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Identification and Characterization of *Phytophthora* Effectors with RNA Silencing Suppression Activity

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by

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ABSTRACT OF THE DISSERTATION

Identification and Characterization of *Phytophthora* Effectors with RNA Silencing Suppression Activity

by

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Dr. Wenbo Ma, Chairperson

Phytophthora are filamentous eukaryotes that contain many important pathogens of plants. These destructive pathogens bring economic losses of billions of dollars per year. My thesis research aims to understand the molecular mechanisms involved in *Phytophthora* pathogenesis. In particular, I am interested in understanding how *Phytophthora* pathogens defeat plant immunity.

Existing evidence have revealed the never-ending molecular arms race between pathogens and their hosts. Recent research discovered that small RNAs play an important regulatory role in plant defense. However, *Phytophthora* pathogens encode effectors with RNA silencing suppression activities to manipulate small RNA-mediated plant defense. In this thesis, I characterized the host target of the *Phytophthora* RNA silencing suppressor 2 (PSR2) and identified additional RNA silencing suppressors from *Phytophthora* species.

In chapter I, I characterized the host target of PSR2. PSR2 was previously identified from *Phytophthora sojae* and specifically affects secondary small interfering RNA (siRNA) accumulation in *Arabidopsis*. Using genetics and molecular biology approaches, I found that PSR2 interacts with the double-stranded RNA binding protein 4 (DRB4) in plant cells. DRB4 has a known function in secondary siRNA biogenesis by partnering with the endonuclease Dicer-like protein 4 (DCL4), which processes long double-stranded RNA (dsRNA) precursors into siRNAs. DRB4 contains two double-stranded RNA binding motifs (dsRBMs) in the N terminus, which are required for the interaction with PSR2. In addition, I also determined that the N-terminus of PSR2 is necessary and sufficient to interact with DRB4. Importantly, this N-terminal region of PSR2 is also necessary and sufficient for the virulence activity of PSR2. These results indicate DRB4 is likely a major virulence target of PSR2 to promote *Phytophthora* infection.

In chapter II, I identified additional effectors that suppress RNA silencing in plants. Previous research on *Phytophthora* has focused on cytoplasmic effectors that contain the "RXLR" motif. In order to examine the prevalence of RNA silencing suppressors in *Phytophthora*, I screened a collection of the Crinkler effectors, which contain a different host-targeting motif. The CRN effectors are also unique in that: 1) they are usually constitutively expressed; 2) they are nuclear-localized. I found two CRNs from *Phytophthora capsici* as potential RNA silencing suppressors. Both effectors are widely spread in other *Phytophthora* species. In particular, CRN36_259 was able to reduce the

accumulation of siRNAs in *Nicotiana benthamiana* while CRN32_283 suppressed RNA silencing without affecting siRNA levels. Interestingly, the nuclear localization of CRN36_259 is required for its RNA silencing suppression activity. In addition, the CRN36_259 knockout mutant of *P. capsici* exhibited altered developmental phenotype and virulence activity. These results suggest that CRN36_259 regulates *Phytophthora* development and virulence, possibly through its function as a suppressor of small RNAs.

This thesis provides fundamental knowledge about *Phytophthora* pathogenesis and sets the foundation to develop resistance against the destructive *Phytophthora* diseases.

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GENERAL INTRODUCTION

***Phytophthora* the "plant destroyers"**

Oomycetes are fungus-like organisms that are evolutionarily related to brown algae and diatoms. Although morphologically similar to filamentous fungi, oomycetes are diploid without a free haploid stage in their life cycle and belong to the Kingdom *Straminipilia* (Judelson and Blanco, 2005) (Tyler, 2009) (Kamoun et al., 2014). Oomycete pathogens include more than 90 *Phytophthora* species, over 100 *Pythium* species, and a broad range of downy mildew pathogens, most of which are plant parasites (Tyler, 2009). In addition, some oomycete pathogens also infect animals, such as pathogens in the order *Saprolegniales* (Tyler, 2009) (Earle and Hintz, 2014).

Phytophthora is a genus that contains many important plant pathogens. The name "*Phytophthora*" means "plant destroyer" in Greek because many *Phytophthora* species are important pathogens that cause devastating diseases of crops. The most destructive infection of *Phytophthora* caused the Irish potato famine in the mid-1840s, drawing researchers' attention to this pathogen. And the culprit, *P. infestans,* caused approximately 1 million deaths due to lack of food (Zadoks, 2008) (Nowicki, 2012). Even today, potato late blight causes approximately $5 billion in losses each year worldwide. In the past decades, there are hundreds of research groups studying *P. infestans* and it

was recently voted as the most important for its scientific and economic importance among 33 plant-pathogenic oomycete species (Kamoun et al., 2014).

There are ~60 identified species of *Phytophthora* and up to 600 unknown species infecting hundreds of plant hosts (Wrather, 1997) (Judelson and Blanco, 2005) (Wrather and Koenning, 2006) (Tyler, 2007) (Brasier and Webber, 2010) (Whitham et al., 2016) (Chang et al., 2017). For example, *P. sojae,* which causes soybean root and stem rot is the second most destructive disease of soybean with an estimated damage of up to $2 billion per year worldwide (Wrather and Koenning, 2006) (Tyler, 2007) (Whitham et al., 2016) (Chang et al., 2017). Unlike *P. sojae*, which only infects soybean, *P. capsici* has a broad host range and infects many vegetable crops including pepper, pumpkin, cucurbit, and beans. Yearly loss by *P. capsici* was estimated to be about $1 billion worldwide (Chen et al., 2013). In addition, many species of hardwood trees and ornamentals worldwide were infected by *P. ramorum* , including the evergreen oak and tanoak along 1500 kilometers of forests along the coast of California and Oregon (Brasier and Webber, 2010) (Kamoun et al., 2014).

Plant pathogens can be generally classified as biotrophs or necrotrophs based on their infection styles. Biotrophic pathogens establish a complex symbiotic relationship with specific hosts and feed on living tissues; in contrast, necrotrophic pathogens kill plant cells and obtain nutrients from dead/collapsed tissues of a broad range of hosts (Spoel et al., 2007). For example, *P. sojae* is a soil-born pathogen and with an initial biotrophic

stage. The disease cycle starts when zoospores are attracted to the soybean roots under high soil moisture condition, losing their flagella and forming a cell wall as zoospore cyst (Stassen and Van den Ackerveken, 2011) (Fawke et al., 2015). The zoospores attach to the surface of the root and geminate hyphae to penetrate into the plant tissue, forming haustoria as the feeding structure. It has been reported that haustoria in fungi could import the nutrients, such as sugars, from the plant host, and *Phytophthora* haustoria likely have a similar function (Voegele et al., 2001) (Stassen and Van den Ackerveken, 2011) (Dou and Zhou, 2012) (Fawke et al., 2015) (Judelson, 2017). Haustoria are also believed to be the infection structure where effector proteins are secreted to promote disease development (Fawke et al., 2015) (Whitham et al., 2016). A fundamental function of these effectors is to manipulate plant immunity (Fawke et al., 2015). *Phytophthora* are hemibiotrophic pathogens. For *P. sojae*, oospores are produced at the end of the biotrophic stage and the switch to a necrotrophic stage leads to death of the plants. The thick-walled oospores will be released and are able to survive in soil, plant debris, or even hostile environment. Whenever the temperature and moisture conditions are optimal, the dormant oospores germinate to form mycelia which produce sporangia and zoospore, starting a new life cycle (Stassen and Van den Ackerveken, 2011) (Fawke et al., 2015) (Judelson, 2017). (Figure i)

Figure i. *Phytophthora sojae* infection cycle.

During the biotrophic stage, *P. sojae* release zoospores (asexual spores) from sporangia into the soil during wet conditions. The zoospores attach to surface of soybean root and encyst, penetrating the plant cell wall by forming vegetative hyphae. Haustoria were developed as side branches from the intercellular hyphae, penetrating inside the host cells. *P. sojae* colonizes the root and stem with the formation of oospores (sexual spores). Upon entering the necrotrophic stage, plant tissue damage, wilting, chlorosis and plant death occurred. Eventually, oospores were released into the soil to start next infection cycle (Stassen and Van den Ackerveken, 2011) (Fawke et al., 2015).

Soil becomes saturated
Form sporangia
Release zoospores
Attach
Penetrate
Colonize
Cyst
Haustoria
Cortex
Vasculature
Rhizodermis
Form Oospores
Necrotrophic
Biotrophic
Wilting and
death of plant,
Dormant status in
soil / plant debris

Overview of plant-pathogen interactions

Constantly challenged by potential microbial pathogens in the surrounding environment, plants have evolved the ability to respond in a timely and efficient manner. The plant innate immune system is divided into two branches (Jones and Dangl, 2006) (Boller and He, 2009). The first branch relies on the recognition of microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) by transmembrane proteins called pattern recognition receptors (PRRs) in plants (Boller and He, 2009) (Monaghan and Zipfel, 2012). Flg22, an active epitope of bacteria flagellin and one of the best-studied PAMPs is recognized by directly binding to the PRR Flagellin Sensing 2 (FLS2) (Felix et al., 1999) (Ma, 2014). Upon activation by flg22, the co-receptor BAK1 was brought to FLS2, resulting in the subsequent transphosphorylation events (Sun et al., 2013) (Ma, 2014). Another well-studied PAMP is infestin 1 (INF1) elicitin secreted from *P. infestans* (Kamoun et al., 1998) (Chaparro-Garcia et al., 2011). Perception of INF1 leads to a series of defense responses, including interaction with a lectin-like receptor kinase protein of *Nicotiana benthamiana* (NbLRK1), leading to cell death (Fawke et al., 2015). This pattern-triggered immunity (PTI) serves as a general or basal defense that prevents colonization from the majority of potential pathogens; however, successful pathogens are able to effectively defeat PTI through the function of effectors. It became clear that effectors are produced by a broad range of plant pathogens and their major function is to suppress host immunity (Dou and Zhou, 2012) (Spoel et al., 2007). For

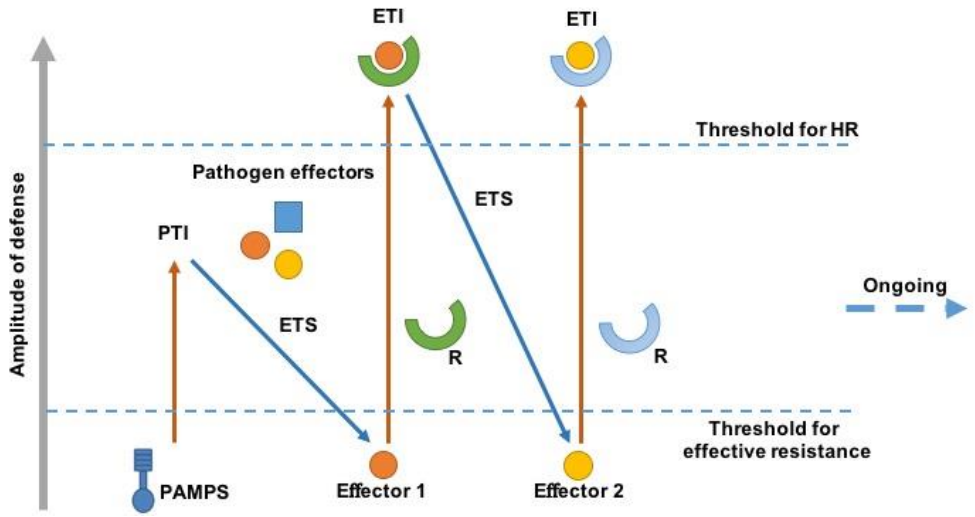
example, gram-negative bacteria deliver type III secreted effectors (T3SEs) into host cells through the type III secretion system (T3SS); fungi and oomycetes deliver effectors through haustoria (Galán et al., 2014) (Stassen and Van den Ackerveken, 2011). As this dissertation focuses on the function of *Phytophthora* effectors, effectors produced by bacteria will not be discussed in detail.

As a counter strategy, plants evolved another layer of defense which depends on the recognition of specific pathogen effectors by resistance (R) proteins in a gene-for-gene manner (Monaghan and Zipfel, 2012) (Dangl et al., 2013). Canonical R proteins share conserved nucleotide-binding leucine-rich repeat (NB-LRR) domains, and the activation of the NB-LRR proteins, usually in the cytoplasm of the plant cells, results in effector-triggered immunity (ETI). ETI often involves programmed cell death called the hypersensitive response (HR), which restricts the spread of biotrophic pathogens from initial infection sites (Boller and He, 2009) (Dangl et al., 2013). Thus, effectors that could trigger HR in plants were usually referred as avirulence proteins (Avr). During the defense response to *Phytophthora*, the gene-for-gene rule discovered in antibacterial resistance is still applied (Anderson et al., 2015). *P. infestans* Avr3a encodes an avirulence effector, which is recognized by the cytoplasmic NB-LRR protein R3a in potato and triggers cell death (Armstrong et al., 2005). This defense, counter-defense, and counter-counter-defense is summarized in a 'zig-zag' model that applies to a broad range of pathosystems (Jones and Dangl, 2006) (Ma and Guttman, 2008) (Pumplin and

Voinnet, 2013) (Fei et al., 2016) (Figure i). Both PTI and ETI involve defense signal transduction through kinases (such as mitogen-activated protein (MAP) kinases) and extensive transcriptional reprogramming, which eventually leads to immunity (Tsuda and Katagiri, 2010).

Figure ii. The zig-zag model of the plant immune system.

Recognition of PAMPs by plant cell-surface receptors triggers broad spectrum PTI, which prevents colonization by most pathogenic microbes. Adapted pathogens deliver effector proteins (Effector 1 in red) that suppress PTI, resulting in effector triggered susceptibility (ETS). In response, plants evolved R proteins that are able to directly or indirectly recognize these effectors and trigger robust defense responses collectively referred to as ETI. Among these responses, a form of programmed cell death called HR prevents proliferation of biotrophic pathogens. The arms race between plant and pathogen continues as microbes acquire new effectors (Effector 2 in yellow) to suppress ETI responses triggered by effector 1 and plants evolve additional R proteins to maintain their recognition capabilities. [adapted from (Jones and Dangl, 2006) (Pumplin and Voinnet, 2013)]



Phytophthora secreted effectors as weaponry

Successful plant pathogens secrete effector proteins to function both in the extracellular space and inside the plant cell. Based on the host target sites, *Phytophothora* effectors are classified as apoplastic effectors and cytoplasmic effectors (Whitham et al., 2016) (Wang et al., 2017). Apoplastic effectors are often cysteine-rich proteins targeting plant hydrolytic enzymes secreted into the extrahaustorial matrix as defense response (Stassen, 2011). One of the best characterized *Phytophthora* apoplastic effectors is EPIC1 from *P. infestans*, which directly targets and inhibits defense-related cysteine protease RCR3 in tomato (Song et al., 2009) (Dong et al., 2014).

Different with apoplastic effectors, cytoplasmic effectors are translocated into plant cells after secreted from the pathogens and target different subcellular compartments (Stassen and Van den Ackerveken, 2011). There are two classes of effectors with host-translocation signals currently known in *Phytophthora*. The major class, RxLR effectors, contains an N-terminal signal peptide, followed by an RxLR domain (arginine, any amino acid, leucine, arginine), and an optional dEER-motif (two glutamic acid residues and an arginine residue). The C-terminal part usually contains the function domain, which is variable among effectors. The RxLR motif is essential for the translocations of effectors into the host cells (Whisson et al., 2007) (Birch et al., 2008) (Tyler, 2009) (Kale et al., 2010). Another class of *Phytophthora* effectors is called the Crinklers (CRNs), which means crinkling and necrosis. The CRN effectors also have the N-terminal signal peptide,

but followed with a conserved N-terminal LxLFLAK motif that is required for translocation. Similar to the RxLR effectors, CRNs have diverse C-terminal effector domains that are presumed to perform different virulence functions in plants. Unlike RxLR effectors, which are restricted to *Phytophthora*, CRNs are believed to be more ancient as they are also produced by other pathogenic oomycetes (Tyler, 2006) (Stam et al., 2013b) (Lamour et al., 2012b).

With the rapid progress on genome sequence analysis, extraordinarily large repertoires of potential cytoplasmic effectors from *Phytophthora* spp. were discovered. Bioinformatic prediction revealed around 563, 400, 357 and 245 RxLR effectors from *P. infestans*, *P. sojae*, *P. capsici* and *P. litchii* respectively (Tyler, 2006) (Whisson et al., 2007) (Haas et al., 2009) (Lamour et al., 2012a) (Ye et al., 2016), and 196, 100, 84 and 14 CRN effectors from *P. infestans*, *P. sojae*, *P.capsici* and *P. litchii* respectively (Stam et al., 2013b) (Ye et al., 2016). Since effectors directly manipulate host cellular processes, they have been used as molecular probes to understand mechanisms by which pathogens cause diseases.

Many studies on RxLR effectors show that they could suppress programmed cell death to interfere with plant defense. For example, AVR3a from *P. infestans* suppresses INF1-triggered cell death by stabilizing plant E3 ubiquitin ligase MPG1, revealing the possible virulence function of Avr3a (Bos et al., 2010). *P. infestans* Avr3b is able to suppress both PTI and ETI, it not only suppresses INF1-induced cell death in *N. benthamiana*, but also

significantly suppresses PITG_22798-triggered cell death in *N. benthamiana* (Zheng et al., 2014) (Wang et al., 2017). *P. infestans* Avr3b is reported to be recognized by NB-LRR R3b of potato when they are co-expressed in *N. benthamiana*. (Li et al., 2011)

RxLR effectors could also disrupt the kinase cascades which are key players in plant immune signaling pathways. For example, eight RxLR effectors from *P. infestans* could suppress flg22-induced immune response by disrupting the MAPK cascade (Zheng et al., 2014). *P. infestans* RxLR effector PexRD2 was reported to interact with MAPK kinase kinase ε (MAPKKKε), a positive regulator of plant immunity. PexRD2 interacts with MAPKKKε at the kinase domain, which is required to trigger cell death, thus manipulating plant defense (King et al., 2014). Transiently expression PexRD2 enhances the susceptibility of *N. benthamiana* to *P. infestans*, possibly due to its inhibitory effect on MAPKKKε (King et al., 2014).

RxLR effectors could also facilitate infection through other mechanisms. *P. infestans* RxLR effector AVRblb2 interrupts with the secretion of papain-like cysteine protease C14 (Bozkurt et al., 2011) and Pi03192 prevents transcription factor NAC targeted by *Phytophthora* (NTP1) and NTP2 from relocating from ER to the nucleus to perturb plant immunity (McLellan et al., 2013). In addition, Avr3b of *P. sojae* is an ADP-ribose/NADH pyrophosphorylase that can increase the susceptibility of *N. benthamiana* to *Phytophthora* infection (Dong et al., 2011) (Kong et al., 2015).

Unlike RxLRs, very few CRNs have been studied for their functions. CRNs were named after a leaf crinkling and necrosis phenotype, for example, CRN8 from *P. infestans* possesses a kinase activity and induces cell death in planta (van Damme et al., 2012); over expression of CRN83_152 in *N. benthamiana* distinctly changes the chromatin organization in nucleus and induces cell death (Stam et al., 2013a) (Stam et al., 2013c). Moreover, CRN20_624 in *P. capsici* not only induces cell death but also exaggerates INF1-induced cell death in *N. benthamiana* (Stam et al., 2013c). However, recent study showed that not all CRNs induce necrosis when expressing in *N. benthamiana* (Stam et al., 2013b). In addition, two CRNs from *P. soja*e, PsCRN63 and PsCRN115, interact with catalases in *N. benthamiana* or soybean and interfere with H_2O_2 homeostasis (Zhang et al., 2015). PsCRN63 alone or PsCRN63 and PsCRN115 together might suppress the immune response in *N. benthamiana* (Zhang et al., 2015). Interestingly, many CRNs, with or without an observable nucleus localization signal (NLS), are located in the host nucleus (Stam et al., 2013b). Whether and how nuclear localization is involved in the virulence activity of CRNs remains unclear.

Small RNAs are essential regulators of gene expression in plants

Plants produce two major classes of endogenous small RNAs, namely microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Chen, 2009) (Pumplin and Voinnet, 2013) (Borges and Martienssen, 2015). miRNAs mediate sequence-dependent post-transcriptional gene silencing (PTGS) by guiding mRNA cleavage and/or translation

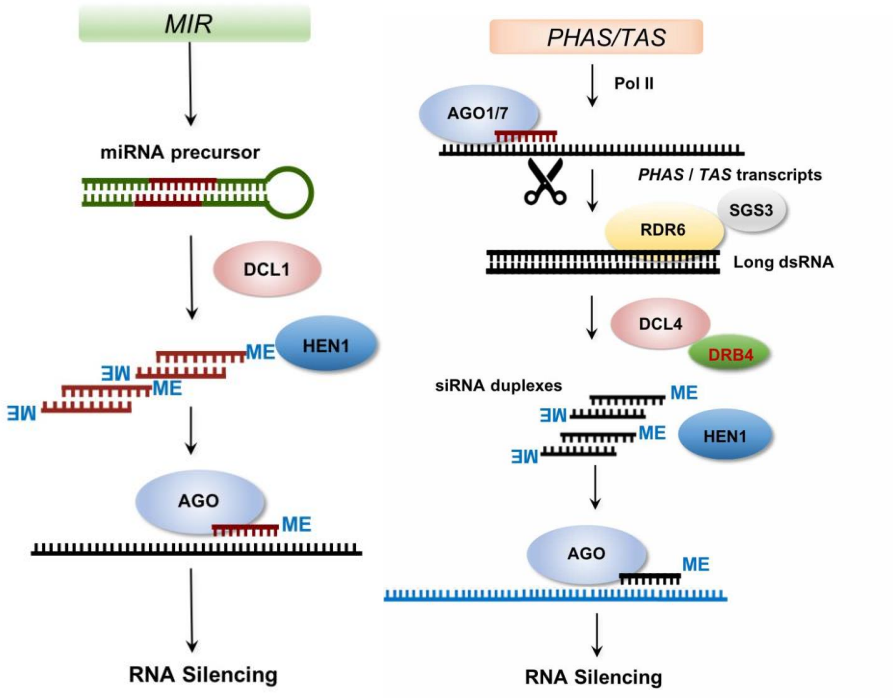
inhibition; whereas siRNAs can also mediate transcriptional gene silencing (TGS) through sequence-dependent DNA modification in addition to PTGS (Chen, 2009) (Bologna and Voinnet, 2014) (Borges and Martienssen, 2015). The core components of plant small RNA silencing pathways include Dicer-like ribonucleases (DCLs) that produce small RNAs from double-stranded precursors, Argonaute (AGO) proteins that form the RNA silencing effector complexes, RNA-dependent RNA polymerases (RDRs) that synthesize long double-stranded precursors, and double-stranded RNA binding proteins (DRBs) that facilitate small RNA biogenesis (Pumplin and Voinnet, 2013) (Bologna and Voinnet, 2014) (Borges and Martienssen, 2015).

miRNAs are produced from endogenous MIR loci where non-protein-coding transcripts are transcribed by RNA polymerase II (Chen, 2009). Normally primary miRNA transcripts (pri-miRNAs) are processed in the nucleus by DCL1 and its dsRNA-binding cofactor protein Hyponastic leaves 1 (HYL1) to generate double-stranded miRNA duplexes. Many of these miRNAs are 21-nt duplexes that include a guide strand and a passenger stand which will be degraded afterwards. Both strands are methylated by the Hua enhancer 1 (HEN1) methyltransferase. The guide strand is then transported into the cytoplasm and loaded into AGO1 to form an RNA-induced silencing complex (RISC) which targets miRNA-complementary mRNAs to induce gene silencing (Chen, 2009) (Huntzinger and Izaurralde, 2011) (Pumplin and Voinnet, 2013).

Distinct from miRNAs, siRNAs are derived from invading nucleic acids such as viruses and transgenes, and endogenous loci such as repeats, transposable elements, and genes (Chen, 2009) (Huntzinger and Izaurralde, 2011) (Pumplin and Voinnet, 2013). Typically, the precursors of siRNAs are long double-stranded RNAs (dsRNAs) synthesized by RDRs facilitated by Suppressor of Gene Silencing 3 (SGS3); and three DCLs in *Arabidopsis* catalyze the formation of 21-nt (DCL4), 22-nt (DCL2), and 24-nt (DCL3) siRNAs. The 21-nt and 22-nt siRNAs guide gene silencing by PTGS; whereas the 24-nt siRNAs lead to TGS through the RNA-directed DNA methylation (RdDM) pathway (Yang and Huang, 2014) (Borges and Martienssen, 2015). (Figure iii)

Figure iii. miRNA and siRNA biogenesis in plants.

The precursors of small RNAs are processed by DCLs to double-stranded small RNA duplexes. Both strands are methylated by HEN1 and then only the guide strand is loaded into AGO proteins to form RISCs to suppress gene expression.



As central players in transcriptional regulation or post-transcriptional regulation of gene expression, small RNAs are involved in many biological processes in eukaryotes. From regulating the first embryonic divisions to controlling meiosis and gametogenesis, from ovule and floral to shoot, root, and leaf development, small RNAs are crucial in plant growth and development (Schauer et al., 2002) (Liu, 2005) (Nodine and Bartel, 2010) (Thompson et al., 2015). In addition, small RNAs contribute to regulation of plant responses to biotic and abiotic stresses (Ruiz-Ferrer and Voinnet, 2009) (Kulcheski et al., 2011).

Plant mutants defective in small RNA silencing exhibit altered disease susceptibility

There is an accumulating body of evidence suggesting that small non-coding RNAs are integral regulators of defense-related gene expression during pathogen infection as well as a pivotal switch that governs the growth/defense tradeoff (Chen, 2009) (Bologna and Voinnet, 2014). Small RNA silencing is a universal and fundamental gene regulation mechanism in eukaryotes that governs cellular processes. In plant immunity, it is well established that virus-induced RNA silencing is critical for anti-viral defense (Pumplin and Voinnet, 2013) (Cao et al., 2014). More recent studies showed that specific small RNAs were differentially accumulated during infection by bacteria, fungal, and oomycete pathogens (Staiger et al., 2012). Small RNAs have also been found to suppress PTI and ETI in the absence of pathogens to avoid auto-immune responses (Yi and Richards, 2007) (Boccara et al., 2014). Furthermore, effectors with small RNA silencing

suppression activity have been identified from bacteria (Navarro et al., 2008) (Thiébeauld et al., 2017) and oomycetes (Qiao et al., 2013). These findings strongly suggest that small RNA silencing is required to establish effective defense responses to a large variety of pathogens.

There are several pieces of evidence that plant mutants defective in small RNA silencing exhibit altered susceptibility upon infection by filamentous pathogens. For example, two *dcl1 Arabidopsis* mutants which have largely reduced miRNA levels exhibited hypersusceptibility to *P. capsici* (Qiao et al., 2015). siRNA-mediated PTGS has been implicated in plant immunity during the infection of other filamentous pathogens (Baulcombe, 2015). For example, upon infection with *Magnaporthe oryzae*, expression of *OsRDR6* and *OsSGS3* was highly induced in a resistant cultivar of rice while such induction was not observed in a susceptible cultivar (Wagh et al., 2016). A similar induction of *RDR1* and *RDR6* was also observed in *Nicotiana glutinosa* during infection with *P. parasitica* and the fungal pathogen *Colletotrichum nicotianae* (Liu et al., 2009) (Yang et al., 2010). These results indicate that RDR6-dependent siRNAs might be positive regulators of anti-fungal (and anti-oomycete) defense. Indeed, *Arabidopsis* mutants *rdr6*, *sgs3,* and *dcl4* were hypersusceptible to *Verticillium dahlia* (Ellendorff et al., 2008). Moreover, an *OsRDR6* mutant, called *shl2-rol*, exhibited larger necrotic lesions after infection with *M. oryzae* (Wagh et al., 2016). Since RDR6 is a key component of long dsRNA synthesis triggered by miRNA for further secondary siRNA production, these

findings indicate secondary siRNAs play an important role in plant defense against filamentous pathogens.

Suppression of host RNAi pathway by pathogen effectors promotes infection

As described in the zig-zag model (Jones and Dangl, 2006), plant hosts and pathogens are engaged in an arms race between plant defense mechanisms and pathogen effector functions. Since small RNA silencing contributes to plant immunity, it is not surprising that pathogens have evolved effectors to interfere with this process for the benefit of infection (Pumplin and Voinnet, 2013).

The best studied pathogen suppressors of host RNA silencing are viral RNA silencing suppressors (VSRs), which are essential for viral infection (Pumplin and Voinnet, 2013). For example, cucumber mosaic virus protein 2b (CMV2b) binds to siRNAs as well as AGO1 and AGO4 to inhibit RNA silencing (Ding, 2010) (Duan et al., 2012); the Polerovirus-encoded F box protein (P0) protein acts as a component of E3 ubiquitin ligase complexes to interact with AGO1 and AGO5 to mediate their degradation (Baumberger et al., 2007) (Bortolamiol et al., 2008). In addition, the bacteria RNA silencing suppressor (BSR) HopT1-1 from *Pseudomonos syringae* was shown to suppress miRNA functions by inhibiting the slicing activity of AGO1 and thereby suppressing PTI response to promote disease (Navarro et al., 2008) (Thiébeauld et al., 2017).

Recently, effectors with RNA silencing suppression activity were also identified from *Phytophthora* (Qiao et al., 2013)(Qiao et al., 2013) (Xiong et al., 2014). Expression of these *Phytophthora* suppressors of RNA silencing (PSRs) promotes *Phytophthora* infection in *Arabidopsis*, soybean, and *N. benthamiana* (Qiao et al., 2013) (Xiong et al., 2014) (Qiao et al., 2015). Further analysis demonstrated that PSR1 affected the biogenesis of both miRNAs and siRNAs by associating with a putative RNA helicase called PINP1, which is involved in the assembly of DCL complexes for small RNA processing. Silencing of *PINP1* in *Arabidopsis* and *N. benthamiana* led to enhanced susceptibility to *P. capsici* and *P. infestans*, respectively, confirming that an intact small RNA pathway is required to establish effective immunity against *Phytophthora* infection (Qiao et al., 2013). Different from PSR1, PSR2 specifically affects the accumulation of siRNAs in *Arabidopsis*; nonetheless, *PSR2*-expressing *Arabidopsis* plants were drastically more susceptible to *P. capsici* (Xiong et al., 2014). In addition, the most recent study revealed another candidate suppressor from *P. infestans*, Pi14054, which is present in a variety of *P. infestans* isolates (Vetukuri et al., 2017). Though the mechanisms by which the PSRs suppress host RNA silencing activity are unknown, the identification of RNA silencing suppressors in *Phytophthora* strongly supports the idea that the small RNA silencing pathway is an integral component of the plant immune system and a conserved target of diverse pathogens to facilitate infection.

Role of pathogen-responsive miRNAs in plant-fungus/oomycete interactions

A key step towards understanding how small RNA silencing regulates plant immunity is to identify specific small RNAs that affect plant defense responses and characterize their function, as well as the function of their target genes, during pathogen infection. Over the years, genome-wide small RNA analyses using microarray and small RNA sequencing have been conducted for several plant-filamentous pathogen interactions. Recent advances in next-generation sequencing have allowed the rapid identification of pathogen-responsive small RNAs and their predicted target genes. Most of these studies looked for differentially accumulated small RNAs upon pathogen infection or pathogen elicitor (i.e. PAMP) treatment; other studies compared small RNA profiles in susceptible vs. resistant plant cultivars or between plants infected by pathogen strains with different levels of virulence. In addition, some small RNA profiling studies were supported with degradome sequencing data for genome-wide analysis of small RNA target genes. Most recent studies focus on the specific cellular processes affected by small RNAs during infection and how small RNAs may modulate plant defense responses.

miRNAs are known to regulate both plant growth and defense response, however, plant growth and defense are antagonistic process, which means plant prioritize the fighting mode over fitness maintenance upon infected by pathogens (Huot et al., 2014). Pathogen-responsive miRNAs are involved to fine-tune gene expression to balance the

defense and growth (Huot et al., 2014) (Curaba et al., 2014). For example, miR393 which targets component in auxin response signaling pathway is increased during the infection of several plants by different pathogens (Xin et al., 2010) (Chen, 2012) (Wong et al., 2014) and acts as a conserved positive regulator of plant defense among different pathosystems. Pathogen-responsive miRNAs could also regulate defense signaling triggered by PRRs. For example, miR390 which may target receptor-like protein kinase (RLKs) was found to be down-regulated upon infection of *Verticillium longisporum* in oilseed rape (Shen et al., 2014) (Baldrich et al., 2014). Furthermore, miRNAs targeting components of RNA silencing pathways also respond to infection. miR168 targeting AGO1 was found to be induced in several plant species during infection of a large variety of fungal and oomycete pathogens (Zhao et al., 2012) (Shen et al., 2014) (Wong et al., 2014) (Zhang et al., 2015). The differential accumulation of those pathogen-responsive miRNAs are likely due to the differential expression of the corresponding *MIR* genes (Zhao et al., 2012) (Baldrich et al., 2014) as a defense response.

Secondary siRNAs regulate defense-related genes

Although not as well studied as miRNAs, emerging evidence suggests that siRNAs are also important regulators of plant immunity. Upon filamentous pathogen infection, extensive changes in siRNAs have been observed from several pathosystems (Lu et al., 2007) (Yin et al., 2012) (Baldrich et al., 2014) (Wong et al., 2014) (Zhao et al., 2015) (Jin and Wu, 2015). Many pathogen-responsive siRNAs are 24-nt in length and derived from

repeat sequences or transposons (Xin et al., 2010) (Zhao et al., 2015). These changes are usually found to be broadly distributed throughout the genome, and thereby representing a general response that affects transposon activities under stress conditions (Lu et al., 2007) (Baldrich et al., 2014) (Jin and Wu, 2015).

A specific class of siRNA that appears to be particularly important for plant defense against fungal and oomycete pathogens is secondary siRNA derived from transcripts targeted by specific miRNAs. Both protein-coding gene including *Pentatricopeptide Repeat Protein* (*PPR*), *NB-LRR* and *MYB*, and non-protein-coding *TAS* loci are able to generate secondary siRNAs (Li et al., 2016) (Cui et al., 2017). In this case, miRNA-mediated cleavage leads to the synthesis of long dsRNAs by RDR6 and SGS3. These dsRNA precursors are then processed to produce secondary siRNAs. Depending on the dicing enzyme, the length of secondary siRNAs can be 21-nt, 22-nt, or 24-nt. The 21-nt/22-nt secondary siRNAs act like miRNAs and repress target gene expression in trans or in cis through PTGS. *Arabidopsis* mutants *rdr6* and *sgs3* which are defective in secondary siRNA production showed compromised resistance to the fungal pathogens *V. dahlia* (Ellendorff et al., 2008); a rice *rdr6* mutant was also more susceptible to *M. oryzae* (Wagh et al., 2016). In addition, the *Phytophthora* RNA silencing suppressor PSR2 specifically interferes with the accumulation of secondary siRNAs in *Arabidopsis* to promote infection (Qiao et al., 2013) (Xiong et al., 2014). These findings suggest that the secondary siRNA pathway is required for plant immunity. In contrast, mutations in

Arabidopsis RDR6 either exhibited unchanged resistance or enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Navarro et al., 2008) (Cao et al., 2014). Therefore, secondary siRNAs seem to be particularly important for plant responses to filamentous pathogens.

Most secondary siRNAs are in phase with the miRNA cleavage site; therefore, they are named phased siRNAs or phasiRNAs (Yang and Huang, 2014) (Fei et al., 2016) (Li et al., 2016). Differential accumulation has been observed in phasiRNAs during infection. For example, levels of phasiRNAs were found to be reduced in cotton roots inoculated with *V. dahlia* (Yin et al., 2012). In addition, miRNAs that can trigger phasiRNA production were also down-regulated in loblolly pine infected with the rust fungus *Cronartium quercuum* f. sp. *fusiforme* (Lu et al., 2007). The best-known class of genes that are targeted by miRNAs and are producing phasiRNAs are the *NB-LRR* genes, which encode resistance proteins essential for ETI (Fei et al., 2013) (Fei et al., 2016). NB-LRR proteins can be further classified into toll/interleukin-1 receptor (TIR-NB-LRRs, TNLs) and coiled-coil (CC-NB-LRRs, CNLs) based on their distinctive N-terminal domains (Eitas and Dangl, 2010). Genes encoding TNLs or CNLs have both been found to be targeted by miRNAs and serve as "*PHAS*" loci to produce phasiRNAs, which subsequently silence additional *NB-LRR* genes *in trans* or *in cis* (Zhai et al., 2011) (Li et al., 2012) (Fei et al., 2013). For example, the conserved miR482 superfamily binds to sequences within the *NB-LRR* genes that encode the highly conserved P-loop motif; therefore, phasiRNAs produced

from the miR482-targeted *NB-LRR* genes are presumably able to repress an amplified number of *NB-LRR* genes (Shivaprasad et al., 2012) (Park and Shin, 2015). In this manner, repression of a large number of *NB-LRR* genes might be accomplished by a few miRNAs through the production of phasiRNAs. The best example of this mechanism has been shown in legumes, where three 22-nt miRNA families may act as master regulators for *NB-LRR* gene expression (Zhai et al., 2011). Not all studies on phasiRNA-triggering miRNAs looked at the associated phasiRNAs during pathogen infection; nonetheless, some of these miRNAs affect plant resistance to a variety of pathogens including viruses, bacteria, fungi, and oomycetes, indicating that they regulate multiple *NB-LRRs*, possibly through triggering phasiRNA production (Shivaprasad et al., 2012) (Li et al., 2012) (Fei et al., 2013) (Park and Shin, 2015). More recently, evidence suggesting phasiRNA-mediated regulation of *NB-LRR* genes during infection of filamentous pathogens has emerged. A summary of these miRNA families is shown in Table i. As negative regulators of *NB-LRRs*, phasiRNA-producing miRNAs are presumably down-regulated upon infection to enhance ETI (Fei et al., 2016).

Consistent with this assumption, decreased levels of miRNAs belonging to the miR482 family have been found in soybean infected with the oomycete pathogen *P. sojae* (Guo et al., 2011) and the fungal pathogen *Phakopsora pachyrhizi* (Kulcheski et al., 2011) in cotton infected with the fungal pathogen *V. dahlia* (Zhu et al., 2013), as well as in tomato infected with the oomycete pathogen *P. infestans* (Luan et al., 2015) and the

fungal pathogen *Fusarium oxysporum* (Ouyang et al., 2014). Concomitant with the reduced parental miRNA levels, a reduction in the accumulation of phasiRNAs and the corresponding induction of *NB-LRR* genes was also observed in *P. sojae*-infected soybean (Zhao et al., 2015). As such, suppression of phasiRNA production during pathogen infection could de-repress *NB-LRR* gene expression to enhance resistance. However, other studies suggested the opposite pattern. For example, the abundance of miR1507 and miR2109, together with their associated phasiRNAs, was increased during the early infection stage of *P. sojae* in soybean; consistently, the expression of their targeted *NB-LRR* genes was repressed (Wong et al., 2014). Similarly, the transcript levels of *MIR9863* and the abundance of miR9863-derived phasiRNAs were also increased in barley infected with the powdery mildew fungal pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*). miR9863 family members regulate the CNL gene *Mla* (*Mildew resistance locus a*), which confers resistance to some *Bgh* isolates (Liu et al., 2014). These results indicate that phasiRNA changes are dynamic during pathogen infection. This is not surprising because precise regulation of *NB-LRR* genes is essential to minimize tissue damage and other fitness costs. The observed changes in phasiRNA accumulation are likely influenced by the specific pathosystem used in a given study, especially differences in susceptibility levels and infection stages. Indeed, down-regulation of miR482 in tomato was only found in a resistant cultivar when infected with the fungal pathogen *F. oxysporum* (Ouyang et al., 2014). Furthermore, in addition to a miR482 family member that was down-regulated in *P. infestans*-infected tomato (as mentioned

above), another member of the same miRNA family was up-regulated (Luan et al., 2015). Further investigation on phasiRNA-based *NB-LRR* regulation will be important to understand the mechanism by which the secondary siRNA pathway contributes to plant immunity.

Table i. miRNAs that trigger secondary siRNAs and their predicted target transcripts in different pathosystems.

miRNA	Pathosystem	Predicted target transcript	Function	Refs.
miR1507	Soybean-*P. sojae*	NB-LRR protein family	Defense response	Guo et al., 2011; Wong et al., 2014
miR1510	Soybean-*P. sojae*	NB-LRR protein family	Defense response	Guo et al., 2011; Zhao et al., 2015
miR1524	Soybean-*P. sojae*	NB-LRR protein family	Defense response	Zhao et al., 2015
miR1536	Soybean-*P. sojae*	NB-LRR protein family	Defense response	Zhao et al., 2015
miR2109	Soybean-*P. sojae*	NB-LRR protein family	Defense response	Wong et al., 2014; Zhao et al., 2015
miR2118	Cotton-*V. dahliae; Soybean-P. sojae; Poplar-M. larici-populina*	NB-LRR protein family	Defense response	Chen and Cao, 2015; Zhao et al., 2015
miR5300	Tomato-*F. oxysporum*; Tomato-*P. infestans*	NB-LRR protein family	Defense response	Ouyang et al., 2014; Luan et al., 2015
miR9863	Wheat-*P. striiformis*	Cysteine-rich receptor-like protein kinase 41	Pathogen perception	Liu et al., 2014

Other than *NB-LRRs*, genes encoding PPR, MYB transcription factors, and other protein families are also known to be targets of miRNAs and their associated phasiRNAs (Fei et al., 2013) (Zhao et al., 2015) (Fei et al., 2016). Similar to *NB-LRRs*, *PPR* genes form a large family with members that can be targeted by miRNAs for phasiRNA production, which in turn regulate additional gene members (Xia et al., 2013). PPRs regulate RNA processing, stability, editing, or translation of proteins that exert key functions in mitochondria and chloroplasts. These organelles are important for plant defense by producing ROS and salicylic acid (SA) (Schwarzländer and Finkemeier, 2013) (Caplan et al., 2015). Recently, 23 *PPR* genes were identified as *PHAS* loci from soybean infected with *P. sojae* (Zhao et al., 2015). PhasiRNAs derived from *PPR* loci were increased during the early stage of *P. sojae* infection, leading to reduced expression of multiple *PPR* genes (Wong et al., 2014). Intriguingly, the expression levels of some phasiRNA-targeting *PPR* genes were significantly higher in a susceptible cultivar during *P. sojae* infection, suggesting a possible correlation between PPRs and plant immunity (Wong et al., 2014).

Although phasiRNAs are believed to be highly effective in regulating large gene families, such as *NB-LRR* and *PPR* genes, other targets related to defense have also been identified. Two 21-nt miRNA families, sly- and stu-miR6022 and nta-miR6021, that are able to trigger phasiRNA production were predicted to target members of the *Hcr9* (*Homologs of Cladosporium fulvum resistance 9)* gene family, which encode membrane-bound proteins with extracellular LRR domains and confer resistance to fungal infection

in tomato (Li et al., 2012). Genes involved in small RNA silencing could also be regulated by phasiRNAs. For example, *DCL2* acts as a *PHAS* locus where phasiRNA production is triggered by miR1507 in *Medicago truncatula* and miR1515 in soybean; and SGS3 could also be targeted by miR2118 for phasiRNA production in soybean (Zhai et al., 2011) (Zhao et al., 2015). As such, phasiRNA biogenesis can be modulated as a feedback loop to achieve precise regulation in response to pathogen infection.

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Chapter I. *Phytophthora* suppressor of RNA silencing 2 (PSR2) suppresses secondary small RNA accumulation in *Arabidopsis* by interacting with Double-stranded RNA Binding protein 4 (DRB4)

ABSTRACT

Phytophthora contains important plant pathogens that cause devastating diseases of crops. Genome sequences of *Phytophthora* pathogens revealed a large number of secreted virulence proteins; some of these so-called effectors function inside the host cells to facilitate colonization and infection. Previously, we discovered that *Phytophthora* produce effectors with RNA silencing suppression activity to promote infection. *Phytophthora* Suppressor of RNA silencing 2 (PSR2) belongs to a conserved RxLR effector family with seven tandem repeats of L-W-Y motifs. Using transgenic *Arabidopsis* plants, PSR2 was found to specifically affect the abundance of secondary small interfering RNAs (siRNAs). In order to understand the virulence mechanism of PSR2, I characterized PSR2-associating protein(s) in plants. My studies show that PSR2 interacts with Double-stranded RNA-Binding protein 4 (DRB4) in the nucleus and cytoplasm. DRB4 has a known function in secondary siRNA biogenesis by partnering with the endonuclease Dicer-like protein 4 (DCL4), which processes long double-stranded RNA precursors into siRNAs. DRB4 has two double-stranded RNA binding motifs (dsRBMs), and my experiments revealed that they are required for the

association with PSR2. In addition, I found that the first two repeat units of PSR2, WY1 and LWY2 are necessary and sufficient for RNA silencing suppression activity and virulence activity. Consistently, WY1 and LWY2 are also required for the interaction with DRB4. Furthermore, the developmental and disease susceptibility phenotypes of a *drb4* mutant of *Arabidopsis* is reminiscent to *PSR2*-expressing plants, demonstrating that DRB4 is a virulence target of PSR2. Collectively, all my results indicate that PSR2 promotes infection by targeting siRNA production through its interaction with DRB4.

INTRODUCTION

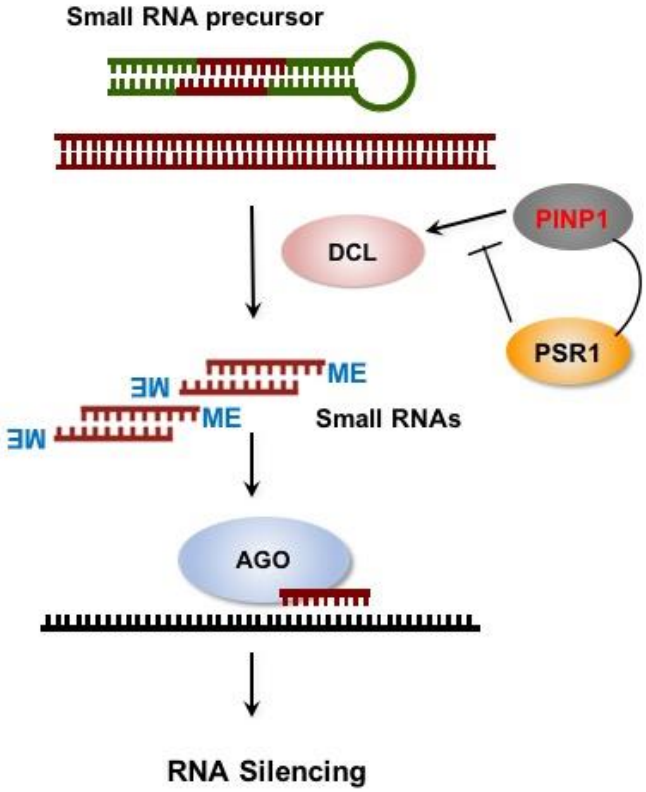
Recently, two RxLR effectors of *P. sojae* were shown to possess RNA silencing suppression activities. In the screening system, 59 *P. sojae* RxLR effetors and green fluorescent protein (GFP) were coexpressed by Agro-infiltration individually in the leaves of *Nicotiana benthamiana* 16c which consecutively express GFP under the control of the *cauliflower mosaic* virus *35S* promoter (Ruiz et al., 1998). The introduction of infiltrated *GFP* gene triggers silencing of both endogenous and exogenous *GFP* genes, resulting in no or very low green fluorescence in the infiltrated area. However, strong fluorescence is observed if *GFP* with virus silencing suppressor or two effectors from *Phytophothora*, indicating that they can suppress the transgene silencing. These *Phytophthora* effectors are PsAvh18 and PsAvh146, then are designated *Phytophthora* suppressor of RNA silencing 1 and 2 (PSR1 and PSR2).

Using transgenic *Arabidopsis* plants over expressing PSR1, the abundance of all the representative endogenous small RNAs was examined. The results show that PSR1 has a general negative impact on both miRNAs and siRNAs biogenesis (Qiao et al., 2015). Further analysis suggests that PSR1 interacts with a conserved nuclear protein namely PSR1-interacting Protein 1 (PINP1) in *Arabidopsis*, soybean and *N. benthamiana*. PINP1 contains an aspartate-glutamate-alanine-histidine-box RNA helicase domain and is highly conserved in eukaryotes. In *Arabidopsis*, PINP1 is required for the accumulation of both miRNAs and siRNAs and probably through facilitating the assembly of dicing

complexes. PSR1 directly interacts with PINP1 in the nucleus of plant cells and interferes with PINP1 function (Qiao et al., 2013) (Qiao et al., 2015). As such, *PINP1*-silenced plants and *PSR1*-expressing transgenic *Arabidopsis* are both hypersusceptible to *Phytophthora* infection (Qiao et al., 2015). A model illustrating the mechanism by which PSR1 affects plant small RNAs biogenesis is presented in Figure 1.1. The fact that PSR1 promote *Phytophthora* infection and the expression of a viral RNA silencing suppressor P19 also leads to increased susceptibility to *P. infestans*, suggesting that small RNA pathway is required for full resistance to *Phytophthora*.

Figure 1.1. A model showing the small RNA suppression mechanism of PSR1 in *Arabidopsis*.

DCL proteins process the small RNA precursors into short small RNAs duplexes. PSR1 interacts with PINP1 to affect the assembly of the dicing complexes, thus affecting the biogenesis of small RNAs.



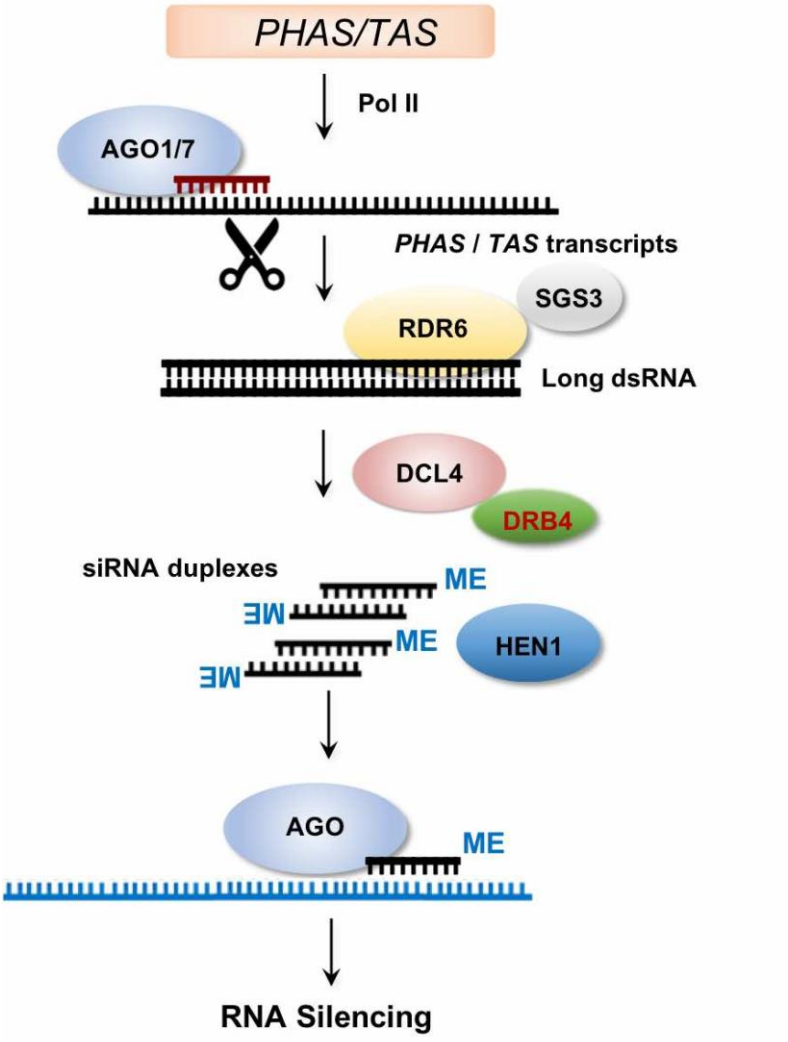
RNA silencing is an essential regulatory mechanism of gene expression in eukaryotes. It is mediated by small RNAs through transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) by guiding mRNA cleavage, translation inhibition or chromatin modification (Chen, 2009) (Bologna and Voinnet, 2014) (Borges and Martienssen, 2015). Major endogenous small RNAs in plants include microRNAs (miRNAs), which are encoded by *MIR* genes, secondary small interfering RNAs (siRNAs), which are derived in a miRNA-dependent manner, and heterochromatic siRNAs (hcsiRNAs), which are produced from repeats and transposable elements (Chen, 2009) (Bologna and Voinnet, 2014) (Borges and Martienssen, 2015).

Different with the general impact of PSR1 on all types of small RNAs, PSR2 specifically affects the accumulation of secondary siRNAs but not miRNAs or hcRNAs in *Arabidopsis thaliana* transgenic plants (Qiao et al., 2013). Among the secondary siRNAs in *Arabidopsis*, most of them are in 21-nt regime, therefore they are also called phased siRNA or phasiRNAs (Kuan et al., 2016). In the old days, phasiRNAs are defined as phased 21-nt siRNAs generated from the non-coding transcripts of the *TAS* loci and function *in trans*, thus, also called trans-acting small interfering RNAs (tasiRNAs). However, upon the advanced analysis of small RNA sequencing recently, more *PHAS* loci were defined as the target of miRNA to generate phasiRNAs, including *NB-LRR*, genes encoding pentatricopeptide repeat proteins (*PPR*) and *MYB* transcription factors, and non-coding *PHAS*/*TAS* loci (Li et al., 2016) (Cui et al., 2017). TasiRNAs production are

triggered by the cleavage of *TAS* loci through AGO1 or AGO7 in a “one-hit” or “two-hits” model (Axtell et al., 2006) (Fei et al., 2013) (Fei et al., 2016). Long dsRNAs are synthesized using cleaved products by RNA-dependent RNA polymerase 6 (RDR6) with the help of suppressor of gene silencing 3 (SGS3) (Chen, 2012) (Pumplin and Voinnet, 2013). The dsRNAs were then processed by Dicer-like 4 (DCL4), together with the assistance of double-stranded RNA binding protein 4 (DRB4) (Fukudome et al., 2011). The produced 21-nt siRNAs function like miRNAs which are incorporated into RNA-induced silencing complexes (RISCs) to guide target mRNA cleavage or translation inhibition (Mallory and Vaucheret, 2006) (Chen, 2009) (Figure 1.2).

Figure 1.2. A model showing the pathway of secondary siRNAs biogenesis in *Arabidopsis*.

Rather than directly binding to target mRNAs, some miRNAs trigger secondary siRNA production by targeting *PHAS* or *TAS* loci. This cleavage is mediated by AGO1 or AGO7, the long dsRNAs are synthesized by RDR6 and SGS3 and diced into small RNA duplexes by DCL4 and DRB4. One strand will be loaded into AGO to induce target gene silencing.



Though PSR2 specifically affects the accumulation of tasiRNAs generated from *TAS1/2* loci, the abundance of miR173, which triggers the production of tasiRNAs from the *TAS1/2* transcripts, is not changed in *PSR2*-expressing *Arabidopsis* plants (Qiao et al., 2013). These data suggest that PSR2 interferes with tasiRNA biogenesis without affecting its parent miRNA. However, the mechanism by which PSR2 interferes with small RNA biogenesis in plants remains unclear. PSR2 promotes *Phytophthora* infection when expressed in *N. benthamiana* and *Arabidopsis*, and *PSR2*-silenced mutants of *P. sojae* exhibited largely reduced virulence on soybean hypocotyls (Qiao et al., 2013). Therefore, tasiRNAs pathway is likely required for plant defense against *Phytophthora*.

In the known tasiRNAs biogenesis pathway, DRB4 is known to interact with DCL4 and is required for the dicing activity of DCL4 to produce 21-nt small RNAs (Hiraguri, 2005; Fukudome et al., 2011). Indeed, reduced accumulation of tasiRNA was observed in both *dcl4* mutant *Arabidopsis* plants and *drb4* plants (Nakazawa et al., 2007; Gasciolli et al., 2005; Curtin et al., 2008; Fukudome et al., 2011). In addition, DRB4 has been shown to positively regulate plant defense (Love et al., 2007; Haas et al., 2008; Qu et al., 2008; Jakubiec et al., 2012; Zhu et al., 2013; Barton et al., 2017). An earlier study showed that the viral translational transactivator protein P6 from *Cauliflower mosaic virus* (CaMV) binds with DRB4 in CaMV-infected cells, indicating that the possibility of DRB4 as a target of VSR (Haas et al., 2008). Later it was reported that DRB4 was required for HR triggered by turnip crinkle virus (TCV) in *Arabidopsis* eco Di-17 (Zhu et al, 2013). The

responsible R protein HRT directly interacts with DRB4 and its abundance was largely reduced in *drb4* mutant plants. Furthermore, the TCV coat protein (CP) also interacts with DRB4 in cytoplasm, and this CP-DRB4 interaction changes the cytoplasmic:nuclear ratio of DRB4 (Zhu et al., 2013). Therefore, DRB4 may be a virulence target of TCV CP protein, which has RNA silencing suppression activity. In another recent study of *drb4 Arabidopsis* mutant infected by (TuMV), *drb4* exhibited higher accumulation of virus compared with wild-type plants, indicating that DRB4 is essential for repressing viral replication (Barton et al., 2017). Since DRB4 plays an important role in anti-viral defense, it is possible that DRB4 is also involved in anti-*Phytophthora* infection.

Phytophthora are notorious for causing important plant diseases. Every year, the economic damage caused by *Phytophthora spp.* is estimated in billions of dollars worldwide. *Phytophthora* are hemibiotrophic pathogens. During the early biotrophic stage, *Phytophthora* secrete effector proteins as key virulence factors through haustoria (Stassen and Van den Ackerveken, 2011) (Dou and Zhou, 2012) (Fawke et al., 2015) (Judelson, 2017). Each *Phytophthora* genome is predicted to encode hundreds of cytoplasmic effectors, which are delivered into the plant cell to enhance disease development (Tyler, 2006) (Whisson et al., 2007) (Haas et al., 2009) (Lamour et al., 2012a) (Ye et al., 2016). These effectors are presumed to target various components of plant immunity.

PSR2 homologs are produced by a variety of *Phytophthora* species, and its homolog from *P. infestans* also possess RNA silencing suppression activity and promote *Phytophthora* infection (Xiong et al., 2014). Both homologs contain tandem repeats including the sequences of W, Y and L motifs, which is a unique structure feature of some oomycete RxLR effectors while it has not been found in fungi or other organisms (Boutemy et al., 2011) (Ye et al., 2015) (Ye and Ma, 2016). W, Y and L motifs are named after highly conserved residues, ranging from 21-30 residues in length (Jiang et al., 2008). For example, PiPSR2 has one more repeat than PsPSR2 (Xiong et al., 2014) (Figure 1.3). This interesting arrangement of PSR2 calls for further investigation on the contribution of the LWY repeat unit(s) to its functions in plant cells.

Figure 1.3. Phylogenetic relationship of PSR2 family effectors in sequenced oomycete genomes.

The phylogenetic tree was constructed using amino acid sequence alignment generated with the ClustalW algorithm using the neighbor-joining method.

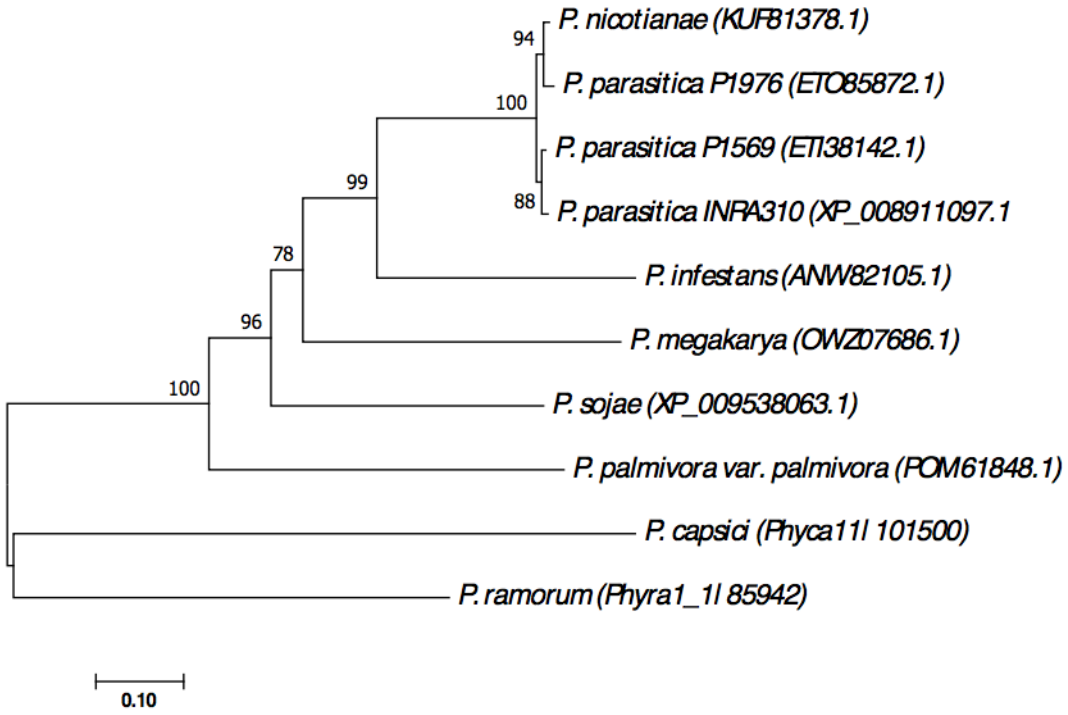
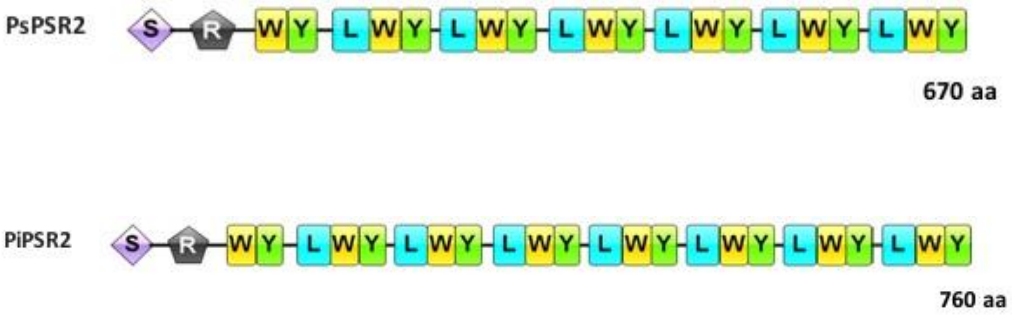


Figure 1.4. Schematic representation of the domain structure of PSR2 proteins.

PsPSR2 has seven tandem repeat units consisting W, Y and/or L motif, whereas PiPSR2 has eight tandem repeat units. In both protein sequences, the first repeat lacks L motif and only contains W and Y motifs. S: secretion signal; R: RxLR domain.



This main purpose of the study in this chapter is to understand the mechanism by which PSR2 interferes with small RNA biogenesis in plants. Using yeast two-hybrid screening, I identified DRB4 as the interacting protein of PSR2 in *Arabidopsis*. This interaction requires the two dsRBMs at the N terminus of DRB4. In addition, I found that the first two repeat units, WY1 and LWY2 play a key role in RNA silencing suppression and virulence activity of PSR2. Importantly, these two repeat units are essential for the interaction of PSR2 with DRB4. Finally, I demonstrate that *drb4 Arabidopsis* mutants exhibited similar developmental and disease phenotypes as PSR2 transgenic *Arabidopsis* plants, indicating that DRB4 is a virulence target of PSR2.

MATERIALS AND METHODS

Microbial strains and plasmids

P. capsici isolate LT263 was cultured on 10% (vol/vol) V8 medium at 25°C in the dark for 3 to 4 days. *Agrobacterium tumefaciens* strain GV3101, *Escherichia coli* strains DH5α and BL21 were grown in Luria-Bertani (LB) medium at 30°C and 37°C respectively as described (Wroblewski et al., 2005). The medium was supplemented with kanamycin at 50 μ g/ml, rifampicin at 50 μ g/ml, or gentamycin at 50 μ g/ml when necessary. The sequences encoding full length or truncated PSR2 were cloned from *P. sojae* using gene-specific primers into the Gateway entry vector TSK108-N3F and subsequently transferred to the destination vector pEG100 using LR reaction (Qiao et al., 2013); sequences encoding full length or truncated DRB4 were cloned from Arabidopsis using gene-specific primers into the Gateway entry vector pENTR1a and subsequently into vector pEG101 using LR reaction (Invitrogen) (Earley et al., 2006). The recombinant plasmids were transformed into *Agrobacterium* GV3101 using freeze-thaw method (Höfgen and Willmitzer, 1988).Constructs and primers used in this study are summarized in Table 1.1 and Table 1.2.

Plant materials and growth conditions

Arabidopsis thaliana seeds were sown in soil and vernalized at 4°C for 3 days. The plants were then grown in a conditioned growth room at 22°C with a 12/12 light/dark regime.

PSR2 was tagged with 3xFlag on the N-terminus and cloned into the vector pEG100 (Qiao et al., 2013). DRB4 under its native promoter was cloned into the vector pGWB640 (Chen Lab). The recombinant plasmids were transformed into *Agrobacterium* and the *Arabidopsis* wildtype (WT) plants were transformed using the floral dip method. *drb4* (SALK_000736) was provided by the Salk T-DNA collection.

Nicotiana benthamiana plants were geminated and grown in a conditioned growth room at 22°C with a 12/12 light/dark regime. *N. benthamiana* 16c is a stable transgenic line consecutively expressing *GFP* under the control of the cauliflower mosaic virus *35S* promoter (Haseloff et al., 1997) (Ruiz et al., 1998).

Sequence analysis of *PSR2* homologs in oomycetes.

The full-length amino acid sequence of *PSR2* was used to search against the genome sequences of different *Phytophthora* spp. for potential homologs (Sequences obtained from Department of Energy Joint Genome Institute database and National Center for Biotechnology Information). Neighbor-joining trees were generated using MEGA 7 with 1,000 bootstraps and other default parameters.

Yeast two-hybrid assay

PSR2, without the N-terminal secretion signal (1-17aa), was cloned into the bait vector pGBKT7–BD. A cDNA library of *Arabidopsis* was constructed in the prey vector pGADT7-

AD. The cDNA library was screened by introducing into the yeast strain AH109 carrying the plasmid *pGBKT7-BD::PSR2*. Yeast transformants carrying both plasmids were selected on a synthetic medium supplemented with dropout solution (SD-2) plates, lacking tryptophan (Trp) and leu (Leucine). Individual colonies were subsequently screened using 100 ul X-α-Gal (20mg/ml) (GoldBio) plus 10mM 3-amino-1,2,4-triazole (3-AT) on a selective medium plate (SD-3) lacking Trp, Leu, histidine (His). Potential PSR2-interacting proteins were then identified by DNA sequencing. The numbers of clones screened is calculated based on the handbook provided by Matchmaker GAL4 two-hybrid system 3 (Clontech).

Full length sequences of potential candidates from *Arabidopsis* were cloned into the prey vector pGADT7-AD. The recombinant plasmid was transformed into yeast strain AH109 harboring *pGBKT7-BD::PSR2*, the transformants were selected on SD-2 plates and followed by SD-4 plate lacking Trp, Leu, His and adenine (Ade) but with X-α-Gal.

dsRNA preparation and dsRNA cleavage assay

The sense and antisense transcripts were synthesized using 0.5 µM home-made T7 RNA polymerase, 5 mM each NTP (Roche), and 0.1 µM plasmid template containing partial GFP gene (100 bp or 500bp, sequences were listed in table 1.3) in 50 µl reactions at 37^{o}C for 3 hours. Then 0.1 U/ µl Turbo DNase (Ambion) were added to the reactions to remove template DNA at 37^{o}C for 15 minutes. Nucleotides and NTPs were removed

using Bio-Spin 6 Columns (BioRad). dsRNAs were extracted with acidic phenol/chloroform (Ambion), precipitated in Isopropanol (FisherChemical), and dissolved in RNase free water. Equal amounts of these ssRNAs were annealed at 90^{o}C for 5 min, followed by turning off the heater for 10 min and incubating for 10 min at room temperature in annealing buffer containing 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl (Fukudome et al., 2011).

For the cleaving assay, [α-^{32}P] UTP-labeled 500-bp dsRNAs were synthesized as described above. 4-week-old Arabidopsis leaves were collected, and proteins were extracted in 1.5 ml/g of extraction buffer containing 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 5 mM DTT, 1 x Protease inhibitor cocktail and 1 mM PMSF at 4°C. The concentration of those ^{32}P-labeled dsRNAs was estimated on 8% native PAGE followed by SybrGold Staining. The dsRNAs (final concentration ~ 0.5 nM) were incubated with 30 µL of crude extracts at 23 °C for 2h in 45ul dsRNA-cleaving buffer containing 30 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4 mM $MgCl_2$, 5 mM ATP and 1 mM GTP. As well, 1 µL of RNaseOUT (Invitrogen) was added to each 40ul reaction. After incubation, the cleavage products were purified by phenol/chloroform (Ambion), precipitated in ethanol and dissolved in RNase-free water. The dsRNAs were then analyzed on 15% denaturing PAGE with 8M urea and detected by autoradiography. The signal of the 500-bp dsRNA bands was used to normalize the signal of small RNAs. Wild type (WT) plant was used as a negative control and the abundance of small RNAs in WT sample were set to 1. Total

proteins were analyzed on SDS-PAGE gel and gel stained with Coomassie Blue (Bio-rad) was used as a loading control.

RNA extraction and northern blotting

Total RNA was extracted from 4-week old Arabidopsis leaves using TRizol reagent followed the company protocol (Ambion). Small RNA northern blotting was performed as described (Park et al., 2002) (KURIHARA, 2005). 5 μg total RNA was loaded for each sample and U6 is used as loading control. The oligonucleotide probes are listed in Table 1.2.

In vitro GST pull-down assay

To construct GST-fusion plasmids, the full-length *DRB4* gene was inserted into the vector pGEX4T-2 (GE Healthcare Life Science). *PSR2* gene was cloned into the vector pRSF, which has Sumo and His in the N-terminus. GST pull-down assay was carried out using GST pull-down protein:protein interaction kit (Pierce) following the manufacturer's instructions. Briefly, GST-DRB4 or Sumo-His-PSR2 were expressed in *E.coli* strain BL21 (RIL). Soluble proteins were incubated with 30ul glutathione agarose beads (Thermo Scientific) for 1 hour at 4°C. The beads were washed with TKET150 (20 mM Tris-HCl (pH=7.5), 150 mM KCl, 0.1 mM EDTA and 0.05% Triton X-100) five times and then incubated with purified Sumo-His-PSR2 proteins at 4°C overnight. The beads then were washed for 5 times and the presence of the PSR2 protein on the beads was detected by

western blotting using anti-His antibodies conjugated with horseradish peroxidase (HRP) (R&D system). GST only was used as a negative control and gel stained with Coomassie Blue (Bio-rad) was used as a loading control.

Protein pull-down assays in planta

Fully extended leaves of *N. benthamiana* plants at the six-leaf stage were infiltrated with *Agrobacterium* harboring WT or truncated *3xFlag-PSR2* and *Agrobacterium* carrying *DRB4-YFP*, or *Agrobacterium* harboring WT or truncated *DRB4-YFP* and *Agrobacterium carrying 3xFlag-PSR2*. The *Agrobacterium* cells were suspended in 10 mM $MgCl_2$ to OD_{600} = 1.0, then the cells suspension that needs to be co-infiltrated were mixed to reach OD_{600} = 0.5. At last, the cells were activated using 10mM MES and 10mM Acetosyringone. After 3 hours induction, the cell suspension was infiltrated into leaves using 1ml syringe. Leaves at 48 hours post infiltration (hpi) were collected for pull-down in planta. Total proteins were extracted using an IP buffer [10% (vol/vol) glycerol, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5mM DTT, 1x protease inhibitor mixture (Roche), 1mM PMSF, and 0.1% CA-630], and the crude extracts (0.5 g/ml) were incubated with either 40 ul anti-Flag (Sigma-Aldrich) or 15 ul anti-GFP (Chromotek) agarose beads at 4°C for 2-12 hours. The immune complexes were washed 5 times with IP buffer [10% (vol/vol) glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5mM DTT, and 0.1% CA-630] detected by anti-Flag (Sigma Aldrich) or anti-GFP antibody (Clontech) using western

blot. YFP protein was used as a negative control and gel stained with Coomassie Blue (Bio-rad) was used as a loading control.

Fluorescence microscopy

To construct plasmids for bimolecular fluorescence complementation (BiFC) assay, *PSR2* without the N-terminal secretion signal (1-17aa) and full-length cDNA of *DRB4* were cloned into the vectors pVYNE and pVYCE, respectively. To examine the subcellular localization of PSR2, full-length cDNA was cloned into the vector pEG104 with an N-terminus YFP tag and pGWB645 to generate N-terminus CFP fusion protein. Full-length cDNA of *DRB4* was cloned into vector pEG101 and pGWB644 to generate C-terminal YFP and C-terminal CFP fusion proteins, respectively. All fusion proteins were transformed into *Agrobacterium* individually and were later infiltrated in 4-week old *N. benthamiana* leaves with an OD_{600} = 0.5. The functional fluorophore was visualized in the infiltrated leaves using a Leica SP5 Laser Scanning Confocal Microscope (Leica) for BiFC a Zessie 880 Inverted Confocal Microscope (Zessie) for subcellular localization at 48 hpi.

RNaseIII digestion assay

YFP-DRB4 and 3xFlag-PSR2 were co-expressed in *N. benthamiana* by Agro-infiltration. Total proteins were extracted using leaves collected at 48 hpi and incubated with anti-Flag agarose beads (Sigma) at 4°C for 4 hours. After 3 times washing, the immune complexes were treated with ShortCut RNaseIII (NEB) and then were washed with IP

buffer for 3 times. Partial complexes were detected by anti-Flag (Sigma Aldrich) or anti-GFP antibody (Clontech) using western blot. YFP protein was used as a negative control and gel stained with Coomassie Blue (Bio-rad) was used as a loading control. Total RNA was extracted from the rest of the complexes using Trizol (Ambion) and then analyzed on 10% denaturing PAGE with 8M urea, followed by SybrGold staining (Invitrogen).

dsRNA binding assay

Recombinant protein YFP-PSR2 was transiently expressed in *N. benthamiana* and the leave samples were collected at 48 hpi. The total proteins were extracted using the extraction buffer described above, plus the treatment of 10 ul RNaseIII (NEB), 10 ul $MnCl_2$ buffer and 10 ul 10x RNaseIII buffer. The immune complexes were pulled-down by 50 ul anti-GFP agarose beads (Chromotek) after 4 hours incubation at 4 °C. After 4 times washing, the 100-bp dsRNAs were incubated with the immune complexes for 35 min at 4 °C in dsRNA-binding buffer containing 30 mM Tris-HCl (pH 7.0), 10 mM NaCl, 20 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM DTT (Fukudome et al., 2011). After washing twice, total RNA in this complex was extracted using phenol/chloroform (Ambion) and precipitated in ethanol at -20 °C overnight and then analyzed on 6% native PAGE, followed by SybrGold Staining (Invitrogen). YFP protein was used as a negative control and DRB4-YFP was used as a positive control.

RNA silencing suppression assays using *N. benthamiana* 16c plants

PSR2 and its derivatives were cloned into the destination vector pEG100. Plasmids were transformed into *Agrobacterium* strain GV3101, and then were transient expressed together with *35S:GFP* in *N. benthamiana* 16c. Green fluorescence was observed using a handheld long-wavelength UV lamp (BlackRay B100AP, UVP) at 5 days after infiltration. *Agrobacterium* carrying the empty vector pEG100 was used as a negative control.

PSR2 protein abundance was examined 2 days after Agro-infiltration in the infiltrated leaves by western blot using anti-PSR2 antibody. The abundance of GFP protein was confirmed after photos were taken by western blot using anti-GFP antibody (Santa Cruz). Empty vector pEG100 was used as a negative control and gel stained with Coomassie Blue (Bio-rad) was used as a loading control.

***Phytophthora* infection assays**

Arabidopsis seedlings for *Phytophthora* infection assay were grown on Murashige and Soog agar containing 1% (wt/vol) sucrose. Roots of 2-week old seedlings were dipped in *P. capsici* isolate LT263 zoospores suspension (1 x 10^5 zoospores per mL) as previously described (Yan Wang 2013). The seedlings were then transferred into soil, photos were taken at 3 days post inoculation (dpi). Detached leaves of 4-week old *Arabidopsis* were inoculated with *P. capsici* isolate LT263 by using 10 µL of zoospores suspension (1 x 10^5 zoospores per mL) as previously described (Wang et al., 2013). Disease severity was

evaluated at 3 dpi using a disease index based on hyphae extension and visualized by Trypan blue staining.

N. benthamiana leaves transiently expressing PSR2 and its derivatives were detached 36 hours after Agro-infiltration and inoculated with mycelium-growing agar plug of *P. capsici* isolate LT263 at the abaxial side, 5 ul water was added in the interface of leave and mycelium plug. Leaves were kept in sealed plates with high humidity in the dark at 25°C. Disease symptoms and lesion sizes were measured at 3 dpi and photos were taken under a handheld long-wavelength UV lamp (BlackRay B100AP, UVP). YFP was used as a negative control.

Table 1.1. Strains or plasmids used in Chapter I.

Strains or Plasmids	Description	Source/reference
Escherichia coli DH5α	F- Φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1, hsdR17(rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1	Invitrogen
Agrobacterium tumefaciens GV3101	Rif^R, $Gent^R$	Wroblewski, 2005
Phytophthora capsici isolate LT263	Isolated from pumpkin, can infect Arabidopsis	Donahoo and Lamour , 2008
Phytophthora capsici isolate 1534	Mating from LT263 and OP97	Stam,2013
pGBKT7BD::PSR2	Yeast BD vector carrying N-terminus c-myc tagged PSR2, Kan^R	This study
pGADT7AD::DRB4	Yeast AD vector carrying N-terminus HA tagged DRB4, Amp^R	This study
pGEX4T-2::DRB4	pGEX4T-2 carrying DRB4, Amp^R	This study
pRSF::PSR2	N-terminal 6xHis-Sumo tagged PSR2, Kan^R	This study
pEG100::3xFlag-PSR2	pEG100 carrying PSR2 tagged with 3xFlag at N-terminus, Kan^R	Qiao et al., 2013
pEG101::DRB4	pEG101 carrying DRB4 tagged with YFP at C-terminus, Kan^R	This study

pGWB640::pDRB4-DRB4	pGWB640 carrying DRB4 tagged with YFP at C-terminus, Spc^R	This study
pEG100::3xFlag-PSR2$^{\Delta WY1}$	pEG100 carrying PSR2 with deletion of WY1 tagged with 3xFlag at N-terminus, Kan^R	This study
pEG100::3xFlag-PSR2$^{\Delta LWY2}$	pEG100 carrying PSR2 with deletion of LWY2 tagged with 3xFlag at N-terminus, Kan^R	This study
pEG100::3xFlag-PSR2$^{\Delta WY1+LWY2}$	pEG100 carrying PSR2 with deletion of WY1 and LWY2 tagged with 3xFlag at N-terminus, Kan^R	This study
pEG100::3xFlag-PSR2$^{WY1+LWY2}$	pEG100 carrying WY1 and LWY2 of PSR2 tagged with 3xFlag at N-terminus, Kan^R	This study
pEG101::DRB4ΔdsRBD1	pEG101 carrying DRB4 with deletion of dsRBM1 tagged with YFP at C-terminus, Kan^R	This study
pEG101::DRB4ΔdsRBD2	pEG101 carrying DRB4 with deletion of dsRBM2 tagged with YFP at C-terminus, Kan^R	This study
pEG101::DRB4ΔdsRBD1+2	pEG101 carrying DRB4 with deletion of dsRBM1 and dsRBM2 tagged with YFP at C-terminus, Kan^R	This study
pEG101::DRB4ΔC	pEG101 carrying DRB4 with deletion of C-terminus tagged with YFP at C-terminus, Kan^R	This study
pGWB644::DRB4	pGWB644 carrying DRB4 tagged with CFP at C-terminus, Spc^R	This study

pGWB645::PSR2	pGWB645 carrying PSR2 tagged with CFP at N-terminus, Spc^R	This study
pEG104::PSR2	pEG104 carrying PSR2 tagged with YFP at N-terminus, Kan^R	This study

Table 1.2. Primers used in Chapter I.

	Primer sequence
pGADT7::DRB4-F	cca cccgggT GATCATGTATACAAAGGTCAAC
pGADT7::DRB4-R	gtg ctcgag TTA TGGCTTCACAAGACGATAG
pGEX4T-2::DRB4-F	cgt ggatcc ATGGATCATGTATACAAAGGTCAAC
pGEX4T-2::DRB4-R	cgctcgagtTGGCTTCACAAGACGATAGG
pRSF::PSR2-F	tcc gaattc atgACACATGCTCCTCCTAACGTT
pRSF::PSR2-R	gcat gatatc TTA CCCCCACCTGACTTTGAAC
pENTR1a::DRB4-F	tca GTC GAC TGG ATC CGG ATGGATCATGTATACAAAGGTCAACTGC
pENTR1a::DRB4-R	tag ata tct cga gtg TGGCTTCACAAGACGATAGGCTA
OV-pENTR1a::DRB4ΔdsRBM1-F	ACGCCACAAAGTCCAGAGGG
OV-pENTR1a::D4RBΔdsRBM1-R	TACATGATCCATGTCGACTGAA
OV-pENTR1a::DRB4ΔdsRBM2-F	AAAAATGGGAACTCGAACCAGAC
OV-pENTR1a::DRB4ΔdsRBM2-R	GGCAACATCAATTCCCTCTGG
OV-pENTR1a::DRB4ΔC-F	CACTCGAGATATCTAGACCCAGCT
OV-pENTR1a::DRB4ΔC-R	GATACTCATGAATGCAACTTTAG
TSK108::DRB4G-F	CACGGTACCGAATTTCTTACGGATCTCGGAC

TSK108::DRB4G-R	ACGGGATCCTGGCTTCACAAGACGATAGGCT
pTSK108N3F::PSR2$^{\Delta WY1}$-F	TCGGAAGCTTCCGCCGTTATG
pTSK108N3F::PSR2$^{\Delta WY1}$-R	TTTCAAACCGGGCTTAGGCTTTA
pTSK108N3F::PSR2$^{\Delta LWY2}$-F	CCCAAAGCCCAAACGACTTTGA
pTSK108N3F::PSR2$^{\Delta LWY2}$-R	GTCGGTCTGCGCGACCGAG
pTSK108N3F::PSR2$^{WY1+LWY2}$-F	CGGAATTCGGAATCAACTTCAGTTCGGTG
pTSK108N3F::PSR2$^{WY1+LWY2}$-R	GCTCTAGA TTA CTTGTTCGACAGCTTCATATAG
T7-gfp100-sen-F	GAAATTAATACGACTCACTATA GGG GAGGGATACGTGCAGGAGAG
gfp100-sen-R	GATCCTGTTGACGAGGGTGT
gfp100-anti-F	GAGGGATACGTGCAGGAGAG
T7-gfp100-anti-R	GAAATTAATACGACTCACTATA GGG GATCCTGTTGACGAGGGTGT
T7-gfp500-ss-F	GAAATTAATACGACTCACTATA GGG GAAAACTACCTGTTCCATGGCCAAC
gfp500-sen-R	GAAGGACCATGTGGTCTCTCTTTTCG
gfp500-anti-F	GAAAACTACCTGTTCCATGGCCAAC
T7-gfp500-anti-R	GAAATTAATACGACTCACTATA GGG GAAGGACCATGTGGTCTCTCTTTTCG
ASRP255	TACGCTATGTTGGACTTAGAA
ASRP1151	AAGTATCATCATTCGCTTGGA

miR161	ACCCCGATGTAGTCACTTTCA
miR173	GTGATTTCTCTCTGTAAGCGA
U6	AGGGGCCATGCTAATCTTCTC
22-nt DNA	GCCAGTTGGTATACTCAGGTGG

Table 1.3. *GFP* sequences used in Chapter I.

***GFP* 100-bp sequence**

GAGGGATACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCT
CGTCAACAGGATC

***GFP* 500-bp sequence**

GAAAACTACCTGTTCCGTGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAGCGGCAC
GACTTCTTCAAGAGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAA
GTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTG
GAATACAACTACAACTCCCACAACGTATACATCATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCAAGACCCGCCACAACATCG
AAGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCC
ACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT

RESULTS

Identification of PSR2-interacting protein(s) in *Arabidopsis*

Previously, PSR2 was identified as *Phytophthora* suppressor of RNA silencing that affects the biogenesis of small RNA in plant host (Qiao et al., 2013). The first key question is that how PSR2 targets the small RNA pathway. To address this question, I screened the potential interacting protein(s) of PSR2 by yeast two-hybrid (Y2H) assays using the MATHCHMAKER GAL4 Two-Hybrid System 3. After five independent transformations, I screened a total of 3.83 million transformants and obtained 507 sequences. These clones were analyzed for frame-shift, common false positive sequences, and autonomous activation. At the end, 26 proteins were considered to be potential interactors of PSR2. Among them, 10 are RNA-binding proteins, and another five can bind to DNA (Table 1.4), indicating that PSR2 may interact with nucleic acid-binding proteins in plants. The interactions of PSR2 with some of these candidates were then further investigated.

Table 1.4. Candidates of PSR2-interacting proteins.

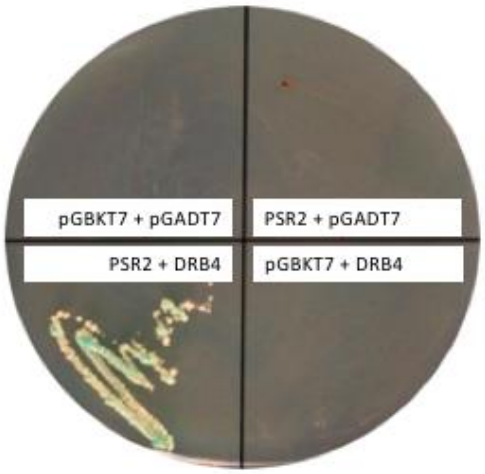
RNA binding proteins	Cloned sequence in cDNA library	Full length
Double-stranded-RNA-binding protein 4	1-975	1008
Spliceosome associated protein SF3B4	1-747	1092
RNA-binding protein 37	1-657	981
SC35-like splicing factor 30	147-348	789
DNA-directed RNA polymerase V subunit 5A	1-663	669
RNA helicase DRH1	162-867	1857
Ribosomal protein S1-like RNA-binding domain-	18-684	1179
Multiple organellar RNA editing factor 3	111-534	735
DEAD/DEAH box helicase	1686-2466	2469
Argonaute 1	2352-3048	3153
DNA binding proteins		
Transcription initiation factor IIA subunit 2	1-321	321
GATA transcription factor 20	1-627	627
Histone H2B	150-453	453
Translesion synthesis polymerase zeta subunit REV7	1-594	648
Homeobox-leucine zipper protein HAT22	415-606	837

DRB4 is a potential candidate of PSR2-interacting protein

From the screening results, I am particular interested in DRB4, which is known to interact with DCL4 and is required for the dicing activity of DCL4 to produce 21-nt tasiRNAs (Fukudome et al., 2011). Moreover, PSR2 was reported to specifically affect the accumulation of secondary siRNAs (Qiao et al., 2013). The yeast colony selected from SD-4 plate together with LacZ filter assay was shown and the empty vector were used as negative controls (Figure 1.5).

Figure 1.5. Interaction of PSR2 with DRB4 in yeast two-hybrid assay.

PSR2 without the N-terminal secretion signal or the RxLR-dEER motif fused to GAL4 DNA binding domain (pGBKT7-BD) was expressed in combination with DRB4 fused to activation domain (pGADT7-AD) in yeast strain AH109. Transformants were selected on media lacking Trp, Leu and His (SD-3 plate) together with 1mM 3'AT, and then individual colonies were grown on selective media lacking Trp, Leu, His and Ade (SD-4 plate) but with x-a-gal at 30°C for 4 days. Empty vector (EV) was used as negative control. Empty vector was used as negative control.

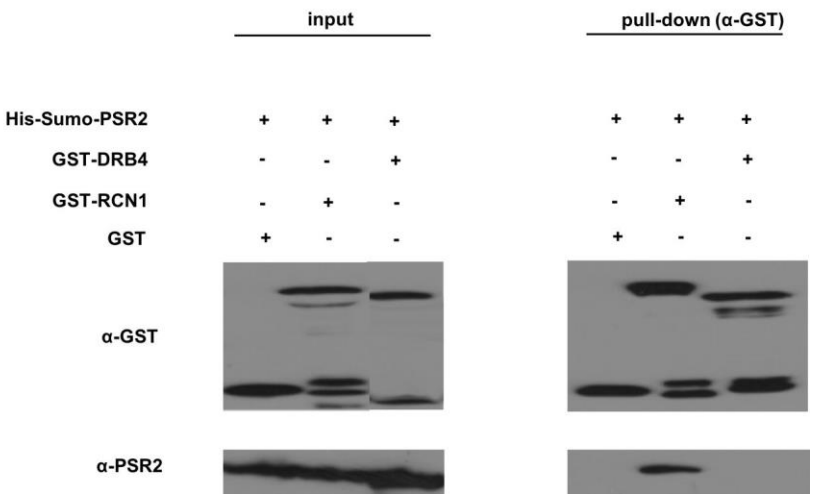


PSR2 does not interact with DRB4 in vitro

To further confirm the interaction between PSR2 and DRB4, GST pull-down assay was also tried to confirm the interaction, GST-tagged DRB4 were immobilized on glutathione-agarose beads and used to pull down full-length PSR2 protein. GST protein was used as a negative control (Figure 1.6). Surprisingly, no interaction between PSR2 and DRB4 was observed, suggesting that PSR2 and DRB4 does not interact in vitro. Thus, I suspect that other components such as proteins or nuclei acids are needed to mediate this interaction, otherwise it is possible that modification or protein structure of DRB4 is changed with presence of PSR2.

Figure 1.6. Interaction of PSR2 with DRB4 in GST pull-down assay.

DRB4 fused with the GST tag and PSR2 protein fused with a Sumo-His tag were expressed in *E. coli* strain BL21 individually. The recombinants were incubated together and the co-precipitation of DRB4 with PSR2 using anti-GST resins was examined by anti-PSR2 antiserum to verify their interaction. RCN1 known as another PSR2-interacting protein that other people is working on is used as a positive control.



PSR2 associates with DRB4 in planta

In order to prove the interaction in vivo, I then carried out Co-immunoprecipitation (Co-IP) and Bimolecular fluorescence complementation analysis (BiFC) assay. Flag-tagged PSR2 and YFP-tagged DRB4 were co-expressed in *N. benthamiana* using Agrobacterium-infiltration and then the total protein were incubated with anti-Flag agarose beads. The western blot result suggests that PSR2 associate with DRB4 in planta, using HopZ1a which is a T3SS effector as a negative control (Figure 1.7). In addition to Co-IP, the interaction was also shown by BiFC assay, using the empty vector as a negative control (Figure 1.8).

Figure 1.7. PSR2 interacts with DRB4 in planta.

3xFlag-PSR2 and DRB4-YFP were expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using anti-Flag agarose beads. The co-precipitated proteins were then detected by western blotting. 3xFlag-HopZ1a C/A was used as a negative control and the gels stained with Coomassie Brilliant Blue (CBB) were used as loading controls. Experiments were repeated four times with similar results.

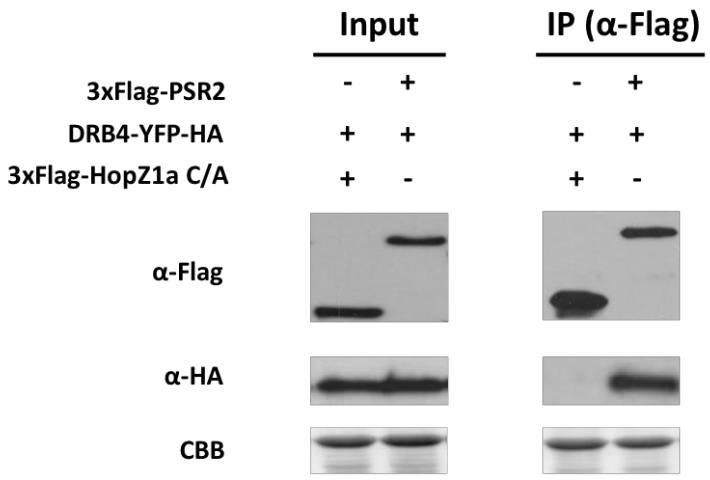
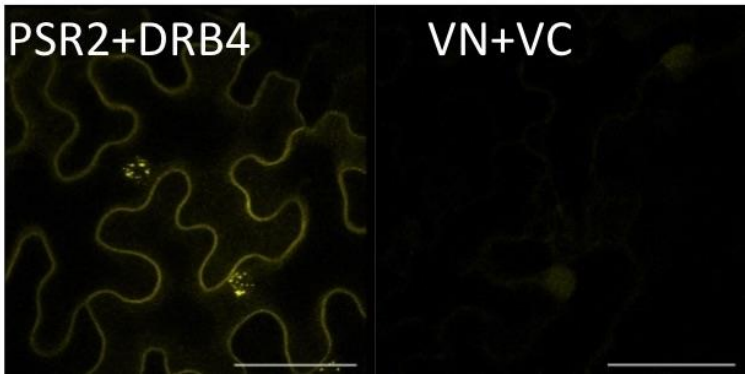


Figure 1.8. PSR2 interacts with DRB4 in nucleus and cytoplasm.

Bimolecular fluorescence complementation analysis showing PSR2/DRB4 interaction in the nucleus and cytoplasm of plant cells. PSR2–nVenus and DRB4–cVenus were co-expressed in *N. benthamiana* through Agro-infiltration. Fluorescence was detected by confocal microscopy at 48 hpi. Empty vector (VN and VC) were used as negative control. Bars = 50 µm.

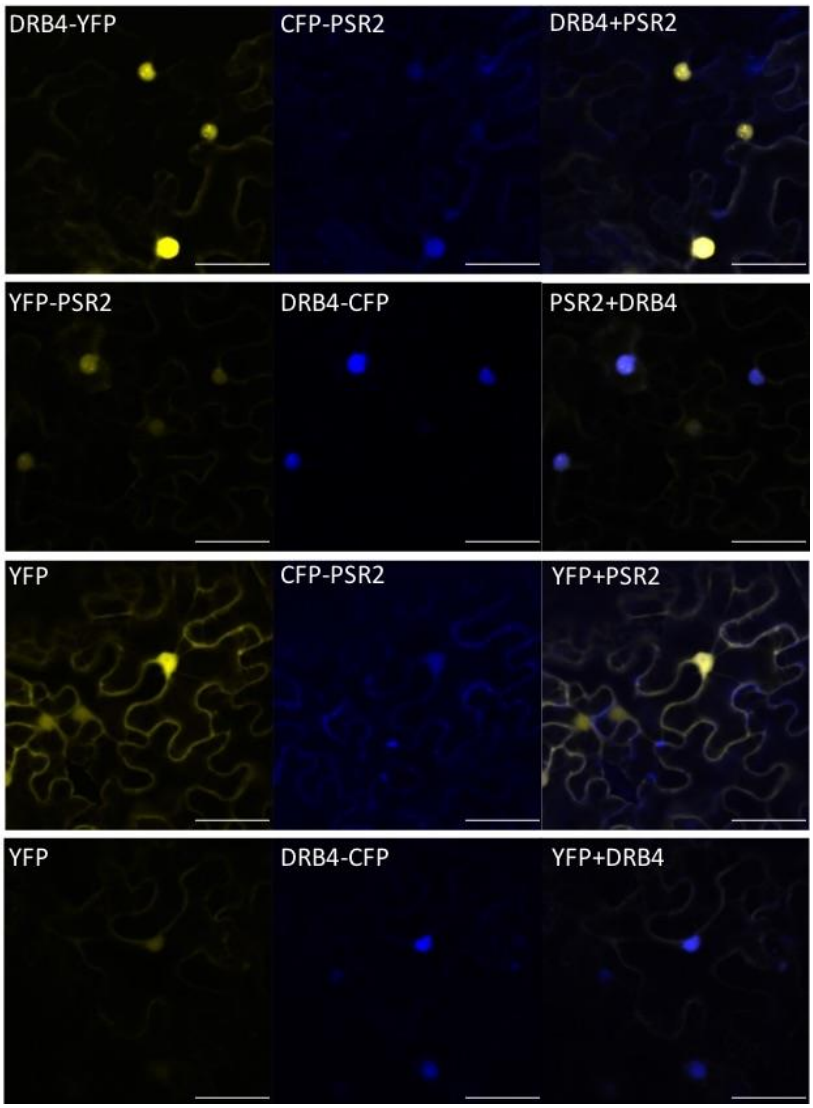


DRB4 associates with PSR2 in nucleus and cytoplasm

To further confirm the sub-cellular localization of DRB4 and PSR2, I co-expressed DRB4 and PSR2 fused with YFP and CFP tags respectively in *N. benthamiana* by Agro-infiltration and then observed using a confocal microscopy. As the fluorescent protein excited by longer wavelength could also be excited by the lower wavelength, the fluorescent tags were also exchanged to eliminate the possibility of false localization, YFP and CFP only were used as negative control. Both PSR2 and DRB4 localize in nucleus and cytoplasm, however, DRB4 had a unique localization in small speckles in the nucleus (Figure 1.9). PSR2 possess this localization only with the presence of DRB4, suggesting that PSR2 is associated with DRB4.

Figure 1.9. DRB4 co-localized with PSR2 in nucleus and cytoplasm.

PSR2 co-localizes with DRB4 in nucleus and cytoplasm. DRB4-YFP and CFP-PSR2 or YFP-PSR2 and DRB4-CFP were co-expressed in *N. benth*amiana through *Agro*-infiltration. Fluorescence was examined at 48 hpi using confocal microscope. YFP was used as negative control. Bars = 50 µm.



Double stranded RNA-binding domains are required for DRB4 interaction with PSR2

It is known that DRB4 is consist of two double-stranded RNA-binding motifs (dsRBMs) and they are required for DCL4-DRB4 interaction (Fukudome et al., 2011). It could be possible that PSR2 interacts with either one or both dsRBMs in planta. Therefore, I generated different deletion mutants of DRB4 and transiently expressed the proteins in *N. benthamiana* by Agrobacterium-infiltration (Figure 1.10). The Co-IP result demonstrates that DRB4 losing either one of the dsRBMs would result in weaker interaction with PSR2, while losing both dsRBMs would abolish this interaction (Figure 1.11). This result provides hint that PSR2 may disrupt the role of DRB4 in dsRNA processing.

Figure 1.10. Schematic representation of the domain structure of DRB4 protein and its derivatives.

DRB4 has two double stranded RNA-binding motifs (dsRBM1 and dsRBM2) on the N-terminus.

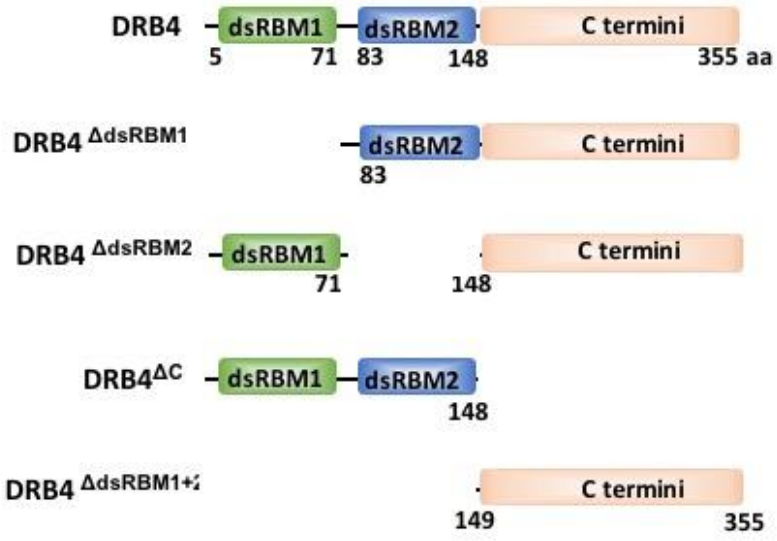
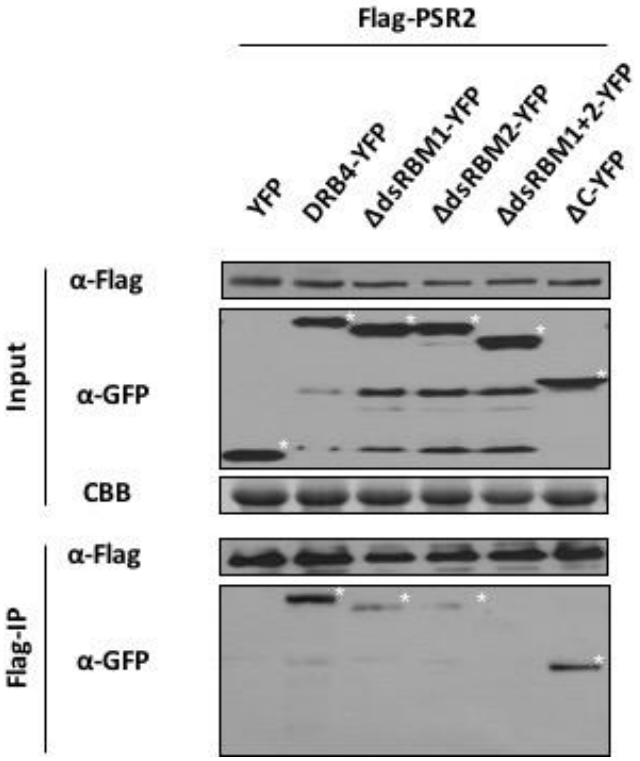


Figure 1.11. PSR2 associates with DRB4 at dsRBMs in planta.

PSR2 and DRB4 or its derivatives were co-expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using either anti-Flag beads. The co-precipitated proteins were then detected by anti-GFP or anti-Flag antibody respectively using western blotting. YFP was used as a negative control and the gels stained with Coomassie Brilliant Blue (CBB) were used as loading control. Experiments were repeated twice with similar results. The bands marked with asterisk are DRB4 and its devatives.



The interaction between PSR2 and DRB4 is not dependent on long dsRNA

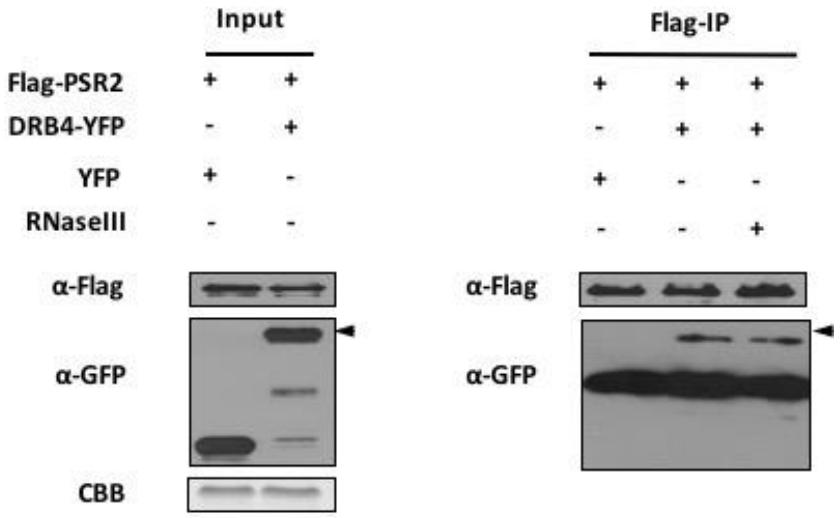
In order to understand whether dsRNA mediates the interaction between PSR2 and DRB4, I introduced RNaseIII which specifically cuts long dsRNA into small RNAs to the Co-IP system described before. After RNaseIII treatment, DRB4 was still successfully pulled down by PSR2, suggesting that PSR2 binding to DRB4 is not depend on dsRNA (Figure 1.12). The co-immune complexes were also detected for long dsRNA after RNaseIII treatment (Figure 1.12). YFP was used as a negative control. Consistent with this result, PSR2 expressed in *N. benthamiana* using Agrobacterium-infiltration was proved not to bind with 100-bp dsRNA in vitro (Figure 1.13). YFP and DRB4 expressed in the same condition were used as negative and positive control respectively. These data suggest that dsRNA is not the component mediating the interaction between PSR2 and DRB4.

Figure 1.12. DRB4-PSR2 interaction is not dependent on dsRNA.

(A) DRB4 interacts with PSR2 with treatment of RNaseIII. PSR2 and DRB4 were co-expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using anti-Flag magnetic beads. The co-precipitated proteins were then detected by anti-Flag antibody respectively using western blotting. YFP was used as a negative control and the gels stained with Coomassie Brilliant Blue (CBB) were used as loading control. Black arrowhead: DRB4-YFP.

(B) dsRNAs were successfully digested by RNaseIII. The immune complexes pulled-down by anti-Flag beads were treated by RNaseIII and then analyzed by 10% denaturing PAGE with 8M urea, followed by SybrGold staining.

A


Input
Flag-IP
Flag-PSR2
DRB4-YFP
YFP
RNaseIII
α-Flag
α-GFP
CBB
α-Flag
α-GFP

B

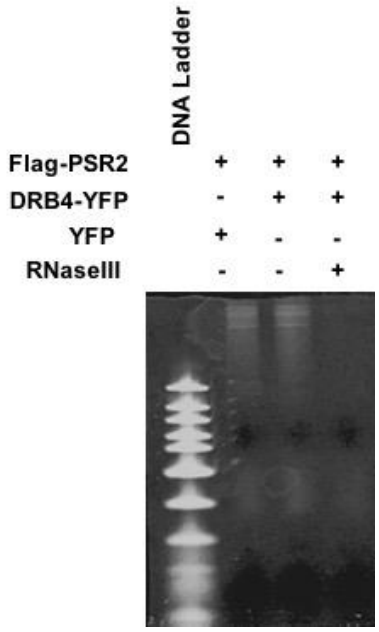
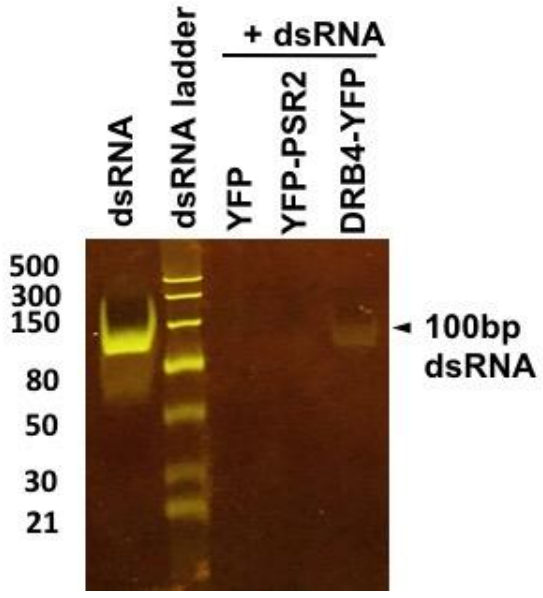

DNA Ladder
Flag-PSR2
DRB4-YFP
YFP
RNaseIII

Figure 1.13. PSR2 does not possess long dsRNA binding activity in vitro.

PSR2 and DRB4 were expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using anti-GFP agarose beads. The co-precipitated proteins were then incubated with 100-bp dsRNA and then analyzed on 6% native PAGE, stained with SybrGold. YFP was used as a negative control and dsRNA ladder was used as a size marker. Black Arrowhead: 100-bp dsRNA. Experiments were repeated twice with similar results.



PSR2 affects secondary siRNA production

Since PSR2 is shown to specifically affect biogenesis of secondary siRNAs (Qiao et al., 2013), I focused my research on the potential interaction of PSR2 with DRB4. DRB4 is known to bind with dsRNA precursor to assist DCL4 in secondary siRNA biogenesis (Xie et al., 2005; Nakazawa et al., 2007; Curtin et al., 2008; Fukudome et al., 2011; Vaucheret et al., 2015). The sequence identified from the *Arabidopsis* cDNA library that was used for Y2H screening is 975 bp in length, almost covering the full length of DRB4 (1008 bp).

It has been reported that *Arabidopsis* crude extract lacking in DRB4 lost the ability to cleave long dsRNA into 21-nt small RNAs (Fukudome et al., 2011). Therefore, I attempted to detect that whether PSR2 in crude extracts from *Arabidopsis* also affect dsRNA cleavage. The dsRNA-cleaving assay using 4-week old *Arabidopsis* mature leaves was performed as described previously (Fukudome et al., 2011). All crude extracts were incubated with 500-bp dsRNA as a substrate, extracts from wild-type (WT) plants produced plenty 21-nt small RNAs while extracts from either transgenic plant over expressing PSR2 or a *drb4* mutant under the same experimental conditions produced a lower level small RNAs (Figure 1.14).

The previous study has shown the reduced secondary siRNAs in this *drb4* mutant and PSR2 transgenic plant, respectively (Fukudome et al., 2011, Qiao et al., 2013). To further confirm this reduction of two lines under one experimental condition, I examined the

siRNAs level again using 4-week old *Arabidopsis* mature leaves. Col-0 (WT) and a complementary line expressing DRB4 under DRB4 native promoter (pDRB4:DRB4) were used as controls. This northern blot analysis demonstrates that secondary siRNAs reduce in both *drb4* mutant and PSR2 transgenic plant while two representative miRNAs remain the same level (Figure 1.15). Collectively, these results support that PSR2 may lead to the reduction of siRNA production by interfering with DRB4 activity.

Figure 1.14. Reduced dsRNA-cleaving activity of *PSR2-expressing plant* and *drb4* mutant crude extracts.

^{32}P-labeled 500-bp dsRNAs were incubated with *Arabidopsis* wild-type (WT), *PSR2-5*, or *drb4* crude extracts for 2 h at 23°C. The cleavage products were analyzed on 15% denaturing PAGE with 8 M urea. A 22-nt DNA end-labeled with ^{32}P and dsRNA ladder stained with SybrGold were used as size markers for 21-nt small RNA. The crude extracts that were analyzed on gels stained with Coomassie Brilliant Blue (CBB) were used as loading control. Black arrow: The 500-bp dsRNA substrates; black arrowhead: 21-nt small RNAs. Experiments were repeated twice with similar results.

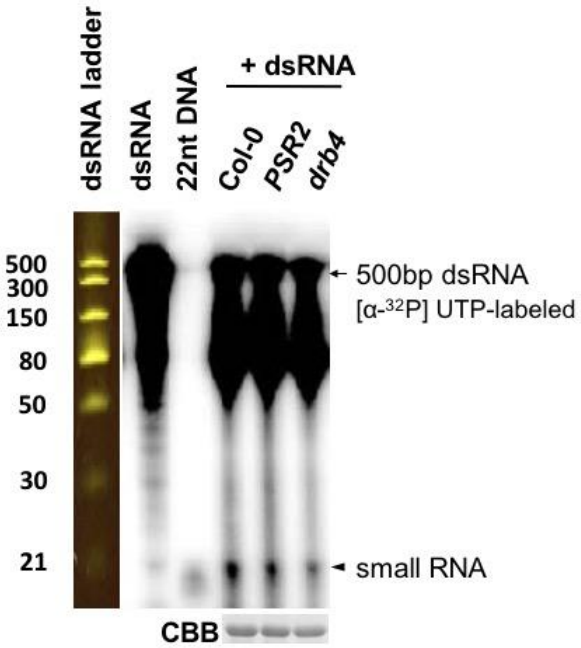
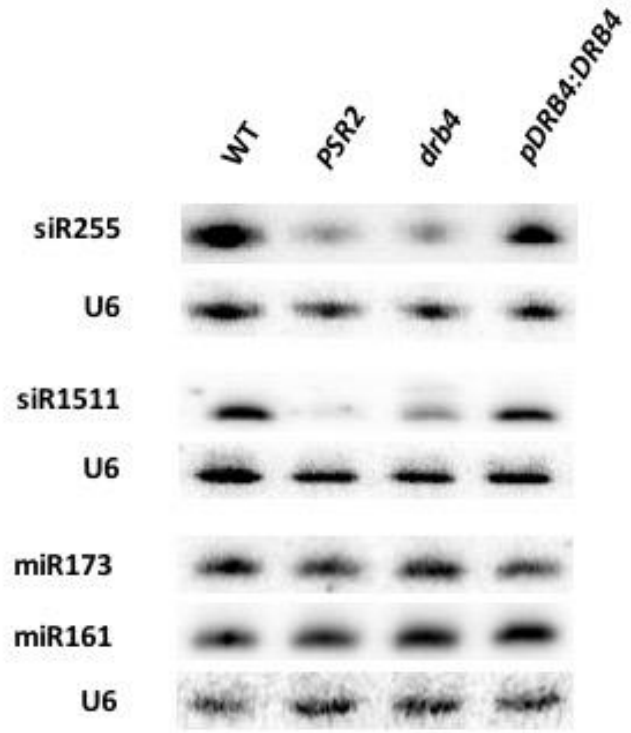


Figure 1.15. tasiRNAs reduced in both *PSR2-expressing plant* and *drb4* mutant.

Northern blotting of tasiRNAs show reduced accumulation in both *drb4* and the *PSR2* transgenic plants. Results from two ta-siRNAs (siR255, siR1511) and two miRNAs (miR173, miR161) are presented. Wild-type (WT) was used as control. U6 serves as the loading control. Numbers below the blots represent the relative abundance of the small RNAs with the levels in WT set to 1. These experiments were repeated three times with similar results.



The first two repeat units are required for PSR2 function and interaction with DRB4

It is reported that PSR2 has seven tandem repeats that each contains three conserved motifs, namely L, W, and Y, and the first repeat units only contains the W and Y motifs (Figure 1.16). Our unpublished structural analysis of PSR2 suggests that it has a general linear structure with each repeat unit forming a highly similar fold. Therefore, it is interesting to investigate where DRB4 binds to PSR2. Truncated mutants of *PSR2* lacking in each unit were cloned in pEG100 vector and co-expressed with GFP in *N. benthamiana* 16c by Agro-infiltration (Figure 1.17). Intriguingly, the mutants $PSR2^{\Delta N}$, $PSR2^{\Delta WY1}$ and $PSR2^{\Delta LWY2}$ lost the ability to suppress GFP-mediated transgene silencing in *N. benthamiana* 16c (Figure 1.18). Consistent with the reduced green fluorescence, the western analysis showed the reduced level of GFP in $PSR2^{\Delta N}$, $PSR2^{\Delta WY1}$ and $PSR2^{\Delta LWY2}$ (Figure 1.19). Empty vector was used as a negative control. The similar expression level of wild-type and truncated mutants of PSR2 in the infiltrated area are also shown using western blot (Figure 1.20).

Figure 1.16. Sequence alignment of repeat units of PSR2 protein.

The aligned sequences are in the order of WY1, LWY2, LWY3, LWY4, LWY5, LWY6, LWY7, from N-terminus to C-terminus of PSR2. WY1 lacks the L motif compared with other units. Numbers on the right mark the position of last amino acid in each row.

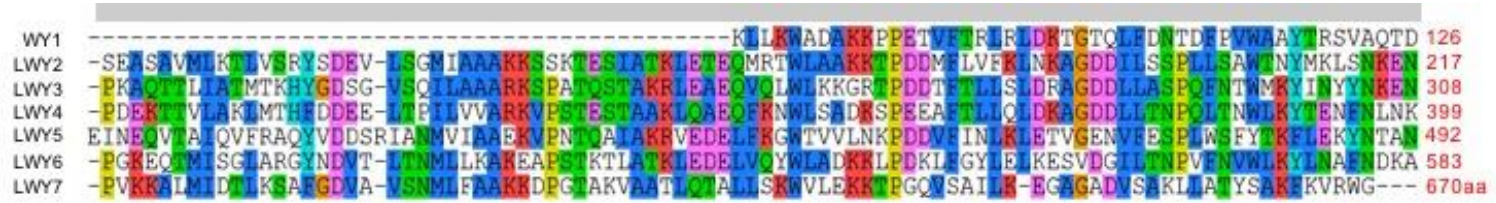


Figure 1.17. Schematic representation of the domain structure of WT and truncated mutants of PSR2 protein.

PSR2 has seven tandem repeat units consisting W, Y and/or L motif. R: RxLR domain.

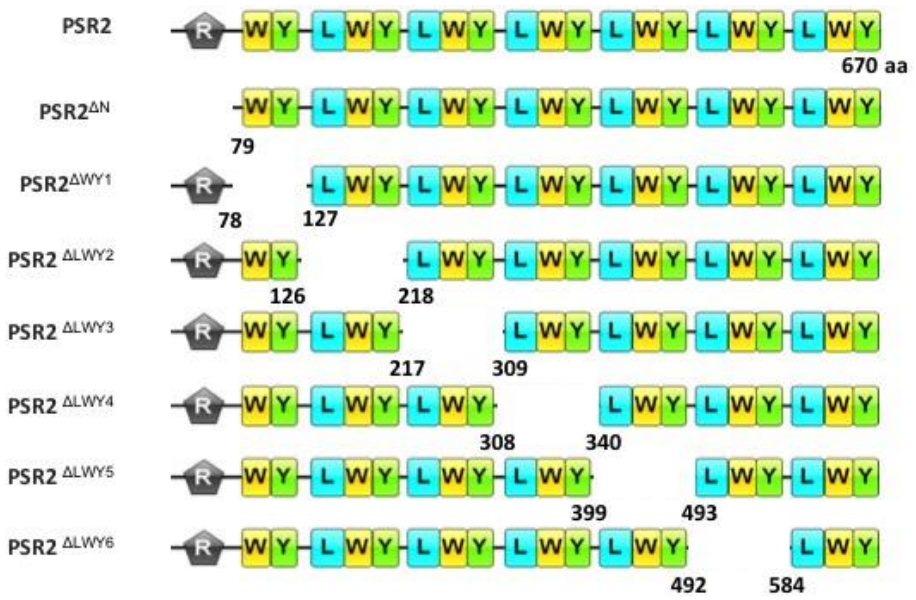


Figure 1.18. The first two repeat units in PSR2 that are required for RNA silencing suppression activity.

The transgene silencing suppression activities of PSR2 and its derivatives were examined in *N. benthamiana* 16c plants, which constitutively expresses *GFP*. The 16c plants were co-infiltrated with Agrobacterium carrying *35S-GFP* and harboring *35S-PSR2*, *35S-PSR2$^{\Delta WY1}$*, *35S-PSR2$^{\Delta LWY2}$*, *35S-PSR2$^{\Delta LWY3}$*, *35S-PSR2$^{\Delta LWY4}$*, *35S-PSR2$^{\Delta LWY5}$*, *35S-PSR2$^{\Delta LWY6}$* *35S-PSR2$^{\Delta LWY7}$*. Pictures were taken 5 days after *Agrobacterium* infiltration. Empty vector (EV) was used as a negative control. Experiments were repeated four times with similar results.

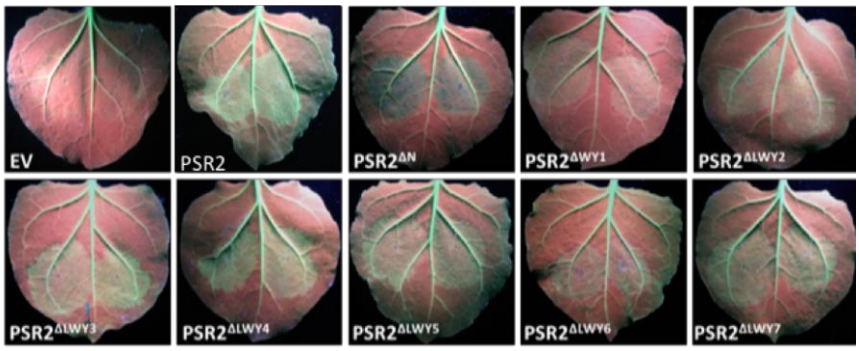


Figure 1.19. Accumulation of GFP protein in infiltrated *N. benthamiana* 16c leaves. PSR2 and GFP were expressed in *N. benthamiana* 16c through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves at 5 dpi and then detected by anti-GFP antibody using western blotting. Empty vector (EV) was used as a negative control. Coomassie Brilliant blue staining (CBB) was used as loading control. Experiments were repeated four times with similar results.

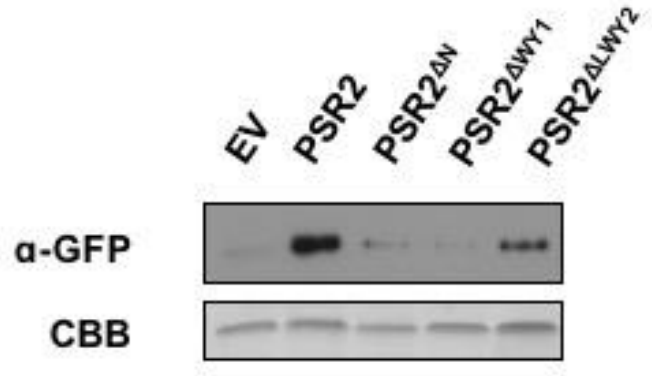
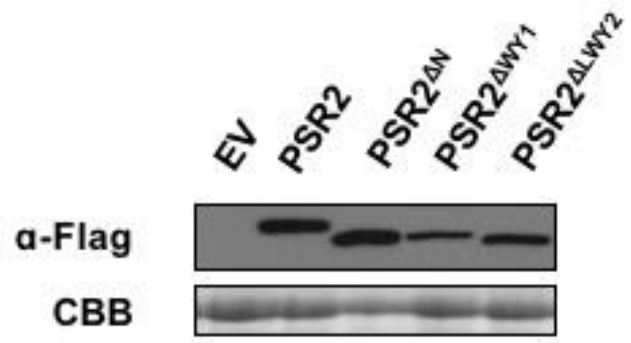


Figure 1.20. Protein abundance of PSR2 or its derivatives in infiltrated *N. benthamiana* 16c leaves.

PSR2 and GFP were expressed in *N. benthamiana* 16c through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves at 2 dpi and then detected by anti-Flag antibody using western blotting. Empty vector (EV) was used as a negative control. Coomassie Brilliant blue staining (CBB) was used as loading control. Experiments were repeated twice with similar results.



Based on the result from RNA silencing suppression assay, I hypothesized that WY1 and LWY2 would also be required for disease enhancement ability of PSR2, I therefore infected *N. benthamiana* transiently expressing wild-type and truncated PSR2 by *P. capsici* medium agar plug. As expected, the mutants were not able to promote *Phytophthora* infection at the same level compared with wild-type PSR2 (Figure 1.21). YFP was used as a negative control and lesion size caused by *P. capsici* infection was shown as well (Figure 1.21). $PSR2^{\Delta N}$ was excluded in this infection analysis as it could cause HR. These results confirmed that WY1 and LWY2 repeat units are required for PSR2 function which is to suppress transgene silencing and promote *Phytophthora* infection in *N. benthamiana*.

Figure 1.21. The first two repeat units in PSR2 that are required for virulence activity.

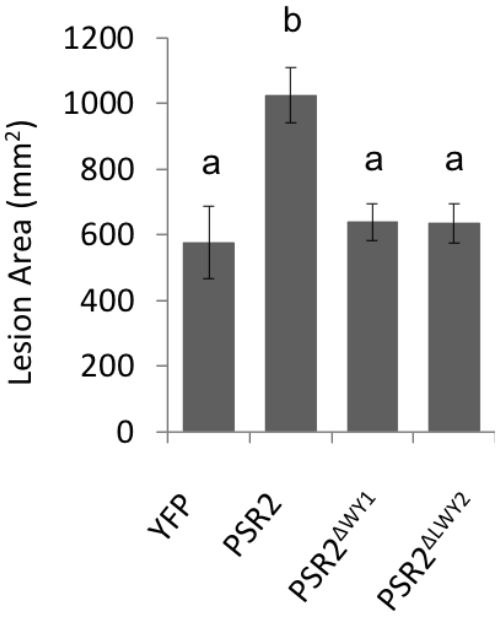
(A) The virulence activities of PSR2 and its derivatives were examined by inoculating *N. benthamiana* expressing PSR2, PSR2$^{\Delta WY1}$, PSR2$^{\Delta LWY2}$. Detached leaves of *N. benthamiana* were inoculated with mycelium-growing agar plug of *P. capsici* strain LT263 36 hours after *Agrobacterium* infiltration. Pictures were taken at 3 dpi. YFP was used as a negative control. Experiments were repeated three times with similar results.

(B) Sizes of lesion caused by *P. capsici* infection on leaves expressing PSR2, PSR2$^{\Delta WY1}$, PSR2$^{\Delta LWY2}$. Error bars are ± SEM. Letters represent differences with statistical significance ($P < 0.01$) as determined by Duncan's multiple range test.

A


YFP
PSR2
PSR2ΔWY1
PSR2ΔLWY2

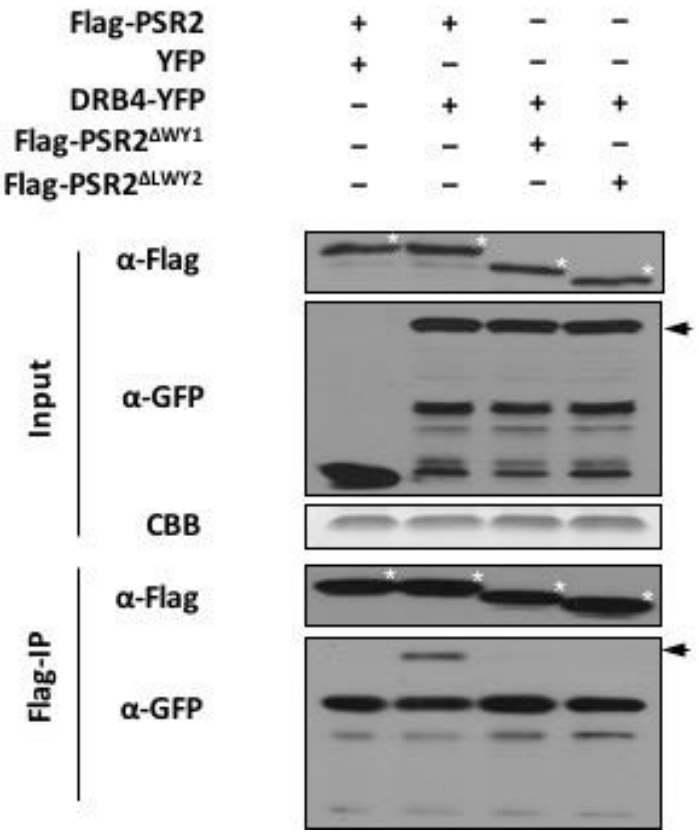
B


Lesion Area (mm²)
1200
1000
800
600
400
200
0
a
b
a
a
YFP
PSR2
PSR2ΔWY1
PSR2ΔLWY2

Since PSR2 interacts with DRB4, and the two repeat units are required for PSR2 function, I speculated that WY1 and LWY2 are required for this interaction. In order to gain a clearer picture, I performed Co-IP to detect the interaction between truncated mutant PSR2 and DRB4. Interestingly, PSR2$^{\Delta WY1}$ and PSR2$^{\Delta LWY2}$ lost the ability to interact with DRB4, suggesting that WY1 and LWY2 are required for the association between PSR2 and DRB4 (Figure 1.22).

Figure 1.22. The first two repeat units in PSR2 that are required for interaction with DRB4.

DRB4 and PSR2 or PSR2$^{\Delta WY1}$ or PSR2$^{\Delta LWY2}$ were co-expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using either anti-Flag beads. The co-precipitated proteins were then detected by anti-GFP or anti-Flag antibody respectively using western blotting. YFP was used as a negative control and the gels stained with Coomassie Brilliant Blue (CBB) were used as loading control. Experiments were repeated twice with similar results. Protein bands marked with asterisks are PSR2 and bands marked with black arrow are DRB4.


Flag-PSR2 + + − −
YFP + − − −
DRB4-YFP − + + +
Flag-PSR2ΔWY1 − − + −
Flag-PSR2ΔLWY2 − − − +
Input
α-Flag
α-GFP
CBB
Flag-IP
α-Flag
α-GFP

The first two repeat units are sufficient for PSR2 function and interaction with DRB4

To further characterize the role of WY1 and LWY2 in PSR2 function, we generated the short version of PSR2 containing WY1 and LWY2 (Figure 1.23). Surprisingly, this short fragment of PSR2 maintained ability to suppress GFP-mediated transgene silencing in *N. benthamiana* 16c (Figure 1.24). And from the western blot analysis, the level of GFP of $PSR2^{WY1+LWY2}$ is slightly weaker than the full length PSR2 (Figure 1.25). Empty vector was used as a negative control. The similar expression level of full length and short fragment of PSR2 in the infiltrated area are also shown using western blot (Figure 1.26).

Figure 1.23. Schematic representation of the domain structure of WT and short fragment of PSR2 protein.

PSR2 has seven tandem repeat units consisting W, Y and/or L motif. R: RxLR domain.

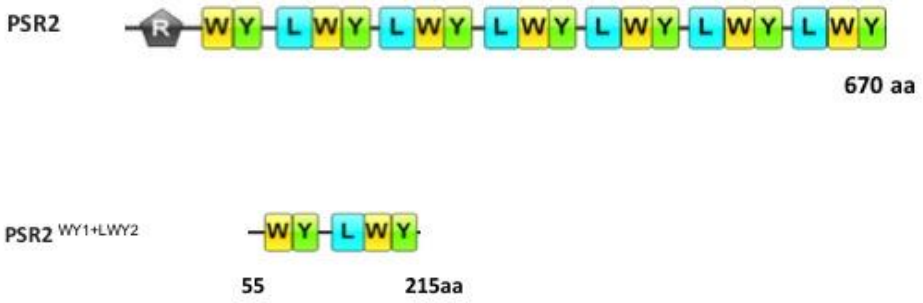


Figure 1.24. The first two repeat units in PSR2 that are sufficient for RNA silencing suppression activity.

The transgene silencing suppression activities of PSR2 and its derivatives were examined in *N. benthamiana* 16c plants. The 16c plants were co-infiltrated with Agrobacterium carrying *35S-GFP* and harboring *35S-PSR2* or *35S-PSR2*$^{WY1+LWY2}$. Pictures were taken 5 days after *Agrobacterium* infiltration. Empty vector (EV) was used as a negative control. Experiments were repeated four times with similar results.

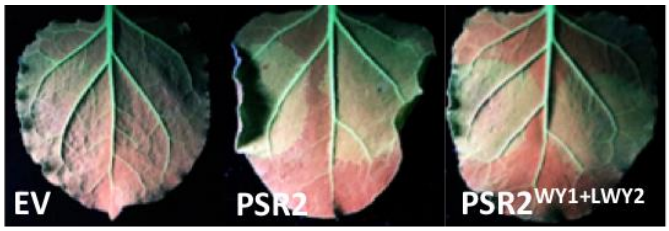


Figure 1.25. Accumulation of GFP protein in infiltrated *N. benthamiana* 16c leaves. PSR2 and 35S-PSR2$^{WY1+LWY2}$ were co-expressed with GFP respectively in *N. benthamiana* 16c respectively through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated area of leaves at 5 dpi and then detected by anti-GFP antibody using western blotting. Empty vector (EV) was used as a negative control. Coomassie Brilliant blue staining (CBB) was used as loading control.

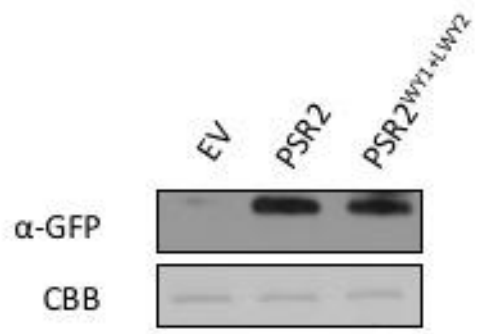
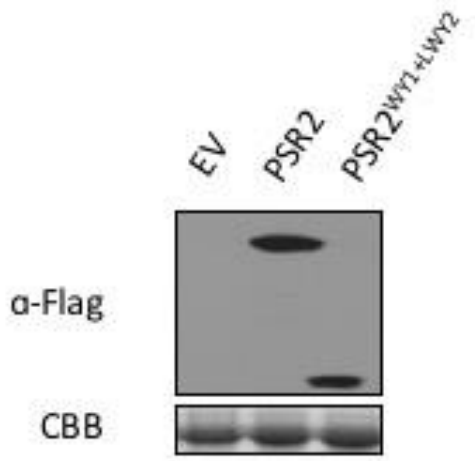


Figure 1.26. Protein abundance of PSR2 and PSR2$^{WY1+LWY2}$ in infiltrated *N. benthamiana* 16c leaves.

PSR2 and 35S-PSR2$^{WY1+LWY2}$ were co-expressed with GFP respectively in *N. benthamiana* 16c through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves at 2 dpi and then detected by anti-Flag antibody using western blotting. Empty vector (EV) was used as a negative control. Coomassie Brilliant blue staining (CBB) was used as loading control.



From the result from RNA silencing suppression assay, I hypothesized that this short version of PSR2 would also be sufficient to promote *Phytophthora* infection, I therefore infected *N. benthamiana* transiently expressing full length and short fragment of PSR2 by *P. capsici* medium agar plug. As expected, the mutants were still able to promote *Phytophthora* infection compared with YFP which was used as a negative control and lesion size caused by *P. capsici* infection was shown as well (Figure 1.27).

Since the two repeat units are sufficient for PSR2 function, I speculated that WY1 and LWY2 are sufficient for the interaction between PSR2 and DRB4. In order to confirm this speculation, I performed Co-IP to detect the interaction between short fragment of PSR2 and DRB4. Expectedly, $PSR2^{WY1+LWY2}$ were still able to pull down DRB4, suggesting that WY1 and LWY2 are sufficient for this association (Figure 1.28).

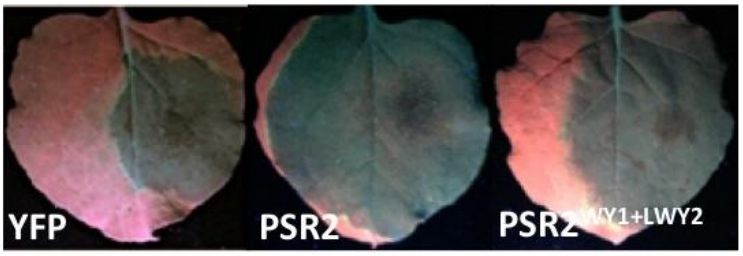
These results confirmed that the N-terminal fragment of PSR2 containing the WY1 and LWY2 repeat units is sufficient for the virulence and RNA silencing suppression activities and is also sufficient for interaction with DRB4.

Figure 1.27. The first two repeat units in PSR2 that are sufficient for virulence activity.

(A) The virulence activities of PSR2 and its derivatives were examined by inoculating *N. benthamiana* expressing PSR2 or PSR2$^{WY1+LWY2}$. Detached leaves of *N. benthamiana* were inoculated with mycelium-growing agar plug of *P. capsici* strain LT263 36 hours after *Agrobacterium* infiltration. Pictures were taken at 3 dpi. YFP was used as a negative control.

(B) Sizes of lesion caused by *P. capsici* infection on leaves expressing PSR2 and PSR2$^{WY1+LWY2}$. Error bars are ± SEM. Letters represent differences with statistical significance ($P < 0.01$) as determined by Duncan's multiple range test. Experiments were repeated three times with similar results.

A


YFP
PSR2
PSR2WY1+LWY2

B

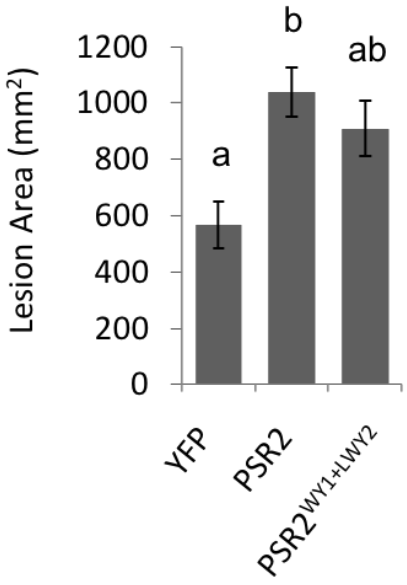
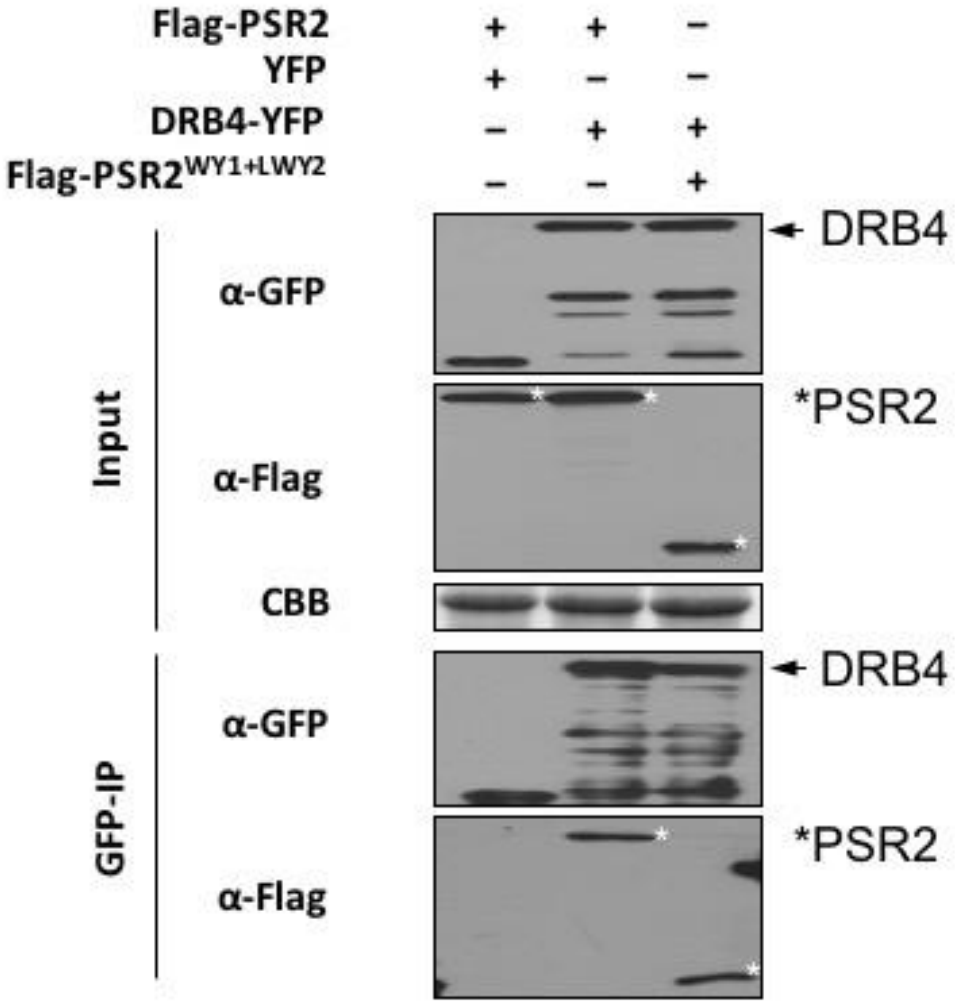

Lesion Area (mm2)
1200
1000
800
600
400
200
0
a
b
ab
YFP
PSR2
PSR2WY1+LWY2

Figure 1.28. The first two repeat units in PSR2 that are sufficient for interaction with DRB4.

PSR2 or PSR2$^{WY1+LWY2}$ and DRB4 were expressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves and the immune complexes were pulled-down using either anti-GFP beads. The co-precipitated proteins were then detected by anti-GFP or anti-Flag antibody respectively using western blotting. YFP was used as a negative control and the gels stained with Coomassie Brilliant Blue (CBB) were used as loading control. Experiments were repeated three times with similar results. Bands marked with asterisks are PSR2 and bands marked with arrow are DRB4.


Flag-PSR2 + + −
YFP + − −
DRB4-YFP − + +
Flag-PSR2WY1+LWY2 − − +
Input
α-GFP
α-Flag
CBB
GFP-IP
α-GFP
α-Flag
DRB4
*PSR2
DRB4
*PSR2

***drb4* mutant of *Arabidopsis* phenocopies *PSR2*-expressing plants**

To further demonstrate that DRB4 is a virulence target of PSR2, I examined the developmental and disease susceptibility phenotypes of a *drb4* mutant of *Arabidopsis*. Both transgenic plant over expressing PSR2 and drb4 exhibit developmental defects, such as narrow and curly leaves (Figure 1.29). The whole plant of 5-week old *Arabidopsis* and individual leaves are shown to compare the different leave shapes. Importantly, the developmental defects were compensated by introducing DRB4 expressed under DRB4 native promoter. A similar leaf phenotype has also been reported in *rdr6* (Peragine et al., 2004), indicating that it is likely associated with secondary siRNA production.

In addition, different experimental designs of infection assay both showed that PSR2 plants and *drb4* mutant are hypersusceptible to *P. capsici*. I first dip the root of 2-week old *Arabidopsis* seedlings in *P. capsici* zoospore suspension ($1x10^5$ zoospore / ml) and then transfer the seedlings into soil for the infection cycle. Both PSR2 plants and *drb4* mutant showed more severe disease symptom, like wilting and rotting, compared with WT and the complementary line (Figure 1.30). The other method is using detached mature leave of 4-week old *Arabidopsis* and droplet of *P. capsici* zoospore suspension (1.10^5 zoospore / ml). The representative leaves were then stained by Trypan blue to clearly show the lesion size (Figure 1.31). All together, these results support that PSR2 targets DRB4 to fulfill its RNA silencing suppression activity and virulence function in *Arabidopsis*.

Figure 1.29. *drb4* mutant of *Arabidopsis* exhibits similar developmental phenotype to *PSR2*-expressing plants.

drb4 mutant and *PSR2*-expressing plants show similar curly leaves phenotype. Photos were taken after 5-week of growth. Wild type plants (WT) and the complimentary line were used as controls.

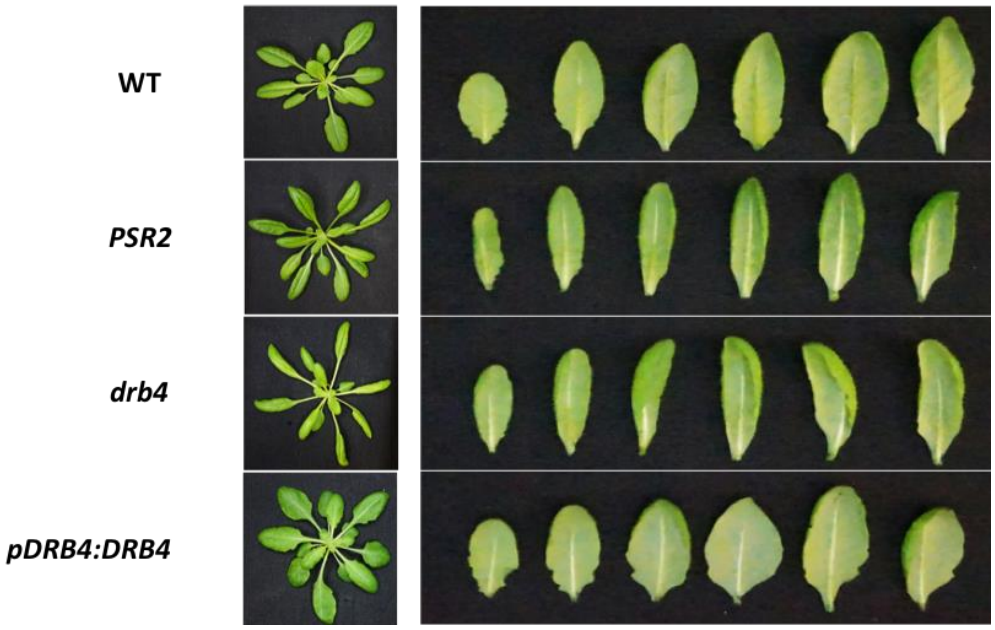


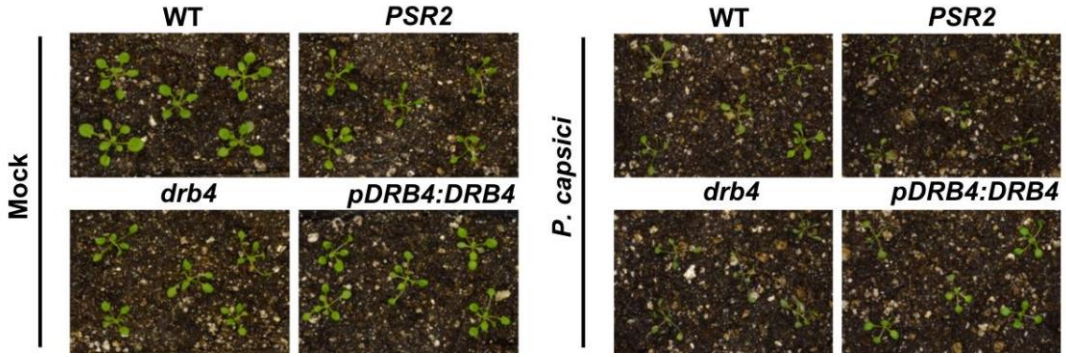
Figure 1.30. Both *drb4* mutant and PSR2 plants exhibit hypersusceptibility to *P. capsici* (seedlings).

(A) Seedlings of 14-day-old wild-type (Col-0), PSR2-5, *drb4* mutant and its complementary line (expressing *DRB4-YFP* under the native *DRB4* promoter) were inoculated with zoospore suspension ($1x10^5$ zoospore per mL). Enlarged photos showing individual seedling. Photos were taken at 3 dpi. Seedlings treated by water (Mock) were used as negative control. Experiments were repeated twice with similar results.

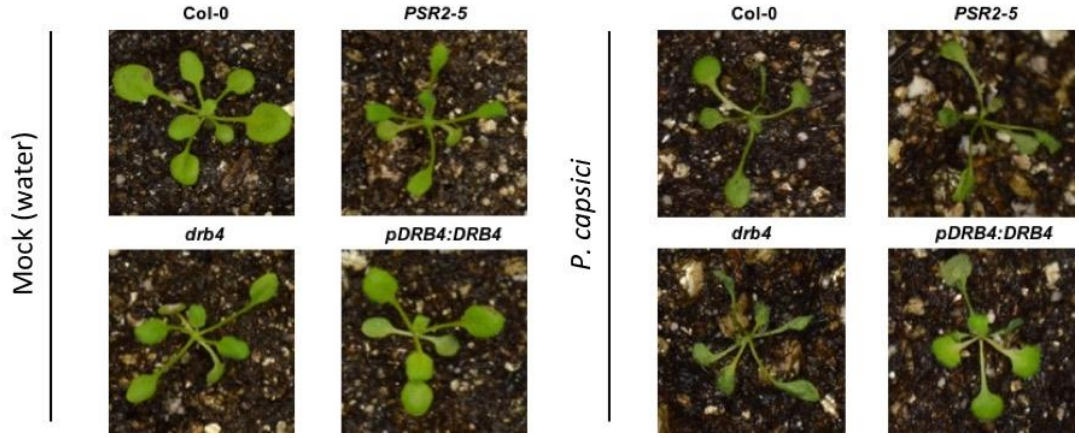
(B) Individual seedlings were shown in enlarged photos.

(C) Seedlings pulled out from the soil were transferred to 8% agar plate for better comparison.

A


WT
PSR2
Mock
drb4
pDRB4:DRB4
WT
PSR2
P. capsici
drb4
pDRB4:DRB4

B


Col-0
PSR2-5
Mock (water)
drb4
pDRB4:DRB4
Col-0
PSR2-5
P. capsici
drb4
pDRB4:DRB4

C

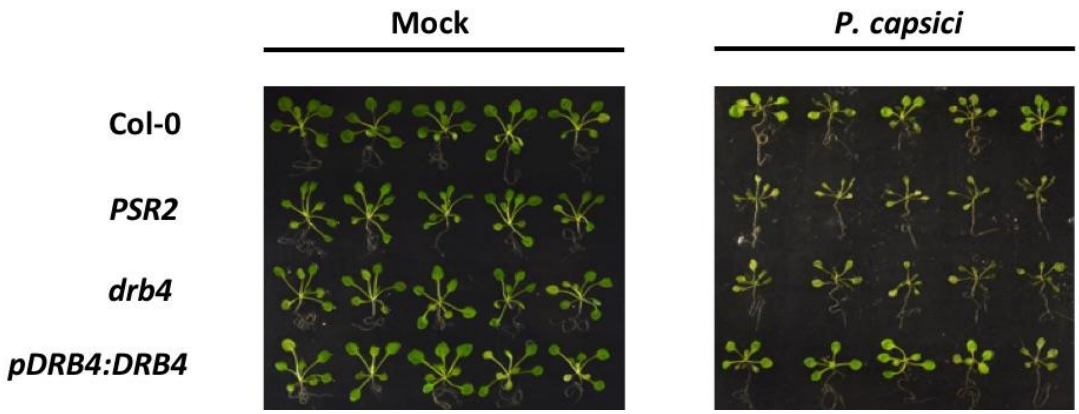
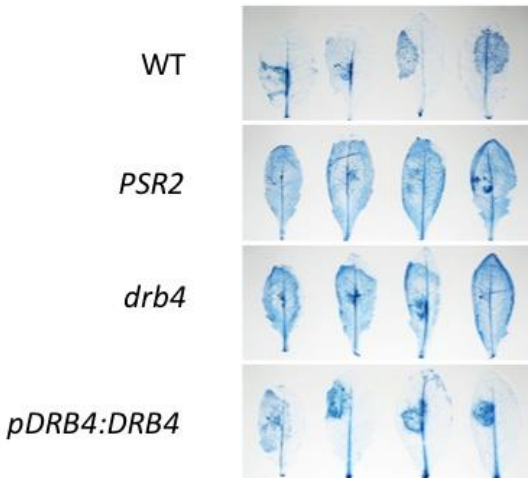


Figure 1.31. A *drb4* mutant of *Arabidopsis* exhibits hypersusceptibility, similar to *PSR2*-expressing plants (mature plant).

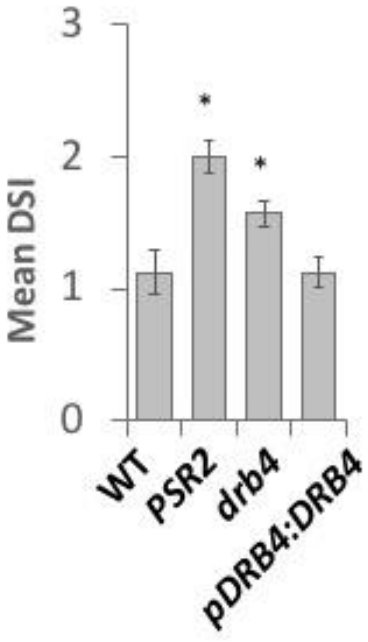
(A) Detached leaves of 4-week-old wild-type (WT), *PSR2-5*, *drb4* mutant and its complementary line (expressing *DRB4-YFP* under the native *DRB4* promoter) were inoculated with zoospore suspension ($1x10^5$ zoospore per mL). Photos were taken at 3 dpi.

(B) Bars show disease severity index (DSI) of inoculated leaves which was determined at 3 days post inoculation (dpi). Values are mean ±SEM. * labels results that are statistically different at $p<0.05$.

A



B



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Chapter II. Identification of Phytophthora Crinklers with RNA silencing suppression activity

ABSTRACT

Phytophthora are important plant pathogens that cause devastating diseases of crops. Genome sequences of *Phytophthora* revealed hundreds of cytoplasmic effectors, which function inside the plant cells to enhance virulence. Recent findings suggested that *Phytophthora* produces RxLR effectors with RNA silencing suppression activity. Here, I examined the Crinklers (CRNs), another family of cytoplasmic effectors that are widely distributed in oomycetes, for their ability to suppress small RNA silencing in plants. CRNs are under-studied compared to RxLR effectors although they are considered a more ancient effector family in oomycetes. Using a functional screen, two CRNs from *Phytophthora capsici* were found to be able to suppress transgene silencing in *Nicotiana benthamiana*. Among them, CRN36_259 was able to reduce the accumulation of siRNAs in *N. benthamiana*; whereas CRN32_283 suppressed RNA silencing without affecting siRNA levels. Despite this difference, *P. capsici* knockout mutants lacking each CRN exhibited reduced virulence activities, indicating that both CRNs promote *Phytophthora* infection. Especially CRN36_259 is very important for *P. capsici* mycelium growth and zoospores production, Interestingly, CRN36_259 is localized in the nucleus when

expressed in plant cells and the nuclear localization is required for its RNA silencing suppression activity. Further analysis on the mechanism by which these CRNs affect small RNA silencing and their virulence function will be discussed.

INTRODUCTION

Phytophthora produces two classes of cytoplasmic effectors, the RxLR effectors and the CRNs. The CRN effectors also have the N-terminal signal peptide but followed with a conserved N-terminal LxLFLAK motif that is required for translocation (Stassen et al., 2011). Similar to the RxLR effectors, CRNs have diverse C-terminal effector domains that are presumed to perform different virulence functions in plants. CRNs were named after a leaf crinkling and necrosis phenotype upon expression in plant cells (Lamour et al., 2012b). For example, CRN8 in *P. infestans* possesses a kinase activity and triggers cell death when expressed in planta (van Damme et al., 2012). CRN20_624 in *P. capsici* exaggerates INF1-induced cell death (Stam et al., 2013b). CRNs widely distributed in *Phytophthora* species, as well as in other oomycetes. Bioinformatic prediction revealed 196, 100, 84 and 14 CRN effectors from *P. infestans*, *P. sojae*, *P.capsici* and *P. litchii* respectively (Stam et al., 2013b) (Ye et al., 2016). No or only a few classical RxLRs are in Phythium ultimum, Albugo candida and Aphanomyces euteiches while CRNs are predicted in those examined species (Gaulin et al., 2008) (Levesque et al., 2010) (Sebastian et al., 2010). Therefore, they are considered to be a more ancient class of effectors compared to the RxLR effectors. However, the function of CRNs is rarely investigated and poorly understood.

Not all CRNs induce necrosis when over-expressing in *N. benthamiana* (Stam et al., 2013a). In fact, some CRNs can even suppress cell death. For example, PsCRN63 from *P.*

sojae could interact with catalases in *N. benthamiana* or soybean to induce programmed cell death while PsCRN115 suppresses the cell death triggered by PsCRN63 (Liu et al., 2011) (Zhang et al., 2014b). In addition, PsCRN161 suppresses cell death triggered by other cell death-inducing elicitors (Rajput et al., 2015). The preliminary functional analysis of few CRNs suggest that they also play a role in modifying cell signaling required for plant immunity.

An interesting feature of CRNs is that many of them are exclusively located in the nucleus when they are expressed in plant cells although some of them do not have predictable nucleus localization signal (NLS) (Stam et al., 2013a). For example, *P. infestans* CRN8 localizes in the nucleus and this localization is required for triggering cell death (van Damme et al., 2012); *P. sojae* PsCRN63 also needs the nucleus localization to trigger cell death (Liu et al., 2011). This nucleus localization indicates that they may directly affect plant gene expression in the transcription or post-transcription levels. For example, CRN83_152 expression distinctly changed the chromatin organization in nucleus (Amaro et al., 2017). Since small RNAs are major regulators of gene expression, I was interested in determining whether some CRNs possess RNA silencing suppression activity, and if so, how they contribute to infection.

To test this hypothesis, I screened CRNs of *P. capsici* for RNA silencing suppression activity. Unlike *P. sojae* which only infects soybean, *P. capsici* has a broad host range and infects many vegetables like tomato, pepper, pumpkin, cucurbit and beans. Due to this

feature, *P. capsici* has spread all over the world and also adapted to fungicides upon evolution (Lamour and Kamoun, 2009) (Lamour et al., 2012a) (Lamour et al., 2012b). Like other *Phytophthora*, *P. capsici* is also a hemibiotrophic pathogen and produces asexual spores to infect plant hosts (Lamour and Kamoun, 2009). *P. capsici* caused significant losses worldwide, estimated to be about $1 billion each year (Lamour et al., 2012a) (Lamour et al., 2012b) (Chen et al., 2013). *P. capsici* also has a large repertoire of cytoplasmic effectors, including 357 RxLR effectors and 84 CRNs (Lamour et al., 2012b) (Stam et al., 2013a) (Stam et al., 2013b). I conducted a screen of 16 CRNs that do not trigger cell death when expressed in *N. benthamiana* and identified two as potential suppressor of RNA silencing. I further characterized the function of these two CRNs in *P. capsici*.

MATERIALS AND METHODS

Microbial strains and plasmids

P. infestans isolate 1306 was cultured on rye sucrose agar plates at 18°C in the dark for 7 to 10 days. *Agrobacterium tumefaciens* strain GV3101, *Escherichia coli* strains DH5α were grown in Luria-Bertani (LB) medium at 30°C and 37°C respectively as described (Wroblewski et al., 2005). The medium was supplemented with kanamycin at 50 ug/ml, rifampicin at 50 ug/ml, or gentamyci n at 50 ug/ml when necessary. Strains used in this study were listed in Table 2.1.

Plant materials and growth conditions

Nicotiana benthamiana plants were geminated and grown in a conditioned growth room at 22°C with a 12/12 light/dark regime. *N. benthamiana* 16c is a stable transgenic line consecutively expressing *GFP* under the control of the cauliflower mosaic virus *35S* promoter (Haseloff et al., 1997) (Ruiz et al., 1998).

Protein expression and detection in N. benthamiana

Fully extended leaves of *N. benthamiana* plants at the six-leaf stage were infiltrated with *Agrobacterium* cell suspension. The *Agrobacterium* cells collected from cell culture were suspended in 10 mM $MgCl_2$ to OD_{600} = 1.0, to be diluted to OD_{600} = 0.5 when necessary. Then the cells were activated using 10 mM MES and 10 mM Acetosyringone. After 3

hours induction, the cell suspension was infiltrated into abaxial side of leaves using needle-less 1 ml syringes. Leaves at 48 hours post infiltration (hpi) were collected using liquid nitrogen for further analysis.

Total proteins of leaves samples were extracted using 2x Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl) and separated by SDS-PAGE. Proteins were then transferred to Polyvinylidene Difluoride (PVDF) membrane (GE Healthcare) using semi-dry transfer cell (Biorad), followed by incubation with GFP antibody (Santa Cruz). The western results were visualized by chemiluminescent substrate (Thermo Scientific). Gel stained by coomassie blue (CBB) was used as loading control.

RNA silencing suppression assays using *N. benthamiana* 16c plants

With the host-targeting sequence at the N-termini deleted, the C-terminus sequences of 16 *Crinklers* (*CRN1_719, CRN2_1137, CRN10_627, CRN11_767, CRN12_997, CRN20_624, CRN22_248, CRN32_283, CRN33_10, CRN36_259, CRN47_135, CRN60_274, CRN79_188, CRN83_152, CRN105_25, CRN125_11*) from *Phythophthora capsici* were cloned into the destination vector pEG100 using Gateway cloning system (Earley et al., 2006). Sequences of *CRN32_283* and *CRN 36_259* are listed in Table 2.1. The recombinant plasmids were transformed into *Agrobacterium* GV3101 using freeze-thaw method (Höfgen and Willmitzer, 1988) and then were transient expressed together with *35S:GFP*

in *N. benthamiana* 16c using the infiltration method described above. Green fluorescence was observed using a handheld long-wavelength UV lamp (BlackRay B100AP, UVP) at 5 days after infiltration. *Agrobacterium* carrying the empty vector (EV) pEG100 was used as a negative control and Cucumber mosaic virus 2b (CMV2b) was used as a positive control. Plasmids and primers used in this study are summarized in Table 2.2 and Table 2.3.

RNA extraction and northern blotting

Total RNA was extracted from infiltrated *N. benthamiana* 16c leaves using TRizol reagent followed the company protocol (Ambion). The abundance of *GFP* siRNA was examined by northern blotting using [α-^{32}P]-labeled random primers that cover full length of the GPF gene. Four µg total RNA was loaded for each sample and U6 was used as loading control. Small RNA northern blotting was performed as described (Park et al., 2002) (Kurihara et al., 2005). Sequences of the oligonucleotide probes are listed in Table 2.3.

Sequence analysis of *CRNs* homologs in oomycetes.

The amino acid sequence of CRN32_283 and CRN 36_259 was used to search against the genome sequences of different *Phytophthora* spp. and *Aphanomyces* spp. for potential homologs (Sequences obtained from Department of Energy Joint Genome Institute

database and National Center for Biotechnology Information). Neighbor-joining trees were generated using MEGA 7 with 1,000 bootstraps and other default parameters.

Real time RT-PCR

The expression pattern of *CRN32_283* and *CRN 36_259* during *P. capsci* infection of *Solanum lycopersicum* was analyzed by real-time RT-PCR (Biorad). Chromosomal DNAs of *P. capsci* were isolated from infected tissues that collected at 0, 8, 16, 24, 48 and 72 hpi and were then used as templates using gene-specific primers. *Tubulins* were used as an internal control.

***Phytophthora* infection assays**

N. benthamiana leaves transiently expressing CRN32_283 and CRN 36_259 were detached 36 hours after Agro-infiltration and inoculated with 25ul sporangia suspension of *P. infestans* isolate 1036 at the abaxial side. Leaves were kept in sealed plates with high humidity in the dark at 18°C. Disease symptoms and lesion sizes were measured at 9 days after inoculation. Empty vector was used as a negative control.

Fluorescence microscopy

To examine the subcellular localization of CRN36_259, C-terminus of CRN36_259 and the mutated CRN36_259 fused with a nuclear export signal (NES) at N-terminus were cloned into the vector pEG104 with an N-terminal YFP tag. All fusion proteins were

transformed into *Agrobacterium* individually and were later infiltrated in 4-week old *N. benthamiana* leaves with an OD_{600} = 0.5. The functional fluorophore was visualized in the infiltrated leaves using a Leica SP5 Laser Scanning Confocal Microscope (Leica) at 48 hpi.

Table 2.1. *CRN* sequences used in Chapter II.

CRN32_283

ATGGAGGATTCAAAACGGCGATGGGAGGAGCTATTGAGTTCTCTAGCGTATAAGGACACCAAAAGACTTTGTTCAAGTACTGGATCTAAG
TGGGAATATCAAGGGGGAAAAGAGCTTGTAGAAACTCTTGCGAAGCCGTTGGTCGACCATTACAACGCTTGGAAATTGGGGAATGAGGA
CAAGCAGAGGCATCCAGTTCCTTTGGTGTTGAGTGGACCTGGAACGGGGAAGTCACGCATGCTGGACGAAATGAAGAATTTGCTGTGTGC
AGCTGCCATAGAATCCAAGGATCAAGCGCTCATCGGCAGAATGAATAGCGCATATATGTTTCGTGTGACGTTCGAGAACGACACTCCAGC
AACCGGTTCATTACTCAATCCAAATATCCCAGAATATGACATCAGCTACCGAATGCTTTACCAACTCTCGACAGAAGAAAAACCGTGGGGA
GATTTCGTTCATAGTTTGAACATGTACCCTCAGAAGTATATCTCTATTGAGAAAGTGATTCGTATCCTGGCTAAACGCGAGGGTGTTGACGT
GAAGAAAATGACAGTGATCTTGTGTGTGGATGGGTTCCAGAAGCTGATGAACGACGGCACGAAAACGTGTGCTTTTTATCGCGTAATGAG
CTCGATCTTCGGTTTTGTTAATTCATCGACAGCATTTTCTGTTTGTGCGTGCTCGTCAACAATTGCGAAACCTGTTATGAAAGTGCTTAGTGT
TTCTGTCCAGAAGTGCCTGTATCTGGTTCCACCTGCATTATGTGGTGAAAAAGTTTTGGAAACACGAACTCCGACCATGAAAGTGTTGGTG
GGAGATATGGGTGGTCATGGTCGAGCGCTGGAGGCATTGGACAGCGTGTTGCGAAAGTTAAATGTGGGTGAGGAGGAAATTGACCCAT
GTTACGTTGTTGATATGGTGAACCGAAAGCTACAGCATGAGTATCCGGGTCTATTTGATAGTCGTGTATTTAAACCCGATACCTGCAAAGA
ATTGGTGTCCGCAATTTTGTTGCAACGACGATATTATGTATCGGATGTCATTGGTGAAACCAATTTGACAGTAGACGAACTTCGAAGCTTC
GGTTTGTTCCGACTGACCAGTAAAGGATGTTTGGAATGCGCCTTCATCTTTTTGGTGGAGTTAATACGGAAAATGTCCAAGTTGGAAGGCG
AACTGGCCAACTTCGACGATCATATCACGCGTTCAGTGACGGCATGGCAGCCAGTTGAGTATTTCGTGGGGTTTTACCGCCAGGTAAAATC
GATTGCTTTCCGCAACACCCCACTTCCCCTGTCAAAATTTCACGCTGGCGCTCGTTTTAGCAACATCGGTGGCGTTTTGATCACCGAGCCGT
CTCCACGCGAACTAGTGGAAGCTGTGCACCAACACGGAACAAAATCCAGCTCTGGAGTCTCGTTGGTCACCACAGATCAACATGGTGACG
TTAAGGTTTCAGATATGCAAACAATCATTATTAACGGCACAAGGGCTTCAGCTGGCAATTTGTTTATGACAGTGGAATTGACGAATTTTGG
CGGCCAGCAAGTCAAGTGCAACGAAGTCATCCAATGCAAGTTTCTGCACACAAAAGCGAAGTTCGACGAGGACGTTTACGCCGCAGAGCG
AGAAAAAGCTGTGGATGACTCGGATGTGTTTTTGCTCATTACGCCAAGTTCAGTAGACGAATTTGATCTTCCTCCAAAATGCGGAATCGTCT
CCGAAAAGGAATTTCGCCAATATTTCGGACCTTTTGCCTCTCGCGCGTTCCGAAGCATATTGGCGCCACCCGATATCAACAAAGCATCATCC
GAGGTACTACGTTTGGTAAAAGGCTTGAGTATCGAAACGGTGGAAAAAATTGTGAAGGAGAGGGCTAAACGGAAGTTTTCAGATGTTGA
AGATGCAGTGGATCGAGTGTGCTCAAACAAGAAATGCAAGACCGCCGAGGATATTCGCCGGGTCCACTCGGATTTGGCGGACGTGGCGG
CTTTCATGAATATTGCCTGGTCGCATCCGCAGTTCGAGAAGTGA

CRN36_259

ATGGATTATGACAGCGACTCAGAAGTGATCCAACAAAGAAAACTGACGTCTTTGGGAAAACTACTAAAACAAAGTGAAGTC
AGTGTCGAGCTACCTGTGCAAGGAGACTTTATGAAGTTGTTCGACTTGACCGATGACGATATTGGGAAAGTATTAGACATC
AAAGCAATTGGTGACATCGTGGGTTTTACTGGTTCCGACTTCTACATACGGAAGGAGATTCTGTGTGTGCTGGAGAATTTCA
AGAAAACCTATCACGATGAGCTTGAAGATGACATAGCGACTAGCAAACAGTTCATTCTCATGGGAAGTCCAGGAACAGGCA
AGAGCTGTATCTTGGCTATGATTTGTTTCTGTATCGCAGTGAAGTACAATCGACCAGTGGTCTGGTTCCGCCATGTTTCTGGT
GGCTATAATCTGACAACTACGCGACTGTTCTATGAAGGCAAATTTTATGCTTGGAAAGACTTCAAGGGAGAGAAATACGAT
CAACTCTTTGATTGTTTGAGAGCTATGGGCGATGTGAAAACGTGCTGGTTCTGTCTTGACGGGATGACCCAGAGTGAAATT
GATCAGAAGAATTGGCTTAACAAGTACAAGGTGTTGGCAAATTCGGGGCCGTTTTCAGTTAAAGCTGAAGCATCGGCGCTC
TTGGAATTGTGTCTTGTACCTTACTGGAGGCAGGAAGACCTGGAAGATGTCGGCAAGCATGAGGGGATGTCAGAGAGCGA
CGTGGCTGCTCGTTTCTTTAAGTCCGGAGGGAGCTTGCGAGATTTTCGTCAGCTTTCTTTTGCTCACGTGAAAGGAGCATTG
AGATCTATTCAATGCTCTAGCCATGTGGAGAAGTTACTCACGAATGTTGGACTTAGTGACGTCGATCAGATTGACTGTATCA
GCATGGGAGGAGTGAAAGATCGCGAAAATGCAGATCACTACGTGGATATAGATATGTGGACCAGCTGTGTGACATCGCAA
GCCGTATTCAACGCCATCGGACATCTTTTGGAGCCCAAGTTTTTTAAGAAGCAGAAGGATGTTGCAAAGGGGATGAACGAC
GCACGTCTCGAAGGTGTTGCTACCAAGGGATATTTCCACGCGTTAGTGCGTCATCACCAACCATTTTGCGTGAAGTTTCGCA
GGTATGACGATGGGAATCGCAGGGACTACCCGAACTGGATGAATGTTATGGAAGAGCTGGGCGTGATCGACTGCAACCAG
TTTCCCTTGAAGAAGATCAAAGTTTCATCCGCTGGTAAGAACATGGAGGAATGTGTAGCTGAAATGGAGGAATGGGCCGGA
AACCCTTCTAAGATGGACTATTGGACCCCCAATACCAGTTTGTGTGAGACTATTGATGCCGTTGCGAAATGGACACTACCCG
ATGATGAAGAGCATTTTTGTCTCCTGCAGTTGACGATGGCCACCAAGCACAAATGTGATGAGAATGTTCTCTGGGAGCTTGT
GCAGCCATTCGTGAAGAAGAACTTGAAAGTGTGCTATATTGCAATTCTTGACGACGAGGCCGATATATTCAAGTTCCGAGTG
GACCCTGCGCAGATCAAGAAACGTGATATCTTGGATAATGTTCCTCTTTATGCCGCGCATTTTGAGAAAGTTGATGTCTCGC
AGTTCATCGCACGAGAGTGGTCGCATCCGCAGTTCGAGAAGTGA

Table 2.2. Strains and Plasmids used in Chapter II.

Strains or Plasmids	Description	Source/reference
Escherichia coli DH5α	F- Φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1, hsdR17(rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1	Invitrogen
Agrobacterium tumefaciens GV3101	Rif^R, $Gent^R$	Wroblewski, 2005
Phytophthora infestans isolate 1306	A1 mating type	Cvitanich and Judelson, 2003
Phytophthora capsici isolate LT263	Isolated from pumpkin, can infect Arabidopsis	Donahoo and Lamour, 2008
Phytophthora capsici isolate 1534	Mating from LT263 and OP97	Stam,2013b
pEG100	pEarleyGate100, a Gateway binary vector with cauliflower mosaic virus *35S* promoter, Kan^R	Earley., 2006
pEG100::CRN1_719	pEG100 carrying CRN1_719, Kan^R	This study
pEG100::CRN2_1137	pEG100 carrying CRN2_1137, Kan^R	This study

pEG100::CRN10_627	pEG100 carrying CRN10_627, Kan^R	This study
pEG100::CRN11_767	pEG100 carrying CRN11_767, Kan^R	This study
pEG100::CRN12_997	pEG100 carrying CRN12_997, Kan^R	This study
pEG100::CRN20_624	pEG100 carrying CRN20_624, Kan^R	This study
pEG100::CRN22_248	pEG100 carrying CRN22_248, Kan^R	This study
pEG100::CRN32_283	pEG100 carrying CRN32_283, Kan^R	This study
pEG100::CRN33_10	pEG100 carrying CRN33_10, Kan^R	This study
pEG100::CRN36_259	pEG100 carrying CRN36_259, Kan^R	This study
pEG100::CRN47_135	pEG100 carrying CRN47_135, Kan^R	This study
pEG100::CRN60_274	pEG100 carrying CRN60_274, Kan^R	This study
pEG100::CRN79_188	pEG100 carrying CRN79_188, Kan^R	This study
pEG100::CRN83_152	pEG100 carrying CRN83_152, Kan^R	This study
pEG100::CRN105_25	pEG100 carrying CRN105_25, Kan^R	This study
pEG100::CRN125_11	pEG100 carrying CRN125_11, Kan^R	This study
pEG104::CRN32_283	pEG104 carrying CRN32_283 with YFP at N-terminus, Kan^R	This study
pEG104::NES-CRN32_283	pEG104 carrying NES-CRN32_283 with YFP at N-terminus, Kan^R	This study
pEG104::nes-CRN32_283	pEG104 carrying nes-CRN32_283 with YFP at N-terminus, Kan^R	This study

pEG104::CRN36_259	pEG104 carrying CRN36_259 with YFP at N-terminus, Kan^R	This study
pEG104::NES-CRN36_259	pEG104 carrying NES-CRN36_259 with YFP at N-terminus, Kan^R	This study
pEG104::nes-CRN36_259	pEG104 carrying nes-CRN36_259 with YFP at N-terminus, Kan^R	This study

Table 2.3. Primers used in Chapter II.

	Primer sequence
pENTR1a::CRNs-R	TAT CTCGAG TCACTTCTCGAACTGCGGG
pENTR1a::CRN1_719-F	TCAGTCGACATGGCACCTGAGAATGAAAGGAAG
pENTR1a::CRN2_1137-F	TCAGTCGACATGAAGCAAGACGGTACCTTAAACG
pENTR1a::CRN10_627-F	TCAGTCGACATG GATCCAGTTCGAATGAAACTTCAGAC
pENTR1a::CRN10_627-R	CTA GATATC TCA ATTAAATGCGCAGCCGCTTTTGG
pENTR1a::CRN11_767-F	TCAGTCGACATGTTGTCTACGGGAGAAGATGTCG
pENTR1a::CRN12_997-F	TCAGTCGACATGGAGGAAAATATGACGGTGGG
pENTR1a::CRN12_997-R	CTAGATATCTCAGAAACGTTTAAGCTTTTTATGCGTTC
pENTR1a::CRN20_624-F	TCAGTCGACATGAAAGGTAAAAACGACCGCTCC
pENTR1a::CRN20_624-R	CGT GAATTC TCA CTTCTCGAACTGCGGG
pENTR1a::CRN22_248-F	TCCGGTACCATGGGGGGTATTGATTGCTCAGT
pENTR1a::CRN33_10-F	TCA GTCGAC ATG ATTAAACTCTTTTGTGCG
pENTR1a::CRN33_10-R	CTA GATATC TCA TAATATTCGACGGAAAAAGCCG

pENTR1a::CRN36_259-F	TCAGTCGACATGGATTATGACAGCGACTC
pENTR1a::CRN47_135-F	TCAGTCGACCGGATTTCACGGTCGATGAA
pENTR1a::CRN60_274-F	TCAGTCGACATGGAGAGCGTTGGACGGACTC
pENTR1a::CRN79_188-F	TCA GTCGAC ATG CAGCAATGGACAATTTCTCAA
pENTR1a::CRN79_188-R	TAT CTCGAG TCA ATCATGCAGTTGATTCAGCAG
pENTR1a::CRN105_25-F	TCAGTCGACATGACTTCTTCGCTAGGAGTGAGAGC
GFP-Random1	GAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTA
GFP-Random2	CTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCT
GFP-Random3	CACAAGTTGGAATACAACTACAACTCCCACAACGTATACATCATGGCCGACAAGCAAAAG
GFP-Random4	AAGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAATTGGCGATGGCC
GFP-Random5	TGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAAC
U6	AGGGGCCATGCTAATCTTCTC
pENTR1a::CRN32_283_F_NES	C TGG ATC CGG ATG CTGGCTTTGAAGTTAGCTGGTTTGGATATC GAGGATTCAAAACGGCG
pENTR1a::CRN32_283_F_mnes	C TGG ATC CGG ATG CTTGCTCTTAAGGCGGCTGGAGCTGATGCT GAGGATTCAAAACGGCG
pENTR1a::CRN-R_NES	GTCTCGAGTGTCAGATATCCAAACCAGCTAACTTCAAAGCCAGCTTCTCGAACTGCGGATGCG
pENTR1a::CRN-R_mnes	GTCTCGAGTGTCAAGCATCAGCTCCAGCCGCCTTAAGAGCAAG CTTCTCGAACTGCGGATGCG
pENTR1a::CRN36_259_F_NES	C TGG ATC CGG ATG CTGGCTTTGAAGTTAGCTGGTTTGGATATC GATTATGACAGCGACTCAGAAG

pENTR1a::CRN36_259_F_mnes C TGG ATC CGG ATG CTTGCTCTTAAGGCGGCTGGAGCTGATGCT GATTATGACAGCGACTCAGAAG

RESULTS

Two CRNs of *P. capsici* possess RNA silencing suppression activity

Bioinformatic analysis of the genome sequence of *P. capsici* has identified 84 CRNs (Stam et al., 2013b). I cloned 16 *CRN* genes, with the host-targeting sequence at the N-termini deleted, in the vector pEG100 (Stam et al., 2013b). These constructs were then introduced into *Agrobacterum tumefacience* strain GV3101. Using Agro-infiltration, these genes were individually expressed in the GFP-expressing transgenic *N. benthamiana* 16c plants together with *Agrobacterum* carrying *35S-GFP*. The expression of the external *GFP* gene induces the production of siRNAs that silence both the endogenous and exogenous *GFP* genes, resulting in no or very low green fluorescence in the infiltrated leaf zone. However, if the external GFP is co-expressed with an RNA silencing suppressor, the production of GFP in the infiltrated area will be recovered (Ruiz et al., 1998; Guo and Ding, 2002).

Using this assay, I screened the 14 CRN effectors and observed the production of GFP at 5 days post inoculation (dpi) under UV light. The well-studied viral RNA silencing suppressor CMV2b was used as the positive control (Guo and Ding, 2002). The results show that CRN32_283 and CRN 36_259 suppressed the *GFP* silencing in *N. benthamiana* 16c (Figure 2.1). Consistent with the reduced green fluorescence, the western analysis showed increased accumulation of GFP proteins in leaves expressing CRN32_283 and

CRN 36_259 (Figure 2.2). Empty vector was used as a negative control and Cucumber mosaic virus 2b (CMV2b) was used as a positive control.

Next, I examined the abundance of GFP siRNAs in *N. benthamiana* 16c leaves co-expressing GFP and the two CRNs using northern blotting. Similar to CMV2b, leaves expressing CRN36_259 exhibited reduced GFP siRNA accumulation; however, the GFP siRNA level in leaves expressing CRN32_283 remained unchanged (Figure 2.3). These results suggest that CRN36_259 may affect small RNA accumulation while CRN32_283 only affects small RNA function.

Figure 2.1. Two *P. capsici* CRN effectors suppress transgene silencing in *N. benthamiana* 16c plants.

CRNs were individually expressed in *GFP*-transgenic *N. benthamiana* 16C plants together with *35S-GFP* using *Agrobacterium*-mediated transient expression. Empty vector (EV) and a well-studied viral RNA silencing suppressor CMV2b were used as the negative and positive control, respectively. Experiments were repeated four times with similar results.

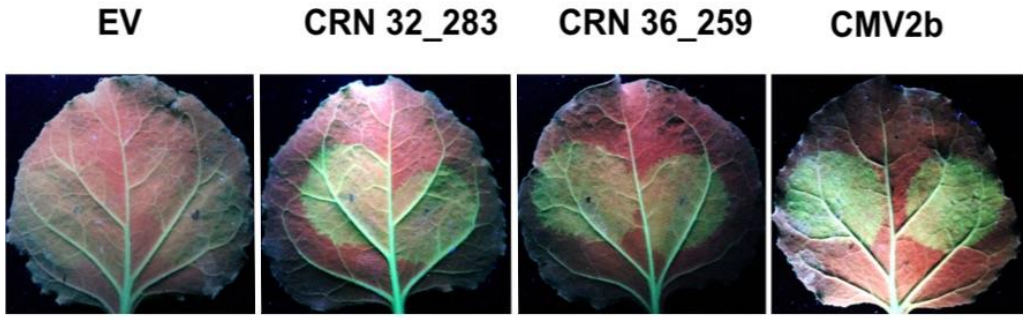


Figure 2.2. Protein abundance of GFP in infiltrated *N. benthamiana* 16C leaves.

Accumulation of GFP protein in infiltrated *N. benthamiana* 16c leaves. PSR2 and GFP were expressed in *N. benthamiana* 16c through *Agrobacterium*-mediated transient expression. Total proteins were extracted from infiltrated leaves at 5 dpi and then detected by anti-GFP antibody using western blotting. EV was used as a negative control and CMV2b was used as a positive control. Coomassie Brilliant blue staining (CBB) was used as loading control. Experiments were repeated four times with similar results.

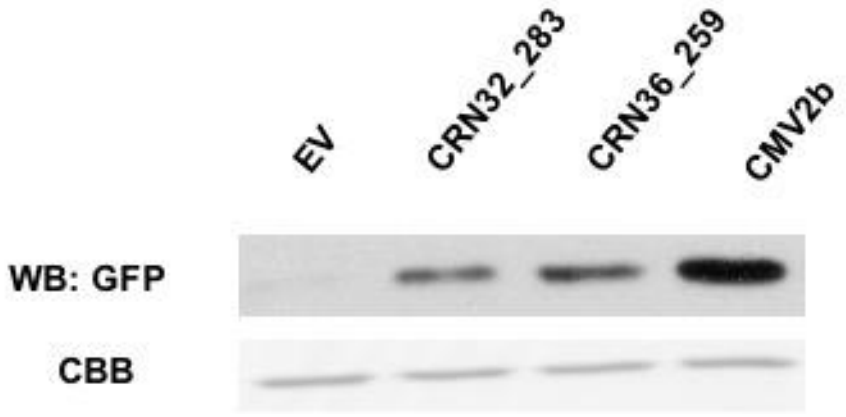
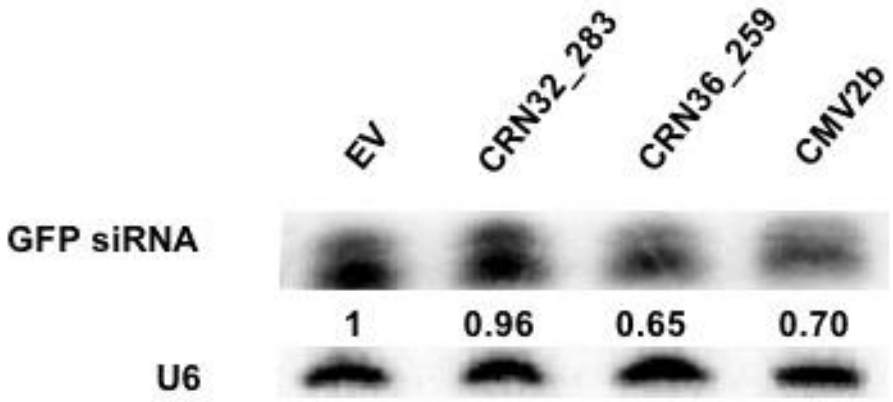


Figure 2.3. Abundance of GFP siRNAs in infiltrated *N. benthamiana* 16C leaves.

Total RNA was extracted and small RNAs were separated by denaturing PAGE. Northern blot showing the accumulation of GFP small interfering RNAs (siRNAs) in infiltrated *N. benthamiana* 16C leaves. EV was used as a negative control and CMV2b was used as a positive control. U6 was used as a loading control. Numbers below the blots represent the relative abundance of the small RNAs with the levels in leaves expressing EV set to 1.



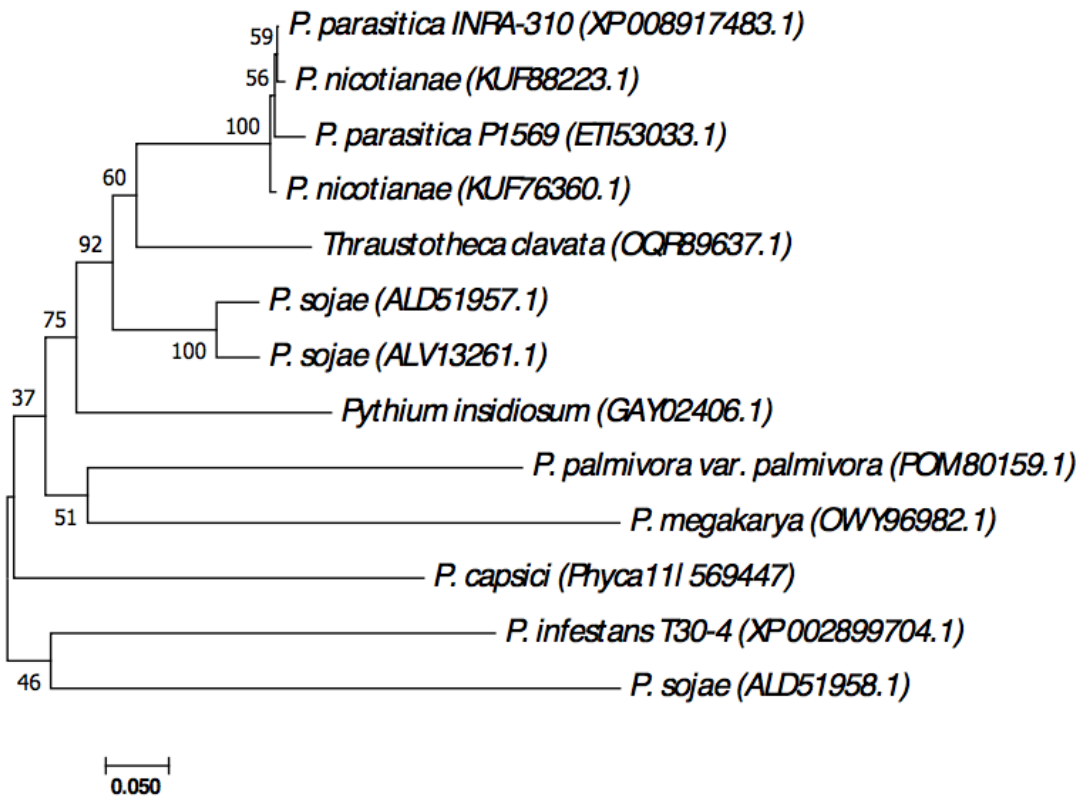
CRN32_283 and CRN36_259 are widespread in oomycetes

It was found that both CRN32_283 and CRN36_259 have homologs present in other *Phytophthora* species, even in another oomycete genus, *Aphanomyces* which contains many animal pathogens (Figure 2.4). Interestingly, the homolog of CRN32_283 found in *P. infestans* were classified as CRN family proteins, and its homolog in *P. sojae* is identified as PsCRN108 with the known function of reprograming expression of plant heat shock proteins (Song et al., 2015). CRN36_259 found in *P. infestans* were classified as CRN family proteins while homologs in other species are hypothetical proteins.

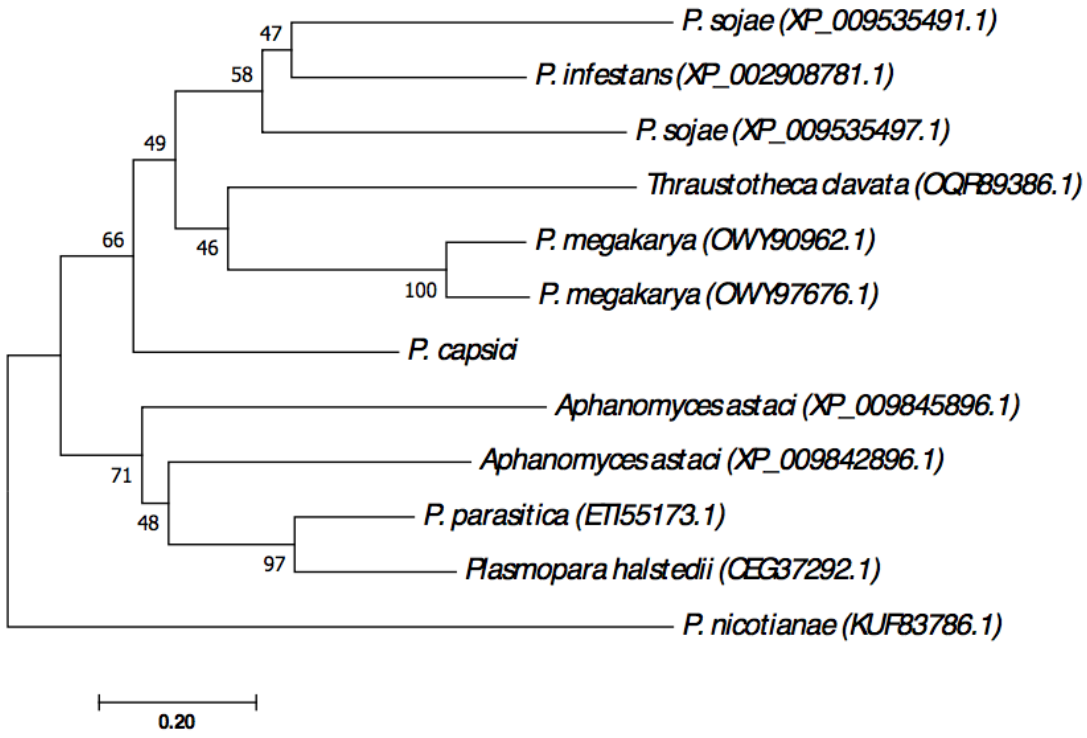
Figure 2.4. Phylogenic analysis showed CRN32_283 and CRN36_259 had homologs present in other *Phytophthora* species.

Neighbor-joining tree of the *CRNs* homologs in various *Phytophthora* species using full-length amino acid sequences. (A) Neighbor-joining tree of CRN32_283. (B) Neighbor-joining tree of CRN36_259.

A



B

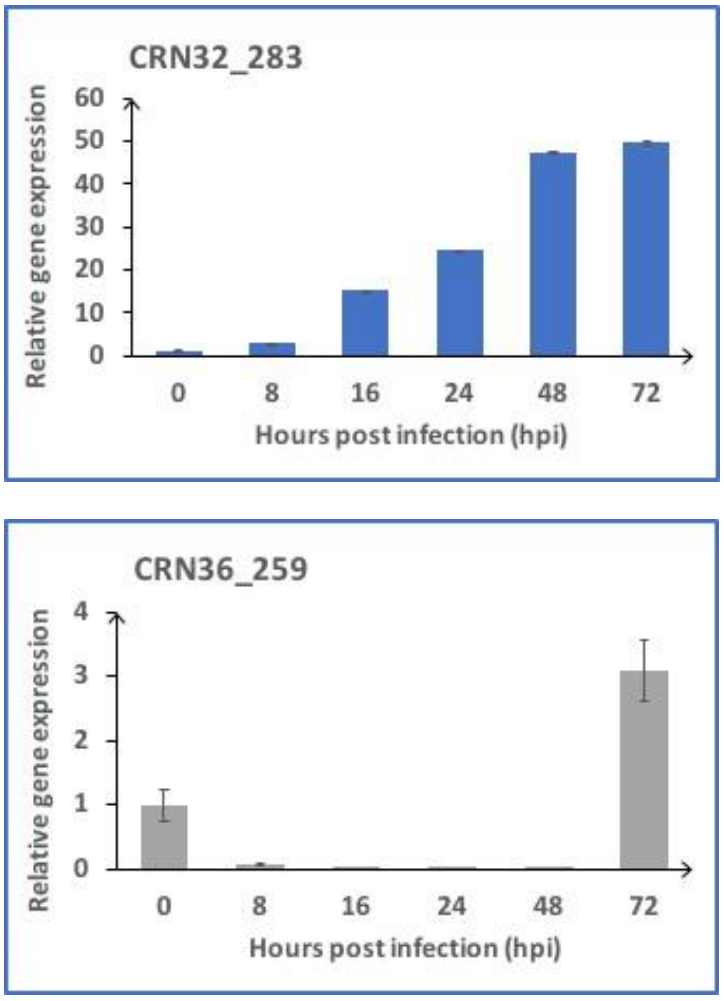

P. sojae (XP_009535491.1)
P. infestans (XP_002908781.1)
P. sojae (XP_009535497.1)
Thraustotheca clavata (OQR89386.1)
P. megakarya (OWY90962.1)
P. megakarya (OWY97676.1)
P. capsici
Aphanomyces astaci (XP_009845896.1)
Aphanomyces astaci (XP_009842896.1)
P. parasitica (ETI55173.1)
Plasmopara halstedii (CEG37292.1)
P. nicotianae (KUF83786.1)
47
58
49
66
46
100
71
48
97
0.20

Expression profile of *CRN32_283* and *CRN36_259* during infection

Using qRT-PCR, I examined the expression pattern of these two CRNs during infection of *Solanum lycopersicum* with *P. capsici*. The results showed that *CRN32_283* and *CRN36_259* have different expression profiles. For *CRN32_283*, the expression level increased gradually throughout the infection (Figure 2.5). The expression at 8 hpi (hours post infection) was approximately three folds compared to that at the beginning of the infection and then reached the maximum level at 48 hpi. On the contrary, *CRN36_259* exhibited a basal expression level at 0 hpi but was repressed till 48 hpi. The expression of CRN36_259 was observed again at 72 hpi, possibly at the completion of the infection cycle (Figure 2.5).

Figure 2.5. Quantitative RT-PCR determining the transcript abundance of *P. capsici* CRN32_283 and CRN36_259 during infection of *S. lycopersicum*.

The cDNA was used as the template and examined at 0, 8, 16, 24, 48 and 72 hours in a time course. Tubulin was used as a negative control. Values are mean ±SD.



CRN32_283 and CRN36_259 promote *Phytophthora* infection

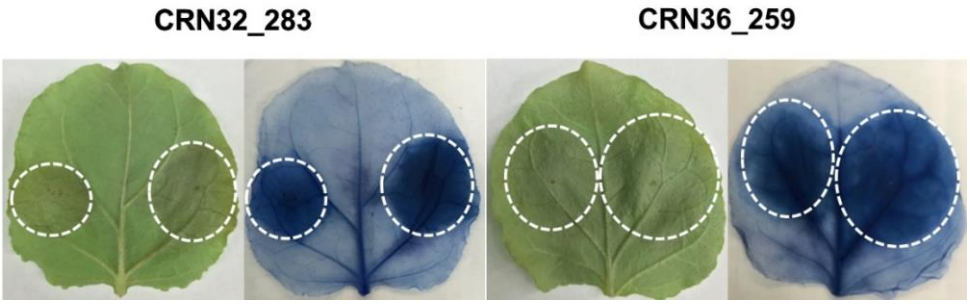
To examine the virulence activity of these two CRNs, I therefore infected *N. benthamiana* transiently expressing CRN32_283 and CRN36_259 by *P. infestans* sporangia. EV was used as a negative control and expressed in the left half of the leaves, CRN32_283 and CRN36_259 were expressed in the right half of the leaves respectively. Lesion size caused by *P. infestans* infection was measured as described previously (Qiao et al., 2013) and visualized using Trypan Blue (Figure 2.6). The white circle was used to show the lesion area, the result suggests that CRN32_283 and CRN36_259 promote *Phytophthora* infection in *N. benthamiana*.

Figure 2.6. Virulence activity of CRN32_283 and CRN36_259 during *Phytophthora* infection.

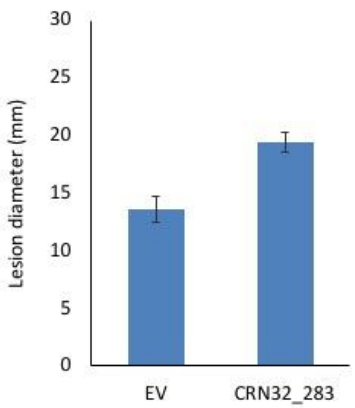
(A) The virulence activities of CRN32_283 and CRN36_259 were examined by inoculating *N. benthamiana* transiently expressing the CRNs (the right half of each leaf) or infiltrated with *Agrobacterium* carrying the empty vector (EV; the left half of each leaf) with *P. infestans*. Detached leaves of *N. benthamiana* were inoculated with zoospore suspension ($1x10^5$ zoospores per ml) of *P. infestans* 36 hours after *Agrobacterium* infiltration. Pictures were taken at 3 dpi. EV was used as a negative control. Disease symptoms were visualized by trypan blue staining.

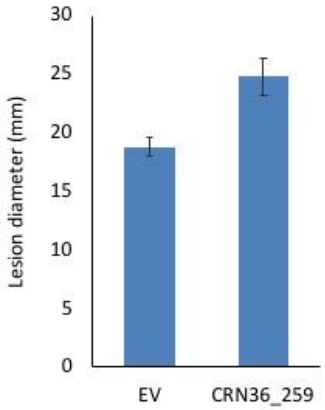
(B) Lesion diameters caused by *P. infestans* infection and error bars are ± SEM.

A


CRN32_283
CRN36_259

B


Lesion diameter (mm)
30
25
20
15
10
5
0
EV
CRN32_283


Lesion diameter (mm)
30
25
20
15
10
5
0
EV
CRN36_259

_CRN36_259_ knockout mutant of _P. capsici_ exhibited reduced hyphae growth and virulence activities

To further confirm the virulence function of CRN32_283 and CRN36_259, I examined the knockout mutants of *P. capsici*. CRN32_283 or CRN36_259 was deleted from the genome of *P. capsici* isolate 1534 using CRISPR-based mutagenesis (Fang et al., 2017) by Zhiwen Wang (visiting Ph.D student from Chinese Agricultural University). Two mutants of CRN36_259 and one mutant of CRN32_283 were obtained after sequencing confirmation, however, the expression level of each effector in those mutants needs to be examined by qRT-PCR. When growing on 10% V8 medium, the *CRN36_259* knockout mutant grew slower than the control and the *CRN32_283* mutant (Figure 2.7). Furthermore, *CRN36_259* knockout mutant was almost abolished for zoospores production but not for CRN32_283, suggesting that CRN36_259 is required for *P. capsici* development. This is consistent with the expression profile of this gene during infection.

The mutants were then used to inoculate *N. benthamiana* leaves to examine the virulence contribution of CRN32_283 and CRN36_259. My results show that the *CRN32_283* mutant was still able to infect *N. benthamiana*, although the virulence was reduced when using zoospores for inoculation (Figure 2.8 and Figure 2.9). On the contrary, the *CRN36_259* mutant showed strongly reduced virulence when the leaves were inoculated with mycelium agar plug (Figure 2.8). In addition, leaves inoculated with zoospore suspension of the *CRN36_259* mutant no longer showed any disease

symptom (Figure 2.9). Note that the *CRN36_259* mutant produced a very small amount of zoospores, which may not be able to establish infection. Collectively, these results suggest that CRN36_259 is an important regulator of hyphal growth and zoospores production, and may also affect virulence of *P. capsici*.

Figure 2.7. Hyphal growth of *P. capsici* knockout mutant.

CRN32_283 or *CRN36_25* was individually knocked out from *P. capsici* strain 1534 using CRISPR/Cas9-based mutagenesis. *P. capsici* 1534 strain containing the Empty Vector (EV) was used as a negative control. *P. capsici* 1534 strain knockout mutants were grown on 10% V8 agar plate at 25 ° incubator for four days in dark condition.

Figure 2.8. Virulence activity of *P. capsici* knockout mutant (Plants infected by mycelium plug).

N. benthamiana leaves were inoculated with mycelium agar plug of *P. capsici* 1534 strain knockout mutants. The *P. capsici* 1534 strain containing Empty Vector (EV) was used as a negative control. Photos were taken at 4 days after inoculation.

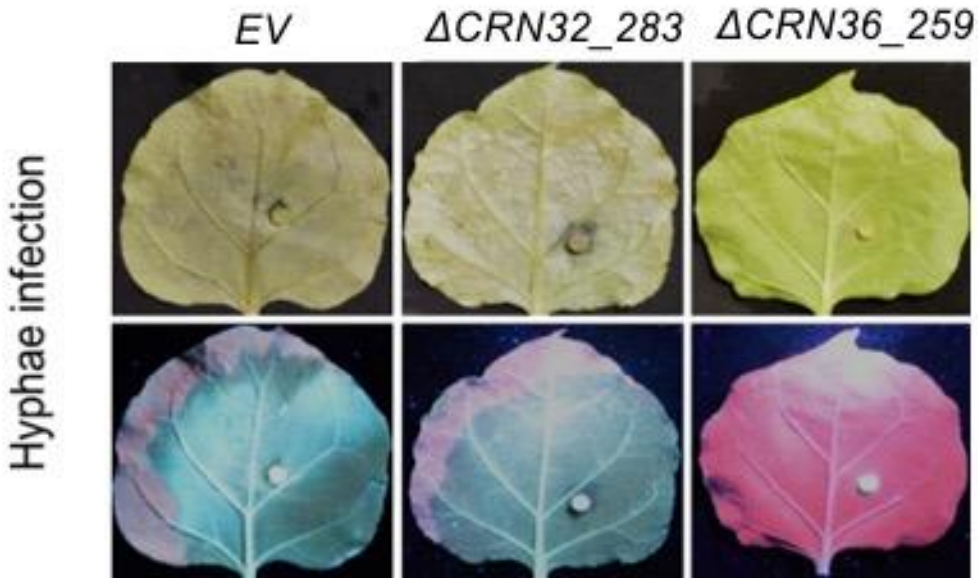
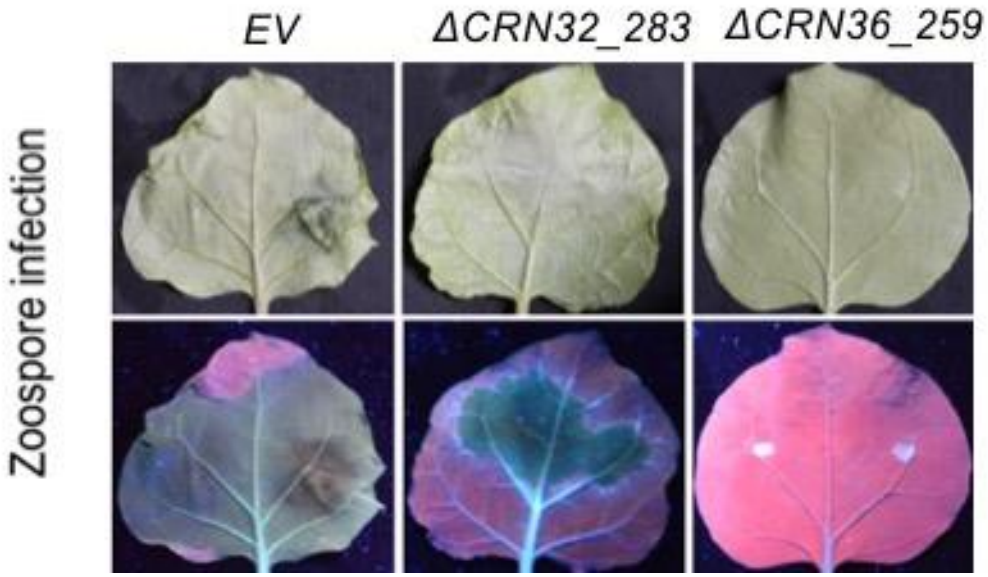


Figure 2.9. Virulence activity of *P. capsici* knockout mutant (Plants infected by zoospores).

N. benthamiana leaves were inoculated with zoospores suspension ($1x10^3$ zoospores per ml) of *P. capsici* 1534 strain knockout mutants. The *P. capsici* 1534 strain containing Empty Vector (EV) was used as a negative control. Photos were taken at 4 days after inoculation.



Nuclear localization of CRN36_259 is required for its RNA silencing suppression activity

Similar to many CRN effectors, both CRN32_283 and CRN36_259 are exclusively located in plant nucleus (Stam et al., 2013b). The nuclear localization has been shown to be important for the function of CRNs. Therefore, I examined whether the nuclear localization of CRN32_283 and CRN36_259 was also required for their RNA silencing suppression activity. Interestingly, neither of these two CRN effectors has predicted nucleus localization signal (NLS) (Stam et al., 2013b). I fused the C-terminal effector domain of CRN32_283 and CRN36_259 to a nuclear export signal (NES) derived from the HIV-1 Rev protein (Schornack et al., 2010) and transiently expressed the recombinant proteins in *N. benthamiana* 16c by *Agrobacterium* infiltration. It turned out that the expression of NES-CRN36_259 was diffused to cytoplasm and leaves expressing NES-CRN36_259 was no longer able to suppress GFP-mediated transgene silencing (Figure 2.10 and Figure 2.11). However, NES-CRN32_283 did not alter any of these activities. This result suggests that RNA silencing suppression activity of CRN36_259 requires its nuclear localization.

Figure 2.10. CRN36_259 is located exclusively in the nucleus.

Confocal microscope images showing the subcellular localization of CRN36_259 when the effector is expressed in *N. benthamiana*. A mutant fused with a nuclear export signal (NES) lost the nuclear localization. Photos were taken at 48 hours after infiltration.

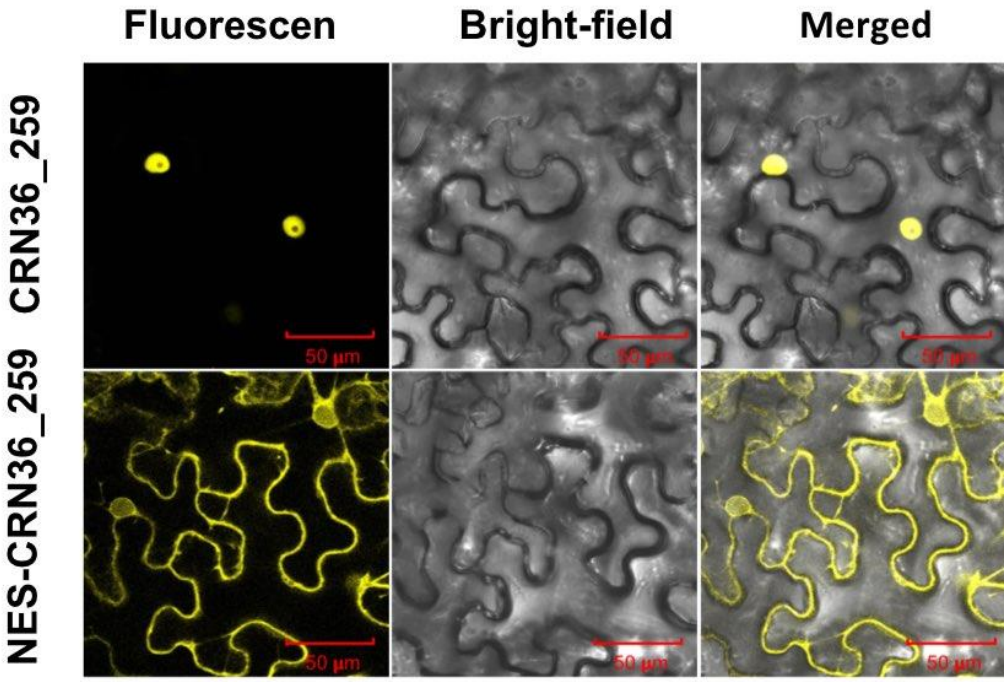
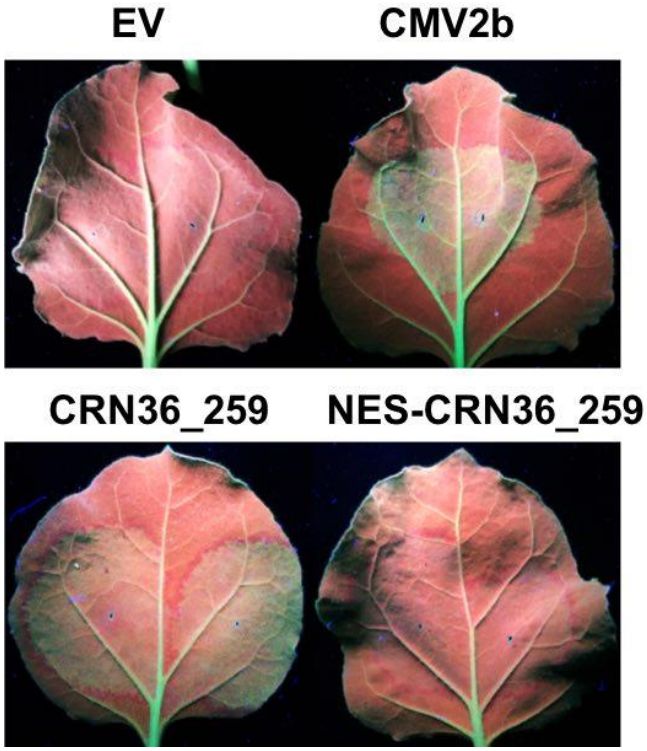


Figure 2.11. The nuclear localization of CRN36_259 is required for its RNA silencing suppression activity.

A mutant fused with a nuclear export signal (NES) and the WT CRN36_259 were co-expressed with 35S:GFP individually in *N. benthamiana* 16C. NES-CRN36_259 which lost the nuclear localization was no longer able to suppress transgene silencing in *N. benthamiana* 16C. EV was used as a negative control and CMV2b was used as a positive control.



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Chapter III. Conclusion and Discussion

Every year, the economic damage caused by *Phytophthora spp.* is estimated in billions of dollars worldwide. For decades, the imperative situation urges researchers to seek novel strategies to control *Phytophthora* diseases, which should be based on a thorough understanding of the pathogenesis.

Although small RNAs are well known to play essential roles in regulating plant growth, development and stress response (Chen, 2009) (Mallory and Vaucheret, 2006), their contribution to plant immunity remains poorly understood. The fact that PSR1 and PSR2, two RxLR effectors from *P. sojae*, manipulate small RNA pathways in plants and promote *Phytophthora* infection suggests that small RNA silencing is a battle ground of plant-*Phytophthora* arms race (Qiao et al., 2013).

Characterization of effector targets provides key mechanisms of effector function in plant hosts. My first focus on the PSR1 target revealed PINP1 as a novel component in the small RNA biogenesis and regulators of plant immunity. Due to the feature of impact on all classes of small RNAs, PSR1-expressing transgenic *Arabidopsis* exhibited severe developmental deficiency, including serrated leaves, dwarfism, late flowering, and reduced seed production (Qiao et al., 2015). As a virulence target of PSR1, *PINP1*-silenced lines exhibited similar developmental phenotype to PSR1-expressing *Arabidopsis*. More importantly, both *PINP1*-silenced lines and PSR1-expressing

Arabidopsis showed reduced abundance of diverse classes of small RNAs and hypersusceptibility to *Phytophthora* infection. It is interesting that the localization of DCL1 and HYL1 which are responsible for endogenous small RNA production is altered in both *PINP1*-silenced lines and PSR1-expressing *Arabidopsis*, indicating that PSR1 associates with PINP1 thus interfering DCL1 activity. Indeed, the nuclear localization of PSR1 is required for its RNA silencing suppression activity and association with PINP1, suggesting its function through DCL1 in the nucleus (Qiao et al., 2015). However, the abundance of DCL1 remains unchanged, how PSR1 affects DCL1 activity is still unknown.

Different with PSR1, PSR2 specifically affects the accumulation of tasiRNAs but not affecting miRNAs or hcsiRNAs in *Arabidopsis* (Qiao et al., 2013). Nonetheless, PSR2-expressing *Arabidopsis* plants are highly susceptible to *P. capsici* infection, indicating that this particular siRNA pathway is important for plant immunity. Furthermore, PSR2 homologs are produced by several *Phytophthora* species, suggesting that this virulence activity might be important in different pathosystems (Xiong et al., 2014).

The secondary siRNAs that triggered by miRNAs-cleavage are widely spread in diverse plants and were predicted to regulate defense-related genes through an amplification effect (Wong et al., 2014) (Ye and Ma, 2016) (Li et al., 2016). For example, one of the secondary siRNAs targets is nucleotide-binding leucine-rich repeat (*NB-LRR*) genes, which encode the canonical disease resistance proteins (Zhai et al., 2011; Shivaprasad et al., 2012; Li et al., 2012; Zhao et al., 2015). Another gene family encoding

pentatricopeptide repeat (PPR) proteins that can influence immune response is also a major target of secondary siRNAs. Comprehensive analyses on the function of the secondary siRNA pathway and specific siRNAs (or their parental miRNAs) are required to fully understand how these siRNAs regulate plant immunity. Importantly, not only the key components for synthesis of long dsRNAs RDR6 and SGS3, but also the dicing enzyme DCL4 have been identified to play a significant role in plant defense (Ellendorff et al., 2008) (Liu et al., 2009) (Yang et al., 2010) (Wagh et al., 2016). It is not surprising that DRB4 which is responsible for dsRNA processing is required for plant immunity against phytopathogens. In this study, *drb4* mutant plants are hypersusceptible to *P. capsici* which is similar with PSR2 transgenic plant. My preliminary data also showed that the expression level of DRB4 in *Arabidopsis* was not altered upon flg22 treatment, intriguingly, the susceptibility of *PSR2*-expressing *Arabidopsis* was not changed upon bacterial infection (Xiong et al., 2014). This data suggests that PSR2 specifically promotes *Phytophthora* infection and the specific role of DRB4 in host resistance against *Phytophthora* but not bacterial infection. More importantly, DRB4 also has been studied for many years in plant defense against virus infection (Haas et al., 2008) (Barton et al., 2017) (Zhu et al., 2013). Together, these pieces of evidence demonstrate that DRB4 is a conserved positive regulator in small RNA biogenesis and immunity in plants.

The experiments showed that DRB4 and PSR2 would not interact in vitro, indicating that other components might be required to mediate this interaction. The RNase digestion

assay suggests that dsRNA is unlikely the mediator. There might be other proteins that are needed in DRB4-PSR2 complex, Mass Spectrometry can be used to analyze other components of the complex, however, it's hard to catch this interaction complex if it's a transient process. To address this concern, samples collected in a time course are required to detect the dynamic cellular process. Another possibility is that DRB4 is subjected to various post-translational modification (PTMs) with the presence of PSR2, facilitating the interaction in plant.

DRB4 contains two dsRBMs which are required for the interaction with DCL4 and as well as long dsRNA binding (Fukudome et al., 2011). The experiments in this study demonstrated that these two dsRBMs are also required for the association between DRB4 and PSR2, implying that PSR2 could disrupt the function of dsRBMs, which is either binding with DCL4 or long dsRNA. If PSR2 abolishes the interaction between DRB4 and DCL4, the dicing activity of DCL4 is then affected. Indeed, the leaves of *dcl4* *Arabidopsis* mutant also exhibited the curly, narrow developmental defects (Nakazawa et al., 2007). To further study the consequence of PSR2-DRB4 interaction, competition assay using Co-IP and in vitro dsRNA binding assay will be performed. To obtain more comprehensive analysis, RNA-seq using DRB4-immunoprecipitated complex from *PSR2*-expressing *Arabidopsis* plants will reveal if PSR2 has impact on dsRNA-binding activity of DRB4. As my preliminary data showed that the accumulation of DRB4 remains unchanged in PSR2 transgenic plant, another possible consequence is that DRB4

localization is altered with the presence of PSR2, as it's described in anti-viral defense (Jakubiec et al., 2011; Zhu et al., 2013). Though the co-localization of DRB4 and PSR2 under confocal microscopy is shown in this study, a quantitative measurement will be helpful to determine the ratio of DRB4 in the nucleus and cytoplasm.

On the other hand, the fact that PSR2 interacts with DRB4 at its N-terminus, the two dsRBMs which are required for DCL4 interaction and long dsRNA-binding leads to a complicated case to engineer DRB4 to avoid the interaction with PSR2. As such, point mutagenesis in these two dsRBMs might result in the loss of DRB4 function in endogenous secondary siRNAs biogenesis. However, it's possible that PSR2 interacts with DRB4 through other protein which directly binds with DRB4 without occupying the pocket responsible for DCL4 and long dsRNA binding. To investigate how PSR2 interrupts DRB4 function is helpful for the research that engineering DRB4 as the tool against *Phytophthora* infection.

In my chapter II, I identified new *Phytophthora* RNA silencing effectors in another class, Crinklers (CRNs). Compared to RxLR effectors, CRN effectors are rather overlooked although they are broadly produced by oomycetes. To date, the virulence functions of CRN effectors are largely unknown. Intriguingly, many CRNs are located in the nucleus of plant cells (Stam et al., 2013a). Besides, neither PSR1 or PSR2 have been found in *P. capsici* yet. Therefore, it is interesting to examine if any CRNs possess RNA silencing suppression activity.

The screening results identified two CRNs from *P. capsici* possess the RNA silencing suppression activity. It is very interesting that the GFP siRNA level in the leaves of *N. benthamiana* 16c expressing CRN32_283 remain unchanged while it reduced in leaves expressing CRN36_259. Besides, these two CRNAs have different expression pattern: CRN32_283 has a gradual increased expression level during the infection, it's very likely that CRN32_283 affects plant small RNA pathway without altering its accumulation; for CRN36_259, following the initial expression at 0 hours post inoculation (hpi), there was a minimal expression between 8 hpi and 48 hpi, then level peaked at 72 hpi which is the late stage of infection. Though more time points of expression level between 0 and 8 hpi should be examined, CRN36_259 is more likely to affect the *Phytophthora* small RNA pathway. Indeed, a *CRN36_259* knockout mutant almost completely lost virulence activity, which is most likely due to the loss of the production of zoospores. It's reported that *Phytophthora sojae* produce two distinct populations of small RNAs including 21-nt and 25-nt small RNAs (Fahlgren et al., 2013). The RNA-seq and small RNA-seq using this CRISPR knockout mutant could help reveal the role of CRN32_283 and CRN36_259.

In this study, using CRISPR-Cas9 system to knockout *CRN32_283* and *CRN36_259* in *P. capsici* respectively, reduced virulence activity was observed from the *CRN32_283* and *CRN36_259* knockout mutant. The *CRN32_283* knockout mutant exhibited reduced virulence activity during zoospores infection while it remained unchanged during hyphae infection. This is probably due to the impact of CRN32_283 on zoospore

production or the dosage of zoospores didn't reach the threshold leading to disease symptom caused by wildtype *P. capsici*. What is more interesting, the hyphae growth and zoospores production of a *CRN36_259* knockout mutant was affected which indicates the special role of CRN36_259 in *P. capsici* development. To confirm these phenotypes were caused by lacking *CRN32_283* and *CRN36_259*, a wild-type copy of *CRN32_283* and *CRN36_259* should be re-introduced to the *P. capsici* knockout mutants respectively. In addition, the presence of CRN32_283 and CRN36_259 in other *Phytophthora* species further indicates that they are essential virulence factors for *P. capsici*.

To further investigate the type(s) of small RNAs that are affected by CRN32_283 and CRN36_259, sRNA-seq using *Arabidopsis* transgenic lines expressing these effectors respectively should be performed. Because small RNAs play an important role in anti-viral defense, I expect the CRN expression will also enhance viral infection as shown for PSRs and VSRs. Further study about these two CRNs will use Mass Spectrometry to identify their interacting proteins involved in suppression of RNA silencing activity. This work will not only define the function of CRN effectors, but also reveal novel virulence mechanisms of oomycetes, including pathogens of both plants and animals.

In summary, my experiments in Chapter I suggest DRB4 as a virulence target of PSR2 in *Arabidopsis*, the results in Chapter II identify two effectors which possess RNA silencing suppression activity as important virulence factor during *Phytophthora* infection.

Collectively, RNA silencing as an essential regulator of plants growth and defense response is a common and effective target of phytopathogens during the endless arms race. In the long run, these findings will provide mechanistic insight to *Phytophthora* pathogenesis and new disease management approaches to battle against the notorious *Phytophothora* diseases.

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