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Conserved C-Terminal Motifs Required for Avirulence and Suppression of Cell Death by *Phytophthora sojae effector* Avr1b [W]

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The sequenced genomes of oomycete plant pathogens contain large superfamilies of effector proteins containing the protein translocation motif RXLR-dEER. However, the contributions of these effectors to pathogenicity remain poorly understood. Here, we show that the *Phytophthora sojae* effector protein Avr1b can contribute positively to virulence and can suppress programmed cell death (PCD) triggered by the mouse BAX protein in yeast, soybean (*Glycine max*), and *Nicotiana benthamiana* cells. We identify three conserved motifs (K, W, and Y) in the C terminus of the Avr1b protein and show that mutations in the conserved residues of the W and Y motifs reduce or abolish the ability of Avr1b to suppress PCD and also abolish the avirulence interaction of Avr1b with the *Rps*1b resistance gene in soybean. W and Y motifs are present in at least half of the identified oomycete RXLR-dEER effector candidates, and we show that three of these candidates also suppress PCD in soybean. Together, these results indicate that the W and Y motifs are critical for the interaction of Avr1b with host plant target proteins and support the hypothesis that these motifs are critical for the functions of the very large number of predicted oomycete effectors that contain them.

INTRODUCTION

Oomycete plant pathogens, including >80 species of *Phytophthora*, are destructive to a vast variety of plants important to agriculture and forestry and to natural ecosystems. The economic damage to crops in the United States by *Phytophthora* species is estimated in the billions of dollars, and worldwide it is many times that (Erwin and Ribiero, 1996). Some species of *Phytophthora*, such as *P. cinnamomi* and *P. parasitica*, each attack hundreds of different plant host species. Others, like the soybean (*Glycine max*) pathogen *P. sojae* and the potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) pathogen *P. infestans*, have narrow host ranges. In addition to *Phytophthora*, the oomycete class Peronosporomycetidae includes many other destructive plant pathogens, such as the downy mildews and >100 species of *Pythium* (Agrios, 2005). The downy mildew pathogen of *Arabidopsis thaliana*, *Hyaloperonospora parasitica*, has been used extensively as a model for understanding plant responses to oomycete infection (Slusarenko and Schlaich, 2003). Despite morphological and physiological resemblances to fungi, oomycetes belong to the kingdom Stramenopila, which includes diatoms and golden brown algae (Sogin and Silberman, 1998).

P. sojae and *P. infestans* are hemibiotrophic, which means they have an initial phase of biotrophic growth followed by a transition to necrotrophy. During the early stages of infection, biotrophic and hemibiotrophic oomycetes form specialized feeding structures called haustoria. Haustoria are formed when hyphae penetrate the host plant cell wall but remain separated from the host cytoplasm by the plant plasma cell membrane (Erwin and Ribiero, 1996; Hahn and Mendgen, 2001; Tyler, 2007). Haustoria are thought to play a key role in exploitation of the plant by actively importing carbon, water, and inorganic nutrients. Haustoria are also likely structures through which pathogens might deliver effector molecules into the plant cell to modulate plant defense circuitry and enable parasitic colonization (Hahn and Mendgen, 2001).

The role of effector proteins in pathogenesis has been characterized most extensively in bacteria (Alfano and Collmer, 2004; Chang et al., 2004; Mudgett, 2005; Chisholm et al., 2006). During animal infection, pathogen effector proteins can alter specific host cell functions, such as phagocytosis, proinflammatory responses, apoptosis, and intracellular trafficking (Mota and

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Cornelis, 2005). In plant pathogenesis, many bacterial effectors suppress defense responses, triggered by either microbe-associated molecular patterns (MAMPs) or by other effectors due to recognition by resistance (R) proteins (Kjemtrup et al., 2000; Alfano and Collmer, 2004; Chang et al., 2004; Mudgett, 2005; Chisholm et al., 2006; Jones and Dangl, 2006). Many bacterial effectors are Cys proteases that presumably target positive regulators of defense (Hotson and Mudgett, 2004). On a similar theme, AvrPtoB from *Pseudomonas syringae* suppresses programmed cell death (PCD) triggered by diverse elicitors, including the mouse BAX protein via a C-terminal E3 ubiquitin ligase domain (Abramovitch et al., 2003, 2006). As an alternative mechanism, several bacterial effectors are transcription factors and can stimulate the expression of host genes that promote disease susceptibility (Arbibe et al., 2007; Kay et al., 2007). Several fungal plant pathogens secrete effector proteins that contribute to virulence (reviewed in Chisholm et al., 2006), including the toxins NIP1 from *Rhynchosporium secalis* and PtrA from *Pyrenophora tritici-repentis* and the chitin binding protein Avr4 from *Cladosporium fulvum*. Several additional secreted proteins are presumed to be effectors since they interact with plant resistance proteins, but their contribution to pathogen virulence has not yet been directly demonstrated. These include the metalloproteases Avr-Pita and Avr-YAMO from *Magnaporthe grisea* and the proteinase inhibitors Avr2 from *C. fulvum* and AvrP123 from *Melampsora lini* (reviewed in Chisholm et al., 2006). Some of these fungal effectors can cross into the plant cell cytoplasm, or are inferred to do so, but the mechanisms of entry are unknown. Many potential effector proteins have been identified in oomycetes through structural and functional genomics studies, including toxins, proteases, and proteinase inhibitors (reviewed in Kamoun, 2007), but the contributions of these proteins to virulence are currently only inferred.

Many plant disease resistance genes that confer resistance against oomycete pathogens encode nucleotide binding site, leucine-rich repeat (NBS-LRR) proteins. These include the *Arabidopsis RPP*1, *RPP*2, *RPP*4, *RPP*5, *RPP*7, *RPP*8, and *RPP*13 genes against *H. parasitica* (Slusarenko and Schlaich, 2003), the lettuce (*Lactuca sativa*) *Dm*3, *Dm*14, and *Dm*16 resistance genes against *Bremia lactucae* (Wroblewski et al., 2007), the potato *R*1 (Ballvora et al., 2002), *Rb*/*RpiBlb*1 (Song et al., 2003; van der Vossen et al., 2003), *RpiBlb*2 (van der Vossen et al., 2005), and *R*3a (Huang et al., 2005) genes against *P. infestans*, and the soybean *Rps*1k (Gao et al., 2005), *Rps*4, and *Rps*6 (Sandhu et al., 2004) genes against *P. sojae*. Five oomycete avirulence genes that interact with these *R* genes in a gene-for-gene manner have been cloned, namely, *P. sojae Avr1b*-1 (Shan et al., 2004), *P. infestans Avr3a* (Armstrong et al., 2005), *H. parasitica ATR13* (Allen et al., 2004) and *ATR1* (Rehmany et al., 2005), and *P. sojae Avr4/6* (D. Dou, S.D. Kale, F.D. Arredondo, and B.M. Tyler, unpublished data). All five of these genes encode small secreted hydrophilic proteins that are recognized by their cognate resistance gene products in the cytoplasm of the plant host (Allen et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). These effector proteins share an N-terminal motif, RXLR-dEER (Rehmany et al., 2005; Birch et al., 2006; Tyler et al., 2006), that is required to carry these proteins across the host plasma cell membrane into the cytoplasm (Whisson et al., 2007). The RXLR-dEER motif resembles the host targeting signal of *Plasmodium* effectors that carries those effectors across the parasitiphorous vacuolar membrane in erythrocytes (Hiller et al., 2004; Marti et al., 2004) and is functionally exchangeable with the *Plasmodium* host targeting signal (Bhattacharjee et al., 2006).

Bioinformatic analysis of the genome sequences of *P. sojae* and *P. ramorum* has identified an extraordinarily large superfamily of predicted proteins with sequence similarity to the *P. sojae Avr1b*-1 and *P. infestans Avr3a* genes (Tyler et al., 2006; Win et al., 2007; Jiang et al., 2008). These genes all encode small secreted proteins that contain an N-terminal RXLR-dEER motif, which leads us to the hypothesis that the proteins can enter host cells and so may be considered as candidate effector proteins (Tyler et al., 2006; Win et al., 2007; Jiang et al., 2008). The members of the superfamily also share sequence similarity in the C-terminal regions, indicating they have a common evolutionary origin (Jiang et al., 2008). In more than half of the members of the superfamily, bioinformatic analysis has identified three conserved motifs, termed W, Y, and L motifs. Many of these effector candidates contain multiple copies of the three motifs, in which case, the three motifs are repeated in tandem as a single W-Y-L module (Jiang et al., 2008).

Since RXLR-dEER–containing proteins can enter plant cells, they are presumed to act to promote the virulence of these pathogens. However, this has not yet been directly demonstrated, and the mechanisms by which they might do this are poorly understood. The oomycete avirulence genes cloned to date are not essential for pathogenicity since virulent races of the respective pathogens exist in which the avirulence genes are either not expressed or have accumulated many mutations. Bos et al. (2006) demonstrated that transient expression of *Avr3a* in *Nicotiana benthamiana* could suppress PCD triggered by the INF1 elicitin protein, which is a MAMP (Nurnberger et al., 2004); this suggests that Avr3a may contribute to the pathogenicity of *P. infestans* by suppressing MAMP-triggered immunity. The C terminus of the protein was required for this activity. However, Avr3a has not been demonstrated directly to be required for or to contribute to the virulence of *P. infestans*.

We show here that overexpression of the *P. sojae* effector gene *Avr1b*-1 measurably increases the virulence of *P. sojae* transformants. We further show that expression of the *Avr1b*-1 gene can suppress PCD triggered by the mouse BAX protein in yeast, soybean, and *N. benthamiana*, identifying a mechanism by which Avr1b may contribute to virulence. We show that the C-terminal W and Y motifs of Avr1b are required for suppression of PCD and for interaction with the Rps1b resistance gene product. Finally, we show that three bioinformatically identified oomycete effector candidates that contain W and Y motifs can also suppress BAX-induced PCD, suggesting that suppression of PCD is a major function of the >214 *P. sojae* effector candidates that contain these motifs.

RESULTS

Avr1b Contributes Positively to Virulence

Loss of *Avr1b*-1 expression does not compromise the virulence of *P. sojae* (Shan et al., 2004), presumably because the genome

encodes a very large number of effectors of similar function. Therefore, to test whether the Avr1b gene product contributes positively to virulence, we produced *P. sojae* transformants that overexpress *Avr1b*-1. We obtained two *P. sojae* transformants (T17 and T20) that contain multiple copies of the *Avr1b*-1 gene and produce high levels of *Avr1b*-1 mRNA (Figures 1A and 1B). Both transformants killed soybean seedlings slightly more quickly than the recipient strain P7076 in a hypocotyl inoculation assay (Figure 1C). To measure the difference quantitatively, we used an assay routinely used to measure quantitative resistance in soybean cultivars (Vega-Sánchez et al., 2005). In this assay, in which the rate of lesion progression up the roots of soybean seedlings is measured, both transformants produced lesions that progressed statistically significantly faster than the recipient ($P < 0.05$; Figure 1D).

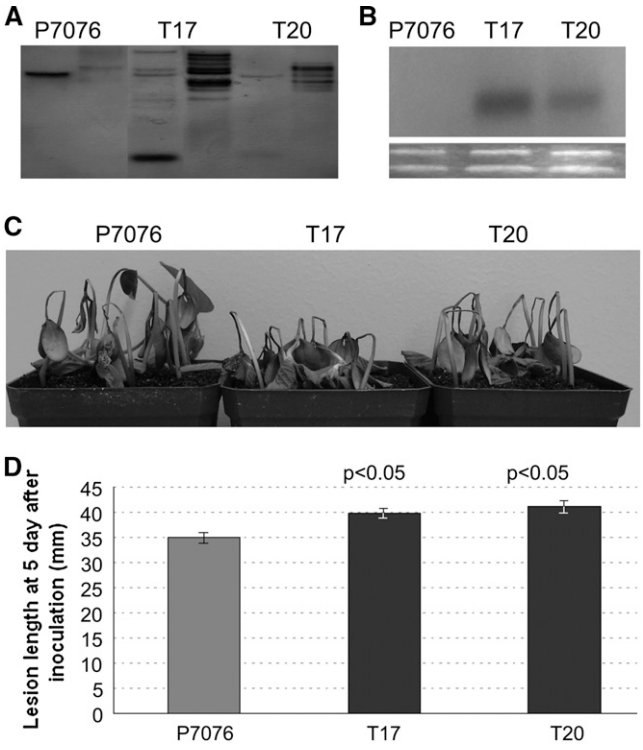

Figure 1. Overexpression of *Avr1b*-1 Confers Increased Virulence on Soybean.

(A) DNA gel blots hybridized with the *Avr1b*-1 probe, demonstrating multiple copies of *Avr1b*-1 transgenes. P7076 is the transformation recipient, and T17 and T20 are two transformants. DNA from each strain was digested with *Kpn*I (lanes 1, 3, and 5) or *Xho*I (lanes 2, 4, and 6). A band around 400 bp is released when transgenic DNA is cut with *Kpn*I.

(B) RNA gel blots of RNA extracted from in vitro grown mycelium and hybridized with an *Avr1b*-1 probe. The bottom panel shows ethidium bromide staining of RNA prior to transfer.

(C) Seven-day-old seedlings of soybean cultivar Williams were inoculated on the hypocotyls and photographed 4 d later.

(D) Root lesion lengths 5 d after inoculation. Lesions on the roots of 30 seedlings inoculated with wild-type or transgenic *P. sojae* were measured in each of five independent experiments. Combined data for all 150 plants are shown. Error bars show SE. P values were determined with a *t* test.

Avr1b Can Suppress PCD Induced by Mouse BAX Protein in Soybean and *Nicotiana*

To explore the mechanisms by which Avr1b contributes to virulence, we tested whether, like many bacterial effectors, Avr1b could suppress PCD induced by the proapoptotic protein BAX. We used a particle bombardment assay that introduces DNA encoding β-glucuronidase (GUS) into soybean leaf cells. When DNA encoding an elicitor of PCD is cotransformed with the *GUS* gene, PCD results in the elimination of most cells expressing *GUS* (Mindrinos et al., 1994; Qutob et al., 2002) (Figures 2A and 2B). When a third gene is introduced on the same particles, the ability of the protein encoded by the gene to suppress PCD can be measured by the restoration of cells expressing *GUS* (Figure 2A). To facilitate the comparison of test and control bombardments, we invented a novel, double-barreled attachment for the Bio-Rad Gene Gun (see Supplemental Figure 1 online) that enables us to shoot two different DNA samples side by side into a leaf in the same shot, which greatly improves the reproducibility of the results (see Supplemental Figure 2 online).

To trigger PCD in soybean cells, we used a mouse *Bax* cDNA that triggers PCD when expressed in *Nicotiana* (Lacomme and Cruz, 1999) and *Arabidopsis* (Baek et al., 2004). Figure 2B and Table 1 show that expression of *Bax* reduced the number of GUS-positive blue patches by 88% (experiment a compared with experiment c in Table 1), confirming that mouse BAX protein can trigger PCD in soybean cells. When *Avr1b*-1 was coexpressed with the *Bax* cDNA, the number of surviving GUS-positive blue patches was tripled, indicating that Avr1b could partially suppress BAX-induced PCD. This result was obtained both when the cobombardment of *Bax* and *Avr1b*-1 was compared directly to cobombardment of *Bax* and empty vector using the double-barreled bombardment (direct assay; Figure 2C, Table 1) and when each mixture was separately compared with a reference consisting of *GUS* plus empty vector (indirect assay) (Table 1). *Avr1b*-1 expression did not increase the number of blue spots when BAX was omitted from the experiment (Figure 2D, Table 1).

To independently confirm that Avr1b could suppress BAX-induced PCD in plant cells, we used *Agrobacterium tumefaciens* cells to deliver a potato virus X (PVX) vector carrying *Avr1b*-1 and the *Bax* cDNA into *N. benthamiana* leaves. *A. tumefaciens*–mediated transient expression was used previously to demonstrate suppression of BAX-induced PCD by bacterial effectors (Jamir et al., 2004) and suppression of elicitin-induced PCD by *P. infestans Avr3a* (Bos et al., 2006). When *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells containing a *Bax* cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter, obvious cell death symptoms were observed (Figures 3A and 3C), confirming that BAX could trigger PCD in *N. benthamiana* cells (Lacomme and Cruz, 1999). However, when the leaves were infiltrated with *A. tumefaciens* cells containing an *Avr1b*-1 gene, 1 d (Figure 3C) or 2 d (Figure 3B) prior to infiltration with the *Bax* cDNA-containing cells, no cell death symptoms were observed except in regions of the leaf that failed to receive *Avr1b*-1 gene–containing cells. *Avr1b*-1 gene expression also protected *N. benthamiana* tissue from cell death triggered by *Bax* cDNA

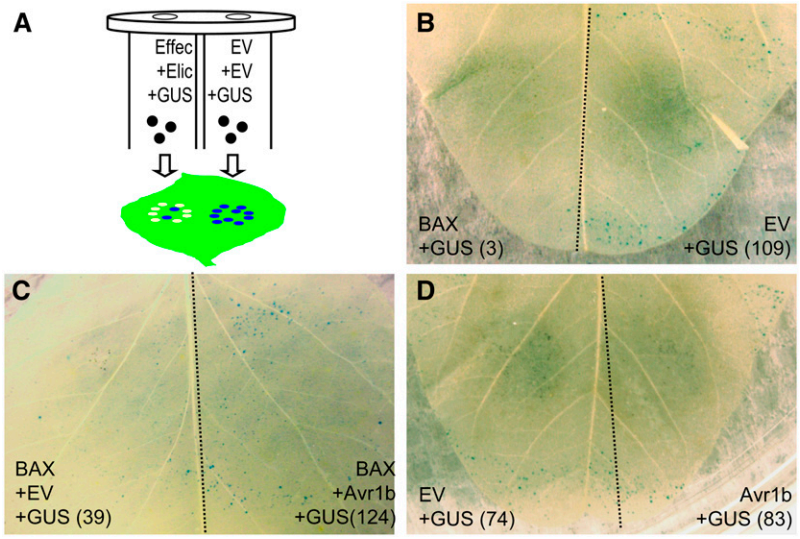

Figure 2. Measuring Suppression of BAX-Mediated PCD by Avr1b Using Double-Barreled Particle Bombardment of Soybean Leaves.

(A) Experimental design using the double-barreled particle bombardment assay. The diagram illustrates the indirect assay in which the number of GUS-positive blue spots produced by a mixture of an effector (e.g., Avr1b) and elicitor (e.g., BAX) is measured as a ratio to an empty vector (EV) control. PCD triggered by BAX protein reduces the appearance of blue spots because the GUS-producing cells are killed. Avr1b partially restores the appearance of blue spots by suppressing PCD.

(B) to **(D)** Leaves bombarded with the pairs of DNA mixtures indicated. Dotted line indicates the position of a divider used to prevent overlap of the two bombardment areas. Numbers of blue spots counted are indicated in parentheses. Blue spots cannot be counted in the central area of a shot due to tissue damage. Conclusions are based on statistical analysis of results from 14 to 16 leaves similar to those illustrated (Table 1).

(B) Ablation of blue spots by BAX-triggered PCD.

(C) Suppression of BAX-triggered PCD by Avr1b.

(D) A control experiment verifying that Avr1b does not significantly increase the number of blue spots in the absence of BAX.

expression when *A. tumefaciens* strains containing the two genes were infiltrated simultaneously (Figure 3A). Prior infiltration of *N. benthamiana* leaves with buffer or with *A. tumefaciens* cells containing a green fluorescent protein (*GFP*) gene did not protect the leaves from BAX-induced PCD (Figure 3C). Furthermore, as expected, infiltrations of *A. tumefaciens* cells containing *Avr1b*-1 alone failed to elicit visible cell death (Figures 3A and 3C). These results support the conclusion from the soybean particle bombardment assay that *Avr1b*-1 gene expression can suppress BAX-induced PCD in plant cells.

Suppression of PCD in Yeast Indicates That Avr1b Has a Highly Conserved Target

To address whether Avr1b targets a host protein specific to plants or a highly conserved protein in the pathway leading to PCD, we tested whether, like some bacterial effectors (Abramovitch et al., 2003), Avr1b can suppress PCD in the yeast *Saccharomyces cerevisiae*. We introduced the *Avr1b*-1 gene into yeast strain W303 under the control of the *GAL*1 promoter and then tested the resistance of several transformants to PCD induced by hydrogen

Table 1. Suppression of BAX-Mediated PCD by Avr1b, Measured by Double-Barreled Particle Bombardment

Experiment	Barrel 1[a]	Barrel 2[a]	Direct Ratio[b]	Indirect Ratio[c]	P Value[d]
a	EV + GUS	EV + GUS	0.99 ± 0.03		
b	Avr1b + GUS	EV + GUS	1.03 ± 0.15	b/a = 1.04	>0.100
c	BAX + GUS	EV + GUS	0.12 ± 0.02		
d	Avr1b + BAX + GUS	EV + EV + GUS	0.34 ± 0.04	d/c = 2.84	<0.001
e	Avr1b + BAX + GUS	EV + BAX + GUS	3.36 ± 0.7		<0.004

[a] Barrels 1 and 2 are physically identical. Half of all the replicates were conducted using the configuration of DNA samples indicated, and half were conducted with the samples reversed between barrels 1 and 2. In all cases, the mass of DNA in each barrel was identical. EV, empty vector.

[b] Ratios between the numbers of spots produced by each barrel. Geometric averages and SE were calculated from log ratios obtained from 14 to 16 pairs of shots.

[c] Comparison of the two averaged ratios from the experiments indicated by the lowercase letters.

[d] P values for the indirect comparisons were calculated from the log ratios using the Wilcoxon rank sum test. P value for the direct comparison (experiment e) was calculated from the log ratios using the Wilcoxon signed ranks test. A significant P value indicates significant suppression of PCD.

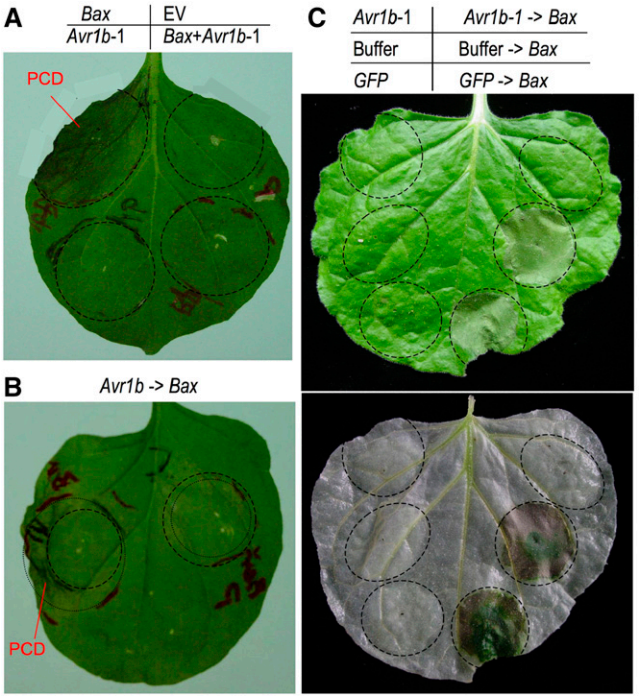

Figure 3. Avr1b Suppresses BAX-Induced Cell Death in *N. benthamiana*.

N. benthamiana leaves were infiltrated with *A. tumefaciens* cells containing a PVX vector carrying a mouse *Bax* cDNA, the *P. sojae Avr1b*-1 gene, a *GFP* gene or an empty vector (EV) within the regions indicated by the dashed lines. Photos were taken 5 or 6 days after the last infiltration. In **(A)**, the *Bax*- and *Avr1b*-1–containing cells were infiltrated at the same time. In **(B)**, *Avr1b*-1–containing cells were infiltrated into the areas marked by the dashed lines, followed 2 d later by infiltration with cells carrying the *Bax* cDNA into the region marked with the dotted line. In **(C)**, buffer or cells carrying the *Avr1b*-1 or *GFP* genes were infiltrated into the leaf, followed after 24 h either by no further challenge (left side) or by infiltration with cells carrying the *Bax* cDNA. The bottom panel shows the same leaf as the top panel after decolorization with ethanol.

peroxide. We observed that Avr1b protected yeast from the PCD induced by 15 mM H_2O_2 compared with control transformants (Figure 4A). We also observed that *Avr1b*-1 expression could weakly suppress PCD induced by BAX (Figure 4B), although the activity is not as strong as the cell death suppressor Bcl2 (see Supplemental Figure 3 online). In yeast, production of reactive oxygen species was shown to play a key role in BAX-induced apoptosis (Madeo et al., 1999). Therefore, we conclude that the components of the PCD machinery of the plants and yeast targeted by Avr1b are substantially conserved.

Identification of Conserved C-Terminal Motifs in Avr1b and Close Paralogs

To identify the regions of Avr1b responsible for mediating the suppression of PCD, we performed an amino acid sequence alignment of Avr1b with *P. infestans* Avr3a (Armstrong et al., 2005) and with Avr1b paralogs that we identified in the genome sequences of *P. sojae*, *P. infestans*, and *P. capsici* (see Supplemental Table 1 online). The genome sequences of *P. ramorum* and *H. parasitica* do not encode any effector-like proteins with strong similarity to Avr1b. As shown in Figure 5A, the sequence alignment identifies three regions of sequence conservation in the C terminus of these proteins, in addition to the secretory leader and RXLR-dEER domain in the N terminus. Two of the conserved regions correspond to W and Y motifs found in more than half of the RXLR-dEER–containing effectors in the *P. sojae* and *P. ramorum* genomes (Jiang et al., 2008). In addition to the W and Y motifs, the alignment reveals a conserved Lys-rich motif that we have called the K motif. Adding an allele of Avr1b to the sequence alignment together with the product of *Avh*1, a highly similar paralog of *Avr1b*-1 found in some strains of *P. sojae* (Shan et al., 2004), reveals that the K, W, and Y regions are also the locations of highly polymorphic residues. These residues are under strong positive selection (Shan et al., 2004; Jiang et al., 2008).

To resolve why the most conserved regions corresponding to the K, W, and Y motifs are also the most polymorphic, we mapped the polymorphic and conserved residues onto the predicted secondary structure of Avr1b protein. The predicted

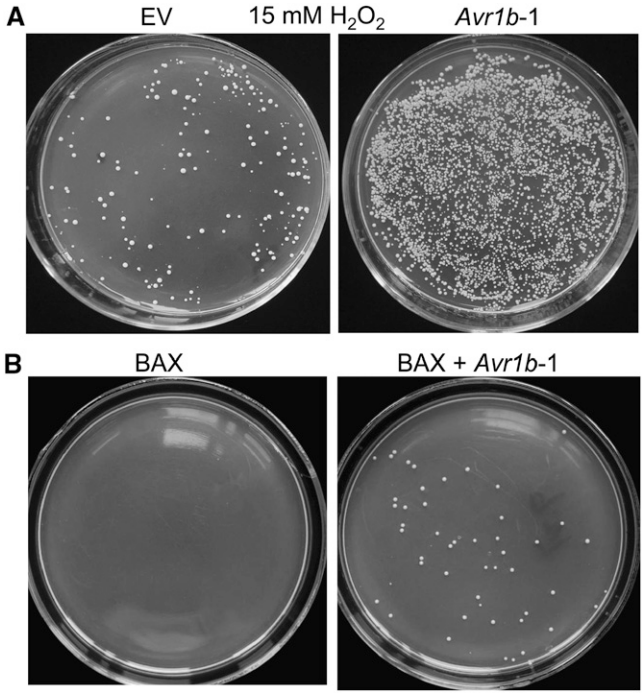

Figure 4. Avr1b Suppresses H_2O_2- and BAX-Induced Cell Death in Yeast.

(A) Yeast cells of strain W303 containing an empty vector (EV) or *Avr1b*-1 under control of the galactose-inducible *GAL*1 promoter were grown in galactose-containing medium, harvested, treated with 15 mM H_2O_2 for 6.5 h, and then diluted and plated as detailed in Methods.
(B) Yeast cells of strain W303 containing the Bax cDNA under control of the galactose-inducible *GAL1* promoter either alone (BAX) or together with *Avr1b*-1 under control of the *GAL1* promoter (BAX + Avr1b) were grown on glucose-containing medium and then harvested, diluted, and plated on galactose-containing medium as detailed in Methods.

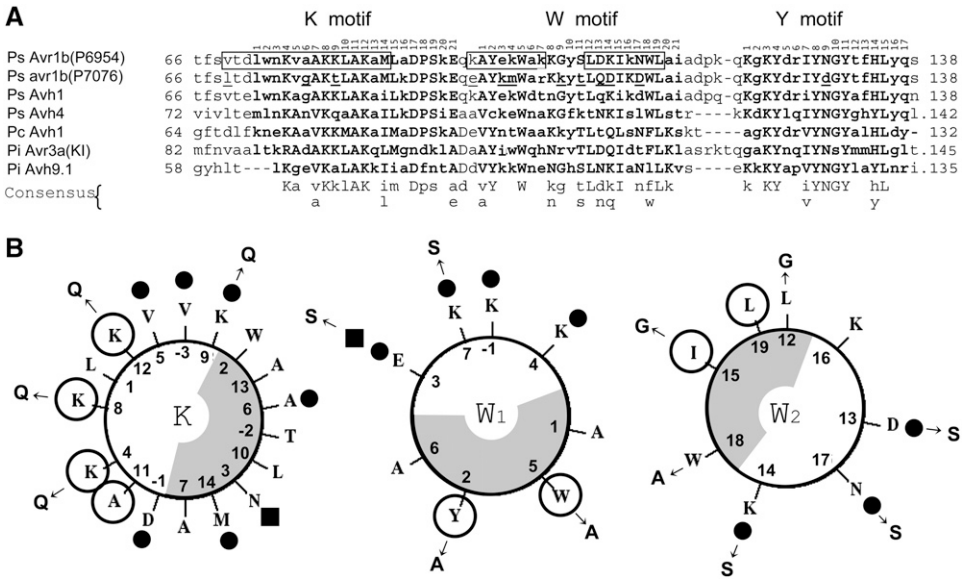

Figure 5. Conserved Motifs in the Avr1b C Terminus Correspond to Predicted Polymorphic Amphipathic α-Helices.

(A) Sequence alignment of C-terminal regions of Avr1b family proteins reveals three conserved C-terminal motifs, K, W, and Y, shown in bold. In the alignment, uppercase letters indicate the most highly conserved residues in the sequences and the consensus. Dots at the end of sequences indicate residues omitted from the figure. Boxed regions indicate predicted α-helices. Ps Avr1b(P6954) sequence is from avirulent strain P6954 (AAM20937); Ps avr1b(P7076) sequence is from virulent strain P7076 (AF449624) (Shan et al., 2004). Underlined sequences are polymorphic among *Avr1b*-1 alleles (Shan et al., 2004). Ps Avh1 (AF449626) and Avh4 indicate Avr1b paralogs in *P. sojae* strain P6497 (Shan et al., 2004; Tyler et al., 2006; Jiang et al., 2008). Pc Avh1 from *P. capsici* is representative of seven nearly identical paralogs (see Supplemental Table 1 online) in the genomic sequence (www.jgi.doe.gov). *P. infestans* Avh9.1 (gene model PITG_05911.1) is representative of seven nearly identical paralogs (see Supplemental Table 1 online) in the genomic sequence (www.broad.mit.edu). *P. infestans* Avr3a (Armstrong et al. 2005) represents five nearly identical paralogs; the KI allele from an avirulent strain (Armstrong et al., 2005) is shown.
(B) Helical wheel projections of the predicted α-helices that overlap the Avr1b K and W motifs. The shaded and unshaded regions indicate hydrophobic and hydrophilic faces of the helices, respectively. Black circles indicate residues polymorphic in *Avr1b*-1 alleles and in *Avh*1. Black squares indicate positions corresponding to polymorphic residues in *P. infestans* Avr3a. Open circles indicate the most conserved residues. Arrows indicate substitutions in the mutants Avr1bK1 (Q substitutions), Avr1bW1 (A and G substitutions), and Avr1bW2 (S substitutions). Numbering refers to positions within each motif as designated in **(A)**.

secondary structure of the C terminus of Avr1b contains three α-helices separated by turns (Shan et al., 2004). One helix corresponds to the K motif and two to the W motif (Figure 5A). Figure 5B shows a helical wheel projection of the residues in the K and W regions. All three helices are predicted to be amphipathic with hydrophobic residues clustered primarily on one side of the helix (shaded in Figure 5B) and hydrophilic residues primarily on the other. The pattern is particularly strong in the two helices associated with the W motif. Figure 5B also shows that in the two helices associated with the W motif, the polymorphic residues are exclusively located on the hydrophilic sides of the two helices, while the most conserved residues are located on the hydrophobic sides. In the K motif helix, the most conserved residues are hydrophilic, and so the hydrophilic side of the helix is well conserved. However, the polymorphic residues form two clusters located ~180° from one another.

Conserved Residues in the W Motif Are Required for Suppression of PCD

To test the roles of the conserved and polymorphic residues of the three conserved C-terminal motifs in the function of Avr1b, we introduced substitution mutations into the residues and tested the ability of genes encoding the mutant Avr1b proteins to suppress BAX-induced PCD using the soybean leaf bombardment assay. Initially five multipoint mutations were introduced into the K, W, and Y motifs. The Avr1bK1 mutation replaced each of the four Lys residues with Gln, which is uncharged but has a similar size to Lys and is hydrophilic (Figures 5B and 6). This mutation did not significantly affect the suppression of PCD by *Avr1b*-1 ($P > 0.1$; Figure 6, Table 2; see Supplemental Table 2 online). The Avr1bW1 mutation replaced the six most highly conserved hydrophobic residues in the W motif with Ala or Gly residues (Figures 5B and 6); this mutant lost all ability to suppress PCD (Figure 6, Table 2; see Supplemental Table 2 online). The Avr1bW2 mutation replaced five of the six polymorphic hydrophilic residues of the W motif with Ser residues (Figures 5B and 6); this mutation did not affect the suppression of PCD (Figure 6, Table 2; see Supplemental Table 2 online). The Avr1bW3 mutation changed four consecutive residues in the second W motif helix to Gly; this also abolished suppression of PCD (Figure 6, Table 2; see Supplemental Table 2 online). The Y1 mutation replaced eight conserved residues in the Y motif; this mutation partially reduced the suppression of PCD (Figure 6, Table 2; see Supplemental Table 2 online). We also tested whether the allele of *Avr1b*-1 found in strain P7076

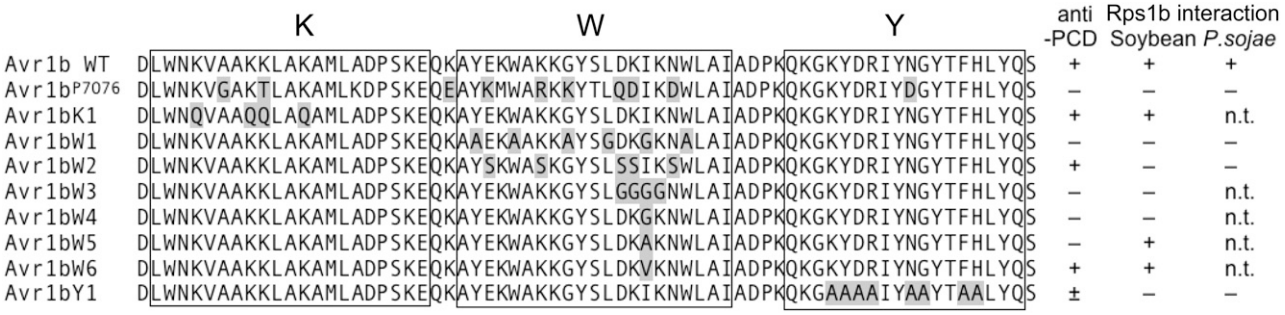

Figure 6. Mutational Analysis of Avr1b C-Terminal K, W, and Y Motifs.

Sequences of the mutants and summary of phenotypes. Mutations are shaded. Avr1b^{P7076} is an allele from virulent strain P7076. Anti-PCD indicates the ability to suppress PCD in the particle bombardment assays. Avirulence was determined using the particle bombardment assay (soybean) and/or in *P. sojae* transformants (*P. sojae*). +, active; −, inactive; ±, partially active; n.t., not tested. Detailed assay data are given in Table 2 and in Supplemental Tables 2 to 4 online.

could suppress PCD; P7076 is virulent against soybean cultivars containing *Rps*1b, and the encoded protein contains substitutions at most of the polymorphic sites in the K, W, and Y motifs. This allele was inactive in suppressing PCD (Figure 6, Table 2; see Supplemental Table 2 online).

A further set of mutations was targeted to a highly conserved Ile residue (position 15 in the W motif; position 109 in the protein) that had been changed to Gly in the Avr1bW1 and Avr1bW3 mutants. I109 was changed to Gly (W4), Ala (W5), and Val (W6). I109G and I109A abolished suppression of PCD, but the more conservative I109V did not (Figure 6, Table 2; see Supplemental Table 2 online).

Conserved and Polymorphic Residues in the W and Y Motifs Are Required for the Functional Interaction of *Avr1b*-1 with *Rps*1b

To determine whether the same residues required for suppression of PCD were also required for the interaction that makes an *Avr1b*-1–expressing *P. sojae* strain avirulent on an

Table 2. Effects of Avr1b Mutations on PCD Suppression and Interaction with Rps1b

	PCD Suppression[b]				Rps1b Interaction				
					Particle Bombardment[c]		*P. sojae* Transformants[d]		
	Direct		Indirect		Rps1b to rps		No. of Transformants	Phenotype	
Mutant[a]	Ratio to Control	P Value	Ratio to Control	P Value	Ratio	P Value		Rps1b	rps
Avr1b^{+}	3.36	<0.001	2.84	<0.001	0.22	<0.001	2	A	V
Avr1b^{P7076}	0.74	>0.5	0.74	>0.1	1.01	>0.1	2	V	V
Avr1bK1	2.41[e]	<0.001	4.87[e]	<0.001	0.12	<0.001	nt		
Avr1bW1	0.99	>0.4	0.68	>0.1	1.02	>0.1	4	V	V
Avr1bW2	3.48	<0.001	3.81	<0.001	0.93	<0.001	4	V	V
Avr1bW3	0.85	>0.5	0.67	>0.1	1.01	>0.1	nt		
Avr1bW4	1.03	>0.2	0.82	>0.1	1.05	>0.1	nt		
Avr1bW5	0.93	>0.5	1.06	>0.1	0.17	<0.001	nt		
Avr1bW6	3.12	<0.001	3.46	<0.001	0.18	<0.001	nt		
Avr1bY1	1.80[f]	<0.01	1.55[g]	<0.05	1.00	>0.1	2	V	V

[a] Sequences of Avr1b mutant proteins given in Figure 6.

[b] PCD suppression was measured using both the direct and indirect assays as described in the Methods and in the legend of Table 1. Significant deviation above 1.0 indicates activity in PCD suppression.

[c] A significant reduction in GUS expression on *Rps*1b leaves compared with *rps* leaves indicates a positive interaction with Rps1b.

[d] Two to four independent transformants carrying each tested mutant *Avr1b*-1 gene were inoculated onto soybean Rps1b or rps seedlings to determine virulence (V) or avirulence (A). To test the P7076 allele, strain P7076 itself was transformed with a GUS gene. More detailed data are presented in Supplemental Table 4 online. nt, not tested.

[e] Not significantly different than wild-type Avr1b ($P > 0.100$).

[f] Significantly different than wild-type Avr1b ($P < 0.025$).

[g] Significantly different than wild-type Avr1b ($P < 0.001$).

*Rps*1b-expressing soybean plant, we introduced *Avr1b*-1 genes carrying the W1, W2, and Y1 mutations into *P. sojae* by transformation. All three mutations abolished the ability of *Avr1b*-1 to make the transformants avirulent upon infection of *Rps*1b-containing cultivars (Figure 6, Table 2; see Supplemental Table 4 online). To confirm these results and to test additional mutations, we used the soybean leaf bombardment assay. In this version of the assay, ablation of the GUS-positive tissue patches is caused by the hypersensitive response (HR) of the soybean tissue to the Avr1b gene product when the resistance gene *Rps*1b is expressed. Wild-type *Avr1b*-1 DNA caused a 78% reduction in the number of GUS-positive spots on *Rps*1b-containing leaves relative to an empty vector control (Table 2, line 1, indirect assay) but caused no reduction on leaves lacking *Rps*1b (rps) (Table 1, line 2, experiment b; Figure 2D; see Supplemental Table 3 online). Of the multipoint mutations, W1, W2, W3, and Y1 all abolished triggering of the HR in the presence of *Rps*1b, but K1 had no effect (Figure 6, Table 2; see Supplemental Table 3 online). Of the five mutations, only W2 abolished the interaction with Rps1b but did not abolish suppression of PCD. The P7076 allele of *Avr1b*-1 (*Avr1b*-1^{P7076}) did not trigger a reaction in *Rps*1b-expressing tissue, as expected since P7076 is virulent on *Rps*1b-containing cultivars. Of the three point mutations at Ile-109, I109G abolished the Rps1b interaction, but I109A and I109V did not (Figure 6, Table 2; see Supplemental Table 3 online). Thus, I109A abolished suppression of BAX-triggered PCD but did not abolish the Rps1b reaction.

Three Bioinformatically Identified Effectors Containing W and Y Motifs Suppress PCD

The experiments described in the previous section demonstrated that residues in the W motif are required for Avr1b to suppress PCD. To determine if other putative RXLR-dEER effectors that contain W motifs also can suppress PCD, we selected two such effectors from the genome sequence of *P. sojae* and one from the genome sequence of *H. parasitica*. One of the *P. sojae* genes, Ps *Avh*331, was selected because it is located 5 kb from *Avr1b*-1. The other two genes, Ps *Avh*163 and Hp *RxL*96, were selected because they display significant sequence similarity with each other. As illustrated in Figure 7A and Supplemental Figure 4 online, all three of these putative effectors contain multiple W and Y motifs, together with an additional conserved motif not found in Avr1b, called an L motif (Jiang et al., 2008). Many predicted oomycete effectors contain multiple modules consisting of adjacent W, Y, and L motifs (W-Y-L modules), though some motifs may be degenerate or missing in some modules (Jiang et al., 2008). All three of these putative effectors could suppress BAX-mediated PCD in the soybean leaf bombardment assay (Figure 7; see Supplemental Table 2 online). Ps Avh331 suppressed PCD even more strongly than Avr1b. Ps Avh331 also could suppress BAX-induced PCD in the *Agrobacterium* infiltration assay in *N. benthamiana* leaves (Figure 7C); the other two genes were not tested in *N. benthamiana*. As an example of an oomycete effector that lacks W, Y, and L motifs, we tested the ability of *P. sojae* avirulence gene Avr4/6 to suppress BAX-induced PCD. Avr4/6 was identified as an RXLR-containing protein encoded in the genetic interval defined

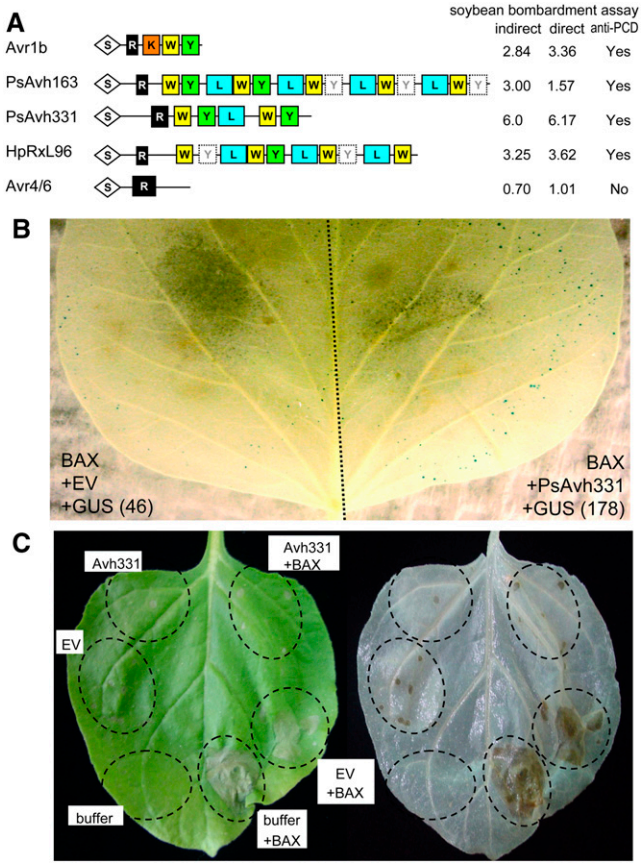

Figure 7. Suppression of BAX-Mediated PCD by Diverse Predicted Effectors Containing W and Y Motifs.

(A) Structure of the predicted effector proteins and their suppression of BAX-mediated cell death in soybean bombardment assays. The positions of each motif are approximately to scale. S, secretory leader; R, RXLR-dEER motif; K, K motif; W, W motif; L, L motif (Jiang et al., 2008). Dotted outlines and gray lettering indicate weak matches to the respective motifs. Each effector was tested for suppression of PCD using both the direct and indirect bombardment assays as described in the Methods and the legend to Figure 2. All effectors with significant suppression activity had P values <0.01 in both assays. Detailed assay data are given in Supplemental Table 2 online.

(B) A leaf bombarded with the pair of DNA mixtures indicated (empty vector [EV]). Dotted line indicates the position of a divider used to prevent overlap of the two bombardment areas. Numbers of blue spots counted are indicated in parentheses. Blue spots cannot be counted in the central area of a shot due to tissue damage. Conclusions are based on statistical analysis of results from 14 to 16 leaves similar to the one illustrated (see Supplemental Table 2 online).

(C) Suppression of BAX-triggered cell death by PsAvh331 confirmed in *N. benthamiana* leaves using *Agrobacterium* infiltration. Buffer or *Agrobacterium* cells carrying PsAvh331 in a PVX vector or an empty vector were infiltrated into the indicated leaf panels. After 2 d, *Agrobacterium* cells carrying BAX in a PVX vector were infiltrated into the same panels as indicated. After 5 d, the leaves were photographed, decolorized in ethanol to visualize the necrotic tissue more clearly, and then photographed again. Two photographs of a representative leaf (before and after decolorization) are shown.

by Whisson et al. (2004). Avr4/6 triggers HR in both *Rps*4 and *Rps*6 soybean plants in the bombardment assay, and silencing of the gene in *P. sojae* eliminates avirulence on both *Rps*4 and *Rps*6 cultivars (D. Dou, S.D. Kale, F.D. Arredondo, and B.M. Tyler, unpublished data). As indicated in Figure 7A, Avr4/6 caused no suppression of BAX-mediated PCD at all.

DISCUSSION

In the first part of this study, we used *P. sojae* overexpression transformants to obtain evidence that avirulence protein Avr1b is an effector that contributes positively to the virulence of the pathogen. In the second part of the study, we demonstrated that Avr1b can suppress PCD triggered in soybean and *N. benthamiana* plants by expression of a mouse *Bax* cDNA, suggesting that one of the mechanisms by which Avr1b contributes to virulence is by suppressing the host plant's HR. We further showed that *Avr1b*-1 gene expression could suppress PCD in yeast, suggesting that the PCD signaling protein(s) targeted by the effector is highly conserved. In the final part of this study, we showed that a conserved C-terminal motif (W motif) found in many predicted oomycete effectors was required for the ability to suppress PCD and that a second motif (Y motif) also may play a role. The hypothesis suggested by these results, namely, that many of the other predicted effectors that contain W motifs may also act to suppress PCD, was reinforced by our confirmation that three predicted effectors containing W and Y motifs (W-Y motif effectors) could suppress PCD, whereas a functional avirulence protein (Avr4/6) that lacked the motif could not. We further demonstrated that many of the residues of Avr1b required for suppression of PCD were also involved in eliciting an Rps1b-mediated HR, though two mutations could be identified that separated the two activities.

This study also introduces two technical improvements that are of general value to researchers studying *P. sojae* and plant–microbe interactions in general. First, we have introduced three major improvements to the efficiency and reliability of *P. sojae* transformation (Judelson et al., 1991), namely, use of a new protoplast transformation procedure (McLeod et al., 2008), use of G418 selection, and the use of the strong constitutive promoter of the *P. sojae* rpL41 ribosomal protein gene. Our second major innovation has been the invention of a double-barreled modification of the Bio-Rad Gene Gun, which enables all quantitative particle bombardment experiments to be internally controlled. This innovation greatly increases the reproducibility and, hence, sensitivity of these experiments.

Avr1b Is an Effector Protein That Can Suppress PCD

Our results show that although Avr1b is not essential to virulence, presumably because the *P. sojae* genome contains a very large number of similar effectors (Tyler et al., 2006; Jiang et al., 2008), its contribution to virulence can be seen when magnified by strong overexpression. Suppression of PCD constitutes an important potential mechanism by which Avr1b may contribute to virulence. A key cytological difference between a compatible and an incompatible interaction between *P. sojae* and soybean is the appearance of many dying plant cells during ingress of *P. sojae* hyphae during the early hours of an incompatible interaction and a paucity of cells containing pathogen haustoria. Haustoria are a major site of nutrition for the pathogen, and suppression of PCD by haustorial cells would be of major benefit to the pathogen.

Plant defenses to infection by biotrophic and hemibiotrophic pathogens are induced by a multilayered set of pathways that respond to common pathogen molecules (MAMPs; also called pathogen-associated molecular patterns) (Nurnberger et al., 2004) and also to specific pathogen effector molecules (Jones and Dangl, 2006). Both MAMP- or PAMP-triggered immunity (MTI or PTI) and effector-triggered immunity (ETI) can be suppressed by bacterial effector proteins (reviewed in Chisholm et al., 2006). There is considerable evidence for extensive overlap and crosstalk between the signaling pathways for MTI and ETI (He et al., 2007), and a number of effectors can interfere with both types of responses (Chisholm et al., 2006). Other bacterial effectors, such as AvrBs3 (Kay et al., 2007) and *Shigella flexneri* OspF (Arbibe et al., 2007), modify host transcription by acting as transcription factors.

Both MTI and ETI responses can include a form of PCD termed the HR. The HR is very effective against obligate biotrophic pathogens, such as downy mildews and viruses. PCD is similarly effective against obligate biotrophic pathogens of animals. Consequently, several animal viruses produce proteins that suppress PCD (Clem, 2007). HR can also be effective against hemibiotrophic pathogens if it is triggered very quickly before the pathogens have switched to necrotrophic growth. Thus, numerous bacterial effectors have been identified that suppress PCD (e.g., Jamir et al., 2004; Nomura et al., 2006). However, if HR is delayed, hemibiotrophic pathogens, especially filamentous ones such as fungi and oomycetes, can grow out of the region affected by HR, resulting in a lethal spreading HR that favors necrotrophic proliferation of the pathogen. *P. sojae* is a hemibiotroph. It typically switches from biotrophic to necrotrophic growth 16 to 24 h following invasion of host tissue (Enkerli et al., 1997). Therefore, the ability of *P. sojae* to suppress or delay the HR of soybean tissue is likely a major component of its pathogenic strategy.

The sequence similarity of the *P. infestans* Avr3a protein to Avr1b, including the presence of W and Y motifs (Figure 5A), suggests that the two effectors have a similar function. Bos et al. (2006) showed that Avr3a could suppress the PCD triggered in *Nicotiana* by the *P. infestans* MAMP INF1. Thus, oomycete W-Y motif effectors may share the ability to suppress PCD associated with MAMP perception. The spectrum of responses that W-Y motif effector proteins can suppress needs to be more fully explored. For example, do such effectors have some specificity as suggested by the fact that Avr3a could not suppress PCD triggered by the MAMP NEP1 (Bos et al., 2006)? Do oomycete pathogens have large numbers of diverse W-Y motif effectors, some with multiple W and Y (and L) motifs, to suppress PCD responses to a wide diversity of MAMPs? Can these effectors suppress PCD triggered by interactions between *R* gene products and effectors that are avirulence proteins?

The Avr1b Target Is Conserved and Lies at or Downstream of the Point at Which BAX Acts

All four W-Y motif effectors that we tested suppressed BAX-induced PCD in soybean, and the two that were tested in

N. benthamiana, Avr1b and Ps Avh331, also suppressed BAX-induced PCD in that plant species. Mouse BAX protein triggers PCD in animal cells by inserting into the mitochondrial outer membrane, destabilizing the mitochondria and releasing cytochrome c and reactive oxygen species. The reactive oxygen species further destabilize the mitochondrial outer membrane, creating a positive feedback loop as more reactive oxygen species are released. Cytochrome c is recognized by ApaF1, leading to the activation of the caspase cascade; ApaF1 is an NBS-LRR protein that has similarity to plant NBS-LRR resistance proteins. In plants, the oxidative burst plays a key role in initiating the HR in response to pathogen infection. Current evidence suggests that H_2O_2 and NO produced by the oxidative burst act together to activate caspases via a mitogen-activated protein kinase pathway. Since Avr1b can suppress PCD in both yeast and plants, it presumably targets a molecule(s) that is common to the PCD signal transduction pathways in both kingdoms.

Avr1b and other W-Y motif effectors could potentially suppress PCD by directly interfering with a host component necessary for PCD or by altering host gene expression or cell physiology to create an antiapoptotic cellular environment. Numerous pathogen proteins have been identified as suppressors of BAX-induced PCD in yeast or plants (reviewed in Hoeberichts and Woltering, 2002; Madeo et al., 2004; see also Abramovitch et al., 2006; Janjusevic et al., 2006). Avr1b and other W-Y motif effectors lack any recognizable sequence similarity to enzymes, such as peroxidases, proteases, or E3 ligases, that could conceivably interfere with PCD signaling. The effectors also lack hydrophobic domains common to anti-apoptotic proteins in the Bcl-2 family or anti-apoptotic proteins, such as *Arabidopsis* At BI-l (for Bax inhibitor-1) (Watanabe and Lam, 2006), the BON family (Yang et al., 2006), and the BON-associated protein family (Yang et al., 2007). Furthermore, the W-Y motif effectors do not resemble any bacterial effector proteins, and most lack canonical nuclear localization signals. However, it is intriguing that many W-Y motif effectors, including the three tested here, have multiple copies of the W-Y-L module and in this regard resemble the IAP proteins that contain multiple zinc finger domains involved in caspase binding (Fesik and Shi, 2001). W-Y-L modules are very diverse in sequence (Jiang et al., 2008). While this diversity may result from coevolutionary conflict with plant resistance gene products (Rehmany et al., 2005; Jiang et al., 2008), it is also possible that the modules have evolved to attack a diversity of targets in the plant defense and/or PCD pathways. Further biochemical and structural studies of Avr1b and other W-Y motif effectors and their interaction with proteins in yeast and plants will be required to uncover their molecular mechanisms of action. These studies may in turn shed additional light on the processes of PCD signaling in these organisms.

C-Terminal Motifs Conserved across the RXLR-dEER Superfamily Are Required for Suppression of PCD

The W, Y, and L motifs are the most prominent C-terminal motifs conserved across the RXLR-dEER superfamily. Of the 397 family members, 214 have at least a W motif, and many have multiple copies of the module consisting of the W, Y, and L motifs (Jiang et al., 2008), though in many modules, one or both of the Y and L motifs cannot readily be recognized (Figure 7A). The predicted secondary structure for the region of Avr1b containing the W and Y motifs is three α-helices separated by turns, two shorter ones spanning the W motif, and one longer one spanning the K motif. The three predicted helices are all amphipathic, suggesting that they form a helical bundle with the hydrophobic faces of the helices turned inwards. Mutagenesis of six conserved hydrophobic residues of the W motif (W1 mutation) abolished the ability of Avr1b to suppress PCD, but mutagenesis of five variable hydrophilic residues (W2 mutation) did not. This suggests that the overall structure of the region carrying the W and Y motifs may be important for suppression of PCD but that none of the exterior residues altered in W2 are required. Mutagenesis of the Y motif significantly reduced the suppression of PCD by Avr1b ($P < 0.001$) but did not abolish it. Thus, the Y motif may not be directly involved in the suppression of PCD, but the Y1 mutation may have partially disrupted the structure of the region containing the W and Y motifs.

Replacement of I109 with Ala (mutation W5; I109A) abolished the ability of Avr1b to suppress PCD. However, the mutation is not predicted to disrupt the structure of the helix, as Ala is a helix-promoting residue, and the mutation did not disrupt the interaction of Avr1b with Rps1b. Thus, Ile-109 may define a site that is important in the suppression of PCD. All three bioinformatically predicted W motif–containing effectors that we tested could suppress BAX-mediated PCD, one of them (Ps Avh331) exceptionally well. The position in the W motif corresponding to Ile-109 is either Val, Leu, or Ile in 85% of the predicted W motifs, and replacement of Ile-109 with Val (W6 mutation) did not disrupt the ability of Avr1b to suppress PCD. At the position corresponding to Ile-109 in the second predicted helix of the W motif in Avr1b, Avr3a also has an Ile residue. Since 174 of the 350 predicted effectors encoded in the *P. sojae* genome contain W motifs and many contain multiple W-Y-L modules, it appears that as much as half of the oomycete effector repertoire may be dedicated to the suppression of host PCD. Very many bacterial effectors can also suppress PCD (e.g., Jamir et al., 2004; Nomura et al., 2006), leading to the hypothesis that suppression of PCD is central to pathogenicity in both bacterial and oomycete plant pathogens.

Avr4/6 lacks any recognizable W, Y, or L motifs, and it was unable to suppress PCD, consistent with its lack of a W motif. Assuming that Avr4/6 contributes to virulence, this observation suggests that Avr4/6 may have a different mechanism than Avr1b for doing so. This may also be true of the 175 other predicted *P. sojae* RXLR-dEER effectors that lack any W, Y, or L motifs. Many of these effectors have relatively well-conserved paralogs in *P. ramorum*, indicating that they are not pseudogenes (Jiang et al., 2008). Further investigation is needed to determine what those functions might be.

The *R* Gene Rps1b Targets the C-Terminal Functional Domain of Avr1b

Mutations in the W motif not only abolish the ability of Avr1b to suppress PCD but also abolish its ability to trigger an Rps1b-mediated HR. The Y1 mutation also abolished triggering of the Rps1b-mediated response. Thus, the *Rps*1b resistance gene in soybean has evolved to respond to the region of Avr1b that is

involved in its ability to suppress PCD. Presumably, *Rps*1b was selected during evolution because it targeted a region of Avr1b that was essential for virulence. The allele of Avr1b found in P7076 has many mutations in the W and Y motifs, and the encoded protein can no longer trigger an Rps1b-mediated response nor suppress PCD. Five of the residues polymorphic in P7076 were targeted in the W2 mutation, and W2 also abolishes the triggering of an Rps1b-mediated response by Avr1b. However, W2 does not interfere with suppression of PCD, suggesting that some of the four other residues in the W motif that are altered in P7076 may play a role in this function of Avr1b. All of the nine DNA sequence changes within the region of the *Avr1b*-1 gene encoding the W motif cause amino acid substitutions, indicating that this part of the protein is under extremely strong positive selection (Shan et al., 2004; Jiang et al., 2008), presumably due to pressure from *Rps*1b and/or other similar resistance genes.

In contrast with Avr1b, *P. infestans* Avr3a has only one polymorphic site in the W motif, and that mutation does not strongly affect the ability of Avr3a to interact with the R3a gene product. The Lys at position 80 in Avr3a near the K motif was more important to the interaction. Furthermore, mutations spanning large regions of the Avr3a protein restored the interaction with R3a (J. Bos and S. Kamoun, unpublished data). It has been proposed that there are two mechanisms by which plant resistance gene products detect the presence of pathogen effectors. One mechanism is direct interaction and the other is indirect interaction. Direct interaction has been documented in the case of the Pi-ta resistance protein of rice (*Oryza sativa*) and the AvrPi-ta avirulence protein of the fungus *Magnaporthe grisea* (Jia et al., 2000), and in the case of the flax resistance protein L and the avirulence protein AvrL567 of the rust fungus *M. lini* (Dodds et al., 2006). Indirect interaction has been hypothesized in the case of many bacterial effectors that appear to be detected by the cognate *R* gene product indirectly through changes in a plant protein targeted by the effector; this situation has been termed the guard hypothesis (Van der Biezen and Jones, 1998; Jones and Dangl, 2006). The W2 mutation abolishes the triggering of the Rps1b-mediated response but does not interfere with suppression of PCD. This suggests that the interaction with Rps1b is not mediated by the PCD-signaling protein that is presumably targeted by Avr1b. It remains to be determined whether Avr1b interacts directly with Rps1b or whether there is another plant protein targeted by Avr1b and guarded by Rps1b. In the case of Avr3a, the same two polymorphisms that abolish the suppression of PCD also abolish the interaction with R3a (Bos et al., 2006), consistent with indirect guard recognition of Avr3a by R3a.

METHODS

Plasmids and Strain Construction

The oligonucleotides used for the following plasmid constructions are documented in Supplemental Table 5 online. *Phytophthora sojae* transformation plasmids pHamAvr1b (full sequence of *Avr1b*-1, AAR05402, driven by Ham34 promoter; Judelson et al., 1991), pHamGUS (modified *Staphylococcus* GUS gene [Cambia *GUSPlus*; www.cambia.org]) driven by Ham34 promoter), and pUN (NptII gene driven by *P. sojae* rpL41 promoter) were constructed as follows. For pHamAvr1b, the NptII gene of pHAMT35N (Judelson et al., 1991) was replaced with *P. sojae Avr1b*-1 using PrimerC and PrimerD. For pHamGUS, the NptII gene of pHAMT35N was replaced with the CAMBIA *GUSPlus* gene using primers GusR and GusF. For pUN, the Ham34 promoter of pHAMT35N was replaced by the *P. sojae* rpL41 promoter (EF681129) using primers UF and UR.

Soybean (*Glycine max*) transient expression plasmids pUCAvr1b (leaderless *Avr1b*-1 driven by the CaMV 35S promoter) and pUCGUS (GUSPlus gene driven by the CaMV 35S promoter) were constructed as follows. For pUCGUS, a cassette containing the CaMV 35S promoter, CAMBIA *GUSPlus* gene, and nos terminator was amplified from pCambia1305.2 using primers GusF_EcoRI and GUSR_HindIII, cleaved with *Eco*RI and *Hin*dIII, and ligated into pUC19. For pUCAvr1b, the hygromycin resistance of pCambia1305.2 was replaced by Avr1b-1 lacking its secretory leader (replaced by ATG codon) using primers Avr1bF and Avr1bR, creating pCambia-mAvr1b. Then, the Avr1b expression cassette, including the double 35S promoter was amplified from pCambia-mAvr1b using primers Avr1b_EcoRI, Avr1b_HindIII, and Avr1b_genegun_KpnI and subcloned into pUC19. These primers also created *Xma*I and *Kpn*I sites for later manipulation of the *Avr1b*-1 gene.

To construct *Avr1b*-1 C-terminal mutants for soybean transient expression, we used a two-step amplification procedure to create an *Avr1b*-1 amplicon containing the desired mutation and then replaced *Avr1b*-1 in pUCAvr1b with the mutant gene. For example, to construct Avr1b K1, we amplified the C terminus of *Avr1b*-1 from pUCAvr1b using oligonucleotide Avr1bK1F in combination with Avr1bR and the N terminus using Avr1bK1R and Avr1bF. Then, the full sequence of Avr1bK1 was obtained by combining the N-terminal and C-terminal amplicons and amplifying them with primers Avr1bF and Avr1bR. We used the same two-step PCR strategy to construct Avr1bW1, Avr1bW2, Avr1bW3, Avr1bW4, Avr1bW5, Avr1bW6, and Avr1bY1 with the following oligonucleotide pairs: Avr1bW1F and Avr1bW1R, Avr1bW2F and Avr1bW2R, Avr1bW3F and Avr1bW3R, Avr1bW4F and Avr1bW4R, Avr1bW5F and Avr1bW5R, Avr1bW6F and Avr1bW6R, and Avr1bYF and Avr1bYR. We cleaved the above eight amplicons with *Nco*I and *Kpn*I and inserted them into *Nco*I- and *Kpn*I-digested pUCAvr1b to obtain pUCAvr1bK1, pUCAvr1bW1, pUCAvr1bW2, pUCAvr1bW3, pUCAvr1bW4, pUCAvr1bW5, pUCAvr1bW6, and pUCAvr1bY1, respectively. We amplified the *Avr1b*-1 allele from *P. sojae* strain P7076 using oligonucleotides Avr1bF and AP7076R. The amplicons obtained were inserted into *Xma*I- and *Kpn*I-digested pUCAvr1b to obtain pUCAvr1bP7076. Plasmids pUCAvr46, pUCBax, pUCHpRxL96, pUCAvh163, and pUCAvh331 were obtained by insertion into *Xma*I- and *Kpn*I-digested pUCAvr1b with the amplicons obtained with oligonucleotides Avr4F and Avr4R, BaxF and BaxR, Hp96F and Hp96R, Avh163F and Avh163R, and Avh331F and Avh331R, respectively.

The identification of the Avr4/6 gene (ABS50087) will be described in detail elsewhere. Briefly, Avr4/6 was identified as the only RXLR-containing gene in the region identified by Whisson et al. (2004). Particle bombardment confirmed an avirulence interaction with both the soybean *Rps*4 and *Rps*6 genes, and silencing of the gene in *P. sojae* showed that it was responsible for the avirulence phenotype of the pathogen on cultivars containing either *Rps*4 or *Rps*6 (D. Dou, S.D. Kale, F.D. Arredondo, and B.M. Tyler, unpublished data).

To make the constructs for *P. sojae* transformation, three amplicons (Avr1bW1, Avr1bW2, and Avr1bY1) were digested with *Nco*I and *Kpn*I and inserted into pHamAvr1b digested with the same enzymes to obtain pHamAvr1bW1, pHamAvr1bW2, and pHamAvr1bY1, respectively. All the plasmids were further confirmed by sequencing at the Virginia Bioinformatics Institute core facility.

To make constructs for the yeast cell death assay, *Avr1b*-1 was amplified from pHamAvr1b with the oligonucleotides AvrYeastF and AvrYeastR. The amplicon was inserted into *Eco*RI- and *Sal*I-digested

pGilda (Clontech). For the PVX assay, *Avr1b-1*, *Avh331*, and Bax were amplified using combinations of oligonucleotides Avr1bPVXF and Avr1bPVXR, Avh331PVXF and Avh331PVXR, or BaxPVXF and BaxPVXR, respectively. Then, the amplicons were cloned using appropriate restriction enzymes into the PVX vector pGR106 (Lu et al., 2003). The constructs were further confirmed by sequence by Invitrogen Biotechnology.

P. sojae Strains, Manipulation, and Inoculation Assay

P. sojae isolates P6954 (Race 1), P6497 (Race 2), P7064 (Race 6), and P7076 (Race 19) (Forster et al., 1994) were routinely grown and maintained on V8 agar (Erwin and Ribiero, 1996). For transformation experiments, pea broth medium was made by autoclaving 120 g of frozen peas in 1 liter of distilled water for 15 min, filtering through four layers of cheesecloth, and then autoclaving again after bringing the volume up to 1 liter.

The following protocol was modified from that kindly provided by A. McLeod and W. Fry (McLeod et al., 2008). Three-day-old *P. sojae* mycelial mats, cultured in pea broth medium, were rinsed and washed in 0.8 M mannitol, then placed in enzyme solution (0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$, 10 mg/mL β-1.3 glucanase [InterSpec 0439-1], and 5 mg/mL cellulysin [Calbiochem 219466]) and incubated for 40 min at 22°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 2×10^6 protoplasts/mL or higher. 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 protoplasts to swell. To each of 1 mL MMg solution, 25 μg transforming DNA was added and incubated for 10 min on ice. For cotransformation experiments, 10 μg of DNA containing the selectable marker and 30 μg of DNA containing the gene of interest were used. Then, three aliquots of 580 μL 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 were added, and the protoplasts were incubated overnight to regenerate. The regenerated protoplasts were suspended in liquid pea agar (40°C) containing 0.5 M mannitol and 50 μg/mL G418 (AG Scientific) and plated. The visible colonies could be observed after 2 to 3 d incubation at 22°C. All transformants were propagated on V8 agar with 50 μg/mL G418 at 22°C.

This procedure is very reliable. Approximately 30 to 100 putative transformants were obtained from each experiment, of which 80% would continue growing through the third round of antibiotic selection. Of these, 20 to 45% were generally confirmed to contain and express the transgene. When cotransformation was used, 4 to 13% of the transformants contained the nonselected plasmid.

Putative *P. sojae* transformants containing wild-type and mutant *Avr1b*-1 transgenes were screened in three steps. First, DNA from each line was amplified with oligonucleotides HamF (see Supplemental Table 5 online) and HamR, which are primers for the Ham34 promoter and terminator, respectively. Next, RT-PCR was performed on RNA extracted from each line using oligonucleotides Avr1bReF and Avr1bReR to confirm *Avr1b*-1 expression. Finally, the mRNA levels derived from the *Avr1b*-1 transgenes were accurately measured by quantitative real-time PCR performed by the Virginia Bioinformatics Institute core facility. To perform DNA gel blot hybridization analysis of *P. sojae* transformants, we purified genomic DNA from mycelium as described by Judelson et al. (1991) and digested 10 μg total genomic DNA with *Kpn*I or *Xho*I purchased from New England Biolabs. The digested DNA was size fractionated on a 0.7% agarose gel followed by transfer to Hybond N^+ nylon membrane (Amersham). For RNA gel blot analysis, total RNA from the transformants and wild-type *P. sojae* were isolated using the RNeasy plant mini kit (Qiagen) according to the manufacturer's recommendations. We electrophoresed 5 to 10 μg total RNA samples in a 1% agarose gel in MOPS running buffer at 45 mV for 50 min. The gel was then stained with an RNA staining buffer for 15 min. The gel was transferred overnight with 20× SSC buffer. DNA probes of *Avr1b*-1 were synthesized using a PCR digoxigenin probe synthesis kit according to the manufacturer's manual (Roche Diagnostics). The membrane was hybridized, and chemiluminescent detection was performed following the kit instructions.

The avirulence phenotypes of selected transformants were evaluated by hypocotyl inoculation (Tyler et al., 1995) using soybean (*Glycine max*) cultivars HARO(1-7) (rps), Haro13 (Harosoy background, *Rps*1b), Williams (rps), and L77-1863 (Williams background, *Rps*1b). The differences between the numbers of surviving plants from *rps* and *Rps*1b cultivars were compared using Fisher's exact test. Only the transformants producing significant differences between rps and *Rps*1b cultivars were judged as avirulent. Each avirulence determination was repeated at least three times. Quantitative virulence of *P. sojae* transformants was measured using a lesion length assay described by Vega-Sánchez et al. (2005) for measuring soybean partial resistance levels against *P. sojae*. There were three replications of 10 plants each within each experiment, and each experiment was repeated five times.

Particle Bombardment Assays for Avirulence and Virulence Phenotypes

Soybean plants were grown in the growth chamber; the day was 12 h at 28°C and the night was 12 h at 25°C. The first (monofoliate) true leaves were selected for bombardment 9 to 14 d after planting.

Plasmid DNA was isolated using Qiagen brand Maxi preparation kits and concentrated to 5 to 6 μg/μL in sterile deionized water. M-10 tungsten particles (Bio-Rad) were washed twice with 95% ethanol and twice with sterile deionized water, then resuspended in 50% sterile glycerol to a concentration of 90 mg/mL. For bombardment, 9 mg of tungsten particles were combined with 50 μg of GUS plasmid DNA (pCambia1305.2) and 50 μg of either test DNA (e.g., pCaAvr1b) or empty vector [pCa-GUS(−) or pUC19] as the control in a total of 100 μL of 25% glycerol on ice in a 0.5 mL centrifuge tube. Sixty-five microliters of 2.5 M $CaCl_2$ was added followed by 25 μL of freshly prepared 0.1 M spermidine. The preparation was vortexed for 2 min and then placed on ice for 20 min. The particle preparation was then concentrated to 30 μL by brief centrifugation.

Bombardment was performed using the Bio-Rad He/1000 particle delivery system with a double-barreled extension attached (see Supplemental Figure 1 online) to enable leaves to be bombarded with two DNA preparations simultaneously. A patent application has been submitted for this device. One microliter of each DNA particle preparation was loaded onto the macrocarrier so that the mixtures were directly over their respective barrels (see Supplemental Figure 1D online). The distance from the stopping screen to the target shelf was 12 cm. The distance between the rupture disk and macrocarrier was set to 3/8 inch. and the highest position was used for the macrocarrier in the macrocarrier assembly; 650 p.s.i. rupture disks were used. The chamber vacuum was 26 p.s.i. The pressure build time was set to 12 to 14 s. The two barrels of the extension were cleaned with 70% ethanol and then dried with compressed air between shots.

The target leaves were bombarded twice: first the petiole-proximal half of the leaf and then the petiole-distal half, resulting in a total of four bombardment sites. A cover was used to prevent overlapping bombardments.

After bombardment, the leaves were incubated for 5 d in darkness at 28°C. The leaves were then stained for 16 h at 28°C using 0.8 mg/mL X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt), 80 mM Na phosphate, pH 7.0, 0.4 mM $K_3Fe(CN)_6$, 0.4 mM

$K_4Fe(CN)_6$, 8 mM Na_2EDTA, 0.8 mg/mL 20% methanol, and 0.06% (v/v) Triton X-100 and then de-stained in 100% methanol. Blue spots were counted using a dissecting microscope at ×5 to ×20 magnification.

To quantitate the avirulence activity of *Avr1b*-1 constructs, DNA carrying the constructs (1.7 μg per shot) was cobombarded into soybean leaves along with DNA carrying a GUS reporter gene (1.7 μg per shot). Avirulence activity was measured as the reduction in the number of blue staining GUS-positive spots in leaves carrying Rps1b compared with leaves lacking Rps1b. The double-barreled device was used to deliver a parallel control shot in every case that contained GUS DNA plus empty vector DNA. For each pair of shots, the logarithm of the ratio of the blue spots with Avr1b to that with the empty vector control was calculated. Each assay consisted of eight pairs of shots and was conducted at least twice. The log ratios from all the *Rps*1b leaves were then compared with those from the non-*Rps*1b leaves using the Wilcoxon rank sum test.

To quantitate suppression of BAX-mediated cell death, two assays were used: an indirect assay and a direct assay. For the indirect assay, *Avr1b*-1 DNA (1.7 μg/shot) was mixed with Bax DNA (pUCBax; 0.83 μg/shot) and GUS DNA (1.7 μg/shot) and bombarded into soybean leaves lacking Rps1b. The control shot in the second barrel was empty vector (pUC19; 2.53 μg/shot) plus GUS DNA (1.7 μg/shot). The log ratios for these shots were then compared with the log ratios obtained when *Avr1b*-1 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 BAX could be monitored in every shot. For the direct assay, Avr1b + BAX + GUS was compared directly with empty vector + BAX + 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 *Avr1b*-1 could be compared with a control (or another *Avr1b*-1 construct) directly on the same leaves. Other effectors or mutants were tested by replacing the *Avr1b*-1 DNA with the relevant DNA.

Agrobacterium tumefaciens Infiltration Assays

A. tumefaciens strain GV3101 (Hellens et al., 2000) was used. For infiltration, recombinant strains were cultured in Luria-Bertani media supplemented with 50 μg/mL kanamycin and then harvested, washed three times in 10 mM $MgCl_2$, and resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.3. Infiltration experiments were performed on 4- to 6-week-old *Nicotiana benthamiana* plants. Plants were grown and maintained throughout the experiments in a greenhouse with an ambient temperature of 25° and high light intensity. *A. tumefaciens* cell suspensions carrying the *Avr1b*-1 gene (pGR106*Avr1b*-1) or PsAvh331 (pGR106*Avh331*) were infiltrated into *N. benthamiana* leaves (Bos et al., 2006) by pressure infiltration; a small nick was placed in each leaf with a razor blade and then 100 μL of cell suspension was infiltrated through the nick using a syringe without a needle. *A. tumefaciens* cells carrying the *Bax* gene (pGR106: *Bax*) were infiltrated into the same site 24 or 48 h later. As controls, *A. tumefaciens* strains carrying *Bax* or *Avr1b*-1 were replaced with *A. tumefaciens* strains carrying empty vectors. Symptom development was monitored from 3 to 8 d after infiltration, and pictures were taken after 5 d. The experiments were repeated at least three times. Although it is likely that PVX replication occurred in the transformed plant cells, resulting in amplified expression of the genes in the PVX vector, no attempt was made to quantitate PVX replication.

Yeast Cell Death Assays

The *Saccharomyces cerevisiae* strain W303 (MATa; ura3-52; trp1Δ2; leu2-3,112; his3-11; ade2-1; can1-100) was used, and the culture and transformation of the cells were performed essentially as described by Kampranis et al. (2000). To test if *Avr1b*-1 could suppress PCD triggered by H_2O_2, *Avr1b*-1 was introduced into W303 cells on the plasmid pGilda under the control of the galactose-inducible *GAL*1 promoter. Cells from W303 and the transformants were pelleted, washed, and resuspended in SD medium [0.17% YNB-AA/AS; 0.5%$(NH_4)_2SO_4$] containing 2% galactose and 1% raffinose as carbon sources (SD/gal/raff/±his) to induce expression of the Avr1b protein from the *GAL*1 promoter. After 12 h of induction, cells were diluted to $OD_{600} = 0.05$ and treated with 15 mM H_2O_2 added to the medium, and cultures were incubated at 30°C with vigorous shaking for 6 h. After treatment, viability was determined by plate counting. Treated and untreated cells were sampled and spread onto YPD medium (1% yeast extract, 2% peptone, 2% dextrose) with 2% agar and then incubated at 30°C for 48 h. The number of colony-forming units from treated cells were compared with the colony-forming units of untreated cells, and photos were taken. All the experiments were repeated least three times.

To test if *Avr1b*-1 expression could suppress PCD triggered in yeast cells by BAX, the plasmid pGildaBax that carries a mouse *Bax* cDNA under the control of the *GAL*1 promoter (Kampranis et al., 2000) was introduced into W303 cells. *Avr1b*-1 was introduced on the plasmid pYES2, also under the control of the *GAL*1 promoter. Cells containing pGildaBax, with or without pYES2*Avr1b*-1, were grown in SD-glucose medium. After 24 h, the cells were harvested and resuspended in water to an OD_{600} of 1.0. One hundred microliters of the cell suspension were then plated on SD-galactose and cultured at 30°C for 3 d, and the colonies were photographed and counted.

Sequence Search, Alignment, and Structure Prediction

The sequenced oomycete genomes were accessed at the following sites: *P. sojae*, *P. ramorum*, and *Hyaloperonospora parasitica* at vmd.vbi.vt.edu, *P. infestans* at www.broad.mit.edu, and *P. capsici* at shake.jgi-psf.org/Phyca1 (Department of Energy Joint Genome Institute). Avr1b paralogs were identified by tBLASTN search (Altschul et al., 1997) with a cutoff of $E = 10^{-7}$. Paralogs were selected with an amino acid similarity of at least 45%. Two such Avr1b paralogs were found in *P. sojae*, seven in *P. capsici*, and eight in *P. infestans*. The two *P. sojae* paralogs were *Avh*1 (Shan et al., 2004) and *Avh*4 (Jiang et al., 2008). The seven paralogs in *P. capsici* were nearly identical, and one was selected for the sequence alignment shown in Figure 4. The paralogs in *P. infestans* fell into two classes with near identity within each class; one from each class, including Avr3a, were selected for the sequence alignment. RXLR-dEER family members PsAvh331 and PsAvh163 were described by Jiang et al. (2008), and HpRxL96 was identified from the *H. parasitica* sequence by the procedures described by Jiang et al. (2008) together with Smith Waterman alignment to identify candidate effectors conserved between *P. sojae* and *H. parasitica*. PsAvh163 and HpRxL96 show 26% identity and 43% similarity over their aligned sequences. Multiple alignments were created using the program ClustalW (Thompson et al., 1994) with minor manual adjustment to optimize the alignment as necessary. Secondary structure prediction was performed using 3D-pssm (Imperial College of Science, Technology and Medicine, London, UK) and by using PredictProtein (www.PredictProtein.org).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Ps *Avh*4 (EU282486), Ps *Avh*331 (EU282487), Ps *Avh*163 (EU282485), Hp *RXL*96 (EU282490), Pc *Avh*1 (EU282489), Pi05911 (EU282488), Ps *RpL*41 (EF681129), and Ps Avr4/6 (ABS50087).

Supplemental Data

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

Supplemental Figure 1. The Double-Barreled Particle Bombardment Device.

Supplemental Figure 2. Correlation between Replicate Bombardments Produced by Double-Barreled Bombardment.

Supplemental Figure 3. Comparison of Ability of Avr1b and Bcl2 to Suppress BAX-Mediated Cell Death in Yeast.

Supplemental Figure 4. Sequences and Motif Structures of Avr and Avh Proteins.

Supplemental Table 1. Details of Avr1b Family Proteins.

Supplemental Table 2. Complete Data for Soybean Bombardment Assays of Suppression of BAX-Mediated PCD by Avr1b-1 Mutants and Other W-Y-Motif Effectors.

Supplemental Table 3. Assay Data for Bombardment Tests of Avr1b Avirulence Function.

Supplemental Table 4. Assay Data for Avirulence Tests of *P. sojae* Stable Transformants.

Supplemental Table 5. Summary of Oligonucleotides Used.

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