Sequence Variants of the Phytophthora sojae RXLR Effector Avr3a/5 Are Differentially Recognized by Rps3a and Rps5 in Soybean

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Abstract
The perception of Phytophthora sojae avirulence (Avr) gene products by corresponding soybean resistance (Rps) gene products causes effector triggered immunity. Past studies have shown that the Avr3a and Avr5 genes of P. sojae are genetically linked, and the Avr3a gene encoding a secreted RXLR effector protein was recently identified. We now provide evidence that Avr3a and Avr5 are allelic. Genetic mapping data from F2 progeny indicates that Avr3a and Avr5 co-segregate, and haplotype analysis of P. sojae strain collections reveal sequence and transcriptional polymorphisms that are consistent with a single genetic locus encoding Avr3a/5. Transformation of P. sojae and transient expression in soybean were performed to test how Avr3a/5 alleles interact with soybean Rps3a and Rps5. Over-expression of Avr3a/5 in a P. sojae strain that is normally virulent on Rps3a and Rps5 results in avirulence to Rps3a and Rps5; whereas silencing of Avr3a/5 causes gain of virulence in a P. sojae strain that is normally avirulent on Rps3a and Rps5 soybean lines. Transient expression and co-bombardment with a reporter gene confirms that Avr3a/5 triggers cell death in Rps5 soybean leaves in an appropriate allele-specific manner. Sequence analysis of the Avr3a/5 gene identifies crucial residues in the effector domain that distinguish recognition by Rps3a and Rps5.

Introduction
Phytophthora sojae is an oomycete and a plant pathogen that infects soybean. It is one of more than 80 species of Phytophthora that cause destructive diseases of a large range of agriculturally and ornamentally important plants and native species in forests and natural ecosystems. P. sojae is a soil-borne pathogen that is highly host-specific to soybean, causing damping-off of seedlings and root rot of older plants, with an estimated annual cost of $1–2 billion worldwide [1,2]. Management of the disease relies in part on the development and proper deployment of soybean varieties with cultivar-specific resistance traits.

Historically, the gene-for-gene model provided a framework to interpret the interaction between cultivar-specific resistance in a host plant and strain-specific virulence in a pathogen [3]. This concept established the association between genes conditioning avirulence in the pathogen and corresponding genes conferring resistance in the host. Now, we understand that pathogen avirulence (Avr) genes encode effectors that normally enable pathogen growth on host plants in the absence of appropriate host resistance (R) genes [4]. Host plant R genes constitute elements of the plant immune system that cause effector-triggered immunity in the presence of corresponding pathogen Avr factors.

The identification of oomycete Avr genes has a recent history that follows the development of advanced molecular and genetic technologies. Inheritance studies of Avr genes in P. sojae provided a basis for genetic mapping in F2 populations [5,6]. This work established that at least three pairs of P. sojae Avr genes are genetically linked, specifically Avr1b and Avr1k, Avr3a and Avr5, and Avr4 and Avr6 [7,8,9,10]. The Avr4 and Avr6 genes have since been identified and found to be encoded by a single locus, Avr4/6 [11]. Other oomycete Avr genes that have been identified include Avr1a, Avr1b, Avr3a, and Avr3c from P. sojae [12,13,14], Avr3a, Avr4, Avr-bb1 and Avr-bb2 from Phytophthora infestans [15,16,17,18], and ATR1, ATR5, and ATR13 from Hyaloperonospora arabidopsidis [19,20,21]. With the exception of ATR5, each of these oomycete Avr genes encode predicted secreted proteins with a conserved motif consisting of RXLR (Arg-any amino acid-Leu-Arg) and variable EER (Glu-Glu-Arg) sequences that occur downstream from the amino terminal signal peptide. The ATR5 sequence is predicted to encode a secreted protein that includes an EER and an RGD (Arg-Gly-Asp) motif, but no canonical RXLR motif. It is...
believed that the RXLR, EER, and RGD motifs are responsible for targeting the effectors into host cells [20,22,23,24]. The RXLR effectors comprise large and rapidly evolving gene families in Phytophthora species. The best-studied species include P. infestans and P. sojae, with an estimated 563 and 396 RXLR effectors, respectively [25,26,27].

The identification of 12 different oomycete Avr genes and the finding that all encode secreted effectors targeted for delivery into host cells has opened up new avenues of investigation. The momentum of research has shifted towards functional studies of the effectors themselves and their mechanism of host-targeting. Nonetheless, the identification and characterization of new Avr factors remains relevant, since they are the crucial virulence determinants in cultivar-specific interactions. Perhaps new patterns will emerge among RXLR effectors that operate as Avr factors. On the practical side, the identification of Avr genes and the predicted RXLR secretome of P. sojae plants carrying the Avr3a or Avr5 resistance genes is alleles. This was confirmed by functional tests based upon transient expression in soybean and determined by a single genetic locus, Rps within the effector domain of Avr3a/5 determines recognition by that the Avr3a and the predicted RXLR secretome of mapping data, genome sequence assemblies, haplotype analysis, Rps soybean plants carrying the crops on the globe. Variation in amino acid sequence factors remains relevant, since they are the crucial virulence results. On the practical side, the identification of P. sojae Avr genes will provide important tools for breeding and disease management in soybean, which is among the economically most important crops on the globe.

In this paper, we show that strain-specific virulence of P. sojae to soybean plants carrying the Rps3a or Rps5 resistance genes is determined by a single genetic locus, Avr3a/5. Based upon genetic mapping data, genome sequence assemblies, haplotype analysis, and the predicted RXLR secretome of P. sojae, we hypothesized that the Avr3a and Avr5 genes are alleles. This was confirmed by functional tests based upon transient expression in soybean and stable transformation of P. sojae. Variation in amino acid sequence within the effector domain of Avr3a/5 determines recognition by Rps3a or by Rps5.

Results

Genetic mapping and haplotype analysis suggest that Avr3a and Avr5 are alleles of a single locus

Previous studies have shown that Avr3a and Avr5 are genetically linked [6,7,9]. The most recent work, showing that Avr3a and Avr5 precisely co-segregate in 100 F2 progeny, resulted in the identification of Avr3a [14]. Based upon fine genetic mapping of Avr5, this gene is predicted to occur within a 78 kb region in scaffold 80 delineated by the molecular markers named ‘Ns2’ and ‘8R’, as shown in Figure 1. To identify candidates for Avr5, predicted RXLR effectors (named Avh genes) and other secreted proteins within this region were catalogued. By examining this space and comparing it with a syntetic region from the Phytophthora ramorum genome assembly, we determined that three different genes encoding predicted RXLR effectors provided the most plausible candidates for Avr5. Thus, Avh38, Avh36, and Avr3a are possible candidate effector genes for Avr5.

Sequence and transcript analyses of these candidate genes were conducted on P. sojae strains P6497 and P7064 in order to detect any polymorphisms because these two strains are different in virulence characteristics. P. sojae P6497 is avirulent on Rps3a and Rps5 while P7064 is virulent on Rps3a and Rps5. The results are summarized in Table 1. This shows that Avh38 and Avh36 sequences are identical in P. sojae strains P6497 and P7064, and that there are no transcriptional polymorphisms for these genes between the two strains. Expression of Avh38 occurs in virulent and avirulent strains, whereas Avh36 transcripts could not be detected in either strain. In contrast, the Avr3a sequence displayed copy number polymorphisms, amino acid changes, and expression differences between P. sojae strains P6497 and P7064, as we previously reported [14]. Thus, Avr3a remains the best candidate for Avr5.

The Avr3a locus is highly polymorphic among P. sojae strains, as summarized in Table 2. Transcriptional differences among strains are sufficient to account for virulence differences on Rps3a but not on Rps5. This is because at least three strains (ACR12, P7076, and ACR20) express Avr3a but are virulent on Rps5. These three strains show differential virulence towards Rps3a and Rps5. Thus, gain of virulence on Rps5 in these strains could result from sequence differences, since Avr3a differs by two amino acids from Avr3aP6497. Compared to the reference strain (P6497) the

Figure 1. Physical map of the P. sojae Avr3a and Avr5 region. Shown are the positions of predicted genes including candidate RXLR effector genes, and synteny with P. ramorum. In P. sojae strain P6497, Avr3a is embedded in a 10.8 kb DNA segment that is present in a tandem array of four copies, which is shown as a box in this illustration (not drawn to scale). Scaffold designations are from v1.0 of the genome sequence.

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sequence of Avr3aACR12 shows changes to adjacent amino acids K64P and A65S within the effector domain of the predicted protein, as shown in Figure 2. The predicted amino acid sequence of Avr3aP7064 is also shown for comparison, but Avr3aP7064 is likely a pseudogene because no transcripts can be detected for this gene in strains that carry it.

Transient expression of Avr3aP6497 triggers cell death in Rps5 soybean leaves

The proteins encoded by P. sojae Avr genes trigger defense responses and cell death when they are recognized by soybean Rps gene products. In order to test whether the various alleles of Avr3a can interact functionally with Rps5, a co-bombardment and transient expression test was performed. Plasmid constructs directing expression of Avr3aP6497, Avr3aP7064, and Avr3aACR12 without native signal peptide were introduced into leaves of an Rps5 soybean cultivar together with a reporter gene (beta-glucoronidase, GUS) to measure cell viability, as shown in Figure 3 (representative photographs shown in Figure S2). Results indicate that Avr3aP6497 interacts with Rps5, as evidenced by a two-fold reduction in GUS staining in Rps5 plants compared to control plants without this resistance gene. In contrast, co-transformation with either Avr3aP7064 or Avr3aACR12 does not reduce GUS expression by this assay, regardless of the presence of Rps5. Past results have shown that Rps3a interacts with Avr3aP6497 and Avr3aACR12 [14]. Thus, the co-bombardment assays provide functional evidence for the hypothesis that Avr3a and Avr5 are alleles.

Over-expression of Avr3aP6497 in P. sojae strain P7074 causes gain of avirulence in the presence of Rps3a and Rps5

In order to obtain additional evidence that the Avr3aP6497 gene product interacts with Rps5, we transferred Avr3aP6497 into protoplasts of P. sojae strain P7074 to produce stable transformants. The strain P7074 carries an Avr3a allele identical in sequence to Table 1.

### Table 1. Summary of Avr5 candidate genes in P. sojae strains P6497 and P7064

| Gene | Sequence polymorphisms | Transcript polymorphisms |
|------|------------------------|--------------------------|
| Avh38 | None apparent | No difference; expression detected in both strains |
| Avh36 | None apparent | No difference; no expression detected in both strains |
| Avr3a | DNA sequence differences causing promoter changes and amino acid substitutions, and copy number variation | Polymorphic; expressed in P6497 but not in P7064 |

1P. sojae strain P6497 is avirulent while strain P7064 is virulent, on soybean plants with the rps5 resistance gene.

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| Table 2. Virulence phenotypes and haplotype analysis of P. sojae strains. |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| **P. sojae strain** | **Virulence** | **Virulence** | **Avr3a copy number** | **Avr3a mRNA** | **Promoter INDEL** | **Avr3a sequence** |
| A | A | 4 | + | – | – | P6497 |
| P6497 | A | A | 4 | + | – | P6497 |
| 25MEK4 | A | A | 4 | + | – | P6497 |
| ACR8 | A | A | 4 | + | – | P6497 |
| ACR9 | A | A | 4 | + | – | P6497 |
| ACR11 | A | A | 4 | + | – | P6497 |
| ACR25 | A | A | 4 | + | – | P6497 |
| ACR10 | V | V | 4 | – | – | P6497 |
| ACR16 | V | V | 4 | – | – | P6497 |
| ACR12 | A | V | 1 | + | – | ACR12 |
| P7076 | A | V | 1 | + | – | ACR12 |
| ACR20 | A | V | 1 | + | – | ACR12 |
| P7064 | V | V | 1 | – | + | P7064 |
| ACR17 | V | V | 1 | – | + | P7064 |
| P7074 | V | V | 1 | – | + | P7064 |
| ACR21 | V | V | 1 | – | + | P7064 |
| ACR24 | V | V | 1 | – | + | P7064 |

1Origins of P. sojae strains are provided in Table S1.
2A, Avirulent; V, virulent.
3The Avr3a gene occurs in a 10.8 kb DNA segment that is present either as one copy or as four tandemly-arrayed copies, as indicated.
4Positive (+) or negative (–) for expression of Avr3a transcripts, as determined by RT-PCR.
5Insertions and deletions (INDEL) within the promoter region of Avr3a gene, as illustrated in Figure S1.
6Three different DNA sequences for the Avr3a open reading frame, indicated according to the representative strain. GenBank accession numbers are as follows: EF587759 (P6497), JF412456 (ACR12), and JF433921 (P7064).

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**Figure 2. Predicted amino acid sequences of Avr3a alleles from three strains of *P. sojae*.** The position of the predicted signal peptide and the RXLR and EER host-targeting sequences are shown. The deduced Avr3a protein sequence in strain P7064 is shown.

Avr3aP7064 and is normally virulent on Rps3a and Rps5. Three stable transformants (P7064:Avr3aP6497-O3, -O4, and -O8) with high expression levels of the transgene Avr3aP6497 were recovered, as shown in Figure 4A. These results show that ectopic expression of Avr3aP6497 with the heterologous Ham34 promoter from *Bremia lactucae* causes mRNA levels to be several-fold greater than in wild type strains that express this gene off the native promoter.

Virulence assays of the wild-type and transformed *P. sojae* strains were performed by two different methods, consisting of stem inoculations of mycelia on light-grown seedlings and zoospore inoculations of etiolated hypocotyls. Results from stem inoculations of light-grown seedlings are shown in Table 3 (representative photographs shown in Figure S2). As expected, wild-type *P. sojae* strain P7074 is virulent in the presence of *Rps*3a and *Rps*5 whereas *P. sojae* strain P6497 is avirulent on each of these genes. Inoculation with *P. sojae* type strains that express this gene off the native promoter.

Silencing of Avr3aP6497 in *P. sojae* strain P6497 causes loss of avirulence in the presence of Rps3a and Rps5. To further test the interaction of Avr3dP6497 and Rps5, we obtained a gene-silenced transformed strain of *P. sojae* P6497. Levels of the Avr3dP6497 transcript are severely reduced in the gene-silenced strain compared to the wild-type strain P6497, as shown in Figure 4B (representative photographs shown in Figure S2). Stem inoculation of light-grown seedlings was performed to test the virulence of the gene-silenced strain compared to wild-type controls, as shown in Table 3. These results show that soybean plants containing Rps3a and Rps5 were mostly killed by strain P6497:Avr3dP6497-S1, whereas plants inoculated with the wild type strain P6497 survived. Furthermore, as shown in Figure 5B, the Avr3dP6497-silenced strain (P6497:Avr3dP6497-S1) produced large water-soaked lesions by the zoospore inoculation assay, similar to strain P7074 which is known to be virulent in the presence of Rps3a and Rps5.

**Figure 3. Expression of Avr3aP6497 in Rps5 soybean plants can trigger cell death.** Results from a co-bombardment assay are shown. The ratio of GUS-positive blue spots following co-bombardment with Avr3aP6497, Avr3aP7064 and Avr3aACR12 compared with the empty vector DNA. Expression constructs for Avr3a alleles were without signal peptides. Soybean lines Williams (rps) and L85-3059 (Rps5) are genetic isolines. Bars represent standard errors from 12-14 replicates each. An asterisk (*) indicates a significant difference (p<0.001) by the Wilcoxon rank sum test.

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**Discussion**

To understand effector-triggered immunity in plants, it is useful to consider the hypothesis that pathogen Avr factors simply represent effectors that have had *R* genes targeted to them and selected for in host plant populations. The completion of the *P. sojae* genome sequence and the discovery of RXLR effectors have facilitated Avr gene identification for this and other oomycete plant pathogens. Nonetheless, Avr gene identification still requires substantial work to create short-lists of candidate genes and to
test their interaction with soybean Rps genes. In this paper, we identify the Avr5 gene from P. sojae. We provide evidence that Avr3a and Avr5 are alleles and represent a single genetic locus, Avr3a/5.

Previous studies have demonstrated that the RXLR motif is necessary and sufficient for delivery of proteins into plant cells [22,23]. Results from our co-bombardment assays indicate that Avr3a/5 is able to trigger cell death in Rps5 plants in an appropriate allele-specific manner, when expressed without a signal peptide. Thus, we conclude that recognition of Avr5 occurs within the plant cell and that Rps5 is a cytoplasmic R-gene.

We have recently described the organization of the Avr3a/5 gene and the haplotypes of the locus among P. sojae strains [14], but it is worthwhile to briefly review these features here. The Avr3a/5 gene is embedded in a 10.8 kb DNA segment that includes four other predicted genes. P. sojae strains may possess one or four copies of this 10.8 kb segment arranged in a tandem array. Gain of virulence on Rps3a is caused by transcriptional differences between strains. However, the underlying causes of the transcriptional polymorphisms appear to differ among the strains. No transcripts of Avr3a/5 can be detected in P. sojae strains ACR10 and ACR16 but nevertheless these strains possess a tandem array of four Avr3a/5 copies that is identical in nucleotide sequence to strains that are transcriptionally active, such as P6497. In other P. sojae strains that do not appear to express the Avr3a/5 gene, such as P7064, Avr3a/5 is present as a single copy gene with rearrangements (insertions and deletions) in the promoter region, compared to the reference strain P6497. Our present hypothesis is that the lack of Avr3a/5 transcripts in strains like ACR10 is an epigenetic effect caused by small RNA mediated gene silencing, whereas in strains like P7064 it is caused by a failure of transcription due to the changes present in the promoter. These theories will require further experimentation to test their validity.

Regardless of the mechanism, it is clear that the loss of Avr3a/5 transcripts in P. sojae causes gain of specific virulence in the presence of either Rps3a or Rps5. Although silencing of Avr3a/5 in transformed P. sojae strain P6497:Avr3aP6497-S1 does not gain full virulence on light-grown seedlings containing Rps5, this might result from some residual transcription and expression of Avr3a/5 appropriate allele-specific manner, when expressed without a signal peptide. Thus, we conclude that recognition of Avr5 occurs within the plant cell and that Rps5 is a cytoplasmic R-gene.
compared to P7074; presumably this level is not high enough to trigger a response in the presence of Rps3a. Silencing of Avr3a/5 did not cause loss of general virulence on Williams, suggesting that Avr3a/5 is dispensable for general virulence. Over-expression of Avr3a/5 in P. sojae strains that do not normally express this gene caused the expected loss of specific virulence in the presence of Rps3a or Rps5. However, the lines over-expressing Avr3a/5 retained partial virulence against light-grown seedlings containing Rps5, but not those containing Rps3a. Possibly the resistance conferred by Rps5 was partially compromised by the unusually high level of Avr3a/5 transcripts in the transformed strains or by other effectors in the genetic background of P7074.

We have also shown differential recognition of Avr3a/5 alleles by Rps3a and Rps5. Crucial residues of the Avr3a/5 protein that are required for recognition by Rps5 were identified. Thus, the K64P and A65S changes result in evasion of recognition by Rps5 but not Rps3a. Isolates of P. sojae with reciprocal characteristics, displaying specific virulence in the presence of Rps3a but not Rps5, have been reported but were not available for the present study [28,29,30]. Field surveys of P. sojae pathotypes demonstrate that strains showing virulence on Rps3a but not Rps5 may exist but nevertheless are rare, as summarized in Table 4. Thus, it is possible that there are additional alleles of the Avr3a/5 locus that are differentially recognized by Rps5 but not Rps3a. In order to resolve this, it will be necessary to isolate the strains, confirm their virulence phenotypes, determine the Avr3a/5 gene sequences, and test their functional interaction with Rps3a and Rps5.

To conclude, we have shown that Avr3a and Avr5 from P. sojae represent a single gene, Avr3a/5. This highly polymorphic locus displays copy number variation, sequence polymorphisms, and transcriptional differences among P. sojae strains. The identification of the P. sojae Avr3a/5 gene will assist soybean breeding programs and pathogen diagnostics, and will lead to more rational disease control measures. This is important because root rot caused by P. sojae is among the most destructive diseases that challenges soybean producers.

Table 4. Virulence phenotypes in the presence of Rps3a and Rps5 of P. sojae field isolates, from three different studies.

| Location | Total isolates | Virulence phenotype | Reference |
|----------|----------------|---------------------|-----------|
|          | n | A-Rps3a | A-Rps5 | V-Rps3a | V-Rps5 | A-Rps3a | V-Rps5 | V-Rps3a | V-Rps5 |
| Michigan | 67 | 27 | 6 | 55 | 31 | 36 | 31 | Kaitany et al., 2001 |
| Ohio     | 289 | 58 | 4 | 16 | 21 | 16 | 21 | Dorrance et al., 2003 |
| China    | 75 | 15 | 3 | 56 | 27 | 56 | 27 | Cui et al., 2010 |

A, Avirulent; V, virulent.

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Materials and Methods

Phytophthora sojae isolates, manipulation, plant materials and virulence tests

Strains of Phytophthora sojae were from the collection at Agriculture and Agri-Food Canada, London, ON. Original sources are provided in Table S1. P. sojae isolates were routinely grown on vegetable juice (V8) agar. Transformation of P. sojae was based on published protocols [31,32], as described below. Genomic PCR screening of all putative transformed strains was performed with CE+p35 F/R primers. A complete primer list is provided in Table S2.

Soybean (Glycine max) cultivars Williams (rps) and the Williams isolate L83–570 (Rps3a) and L85–3059 (Rps5) used to evaluate the virulence of P. sojae cultures were from the collections at Agriculture and Agri-Food Canada (London, Ontario) or Virginia Tech. Stem inoculation of light grown plants was as described [13]. Inoculation of etiolated hypocotyls with P. sojae zoospores was carried out as described [33,34]. Each treatment contained at least 20 plants. All inoculated seedlings were incubated in the dark at 25°C under 80% humidity. The virulence tests were repeated at least three times, independently. Fischer’s exact test or Duncan’s multiple range test were performed to determine significance of the results.

Generation of F1 and F2 progeny

Cultures of F1 hybrids were derived from crosses of P6497×P7064. Oospores from were produced by co-cultivation of the parental strains on 2.5% (v/v) vegetable juice (V8) agar plates supplemented with 10 mg/ml beta-sitosterol. Cultures were kept at 25°C in darkness for at least 20 d to produce mature oospores. Oospores were isolated by placing a mature culture in a sterile blender (Waring) with 100 ml of 4°C sterile distilled water (SDW). The culture was macerated for 2 min and sieved twice through a sterile 75 mm nylon mesh. The filtrate was collected in 2 to 5 ml of SDW. The mixture was incubated at 37°C for 16 h. Oospores were washed twice with SDW prior to re-suspending the pellet in 2 to 5 ml of SDW. Approximately 500 oospores were spread onto 9 cm 2.5% (v/v) water agar plates supplemented with beta-sitosterol (10 mg/ml) and rifampicin (10 mg/ml). Plated oospores were incubated in the dark at 25°C for at least four days before being checked for germination. Germinating oospores were observed using a stereo microscope (60× magnification) and transferred to separate 9 cm 2.5% (v/v) V8 agar plates supplemented with rifampicin (10 mg/ml). Hybrid F1 progeny were identified using co-dominant DNA markers that are polymorphic between the parents P6497 and P7064. For production of F2 progeny, oospores were generated from F1 cultures and isolated as described above. A total of 100 F2 progeny were isolated for virulence screening and genetic mapping.

Plasmid construction, transformation, and plant transient expression assays

For transformation experiments on P. sojae, each of the Avr3a alleles was amplified from genomic DNA and the resulting PCR products were separated by agarose gel electrophoresis, excised and purified (QiAquick Gel Extraction Kit, Qiagen) prior to cloning. Purified PCR products of Avr3a were then subcloned into the stable transformation vector CE+p35, which is a construct of pUC19 with the Hom34 promoter and terminator of B. lactucae [35]. Sub-cloning of Avr3a for transcription of the positive (sense) strand was used for overexpression whereas orientation of Avr3a for transcription of the negative (anti-sense) strand was used for gene silencing. The CE+p35:Avr3a and a helper plasmid CE+p35:apII gene (confering resistance to Geneticin) were delivered into P. sojae protoplasts by using a polyethylene glycol mediated co-transformation protocol [31,32]. The transformed cultures were selected on para nitrophenol (30 μg/ml Geneticin and propagated in pea broth medium suspended with 30 μg/ml Geneticin, and subject to quantitative real time PCR (qRT-PCR) analysis.

For transient expression by particle bombardment, each of the Avr3a/5 alleles were sub-cloned into pFF19 plant transient expression vector containing the 35S promoter. Recombinant plasmids were purified for verification by DNA sequencing and for plant transient expression assays. Particle bombardment assays were carried out using a double-barrelled extension of the Bio-Rad He/1000 Particle Delivery System [31]. To measure the activity of Avr35 candidates in triggering Rp5-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 Rp5 compared with leaves lacking either resistance gene. The double-barrelled device was used to deliver a parallel control shot for the expression of GUS DNA plus empty vector (EV) DNA. For each pair of shots, the logarithm of the ratio of the number of blue spots with various Avr5 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 Rp5 leaves were then compared with those from the non-Rp5 leaves using the Wilcoxon rank sum test [31].

Nucleic acid isolation and assay of Avr5 mRNA levels

Genomic DNA of P. sojae was isolated from mycelia grown in 10% vegetable juice (V8) liquid medium according to published methods [6]. Total RNA was isolated using the NucleoSpin RNA II RNA extraction kit (Macherey-Nagel) following the procedures described by the manufacturer. The integrity of total RNA was confirmed using agarose gel electrophoresis.

To investigate the expression efficiency of the Avr3aP6497 transgene in the transformants, qRT-PCR was performed according to the following steps. First-strand cDNA was synthesized using M-MLV reverse transcriptase (RNase-free) and oligo (dT) 18-mer primer (Invitrogen, USA). The qRT-PCR was conducted in 20 μl reactions including 20 ng of cDNA, 0.2 μM gene-specific primer for Avr3a or the reference Actin gene, 10 μl of SYBR Premix ExTaq (TaKaRa, Japan), and 6.8 μl of distilled H2O. The reactions were performed on a real-time PCR instrument (Applied Bio-systems, PRISM 7300, Foster City, CA, USA) 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. Computer software (7300 System Sequence Detection, version 1.4) was used to obtain the expression level of each sample relative to the Actin control. Normalization and comparison of mean Ct values were performed as described [96]. The experiment was conducted twice, each with three independent biological replicates.

Data Deposition

The sequence data for the Avr3a/5 alleles have been deposited to NCBI GenBank under the accession numbers EF587759, JF412456 and JF433921.
Supporting Information

Table S1 List of P. sojae strains used in this study.

Table S2 Oligonucleotide primers used in this study.

Figure S1 Comparison of the Avr3a structural gene in P. sojae strains P6497, P7064, and ACRI2, illustrating promoter region insertions and deletions.

Figure S2 Representative photographs from co-bombardment and virulence assays performed on soybean plants. (A) Photographs of soybean LBS-3059 (Rp5) leaves, after co-bombardment of GUS reporter together with control plasmid or selected Avr3a alleles. Numbers in parenthesis indicate total GUS positive spots counted for each treatment, in this experiment (B) Photographs of light-grown soybean plants after inoculation with P. sojae wild-type and transformed strains, as indicated. (C) Photographs of etiolated soybean hypocotyls after inoculation with P. sojae wild-type and transformed strain, as indicated.

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