr4ReR* following the programs of 35 cycles of 45 s at 94°C, 45 s at 56°C, and 60 s at 72°C, with the *Actin* gene as the reference. Three of the *Avr4/6*-silenced transformants were further validated by quantitative real-time RT-PCR assay with the same steps as above except using the Bio-Rad i-cycler (BioRad, Hercules, CA, U.S.A.) and SYBR Green I PCR Mastermix (Applied Biosystems).

ACKNOWLEDGMENTS

We thank A. McLeod and W. Fry (Cornell University) for providing their *P. sojae* transformation protocol prior to publication, and T. Anderson (Agriculture Canada) and S. Maroof (Virginia Tech) for soybean seed. This work was supported by grants to B. M. Tyler from the National Research Initiative of the United States Department of Agriculture Cooperative State Research, Education and Extension Service, grant numbers 2002-35600-12747 and 2007-35319-18100, and from the United States National Science Foundation, grant number MCB-0242131, by funds from the VBI, and by funds (to D. Dou) from Key Project of Chinese Ministry of Education (E200909). Funding for S. Basnayake, S. Whisson, A. Drenth, and D. Maclean was provided by the Australian Research Council and the Cooperative Research Centre for Tropical Plant Protection; S. Whisson is currently supported by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD).

LITERATURE CITED

- Allen, R. L., Bittner-Eddy, P. D., Grenville-Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E., and Beynon, J. L. 2004. Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. *Science* 306:1957-1960.
- Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I. B., Venter, E., Avrova, A. O., Rehmany, A. P., Bohme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Frasers, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. J. 2005. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 102:7766-7771.
- Bannai, H., Tamada, Y., Maruyama, O., Nakai, K., and Miyano, S. 2002. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* 18:298-305.
- Basnayake, S., Maclean, D. J., Whisson, S. C., and Drenth, A. 2009. Identification and occurrence of the LTR-copia-like retrotransposon, PSCR and other copia-like elements in the genome of *Phytophthora sojae*. *Curr. Genet.* 55:521-536.
- Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340:783-795.
- Bos, J. I. B., Kanneganti, T. D., Young, C., Cakir, C., Huitema, E., Win, J., Armstrong, M. R., Birch, P. R. J., and Kamoun, S. 2006. The C-terminal half of *Phytophthora infestans* RXLR effector *Avr3a* is sufficient to trigger *R3a*-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*. *Plant J.* 48:165-176.
- Catanzariti, A. M., Dodds, P. N., Lawrence, G. J., Ayliffe, M. A., and Ellis, J. G. 2006. Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell* 18:243-256.
- Chamnanpant, J., Shan, W. X., and Tyler, B. M. 2001. High frequency mitotic gene conversion in genetic hybrids of the oomycete *Phytophthora sojae*. *Proc. Natl. Acad. Sci. U.S.A.* 98:14530-14535.
- Chen, X. R., Shen, G., Wang, Y. L., Zheng, X. B., and Wang, Y. C. 2007. Identification of *Phytophthora sojae* genes upregulated during the early stage of soybean infection. *FEMS (Fed. Eur. Microbiol. Soc.) Lett.* 269:280-288.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Ayliffe, M. A., and Ellis, J. G. 2004. The *Melampsora lini* *AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell* 16:755-768.

- Dong, S., Qutob, D., Tedman-Jones, J., Kuflu, K., Wang, Y., Tyler, B. M., and Gijzen, M. 2009. The *Phytophthora sojae* avirulence locus *Avr3c* encodes a multi-copy RXLR effector with sequence polymorphisms among different pathogen strains. *PLoS ONE* 4:e5556.
- Dou, D., Kale, S. D., Wang, X., Jiang, R. H. Y., Bruce, N. A., Arredondo, F. D., Zhang, X., and Tyler, B. M. 2008a. RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20:1930–1947.
- Dou, D., Kale, S. D., Wang, X., Chen, Y., Wang, Q., Wang, X., Jiang, R. H. Y., Arredondo, F. D., Anderson, R., Thakur, P., McDowell, J., Wang, Y., and Tyler, B. M. 2008b. Carboxy-terminal motifs common to many oomycete RXLR effectors are required for avirulence and suppression of BAX-mediated programmed cell death by *Phytophthora sojae* effector Avr1b. *Plant Cell* 20:1118–1133.
- Erwin, D. C., and Ribiero, O. K. 1996. *Phytophthora* Diseases Worldwide. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Förster, H., Coffey, M. D., Elwood, H., and Sogin, M. L. 1990. Sequence analysis of the small subunit ribosomal RNAs of 3 zoospore fungi and implications for fungal evolution. *Mycologia* 82:306–312.
- Förster, H., Tyler, B. M., and Coffey, M. D. 1994. *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. *Mol. Plant-Microbe Interact.* 7:780–791.
- Gao, H., Narayanan, N. N., Ellison, L., and Bhattacharyya, M. K. 2005. Two classes of highly similar coiled coil-nucleotide binding-leucine rich repeat genes isolated from the *Rps1-k* locus encode *Phytophthora* resistance in soybean. *Mol. Plant-Microbe Interact.* 18:1035–1045.
- Gijzen, M., Förster, H., Coffey, M. D., and Tyler, B. M. 1996a. Cosegregation of *Avr4* and *Avr6* in *Phytophthora sojae*. *Can. J. Bot.* 74:800–802.
- Gijzen, M., Macgregor, T., Bhattacharyya, M., and Buzzell, R. 1996b. Temperature induced susceptibility to *Phytophthora sojae* in soybean isolines carrying different *Rps* genes. *Physiol. Mol. Plant Pathol.* 48:209–215.
- Harper, J. T., Waanders, E., and Keeling, P. J. 2005. On the monophyly of chromalveolates using a six-protein phylogeny of eukaryotes. *Int. J. Syst. Evol. Microbiol.* 55:487–496.
- Jiang, R. H., Tyler, B. M., and Govers, F. 2006. Comparative analysis of *Phytophthora* genes encoding secreted proteins reveals conserved synteny and lineage-specific gene duplications and deletions. *Mol. Plant-Microbe Interact.* 19:1311–1321.
- Jiang, R. H. Y., Tripathy, S., Govers, F., and Tyler, B. M. 2008. RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving super-family with more than 700 members. *Proc. Natl. Acad. Sci. U.S.A.* 105:4874–4879.
- Joosten, M. H., Cozijnsen, T. J., and De Wit, P. J. 1994. Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature* 367:384–386.
- Judelson, H., Tyler, B. M., and Michelsmore, R. W. 1991. Transformation of the oomycete pathogen, *Phytophthora infestans*. *Mol. Plant-Microbe Interact.* 4:602–607.
- Lee, J. Y., Colinas, J., Wang, J. Y., Mace, D., Ohler, U., and Benfey, P. N. 2006. Transcriptional and posttranscriptional regulation of transcription factor expression in *Arabidopsis* roots. *Proc. Natl. Acad. Sci. U.S.A.* 103:6055–6060.
- Leitz, R. A., Hartman, G. L., Pedersen, W. L., and Nickell, C. D. 2000. Races of *Phytophthora sojae* on soybean in Illinois. *Plant Dis.* 84:487.
- Lokossou, A. A., Park, T.-H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., and Whisson, S. C., Birch, P. R. J., Visser, R. G. F., Jacobsen, E., and van der Vossen, E. A. G. 2009. Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. *Mol. Plant-Microbe Interact.* 22:630–641.
- Ludwig, E. H., Mahley, R. W., Palaoglu, E., Ozbayrakci, S., Balestra, M. E., Borecki, I. B., Innerarity, T. L., and Farese, R. V., Jr. 2002. Dgat1 promoter polymorphism associated with alterations in body mass index, high density lipoprotein levels and blood pressure in Turkish women. *Clin. Genet.* 62:68–73.
- MacGregor, T., Bhattacharyya, M., Tyler, B. M., Bhat, R., Schmitthenner, A. F., and Gijzen, M. 2002. Genetic and physical mapping of *Avr1a* in *Phytophthora sojae*. *Genetics* 160:949–959.
- May, K. J., Whisson, S. C., Zwart, R. S., Searle, I. R., Irwin, J. A. G., Maclean, D. J., Carroll, B. J., and Drenth, A. 2002. Inheritance and mapping of eleven avirulence genes in *Phytophthora sojae*. *Fungal Genet. Biol.* 37:1–12.
- Nakai, K., and Horton, P. 1999. Psort: A program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* 24:34–36.
- Nicholls, H. 2004. Stopping the rot. *PLoS Biol* 2:E213.
- Oh, S. K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I., Liu, H. Y., van Damme, M., Morgan, W., Choi, D., van der Vossen, E. A., Vleeshouwers, V. G., and Kamoun, S. 2009. In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. *Plant Cell* 21:2928–2947.
- Orbach, M. J., Farrall, L., Sweigard, J. A., Chumley, F. G., and Valent, B. 2000. A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. *Plant Cell* 12:2019–2032.
- Pieterse, C. M. J., Van West, P., Verbakel, H. M., Brasse, P. W. H. M., Van Den Berg-Velthuis, G. C. M., and Govers, F. 1994. Structure and genomic organization of the IpiB and IpiO gene clusters of *Phytophthora infestans*. *Gene* 138:67–77.
- Qutob, D., Tedman-Jones, J., Dong, S., Kuflu, K., Pham, H., Wang, Y., Dou, D., Kale, S. D., Arredondo, F. D., Tyler, B. M., and Gijzen, M. 2009. Copy number variation and transcriptional polymorphisms of *Phytophthora sojae* RXLR effector genes *Avr1a* and *Avr3a*. *PLoS ONE* 4:e5066.
- Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R., and Beynon, J. L. 2005. Differential recognition of highly divergent downy mildew avirulence gene alleles by *Rpp1* resistance genes from two *Arabidopsis* lines. *Plant Cell* 17:1839–1850.
- Ryley, M. J., Obst, N. R., Irwin, J. A. G., and Drenth, A. 1998. Changes in the racial composition *Phytophthora sojae* in Australia between 1979 and 1996. *Plant Dis.* 82:1048–1054.
- Sandhu, D., Gao, H., Cianzio, S., and Bhattacharyya, M. K. 2004. Deletion of a disease resistance nucleotide-binding-site leucine-rich repeat-like sequence is associated with the loss of the *Phytophthora* resistance gene *Rps4* in soybean. *Genetics* 168:2157–2167.
- Schneider, D. S. 2002. Plant immunity and film noir: what gumshoe detectives can teach us about plant-pathogen interactions. *Cell* 109:537–540.
- Shan, W. X., Cao, M., Leung, D., and Tyler, B. M. 2004. The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene *Rps1b*. *Mol. Plant-Microbe Interact.* 17:394–403.
- Smith, S. M., Fulton, D. C., Chia, T., Thorncroft, D., Chapple, A., Dunstan, H., Hylton, C., Zeeman, S. C., and Smith, A. M. 2004. Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis* leaves. *Plant Physiol.* 136:2687–2699.
- Sohn, K. H., Lei, R., Nemri, A., and Jones, J. D. 2007. The downy mildew effector proteins Atr1 and Atr13 promote disease susceptibility in *Arabidopsis thaliana*. *Plant Cell* 19:4077–4090.
- Sokal, R. R., and Rohlf, F. J. 1995. Biometry. The Principles and Practice of Statistics in Biological Research. W.H. Freeman and Company, New York.
- Stacey, G. 2008. Soybean Genomics. Springer, New York.
- Tyler, B. M. 2007. *Phytophthora sojae*: Root rot pathogen of soybean and model oomycete. *Mol. Plant Pathol.* 8:1–8.
- Tyler, B. M. 2008. Genomics of fungal- and oomycete-soybean interactions. Pages 243–267 in: Soybean Genomics. G. Stacey, eds. Springer, New York.
- Tyler, B. M., Förster, H., and Coffey, M. D. 1995. Inheritance of avirulence factors and restriction-fragment-length-polymorphism markers in outcrosses of the oomycete *Phytophthora sojae*. *Mol. Plant-Microbe Interact.* 8:515–523.
- Tyler, B. M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R. H., Aerts, A., Arredondo, F. D., Baxter, L., Bensasson, D., Beynon, J. L., Chapman, J., Damasceno, C. M., Dorrance, A. E., Dou, D., Dickerman, A. W., Dubchak, I. L., Garbelotto, M., Gijzen, M., Gordon, S. G., Govers, F., Grunwald, N. J., Huang, W., Ivors, K. L., Jones, R. W., Kamoun, S., Krampis, K., Lamour, K. H., Lee, M. K., McDonald, W. H., Medina, M., Meijer, H. J., Nordberg, E. K., Maclean, D. J., Ospina-Giraldo, M. D., Morris, P. F., Phuntumart, V., Putnam, N. H., Rash, S., Rose, J. K., Sakihama, Y., Salamov, A. A., Savidor, A., Scheuring, C.F., Smith, B. M., Sobral, B. W., Terry, A., Torto-Alalibo, T. A., Win, J., Xu, Z., Zhang, H., Grigorov, I. V., Rokhsar, D. S., and Boore, J. L. 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313:1261–1266.
- Van den Ackerveken, G. F. J. M., van Kan, J. A. L., and de Wit, P. J. G. M. 1992. Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *Plant J.* 2:359–366.
- van Poppel, P. M. J. A., Guo, J., van de Vondervoort, P. J. I., Jung, M. W. M., Birch, P. R., Whisson, S. C., and Govers, F. 2008. The *Phytophthora infestans* avirulence gene *Avr4* encodes an RXLR-DEER effector. *Mol. Plant-Microbe Interact.* 21:1460–1470.
- van West, P., Kamoun, S., van 't Klooster, J. W., and Govers, F. 1999. Inter-nuclear gene silencing in *Phytophthora infestans*. *Mol. Cell* 3:339–348.
- Vleeshouwers, V. G. A. A., Rietman, H., Krennek, P., Champouret, N.,

- Young, C., Oh, S.-K., Wang, M., Bouwmeester, K., Vosman, B., Visser, R. G. F., Jacobsen, E., Govers, F., Kamoun, S., and van der Vossen, E. A. G. 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *PLoS ONE* 3:e2875.
- Whisson, S. C., Drenth, A., Maclean, D. J., and Irwin, J. A. 1994. Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Curr. Genet.* 27:77-82.
- Whisson, S. C., Drenth, A., Maclean, D. J., and Irwin, J. A. G. 1995. *Phytophthora sojae* avirulence genes, RAPD and RFLP markers used to construct a detailed genetic linkage map. *Mol. Plant-Microbe Interact.* 8:988-995.
- Whisson, S. C., Basnayake, S., Maclean, D. J., Irwin, J. A., and Drenth, A. 2004. *Phytophthora sojae* avirulence genes *Avr4* and *Avr6* are located in a 24kb, recombination-rich region of genomic DNA. *Fungal Genet. Biol.* 41:62-74.
- Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., West, P. V., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. J. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450:115-119.
- Wrather, J. A., and Koenning, S. R. 2006. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *J. Nematol.* 38:173-180.
- Zhou, L., Mideros, S. X., Bao, L., Hanlon, R., Arredondo, F. D., Tripathy, S., Krampis, K., Jerauld, A., Evans, C., St. Martin, S. K., Maroof, S. M. A., Hoeschele, I., Dorrance, A. E., and Tyler, B. M. 2009. Infection and genotype remodel the entire soybean transcriptome. *BMC Genomics* 10:49.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Department of Energy Joint Genome Institute's *Phytophthora sojae* genome database: www.jgi.doe.gov
 iPSORT service: hc.ims.u-tokyo.ac.jp/iPSORT
 National Center for Biotechnology Information Blast alignment: www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
 SignalP 3.0 server: www.cbs.dtu.dk/services/SignalP
 PSORTII Prediction: psort.nibb.ac.jp/form2.html
 The Virginia Bioinformatics Institute microbial database: vmd.vbi.vt.edu