Deletion of the *Phytophthora sojae* Avirulence Gene *Avr1d* Causes Gain of Virulence on *Rps1d*

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Phytophthora sojae is an oomycete and a pathogen of soybean that causes root rot. During infection *P. sojae* delivers effector proteins into host cells to foster disease. However, effector-triggered immunity (ETI) results when pathogen factors are recognized by host resistance (R) proteins. We have now identified the *P. sojae* *Avr1d* gene, which encodes a predicted effector protein with the amino acid motif Arg-X-Leu-Arg (RXLR). Genetic mapping of 16 different *P. sojae* isolates and of a segregating F₂ population of 40 individuals shows that the predicted RXLR effector gene *Avh6* precisely cosegregates with the *Avr1d* phenotype. Transient expression assays confirm that *Avr1d* triggers cell death specifically in *Rps1d* soybean plants. The *Avr1d* gene is present in *P. sojae* strains that are avirulent on *Rps1d*, whereas the gene is deleted from the genome of virulent strains. Two sequence variants of the *Avr1d* gene encoding different protein products occur in *P. sojae* strains, but both are recognized by *Rps1d* and cause ETI. Liposome binding assays show that *Avr1d* has affinity for phosphatidylinositol 4-phosphate and that binding can be disrupted by mutation of lysine residues in the carboxy-terminal effector domain of the protein. The identification of *Avr1d* aids pathogen diagnostics and soybean cultivar development.

Plant pathogens must overcome preformed and induced defenses in order to successfully colonize and reproduce on host plants. Induced host-plant defenses are activated by danger signals that arise from pathogen growth (Thomma et al. 2011). Effector proteins are secreted by pathogens to suppress host defenses and foster disease (Bozkurt et al. 2012). Nonetheless, pathogen effectors can become self-defeating when plant immune systems recognize their presence. This interaction can be genetically tracked in the host and pathogen. In such cases, host plant resistance (*R*) genes and pathogen avirulence (*Avr*) genes encode the immune and effector system components that determine the disease outcome, either resistance or susceptibility (Jones and Dangl 2006).

Nucleotide sequence data are available in the GenBank database under accession numbers KC004057 to KC004058.

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The interaction between soybean and the root rot pathogen *Phytophthora sojae* follows the *R*- and *Avr*-gene model (Gijzen and Qutob 2009). Soybean *R* genes that confer strain-specific resistance to *P. sojae* are known as *Rps* genes. Complementary *P. sojae* *Avr* genes encode effector proteins that potentially can elicit rapid and robust host immune responses via a process known as effector triggered immunity (ETI). The identification of *Avr* genes from *P. sojae* and other oomycete plant pathogens provides practical tools for crop breeding, pathogen diagnostics, and disease management (Vleeshouwers et al. 2011; Rietman et al. 2012). Known *P. sojae* *Avr* genes include *Avr1a* (Qutob et al. 2009), *Avr1b* (Shan et al. 2004), *Avr3a/5* (Dong et al. 2011a), *Avr3b* (Dong et al. 2011b), *Avr3c* (Dong et al. 2009), and *Avr4/6* (Dou et al. 2010). Each of these *Avr* genes is predicted to encode a secreted protein containing an RXLR (Arg-any amino acid-Leu-Arg) and dEER (Asp-Glu-Glu-Arg) motif downstream from the signal peptide. The RXLR and associated dEER motif are proposed to function in targeting the effector for delivery to the host cell cytoplasm (Dou et al. 2008; Whisson et al. 2007). Whole genome analysis has demonstrated that RXLR effectors constitute huge and rapidly evolving gene families in *Phytophthora* species (Jiang et al. 2008; Win et al. 2007). For example, the *P. sojae* and *Phytophthora infestans* genomes each possess some 350 to 560 distinct genes encoding RXLR effectors (Haas et al. 2009; Tyler et al. 2006). Predicted *P. sojae* RXLR effectors that are not known to be *Avr* genes are named *Avirulence homolog* (*Avh*) genes (Wang et al. 2011).

Secreted RXLR effectors have the ability to enter host cells, but it is not clear how this occurs. Some investigators have suggested that binding to phospholipids such as phosphatidylinositol 3-phosphate [PtdIns(3)P] is important for host cell targeting (Kale et al. 2010), while others have discounted this hypothesis (Wawra et al. 2012). The RXLR motif was proposed to function in PtdIns(3)P binding (Kale et al. 2010), but subsequent results indicate that most of the lipid-binding affinity resides downstream in the carboxy (C)-terminal effector domain of the protein rather than the RXLR sequence (Sun et al. 2013; Wawra et al. 2012; Yaeno et al. 2011).

The purpose of the present study was to identify the *P. sojae* *Avr1d* gene. This was accomplished by genetic mapping and functional genomic approaches. The *P. sojae* *Avr1d* gene corresponds to a predicted protein with a signal peptide and an RXLR motif. Deletions of the *Avr1d* structural gene cause gain of virulence in *P. sojae* strains that are able to defeat the soybean *Rps1d* resistance gene. Expression and purification of recombinant *Avr1d* protein was performed to test the lipid-binding properties of the protein. Results indicate that *Avr1d* binds to phosphatidylinositol-4-phosphate [PtdIns(4)P] rather

than to PtdIns(3)P. Lipid binding could be disrupted by mutation of selected lysine residues in the C-terminal effector domain but not by mutation of the RXLR motif.

RESULTS

Avr1d segregates as a single dominant gene in *P. sojae* cross ACR10 × P7076.

To test for the segregation of the predicted *Avr1d* gene, we performed a cross between *P. sojae* strains ACR10 (avirulent on *Rps1d*) and P7076 (virulent on *Rps1d*). A total of 20 F₁ hybrids were tested, and each displayed avirulence towards *Rps1d*. A population of 40 individuals developed from one F₁ progeny was used to determine the segregation of *Avr1d* in the F₂ generation. Virulence segregated as a qualitative trait with the ratio of avirulent/virulent F₂ cultures being 32:8. This ratio is consistent with the expected value of 3:1, as predicted for a single dominant *Avr1d* gene (chi-squared probability = 0.46).

The predicted RXLR effector *Avh6* cosegregates with *Avr1d*.

Known *Avr* genes from *Phytophthora* species possess common characteristics that provide a starting point for searches of unidentified *Avr* genes. To begin the hunt for *Avr1d*, we made the following hypotheses. It encodes a secreted protein with RXLR motif; it is expressed in avirulent strains of the pathogen; and it is polymorphic in sequence or expression between strains that differ in virulence on plants with *Rps1d*. A total of 497 different *Avh* genes have been identified from whole-genome sequence data of *P. sojae* reference strain P6497 (Wang et al. 2011). Transcript analysis provides evidence for expression of 119 of these predicted RXLR effectors. Genomic resequencing of three additional *P. sojae* strains, P7064, P7074, and P7076, indicates that expressed *Avh* sequences are often polymorphic (Wang et al. 2011). Strains that differ in their virulence phenotype to particular *Rps* genes would be expected to display corresponding differences in the sequence or expression of the corresponding *Avr* gene. Thus, by analysis of *Avh* gene expression and sequence polymorphisms and comparison with strain-specific virulence on particular *Rps* genes, candidate *Avr* effectors can be identified *a priori*.

Table 1. Testing of 15 candidate *Avirulence homolog* (*Avh*) genes for cosegregation with *Avr1d*

Candidate	<i>P. sojae</i> strain ^a			<i>Avr1d</i> locus ^d
	P7064 ^b	P7074 ^c	P7076 ^c	
<i>Avh6</i>	SP	Absent	Absent	Linked
<i>Avh15</i>	SP	NP	SP	Unlinked
<i>Avh20</i>	NP	SP	SP	Unlinked
<i>Avh51</i>	NP	NP	SP	Unlinked
<i>Avh67</i>	NP	SP	SP	Unlinked
<i>Avh71</i>	NP	SP	SP	Unlinked
<i>Avh161</i>	NP	NP	SP	Unlinked
<i>Avh186</i>	ND	SP	SP	Unlinked
<i>Avh225</i>	NP	NP	NP	Unlinked
<i>Avh238</i>	NP	SP	SP	Unlinked
<i>Avh288</i>	ND	SP	SP	Unlinked
<i>Avh308</i>	SP	SP	SP	Unlinked
<i>Avh320</i>	SP	NP	SP	Unlinked
<i>Avh343</i>	NP	SP	NP	Unlinked
<i>Avh362</i>	NP	NP	SP	Unlinked

^a SP, sequence polymorphisms between tested strain and reference strain P6497; Absent, sequence absent in the tested *P. sojae* strain; NP, no sequence polymorphisms between tested strain and reference strain P6497; ND, not determined

^b *P. sojae* strain avirulent on *Rps1d*.

^c *P. sojae* strain virulent on *Rps1d*.

^d Result of linkage analysis for segregation of candidate *Avh* genes and *Avr1d* phenotype using ACR10 × P7076 F₂ progeny.

This rationale guided our selection of 15 predicted RXLR effectors as potential candidate genes for *Avr1d* (Table 1). These 15 candidate genes display strain-specific polymorphisms that are consistent with a possible role in determining virulence on plants carrying *Rps1d*; however, not all of the candidates were among the 119 RXLR effectors with evidence for expression. Molecular DNA markers based upon presence or absence of amplified sequences or upon cleaved amplified polymorphic sequences were developed for each of the 15 candidate *Avh* genes. The DNA markers were validated as polymorphic in parental strains ACR10 and P7076 prior to testing the 40 progeny of the segregating F₂ population. Results from this analysis indicate that the predicted RXLR effector gene *Avh6* cosegregates with *Avr1d* (Table 1; Supplementary Fig. S1). The *Avh6* gene lies on a contiguous sequence named Scaffold_5 in the *P. sojae* reference genome assembly V5.0.

It was necessary to further characterize the region around *Avh6* to verify that the reference genome assembly from *P. sojae* P6497 provides a useful physical map for segregation of this gene in ACR10 × P7076. Furthermore, we noted that four other predicted RXLR effector genes, *Avh73*, *Avh272*, *Avh282* and *Avh283*, occur in the vicinity of *Avh6* in the reference genome. We sought to create a fine genetic map of the region to delineate the segment containing *Avr1d*, preferably using more informative codominant markers. The *Avh6* DNA marker is not optimal for mapping because it is a dominant marker that detects presence or absence of the gene and cannot distinguish heterozygous- from homozygous-dominant genotypes. Thus, an additional 28 codominant DNA markers flanking the *Avh6* gene were developed. The DNA markers were designed based upon sequence polymorphisms between *P. sojae* reference strain P6497 and resequenced strain P7076 and were validated by scoring in the parental strains ACR10 and P7076. Results from this analysis are shown in Figure 1 and Supplementary Figure S2. The 28 DNA markers flanking *Avh6* produced a genetic map in the ACR10 × P7076 F₂ progeny that is consistent with their physical location in reference genome P6497. With the exception of *Avh6*, which cosegregates with *Avr1d*, all other *Avh* genes in the region display recombination with *Avr1d*. Furthermore, analysis of *Avh73*, *Avh272*, and *Avh283* for sequence differences indicates that these predicted RXLR effector genes are not good candidates for *Avr1d* because they do not display any polymorphisms among four *P. sojae* strains that differ in virulence against *Rps1d* (Wang et al. 2011). The *Avh282* gene is polymorphic and is among the 119 RXLR effectors with evidence for expression (Wang et al. 2011), but this gene does not cosegregate with *Avr1d*. Thus, the *Avh6* gene remains the best candidate for *Avr1d* because it precisely cosegregates with *Avr1d* and it displays the presence or absence polymorphisms that are consistent with *Rps1d* virulence phenotypes in the reference and resequenced strains of *P. sojae*.

Haplotype and transcript analysis

Avh6 matches *Avr1d* phenotypes among *P. sojae* strains.

Whole-genome sequence information for four different *P. sojae* strains suggested that the *Avh6* gene is polymorphic in coding sequence between strains P6497 and P7064 and that it additionally displays presence or absence polymorphisms among strains (Table 1). These observations were verified by analysis of 16 different *P. sojae* strains within our own collection, as shown in Figure 2. The *Avh6* gene encodes a predicted protein of 125 amino acids, including a signal peptide, RXLR-deER sequences, and a W-motif, as shown in Figure 2A. Using the *Avh6* DNA marker to score for the presence or absence of this gene in the 16 strains, we found that 11 strains carry a copy of *Avh6* and are avirulent against *Rps1d*, whereas five strains are lacking an *Avh6* gene and are virulent towards *Rps1d*.

(Fig. 2B). Haplotype mapping of *Avh6* and associated genetic markers from the 16 strains provides further evidence that *Avh6* corresponds to *Avr1d* (Supplementary Fig. S3). Among the 11 strains carrying a copy of *Avh6*, two sequence variants of *Avh6* were identified that encoded slightly different protein products (Fig. 2A). The most variable region of the protein was found within the effector domain. The *Avh6-1* allele was present in seven strains, while the *Avh6-2* allele was found in four strains (Fig. 2B).

To test for the presence of the *Avh6* transcript, reverse transcriptase polymerase chain reaction (RT-PCR) was performed, using template mRNA isolated from the 16 *P. sojae* isolates within our collection. As expected, transcripts of *Avh6* are present in strains avirulent towards *Rps1d* but not in the corresponding virulent strains (Fig. 2B).

Transient expression in soybean confirms that *Avh6* is *Avr1d*.

Effector proteins expressed by *P. sojae* *Avr* genes trigger cell death in soybean plants that carry matching *Rps* genes. In order to test whether *Avh6* can trigger cell death in an *Rps1d* soybean plant, transient expression of *Avh6* together with a reporter gene was performed in a cobombardment assay. Plasmid constructs of *Avh6* excluding the signal peptide were bombarded into soybean leaves together with the glucuronidase (*GUS*) gene to measure cell death. This was done for each of the *Avh6* alleles, *Avh6-1* and *Avh6-2*, in the soybean isolines Harosoy (*rps1d*) and Haro16 (*Rps1d*). Expression of each of the *Avh6* alleles triggered cell death in Haro16 (*Rps1d*) but not in Harosoy (*rps1d*) leaves, as shown in Figure 3A and Supplementary Figure S4. Results from this

Fig. 1. Physical and genetic map of the *Avh6* region. The linear map shows the position of *Avirulence homolog* (*Avh*) genes occurring on a segment of *Phytophthora sojae* Scaffold_5 (assembly V5.0). Scale is in kilobase pairs (kb). The table below provides the exact position of selected DNA markers in base pairs (bp) and the corresponding recombination frequencies (RF) with *Avr1d* phenotype within the F₂ population.

Fig. 2. The predicted RXLR effector *Avh6* corresponds to *Avr1d*. **A**, Primary sequence of *Avh6* alleles. Two sequence variants of *Avh6* were identified among *Phytophthora sojae* strains, as shown. The signal peptide domain is underlined. Black arrows denote the putative signal peptide cleavage site. The RXLR dEER motif is highlighted. The W-like domains are boxed with signature residues indicated below each protein. **B**, Expression of *Avh6* in *Phytophthora sojae* strains cosegregates with *Avr1d* phenotype. Virulence of *P. sojae* strains on *Rps1d* plants is shown as virulent (V) or avirulent (A). Polymerase chain reaction (PCR) analysis was performed on genomic DNA to test for the presence of the *Avh6* gene in different *P. sojae* strains. Reverse transcriptase-PCR (RT-PCR) analysis was performed using cDNA from mRNA, to test for transcripts of *Avh6* and a control gene (*Actin*). Samples of total RNA isolated from each strain, showing the small and large subunit of rRNA, are also shown (RNA).

functional assay indicate that there is a specific interaction between *Avh6* and *Rps1d*, and we conclude that *Avh6* corresponds to *Avr1d*.

To determine the influence of the RXLR motif on host targeting, as assessed by the cobombardment assay, we tested *Avr1d* constructs with the RFLR (Arg-Phe-Leu-Arg) sequence mutated to AAAA (Ala-Ala-Ala-Ala). This was performed for each of the two *Avr1d* open reading frames, with the signal peptides intact. Results shown in Figure 3B and Supplementary Figure S5 indicate that the RFLR^{AAAA} mutation does not change the outcome, compared with wild-type protein sequences. Cell death was triggered equally well in *Rps1d* plants by transient expression of the RFLR^{AAAA} mutant and wild-type *Avr1d* protein constructs.

Deletion of the *Avr1d* structural gene causes gain of virulence on *Rps1d*.

To compare the *Avr1d* genomic region in strains of *P. sojae* that are either virulent or avirulent towards *Rps1d*, we performed an alignment of sequence reads to the assembled genomic segment (*P. sojae* V5.0). The sequence reads were from whole-genome shotgun sequencing. The extent of sequence read coverage along the 7-kb segment encompassing the *Avr1d* gene is clearly different between the virulent and avirulent strains of *P. sojae*, as shown in Figure 4. In *Rps1d*-virulent strains P7074 and P7076, there is no sequence read coverage whatsoever along a 1.53-kb segment that includes the *Avr1d* gene, while this region is covered by sequence reads in the *Rps1d*-avirulent strains P6497, P7064, and ACR10. This result indicates that gain of virulence to *Rps1d* and the presence or absence polymorphism of *Avr1d* are due to a 1.53-kb deletion that includes the entire *Avr1d* structural gene.

Avr1d binds to phosphatidylinositol 4-phosphate.

Oomycete effector proteins are known to bind to phospholipids, especially PtdIns(3)P. To investigate the affinity of *Avr1d* towards phospholipids, recombinant protein was produced, purified, and tested by lipid blot and liposome binding assays. Lipid blot assays suggested that *Avr1d* could bind to PtdIns(4)P, so this was further tested and compared with PtdIns(3)P binding by more reliable liposome-based binding methods. Liposomes are made from phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and lesser amounts of additional lipids such as PtdIns(3)P or PtdIns(4)P are added to test specificity. Results shown in Figure 5 indicate that *Avr1d* has a high affinity for liposomes containing PtdIns(4)P, whereas its binding to liposomes containing PtdIns(3)P did not differ from the binding to control liposomes containing only PC and PE. A control protein (Vam7pPX) known to bind to PtdIns(3)P displayed affinity for PtdIns(3)P and, to a lesser extent, PtdIns(4)P.

To determine whether the RXLR motif or C-terminal Lys residues are important to the PtdIns(4)P binding of *Avr1d*, we created two different mutant proteins. In one mutant, the RFLR sequence of *Avr1d* was mutated to EEEE (Glu-Glu-Glu-Glu). The other mutant was designed to change three different Lys (K) residues in the C-terminal effector domain to Glu (E), specifically K104E, K105E, and K107E. These residues (K104, K105, and K107) were chosen based on their position in a predicted W-like motif within the effector domain. Results show that the RFLR^{EEEE} mutations do not affect the phospholipid-binding properties of *Avr1d*, whereas the K104, K105, and K107E changes drastically altered the lipid-binding characteristics (Fig. 5). Only trace amounts of the K104, K105, and K107E mutant of *Avr1d* bound to liposomes containing

Fig. 3. Measurement of cell death in soybean leaves triggered by transient expression of *Avr1d*. **A**, Transient expression of *Avr1d* triggers a *Rps1d*-specific cell death. Leaves of Harosoy (*rps1d*) and Haro16 (*Rps1d*) were cobombarded with plasmids carrying a reporter glucuronidase gene (*GUS*) and a test gene, as indicated. Expression of *Avr1d* alleles from *Phytophthora sojae* strains P6497 and P7064 is shown (signal peptides were removed from *Avr1d* constructs). Volume intensity of GUS-positive spots was measured from three sections of each leaf, using software. Error bars represent standard deviation for 18 measurements from two independent biological replications. **B**, Mutation of the *Avr1d* RXLR motif does not alter the level of cell death for *Avr1d* constructs expressed with native signal peptide. Leaves of Harosoy (*rps1d*) and Haro16 (*Rps1d*) were cobombarded with plasmids carrying a reporter gene (*GUS*) and a test gene, as indicated. Expression of the full-length *Avr1d* with native signal peptide is shown for wild-type (WT) and RXLR-mutated (RFLR^{AAAA}) sequences for *Avh6* alleles from *Phytophthora sojae* strains P6497 and P7064. Volume intensity of GUS-positive spots was measured as described above.

PtdIns(4)P. The K104, K105, and K107E mutant also showed reduced affinity to the PtdIns(3)P liposomes.

DISCUSSION

Management of soybean root rot caused by *P. sojae* relies on the development of resistant cultivars (Sugimoto et al. 2012). Single dominant *Rps* genes are powerful resources in breeding programs, but their utility in the field may be defeated by the emergence of new pathogen strains. Molecular characterization of *P. sojae* Avr genes delivers valuable tools to investigate the basis of host resistance and for the development of precise pathogen diagnostic methods (Vleeshouwers et al. 2011). The primary objective of the present study was to identify the *P. sojae* Avr1d gene. This was accomplished by genetic mapping of candidate genes selected from a comparative analysis of whole-genome sequence data. Another research group, working independently and using a cross *P. sojae* strain different from that used in our study, has simultaneously provided their

own experimental evidence that *Avh6* corresponds to *Avr1d* (Yin et al. 2013). These separate but matching results provide an added level of confidence in our main conclusion regarding the identity of *Avr1d*.

Previous inheritance studies in *P. sojae* indicated that avirulence to *Rps1d* segregates as a single dominant gene (May et al. 2002; Tyler et al. 1995). We confirmed these results in our analysis of progeny from a cross of *P. sojae* strains ACR10 and P7076. Phenotypes conditioned by *P. sojae* Avr genes are inherited as dominant traits, but there are important exceptions. For example, in a cross that was identical to that used in this study, we previously demonstrated non-Mendelian interactions between *Avr3a* alleles resulting from transgenerational effects on gene expression (Qutob et al. 2013). We now show that *P. sojae* ACR10 possesses an *Avr1d* gene that follows Mendelian rules in outcrosses with P7076. This indicates that the transgenerational effects on *Avr3a* expression do not affect *Avr1d* and that there is nothing inherently abnormal or dysfunctional in ACR10 × P7076 progeny with regard to Avr effector gene expression in general.

The selection of candidate *Avh* genes as an entry point for Avr gene identification in *P. sojae* is now well-established (Dong et al. 2009, 2011a and b). Our choice of 15 candidate

Fig. 4. Sequence read coverage of the *Avr1d* region from five different *Phytophthora sojae* strains. A 7.0-kb segment containing *Avr1d* and three other predicted genes is from the *Phytophthora sojae* reference genome assembly V5.0. Raw sequence reads from whole-genome shotgun sequencing of the reference strain (P6497) or from resequencing of strains P7064, ACR10, P7074, and P7076 were aligned to the reference assembly of this segment to determine read coverage. Within the 7.0-kb segment, a 1.53-kb section including the entire *Avr1d* gene is entirely lacking sequence reads in *P. sojae* strains P7074 and P7076.

Fig. 5. The effector domain of *Avr1d* binds to PtdIns(4)P. **A**, Shown is a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of a liposome protein-binding assay. Control liposomes or liposomes with added PtdIns(3)P or PtdIns(4)P were prepared and incubated with test proteins as indicated. The Vam7p PX is a control protein known to bind to PtdIns(3)P. Wild-type *Avr1d* is indicated as *Avr1d*. Altered versions of *Avr1d* with mutated RXLR motif or C-terminal Lys residues are indicated as RFLR^{EEEE} and K104E,105,107E, respectively. After ultracentrifugation the pellet (P) and supernatant (S) fractions were analyzed by SDS-PAGE and proteins were visualized by Coomassie staining. kD indicates the size of proteins as measured in kilodaltons. **B**, *Avr1d* binds to PtdIns(4)P. Measurement of liposome-bound versus soluble protein by image analysis of SDS-PAGE results. The ratio of protein in the pellet versus the supernatant fraction is shown. Error bars represent the standard deviation from three independent biological replicates.

Avh genes was guided by existing sequence and expression data. It was fortunate that *Avh6* was among those selected because the candidate gene approach is not an infallible method for discovery of unknown *Avr* gene targets. Choosing candidate genes relies on imperfect assumptions and incomplete data. Nevertheless, it has the potential to facilitate and accelerate target discovery, as was the case with *Avr1d*.

The presence or absence polymorphism of *Avr1d* among *P. sojae* strains provides another example of the dispensability of most *Avr* genes and, in particular, of individual RXLR effectors. This illustrates that even highly expressed effectors can be expendable, since *Avr1d* (*Avh6*) was shown in a previous study to be among the most strongly induced during infection of all the RXLR effectors in the *P. sojae* genome (Wang et al. 2011).

Transient expression analysis by cobombardment with a reporter is a long-standing and widely used technique to evaluate *R*- and *Avr*-gene interactions (Allen et al. 2004; Jia et al. 2000; Mindrinos et al. 1994). This method is particularly valuable for analysis of candidate genes in soybean, because its leaves are recalcitrant to infiltration and *Agrobacterium*-based transformation assays. Expression of each of the two different *Avr1d* alleles in the cobombardment assay triggered cell death but only in plants carrying the *Rps1d* gene. Thus, the results from this functional test provide a validation of the genetic mapping and haplotype analysis that led to the identification of *Avr1d*. We also performed further cobombardment experiments to test the role of the RXLR motif in the host targeting of *Avr1d*. Our hypothesis was that mutation of the RXLR motif of *Avr1d* would reduce cell death triggered by transient expression because host targeting would be impaired in cases in which the signal peptide was retained. Implicit in this assumption is that *Rps1d* occurs in the cell cytoplasm and that the RXLR motif is important for reentry of secreted protein into the cell. However, the results were negative and the RFLR^{AAAA} mutation did not reduce cell death to any measureable degree. It is possible that high-level ectopic expression of *Avr1d* causes translated protein to escape the secretory pathway and directly enter the cytoplasm; or perhaps, *Avr1d* is efficiently secreted, but reentry of the protein into the cell cytoplasm occurs despite the RFLR^{AAAA} mutation. Other explanations are possible, especially since the *Rps1d* gene has not been identified and it is not known where or how ETI is activated by *Avr1d*. Thus the only conclusion that we can draw from this experiment is that an RFLR^{AAAA} mutation of *Avr1d* has no effect on its ability to activate ETI under the conditions described.

Mutations of the RXLR motif also failed to alter the lipid-binding properties of *Avr1d*, as assessed by liposome-binding studies. The binding of recombinant *Avr1d* to PtdIns(4)P was surprising because other oomycete RXLR effectors have shown a preference for PtdIns(3)P. Each of these phospholipids, PtdIns(4)P and PtdIns(3)P, participate in a variety of cellular functions, including protein trafficking. In animal cells, PtdIns(4)P is enriched in the Golgi apparatus, while PtdIns(3)P is most abundant in the endosomal membrane system (Mayering 2012). Recent work using murine cells has suggested a role for PtdIns(4)P in maintaining the polyanionic character of membrane lipids that is necessary for electrostatic interactions with basic amino acids of protein cargo (Hammond et al. 2012). The affinity of RXLR effectors towards PtdIns(3)P was initially proposed to be mediated by the RXLR motif itself (Kale et al. 2010), but the consensus now is that this occurs through basic amino acids in the C-terminal domain (Sun et al. 2013; Wawra et al. 2012; Yaeno et al. 2011). There is, however, no such consensus on any hypothesis regarding the significance or biological function of lipid binding by RXLR effectors. Suggestions that lipid binding directs effector targeting in one or both the host or pathogen protein traffick-

ing systems are plausible but controversial (Bhattacharjee et al. 2012; Kale et al. 2010; Sun et al. 2013; Wawra et al. 2012; Yaeno et al. 2011). Structural models for several RXLR effectors revealed a common three-helix fold called the WY domain, and this has been proposed as an adaptable scaffold for functional diversification (Win et al. 2012). However, the WY domain represents an unusual example for phosphoinositide binding, and the molecular interactions that coordinate this process are not known (Jiang et al. 2013). Our results show that *Avr1d* binds to PtdIns(4)P and that this interaction can be disrupted by mutation of Lys residues in the C-terminal domain but not by mutation of the RXLR motif.

In conclusion, we have identified the *P. sojae Avr1d* gene and shown that it encodes an RXLR effector protein. Two alleles of *Avr1d* that differ in predicted amino acid sequence were discovered; however, both alleles activate ETI in soybean plants carrying the *Rps1d* gene. *P. sojae* strains able to defeat *Rps1d* lack the *Avr1d* gene entirely. This presence or absence polymorphism of *Avr1d* among *P. sojae* strains is easily measured and will aid diagnostics. These results provide new tools for management of soybean root rot caused by *P. sojae*, a widespread and damaging disease of the world's foremost legume grain crop.

MATERIALS AND METHODS

Plant materials, *Phytophthora sojae* strains and virulence scoring.

Soybean (*Glycine max*) cultivars Haro16 (*Rps1d*) and Harosoy (*rps1d*) from the collection at Agriculture and Agri-Food Canada (London, ON, Canada) were used to evaluate the virulence of pathogen cultures. Ten soybean seeds were sown in 10-cm pots (a minimum of three pots per isolate) containing soilless mix (Pro-Mix 'BX', Premier Horticulture Ltd, Rivière-du-Loup, Canada). Pots were watered with 3 mg of 20-20-20 (N-P-K) fertilizer per liter. Plants were grown in a controlled growth chamber with supplementary light (16-h photoperiod with 25°C day and 16°C night temperatures) for 1 week before inoculations.

Phytophthora sojae isolates were from the collection at Agriculture and Agri-Food Canada. The origin of each strain has been described (Dong et al. 2011a; Qutob et al. 2009). The sexual cross of *P. sojae* strains ACR10 and P7076 and the generation of F₁ and F₂ progeny has been described (Qutob et al. 2013). All of the isolates were routinely maintained on 2.5% (vol/vol) vegetable juice (V8) medium at 25°C in the dark. *P. sojae* cultures were transferred to 0.9% (vol/vol) V8 agar plates for 5 to 7 days prior to green plant inoculations. Methods of hypocotyl inoculation and virulence scoring have been described (Qutob et al. 2009). Three independent replicates of the virulence assay were performed for each culture tested.

Genetic mapping.

A total of 40 F₂ progeny were selected for genetic mapping of the *Avr1d* interval. Genome sequence data from the reference strain P6497 and from parental strains ACR10 and P7076 were analyzed to design 28 codominant and one dominant DNA marker in the vicinity of the candidate gene *Avh6*. Details of PCR product sizes in two strains and the restriction enzyme used for each marker are provided in Supplementary Table S1. A selection of 11 of the DNA markers were also scored in 16 different *P. sojae* isolates for the haplotype analysis.

Nucleic acid isolation and RT-PCR.

The purification of RNA from *P. sojae* mycelium and transcript analysis by RT-PCR was carried out as previously described (Qutob et al. 2000). Total RNA was treated with

DNase I (Invitrogen, Carlsbad, CA, U.S.A.). The RT-PCR was conducted on 1 µg of RNA, using a reverse transcriptase (SuperScriptIII, Invitrogen) system according to the manufacturer's instructions. Genomic DNA from *P. sojae* mycelium was isolated by phenol chloroform extraction and ethanol precipitation (Tyler et al. 1995). Genomic PCR amplifications were performed using 15 ng of DNA + 0.5 µM primers + 0.25 mM dNTPs + *Taq* + buffer to obtain conditions for PCR and the following program: 94°C for 2 min, 40 cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 30 to 60 s (varied with the PCR product size), and a final extension of 72°C for 10 min. A *P. sojae* *actin* gene was used as a control.

Transient expression assays.

Soybean plants were grown in the growth chambers as described above. Leaves from 14-day-old plants were harvested for cobombardment. The two different *Avr1d* open reading frames (Avh6P6497 and Avh6P7064), but lacking the signal peptide, were amplified using specific primers and cloned into the 35S promoter-derived plant expression vector pFF19, using *Bam*HI and *Pst*II restriction sites. Similarly, constructs for full-length wild-type *Avr1d* alleles with signal peptide (FLAvr1dP6497 and FLAvr1dP7064), and the corresponding RFLR-mutated *Avr1d* synthetic sequences (FLAvr1dP6497-RFLR^{AAAA} and FLAvr1dP7064RFLR^{AAAA}) were also cloned into the pFF19 vector, using *Bam*HI and *Sph*I restriction sites, respectively. Soybean leaves of cultivar Harosoy (*rps1d*) or Haro16 (*Rps1d*) were transformed by cobombardment with plasmids encoding a GUS reporter gene and a test construct, using tungsten beads. Cobombardment assays were performed as previously described (Qutob et al. 2002, 2006). For the control, the test gene corresponded to a frame-shift mutant of *Avr1a*. Leaves were washed in lactic acid/phenol/water (1:1:1) and were photographed using a digital camera. Sample images were obtained and processed as previously described (Qutob et al. 2009), and the volume of GUS-positive spots was calculated as the intensity (measured in absorbance units) multiplied by the area (square millimeter).

Sequence analysis.

Genome sequence data for the *P. sojae* reference strain P6497 and resequenced strains P7064, P7074, P7076 were from the Joint Genome Institute database and the VBI Microbial database, respectively. Resequencing of strain ACR10 was performed by The Centre for Applied Genomics (Sick Kids Hospital, Toronto, Canada). Aligning sequence reads from the *P. sojae* strains to a 7-kb segment of reference genome assembly encompassing the *Avr1d* region was performed using the Barrows-Wheeler Alignment and Samtools software (Li and Durbin 2009; Li et al. 2009). Amino acid alignment was determined using software (Lasergene 6.0 MegAlign module). The SignalP 4.0 server was used for secretion signal peptide prediction.

Protein expression and purification.

For recombinant protein expression, *Avr1d* from *P. sojae* strain P6497 was defined as wild-type. The RFLR^{EEEE} and K104, K105, and K107E mutant sequences were generated and cloned into the pSDK1 vector for expression in *Escherichia coli* (Kale et al. 2010). The Vacuolar morphogenesis protein 7 Phox homology domain provided a positive control for PdIns(3)P binding. Expression plasmids and protocols were provided by B. Tyler and have been previously described (Kale et al. 2010). Recombinant *Avr1d* proteins were expressed in fusion with an N-terminal glutathione *S*-transferase and C-terminal green fluorescent protein, and poly-histidine tags. Plasmids were transformed in BL21(DE3) *E. coli* cells and were grown in Luria-Bertani media at 37°C, until cells reached

an optical density of 0.7 to 0.9 absorbance units. The proteins were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and then, cell growth was continued at 16°C for 12 to 16 h. Harvested cells were lysed by sonication in autoclaved chilled lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 8.0). Lysed cells were centrifuged at 10,000 × *g* for 30 min at 4°C. Proteins collected from the supernatant were purified using a column containing Ni-NTA superflow (Qiagen, Basel, Switzerland). The column was then washed (50 mM sodium phosphate, 300 mM sodium chloride, 60 mM imidazole, pH 8.0) and was eluted (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, pH 8.0). Purified proteins were then concentrated by using 10-kDa cut-off centrifugal filter devices (Millipore, Bedford, MA, U.S.A.). Protein concentration was calculated using a microplate reader (Bio-Rad protein assay; Bio-Rad, Munich).

Liposome-protein binding assay.

Lipids were dissolved in an organic solvent according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, U.S.A.). Liposomes were prepared by mixing equal mass amounts of PC and PE. Additional test lipids PtdIns(3)P or PtdIns(4)P were added to 10% (wt/wt) concentration. Control liposomes contained only PC and PE. Lipid films were generated by drying the lipid mixture under vacuum overnight at room temperature. Lipid films were hydrated in Tris buffered saline I (20 mM Tris HCl, pH 6.8, 100 mM NaCl, 1 mM NaN₃) to 1 mg/ml at 68°C for 1 h, and then, were subjected to three freeze-thaw cycles (immersion in liquid nitrogen, then thawed at 25°C). Large unilamellar vesicles were formed by extruding the lipid suspension through an Avanti extruder with a 0.2-µm filter at 68°C (20 cycles). Liposomes were collected by centrifugation at 40,000 × *g* for 20 min using an ultracentrifuge and were resuspended in Tris-buffered saline II (20 mM Tris HCl, pH 5.8, 50 mM NaCl, 1 mM NaN₃) to a final concentration of 5 µg/µl. Proteins were centrifuged at 40,000 × *g* for 20 min at 4°C to remove protein aggregates. Purified protein (10 µg) was incubated with 200 µg of liposome for 30 min at 4°C. Protein-liposome mixtures were centrifuged at 40,000 × *g* for 20 min at 4°C. Pellets containing liposome-bound proteins and supernatants containing free proteins were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were stained with Coomassie-blue using standard methods.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Joint Genome Institute database: genome.jgi-psf.org
 SignalP 4.0 server: www.cbs.dtu.dk/services/SignalP
 Virginia Bioinformatics Institute microbial database: www.vbi.vt.edu/resources_and_tools