UNIVERSITY OF CALIFORNIA
RIVERSIDE

Small RNA Regulation During *Phytophthora Sojae* Infection in Soybean

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

Doctor of Philosophy

in

Cell, Molecular and Developmental Biology

by

James Tac Wong

December 2013

Dissertation Committee:

Dr. Wenbo Ma, Chairperson
Dr. Howard S. Judelson
Dr. Xuemei Chen

The Dissertation of James Tac Wong is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and respect to my advisor Dr. Wenbo Ma for her mentorship, guidance and support on this project for the past five years. She has been an encouraging and critical figure in my research and my development as a scientist and I am grateful for the opportunity to have trained in her laboratory.

I wish to thank Dr. Xuemei Chen for providing her expertise as my research advisor and dissertation committee member. My gratitude extend to her lab members, Dr. Shengben Li, Dr. Theresa Dinh, Lei Gao, Yu Yu and Jane Jia for their experimental and technical support. I am thankful to Dr. Howard Judelson for providing me with valuable guidance and advice as my dissertation and research advisor, as well as Audrey Fong for her assistance in *Phytophthora* experimental protocols.

My accomplishments are made possible with help and encouragement from all the previous and current members of Wenbo's laboratory. I am appreciative of Dr. Huanbin Zhou and Robyn Morgan for their help during my first year as a graduate student. I am especially grateful to Dr. Yongli Qiao, Dr. Jinxia Shi, Dr. Shushu Jiang, Qin Xiong, Ka-Wai Ma, Yi Zhai, Shuyi Duan, Ariel Kuan, Yuanchun Wang and Christina Flores for their support in and outside the lab. I could not have asked for a better group of colleagues for work and play. I am also thankful to my undergraduate research trainees, Vicky Chan and Daisy Cruz, for their assistance to my research.

In addition, I have to acknowledge Dr. Jason Stajich, Dr. Thomas Girke, Dr. Yang Yang and Dr. Blake Meyers for their contributions to my small RNA data processing and bioinformatic analyses. I am thankful to Dr. Caroline Roper, Dr. Katherine Borkovich, and Dr. Venugopala Gonehal for allowing me to utilize their laboratory supplies and equipment. I wish to thank Dr. Yuanchao Wang, Dr. Guilang Tang and Dr. Brett Tyler for providing invaluable supplies and materials for my experiments. I am deeply thankful to the administrative staff of the CMDB graduate program and the Department of Plant Pathology and Microbiology, especially Kathy Redd, Nancy Ferguson and Joann Braga, for their indispensable support for the past five years.

None of this would be possible without the support, encouragement and love from my family. Most importantly, I dedicate this work to my wife Rhiannon for her sacrifice, endless love and spiritual support throughout my academic career.

ABSTRACT OF THE DISSERTATION

Small RNA Regulation During *Phytophthora sojae* Infection in Soybean

by

James Tac Wong

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, December 2013
Dr. Wenbo Ma, Chairperson

Plant endogenous small RNA pathways generate non-coding regulatory RNAs that regulate gene expression through target mRNA cleavage, translation inhibition or chromosomal modifications. Regulation of small RNAs and their targets during pathogen infection is tightly controlled to promote defensive mechanisms against disease progression. The oomycete pathogen, *Phytophthora sojae* is a principal infectious agent of soybean. To date, there is limited information on small RNAs that regulate defense responsive genes against *P. sojae*.

Infection response in plants is evidently regulated in part by small RNAs. High-throughput sequencing of small RNA libraries constructed from *P. sojae*-infected and mock-infected soybean roots and subsequent computational analysis revealed approximately 324 known soybean miRNAs and 109 potential novel soybean miRNAs

that differentially accumulate between the *P. sojae*-infected and mock-infected samples. Of these, 8 conserved miRNAs and 2 novel miRNAs were verified by Northern blot analysis. Targets of the miRNAs displayed abundance changes respective to their complementary miRNA's levels.

The down-regulation of the conserved miR393 by target mimicry points to a positive regulatory role for miR393 during pathogen response. In addition, we noted the induction of miRNA-directed expression of phasiRNAs from multiple NB-LRR loci. These results indicate a pool of miRNAs specific in responding to *P. sojae* infection. Our study identified multiple conserved and novel soybean miRNAs with potential defensive roles against *P. sojae*. Our data demonstrates that plant response to pathogen infection is complex and multi-layered. Further study of small RNAs involved in defense regulation may contribute to combating *Phytophthora* diseases.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. SMALL RNAS THAT ARE DIFFERENTIALLY ACCUMULATED IN SOYBEAN ROOTS DURING INFECTION BY *PHYTOPHTHORA SOJAE*

1.1 ABSTRACT

Oomycetes are a diverse group of eukaryotic organisms, of which many are pervasive plant pathogens that cause economically significant diseases worldwide. In particular, *Phytophthora spp*. are plant destroyers that cause billions of dollars of annual loss worldwide. However, our understanding of the pathogenesis of *Phytophthora* diseases is rather limited. My research aims to investigate the role of small RNAs in soybean in response to infection by *Phytophthora sojae*, which is second most devastating soybean pathogen and causes the severe root and stem rot disease. Plants produce two classes of small RNAs, microRNA (miRNA) and small interfering RNA (siRNA), that regulate important processes including development and immunity. Currently very few studies have investigated small RNA expression in plants during *Phytophthora* infection. By analyzing the small RNA population at the early stage of infection in soybean roots, I identified eight known and two novel miRNAs that are differentially expressed upon *P. sojae* infection. These miRNAs directly target genes regulating resistance and hormone signaling, which may play a role in plant immunity. This research provides greater understanding of the gene expression and metabolic changes regulated by miRNAs that occur during pathogen infection.

1.2 INTRODUCTION

1.2.1 Oomycete plant pathogens

The Oomycete lineage is comprised of filamentous eukaryotic microorganisms, many of which are plant pathogens. Although morphologically similar to fungi, molecular and biochemical data confirmed that the Oomycetes are in the Stramenopiles phylum and closely related to brown algae and diatoms (Baldauf, 2000; Haas et al., 2009). Among the Oomycetes, there are more than 90 *Phytophthora* species that cause disease in plants. *Phytophthora* species are some of the most destructive hemibiotrophic plant pathogens.

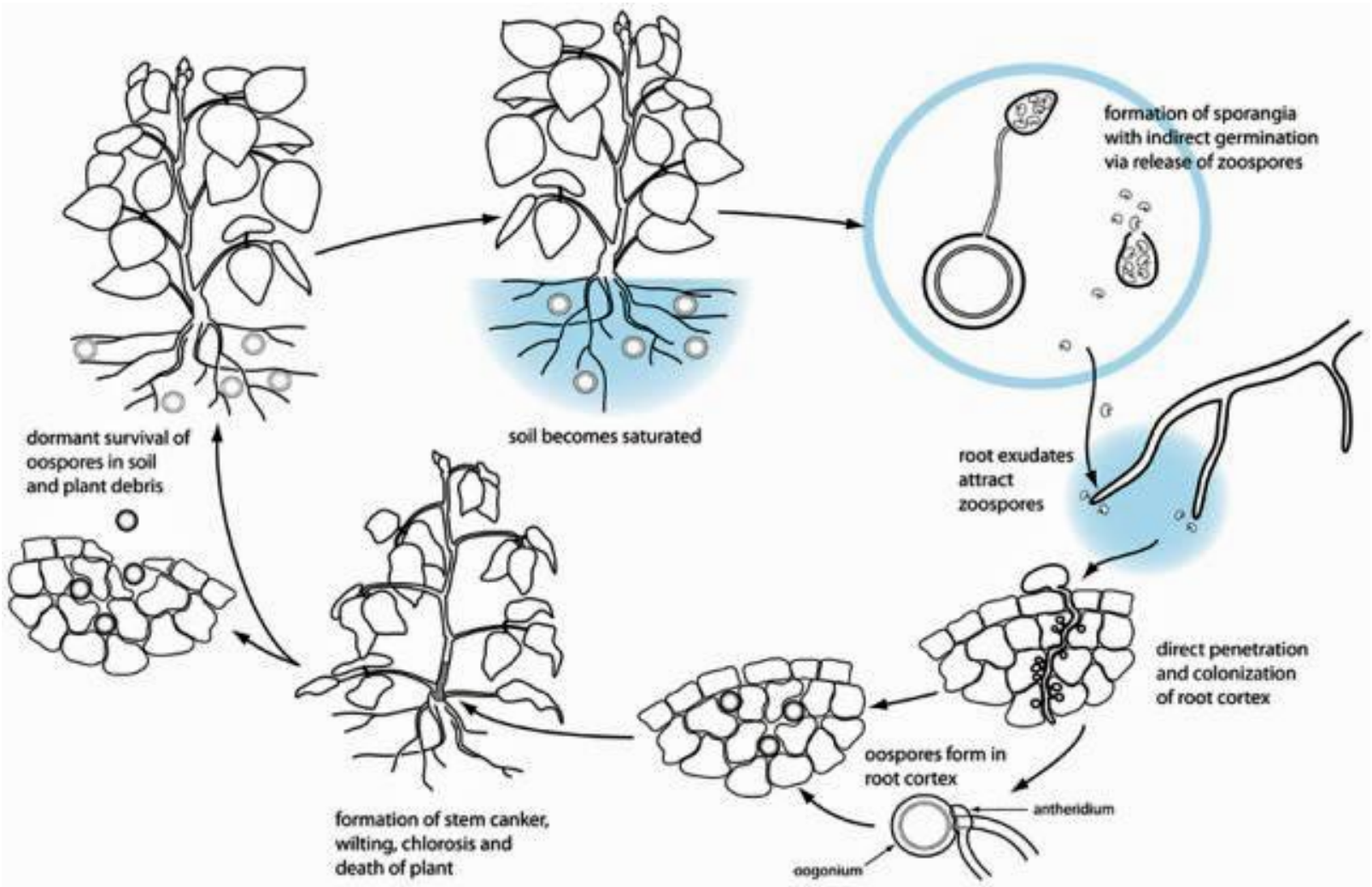
The economic damage generated by *Phytophthora* is estimated in the tens of billions of dollars per year, including cost for loss of crops and ornamental plants and methods to control the diseases (Erwin and Ribeiro, 1996; Wrather and Koenning, 2006). The most well-studied *Phytophthora* is *Phytophthora infestans*, the causal agent of late blight in potatoes and tomatoes (Birch and Whisson, 2001; Haas et al., 2009). *P. infestans* is renowned as the cause of the Irish Potato Famine. The devastation resulted in drastic social, economical and agricultural changes including the loss of approximately 1.5 million lives and mass emigration of much of Ireland's population (Ristaino, 2002). Importantly, *Phytophthora* diseases remain difficult to control due to a lack of understanding on their modes of pathogenesis.

The infection cycle of *Phytophthora* species begins when motile zoospores encyst and germinate on the plant host surface. The germinated hyphae penetrate into the plant tissue and colonize the intercellular space. Feeding structures called haustoria

are then formed, which facilitate infection and disease progression (Hardham, 2001) (Figure 1.1). During this biotrophic infection stage, *Phytophthor*a secretes effector proteins from the haustoria to suppress host immunity and lives together with the host. *Phytophthora* are hemibiotrophic pathogens. Following the biotrophic infection stage, the infection enters the necrotrophic stage, which is hallmarked by the death of the infected plant cells and the appearance of disease symptoms. After host tissue degradation, reproductive structures called sporangia are formed and zoospores are released to start the next infection cycle.

Some *Phytophthora* have a wide range of hosts, for example *Phytophthora ramorum* has gained notoriety for the death of thousands of oak and tanoak trees in California and Oregon, but can also infect many other ornamental plant families. *Phytophthora capsici* is the most broad range pathogen, capable of infecting hosts in the Cucurbitaceae, Fabaceae and Solanaceae families (Hausbeck and Lamour, 2004). *Phytophthora sojae*, however, has a narrow host range and primarily infects soybean. *P. sojae* is the causal agent of soybean root and stem rot disease. Following encystment on root tissue, *P. sojae* enters a biotrophic growth phase, during which hyphae penetrate into the root cortex and form haustorial structures that import nutrients and export virulence proteins to aid in disease development. Late biotrophic phase is recognized by *P. sojae* colonization in the root vascular tissue and development of reproductive structures. Necrotrophic growth is typified by extracellular and intracellular damage. After host tissue degradation, *P. sojae* mycelia and reproductive spores are released to continue its life cycle. (Dorrance et al., 2008; Qutob et al., 2000; Thomas et al., 2007; Tyler, 2007).

Figure 1.1 *Phytophthora sojae* disease cycle. *P. sojae* formation of sporangia produces zoospores that are released into the soil during wet conditions. Infection of root by a zoospore leads to encystment and appressorium formation on the root surface. Biotrophic phase follows hyphae growth, direct penetration into root cortex followed by haustoria formation and colonization. Necrotrophic phase exhibits root tissue damage, wilting, chlorosis and plant death. Oospores produced in the infected tissues are released into the soil to infect additional plants. (Adapted from www.apsnet.org)


formation of sporangia
with indirect germination
via release of zoospores
soil becomes saturated
dormant survival of
oospores in soil
and plant debris
root exudates
attract
zoospores
direct penetration
and colonization
of root cortex
oospores form in
root cortex
antheridium
oogonium
formation of stem canker,
wilting, chlorosis and
death of plant

1.2.2 Small RNAs are essential regulators in plants

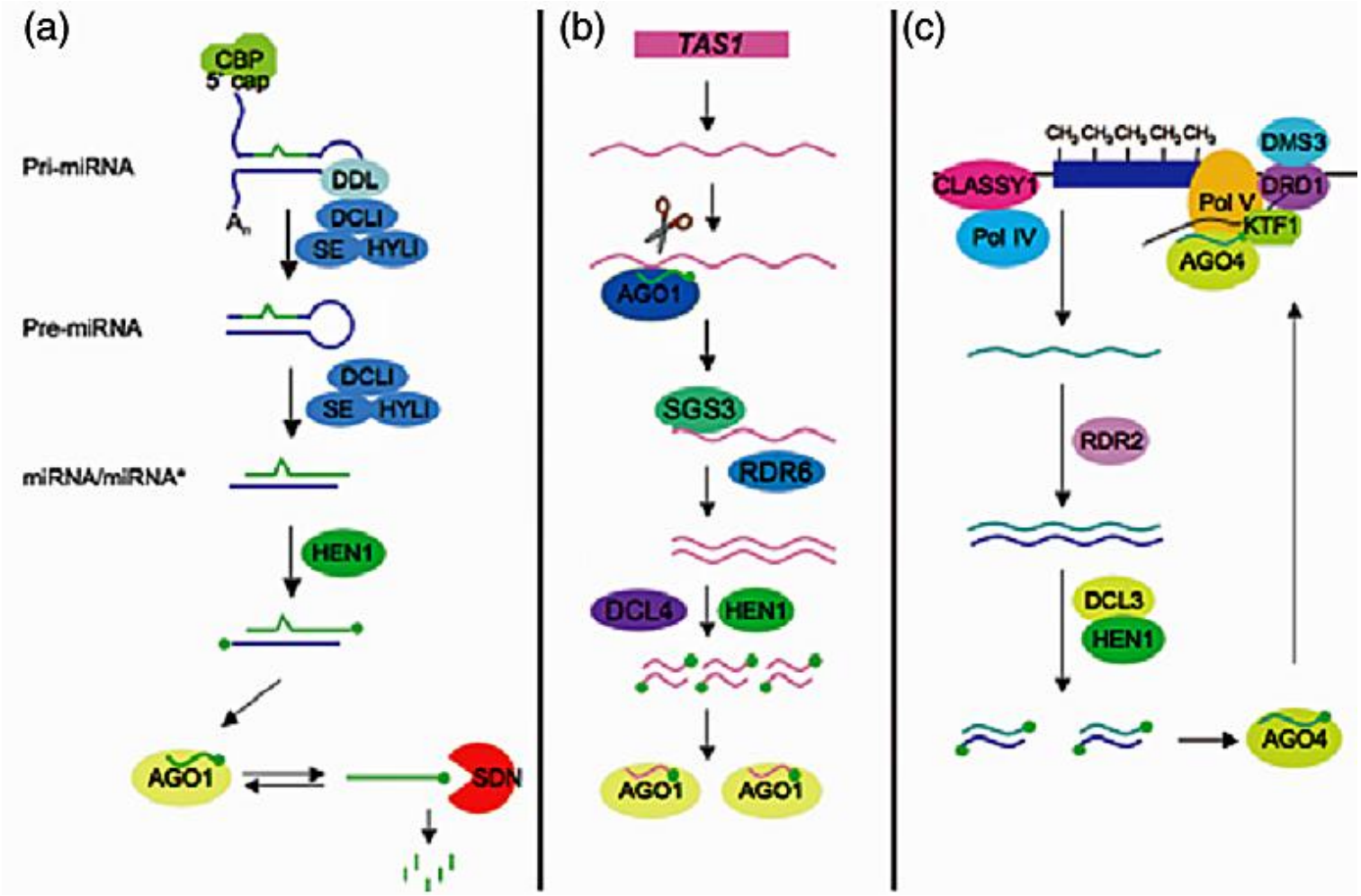
The discovery of small RNAs as genomic regulatory mechanisms has profoundly altered our understanding of the complexity of cellular regulatory pathways. Small RNAs are non-coding short RNA species that are produced by eukaryotic organisms. Small RNAs mediate silencing of genes in a sequence-specific manner. Initially discovered in plants as co-suppressors of transgene expression (Napoli et al., 1990) and later identified to be antisense RNA that function in post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999), we now know small RNAs have essential regulatory roles in many endogenous plant processes including development, metabolism, and responses to biotic and abiotic stresses (Bartel, 2004; Ghildiyal and Zamore, 2009; Khraiwesh et al., 2010; Kulcheski et al., 2011; Mallory and Vaucheret, 2006; Padmanabhan et al., 2009; Ruiz-Ferrer and Voinnet, 2009; Sunkar, 2010; Voinnet, 2009). Plants produce two main classes of endogenous small RNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Figure 1.2). They are generated from double stranded RNAs (dsRNAs) precursors, which are processed by DICER-LIKE (DCL) proteins into mature small RNAs. Small RNAs repress gene expression through complementary base pairing with the target transcripts, resulting in degradation of transcripts and/or translational repression (Ambros, 2004; Bartel, 2009; Cai et al., 2009; Hamilton et al., 2002; Voinnet, 2009). In addition to post-transcriptional gene silencing, siRNAs can also induce epigenetic modifications of the chromatins, leading to transcriptional gene silencing (TGS) (Robert-Seilaniantz et al., 2011; Zhai et al., 2011). Both miRNA and siRNA have been implicated in regulating plant defense.

Figure 1.2 Three distinct endogenous small RNA pathways. (Adapted from Chen., 2010)

(a) The biogenesis of a microRNA (miRNA) begins when a primary miRNA (pri-miRNA) is processed into a pre-miRNA and then processed into a miRNA/miRNA* duplex. The duplex undergoes methylamine by the small RNA methyltransferases HEN1. One miRNA strand is bound by AGO1 to form the RISC complex. Mature miRNAs are turned over by the SDN1 family of small RNA exonucleases.

(b) The biogenesis of trans-acting small interfering RNAs (ta-siRNAs). A non-coding ta-siRNA-generating sequence (TAS) is transcribed into a single stranded RNA, which is targeted by a miRNA for cleavage. Following cleavage, one of the fragments acts as a template for the synthesis of double-stranded RNA (dsRNA) by RDR6. The dsRNA is processed into phased siRNAs by DCL4. Similar to miRNAs, some of the siRNAs are bound by AGO1 and regulate other mRNAs in trans.

(c) Biogenesis and function of heterochromatic siRNAs. A heterochromatic region is transcribed by Pol IV into a single stranded RNA, which is used to generate dsRNA by RDR2. The dsRNA is processed into 24-nt siRNAs and are bound by AGO4. The AGO4/siRNA complex is recruited to a homologous genetic locus by RNA Pol V generated transcripts. DNA and histone modification complexes induce heterochromatin formation of the locus.


(a)
CBP
5' cap
Pri-miRNA
DDL
DCLI
SE
HYLI
Pre-miRNA
DCLI
SE
HYLI
miRNA/miRNA*
HEN1
AGO1
SDN
(b)
TAS1
AGO1
SGS3
RDR6
DCL4
HEN1
AGO1
AGO1
(c)
DMS3
CLASSY1
DRD1
Pol V
KTF1
Pol IV
AGO4
RDR2
DCL3
HEN1
AGO4

miRNA biogenesis

miRNAs are encoded in plant genomes as *MIR* genes. The primary miRNA transcripts (pri-miRNA) are produced by RNA polymerase II and can extend up to 2 kilobases in length. pri-miRNAs form a characteristic hairpin structure, which is subsequently processed by the DICER-LIKE 1 (DCL1) protein complex into dsRNA duplexes and then methylated by HUA ENHANCER 1 (HEN1) in the nucleus (Lee et al., 2004; Park et al., 2002; Yang et al., 2006; Yu et al., 2005). The methylated miRNA duplexes are exported to the cytoplasm by an exportin homolog, HASTY (HST). Mature miRNAs, usually 18-22 nt in length, are incorporated into the RNA-Induced Silencing Complex (RISC) by binding to the ARGONAUTE 1 (AGO1) protein, which guides the recruitment of target gene transcripts for degradation and/or translational repression (Bartel, 2004; Baulcombe, 2004; Chen, 2009).

miRNAs play central roles in regulating development, reproduction, and stress responses. A large number (hundreds to thousands) of miRNAs have been identified in the model plant *Arabidopsis thaliana* and other agriculturally important plant species, including maize, rice, tomato, tobacco, wheat, oranges, grapes, peanuts and soybean. Some miRNAs are conserved in different plant species; others are species-specific (Axtell and Bartel, 2005; Rhoades et al., 2002; Zhang et al., 2007). Thanks to the advent of high-throughput sequencing and the completion of whole genome sequences, there are rapid discoveries of new miRNAs. The recent release from the miRNA database, miRBASE 19 (www.miRBASE.org), contains over 25,000 mature miRNAs sequences from 193 species, reflecting an increase of approximately 4000

new miRNAs within the past ten months. However, further characterization of the conserved and newly identified miRNAs has progressed slowly.

siRNA biogenesis

siRNAs, ranging in length from 21-30 nt, are derived from invading nucleic acids such as viruses and transgenes, and from endogenous loci such as repeats, transposable elements, and genes (Baulcombe, 2004; Diaz-Pendon et al., 2007; Mallory and Vaucheret, 2006; Padmanabhan et al., 2009; Plasterk, 2006; Voinnet, 2009). Endogenous plant siRNAs originate from dsRNA precursors generated by cellular RNA dependent RNA polymerases (RDRs) and are further processed by DICER complexes to produce siRNA duplexes (Baulcombe, 2004; Cerutti and Casas-Mollano, 2006; Chellappan and Jin, 2009; Mallory and Vaucheret, 2006; Xie et al., 2004; Zhang et al., 2012). Major endogenous siRNAs include the 21 nt trans-acting siRNAs (ta-siRNAs) and the 24 nt heterochromatic siRNAs (hc-siRNAs) (Bartel, 2004; Chen, 2009), both of which function to silence gene expression either through the RISC complexes or by inducing DNA methylation,

Ta-siRNAs are a small fraction of secondary siRNAs generated from transcripts that are targeted by specific miRNAs (Allen et al., 2005; Axtell et al., 2006; Bartel, 2005; Cuperus et al., 2010). In *Arabidopsis*, miRNAs trigger the cleavage of *TAS* transcripts through the endonucleolytic (slicer) activity of AGO1 or AGO7, and the cleavage fragments then serve as templates to produce dsRNAs through the activities of RDR6 (Cuperus et al., 2010; Howell et al., 2007; Qu et al., 2008). The produced dsRNAs are further processed to produce siRNAs by DCL4.

These RDR6-dependent siRNAs can regulate target genes just like miRNAs, but they can silence other genes in trans based on sequence homology and are therefore called trans acting siRNAs.

The previous definition for ta-siRNAs was very stringent by referring to secondary siRNAs produced from a small number of known *TAS* loci in *Arabidopsis* (Axtell et al., 2006; Chen et al., 2007; Howell et al., 2007). However, recent analyses of small RNA sequencing data uncovered hundreds of loci in various plants that produce secondary siRNAs from miRNA target transcripts, similar to ta-siRNAs (McHale et al., 2013; Zhai et al., 2011). These small RNAs are referred as phased siRNAs, or phasiRNAs, because they are in 21 nt register from one another. In particular, 21 nt phasiRNAs have been shown to regulate canonical plant resistance (R) genes that contain the nucleotide binding leucine-rich repeat (NB-LRR) domains (Cuperus et al., 2010; McHale et al., 2013; Robert-Seilaniantz et al., 2011; Zhai et al., 2011). Therefore, phasiRNAs might be important regulators of plant defense.

1.2.3 Small RNA-mediated plant defense against pathogen infection

Virus induced small RNAs

Small RNA-mediated resistance is best-studied as the primary defense mechanism against RNA viruses in plants and animals (Li et al., 2013). Initially described as a post-transcriptional gene silencing (PTGS) mechanism, small RNAs were observed to accumulated in plants during viral infections (Hamilton and Baulcombe, 1999; Szittya et al., 2002). The elevated small RNA levels corresponded to reduced viral titers and transient immunity to subsequent infections by the same

virus (Li and Ding, 2001; Ratcliff et al., 1997; Szittya et al., 2002). It is now well documented that virus-infected host cells generate siRNAs from the double-stranded viral RNAs as precursors. These siRNAs then induce the cleavage of the viral RNA, thereby limiting viral infections. (Baulcombe, 2004; Chen, 2009; Ding and Voinnet, 2007). Recently, He et al., demonstrated *Brassica rapa* miR1885 was induced by Turnip mosaic virus (TMV) infection (He et al., 2008). This result suggests that both miRNA and siRNA play essential roles in anti-viral immunity in plants.

Bacteria induced small RNAs

miRNA expression changes have been shown to occur during bacterial, fungal and oomycete infection (Chen, 2009; Guo et al., 2011; Lu et al., 2007; Navarro, 2006; Ruiz-Ferrer and Voinnet, 2009). *Arabidopsis thaliana* infected by the bacterial pathogen *Pseudomonas syringae* exhibits increased expression of miRNAs that target phytohormone pathways including auxin, asbscisic acid and jasmonates (Fahlgren et al., 2007; Navarro, 2006; Zhang et al., 2011). In particular, the bacterial flagellin (flg22) induces the conserved miRNA miR393, which represses the expression of auxin receptor genes *TIR1*, *AFB2* and *AFB2* (Navarro et al., 2006; Parry et al., 2009; Si-Ammour et al., 2011). Over-expression of miR393 in *Arabidopsis* leads to enhanced resistance to bacteria infection (Navarro et al., 2006), suggesting that miR393 is a positive regulator of plant basal defense. The endogenous siRNA, nat-siRNAATGB2, is derived from a pair of natural antisense transcripts within a Rab2-like GTP-binding protein (ATGB2) gene and a Pentaticopeptide repeat protein-like (PPRL) gene. Nat-siRNAATGB2 is specifically induced in *Arabidopsis* by the type III effector AvrRpt2 of *P. syringae* and regulates AvrRpt2-triggered hypersensitive

response (HR) (Katiyar-Agarwal et al., 2006). Taken together, it is evident that small RNAs have important roles in the regulation of plant defense against bacterial pathogens.

Small RNAs induced by fungal pathogens

Small RNA-mediated gene regulation is also employed in plants during infection by eukaryotic pathogens such as fungi. *Arabidopsis Suppressor of Gene Silencing* (*sgs*) mutants have a defect in the RNA silencing machinery, and exhibit susceptibility to *Verticillium* infection (Ellendorff et al., 2009), suggesting that certain endogenous small RNAs play positive roles in plant defense. The destructive fungal pathogen, *Cronartium quercuum*, induced changes in miRNAs of loblolly pine (*Pinus taeda*). 10 miRNA families that potentially target transcription factors, auxin signaling factors and resistance genes were found to be suppressed in the galled stems induced by the pathogen (Lu et al., 2007). In addition, levels of the target transcripts of these repressed miRNAs were increased in the tissue above the galls, suggesting the miRNA changes in the infected region may stimulate immunization of the surrounding tissues (Lu et al., 2007).

Small RNA changes induced by Phytophthora

Infection by *Phytopthora* has also been reported to induce small RNA changes in the host. miRNA microarray analyses in soybean infected with *P. sojae* revealed 42 miRNAs that showed differentially accumulation (Guo et al., 2011). Of particular interest, miRNAs regulating resistant genes, such as miR482, miR1507, miR1508 and miR1510, were shown to be differentially expressed in the infected tissue (Guo,

2011). Furthermore, a recent study on miR482 in *Solanaceae* plants suggests that miR482 regulates R genes that confer resistance to *P. infestans* (Li et al., 2012). However, genome-wide analyses of small RNA profiling in plants during *Phytophthor*a infection has not been conducted.

1.2.4 Pathogen effectors target host small RNA biogenesis

Effectors are essential virulence proteins

Microbial pathogens have numerous mechanisms to facilitate infection. One important strategy is the secretion of virulence proteins, called effectors, that suppresses host defense. Many effectors can enter the host cells and directly target their specific substrates in order to modulate host response. The arms race between plants and pathogens have driven plants to evolve resistance (R) genes that are able to directly or indirectly recognize specific effectors and trigger a rapid and localized programmed cell death, also known as a hypersensitive response (HR) (Greenberg, 1997; Jones and Dangl, 2006). The effectors that can trigger HR are designated avirulence factors (Avr). Thus far, the molecular basis of the virulence function as well as the HR-triggering activity of a majority of these cytoplasmic effectors *in planta* remains unknown.

Because of their essential role in disease development, extensive research has been conducted to identify effector genes and to understand the molecular mechanisms underlying their functionality. The best studied effectors are the type III effectors (T3E) that are secreted through the specialized protein secretion apparatus type III secretion system (T3SS) of Gram negative bacteria. Numerous studies have

revealed that the fundamental function of T3Es is to weaken host resistance by targeting a variety of host processes including the defense signaling pathway, defense hormone production and signaling, and antimicrobial compound production and secretion (Alfano and Collmer, 2004; Galán and Wolf-Watz, 2006).

An extraordinarily large repertoire of effectors has been identified in *Phytophthora*, compared to bacterial pathogens, which typically produce 20-40 T3Es. Genome sequence analyses of *Phytophthora spp*. suggests that these eukaryotic pathogens produce hundreds of effectors. Sequence analyses of *Phytophthora* effectors revealed two motifs at the N-termini - a Sec secretion signal, which allows them to be secreted from the haustoria to the extra-haustorial space, and a host-targeting motif, which directs the translocation of the secreted effectors into host cells. Similar to bacterial T3Es, *Phytophthora* effectors are also believed to mainly function to suppress plant defense (Bos et al., 2010; Jiang et al., 2008; Kelley et al., 2010; Tyler, 2006; Win et al., 2012).

Effectors modulate the small RNA pathways in plants

Because small RNAs regulate plant defense, it is not surprising that pathogens have evolved effectors to disrupt host RNA silencing machinery in order to facilitate infection. Indeed, many plant viruses, especially RNA viruses, encode proteins that suppress small RNA-mediated antiviral defenses. These Viral Suppressors of RNA silencing (VSRs) play important virulence functions that allow successful viral infection (Diaz-Pendon et al., 2007; Voinnet, 2005). Bacterial pathogens also produce T3Es, called Bacterial Suppressors of RNA silencing (BSRs), which manipulate plant

small RNA pathways during infection. Three *P. syringae* effectors, AvrPto, AvrPtoB and HopT1-1, can decrease the abundance of miR393, which is involved in basal defense in *Arabidopsis*. HopT1-1 is thought to manipulate the miRNA pathway by directly targeting AGO1 and suppressing its slicing and miRNA-directed translational inhibition activities (Navarro et al., 2008).

Although it is unclear whether small RNAs are involved in defense response during *Phytophthora* infection, recent findings suggest that this might be the case because two *Phytophthora* effectors have recently been shown to possess RNA silencing suppression activity (Qiao et al., 2013). These *Phytophthora* Suppressors of RNA silencing (PSRs) repress small RNA biogenesis in plant hosts and significantly enhance *Phytophthora* infection. This finding indicates that small RNAs are integral regulators in plant resistance during *Phytophthora* infection (Qiao et al., 2013). In this chapter, I performed experiments to identify specific small RNAs, especially miRNAs, in soybean roots that may regulate defense-associated genes during the infection of *P. sojae*.

1.3. MATERIALS AND METHODS

1.3.1 Plant growth and Phytophthora sojae culture

Soybean cultivars Harosoy and Williams 82 were used as susceptible and resistant hosts, respectively. Williams 82 contains the R gene *Rsps1k* (Gao et al., 2005) and is therefore resistant to *P. sojae* isolate P6497, which produces the effector Avr1k (race2). Harosoy is susceptible to *P. sojae* P6497 since it lacks the *Rsps1k* gene.

Soybean seeds were sterilized in 10% bleach solution and transferred to sterile Petri dishes lined with filter paper. Seeds were kept moist by a daily addition of 1 mL sterile distilled water and germinated in the dark for one week. Germinated seeds were transferred to sterile pouches infused with B&D nutrient solution (1000 μM $CaCl_2 \bullet 2H_2O$, 500 μM KH_2PO_4, 10 μM Fe-Citrate, 250 μM $MgSO_4 \bullet 7H_2O$, 1500 μM K_2SO_4, 1 μM $MnSO_4 \bullet H_2O$, 2 μM H_3BO_4, 0.5 μM $ZnSO_4 \bullet 7H_2O$, 0.2 μM $CuSO_4 \bullet 5H_2O$, 0.1 μM $CoSO_4 \bullet 7H_2O$, 0.1 μM $Na_2MoO_4 \bullet 2H_2O$), and grown in a growth chamber (28ºC at a 12-hour light cycle) for about two weeks until the primary roots were approximately two inches in length (Subramanian et al., 2008).

P. sojae P6497 is cultured at room temperature in the dark on V8 media (Erwin and Ribeiro, 1996). Soybean roots were infected with *P. sojae* by placing mycelial plugs on top of the roots as previously described (Zhou et al., 2009). Inoculated roots were placed in the dark for eight hours before the infected tissues were collected for RNA extraction. Mock-treated roots were treated in the same way, but with sterile agar plugs.

1.3.2 RNA extraction and Small RNA library construction

Total RNAs were extracted, as described by (MacRae, 2007; Simms et al., 1993), from the *P. sojae*-infected and mock-inoculated root tissues at 8 and 24 hours post infection (hpi). 100 milligrams of root tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen. The ground tissue was transferred to a conical tube and mixed with 1mL of TRIzol Reagent (Life Technologies). 200 μL of chloroform was added to the TRIzol mixture, vortexed, and incubated at room temperature for 5 minutes. The samples were centrifuged at 10,000 rpm (12,000 *g*) for 15 minutes at 4°C. The aqueous phase was transferred to a sterile conical tube and mixed with 500 μL isopropyl alcohol to precipitate the RNA. The samples were incubated at room temperature for 10 minutes and centrifuged at 10,000 rpm (12,000 G) for 10 minutes at 4°C. The RNA precipitate was washed with 2 mL of 75% ethanol and allowed to air dry for 5 to 10 minutes. RNA pellet was dissolved in diethyl-pyrocarbonate (DEPC)-treated water (autoclaved 0.1% DEPC water). RNA quality and concentration was determined by A260 measurement with the Nanodrop 1000 spectrophotometer (Thermo Scientific).

Small RNAs were fractionated by electrophoresis with a 6% TBE precast gel (Invitrogen). 5 μg of total RNAs was loaded into the gel submerged in 1X TBE Running Buffer and resolved at 200 V for 1 hour. The gel was stained with 0.01% ethidium bromide and small RNAs in the range of 18-30 nt were recovered from the gel. Total small RNAs were isolated with a RNA purification kit (Life Technologies) and subjected to library construction with the Illumina TruSeq Small RNA Sample Prep Kit for high throughput sequencing.

1.3.3 Small RNA data processing

Small RNA sequences obtained from Illumina sequencing were computationally processed through a small RNA data analysis pipeline as described by (Barrera-Figueroa et al., 2011). Raw small RNA sequences were sorted into individual files according to the 5' barcode. Adaptor sequences were trimmed and the small RNA sequences were filtered for quality scores and a size range between 18 nt and 25 nt. Sequences that match known plant rRNAs, tRNAs, snRNAs, and snoRNAs were removed. The remaining sequences were mapped to the soybean genome (DOE-JGI Glycine max gene index Ver.8) using the Short Oligonucleotide Analyses Package (SOAP) alignment software (Li et al., 2008). Reads that perfectly aligned to the genome were retained and further analyzed for miRNA and phasiRNA identification.

1.3.4 Identification of miRNAs

Known miRNAs were identified by aligning to annotated miRNAs database (www.mirbase.org Release 19) (Griffiths-Jones et al., 2008) using FASTA (version 3.6) (Pearson, 1999). miRNAs with less than two nucleotide differences were classified to the same family. Potential miRNA sequences that did no match to a known miRNA family were further analyzed as novel miRNAs.

Potential novel miRNAs identified from the small RNA libraries were submitted for secondary structure analyses. DNA segments of 100-300bp covering the novel miRNA sequence were taken for secondary structure prediction using UNAFold (Markham and Zuker, 2008). Candidates were considered genuine miRNAs when

they met the following criteria: (1) the free energy of the predicted secondary RNA structure is no more than -35 kcal/mol; (2) the number of mismatches between the putative miRNA and miRNA* is not greater than 4; (3) the number of asymmetrical bulges in the stem region is not greater than 1 and the size of each asymmetrical bulge is less than 2; (4) strand bias of putative miRNAs requires that small RNA reads map to the positive strand of the hairpin DNA segment account for at least 80% of all mapped reads; (5) putative miRNA sequences that map to the miRNA and miRNA* loci account for at least 75% of the reads within the loci (Meyers et al., 2008; Thakur et al., 2011).

1.3.5 Identification of phasiRNAs

PhasiRNA analyses were performed as described previously (De Paoli et al., 2009). We identified 21 nt in-phase signatures from the small RNA sequencing data. The numbers of signatures were counted within 210 bp (10 phases) through the University of Delaware Legume Next-Gen Sequence DataBases soybean sRNA database (http://mpss.udel.edu/soy_sbs/). Genomic regions with phasing scores greater than 15 were considered *PHAS* loci. These regions were then mapped to the soybean genome sequence to determine the identities of the phasiRNA-generating loci.

1.3.6 Northern blot analyses

Approximately 5 to 10 μg of total RNAs were extracted from *P. sojae*-infected and mock-inoculated soybean roots using a TRIzol based extraction method (de Fátima Rosas-Cárdenas et al., 2011; MacRae, 2007; Simms et al., 1993). RNAs were

separated using polyacrylamide gel electrophoresis (PAGE) (6.3 g urea, 1.5 mL 5X TBE, 5.63 mL 40% acrylamide/bis-acrylamide (29:1), 120 μL 10% ammonium persulfate, 9 μL TEMED) at 150 V for 1.5 hours in 0.5X TBE buffer. RNA was then transferred to a nylon membrane (GE Healthcare Life Sciences Amersham Hybond-NX) in a semi-dry transfer system (Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell) at 300 mA per membrane for 1 hour. Transferred RNA were chemically crossed linked onto the membrane with EDC solution (122.5 μL 12.5 M 1-methylimidazole, 0.373 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, pH to 8.0 with 1 N HCl, bring to a final volume of 12 mL) at 60°C for 1.5 hours. The membrane was washed three times with distilled water and dried at 80°C or 30 minutes.

Oligonucleotides complementary to specific miRNA sequences were synthesized and end-labelled with γ-^{32}P ATP (0.5 μL 100 μM oligo probe, 2.5 μL 10X PNK Buffer, 2.5 μL T4 Polynucleotide Kinase, 2.5 μL γ-^{32}P ATP, 25 μL final volume). The labelled oligonucleotides were used as probes for hybridization. Membranes were incubated with the hybridization buffer (5X SSC, 20 mM Na_2HPO4, 7% Sodium dodecyl sulfate, 2X Denhardt's Solution) at 65°C for 2 hour. Probes were denatured at 85°C for 5 minutes and added directly into the hybridization buffer. The members were incubated with probes overnight at 50°C.

Following hybridization, the membrane was rinsed with wash buffer (2X SSC, 0.1% SDS), wrapped in saran wrap, placed into a phosphor screen cassette system and exposed overnight. Phosphor screens were scanned with the Molecular Dynamics Typhoon imager and images processed with the ImageQuant TL software (GE Healthcare Life Sciences).

1.3.7 Quantitative RT-PCR of miRNA target transcripts

Target prediction of soybean small RNAs was performed utilizing the online plant small RNA analyses server: PsRNATarget (http://plantgrn.noble.org/psRNATarget). Submitted small RNA sequences were scored against the soybean genome with at an expectation cut-off threshold of 3.0. The complementary length range was set to 20 bp and the allowed energy to un-pair the target site set to 25 UPE. Up to ten predicted target transcripts were selected for transcript detection using semi-quantitative RT-PCR. Working primers were used for quantitative RT-PCR.

Quantitative RT-PCR (qRT-PCR) was performed using iQ™ SYBR® Green Supermix and a CFX96 Real-Time PCR Detection System (BioRad). DNA was removed from 5 µg of total RNA with DNase I (Fermentas). DNA free RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega) at 48ºC for 2 hours.

Gene-specific primers (Table 1.1) were designed to flank the predicted miRNA-binding site of the target genes. The abundance of the miRNA target transcripts was determined by qRT-PCR using a real time PCR machine (BioRad CFX-96). *GmUBI* was used as the internal control.

Table 1.1 Primers designed for qRT-PCR of microRNAs predicted targets.

microRNAs	Genomic ID	Forward Primers	Reverse Primers
Gma-miR166	Glyma05g30000	AGTGGTCAACACCAACCGCA	ATTGTGCCCCCATTCCCTGC
	Glyma05g06070	CCATTACCACCTCCTCAAACTC	TACCTCCTCTGCCGGATAAT
	Glyma07g01950	GTGACGAGTGGTCAGCAGCAC	ATGCTGCACAGGAGGCATGC
	Glyma08g13110	TGGTAGTGAGTGGTCAACGCCA	ATCGTGCCCCCATTCCCTGT
	Glyma08g21620	AGCTGTGAGGAGTGGTCAGCA	TGGTTCCACCATTTGCTGTGGG
	Glyma11g20520	GCTTCAGACCGTGAACCGCA	AGGCTCCGACAGTCACGGAA
Gma-miR168	Glyma09g29720	ACTGCTTCTGCAGGTACTACCACA	GCTCGAATGGTGCCCCCATTC
	Glyma16g34300	CACATCCTGGAGAGGATTCAAG	GGCACAAACTAAACCAGCATAC
Gma-miR319	Glyma08g10350	CGGATCCACTGTACCTTTCTTC	GAACTGATGCTGGTGATGGT
	Glyma13g29160	GATCGTCCAACCTACCTTTCTT	GGTGATGGTTGTTGCTGTTATT
	Glyma13g34690	GGTTATGGTCAAGGCCAGTAT	CAGATAGTGATGATGCCGATGA
	Glyma15g09910	GGGCCTTGCTGGTTACAATA	TGGAGGATCCGTCGACTAAA
Gma-miR393	Glyma02g17170	TTCCCTTTCTGGGCTTCTAAC	ATGGAGTCCCAAATCGCTATC
	Glyma10g02630	GATCCCTTTGGATGTCCTCTTG	TACTCTCCGGCCTTGAATCT
	Glyma16g05500	ACGGACGTAGGGAAGTATGA	ACATTCAACCTCGGCATCTT
	Glyma19g39420	GCCTATCCCTATCTGGTCTTCT	CCCAAATCACTATCTCCAGCAA
Gma-miR482	Glyma12g28730	CAAACACCCGCCTTCCTAAT	CGCGCATGCTCTGGTATAA
	Glyma12g36510	CACTGCAATCTGTGAGGAAGA	GGTGTCAGCAACGTCTCTATT
Gma-miR2109	Glyma03g14900	CAGGCATGCCATCTCCTATT	TGAGGACCTTCCAAAGCTTCGAAGT
	Glyma16g10080	GGTCAGGGACATTCAGTACATT	TGCCTGTGGATAGTGATTGCTGCA
	Glyma16g34000	CTGTGCATCCTTGACTTCCT	TCAGGAACTGTATGAGGCTGGAGGA
Gma-miR1507	Glyma04g29220	GTTGGATTCCCTGCGAGATAA	CTCATCATGCCACCACCATAA
	Glyma06g39720	CTCACCAATTTGCGTCGCCTTGAA	CAAATCCACTGCCAATGCATCCGA
	Glyma06g47650	TGAGACGCTCTCGAGCAAGTTGAA	ATCAAGCACAGCAACCTTGACAGC
	Glyma13g25950	TAGTGGTGTTGGCGTTGGATCAGA	ATCCCACCCATGCCCACAATAGAA
	Glyma15g37290	GCATTAGAATGCGTTGGTGGTGCT	AGCTTGGATGGAGAGGAGCTTGTT
Gma-miR3522	Glyma12g01290	GGTGCTCCTACTCCACCCCAG	GAACAGGGGGTGTAGGCTGGT
	Glyma16g05240	CTTCCCAAGGCATAGTCCCTGGG	GCCAGAAGAGCATGCACCACGG
Gm13_14875340	Glyma05g21680	TCCTCCGAGTGCTTCTTTGGGATT	ACCGGAGTACGTGGTCACAACAAT
	Glyma17g18040	GCTTATGGACTACGCTATCTCAC	TAGAGTCAAGCAACTCCGTTATG
Gm13_15666134	Glyma05g08230	TCACGCTTCCAAACAGCAACACAG	CCATCAAGAGGCAATGGCATGGTT
	Glyma08g24960	GCAAGCATTGCATACCATCTATT	CTCAGCTTCCATCTCAGTAACC
	Glyma15g10140	GCATGAAGGAGAGTGCTTAGAT	GGGCATGAGGATCTCTTCTAAC

1.4 RESULTS

1.4.1 Small RNA profiling in P. sojae-infected soybean roots

Soybean roots were inoculated with the *P. sojae* strain P6497 (race 2) in order to identify small RNAs that were differentially accumulated during *P. sojae* infection. We used two soybean cultivars Harosoy and Williams 82 as the susceptible and resistant host, respectively. Williams 82 carries the resistance gene *Rps1K*, which activates defense response upon the recognition of an effector Avr1K produced by *P. sojae* strain P6497 (Dorrance et al., 2004; Gao et al., 2005).

Roots of soybean seedlings were inoculated with *P. sojae* mycelial plugs as previously described (Zhou et al., 2009). Small RNA profiles were analyzed in the infected and mock-inoculated tissues at 8 hours post inoculation (hpi). The rationale in selecting 8 hpi for tissue collection is to examine small RNA changes at an early infection stage. Base on my microscopic analyses of the *P. sojae* infection process, I found *P. sojae* hyphae penetration into soybean root tissue to occur at approximately 4-8 hpi for Harosoy and Williams 82 (Figure 1.3). Oospore development usually occurs at approximately 16-24 hpi for the susceptible host Harosoy. Although Williams 82 is resistant to *P. sojae* P6497, a small number of oospores at the inoculation sites (i.e., no hyphae extension from the infection sites) could be observed at round 36 hpi.

In order to monitor small RNA changes at the early infection stages before oospore development and tissue necrosis, total RNAs were extracted from the infected root tissues at 8 hpi, when extensive hyphae penetration into the root cortex layer was

observed (Figure 1.3). Libraries were constructed using 18-30 nt small RNAs and then subjected to Illumina sequencing. Roots inoculated with clean agar plugs were used as mock-treated controls.

We obtained more than 10 million high quality reads from each library (Table 1.2). These reads were aligned to the published soybean genome (http://www.phytozome.net/soybean.php). Sequences that perfectly aligned to the genome were further filtered to remove ribosomal RNAs, transfer RNAs and small nucleolar RNAs. The remaining sequences were retained as potential small RNAs for miRNA and siRNA identification.

In general, 324 miRNAs belonging to 109 known miRNA families were identified using the miRBase Release 19 (www.mirbase.org; Griffiths-Jones et al., 2008). Sixty-nine potential novel miRNAs were also found using a recently developed bioinformatic pipeline (Barrera-Figueroa et al., 2011). These miRNAs range from 18 to 24 nt in length; and as expected for miRNAs, they have a clear size bias of 21 nt (Figure 1.4). The number of miRNA species in the mock-treated susceptible cultivar Harosoy appeared to be smaller than the number of miRNAs in the *P. sojae*-infected tissues; however, I did not observe significant changes in the number of miRNA species produced by the resistant cultivar Williams 82 between the mock-treated and the *P. sojae*-infected samples (Figure 1.4).

Figure 1.3 Microscopic analyses of *P. sojae* infection in soybean roots.

The susceptible soybean cultivar Harosoy was inoculated with agar plugs with growing *P. sojae* hyphae. A GFP-expressing *P. sojae* strain P6497 was used for infection. The inoculated roots were examined at 0, 8 and 24 hpi. The images were taken using 10X magnification at 0 hpi, and 40X magnification at 8 and 24 hpi. At 0 hpi, bar equals 0.2mm. 8 hpi, 24hpi, bar equals 0.05mm.

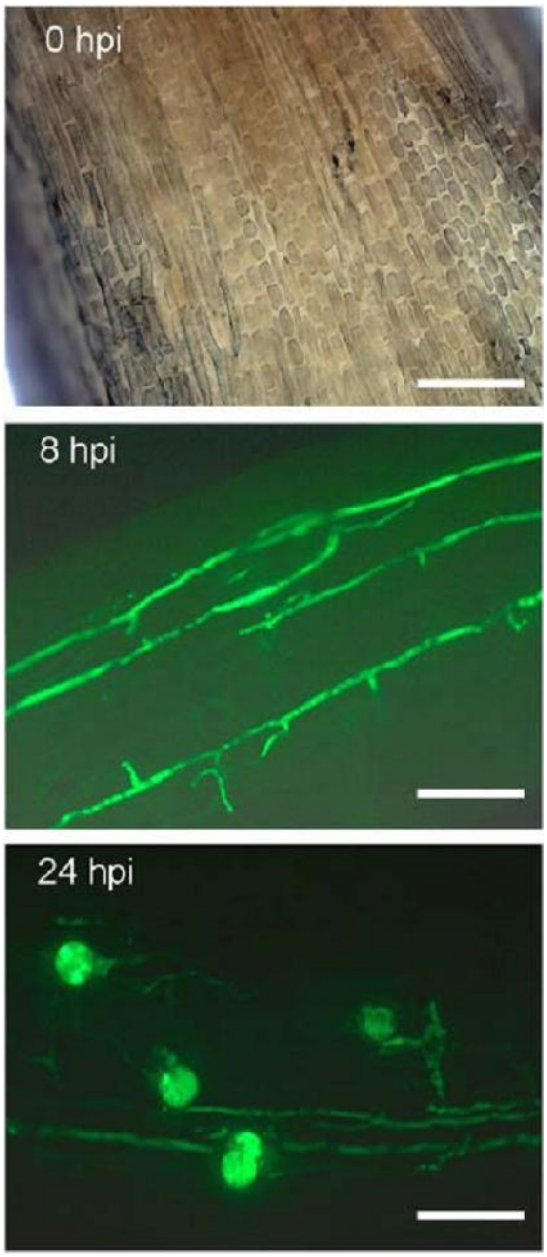
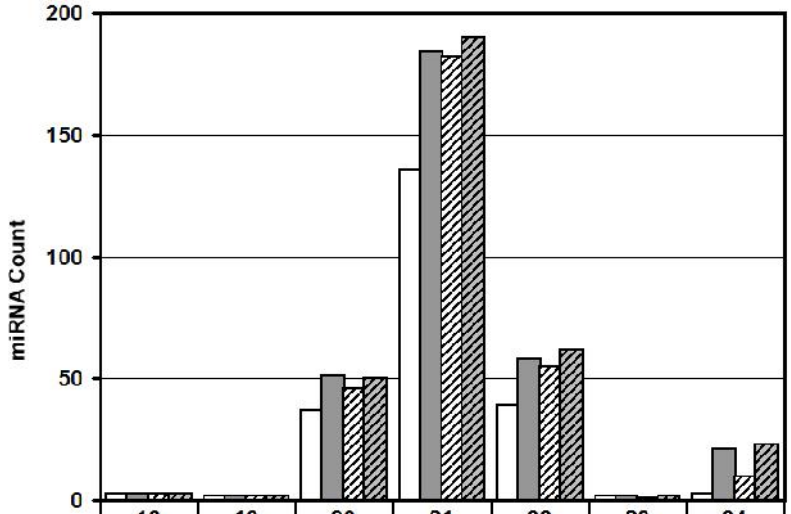

0 hpi
8 hpi
24 hpi

Table 1.2 Small RNA reads obtained from each small RNA library.

Libraries	Total sequences	Reads matched to soybean genome	Reads matching t/r/sn/snoRNA	Small RNA reads
8 hpi Harosoy Mock-treated	11,091,200	4,648,086	3,483,481	1,164,605
8 hpi Harosoy *P. sojae*-infected	13,145,492	8,705,618	5,636,119	3,069,499
8 hpi Williams82 Mock-treated	11,556,525	5,192,259	3,537,199	1,655,060
8 hpi Williams82 *P. sojae*-infected	10,882,558	8,432,412	5,009,415	3,422,997

Figure 1.4 miRNA profiling in soybean roots infected with *P. sojae*.

The susceptible cultivar Harosoy and the resistant cultivar Williams 82 were inoculated with *P. sojae* strain P6497. Small RNAs were extracted from the infected tissues at eight hours post inoculation and analyzed by Illumina sequencing. Numbers of conserved and novel soybean miRNAs in soybean roots and their size distributions are presented.



miRNA Length (nt)	18	19	20	21	22	23	24
Harosoy Mock-treated	3	2	37	136	39	2	3
Harosoy P.sojae Infected	3	2	51	184	58	2	21
Williams82 Mock-treated	3	2	46	182	55	1	10
Williams82 P.sojae Infected	3	2	50	190	62	2	23

1.4.2 Identification of miRNAs differentially expressed during exposure to P. sojae

Relative abundances of miRNAs were determined by comparing the normalized number of reads for a specific miRNA in the *P. sojae*-infected tissues to mock-treated samples. miRNA reads in each sample were normalized using the total number of small RNA reads in the particular library.

Overall, I observed extensive changes in the abundance of specific small RNAs in soybean roots in response to *P. sojae* infection, indicating that significant physiological changes induced by the pathogen are occurring shortly after pathogen exposure. Furthermore, *P. sojae*-induced small RNA changes are similar in the susceptible Harosoy and the resistant Williams 82 (Table 1.2). This is somehow expected because these changes in the early infection stage may reflect basal resistance that is conserved in both susceptible and resistant hosts.

Known soybean miRNAs that are altered upon P. sojae infection

Following bioinformatic analyses of the small RNA libraries, I found 14 known miRNAs and ten novel miRNAs that showed altered accumulations in one or both cultivars (Table 1.3). Northern blotting using samples from independent experiments confirmed the enhanced accumulation of miR393, miR166, miR1507, miR2109, and miR3522 in both cultivars (Figure 1.5, Table 1.3). These changes reflected the same trends as indicated by the sequencing analyses. In contrast, miR168, miR319 and miR482 were confirmed to be repressed during *P. sojae* infection (Figure 1.6, Table 1.3), which was contrary to the small RNA sequencing data. The induction of miR166 and repression of miR482 in *P. sojae*-infected soybean

were also observed in a microarray analyses reported previously (Guo et al., 2011). Several miRNAs that showed differential expressions based on the small RNA sequencing analyses, such as miR164, miR167, miR1508, miR15010 and miR1513, did not exhibit significant differences that could be consistently observed in northern blot or produce detectable signals in the blots (Figure 1.7). Northern blots were performed with independent samples and the relative miRNA levels are presented by average ± standard deviation (Table 1.4).

Novel soybean miRNAs that are altered upon P. sojae infection

In addition to known miRNAs, 69 potential novel miRNAs were identified from small RNA sequencing based on the established criteria (Meyers et al., 2008) (Table 1.5). All of these potential novel miRNAs were predicted to form stem-loop structures from their *MIR* transcripts (Figure 1.8).

Among them, ten novel miRNAs with relatively higher abundances (greater than 100 transcripts per million) showed altered accumulation in the *P. sojae*-infected roots in the sequence analyses (Table 1.6). Northern blotting confirmed the induction of Gma13_15666134 and the repression of Gma13_14875340 during *P. sojae* infection (Figure 1.9). Northern blotting failed to validate the abundance changes in the majority of potential novel miRNAs. This is likely due to low expression levels of these miRNAs.

Table 1.3 Conserved miRNAs that exhibit differential expression from small RNA sequencing analyses. Soybean miRNAs that exhibited larger than two fold changes in root tissues inoculated by *P. sojae* at 8 hours post inoculation. The abundance of specific miRNAs in each sample is represented by normalized reads (per million small RNA sequences) from Illumina sequencing.

Soybean miRNAs	**miRNA Sequence**	**Harosoy 8 hpi**		**Williams82 8 hpi**	
relative expression in TPM		Mock Inf.	*P.sojae* Inf.	Mock Inf.	*P.sojae* Inf.
miR160	TGCCTGGCTCCCTGTATGCCA	1.5	14.7	30.6	45.1
miR164	TGGAGAAGCAGGGCACGTGCA	3.0	18.3	9.8	25.4
miR166a/b/c	TCGGACCAGGCTTCATTCCCC	30.7	1233.7	4.6	134.2
miR166d	TCTCGGACCAGGCTTCATTCC	682.2	3204.0	890.9	1824.7
miR167	TGAAGCTGCCAGCATGATCTA	0.6	2.0	1.2	4.6
miR168	TCGCTTGGTGCAGGTCGGGAA	78.1	285.0	86.7	362.8
miR319	TTGGACTGAAGGGAGCTCCC	1055.9	2185.0	24.5	161.3
miR393	TCCAAAGGGATCGCATTGAT	0.2	5.5	0.2	0.9
miR482	TTCCCAATTCCGCCCATTCCTA	15.5	226.5	28.7	169.0
miR1507	TCTCATTCCATACATCGTCTGA	136.8	1368.7	353.4	2082.4
miR1508	TAGAAAGGGAAATAGCAGTTG	111.4	634.8	285.0	718.1
miR1510	AGGGATAGGTAAAACAATGAC	0.0	0.3	30.6	140.8
miR1513	TGAGAGAAAGCCATGACTTAC	28.0	156.5	12.9	52.3
miR2109	TGCGAGTGTCTTCGCCTCTGA	99.4	395.6	135.4	349.2
miR3522	TGAGACCAAATGAGCAGCTGA	14.4	44.1	20.0	39.4

Table 1.4 Summary of results from northern blots, which were used to confirm the differential accumulations of miRNAs in soybean roots from independent biological replicates. The abundances of miRNAs were quantified by determining relative signal densities in *P. sojae*-infected samples on northern blots in comparison to the mock-treated samples. “n” is the number of independent experiments that were performed for individual miRNAs. Relative miRNA levels are presented by average ± standard deviation. Statistically significant results are labeled with *.

Known soybean miRNA accumulation in *P. sojae*-infected samples at 8 hpi

Harosoy	**miR160**	1.1 ±0.1	n=3	**miR168**	0.6 ±0.2*	n=4	**miR393**	1.8 ±0.3*	n=14
Williams 82		1.0 ±0.2	n=3		0.7 ±0.2*	n=4		1.6 ±0.2*	n=14
Harosoy	**miR164**	0.8 ±0.2	n=3	**miR482**	0.8 ±0.2*	n=4	**miR166**	2.0 ±0.4*	n=4
Williams 82		1.0 ±0.3	n=3		0.6 ±0.1*	n=4		1.7 ±0.3*	n=4
Harosoy	**miR167**	0.8 ±0.3	n=3	**miR319**	0.8 ±0.2*	n=3	**miR1507**	1.4 ±0.3*	n=4
Williams		0.9 ±0.1	n=3		0.7 ±0.1*	n=3		1.5 ±0.2*	n=4
Harosoy	**miR1510**	1.0 ±0.2	n=3	**miR3522**	1.4 ±0.2*	n=4	**miR2109**	1.9 ±0.4*	n=8
Williams 82		0.9 ±0.2	n=3		1.5 ±0.2*	n=4		1.6 ±0.2*	n=8

Novel soybean miRNA accumulation in *P. sojae*-infected samples at 8 hpi

Harosoy	**Gm13_14875340**	0.5 ±0.1*	n=3
Williams 82		0.6 ±0.2*	n=3
Harosoy	**Gm13_15666134**	1.6 ±0.2*	n=3
Williams		1.7 ±0.2*	n=3

Figure 1.5 Specific miRNAs that were induced in soybean roots infected by *P. sojae*.

Northern blots showing miRNA abundances in (-) mock-treated and (+) *P. sojae*-infected tissues at 8 hpi. U6 served as a loading control in the blots. Numbers below each blot image represent abundance of the miRNA relative to the mock-treated sample.

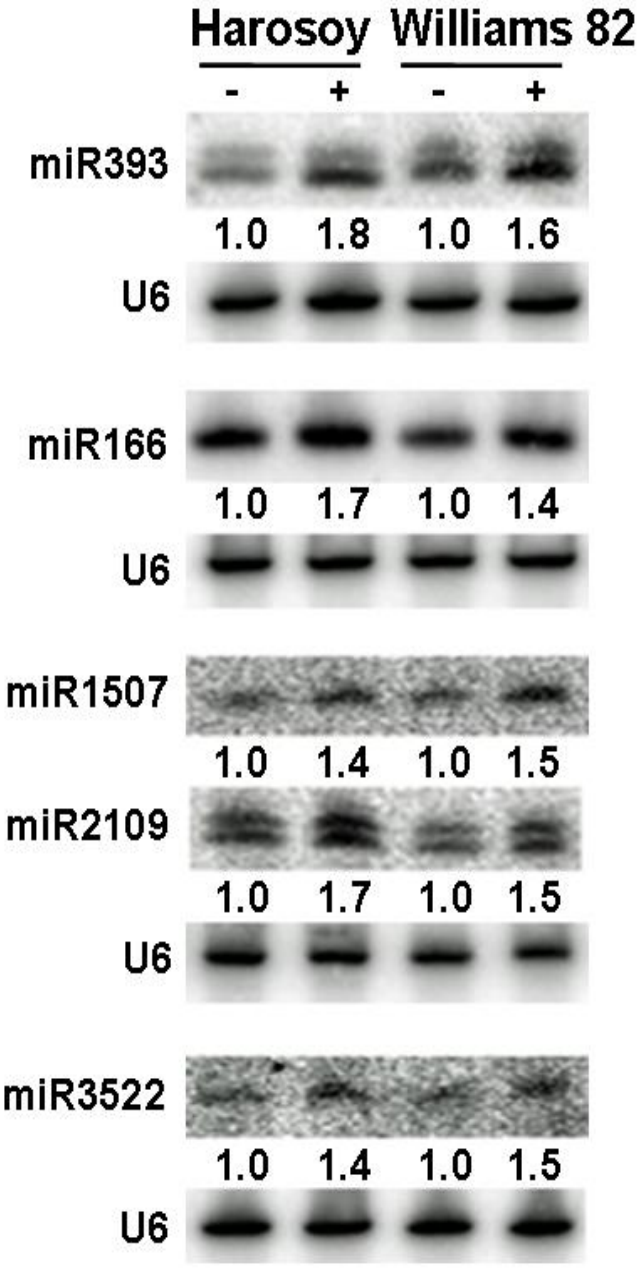

Harosoy
Williams 82
- + - +
miR393
1.0 1.8 1.0 1.6
U6
miR166
1.0 1.7 1.0 1.4
U6
miR1507
1.0 1.4 1.0 1.5
miR2109
1.0 1.7 1.0 1.5
U6
miR3522
1.0 1.4 1.0 1.5
U6

Figure 1.6 miRNAs with reduced abundance in roots infected by *P. sojae*.

Northern blots showing miR168, miR319 and miR482 decreased in abundances in the (+) *P. sojae*-infected roots when compared to the (-) mock-treated roots at 8 hpi. U6 served as a loading control in the blots. Numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample.

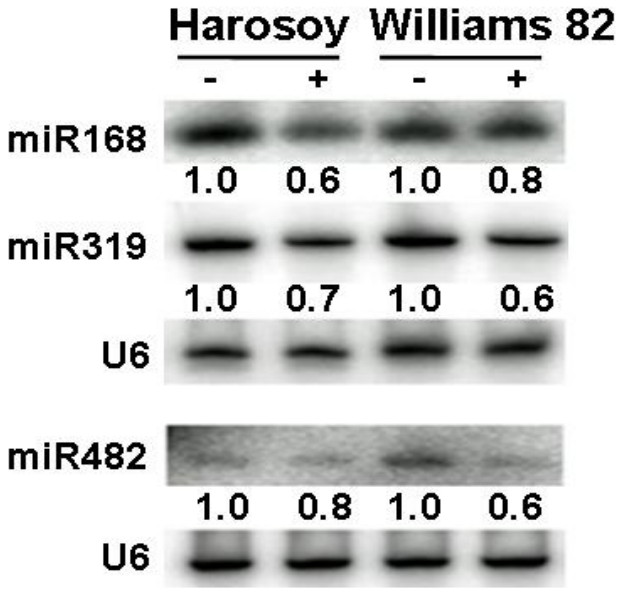

Harosoy
Williams 82
-
+
-
+
miR168
1.0
0.6
1.0
0.8
miR319
1.0
0.7
1.0
0.6
U6
miR482
1.0
0.8
1.0
0.6
U6

Figure 1.7 Soybean miRNAs that did not display abundance changes when infected by *P. sojae*.

Northern blots of miRNAs in (-) mock-treated and (+) *P. sojae*-infected tissues at 8 hpi. U6 served as a loading control in the blots. Numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample.

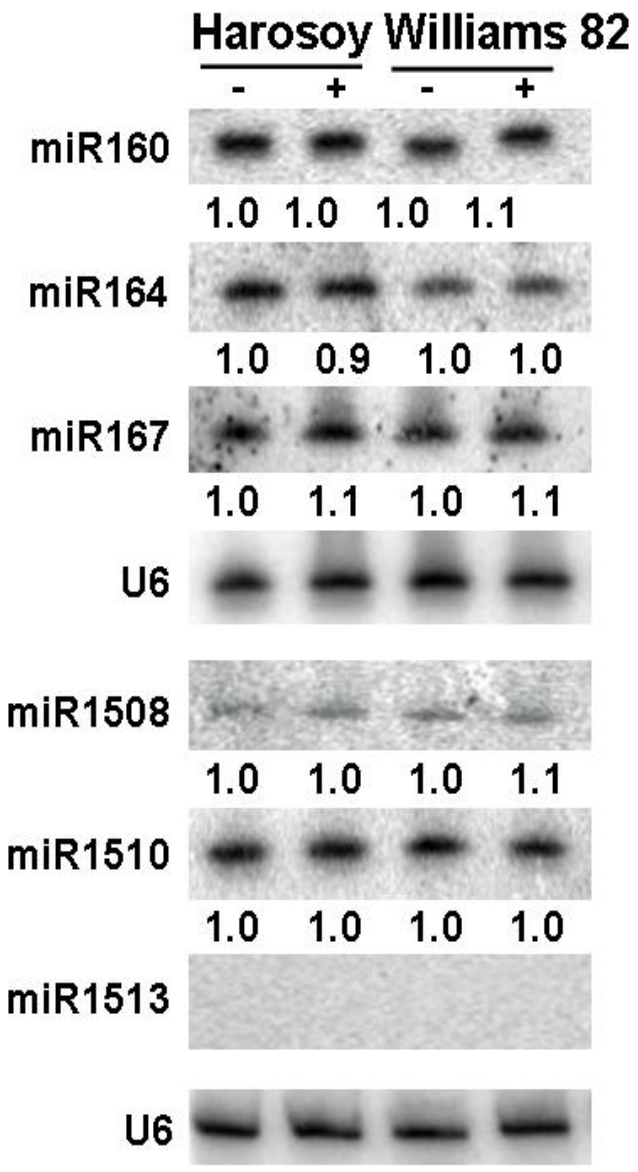

Harosoy Williams 82
- + - +
miR160
1.0 1.0 1.0 1.1
miR164
1.0 0.9 1.0 1.0
miR167
1.0 1.1 1.0 1.1
U6
miR1508
1.0 1.0 1.0 1.1
miR1510
1.0 1.0 1.0 1.0
miR1513
U6

Table 1.5 Novel miRNAs predicted from small RNA sequencing data.

69 putative novel miRNAs were identified in the sequencing data. The abundance of novel miRNAs in each sample is represented by normalized reads per million small RNA sequences.

miRNA ID	Mature miRNA	Length	Harosoy		Williams 82	
			Mock Infected	*P. sojae* Infected.	Mock Infected	*P. sojae* Infected
Gma20_32656628	TTGTGGCTGAAATCACTGTTGC	22	0.6	4.4	1	3.4
Gma19_49128397	TTAGGATGCACACTTTTTGAGGA	23	0.4	4.1	0.2	3
Gma19_1921586	GTCATCTTTCCTCGGCTGAAG	21	3.4	9.1	0.1	2
Gma19_1831364	TTGACGGGAGATTTCTGA	18	59.2	99.1	47	29.6
Gma18_50625462	TTTAGGACTATGGTTTGGACG	21	0	1.3	0.8	2.7
Gma18_458726	GTTGAGATGGCCGAGTTGGT	20	2.8	13	5.4	9.3
Gma18_42254410	TATAGGACCGATGTAGAATGTGTT	24	0	0	0.4	5.5
Gma18_21161222	GTGGTATCAGGTCCTGCTTCA	21	0.2	14.2	3.3	13.8
Gma18_10413938	TGGGGCTTGATCCAAGATAGG	21	0	0.7	0.2	2.1
Gma17_38887592	GTCCGTCGTGAAAAGGGA	18	1.7	2.5	0.2	1.5
Gma17_23985091	GCTAGTCTGCGCTCTTTGGA	20	0.9	3.6	0	1.5
Gma17_23949165	CGTTGGGAGCCAACTTGGA	19	2.2	0.1	0.4	0.1
Gma17_14170501	TTAGCTTCTTTCACCTTTCCC	21	0.2	3.2	2.3	7.7
Gma17_14017387	CGTGACTGTCATAGAATATATTGT	24	0	0.2	0.4	0.9
Gma16_7236789	GGCCGAAGATGAAGAGCT	18	405.3	282	164.1	135.8
Gma16_316395	CCGAATTTGGATGTTGACTGTT	22	0	1.3	0.2	0.7
Gma16_31518897	TGGAGGGATAGGTAAAACAATG	22	0	0.3	30.6	140.8
Gma16_31234017	TGTTGTAAGCACATCTGAGTC	21	0	1.6	0.8	1.3
Gma16_2342983	TGCGAGAGGCACGGGGTT	18	5.4	2.5	0.2	0
Gma15_17904655	CTAACGACAGGTAGTAAATTGA	22	0	0.7	0.6	1.9

miRNA ID	Mature miRNA	Length	Harosoy		Williams 82	
			Mock Infected	*P. sojae* Infected.	Mock Infected	*P. sojae* Infected
Gma15_14150075	TGCCGGCAAGTTTCTCTTGGC	21	0.4	2.2	0.8	3.1
Gma14_6664512	ACTACTATCATGATTGTCATC	21	0.2	1.3	0	0.4
Gma14_6304165	TTCGTCGCTCAGATTAACTTCATC	24	0.2	2.6	0.6	1.9
Gma14_31319908	TTCGTCCTTAAAGTGCTTTAG	21	0.2	0.6	2.5	6.8
Gma14_13971426	ATTCAAGATAGCTGTGGAAAA	21	45.2	68	3.9	12.6
Gma13_8342483	GTTTGACTCTTGTTGGGCTTA	21	0	0.8	0.2	0.6
Gma13_26271122	CGATGTTGGTGAGGTTCAATC	21	0.2	3.1	1.2	3.2
Gma13_15650892	GGAGTCTGACATGTGTGCG	19	828.1	209.4	2008.8	796
Gma13_15673743	GAGTCTGACATGTGTGCG	18	11246.6	12419.6	5834.5	4612.6
Gma13_14875340	CGTCATCATCGCGATTGTGGA	21	767.2	981.9	934.9	568.6
Gma12_6421257	GTTGCTGTGGTGTAGTGG	18	6.2	10.2	60.7	140.9
Gma12_36220615	AGAGGCGCAGATTGAAAT	18	4.3	1.5	6	3.4
Gma12_3536333	TTTCGTCCTTGTGATACTTTAGAT	24	0	0.2	0.4	2.4
Gma12_1600588	AGAGCGTTGTGCTAATAAGCTGAA	24	0	0.9	0	0.7
Gma11_70334	TGTCTGAGATAAGGTTCCTGA	21	0.9	2.8	0.4	1.1
Gma11_33167344	TTCTTGATCCATGCTTCCAGT	21	0	0	0.2	2.1
Gma11_32789083	CATGGAAGTGAATCGGGTGAC	21	1.3	6.7	1.3	4.4
Gma10_2905312	GGAATGTCGTTTGGTTCGAGA	21	687.8	1534.1	762.9	1684
Gma09_38348101	CCCGTCTTAGTATAGTTCACT	21	0	0	6.7	14.1
Gma09_28264416	TGAGAATTTGGCCTCTGTCCA	21	0.9	8.3	2.7	8.1

miRNA ID	Mature miRNA	Length	Harosoy		Williams 82	
			Mock Infected	*P. sojae* Infected.	Mock Infected	*P. sojae* Infected
Gma09_16565938	GAGGTGTTTGGGATGAGAGAA	21	51.2	181	6.4	53.1
Gma09_1261002	GCATACAGGGAGTCAAGCAGA	21	11.2	52.4	2.1	5.3
Gma08_18770402	GGCGACATGGATGGGGGT	18	30.3	0.9	5.6	0.5
Gma08_11433011	TGCTCATTTTTAGTCCTGTAAGT	23	0	0.2	0.4	0.8
Gma07_40555385	CCGTTTGAGAAATAGCACTCA	21	0.2	2.6	0.8	1.8
Gma07_10003195	TTACCGATAAAGAGCTACGAG	21	0.2	0.8	1.3	1.8
Gma07_10001904	TTTATCAGTAGCATCATCATC	21	0	1.8	0.8	2.4
Gma06_50369106	TTTGAAAGGCTGATTTGATAG	21	0.2	1.8	0.4	0.7
Gma05_9279508	TTATAGTCTGACATCTGGAAT	21	0.2	11.1	8.5	27.9
Gma05_7842998	ACTCGTTGTCACTTTAGTCCT	21	0	1.6	0.2	0.9
Gma04_869294	TTAAAGTGCTTCACTTTGTGG	21	0	0.6	0.6	1.3
Gma04_35428793	CATCGTTGACGCTGACTGTACG	22	16.8	119	25.6	88.2
Gma04_28578961	GGCGTAGATCCCCACAACAGT	21	0	1.3	0.6	1.2
Gma03_45714412	AAATGATCGGATTTTGTTTTGGGT	24	0.2	2.1	0.2	1.5
Gma04_5435452	TGAATGGTGAGTATGAGGAGT	21	0.4	1.1	0.2	0.4
Gma03_38272198	AAATGAATGGTGAGGATGAAGAGT	24	0.2	3.3	0	0.2
Gma03_30966758	TCATAATTTGTGGTAGAGG	19	0	0.1	3.3	0.8
Gma03_25186870	TGTTAGTGATAAGGCGTGATG	21	0.4	1.7	0.8	1.7
Gma03_19646929	CATTGTCAGGTGGGGAGT	18	5.4	57.7	99.8	136.1
Gma02_8454921	CCAAAGTTGGGCTTAAGCTGTA	22	0	0.7	0.4	2.3

miRNA ID	Mature miRNA	Length	Harosoy		Williams 82	
			Mock Infected	*P. sojae* Infected.	Mock Infected	*P. sojae* Infected
Gma02_6599292	AGGGATAGGTAAAACAACTA	20	104.3	399.9	45.5	161.5
Gma02_48422521	TCTTGACTTTGGACTTTTGGGT	22	3.2	24	1.9	4.5
Gma02_46876641	AAGAATGACTTGCCGGAATGCATGC	25	0.2	0.6	0.4	1.7
Gma01_7195769	GGAGTGAATCTGAGAACACAAG	22	11	81.1	7.9	24.3
Gma02_10988445	GAGTGGATCTGAGAACACAAGG	22	1.7	11.6	11.7	38.3
Gma01_37228378	TGTGTGTGCTGTGTGTGC	18	1.2	0.4	7	1
Gma01_55781583	CAGGTGATTCGTAAAACTCAC	21	0.2	3.7	2.3	5.6
Gma01_50648993	AGAAGAAAAAAGATGAGATGAACT	24	0.4	2.2	0.4	1.2
Gma01_24948436	TGAAAAATTCATGGATCAGT	20	1.7	8.3	1	1.8

Table 1.6 Ten potential novel miRNAs exhibit differential accumulation in *P. sojae*-infected soybean roots.

Potential novel soybean miRNAs that exhibited fold changes in root tissues inoculated by *P. sojae* at 8 hours post inoculation are listed. The abundance of specific miRNAs in each sample is represented by normalized reads (per million small RNA sequences) from Illumina sequencing.

Novel Soybean miRNAs	**Sequence**	**Harosoy**		**Williams82**	
		Mock Inf.	***P.sojae* Inf.**	**Mock Inf.**	***P.sojae* Inf.**
Gma19_1831364	TCAGAAATCTCCCGTCAA	59.2	99.1	47	29.6
Gma16_7236789	AGCTCTTCATCTTCGGCC	405.3	282.0	164.1	135.8
Gma13_15666134	GGAGTCTGACATGTGTGCG	828.1	209.4	2008.8	796
Gma13_15673744	CGCACACATGTCAGACTC	11246.6	12419.6	5834.5	4612.6
Gma13_14875340	TCCACAATCGCGATGATGACG	767.2	981.9	934.9	568.6
Gma12_6421257	CCACTACACCACAGCAAC	6.2	10.2	60.7	140.9
Gma10_2905312	TCTCGAACCAAACGACATTCC	687.8	1534.1	762.9	1684
Gma05_9956321	GCCGACGGGTTCGGGACTGGG	187.6	174.7	63.2	87.6
Gma03_19646929	ACTCCCCACCTGACAATG	5.4	57.7	99.8	136.1
Gma02_6599292	TAGTTGTTTTACCTATCCCT	104.3	399.9	45.5	161.5

Figure 1.8 Secondary structure analyses of novel miRNAs.

Secondary structures were predicted for potential novel miRNA-generating transcripts using UNAfold. Representative secondary structures of four potential novel miRNAs are presented. Red lines label the positions of the miRNA sequences.

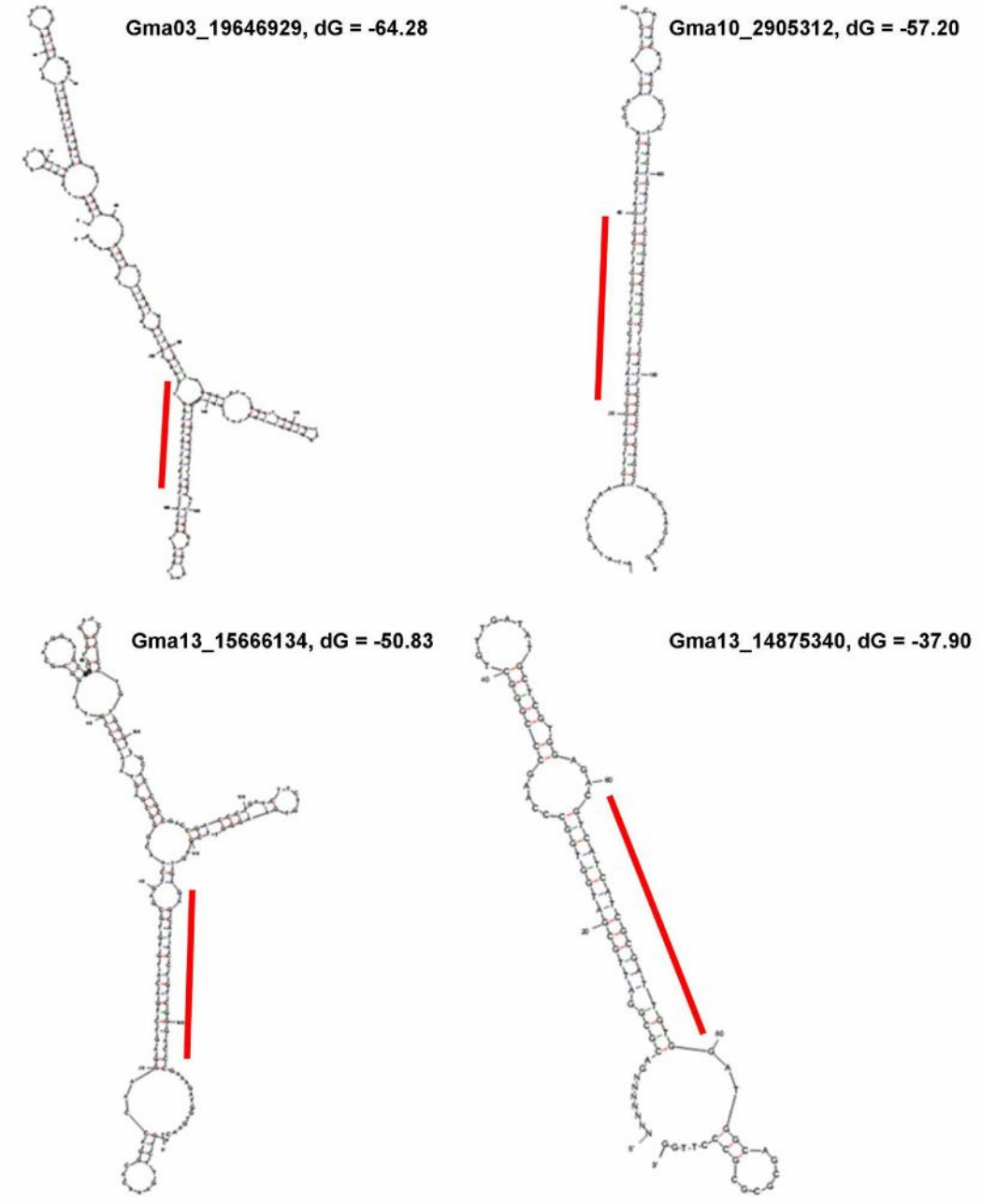
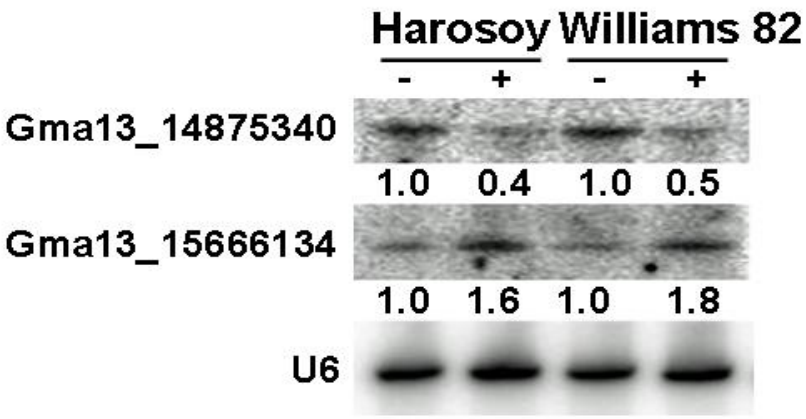

Gma03_19646929, dG = -64.28
Gma10_2905312, dG = -57.20
Gma13_15666134, dG = -50.83
Gma13_14875340, dG = -37.90

Figure 1.9 Putative novel miRNAs that differentially accumulate during *P. sojae* infection.

Northern blots showing miRNA abundances in (-) mock-treated and (+) *P. sojae*-infected tissues at 8 hpi. Gma13_14875340 was reduced while Gma13_15666134 abundance increased. U6 served as a loading control in the blots. Numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample.


Harosoy
Williams 82
- + - +
Gma13_14875340
1.0 0.4 1.0 0.5
Gma13_15666134
1.0 1.6 1.0 1.8
U6

1.4.3 miR166 and miR393 are induced by heat-inactivated *P. sojae* cells

One branch of plant immunity system is based on the recognition of pathogen- or microbe-associated molecular patterns (PAMP/MAMPs), which leads to *PAMP-triggered immunity* (PTI) (Boller and He, 2009; Zipfel, 2009). PTI, broadly referred as the basal defense in plants, restricts the growth of the vast majority of potential pathogens encountered by plants in the surrounding environments (Boller and He, 2009; Jones and Dangl, 2006). It was previously reported that the major PAMP, flg22, of the bacterial pathogen *Pseudomonas syringae* can induce miR393, as a PTI response (Navarro et al., 2006; Zhang et al., 2011).

Phytophthora are known to trigger plant defense response through recognition of cell surface elicitors, such as beta-glucans, NPP1 and PEP-13 (Brunner et al., 2002; Day and Graham, 2007; Fellbrich et al., 2002). In order to determine whether the changes observed in the accumulation of specific miRNAs were PTI responses upon soybean perception of *P. sojae* PAMPs, I treated soybean roots with heat-inactivated *P. sojae* cells (also called the cell wall prep) as previously described (West, 1981). Northern blotting showed that miR166 and miR393 were again induced at eight hours after exposure to the cell wall prep (Figure 1.10, Table 1.7). These data suggest that the induction of miR166 and miR393 is a soybean defense response triggered by certain cell wall component(s), or PAMPs, of *P. sojae*.

Different from miR393 and miR166, miR1507 and miR2109 were only induced by *P. sojae*, but not by the cell wall prep (Figure 1.11, Table 1.7), suggesting that they were triggered by the actual infection process, but not by PAMPs. No signal

was detected when blotting for miR3522. This is likely due to low abundance of miR3522 in roots, especially without infection.

Table 1.7 Summary of results from northern blots, which were used to confirm the differential accumulations of miRNAs in soybean roots from independent biological replicates. The abundances of miRNAs were quantified by determining relative signal densities in *P. sojae* cell wall prep-treated samples on northern blots in comparison to the mock-treated samples. “n” is the number of independent experiments that were performed for individual miRNAs. Relative miRNA levels are presented by average ± standard deviation. Statistically significant results are labeled with *.

Soybean miRNA accumulation in responding to *P. sojae* cell wall prep

Harosoy	**miR160**	1.1 ±0.2	n=2	**miR393**	1.8 ±0.3*	n=4
Williams 82		1.1 ±0.2	n=2		1.7 ±0.2*	n=4
Harosoy	**miR166**	1.4 ±0.2*	n=4	**miR2109**	1.2 ±0.3	n=5
Williams 82		1.5 ±0.3*	n=4		1.1 ±0.3	n=5
Harosoy	**miR168**	0.9 ±0.3	n=4	**miR1507**	0.9 ±0.2	n=3
Williams		0.8 ±0.2	n=4		1.0 ±0.2	n=3
Harosoy	**miR482**	1.0 ±0.2	n=2	**miR1510**	1.0 ±0.3	n=2
Williams 82		1.0 ±0.2	n=2		0.9 ±0.1	n=2

Figure 1.10 miR166 and miR393 were induced by heat-inactivated *P. sojae* cells.

P. sojae hyphae were collected from agar plates and boiled for 5 minutes in water. Boiled cell debris were re-collected by centrifugation, cooled down to room temperature, and then used to inoculate soybean roots. The abundance of miR166 and miR393 were determined by northern blotting eight hours after the roots were exposed to the heat-inactivated cells (+) or water (-). U6 served as a loading control; and numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample. This experiment was repeated three times with similar results.

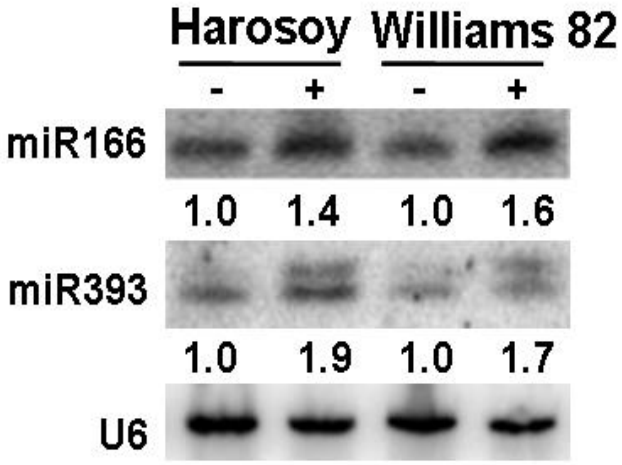
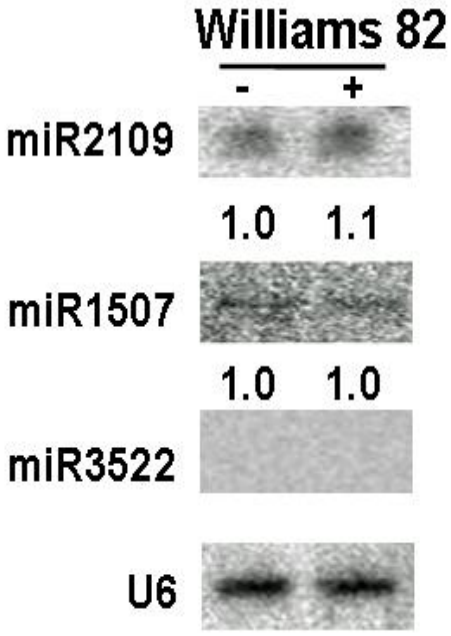

Harosoy
Williams 82
- + - +
miR166
1.0 1.4 1.0 1.6
miR393
1.0 1.9 1.0 1.7
U6

Figure 1.11 miR1507 and miR2109 did not change in roots exposed to heat-inactivated *P. sojae* cells.

The abundance of miR1507, miR2109 and miR3522 were determined by northern blotting eight hours after the roots were exposed to the heat-inactivated *P. sojae* cells (+) or water (-). U6 served as a loading control and the numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample. This experiment was repeated twice to verify results.


Williams 82
-
+
miR2109
1.0
1.1
miR1507
1.0
1.0
miR3522
U6

1.4.4 miRNA target genes also exhibit changes upon P. sojae infection

Small RNAs repress their target gene expression. Therefore, an increase in miRNA levels would likely lead to decrease in the abundance of the target transcripts, and a decrease in miRNA levels should enhance the accumulation of target transcripts.

I examined the transcript levels of genes targeted by the miRNAs that were differentially accumulated in *P. sojae*-infected tissues using quantitative RT-PCR (qRT-PCR) (Table 1.8). Target genes of the differentially expressed soybean miRNAs were predicted utilizing the plant small RNA analyses server: PsRNATarget (http://plantgrn.noble.org/psRNATarget). For the conserved miRNA, their predicted targets in soybean were also matched to the previously identified targets from *Arabidopsis* and other plants for confirmation.

The transcript abundance of two to five predicted target genes was examined for each miRNA. The majority of these genes indeed exhibit changes that are consistent with the corresponding differential expression of the miRNA in *P. sojae*-infected soybean roots. For example, I observed reduced abundance of the predicted target genes of miR166 and miR393, which were induced by *P. sojae* (Figure 1.12). The families of miR166 have known roles in plant growth and development, while miR393 regulates the auxin signaling pathway. miR166 targets the Class III homeodomain leucine zipper (HD-ZIP III) genes (Subramanian et al., 2008; Zhang et al., 2008; Zhang et al., 2011), which encodes CORONA, REV, PHV and PHB. HD-ZIP III proteins have been shown to influence auxin signaling (Itoh et al., 2008;

Prigge et al., 2005). The targets of miR393 are auxin receptor proteins, including TIR1, AFB1, AFB2 and AFB3 in *Arabidopsis* (Chen et al., 2011; Feng et al., 2010; Li et al., 2010; Navarro, 2006; Si-Ammour et al., 2011; Subramanian et al., 2008; Vidal et al., 2010; Zhang et al., 2011).

The target transcripts of the other three induced miRNAs, miR1507, miR2109 and miR3522, also exhibited the expected decrease in *P. sojae*-infected samples (Fig. 1.13 and Fig. 1.14). Among them, miR1507 and miR2109 are particularly interesting because they are predicted to target nucleotide binding-leucine rich repeat (*NB-LRR*) genes, which are canonical plant resistance (R) genes. Consistent with the enhanced accumulation of miR1507 and miR2109, several *NB-LRR* genes that are predicted to be targeted by these miRNAs were repressed by *P. sojae* infection (Figure 1.13).

Another miRNA, miR482, was also predicted to target one *NB-LRR* gene *Glyma12g36510* (Table 1.8). miR482 was repressed during *P. sojae* infection; however, the transcript level of Gma12g36510 was only moderately increased in the resistant host Williams 82, but remained unchanged in the susceptible host Harosoy (Fig. 1.14). Therefore, it seems like *P. sojae* infection leads to a general reduction in the expression levels of *NB-LRR* genes. Furthermore, the target genes of miR168 and miR319 did not show significant changes at the transcription level (Figure 1.15), although these two miRNAs were both down-regulated in *P. sojae*-infected tissues. It is possible that the repression of miR168 and miR319 may lead to enhanced expression of other targets that I did not test.

Corresponding transcript changes of the predicted targets of the two novel miRNAs that exhibit differential expression were also detected by qRT-PCR (Figure 1.16). Gm13_14875340 targets members of the *GH3* gene family, which encodes acyl acid amino synthetases that catalyze the synthesis of amino acid conjugates of auxin and jasmonates (Staswick et al., 2002; Woodward and Bartel, 2005). Gm13_ 14875340 was repressed during the early infection of *P. sojae*, consistent with the increased expression of the *GH3* family gene *Glyma05g21680*. Notably, there were abundance changes only in Williams 82 but not in Harosoy. It may be possible that cultivar differences are contributing to the disparity, but further examination is required. In Arabidopsis, over-expression of a *GH3* gene *WES1* leads to auxin defective phenotypes (Park et al., 2007). It is therefore possible that this novel miRNA represses the auxin signaling pathway during *P. sojae* infection. This activity is consistent with the effect of miR393, whose induction by *P. sojae* also leads to the repression of auxin signaling.

One of the predicted targets of the novel soybean miRNA Gm13_ 15666134 encodes a voltage ligand-gated potassium ion channel (Table 1.8). Consistent with the induction of Gm13_ 15666134, the predicted target *Glyma05g08230* is repressed in *P. sojae*-infected roots. Potassium ion channels have critical roles in plants responding and adapting to biotic and abiotic stresses (Jaspers and Kangasjärvi, 2010; Uozumi and Schroeder, 2010). Therefore, this novel soybean miRNA could also regulate soybean defense by modulating the function of potassium ion channels in responding to *P. sojae* infection.

Table 1.8 Predicted soybean targets of miRNAs that were confirmed to accumulate differentially in roots during *P. sojae* infection.

Conserved and novel miRNAs were submitted for target transcript prediction using the plant small RNA analyses server: PsRNATarget, (http://plantgrn.noble.org/psRNATarget).

Soybean miRNA	Predicted Target Gene	Target Gene Product
gma-miR166	Glyma05g30000	HD-ZIP Transcription Factor
	Glyma05g06070	APRR2 Transcription Factor
	Glyma07g01950	HD-ZIP Transcription Factor
	Glyma08g13110	HD-ZIP Transcription Factor
	Glyma08g21620	HD-ZIP Transcription Factor
	Glyma11g20520	HD-ZIP Transcription Factor
gma-miR168	Glyma09g29720	Argonaute1
	Glyma16g34300	Argonaute1
gma-miR319	Glyma08g10350	TCP family transcription factor
	Glyma13g29160	TCP family transcription factor
	Glyma13g34690	TCP family transcription factor
	Glyma15g09910	TCP family transcription factor
gma-miR393	Glyma02g17170	F-Box/Leucine Rich Repeat Protein
	Glyma10g02630	F-Box/Leucine Rich Repeat Protein
	Glyma16g05500	F-Box/Leucine Rich Repeat Protein
	Glyma19g39420	F-Box/Leucine Rich Repeat Protein
gma-miR482	Glyma12g28730	Glycogen Synthase Kinase 3
	Glyma12g36510	Nucleotide-Binding Leucine Rich Repeat Protein
gma-miR2109	Glyma03g14900	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma16g10080	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma16g34000	Nucleotide-Binding Leucine Rich Repeat Protein
gma-miR1507	Glyma04g29220	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma06g39720	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma06g47650	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma13g25950	Nucleotide-Binding Leucine Rich Repeat Protein
	Glyma15g35920	Nucleotide-Binding Leucine Rich Repeat Protein
gma-miR3522	Glyma12g01290	Glycosyl Hydrolase
	Glyma16g05240	Sec31 Transport Protein
Gm13_14875340	Glyma05g21680	GH3 Auxin Responsive Protein
	Glyma17g18040	GH3 Auxin Responsive Protein
Gm13_15666134	Glyma05g08230	Voltage Ligand-Gated Potassium Channel
	Glyma08g24960	Voltage Ligand-Gated Potassium Channel
	Glyma15g10140	Voltage Ligand-Gated Potassium Channel

Figure 1.12 The expression of the target genes of miR166 and miR393 were repressed during *P. sojae* infection.

Transcript abundances of the predicted targets of miR166 and miR393 were determined by quantitative RT-PCR. *GmUBI* was used as the internal standard. Relative expression levels were determined by comparing the normalized transcript levels between *P. sojae*-infected and mock-treated samples. Values represent averages from three independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).

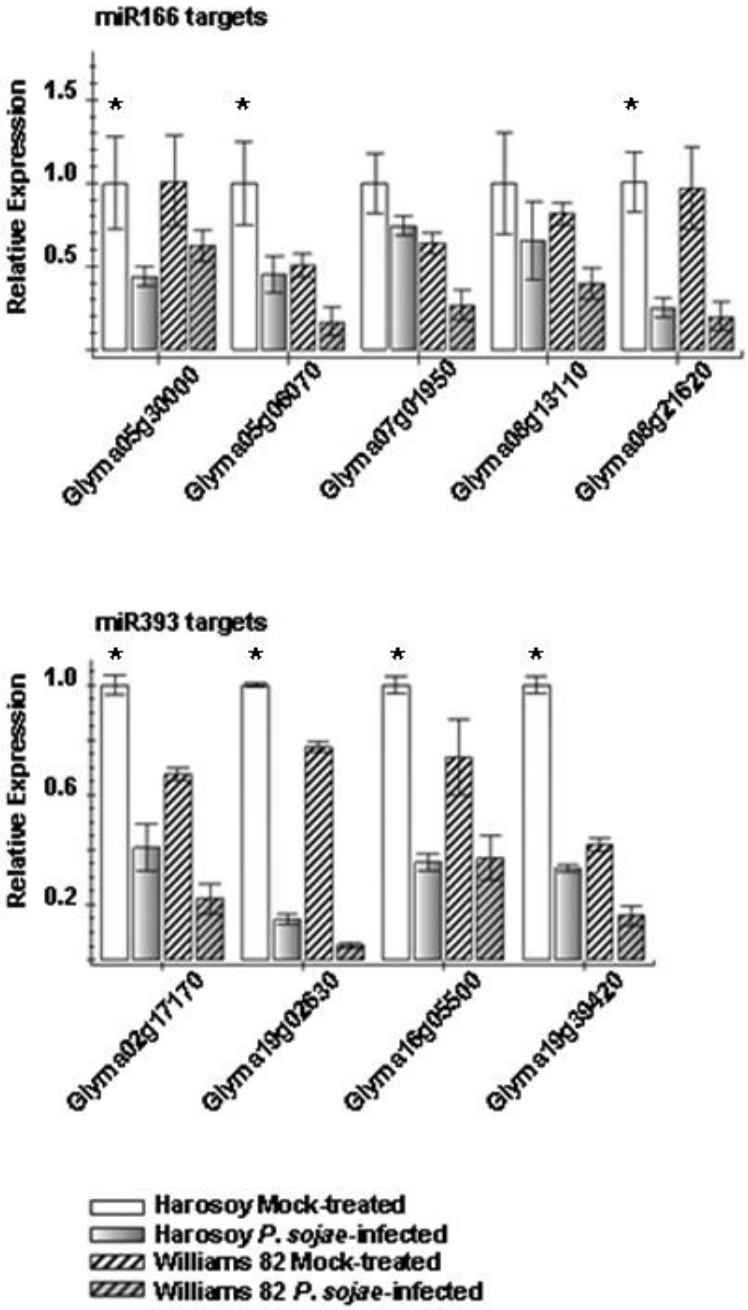

miR166 targets
Relative Expression
1.5
1.0
0.5
*
*
*
Glyma05g30000
Glyma05g06070
Glyma07g01950
Glyma08g13110
Glyma08g21620
miR393 targets
Relative Expression
1.0
0.6
0.2
*
*
*
*
Glyma02g17170
Glyma19g02630
Glyma16g05500
Glyma19g39420
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 1.13 *NB-LRR* genes targeted by miR1507 and miR2109 were repressed during *P. sojae* infection.

Relative transcript abundances were evaluated with qRT-PCR using *GmUBI* as an internal control. Values represent averages from two independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).

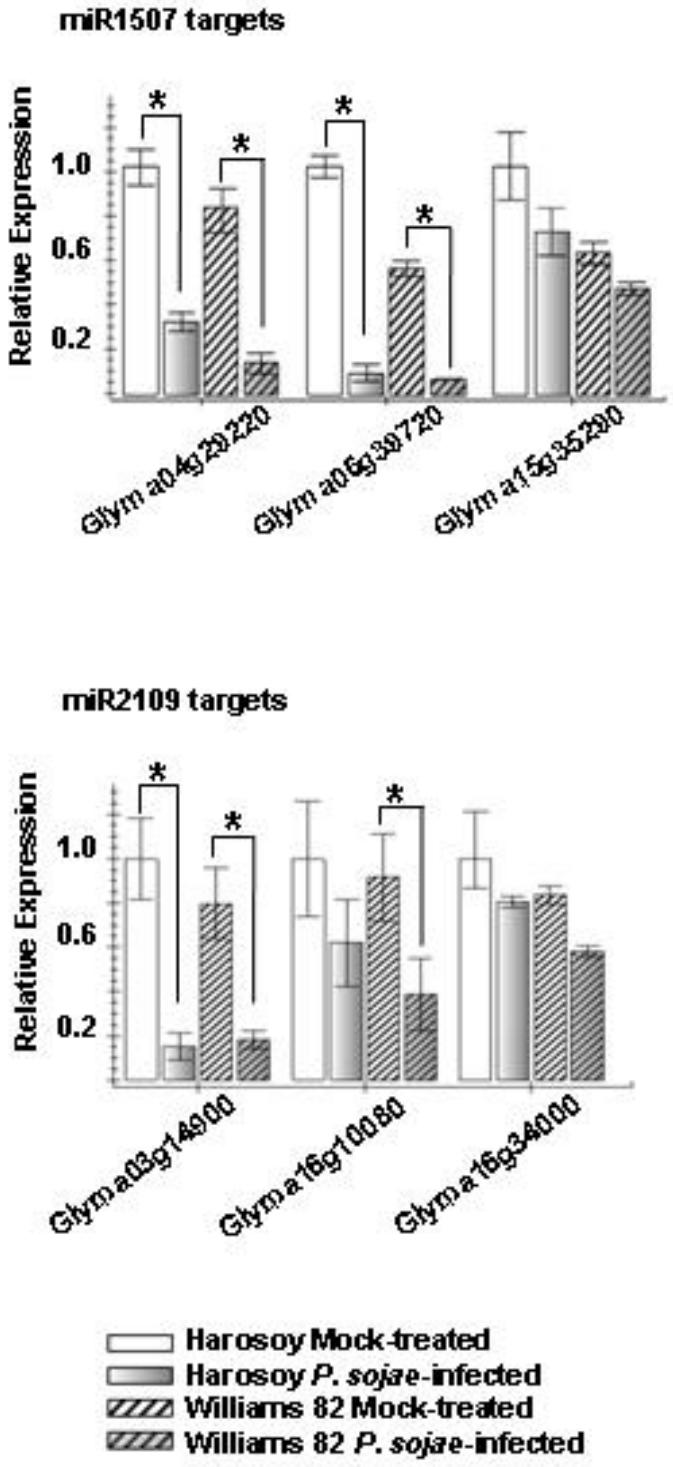

miR1507 targets
Relative Expression
1.0
0.6
0.2
*
Glym a04g29220
Glym a06g30720
Glym a15g35290
miR2109 targets
Relative Expression
1.0
0.6
0.2
*
Glym a03g14900
Glym a16g10080
Glym a16g34000
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 1.14 Quantitative RT-PCR analyses on the expression levels of miR482 and miR3522 target genes in soybean roots infected with *P. sojae*.

The soybean targets of the conserved miRNAs were determined by qRT-PCR in mock-treated and *P. sojae*-infected roots at 8 hpi. Relative transcript abundance was evaluated using *GmUBI* as an internal control. * represents statistically significant difference.

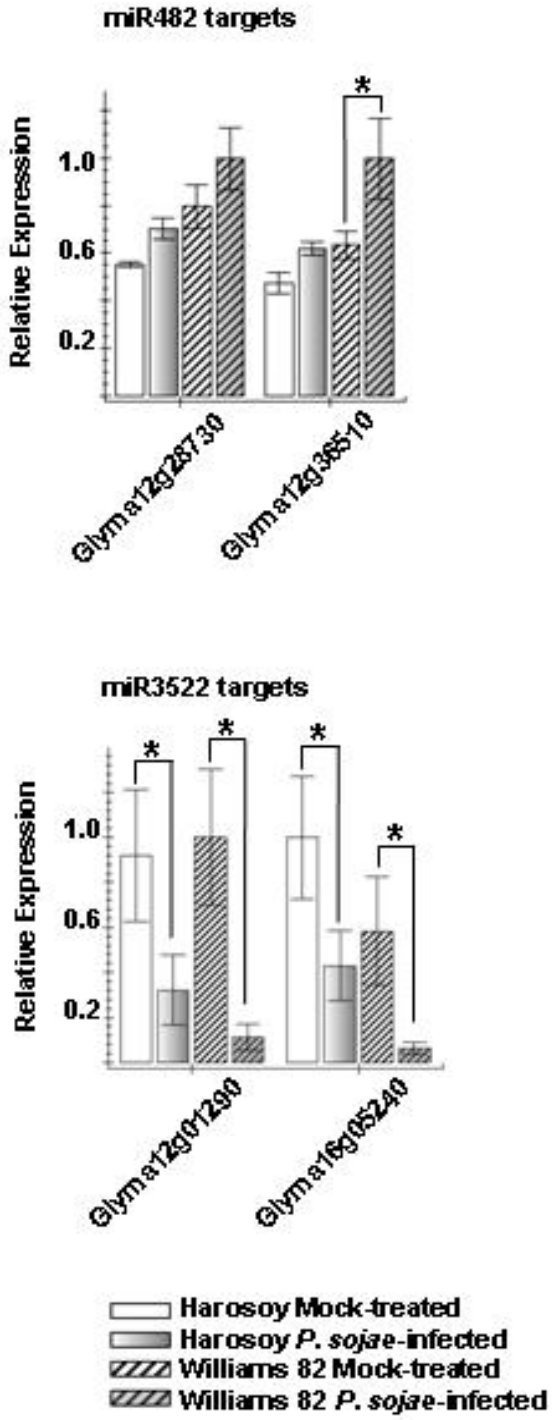

miR482 targets
Relative Expression
1.0
0.6
0.2
*
Glyma12g28730
Glyma12g36510
miR3522 targets
Relative Expression
1.0
0.6
0.2
*
*
*
*
Glyma12g01290
Glyma16g05240
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 1.15 Quantitative RT-PCR analyses on the expression levels of miR168 and miR319 target genes in soybean roots infected with *P. sojae*.

The soybean targets of conserved miRNAs were determined by qRT-PCR in mock-treated and *P. sojae*-infected roots at 8 hpi. Relative transcript abundance was evaluated using *GmUBI* as an internal control. * represents statistically significant difference.

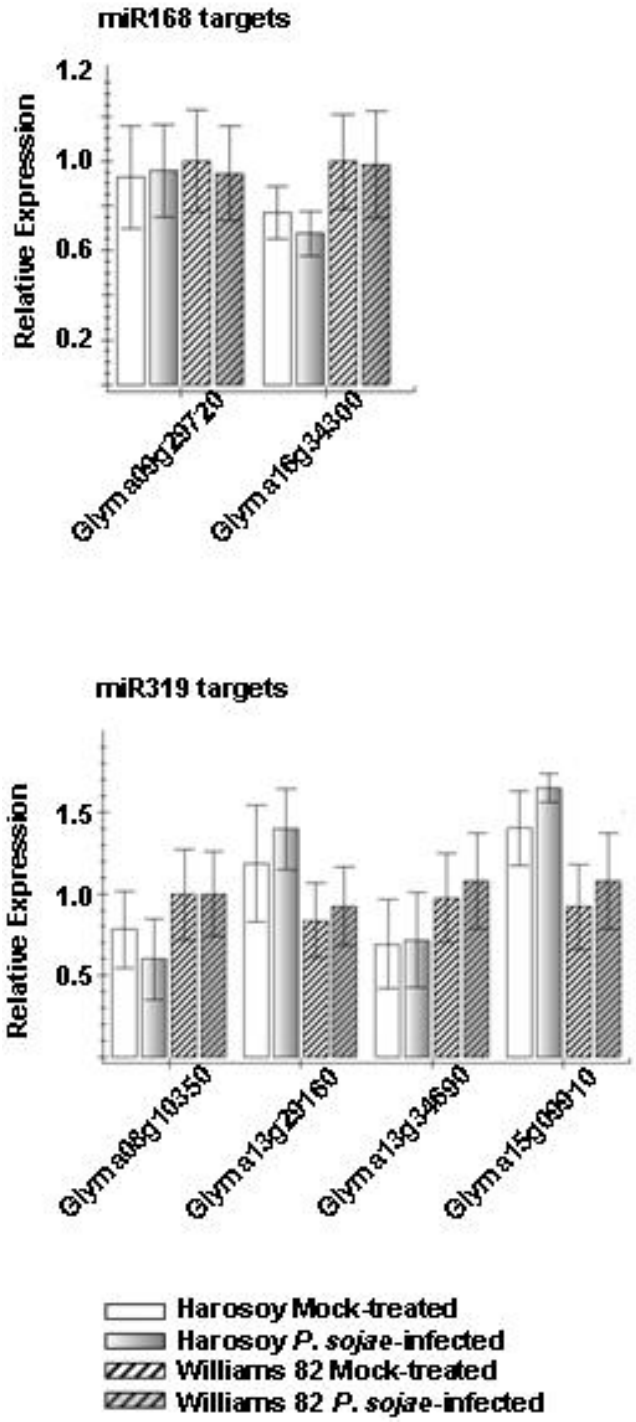
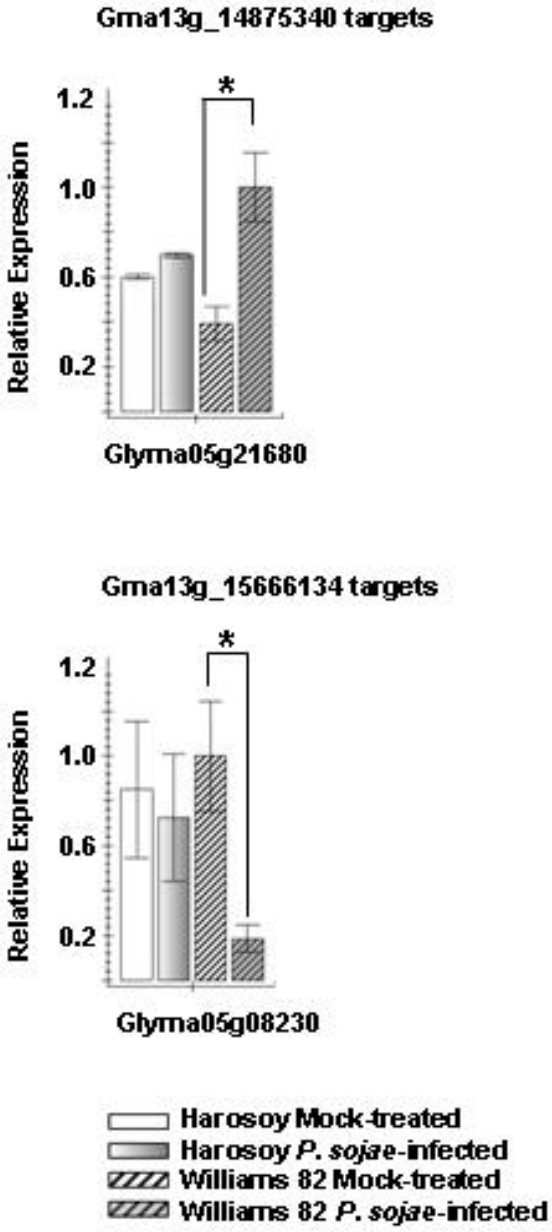

miR168 targets
Relative Expression
1.2
1.0
0.6
0.2
Glyma09g29720
Glyma16g34300
miR319 targets
Relative Expression
1.5
1.0
0.5
Glyma08g10350
Glyma13g29160
Glyma13g34690
Glyma15g09910
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 1.16 Quantitative RT-PCR analyses on the expression levels of novel miRNAs Gma13g_14875340 and Gma13g15666134 target genes in soybean roots infected with *P. sojae*.

The soybean targets of the novel miRNAs were determined by qRT-PCR in mock-treated and *P. sojae*-infected roots at 8 hpi. Relative transcript abundance was evaluated using *GmUBI* as an internal control. * represents statistically significant difference.


Gma13g_14875340 targets
Relative Expression
1.2
1.0
0.6
0.2
*
Glyma05g21680
Gma13g_15666134 targets
Relative Expression
1.2
1.0
0.6
0.2
*
Glyma05g08230
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

1.5 DISCUSSION

1.5.1 Specific miRNA changes as defense responses in soybean

The research aiming to understand small RNA biogenesis and regulatory mechanisms has been rapidly increasing over the past several decades. The vast knowledge accumulated indicates a very complex and dynamic function for small RNAs in plants and animals. During infection, the host utilizes small RNA pathways to block pathogen infection and control disease development. However, pathogens have evolved mechanisms to modulate small RNA biogenesis and suppress host immunity. In plants, small RNAs have been shown as an important defense mechanism against viruses and bacteria. Changes of specific small RNAs in response to these pathogens enhance host survivability either by directly targeting pathogen RNAs or by regulating host resistance genes.

Using small RNA sequencing and experimental confirmation with northern blots and qRT-PCR, I identified abundance changes of eight known miRNAs and two novel soybean miRNAs in the roots infected with an economically important oomycete pathogen *P. sojae*. This study represents the first genome-scale small RNA profiling during *Phytophthora* infection. These data demonstrate distinct regulations of specific miRNAs in response to *P. sojae* infection. The small RNA changes lead to altered expression of defense-associated genes, suggesting that specific small RNAs are important regulators of plant immunity. Though this study focused on miRNAs, our small RNA library remains a vast resource for the identification of additional novel soybean miRNAs and siRNAs.

1.5.2 *P. sojae* PAMPs trigger miR393 and miR166 expression.

miR166 and miR393 are conserved miRNAs that primarily regulate plant development. miR393 represses auxin signaling by targeting the TIR/AFB auxin receptors (Chen et al., 2011; Feng et al., 2010; Si-Ammour et al., 2011; Vidal et al., 2010). The function of miR393 in plant immunity has been studied in *Arabidopsis* using *MIR393*-overexpressing lines, which exhibited enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Navarro et al., 2006) and the oomycete pathogen *Hyaloperonospora parasitica* (Robert-Seilaniantz et al., 2011). Interestingly, miR393 was induced by heat-inactivated *P. sojae* cells, indicating that the induction was likely triggered by PAMPs. *Phytophthora* cell surface peptide β-glucans and PEP-13 have been reported to elicit defense response including the accumulation of phytoalexins and the activation of salicylic acid (SA) signaling pathways (Brunner et al., 2002; Day and Graham, 2007; Fellbrich et al., 2002). Although it remains to be determined which cell wall component(s) elicits the induction of miR393, my results support a function of this miRNA in establishing PAMP-triggered immunity in soybean. Indeed, miR393 can be induced by the major PAMP, flagellin, of the bacterial pathogen *P. syringae* in *Arabidopsis* (Navarro et al., 2006). Collectively, these data suggest that miR393 may play a conserved role in plant basal defense upon the detection of a broad range of microbial pathogens.

Similar to miR393, miR166 was also induced by heat-inactivated *P. sojae* cells. miR166 targets the Class III homeodomain leucine zipper (HD-ZIP III) genes and regulates organ polarity, vascular development and lateral root development (Prigge et al., 2005). It is possible that the induction of miR166 could stall plant

growth so that infected plants could devote more energy to establishing a defense response. In *Arabidopsis*, miR166 was up regulated when exposed to a nonvirulent *P. syringae* mutant (Zhang et al., 2011). Another conserved miRNA, miR160, was also indicated to be induced in *Arabidopsis* by *P. syringae* (Fahlgren et al., 2007), but remained unchanged in our systems. These data suggests conserved and specific roles of miRNAs in different plants challenged by different pathogens.

1.5.3 Soybean miRNAs regulate *NB-LRR* genes during *P. sojae* infection.

NB-LRRs are immune receptors that recognizes specific pathogen proteins, such as effectors, and elicit robust defense response (Eitas and Dangl, 2010; Jones and Dangl, 2006; McHale et al., 2006; Swiderski et al., 2009). The activation of NB-LRR proteins often associates with programmed cell death, which restricts the spread of the pathogen from the infection sites; on the other hand, mis-regulation of NB-LRRs leads to tissue damage and auto-immune diseases (McHale et al., 2006; Swiderski et al., 2009). Some NB-LRRs were found to be involved in other cellular signaling pathway, such as stress tolerance and development (Tameling and Joosten, 2007). In soybean, NB-LRR proteins can also regulate nodulation through the symbiotic relationship with rhizobia (Kulcheski et al., 2011; Subramanian et al., 2008). In particular, the soybean NB-LRRs Rj2 and Rfg1 are involved in host specific recognition of *Bradyrhizobium japonicum* (Yang et al., 2010).

A number of soybean miRNAs, including miR482, miR1507, miR1509, miR1510 and miR2109 have been predicted to target the *NB-LRR* genes. Among them, both miR1507 and miR2109 are induced during *P. sojae* infection.

Furthermore, miR1509 and miR1510 were found to be up-regulated based on the small RNA sequencing data, although these changes were not confirmed by northern blotting. These data suggest a general down-regulation of *NB-LRR* genes by miRNAs in *P. sojae*-infected soybean.

22 nt miRNAs can trigger the production of secondary siRNAs from their target transcripts. These siRNAs are referred as phasiRNAs because they are in 21 nt register from one another. miR1507 and miR2109 are 22 nt in length in *Medicago truncatula* and are able to trigger the production of phasiRNAs from their target *NB-LRR* loci (Zhai et al., 2011). As such, these miRNAs could be central regulators of a potentially large number of *NB-LRR* genes.

miR1507 is a conserved 22 nt miRNA in legumes. Although produced by both soybean and *M. truncatula*, miR2109 was thought to be predominantly 21 nt in soybean (Zhai et al., 2011). However, our sequencing data and northern blotting analyses detected both 21 and 22 nt forms of miR2109 in soybean, and both forms were induced during *P. sojae* infection (Figure 1.5). The presence of the 22 nt form raised the possibility that miR2109 may also trigger the production of phasiRNAs from its target *NB-LRR* loci in soybean. Therefore, it is likely that miR1507 and miR2109 can regulate a large group of *NB-LRR* genes through phasiRNA production in *P. sojae*-infected soybean.

Genome sequence analyses revealed hundreds of potential *NB-LRR* genes in soybean (Innes et al., 2008; Zhai et al., 2011). Since activation of NB-LRRs leads to a fitness penalty (Day and Graham, 2007; Jones and Dangl, 2006; Rafiqi et al., 2009),

precise regulation of *NB-LRR* gene expression is of significant importance for effective defense against the pathogens. I hypothesize that in the early stages of infection, it is in the best interest of the plant to maintain NB-LRR proteins in low levels to avoid the fitness penalty. It is possible that soybean may use miRNAs, and possibly phasiRNAs, to fine tune the expression levels of *NB-LRR* genes, especially at the early infection stages by *P. sojae*.

1.5.4 Conclusions

This study represents the first genome-scale small RNA profiling in plants during *Phytophthora* infection. I identified specific soybean miRNAs that are differentially accumulated in soybean roots infected by the economically important pathogen *P. sojae*. These data demonstrate that the small RNA changes lead to altered expression of defense-associated genes and suggest that specific small RNAs are important regulators of plant immunity.

Many aspects of the molecular and genetic mechanisms of small RNA mediated plant defense remain unclear. For example, how small RNAs are regulated in response to pathogens and how these changes confer resistance are largely unknown. Further research is needed to gain additional insight into the roles of small RNAs during plant-pathogen interactions.

1.6 REFERENCES

Alfano, J.R., and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annual review of Phytopathology* 42, 385-414.

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121, 207-221.

Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431, 350-355.

Axtell, M.J., and Bartel, D.P. (2005). Antiquity of microRNAs and their targets in land plants. *The Plant Cell Online* 17, 1658-1673.

Axtell, M.J., Jan, C., Rajagopalan, R., and Bartel, D.P. (2006). A two-hit trigger for siRNA biogenesis in plants. *Cell* 127, 565-577.

Baldauf, S.L. (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290, 972-977.

Barrera-Figueroa, B.E., Gao, L., Diop, N.N., Wu, Z., Ehlers, J.D., Roberts, P.A., Close, T.J., Zhu, J.K., and Liu, R. (2011). Identification and comparative analyses of drought-associated microRNAs in two cowpea genotypes. *BMC Plant Biology* 11, 127.

Bartel, B. (2005). MicroRNAs directing siRNA biogenesis. *Nature Structural and Molecular Biology* 12, 569.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215-233.

Baulcombe, D. (2004). RNA silencing in plants. *Nature* 431, 356-363.

Birch, P.R., and Whisson, S.C. (2001). *Phytophthora infestans* enters the genomics era. *Molecular Plant Pathology* 2, 257-263.

Boller, T., and He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324, 742.

Bos, J.I.B., Armstrong, M.R., Gilroy, E.M., Boevink, P.C., Hein, I., Taylor, R.M., Zhendong, T., Engelhardt, S., Vetukuri, R.R., and Harrower, B., (2010). *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proceedings of the National Academy of Sciences* 107, 9909-9914.

Brunner, F., Rosahl, S., Lee, J., Rudd, J.J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D., and Nurnberger, T. (2002). Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases. *The EMBO Journal* 21, 6681-6688.

Cai, Y., Yu, X., Hu, S., and Yu, J. (2009). A Brief Review on the Mechanisms of miRNA regulation. *Genomics, Proteomics & Bioinformatics* 7, 147-154.

Cerutti, H., and Casas-Mollano, J.A. (2006). On the origin and functions of RNA-mediated silencing: from protists to man. *Current Genetics* 50, 81-99.

Chellappan, P., and Jin, H. (2009). Discovery of plant microRNAs and short-interfering RNAs by deep parallel sequencing. *Plant Hormones*, Humana Press, 121-132.

Chen, H.M., Li, Y.H., and Wu, S.H. (2007). Bioinformatic prediction and experimental validation of a microRNA-directed tandem trans-acting siRNA cascade in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 104, 3318-3323.

Chen, X. (2009). Small RNAs and their roles in plant development. *Annual Review of Cell and Developmental Biology* 25, 21-44.

Chen, Z.H., Bao, M.L., Sun, Y.Z., Yang, Y.J., Xu, X.H., Wang, J.H., Han, N., Bian, H.W., and Zhu, M.Y. (2011). Regulation of auxin response by miR393-targeted transport inhibitor response protein 1 is involved in normal development in *Arabidopsis. Plant Molecular Biology* 77, 619-629.

Cuperus, J.T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R.T., Takeda, A., Sullivan, C.M., Gilbert, S.D., Montgomery, T.A., and Carrington, J.C. (2010). Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. *Nature Structural & Molecular Biology* 17, 997-1003.

Day, B., and Graham, T. (2007). The plant host–pathogen interface. *Annals of the New York Academy of Sciences* 1113, 123-134.

De Paoli, E., Dorantes-Acosta, A., Zhai, J., Accerbi, M., Jeong, D.H., Park, S., Meyers, B.C., Jorgensen, R.A., and Green, P.J. (2009). Distinct extremely abundant siRNAs associated with cosuppression in petunia. *RNA* 15, 1965-1970.

Diaz-Pendon, J.A., Li, F., Li, W.X., and Ding, S.W. (2007). Suppression of antiviral silencing by Cucumber Mosaic Virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *The Plant Cell Online* 19, 2053-2063.

Ding, S.W., and Voinnet, O. (2007). Antiviral immunity directed by small RNAs. *Cell* 130, 413-426.

Dorrance, A., Jia, H., and Abney, T. (2004). Evaluation of soybean differentials for their interaction with *Phytophthora sojae*. *Plant Health Progress* 10.

Dorrance, A., Berry, S.A, and Andeerson, T.R. (2008). Isolation, storage, pathotype characterization, and evaluation of resistance for *Phytophthora sojae* in soybean. *Plant Health Progress* 10, 1094.

Eitas, T.K., and Dangl, J.L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. *Current Opinion in Plant Biology* 13, 472-477.

Ellendorff, U., Fradin, E.F., de Jonge, R., and Thomma, B.P. (2009). RNA silencing is required for *Arabidopsis* defence against *Verticillium* wilt disease. *Journal of Experimental Botany* 60, 591-602.

Erwin, D.C., and Ribeiro, O.K. (1996). *Phytophthora* diseases worldwide. American Phytopathological Society (APS Press).

Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., and Dangl, J.L. (2007). High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE* 2, e219.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M., and Nurnberger, T. (2002). NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. *The Plant Journal* 32, 375-390.

Feng, X.M., Qiao, Y., Wang, S.S., and Hao, Y.J. (2010). Overexpression of *Arabidopsis* At-miR393a gene alters the sensitivity to auxin in tomato. *Scientia Horticulturae* 124, 165-169.

Galán, J.E., and Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444, 567-573.

Gao, H., Narayanan, N.N., Ellison, L., and Bhattacharyya, M.K. (2005). Two classes of highly similar coiled-coil nucleotide-binding leucine-rich repeat genes isolated from the *Rps1-k* locus encode *Phytophthora* resistance in soybean. *Molecular Plant-Microbe Interactions* 18, 1035-1045.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. *Nature Reviews Genetics* 10, 94-108.

Greenberg, J.T. (1997). Programmed cell death in plant-pathogen interactions. *Annual Review of Plant Biology* 48, 525-545.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. *Nucleic Acids Research* 36, D154-D158.

Guo, N., Ye, W.W., Wu, X.L., Shen, D.Y., Wang, Y.C., Xing, H., and Dou, D.L. (2011). Microarray profiling reveals microRNAs involving soybean resistance to *Phytophthora sojae*. *Genome* 54, 954-958.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H.Y., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T. *et al.* (2009). Genome sequence and analyses of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461, 393-398.

Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. *The EMBO Journal* 21, 4671-4679.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-952.

Hardham, A.R. (2001). The cell biology behind *Phytophthora* pathogenicity. *Australasian Plant Pathology* 30, 91-98.

Hausbeck, M.K., and Lamour, K.H. (2004). *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Disease* 88, 1292-1303.

He, X.F., Fang, Y.Y., Feng, L., and Guo, H.S. (2008). Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR–NBS–LRR class *R* gene-derived novel miRNA in *Brassica*. *FEBS Letters* 582, 2445-2452.

Howell, M.D., Fahlgren, N., Chapman, E.J., Cumbie, J.S., Sullivan, C.M., Givan, S.A., Kasschau, K.D., and Carrington, J.C. (2007). Genome-wide analyses of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 pathway in *Arabidopsis* reveals dependency on miRNA-and tasiRNA-directed targeting. *The Plant Cell Online* 19, 926-942.

Innes, R.W., Ameline-Torregrosa, C., Ashfield, T., Cannon, E., Cannon, S.B., Chacko, B., Chen, N.W., Couloux, A., Dalwani, A., and Denny, R. (2008). Differential accumulation of retroelements and diversification of NB-LRR disease resistance genes in duplicated regions following polyploidy in the ancestor of soybean. *Plant Physiology* 148, 1740-1759.

Itoh, J.I., Hibara, K.I., Sato, Y., and Nagato, Y. (2008). Developmental role and auxin responsiveness of class III homeodomain leucine zipper gene family members in rice. *Plant Physiology* 147, 1960-1975.

Jaspers, P., and Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. *Physiologia plantarum* 138, 405-413.

Jiang, R.H.Y., Tripathy, S., Govers, F., and Tyler, B.M. (2008). RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proceedings of the National Academy of Sciences* 105, 4874-4879.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. *Nature* 444, 323-329.

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Zhu, J.K., Staskawicz, B.J., and Jin, H. (2006). A pathogen-inducible endogenous siRNA in plant immunity. *Proceedings of the National Academy of Sciences* 103, 18002-18007.

Kelley, B.S., Lee, S.J., Damasceno, C.M.B., Chakravarthy, S., Kim, B.D., Martin, G.B., and Rose, J.K.C. (2010). A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. *The Plant Journal* 62, 357-366.

Khraiwesh, B., Arif, M.A., Seumel, G.I., Ossowski, S., Weigel, D., Reski, R., and Frank, W. (2010). Transcriptional control of gene expression by microRNAs. *Cell* 140, 111-122.

Kulcheski, F.R., de Oliveira, L.F., Molina, L.G., Almerão, M.P., Rodrigues, F.A., Marcolino, J., Barbosa, J.F., Stolf-Moreira, R., Nepomuceno, A.L., and Marcelino-Guimarães, F.C., and Margis, R. (2011). Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genomics* 12, 307.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *The EMBO Journal* 23, 4051-4060.

Li, F., Pignatta, D., Bendix, C., Brunkard, J.O., Cohn, M.M., Tung, J., Sun, H., Kumar, P., and Baker, B. (2012). MicroRNA regulation of plant innate immune receptors. *Proceedings of the National Academy of Sciences* 109, 1790-1795.

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics* 24, 713-714.

Li, W.X., and Ding, S.W. (2001). Viral suppressors of RNA silencing. *Current Opinion in Biotechnology* 12, 150-154.

Li, Y., Lu, J., Han, Y., Fan, X., and Ding, S.W. (2013). RNA interference functions as an antiviral immunity mechanism in mammals. *Science* 342, 231-234.

Li, Y., Zhang, Q., Zhang, J., Wu, L., Qi, Y., and Zhou, J.M. (2010). Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiology* 152, 2222-2231.

Lu, S., Sun, Y.H., Amerson, H., and Chiang, V.L. (2007). MicroRNAs in loblolly pine (*Pinus taeda L.*) and their association with fusiform rust gall development. *The Plant Journal* 51, 1077-1098.

MacRae, E. (2007). Extraction of plant RNA. *Protocols for Nucleic Acid Analyses by Nonradioactive Probe*s, Humana Press, pp. 15-24.

Mallory, A.C., and Vaucheret, H. (2006). Functions of microRNAs and related small RNAs in plants. *Nature Genetics* 38, S31-S36.

Markham, N.R., and Zuker, M. (2008). UNAFold. *Bioinformatics*, Humana Press, pp. 3-31.

McHale, L., Tan, X., Koehl, P., and Michelmore, R.W. (2006). Plant NBS-LRR proteins: adaptable guards. *Genome Biology* 7, 212.

McHale, M., Eamens, A.L., Finnegan, E.J., and Waterhouse, P.M. (2013). A 22-nt artificial microRNA mediates widespread RNA silencing in *Arabidopsis*. *The Plant Journal* 76, 519-529.

Meyers, B.C., Axtell, M.J., Bartel, B., Bartel, D.P., Baulcombe, D., Bowman, J.L., Cao, X., Carrington, J.C., Chen, X., and Green, P.J. (2008). Criteria for annotation of plant MicroRNAs. *The Plant Cell Online* 20, 3186-3190.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible cosuppression of homologous genes in trans. *The Plant Cell Online* 2, 279-289.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312, 436-439.

Navarro, L., Jay, F., Nomura, K., He, S.Y., and Voinnet, O. (2008). Suppression of the microRNA pathway by bacterial effector proteins. *Science* 321, 964-967.

Padmanabhan, C., Zhang, X., and Jin, H. (2009). Host small RNAs are big contributors to plant innate immunity. *Current Opinion in Plant Biology* 12, 465-472.

Park, J.E., Seo, P.J., Lee, A.K., Jung, J.H., Kim, Y.S., and Park, C.M. (2007). An *Arabidopsis* GH3 gene, encoding an auxin-conjugating enzyme, mediates phytochrome B-regulated light signals in hypocotyl growth. *Plant and Cell Physiology* 48, 1236-1241.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. (2002). CARPEL FACTORY, a DICER homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana* . *Current Biology* 12, 1484-1495.

Parry, G., Calderon-Villalobos, L.I., Prigge, M., Peret, B., Dharmasiri, S., Itoh, H., Lechner, E., Gray, W.M., Bennett, M., and Estelle, M. (2009). Complex regulation of the TIR1/AFB family of auxin receptors. *Proceedings of the National Academy of Sciences* 106, 22540-22545.

Pearson, W.R. (1999). Flexible sequence similarity searching with the FASTA3 program package. *Bioinformatics Methods and Protocols*, Humana Press, pp. 185-219.

Plasterk, R.H. (2006). MicroRNAs in animal development. *Cell* 124, 877-881.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., and Clark, S.E. (2005). Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. *The Plant Cell Online* 17, 61-76.

Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., Wang, X., Liu, X., Xiang, Q., Jiang, S., Zhang, F., Wang, Y., Judelson, H.S., Chen, X., and Ma, W. (2013). Oomycete pathogens encode RNA silencing suppressors. *Nature Genetics* 45, 330-333.

Qu, F., Ye, X., and Morris, T.J. (2008). *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proceedings of the National Academy of Sciences* 105, 14732-14737.

Qutob, D., Hraber, P.T., Sobral, B.W., and Gijzen, M. (2000). Comparative analyses of expressed sequences in *Phytophthora sojae*. *Plant Physiology* 123, 243-254.

Rafiqi, M., Bernoux, M., Ellis, J.G., and Dodds, P.N. (2009). In the trenches of plant pathogen recognition: Role of NB-LRR proteins. *Seminars in Cell & Developmental Biology* 20(9), Academic Press, pp. 1017-1024.

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997). A similarity between viral defense and gene silencing in plants. *Science* 276, 1558-1560.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. *Cell* 110, 513-520.

Ristaino, J.B. (2002). Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. *Microbes and Infection* 4, 1369-1377.

Robert-Seilaniantz, A., MacLean, D., Jikumaru, Y., Hill, L., Yamaguchi, S., Kamiya, Y., and Jones, J.D.G. (2011). The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. *The Plant Journal* 67, 218-231.

Ruiz-Ferrer, V., and Voinnet, O. (2009). Roles of plant small RNAs in biotic stress responses. *Annual Review of Plant Biology* 60, 485-510.

Si-Ammour, A., Windels, D., Arn-Bouldoires, E., Kutter, C., Ailhas, J., Meins, F., and Vazquez, F. (2011). miR393 and secondary siRNAs regulate expression of the TIR1/AFB2 auxin receptor clade and auxin-related development of *Arabidopsis* leaves. *Plant Physiology* 157, 683-691.

Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993). TRIzol: A new reagent for optimal single-step isolation of RNA. *Focus* 15, 532-535.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *The Plant Cell Online* 14, 1405-1415.

Subramanian, S., Fu, Y., Sunkar, R., Barbazuk, W.B., Zhu, J.K., and Yu, O. (2008). Novel and nodulation-regulated microRNAs in soybean roots. *BMC Genomics* 9, 160.

Sunkar, R. (2010). MicroRNAs with macro-effects on plant stress responses. *Seminars in Cell & Developmental Biology* 21, 805-811.

Swiderski, M.R., Birker, D., and Jones, J.D. (2009). The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. *Molecular Plant-Microbe Interactions* 22, 157-165.

Szittya, G., Molnár, A., Silhavy, D., Hornyik, C., and Burgyán, J. (2002). Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. *The Plant Cell Online* 14, 359-372.

Tameling, W.I., and Joosten, M.H.A.J. (2007). The diverse roles of NB-LRR proteins in plants. *Physiological and Molecular Plant Pathology* 71, 126-134.

Thakur, V., Wanchana, S., Xu, M., Bruskiewich, R., Quick, W., Mosig, A., and Zhu, X.G. (2011). Characterization of statistical features for plant microRNA prediction. *BMC Genomics* 12, 108.

Thomas, R., Fang, X., Ranathunge, K., Anderson, T.R., Peterson, C.A., and Bernards, M.A. (2007). Soybean root suberin: anatomical distribution, chemical composition, and relationship to partial resistance to *Phytophthora sojae*. *Plant Physiology* 144, 299-311.

Tyler, B.M. (2006). *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313, 1261-1266.

Tyler, B.M. (2007). *Phytophthora sojae*: root rot pathogen of soybean and model oomycete. *Molecular Plant Pathology* 8, 1-8.

Uozumi, N., and Schroeder, J.I. (2010). Ion channels and plant stress: Past, Present, and Future. *Ion Channels and Plant Stress Responses*, Humana Press, pp. 1-22.

Vidal, E.A., Araus, V., Lu, C., Parry, G., Green, P.J., Coruzzi, G.M., and Gutiérrez, R.A. (2010). Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 107, 4477-4482.

Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. *Nature Reviews Genetics* 6, 206-220.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell* 136, 669-687.

West, C.A. (1981). Fungal elicitors of the phytoalexin response in higher plants. *Naturwissenschaften* 68, 447-457.

Win, J., Krasileva, K.V., Kamoun, S., Shirasu, K., Staskawicz, B.J., and Banfield, M.J. (2012). Sequence divergent RXLR effectors share a structural fold conserved across plant pathogenic oomycete species. *PLoS Pathogens* 8, e1002400.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Annals of Botany* 95, 707-735.

Wrather, J.A., and Koenning, S.R. (2006). Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology* 38, 173.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. *PLoS Biology* 2, e104.

Yang, S., Tang, F., Gao, M., Krishnan, H.B., and Zhu, H. (2010). R gene-controlled host specificity in the legume-rhizobia symbiosis. *Proceedings of the National Academy of Sciences* 107, 18735-18740.

Yang, Z., Ebright, Y.W., Yu, B., and Chen, X. (2006). HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2′ OH of the 3′ terminal nucleotide. *Nucleic Acids Research* 34, 667-675.

Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R., and Chen, X. (2005). Methylation as a crucial step in plant microRNA biogenesis. *Science* 307, 932-935.

Zhai, J., Jeong, D.H., De Paoli, E., Park, S., Rosen, B.D., Li, Y., Gonzalez, A.J., Yan, Z., Kitto, S.L., Grusak, M.A., Jackson, S.A., Stacey, G., Cook, D.R., Green, P.J., Sherrier, D.J., and Meyers, B.C. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes & Development* 25, 2540-2553.

Zhang, B., Pan, X., and Stellwag, E.J. (2008). Identification of soybean microRNAs and their targets. *Planta* 229, 161-182.

Zhang, B., Wang, Q., and Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. *Journal of Cellular Physiology* 210, 279-289.

Zhang, W., Gao, S., Zhou, X., Chellappan, P., Chen, Z., Zhou, X., Zhang, X., Fromuth, N., Coutino, G., and Coffey, M. (2011). Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Molecular Biology* 75, 93-105.

Zhang, X., Xia, J., Lii, Y.E., Barrera-Figueroa, B.E., Zhou, X., Gao, S., Lu, L., Niu, D., Chen, Z., and Leung, C. (2012). Genome-wide analyses of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. *Genome Biology* 13, R20.

Zhou, L., Mideros, S.X., Bao, L., Hanlon, R., Arredondo, F.D., Tripathy, S., Krampis, K., Jerauld, A., Evans, C., St Martin, S.K., Maroof, M.A.S., Hoeschele, I., Dorrance, A.E., and Tyler, B.M. (2009). Infection and genotype remodel the entire soybean transcriptome. *BMC Genomics* 10, 49.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Current Opinion in Plant Biology* 12, 414-420.

CHAPTER 2. MIR393 CONTRIBUTES TO SOYBEAN RESISTANCE AGAINST *PHYTOPHTHORA SOJAE*

2.1 ABSTRACT

Small RNAs have essential regulatory roles in gene expression in eukaryotes. Plants produce two major classes of small RNAs called microRNAs (miRNAs) and small interfering RNAs (siRNAs). Studies have shown that miRNAs are central players in plant development, metabolism and stress response. In this chapter I investigated the function of the conserved miRNA, miR393, in soybean during infection by the oomycete pathogen *Phytophthora sojae*. *P. sojae* causes soybean stem and root rot, the second most devastating disease of soybean. My previous experiments revealed an increase in miR393 abundance in *P. sojae*-infected soybean roots. Here, I analyzed the function of miR393 using the Short Tandem Target Mimic (STTM) technology. By expressing the STTM construct in soybean hairy roots, I successfully knocked down the levels of mature miR393. Importantly, these roots are highly susceptible to *P. sojae*, suggesting that miR393 is a positive regulator of soybean defense. I further studied the mechanism by which miR393 regulates defense response against *P. sojae* and found that roots with decreased miR393 also exhibit largely reduced expression of genes required for phytoalexin production. This study demonstrates an important function of miR393 in soybean defense against *P. sojae*, probably by regulating the production of phytoalexins.

2.2 INTRODUCTION

2.2.1 Small RNAs regulate plant defense

Small RNAs have essential regulatory roles in various important plant processes including development, metabolism, and responses to biotic and abiotic stresses (Bartel 2004; Bartel 2009; Chen 2009; Kulcheski et al., 2011; Khraiwesh et al., 2012). miRNA changes have been shown to occur during bacterial, fungal and oomycete infection (Navarro et al., 2006; Park et al. 2007; Ruiz-Ferrer and Voinnet, 2009; Guo et al., 2011). *Arabidopsis thaliana* infected with the bacterial pathogen *Pseudomonas syringae* exhibits increased expression of miRNAs that target phytohormone pathways including auxin, asbscisic acid and jasmonates (Navarro et al., 2006; Dunoyer et al., 2006; Fahlgren et al., 2007). In particular, the bacterial flagellin (flg22) induces the abundance of the conserved miRNA miR393, which is a positive regulator of defense to bacterial infection in *Arabidopsis* (Navarro et al., 2006; Dunoyer et al,. 2006).

Recent evidence in our laboratory suggests small RNAs may regulate the defense response in soybean during the infection of the oomycete pathogen *Phytophthora sojae*. Firstly, two *P. sojae* effectors can suppress small RNA biogenesis and promote infection (Qiao et al., 2013). Furthermore, small RNA profiling in soybean roots inoculated with *P. sojae* identified ten miRNAs that are differentially expressed at the early infection stage. In particular, miR393, along with six other miRNAs, was up-regulated in *P. sojae*-infected soybean roots. Finally, miR393 could also be induced by the cell wall prep of *P. sojae*. All together, these pieces of evidence indicate a potential role of miR393 in PAMP-induced immunity

(PTI). In this Chapter, I performed experiments to determine the function of miR393 in soybean defense during *P. sojae* infection.

2.2.2 miR393 regulates auxin response

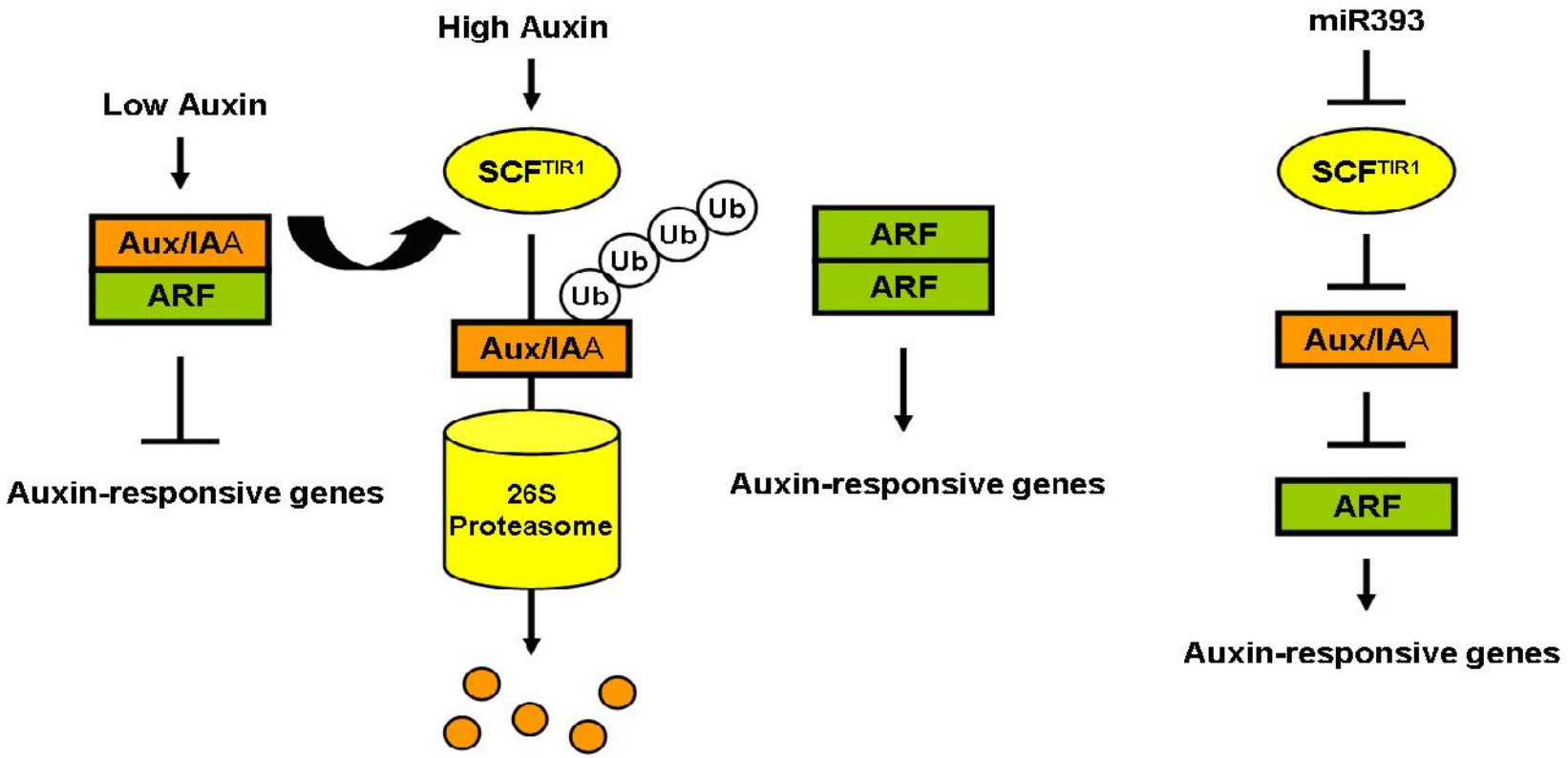
miR393 is a conserved miRNA found in multiple plant species and known to target the key negative regulators of the auxin signaling pathway. Auxin is an essential phytohormone that is synthesized from tryptophan and processed to indole-3-acetic acid (IAA) (Bartel, 1997). It is required for cell elongation, and the maintenance of the primary meristems and axillary meristems. Auxin transport and localization influence organogenesis, structural plasticity, apical dominance, and phototrophism. As such, this hormone contributes to numerous plant processes in plant growth and development (Reed et al., 1998; Brady et al., 1998; Teale et al., 2006; Paponov et al., 2006; Mockaitis and Estelle, 2008).

A major regulatory mechanism of auxin signaling in plants involves the transcriptional repressors Aux/IAA. In the absence of IAA, Aux/IAA dimerizes with Auxin Response Factors (ARFs) to inhibit the transcriptional activation of auxin responsive genes. Auxin promotes the protein-protein interaction of Aux/IAA with the Transport Inhibitor Response1 (TIR1) protein and the Auxin Signaling F-box (AFB) protein, which are subunits of the SKIP/CULLIN/F-BOX (SCF)-type ubiquitin ligases. TIR1/AFB then target Aux/IAA for ubiquitinylation and the subsequent degradation by the 26S proteosome. Following auxin triggered degradation of Aux/IAA, ARFs form homodimmers and activate auxin response gene expression (Figure 2.1) (Gray et al., 2001; Kepinski et al., 2001; Mallory et al., 2005; Bartel et al., 2005; Tan et al., 2007).

The auxin signaling pathway is subjected to regulation by several miRNAs. miR160 and miR167 target auxin response factors. ARF1, ARF10, ARF16 and ARF17 are regulated by miR160, while ARF6 and ARF8 are regulated by miR167 (Rhoades et al., 2002; Rubio-Somoza et al., 2009). miR393 directly targets the transcription repressors TIR1/AFB. Increased expression of miR393 leads to the cleavage of TIR1/AFB transcripts, thereby suppressing auxin signaling (Navarro et al., 2006; Feng et al., 2010; Vidal et al., 2010; Si-Ammour et al., 2011).

Figure 2.1 Auxin signaling pathway and regulation by miR393.

When auxin concentrations are low, Aux/IAA repressors associate with the ARF transcriptional activators and repress expression of auxin responsive genes. When auxin concentrations increase, auxin binds to the TIR1 receptor in the SCF^{TIR1} complex, leading to recruitment of Aux/IAA to the SCF^{TIR1} complex. The Aux/IAA repressors are subjected to ubiquitinylation-mediated degradation and the auxin signaling pathway is activated.miR393 mediated regulation of the auxin signaling pathway suppresses


High Auxin
Low Auxin
SCFTIR1
Aux/IAA
ARF
Ub
Ub
Ub
Aux/IAA
26S
Proteasome
Auxin-responsive genes
ARF
ARF
Auxin-responsive genes
miR393
SCFTIR1
Aux/IAA
ARF
Auxin-responsive genes

2.2.3 Auxin is manipulated during pathogen infection

The role of auxin in cell growth makes it a valuable target for biotrophic pathogens that feed on plant nutrients. Auxin production and transport have been shown to be manipulated during pathogen infection. Many plant pathogens, such as *Agrobacterium* spp. and *Pseudomonas* spp produce indole-3-acetamide (IAM), an intermediate in the IAA synthesis pathway. Auxins produced by bacteria can induce plant growth and the leakage of nutrients, which benefit bacterial multiplication (Camilleri and Jouanin, 1991; Glickmann et al., 1998).

High levels of auxin can enhance disease progression in *Arabidopsis* plants infected with the bacterial pathogen *P. syringae* (Navarro et al., 2006; Chen et al., 2007; Kazan and Manners, 2009). Elevated levels (approximately three folds) of auxin have been observed in *Arabidopsis* infected with *P. syringae* strain DC3000 at 48 and 96 hours post inoculation (Chen et al., 2007). Auxin accumulation was also observed in rice and *Arabidopsis* infected by another bacterial pathogen *Xanthomonas oryzae* (Fett et al., 1987; O'Donnell et al., 2003; Ding et al., 2008).

Since auxin seems to be a negative regulator of plant defense, plants employ miRNAs to suppress the auxin pathway in response to pathogen infection to enhance resistance. miR160, miR167 and miR393 were all found to be induced in *Arabidopsis* after the infection of *P. syringae* (Fahlgren et al., 2007; Li et al., 2010). miR393 can be induced after exposure to the bacterial flagellin, which is a major Pathogen-associated molecular pattern (PAMP) of *P. syringae.* Furthermore, over-expression of miR393 enhances plant defense, suggesting that the induction of these miRNAs is a basal defense mechanism against bacterial infection (Navarro et al., 2006; Navarro et al., 2008). Consistently, *Arabidopsis* mutants *dcl1* and *hen1*, which lack essential

enzymes in miRNA biogenesis, showed compromised resistance to *P. syringae* infection, confirming an important role of miRNAs in plant defense (Navarro et al., 2008).

2.2.3 miR393 regulates antimicrobial metabolite production in plants

Although the direct targets of miR393 are believed to be the auxin regulators TIR1/AFB, miR393 was found to also regulate secondary metabolism in plants, presumably in an indirect manner.

Plants produce antimicrobial compounds in response to pathogen infection. Well known examples of antimicrobial metabolites are phytoalexins, which contribute to defense against many phytopathogenic organisms. Phytoalexins have been shown to possess antimicrobial activities against viruses, bacteria, fungi and *Phytophthora* (Yoshikawa et al., 1978; Kuc, 1995; Osbourn, 1999; Brunner et al., 2002).

Studies on signaling pathways that regulate the major *Arabidopsis* phytoalexin, camalexin, revealed variable effects on plant resistance depending on the specific infecting pathogen (Glazebrook, 2005; Ahuja et al., 2012). Camalexin is an indole derivative. Camalexin biosynthetic genes are induced in response to the bacterial pathogen *P. syringae* (Zhou et al., 1999; Heck et al., 2003) and necrotrophic fungal pathogens, including *Sclerotinia sclerotiorum*, *Alternaria brassica*e and *Botrytis cinerea* (Zhou et al., 1999; Ferrari et al., 2003; Walley et al., 2008; Rowe et al., 2010; Stotz et al., 2011). However, a defect in camalexin production did not affect *Arabidopsis* resistance to *P. syringae*; although mutants impaired in camalexin accumulation allowed enhanced disease progression of the necrotrophic fungal pathogens (Zhou et al., 1999; Ferrari et al., 2003; Heck et al., 2003; Stotz et al., 2011).

Recent studies have suggested that miR393 could indirectly modulate secondary metabolism in infected *Arabidopsis* plants to enhance the production of antimicrobial compounds that specifically target biotrophic pathogens (Robert-Seilaniantz et al., 2011). Over expression of miR393 suppresses auxin signaling and directs metabolic synthesis toward glucosinates and away from camalexin. This leads to increased resistance to biotrophic pathogens, such as *P. syringae*, but decreased resistance to necrotrophs (Kazan and Manners, 2009; Robert-Seilaniantz et al., 2011).

Soybean produces glyceollins, another major class of plant phytoalexins that are isoflavonoid derivatives. Glyceollin production is induced by *P. sojae* infection, and the external application of glyceollins inhibits *P. sojae* growth in artificial media (Lozovaya et al., 2004; Lozovaya et al., 2007; Lygin et al., 2010). Glyceollin synthesis requires the enzymes isoflavone synthase (IFS) and chalcone reductase (CHR). Silencing of *IFS* or *CHR* in soybean leads to decreased glyceollin levels and enhanced susceptibility to *P. sojae* infection (Graham et al., 2007).

Because miR393 affects the accumulation of camalexin in *Arabidopsis* (Robert-Seilaniantz et al., 2011), it is interesting to examine whether miR393 can regulate glyceollin production in soybean during *P. sojae* infection.

2.3 MATERIALS AND METHODS

2.3.1 Soybean hairy roots induction

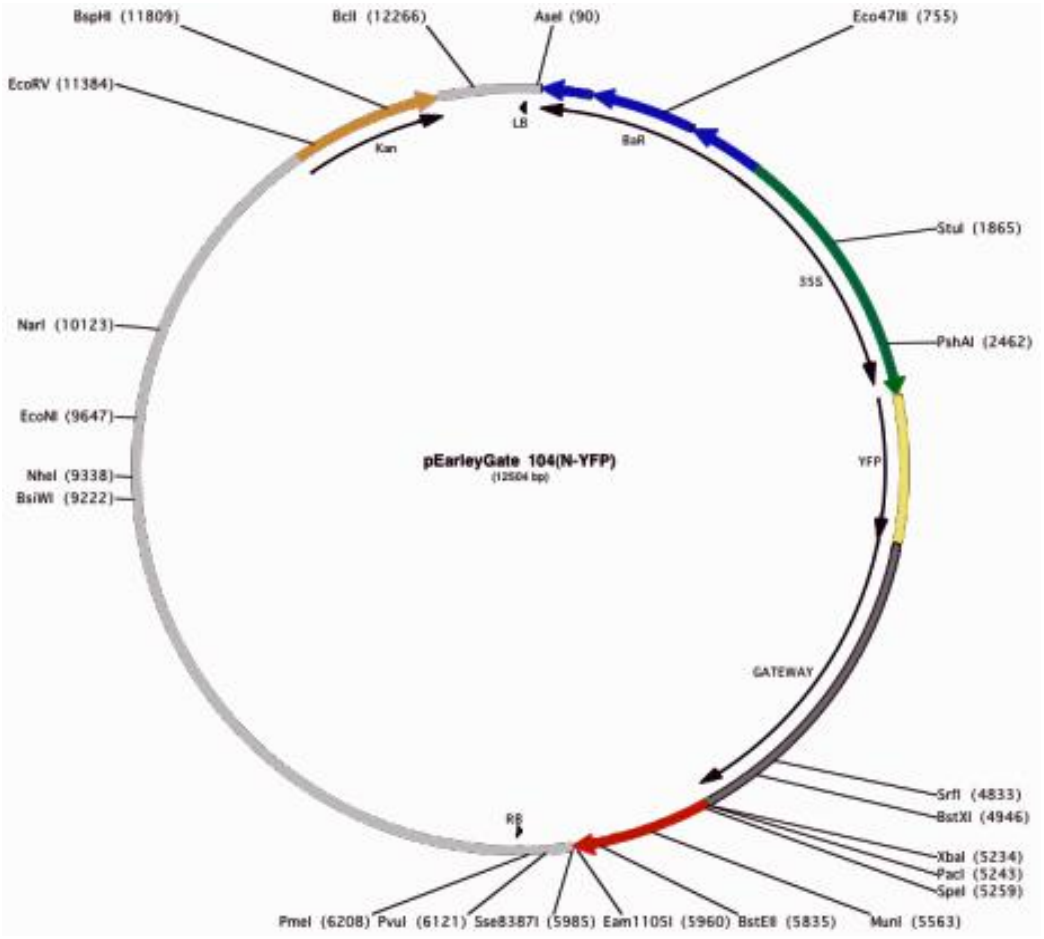
Soybean hairy roots were induced by *Agrobacterium rhizogenes* strain K599. *A. rhizogenes* was grown and maintained with Luria-Bertani medium and supplemented with antibiotics for plasmid maintenance. Transformation of *A. rhizogenes* was performed using the freeze-thaw method as previous described (Chen et al., 1994). *A. rhizogene* K599 cells in a 1.5 mL centrifuge tube were frozen in liquid nitrogen for 1 minute and thawed at 37ºC. One μg of plasmid DNA is added to the competent cells, which were incubated at 30ºC for 5-6 hours with shaking at 200 rpm. The transformation mix was then plated on medium supplemented with antibiotics to select for transformants.

A. rhizogenes K599 containing the plasmid pEG104 (Figure 2.2) or the STTM393 construct were grown in Luria-Bertani broth at 25 ºC for 2 days. The cells were collected by centrifugation at 10,000 rpm and re-suspended in 10 mM $MgSO_4$ to a final OD_{600} of 0.3. The cell suspensions were then used for the inoculation of soybean cotyledons and the induction of hairy following an established protocol (Subramanian et al., 2005).

Soybean cotyledons were removed from 5-7 day old seedlings and surface sterilized by wiping with 70% ethanol. A circular incision was made near the petiole end of each cotyledon and the wounded cotyledons were placed in a petri plate containing 1% water agar. 20 μL of the *A. rhizogenes* cell suspension were spotted on the wounds. Cotyledons were either inoculated with individual *A. rhizogenes* cell suspensions or a 1:1 mixture of *A. rhizogenes* carrying STTM393 or pEG104

respectively. The plates were wrapped in Parafilm to maintain high humidity and incubated in a plant growth chamber at 22ºC on a 12 hr. light cycle. Hairy roots development was monitored during a period of four weeks and the roots that exhibited yellow fluorescence under a fluorescent microscope (Leica Microsystems) were used for northern blotting and *P. sojae* infection.

Figure 2.2 pEARLEYGATE binary vector utilized in the transformation of *A. rhizogenes.*


BspHI (11809)
BclI (12266)
AseI (90)
Eco47III (755)
EcoRV (11384)
LB
Kan
BsR
StuI (1865)
35S
NarI (10123)
PshAI (2462)
EcoNI (9647)
pEarleyGate 104(N-YFP)
(12504 bp)
YFP
NheI (9338)
BsiWI (9222)
GATEWAY
SrfI (4833)
BstXI (4946)
RB
XbaI (5234)
PacI (5243)
SpeI (5259)
PmeI (6208)
PvuI (6121)
Sse8387I (5985)
Eam1105I (5960)
BstEII (5835)
MunI (5563)

2.3.2 Construction of the STTM393 construct

The STTM393 construct was obtained from Dr. Guiliang Tang (Michigan Technological University). The construct is composed of two tandem gma-miR393 binding sites linked by a 88 nt spacer (Tang et al., 2012; Yan et al., 2012). The spacer was synthesized first and used as template to amplify the core sequence of STTM393 using the forward primer, CATTTGGAGAGGACAGCCCAAGCTTGATCAATGCGACTATCCCTTTGGAG TTGTTGTTGTTATGGTCTAG, and reverse primer, CTGGTGATTTCAGCGTACCGAATTCTCCAAAGGGATAGTCGCATTGATCAT TCTTCTTCTTTAGACCATA. The gma-miR393 binding site contains miR393 perfect complementary binding sequences with the exception of the "CUA" bulges that prevent miR393 mediated cleavage of the STTM393 transcript (Figure 2.3). The core sequence of STTM393 was inserted between the *35S* promoter and the Nos-polyA terminator in a reconstructed pCAMBIA1300 vector (Figure 2.4). The recombinant plasmid was transformed into *A. rhizogenes* K599 for hairy root induction.

Figure 2.3 A diagram showing the design of STTM393 construct.

The STTM393/393 construct is composed of two tandem binding sites for miR393 that are linked by a 88 nt spacer. The STTM393/393 is expressed using a 35S promoter and a Nos-polyA terminator. The miR393 binding sites on STTM393/393 contain miR393 perfect binding sequences except the “CUA” bulge that prevents the cleavage of STTM393/393 by miR393. Binding of STTM393/393 by miR393 leads to the degradation of mature miR393 in plants.

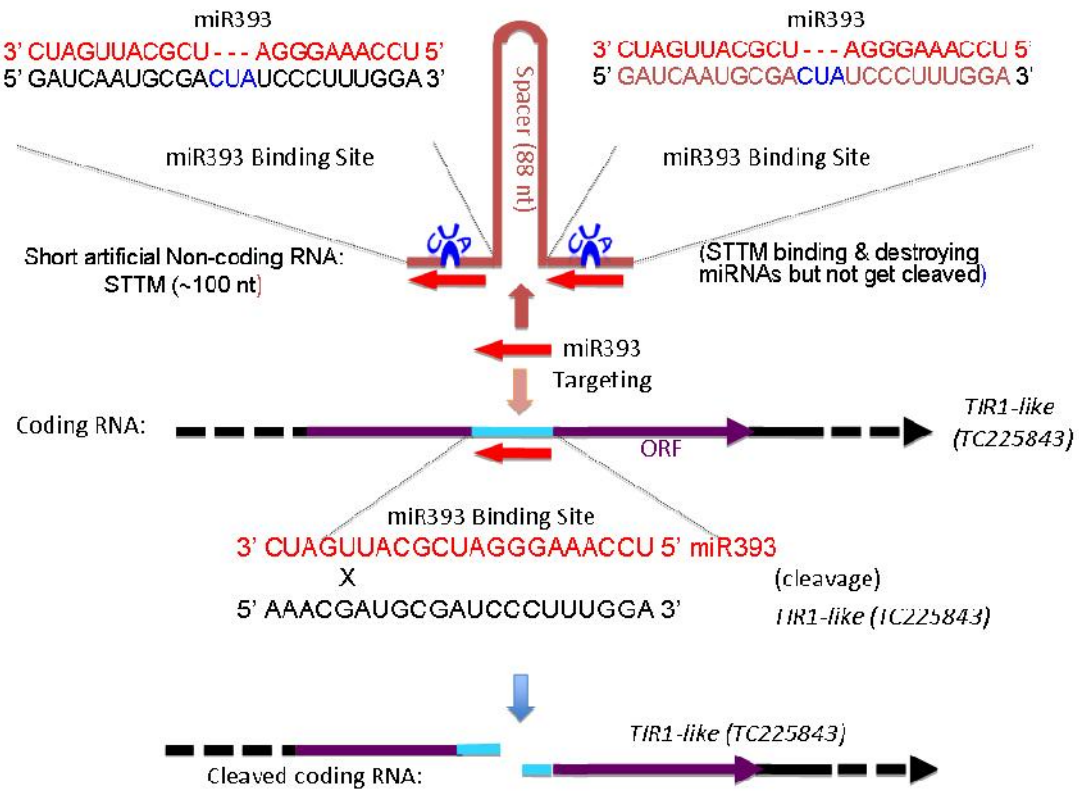
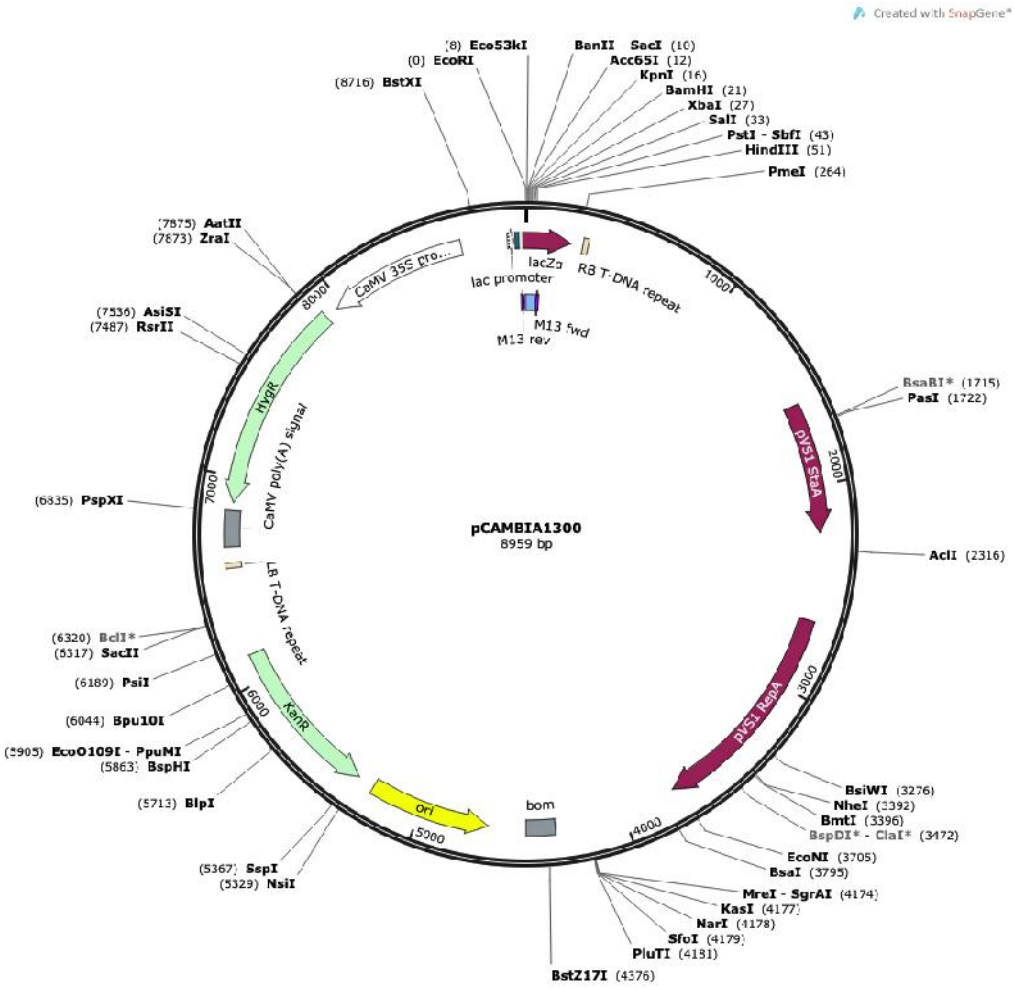

miR393
3' CUAGUUACGCU - - - AGGGAAACCU 5'
5' GAUCAAUGCGACUAUCCCUUUGGA 3'
miR393
3' CUAGUUACGCU - - - AGGGAAACCU 5'
5' GAUCAAUGCGACUAUCCCUUUGGA 3'
Spacer (88 nt)
miR393 Binding Site
miR393 Binding Site
Short artificial Non-coding RNA:
STTM (~100 nt)
(STTM binding & destroying
miRNAs but not get cleaved)
miR393
Targeting
Coding RNA:
ORF
TIR1-like
(TC225843)
miR393 Binding Site
3' CUAGUUACGCUAGGGAAACCU 5' miR393
X
5' AAACGAUGCGAUCCCUUUGGA 3'
(cleavage)
TIR1-like (TC225843)
TIR1-like (TC225843)
Cleaved coding RNA:

Figure 2.4 Physical map of the binary vector, pCambia1300, which houses the STTM393 construct.


Created with SnapGene®
(8) Eco53kI
(0) EcoRI
(8716) BstXI
BanII SacI (10)
Acc65I (12)
KpnI (16)
BamHI (21)
XbaI (27)
SalI (33)
PstI - SbfI (43)
HindIII (51)
PmeI (264)
(7875) AatII
(7873) ZraI
(7536) AsiSI
(7487) RsrII
(6835) PspXI
(6320) BclI*
(5317) SacII
(6189) PsiI
(6044) Bpu10I
(5905) Eco0109I - PpuMI
(5863) BspHI
(5713) BlpI
(5367) SspI
(5329) NsiI
BstZ17I (4376)
PluTI (4181)
SfoI (4179)
NarI (4178)
KasI (4177)
MreI - SgrAI (4174)
BsaI (3795)
EcoNI (3705)
BspDI* - ClaI* (3472)
BmtI (3396)
NheI (3392)
BsiWI (3276)
AclI (2316)
PasI (1722)
BsaBI* (1715)
lacZα
lac promoter
M13 fwd
M13 rev
RB T-DNA repeat
CaMV 35S pro...
hygR
CaMV poly(A) signal
LB T-DNA repeat
KanR
ori
bom
pVS1 StrA
pVS1 RepA
pCAMBIA1300
8959 bp
1000
2000
3000
4000
5000
6000
7000
8000

2.3.3 Evaluation of the transcript abundance of isoflavone biosynthetic genes

Quantitative RT-PCR (qRT-PCR) was performed using iQ™ SYBR® Green Supermix and a CFX96 Real-Time PCR Detection System (BioRad). Total RNA was extracted using Trizol, and DNA was removed with DNase I (Fermentas). 5 μg of total RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative PCR was performed using gene specific primers for *GmHID1* (Glyma01g45020) and *GmIFS1* (Glyma07g32330). *GmUBI* (Glyma20g27950) was used as the internal control.

	Forward Primer	**Reverse Primer**
GmHID1	GGCGAAGGAGATAGTGAAAGAGC	GTGTGGTGGGATTTGGGAAGG
GmIFS1	ACCGAGGAGCTTCTCAAATG	TGAAGTCAGTGAGGCTGTATTC
GmUbi3	GACCAGCAGCGGCTGATTT	GGACAAGGTGAAGGGTTGAT

2.3.4 P. sojae infection assay on hairy roots

Hairy roots expressing YFP or YFP+STTM393 were removed from the cotyledons and inoculated with *P. sojae* zoospores induced from a *P. sojae* P6497 strain carrying the plasmid pTOR::*GFP*, which confers constitutive expression of GFP (Yuanchao Wang, unpublished data). *P. sojae* was grown for three days in Petri-dishes containing 10% V8 broth at 25°C. The mycelia were rinsed twice, and then flooded with sterile distilled water overnight at 25°C to release the zoospores. Detached hairy roots were immersed in the zoospore suspension (approximately 5000 zoospores/μL) for 60 seconds.

Inoculated roots were placed in 1% water agar plates and incubated at room temperature in the dark. Disease progression was monitored at 8, 24, 36, and 48 hpi. The number of roots that allowed oospore development were numerated using a Leica MZFLIII stereo microscope. The biomass of *P. sojae* hyphae was also determined at 48 hpi by quantitative PCR using primers amplifying the *Phytophthora cox* spacer region and the *actin* gene (Duo et al., 2008; Grünwald et al., 2011).

	Forward primer	**Reverse primer**
PsCOX	AAAAGAGAAGGTGTTTTTTATGGA	GCAAAAGCACTAAAAATTAAATATAA
PsACT	ACTGCACCTTCCAGACCATC	CCACCACCTTGATCTTCATG

2.4 RESULTS

2.4.1 Repression of miR393 in soybean hairy roots expressing STTM393

The up-regulation of miR393 in multiple plant species following pathogen exposure indicates a conserved mechanism wherein miR393 induction promotes resistance to pathogens. I hypothesized that a reduction of gma-miR393 would enhance *P. sojae* infection of soybean plants. To evaluate the contributions of miR393 in soybean defense against *P. sojae*, we employed the Short Tandem Target Mimic (STTM) technique (Tang et al., 2012; Yan et al., 2012) to knock down the mature miR393 levels in soybean roots.

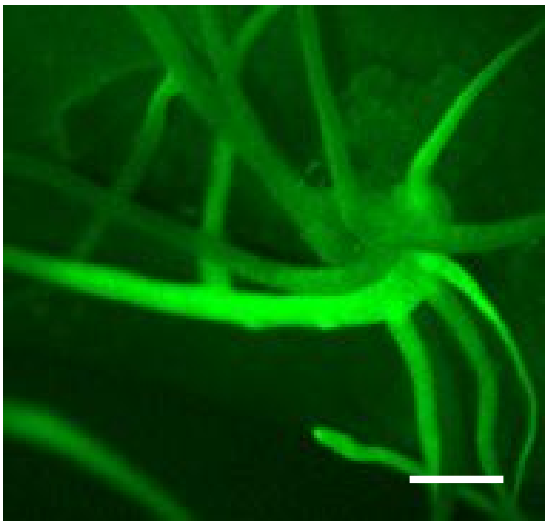
Functional analyses of miRNAs are technically challenging especially when they are generated from multiple *MIR* loci. Soybean has a tetraploid genome with 12 potential *MIR393* loci (Turner et al., 2012); it is therefore impracticable to generate soybean mutants with the *MIR393* knocked out or silenced. We generated hairy roots with reduced levels of mature miR393 using a STTM construct, which contains two binding sites of miR393. The STTM technology is based on the target mimicry phenomenon discovered in *Arabidopsis*, wherein the transcript of *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*) regulates miR399 (Franco-Zorrilla et al., 2007; Yan et al., 2012). The *IPS1* transcript is partially complementary to miR399, leaving a 3 nt bulge between the two RNA strands. As such, *IPS1* transcript can not be cleaved by miR399; rather the formation of *IPS1*/miR399 duplex leads to the suppression of miR399 activity. Similarly, a STTM construct, designed to knock down a specific miRNA, carries two binding sites of the miRNA that can not be degraded by the miRNA. When expressing in plant cells, the STTM transcripts specifically form

complexes with the target miRNA, which triggers the degradation, and thus reduced abundance, of the miRNA.

Using *Agrobacterium rhizogenes*-mediated hairy root system (Subramanian et al., 2005), we generated transgenic soybean roots expressing the *35S-STTM393* construct. Cotyledons were inoculated with *A. rhizogenes* strain K599 harbouring two plasmids, one carrying *35S-STTM393* and the other carrying *35S-YFP*. Roots expressing *35S-STTM393* were isolated based on the expression of yellow fluorescence because it is known that not all hairy roots express the exogenous gene (Figure 2.5). Northern blotting demonstrated that the *STTM393*-expressing roots showed an approximately 70% reduction in the level of miR393 compared to roots only expressing YFP, confirming that the STTM393 construct effectively targeted miR393 for destruction (Figure 2.6).

Figure 2.5 Transformed soybean hairy root are visualized by fluorescent microscopy. Hairy roots were induced by *Agrobacterium rhizogenes* carrying *35S-YFP* or *35S-YFP+35S-STTM393*. Transgenic roots were selected by the production of yellow fluorescence as shown in the images (5X magnification; bar equals 4 mm). Knocking down miR393 seems to affect the development of hairy roots. This is reflected by the observations that fewer hairy roots were generated from soybean cotyledons expressing the *STTM393* construct.

YFP



YFP+
STTM393

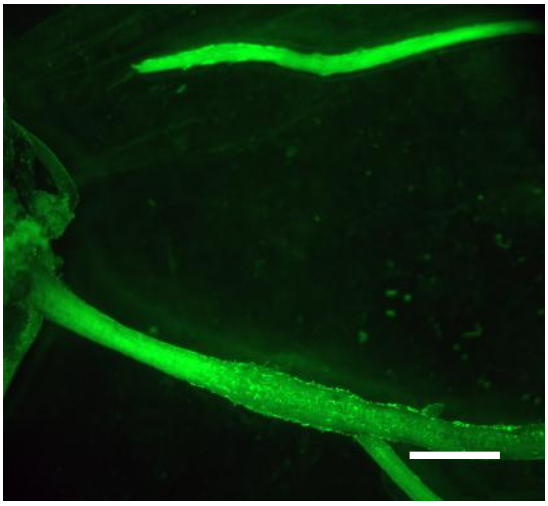
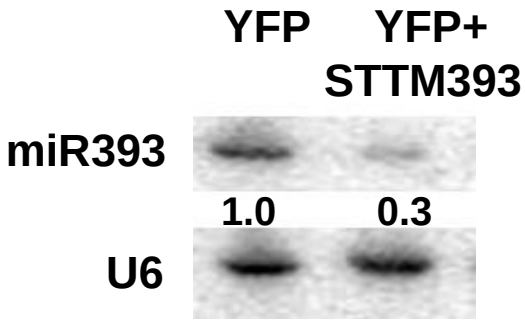


Figure 2.6 miR393 levels were largely decreased in soybean roots expressing the *STTM393* construct.

Northern blotting was used to determine the abundance of miR393 in the transgenic hairy roots. U6 served as a loading control. Numbers below each blot image represents the abundance of the miRNA relative to the mock-infected sample. This experiment was repeated twice with similar results. Mean standard deviation is ±2.


YFP
YFP+ STTM393
miR393
1.0
0.3
U6

2.4.2 miR393 knocked down roots were hypersusceptible to P. sojae

I then examined *P. sojae* infection in the *35S-YFP* and *STTM393*-expressing roots, which were dip-inoculated with zoospore suspensions. I used a transgenic line of *P. sojae* P6497 that expresses green fluorescence protein (GFP) to facilitate the infection progression analysis using microscopy. Numbers of roots that supported hyphae penetration and oospore development at the inoculated area were numerated at 24, 36 and 48 hpi. Note that although Williams 82 is a resistant host, hyphae penetration and oospore development could still be observed in a small region of the inoculated roots, but the infection was restricted to the inoculated tissues in these roots.

My experiments showed that the *STTM393*-expressing roots were hyper-susceptible to *P. sojae*. In Williams 82, I observed both accelerated and enhanced oospore development in roots expressing *STTM393* (Table 2.1 and Figure 2.7). Enhanced susceptibility of *STTM393*-expressing roots was also evident in the susceptible cultivar Harosoy, as the development of oospores was accelerated (Table 2.1). The difference of *STTM393*-expressing roots and the control in Harosoy was not as significant as in Williams 82. This was expected because *P. sojae* P6497 is already a potent pathogen of Harosoy.

Enhanced infection in *STTM393*-expressing roots was also supported by an increase in *Phytophthora* hyphae biomass, which was evaluated in the infected tissues at 48 hpi. The abundance of *P. sojae*-specific DNA *PsCOX* and *PsACT* was quantified using qPCR, which showed an approximately two fold increase in the

STTM393-expressing roots (Figure 2.8). Taken together, these data strongly suggest that miR393 is a positive regulator of soybean defense against *P. sojae* infection.

Table 2.1 miR393 contribute to defense against *P. sojae* infection.

Soybean hairy roots expressing YFP or *STTM393* were inoculated with zoospores of the *P. sojae* strain P6497. Number of infected hairy roots, represented by the formation of *P. sojae* oospores, in the soybean cultivars Harosoy (susceptible) and Williams 82 (resistant) at 48 hours post inoculation.

No. of infected roots in Harosoy

	Repeat 1	**Repeat 2**	**Repeat 3**
YFP	11/11	7/9	17/25
YFP + STTM393	11/12	9/9	18/24

No. of infected roots in Williams 82

	Repeat 1	**Repeat 2**	**Repeat 3**
YFP	11/22	6/15	4/16
YFP + STTM393	15/17	11/12	16/20

Figure 2.7 miR393 is a positive regulator of soybean defense against *P. sojae* infection.

Hairy roots of soybean cultivar Williams 82 expressing YFP or *STTM393* were inoculated with zoospores of the *P. sojae* strain P6497 expressing GFP. Fluorescent microscopy was used to monitor the disease progression of *P. sojae* on transformed hairy roots at 24, 36, and 48 hours post inoculation (hpi). Note that *STTM393*-expressing roots exhibited accelerated and enhanced formation of oospores. The red arrow labels *Phytophthora* hyphae and the yellow arrow labels oospores. Bar equals 1mm.

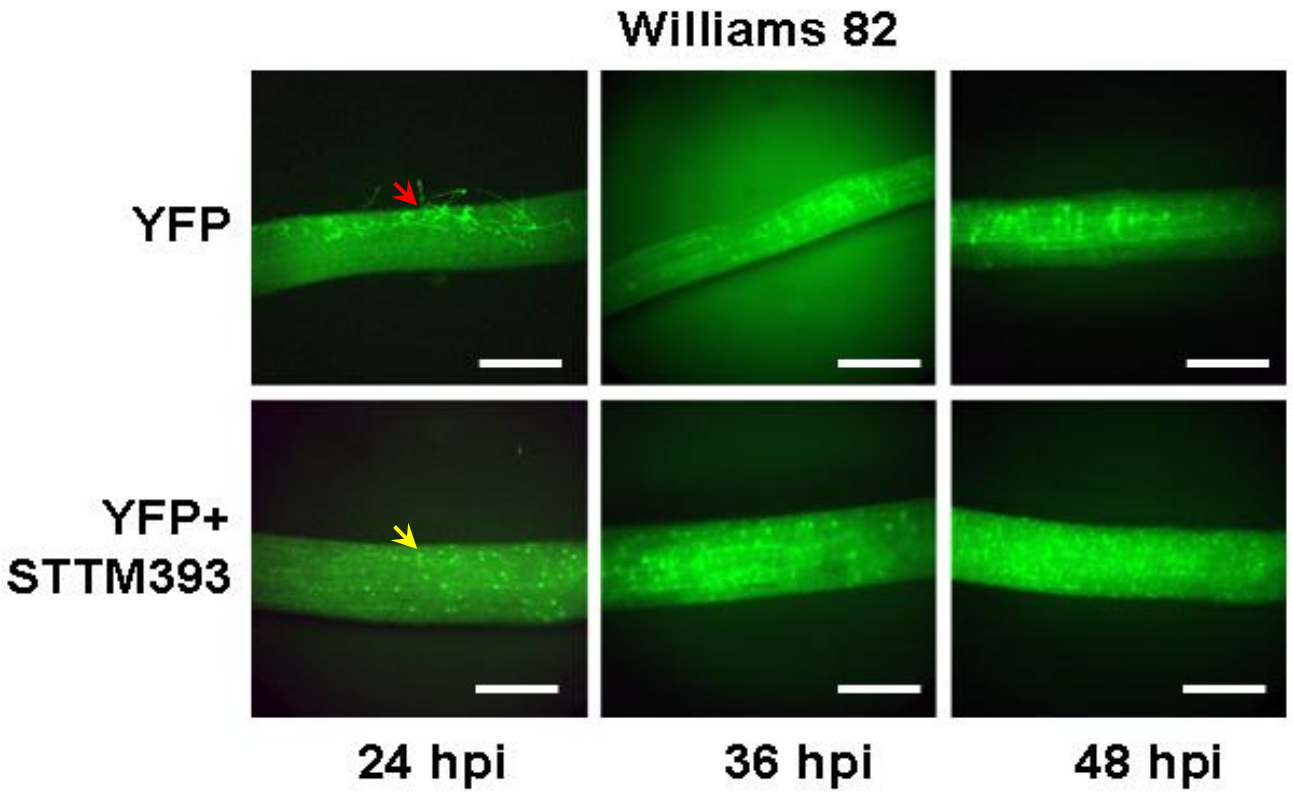
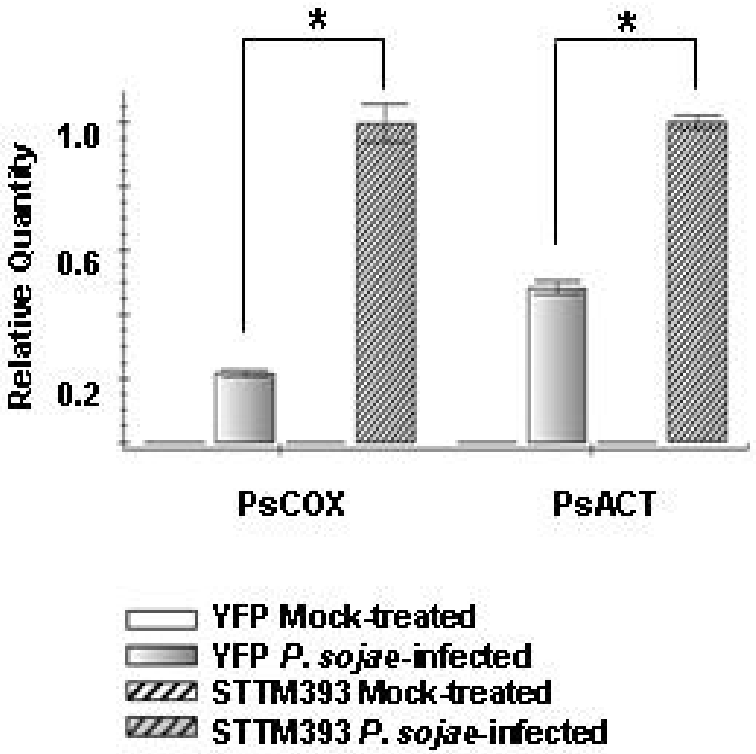

Williams 82
YFP
YFP+
STTM393
24 hpi
36 hpi
48 hpi

Figure 2.8 *STTM393*-expressing soybean roots were hyper-susceptible to *P. sojae*. *P. sojae* biomass was evaluated by qPCR amplifying *P. sojae*-specific DNA fragments *PsCOX* and *PsACT* at 24 hpi. The soybean gene *GmUBI* was used as an internal control. * denotes data with statistically significant differences.


*
*
Relative Quantity
1.0
0.6
0.2
PsCOX
PsACT
YFP Mock-treated
YFP P. sojae-infected
STTM393 Mock-treated
STTM393 P. sojae-infected

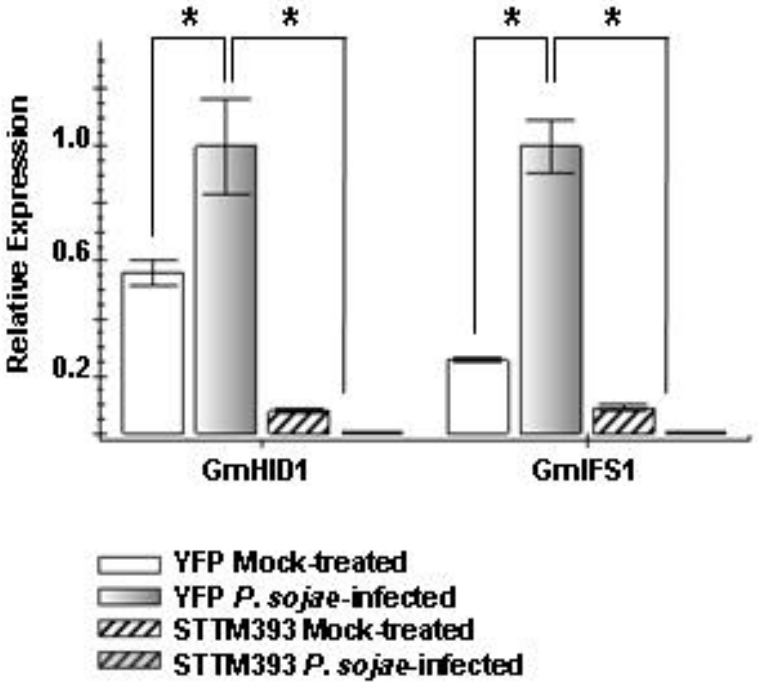
2.4.3 miR393 regulates the expression of isoflavonoid biosynthetic genes

I next seek to understand the underlying mechanism of miR393-mediated resistance in soybean during *P. sojae* infection. miR393 is known to directly repress auxin receptor genes; however, a recent report suggested that miR393 can also modulate secondary metabolism in infected *Arabidopsis* plants to enhance the production of antimicrobial compounds that specifically target biotrophic pathogens (Robert-Seilaniantz et al., 2011). Soybean produces glyceollins that can inhibit *P. sojae* growth (Lygin et al., 2010). I therefore examined potential impacts of miR393 on glyceollin production by evaluating the expression of genes required for the biosynthesis of isoflavones, which are precursors of glyceollins. In particular, I measured the transcript levels of *GmHID1* and *GmIFS1*, which encode enzymes catalyzing two consecutive steps in the isoflavone biosynthetic pathway (Lygin et al., 2010; Lozovaya et al., 2007).

Using qRT-PCR, I found that both *GmHID1* and *GmIFS1* were significantly induced by *P. sojae* at 8 hpi in soybean (Figure 2.9). Remarkably, *STTM393*-expressing roots exhibited drastically reduced expression of these two genes with or without *P. sojae* infection (Figure 2.9). These data demonstrated that miR393 positively regulates isoflavonoid biosynthesis, which is required for the production of glyceollins. A potentially reduced level of glyceollins in the *STTM393*-expressing roots may then contribute, at least partially, to the enhanced susceptibility to *P. sojae*.

Figure 2.9 miR393 regulates isoflavone synthesis.

STTM393-expressing soybean roots exhibited reduced expression of *GmHID1* and *GmIFS1* as determined by qRT-PCR. Values represent averages from two independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).


*
*
*
*
Relative Expression
1.0
0.6
0.2
GmHID1
GmIFS1
YFP Mock-treated
YFP P. sojae-infected
STTM393 Mock-treated
STTM393 P. sojae-infected

2.5 DISCUSSION

The highly conserved miR393 primarily regulates plant development through targeted degradation of TIR1/AFB auxin receptors and repression of auxin signaling. The role of miR393 in plant defense has also been reported in *Arabidopsis*. Over-expression of miR393 in *Arabidopsis* leads to enhanced resistance against the bacterial pathogen *P. syringae* (Navarro et al., 2006) and the oomycete pathogen *Hyaloperonospora parasitica* (Robert-Seilaniantz et al., 2011). Accumulation of miR393 was triggered by exposure to microbial PAMPs, such as flagellin of *P. syringae* (Navarro et al., 2006). My previous results showed that miR393 is also induced in soybean root after exposure to heat-inactivated *P. sojae*, suggesting that miR393 is induced by *Phytophthora* PAMPs. Cell surface proteins of *Phytophthora* PEP-13 and glucans can trigger plant defense responses, such as the production of phytoalexin and the activation of the salicylic acid signaling pathway (Brunner et al., 2002). Although we do not know which PAMP in *P. sojae* heat-inactivated cells is responsible for the induction of miR393, my results are consistent with a role of miR393 in plant basal defense.

Target mimicry proves to be a useful tool in determining function of specific miRNAs. Using the STTM393 construct, we effectively knocked down the levels of mature miR393 in soybean roots. Consistent with the prior observations in *Arabidopsis* during bacterial infection, soybean with reduced miR393 levels exhibited significantly enhanced susceptibility to *P. sojae*. Taken together, these findings suggest that miR393 positively regulates plant defense against bacterial, fungal and

oomycete pathogens (Navarro et al., 2006, Zhang et al., 2011, Robert-Seilentz et al., 2011).

As a result from the reduced miR393 levels, *P. sojae* mycelium invaded the *STTM393*-expressing root tissue at an earlier time point and developed a greater number of oospores when compared to roots only expressing YFP. It is likely that miR393 positively regulates the expression of defense-associated genes in plants to attenuate pathogen infection. Some of these genes might be involved in the regulation and biosynthesis of phytoalexins in the plant. Plants produce phytoalexins as antimicrobial compounds. In soybean, the major phytoalexins are glyceollins, which are produced from isoflavones as precursors. External application of glyceollins inhibits the growth of *P. sojae* in vitro (Lygin et al., 2010). My data showed that the isoflavone biosynthetic genes, and likely the production of isoflavones, are induced in soybean roots at the early stage of *P. sojae* infection. These data suggest a direct inhibitory effect of glyceollins on *P. sojae*. In the *STTM393*-expressing roots, two key genes in the isoflavone biosynthetic pathway were no longer induced by *P. sojae*. Suppression of miR393 activity likely leads to the inability of the plants to accumulate glyceollins, and may subsequently increase their susceptibility to *P. sojae* infection.

Taken together, my experiments suggest that miR393 plays an important role as a positive regulator of basal defense in soybean against *P. sojae*. Further experiments using soybean hairy roots over-expressing miR393 will provide additional evidence to confirm this conclusion.

2.6 REFERENCES

Ahuja, I., Kissen, R., and Bones, A.M. (2012). Phytoalexins in defense against pathogens. *Trends in Plant Science* 17(2): 73-90.

Bartel, B. (1997). Auxin biosynthesis. *Annual Review of Plant Biology* 48(1): 51-66.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116(2): 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136(2): 215-233.

Brunner, F., Rosahl, S., Lee, J., Rudd, JJ., Geller, C., Kauppinen, S., Rasmussen, G., Scheel, D., and Nurnberger, T. (2002). Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases. *The EMBO Journal* 21(24): 6681-6688.

Camilleri, C., and Jouanin, L. (1991). The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine-type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. *Molecular Plant-Microbe Interact* 4: 155-162.

Chen, H., Nelson, R., and Sherwood, J.L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. *Biotechniques* 16(4): 664-668, 670.

Chen, X. (2009). Small RNAs and their roles in plant development. *Annual Review of Cell and Developmental Biology* 25(1): 21-44.

Chen, Z., Agnew, J.L., Cohen., J.D., He, P., Shan, L., Sheen, J., and Kunkel, B.N. (2007). *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proceedings of the National Academy of Sciences* 104(50): 20131-20136.

Ding, X., Cao, Y., Huang, L., Zhao, J., Xu, C., Li, X., and Wang, S. (2008). Activation of the indole-3-acetic acid–amido synthetase GH3-8 suppresses expansin expression and promotes salicylate-and jasmonate-independent basal immunity in rice. *The Plant Cell Online* 20(1): 228-240.

Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Scott, A.G., and Carrington, J.C. (2007). High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. PLoS ONE 2(2): e219.

Feng, X.M., Qiao, Y., Wang, S.S., and Hao, Y.J. (2010). Overexpression of *Arabidopsis* AtmiR393a gene alters the sensitivity to auxin in tomato. *Scientia Horticulturae* 124(2): 165-169.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausubel, F.M. (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *The Plant Journal* 35(2): 193-205.

Fett, W.F., Osman, S.F., and Dunn, M.F. (1987). Auxin production by plant-pathogenic pseudomonads and xanthomonads. *Applied and Environmental Microbiology* 53(8): 1839-1845.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., and Paz-Ares, J. (2007). Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics* 39(8): 1033-1037.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*. 43: 205-227.

Glickmann, E., Gardan, L., Jacquet., S., Hussain, S., Elasri, M., Petit, A., and Dessaux, Y. (1998). Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions* 11(2): 156-162.

Graham, T.L., Graham, M.Y., Subramanian, S., and Yu, O. (2007). RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in *Phytophthora sojae* infected tissues. *Plant Physiology* 144(2): 728-740.

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. *Nature* 414(6861): 271-276.

Guo, N., Ye, WW., Wu, X.L., Shen, D.Y., Wang, Y.C., Xing, H., and Dou, D.L. (2011). Microarray profiling reveals microRNAs involving soybean resistance to *Phytophthora sojae*. *Genome* 54: 954-958.

Heck, S., Grau, T., Buchala, A., Metraux, J.P., and Nawrath., C. (2003). Genetic evidence that expression of NahG modifies defence pathways independent of salicylic acid biosynthesis in the *Arabidopsis–Pseudomonas syringae* pv. tomato interaction. *The Plant Journal* 36(3): 342-352.

Kazan, K., and Manners, J.M. (2009). Linking development to defense: auxin in plant–pathogen interactions. *Trends in Plant Science* 14(7): 373-382.

Khraiwesh, B., Zhu, J.K., and Zhu, J. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1819(2): 137-148.

Kuc, J. (1995). Phytoalexins, stress metabolism, and disease resistance in plants. *Annual Review of Phytopathology* 33(1): 275-297.

Kulcheski, F.R., de Oliveira, L.F., Molina, L.G., Alerão, M.P., Rodrigues, F.A., Marcolino, J., Barbosa, J.F., Stolf-Moreira, R., Nepomuceno, A.L., and Marcelino-Guimarães, F.C., and Margis, R. (2011). Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genomics* 12(1): 307.

Li, Y., Zhang, Q., Zhang, J., Wu, L., Qi, Y., and Zhou, J.M. (2010). Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiology* 152(4): 2222-2231.

Lozovaya, V.V., Lygin, A.V., Zernova, O.V., Li, S., Hartman, G.L., and Widholm, J.M. (2004). Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. *Plant Physiology and Biochemistry* 42(7-8): 671-679.

Lozovaya, V.V., Lygin, A.V., Zernova, O.V., Ulanov, A.V., Li, S., Hartman, G.L., and Widholm, J.M. (2007). Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. *Planta* 225(3): 665-679.

Lygin, A.V., Hill, C.B., Zernova, O.V., Crull, L., Widholm, J.M., Hartman, G.L., and Lozovaya, V.V. (2010). Response of soybean pathogens to glyceollin. *Phytopathology* 100(9): 897-903.

Mallory, A. C., D. P. Bartel, et al. (2005). MicroRNA-directed regulation of *Arabidopsis* AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *The Plant Cell Online* 17(5): 1360-1375.

Mockaitis, K. and Estelle, M. (2008). Auxin receptors and plant development: a new signaling paradigm. *Annual Review of Cell and Developmental Biology* 24: 55-80.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312(5772): 436-439.

Navarro, L., Jay, F., Nomura, K., He, S.Y., and Voinnet, O. (2008). Suppression of the microRNA pathway by bacterial effector proteins. *Science* 321(5891): 964-967.

O'Donnell, P. J., Schmelz, E.A., Moussatche, P., Lund, S.T., Jones, J.B., and Klee, H.J. (2003). Susceptible to intolerance–a range of hormonal actions in a susceptible *Arabidopsis* pathogen response. *The Plant Journal* 33(2): 245-257.

Osbourn, A.E. (1999). Antimicrobial phytoprotectants and fungal pathogens: a commentary. *Fungal Genetics and Biology* 26(3): 163-168.

Park, J.E., Park, J.Y., Kim, Y.S., Staswick, P.E., Jeon, J., Yun, J., Kim, S.Y., Kim, J., Lee, Y.H., and Park, C.M. (2007). GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. *Journal of Biological Chemistry* 282(13): 10036-10046.

Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., Wang, X., Liu, X., Xiang, Q., Jiang, S., Zhang, F., Wang, Y., Judelson, H.S., Chen, X., and Ma, W. (2013). Oomycete pathogens encode RNA silencing suppressors. *Nature Genetics* 45(3): 330-333.

Reed, R.C., Brady, S.R., and Muday, G.K. (1998). Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. *Plant Physiology* 118(4): 1369-1378.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. *Cell* 110(4): 513-520.

Robert-Seilaniantz, A., MacLean, D., Jikumaru, Y., Hill, L., Yamaguchi, S., Kamiya, Y., and Jones, J.D.G. (2011). The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. *The Plant Journal* 67(2): 218-231.

Rowe, H.C., Walley, J.W., Corwin, J., Chan, E.K.F., Dehesh, K., and Kliebenstein, D.J. (2010). Deficiencies in jasmonate-mediated plant defense reveal quantitative variation in *Botrytis cinerea* pathogenesis. *PLoS Pathogens* 6(4): e1000861.

Rubio-Somoza, I., Cuperus, J.T., Weigel, D., and Carrington, J. (2009). Regulation and functional specialization of small RNA-target nodes during plant development. *Current Opinion in Plant Biology* 12(5): 622-627.

Ruiz-Ferrer, V., and Voinnet O. (2009). Roles of plant small RNAs in biotic stress responses. *Annual Review of Plant Biology* 60(1): 485-510.

Si-Ammour, A., Windels, D., Arn-Bouldoires, E., Kutter, C., Ailhas, J., Meins, F., and Vazquez, F. (2011). miR393 and secondary siRNAs regulate expression of the TIR1/AFB2 auxin receptor clade and auxin-related development of *Arabidopsis* leaves. *Plant Physiology* 157(2): 683-691.

Stotz, H.U., Jikumaru, Y., Shimada, Y., Sasaki, E., Stingl, N., Mueller, M.J., and Kamiya, Y. (2011). Jasmonate-dependent and COI1-independent defense responses against *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*: auxin is part of COI1-independent defense signaling. *Plant and Cell Physiology* 52(11): 1941-1956.

Subramanian, S., Graham, M.Y., Yu, O., and Graham, T.L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. *Plant Physiology* 137(4): 1345-1353.

Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* 446(7136): 640-645.

Tang, G., Yan, J., Gu, Y., Qiao, M., Fan, R., Mao, Y., and Tang, X. (2012). Construction of short tandem target mimic (STTM) to block the functions of plant and animal microRNAs. *Methods* 58(2): 118-125.

Teale, W.D., Paponov, I.A., and Palme, K. (2006). Auxin in action: signaling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology* 7(11): 847-859.

Turner, M., Yu, O., and Subramanian, S. (2012). Genome organization and characteristics of soybean microRNAs. *BMC Genomics* 13(1): 169.

Vidal, E.A., Araus, V., Lu, C., Parry, G., Green, P.J., Coruzzi, G.M., and Gutiérrez, R.A. (2010). Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 107(9): 4477-4482.

Walley, J.W., Rowe, H.C., Xiao, Y., Chehab, E.W., Kliebenstein, D.J., Wagner, D., and Dehesh, K. (2008). The chromatin remodeler SPLAYED regulates specific stress signaling pathways. *PLoS Pathogens* 4(12): e1000237.

Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X., Chen, X., and Tang, G. (2012). Effective small RNA destruction by the expression of a short tandem target mimic in *Arabidopsis*. *Plant Cell* 24(2): 415-427.

Yoshikawa, M., Yamauchi, K., and Masago, H. (1978). Glyceollin: its rôle in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. *Physiological Plant Pathology* 12(1): 73-82.

Zhou, N., Tootle, T.L., and Glazebrook, J. (1999). *Arabidopsi*s PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *The Plant Cell Online* 11(12): 2419-2428.

CHAPTER 3. PHASED SMALL INTERFERING RNAS (PHASIRNAS) REGULATE PLANT IMMUNITY

3.1 ABSTRACT

Plant innate immunity is a counter measure to prevent infection following the recognition of a potential pathogen. Immune response is initiated following the perception of pathogen-associated molecular patterns, known as PAMPs. PAMP-triggered immunity (PTI) is considered the basal plant defense that protects plants from the majority of potential pathogens. Another branch of plant immunity is effector-trigger immunity (ETI), which is activated when plants detect specific effector proteins secreted from a pathogen into the host cell. Recognition of effectors leads to the activation of intracellular receptors that often contain a conserved nuclear-binding leucine rich repeat (NB-LRR) domain. ETI usually associates with rapid but restricted programmed cell death within the infected region, also known as the hypersensitive response (HR).

Recent research suggests an integral role of plant small RNAs in the regulation of immune response. I analyzed the small RNA profile in soybean roots infected with the oomycete pathogen *Phytophthora sojae*. This analysis revealed specific microRNAs (miRNAs) and small interfering RNAs (siRNAs) that are differentially expressed during infection. Interestingly, I found a significant induction of secondary siRNAs that are produced from miRNA-targeted loci in *P. sojae*-infected plants. These so-called phased siRNAs, or phasiRNAs, mainly regulate genes encoding NB-LRR proteins and the pentatricopeptide repeat (PPR) proteins. Since both classes of proteins are potentially involved in plant immunity, their regulation by miRNAs and phasiRNAs might be an important mechanism in plant defense.

3.2 INTRODUCTION

3.2.1 Plant defense mechanism against pathogens

PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) are two major branches of plant innate immunity that have been evolved to ward off pathogens. PTI, as a basal defense, is induced by the recognition of pathogen-associated molecular patterns (PAMPs), which are conserved and often indispensable elicitors on the cell surface of a pathogen. PAMP recognition is accomplished by the membrane bound pattern recognition receptors (PRR) proteins with extracellular ligand-binding domains (Zipfel and Robatzek, 2010). Activation of PTI elicits defense signal transduction that leads to immune responses including the reactive oxygen species (ROS) production, cell wall fortification and the secretion of antimicrobial compounds (Zhang and Zhou, 2010).

So far, PAMPs have been identified from bacteria, fungi and oomycetes. For example, flagellin, elongation factor Tu (EF-Tu) and lipopolysaccharides (LPS) are well-known PAMPs of bacteria (Zhang and Zhou, 2010; Zipfel and Robatzek, 2010); chitin, ergosterol and beta-glucans in fungi (Granado et al., 1995; Kishimoto et al., 2011), and PEP-13 and beta-glucans in *Phytophthora* (Brunner et al., 2002; Klarzynski et al., 2000) have also been shown to trigger plant defense response. The best characterized PAMP is the flg22 peptide of the amino terminus of bacterial flagellin. Flg22 directly binds to the *Arabidopsis* PRR FLS2 (Boller and He, 2009; Zipfel, 2009), and together with the co-receptor BAK1, this receptor complex activates defense signaling (Sun et al., 2013).

ETI depends on the recognition of specific effector proteins of pathogens and the activation of their cognate intracellular Resistance (R) proteins (Jones and Dangl,

2006). Pathogens secrete effectors as virulence factors, many of which can enter the host cell. Although the fundamental function of pathogen effectors is to overcome host defense, plants have evolved R proteins, which detect the activities of specific effectors and trigger robust defense response. ETI often lead to a hypersensitive response (HR) with the hallmark of localized cell death in the infected region, which effectively restricts the spread of the pathogen. The recognized effectors are then designated avirulence proteins (Avr).

Canonical R proteins are intracellular receptors containing the nucleotide-binding leucine-rich repeat (NB-LRR) domain. Some R proteins directly bind to the corresponding effector, such as the Pi-ta protein in rice that recognizes AvrPita produced by the fungal pathogen *Magnaporthe oryzae* (Huang et al., 2008; Kamoun, 2007). More often, R proteins recognize the presence of effectors in an indirect manner. The indirect recognition of effectors is illustrated as the Guard Model or the Decoy Model (Dodds and Rathjen, 2010; van der Hoorn and Kamoun, 2008). For example, the type III effectors AvrRpm1, AvrB and AvrRpt2, produced by the bacterial pathogen *P. syringae*, are recognized by NB-LRR proteins RPM1 and RPS2, respectively, in *Arabidopsis*. However, these effectors do not directly interact with the R proteins; rather they all target an *Arabidopsis* protein called RIN4. AvrB and AvrRpm1 induce the phosphorylation of RIN4, which is monitored by RPM1; AvrRpt2 hydrolyzes RIN4 with a cysteine protease activity, and the degradation of RIN4 is monitored by RPS2. The activation of RPM1 and RPS2 due to the modifications of RIN4 leads to ETI; and RIN4 is considered the "guardee" of these R proteins (Day et al., 2005; Ritter and Dangl, 1996).

3.2.2 The role of NB-LRRs in plant immunity

Most R proteins have a C-terminal leucine rich repeat (LRR) domain, a central nucleotide binding (NB) domain and a N-terminal domain that either has a coiled-coil (CC) motif or a toll/interleukin 1 receptor (TIR) domain (Caplan et al., 2008; Eitas and Dangl, 2010; Takken et al., 2006).

The N-terminal TIR1 or CC domains have been shown to suppress the activation of the NB-LRR proteins. For example, a truncated mutant of the *Arabidopsis* R protein RPS5 with the CC domain deleted promotes HR in the absence of its guardee PBS1, which could be modified by a bacterial effector AvrPphB (Qi et al., 2012). In addition, swapping of LRR domain between two different but paralogous tomato R proteins, Mi-1.1 and Mi-1.2, leads to constitutive activation of the NB-LRRs and disease-like symptoms (Hwang and Williamson, 2003; Van Ooijen et al., 2008). The NB-LRR domain is involved in protein-protein interactions. Interaction with the effector or the guardee at the LRR domain triggers a conformational change followed by a second conformation change at the N-terminus of either the TIR or CC domain in order to activate downstream signaling (Takken and Tameling, 2009).

Some NB-LRRs are relocated from the cytoplasm to the nucleus following activation and may directly affect gene expression (Burch-Smith et al., 2007; Shen and Schulze-Lefert, 2007). The *Arabidopsis* TIR-NB-LRRs, RRS1 and RPS4, and the barley CC-NB-LRR MLA10 require nuclear localization to activate defense responses (Burch-Smith et al., 2007; Cheng et al., 2009; Shen and Schulze-Lefert, 2007). RRS1 and RPS4 also contain DNA binding domains and therefore were hypothesized to function as transcription factors to directly induce defense-associated gene expression.

3.2.3 MiRNA regulation of NB-LRR genes

NB-LRR proteins are tightly regulated in plants since their activation often leads to programmed cell death. Over-expression of *NB-LRR* genes causes auto-immune symptoms (Eitas and Dangl, 2010; Gabriëls et al., 2007). Therefore, precise regulation of the expression of *NB-LRR* genes is necessary to ensure proper resistance. Mechanisms of *NB-LRR* gene regulation at the post-transcriptional level include alternative splicing, such as the *Arabidopsis RPS4* (Zhang and Gassmann, 2007), and small RNA-mediated regulation.

One of the first experimental evidence suggesting that small RNAs have significant contributions to *NB-LRR* gene regulation came from a study on the N protein, which confers resistance to the tobacco mosaic virus (TMV) in *Nicotiana benthamiana* (Li et al., 2012). Li et al discovered that during TMV infection, *N. benthamiana* plants produce two miRNAs, miR6019 and miR6020, that target the transcripts of the *N* gene and lead to their cleavage (Li et al., 2012).

Following this discovery, several other miRNAs were also identified to regulate *NB-LRR* genes. One of them is miR482, which is a conserved miRNA family that is predicted to regulate a variety of targets including *NB-LRR* genes (Li et al., 2010; Shivaprasad et al., 2012; Zhai et al., 2011). The miR482 family has approximately 30 isoforms in multiple plant species (Shivaprasad et al. 2012). miR482 potentially regulates at least 58 *NB-LRR* targets in tomato (Eckardt, 2012; Li et al., 2012; Shivaprasad et al., 2012) and it was down-regulated in tomato plants infected with Turnip Crinkle Virus (TCV), Cucumber Mosaic Virus (CMV) and Tobacco Rattle Virus (TRV) (Shivaprasad et al., 2012). I identified one *NB-LRR* gene

that is potentially targeted by miR482 in soybean. My experiments showed that miR482 level was also reduced in soybean roots infected with the oomycete pathogen *Phytophthora sojae* (Chapter 1 of this thesis). On the contrary, miR482 was induced in soybean during nodulation (Li et al., 2012). These results indicate that miR482 may regulate plant defense response through its regulatory activity of *NB-LRR* genes.

My analysis on the small RNA profiles in soybean roots infected with the *P. sojae* revealed several miRNAs that primarily regulate *NB-LRR* genes and are induced during infection. Four miRNAs, miR1507, miR1509, miR1510 and miR2109 were all found to be up-regulated in *P. sojae*-infected roots based on small RNA sequencing data; and the induction of miR1507 and miR2109 was also confirmed by northern blotting. Furthermore, I also observed reduced expression of several predicted *NB-LRR* targets of miR1507 and miR2109 in *P. sojae*-infected soybean roots, consistent with the increased abundance of the miRNAs. These data demonstrate that *NB-LRR* genes are subjected to the regulation by multiple miRNAs in soybean during pathogen infection.

3.2.4 phasiRNA production from miRNA-targeted NB-LRR loci

An interesting perspective of NB-LRR targeting miRNAs is that many of them are 22 nt in length, although plant miRNAs are predominantly 21 nt long. 22 nt miRNAs can trigger the production of secondary siRNAs from their target transcripts. These siRNAs are referred as phasiRNAs because they are in 21 nt register from one another (Allen et al., 2005; Chen, 2010; Cuperus et al., 2010; McHale et al., 2013).

Secondary siRNAs are a class of small siRNAs produced by miRNA directed cleavage of a *TAS* or *PHAS* transcript (Allen et al., 2005; Cuperus et al., 2010;

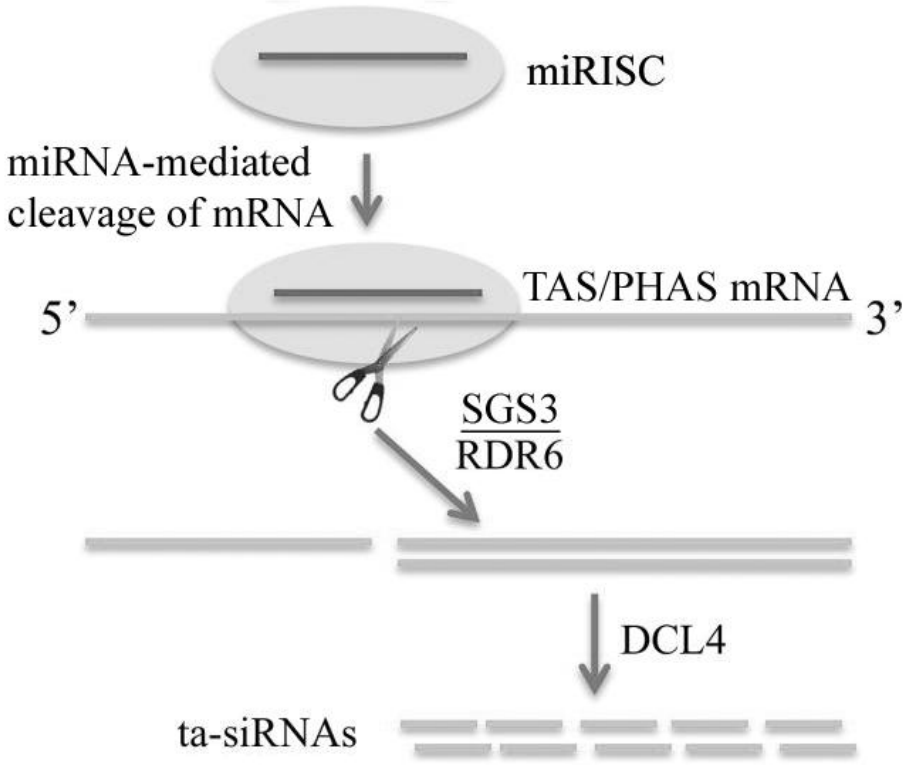
McHale et al., 2013; Vazquez et al., 2004; Yoshikawa et al., 2005). In *Arabidopsis*, there are four *TAS* gene families, whose transcripts can be targeted by the 22 nt miRNAs miR173, miR390 or miR828 (Cuperus et al., 2010). The *TAS* transcripts are then recruited to the RISC complex (Fei et al., 2013), where the miRNA-mediated cleaved transcript fragments are used as templates for the synthesis of double-stranded RNAs by RNA POLYMERASE 6 (RDR6). DICER-LIKE 4 (DCL4) then cleaves the dsRNA into 21 nt siRNAs. These siRNAs can regulate the expression of genes with restricted sequence similarity with their parent loci in trans and therefore designated trans-acting siRNAs (tasiRNAs). The biosynthesis of phasiRNAs are the same with that of the tasiRNAs, but they may regulate gene expression in *cis* and/or in *trans* (Zhai et al., 2011). Genes that generate phasiRNAs are called *PHAS* loci. (Figure 3.1) (Cuperus et al., 2010; Gasciolli et al., 2005; Howell et al., 2007).

Specific phasiRNAs target plant defense genes, including *NB-LRR* (Cuperus et al., 2010). It is suggested that a major class of *PHAS* loci are *NB-LRR* genes in legumes (Shivaprasad et al., 2012; Zhai et al., 2011). Approximately 70% of the identified *PHAS* loci in the model legume *Medicago truncatula* encode potential NB-LRR proteins. Most of the phasiRNA-generating *NB-LRR* genes in *M. truncatula* are targets of miR1507, miR2109 and miR2118 (Zhai et al. 2011). As such, these miRNAs could be central regulators of a potentially large number of *NB-LRR* genes.

Intriguingly, both miR1507 and miR2109 were found to be up-regulated in *P. sojae*-infected soybean roots (Chapter 1 of this thesis). These results lead to our hypothesis that the induction of phasiRNAs from *NB-LRR* genes may potentially lead to regulation of a large number of *NB-LRR* genes during *P. sojae* infection.

Figure 3.1 Biogenesis pathways for tasiRNAs and phasiRNAs.

miRNA-directed cleavage of a *TAS* or *PHAS* transcript provides templates for RDR6-dependent synthesis of dsRNA, which is further processed by DCL4 into 21-nt in-phased siRNAs (adapted from Chen, 2012).


miRISC
miRNA-mediated
cleavage of mRNA
TAS/PHAS mRNA
5'
3'
SGS3
RDR6
DCL4
ta-siRNAs

3.2.5 Pentatricopeptide repeat (PPR) genes contribute to plant defense

Another gene family that has been known to be regulated by secondary siRNAs in plants encodes proteins containing the Pentatricopeptide Repeat (PPR) motif. The *TAS1* and *TAS2* tasiRNAs, generated by the 22 nt miR173 (Cuperus et al., 2010; Fei et al., 2013; Howell et al., 2007; Yoshikawa et al., 2013), target several *PPR* genes (Allen et al., 2005; Howell et al., 2007). There are approximately 450 *PPR* genes in *Arabidopsis*, comprising of approximately 1% of the nuclear genomes. Although the function of most PPR proteins have yet to be determined, some of them were demonstrated to be involved in regulating mitochondrial and chloroplast functions (Fujii and Small, 2011; Schmitz-Linneweber and Small, 2008).

Recent findings showed that PPR proteins play critical roles in post transcriptional regulation of organelle gene expression through their RNA editing activity (Kobayashi et al., 2012; Kotera et al., 2005; Lurin et al., 2004; O'Toole et al., 2008). The PPR domains in different PPR proteins share sequence similarity and arise in related gene regions, suggesting that they may have evolved from a small number of ancestral genes and subjected to conditions that promoted gene expansion and diversification. Similarly, *NB-LRR* proteins are also highly expanded and diversified in plants (Caplan et al., 2008; Eitas and Dangl, 2010; Shivaprasad et al., 2012). It is intriguing to hypothesize that phasiRNA-mediated regulation is an effective strategy to regulate large gene families.

PPR proteins have also been implicated to regulate plant defense. In *Arabidopsis*, PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl (PGN) positively regulates resistance against the necrotrophic fungal pathogen *Botrytis cinerea*. Loss of *PGN* function results in enhanced susceptibility to

B. cinerea infections (Laluk et al., 2011). In addition, the *Arabidopsis* LOVASTATIN INSENSITIVE1 (LOI1) is a PPR protein that regulates isoflavonoid biosynthesis in response to *B. cinerea* infection (Kishimoto et al., 2005; Kobayashi et al., 2007). *LOI1* mutant plants have decreased sensitivity to isoflavones and the phytotoxin lovastatin resulting in enhanced susceptibility to *B. cinerea* (Kishimoto et al, 2005; Kobayashi et al., 2007).

Recently, our laboratory identified an effector from *P. sojae*, called Phytophthora Suppressor of RNA silencing 2 (PSR2), that specifically interferes with the biogenesis of tasiRNAs produced from *TAS1* and *TAS2* in *Arabidopsis* (Qiao et al., 2013). PSR2 significantly promotes *Phytophthora* infection, indicating that phasiRNAs regulating *PPR* gene expression could contribute to defense response in plants.

3.3 MATERIALS AND METHODS

3.3.1 Soybean germination and growth

Harosoy and Williams 82 seedlings were sterilized in 10% bleach and germinated in a petri dish lined with moist filter paper. Seedlings were incubated in the dark at room temperature for approximately one week. Germinated seeds were transferred to sterile pouches infused with B&D nutrient solution and grown in a growth chamber (28ºC at a 12 hour light cycle) for about two weeks until the primary roots were approximately two inches in length (Subramanian et al., 2008).

Phytophthora sojae strain P6497 is cultured at room temperature in the dark on V8 media for approximately one week. Soybean roots were infected with *P. sojae* by placing mycelial plugs on top of the roots as previously described (Zhou et al., 2009). Inoculated roots were placed in the dark for 8 and 24 hours before the infected tissues were collected for RNA extraction. Mock-treated roots were treated in the same way, but with sterile agar plugs.

3.3.2 Total RNA Extraction

Up to 200 mg of root tissue was used for total RNA was extracted from *P. sojae*-infected or mock-treated root tissues by Trizol (Invitrogen) as described in Chapter 1.3.2. RNA quality and concentration was determined by spectrophotometer (Thermo Scientific Nanodrop 1000).

3.3.3 Identification of phasiRNAs

PhasiRNA analysis was performed as described previously (De Paoli et al., 2009). We identified 21-nt in-phase signatures from the small RNA libraries. The numbers of signatures were counted within 210 bp (10 phases) through the University of Delaware Legume Next-Gen Sequence DataBases soybean sRNA database (http://mpss.udel.edu/soy_sbs/). Genomic regions with phasing scores greater than 15 were considered as *PHAS* loci. These regions were then mapped to the soybean genome sequence to determine gene identities.

3.3.4 Northern blotting of small RNAs

Five μL of total RNA was fractionated by polyacrylamide urea gels and then transferred to a Hybond-NX nylon membrane. The RNA samples were hybridized with γ-^{32}P-labelled probes with sequences complementary to specific miRNAs. miRNA probes were generated from synthesized oligonucleotides with the antisense sequence of targeted miRNAs. phasiRNA probes were generated using amplified 300-500 bp PCR products that encompass the phasiRNA-generating regions (Table 3.1).

Following overnight hybridization, membranes were placed into a cassette and exposed to a phosphor screen for at least 12 hours. Phosphor screens were scanned with the Molecular Dynamics Typhoon imager and images processed with the ImageQuant TL software (GE Healthcare Life Sciences).

Table 3.1 Primers used for qRT-PCR and the amplification of phasiRNA probes.

Gene ID	Forward Primers	Reverse Primers
***Pentatriopeptide Repeat* (*PPR*) genes**		
Glyma09g07290	GTTTGATGAAGCTTTGGCCATTA	GGAGAAGTTTCTCGGCCTTATC
Glyma16g28020	TTCCTGATGCAGCGACATATAG	TCAGGGAGAATTACTACTGCCTTGAGTC
Glyma16g31950	GATTACTGAGGAAGCTGGAAGG	GAGTACACATCACAGGCATCTC
Glyma16g32030	AAGGGTGGAAGACTTGAGAATG	AACAGGCCCGCTTTACAA
phasiRNA Probes		
Glyma06g39720	TGGGAATTTTCAGAAGAGGAC	GAGATAAAACCCGTAAGAACT
Glyma15g35920	AGGTGATAGTGGAGACCTAGAA	TGTTGTCCTAGATATGGGCATAAA
Glyma03g14900	AAGGACTGTAAGAGCTAATGG	TCCAACAAACGTGTAACATTT
Glyma16g34000	CCATCTTTGAAATGATAGCCAGAC	AATAATGGCTGCGACAACAC

3.3.5 Quantitative RT-PCR of miRNA target transcripts

Prediction of soybean small RNAs target transcripts was performed utilizing the online plant small RNA analysis server: PsRNATarget (http://plantgrn.noble.org/psRNATarget). Small RNA sequences were submitted and scored against the soybean genome with at an expectation cut-off threshold of 3.0. The complementary length range was set to 20 bp and the allowed energy to unpair the target site set to 25UPE.

The abundance of the miRNA target transcripts was determined by qRT-PCR using iQ™ SYBR® Green Supermix and a real time PCR machine (BioRad CFX-96).. Five µg of purified RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega). Gene-specific primers (Table 3.1) were designed to flank the predicted miRNA-binding site of the target genes. *GmUBI* was used as the internal control.

3.4 RESULTS

3.4.1 miR1507 and miR2109 regulate *NB-LRR* genes and trigger phasiRNA production during *P. sojae* infection

Among the miRNAs that were differentially accumulated in the *P. sojae*-infected soybean roots, miR1507 and miR2109 are interesting because they are predicted to target *NB-LRR* genes. Consistent with the enhanced accumulation of miR1507 and miR2109, several *NB-LRR* genes that are predicted to be targeted by these miRNAs were repressed during early *P. sojae* infection (Table 1.7, Figure 1.13).

miR1507 is a conserved 22 nt miRNA in legumes that is known to trigger the production of phasiRNAs from their target transcripts in soybean. Consistent with previous reports, I found that miR1507 was exclusively 22 nt in length in soybean with or without *P. sojae* infection (Table 1.3). To examine the production of miR1507-dependent phasiRNAs, we calculated the Phasing score (Zhai et al., 2011) of each miR1507 target locus using the Illumina sequencing data. This analysis revealed two phasiRNA-generating *NB-LRR* loci *Glyma04g29220* and *Glyma06g39720*, from which the production of phasiRNAs was particularly evident in the *P. sojae*-infected roots (Fig 3.2). I further confirmed the enhanced accumulation of phasiRNAs derived from *Glyma06g39720* in *P. sojae*-infected tissues using northern blotting (Figure 3.4). These siRNAs are clearly "phased" in 21 nt intervals from the predicted cleavage site of miR1507 (Figure 3.5).

phasiRNAs could act in cis to repress the target loci or in trans to regulate a potentially large number of genes. Consistent with the induction of both miR1507 and

the phasiRNAs, *Glyma06g39720* exhibited a much greater level of reduction in transcript abundance compared to another miR1507-targeted *NB-LRR* gene *Glyma15g35920* (Figure 3.3), from which the production of phasiRNAs was not detected (Figure 3.2, Figure 3.5). These data suggest that miR1507-depedent phasiRNA production was induced to regulate *NB-LRR* gene expression during *P. sojae* infection.

Figure 3.2 phasiRNA-generating loci were identified from miR1507 target NB-LRR genes. Phasing scores were calculated from all the predicted target loci of these two miRNAs with three representative loci from each miRNA presented in the graphs. Phasing scores >15 are considered as PHAS loci.

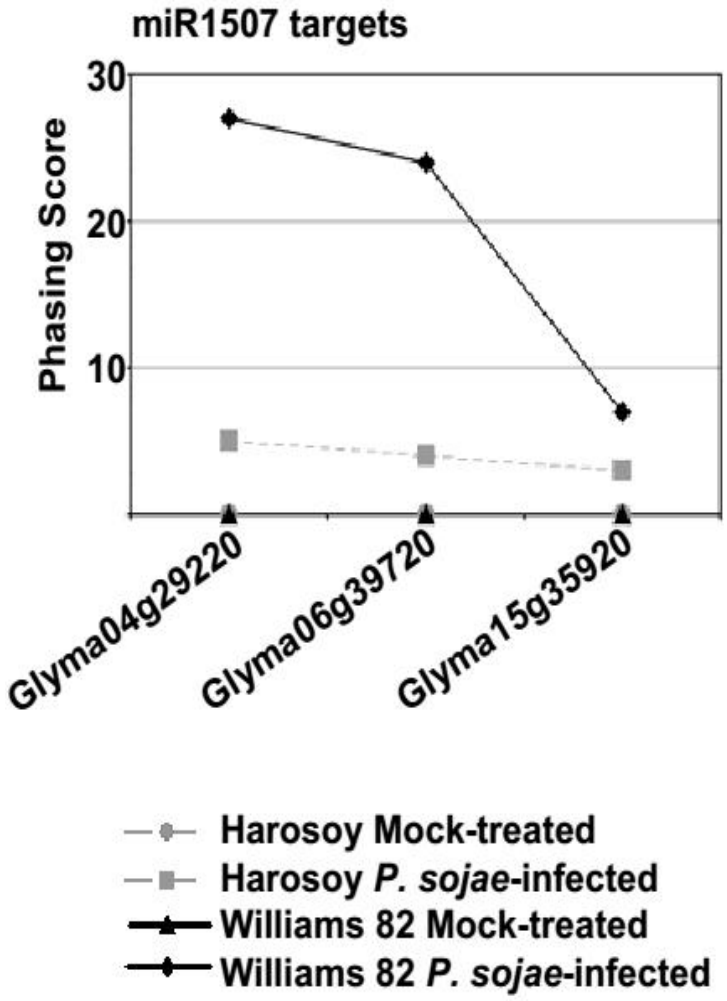

miR1507 targets
Phasing Score
30
20
10
Glyma04g29220
Glyma06g39720
Glyma15g35920
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 3.3 *NB-LRR* genes targeted by miR1507 were repressed during *P. sojae* infection. Relative transcript abundances were evaluated with qRT-PCR using *GmUBI* as an internal control. Values represent averages from two independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).

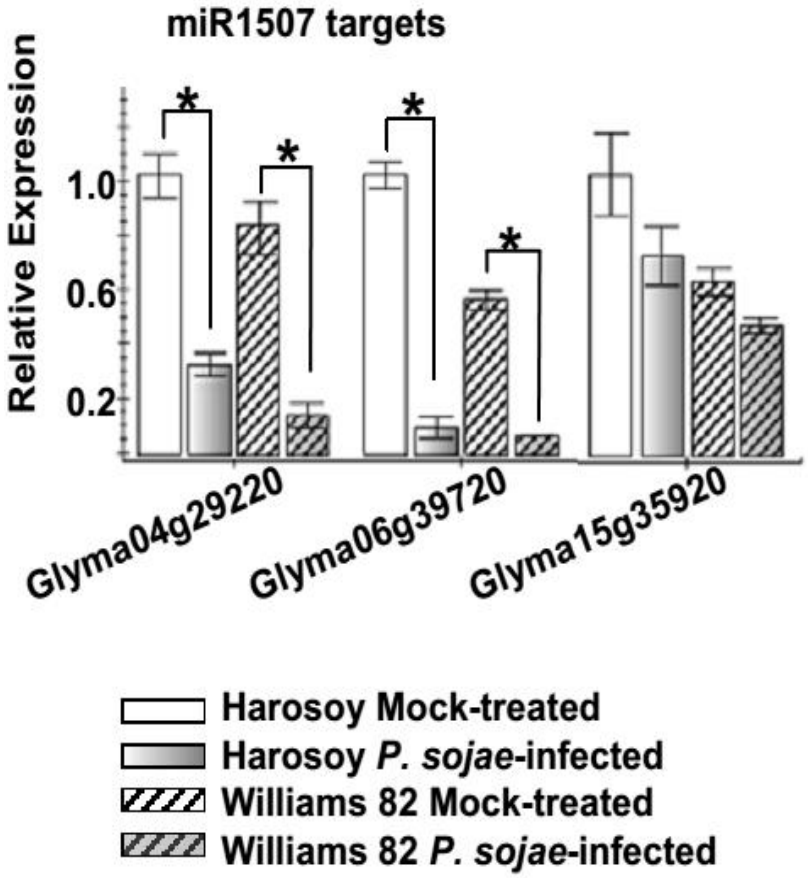

miR1507 targets
Relative Expression
1.0
0.6
0.2
*
*
*
*
Glyma04g29220
Glyma06g39720
Glyma15g35920
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 3.4 miRNA1507 and phasiRNAs derived from specific miR1507 target *NB-LRR* loci were induced during *P. sojae* infection. The mean standard deviation for miR1507 and *Glyma06g39720* is ±2.

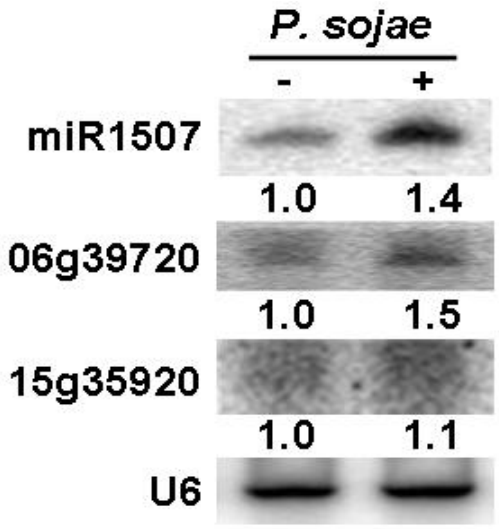
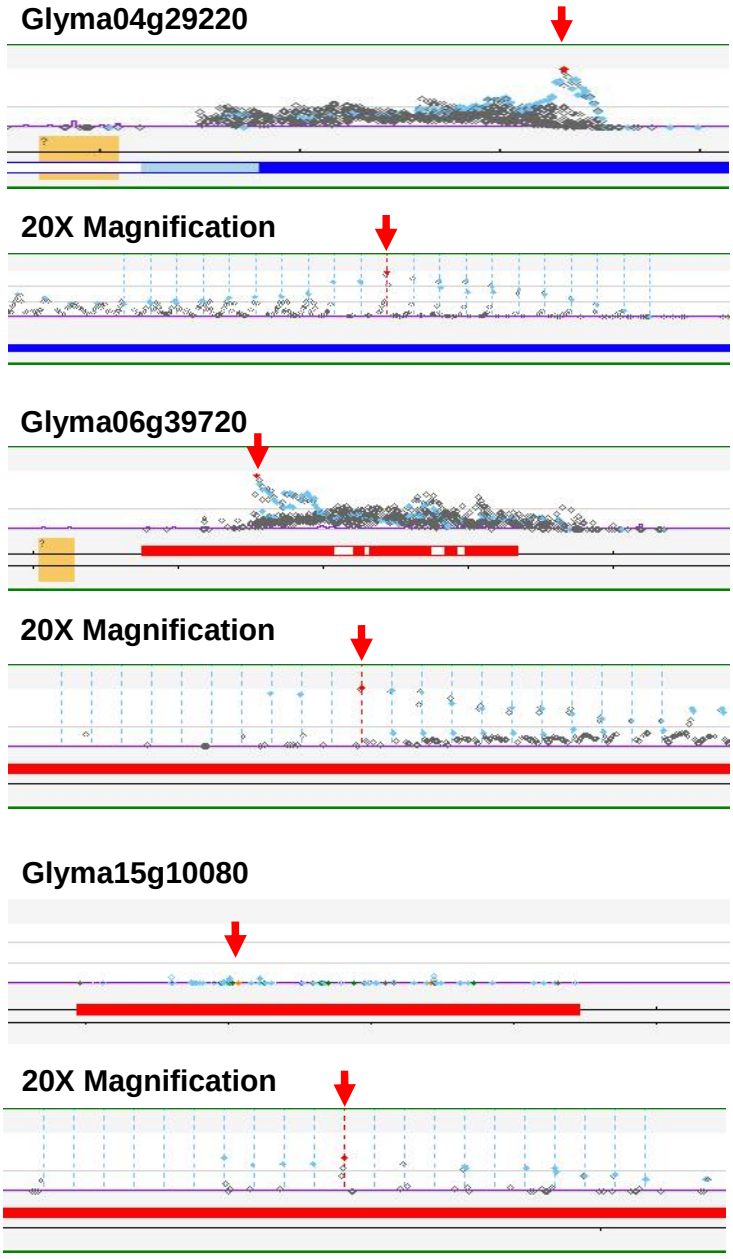

P. sojae
-
+
miR1507
1.0
1.4
06g39720
1.0
1.5
15g35920
1.0
1.1
U6

Figure 3.5 phasiRNAs generated from the miR1507 target *Glyma04g29220* show 21 nt in-phase signatures following the cleavage sites (shown as the red arrows) of their parent miRNA. phasiRNAs are shown as blue dots in a ten cycle interval of 21 nt.


Glyma04g29220
20X Magnification
Glyma06g39720
20X Magnification
Glyma15g10080
20X Magnification

Although produced by both soybean and *Medicago truncatula*, miR2109 was thought to be 22 nt in *M. truncatula*, but predominantly 21 nt in soybean (Zhai et al., 2011). Our sequencing data and northern blots detected both 21 and 22 nt forms of miR2109 in soybean, and both forms were induced during *P. sojae* infection (Figure 3.6). The presence of the 22 nt form raised the possibility that miR2109 may also trigger the production of phasiRNAs from its target *NB-LRR* loci in soybean.

Indeed, small RNA sequencing data revealed three *NB-LRR* genes that may be targeted by miR2109 and potentially produce 21 nt in-phased siRNAs (Figure 3.7, Figure 3.8). I confirmed the production of phasiRNAs from one *NB-LRR* gene (*Glyma03g14900*), especially during *P. sojae* infection (Figure 3.6). Similar to the miR1507 target *Glyma06g39720*, the transcript level of *Glyma03g14900* was also significantly lower than other miR2109-targeted loci *Glyma16g10080* and *Glyma16g34000* (Figure 3.9), from which we did not detect significant phasiRNA production (Figure 3.6, Figure 3.7). These data indicate that the expression of *Glyma03g14900* may be under the regulation of both miR2109 and the corresponding phasiRNAs.

Figure 3.6 miR2109 and the phasiRNAs derived from specific target *NB-LRR* loci were induced during *P. sojae* infection. Mean standard deviations for miR2109 and *Glyma03g14900* are ±3 and ±4, respectively.

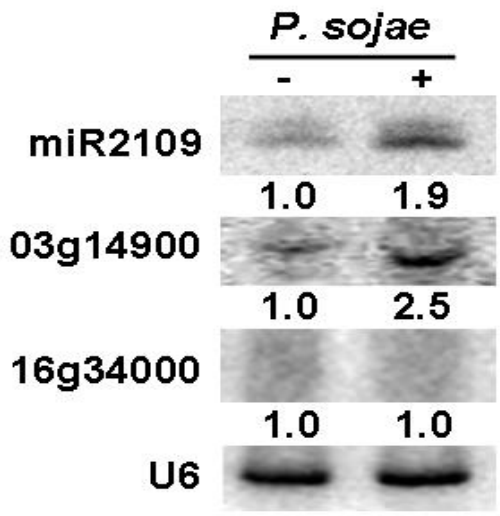

P. sojae
-
+
miR2109
1.0
1.9
03g14900
1.0
2.5
16g34000
1.0
1.0
U6

Figure 3.7 phasiRNA-generating loci were identified from miR2109 target *NB-LRR* genes. Phasing scores were calculated from all the predicted target loci of miR2109 with three representative loci presented in the graph. Phasing scores >15 are considered as *PHAS* loci.

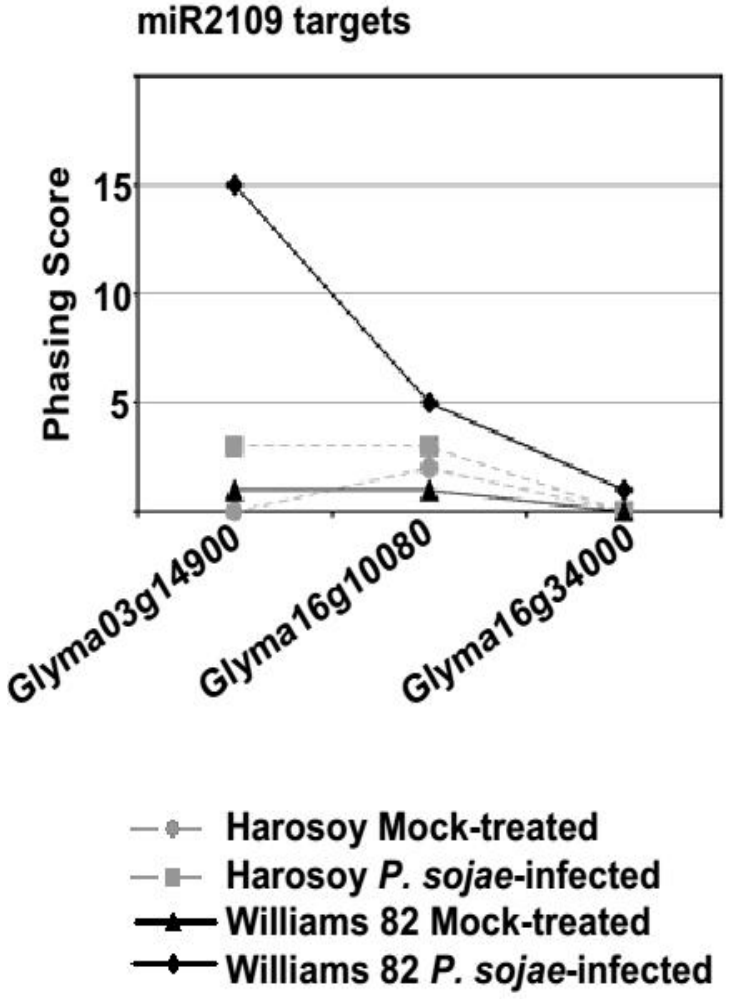
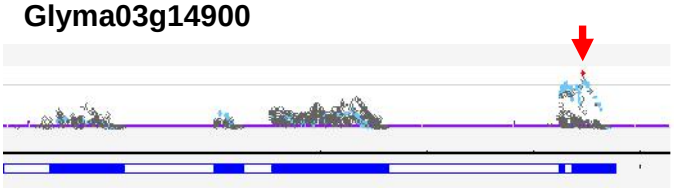
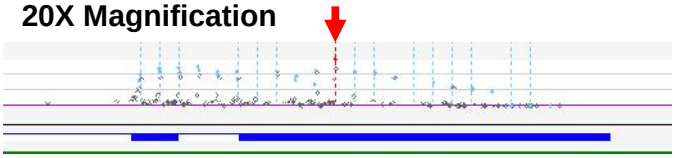

miR2109 targets
Phasing Score
15
10
5
Glyma03g14900
Glyma16g10080
Glyma16g34000
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Figure 3.8 phasiRNAs generated from the miR2109 target *Glyma03g14900* show 21 nt in-phase signatures following the cleavage sites (shown as the red arrows) of their parent miRNA. Cleavage of NB-LRR targets *Glyma16g10080* and *Glyma16g34000* did not generate substantial abundance of phasiRNAs. phasiRNAs are shown as blue dots in a ten cycle interval of 21 nt.

Glyma03g14900

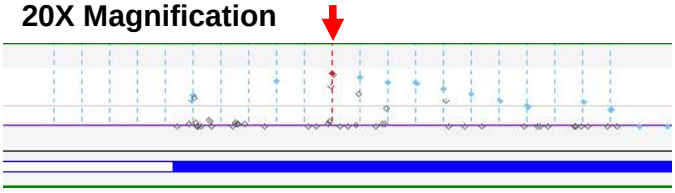


20X Magnification

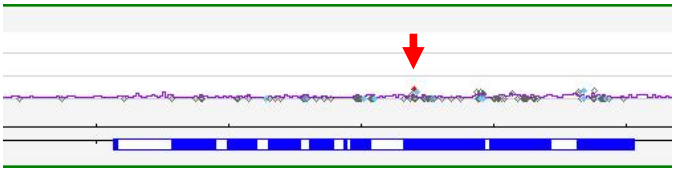


Glyma16g10080

20X Magnification



Glyma16g34000



20X Magnification

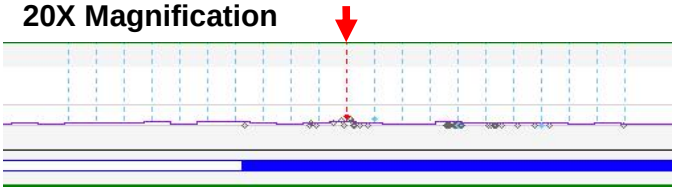
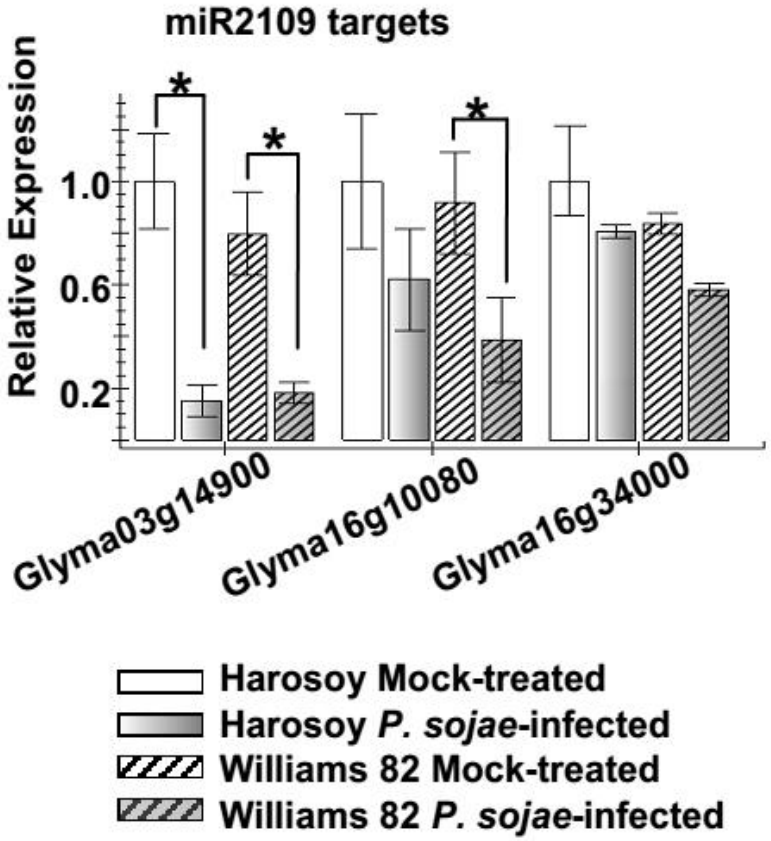


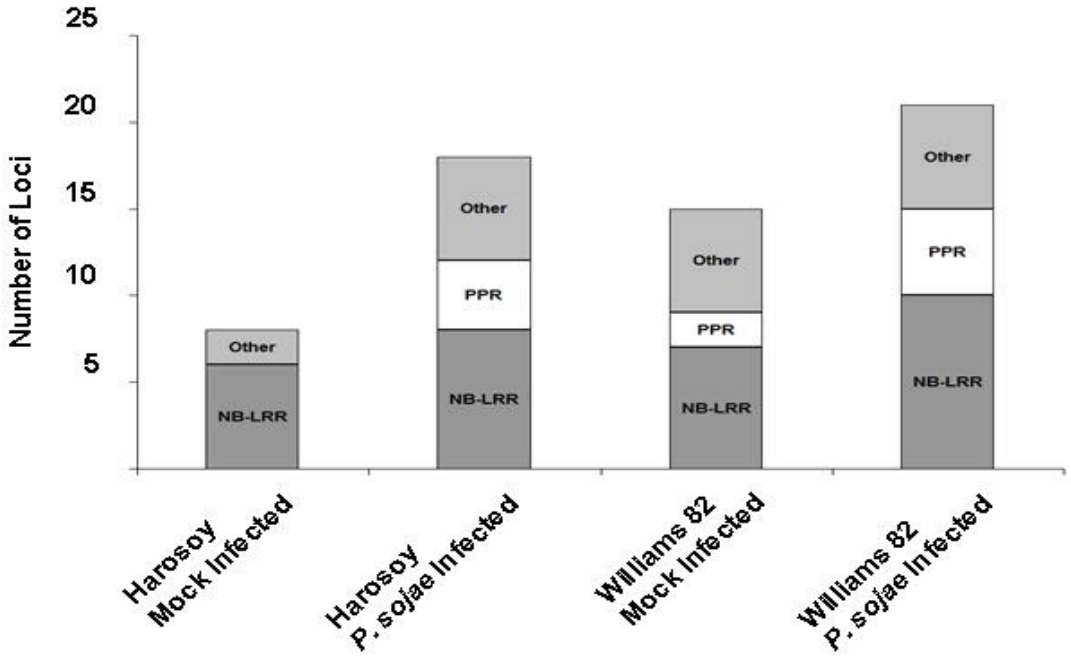
Figure 3.9 *NB-LRR* genes targeted by miR2109 were repressed during *P. sojae* infection. Relative transcript abundances were evaluated with qRT-PCR using *GmUBI* as an internal control. Values represent averages from two independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).


miR2109 targets
Relative Expression
1.0
0.6
0.2
*
*
*
Glyma03g14900
Glyma16g10080
Glyma16g34000
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

3.4.2 Genome-scale analysis of phasiRNAs in soybean during P. sojae infection

Because phasiRNA production from specific loci was enhanced in *P. sojae*-infected tissues, we next performed a genome-scale analysis on *PHAS* loci in soybean during *P. sojae* infection. Genes with Phasing score greater than 15 were identified and categorized into three classes: *NB-LRR* genes, *PPR* genes and other (non *NB-LRR* or *PPR*) (Figure 3.9). In each sample, phasiRNAs were predominantly generated from *NB-LRR* and *PPR* loci. Intriguingly, *P. sojae* infection led to increased numbers of *PHAS* loci in both susceptible and resistant plants (Figure 3.10). These data suggest that phasiRNAs may play a role in regulating defense response by repressing the expression of specific *NB-LRR* and *PPR* genes during infection.

Figure 3.10 Functional classification of *PHAS* loci in soybean roots. phasiRNAs were predominantly generated from *NB-LRR* genes and *pentatriopeptide repeat* (*PPR*) genes.


25
20
15
10
5
Number of Loci
Other
NB-LRR
Other
PPR
NB-LRR
Other
PPR
NB-LRR
Other
PPR
NB-LRR
Harosoy
Mock Infected
Harosoy
P. sojae Infected
Williams 82
Mock Infected
Williams 82
P. sojae Infected

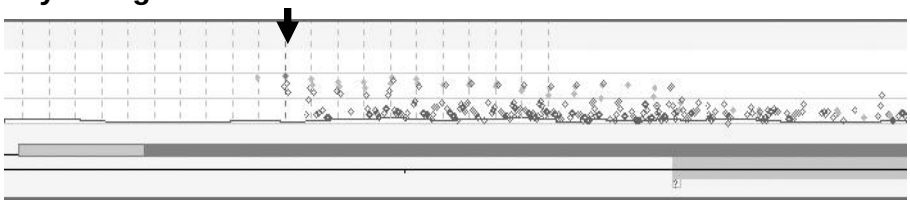
3.4.3 PPR genes generate phasiRNAs following P. sojae infection

In addition to phasiRNAs derived from *NB-LRR* loci, *P. sojae* infection also induced the production of phasiRNAs from *PPR* genes. PPR proteins belong to a large sequence-specific RNA binding protein family that regulates the expression of key genes in mitochondria and chloroplasts (Kotera et al., 2005; Prikryl et al., 2011; Zehrmann et al., 2011).

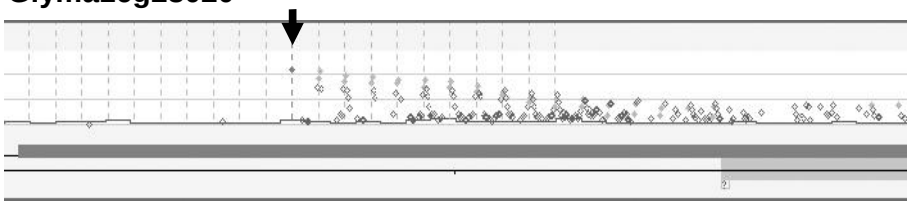
Sequencing analysis our library identified four potential *PPR* phasiRNA-generating loci, *Glyma09g07290, Glyma16g28020, Glyma16g31950 and Glyma16g28020* (Figure 3.11). Quantitative qRT-PCR was performed to verify small RNA mediated cleavage of the *PPR* transcripts. Indeed, I observed significantly reduced expression of all the *PPR* genes that were tested during *P. sojae* infection in both susceptible and resistant cultivars at 8 hpi (Figure 3.12). This is consistent with the enhanced accumulation of phasiRNAs at this early infection stage (Table 3.2). Interestingly, three out of the four *PPR* genes that were repressed at 8 hpi were de-repressed at 24 hpi in the susceptible Harosoy roots, but remained repressed in the resistant Williams 82 roots (Figure 3.12). These data revealed a potential correlation between low levels of *PPR* gene expression and plant resistance.

Figure 3.11. phasiRNAs were generated from four *PPR* loci with clear 21 nt in-phase signatures following the cleavage sites of miR1508, which are shown as the arrows. phasiRNA abundance are shown as dots in a ten cycle interval of 21 nt.

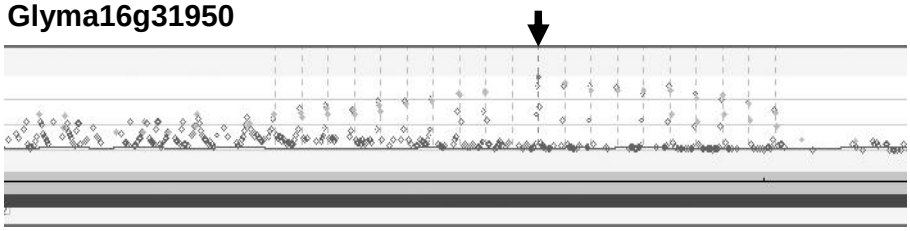
Glyma09g07290



Glyma16g28020



Glyma16g31950



Glyma16g28020

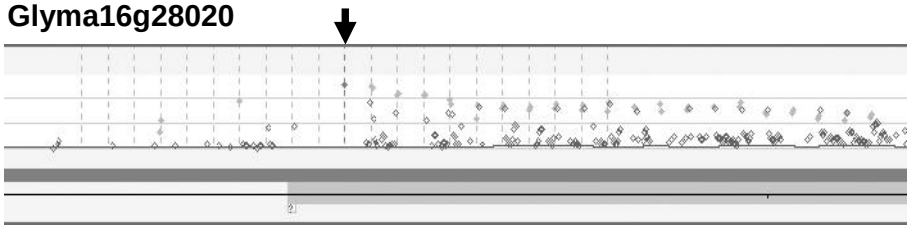


Figure 3.12 *Pentatricopeptide Repeat* genes were repressed during *P. sojae* infection, corresponding to the induction of phasiRNAs.

Transcript levels of *PPR* genes were repressed in *P. sojae*-infected roots at 8 hpi. At 24 hpi, the *PPR* genes were de-repressed in the susceptible cultivar Harosoy, but remained repressed in the resistant cultivar Williams 82. Relative transcript abundance was evaluated by qRT-PCR using *GmUBI* as an internal control. Values represent averages from two independent biological replicates and error bars represent standard deviations. * denotes statistically significant difference ($p<0.01$).

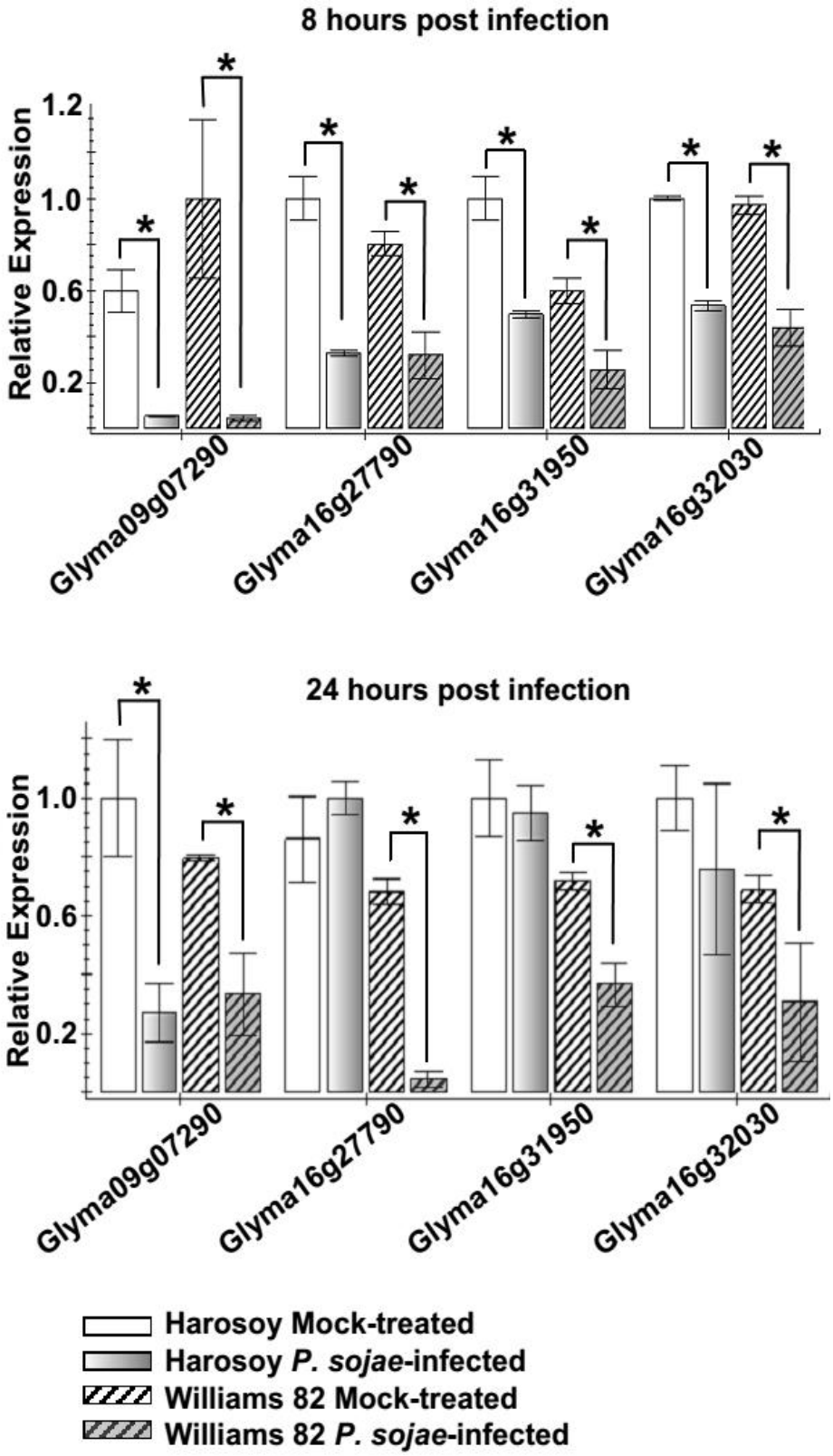
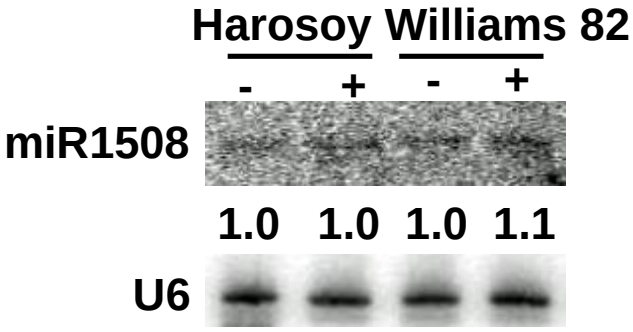

8 hours post infection
Relative Expression
1.2
1.0
0.6
0.2
Glyma09g07290
Glyma16g27790
Glyma16g31950
Glyma16g32030
24 hours post infection
Relative Expression
1.0
0.6
0.2
Glyma09g07290
Glyma16g27790
Glyma16g31950
Glyma16g32030
Harosoy Mock-treated
Harosoy P. sojae-infected
Williams 82 Mock-treated
Williams 82 P. sojae-infected

Table 3.2 phasiRNAs generated from *PPR* loci exhibited enhanced accumulation in soybean roots infected with *P. sojae*.

Normalized reads (per million) of phasiRNAs				
***PHAS* loci**	**Harosoy 8 hpi**		**Williams 82 8 hpi**	
	Mock-treated	*P. sojae*-infected	Mock-treated	*P. sojae*-infected
Glyma09g07290	62	211	76	253
Glyma16g27790	30	133	65	143
Glyma16g31950	70	218	82	192
Glyma16g32030	21	67	52	146

Our data showed that phasiRNAs produced from *PPR* genes were potentially triggered by a 23 nt miRNA, miR1508 (Turner et al., 2012; Xia et al., 2013) (Figure 3.11). This result is consistent with a recent soybean degradome analysis, which suggested miR1508 as a trigger for phasiRNA production (Hu et al., 2012). It is worth noting that the level of miR1508 was not altered by *P. sojae* infection (Fig 3.13); therefore, *P. sojae* infection may specifically induce the production of phasiRNAs from the *PPR* loci without changing their parent miR1508. These data raised the interesting possibility that the phasiRNA biosynthetic pathway might be involved in defense response.

Figure 3.13 miR1508 accumulation was not changed by *P. sojae* infection. miR1508 abundances in the mock-treated and the *P. sojae*-infected tissues were analyzed by northern blotting at 8 hours post inoculation. U6 served as a loading control in the blots. Numbers below the blot images represent relative abundance of the miR1508. This experiment was repeated three times with similar results and a standard mean of ±1.


Harosoy Williams 82
- + - +
miR1508
1.0 1.0 1.0 1.1
U6

3.5 DISCUSSION

3.5.1. NB-LRR genes are regulated by miRNAs

The interaction between a pathogen effector protein and a corresponding R protein, typically containing the conserved NB-LRR domain, in the host is the basis of the gene-for-gene disease resistance in plants (Shivaprasad et al., 2012; Tameling and Joosten, 2007). NB-LRR proteins are canonical Resistance (R) proteins, which directly or indirectly detect specific pathogen effectors and trigger the effector-triggered immunity (ETI). ETI often lead to localized programmed cell death in the infected region, which effectively restricts the spread of the pathogen. As such, NB-LRR proteins play essential role in plant resistance.

In addition to pathogenic interactions, NB-LRRs also participate in symbiotic interactions between plants and microorganisms including nodulation of soybean roots by rhizobia (Kulcheski et al., 2011; Subramanian et al., 2008). Specifically, Yang et al. showed the soybean NB-LRRs Rj2 and Rfg1 are involved host specific recognition of *Bradyrhizobium japonicum* (Yang et al., 2010). An increase of miR482 accumulation in soybean roots during nodulation potentially targets some *NB-LRR* genes to promote the symbiotic interaction rather than defense response (Li et al., 2012).

Infection by *P. sojae* at 8 hpi appears to reduce miR482 levels but induce other *NB-LRR* targeting miRNAs, miR1507 and miR2109. Consistently, I observed decreased expression levels of several *NB-LRR* genes that are potentially regulated by miR1507 and miR2109 at 8 hpi. These data suggest that plants rely on miRNAs to suppress *NB-LRR* gene expression during the early stages of *P. sojae* infection. Activation or over-expression of NB-LRR proteins often lead to programmed cell

death, which is a significant fitness and energy cost to the plant. Therefore, NB-LRRs must be tightly and precisely regulated during infection. I hypothesized that plants have multilayered defensive systems during pathogen infection, and the control of NB-LRRs is coordinated with other mechanisms at different infection stages to mount effective defense. miRNAs may play an important role in the precise regulation of *NB-LRR* gene expression.

3.5.2. phasiRNAs produced from NB-LRR loci

Another class of soybean small RNAs that were significantly induced by *P. sojae* was phasiRNAs. Recent analyses of small RNA populations in legumes during mutually beneficial interactions with rhizobia and mycorrhizae revealed that the majority of the phasiRNA-generating (*PHAS*) loci are *NB-LRR* genes (Zhai et al., 2011). In *Medicago truncatula*, these *PHAS* loci are mainly targeted by three 22 nt miRNA families, i.e. miR1507, miR2109 and miR2118. miR1507 targets *NB-LRR* transcript in soybean, tobacco, tomato and *Medicago* plant species and have been shown to generate phasiRNAs in *M. truncatula* and soybean (Li et al., 2012; Zhai et al., 2011). In both *M. truncatula* and soybean, induction of miR1507 leads to enhanced production of phasiRNAs.

Soybean miR2109 was believed to be 21 nt in length and therefore could not trigger phasiRNAs (Zhai et al., 2011). However, my sequencing data revealed miR2109 isoforms that are 20, 21 and 22 nt in length (data not shown). Though it is unknown if the 21 nt form of miR2109 could trigger phasiRNA production, it is likely that the 22 nt isoform of miR2109 is the parent of the phasiRNAs.

I observed a significant portion of *PHAS* loci to be *NB-LRR* genes that are targets of miR1507 and miR2109, which were induced during *P. sojae* infection. Furthermore, I found induced production of phasiRNAs in *P. sojae*-infected tissues from specific *NB-LRR* loci. It is also evident that phasiRNAs derived from the *PHAS* loci act both in cis and in trans to repress *NB-LRR* gene expression. The induction of miR1507, miR2109 and their corresponding phasiRNAs led to decreased expression of multiple *NB-LRR* genes during early infection of *P. sojae* in soybean. It is possible that plants may use miRNAs and phasiRNAs to fine tune the expression levels of *NB-LRR* genes, especially at the early infection stages, and the precise regulation of *NB-LRR* gene expression may be of significant importance for effective defense against the pathogens.

22 nt miRNAs and their derived phasiRNAs have also been shown to regulate *NB-LRR* genes in potato during viral infection (Li et al., 2012). Taken together, these findings suggested a significant regulatory role of phasiRNAs in plant immune response to both viral and oomycete pathogens.

3.5.3. phasiRNAs produced from PPR loci

In addition to phasiRNAs derived from *NB-LRR* loci, *P. sojae* infection also induced the production of phasiRNAs from *PPR* genes, which were repressed at the early infection stage. PPR proteins belong to a large sequence-specific RNA binding protein family that regulates the expression of key genes in mitochondria and chloroplasts (Kotera et al., 2005; Prikryl et al., 2010). In *Arabidopsis, PPR* genes are

regulated by trans-acting siRNAs (tasiRNAs) generated from the *TAS1* and *TAS2* loci (Howell et al., 2007). It was recently shown that siRNA-mediated regulation of *PPR* genes is a conserved mechanism in eudicots: *PPR* genes are regulated either by phasiRNAs generated from *PPR PHAS* loci or by tasiRNAs generated from the *TAS* loci (Xia et al., 2013). Furthermore, miR1509, which belongs to the evolutionarily conserved miR173 family, was proposed to regulate *PPR* genes in soybean by triggering tasiRNA production from intermediate *TAS*-like genes Our data showed that phasiRNAs produced from *PPR* genes were triggered by a 23 nt miRNA, miR1508 (Fig. S5b). This result is consistent with a recent soybean degradome analysis, which suggested miR1508 as a trigger for phasiRNA production (Hu et al., 2013). It is worth noting that the level of miR1508 was not altered by *P. sojae* infection; therefore, *P. sojae* infection may specifically induce the production of phasiRNAs from the *PPR* loci without changing their parent miR1508. These data raised the interesting possibility that the phasiRNA biosynthetic pathway might be involved in defense response.

If phasiRNAs regulating *NB-LRR* and/or *PPR* genes are important regulators of plant defense, successful pathogens may have evolved virulence factors to target the production of phasiRNAs. Indeed, our lab recently identified a *P. sojae* effector PSR2 that specifically suppresses the biogenesis of tasiRNAs from the *TAS1* and *TAS2* loci in *Arabidopsis* (Qiao et al., 2013). Silencing of *PSR2* in *P. sojae* drastically reduced the virulence of the pathogen in soybean, demonstrating that it is an essential effector that promotes pathogenesis. Consistent with the expression profile of *PSR2*, which reaches its maximum level at around 16 hpi (Qiao et al., 2013), the *PPR* genes were

de-repressed at 24 hpi in the susceptible host Harosoy, which could be due to the phasiRNA suppression activity of PSR2. On the contrary, the *PPR* genes remained repressed at 24 hpi in the resistant cultivar Williams 82. It is intriguing to propose that soybean employs specific phasiRNAs to regulate the expression of defense-associated genes, such as *NB-LRR* and *PPR* genes, during *P. sojae* infection; and pathogens have evolved effectors, such as PSR2, to disturb this regulatory mechanism for the benefit of infection. Functional characterization of phasiRNAs in soybean defense will provide further evidence on the role of phasiRNAs during *P. sojae* infection.

3.5.4 Conclusion

The requirements for defense mechanisms and selective pressure from pathogens have resulted in rapid evolution and diversification of genes that encode crucial defense-related proteins, such as NB-LRRs and possibly PPRs. These genes are known to be regulated by small RNAs to ensure their precise expression. I performed an extensive genome-wide analysis of soybean small RNAs in response to *P. sojae* infection. This lead to the identification of miRNAs and their derived phasiRNAs that regulate *NB-LRR* and *PPR* genes and may potentially contribute to plant defense. The generation of secondary siRNAs seems to be an effective mechanism to target large gene families wherein a small number of miRNAs regulate a large number of related targets. This mechanism may allow for precise and rapid global control of gene expression during pathogen infection.

3.6. FUTURE DIRECTIONS

Phytophthora diseases have caused enormous economical damage to agriculture. Consistent with our recent findings on *Phytophthora* effectors that inhibit small RNA biogenesis, my experiments revealed important regulatory roles of specific miRNAs and phasiRNAs in plant immunity. This work leads to interesting future directions that will provide further insights into small RNA-mediated regulation of plant defense.

Firstly, small RNAs selected for further verification and function analysis were based on computational analyses of the small RNA sequencing data, which revealed small RNA species with significant changes in abundance. However, out of the 16 miRNAs that were computationally predicted to have a significant fold changes, only eight were confirmed to be differentially expressed by Northern blotting. Clearly, there are restrictions in screening altered small RNAs using Illumina data. Further examinations on additional miRNAs are needed to obtain a better view of small RNA changes during *P. sojae* infection.

Further time-course analysis of these specific miRNAs and phasiRNAs will be pursued to elucidate mechanistic details of small RNA-mediated defense regulation during *Phytophthora* infection. In this study, experimental data and analysis were based on soybean response to *P. sojae* at 8 hpi. Although both the susceptible and resistant soybean cultivars were examined, I did not observe significant differences in small RNA changes in these hosts. This observation is consistent with the assumption that the small RNAs changes at this early stage represent the plant defense response. We have generated small RNA libraries at 24 hpi. Although a detailed study has yet to

be performed, preliminary examination of these 24 hpi small RNA sequences revealed evident differences in small RNA profiles between the susceptible Harosoy and the resistant Williams 82 cultivars. This is likely due to the effector-triggered immunity established in Williams 82 that is absent in Harosoy. It could also be due to the activity of *Phytophthora* effectors with inhibitory effects on small RNA biogenesis. In depth analysis of the small RNA changes at 24 hpi in comparison to 8 hpi will no doubt provide additional insight into the regulatory processes during the dynamic interaction between soybean and *P.sojae*.

Using the new STTM technique, I showed that knocking down miR393 levels in soybean roots greatly enhanced the susceptibility to *P. sojae*, partly due to defects in the production of antimicrobial compounds. Functional characterization on other miRNAs, especially miR1507 and miR2109, which regulate *NB-LRR* genes, should be conducted to elucidate the functions of these miRNAs and their derived phasiRNAs in plant defense. STTM-mediated knocking down and over-expression of the corresponding *MIR* genes in soybean hairy roots will provide important information on the roles of in plant defense.

3.7 REFERENCES

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-Directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121, 207-221.

Boller, T., and He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324, 742.

Burch-Smith, T.M., Schiff, M., Caplan, J.L., Tsao, J., Czymmek, K., and Dinesh-Kumar, S.P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. *PLoS Biology* 5, e68.

Caplan, J., Padmanabhan, M., and Dinesh-Kumar, S.P. (2008). Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. *Cell Host & Microbe* 3, 126-135.

Chen, X. (2010). Small RNAs–secrets and surprises of the genome. *The Plant Journal* 61, 941-958.

Cheng, Y.T., Germain, H., Wiermer, M., Bi, D., Xu, F., García, A.V., Wirthmueller, L., Després, C., Parker, J.E., and Zhang, Y. (2009). Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in *Arabidopsis*. *The Plant Cell Online* 21, 2503-2516.

Cuperus, J.T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R.T., Takeda, A., Sullivan, C.M., Gilbert, S.D., Montgomery, T.A., and Carrington, J.C. (2010). Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. *Nature Structural & Molecular Biology* 17, 997-1003.

Day, B., Dahlbeck, D., Huang, J., Chisholm, S.T., Li, D., and Staskawicz, B.J. (2005). Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. *The Plant Cell Online* 17, 1292-1305.

De Paoli, E., Dorantes-Acosta, A., Zhai, J., Accerbi, M., Jeong, D.H., Park, S., Meyers, B.C., Jorgensen, R.A., and Green, P.J. (2009). Distinct extremely abundant siRNAs associated with cosuppression in petunia. *RNA* 15, 1965-1970.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics* 11, 539-548.

Eckardt, N.A. (2012). A microRNA cascade in plant defense. *The Plant Cell Online* 24, 840-840.

Eitas, T.K., and Dangl, J.L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. *Current Opinion in Plant Biology* 13, 472-477.

Fei, Q., Xia, R., and Meyers, B.C. (2013). Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. *The Plant Cell Online* 25, 2400-2415.

Fujii, S., and Small, I. (2011). The evolution of RNA editing and pentatricopeptide repeat genes. *New Phytologist* 191, 37-47.

Gabriëls, S.H., Vossen, J.H., Ekengren, S.K., Ooijen, G.V., Abd-El-Haliem, A.M., Berg, G., Rainey, D.Y., Martin, G.B., Takken, F.L., and Wit, P.J.D. (2007). An NB-LRR protein required for HR signaling mediated by both extra- and intracellular resistance proteins. *The Plant Journal* 50, 14-28.

Gasciolli, V., Mallory, A.C., Bartel, D.P., and Vaucheret, H. (2005). Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Current Biology* 15, 1494-1500.

Howell, M.D., Fahlgren, N., Chapman, E.J., Cumbie, J.S., Sullivan, C.M., Givan, S.A., Kasschau, K.D., and Carrington, J.C. (2007). Genome-wide analysis of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 pathway in *Arabidopsis* reveals dependency on miRNA-and tasiRNA-directed targeting. *The Plant Cell Online* 19, 926-942.

Hu, Z., Jiang, Q., Ni, Z., Chen, R., Xu, S., and Zhang, H. (2012). Analyses of a *Glycine max* degradome library identify microRNA targets and microRNAs that trigger secondary siRNA biogenesis. *Journal of Integrative Plant Biology* 55, 160-176.

Huang, C.L., Hwang, S.Y., Chiang, Y.C., and Lin, T.P. (2008). Molecular evolution of the *Pi-ta* gene resistant to rice blast in wild rice (*Oryza rufipogon*). *Genetics* 179, 1527-1538.

Hwang, C.F., and Williamson, V.M. (2003). Leucine-rich repeat-mediated intramolecular interactions in nematode recognition and cell death signaling by the tomato resistance protein Mi. *The Plant Journal* 34, 585-593.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. *Nature* 444, 323-329.

Kamoun, S. (2007). Groovy times: filamentous pathogen effectors revealed. *Current Opinion in Plant Biology* 10, 358-365.

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2005). Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. *Plant and Cell Physiology* 46, 1093-1102.

Kobayashi, K., Kawabata, M., Hisano, K., Kazama, T., Matsuoka, K., Sugita, M., and Nakamura, T. (2012). Identification and characterization of the RNA binding surface of the pentatricopeptide repeat protein. *Nucleic Acids Research* 40, 2712-2723.

Kobayashi, K., Suzuki, M., Tang, J., Nagata, N., Ohyama, K., Seki, H., Kiuchi, R., Kaneko, Y., Nakazawa, M., and Matsui, M. (2007). LOVASTATIN INSENSITIVE 1, a novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in *Arabidopsis*. *Plant and Cell Physiology* 48, 322-331.

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* 433, 326-330.

Kulcheski, F.R., de Oliveira, L.F.V., Molina, L.G., Almerão, M.P., Rodrigues, F.A., Marcolino, J., Barbosa, J.F., Stolf-Moreira, R., Nepomuceno, A.L., Marcelino-Guimarães, F.C., and Margis, R. (2011). Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genomics* 12, 307.

Laluk, K., AbuQamar, S., and Mengiste, T. (2011). The *Arabidopsis* mitochondria-localized pentatricopeptide repeat protein PGN functions in defense against necrotrophic fungi and abiotic stress tolerance. *Plant Physiology* 156, 2053-2068.

Li, F., Pignatta, D., Bendix, C., Brunkard, J.O., Cohn, M.M., Tung, J., Sun, H., Kumar, P., and Baker, B. (2012). MicroRNA regulation of plant innate immune receptors. *Proceedings of the National Academy of Sciences* 109, 1790-1795.

Li, H., Deng, Y., Wu, T., Subramanian, S., and Yu, O. (2010). Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. *Plant Physiology* 153, 1759-1770.

Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C., Caboche, M., Debast, C., Gualberto, J., and Hoffmann, B. (2004). Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *The Plant Cell Online* 16, 2089-2103.

McHale, M., Eamens, A.L., Finnegan, E.J., and Waterhouse, P.M. (2013). A 22-nt artificial microRNA mediates widespread RNA silencing in *Arabidopsis*. *The Plant Journal* 76, 519-529.

O'Toole, N., Hattori, M., Andres, C., Iida, K., Lurin, C., Schmitz-Linneweber, C., Sugita, M., and Small, I. (2008). On the expansion of the pentatricopeptide repeat gene family in plants. *Molecular Biology and Evolution* 25, 1120-1128.

Prikryl, J., Rojas, M., Schuster, G., and Barkan, A. (2011). Mechanism of RNA stabilization and translational activation by a pentatricopeptide repeat protein. *Proceedings of the National Academy of Sciences* 108, 415-420.

Qi, D., DeYoung, B.J., and Innes, R.W. (2012). Structure-function analysis of the coiled-coil and leucine-rich repeat domains of the RPS5 disease resistance protein. *Plant Physiology* 158, 1819-1832.

Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., Wang, X., Liu, X., Xiang, Q., Jiang, S., Zhang, F., Wang, Y., Judelson, H.S., Chen, X., and Ma, W. (2013). Oomycete pathogens encode RNA silencing suppressors. *Nature Genetics* 45, 330-333.

Ritter, C., and Dangl, J.L. (1996). Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes. *The Plant Cell Online* 8, 251-257.

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends in Plant Science* 13, 663-670.

Shen, Q.H., and Schulze-Lefert, P. (2007). Rumble in the nuclear jungle: compartmentalization, trafficking, and nuclear action of plant immune receptors. *The EMBO Journal* 26, 4293-4301.

Shivaprasad, P.V., Chen, H.M., Patel, K., Bond, D.M., Santos, B.A.C.M., and Baulcombe, D.C. (2012). A microRNA superfamily regulates nucleotide-binding site leucine-rich repeats and other mRNAs. *The Plant Cell* 24, 859-874.

Subramanian, S., Fu, Y., Sunkar, R., Barbazuk, W.B., Zhu, J.K., and Yu, O. (2008). Novel and nodulation-regulated microRNAs in soybean roots. *BMC Genomics* 9, 160.

Sun, Y., Li, L., Macho, A.P., Han, Z., Hu, Z., Zipfel, C., Zhou, J.M., and Chai, J. (2013). Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* 342, 624-628.

Takken, F.L., and Tameling, W.I. (2009). To nibble at plant resistance proteins. *Science* 324, 744.

Takken, F.L., Albrecht, M., and Tameling, W.I. (2006). Resistance proteins: molecular switches of plant defense. *Current Opinion in Plant Biology* 9, 383-390.

Tameling, W. I., and Joosten, M.H.A.J. (2007). The diverse roles of NB-LRR proteins in plants. *Physiological and Molecular Plant Pathology* 71, 126-134.

van der Hoorn, R.A., and Kamoun, S. (2008). From guard to decoy: a new model for perception of plant pathogen effectors. *The Plant Cell Online* 20, 2009-2017.

van Ooijen, G., Mayr, G., Kasiem, M.M., Albrecht, M., Cornelissen, B.J., and Takken, F.L. (2008). Structure–function analysis of the NB-ARC domain of plant disease resistance proteins. *Journal of Experimental Botany* 59, 1383-1397.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.L., Bartel, D.P., and Crété, P. (2004). Endogenous trans-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. *Molecular Cell* 16, 69-79.

Xia, R., Meyers, B.C., Liu, Z., Beers, E.P., Ye, S., and Liu, Z. (2013). MicroRNA superfamilies descended from miR390 and their roles in secondary small interfering RNA biogenesis in Eudicots. *The Plant Cell Online*.

Yang, S., Tang, F., Gao, M., Krishnan, H.B., and Zhu, H. (2010). R gene-controlled host specificity in the legume-rhizobia symbiosis. *Proceedings of the National Academy of Sciences* 107, 18735-18740.

Yoshikawa, M., Iki, T., Tsutsui, Y., Miyashita, K., Poethig, R.S., Habu, Y., and Ishikawa, M. (2013). 3′ fragment of miR173-programmed RISC-cleaved RNA is protected from degradation in a complex with RISC and SGS3. *Proceedings of the National Academy of Sciences* 110, 4117-4122.

Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S. (2005). A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. Genes & Development 19, 2164-2175.

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2011). PPR proteins network as site-specific RNA editing factors in plant organelles. *RNA Biology* 8, 67-70.

Zhai, J., Jeong, D.H., De Paoli, E., Park, S., Rosen, B.D., Li, Y., Gonzalez, A.J., Yan, Z., Kitto, S.L., Grusak, M.A., Jackson, S.A., Stacey, G., Cook, D.R., Green, P.J., Sherrier, D.J., and Meyers, B.C. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes & Development* 25, 2540-2553.

Zhang, J., and Zhou, J.M. (2010). Plant immunity triggered by microbial molecular signatures. *Molecular Plant* 3, 783-793.

Zhang, X.C., and Gassmann, W. (2007). Alternative splicing and mRNA levels of the disease resistance gene RPS4 are induced during defense responses. *Plant Physiology* 145, 1577-1587.

Zhou, L., Mideros, S.X., Bao, L., Hanlon, R., Arredondo, F.D., Tripathy, S., Krampis, K., Jerauld, A., Evans, C., St Martin, S.K., Maroof, M.A.S., Hoeschele, I., Dorrance, A.E., and Tyler, B.M. (2009). Infection and genotype remodel the entire soybean transcriptome. *BMC Genomics* 10, 49.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Current Opinion in Plant Biology* 12, 414-420.

Zipfel, C., and Robatzek, S. (2010). Pathogen-associated molecular pattern-triggered immunity: Veni, Vidi...? *Plant Physiology* 154, 551-554.