The *Phytophthora sojae* *Avr1d* Gene Encodes an RxLR-dEER Effector with Presence and Absence Polymorphisms Among Pathogen Strains

Weixiao Yin,¹ Suomeng Dong,^{1,2} Luchong Zhai,¹ Yachun Lin,¹ Xiaobo Zheng,^{1,2} and Yuanchao Wang^{1,2}

¹College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China; ²Key Laboratory of Integrated Management of Crop Diseases and Pests (Nanjing Agricultural University), Ministry of Education, Nanjing 210095, China

Submitted 4 February 2013. Accepted 9 April 2013.

Soybean root and stem rot is caused by the oomycete pathogen *Phytophthora sojae*. The interaction between *P. sojae* and soybean fits the “gene-for-gene” hypothesis. Although more than 10 *P. sojae* avirulence (*Avr*) effectors have been genetically identified, nearly half of genetically defined *avr* genes have been cloned. In a previous bioinformatic and global transcriptional analysis, we identified a *P. sojae* RxLR effector, *Avr1d*, which was 125 amino acids in length. Mapping data demonstrated that *Avr1d* presence or absence in the genome was co-segregated with the *Avr1d* avirulence phenotype in F₂ populations. Transient expression of the *Avr1d* gene using co-bombardment in soybean isogenic lines revealed that this gene triggered a hypersensitive response (HR) in the presence of *Rps1d*. Sequencing of *Avr1d* genes in different *P. sojae* strains revealed two *Avr1d* alleles. Although polymorphic, the two *Avr1d* alleles could trigger *Rps1d*-mediated HR. *P. sojae* strains carrying either of the alleles were avirulent on *Rps1d* soybean lines. *Avr1d* was upregulated during the germinating cyst and early infection stages. Furthermore, transient expression of *Avr1d* in *Nicotiana benthamiana* suppressed BAX-induced cell death and enhanced *P. capsici* infection. *Avr1d* also suppressed effector-triggered immunity induction by associating with *Avr1b* and *Rps1b*, suggestive of a role in suppressing plant immunity.

Soybean (*Glycine max* L. (Merrill)) is an important food and fuel crop worldwide. *Phytophthora* root and stem rot of soybean caused by *Phytophthora sojae* Kaufm. & Gerd. (syn. *P. megasperma* f. sp. *glycinea*) is one of the most destructive soybean diseases, with annual yield losses of up to US\$300 million in North America (Wrather and Koenning 2006) and approximately US\$1 to 2 billion dollars worldwide (Nicholls 2004; Tyler 2007). The genus *Phytophthora* contains more than 80 species that cause destructive diseases in many agricultural and ornamental plants as well as in forests. The *P. sojae*–soybean interaction is a useful model of development, and can be used to obtain advanced genetic information as

well as the genome sequence of *P. sojae* (Tyler et al. 2006) and soybean (Schmutz et al. 2010).

Plants rely on innate immunity and systemic signals to detect specific infection-related substances. The basal defense pathway involves conserved microbial elicitors called pathogen-associated molecular patterns (PAMPs), which are recognized by host receptor proteins called pattern-recognition receptors that trigger PAMP-triggered immunity (PTI) (Dodds and Rathjen 2010; Jones and Dangl 2006). Pathogens deliver secreted proteins (effectors) to suppress PTI, which results in effector-triggered susceptibility. As a consequence, the given effector is recognized by specific receptor proteins that induce effector-triggered immunity (ETI) (Dodds and Rathjen 2010). The pathogen effectors recognized by plant receptor proteins have been termed avirulence (*Avr*) effectors. ETI is an accelerated and amplified PTI response and often involves a form of localized cell death called the hypersensitive response (HR) (Dodds and Rathjen 2010; Jones and Dangl 2006). Thus, the interaction between pathogen *Avr* proteins and host receptor proteins determines the outcome of infection. Recently, however, the circumscription between PTI and ETI is becoming more and more blurred.

The interaction between *P. sojae* and soybean follows the “gene-for-gene” hypothesis. Control of the disease-based monogenic resistance encoded by resistance to *P. sojae* (*Rps*) genes is the primary defense strategy. To date, 15 *Rps* genes have been mapped to eight genomic loci, including five at the *Rps1* locus and three at the *Rps3* locus (Burnham 2003; Tyler 2007, 2008). Only *Rps1k* has been identified and characterized from soybean, which includes two classes of similar genes encoding coiled-coil nucleotide-binding leucine-rich repeat (NB-LRR) proteins, and confers *P. sojae* resistance in soybean (Gao et al. 2005).

Recently, specific *Avr* effector genes have been identified and characterized from oomycete plant pathogens. These include *Avr3a*, *Avr4*, *Avrblb1*, *Avrblb2*, and *Avr2* from *P. infestans* (Armstrong et al. 2005; Gilroy et al. 2011; Oh et al. 2009; van Poppel et al. 2008; Vleeshouwers et al. 2008); *Avr1b*, *Avr1a*, *Avr3a/5*, *Avr3c*, *Avr4/6*, and *Avr3b* from *P. sojae* (Dong et al. 2009, 2011a,b; Dou et al. 2010; Qutob et al. 2009; Shan et al. 2004); and *ATR1*, *ATR13*, *ATR5*, and *ATR39-1* from *Hyaloperonospora arabidopsidis* (Allen et al. 2004; Bailey et al. 2011; Goritschnig et al. 2012; Rehmany et al. 2005).

One common characteristic of these *Avr* effectors is that they contain a secretion signal peptide followed by the amino acid motif RxLR (Arg-X-Leu-Arg), in which X could be any amino acid. The RxLR motif plays an important role in delivering the protein effector inside host cells in the absence of the

Corresponding author: Y. Wang; Telephone: +86 25 84399071; E-mail: wangyc@njau.edu.cn

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary tables and one supplementary figure are published online and also that Figures 2, 4, 6, and 7 appear in color online.

pathogen (Bhattacharjee et al. 2006; Dou et al. 2008a; Whisson et al. 2007). The RxLR effectors are included in large genomic families in *Phytophthora* spp. and *H. arabidopsidis* (Baxter et al. 2010; Haas et al. 2009; Jiang et al. 2008; Tyler et al. 2006). More than 400 RxLR effectors have been predicted and termed avirulence homolog (*Avh*) genes in *P. sojae* (Jiang et al. 2008). This information provides an important resource for identifying putative *Phytophthora* Avr effectors.

In the present study, we identified an Avr effector, *Avr1d*, based on a genetic map. Transient expression of the *Avr1d* gene in soybean revealed that it could trigger an HR in the presence of *Rps1d*. *Avr1d* genes are absent in virulent *P. sojae* strains, and sequencing of avirulent strains revealed two *Avr1d* alleles. Although polymorphic, the two *Avr1d* alleles could trigger *Rps1d*-mediated HR. *Avr1d* was upregulated during the germinating cyst stage and early infection. Transient expression of *Avr1d* in *Nicotiana benthamiana* could suppress cell-death-induced BAX and promote *P. capsici* infection. *Avr1d* could also suppress ETI induction by interacting with *Avr1b* and *Rps1b* in soybean.

RESULTS

Identification of an RxLR effector that co-segregates with the *Avr1d* phenotype.

Based on our current knowledge of *Phytophthora* Avr effectors, we hypothesized that *P. sojae* RxLR effectors are expressed in avirulent strains. These effectors showed transcriptional and sequence polymorphisms in virulent strains, which could be used for Avr effector screening (Dong et al. 2011a).

The genome sequence of *P. sojae* isolates P6497, P7064, P7074, and P7076 have recently been obtained (Wang et al. 2011), and the four strains were delineated into four major genetic lineages according to genomic and mitochondrial restriction fragment length polymorphism data (Förster 1994). Considering the identical virulent or avirulent phenotypes of these isolates (Supplementary Table S1), the candidates for *Avr3b* were also useful for *Avr1d* (Dong et al. 2011a). Thus, *Avh20*, *Avh113*, *Avh238*, *Avh258*, *Avh288*, and *Avh6* are candidate effectors for *Avr1d*. Although *Avh6* is absent from virulent strains, it is also considered polymorphic.

To perform genetic mapping and identify *Avr1d*, we crossed the two isolates P6497 and P7076 to generate F₁ heterozygote individuals. Then, one of these individuals was selected for self-crossing to generate a total of 71 F₂ populations (Dong et al. 2011a). Based on virulence assays of the F₂ progeny on soybean plants carrying the resistance gene *Rps1d*, 53 F₂ individuals were avirulent and 18 were virulent. The segregation ratio for *Avr1d* fit a 3:1 ratio ($\chi^2 = 0.005$, $P < 0.05$), suggesting that the *Avr1d* avirulent phenotype was conferred by a single dominant allele, consistent with a previous report (May et al.

2002). The genomic polymorphisms associated with these candidate effectors were used as cleaved amplified polymorphic (CAP) DNA markers to identify the polymorphism that included *Avr1d* (Dong et al. 2011a). The results were suggestive of co-segregation of the *Avh6* genotype with the *Avr1d* avirulent phenotype among the F₂ population (Fig. 1). Another marker, CAPSAvh282, which was designed based on the polymorphism of the nearest RxLR effector approximately 1.5 Mb from *Avh6*, showed 22.54% recombination with the *Avr1d* avirulent phenotype (Supplementary Table S2). These data indicate that *Avh6* is localized to the *Avr1d* genetic locus, and suggest that *Avh6* is *Avr1d*. Also, another across ACR10 and P7076 showed that *Avh6* co-segregates with *Avr1d* (Na et al. 2013).

Transient expression of *Avh6* in soybean leaves triggers *Rps1d*-dependent cell death, and the C terminus (but not the N terminus) of *Avr1d* can be recognized by *Rps1d*.

To determine whether *Avh6* is the *P. sojae* avirulent effector *Avr1d*, a double-barreled co-bombardment assay was used to express *Avh6* without the signal peptide in soybean leaves containing *Rps1d*, using β -glucuronidase (GUS) as a marker of cell vitality. HR can be detected by co-expression with a reporter gene; the gene encoding GUS was used in this experiment (Dou et al. 2008b). Plant leaves containing *Rps1d* ('Harol6' and 'PI103091' soybean, *Rps1d*) had significantly fewer blue spots compared with the empty vector (EV) control, which was consistent with an HR. No difference in GUS activity was observed in the differential leaves lacking *Rps1d* ('Williams' soybean, *rps*) (Fig. 2A). The differences in ratios between the *Rps1d* cultivars compared with the *rps* cultivars were statistically significant, with a P value < 0.01 between 'Harol6' and 'Williams' (Fig. 2B). These results suggest that *Avh6* triggers cell death independent of *Rps1d*; thus, *Avh6* is likely the Avr effector *Avr1d*.

To identify the recognized region of *Avr1d*, we created N-terminal (from the 16th site to the end of the dEER motif) and C-terminal (from the first amino acid after the dEER motif to the stop codon) deletion mutants and tested their ability to trigger *Rps1d*-dependent cell death in a bombardment assay. The N-terminal domain was not recognized, whereas stronger cell death was observed with the C-terminal mutant of *Avr1d* compared with the EV (Fig. 2C; Supplementary Fig. S1B). These results indicate that recognition of *Avr1d* is not dependent on the N terminus of the protein but, rather, the C terminus, which contains predicted W motifs (Wang et al. 2011).

Avr1d genes are absent in virulent isolates and contain sequence polymorphisms.

Oomycete Avr effectors often show significant sequence polymorphisms among field populations (Allen et al. 2004, 2008;

Fig. 1. Co-segregation of *Avh6* with the *Avr1d* virulence phenotype in F₂ progeny from a cross between *Phytophthora sojae* isolates P6497 and P7076. A cross of *P. sojae* P6497 × P7076 generated the F₂ progeny. The population were scored as virulent (V) or avirulent (A) on *Rps1d* soybean plants. Cleaved amplified polymorphic (CAP) marker designed specific for *Avh6* was scored using genomic DNA from the parents and F₂ progeny. Shown is a photograph of an ethidium-bromide-stained agarose gel of the CAP marker results.

Cui et al. 2011; Dong et al. 2009; Shan et al. 2004). To investigate the polymorphisms in *Avr1d* that may explain the loss of avirulent phenotypes in virulent isolates, polymerase chain reaction (PCR) primer pairs located up- and downstream of the *Avr1d* coding region were used to amplify *Avr1d* from a collection of 24 *P. sojae* isolates (including the 2 parental isolates P6497 and P7076), 12 of which are virulent on *Rps1d* soybean and 12 which are avirulent (Supplementary Table S3). PCR amplification was not observed from the virulent isolates whereas PCR products of the expected size were obtained from the avirulent isolates (Fig. 3A). Amplification was also detected for the parental isolate P6497, which is avirulent to *Rps1d*. However, another virulent parental isolate, P7076, was not detected. Detection of the parental isolates was consistent with the other 22 isolates.

PCR amplification products generated with the primer set from the tested isolates were sequenced. Four alleles from the avirulent isolates shared an identical protein sequence that we

designated *Avr1d*, while several amino acid polymorphisms were observed in the predicted mature protein sequences derived from the other isolates (Fig. 3B). The isolates with the mutated sequences were avirulent, although there were several mutations outside of the W domain, indicating that this polymorphism is unlikely to specify differential recognition by *Rps1d*.

Considering the large number of amino acid mutations in avirulent isolates, we examined whether these polymorphisms in *Avr1d* are recognized by *Rps1d*. *Avr1d* from isolate P7064 (minus the SP-encoding sequence) was expressed in soybean leaves containing *Rps1d*. We found that *Avr1d*^{P7064} was recognized by *Rps1d* (Fig. 4), and demonstrated that these mutated amino acid sites are not differentially recognized by *Rps1d*.

Transcription of *Avr1d* during infection.

Next, we examined whether *Avr1d* transcription was induced during infection and was involved in *P. sojae*–soybean interac-

Fig. 2. Expression of *Avr1d*^{P6497} in *Rps1d* ('Haro16') can trigger cell death, and the C terminus (but not the N terminus) is recognized by *Rps1d*. **A**, Direct comparison of bombardment with *Avr1d*^{P6497} and empty vector (EV) into the same leaf of soybean. Soybean leaves of 'Haro16' and 'PI10309' (*Rps1d*) and 'Williams' (*rps*) were bombarded using the double-barreled device; *Avr1d*^{P6497} DNA-bearing particles were delivered into one side of the leaf and control (EV) DNA to the other; both sides were co-bombarded with a plasmid mixture consisting of a glucuronidase reporter gene (β -glucuronidase [GUS]) expression vector DNA. **B**, Percentage rate of GUS-positive blue spots following bombardment with *Avr1d*^{P6497} compared with the EV DNA. Data are the means of eight independent experiments. Two asterisks means $P < 0.01$. **C**, Mutation analysis of the region of *Avr1d*^{P6497} showed that C terminal can be recognized by *Rps1d*. Summary of deletion mutants of *Avr1d*, and the recognition determined by double-barreled particle bombardment.

tions. Real-time reverse-transcriptase (RT)-PCR was performed to examine the *Avr1d* transcriptional profile at different stages of the life cycle, including the infection stage. To define the transcriptional pattern of *Avr1d*, we extracted total RNA from mycelia, zoospores, cysts, germinating cysts, and infected susceptible soybean leaves at 1.5, 3, 6, 12, and 24 h postinfection (hpi). The results indicated that *Avr1d* was expressed during infection, and the transcript levels were elevated in germinating cysts and during the mycelia and zoospores stages (Fig. 5). This suggests that *Avr1d* is induced in preinfection structures and during infection.

N. benthamiana transiently expressing *Avr1d* has increased susceptibility to *P. capsici*.

Several oomycete effectors can suppress aspects of plant immunity and promote disease susceptibility (Dong et al. 2011a; Sohn et al. 2007; Wang et al. 2011; Yu et al. 2012). To determine whether *Avr1d* enhances virulence, we transiently expressed recombinant *Avr1d* (without a signal peptide) in *N. benthamiana* using agroinfiltration with a *Potato virus X* (PVX) vector. *P. capsici* (which is the pathogen of *N. benthamiana*) was used to challenge the infiltrated tissue. We infiltrated *Agrobacterium tumefaciens* carrying *Avr1d* on one side of the leaf and green fluorescent protein (GFP) on the other side as a control. Both sides were inoculated with an agar plug (5 by 5 mm) containing freshly grown mycelia of *P. capsici* after 48 h of infiltration. The diameter of the disease lesion was larger on leaves infiltrated with *Avr1d*^{P6497} compared with GFP at 36 hpi (Fig. 6B). Trypan blue staining was performed to examine the distribution of hyphae in infected plant tissues. At 16 hpi, abundant *P. capsici* mycelia were observed in *Avr1d*-expressing tissues but few mycelia were present in the region expressing GFP (Fig. 6A). To accurately examine *P. capsici* infection, we measured the ratios of *P. capsici* DNA to *N. benthamiana* DNA using real-time PCR to determine the *P. capsici* biomass in the infected plant tissues. The *P. capsici*/*N. benthamiana* biomass ratio was significantly higher in *Avr1d*^{P6497}-infiltrated leaves than in GFP leaves (Fig. 6C). Next, total protein was extracted and Western blot analysis was performed on extracts

from *N. benthamiana* leaves 48 h after agroinfiltration, which expressed *Avr1d*^{P6497} and GFP. Because *Avr1d*^{P7064} is also recognized by *Rps1d*, we wanted to examine the virulence of *Avr1d*^{P7064}. Thus, the *N. benthamiana* leaves expressing *Avr1d*^{P7064} were infected with *P. capsici* and monitored. Both the lesion size and biomass data indicated that *Avr1d*^{P6497} and *Avr1d*^{P7064} expression in planta enhanced the susceptibility of *N. benthamiana* to *P. capsici* whereas, compared with *Avr1d*^{P6497}, *Avr1d*^{P7064} showed less ability to contribute to virulence (Fig. 6E to H).

Avr1d suppresses cell death induced by BAX and ETI.

Previous studies have shown that *Avr1d* suppresses cell death induced by the pro-apoptotic mouse protein BAX (Wang et al. 2011). We generated PVX-*flag::Avr1d*^{P6497} and PVX-*flag::Avr1d*^{P7064}, which can also be recognized by *Rps1d*. *Agrobacterium* cells carrying either of the *Avr1d* effector genes were infiltrated into *N. benthamiana* leaves 24 h prior to the leaf tissue being challenged with cells carrying BAX with a hemagglutinin (HA) tag (PVX vector with an HA tag). When the leaves were infiltrated with *A. tumefaciens* cells containing an *Avr1d*^{P6497} gene prior to infiltration with the BAX-containing cells, no cell death symptoms were observed. *Avr1d*^{P7064} also showed the same symptoms, suggesting that these genes suppressed cell death induced by BAX (Fig. 7A). Cell death symptoms were observed after infiltration of *N. benthamiana* leaves with *A. tumefaciens* cells containing a GFP gene or infiltration with BAX alone (Fig. 7A). These results indicate that polymorphisms of *Avr1d* did not interfere with the suppression of cell death.

Previous studies have shown that *Avr1d* suppresses cell death induced by interactions between *Avr3a* and *R3a* (Wang et al. 2011); thus, we examined whether *Avr1d* could suppress ETI in soybean using double-barreled bombardment. This experiment involved measuring ETI-associated cell death triggered by the *P. sojae* Avr effector *Avr1b* in the presence of soybean *Rps1b* and in the presence or absence of *Avr1d*. The comparison of cell death triggered by co-bombardment of *Avr1b* and EV compared with *Avr1b* and *Avr1d*^{P6497} on *Rps1b*

Fig. 3. *Avr1d* genes are absent in virulent isolates and contain sequence polymorphisms. **A**, Presence or absence polymorphisms across 12 virulent and 12 avirulent *Phytophthora sojae* isolates. Polymerase chain reaction amplification of the control gene *Actin* is indicated. **B**, Protein alignment of *Avr1d*, revealing amino acid polymorphisms between the two mature proteins. The RXLR and EER motifs and predicted W motif (dark boxes) are indicated.

soybean indicated that Avr1b-triggered cell death was suppressed in the presence of Avr1d^{P6497} (Fig. 7C and D).

DISCUSSION

Avirulent effectors from oomycete, fungal, and bacterial pathogens control race-cultivar compatibility and ETI by interacting with their host receptor genes (Dodds and Rathjen 2010; Jones and Dangl 2006). The traditional methods of map-based cloning are inefficient and time consuming. It is important to identify numerous Avr effectors from a diverse collection of proteins with few common sequence signatures. For oomycetes, identification of the Avr effectors in the early stages and the development of bioinformatics led to the discovery of the RxLR host-targeting motif (Dou et al. 2008a; Jiang et al. 2008; Shan et al. 2004). Almost all Avr effectors identified in three species of oomycete plant pathogens in the past few years possess the RxLR motif (Bailey et al. 2011; Dong et al. 2009; Goritschnig et al. 2012; Oh et al. 2009; Qutob et al. 2009; Vleeshouwers et al. 2008). The study of RxLR effectors has increased our understanding of their function and interactions with plant immunity systems.

The traditional approach to identify Avr effectors relies heavily on genetic mapping, and most methods of identifying oomycete and fungal Avr effectors have applied genetic mapping or

genomic subtraction techniques. Discovery of the RxLR host-targeting motif in oomycetes has promoted the cloning of novel Avr effectors. The combination of mapping and screening strategies has provided a rapid method for identifying new Avr effectors such as the *P. sojae* Avr effectors Avr3a, Avr3c, and Avr3b, which were identified by combining RxLR effector prediction with transcriptional patterns (Dong et al. 2009, 2011a; Qutob et al. 2009). Here, using RxLR effector prediction (Jiang et al. 2008), transcriptional data (Dong et al. 2009; Ye et al. 2011), and sequence polymorphism analysis, we identified RxLR as an Avr1d candidate. CAP markers were designed to identify an RxLR effector gene as the Avr1d from *P. sojae*. This combination of methods could significantly accelerate the identification of additional oomycete and fungal avirulent effectors.

Previous studies have demonstrated that oomycete RxLR effector proteins enter host cells across the plasma cell membrane using the RxLR motif in the absence of the pathogen (Dou et al. 2008a; Whisson et al. 2007). Whether the RxLR motif plays an important role in effector translocation remains unknown (Kale et al. 2010; Yaeno et al. 2011). Most Avr effector proteins identified from *H. arabidopsidis*, *P. infestans*, and *P. sojae* contain an RxLR motif, suggesting that this motif is an important characteristic of Avr effectors in oomycetes. The avirulent effector products AvrL567 and AvrM from the flax rust *Melampsora lini* are internalized into the plant cell cytosol in the absence of the pathogen. Although they share RxLR-like sequences, the primary sequence features of the uptake regions are not conserved (Rafiqi et al. 2010). The AVR-Pita and AvrPiz-t proteins from *Magnaporthe oryzae* are recognized by intracellular receptor proteins (Jia et al. 2000; Li et al. 2009). Also, the Avr2 protein from *Fusarium oxysporum* f. sp. *lycopersicon* is recognized intracellularly by the tomato I-2 resistance protein (Houterman et al. 2009). Expression of the Avr1d protein minus the signal peptide in plants resulted in Rps1d-dependent HR, indicating that recognition occurs within the host cell, as has been shown for other RxLR effectors. The most likely reason for this is that the receptor proteins encode intracellular NB-LRR motifs (Krasileva et al. 2010; Sandhu et al. 2004) such as *Rps1k* (Gao et al. 2005).

Positive selection, which is observed for many Avr effectors (Armstrong et al. 2005; Cui et al. 2011; Wang et al. 2007; Yoshida et al. 2009), highlights the evolutionary adaptation to

Fig. 4. Avr1d^{P7064} can be recognized by Rps1d. **A**, Direct comparison of bombardment with Avr1d^{P7064} and empty vector (EV) into the soybean leaves of 'Haro16' (*Rps1d*) and 'Williams' (*rps*). **B**, Percentage rate of β -glucuronidase- (GUS) positive blue spots following bombardment with Avr1d^{P7064} compared with the EV. Data are the means of eight independent experiments. Two asterisks means $P < 0.01$.

Fig. 5. Transcriptional level of Avr1d during different stages of the life cycle. Avr1d transcript levels in different developmental and infection stages measured by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from mycelia (MY), zoospores (SP), zoospores (ZO), cysts (CY), germinating cysts (GC), and infected soybean leaves at 1.5, 3, 6, 12, and 24 h postinfection. All inoculations for RNA isolation were performed on susceptible soybean 'Williams'. Real-time RT-PCR analysis employed primers specific for Avr1d and the *Actin* gene. Transcript level represents the *Phytophthora sojae* Avr1d mRNA levels compared with *Actin* mRNA levels. Bars represent standard errors from two independent replicates each.

overcome the plant immune system. Pathogens have evolved several distinct mechanisms to evade Avr effector protein recognition by cognate receptor proteins. Loss of Avr effector proteins in pathogens have occurred by partial or complete de-

letions, point mutations, transposon insertions, and transcriptional variation (Catanzariti et al. 2006; Dodds et al. 2004; Jia et al. 2000; Orbach et al. 2000; Stergiopoulos et al. 2007; Zheng et al. 2011).

Fig. 6. Tobacco transiently expressing Avr1d is more susceptible to *Phytophthora capsici*. Each side of *Nicotiana benthamiana* leaves was infiltrated with *Agrobacterium tumefaciens* cells carrying T-DNAs encoding green fluorescent protein-flag or Avr1d-flag. Leaves were inoculated with *P. capsici* mycelia on the infiltrated regions after 48 h of infiltration. **A** to **D**, Expression of Avr1d^{P6497}; **E** to **H**, expression of Avr1d^{P7064}. **A**, Treated leaves were stained with Trypan blue 16 h postinoculation (hpi) and decolorized with chloral hydrate. Bar, 500 µm. **B**, Leaves were photographed 36 hpi. **C**, Real-time polymerase chain reaction measurement of pathogen and plant DNA ratios were used to determine *P. capsici* biomass in infected plant tissues. DNA from *P. capsici*-infected regions was isolated at 36 hpi. Two asterisks means $P < 0.01$. **D**, Western blot analysis of proteins from *N. benthamiana* leaves 48 h after agroinfiltration. Total proteins were stained with Ponceau S. Experiments were repeated for three times with same results. **E** to **H**, Avr1d^{P7064} can also promote the infection.

In oomycetes, amino acid substitutions have been observed in alleles of Avr1b-1 and Avr3c from *P. sojae* (Cui et al. 2010; Dong et al. 2009; Shan et al. 2004), Avr3a and Avr4 from *P. infestans* (Armstrong et al. 2005; van Poppel et al. 2008), and ATR1 and ATR13 from *H. arabidopsidis* (Allen et al. 2004; Rehmany et al. 2005). In addition, Avr1b-1 and Avr2 transcrip-

tional differences and deletions have contributed to virulence in plants containing the cognate receptor genes (Cui et al. 2011; Gilroy et al. 2011; Shan et al. 2004). In our study, PCR and genome sequencing suggested that *Avr1d* is deleted from the genomes of virulent isolates. In addition, numerous point mutations have been identified in avirulent isolates (Fig. 3).

Fig. 7. Avr1d suppress cell death induced by BAX and Avr1b. **A**, *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* cells carrying green fluorescent protein (GFP)-Flag, Avr1d^{P6497}-Flag, and Avr1d^{P7064}-Flag. Then, BAX-hemagglutinin (HA) was expressed in the same infiltrate site after 24 h. These experiments were repeated three times with at least three leaves for one time with same results. **B**, Western blot analysis of proteins from *N. benthamiana* leaves 48 h after agroinfiltration. GFP, Avr1d^{P6497}, and Avr1d^{P7064} were fused with Flag tag, while BAX was fused with HA tag. Total proteins were stained with Ponceau S. **C**, Avr1d^{P6497} suppresses effector-triggered immunity triggered by *Phytophthora sojae* effector Avr1b. The direct comparison of cell death triggered by co-bombardment of Avr1b + empty vector (EV) compared with Avr1b + Avr1d^{P6497} on *Rps1b* soybean. **D**, Percentage rate of β -glucuronidase (GUS)-positive blue spots following bombardment with Avr1d^{P6497} compared with the EV. Data are the means of eight independent experiments. Two asterisks means $P < 0.01$.

One possible explanation for this is that the mutation cannot escape recognition by *Rps1d*, which was also verified by the co-bombardment assay. In addition, positive selection of receptor proteins has not yet been explored. Only one allele was observed in the mutated isolates, which was also found in *Avr3b* from *P. sojae* (Dong et al. 2011a). The RxLR effectors also contain carboxyl-terminal effector domains called K, I, W, and Y motifs, which are involved in interactions with receptor genes and the suppression of host programmed cell death (PCD) (Dou et al. 2008b; Jiang et al. 2008; van Poppel et al. 2008). Thus, the C-terminal domains are highly divergent and do not typically have high sequence similarity to other proteins. *Avr1d* was recognized by *Rps1d* through the C-terminal region (but not the N-terminal region) (Fig. 2), which was consistent with previous reports indicating that the C-terminal sequences of most oomycete avirulent effectors show positive selection (Dong et al. 2011a; Dou et al. 2008b; Oh et al. 2009). The C terminus of *Avr1d* contained a predicted W motif, which is important for function and recognition. Although there are several amino acid differences between *Avr1d*^{P6497} and *Avr1d*^{P7064} within the W motif (Fig. 2), it plays an important role in function and recognition. Although there are differences in the protein sequence between *Avr1d*^{P6497} and *Avr1d*^{P7064}, both of the alleles can be recognized by *Rps1d*. It shows that these mutation do not affect the protein fold and the critical surface-recognized residues do not change. Bacterial, fungal, and oomycete pathogens secrete effectors into host cells. Some of these effectors play a role in the suppression of the immune signaling pathway and the alteration of plant hormone signaling (Birch et al. 2008; Ellis et al. 2009; Panstruga and Dodds 2009; Tyler 2009). Some effectors such as SNE1 from *P. infestans* can suppress PCD (Kelley et al. 2010). Several RxLR effectors can manipulate host defense responses by suppressing PCD (Dong et al. 2011a; Dou et al. 2008b; Oh et al. 2009; Wang et al. 2011). Previous reports have shown that *Avr1d* can suppress PCD triggered by the BAX protein and Avh238 and Avh241 effector proteins from *P. sojae* in *N. benthamiana* cells (Wang et al. 2011). Therefore, *Avr1d* likely positively contributes to pathogenesis via suppressing ETI. In our assays, expression of *Avr1d* in *N. benthamiana* cells without its signal peptide resulted in virulent activities, suggesting that *Avr1d* functions normally inside plant cells; thus, it should be able to enter plant cells during infection. Further studies are required to identify potential host targets of *Avr1d* and to investigate the role of *Avr1d* in promoting *P. sojae* disease development.

MATERIALS AND METHODS

P. sojae strains, culture, and pathogenicity assays.

All isolates and the P6497 × P7076 F₁ and F₂ progeny used in this study were routinely maintained on 2.5% vegetable juice (V8) medium at 12°C in the dark (Qutob et al. 2000). All isolates were grown on plates containing V8 juice agar (10% V8 juice filtered through four layers of cheesecloth, 1% CaCO₃, and 1.5% agar) for pathogenicity assays at 25°C in the dark, or grown in 10% V8 juice at 25°C for the production of mycelia to be used for DNA and RNA preparation.

The virulence of *P. sojae* isolates was evaluated by hypocotyl inoculation (Cui et al. 2010) using soybean ‘Haro16’ (*Rps1d*) and ‘Williams’ (*rps*) (B. M. Tyler, Oregon State University, Corvallis, OR, U.S.A.). Pathogenicity determination was repeated twice with each isolate. Strains were considered avirulent if more than 70% of the inoculated seedlings survived, virulent if 30% or less of the seedlings survived, and intermediate if 30 to 70% of the seedlings survived (Cui et al. 2010; Jackson et al. 2004).

Generation of the F₂ mapping population.

F₁ hybrids were derived from P6497 (*Avr1d*) × P7064 (*avr1d*) crosses. Oospores from F₁ hybrids and F₂ population were produced as described previously (Dong et al. 2011a; Qutob et al. 2009). In total, 71 F₂ progeny were used to establish an expanded mapping population to examine *Avr1d* candidate effectors. For each F₂ individual, virulence was scored on ‘Williams’ (*rps*) and ‘Haro16’ (*Rps1d*) plants, and DNA samples from mycelia were isolated for genotyping. DNA isolation was performed using a fast DNA isolation kit (Axygen Biotech, Hangzhou City, China).

RNA extraction and SYBR green real-time RT-PCR assay.

P. sojae mycelia, zoospores, and cysts were prepared as described previously (Hua et al. 2008). Cysts germinating at 25°C for 6 h were used for this study. For RNA samples, infection assays were performed by sandwiching *P. sojae* mycelia between pairs of soybean leaves (Chen et al. 2007). Total RNA samples from mycelia, zoospores, cysts, germinating cysts, and mycelia-infected plant tissue samples were extracted using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, U.S.A.). Then they were transcribed to cDNA using a PrimeScript RT-PCR Kit (TaKaRa Inc., Dalian, China) following the manufacturer’s protocol. SYBR green real-time RT-PCR assays were performed for *Avr1d* transcript profiling analysis. Primer pairs of gene-specific and reference *Actin* genes were designed. PCR reactions (20 µl) included 20 ng of cDNA, 0.2 mM each primer, and 10 µl of SYBR Premix Ex-Taq (TaKaRa Inc.). Reactions were performed on an ABI PRISM 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) under the following conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 31 s; followed by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain melt curves. The 7500 System Sequence Detection Software (version 1.4) was used to obtain the relative expression levels of each sample. The real-time RT-PCR assay was repeated three times.

Plasmid construction and particle bombardment assays.

Avr1d was amplified from protein sequences encoding from the 16th site to the stop codon from genomic DNA of *P. sojae* isolate P6497 with gene-specific primers. The *Avr1d* amplicon was digested with *Bam*HI/*Sph*I and inserted into plant transient expression plasmid pFF19::GUS (replacing GUS with *Avr1d*, creating pFF19::*Avr1d*). Particle bombardment assays were performed using a double-barreled extension of the Bio-Rad He/1000 Particle Delivery System (Dou et al. 2008b). Specific cell death activity was measured as a reduction in the number of blue-stained GUS-positive spots in leaves carrying *Rps1d* compared with leaves lacking either resistance gene. The double-barreled device was used to deliver a parallel control shot in every case that contained GUS DNA plus EV DNA. For each pair of shots, the percentage of blue spots for various *Avr1d* constructs compared with the EV control was calculated. Each assay consisted of eight pairs of shots.

A. tumefaciens infiltration assays for fluorescence.

Constructs were introduced into *A. tumefaciens* strain GV3101 by electroporation. Individual colonies were verified by PCR using vector primers. Recombinant strains of *A. tumefaciens* were grown in Luria-Bertani medium-plus for 48 h, harvested, and washed with 10 mM MgCl₂ three times, then resuspended in 10 mM MgCl₂ to achieve a final optical density of 0.4. Plants were grown in a greenhouse for 4 to 6 weeks. GFP fluorescence in *N. benthamiana* leaves was detected using a fluorescence microscope (IX71; Olympus, Tokyo).

Protein extraction and Western blots.

Agrobacterium-infiltrated *N. benthamiana* plants were grown in a greenhouse for 48 h. In total, 400 mg of treated leaf tissue was ground in liquid nitrogen and added to a tube with 1 ml of extraction buffer (number P0013F; Beyotime, Jiangsu, China) in the presence of 0.1 mM protease inhibitor phenylmethylsulfonyl fluoride (number ST506; Beyotime), shaken on a vortex intensely for 60 s, and then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube. A standard sodium dodecyl sulfate polyacrylamide gel electrophoresis protocol was used for protein separation. Proteins were transferred onto polyvinylidene difluoride membranes using a semi-wet apparatus (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Then, the membrane was blocked using phosphate-buffered saline (PBS; pH 7.4) with 5% nonfat dry milk for 30 min at room temperature with 50 rpm shaking, followed by one wash with PBS with 0.1% Tween 20 (PBST). Antibodies were added to PBST with 3% nonfat dry milk (PBSTM) at a ratio of 1:5,000 and incubated at room temperature for 90 min or more, followed by three washes (5 min each) with PBST. Then, the membrane was incubated with a goat anti-mouse IRDye 800CW (Odyssey, number 926-32210; Li-Cor, Lincoln, NE, U.S.A.) at a ratio of 1:10,000 in PBSTM at room temperature for 30 min with 50 rpm shaking. The membrane was washed three times (5 min each) with PBST and one time for 5 min with PBS, then visualized using Odyssey with excitation at 700 and 800 nm.

ACKNOWLEDGMENTS

This work was supported by the China National Funds for Distinguished Young Scientists (31225022) and the Special Fund for Agro-scientific Research in the Public Interest (201303018) from China to Y. Wang. We thank B. Tyler (Oregon State University, U.S.A.) for sharing 454 sequencing data for *P. sojae* strains; L. Cui for helpful suggestion in sequence analysis and virulence test; M. Gijzen (Agriculture and Agri-Food Canada, Canada) for providing vector pFF19::GUS; and Z. Zhang (Nanjing Agricultural University, China) for sharing plant transient expression experience. W. Yin, S. Dong, Y. Wang, and X. Zheng conceived and designed experiment; W. Yin, S. Dong, L. Zhai, and Y. Lin performed the experiments; W. Yin, S. Dong, Y. Wang, and X. Zheng analyzed the data; and W. Yin, S. Dong, Y. Wang, and X. Zheng wrote the article.

LITERATURE CITED

- Allen, R. L., Bittner-Eddy, P. D., Grenville-Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E., and Beynon, J. L. 2004. Host-parasite co-evolutionary conflict between *Arabidopsis* and downy mildew. *Science* 306:1957-1960.
- Allen, R. L., Meitz, J. C., Baumber, R. E., Hall, S. A., Lee, S. C., Rose, L. E., and Beynon, J. L. 2008. Natural variation reveals key amino acids in a downy mildew effector that alters recognition specificity by an *Arabidopsis* resistance gene. *Mol. Plant Pathol.* 9:511-523.
- Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I., Venter, E., Avrova, A. O., Rehmany, A. P., Bohme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. 2005. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 102:7766-7771.
- Bailey, K., Çevik, V., Holton, N., Byrne-Richardson, J., Sohn, K. H., Coates, M., Woods-Tör, A., Aksoy, H. M., Hughes, L., Baxter, L., Jones, J. D. G., Beynon, J., Holub, E. B., and Tör, M. 2011. Molecular cloning of *ATRSEmoy2* from *Hyaloperonospora arabidopsidis*, an avirulence determinant that triggers RPP5-mediated defense in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 24:827-838.
- Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A., Anderson, R., Badejoko, W., Bittner-Eddy, P., Boore, J. L., Chibucos, M. C., Coates, M., Dehal, P., Delehaunty, K., Dong, S., Downton, P., Dumas, B., Fabro, G., Fronick, C., Fuerstenberg, S. I., Fulton, L., Gaulin, E., Govers, F., Hughes, L., Humphray, S., Jiang, R. H., Judelson, H., Kamoun, S., Kyung, K., Meijer, H., Minx, P., Morris, P., Nelson, J., Phuntumart, V., Qutob, D., Rehmany, A., Rougon-Cardoso, A., Ryden, P., Torto-Alalibo, T., Studholme, D., Wang, Y., Win, J., Wood, J., Clifton, S. W., Rogers, J., Van den Ackerveken, G., Jones, J. D., McDowell, J. M., Beynon, J., and Tyler, B. M. 2010. Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* 330:1549-1551.
- Bhattacharjee, S., Hiller, N. L., Liolios, K., Win, J., Kanneganti, T. D., Young, C., Kamoun, S., and Haldar, K. 2006. The malarial host-targeting signal is conserved in the Irish potato famine pathogen. *PLoS Pathog.* 2:e50. Published online.
- Birch, P. R., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L., and Whisson, S. C. 2008. Oomycete RXLR effectors: Delivery, functional redundancy and durable disease resistance. *Curr. Opin. Plant Biol.* 11:373-379.
- Burnham, K. D. 2003. *Rps8*, a new locus in soybean for resistance to *Phytophthora sojae*. *Crop Sci.* 43:101.
- Catanzariti, A. M., Dodds, P. N., Lawrence, G. J., Ayliffe, M. A., and Ellis, J. G. 2006. Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell* 18:243-256.
- Chen, X., Shen, G., Wang, Y., and Zheng, X. 2007. Identification of *Phytophthora sojae* genes upregulated during the early stage of soybean infection. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 269:280-288.
- Cui, L., Yin, W., Tang, Q., Dong, S., Zheng, X., Zhang, Z., and Wang, Y. 2010. Distribution, pathotypes, and metalaxyl sensitivity of *Phytophthora sojae* from Heilongjiang and Fujian Provinces in China. *Plant Dis.* 94:881-884.
- Cui, L., Yin, W., Dong, S., and Wang, Y. 2012. Analysis of polymorphism and transcription of the effector gene *Avr1b* in *Phytophthora sojae* isolates from China virulent to *Rps1b*. *Mol. Plant Pathol.* 13:114-122.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11:539-548.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A.-M., Ayliffe, M. A., and Ellis, J. G. 2004. The *Melampsora lini* *AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell Online* 16:755-768.
- Dong, S., Qutob, D., Tedman-Jones, J., Kuflu, K., Wang, Y., Tyler, B. M., and Gijzen, M. 2009. The *Phytophthora sojae* avirulence locus *Avr3c* encodes a multi-copy RXLR effector with sequence polymorphisms among pathogen strains. *PLoS One* 4:e5556. Published online.
- Dong, S., Yin, W., Kong, G., Yang, X., Qutob, D., Chen, Q., Kale, S. D., Sui, Y., Zhang, Z., Dou, D., Zheng, X., Gijzen, M., M. Tyler, B., and Wang, Y. 2011a. *Phytophthora sojae* avirulence effector *Avr3b* is a secreted NADH and ADP-ribose pyrophosphorylase that modulates plant immunity. *PLoS Pathog.* 7:e1002353. Published online.
- Dong, S., Yu, D., Cui, L., Qutob, D., Tedman-Jones, J., Kale, S. D., Tyler, B. M., Wang, Y., and Gijzen, M. 2011b. Sequence variants of the *Phytophthora sojae* RXLR effector *Avr3a/5* are differentially recognized by *Rps3a* and *Rps5* in soybean. *PLoS One* 6:e20172. Published online.
- Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., Zhang, X., and Tyler, B. M. 2008a. RXLR-mediated entry of *Phytophthora sojae* effector *Avr1b* into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20:1930-1947.
- Dou, D., Kale, S. D., Wang, X., Chen, Y., Wang, Q., Jiang, R. H., Arredondo, F. D., Anderson, R. G., Thakur, P. B., McDowell, J. M., Wang, Y., and Tyler, B. M. 2008b. Conserved C-terminal motifs required for avirulence and suppression of cell death by *Phytophthora sojae* effector *Avr1b*. *Plant Cell* 20:1118-1133.
- Dou, D., Kale, S. D., Liu, T., Tang, Q., Wang, X., Arredondo, F. D., Basnayake, S., Whisson, S., Drenth, A., Maclean, D., and Tyler, B. M. 2010. Different domains of *Phytophthora sojae* effector *Avr4/6* are recognized by soybean resistance genes *Rps4* and *Rps6*. *Mol. Plant-Microbe Interact.* 23:425-435.
- Ellis, J. G., Rafiqi, M., Gan, P., Chakrabarti, A., and Dodds, P. N. 2009. Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. *Curr. Opin. Plant Biol.* 12:399-405.
- Förster, H. 1994. *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. *Mol. Plant-Microbe Interact.* 7:780.
- Gao, H., Narayanan, N. N., Ellison, L., and Bhattacharyya, M. K. 2005. Two classes of highly similar coiled coil-nucleotide binding-leucine rich repeat genes isolated from the *Rps1-k* locus encode *Phytophthora* resistance in soybean. *Mol. Plant-Microbe Interact.* 18:1035-1045.
- Gilroy, E. M., Breen, S., Whisson, S. C., Squires, J., Hein, I., Kaczmarek, M., Turnbull, D., Boevink, P. C., Lokossou, A., Cano, L. M., Morales, J., Avrova, A. O., Pritchard, L., Randall, E., Lees, A., Govers, F., van West, P., Kamoun, S., Vleeshouwers, V. G., Cooke, D. E., and Birch, P. R. 2011. Presence/absence, differential expression and sequence poly-

- morphisms between PiAVR2 and PiAVR2-like in *Phytophthora infestans* determine virulence on R2 plants. *New Phytol.* 191:763-776.
- Goritschnig, S., Krasileva, K. V., Dahlbeck, D., and Staskawicz, B. J. 2012. Computational prediction and molecular characterization of an oomycete effector and the cognate *Arabidopsis* resistance gene. *PLoS Genet.* 8:e1002502. Published online.
- Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grunwald, N. J., Horn, K., Horner, N. R., Hu, C. H., Huitema, E., Jeong, D. H., Jones, A. M., Jones, J. D., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., Maclean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J., Morgan, W., Morris, P. F., Munro, C. A., O'Neill, K., Ospina-Giraldo, M., Pinzon, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R., Whisson, S. C., Judelson, H. S., and Nussbaum, C. 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461:393-398.
- Houterman, P. M., Ma, L., van Ooijen, G., de Vroomen, M. J., Cornelissen, B. J., Takken, F. L., and Rep, M. 2009. The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J.* 58:970-978.
- Hua, C., Wang, Y., Zheng, X., Dou, D., Zhang, Z., Govers, F., and Wang, Y. 2008. A *Phytophthora sojae* G-protein α subunit is involved in chemotaxis to soybean isoflavones. *Eukaryot. Cell* 7:2133-2140.
- Jackson, T. A., Kirkpatrick, T. L., and Rupe, J. C. 2004. Races of *Phytophthora sojae* in Arkansas soybean fields and their effects on commonly grown soybean cultivars. *Plant Dis.* 88:345-351.
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:4004-4014.
- Jiang, R. H., Tripathy, S., Govers, F., and Tyler, B. M. 2008. RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc. Natl. Acad. Sci. U.S.A.* 105:4874-4879.
- Jones, J. D., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., Arredondo, F. D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C. B., Shan, W., and Tyler, B. M. 2010. External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* 142:284-295.
- Kelley, B. S., Lee, S. J., Damasceno, C. M., Chakravarthy, S., Kim, B. D., Martin, G. B., and Rose, J. K. 2010. A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. *Plant J.* 62:357-366.
- Krasileva, K. V., Dahlbeck, D., and Staskawicz, B. J. 2010. Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22:2444-2458.
- Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z., Zhao, Q., Feng, Q., Zhang, H., Wang, Z., Wang, G., Han, B., and Zhou, B. 2009. The *Magnaporthe oryzae* avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene Piz-t. *Mol. Plant-Microbe Interact.* 22:411-420.
- May, K. J., Whisson, S. C., Zwart, R. S., Searle, I. R., Irwin, J. A., Maclean, D. J., Carroll, B. J., and Drenth, A. 2002. Inheritance and mapping of 11 avirulence genes in *Phytophthora sojae*. *Fungal Genet. Biol.* 37:1-12.
- Na, R., Yu, D., Qutob, D., Zhao, J., and Gijzen, M. 2013. Deletion of the *Phytophthora sojae* avirulence gene Avr1d causes gain of virulence on Rps1d. *Mol. Plant-Microbe Interact.* 26:969-977.
- Nicholls, H. 2004. Stopping the rot. *PLoS Biol.* 2:E213. Published online.
- Oh, S. K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I., Liu, H. Y., van Damme, M., Morgan, W., Choi, D., Van der Vossen, E. A., Vleeshouwers, V. G., and Kamoun, S. 2009. In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. *Plant Cell* 21:2928-2947.
- Orbach, M. J., Farrall, L., Sweigard, J. A., Chumley, F. G., and Valent, B. 2000. A telomeric avirulence gene determines efficacy for the rice blast resistance gene Pi-ta. *Plant Cell* 12:2019-2032.
- Panstruga, R., and Dodds, P. N. 2009. Terrific protein traffic: The mystery of effector protein delivery by filamentous plant pathogens. *Science* 324:748-750.
- Qutob, D., Hraber, P. T., Sobral, B. W., and Gijzen, M. 2000. Comparative analysis of expressed sequences in *Phytophthora sojae*. *Plant Physiol.* 123:243-254.
- Qutob, D., Tedman-Jones, J., Dong, S., Kuflu, K., Pham, H., Wang, Y., Dou, D., Kale, S. D., Arredondo, F. D., Tyler, B. M., and Gijzen, M. 2009. Copy number variation and transcriptional polymorphisms of *Phytophthora sojae* RXLR effector genes Avr1a and Avr3a. *PLoS One* 4:e5066. Published online.
- Rafiqi, M., Gan, P. H., Ravensdale, M., Lawrence, G. J., Ellis, J. G., Jones, D. A., Hardham, A. R., and Dodds, P. N. 2010. Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell* 22:2017-2032.
- Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R., and Beynon, J. L. 2005. Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two *Arabidopsis* lines. *Plant Cell* 17:1839-1850.
- Sandhu, D., Gao, H., Cianzio, S., and Bhattacharyya, M. K. 2004. Deletion of a disease resistance nucleotide-binding-site leucine-rich-repeat-like sequence is associated with the loss of the *Phytophthora* resistance gene Rps4 in soybean. *Genetics* 168:2157-2167.
- Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X. C., Shinozaki, K., Nguyen, H. T., Wing, R. A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R. C., and Jackson, S. A. 2010. Genome sequence of the palaeopolyploid soybean. *Nature* 463:178-183.
- Shan, W., Cao, M., Leung, D., and Tyler, B. M. 2004. The Avr1b locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene Rps1b. *Mol. Plant-Microbe Interact.* 17:394-403.
- Sohn, K. H., Lei, R., Nemri, A., and Jones, J. D. 2007. The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in *Arabidopsis thaliana*. *Plant Cell* 19:4077-4090.
- Stergiopoulos, I., De Kock, M. J., Lindhout, P., and De Wit, P. J. 2007. Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. *Mol. Plant-Microbe Interact.* 20:1271-1283.
- Tyler, B. M. 2007. *Phytophthora sojae*: Root rot pathogen of soybean and model oomycete. *Mol. Plant Pathol.* 8:1-8.
- Tyler, B. M. 2008. Genomics of fungal- and oomycete-soybean interactions. Pages 243-267 in: *Genetics and Genomics of Soybean*. N. S. Iacobellis, A. Collmer, S. W. Hutcheson, J. W. Mansfield, C. E. Morris, J. Murillo, N. W. Schaad, D. E. Stead, G. Surico, and M. Ullrich, eds. Kluwer Academic Springer, New York.
- Tyler, B. M. 2009. Entering and breaking: Virulence effector proteins of oomycete plant pathogens. *Cell Microbiol.* 11:13-20.
- Tyler, B. M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R. H., Aerts, A., Arredondo, F. D., Baxter, L., Bensasson, D., Beynon, J. L., Chapman, J., Damasceno, C. M., Dorrance, A. E., Dou, D., Dickerman, A. W., Dubchak, I. L., Garbelotto, M., Gijzen, M., Gordon, S. G., Govers, F., Grunwald, N. J., Huang, W., Ivors, K. L., Jones, R. W., Kamoun, S., Krampis, K., Lamour, K. H., Lee, M. K., McDonald, W. H., Medina, M., Meijer, H. J., Nordberg, E. K., Maclean, D. J., Ospina-Giraldo, M. D., Morris, P. F., Phuntumart, V., Putnam, N. H., Rash, S., Rose, J. K., Sakihama, Y., Salamov, A. A., Savidor, A., Scheuring, C. F., Smith, B. M., Sobral, B. W., Terry, A., Torto-Alalibo, T. A., Win, J., Xu, Z., Zhang, H., Grigoriev, I. V., Rokhsar, D. S., and Boore, J. L. 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313:1261-1266.
- van Poppel, P. M., Guo, J., van de Vondervoort, P. J., Jung, M. W., Birch, P. R., Whisson, S. C., and Govers, F. 2008. The *Phytophthora infestans* avirulence gene Avr4 encodes an RXLR-dEER effector. *Mol. Plant-Microbe Interact.* 21:1460-1470.
- Vleeshouwers, V. G., Rietman, H., Krenek, P., Champouret, N., Young, C., Oh, S. K., Wang, M., Bouwmeester, K., Vosman, B., Visser, R. G., Jacobsen, E., Govers, F., Kamoun, S., and Van der Vossen, E. A. 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *PLoS One* 3:e2875. Published online.
- Wang, C.-I. A., Guncar, G., Forwood, J. K., Teh, T., Catanzariti, A.-M., Lawrence, G. J., Loughlin, F. E., Mackay, J. P., Schirra, H. J., Anderson,

- P. A., Ellis, J. G., Dodds, P. N., and Kobe, B. 2007. Crystal structures of flax rust avirulence proteins AvrL567-A and -D reveal details of the structural basis for flax disease resistance specificity. *Plant Cell Online* 19:2898-2912.
- Wang, Q., Han, C., Ferreira, A. O., Yu, X., Ye, W., Tripathy, S., Kale, S. D., Gu, B., Sheng, Y., Sui, Y., Wang, X., Zhang, Z., Cheng, B., Dong, S., Shan, W., Zheng, X., Dou, D., Tyler, B. M., and Wang, Y. 2011. Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell* 23:2064-2086.
- Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450:115-118.
- Wrather, J. A., and Koenning, S. R. 2006. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *J. Nematol.* 38:173-180.
- Yaeno, T., Li, H., Chaparro-Garcia, A., Schornack, S., Koshiba, S., Watanabe, S., Kigawa, T., Kamoun, S., and Shirasu, K. 2011. Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. *Proc. Natl. Acad. Sci. U.S.A.* 108:14682-4687.
- Ye, W., Wang, X., Tao, K., Lu, Y., Dai, T., Dong, S., Dou, D., Gijzen, M., and Wang, Y. 2011. Digital gene expression profiling of the *Phytophthora sojae* transcriptome. *Mol. Plant-Microbe Interact.* 24:1530-1539.
- Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Tosa, Y., Chuma, I., Takano, Y., Win, J., Kamoun, S., and Terauchi, R. 2009. Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. *Plant Cell* 21:1573-1591.
- Yu, X., Tang, J., Wang, Q., Ye, W., Tao, K., Duan, S., Lu, C., Yang, X., Dong, S., Zheng, X., and Wang, Y. 2012. The RxLR effector Avh241 from *Phytophthora sojae* requires plasma membrane localization to induce plant cell death. *New Phytol.* 196:247-260.
- Zheng, Y., Zheng, W., Lin, F., Zhang, Y., Yi, Y., Wang, B., Lu, G., Wang, Z., and Wu, W. 2011. AVR1-CO39 is a predominant locus governing the broad avirulence of *Magnaporthe oryzae* 2539 on cultivated rice (*Oryza sativa* L.). *Mol. Plant-Microbe Interact.* 24:13-17.