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LARGE-SCALE BIOLOGY ARTICLE

Transcriptional Programming and Functional Interactions within the *Phytophthora sojae* RXLR Effector Repertoire [C][W][OA]

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The genome of the soybean pathogen *Phytophthora sojae* contains nearly 400 genes encoding candidate effector proteins carrying the host cell entry motif RXLR-dEER. Here, we report a broad survey of the transcription, variation, and functions of a large sample of the *P. sojae* candidate effectors. Forty-five (12%) effector genes showed high levels of polymorphism among *P. sojae* isolates and significant evidence for positive selection. Of 169 effectors tested, most could suppress programmed cell death triggered by BAX, effectors, and/or the PAMP INF1, while several triggered cell death themselves. Among the most strongly expressed effectors, one immediate-early class was highly expressed even prior to infection and was further induced 2- to 10-fold following infection. A second early class, including several that triggered cell death, was weakly expressed prior to infection but induced 20- to 120-fold during the first 12 h of infection. The most strongly expressed immediate-early effectors could suppress the cell death triggered by several early effectors, and most early effectors could suppress INF1-triggered cell death, suggesting the two classes of effectors may target different functional branches of the defense response. In support of this hypothesis, misexpression of key immediate-early and early effectors severely reduced the virulence of *P. sojae* transformants.

INTRODUCTION

Oomycete plant pathogens are destructive to a vast variety of plants important to agriculture, forestry, and natural ecosystems. Examples include *Phytophthora infestans* causing late blight of potato, responsible for the Irish potato famine; *Phytophthora ramorum*, responsible for sudden oak death; and *Phytophthora sojae*, responsible for root and stem rot of soybean. *P. sojae* is one of the most important pathogens of soybean (*Glycine max*), causing losses of $1 to $2 billion per year worldwide (Tyler, 2007). *P. sojae* has a narrow host range and is restricted primarily to soybean. Resistant cultivars carrying major resistance (*R*) genes against *P. sojae* (*Rps* genes) have been a cornerstone for management of the pathogen for 50 years (Schmitthenner, 1985; Buzzell et al., 1987). Currently, 14 *Rps* genes at eight loci are known (Buzzell and Anderson, 1992; Burnham et al., 2003; Sandhu et al., 2005). The *Rps* genes function in a gene-for-gene interaction with avirulence genes in *P. sojae* (Whisson et al., 1994; Tyler et al., 1995; Gijzen et al., 1996; May et al., 2002; Tyler, 2009).

Effector-triggered immunity (ETI) forms one of two overlapping branches of the plant defense system that responds to infection by pathogens. ETI is triggered by recognition of pathogen effector molecules by the plant defense system. In many cases, recognition is mediated by plant proteins that are products of genetically defined *R* genes. Natural selection favors pathogens that can avoid ETI as a result of loss or diversification of the recognized effector(s) and/or by acquisition or activation of additional effectors that can suppress ETI (Jones and Dangl, 2006). R proteins may recognize pathogen effectors either directly or indirectly, resulting in disease resistance and usually a hypersensitive cell death response (HR) at the infection site (Chisholm et al., 2006). The second branch of the defense system responds to conserved microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) and is referred to as PAMP-triggered immunity (PTI) (Felix et al., 1993; Medzhitov and Janeway, 1997). Recognition of PAMPs is mediated by transmembrane pattern recognition receptors, and signaling is propagated by downstream mitogen-activated protein kinase (MAPK) cascades (Boller and Felix, 2009; Boller and He, 2009). Some PAMPs, such as bacterial flagellin and lipopolysaccharides, do not trigger

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HR in plant hosts (Mishina and Zeier, 2007). However, some other PAMPs may induce a vigorous defense response and HR. For example, *Phytophthora* elicitin proteins trigger an HR in species of the genus *Nicotiana* and a few other genera (Kamoun et al., 1998a). Elicitins are small (10 kD) disulfide-bonded sterol carrier proteins produced by all *Phytophthora* species. The responses of tobacco (*Nicotiana tabacum*) tissues to the elicitins cryptogein and INF1 from *Phytophthora cryptogea* and *P. infestans*, respectively, have been characterized extensively (Boissy et al., 1996; Kamoun et al., 1998a, 1998b; Kanzaki et al., 2003; Lherminier et al., 2003; Huitema et al., 2005; Lochman et al., 2005; Kanneganti et al., 2006; Svozilová et al., 2009). The extent to which ETI and PTI involve distinct mechanisms is still an open question (Jones and Dangl, 2006; Lee et al., 2009; Thomma et al., 2011).

Mitogen-activated protein kinase (MAPK) cascades mediate the activation of PTI and contribute to the activation of ETI (Kovtun et al., 1998; Ligterink and Hirt, 2001; Jin et al., 2002; Rodriguez et al., 2010). MAPK cascades consist of at least three protein kinases: a MAPK kinase kinase phosphorylates and activates a MAPK kinase, which in turn activates a MAPK by phosphorylation. The *Arabidopsis thaliana* MAPK kinase kinase, MEKK1 (Mizoguchi et al., 1998; Covic et al., 1999), is required for flg22-induced activation of MPK4 (Suarez-Rodriguez et al., 2007), which interacts with MKK1 and MKK2 and negatively regulates plant innate immunity (Gao et al., 2008). MKK1 and MKK2 are also required for the activation of MPK3 and MPK6 in response to flg22 (Mészáros et al., 2006; Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009).The tobacco MEKK1 ortholog NPK1 is involved in responses mediated by the resistance genes *N*, *Bs2*, and *Rx* (Kovtun et al., 1998; Jin et al., 2002). MAPKs appear to exert their control of plant defenses via a network of transcription factors, especially those of the *WRKY* family (Pandey and Somssich, 2009).

Effector proteins are secreted by many plant pathogens to promote infection (Alfano and Collmer, 2004; Chang et al., 2004; Göhre and Robatzek, 2008; Torto-Alalibo et al., 2009). Gram-negative bacterial plant pathogens, such as *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas campestris*, deliver 15 to 30 effectors per strain into host cells using type III secretion systems to suppress PTI and ETI, including the HR (Kjemtrup et al., 2000). These type III effectors use diverse strategies to alter host immunity. One strategy is to destabilize host protein components, either by direct cleavage or by modulating ubiquitination (Axtell et al., 2003; Colby et al., 2006; Ade et al., 2007; Chosed et al., 2007). Type III effectors can also modify host transcription (Kay et al., 2007; Römer et al., 2007; Sugio et al., 2007) or RNA metabolism (Fu et al., 2007). The third major strategy is to inhibit the kinases involved in plant defense signaling, especially MAPK pathways (Zhang et al., 2007; Block et al., 2008; Xiang et al., 2008).

Bioinformatic analyses of the draft genome sequences of *P. sojae*, *P. ramorum*, *P. infestans*, and *Hyaloperonospora arabidopsidis* (Tyler et al., 2006; Haas et al., 2009; Baxter et al., 2010) have identified an extraordinarily large superfamily of candidate effector proteins (134 to 565 per genome), with sequence similarity to cloned oomycete avirulence genes, including *P. sojae Avr1b*-1 (Shan et al., 2004), *P. infestans Avr3a* (Armstrong et al., 2005), and *H. arabidopsidis ATR1* (Rehmany et al., 2005) and *ATR13* (Allen et al., 2004). These Avirulence Homolog (Avh) genes all encode small secreted proteins with a shared N-terminal RxLR-dEER motif (Rehmany et al., 2005; Jiang et al., 2008), which acts to carry the proteins into the host cell cytoplasm (Whisson et al., 2007; Dou et al., 2008a) through binding to cell surface phosphatidylinositol-3-phosphate (Kale et al., 2010). Thus, presence of the RXLR-dEER motifs in the Avh proteins makes them all candidate effectors. The importance of the Avh proteins is underlined by the finding that 10 recently cloned oomycete *Avr* genes all encode RXLR-dEER proteins, including *Avr1a*, *Avr3a*, *Avr3c*, and *Avr4/6* from *P. sojae* (Shan et al., 2004; Dong et al., 2009; Qutob et al., 2009; Dou et al., 2010), *Avr2*, *Avr4*, *Avr-blb1*, and *Avr-blb2* from *P. infestans* (Armstrong et al., 2005; van Poppel et al., 2008; Vleeshouwers et al., 2008; Oh et al., 2009), and ATR1 and ATR13 from *H. arabidopsidis* (Allen et al., 2004; Rehmany et al., 2005). Due to selection pressure from the hosts, the avirulence genes show extensive variations, including amino acid changes indicative of strong positive selection, gene truncations and deletions, and transcriptional silencing (Jiang et al., 2008; Qutob et al., 2009).

The Avh proteins are presumed to contribute to virulence. However, the molecular mechanisms by which they act are currently unclear. The Avh proteins lack significant sequence similarity to any known proteins (other than oomycete Avr proteins) (Jiang et al., 2008). Functional assays have demonstrated that four Avr proteins positively contribute to biotrophic growth inside susceptible host cells. High-level constitutive expression of Avr1b in *P. sojae* transformants made the strains more virulent on soybean, indicating that *Avr1b*-1 contributes positively to virulence (Dou et al., 2008b). Avr1b and Avh331 from *P. sojae* could suppress programmed cell death (PCD) triggered in soybean, *Nicotiana benthamiana*, and *Saccharomyces cerevisiae* cells by the pro-apoptotic protein BAX, suggesting that suppression of defense-related host cell death is one mechanism by which these two effectors contribute to virulence (Dou et al., 2008b). Avr3a is required for full virulence of *P. infestans* (Bos et al., 2010) and could suppress cell death induced by the PAMP INF1, suggesting that this as a virulence function for Avr3a (Bos et al., 2006). Expression of *ATR13* from *H. arabidopsidis* in plant cells suppressed PAMP-triggered reactive oxygen species production and callose deposition (Sohn et al., 2007). *H. arabidopsidis* ATR1 could also contribute to virulence when delivered by *Pst* DC3000 (Sohn et al., 2007). Here, we report a systematic functional characterization of a large sample of the Avh proteins encoded in the *P. sojae* genome. The results reveal that most of the proteins have the potential to suppress PCD triggered by effectors and a PAMP. However, the expression of these potential effectors during *P. sojae* infection is dominated by a small number of proteins whose program of expression appears coordinated to maximize suppression of host defenses.

RESULTS

Large Numbers of *P. sojae* Effectors Can Suppress BAX-Triggered PCD

The PCD triggered in plants by the pro-apoptotic mouse protein BAX physiologically resembles that associated with the

defense-related HR (Lacomme and Santa Cruz, 1999; Baek et al., 2004). As a result, the ability to suppress BAX-triggered PCD (BT-PCD) has proved a valuable initial screen for pathogen effectors capable of suppressing defense-associated PCD (Abramovitch et al., 2003; Jamir et al., 2004; Dou et al., 2008b). Therefore, to begin to characterize the predicted *P. sojae* effector repertoire, we screened ~40% of *P. sojae* effectors for their ability to suppress BT-PCD. The effectors were selected on the basis of at least one of three criteria, namely, that they were encoded by identified avirulence genes, that they were highly expressed during infection, or that they contained C-terminal W motifs (Jiang et al., 2008) previously shown to be associated with effectors that could suppress BT-PCD (Dou et al., 2008b).

A total of 169 effector genes (see Supplemental Data Set 1 online) were screened using an *Agrobacterium tumefaciens*–mediated transient expression assay in *N. benthamiana*. *Agrobacterium* strains carrying each effector gene were infiltrated into *N. benthamiana* leaves either simultaneously with a strain carrying the *BAX* gene or 12 or 24 h prior to infiltration of the BAX-carrying strain (i.e., three different assays for each effector, each done with at least three replications) (Figure 1A). The degree of PCD triggered by BAX in each case was scored after 72 h on a three-point qualitative scale (consistent suppression, partial suppression, or no suppression). Of the 169 effectors screened, 107 consistently suppressed BT-PCD when infiltrated 24 h prior to BAX, 20 partially suppressed BT-PCD, and 31 had no activity (Figure 1C; see Supplemental Data Set 1 online). When infiltrated at the same time as BAX, only 6 and 19 could completely or partially suppress BT-PCD, respectively. When infiltrated 12 h prior to BAX, 41 and 38 could completely or partially suppress BT-PCD, respectively. Integrating the results from the three assays, a six-point scale was assigned to each effector: two points for consistent suppression and one point for partial suppression at each time point (Figure 1D; see Supplemental Data Set 1 online).

It is unlikely that suppression of BT-PCD resulted from inhibition of BAX DNA delivery or PVX replication after the second *A. tumefaciens* infiltration because prior infiltration with *A. tumefaciens* cells carrying an enhanced green fluorescent protein (*eGFP*) gene instead of an *Avh* gene never suppressed BT-PCD (e.g., Figures 1A and 1E). To further validate that suppression of BT-PCD did not result from suppression of the accumulation of the BAX protein (either at the transcriptional, translational, or posttranslational level), protein immunoblots were performed to determine the amount of BAX protein accumulated in tissues showing suppression of BT-PCD compared with those not showing suppression. As illustrated in Figure 1B, the level of BAX protein was identical in tissues showing suppression and those not showing suppression. Prior infiltration of leaf tissue with *A. tumefaciens* cells carrying *eGFP* reduced the level of BAX protein accumulated when there was a 16- or 24-h delay compared with a 0- or 12-h delay (Figure 1F); this reduction likely partially explains why many more effectors could suppress BT-PCD given a 24-h delay compared with a 0- or 12-h delay (Figure 1C). One reason for the reduced BAX protein levels was revealed when *A. tumefaciens* cells carrying *DsRed*, encoding red fluorescent protein, were substituted for those carrying a *BAX* gene; as the time delay increased, fewer cells in the tissue expressed the second protein (DsRed) (Figures 1G and 1H). The fact that most cells expressed either *eGFP* or *DsRed* but only ~10% expressed both (Figure 1H) suggests that many of the effectors were acting to suppress the spread of BT-PCD throughout the infiltrated tissue rather than preventing the initial triggering of PCD by BAX.

A small number (11) of the effectors could trigger cell death, chlorosis, or mottling in *N. benthamiana* leaves themselves, suggesting either that they were recognized by the defense machinery of *N. benthamiana* or that they could act as toxins (Figure 2A). All of these effectors were tested for the ability to trigger PCD in soybean leaf cells, using double-barreled particle bombardment assays (Dou et al., 2008b). All the effectors, except Avh163, triggered PCD in soybean leaves (Figure 2B), suggesting that these proteins trigger similar responses in diverse plant families.

Suppression of INF1- and Effector-Triggered PCD by *P. sojae* Effectors

To extend the screen of the effectors to the suppression of PCD triggered by pathogen molecules, 49 effectors were screened for the ability to suppress PCD triggered by INF1 (PT-PCD) and by the *P. sojae* effectors Avh238 and Avh241 (ET-PCD) using the *Agrobacterium* infiltration assay (Table 1, Figure 3). Avh238 and Avh241 were selected because they produced highly reproducible cell death responses and because one (Avh241) had W motifs and one (Avh238) did not. The 49 effectors were selected based on their high expression level during infection, on whether they had a BT-PCD suppression score of 2 or more, and/or on whether they were encoded by avirulence genes. The P7076 allele of Avh238 was added later in the study (see below). Of the 49 effectors selected, 43 had the ability to suppress BT-PCD. A uniform delay of 16 h was used between effector infiltration and the PCD challenge (Avh238, Avh241, INF1, or BAX control); selected effectors were retested using a 24-h delay. The cell death phenotype was scored 5 d after the second infiltration. Of the effectors that could suppress BT-PCD, 23 (53%) also could suppress PCD triggered by INF1 and by both effectors, while 16 could only suppress PCD triggered by the two effectors, but not by INF1 (Table 1, Figure 3). All of the effectors that suppressed BT-PCD could also suppress PCD triggered by at least one effector. Overall, 22 could suppress PCD triggered by at least one effector but not by INF1. Most effectors that could suppress ET-PCD showed no specificity Avh238- or Avh241-triggered cell death; exceptions were Avh277, PsAvr3a, Avh158, Avh270 (specific for Avh238), Avh1, and Avh140 (partially specific for Avh241). Of the eigth effectors that could not suppress BT-PCD, two (Avh36 and Avr3c) could not suppress any elicitor of PCD, while the others could suppress some combination of effector- and/or INF1-triggered PCD.

Polymorphism Profiles of *P. sojae* Effectors

Effectors that trigger plant defense responses, such as those encoded by avirulence genes, often exhibit a high level of polymorphism within the species as a result of selection for alleles that do not trigger responses deleterious to the pathogen. To identify polymorphisms in *P. sojae* effector genes, the genomes of three

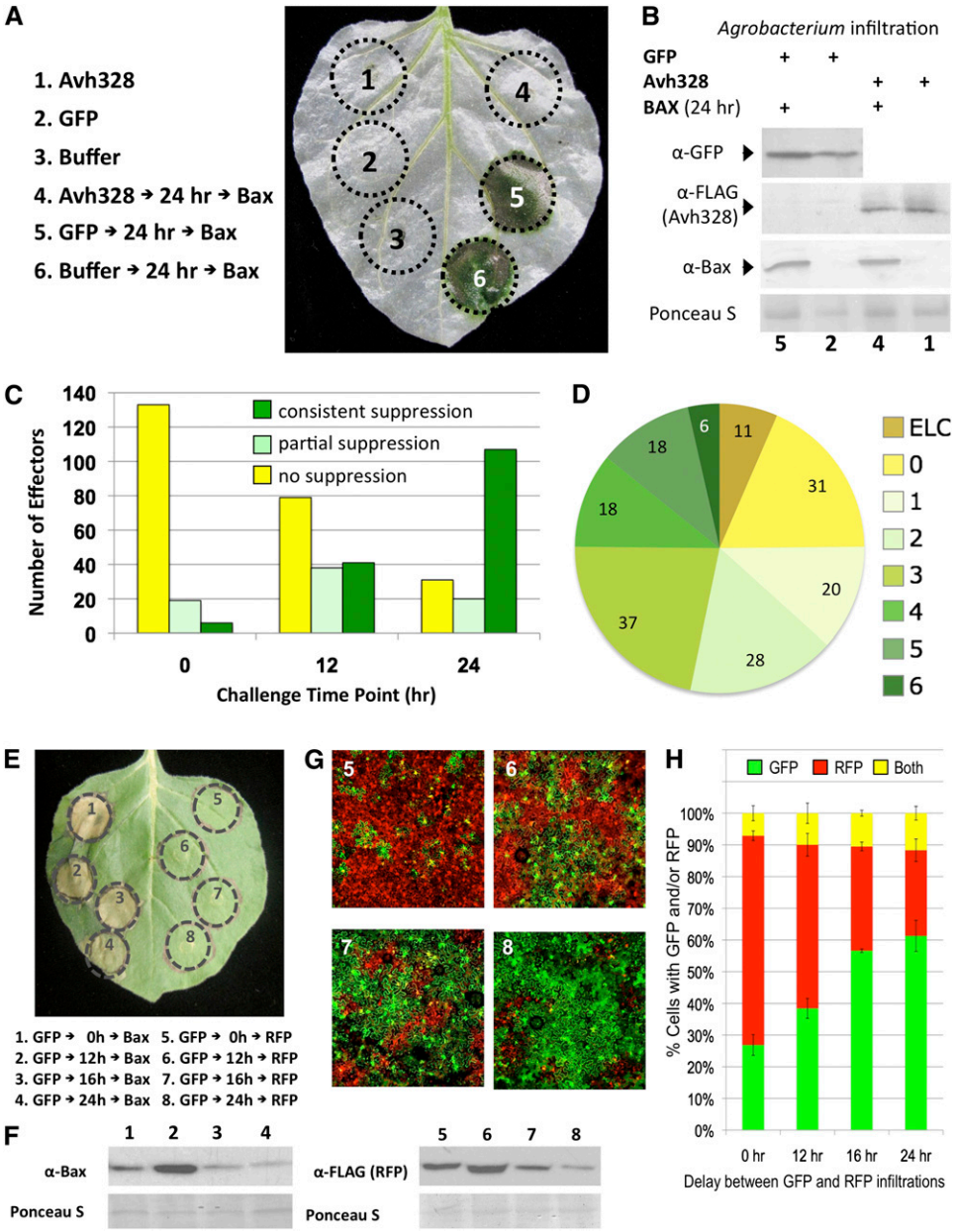


Figure 1. Suppression of PCD by *P. sojae* Effectors.

(A) Assay for suppression of BAX-triggered cell death (BT-PCD) in *N. benthamiana* by *P. sojae* effectors (example Avh328). *N. benthamiana* leaves were infiltrated with buffer or *A. tumefaciens* cells containing a vector carrying the effector gene or a control gene (e*GFP*), either alone or followed 24 h later with *A. tumefaciens* cells carrying a mouse *Bax* cDNA. Photos were taken at 5 d after infiltration after decolorization with ethanol.

(B) Protein immunoblot analysis of eGFP, Avh328, and Bax protein levels in plant tissues treated as in **(A)**. Proteins were extracted 60 h after the last infiltration. Equal amounts of protein lysate were loaded in each lane, as verified by Ponceau S staining. Numbers below the lanes match treatment numbers in **(A)**.

(C) Suppression of BAX-triggered PCD by preinfiltration with RXLR effector genes. *A. tumefaciens* cell lines, each carrying 1 of the 169 predicted RXLR effector genes, were separately infiltrated in *N. benthamiana* leaves, 0, 12, or 24 h prior to infiltration with cells carrying BAX cDNA and then scored for consistent or partial suppression of BT-PCD after 5 d.

(D) Distribution of BT-PCD suppression activity by predicted effectors. A six-point scale was assigned to each effector: two points were assigned for consistent suppression at any time point and one point for partial suppression. ELC, effectors eliciting cell death.

(E) to **(H)** Levels and distribution of proteins expressed following the two-step infiltration protocol used to screen the *P. sojae* effectors. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells containing a vector carrying *eGFP*, followed 0 h (i.e., simultaneously), 12, 16, or 24 h later with *A. tumefaciens* cells carrying DNA encoding Bax or FLAG-tagged red fluorescent protein (DsRed-FLAG).

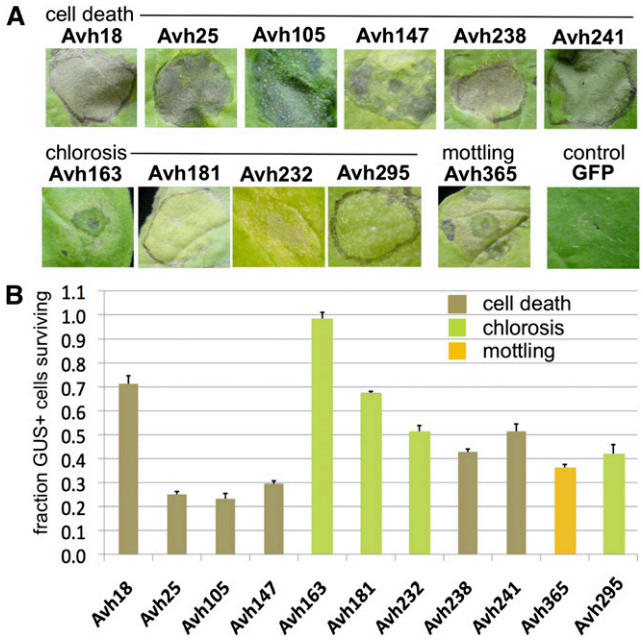


Figure 2. Effectors That Trigger Cell Death Symptoms in *N. benthamiana* and Soybean.

(A) Tissue responses triggered in *N. benthamiana* by *P. sojae* effectors. Avh18, Avh25, Avh105, Avh147, Avh238, and Avh241 triggered cell death; Avh163, Avh181, Avh295, and Avh365 induced chlorosis; and Avh232 triggered mottling. Representative photos were taken 5 d after infiltration.

(B) Effector-triggered PCD measured in soybean using double-barreled particle bombardment of leaves. The number of GUS-positive blue spots surviving in the presence of DNA encoding each effector was measured as a ratio to an empty vector control. PCD triggered by an effector protein reduces the numbers of blue spots because the GUS-producing cells are killed. All effectors except Avh163 produced a statistically significant ($P < 0.001$) reduction in blue spots as determined by the Wilcoxon signed ranks test. Bars are color-coded based on symptoms triggered in *N. benthamiana*.

P. sojae isolates were sequenced to 6- or 13-fold coverage using the 454 Life Sciences Titanium technology (summarized in Supplemental Table 1 online). The three isolates, P7064 (race 7), P7074 (race 17), and P7076 (race 19), together with the isolate from which the existing draft genome sequence was derived (P6497; race 2), comprise the four major genotypes of *P. sojae*, encompassing nearly all the genetic variation that occurs in the species (Forster et al., 1994). Of the 391 effectors predicted to be encoded in the P6497 genome (Jiang et al., 2008), reliable 454 assemblies could be obtained for 378 of them, including 18 that were duplicates of another effector gene; the remainder had poor coverage and/or the presence of close nonidentical paralogs interfered with reliable the assembly. Of the 378 assembled, 147 had small numbers (1 to 5) of nucleotide substitutions among any of the four *P. sojae* lineages (see Supplemental Data Set 2 online). Among this group, the frequency of substitutions closely followed a Poisson distribution (Figure 4A), indicating a uniform rate of accumulation of mutations across the four lineages of 1.52 per gene. However, 164 genes showed no substitutions whatsoever, far in excess of the number predicted from the Poisson distribution. Presumably, many alleles were lost due to the founder effect associated with the origination of the four *P. sojae* lineages and subsequent generations of inbreeding by this homothallic species (Forster et al., 1994). A much larger number of genes (50 genes) than predicted from the Poisson distribution showed large numbers of substitutions (six or more), suggesting that these genes were under positive selection (Table 2, Figures 4B and 4C). Individually, within this group, 18 of the 50 genes had ratios of nonsynonymous substitutions (dN) to synonymous substitutions (dS) statistically significantly >1.0. Of the eight effectors that triggered PCD in *N. benthamiana* and for which assemblies were obtained, six were polymorphic and three showed high levels of polymorphisms in at least one isolate, for example, Avh238 (Figure 4C). Very few (five) effector genes were entirely missing from any isolate; however, one of these was *Avh*6, which is one of the most strongly constitutively expressed effectors in P6497 (see next section). A paralog of *Avh*6, *Avh*32, was present in isolates lacking *Avh*6 but was missing from one isolate that retained *Avh*6, suggesting the two effectors may be redundant. Because most of the effector genes are quite short and because of the multiple testing adjustment, the individual tests for positive selection lacked statistical power. Therefore, as an alternative, the genes were grouped according to the number of mutations or according to different functional groups and then two group-wise tests for positive selection were conducted. One test used the Wilcoxon signed ranks test to examine if dN exceeded dS more frequently among the genes in a group than expected at random. In the other test, the protein sequences of the most divergent alleles were concatenated and the differences between the dN and dS values of the concatenated sequences were examined using a *t* test. The results revealed evidence for positive selection in groups of genes with seven or more mutations in the four *P. sojae* lineages, in avirulence genes, elicitor genes, and in highly expressed effector genes. No

Figure 1. (continued).

(E) Cell death symptoms at 5 d after infiltration.

(F) Same experiment as **(E)**, but proteins were extracted from the infiltrated regions 60 h after the second infiltration and probed with anti-Bax or anti-FLAG antibodies.

(G) Same experiment as **(E)**, but the infiltrated regions were examined by confocal microscopy 5 d after the second infiltration. Red and green channels were merged in these photographs.

(H) The experiment illustrated in **(E)** and **(G)** was repeated three times on different days. At each time point, 400 to 600 cells from multiple fields were counted 4 d after the last infiltration for presence of eGFP and/or DsRed, and the average percentage of each category was plotted. Bars indicate SE.

Table 1. Suppression of Cell Death Triggered by BAX, Effectors, and a PAMP

		Suppression of Cell Death[c]				
Effector (Allele)[a]	Preinfiltration Time[b]	BAX	Avh238	Avh241	INF1	Notes
Avr1b[d]	24 h	+	n.t.	n.t.	n.t.	Intermittently triggers cell death[d]
Avr3a	16 h	+	+	–	–	Was Avh92 and Avh370
Avr3c	16 h	–	–	–	–	Was Avh27
	24 h	–	n.t.	n.t.	n.t.	
Avr4/6	16 h	+	+	+	–	Was Avh171
Avh1	16 h	+	–	–	–	
	24 h	+	±	+	n.t.	
Avh5	16 h	+	+	+	+	
Avh6	16 h	+	+	+	–	
Avh7b1	16 h	+	+	+	+	
Avh8	16 h	+	+	+	–	
Avh16	16 h	+	+	+	–	
Avh23	16 h	+	+	+	+	
Avh29	16 h	+	+	+	–	
Avh36	16 h	–	–	–	–	
	24 h	–	n.t.	n.t.	n.t.	
Avh38	16 h	+	+	+	+	
Avh52	16 h	+	+	+	+	
Avh62	16 h	+	+	+	+	
Avh64	16 h	+	+	+	+	
Avh94a1	16 h	+	+	+	+	Was Avh94
Avh98a	16 h	+	+	+	+	Was Avh98
Avh107	16 h	+	+	+	–	
Avh109	16 h	+	±	±	+	
Avh110	16 h	+	+	+	+	
Avh115	16 h	+	+	+	+	
Avh138	16 h	+	–	–	–	
	24 h	+	±	±	n.t.	
Avh140	16 h	–	–	+	–	
	24 h	–	±	+	n.t.	
Avh141	16 h	+	+	+	–	
Avh158	16 h	+	+	–	–	
	24 h	+	+	–	–	
Avh172	16 h	+	+	+	–	
Avh180	16 h	+	+	+	+	
Avh181	16 h	n.t.	n.t.	n.t.	–	Triggers cell death[e]
Avh183	16 h	+	+	+	–	
Avh196	16 h	+	+	+	+	
Avh197	16 h	+	+	+	–	
Avh231	16 h	+	+	+	+	
Avh238 (P6497)	16 h	n.t.	n.a.	n.t.	+	Triggers cell death [e]
Avh238 (P7076)	16 h	n.t.	–	–	n.t.	
	24 h	–	–	–	+	
Avh240	16 h	+	+	+	+	
Avh256	16 h	+	+	+	–	
Avh260	16 h	+	+	+	–	
Avh263	16 h	+	+	+	+	
Avh270	16 h	+	+	–	–	
Avh277	16 h	–	+	–	–	
Avh283	16 h	+	+	+	+	
Avh299	16 h	+	+	+	–	
Avh320	16 h	+	+	+	+	
Avh324	16 h	+	+	+	–	
Avh328	16 h	+	+	+	+	

(Continued)

Table 1. (continued).

Effector (Allele)[a]	Preinfiltration Time[b]	Suppression of Cell Death[c]				Notes
		BAX	Avh238	Avh241	INF1	
Avh331	16 h	+	+	+	+	Candidate Avr1k
Avh333	16 h	–	–	+	+	
	24 h	–	+	+	n.t.	
Avh345	16 h	+	+	+	+	
Avh360	16 h	+	+	+	+	
Avh432	16 h	–	+	+	–	

[a]Includes 49 effectors that were screened for suppression of ET-PCD and PT-PCD, plus data for Avr1b from Dou et al. (2008b) and for Avh181 and Avh238^{P6497}, which were tested for suppression of PT-PCD in combination with Avh172, which suppresses the PCD triggers by the effectors themselves.

[b]Time elapsed between infiltration with strain carrying effector gene and infiltration with strain carrying the cell death elicitor gene.

[c]+, Consistent suppression; ±, partial suppression; –,no suppression; n.t., not tested; n.a., not applicable. The cell death phenotype was scored 5 d after the second infiltration.

[d]Data from Dou et al. (2008b). In this study, Avr1b intermittently triggered weak PCD in *N. benthamiana*, making it difficult to consistently assay cell death suppression.

[e]The cell death triggered by these effectors was suppressed by infiltration of a strain carrying Avh172 4 h beforehand, enabling assay of suppression of INF1-triggered PCD. Avh172 does not suppress INF1-triggered cell death.

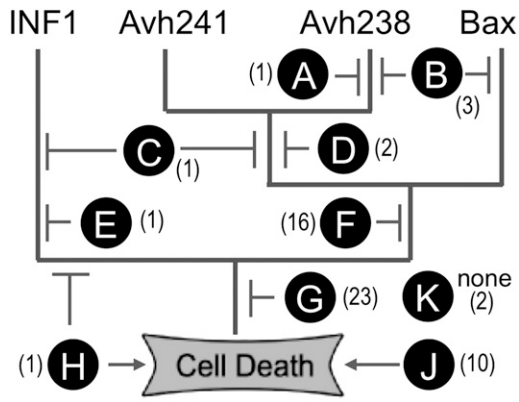
significant evidence for positive selection was found in groups of genes with six or less mutations.

Transcriptional Profiles of *P. sojae* Effectors

Since the effectors identified in the *P. sojae* genome are predicted to mediate infection, we examined the transcriptional profile of the effectors using the Affymetrix SoyChip, which contains probe sets for 15,421 predicted *P. sojae* genes, including 181 for RXLR effector genes (the chip was constructed in 2005 before the RXLR set was fully defined). Soybean hypocotyls of the highly susceptible cultivar Sloan were inoculated with *P. sojae* mycelia from isolate P6594 (race 1), and the inoculated region was harvested for RNA extraction 3, 6, and 12 h later. P6594 is genetically near-identical to P6497 but expresses *Avr1b*-1 (Forster et al., 1994; Shan et al., 2004). As a control, RNA was extracted from a sample of the mycelium that was used for inoculation. Three biological replicates of the experiments were carried. The data were background subtracted using GeneChip Robust Multiarray Averaging (GC-RMA) and then normalized using a GC-RMA quantile normalization algorithm that was modified to account for the fact that variable amounts of pathogen mRNA (4 to 7%) were present in the RNA samples. Expression of 3219 *P. sojae* genes could be detected by 12 h of infection (false discovery rate [FDR] < 0.05), of which 20 were predicted RXLR effector genes.

The majority of these 20 effector genes were induced during infection, though the level of induction varied widely, from <2-fold to nearly 120-fold (Table 3). Seven effector genes were expressed at very high levels (level more than 6.0) even in the inoculum, and many cases were further increased somewhat during infection; we term these effectors immediate-early effectors (Table 3). One of these was *Avr1b*-1, which was induced 8-fold during infection. Four were expressed at very low levels in the inoculum but were then very strongly induced (25- to 120-fold) by 12 h; we term these early effectors. Among these four was *Avh*238, which triggered PCD in both *N. benthamiana* and soybean leaves. (We reserve the term late effectors for those expressed during the necrotrophic phase of infection, which were not examined here.) The remaining nine effector genes detected showed <15-fold induction following infection but overall were more weakly expressed than the other immediate-early genes (level <8.0 at 12 h); we termed these weakly expressed immediate-early effectors. All of the 20 strongly expressed effectors could suppress both BT-PCD and ET-PCD, except for Avh238, which could not suppress ET-PCD; Avr1b, Avh181, and Avh163 could not be assayed because they triggered PCD themselves (Tables 1 and 3). However, only 3/6 immediate-early effectors and 1/7 weakly expressed immediate-early effectors could suppress PT-PCD (Table 3). By contrast, all four early effectors could suppress PT-PCD (Table 3), suggesting that the early effectors are relatively more specialized for suppression of PT-PCD. Of the 20 genes, eight showed evidence for positive selection (Table 3).

To validate the expression patterns identified by the microarray data, the experiment was repeated 4 years later using a different cultivar (Williams), a different isolate (P6497), a different location (Nanjing Agricultural University instead of Virginia Tech), and a different assay (quantitative RT-PCR [qRT-PCR]). Thirteen of the most highly expressed effector genes' expression was assayed. Three housekeeping genes, identified from the microarray data as constitutively expressed, were used jointly as a reference to relate the qRT-PCR data to the microarray data. Figure 5 shows that for 12 effectors the levels and programs of expression were very similar between the two experiments, with the exception of the 12-h time point when several effector mRNA levels dropped in the RT-PCR experiment; this may have reflected the overall faster appearance of symptoms in the RT-PCR experiment. One effector (Avh38) showed an apparent expression polymorphism between the two strains; although the program was nearly identical in the two strains, the average expression level was 30-fold lower in P6497.



A: Avh277
B: PsAvr3a, Avh158, Avh270
C: Avh333
D: Avh140, Avh432
E: Avh238^{P7076}
F: Avh1, Avh6, Avh8, Avh16, Avh29, Avh107, Avh138, Avh141, Avr4/6, Avh172, Avh183, Avh197, Avh256, Avh260, Avh299, Avh324
G: Avh5, Avh7b1, Avh23, Avh38, Avh52, Avh62, Avh64, Avh94a1, Avh98a, Avh109, Avh110, Avh115, Avh180, Avh196, Avh231, Avh240, Avh263, Avh283, Avh320, Avh328, Avh331, Avh345, Avh360
H: Avh238^{P6497}
J: Avh18, Avh25, Avh105, Avh147, Avh163, Avh181, Avh223, Avh241, Avh295, Avh365
K: Avr3c, Avh36

Figure 3. Classification of Effectors Based on Suppression of Cell Death Triggered by BAX, Effectors, and the PAMP INF1.

Effectors were classified based on their partial or consistent suppression of cell death when infiltrated 16 or 24 h prior to the elicitor (more details in Table 3). T bar, partial or consistent suppression of cell death; arrow, triggering of cell death. Numbers of genes in each category are shown in parentheses. Categories: A, suppresses only Avh238-triggered cell death; B, suppresses only Avh238- and BAX-triggered cell death; C, suppresses cell death triggered by INF1 or either effector, but not by BAX; D, only suppresses cell death triggered by either effector; E, suppresses only INF1-triggered cell death; F, suppresses cell death triggered by either effector or by BAX, but not by INF1; G, suppresses cell death triggered by either effector, INF1 or BAX; H, suppresses cell death triggered by INF1, triggers cell death itself; J, triggers cell death in *N. benthamiana* itself; K, neither suppresses nor triggers cell death.

Cooperation among *P. sojae* Effectors

Of the 16 immediate-early effectors (seven strongly expressed and nine weakly expressed), all 14 that could be tested could suppress the PCD triggered by Avh238 (as well as Avh241) (Table 3). Since *Avh*238 was weakly expressed at time zero but was the most strongly expressed effector by 12 h, this observation suggested the hypothesis that a key function of the immediate-early effectors was to suppress plant defenses triggered by effectors expressed later in infection, such as Avh238. Since all three early effectors that were strongly induced at 12 h, other than Avh238, which triggers PCD itself, could suppress INF1-triggered PCD, we further hypothesized that the role of the early effectors might be more focused on suppression of PTI.

To test this hypothesis, we performed a sequential *Agrobacterium* infiltration experiment designed to recapitulate the program of effector expression during infection. For this experiment, we used Avh238 together with Avh172. *Avh*172 was strongly expressed in the inoculum, could suppress Avh238-triggered PCD, but could not suppress INF1-triggered PCD. *Agrobacterium* cells carrying *Avh*172 were infiltrated into *N. benthamiana* leaves. Four hours later, *Agrobacterium* cells carrying *Avh*238 were infiltrated into the same region, and then a further 8 h later *Agrobacterium* cells carrying *INF*1 were infiltrated into the same region. Control infiltrations were performed on the same leaves in which one, two, or all three of the effector or *INF*1 genes were replaced by a gene encoding *eGFP*. As shown in Figure 6A, under these conditions, Avh172 could suppress the PCD triggered by Avh238, but not the PCD triggered by INF1. Coinfiltration of *Avh*238 and *INF*1 in the absence of *Avh*172 led to PCD, as both proteins trigger PCD. However, when *Avh*172 was infiltrated into the leaves prior to the successive infiltrations with *Avh*238 and *INF*1, no PCD was observed, suggesting that Avh238 could suppress the PCD triggered by INF1, but only when the plant cells were prevented from engaging in PCD in response to Avh238 itself. We used protein immunoblots to confirm that in every case suppression of PCD triggered by Avh238 and INF1 did not result from a reduction in the accumulation of the relevant protein. Similar cooperation experiments with Avh6 were uninformative because Avh6 could not suppress PCD triggered by Avh238 when there was only a 4-h gap between infiltrations (see Supplemental Figure 1 online). In the case of Avh181, both Avh6 and Avh172 could suppress the PCD triggered by Avh181, even when coinfiltrated with Avh181, indicating cooperation (Figures 6B and 6C). However, even when the PCD triggered by Avh181 was suppressed, Avh181 showed no ability to suppress PCD triggered by INF1 (Figures 6B and 6C). Thus, Avh181 may have a different function than suppressing PCD triggered by PAMPs such as INF1 or may simply be unable to suppress the PCD triggered by INF1 in particular.

To confirm further that Avh238 could suppress INF1-triggered PCD, we searched for alleles of Avh238 that did not trigger PCD in *N. benthamiana*. The Avh238 alleles in P7074 and P7076 show large numbers of substitutions relative to that in Avh238 (Figure 4C). The Avh238 allele found in P7074 triggered PCD in *N. benthamiana* just like the P6497 allele. However, the P7076 allele (Avh238^{P7076}) did not trigger PCD in *N. benthamiana*. Avh238^{P7076} could suppress the PCD triggered by INF1 (Figures 6D and 6E), confirming this activity of Avh238. To further explore the mechanisms by which Avh238 suppressed INF1-triggered PCD, we tested if Avh238^{P7076}could suppress PCD triggered by overexpression of the MAPK genes NPK1 and MKK1. MKK1 encodes a MAP kinase kinase and NPK1 encodes a MAP kinase kinase kinase that functions to transduce PAMP-triggered signals (Kovtun et al., 1998; Jin et al., 2002; Mészáros et al., 2006; Gao et al., 2008). Genes encoding full-length MKK1 and the N terminus of NPK1 (residues 1 to 373; NPK1^{Nt}) could trigger PCD when introduced by agroinfiltration (Figures 7A and 7B). Avh238^{P7076} could suppress the PCD triggered by both MAPKs,

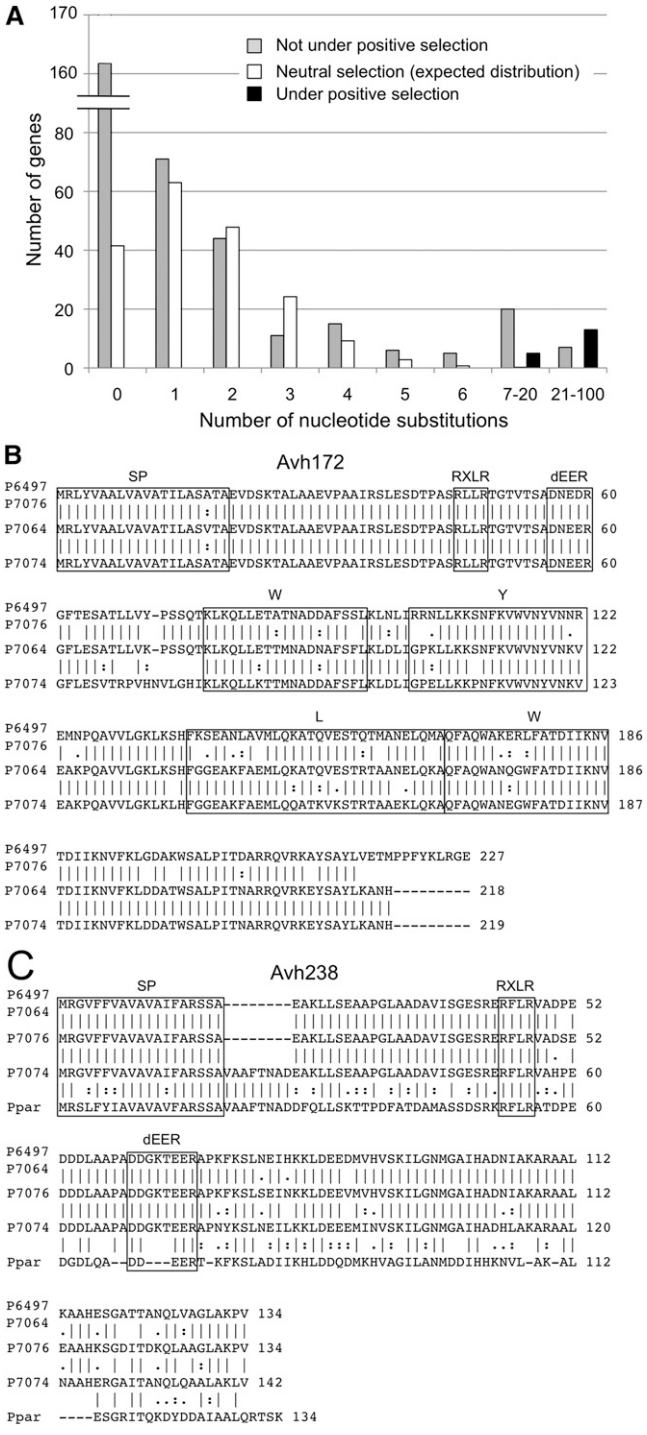


Figure 4. Effector Sequence Polymorphisms among Four Genotypes of *P. sojae*.

(A) Distributions of DNA sequence polymorphisms among 255 effectors. Expected distribution for genes under neutral selection calculated by fitting a truncated Poisson distribution ($\lambda = 1.52$) to the frequencies of genes with one to five nucleotide substitutions.

(B) and **(C)** Amino sequence alignments for two strongly expressed effectors under positive selection. SP, signal peptide; RXLR, dEER, W, Y, and L indicate motifs conserved among RXLR effectors (Jiang et al., 2008); Pp, *P. parasitica* sequence from three appressorium ESTs (GenBank accession numbers FK935883.1, FK937001.1, and FK935761.1).

suggesting that it acted at a point in the signaling pathway downstream of the two kinases. Protein immunoblots validated that accumulation of the MAPK proteins was not inhibited relative to the *eGFP* control. However, Avh238^{P7076} could not suppress PCD triggered by BAX or by the effectors Avh238^{P6497} and Avh241 (Table 1), suggesting that it was not targeting a step common to all PCD pathways.

To test whether Avh172 and Avh6 could also suppress ET-PCD mediated by the products genetically defined *R* genes, *Agrobacterium* cells carrying either of the effector genes were infiltrated into *N. benthamiana* leaves 24 h prior to the leaf tissue being challenged with a mixture of cells carrying the *P. infestans Avr3a* gene, the potato (*Solanum tuberosum*) *R*3a gene, or with appropriate *eGFP* controls. As shown in Figures 6F and 6G, both Avh172 and Avh6 could suppress ET-PCD mediated by R3a in the presence of Avr3a. To validate that Avh172 and Avh6 could suppress ET-PCD in soybean as well as *N. benthamiana*, we used double-barrel particle bombardment experiments to test if coexpression of Avh6 or Avh172 could suppress PCD triggered by Avh238, Avh241, or Avh181. In every case, both Avh6 and Avh172 could suppress the PCD triggered by these three effectors (Figure 6H). The bombardment experiments also revealed that both Avh172 and Avh6 could suppress ET-PCD mediated by soybean *R* gene *Rps*4 in the presence of *P. sojae* Avr4/6. Thus, both Avh172 and Avh6 could suppress ET-PCD in both *N. benthamiana* and soybean, whether or not ET-PCD was mediated by a genetically defined *R* gene. Avh172 was further tested for its ability to suppress PCD triggered in *N. benthamiana* by MKK1 and NPK1^{Nt}. Avh172 could suppress PCD triggered by MKK1 but not by NPK1^{Nt} (Figures 7C and 7D).

Misexpression of Key *P. sojae* Effectors Disrupts Infection

Given the very strong expression and/or induction of Avh238 and Avh172 (Table 3), the extensive polymorphisms found in these effectors (Figures 4B and 4C), and the cooperation evident between them (Figure 6), we hypothesized that these effectors (among others) may play an important role during *P. sojae* infection. In order to test this hypothesis, we modified the expression of these two effectors by DNA transformation and then quantified pathogen proliferation during the first 12 h of infection, which represents the biotrophic phase, using qPCR measurement of host and pathogen DNA levels.

Since Avh238 was expressed at a low level at the outset of infection, we forced high-level constitutive expression of Avh238^{P6497} (the allele that triggers PCD) by introducing multiple copies of the gene under the control of the strong constitutive promoter (HAM34). As shown in Figure 8A, the expression of Avh238 during infection was elevated compared with the wild type (parent strain P6497 not transformed) in two transformants, OX41 and OX53 (around 20-fold), whereas no increase was obtained in a third transformant, OX43. As shown in Figure 8B, infection of soybean hypocotyls by OX41 and OX53, but not

Table 2. Evidence of Positive Selection in Different Categories of Effector Genes

Category[a]	Genes	Individual Genes under Positive Selection[b]	Groupwise Wilcoxon Test P Value[c]	Concatenated Sequences[d]			
				dN	dS	dN/dS	Adjusted P Value
1–5 Mutations	147	0	≤0.24000	0.0007	0.0006	1.08	0.37
6 Mutations	5	0	≤0.24000	0.0051	0.0038	1.40	0.39
7–20 Mutations	25	5	≤0.00024	0.0100	0.0049	2.00	5.5 E-08
21–100 Mutations	20	13	≤0.00160	0.0290	0.0220	1.30	6 E-05
Avr genes	6	4	0.03100	0.0220	0.0057	3.90	1.6 E-09
Elicitor genes	8	3	≤0.00098	0.0097	0.0029	3.40	2.3 E-11
Highly expressed	20	7	≤0.00017	0.0120	0.0030	4.00	<E-11

[a]Numbers of mutations calculated from a multiple sequence alignment among the four alleles of each effector. *Avr* genes include Avr1k candidate Avh331. Elicitor genes are as in Figure 1D. Highly expressed genes are as listed in Table 3.
[b]Individual genes in which at least one pair of alleles exhibits a dN/dS ratio significantly >1.0 (FDR-adjusted P value < 0.05).
[c]For each gene within the group, the most divergent pair of alleles was selected and dN and dS values calculated. Then, all the dN and dS pairs were compared using the Wilcoxon signed ranks test. A significant P value indicates the presence of positive selection within the group as a whole.
[d]The most divergent pairs of alleles of each gene were concatenated and dN and dS calculated between the two concatenates.

OX43, was greatly reduced relative to the parent strain P6497 (wild type). This result suggested that premature expression of Avh238 interfered with the normal program of infection and that the plant response triggered by Avh238 was an effective innate immune response.

To test the hypothesis that Avh172 played a positive role in infection by suppressing the plant response to effectors such as Avh238, we silenced Avh172 by introducing sense constructs in which Avh172 was driven by the strong constitutive promoter *HAM34*; in *P. sojae*, a strongly expressed sense transgene sometimes leads to overexpression and sometimes to silencing. Four stable transformants were recovered in which the constitutive level of Avh172 mRNA was reduced to <35% of the parent strain P6497 (Figure 8C). All four of the transformants showed significantly reduced virulence on soybean hypocotyls as quantitated by qPCR measurement of host and pathogen DNA levels (Figure 8D).

To test the positive contribution of Avh238 to virulence, we attempted to silence the gene; however, only one stable transformant (SS11) with reduced levels of *Avh*238 mRNA could be recovered. Therefore, we used transformation with double-stranded RNA to silence *Avh*238 transiently (Whisson et al., 2005). In *P. sojae*, maximal silencing is achieved between 8 and 15 d following transformation, allowing ample time to propagate the mycelium of individual candidate transformants and measure mRNA levels prior to inoculating plants. As shown in Figure 8E, five lines with <30% of normal *Avh*238 mRNA levels were obtained, together with one line that showed no reduction, compared with a mock-transformed line [CK(TS)]. As shown in Figure 8F, all five lines with reduced *Avh*238 mRNA levels displayed significantly reduced virulence compared with the mock-transformed line, while the line with normal *Avh*238 mRNA levels displayed normal virulence. In accord with this observation, the stable transformant SS11 also displayed significantly reduced virulence compared with the nontransformed parent strain P6497 [WT(SS)].

DISCUSSION

The *P. sojae* genome contains genes encoding ~400 potential RXLR effectors, raising key questions regarding their shared contributions to virulence. Integrating the diverse information presented here regarding the *P. sojae* effector repertoire, several key points emerge. First, most members of the bioinformatically identified repertoire are at least potentially functional based on their ability to suppress PCD triggered by BAX, effectors, and one PAMP; since most of these assays were conducted in the nonhost *N. benthamiana*, these effectors must presumably interact with well-conserved components of the plant defense machinery. Although it is possible that use of a nonhost species could result in some responses not relevant to *P. sojae* infection of soybean, (e.g., triggering of PCD in *N. benthamiana* but not in soybean by Avh163), the *N. benthamiana* model system has been used successfully to characterize many bacterial effectors. Furthermore, the responses of *N. benthamiana* to several *P. sojae* effectors were validated in soybean (Dou et al., 2008b; Figures 2 and 6).

Second, although many *P. sojae* effectors appear potentially able to contribute to virulence, the transcriptional data suggest that the products of a small number of effector genes may dominate the pool of effector proteins (Table 3, Figure 9). The fact that many of these highly expressed effectors are evolving rapidly under strong positive selection (Figure 9, Table 3) and that some of them trigger plant defenses (Figure 2) and/or have been targeted by soybean Rps proteins (i.e., are encoded by avirulence genes; Shan et al., 2004; Dou et al., 2010) (Table 3, Figure 9A) supports the hypothesis that they play key roles in infection. The deleterious effect on virulence from silencing two of the strongly expressed effectors, Avh238 and Avh172 (Figure 8), suggests that, despite the potential redundancy in the effector repertoire, several effectors are individually indispensible for full virulence. Avh238 is partially conserved in the tobacco pathogen *Phytophthora parasitica* (Figure 4C), further emphasizing its importance, and perhaps explaining why *N. benthamiana*, as well as soybean, responds to Avh238 with a defense reaction (convincing homologs of other effectors that triggered ET-PCD or of Avh172 could not be found in the *P. parasitica* EST data set in GenBank). There are at least 20 major effector genes, based on expression levels detectable with the SoyChip microarray, and possibly an equal number among those not on the chip. Thus,

Table 3. Effector Genes Most Strongly Expressed during Soybean Infection, and Their Properties

		Time after Inoculation				Induction (0–12 h)	FDR Adj. P Value[a]	Class[b]	BT-PCD Suppr.[c]	ET-PCD Suppr.[d]	PT-PCD Suppr.[e]	Polymorphism
Gene	Affymetrix ID	0 h	3 h	6 h	12 h							S,N[f]
Effector genes												
Avh238	PsAffx.C181000032_s_at	5.1[g]	8.0	9.9	12.0	120.9	0.003	E	0	0	+	0,26,id*
Avh6	PsAffx.CL641Contig1_s_at	9.3	8.3	10.9	11.6	4.9	0.003	IE	2	2	−	1,16,Δ*
Avh52	PsAffx.CL2570Contig1_at	8.2	8.4	10.3	11.4	9.3	0.001	IE	2	2	+	0,7(*)
Avh172	PsAffx.CL2601Contig1_at	7.5	5.6	7.9	10.5	8.1	0.005	IE	1	2	−	5,48,fs*
Avh260	PsAffx.CL1110Contig1_at	9.3	8.7	8.8	10.1	1.8	0.175	IE	3	2	−	2,1
Avh94a,b	PsAffx.C431000001_s_at	4.2	5.8	7.2	9.8	48.8	0.000	E	2	2	+	0,0
Avr1b	PsAffx.Avr1b-1_s_at	6.8	6.2	8.5	9.8	7.9	0.025	IE	2	n.t.[h]	n.t.	2,20*
Avh240	PsAffx.C241000001_at	4.1	6.0	7.6	9.7	47.1	0.002	E	2	2	+	1,0
Avh38	PsAffx.CL481Contig1_at	8.8	9.8	8.6	9.6	1.7	0.122	IE	2	2	+	0,0
Avh109	PsAffx.C82000047_at	6.1	6.5	7.7	9.3	8.9	0.009	IE	2	(2)	+	0,1
Avh23	PsAffx.C74000070_at	4.2	6.4	7.6	8.9	25.8	0.001	E	2	2	+	0,0
Avh115	PsAffx.C124000032_at	5.1	6.4	6.8	7.9	6.8	0.035	IEW	2	2	+	1,2
Avh181	PsAffx.C59000079_at	4.2	4.7	4.8	7.4	9.2	0.002	IEW	n.t	n.t.	−	2,25,id*
Avh180	PsAffx.C214000005_at	3.9	5.0	5.7	7.3	10.3	0.005	IEW	3	2	+	0,0
Avh8	PsAffx.Avr1b-8_at	5.6	7.6	7.8	6.9	2.5	0.008	IEW	2	2	−	0,1
Avh158	PsAffx.C12000039_at	3.4	4.2	4.2	6.7	9.9	0.026	IEW	1	2	−	2,33*
Avh16	PsAffx.C6000196_s_at	3.9	5.5	5.8	6.6	6.4	0.008	IEW	5	2	−	0,0
Avh29	PsAffx.C25000003_at	2.7	4.3	4.7	6.5	13.4	0.025	IEW	2	2	−	2,21*
Avh163	PsAffx.C132000002_at	3.7	4.8	5.3	6.3	5.9	0.011	IEW	n.t.	n.t.	n.t.	8,83,id*
Avr4/6	PsAffx.C35000086_at	5.1	4.7	4.9	6.1	1.9	0.028	IEW	2	2	−	0,0
Housekeeping genes												
Actin[i]	AFFX-r2-Ps-actin-3_at	12.8	13.0	13.0	13.3	1.4	0.004					
rpL13a[i]	GmaAffx.614.1.S1_at	12.0	12.2	12.1	12.5	1.4	0.008					
rpS5[i]	PsAffx.CL3Contig5_at	13.4	14.6	14.5	14.3	1.8	0.029					
rpS14	PsAffx.CL14Contig1_at	13.5	13.5	13.6	13.8	1.2	0.088					
Ubiquitin	PsAffx.CL7Contig1_at	12.8	12.8	12.9	12.9	1.1	0.029					

[a]P value adjusted for FDR control (Benjamini and Hochberg, 1995) across the 25 tests for significant induction.

[b]IE, immediate early; E, early; IEW, weakly expressed immediate-early gene.

[c]Suppression of BAX-triggered cell death, scored on the scale described in Figure 1D.

[d]Suppression of effector-triggered cell death. 0, Neither Avh238 or Avh241; 2, both Avh238 and Avh241; (2), both, but weakly.

[e]Suppression of INF1-triggered cell death. +, Suppression; −, no suppression.

[f]Polymorphisms present among the four isolates of *P. sojae*. S, synonymous substitutions; N, nonsynonymous substitutions; Δ, gene deleted from two isolates; fs, frameshift near the 3′ end; id, small indel; *, positive selection significant (FDR-adjusted $P < 0.05$); (*), adjusted P value for Avh52 was 0.08.

[g]Background-subtracted, quantile-normalized hybridization signal (log_2 scale) as determined by microarray analysis. Means of the three replicates.

[h]n.t., not tested because the effector triggered cell death in *N. benthamiana.*

[i]Genes used as reference for the qRT-PCR data shown in Figure 5. GmaAffx.614.1.S1_at was originally misannotated as a soybean gene due to its presence in a *P. sojae*–infected soybean EST library.

5 to 10% of the genes in the predicted effector repertoire may make major contributions to virulence.

Third, among the most strongly expressed effector genes, two general patterns of expression are evident: immediate-early genes that are already expressed strongly at the outset of infection, and early genes that are strongly induced during the first 6 to 12 h of infection (Table 3, Figures 9A and 9B). This timing corresponds to the appearance of haustoria in the infected tissue; few haustoria are observed at 3 h (and none, of course, at 0 h), but by 12 h haustoria are abundant. Thus, generally speaking, immediate-early effectors are produced by the pathogen in the absence of haustoria, while early effectors are produced by the pathogen in the presence of haustoria, although we have no data on whether early effector expression actually is confined to haustoria. A further set of genes shows the same induction pattern as the immediate-early genes but are more weakly expressed (Figure 9B). Our data suggest some functional differences between immediate-early effectors (including weakly expressed ones) and early effectors. Only 31% of immediate-early effectors (5/16, including weakly expressed ones) could suppress PT-PCD, whereas 100% (4/4) of early effectors could suppress PT-PCD. Immediate-early effector Avh172 could suppress ET-PCD but not PT-PCD, while early effector Avh238 could suppress PT-PCD but not ET-PCD. Figure 9C shows the aggregate transcript levels for effectors able to suppress ET-PCD and PT-PCD, based on those effector transcripts detectable on the Affymetrix GeneChip. Transcripts of effectors able to suppress ET-PCD exceeded those of effectors able to suppress PT-PCD by 2.6-fold at the outset of infection, but by 12 h, the numbers of transcripts were nearly identical due to the stronger induction of

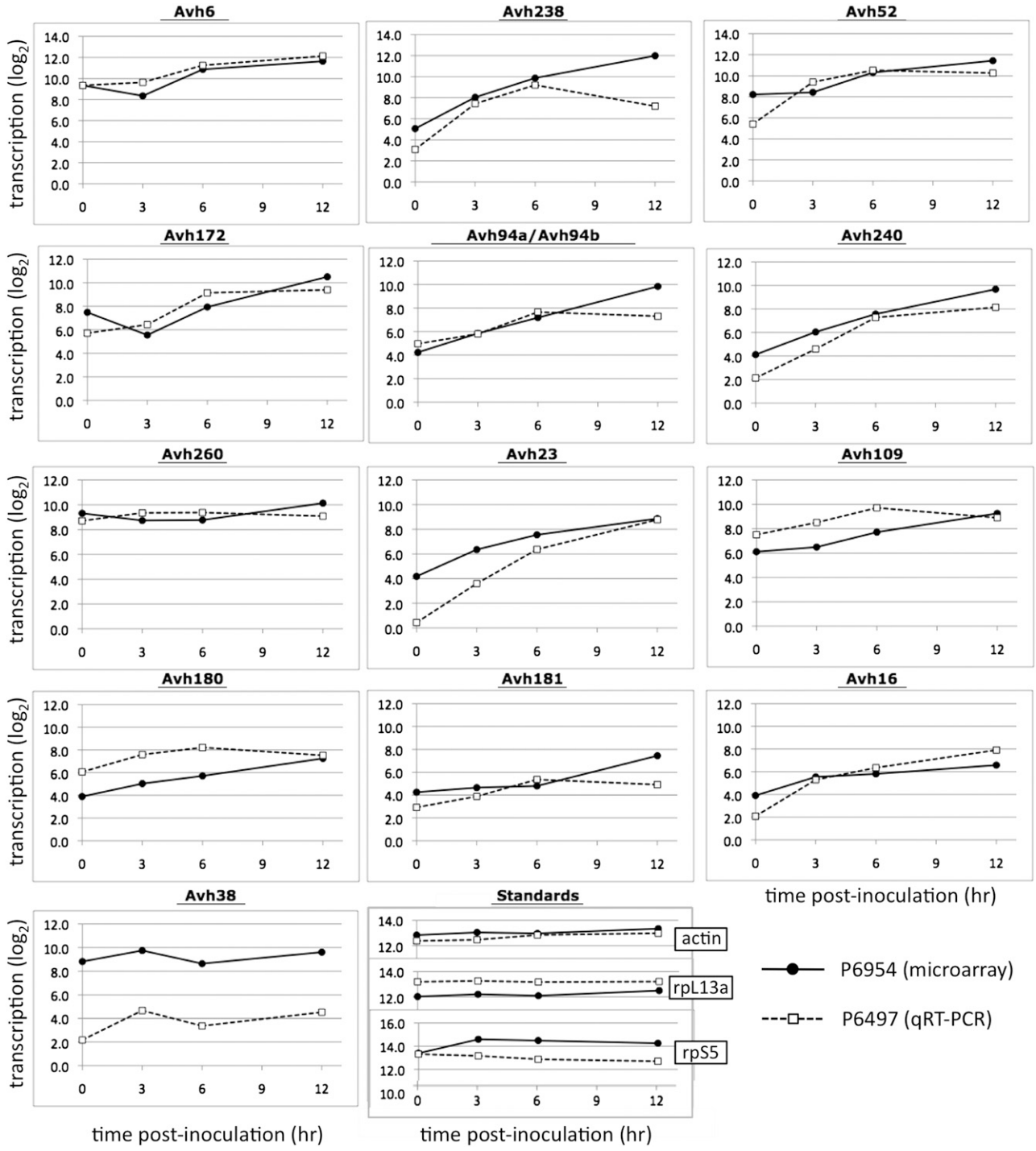


Figure 5. Expression Patterns of 13 Effectors during Infection.

Expression in P6497 was determined by microarray analysis. Expression in P6497 was determined by qRT-PCR. Transcription values represent the $\log_2$-transformed, background-subtracted, quantile-normalized microarray hybridization intensities. qRT-PCR measurements were placed on the same scale as the microarray data by comparison to the averages of the microarray measurements of expression of the three housekeeping gene standards, as detailed in Methods.

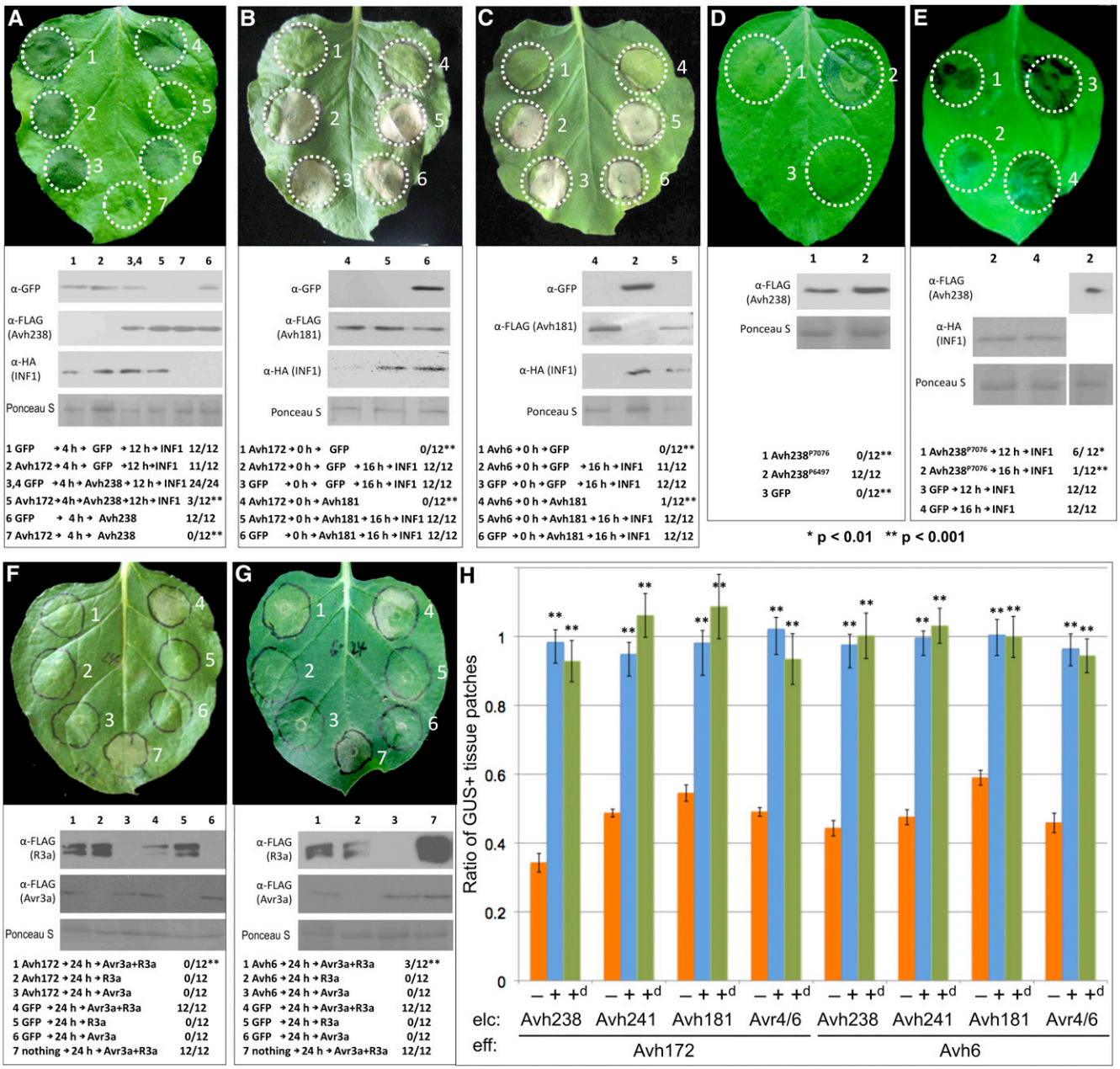


Figure 6. Functional Interactions among Effectors Suppressing Cell Death.

(A) to **(G)** *A. tumefaciens* cells carrying DNA encoding the indicated proteins were infiltrated at the time intervals indicated. Leaves were photographed 96 h after the last infiltration. In parallel experiments, proteins were extracted from the coinfiltrated areas and subjected to protein gel blot analysis using the indicated antibodies. Protein levels were checked by Ponceau S staining. Experiments were replicated in 12 leaves and the numbers of leaves showing cell death in more than 20% of the infiltrated area counted. Treatments showing reduction in cell death were compared statistically to the appropriate control (absence of suppressing effector) using a one-tailed Fisher's exact test with no multiple testing adjustment. *P < 0.01; **P < 0.001.

(A) Suppression of INF1-induced cell death by Avh238 is revealed when Avh172 suppresses Avh238-triggered cell death.

(B) and **(C)** Avh6 and Avh172 can suppress cell death triggered by Avh181, revealing that Avh181 cannot suppress INF1-induced cell death.

(D) Avh238 allele from P7076 does not trigger cell death in *N. benthamiana.*

(E) Avh238 allele from P7076 can suppress INF1-triggered cell death if infiltrated 12 or 16 h beforehand.

(F) and **(G)** Avh6 and Avh172 can suppress cell death triggered by coexpression of *P. infestans* Avr3a and potato R3a. Different gels were used to fractionate and detect R3a (140 kD) and Avr3a (13 kD). Proteins were extracted 96 h after the second infiltration.

(H) Suppression of effector-triggered PCD in soybean by Avh172 and Avh6, measured by indirect and direct particle bombardment assays. Indirect assays: the number of GUS-positive blue spots surviving in the presence of DNA encoding each cell death elicitor in the presence (+ blue bars) or

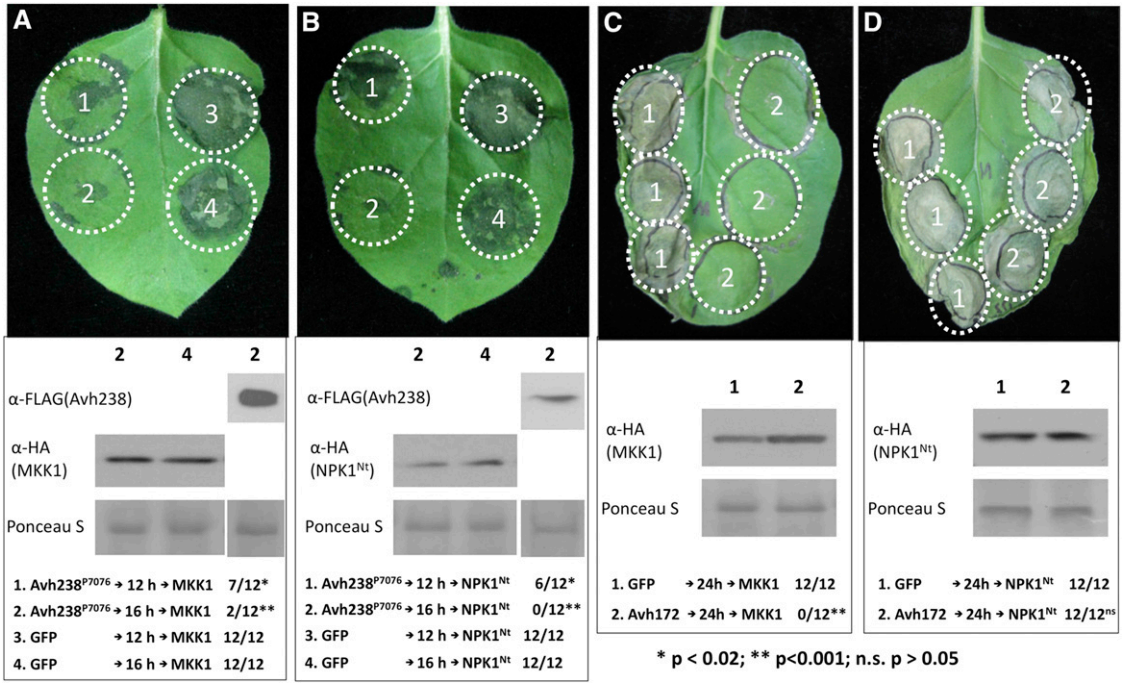


Figure 7. Avh238 and Avh172 Can Suppress MAPK-Triggered Cell Death.

Experiments were performed as described in Figure 6. Treatments showing reduction in cell death were compared statistically to the appropriate control (absence of suppressing effector) using a one-tailed Fisher's exact test with no multiple testing adjustment. *P < 0.02; **P < 0.001; n.s., not significant.

(A) and **(B)** Avh238 allele from P7076 can suppress cell death triggered by MKK1 **(A)** and the N-terminal domain of NPK1 **(B)**.

(C) and **(D)** Avh172 can suppress can suppress cell death triggered by MKK1 **(C)** but not cell death triggered by the N-terminal domain of NPK1 **(D)**.

[See online article for color version of this figure.]

transcripts of effectors suppressing PT-PCD (11-fold compared with 5-fold). Together, these results suggest the hypothesis that functions of the immediate-early effectors are more heavily concentrated on suppressing ETI in preparation for the arrival of the early effectors, whose function is more heavily concentrated on suppressing PTI. This might represent a preemptive strategy by the pathogen in blocking effector-triggered responses rather than a reactive strategy. Although some PAMPs are undoubtedly present at the outset of infection, it may be that early PAMP-triggered responses are less effective against the pathogen than effector-triggered responses and/or the low biomass of the pathogen results in weak PAMP-triggered responses; thus, the pathogen has evolved more immediate countermeasures against the more sensitive and vigorous ETI. A further explanation for delayed expression of effectors that suppress PTI might be that the stimulation of endocytosis that accompanies PTI (Robatzek, 2007) may benefit the pathogen by speeding the entry of its effectors (Kale et al., 2010); thus, it would benefit the pathogen to deliver its ETI-suppressing effectors first, before effector entry is slowed by the delivery of PTI-suppressing effectors. We are currently testing this hypothesis. This work is confined to a single PAMP, INF1, and a single response, cell death. Further experiments that examine wider diversities of PAMPs, effectors, and responses will be required to ascertain if our proposed two-phase delivery hypothesis is more generally true.

The functions of the 80% of effector genes that are weakly expressed during infection remain an enigma. Based on the functional screen, many of these genes retain the ability to suppress PCD, especially effector-triggered PCD. Furthermore, these genes include a number of known avirulence genes that have been targeted by *Rps* genes, for example, *Avr1a* (Qutob et al., 2009), *Avr3a* (Qutob et al., 2009), and the *Avr1k* candidate *Avh*331 (Kale

Figure 6. (continued).

absence (− orange bars) of the suppressing effector was measured as a ratio to an empty vector control. Direct assays: (+^{d} green bars) indicates a direct comparison in which one barrel delivered DNA encoding elicitor + effector and the other barrel delivered DNA encoding elicitor alone (empty vector DNA was used to equalize DNA amounts); the ratio obtained (effector/no effector) was then multiplied by the cell survival in the presence of elicitor alone (i.e., orange bar) to enable comparison to the results of the indirect assays. Asterisks indicate statistically significant (P < 0.001) reduction in blue spots as determined by the Wilcoxon signed ranks test. All bombardments were done with soybean cultivar Williams, except for Avr4/6 for which L85-2352 (containing *Rps*4) was used. In all cases, bars indicate SE obtained from 14 to 16 replicates.

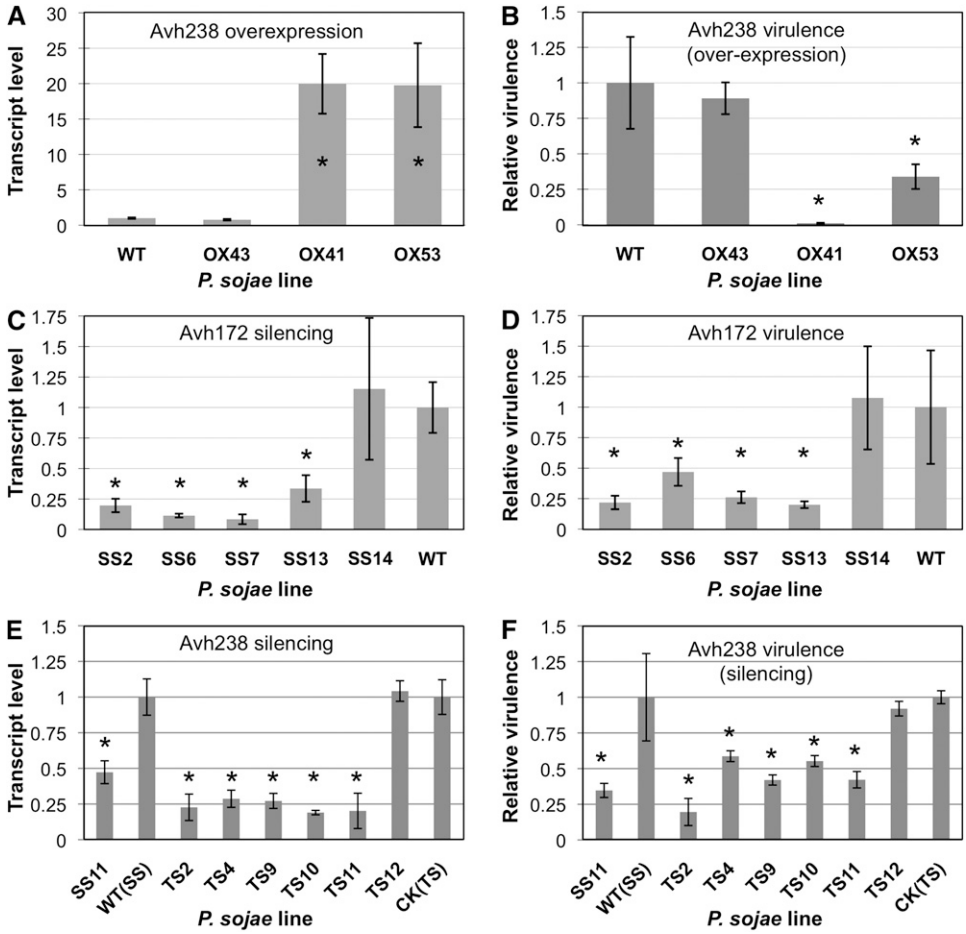


Figure 8. Misexpression of Avh238 or Avh172 Reduces *P. sojae* Virulence.

Effector mRNA levels (**[A]**, **[C]**, and **[E]**) were determined 12 h after mycelial inoculation by qRT-PCR compared with *P. sojae* actin mRNA levels and then normalized to a nontransformed control. Standard errors from three technical replicates are shown. Virulence (**[B]**, **[D]**, and **[F]**) was determined using qPCR to measure the ratios of *P. sojae* and soybean DNA sequences 12 h after zoospore inoculation of etiolated seedlings. In each case, error bars represent SE from three technical replicates. Values marked with an asterisk are significantly different than the untransformed control ($P < 0.001$; no multiple testing adjustment). WT and WT(SS) indicate the nontransformed parent strain P6497. CK(TS) is a line subjected to the transient transformation procedure without dsRNA.

(A) and **(B)** Overexpression of Avh238 in *P. sojae* stable transformants.

(C) and **(D)** Silencing of Avh172 in *P. sojae* stable transformants.

(E) and **(F)** Stable (SS) and transient (TS) silencing of Avh238.

et al., 2010). These observations suggest that the genes have a functional role in infection. On the other hand, based on sequencing of additional *P. sojae* isolates, many of these genes are evolving under neutral selection. This fact, together with their weak transcription levels, suggests that these genes are individually dispensable. A model that might account for the enigma could be that this large silent majority of effectors are individually dispensable but in aggregate are indispensible. One may speculate that a large diverse pool of weakly expressed effectors is less likely to be effectively targeted by *R* genes. Furthermore, any effectors that are nonetheless targeted by *R* genes could be readily lost from the repertoire without a serious reduction of virulence; two such examples are *Avr1a* and *Avr3a* (Qutob et al., 2009). A further point that remains to be investigated is whether some of the genes that are weakly expressed in P6497 may be strongly expressed in other *P. sojae* strains or under other infection conditions (e.g., late infection or infection of other tissues). Also unknown are the actual concentrations of the individual effector proteins inside host cells and their specific activities toward their plant targets; the former is a function of translation efficiency, secretion, host cell entry, and stability inside and outside of the pathogen and host cells.

Our survey of *P. sojae* effectors greatly extends previous reports that individual oomycete effectors can suppress PCD and other responses triggered by BAX, effectors, and PAMPs (Bos et al., 2006; Sohn et al., 2007; Dou et al., 2008b; Kelley et al., 2010). Oh et al. (2009) described a smaller survey of 32 effector genes from *P. infestans* selected on the basis of representation in

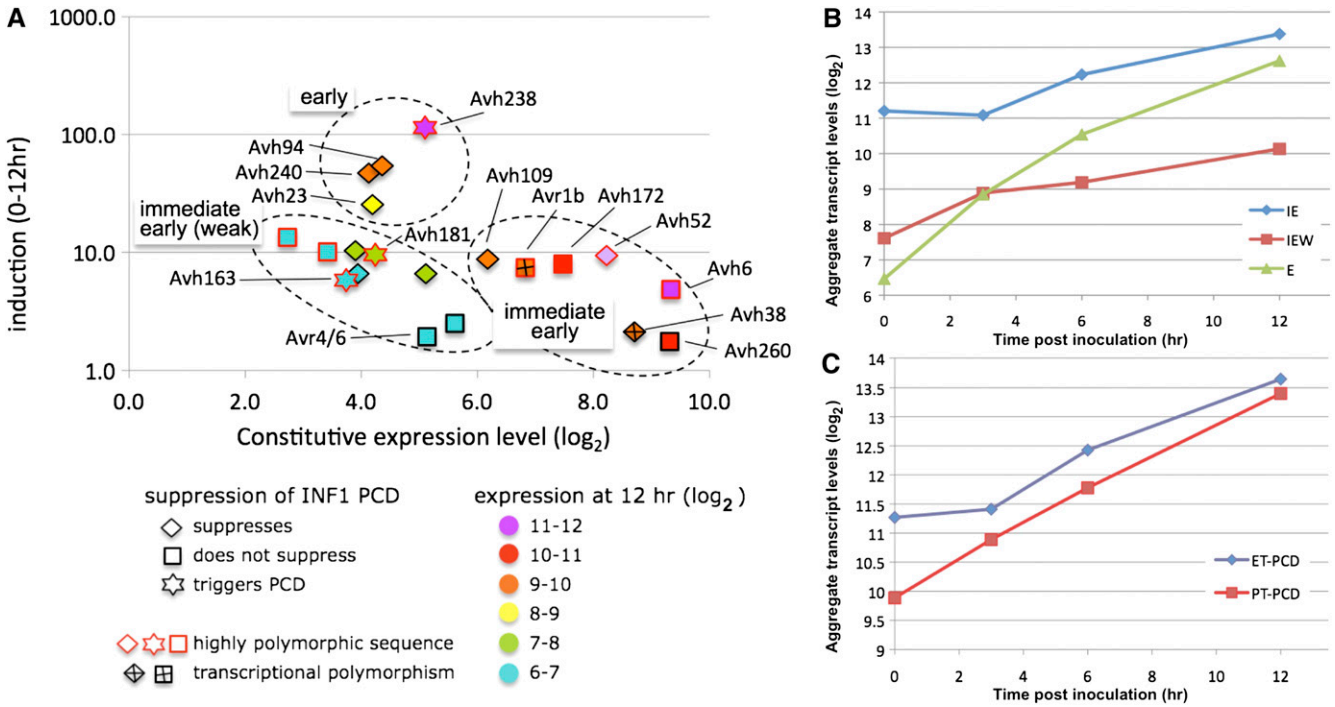


Figure 9. Functional Properties of Key *P. sojae* Effectors.

(A) Properties of the 20 most strongly expressed RXLR effectors (detected by microarrays) are summarized. Avh238 can suppress INF1 PCD but Avh181 cannot when the PCD triggered in *N. benthamiana* by each effector is suppressed. Avr1b triggers an inconsistent PCD in *N. benthamiana*. Avh238 and Avh181 also trigger PCD in soybean, but Avh163 and Avr1b do not. Effectors were not systematically screened for transcriptional polymorphisms.

(B) Aggregate transcript levels, estimated from microarray analysis, of immediate-early effectors (IE), weakly expressed immediate-early effectors (IEW) and early effectors (E).

(C) Aggregate transcript levels, estimated from microarray analysis, of effectors that can suppress PT-PCD and ET-PCD.

an infection EST library. These showed a similar distribution of polymorphisms as the *P. sojae* effectors, suggesting that the fixation rate of mutations in RXLR effector genes is similar in the two *Phytophthora* species. In particular, in both species, the most highly expressed effectors are subject to the strongest positive selection.

Our findings that most of the tested *P. sojae* effectors could suppress ET-PCD (45/49 could suppress PCD triggered by at least one effector; Table 1) and about half of those (25/45) could also suppress PT-PCD closely parallel the findings of Guo et al. (2009) that the majority of the 36 type III secreted effectors from *P. syringae* pv tomato (*Pst*) strain DC3000 could suppress effector-triggered PCD. Furthermore, the *Pst* effectors displayed a spectrum of ET-PCD suppression activity, similar to the spectrum we observed for suppression of BT-PCD. Six of the *Pst* effectors with the strongest ETI suppression were also shown to suppress PTI. The very large investment in effectors that can suppress PCD underlines the importance of the initial biotrophic phase of *P. sojae* infection, despite the fact that this phase is relatively short (15 to 20 h) (Enkerli et al., 1997). It will be interesting to determine if these effectors have any role in the subsequent necrotrophic phase. Since the majority of *P. sojae* effectors suppress both ET-PCD and PT-PCD, as well as BT-PCD, each effector must target some step common to all these PCD pathways. One possibility is that this common step is the spread of PCD from cell to cell within the infiltrated region. This idea is supported by the *eGFP* and *DsRed* coexpression experiments shown in Figures 1E and 1G that suggested that in the *N. benthamiana* agroinfiltration experiments, many cells likely express either the PCD elicitor or the PCD suppressor, but not both. In the context of a real infection in which *P. sojae* hyphae are infiltrating soybean tissue, suppressing the spread of cell death (or other defense responses) would be of major advantage to the pathogen, especially if the effectors had the ability to spread though the apoplast (e.g., Shan et al., 2004; Kale et al., 2010) or though the symplast (e.g., Khang et al., 2010). Alternatively, some effectors may target multiple steps in different pathways.

Although our survey of *P. sojae* effectors necessarily has some limitations, such as the use of overexpression to detect phenotypes, the use of a nonhost plant for much of the screen, the limited number of phenotypic assays used, and many effectors still to be examined, this study provides a valuable systems scale investigation of the effector repertoire of an oomycete pathogen. The key conclusions are that the majority of the effector proteins encoded in the genome have the potential to suppress plant defense, particularly PCD, but that a small minority of strongly

expressed, rapidly evolving, principal effectors may make large indispensible contributions. Furthermore, the expression of several principal effectors appears to be timed to suppress first ETI and then PTI. The plant proteins targeted by these principal effectors are obviously of immediate interest. However, the silent majority of secondary effectors are also of interest, in particular, the question of whether they target the same or diverse proteins or pathways and whether there are synergistic interactions among these effectors that magnify their impact.

METHODS

Construction of Recombinant *Agrobacterium tumefaciens* Binary PVX Vectors

Since none of the tested *Avh* genes contained predicted introns, we amplified each of the genes directly from genomic DNA from the *Phytophthora sojae* isolate P6497 (race 2) (Forster et al., 1994) using high-fidelity DNA polymerase (PrimeSTAR HS DNA Polymerase; TaKaRa Bio) and using the indicated primers (see Supplemental Data Set 1 online). Additional alleles were amplified from DNA of isolates P7064 (race 7), P7074 (race 17), and P7076 (race 19) (Forster et al., 1994). The PCR products were digested with the appropriate enzymes (see Supplemental Data Set 1 online) and cloned into the PVX vector pGR107 (Jones et al., 1999). The *INF1* gene was amplified from genomic DNA of *Phytophthora infestans* isolate 88069 (Kamoun et al., 1998a) and cloned into PVX vector pGR107. NbMKK1 and NbNPK1^{Nt} (residues 1 to 373) were amplified from *Nicotiana benthamiana* cDNA using indicated primers (see Supplemental Table 2 online) and cloned into pGR107. The constructs were confirmed by sequencing at Invitrogen. Recombinant binary plasmids were maintained and propagated in *Escherichia coli*, strain JM109, and grown with 50 μg/mL kanamycin and 12.5 μg/mL tetracycline.

A. tumefaciens Infiltration Assays and Confocal Microscopy

Constructs were introduced into *A. tumefaciens* strain GV3101 by electroporation (Hellens et al., 2000; Wise et al., 2006) and transformants selected with tetracycline (12.5 μg/mL) and kanamycin (50 μg/mL). Individual colonies were verified by PCR using vector primers. For infiltration into leaves, recombinant strains of *A. tumefaciens* were grown in Luria-Bertani media plus 50 μg/mL kanamycin for 48 h, harvested, and washed with 10 mM $MgCl_2$ three times, resuspended in 10 mM $MgCl_2$ to a final OD_{600} of 0.4, and then incubated at room temperature for 1 to 3 h prior to infiltration. *N. benthamiana* plants were grown in the greenhouse for 4 to 6 weeks at 25°C during day time (16 h) and 16°C at night (8 h). For pressure infiltration into *N. benthamiana* leaves (Bos et al., 2006), a small nick was placed in each leaf with a needle and then 100 μL of *A. tumefaciens* cell suspension was infiltrated though the nick using a syringe without a needle.

To assay suppression of BT-PCD, *A. tumefaciens* cells carrying an *Avh* gene were infiltrated initially. Then, the same infiltration site was challenged with *A. tumefaciens* cells carrying the *Bax* gene 0, 12, or 24 h after the initial inoculation. *A. tumefaciens* strains carrying *Bax*, or *Avh* or *eGFP* genes alone, were infiltrated in parallel as controls. Symptom development was monitored visually 3 to 8 d after infiltration. Scoring was done and photographs were taken at 5 d. Each assay consisted of at least three plants inoculated on three leaves (total of nine leaves) on at least two different dates (usually two plants on date 1 and one plant on date 2, then more plants at later dates if the first three were not in full agreement).

Similar procedures were used for other cell death suppression assays, except that *A. tumefaciens* cells carrying *Bax*, *Avh238*, *Avh241*, and *INF1* were infiltrated 16 or 24 h after the *Avh* genes were infiltrated. Symptoms were scored daily and typically started developing from 3 to 10 d after the first inoculation and pictures were taken at 6 d.

For fluorescent protein expression assays, *A. tumefaciens* cells carrying the *DsRed* gene were infiltrated 0, 12, 16, or 24 h after *A. tumefaciens* cells carrying the *eGFP* genes were infiltrated. Tissue was examined by confocal microscopy 4 to 5 d after the second infiltration. Fluorescence of eGFP and DsRed was simultaneously captured (excitation, 488 and 558 nm; emission, 505 to 530 nm and 570 to 610 nm, respectively) using a Carl Zeiss LSM 700 laser scanning microscope with a ×10 objective lens. The images from the two channels were merged by the ZEN 2009 software from Carl Zeiss.

Protein Immunoblotting

Agroinfiltrated *N. benthamiana* leaves were harvested at 4 d after inoculation, and protein extracts were prepared by grinding 400 mg of leaf tissue in 1 mL RIPA lysis and extraction buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) in the presence of 10 μL protease inhibitor mixture (Thermo Scientific Pierce; No.78410) and 1 mM phenylmethylsulfonyl fluoride. Proteins from the sample lysates were fractionated using SDS-PAGE. The amount of lysate loaded was adjusted depending on the expression level of each protein (determined in pilot experiments) but was always consistent within an experiment. After electrophoresis, proteins were transferred from the gel to an Immobilon-P^{SQ} polyvinylidene difluoride membrane. The membrane was washed in at least 0.5 mL/cm^2 of sterile distilled water for 2 to 3 min with mild agitation (~50 to 60 rpm). To enhance protein binding of low molecular mass proteins (<30 kD), methanol was added in the transfer buffer to a final concentration of 35 to 40% and current was reduced. To enhance protein binding of high molecular mass proteins (for example, R3a, ~140 kD), the transfer duration was extended to 3 h and low concentrations of SDS (<0.01% w/v) were included in the transfer buffer. The membrane was then blocked using PBS plus 3% nonfat dry milk (PBSM) for 30 min at room temperature with agitation, followed by one wash with 0.5 mL/cm^2 of PBSM. Mouse anti-GFP, -FLAG, -HA, or -Bax monoclonal antibodies (Sigma-Aldrich) were added to the PBSM to a final concentration of 1 μg/mL and the filter incubated at room temperature for 60 min, followed by three washes (5 min each) in PBS containing 0.05% Tween 20 (PBST). The membrane was then incubated with goat anti-mouse IgG-Peroxidase conjugate (Sigma-Aldrich) at the recommended concentration in PBSM at room temperature for 30 min with gentle agitation. The antibody concentration was adjusted to maximize detection sensitivity and to minimize background, usually in the range 1:10,000 to 1:30,000 dilution. The membrane was washed three times for 5 min each in at least 0.5 mL/cm^2 PBST. The membrane was then treated with the peroxidase substrate Chemiluminescent Peroxidase Substrate-1 (Thermo Scientific Pierce; No.34080) for 5 min without agitation, drained briefly, and then wrapped in plastic film. The wrapped blot was then exposed to BioMax Light film (Kodak XBT-1) for a range of times from several seconds up to 10 min depending on the signal observed.

Microarray Analysis of *Avh* Gene Expression

Agar plugs of *P. sojae* isolate P6954 (race 1) were transferred to Petri dishes containing V8 liquid medium and incubated at 25°C. P6954 is genetically near-identical to P6947 (Forster et al., 1994), but unlike P6947, P6954 expresses Avr1b. After 2 d, the mycelia were transferred to Petri dishes in water to wash away excess V8 medium. Seedlings of highly susceptible soybean (*Glycine max*) cultivar Sloan were grown in the greenhouse with natural light for 9 d. After harvesting, seedling roots were washed gently to remove soil and then placed on moist cloth. Washed *P. sojae* mycelia were used to inoculate seedling hypocotyls: a small (~1-cm long) longitudinal slit was cut near the top half of the hypocotyls, and the mycelia were placed inside the cut. After 3, 6, or 12 h, the tissue

surrounding the infected hypocotyl slits was harvested. As a zero hour control, pure P6954 mycelial inocula were also harvested right after the wash step (no infection). All samples were transferred to liquid nitrogen and frozen at –80°C. The entire experiment was replicated three times (i.e., on different days). RNA was isolated from infected tissue and from mycelia as described (Zhou et al., 2009). Labeling of cRNA and hybridization to Affymetrix Soybean GeneChips were conducted as described (Zhou et al., 2009). The Affymetrix chips contain probe sets for 15,421 *P. sojae* gene transcripts and 7431 *Heterodera glycines* (nematode) cDNAs, as well as 35,611 soybean transcripts. Low-level analysis of the raw GeneChip data began with identifying undetectable *P. sojae* genes based on the Affymetrix Microarray Suite version 5 (MAS5) algorithm for calls (as implemented in the Bioconductor package *affy*; http://bioconductor.org/packages/2.0/bioc/html/affy.html). The default parameter τ (0.015) in the MAS5 present call was used, and a probe set was declared to be detectable (or present) if it had a present call in all three arrays of at least one time point, other than the zero hour time point. This cutoff was validated using the nematode probe set. Only 0.7% of nematode genes were called present under this criterion, whereas 20.9% of the *P. sojae* genes were called present, an FDR of 0.03%. Next, preprocessing was performed, consisting of GC-RMA background correction, quantile normalization, and computation of gene summary values from the corrected probe-level data. Background correction was performed with the model-based procedure (Wu et al., 2004) using sequence information as implemented in the Bioconductor package *gcrma* (http://bioconductor.org/packages/2.0/bioc/html/gcrma.html). Quantile normalization (Bolstad et al., 2003) and Tukey's median-polish algorithm-based gene summary values were computed using a novel modification to the algorithm that takes into account the fact that the *P. sojae* RNA is a small fraction of the total RNA in the samples from infected tissue. The modified procedure was as follows: GC-RMA quantile normalization was first performed on the zero time point samples that derived from pure *P. sojae* mycelia. The synthetic distribution created from the pure mycelia data was then used (instead of the usual internal synthetic distribution) to normalize the ranked probe values from the infected tissue data, thus assuring the distributions of *P. sojae* probe values from infected tissue and pure mycelia were the same. Next, all *P. sojae* probe sets identified by MAS5 as undetectable were eliminated, and median polish was performed to summarize expression for the remaining detectable probe sets. The success of this approach was validated by the generally consistent expression values obtained for constitutive housekeeping genes (Table 3). The statistical significance of the difference in transcript levels between the 0- and 12-h time points of the 20 detectable RXLR effector genes and five housekeeping genes was determined using a *t* test on the quantile-normalized signal intensities, with the multiple test adjustment of Benjamini and Hochberg (1995) across the 25 tests for significant induction.

P. sojae Isolates, Transformation, and Characterization

P. sojae isolates P6954 (race 1), P6497 (race 2), P7064 (race 7), P7074 (race 17), and P7076 (Race19) (Forster et al., 1994) were routinely grown and maintained on V8 agar (Erwin and Ribeiro, 1996). For stable transformation, *Avh*238 and *Avh*172 were amplified by PCR and ligated into vector pHAM34 (Judelson et al., 1991) digested with *Sma*I. Stable transformation was performed as described (Dou et al., 2008b). *P. sojae* transformants were selected in pea broth medium plus 30 μg/mL geneticin and were cultured in V8 liquid medium for 3 d. The mycelia were harvested, frozen in liquid nitrogen, and ground to a powder for DNA or RNA extraction. Genomic DNA was isolated from mycelium (Judelson et al., 1991) and screened for *Avh*238 and *Avh*172 transgenes by PCR using primers HamF and HamR (see Supplemental Table 1 online). RNA was extracted from infected plant tissues using the PureLink RNA mini kit (Invitrogen), and *Avh*238 and *Avh*172 transcript levels were screened by qRT-PCR (see below).

Particle Bombardment Assays

Double-barrel particle bombardment assays of cell death–promoting activity (Figure 2) using soybean leaves were conducted as described (Dou et al., 2008b). Reference bombardments were loaded with 1.7 μg of β-glucuronidase (GUS) DNA (pUCGUS) plus 1.7 μg of empty vector DNA (pUC19), and test bombardments were loaded with 1.7 μg of GUS DNA and 1.7 μg of DNA encoding mature effector proteins (see Supplemental Table 3 online). Fourteen to sixteen paired bombardments were conducted, and the results were analyzed using the Wilcoxon signed ranks test (Dou et al., 2008b).

To assay suppression of cell death–promoting activity (Figure 6), two assays were used: an indirect assay and a direct assay. For the indirect assay, DNA encoding the suppressor (pUCAvh172 or pUCAvh6; 1.7 μg/shot) was mixed with DNA encoding the cell death elicitor (pUCAvh238, pUCAvh241, pUCAvh181, or pUCAvr4/6; 1.7 μg/shot) and GUS DNA (1.7 μg/shot) and bombarded into soybean leaves lacking or containing Rps4. The plasmids are described in Supplemental Table 2 online. The control shot in the second barrel was empty vector (pUC19; 3.4 μg/shot) plus GUS DNA (1.7 μg/shot). The log ratios for these shots were then compared with the log ratios obtained when the suppressor DNA was replaced by empty vector DNA; 14 to 16 pairs of shots were performed for each comparison, and the results were evaluated using the Wilcoxon rank sum test. The indirect assay had the advantage that the level of PCD triggered by each elicitor could be monitored in every shot. For the direct assay, suppressor + elicitor + GUS was compared directly with empty vector + elicitor + GUS in the second barrel. The log ratios obtained were then tested for significance using the Wilcoxon signed ranks test; again, 14 to 16 pairs of shots were performed. The direct assay had the advantage that the activity of the suppressor could be compared with the control directly on the same leaves.

Genome Sequencing

Genomic DNAs from P7064, P7074, and P7076 were sequenced by the Virginia Bioinformatics Institute's Core Laboratory facility using the 454 Life Science Titanium platform according the manufacturer's standard protocols. The reads were trimmed and a provisional assembly was produced using the Newbler Assembler (v 2.0.00.20) with the default settings. Read and assembly statistics are described in Supplemental Table 1 online. The provisional assemblies may be accessed at vmd.vbi.vt.edu.

Manual inspection of the *Avh* genes in the Newbler assemblies revealed frequent problems where coverage was low or where multiple similar paralogs existed in the genome. Therefore, to produce a high-quality assembly of each Avh allele in the three 454-sequenced isolates (P7064, P7074, and P7076), BLASTn was used with an E-value cutoff of 1E-40 to search the raw 454 reads from each isolate using the reference sequence from P6497 as a query. The reads identified for each gene were manually assembled using SeqMan from the Lasergene software package. For single-copy genes, reads with <95% identity were excluded from the assembly, while for genes with multiple closely similar paralogs, reads with <98% identity were excluded from any assembly. Frameshift mutations that occurred within or close to a homopolymer run were assumed to result from 454-sequencing errors even when present in multiple reads and were manually corrected. In the process of analyzing the P7064, P7074, and P7076 sequences, the original *Avh* gene list of Jiang et al. (2008) was updated (see Supplemental Data Set 2 online). In particular, a number of genes with frameshift mutations that were originally assumed to contain sequencing errors were confirmed as pseudogenes or spliced genes because the frameshifts were present in the three sets of 454 sequences.

Polymorphism Analysis

To identify and count polymorphisms, ClustalW was used to create multiple sequence alignments, which were then manually adjusted to

minimize the number of implied mutations. At each nucleotide position in the alignment, the number of different bases was counted and then this count was summed over all positions in the gene and subtracted by the length of the gene to obtain a minimum estimate of the number of mutations occurring in the genes among the four lineages. In no case was the same amino acid substitution assumed to have occurred more than once, which may have resulted in a very slight underestimate of the numbers of mutations in some genes.

To estimate the presence of negative or positive selection, we used YN00 from the PAML package to calculate dN and dS for each pair of *Avh* alleles (P6497 allele to one of the other three isolates), after eliminating any alignment gaps. The statistical significance of the difference between dN and dS was estimated using the standard errors output from PAML for a *t* test with an FDR-adjusted (Benjamini and Hochberg, 1995) P value ≤ 0.05.

Two methods were used to test for positive selection in groups of genes. In the first method, the amino acid sequences of the encoded proteins were concatenated into two super proteins containing each allele pair showing differences. Then, the difference between dN and dS was tested statistically as described above. In the second method, the differences between the dN and dS values of each gene in a group were tested as a group using the Wilcoxon signed ranks test with a P value ≤ 0.05.

Synthesis of *Avh*238 Double-Stranded RNA and Transient Silencing

Pairs of primers were designed for double-stranded RNA (dsRNA) synthesis. Synthesis of *Avh*238 dsRNA was performed using the Megascript RNAi kit (Ambion) following the manufacturer's protocol and using primers Avh238 dsRNA F and Avh238 dsRNA R (see Supplemental Table 2 online). The integrity of the dsRNA was examined by agarose gel electrophoresis, and the yield (typically 100 to 200 μg) was determined by spectrophotometry.

The transformation was performed by the method of Mcleod et al. (2008) with the following modification: 2-d-old *P. sojae* mycelial mats, cultured in pea broth medium, were rinsed and washed in 0.8 M mannitol and then placed in enzyme solution (0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$, 7.5 mg/mL lysing enzyme [from *Trichoderma harzianum*; Sigma-Aldrich L1412], and 3 mg/mL cellulase [from *Trichoderma reesei*; Sigma-Aldrich C8546]) and incubated for 40 min at 25°C with 100 rpm shaking. The protoplasts were harvested by centrifugation at 1500 rpm for 3 min and resuspended in W5 solution (5 mM KCl, 125 mM $CaCl_2$, 154 mM NaCl, and 31 mg/mL glucose) at a concentration of 1×10^6 protoplasts/mL. After 30 min, the protoplasts were centrifuged at 1500 rpm for 4 min and resuspended in an equal volume of MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.7) to allow the protoplasts to swell. Then the protoplasts were diluted to 5000 to 10,000 per 1mL in MMg solution, ~100 μg dsRNA was added, and the mixture incubated for 10 min on ice. Then, three aliquots of 580 mL each of freshly made polyethylene glycol solution (40% [v/v] polyethylene glycol 4000, 0.3 M mannitol, and 0.15 M $CaCl_2$) were slowly pipetted into the protoplast suspension and gently mixed. After 20 min incubation on ice, 10 mL pea broth containing 0.5 M mannitol was added, and the protoplasts were incubated overnight to regenerate. The regenerated protoplasts were suspended in pea broth containing 1% agar (40°C) containing 0.5 M mannitol and plated. Visible regenerating colonies could be observed after 24 h incubation at 25°C. Twenty single regenerating colonies were selected and propagated on V8 agar for further analysis.

Phenotypic Assays of *P. sojae* Transformants on Soybean by Real-Time PCR Quantification of Pathogen DNA

For plant infection assays, *P. sojae* zoospores from wild-type and transformant lines were produced as follows. Stationary mycelial cultures were grown in liquid V8 broth in 90-mm Petri dishes at 25°C in the dark for 3 d. The hyphae were repeatedly washed with sterile distilled water and then incubated in the dark at 25°C for 4 to 8 h until sporangia developed on most of the hyphae and zoospores were released. Soybean seedlings of cultivar Williams were grown in the dark for 4 d at 25°C (16 h per day) and 16°C (8 h per day). Hypocotyls of etiolated soybean seedlings (9 to 12 per assay) were inoculated 2 to 3 cm from the base of the cotyledon with 200 zoospores and then incubated in the dark for 12 h at 25°C.

To measure pathogen virulence, the ratio of *P. sojae* DNA to soybean DNA in the infected tissues was determined using quantitative real-time PCR. Soybean tissue infected with *P. sojae* was harvested from 1 cm above the inoculation site to 1 cm below. Tissue samples from five seedlings were pooled, and total genomic DNA was extracted using a DNeasy plant mini kit (Qiagen) following the recommended protocol. *P. sojae act*A gene (VMD GeneID:108986) was used as the pathogen target, and soybean housekeeping gene *CYP2* (TC224926) was used as the host target gene. PCR reactions (20 μL) included 20 ng DNA, 0.2 μM of each primer (see Supplemental Table 2 online), and 10 μL SYBR *Premix ExTaq* (TaKaRa Bio). Reactions were performed on a ABI PRISM 7300 Fast real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 31 s to calculate cycle threshold (Ct) values, followed by 95°C for 15 s, 60°C for 1 min, then 95°C for 15 s to obtain melt curves. The infestation level of the wild-type strain p6497 (WT) was assigned the value 1.0, and the levels of the transformants were related to P6497 using ABI SDS Software V1.4. All qPCR assays for infection levels were performed in three technical replicates.

RNA Extraction and Transcript Level Analysis

To measure the transcript levels of effector genes by qRT-PCR, mycelia and infected plant tissue samples were prepared as for microarray analysis, except that the soybean cultivar Williams was used for infection and *P. sojae* isolate P6497 was used. Total cellular RNA was extracted using the PureLink RNA mini kit (Invitrogen) following the recommended protocol. For screening RNA levels in *P. sojae* transformants, first-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's directions.

For gene transcription level analysis, SYBR green qRT-PCR assays were performed. Primer pairs (see Supplemental Table 2 online) were designed to anneal specifically to each of the selected genes. Three housekeeping genes of *P. sojae* were used as endogenous controls, namely, actin (JGI/VMD GeneID:109046), rpL13a (JGI/VMD GeneID: 109358), and rpS5 (JGI/VMD GeneID:144810). PCR reactions (20 μL) included 20 ng cDNA, 0.2 μM of each primer, and 10 μL SYBR *Premix ExTaq* (TaKaRa Bio). Reactions were performed on a ABI PRISM 7300 Fast real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 31 s to calculate cycle threshold (Ct) values, followed by 95°C for 15 s, 60°C for 1 min, and then 95°C for 15 s to obtain melt curves. The expression of each gene relative to average Ct values of the three housekeeping genes ($\Delta Ct = Ct_{gene} - Ct_{HKaverage}$) was determined using the ABI 7300 system sequence detection software (version 1.4) (Cui et al., 2009; Schuhmacher et al., 2010). To enable direct comparisons with the microarray data, each ΔCt was added to the averaged GC-RMA–normalized expression values (which are $\log_2$ transformed) of the same three housekeeping genes at the relevant time point from the microarray analysis.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: microarray data sets, Gene Expression Omnibus GSM566765 to GSM566776; 454 read data, GenBank SRP002477; *Avh* gene models and alleles, listed in Supplemental Data Sets 2 and 3 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Functional Interactions between Avh6 and Avh238.

Supplemental Table 1. Read and Assembly Statistics for 454 Sequencing.

Supplemental Table 2. Primers Used for Virulence Assays and RT-PCR Assays and for Miscellaneous Constructs.

Supplemental Table 3. Plasmid Constructs Used in the Study.

Supplemental Data Set 1. Suppression of BT-PCD by Avh Proteins.

Supplemental Data Set 2. Nucleotide Sequence Polymorphisms among Avh Genes.

Supplemental Data Set 3. Updated List of *P. sojae* P6497 Effectors.

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AUTHOR CONTRIBUTIONS

B.M.T. and Y.W. conceived the research. B.M.T., Y.W., D.D., X.Z., W.S., and Q.W. designed the research. Q.W., C.H., A.O.F., S.D.K., B.G., Y.-T.S., Y.S., X.W., Z.Z., and B.C. performed the experiments. Q.W., W.Y., A.O.F., S.T., S.D., and B.M.T. analyzed the data. S.T. contributed new computational tools. B.M.T., Y.W., and Q.W. wrote the article with input from all of the authors.

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