Phytophthora* Suppressor of RNA Silencing 2 Is a Conserved RxLR Effector that Promotes Infection in Soybean and *Arabidopsis thaliana

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The genus *Phytophthora* consists of notorious and emerging pathogens of economically important crops. Each *Phytophthora* genome encodes several hundreds of cytoplasmic effectors, which are believed to manipulate plant immune response inside the host cells. However, the majority of *Phytophthora* effectors remain functionally uncharacterized. We recently discovered two effectors from the soybean stem and root rot pathogen *Phytophthora sojae* with the activity to suppress RNA silencing in plants. These effectors are designated *Phytophthora* suppressor of RNA silencing (PSRs). Here, we report that the *P. sojae* PSR2 (PsPSR2) belongs to a conserved and widespread effector family in *Phytophthora*. A PsPSR2-like effector produced by *P. infestans* (PiPSR2) can also suppress RNA silencing in plants and promote *Phytophthora* infection, suggesting that the PSR2 family effectors have conserved functions in plant hosts. Using *Agrobacterium rhizogenes*-mediated hairy roots induction, we demonstrated that the expression of PsPSR2 rendered hypersusceptibility of soybean to *P. sojae*. Enhanced susceptibility was also observed in PsPSR2-expressing *Arabidopsis thaliana* plants during *Phytophthora* but not bacterial infection. These experiments provide strong evidence that PSR2 is a conserved *Phytophthora* effector family that performs important virulence functions specifically during *Phytophthora* infection of various plant hosts.

Phytophthora spp. are oomycete pathogens responsible for many devastating diseases on important crops, including potato, tomato, melon, and soybean. The potato pathogen *Phytophthora infestans* caused the Irish Famine in the 1840s and remains a serious problem worldwide; *P. sojae* is the second most destructive pathogen of soybean, with an average annual loss of \$200 million in the United States (Tyler 2007). To date, battling *Phytophthora* diseases is challenged by a lack of understanding of the mechanisms of pathogenesis.

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Plants and microbial pathogens are engaged in an endless arms race. The central concept of this battle is the activation of immune responses upon pathogen perception and the subversion of plant defense by virulence factors produced by successful pathogens. The first layer of plant defense is based on the recognition of pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns. Activation of membrane-located pattern recognition receptor (PRR) complexes initiates signal transduction and transcriptional reprogramming, leading to various defense responses (Boller and Felix 2009; Monaghan and Zipfel 2012; Sun et al. 2013). PAMP-triggered immunity (PTI), broadly considered to be a general defense response, restricts the growth of the vast majority of potential pathogens encountered by the plants in their surrounding environment (Boller and He 2009; Jones and Dangl 2006; Zipfel 2009). However, successful pathogens produce virulence factors to effectively suppress PTI. In particular, a multitude of secreted proteins, called effectors, can effectively subvert immune responses, including PTI (Dou and Zhou 2012; Gheysen and Mitchum 2011; Giraldo and Valent 2013; Ma et al. 2011; Stassen and Van den Ackerveken 2011; Torto-Alalibo et al. 2009). Indeed, effectors have been identified from a broad range of parasites, including viruses, bacteria, fungi, oomycetes, protozoa, insects, and nematodes and play essential roles in pathogenesis (Dangl et al. 2013; Lindeberg et al. 2012; Torto-Alalibo et al. 2009).

Recent progress on genome sequence analysis and the identification of the host-targeting signal has facilitated the discovery of exceptionally large repertoires of potential effectors from oomycetes (Haas et al. 2009; Kale et al. 2010; Pais et al. 2013; Thines and Kamoun 2010; Tyler et al. 2006; Whisson et al. 2007), especially *Phytophthora* spp. As hemibiotrophic pathogens, *Phytophthora* spp. form specialized feeding structures called haustoria during the initial biotrophic growth phase (Panstruga and Dodds 2009; Talbot 2007; Whisson et al. 2007). Effectors are secreted from haustoria into the extra-haustoria space by the signal-peptide-based secretion system; and the so-called cytoplasmic effectors can subsequently enter plant cells, dependent on the “host-targeting motif” (Bhattacharjee et al. 2006; Birch et al. 2006, 2008; Lu et al. 2012). A major class of cytoplasmic effectors contain an N-terminal RxLR domain (“x” represents any amino acid) following the secretion signal (Kale et al. 2010; Raffaele and Kamoun 2012; Whisson et al. 2007). Bioinformatic prediction using the presence of the secretion signal and the RxLR motif revealed hundreds of cytoplasmic effectors in each genome of *Phytophthora* spp. (Haldar et al. 2006; Jiang et al. 2008; Pais

et al. 2013; Raffaele and Kamoun 2012; Win et al. 2007). This remarkably large number of effectors suggests complex defense-counter-defense crosstalks between host plants and *Phytophthora* spp.

Following the identification of numerous effectors from *Phytophthora* genomes, substantial efforts have been devoted to understanding their virulence activity during infection. Recent reports showed that, similar to effectors produced by viral and bacterial pathogens, a fundamental function of RxLR effectors is to subvert plant defense (Anderson et al. 2012; King et al. 2014; Qiao et al. 2013). Many RxLR effectors can suppress programmed cell death induced by the pro-apoptotic protein BAX in yeast and the *Phytophthora* infestans 1 (INF1) in *Nicotiana benthamiana* (Bos et al. 2006; Wang et al. 2011). The underlying mechanisms by which specific RxLR effectors facilitate *Phytophthora* infection have also been studied. For example, *P. infestans* RxLR effector AVRblb2 promotes infection by preventing the secretion of the papain-like cysteine protease C14 from host cells to the apoplast (Bozkurt et al. 2011); Avr3a of *P. infestans* perturbs the stability of the plant E3 ubiquitin ligase CMPG1, which is required for INF-triggered cell death (Bos et al. 2010; Gilroy et al. 2011); Avr3b of

P. sojae functions as an NADH and ADP-ribose pyro-phosphorylase in plants and is required for the full virulence of *P. sojae* in soybean (Dong et al. 2011); and the *P. parasitica* effector penetration-specific effector 1 (PSE1) facilitates infection on *Arabidopsis thaliana* by interfering with auxin physiology (Evangelisti et al. 2013).

Recently, we identified two RxLR effectors from *P. sojae* that can suppress RNA silencing in plants (Qiao et al. 2013). As a universal gene regulation mechanism in eukaryotes, RNA silencing depends on 20- to 30-nucleotide (nt) small RNAs to guide sequence-specific repression of target genes. These *P. sojae* *Phytophthora* suppressors of RNA silencing (PsPSR) inhibit small RNA biogenesis and promote *P. infestans* infection when transiently expressed in *N. benthamiana* (Qiao et al. 2013). Furthermore, transient silencing of *PsPSR2* in *P. sojae* led to significantly reduced virulence on soybean hypocotyls, suggesting that inhibiting host RNA silencing pathways is an important virulence strategy of *P. sojae* (Qiao et al. 2013).

In this work, we performed detailed analysis on the prevalence of *PsPSR2* homologs in *Phytophthora* spp. and the virulence function of *PsPSR2*. We first analyzed the prevalence of *PsPSR2* homologs using the genome sequences of several

Fig. 1. Phylogenetic relationship of *PSR2* family effectors in sequenced oomycete genomes. **A**, Neighbor-joining tree of the *PsPSR2* homologs in *Hyaloperonospora arabidopsidis* and various *Phytophthora* spp. using full-length amino acid sequences. **B**, Inferred phylogeny of the related species based on internal transcribed spacer (ITS) sequences. Bootstrap support is indicated near each node. GenBank ID numbers of ITS are presented in parentheses. Congruent species in both trees were connected with dotted lines.

Fig. 2. PiPSR2 suppresses transgene-mediated green fluorescent protein (GFP) silencing in *Nicotiana benthamiana* and promotes *Phytophthora infestans* infection. **A**, Local RNA silencing suppression in *N. benthamiana* 16c transiently expressing GFP and PiPSR2, PsPSR1, PsPSR2, or CMV2b. Empty vector pEG100 (EV) was used as a negative control. Pictures were taken at 4 days after *Agrobacterium* infiltration. **B**, Accumulation of GFP small interfering (si)RNA in the infiltrated *N. benthamiana* 16c leaves. Numbers below the Northern blot represent the relative abundance of siRNA, with the level in the leaves expressing only GFP set to 1. U6 was used as a loading control. The sample of “mock” treatment was from tissues without *Agrobacterium* infiltration. This experiment was repeated three times with similar results. **C**, Expression of PiPSR2 in *N. benthamiana* enhances the infection of *P. infestans*. Wild-type *N. benthamiana* leaves were infiltrated with *Agrobacterium* carrying pEG104 (expressing the yellow fluorescent protein gene [YFP]) in one half and pEG101::PiPSR2 in the other half. Leaves were subsequently inoculated with zoospore suspension of *P. infestans* 1306 (10^4 zoospores/ml) 24 h after the agroinfiltration. Images of disease symptoms (left) and lesions areas visualized after trypan blue staining (right) were taken at 5 days post zoospore inoculation. Average lesion area on leaves expressing YFP or PiPSR2 was presented. Error bars represent standard errors and ** indicate values that are statistically different ($P < 0.001$, *t* test). Experiments were repeated three times with similar results.

Phytophthora spp. We found that PSR2 is a conserved RxLR effector family that is widely distributed in *Phytophthora* spp. PiPSR2, a close homolog of PsPSR2 in *P. infestans* (PITG_15152), was also able to suppress RNA silencing activity when expressed in *N. benthamiana*. Moreover, expressing PiPSR2 enhanced the susceptibility of *N. benthamiana* to *Phytophthora* infection. We then monitored the infection process of *P. sojae* in soybean roots using a green fluorescence protein (GFP)-expressing transformant of *P. sojae* P6497. Using this natural pathosystem, we showed that *PsPSR2*-expressing soy-

bean roots were hypersusceptible to *P. sojae*. Expression of *PsPSR2* in *Arabidopsis* also led to largely enhanced susceptibility to *P. capsici* isolate LT263. Interestingly, *PsPSR2*-expressing *Arabidopsis* plants are not altered in their susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 or its nonpathogenic *hrcC* mutant, suggesting that PsPSR2 specifically promotes *Phytophthora* infection. These data suggest that PSR2 family RxLR effectors may play a fundamental virulence function by suppressing host RNA silencing processes in various plants.

Fig. 3. Infection process and *PsPSR2* expression pattern of *Phytophthora sojae* P6497G in soybean roots. **A**, Microscopic analysis of the progression of *P. sojae* infection in soybean roots. Roots of 10-day-old soybean seedlings ('Harosoy') were inoculated with zoospores of the green fluorescent protein (GFP)-labeled *P. sojae* P6497G. Development of *P. sojae* in the inoculated roots was monitored during a time course; hpi = hours postinoculation. Pictures on the left panel show the general distributions of hyphae or oospores in the roots and pictures on right panel are representative enlarged views of the same roots showing detailed developmental stages. Purple arrows label papilla- or finger-like feeding structures of *P. sojae* and white arrows label oospores formed in infected root tissue. Size bars in the pictures of the left panel represent 100 μ m and those in the right panel represent 20 μ m. **B**, Transcript abundances of *PsPSR2* and another RxLR effector *Avr3b* in *P. sojae*-infected soybean roots. Relative expression levels of *PsPSR2* and *Avr3b* were determined by quantitative reverse-transcriptase polymerase chain reaction using the *P. sojae* housekeeping gene *actinA* as an internal standard. Values are means $\pm$ standard deviations (as error bars) ($n = 3$). This experiment was repeated three times with similar results.

Fig. 4. *PsPSR2* was successfully expressed in soybean hairy roots. **A**, Hairy roots generated from soybean cotyledons after inoculation with *Agrobacterium rhizogenes* K599 carrying pEG104 or pEG101::*PsPSR2*. Image was taken at 30 days after inoculation. **B**, Yellow fluorescence observed from a small portion of *Agrobacterium*-induced hairy roots. Roots were examined under a fluorescence microscope. Purple arrows label fluorescent roots and the blue arrows indicate roots not producing fluorescence. **C**, Western blotting confirming the expression of *PsPSR2*-YFP-HA fusion proteins in the fluorescent hairy roots. Total proteins were extracted from the fluorescent or nonfluorescent roots and *PsPSR2*-YFP-HA was detected using anti-HA antibody. *PSR1*-HA expressed in *Nicotiana benthamiana* was used as a control. The protein gel was stained with Coomassie blue as the loading control. Experiments were repeated three times with similar results.

RESULTS

PsPSR2 belongs to a conserved RxLR effector family in *Phytophthora* spp.

In order to understand the conservation and distribution of *PsPSR2*-like genes in *Phytophthora* spp., we analyzed the genome sequences of eight *Phytophthora* spp., including *P. sojae* (Tyler et al. 2006), *P. capsici* (Lamour et al. 2012), *P. infestans* (Haas et al. 2009), *P. parasitica*, *P. ramorum* (Tyler et al. 2006), *P. cinnamomi* var. *robiniae*, *P. melonis*, and *P. litchi*. Remarkably, we identified at least one *PsPSR2* homolog in each of the *Phytophthora* genomes that were examined. All of these *PsPSR2* homologs encode proteins containing the conserved N-terminal signal peptide and the RxLR-dEER motif (Supplementary Table 1), suggesting that they are cytoplasmic effectors. In addition, a *PsPSR2*-like effector gene (*Hyaar_807752*) was also found in the genome of *Hyaloperonospora arabidopsidis*, which is an obligate oomycete pathogen. Unlike the *PsPSR2* homologs in *Phytophthora* spp., the deduced amino acid sequence of *Hyaar_807752* only has a conserved N-terminal secretion signal and a dEER-like motif (DEDEE DER) but does not have a typical RxLR motif. Although it remains to be determined whether *Hyaar_807752*, indeed, encodes a cytoplasmic effector, these data strongly suggest that the *PsPSR2* family of RxLR effectors is widely distributed in *Phytophthora* spp. and possibly also present in other oomycete pathogens.

Phylogenetic analysis placed the *PsPSR2* homologs into three major clades (Fig. 1A), which are highly congruent to the species phylogeny of the genus *Phytophthora* based on internal transcribed spacer (ITS) sequences (Fig. 1B). Two clades—one including *P. sojae*, *P. cinnamomi*, and *P. melonis* and the other including *P. infestans* and *P. parasitica*—are identical in these trees, indicating that *PsPSR2*-like effectors belong to an evolutionarily conserved family that has diverged together with the speciation of *Phytophthora*. The prevalence of *PsPSR2* homologs in *Phytophthora* spp. supports the argument that this effector family may play a fundamental virulence function.

A *PsPSR2* homolog in *P. infestans* is also an RNA silencing suppressor.

To investigate whether RNA silencing suppression activity is a conserved function of the *PsPSR2* effector family, we examined a close homolog of *PsPSR2* in *P. infestans* (PITG_15152) using the transgenic *N. benthamiana* line 16c, which constitutively expresses GFP, as previously described (Brigneti et al. 1998). Agroinfiltration of 16c leaves with 35S-GFP triggered transgene silencing of GFP at 4 days postinfiltration (Fig. 2A). As expected, co-expression of known RNA silencing suppressors *PsPSR1*, *PsPSR2*, or *Cucumber mosaic virus protein 2b* (*CMV2b*) with 35S-GFP led to the recovery of green fluorescence (Fig. 2A). Similar to these known RNA silencing suppressors, co-expression of *PiPSR2* with 35S-GFP also suppressed GFP silencing, as indicated by the appearance of green fluorescence in the infiltrated tissue (Fig. 2A). Northern blots confirmed that the expression of *PiPSR2* significantly decreased the accumulation of GFP small RNAs (Fig. 2B). These data demonstrate that, similar to *PsPSR2*, *PiPSR2* possesses RNA silencing suppression activity by inhibiting the biogenesis of small RNAs.

To investigate whether *PiPSR2* could promote *Phytophthora* infection, we expressed either yellow fluorescence protein (YFP) or *PiPSR2*-YFP-HA in *N. benthamiana* leaves, which were subsequently inoculated with zoospores of *P. infestans* 1306. At 5 days postinoculation (dpi), the lesion sizes were evaluated as an indication of plant susceptibility (Fig.

2C). Our results clearly demonstrated that expression of *PiPSR2* enhanced the susceptibility of *N. benthamiana* to *P. infestans*.

Infection process in soybean roots determined by a GFP-labeled *P. sojae* transformant P6497G.

In order to monitor *P. sojae* infection in soybean roots using a fluorescence microscope, we generated a GFP-labeled transformant by introducing the recombinant plasmid pTOR::AcGFP1 into wild-type *P. sojae* P6497. Multiple transformants that stably produce green fluorescence were examined for virulence activity in soybean hypocotyls (Supplementary Fig. 1). One transformant, designated P6497G, showed similar virulence compared with the wild-type strain and was used for further experiments (Supplementary Fig. 2).

Using the P6497G strain, we characterized the infection process of *P. sojae* in soybean roots (Fig. 3A). At 6 h postinoculation (hpi), we observed invasive hyphae that had penetrated root tissues. At 12 hpi, we could observe papilla- or finger-like structures (Fig. 3A, purple triangles), which were formed from invasive hyphae. These could function as haustoria for effector delivery. Extensive hyphae development in the intercellular space of root cells was observed at 24 hpi. At 36 hpi, the inoculated root tissues were filled with hyphae and a small amount of oospores (Fig. 3A, white triangles) were developed. Numerous oospores were formed in the root tissues at 48 hpi (Fig. 3A; Supplementary Fig. 3).

Expression profile of *PsPSR2* in *P. sojae* during infection of soybean roots.

We next analyzed the expression profile of *PsPSR2* during *P. sojae* infection. Transcript abundance of *PsPSR2* was determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using the *Phytophthora actinA* gene (VMD GeneID: Ps108986) as an internal standard. To compare the expression pattern and level of *PsPSR2* with other *P. sojae* effectors, we also examined the expression of the RxLR effector *Avr3b*, which was previously reported to be strongly induced during *P. sojae* infection of soybean leaves (Dong et al. 2011). qRT-PCR results showed that *PsPSR2* did not express until 24 hpi but quickly reached the maximum expression level at approximately 36 hpi (Fig. 3B). The abundance of *PsPSR2* transcripts exhibited a more than twofold decrease at 48 hpi. These data demonstrate that *PsPSR2* is expressed at specific infection stages in a transient manner. In contrast to *PsPSR2*, the expression of *Avr3b* could be detected throughout the infection and as early as 6 hpi. The maximum expression of *Avr3b* was observed at 24 hpi, and the abundance of *Avr3b* transcripts was then decreased by approximately threefold at 36 hpi (Fig. 3B). Compared with *Avr3b*, *PsPSR2* was expressed relatively late during the infection process and at a much weaker (approximately 100-fold) level. In regard to the corresponding infection stages, *PsPSR2* expression seems to coincide with the completion of hyphae extension and the onset of oospore formation at 36 hpi (Fig. 3A). In contrast, the highest expression of *Avr3b* coincided with the extensive development of invasive hyphae at 24 hpi (Fig. 3A).

PsPSR2-expressing soybean roots were hypersusceptible to *P. sojae*.

In order to investigate the virulence function of *PsPSR2*, we generated transgenic roots of 'Harosoy' soybean using *Agrobacterium rhizogenes*-mediated ectopic root induction. A fragment of *PsPSR2* gene encoding a truncated protein without the first 20 amino acids (aa), which contains the secretion signal, was cloned into the binary vector pEG101 (Earley et al. 2006). The recombinant plasmid was introduced into *A. rhizogenes*

K599, which induces hairy roots from soybean cotyledons (Kereszt et al. 2007; Subramanian et al. 2005). In pEG101, *PsPSR2* is under the control of the 35S promoter and in-frame fused to an *YFP* gene and an *HA* tag at the C-terminus. Hairy roots generated from the cotyledons inoculated with *A. rhizogenes* K599 carrying the plasmid pEG104, which harbors 35S-*YFP*, were used as controls.

Hairy roots were developed from the inoculation sites on the cotyledons at 4 to 5 weeks postinoculation (Fig. 4A). As previously reported, only a small portion of the ectopic roots expressed foreign genes (Subramanian et al. 2005); therefore, we used fluorescence microscopy to separate roots that expressed *YFP* or *PsPSR2*-*YFP*-*HA* for *P. sojae* infection. Production of yellow fluorescence was observed in approximately 45% of the roots (Fig. 4B), and the expression of *PsPSR2*-*YFP*-*HA* in these fluorescent roots was further confirmed by Western blotting (Fig. 4C). These roots were detached from the cotyledons and inoculated with zoospore suspensions of *P. sojae* P6497G.

We first determined the ratio of the inoculated roots that allowed successful infection, which was indicated by hyphae development beyond the inoculated region at 24 hpi. In three independent experiments, 50 to 58% of the *YFP*-expressing roots allowed successful infection by *P. sojae* P6497G, whereas a significantly higher percentage (71 to 79%) of the *PsPSR2*-expressing roots were successfully infected (Fig. 5A). We then determined the biomass of *P. sojae* in the inoculated roots using qPCR, which determined the relative abundance of *P. sojae*-specific DNA, represented by the *actinA* gene of *P. sojae*. Consistent with the higher infection rates, a greater biomass of *P. sojae* was accumulated in *PsPSR2*-expressing roots throughout the infection process, with the most significant enhancement observed at 36 and 48 hpi (Fig. 5B). Taken together, these data demonstrated that the expression of *PsPSR2* significantly enhanced the susceptibility of soybean to *P. sojae*.

***PsPSR2* transgenic *Arabidopsis* plants exhibit enhanced susceptibility to *P. capsici*.**

Previously, we generated transgenic *Arabidopsis* plants expressing *PsPSR2* under the *Cauliflower mosaic virus* (CaMV) 35S promoter (Qiao et al. 2013). Further experiments revealed that these *PsPSR2*-expressing plants were defective in the accumulation of trans-acting small interfering RNAs (ta-siRNAs), which is a specific group of secondary siRNAs. However, whether the *PsPSR2*-expressing *Arabidopsis* plants were also defective in defense during *Phytophthora* infection was not determined. Using a recently developed pathosystem in *Arabidopsis* (Wang et al. 2013a), we examined the susceptibility of *PsPSR2*-expressing *Arabidopsis* plants to *P. capsici* isolate LT263, which can develop infection and cause disease symptoms on the leaves of *Arabidopsis* ecotype Col-0.

Based on a previous report (Wang et al. 2013a), more than 90% of *Arabidopsis* leaves developed severe disease symptoms (disease severity index [DSI] = 3) 3 days after the inoculation with mycelial plugs of *P. capsici* LT263. In order to better evaluate potential changes in the susceptibility of *PsPSR2*-expressing plants compared with wild-type Col-0, we modified the published protocol by inoculating detached *Arabidopsis* leaves with droplets of zoospore suspensions.

Disease phenotypes of the inoculated leaves varied from no infection (DSI = 0) to complete wilting with water soaked lesions (DSI = 3) at 4 dpi (Fig. 6A). In wild-type Col-0, we consistently observed approximately 40 to 50% of inoculated leaves that supported successful infection by *P. capsici* LT263 (Fig. 6B; Supplementary Fig. 4), with a mean DSI of approximately 1 (Supplementary Fig. 5). However, two independent *Arabidopsis* transgenic lines, *PSR2*-5 and *PSR2*-10, exhibited significant hypersusceptibility, with more than 70% of the inoculated leaves showing a DSI greater than 1 (Fig. 6B). The average DSI values of two independent experiments for *PSR2*-5 and *PSR2*-10 were 2.1 and 1.8, respectively. In particular, a

Fig. 5. *PsPSR2* promotes *Phytophthora sojae* infection in soybean roots. **A**, Summary of the numbers of roots expressing either yellow fluorescent protein (*YFP*) or *PsPSR2*-*YFP*-*HA* that were successfully infected by *P. sojae* in three independent experiments. In each treatment, 24 roots were dip-inoculated in three tubes (eight roots per tube), each containing 400 μ l of zoospore suspension (10^4 zoospores/ml) of the *P. sojae* P6497G. Numbers of roots supported hyphae expansion beyond the inoculated region were numerated at 24 h postinoculation (hpi) using fluorescence microscopy. **B**, *P. sojae* biomass determined by quantitative polymerase chain reaction (qPCR) in inoculated soybean hairy roots expressing *YFP* or *PsPSR2*-*YFP*. Total DNA was extracted from inoculated soybean roots and used as templates for qPCR using primers amplifying the *actinA* gene of *P. sojae*. Soybean *CYP2* gene was used as the internal standard. Values are means $\pm$ standard deviations (as error bars) ($n = 3$). Significantly increased hyphae accumulation in the *PsPSR2*-*YFP*-expressing roots compared with the *YFP*-expressing roots were labeled with ** or *, representing t test $P < 0.001$ or 0.05, respectively. This experiment was repeated three times with similar results.

significantly larger number of the infected leaves from PSR2-5 and PSR2-10 plants (approximately 60 and 48%, respectively) were completely wilted, equivalent to a DSI of 3; in contrast, only 25% of the inoculated leaves from wild-type Col-0 were classified in this category (Fig. 6B).

To further confirm the enhanced susceptibility of *PsPSR2*-expressing *Arabidopsis* plants to *P. capsici*, we quantified the number of zoosporangia developed from each inoculated leaf at 5 dpi using trypan blue staining followed by microscopy. Based on the number of zoosporangia observed on leaf surface, the inoculated leaves were classified into four categories (0 to 450, 451 to 960, 961 to 1,440, and >1,440 zoosporangia/cm²). Consistent with the exacerbated disease symptoms, a higher number of zoosporangia was formed on the inoculated leaves of PSR2-5 and PSR2-10 plants compared with wild-type Col-0 (Fig. 6C). In addition, qPCR quantifying the biomass of *P. capsici* also showed that PSR2-5 and PSR2-10 plants, especially PSR2-5 plants, allowed significant enhancement of *Phytophthora* biomass accumulation as early as 36 hpi, when *P. capsici*-specific DNA was in a very low abundance in wild-type plants (Fig. 6D). Collectively, these re-

sults strongly suggest that the expression of *PsPSR2* leads to largely enhanced susceptibility of *Arabidopsis* to *P. capsici* LT263.

In all of the above-described experiments, we used a third transgenic line, PSR2-18, which expresses *PsPSR2* at a very low level, as an additional control (Qiao et al. 2013) (Supplementary Fig. 6). This is important because PSR2-18 plants did not show the deficiency of accumulating ta-siRNAs as observed in the other two transgenic lines, PSR2-5 and PSR2-10 (Qiao et al. 2013). Consistently, PSR2-18 also exhibited a similar susceptibility to *P. capsici* infection as wild-type Col-0 plants (Fig. 6). Furthermore, *PsPSR2* is expressed at a higher level in PSR2-5 than in PSR2-10. As a result, PSR2-5 was more susceptible to *P. capsici* than PSR2-10 in all these experiments (Fig. 6). For example, the qPCR assay showed as much as twofold higher biomass of *P. capsici* in PSR2-5 than in PSR2-10 (Fig. 6D). Taken together, using the *PsPSR2*-expressing transgenic *Arabidopsis* lines, our experiments demonstrated a strong correlation between the expression levels of *PsPSR2* and the enhanced susceptibility to *P. capsici*.

Fig. 6. *PsPSR2*-expressing *Arabidopsis* plants are hypersusceptible to *Phytophthora capsici* isolate LT263. **A**, Disease symptoms on wild-type *Arabidopsis* ecotype Col-0 leaves exhibiting various disease severity. Detached adult leaves were inoculated with a zoospore suspension (10^5 zoospores/ml) of *P. capsici* isolate LT263. Disease severity index (DSI) was determined at 4 days postinoculation (dpi). In each panel, the image on the left shows disease symptoms and the image on the right shows the same leaf after trypan blue staining. **B**, Disease severity of *Arabidopsis* plants inoculated with *P. capsici* isolate LT263. Thirty leaves from each *Arabidopsis* line (wild type Col-0 and three independent *PsPSR2*-expressing transgenic lines [PSR2-5, PSR2-10, and PSR2-18]) were inoculated with zoospore suspensions. **C**, Zoosporangia formation on *P. capsici*-inoculated leaves of wild type (Col-0) and *PsPSR2*-expressing *Arabidopsis* plants. Each inoculated leaf was stained with trypan blue and examined under a microscope at 5 dpi. Based on the number of zoosporangia, the leaves were classified into four groups: 0 to 480, 481 to 960, 961 to 1,440, or >1,440 zoosporangia/cm². Percentages of leaves in each of the four groups are presented. **D**, Biomass of *P. capsici* determined by quantitative polymerase chain reaction (qPCR) in infected *Arabidopsis* leaves using primers amplifying the internal transcribed spacer region of *P. capsici*. The *Arabidopsis* gene encoding the large subunit of RuBisCo was used as the internal standard. Values are means $\pm$ standard deviations (as error bars) ($n = 3$). Different letters indicate significant differences, as statistically analyzed by Fisher's protected least significant difference test ($P < 0.05$). Experiments were repeated two times with similar results. **E**, *Phytophthora*-induced *pathogenesis-related 1* (*PR1*) expression is abolished in *PsPSR2*-expressing *Arabidopsis* plants. Abundances of *PR1* transcripts in *P. capsici*-infected *Arabidopsis* leaves were determined by reverse-transcriptase qPCR at 0, 12, and 24 h post zoospore inoculation (hpi). The *Arabidopsis* *UBQ10* gene was used as the internal standard. Values are means $\pm$ standard deviations (as error bars) ($n = 3$). Significant reductions on the *PR1* transcript levels in *PsPSR2*-expressing plants compared with wild type plants were labeled with *, representing t test $P < 0.05$. This experiment was repeated two times with similar results.

Salicylic acid pathway is inhibited in *PsPSR2*-expressing *Arabidopsis* plants during *Phytophthora* infection.

In order to further understand the mechanism by which *PsPSR2* promotes *Phytophthora* infection, we analyzed the expression of the *pathogenesis-related 1 (PR1)* gene, which is a marker for the salicylic acid (SA)-dependent defense pathway (Wildermuth et al. 2001), in *PsPSR2*-expressing and wild-type *Arabidopsis* plants during *P. capsici* infection. Previous reports indicate that *PR1* is induced by *P. capsici* infection in *Arabidopsis* and that the SA signaling pathway is required for defense response (Wang et al. 2013a and b). We also observed increased accumulation of *PR1* transcripts in wild-type Col-0 plants upon *P. capsici* infection at 12 and 24 hpi; however, *PR1* induction was completely abolished in the *PsPSR2*-expressing lines (Fig. 6E). Thus, *PsPSR2* may function as a negative regulator in SA-dependent defense responses.

PsPSR2 does not promote bacterial infection.

Experiments described previously (Qiao et al. 2013) and in this report strongly suggest that *PsPSR2* promotes *Phytophthora* infection in *N. benthamiana*, soybean, and *Arabidopsis*; therefore, we next examined whether *PsPSR2* could also promote bacterial infection. For this purpose, we monitored the bacterial titer in *PsPSR2*-expressing and wild-type *Arabidopsis* plants after inoculation with the virulent *Pseudomonas syringae* pv. *tomato* DC3000 or a nonpathogenic mutant, DC3000 – *hrcC*–. The *hrcC* mutant can no longer inhibit PTI responses due to its inability to inject effector proteins through the type III secretion system (Boccardo et al. 2014; Bonas 1994; Heese et al. 2007; Roine et al. 1997). Three days after the plants were inoculated with DC3000 or the *hrcC* mutant, no significant changes in bacterial population was observed in *PSR2-5* or *PSR2-10* plants (Supplementary Fig. 7). These experiments suggest that *PsPSR2* specifically promotes *Phytophthora* infection.

DISCUSSION

A broad range of parasites, including viruses, bacteria, fungi, oomycetes, protozoa, insects, and nematodes, subvert host immunity through the functions of effectors (Dangl et al. 2013). These virulence proteins directly manipulate specific physiological processes or signaling pathways in host cells for the benefit of infection. Although tremendous efforts have been committed to study the functions of bacterial and viral effectors, we are only starting to understand how oomycete and fungal effectors promote disease development. This is extremely important because genome sequence analysis revealed an enormous number of effector proteins produced by these eukaryotic pathogens but the functions of these effectors are largely unknown.

We recently identified two *Phytophthora sojae* effectors with RNA silencing suppression activity in plants (Qiao et al. 2013). In this article, we report detailed characterizations of *PsPSR2* using two pathosystems: the natural soybean root–*P. sojae* interaction and the model *Arabidopsis* leaf–*P. capsici* interaction. In both pathosystems, expression of *PsPSR2* in plants rendered hypersusceptibility to *Phytophthora* infection. This result is consistent with our previous data, showing that transient expression of *PsPSR2* in *N. benthamiana* enhanced the infection of *P. infestans* (Qiao et al. 2013).

RNA silencing is a universal gene regulation mechanism in eukaryotes that impacts many processes, including plant immunity, through the function of small RNAs. Plants produce two major types of small RNAs, microRNAs (miRNAs) that are encoded by endogenous *MIR* genes and siRNAs that are derived from invading nucleic acids such as viruses and trans-

genes, and endogenous loci such as repeats, transposable elements, and genes (Chen 2010). In *Arabidopsis*, *PsPSR2* specifically affects the biogenesis of a small fraction of siRNAs called ta-siRNAs, which are secondary siRNAs generated from miRNA-targeted transcripts (Qiao et al. 2013). Interestingly, ta-siRNAs were proposed to regulate the canonical plant resistance genes in legumes and *N. benthamiana* (Li et al. 2012; Shivaprasad et al. 2012; Wong et al. 2014; Zhai et al. 2011), suggesting a direct link between ta-siRNAs and plant defense regulation. However, whether *PsPSR2* was able to promote *Phytophthora* infection in *Arabidopsis* could not be tested until the recent development of the *Arabidopsis*–*P. capsici* pathosystem (Wang et al. 2013a).

Here, we showed that *PsPSR2*-expressing *Arabidopsis* plants were hypersusceptible to *P. capsici* isolate LT263. Importantly, the enhanced susceptibility of *PsPSR2*-expressing *Arabidopsis* plants was only observed in two independent transgenic lines (i.e., *PSR2-5* and *PSR2-10*) that also exhibited significantly reduced abundances of ta-siRNAs (Qiao et al. 2013). In contrast, a third *PsPSR2*-expressing *Arabidopsis* line, *PSR2-18*, that was not defective in ta-siRNA accumulation due to a low expression level of *PsPSR2* also showed unaltered susceptibility to *P. capsici*. These data show a correlation between reduced levels of ta-siRNAs and enhanced susceptibility of *Arabidopsis*, suggesting that *PsPSR2* may promote infection by interfering with specific ta-siRNAs in plants. Our ongoing effort is to examine the function of ta-siRNAs in regulating plant defense during *Phytophthora* infection. Moreover, *PsPSR2*-expressing plants did not exhibit altered resistance to the bacterial pathogen *Pseudomonas syringae*, consistent with the argument that the virulence activity of *PsPSR2* is specific to *Phytophthora* infection.

A previous report showed that SA-responsive genes were induced in *Arabidopsis* upon infection with *Phytophthora capsici* (Wang et al. 2013a and b). Similarly, we also observed enhanced accumulation of *PR1* transcripts in wild-type plants inoculated by *P. capsici*. Remarkably, the *PR1* induction was completely abolished in the *PsPSR2*-expressing plants, indicating that *PsPSR2* may promote *Phytophthora* infection by interfering with the SA-dependent defense pathway.

Effectors are well known to be fast evolving. Different species of pathogens usually produce largely divergent effector repertoires, whose evolution is shaped by the adaptation to infect specific hosts (Chisholm et al. 2006; Dong et al. 2014; Ma and Guttman 2008; Ma et al. 2006; Stavrinides et al. 2008). Extensive genetic and expression polymorphisms have been observed in *Phytophthora* RxLR effectors and conserved effectors are rare (Baxter et al. 2010). In a comparative analysis on predicted RxLR effectors produced by *H. arabidopsidis*, *P. sojae*, and the sudden oak death pathogen *P. ramorum*, as few as only four trios of genes were revealed to be 1:1:1 orthologous in all three genomes (Win et al. 2007). Limited overlaps in the sets of effectors were also observed when comparing any of the two genomes, and the sequences with similarity often involved the secretion signal peptide and the RxLR motif rather than the C-terminal effector region (Win et al. 2007). Although *PsPSR2* was originally identified from *P. sojae*, sequence analysis revealed that it is a conserved RxLR effector produced by most, if not all, *Phytophthora* spp. In addition to *Phytophthora* spp., we also found a homologous gene in the obligate oomycete pathogen *H. arabidopsidis*, indicating that *PsPSR2* might be an ancient virulence gene of oomycetes. Importantly, PiPSR2, a close homolog of *PsPSR2* in *P. infestans*, also possesses the activities to suppress RNA silencing and promote *Phytophthora* infection. Furthermore, *PsPSR2* was found to be highly conserved with no polymorphism in the four major races of *P. sojae* (Wang et al. 2011),

although many effector genes are strain specific (Haas et al. 2009). The prevalence and sequence or function conservation in various *Phytophthora* spp. and *P. sojae* races strongly suggests an essential virulence function of the PSR2 effector family, likely through its inhibitory effects on host small RNA biogenesis during infection.

MATERIALS AND METHODS

Plant materials.

N. benthamiana plants were grown at 22°C with a 16-h photoperiod. Fully expanded leaves at the six-leaf stage were infiltrated with *A. tumefaciens* strains carrying binary vectors harboring *35S::GFP* or *35S::PSR2* effector genes.

Soybean (*Glycine max*) Harosoy, a susceptible host of the *P. sojae* P6497, was used in all the *P. sojae* infection experiments. Soybean seed were surface sterilized and germinated as previously described (Morgan et al. 2010). Briefly, seed were soaked in 10% bleach solution for 10 min and then rinsed thoroughly with sterile water five to six times. Approximately 25 seeds were placed on top of two layers of wet Whatman filter paper in a sterile glass petri plate. The seed were covered with two additional layers of filter paper and germinated in the dark at room temperature for 4 days. Sterile water was added as needed to keep the filter paper moist. The germinated seed were planted into sterile vermiculites (Crop Production Services) supplemented with Peter's complete Nutrient solution (1g/liter; Crop Production Services) and grown in a growth chamber with a constant temperature of 22°C and a 12-h photoperiod.

Arabidopsis thaliana seed were sown in soil and stratified at 4°C for 3 days. The plants were grown in a conditioned growth room (19 to 21°C, 16-h photoperiod, and relative humidity of 75 to 80%).

Bacteria and *Phytophthora* strains and growth conditions.

Bacteria and *Phytophthora* strains used in this article are listed in Supplementary Table 2.

Agrobacterium strains were grown on Luria-Bertani (LB) medium at 28°C for 2 days. The medium was supplemented with kanamycin at 50 µg/ml, rifampicin at 50 µg/ml, or gentamycin at 50 µg/ml when necessary.

Pseudomonas syringae strains were grown overnight at 28°C on King's B medium (protease peptone at 20 g liter⁻¹, K₂HPO₄ at 1.5 ml liter⁻¹, and glycerol at 15 ml liter⁻¹) supplemented with rifampicin (50 µg/ml).

P. sojae P6497 was cultured in 10% (vol/vol) V8 medium. The GFP-labeled transformant *P. sojae* P6497G was grown in 10% V8 medium supplemented with geneticin at 40 µg/ml. *P. capsici* isolate LT263 was cultured in 20% V8 agar plates. They were grown at 25°C in the dark for 3 to 4 days. *P. infestans* 1306 was cultured on rye agar at 18°C for 10 days.

Plasmid construction and bacterial transformation.

A fragment of *PiPSR2* (*PITG_15152*), excluding the sequences encoding the N-terminal secretion signal (1 to 22 aa), was cloned from *P. infestans* 1306 using gene-specific primers (Supplementary Table 3). The *PsPSR2* gene without the first 60 nt encoding the secretion signal was cloned from *P. sojae* P6497 using gene-specific primers. PCR products were cloned into the Gateway entry vector pENTR1A (Invitrogen), and subsequently transferred to the destination vector pEG100 and pEG101 (Earley et al. 2006) using Gateway LR reaction. The resulting recombinant plasmids were transformed into *Agrobacterium rhizogenes* K599 (Savka et al. 1990) or *A. tumefaciens* GV3101 (Wroblewski et al. 2005) using the freeze-thaw method (Hofgen and Willmitzer 1988). The confirmed transformants were then used for transiently expressing the corresponding effector genes

in *N. benthamiana* or soybean using previously described protocols (Kereszt et al. 2007; Zhou et al. 2011).

Sequence analysis of *PsPSR2* homologs in oomycetes.

The amino acid sequence of *PsPSR2* was used to search against the genome sequences of different *Phytophthora* spp. and *H. arabidopsidis* for potential homologs. We used the program tblastn with an *E* value of 1e-30 as the cut-off. The closest homolog of *PsPSR2* from each species was determined by the lowest *E* value.

Genome sequence of *P. sojae* (v1.1), *P. ramorum* (v1.1), and *P. capsici* (v11.0) was obtained from the Department of Energy Joint Genome Institute database. Genome sequences of *P. infestans* (release of 6/15/2009) and *P. parasitica* (release of 3/26/2013) were downloaded from the Broad Institute website, and the genome sequence of *H. arabidopsidis* (v8.3) was obtained from the VBI Microbial Database. Genome sequences of *P. melonis*, *P. cinnamomi* var. *robiniae*, and *P. litchii* are currently unpublished (Y. Wang, Nanjing Agricultural University).

Neighbor-joining trees were generated using MEGA 5 with 1,000 bootstraps and other default parameters.

RNAi suppression assay of *PiPSR2*.

N. benthamiana 16c leaves were infiltrated with *A. tumefaciens* strains carrying *35S-GFP* and one of the following constructs: *35S-CMV2b*, *35S-PsPSR1*, *35S-PsPSR2*, and *35S-PiPSR2*. Green fluorescence was visualized at 5 dpi using a handheld long-wavelength UV lamp (Blak-Ray B-100AP). The abundance of *GFP* siRNA was examined at 4 days after *Agrobacterium* infiltration by RNA blotting using [α -³²P]-labeled random primes that cover full length of the *GFP* gene. U6 served as a loading control.

P. infestans infection assay.

N. benthamiana leaves (4 to 5 weeks old) were infiltrated with a cell suspension of *A. tumefaciens* at an optical density at 600 nm (OD₆₀₀) of 0.3. The *A. tumefaciens* strains carry either the vector pEG104, which expresses YFP, or pEG101::*35S-PiPSR2*, which expresses PiPSR2-YFP-HA. At 24 h after agroinfiltration, the leaves were detached and inoculated with zoospore suspensions of *P. infestans* 1306 (10⁴ spores/ml) by dropping 50 µl of the suspension onto the abaxial side. Inoculated leaves were incubated for 5 days on 0.8% agar plate before lesions from *P. infestans* infection were measured.

Generation of GFP-labeled *P. sojae* P6497G.

The *AcGFP1* gene was amplified from pACGFP1 (Clontech) using primers AcGFP-ClaI-F and AcGFP-SacII-R and cloned into the vector pTOR::*mRFP* (Whisson et al. 2007) by replacing the *mRFP* gene. pTOR::*AcGFP1* was introduced into *P. sojae* P6497 using polyethylene glycol-mediated protoplast transformation, as previously described (Zhang et al. 2012). Putative transformants were selected on 10% V8 medium containing geneticin at 40 µg/ml. Several transformants were examined for GFP production using a fluorescent microscope (Olympus Fluorescent Binocular Microscope SZX16 with excitation wavelength 460 to 480 nm and emission wavelength 495 to 540 nm) and virulence activity using the soybean hypocotyl infection assay (Zhang et al. 2012). One transformant with relatively strong GFP production in all developmental stages and similar virulence on soybean compared with wild-type P6497 was selected for further experiments. This transformant was designated P6497G.

Soybean cotyledon transformation with *A. rhizogenes*.

Soybean cotyledons were inoculated with *A. rhizogenes* K599 carrying pEG104 or pEG101::*PsPSR2*, as previously de-

scribed (Kereszt et al. 2007; Savka et al. 1990). Individual cotyledons were collected from 10-day-old soybean seedlings. Detached cotyledons were surface sterilized with 70% ethanol before a small, roughly circular (approximately 0.4 cm in diameter) cut was made in each cotyledon at approximately 0.3 cm from the petiole end. The wounded cotyledons were then transferred to a sterile petri plate containing 0.8% agar.

A. rhizogenes cells grown in LB medium supplemented with kanamycin were washed and resuspended in 10 mM MgSO₄ to a final concentration of OD₆₀₀ = 0.3. The cell suspension (20 µl) was directly spotted onto the wounded site of each cotyledon. The inoculated cotyledons were incubated in a growth chamber at 22°C and a regime of 16 h of light and 8 h of darkness, with high humidity. Hairy roots usually started to generate from the wounded sites at approximately 3 weeks after inoculation.

Confirmation of *PsPSR2* expression in hairy roots.

pEG104 carries the *YFP* gene under the control of the CaMV 35S promoter; and pEG101::*PsPSR2* carries the *PsPSR2-YFP-HA* gene that is also under the control of the 35S promoter. We used a fluorescence microscope to select *YFP*- or *PsPSR2-YFP-HA*-expressing roots. Yellow fluorescence was detected in hairy roots using a fluorescence stereomicroscope (Leica MZ FLIII) with a GFP2 filter (excitation 480/40 nm, emission 510 nm). The expression of *PsPSR2-YFP-HA* proteins in the fluorescent roots was also confirmed by Western blotting. In this experiment, total proteins were extracted from the ground root tissues using 2× Laemmli buffer (Laemmli 1970) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. *PsPSR2-YFP-HA* proteins were detected using an anti-HA antibody (Genetex).

P. sojae infection of soybean hairy roots.

P. sojae zoospores used for soybean infection assays were induced as previously described (Erwin and Ribeiro 1996). Zoospores were filtered through one layer of Miracloth (Calbiochem) and adjusted to desired concentrations (10⁴ or 10⁶ zoospores/ml) using sterile mineral water.

Hairy roots expressing *YFP* or *PsPSR2-YFP* with similar lengths (approximately 3.5 cm) were excised from cotyledons and inoculated by dipping into a 1.5-ml Eppendorf tube containing 400 µl of zoospore suspensions (10⁴ zoospores/ml) of the *P. sojae* P6497G for 5 min. For each treatment, 24 roots were treated in three tubes. Inoculated roots were placed in plastic plates containing 0.8% agar and incubated in the dark with high humidity. The number of roots that allowed hyphae extension beyond the inoculation region (approximately 12 mm from the root tips, corresponding to 400 µl of zoospore suspension that was used for inoculation) was numerated at 24 hpi. *P. sojae* biomass was determined by qPCR using primers designed to amplify the *actinA* gene (VMD GeneID: Ps108986). Total DNA was extracted from *P. sojae*-infected root tissues using a DNeasy plant mini kit (Qiagen). The soybean house-keeping gene *CYP2* (TC224926) was used as the internal standard for normalization. PCR reactions were performed on a CFX96 Touch Real-Time PCR detection system (Bio-Rad).

Infection progression was monitored by microscopy at 6, 12, 24, 36, and 48 h after detached roots were inoculated with a higher concentration of zoospore suspensions (10⁶ zoospores/ml). Development of *P. sojae* in soybean roots was visualized using a Zeiss Imager.A1 fluorescence microscope.

P. capsici infection assay of *Arabidopsis* leaves.

Adult leaves from 4-week-old *Arabidopsis* plants, including wild-type Col-0 and three transgenic lines carrying 35S-3×*FLAG-PsPSR2* (PSR2-5, PSR2-10, and PSR2-18), were

inoculated by *P. capsici* isolate LT263. Note that PSR2-18 did not exhibit a deficiency in ta-siRNA accumulation, presumably due to a low expression level of *PsPSR2* (Qiao et al. 2013).

In each experiment, 30 adult leaves with similar sizes were excised from approximately 10 plants of each line and placed in plates containing 0.8% agar. The leaves were inoculated with 10 µl of zoospore suspension (10⁵ zoospores/ml) as a droplet on the abaxial side. The plates were wrapped with Parafilm and incubated at 22°C in the dark during the first 24 h after inoculation, then transferred to a growth chamber at 22°C with 12-h photoperiod.

Disease severity was evaluated at 4 dpi using a DSI on a scale of 0 to 3. Leaves with no visible disease symptoms or only small necrotic flecks restricted to the inoculation area were classified as a DSI score of 0. A leaf with a DSI score of 1 means that it had lesions spreading from the inoculation spot but the lesion only covered less than 50% of the leaf. A DSI score of 2 means that the lesion covered 50 to 75% of the leaf surface. A DSI score of 3 means that the leaf was completely wilted and fully covered with lesions. The mean DSI was calculated as previously described (Wang et al. 2013a).

The susceptibility of *Arabidopsis* to *P. capsici* isolate LT263 was also evaluated by the number of zoosporangia generated on the leaf surface. The inoculated leaves were stained with trypan blue at 5 dpi, as previously described (Bouwmeester et al. 2011). Zoosporangia were numerated under a Zeiss Imager.A1 fluorescence microscope using a hemacytometer. The numbers of zoosporangia per square centimeter were then determined.

The biomass of *P. capsici* in the inoculated *Arabidopsis* leaf tissues was determined by qPCR during a time course (24, 36, 60, and 72 hpi) using *Phytophthora*-specific primers amplifying the ITS region (Silvar et al. 2005). The primer pair AtRub-F4 and AtRub-R4 amplifying the gene encoding the large sub-unit of RuBisCo was used as the internal standard.

Bacterial infection assay of *Arabidopsis*.

Pseudomonas syringae pv. *tomato* strains DC3000 or the *hrcC* mutant were grown overnight and resuspended in 10 mM MgSO₄ to a final concentrations of 5 × 10⁴ and 5 × 10⁵ CFU/ml, respectively. *Arabidopsis* leaves were infiltrated with bacteria suspensions using a needleless syringe. Leaves were harvested immediately (0 dpi) or 3 dpi and examined for in planta bacterial population, as previously described (Jiang et al. 2013).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Department of Energy Joint Genome Institute: genome.jgi.doe.gov
 Broad Institute: www.broadinstitute.org
 Virginia Bioinformatics Institute Microbial database: eumicrobedb.org
 MEGA 5 software: www.megasoftware.net