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Characterization of the *Phytophthora Sojae* RNA Silencing Repressors

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Cell, Molecular and Developmental Biology

by

Cristina Flores

September 2012

Thesis Committee:
Dr. Wenbo Ma, Chairperson
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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE THESIS

Characterization of the *Phytophthora Sojae* RNA Silencing Repressors

By

Cristina Flores

Master of Science, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, September 2012
Dr. Wenbo Ma, Chairperson

Phytophthora sojae is an oomycete pathogen that causes soybean *(Glycine max)* stem and root rot, the second most destructive soybean disease. *P. sojae* belongs to the genus *Phytophthora* which includes other important plant pathogens such as the potato late blight pathogen *Phytophthora infestans* and sudden oak death pathogen *Phytophthora ramorum*. These eukaryotic pathogens cause billions of dollars of crop damage each year. However, their pathogenesis is poorly understood.

As many other plant pathogens, *Phytophthora* utilizes effector proteins to suppress host defense and cause disease. *P. sojae* is predicted to produce more than 400 effectors that contain the conserved host cell entry motif RxLR-dEER; however, most of these effectors are functionally uncharacterized. In order to understand the mechanisms underlying effector-mediated suppression of host defense, a screen was conducted in our laboratory to identify effectors targeting the RNA silencing pathway. Using a post-transcriptional gene silencing (PTGS) assay, two **<u>P</u>**hytophthora **<u>S</u>**uppressors of **<u>R</u>**NA

silencing effectors (PSRs) were indentified. The main goal of my thesis work is to investigate the virulence function of PSRs during pathogen infection. My experiments showed that PSR1 enhances the virulence of Potato Virus X in *Nicotiana benthamiana*. Furthermore, PSR1 and PSR2 may also enhance the virulence of *P. sojae* by accelerating the sporulation of the pathogen in soybean roots. Taken together, these results suggest that *Phytophthora* pathogens have evolved effectors that promote infection by targeting the RNA silencing pathway in plant hosts.

Understanding how these effector proteins target different functional branches of the plant immune systems would advance our understanding of *Phytophthora* pathogenesis. This knowledge will contribute to the development of disease resistant crops.

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INTRODUCTION

Phytophthora sojae: The life cycle of an eukaryotic pathogen

Phytophthora sojae is an oomycete with a genome size of 95 Mbp and belongs to the kingdom of Stramenopiles which includes brown algae and diatom (Tyler et al., 2007). *P. sojae* is hemibiotrophic pathogen and causes stem and root rot disease in soybean (*Glycine max*). It is estimated that this pathogen is responsible for millions of dollars of crop damage each year (Wrather and Koenning, 2006). Environmental conditions play a key role in the development and growth of *P. sojae* in soybean. Under favorable conditions, which usually involve flooding, the pathogen can germinate and infect its host. This infection can then spread from the original site of infection in the roots to the stem and the rest of the plant, resulting in the ultimate death of the plant.

The life cycle of *P. sojae* can be divided into asexual and sexual stages in which the pathogen exists in different morphological forms, which allow the pathogen to either infect the plants or remain dormant in soil under unfavorable conditions (**Figure 1**). The production of oospores occurs in the sexual stage and the resulting thick-cell walled spores can remain dormant in soil for years or in between growing seasons (Tyler et al., 2007). In the asexual stage, zoospores, cysts, mycelia and sporangia are produced and responsible for the spread of the disease in soybean fields (Savidor et al., 2008). The cell-wall-less zoospores are very sensitive to soybean isoflavones daidzein and genistein in the nanomolar range (Morris and Ward 1992 and Tyler et al., 1996). These isoflavones and other nutrients released by soybean roots attract the zoospores to the primary

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infection sites (Morris and Ward 1992 and Tyler et al., 1996). A putative G-coupled protein receptor GRP11 was suggested to play a key role in the virulence of *P. sojae* in the asexual stage by facilitating the encystment and germination of zoospores (Wang et al., 2010). Zoospores can differentiate to form an adhesive cyst (Tyler et al., 2007). This adhesive cyst can germinate to produce a hyphae or secondary zoospore which can infect the plants (Tyler et al., 2007). The hyphae allow the penetration of the soybean root tissue and in less than ten hours the feeding structures called haustoria are produced and facilitate the acquisition of nutrients from the host (Torto-Alabido et al., 2007). At 24 hours post-infection, many plants cells start dying since the deep cell layers within the roots have been colonized by hyphae (Torto-Alabido et al., 2007). *P. sojae* is hemibiotrophic and secretes the *P. sojae* necrosis-inducing protein (NIP) to accelerate host cell death, which facilitates its transition from biotrophy to necrotrophy (Qutob et al., 2002). Prior to the 48 hour time point after infection the host tissue collapses due to the macerations caused by the water-soaked lesions (Bhattacharyya and Ward 1986, Moy et al., 2004, Stossel et al., 1980 and Torto-Alabido et al., 2007). At this time point the pathogen transitions into necrotrophy from biotrophy and the host plant dies. At this time point sporulation also occurs and oospores are produced and released into the environment. The cycle can then be repeated with other plants.
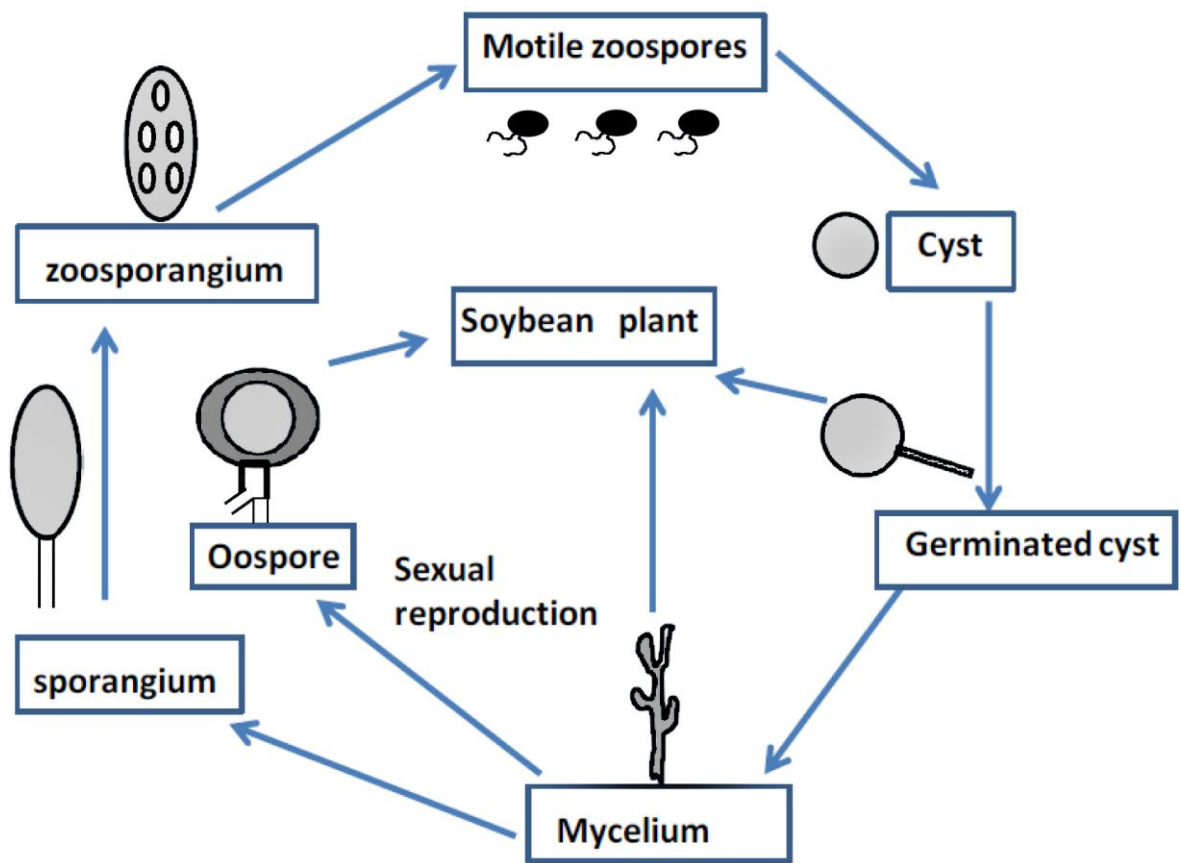
Figure 1. The life cycle of *Phytophthora sojae*. The life cycle initiates with motile zoospores which can form a cyst and germinate to infect soybean roots and produce mycelium. Mycelium can give rise to sporangium which can produce zoosporangium and release motile zoospores. During sexual reproduction, oospores are produced to allow *P. sojae* to remain dormant in the soil.

Subversion of plant defenses by plant pathogens

Pathogens and plants are engaged in an arms race

Plants have evolved diverse defense responses including cell death, cell wall thickening, and the productions of reactive oxygen species, defense hormones including salicylic acid, jasmonates and ethylene, and antimicrobial compounds to protect themselves from microbial pathogens   (Lawton et al., 1995; Lamb and Dixon 1997; Niki et al., 1998 and Livaja et al., 2008) (**Figure 2**).

Plants have two functional branches of plant defense: the Pathogen-associated Molecular Pattern-triggered immunity (PTI) and the Effector-triggered immunity (ETI) (Jones and Dangl, 2006). These two branches are overlapping and respond to different elicitors. PTI responds to conserved microbial structures of potential pathogens and protects plants from the vast majority of attempted infections (Jones and Dangl, 2006 and Boller and Felix 2009). ETI responds to effector proteins that are secreted from the pathogens and function inside the host cells (Jones and Dangl, 2006). The arms race between pathogens and hosts is best illustrated by the zigzag model proposed by Jones and Dangl (2006). According to this model, there are four phases during the arms race between plants and pathogens. In the first phase, pathogen-associate molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on the plasma membrane of plant cells. The activation of PRRs initiates signal transduction pathways that leads to defense responses called the basal defense. In the second phase, a successful pathogen secretes effectors that suppress PTI and thus allowing it to successfully colonize the host. In the

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third phase, plant evolved cytoplasmic resistance (R) proteins which contain a conserved nucleotide binding-leucine rich repeat (NB-LRR) motif that directly or indirectly recognize specific effectors and trigger ETI. ETI is usually associated with a rapid programmed cell death, called hypersensitive response (HR). However, successful pathogens could evade or suppress ETI by effector evolution, as illustrated in the fourth phase. In summary, plants and pathogens are engaged in an endless evolutionary arms race with the central players being pathogen effectors and the plant defense mechanisms.
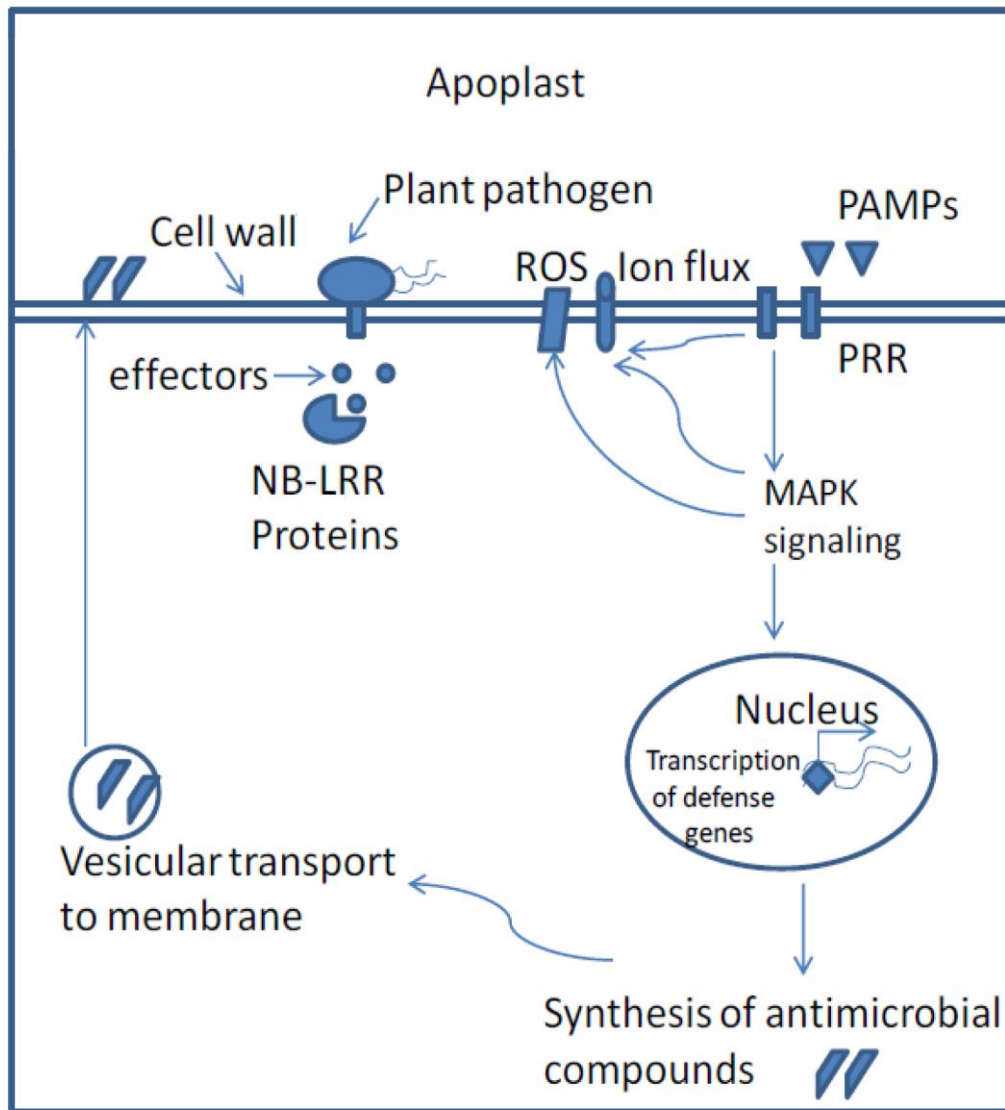
Figure 2. Different plant defense mechanisms triggered by Pathogen-associated molecular patterns (PAMPs) and effectors.

This figure is modified from Göhre and Robatzek, 2008.

RNA silencing as a plant defense mechanism

RNA silencing is a conserved and important mechanism for gene regulation in eukaryotes (Meister and Tuschl 2004). A central component of RNA silencing is the small RNAs, which are single-stranded RNA molecules generally 20-30 nt in length. Two major types of small RNA molecules, microRNA (miRNA) and small interference RNA (siRNA) have been identified from plants (**Figure 3**). Small RNAs guide the sequence-specific cleavage of target RNAs and/or suppress the translation process of their RNA targets. In this way, small RNAs regulate gene expression (Baumberger and Baulcombe 2005).

Past studies have suggested that RNA silencing plays an important role in plant defense against RNA viruses (Brigneti et al., 1998). Double-stranded viral RNAs are detected by plant hosts as invading genes and used as templates to produce siRNAs, which then attack the viral genome by AGONAUTE (AGO)-mediated cleavage, and thereby restricting viral replication (**Figure 3**). miRNA has also been reported to regulate plant defense against pathogens. For example, the production of miRNA393 is induced by the major PAMP flg22 of bacterial pathogens during bacterial infection (Navarro et al., 2006). miRNA393 down-regulates the expression of the auxin receptor genes and inhibits auxin signaling, which results in increased resistance of Arabidopsis to bacterial infection (Navarro et al., 2006).

Figure 3. RNA silencing Pathways resulting in RNA cleavage, translational repression and chromatin modification. Viral suppressors target different steps of this pathway to promote infection.
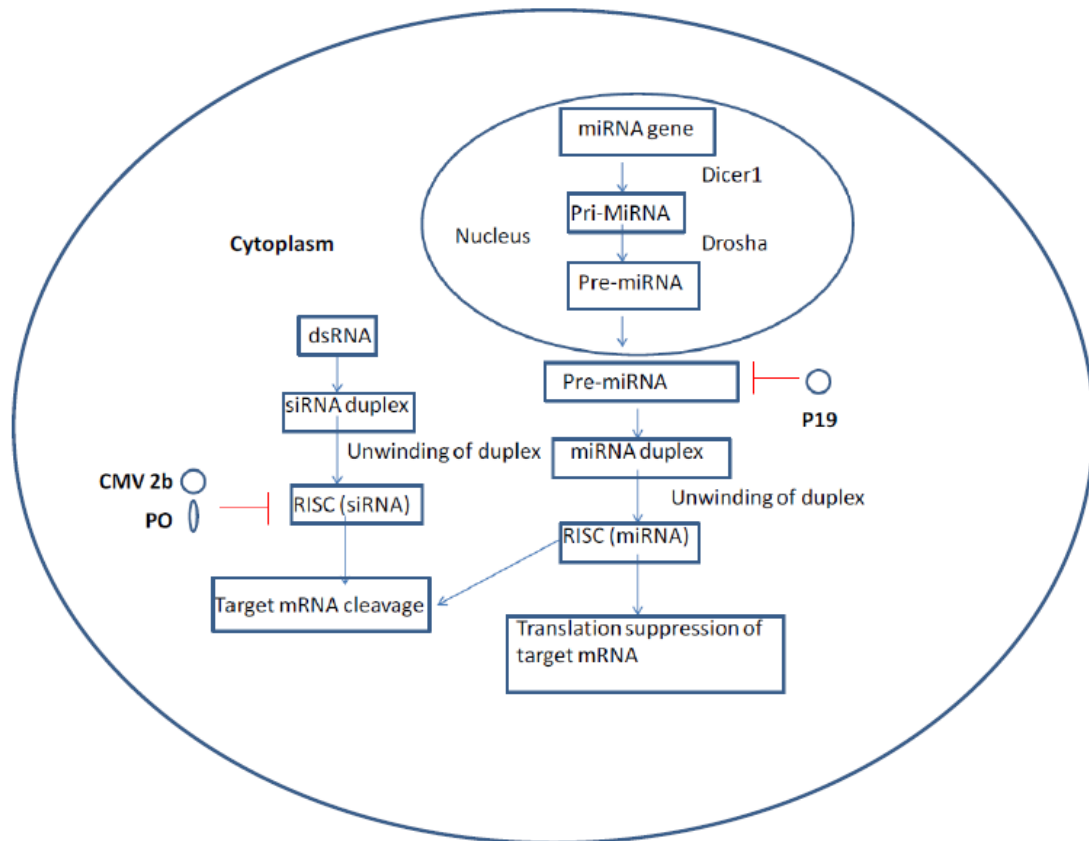
This figure is modified from Mak 2005 and Chellappan et al 2005.

RNA silencing suppressors promote viral infection

Some plant pathogens, especially RNA viruses, have evolved effectors to subvert plant defense by suppressing RNA silencing. Cucumber mosaic virus produces the RNA silencing suppressor 2b (CMV2b), which has been shown to inhibit plant RNA silencing pathway by binding to and sequestering siRNAs. This binding and sequestration of siRNAs is further promoted by the AGO1-binding activity of CMV2b (Vargason et al., 2003 and Zhang et al., 2006). Because siRNAs are signals for the systemic RNA silencing, which spread from the initial infection sites to the rest of the plant, CMV2b also prevents systemic RNA silencing by sequestering siRNA (Guo et al., 2002). Polerovirus produces another RNA silencing suppressor called P0. Similar to CMV2b, P0 also targets AGO1; but different from CMV2b, P0 induces the degradation of AGO1 and therefore suppress small RNA-mediated cleavage of target RNAs (Baumberger et al., 2007). Other viral suppressors target different components of the RNA silencing pathway (Burgyan and Havelda, 2011). Ultimately, by suppressing RNA silencing, viruses are able to colonize the hosts and cause disease.

Bacteria are also known to secrete effectors that target plant RNA silencing pathway. In a past study, it was shown that certain miRNA-deficient mutants of *Arabidopsis* are more susceptible to the infection of the type III secretion system-defective mutant of *Pseudomonas syringae*, which was unable to multiply and cause disease in the wild-type plants (Navarro et al., 2008*).* This study also suggested that a type III secreted effector HopT1-1 may suppress miRNA function by inhibiting the slicing activity of AGO1

(Navarro et al., 2008). However, whether HopT1-1 can suppress plant defense and/or promote bacterial infection remains unclear.

So far, no studies have been done to study effector proteins secreted by eukaryotic pathogens that target the RNA silencing pathway. This thesis is one of the earliest attempts to characterize **_P_**_hytophthora_ **S**uppressors of **R**NA silencing effectors (PSRs).

P. sojae effector proteins

P. sojae effector proteins

It has been shown plant pathogens secrete effector proteins to promote infection (Göhre et al., 2008). As many other successful plant pathogens, _P. sojae_ secretes a diverse repertoire of effector proteins into the apoplast and cytoplasm of the host. These effectors play a fundamental role in weakening plant defense and in facilitating infection. The apoplastic effectors mainly function as protease inhibitors that target the proteases secreted by the plants. For instance, AVRblb2 is secreted by _Phytophthora infestans_ and targets the host papain-like cysteine protease C14 (Bozkurt et al., 2011). The cleavage of this protease results in increased susceptibility of _N. benthamiana_ plants to _Phytophthora infestans_ by allowing the hypha of this pathogen to grow in the apoplast (Bozkurt et al., 2011).

Among the proteins that are targeted into the cytoplasms there are two main categories, the crinklers (CRN) and the RxLR effectors. There are about 61-451 genes coding for

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potential CRN effectors that contain the conserved motif known as FLAK (Phenalanine,

Leucine, Alanine and Lysine respectively) following the N-terminal secretion signal

peptide (Tyler et al., 2006; Haas et al., 2009 and Win et al., 2007). A recent study of the

cypoplasmic CRN effector PsCRN115 secreted by *P. sojae* indicated that it plays a key

role in suppressing host defense by inhibiting cell death (Liu et al., 2011). PsCRN115 is

capable of suppressing cell death elicited by the *P. sojae* necrosis-inducing protein

(PsojNIP) or PsCRN63 (Liu et al., 2011). *P. sojae* with PsCRN115 silenced were unable

to suppress cell death or callose deposition (Liu et al., 2011).

P. sojae effector proteins with the host cell entry motif RxLR-dEER

Bioinformatic analyses predicted around 400 *P. sojae* genes encoding proteins carrying

the N-terminal RxLR-dEER motif (Jiang et al., 2008). Some of these candidate effectors

also have the conserved 21-30 amino acid motifs known as the W, Y, and K in their C-

terminal (Jiang et al., 2008). The RxLR-dEER motif is required for the proteins to enter

the host cells via plasma membrane (Whisson et al., 2007 and Dou et al., 2008b).

However, the mechanism(s) by which oomycetes utilize the RxLR-dEER motif to

translocate the effector proteins into host cytoplasm remain unclear. A study by Kale et al

(2010) suggested that RxLR-dEER motif might bind to phosphatidyinositol-3-phosphate

(PI-3-P) on the surface of eukaryotic cells, which induces subsequent receptor-mediated

endocytosis that would then allow their translocation to the cytoplasm of the host cells. A

later study however showed that the C-terminal domain of the effector protein was

responsible for the PI3P binding and that this binding had no significant role in the

translocation of the effector into the host (Yaeno et al., 2011). A most recent study

suggested the Host-targeting protein 1 (SpHtp1) secreted by the fish pathogen

Saprolegnia parasitica might enter the host cells in a tyrosine-O-sulphate dependent

manner (Wawra et al., 2012). Whether the effectors secreted by *P. sojae* uses the same

mode of action still remains to be investigated.

P. sojae regulates its transcriptional programming of effectors to potentially enhance

virulence and/or avoid the host defense machinery. These effectors appear to be

expressed at different time points during the infection process and target different

functional branches of the host immunity (Wang et al., 2011). A recent study showed that

most of the 169 effectors tested by the authors could inhibit programmed cell death

triggered by Bcl-2–associated X protein (BAX)- or infestin (IFN1)-induced cell death,

indicating that the fundamental function of *Phytophthora* effectors is to suppress plant

defense (Wang et al., 2011). More importantly, alteration of the expression levels of these

effectors reduced the virulence of *P. sojae*, suggesting that the transcription programming

of these effectors must be highly controlled by the pathogen in order to successfully

infect soybean.

One of the most well characterized effectors secreted by *P. sojae* is Avr1b. An early

study suggested that *Avr1b-1* transcript is highly abundant at a later stage of the infection

process (Valer et al., 2006). Subsequent studies demonstrated that Avr1b inhibited

programmed cell death triggered by BAX and enhanced *P. sojae* infection in soybean

(Dou et al., 2008a). The C-terminus of Avr1b contains the conserved K, W, and Y motifs which are required for suppressing BAX-triggered cell death (Dou et al., 2008a). Another effector protein secreted by *P. sojae* that has been recently cloned and characterized is Avr3b. Avr3b carries a Nudix hydrolase motif in the C-terminus, which confers the ADP-ribose/NADH pyrophosphatase activity (Dong et al., 2011). Nudix hydrolase is a housekeeping enzyme that regulates the amount of toxic molecules and signal intermediates within a plant cell (Dong et al., 2011; Bessman et al., 1996 and Perraud et al., 2005). The enzymatic activity of Avr3b plays a key role in the pathogenicity of *P. sojae*; this observation is consistent with previous studies in *Arabidopsis* which suggested that ADP-ribose/NADH pyrophosphatases may act as negative regulators of plant immunity (Bartsch et al., 2003). Therefore, Avr1b and Avr3b appear to enhance the virulence of *P. sojae* by targeting two distinct components of plant defense.

Other members of the *Phytophthora* genus also secrete effectors carrying the RxLR-dEER motif with plant defense suppression functions. The *Phytophthora infestans* RxLR effector AVR3a has been demonstrated to suppress INF1-induced cell death in *Nicotiana benthamania* (Bos et al., 2006). In a later study, Dr. Bos and colleagues showed that AVR3a suppresses INF-induced cell death by binding to the E3 ubiquitin ligase CMPG1, which plays a key role in the apoptosis process (Bos et al., 2010).

Although several studies have been done to study how these effectors suppress Effector-triggered Immunity (ETI) or PAMP-triggered Immunity (PTI), the molecular

mechanisms underlying the functions of the majority of these effectors still remains
unknown. In an effort to understand the function of *Phytophthora* effectors, Dr. Yongli
Qiao in our laboratory conducted a screen and identified two RxLR effectors from *P.
sojae* that have the RNA silencing suppression activity (**Figure 4**). In his screen, green
fluorescent protein (GFP) and *Phytophthora* effectors were co-infiltrated into the
transgenic *N. benthamiana* 16C plants, which constitutively express GFP. Exogenously
expressed GFP through *Agrobacterium*-mediated transient expression would induce
transgene-mediated RNA silencing and result in loss of green fluorescence in the
infiltrated area (Voinnet and Baulcombe, 1997). However, co-expression of RNA
silencing suppressors can recover the production of GFP in the infiltrated tissues. Using
this assay, Dr. Qiao identified two RxLR effectors (PsAvh18 and PsAvh146), named
***P**hytophthora* **S**uppressors of **R**NA Silencing (PSRs) 1 and 2, that significantly
suppressed *GFP* silencing when compared to the control empty vector pEG100 (**Figure
4**). The goal of my research was to investigate the virulence functions of these two PSRs
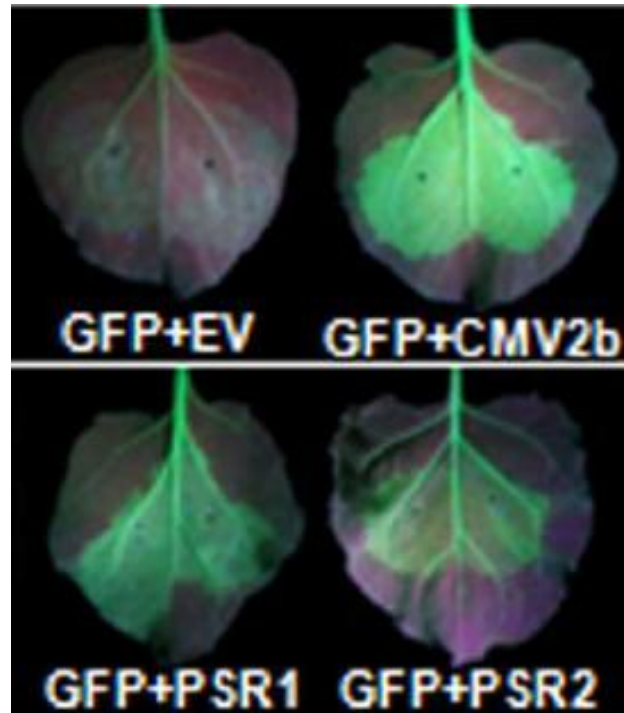during pathogen infection.

Figure 4. PSR1 and PSR2 are able to suppress GFP silencing in *Nicotiana benthamiana*. PSR1 and PSR2 were co-expressed with GFP in the transgenic 16c plants of *N. benthamiana*. Similar to the well studied viral RNA silencing suppressor CMV2b, PSR1 and PSR2 were able to suppress GFP silencing two days after infiltration.

This figure is courtesy of Dr. Yongli Qiao.

<u>OBJECTIVE</u>

Understanding the molecular mechanism which *P. sojae* effectors utilize to enhance disease development will provide important insights of its pathogenesis. Prior to our screen, no eukaryotic effector proteins targeting the host RNA silencing pathway were known. The main goal of this thesis is to characterize the virulence function of the two PSRs during pathogen infection. To accomplish this aim the following objectives were pursued:

1) The first objective was to investigate the virulence function of PSRs during viral and *Phytophthora* infection. I wanted to determine whether PSR1 enhances the infection of Potato Virus X and test whether PSR1 and PSR2 promote disease progression during *P. sojae* infection of soybean roots.

2) The second objective was to investigate the mechanism of RNA silencing suppression activity of PSR1. In particular, I wanted to examine whether PSR1 directly binds to AGO1, which plays a key role in small RNA-mediated cleavage of target transcripts.

By investigating the virulence function of PRS1 and PRS2, we wanted to begin unraveling the role of RNA silencing during the arms race between plants and *Phytophthora*.

MATERIALS AND METHODS

Plants and growth conditions

Nicotiana benthamiana plants were grown in a temperature-controlled greenhouse at 25ºC. Soybean cv. Williams 82 seeds were surface sterilized by soaking in 10% bleach solution for ten minutes. After ten minutes the seeds were rinsed three times with sterile water. 25-30 seeds were placed on top of two layers of Whatman filter paper in a sterile glass petri plate. One mL of sterile water was added to the filter paper, the seeds were covered by two additional layers of Whatman filter paper before closing it. In this manner the seeds were surrounded by moist filter paper which allowed to the seeds to germinate in the dark. The closed plate was covered with aluminum foil and placed in the dark for one week at room temperature. Sterile water was added as needed to keep the filter paper moist. After a week the germinated seeds were planted in autoclaved vermiculite soil (Crop production services) supplemented with Peter's complete Nutrient solution (1g/1L, Crop production services). The plants were allowed to grow at 22 ºC and 12-hour light cycle for 5-6 days before the cotyledons were harvested for inoculation with *Agrobacterium rhizogenes* strain K599 (Savka et al., 1990) to produce hairy roots, which were then infected with *P. sojae*.

Microbial strains and growth conditions

Bacterial and oomycete strains used in this thesis are listed in Table 1. *P. sojae* strain P6497 was grown on V8 medium (20% V8 juice, 0.02 $CaCO_3$, 1.5% agar/L distilled water) at 25ºC in the dark. *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes* and *E.*

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coli strains were grown on Luria-Bertani agar plates supplemented with 50 μg/mL kanamycin, 50 μg/mL rifampicin, 50 μg/mL gentamycin or 5 μg/mL tetracycline when necessary. The strains were stored in 80% glycerol at -80ºC.

Table 1: Bacterial strains and plasmids.

Strain or plasmid	Characteristics[*]	Source or reference
Escherichia coli DH5α	F- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1, *hsd*R17(rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen, CA
Escherichia coli BL21	Carries the lambda DE3 lysogen	Invitrogen, CA
Agrobacterium tumefaciens GV3101(pMP90)	Deletion of T-DNA of pTiC58, Rif[R], Gent[R]	Holsters et al., 1980
Phytophthora sojae P6497	Race 2	Forster et al., 1994
Agrobacterium rhizogenes K599	Causitive agent of hairy root disease in plants, used to transform soybean cotyledons, contains root inducing (Ri) plasmids	Savka et al., 1990
pGEX	Vector contains a tac promoter which allows high levels of IPTG inducible expression of GST-tagged recombinant proteins and an internal *lacI^q* gene for use in any *E. coli* host.	Kaelin et al., 1992
pGEX*::PSR1*	pGEX carrying *PSR1* gene, Amp[R]	This study
pEG100	pEarleyGate100, a Gateway binary vector with cauliflower mosaic virus *35S* promoter, Kan[R]	Earley et al., 2006
pGR106	Binary vectors with cauliflower mosaic virus *35S* promoter, encoding PVX coat protein, Kan[R]	Lu et al., 2003
pGR106::*PSR1*	pGR106 carrying *PSR1* gene, Kan[R]	This study
pGR106::*ΔPSR1*	pGR106 carrying Δ*PSR1* gene with an early stop codon, Kan[R]	This study
pENTR[TM]1A	Gateway[TM] entry vector used in LR reactions,contains *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a gateway destination	Invitrogen,CA

	vector, Kan[R]	
pEG104	Gateway[TM] destination vector for translational fusion with YFP, contains a strong 35S promoter and Kan[R]	Earley et al,. 2006
pEG101	Gateway[TM] destination vector for translational fusion with YFP, contains a strong cauliflower mosaic virus (CaMV) 35S promoter and Kan[R]	Earley et al., 2006
pEG101::*PSR1*	pEG101 carrying the *PSR1* gene in-frame fused to YFP at the N-terminus, Kan[R]	This study
pEG101::*PSR2*	pEG101 carrying the *PSR2* gene in-frame fused to YFP at the N-terminus,, Kan[R]	This study

Table 2: Primers used in this thesis.

	Primer name	Primer sequence (5' to 3')
PVX assay	**pGR106::*PSR1*** pGRNSPSR1ClaI-F	CTACATCGATATGACTAAACCGTCGACGGAGGC
	pGR106::*ΔPSR1* pGRΔPSR1ClaI-F	CTACATCGATATGACTAAACCGTAGACGGAGGC
	pGR106::*PSR1* pGRNSPSR1NotI-R	CTACGCGGCCGCTTTGTTCTAGCCACGCCTTGT
IPTG induction of GST-PSR1 in *E. coli*	**pGEX::*PSR1*** 5' pGEX primer	GGG-CTGGCAAGCCACGTTTGGTG
	PsAvh18-R	TTTGTTCTAGCCACGCCT
	PsAvh18-F	ATGACTAAACCGTCGACG
***Agrobacterium rhizogenes*-mediated hairy root induction**	**pEG101** 35S promoter F	CCA CTA TCC TTC GCA AGA CC
	pEG101::*PSR1* PsAvh18-EcoRVGFP-R	CTACGATATCTTTGTTCTAGCCACGCCT
	pEG101::*PSR2* PsAvh146-EcoRVGFP-R	CTACGATATCTCCCCCACCTGACTTTGAACTT
qPCR	**Ubiquitin Gene in soybean** Ubi +67	AGATTACGAAACCGCCAACTACC
	Ubiquitin Gene in soybean Ubi -265	GGAAGGAGGAGTGGGTGTAGG
	Cox spacer region between *cox*1 and *cox* 2 FMPhy-8b	AAAAGAGAAGGTGTTTTTTATGGA
	Cox spacer region between *cox*1 and *cox* 2 FMPhy-10b	GCAAAAGCACTAAAAATTAAATATAA

IPTG induction of PSR1 expression in *E. coli* cells

DNA fragments coding for *PSR1* (300 bp) was amplified via PCR using gene specific

primers PsAvh18-F and PsAvh18-R **(Table 2).** The size of the PCR products was verified

using 2% agarose gel. The PCR products were ligated to pGEX-4T-2 vector (**Figure 5**) at

the *Bam*HI and *Eco*RI restriction sites (GE life sciences) and the ligation products were

then used to transform *E. coli* BL21 competent cells. Positive clones were confirmed by

colony PCR using 5' pGEX primer and PsAvh18-EcoRVGFP-R (PSR1). *E. coli* BL21

cells carrying pGEX::*PSR1* were cultured overnight in 5 mL of LB medium

supplemented with ampicillin. 100μL of this overnight culture was used to inoculate two

tubes containing 5 mL of fresh LB medium containing ampicillin. The samples were

placed in a 37°C incubator with shaking for two hours until OD_{600} was at around 0.4-0.6.

2.5 μL of 1M IPTG was added to one of the tubes and both samples were incubated with

shaking for another 4-5 hours at 37°C. 5 mL of IPTG-induced and un-induced cells were

pelleted by centrifugation and 200 μL of 2 x Laemmli Loading Buffer (4% SDS, 20%

Glycerol, 0.12M Tris pH 6.8 and 10% Bromophenol Blue) was used to resuspend the

cells. The samples were boiled for five minutes and then placed on ice for ten minutes

before loading into a 12% SDS PAGE gel. The gel was stained with Commassie Brilliant

Blue to observe the specific protein band of PSR1.
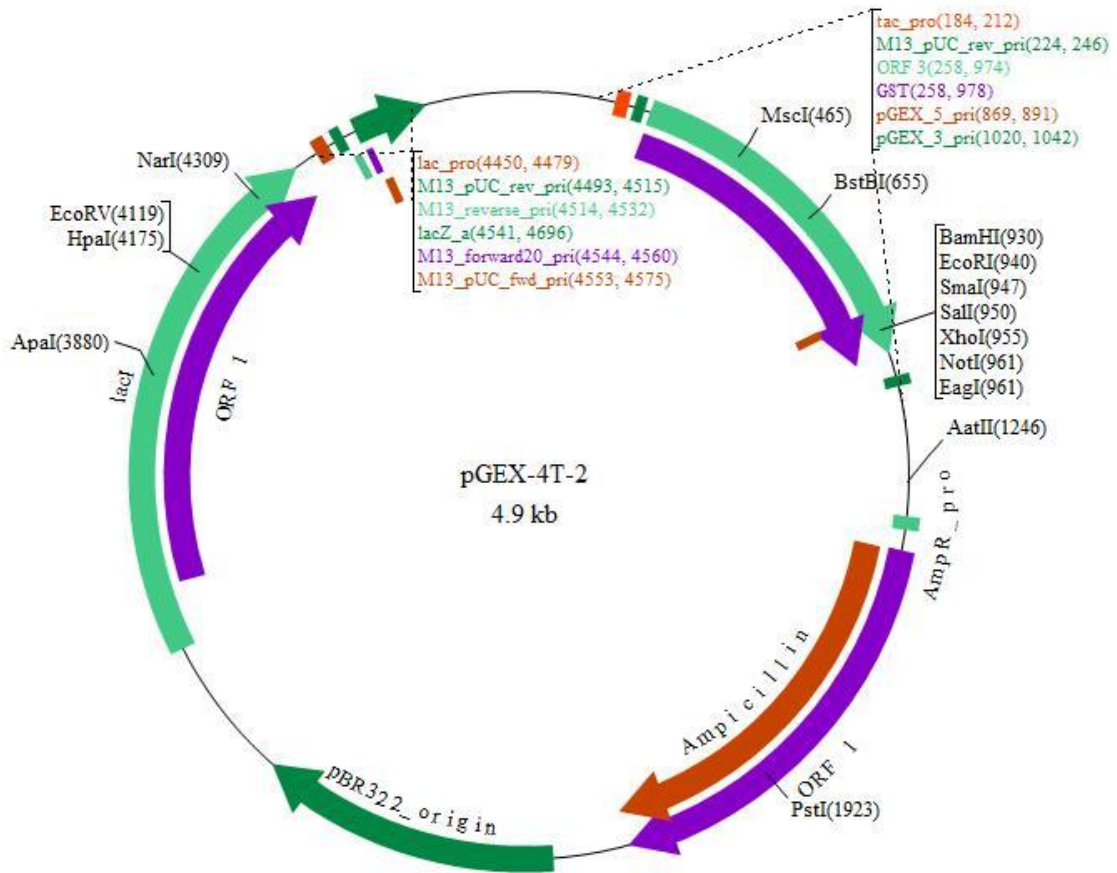
Figure 5. Physical map of pGEX-4T-2. This vector contains a *tac* promoter which allows high levels of IPTG inducible expression of GST-tagged recombinant proteins and an internal *lacI^q* gene for use in *E. coli*. *PSR* genes were digested with *Eco*RI and *Bam*HI and cloned into the respective restriction site.

Online source: http://www.biovisualtech.com/bvplasmid/pGEX-4T-2.htm

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Protein extraction of FLAG-tagged AGO1 from *Arabidopsis thaliana*

To examine the direct interaction between PSR1 and AGO1, AGO1 proteins were extracted from transgenic *Arabidopsis thaliana* plants over-expressing AGO1-FLAG. Influorescence tissues from the transgenic Arabidopsis plants were collected and ground to a fine powder with liquid nitrogen. About two grams of the powder were placed in a 5 mL tube and immediately mixed with 2 mL of extraction buffer (5M NaCl, 1M Tris-HCl pH 7.5, 50% glycerol, NP-40, Protease Inhibitor cocktail, and PMSF) on ice. The protein extract was then added to PSR1-bound affinity beads for immunoprecipitation assays.

Western blot analysis for FLAG-tagged AGO1.

2 x Laemmli Loading Buffer was added to 200 μL of the total protein extract from plants over-expressing AGO1-FLAG. The samples were boiled for five minutes before loading into a 12% SDS PAGE gel. An AGO1-specific antibody (Courtesy of Dr. Xuemei Chen) and an anti-FLAG antibody (Santa Cruz Biotechnology) as primary antibodies (1:1000) and goat anti-mouse IgG-HRP (1:7500) as secondary were used to detect AGO1-FLAG protein, which has a predicted size of about 130 kDa (Baumberger et al., 2007). Same western blot analysis was done for pull-down output.

Pull-down of AGO1-FLAG with GST-PSR1

Pull-down of AGO1 using GST-PSR1 was done using the ProFound Pull-Down GST protein: Protein Interaction kit (Thermo Scientific) following the manufacturer's instructions with small modifications. Columns were equilibrated with immobilized

glutathione (GST resin) by adding 50 μL of 50% GST resin slurry (Thermo scientific).

The columns were washed five times using 400 μL of washing solution which included

TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) and ProFound Lysis Buffer mixed at

1:1 ratio). Cells over-expressing GST-PSR1 were collected from 250 mL of IPTG-

induced culture and 5 mL of lysis buffer (Thermo Scientific) was added before the cells

were sonicated at 4°C. The soluble proteins were separated from the cell debris by

centrifugation and 300 μL of the cell homogenates were added into the each column

containing the washed GST resins. The cell homogenates were sufficiently mixed with

the GST resins in the columns by incubating at 4°C for 30 minutes with gentle rocking on

a rotating platform. After 30 minutes, the columns were placed on a collection tube for

centrifugation at 1,250g for 30 seconds. The flow through was collected and placed on

ice; this sample was used as the input. The GST resin was then washed five times using

the same washing buffer. For each washing step, 400 μL of washing buffer was added to

the column and the column was placed in the rotating platform for two minutes. After

two minutes, the column was placed on collection tube and centrifuged at 1,250g for 30

seconds.

For prey protein capture, 800 μL of the total protein extract from *Arabidopsis thaliana*

over-expressing AGO1-FLAG was added to the PSR1-bound GST column. The columns

were placed on a rotating platform at 4°C for 6 hours or overnight to ensure a good mix

of AGO1-FLAG with the PSR1-bound resins. After the incubation, the columns were

centrifuged at 1,250g for 30 seconds. The flow through was collected as flow through.

The column was then washed for five times using 400 μL of washing solution and 50 μL of the flow through from each wash was collected. In order to elute GST-PSR1 and AGO1-FLAG, 100 mM Glutathione Elution Buffer was used. The Glutathione Elution Buffer was prepared by mixing 31 mg of Glutathione with 1 mL of the washing solution (TBS:ProFound Lysis Buffer with 1:1 ratio). 250 μL of 100 mM Glutathione Elution Buffer was added to the column and incubated at 4°C for five minutes in a rotating platform. After five minutes, the column was placed on a collection tube and centrifuged at 1,250g for 30 seconds. The eluted sample was used as the pull-down sample for the western blots.

Samples collected before (input) and after (pull-down) elution were run in a 12% SDS PAGE gel. AGO1 and PSR1 proteins were detected by western blots using the anti-AGO1 and anti-GST antibodies. A goat anti-mouse IgG-HRP antibody was used as the secondary antibody (1:7500, Santa Cruz Biotechnology).

Detection of GST tagged PSR1 and GST.

2X Laemmli Loading Buffer (4% SDS, 20% Glycerol, 0.12M Tris pH 6.8 and 10% Bromophenol Blue) was added to the IPTG-induced sample from the pull-down and 2 μL was loaded in a 12% SDS PAGE gel. The western blot analysis was done using a GST primary antibody (1:1000) and goat anti-rabbit IgG (HRP) secondary antibody (1:7500) to detect the GST -tagged proteins and GST. GST has a size of about 26 kDa and GST-PSR1 has a size of about 36kDa.

Potato Virus X infection assay

PCR products of *PSR1* and *ΔPSR1* (contained an early stop codon at 3 aa into the *PSR1* open reading frame) were digested with *Cla*I and *Not*I and then ligated into the binary vector pRG106, which carries the full genome of Potato Virus X (PVX) (Lu et al., 2003, **Figure 6**). The positive recombinant plasmids were verified by colony PCR using the primers pGRNSPSR1ClaI-F or pGRΔPSR1ClaI-F and pGRNSPSR1NotI-R (Table 2). pRG106::*PSR1* and pRG106::*ΔPSR1* were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The positive transformants were confirmed using colony PCR using the primers pGRNSPSR1ClaI-F or pGRΔPSR1ClaI-F and pGRNSPSR1NotI-R and then used to inoculate the leaves of 3 or 4-week-old wild-type *N. benthamiana* plants. The confirmed transformants were cultured in LB medium supplemented with 50 μg/mL kanamycin, 50 μg/mL rifampicin, 50 μg/mL gentamycin and 5 μg/mL tetracycline for two days. After two days, the cell pellet was collected and washed with 10 mM $MgCl_2$. The cells were then resuspended in the infiltration buffer (10 mM $MgCl_2$, 10mM MES and 100mM acetosyringone) to reach a final concentration of OD_{600} = 1.0. The cell suspensions were infiltrated into *N. benthamiana* leaves using the pressure infiltration method (Bos et al., 2006). 1 mL of the cell suspension was infiltrated from the lower part of the plant to distribute the solution through the leaves. The excess solution was dried using a Kimwipe. Viral disease symptoms were observed two weeks post infiltration.
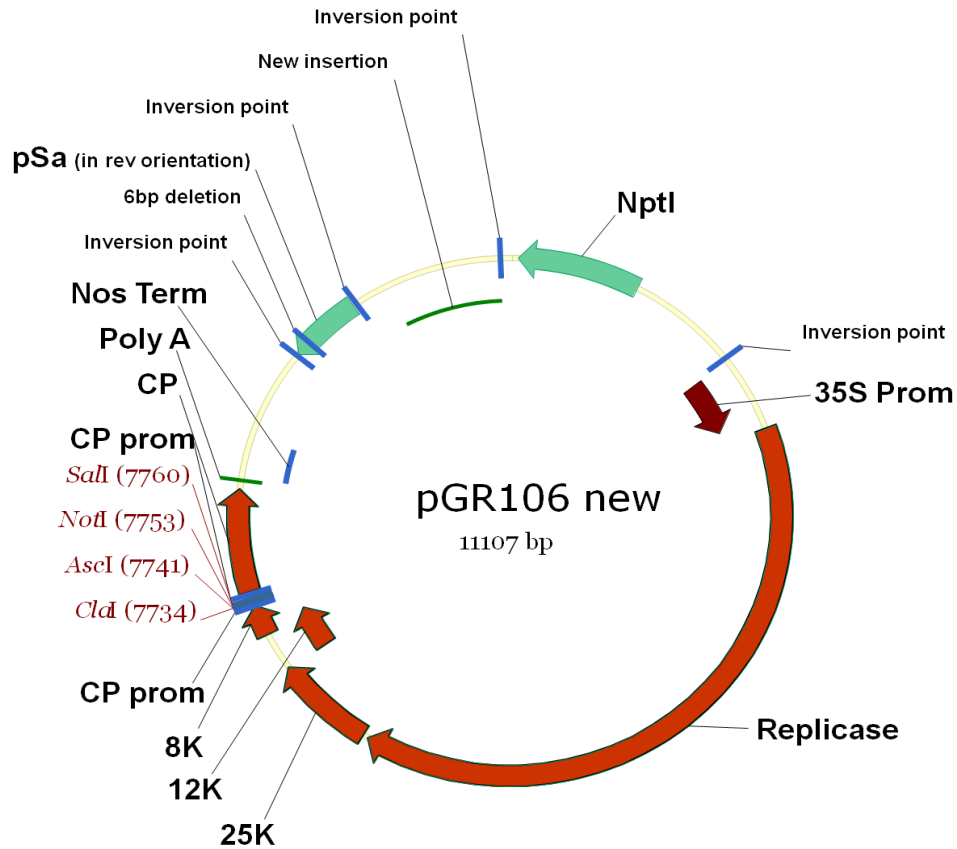
Figure 6. Physical map of vector pGR106. PCR products of the *PSR1* and *ΔPSR1* (contained an early stop codon at 3 aa into the *PSR1* open reading frame) were digested with *Cla*I and *Not*I and then ligated into pRG106, which carries the full genome of Potato Virus X (PVX).

This figure is adapted from Dr. David Baulcombe's laboratory website.
Online source:

http://www.plantsci.cam.ac.uk/research/baulcombe/sequencedata.html

Construction of pEG101 recombinant vectors and Transformation of *Agrobacterium rhizogenes*

Transgenic soybean roots over-expressing PSR1 and PSR2 were generated using

Agrobacterium rhizogenes-mediated hairy roots induction (Subramanian et al., 2005).

PCR products of *PSR1* were ligated into the pENTR vector (Invitrogen) at the *Eco*RI and

*Eco*RV restriction sites, and then transferred into the destination vector pEG101 (Early et

al., 2006, **Figure 7**) using Gateway LR reaction. The LR reaction was done by following

the manufacturer's protocol with small modifications (**Table 3**). 150 ng of the entry

plasmid DNA (pENTR::PSR) were mixed with 150 ng of the destination vector pEG101

DNA and 1 μL of LR Clonase (Invitrogen) to give the total reaction volume of 9 μL.

After one hour incubation at room temperature, 1 μL of Proteinase K was added to each

reaction to terminate the LR reaction. The samples were mixed by vortex and incubated

at 37°C for 10 minutes. In the resulting recombinant plasmid pEG101::*PSR1* and

pEG101::*PSR2*, *PSRs* are under the control of the cauliflower mosaic virus (CaMV) 35S

promoter and in-frame fused to yellow fluorescence protein (YFP). The recombinant

constructs were introduced into *Agrobacterium rhizogenes* strain K599 using the freeze-

thaw method (Hofgen and Willmitzer, 1988). The samples were cultured in LB broth

with 50 μg/mL kanamycin and the positive clones were verified using the following set

of primers PsAvh18-EcoRVGFP-R (PSR1) or PsAvh146-EcoRVGFP-R (PSR2), and

specific primer for 35S promoter (Table 2). Positive clones were then used to inoculate

soybean cotyledon

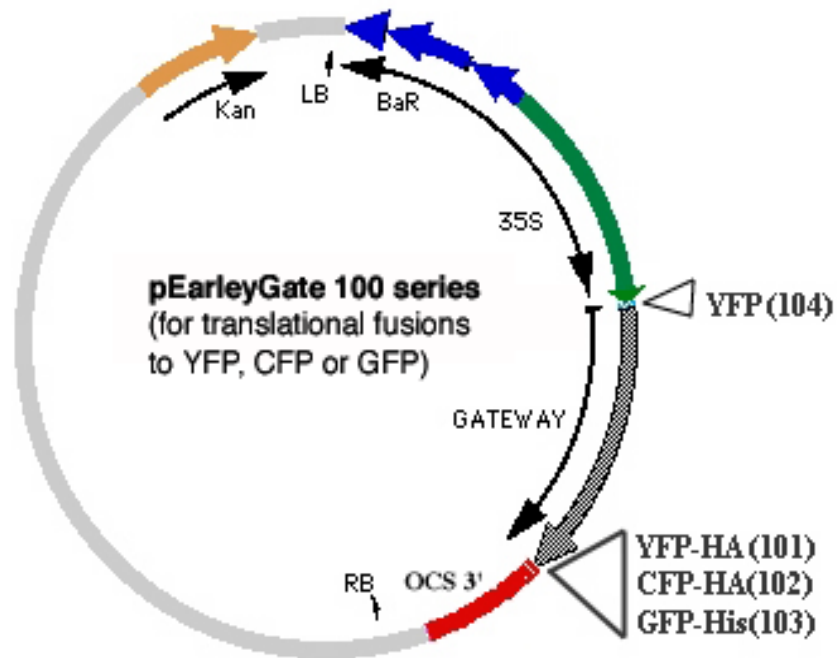**Figure 7. The physical map of the pEarlyGate (pEG) vectors. PCR products coding for PRS1 and PRS2 were ligated into pEG101 to create N-terminal fusions with YFP. pEG104 was used as the YFP only control.**

This figure is adapted from Earley et al 2006.
Online source:
http://sites.bio.indiana.edu/~pikaardlab/pEarleyGate%20plasmid%20vectors%20co
py/Table%20of%20vectors.html

Table 3: Gateway LR clonase reaction

Reagent	Amount used (μL)
Entry clone PSRs (60 ng)	2.5
Destination Vector pEG101 (250 ng)	0.6
TE Buffer pH 8.0	5.9
LR Clonase	1
Total volume	9

Soybean cotyledon transformation with *Agrobacterium rhizogenes*

Agrobacterium rhizogenes strain K599 carrying the plasmids pEG101, pEG101::*PSR1*,

pEG101::*PSR2* or pEG104 were grown in 5 mL of LB broth with 50 µg/mL kanamycin

at 25 ºC for one day. The cells were spun down at 2,500g for 15 minutes, washed with10

mM $MgSO_4$ once, and resuspended in 10 mM $MgSO_4$ to a final concentration of

OD_{600}=0.3. Cotyledons collected from six-day soybean seedlings were surface sterilized

using 70% ethanol. A sterilized razor was then used to make a small circular excision

around 0.4 cm in diameter on the adaxial side about 0.3 cm from the petiole end of the

cotyledon (Subramanian et al., 2005). The wounded cotyledons were then transferred into

a square Petri dish and placed on top of a thick layer of filter paper wetted with sterilized

water. 20 µL of the *Agrobacterium rhizogenes* cell suspension was spotted to the cut

surface. The inoculated cotyledons were sealed in the Petri dish with Parafilm and

incubated at room temperature under the light for a 12 hour cycle. Hairy roots generated

from the wounded area will begin to appear after three weeks.

Microscopic analysis of YFP expression in hairy roots

It has been shown that only some of the hairy roots would ectopically express the foreign

genes introduced by *Agrobacterium rhizogenes* (Subramanian et al., 2005). Because

PSR1 and *PSR2* are in-frame fused to an *YFP* gene, the expression of YFP can be used to

distinguish hairy roots expressing the PSR-YFP fusion proteins. After four weeks of

Agrobacterium rhizogenes inoculation, hairy roots generated from the inoculated

cotyledons were screened for yellow fluorescence using a fluorescent microscope with an

YFP filter, which is 510/20 excitation and 560/40 emission. The hairy roots over-expressing PSR1-YFP were then infected with *P. sojae* strain P6497.

Western Blot analysis for YFP expression in hairy roots

Western blots were conducted to further confirm the expression of PSR-YFP fusion proteins in hairy roots that exhibited yellow fluorescence from the microscopic analysis. A GFP antibody (1:1000, Genetex) was used as the primary antibody and a goat anti-rabbit IgG-HRP (1:10000, Santa Cruz Biotechnology) was used as the secondary antibody to confirm the expression of PSRs in hairy roots.

Phytophthora sojae **infection assay**

Hairy roots expressing YFP or PSR-YFP were infected with *P. sojae* race 2 strain P6497. Blocks of V8 agar with growing *P. sojae* mycelium were placed on top of the selected hairy roots that have been confirmed for YFP expression. Infected roots were placed in Petri dish with high humidity and disease progression was monitored at different time points (24, 48, 72, 96, 120 hours after inoculation). About 5 mm of root tissue from each infected hairy roots that was completely covered the agar block was separated and washed thoroughly with distilled water before been placed on top of a glass slide. 20 μL of 0.005% organic aniline blue solution (0.05 grams of aniline dissolved in 100 mL of water) was added to the slide and used to stain *P. sojae* inside the infected roots by heating the slide for five seconds over a Bunsen burner. After five minutes of staining, the dye was removed using a Kimwipe, and the roots were washed sufficiently with

distilled water and placed on another glass slide with a drop of sterile water for visualization using a light compound Zeiss microscope.

Extraction of genomic DNA from infected soybean roots

Genomic DNA was extracted using a phenol extraction method. *P. sojae*-infected root tissues were grinded into powder in liquid nitrogen. DNA Extraction buffer (0.2M Tris pH 8.5, 0.25M NaCl, 25mM EDTA, and 0.5% SDS) was subsequently added to the fine powder. The samples were boiled for 5 minutes and placed on ice before adding 0.7 mL of phenol and 0.3 mL of chloroform. The samples were placed at room temperature for one hour and then were centrifuged at 7500 rpm. The supernatant was transferred to a new tube with 1:1 volume of chloroform. The sample was centrifuged again at 7500 rpm and 0.6 volumes of isopropanol were added to the supernatant to precipitate DNA. After 30 minutes of incubation on ice, DNA was pelleted by centrifugation and washed with 2 mL of 70% ethanol. After the pellet was allowed to dry, it was suspended in 50 μL of milliQ water. The DNA concentration and purity was determined using Nanodrop with OD280/260 at around 2.8.

Quantitative PCR

Quantitative PCR of the DNA content of *P. sojae* in the infected tissue was done to evaluate disease progression. These primers amplify the *Cox* spacer region between the *cox1* and *cox2* genes of *Phytophthora* and have been shown to specifically bind to *Phytophthora* DNA in infected plant tissues (Grünwald et al., 2011, **Figure 8**). About

100 ng of total genomic DNA extracted from uninfected and *P. sojae* infected soybean

roots that express YFP or PSR-YFP were used as templates. Ub+67 and Ubi -265 primers

(**Table 2**) were used to amplify the housekeeping ubiquitin gene in soybean root, whose

abundance was used as an internal control to normalize the DNA content of
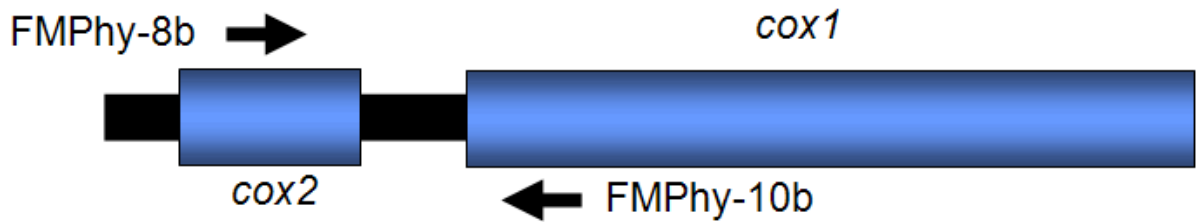
Phytophthora.

Figure 8. A diagram of Cox spacer region between genes *cox2* and *cox1*. The primer annealing sites are in the flanking gene sequences and are specific for the amplification of *Phytophthora* DNA.

This figure is adapted from Grünwald et al 2011.
Online source: http://phytophthora-id.org/Protocols

Table 4: qPCR Master Mix

Reagent	Amount used (μL)
SYBR Green	5
Forward Primer	0.4
Reverse Primer	0.4
Water	1.2
Genomic DNA (100 ng)	8

ABBREVATIONS

PCR- Polymerase Chain Reaction

PSR- *Phytophthora* Suppressors of RNA silencing

qPCR- quantitative Polymerase Chain Reaction

YFP- Yellow Flourescence Protein

GFP- Green Flourescence Protein

PVX- Potato Virus X

HRP- Horseradish peroxidase

IPTG- Isopropyl B-D-1 thiogalatopyranoside

 BAX- Bcl-2–associated X protein

NB-LRR- Nucleotide binding-leucine rich repeat

CRN- crinkling- and necrosis-inducing proteins

CMV- Cucumber mosaic virus

RESULTS

I.   PSR1 does not interact with AGO1

Many viral RNA silencing suppressors such as the Turnip crinckle virus (TCV) P38 and

CMV2b have been shown to directly target AGO1 (Azevedo et al., 2010 and Zhang et al.,

2006). AGO1 is a key component of the RNA Induced Silencing Complex (RISC)

(Baumberger et al., 2007). By binding to AGO1, P38 and CMV2b suppress RNA

silencing. In order to examine whether PSR1 had a direct interaction with AGO1 in order

to suppress RNA silencing, I performed a semi-in vitro pull-down assay using GST-

tagged PSR1 as the bait and FLAG-tagged AGO1 as the prey.

The GST-tagged PSR1 proteins were expressed in *E. coli* BL21 cells. *PSR1* gene was

cloned into pGEX vector, which has the GST epitope fused to the N-terminal of PSR1;

and the resulting recombinant plasmid pGEX::*PSR1* was then used to transform *E. coli*

BL21 competent cells. Positive clones were verified using colony PCR (**Figure 9**). GST-

PSR1 gene expression was highly induced by 1mM of IPTG (**Figure 10**). The cell

homogenates from *E. coli* BL21 over-expressing GST-PSR1 were incubated with the

GST-binding glutathione resins at 4ºC for 30 minutes. The PSR1-bound GST resins were

then incubated with total proteins extracted from the transgenic *Arabidopsis* plants over-

expressing FLAG-AGO1 at 4ºC for 6 hours or overnight. I used AGO1 produced by

transgenic *Arabidopsis* line since AGO1 has never been successfully expressed in *E. coli*.

Co-precipitation of AGO1 with PSR1 could then be detected using an AGO1 specific

antibody and an anti-FLAG antibody. Although I was able to detect AGO1 from input

(**Figure 11**) and PSR1 from the eluted samples using anti-GST antibody, I was unable to

detect AGO1 in the output (**Figure 12**). My results suggest that PSR1 does not interact

with PSR1, at least under the conditions that I have used in this experimental design.
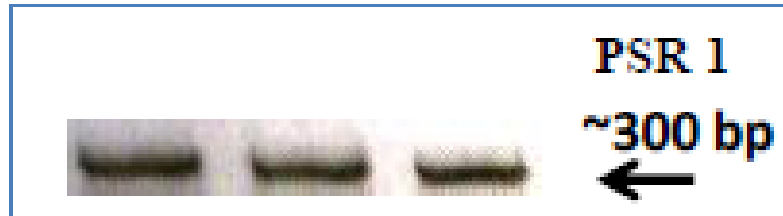
Figure 9. PCR confirmation of *E. coli* BL21 transformants carrying the plasmid pGEX::*PRS1*. Positive clones were confirmed by colony PCR using 5' pGEX primer and PsAvh18 (PSR1). A band about 300 bp was expected and observed.
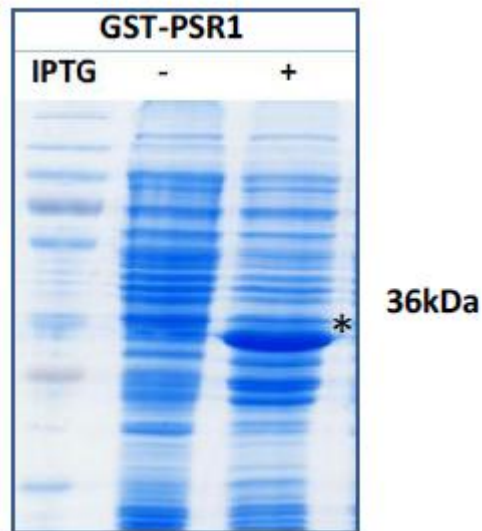
Figure 10. Induction of GST tagged PSR1 (36 kDa) using 1 mM IPTG in *E. coli* BL21 cells. A 12% SDS PAGE gel was used and stained with Commassie Brilliant Blue to observe the specific protein band of PSR1. (-) indicates non-induced samples whereas (+) indicates IPTG-induced samples. Asterisks indicate the 36kDa GST-PSR1 band.

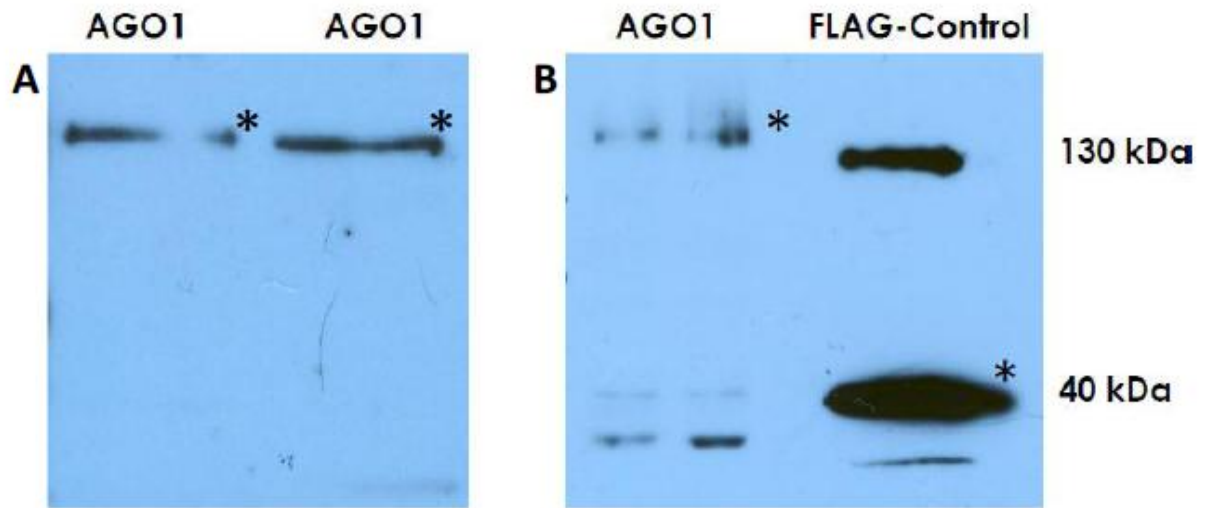**Figure 11. Detection of AGO1-FLAG in total proteins extracted from *Arabidopsis*.**
(A) Detection of AGO1 using an AGO1 specific primary antibody (1:1000) (a gift
from Dr. Xuemen Chen's laboratory). A band of about 130 kDa was detected in the
two loaded samples. (B) Detection of the 130 kDa AGO1-FLAG using anti-FLAG
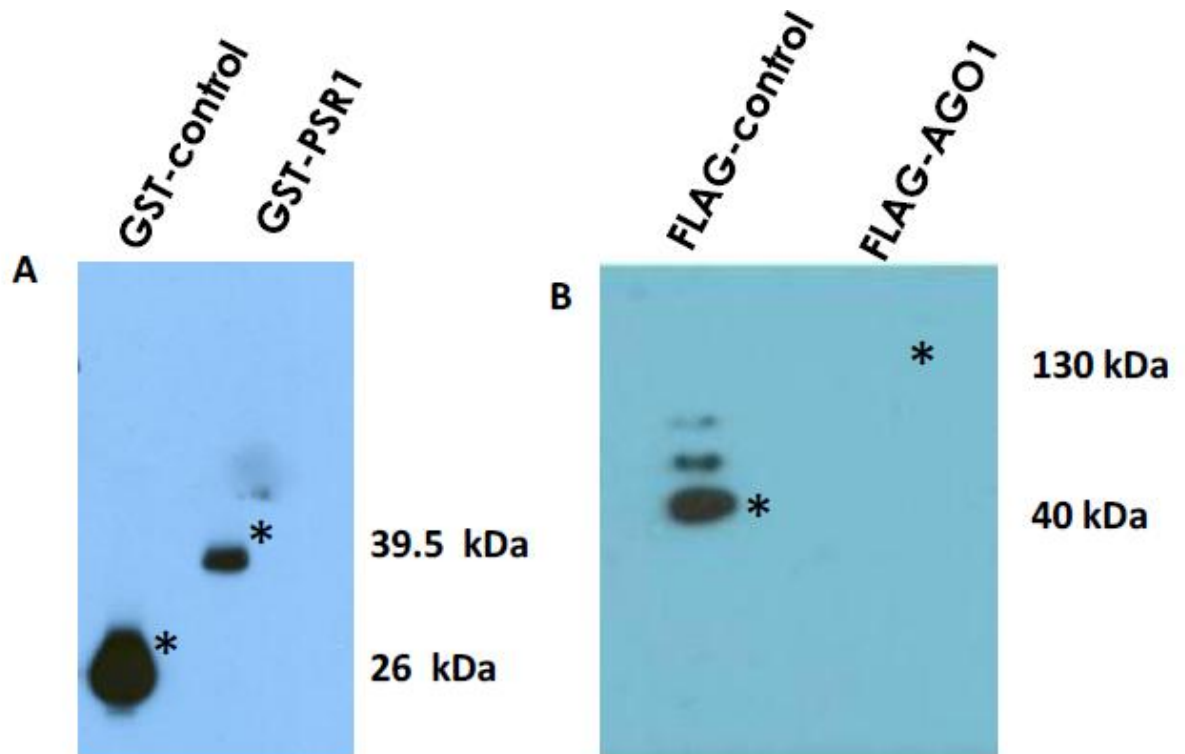**antibody. A 40 kDa FLAG-tagged protein was used as a positive control.**

Figure 12. Western blot analysis of the output of the pull-down assay.

(A) Detection of GST tagged PSR1 from the pull-down assay. 2 µL of eluted samples were loaded in a 12% SDS PAGE gel. The western blot analysis was done by using the GST primary antibody (1:1000) and goat anti-rabbit IgG (HRP) secondary antibody (1:7500) to detect the GST-PSR1 and GST. GST has a size of about 26 kDa and GST-PSR1 has a size of 36 kDa.

(B)Detection of FLAG-AGO1 in the output after co-immunoprecipitation with GST-PSR1. The anti-FLAG antibody (1:1000) as primary antibody and goat anti-mouse IgG-HRP secondary antibody (1:7500) were used to detect the AGO1-FLAG protein, which has a predicted size of about 130 kDa. No band was detected in the eluted sample. A FLAG-tagged control protein with a size of 40 kDa was used as a control.

II. PSR1 enhances the infection of PVX in *Nicotiana benthamiana*

PVX infection assay has been commonly used to determine the virulence function of viral effectors (Brigneti et al., 1998). When PVX infects *Nicotiana benthamiana* alone, it causes minor disease symptoms. However, in the presence of viral RNA silencing suppressors, such as CMV2b, the recombinant PVX can cause more severe symptoms because it impairs the host defense mechanism of RNA silencing (Brigneti et al., 1998). In order to determine whether PSR1 could also enhance the virulence of PVX in *N. benthamiana* plants, I made a recombinant virus using the PVX vector pRG106 (Brigneti et al., 1998). The construct pRG106::*PSR1* (PVX::PSR1) was transformed into *Agrobacterium tumefaciens* strain GV3101 and the resulting *Agrobacterium* strains were then used to infiltrate the leaves of *N. benthamiana* plants. Two weeks post-inoculation, the plant inoculated with PVX::PSR1 showed significantly enhanced disease symptoms (stunting and death of the plants) compared to the wild-type PVX (**Figure 13**), indicating that PSR1 significantly increases the virulence of PVX.

To test whether this phenotype was due to the function of PSR1 protein, but not to the extra RNA sequence in the PVX virus, an early stop codon was introduced into PSR1 to make pRG106::Δ*PSR1* (PVX::ΔPSR1). The plants inoculated with PVX::ΔPSR1 displayed similar disease symptoms comparable to that of the plants inoculated with the wild-type PVX (**Figure 13**). These results suggest that a functional PSR1 protein is required for the enhancement of viral virulence and that the enhanced disease phenotype is not due to the presence of the PSR1 coding sequence in the PVX RNA. PVX::PSR2

also showed enhanced viral disease symptoms when compared to PVX (data from Dr. Yongli Qiao, not shown here). These data suggest that PSR1 and PSR2 can promote viral infection through their RNA silencing suppression activity.

Wild type   **PVX**   **PVX::PSR1**   **PVX::ΔPSR1**

Figure 13. PSR1 enhances the virulence of PVX in *Nicotiana benthamiana*. PVX is a weak pathogen of *N. benthamiana*. However, PSR1 greatly enhances the viral disease symptoms. On the contrary, PVX carrying the ΔPSR1 which contained an early stop codon showed similar weak disease symptoms as the wild-type PVX. The pictures were taken two weeks after inoculation.

III. The effect of PSRs on *P. sojae* infection of soybean roots

Both PSR1 and PSR2 were shown to inhibit the RNA silencing pathway and enhance the virulence of PVX. However, a more important virulence assay would be to examine whether PSR1 and PSR2 could promote the infection of *Phytophthora*. Therefore, I investigated the potential virulence function of PSR1 and PSR2 during infection of *P. soaje* in soybean roots.

I first over-expressed PSR1 and PSR2 in soybean roots using *Agrobacterium rhizogenes*-mediated hairy root induction. PCR products coding for PSR1 and PSR2 were ligated into the binary vector pEG101 which results in the expression of the fusion proteins with an YFP tag fused to the N-terminal of PSRs (**Figure 7**)**.** This YFP tag would allow us to distinguish the hairy roots expressing PSRs because it is known that not all hairy roots express the exogenous gene (Subramanian et al., 2005). The resulting plasmids pEG101::*PSR1* and pEG101::*PSR2* were then used to transform *Agrobacterium rhizogenes* strain K599. Positive clones were verified by PCR using a primer that amplifies the CaMV 35S promoter in the pEG101 vector and the PsAvh18-EcoRVGFP-R or PsAvh146-EcoRVGFP-R primers that are specific for each effector genes respectively (**Table 2** and **Figure 14**). After confirmation, the transformants were used to inoculate cotyledons isolated from six day-old soybean seedlings. The infected cotyledons were placed in a plastic container for four weeks to generate hairy roots from the infected sites (**Figure 15**). About 30% of the hairy roots over-expressed YFP and were selected using a fluorescence microscope (**Figure 16**). Western blots were also used to further confirm the

expression of YFP (from hairy roots induced by *A. rhizogenes* carrying pEG104) or

PSRs-YFP (from hairy roots induced by *A. rhizogenes* carrying pEG101::*PSR1* or

pEG101::*PSR2*) in some of the YFP-expressing roots (**Figure 17**). The selected YFP-

expressing roots were then inoculated with *P. sojae* strain P6497. A block of V8 medium

agar containing growing *P. sojae* mycelium was placed on top of the YFP-expressing

roots and the development of *P. sojae* during infection was monitored at different time

points using microscope. In general, *P. sojae* starts to produce oospores at 48 hours post

infection in the soybean roots over-expressing YFP. However, oospores were observed

inside the roots that over-express PSR1-YFP at 24 hours post infection (**Figure 18**).

Unlike PSR1, PSR2 was unable to accelerate the development of oospores, which were

only observed at 48 hours post infection in roots over-expressing PSR2-YFP. However,

at this time point, a higher percentage of roots expressing PSR2-YFP had oospores than

those expressing YFP (**Figure 19**). These data suggest that both PSR1 and PSP2 could

enhance oospore development during infection.

Finally, I investigated whether the expression of PSRs is sufficient to increase the amount

of *P. sojae* in the infected root tissues by performing qPCR using *Phytophthora* specific

primers FMPhy-8b and FMPhy-10b (Grünwald et al., 2011). The ubiquitin gene of

soybean was used for the normalization of DNA content in the infected tissues. My qPCR

results showed no difference on the DNA content of *P. sojae* in roots expressing PSR1,

PSR2, or YFP (**Figure 20**). These results indicate that PSR1 and PSR2 do not accelerate

pathogen multiplication in soybean roots.

Figure 14. Colony PCR was used to verify *Agrobacterium rhizogenes* strain K599 transformed with pEG101::*PSR*.

(A) A PSR1 band of a size about 300 bp was detected in *A. rhizogenes* transformants using a primer specific for the CaMV 35S promoter sequence in the pEG101 vector and a PSR1-specific gene primer PsAvh18-EcoRVGFP-R.

(B) A PSR2 band of a size about 2 kb was detected in *A. rhizogenes* transformants using a primer specific for the CaMV 35S promoter sequence in the pEG101 vector and the PSR2-specific gene primerPsAvh146-EcoRVGFP-R.

Figure 15. Hairy roots induction in soybean cotyledons. (A) 0 hour post *A. rhizogenes*-inoculation (B) 2.5 weeks post *A. rhizogenes*-inoculation (C) 3-4 weeks post *A. rhizogenes*-inoculation.
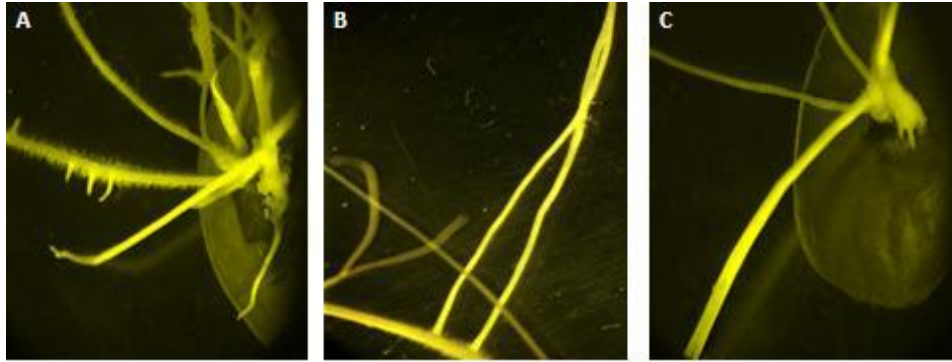
Figure 16. Soybean hairy roots expressing YFP or PSR-YFP produce yellow fluorescence. A fluorescent grey stereo microscope with an YFP filter was used to visualize: (A) YFP expression in hairy roots induced by *A. rhizogenes* carrying the empty vector pEG104; (B) YFP expression in hairy roots induced by *A. rhizogenes* carrying pEG101::*PSR1*; and (C) YFP expression in hairy roots induced by *A. rhizogenes* carrying pEG101::*PSR2*. The YFP is 510/520 excitation and 560/640 emission.
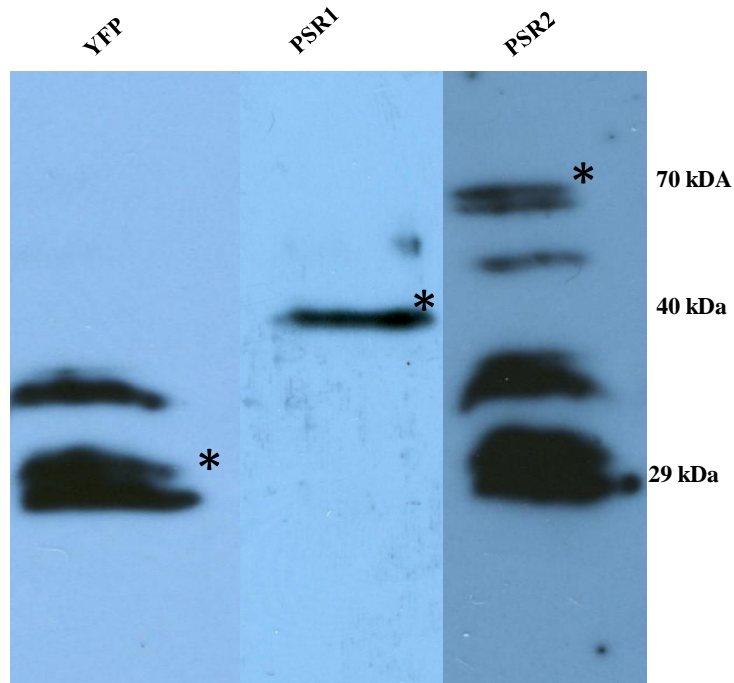
Figure 17. Western blots showing the expression of YFP or PSR-YFP in hairy roots. A GFP antibody (1:1000) and a goat-anti-rabbit IgG (HRP) secondary antibody (1:10000) were used as primary and secondary antibody respectively. Asterisks indicate the size of each protein. YFP protein has a size of about 29 kDa. PSR1-YFP has an approximate size of 40 kDa and PSR2-YFP has a size of 70 kDa.
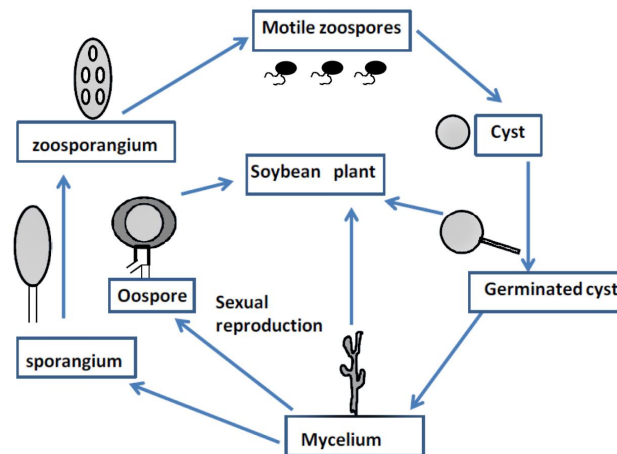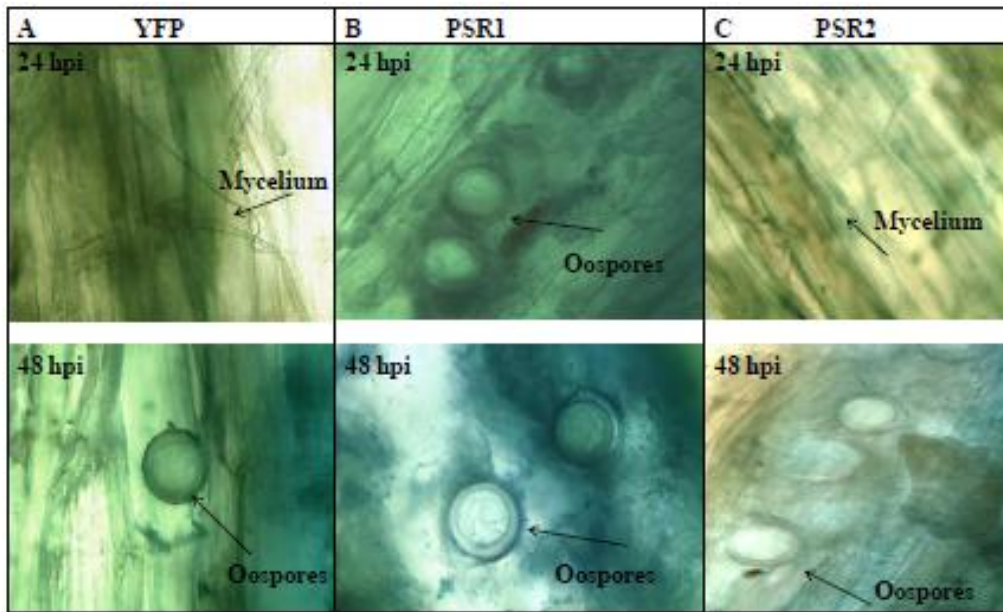
Figure 18. PSR1 accelerates oospore development in soybean roots expressing YFP or PSR-YFP at 24 and 48 hours post infection. Roots were stained using 0.005% organic aniline blue solution and visualized using a light compound Zeiss microscope (A) YFP-expressing roots infected with *P. sojae* only had mycelium at 24 hours post infection and oospores at 48 hours post infection; (B) PSR1-YFP expressing roots infected with *P. sojae* had oospores at 24 and 48 hours post infection; (C) PSR2-YFP expressing roots infected with *P. sojae* had mycelium at 24 hours post infection and oospores at the 48 hours post infection.
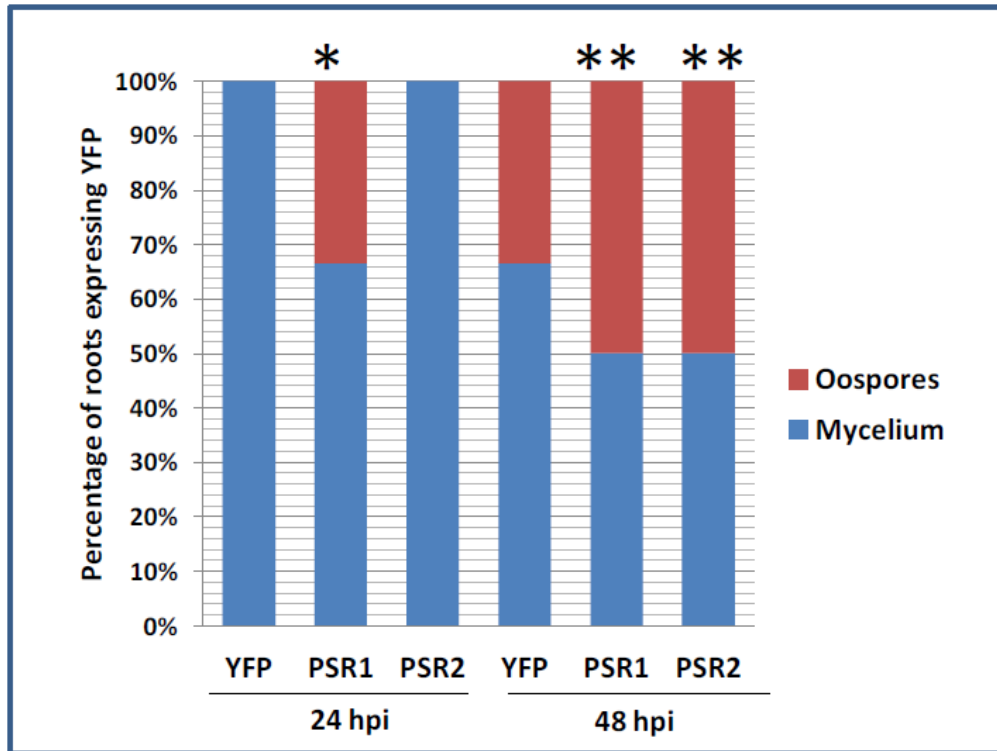
Figure 19. Number of transgenic roots expressing YFP (pEG104), PSR1-YFP (pEG101::PSR1) or PSR2-YFP (pEG101::PSR2) that allowed *P. sojae* to develop oospores at 24 and 48 hours post-inoculation (hpi). Figure shows the average of three replicated. Six roots were analyzed in each experiment and the experiment was repeated three times with similar results. (*) indicates statistical significance whereas (**) indicates no significant difference.

Figure 20. Quantitative PCR of *P. sojae* DNA in the infected hairy roots expressing YFP or PSR1-YFP. FMPhy-8b and FMPhy-10b primers were used to quantify the DNA content of *P. sojae* in the infected tissues at the 48 hpi to evaluate disease progression.

DISCUSSION

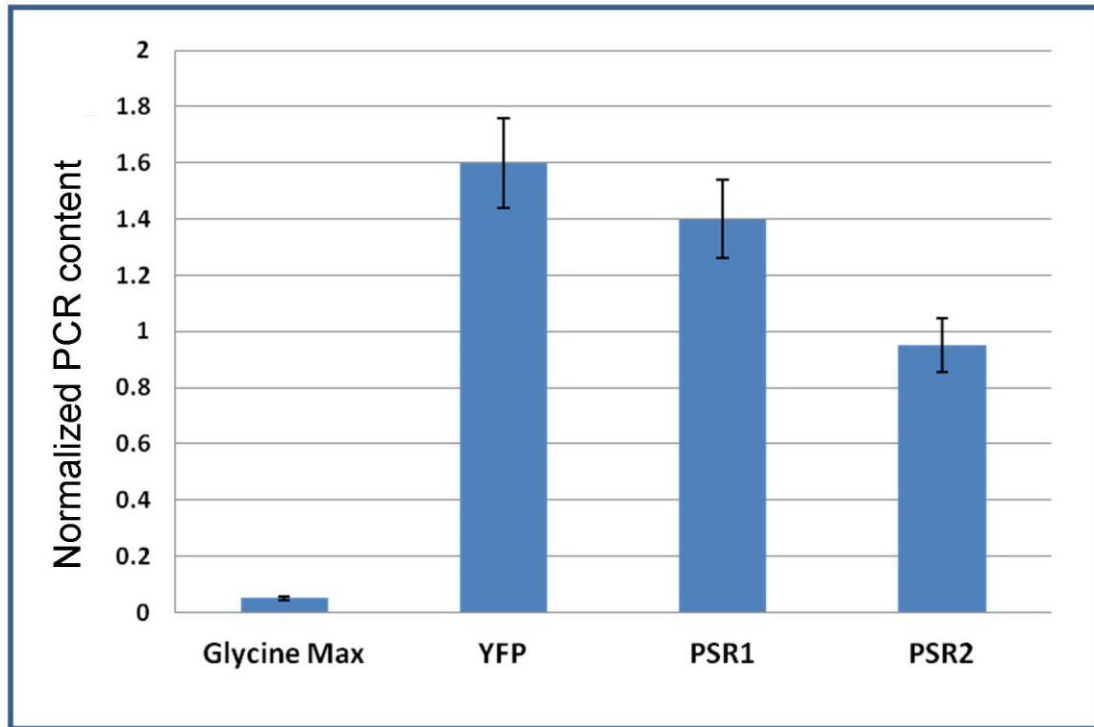Understanding the molecular bases of the complex interaction between *P. sojae* and soybean would allow the development of efficient management strategies to prevent *P. sojae* from causing millions of dollars of losses in the soybean industry. The central players of this interaction are the effector proteins secreted by *P. sojae* and the plant defense mechanisms. *Phytophthora* produces a large number (400-600) of effectors, whose expression is highly regulated (Jiang et al., 2008a and Wang et al 2011). Furthermore, these effectors appear to target different components of the plant immune system (Wang et al., 2011). Research in our laboratory identified *Phytophthora* effectors that can suppress RNA silencing pathway. This thesis presented one of the earliest characterizations of two *Phytophthora* Suppressors of RNA silencing effectors (PSRs) during pathogen infection. My results suggest that PSRs enhance the virulence of PVX in *N. benthamiana* and the infection of *P. sojae* in soybean.

RNA silencing suppressors have been found in many RNA viruses (Burgyan and Havelda, 2011). Furthermore, a type III secreted effector of the bacterial pathogen *Pseudomonas syringae* was also reported to repress the function of microRNAs (Navarro et al., 2008). The two PSRs are the first example of RNA silencing suppressors produced by eukaryotic pathogens. Viral effectors mainly bind to small RNAs and/or AGO1 in order to suppress RNA silencing. So far, the molecular mechanisms underlying the RNA silencing suppression activity of PSRs are unknown. My results from the pull-down experiment suggest that PSR1 does not directly interact with AGO1. It is therefore likely

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that PSR1 might target other component(s) of the RNA silencing pathway. Dr. Yongli Qiao in our laboratory also showed that PSRs do not interact with small RNAs, suggesting that the PSRs use different mechanisms from the viral RNA silencing suppressors to suppress RNA silencing. Further experiments will unravel these potentially novel mechanisms.

PSR1 and PSR2 seem to target the RNA silencing pathway via different mechanisms. PRS1 is a stronger RNA silencing suppressor compared to PSR2 and my results showed that PSR1 accelerates the oospore development more robustly than PSR2 during *P. sojae* infection of soybean roots. Although there might be functional redundancy of these effectors, it is possible that they function synergistically during infection. Preliminary data from our laboratory showed that *PSR1* and *PSR2* are expressed at different infection stages and at different levels (Dr. Yongli Qiao, unpublished data). Therefore, PSR1, PSR2, and possibly other yet identified PSRs, may work synergistically to inhibit the host defense by targeting different components of the RNA silencing pathway at different infection stages.

Together with Dr. Yongli Qiao, we showed that PRS1 and PSR2 are able to enhance PVX virulence. Additional studies done by Dr. Qiao also showed that these PSRs enhance the infection of *Phytophthora infestans* in *N. benthamiana*. Furthermore, my experiments demonstrated that PSR1, and possibly PSR2, accelerate the oospore development during *P. sojae* infection of soybean roots. All these results suggest PSRs

promote pathogen infection through their RNA silencing suppression activity. These data also suggest that RNA silencing pathway is likely involved in the regulation of plant defense against *Phytophthora* infection. *Phytophthora* infection assays using RNA silencing-deficient mutant plants will help test this possibility.

CONCLUSIONS

P. sojae is responsible for millions of dollars of losses in the soybean industry therefore it is imperative to elucidate the virulence mechanism of this pathogen in order to control the disease. As many other successful plant pathogens, *P. sojae* secretes a diverse repertoire of effector proteins into the apoplast and cytoplasm of host cells. These effectors play a fundamental role in the weakening of plant defense and facilitate infection. The identification of RNA silencing suppressors in *P. sojae* is exciting because it indicates a role of RNA silencing pathway in plant defense against *Phytophthora* infection. Here I showed that PSR1 enhances PVX viral infection in *N. benthamiana* and accelerates the sporulation of *P. sojae* in soybean roots. These data suggest that *P. sojae* has evolved effectors to promote infection by targeting the RNA silencing process at different time points in the infection process.

Future studies need to be conducted to continue elucidating the molecular mechanism underlying the functions of PRS1 and PSR2 based on their ability to suppress of RNA silencing. Future studies which include *Phytophthora* infection assays using RNA silencing deficient mutant plants need to be done to demonstrate that RNA silencing is a defense a mechanism against *P. sojae*. Studies involving *P. sojae* transformants silenced for PSR1 or PSR2 can also help determine the extent in which these two PRSs work synergistically to inhibit the host defense. P. *sojae* secretes a diverse repertoire of effector proteins many of which remain uncharacterized. It would also be interesting to investigate whether other effector proteins enhance the RNA silencing suppressing

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activity of PSR1 and PSR2. These studies would elucidate the virulence mechanism of this destructive plant pathogen and contribute to the development of resistant crops.

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