The Turnip mosaic virus and its effects on Arabidopsis thaliana gene expression

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The Turnip mosaic virus and its effects on Arabidopsis thaliana gene expression

by

Brian Anthony Campbell

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
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CHAPTER 1. GENERAL INTRODUCTION

1.1 Significance and Objectives

Viruses and their hosts are engaged in a constantly evolving arms race. Understanding viruses has numerous practical applications, such as the development of vaccines, improving disease resistance, and understanding the basis of host specificity (Hughes, 2009). Although many mysteries surrounding plant-virus interactions have been solved, many questions remain unanswered. Our lab is interested in elucidating the connection host genes have with viral pathogenesis and symptom onset. Specifically, developing a better understanding of the mechanisms the regulate the expression of genes with functions related to cell wall synthesis and maintenance, hormone biosynthesis, chloroplast function, and sulfur uptake and utilization, after TuMV (*Turnip mosaic virus*) infection. Because these genes are down-regulated and their functions are positively associated with plant growth, additional studies are needed to establish whether or not their decreased expression causes symptoms or if they are just correlated with symptoms.

My dissertation is primarily based on three objectives: analyzing the transcripts and small RNAs (sRNAs) of genes correlated with disease symptomology, quantifying their expression in wild-type and RNA silencing defective *Arabidopsis thaliana* plants, and characterizing various TuMV viruses lacking RNA silencing suppressor activity. The first objective was based on Yang and associates’ (2007) findings that genes related to cell wall function, hormone biosynthesis, chloroplast function, and sulfur uptake and utilization are down-regulated post-pathogen challenge. sRNAs derived from those genes were identified using computational methods and a previously published data set (Garcia-Ruiz et al., 2010). Subsequently, gene expression was monitored. Objective two was based on preliminary data from our lab which suggests the above-
mentioned genes are targeted for silencing via a DICER-LIKE 3 (DCL3)-dependent mechanism. The third objective aimed to uncouple virus accumulation from disease symptom progression (e.g. symptomology), as previously demonstrated in other plant-pathogen systems (Shiboleth et al., 2007, Wu et al., 2010). It is my hope that the data presented in this dissertation will lead to standardized comparative studies between debilitated potyviruses and their hosts, because a consensus time point for harvesting systemically infected tissue is sorely lacking. This information has the potential to further invoke critical thought about helper-component proteinase (HC-Pro), the multi-functional RNA silencing suppressor of the Potyviridae family, because one or more mechanisms exist to facilitate successful infection across its broad host range as evidenced by our gene expression data. This introduction provides an overview of relevant plant viruses, viral immunity (e.g. plant defense), and RNA silencing suppressor activity (e.g. virus counter-defense).

1.2 Potyvirus

Potyvirus particles are 700 – 750 nm in length, each of which contains one copy of the genome, a single-stranded, positive-sense RNA molecule approximately 10 Kb in length (Oshima et al., 2006). The genome has untranslated regions flanking each end of the open reading frame (Oshima et al., 2006).

1.2.1 Turnip Mosaic Virus

TuMV, a member of the Potyviridae family, is ranked among the five most damaging plant-pathogenic viruses on vegetable crops in the world (Tomlinson 1987). In many cultivated Brassica spp., TuMV causes a variety of leaf symptoms including mottles, mosaics, and black necrotic ring spots (Tomlinson, 1970). The virus is sap-transmitted in a non-persistent manner by aphids (Tomlinson and Ward, 1978). Symptom variation mainly depends on the virulence of the
virus and on the susceptibility or resistance of the host (Tomlinson and Ward, 1978). TuMV isolates have been classified into four groups based on their phylogenetic relationships and pathogenicity (Nomura et al., 2004). Although TuMV infects economically important Brassica crops, it induces a number of developmental defects in vegetative and reproductive organs in A. thaliana.

Three virus-encoded proteinases, P1, HC-Pro, and nuclear inclusion a protein (NIa), proteolytically process TuMV polyproteins into at least 10 mature protein products (Fig. 1; Nicolas and Laliberte, 1992; Oshima et al., 2006). Specifically, P1 and HC-Pro autocatalytically cleave their respective C-termini, whereas NIa is responsible for processing the seven remaining junctions (Yoon et al., 2000). P1 has the ability to bind ssRNA and ssDNA, with a high affinity for secondary structure (Soumounou and Laliberte, 1994). P3 is known to cause a systemic hypersensitive reaction or mosaic patterning via gene-for-gene interaction with TuNI (TuMV necrosis inducer) based on host and viral factors in A. thaliana ecotype Landsberg erecta (Kim et al., 2010). Previous studies by others have also shown P3 is an important factor during infection and in determining host range as a symptom determinant in Brassicas (Jenner et al. 2003). Although P3 is poorly understood, its protein products are thought to be involved in virus replication, accumulation, symptom development, resistance breaking, and cell-to-cell movement (Suehiro et al. 2004). PIPO, a protein translated in the +2 frame from a short open reading frame overlapping with the P3 ORF, is required for infection, and has been implicated in intercellular movement (Chung et al., 2008, Wei at al., 2010). 6k1 has no known function. The cylindrical inclusion (CI) protein displays RNA-dependent ATPase activity characteristic of RNA helicases, but is also a virulence determinant (Nicolas Laliberte, 1992; Jenner et al., 2000). The 6k2-VPg-Pro polyprotein, through its hydrophobic 6k2 domain forms a cytoplasmic vesicle derived from
the ER (Cotton et al., 2009). It has been suggested that translation of viral RNA occurs within these vesicles (Cotton et al., 2009). VPg is the N-terminal domain of NiA-Pro and has been implicated in resistance breaking (Nicols and Laliberte, 1992; Gallois et al., 2010). NiB is the core replicase and the coat protein monomers self-assemble to form the capsid (Nicols and Laliberte, 1992).

1.2.2 Zucchini Yellow Mosaic Virus

As early as 1973, **Zucchini yellow mosaic virus** (ZYMV) symptoms – reduced growth, yellowing, mosaic and blistering leaves, fruit distortion, fruit malformation, necrosis, and plant death – were being described (Lisa et al., 1981). Sap from infected zucchini squash (**Cucurbita pepo** L.) was used to inoculate a variety of plants and Koch’s postulates were applied to confirm a novel virus was isolated (Lisa et al., 1981). The novel virus, ZYMV, was characterized as a potyvirus belonging to the **Potyviridae** family (Lisa et al., 1981). ZYMV is a positive single-stranded RNA virus, 9593 nt long, and is transmitted by aphids in a non-persistent manner (Gal-On 2007; Lisa et al., 1981). ZYMV can also cause severe symptoms in other cucurbits such as melon (**Cucumis melo** L.), cucumber (**Cucumis sativus** L.), watermelon (**Citrullus lanatus** Schad.), and pumpkin (**Cucurbita maxima** L., **C. moschata** L.) (Gal-On 2007).

1.3 RNA Silencing

Double-stranded RNA can originate from virus replication, secondary structure within viral RNA, transcription of inverted repeat sequences, or convergent transcription (Voinnet, 2001). These dsRNAs are processed into microRNAs (miRNAs) and small-interfering RNAs (siRNAs) that are 21-nt, 22-nt, 24-nt, and 21-nt in length by DICER-like1 (DCL1), DICER-like2 (DCL2), DICER-like3 (DCL3), and DICER-like4 (DCL4), respectively. DCL1 catalyzes cleavage of intergenic/intronic fold-back precursors to release miRNAs (Chen, 2008). Most
Arabidopsis miRNAs function in association with ARGONAUTE1 (AGO1; Vaucheret et al., 2004). DCL2 processes natural antisense transcript siRNAs that regulate stress response (Mossiard and Voinnet, 2006). DCL3 siRNA products guide heterochromatin formation and transcriptional repression of transposons and DNA repeats (Mossiard and Voinnet, 2006). These siRNAs function through AGO4/PoIV complexes to direct DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) dependent RNA-directed DNA methylation at cytosine positions in a CNN context (Cao and Jacobsen, 2002). DCL4 has been implicated in RNA silencing and producing trans-acting siRNAs that control development timing and leaf polarity (Mossiard and Voinnet, 2006).

All DICER-like proteins have been implicated in plant viral defense, also known as RNA silencing. DCL1 negatively regulates DCL2, DCL3, and DCL4 (Mossiard and Voinnet, 2004). Twenty-four-nt viral-derived small RNAs (vsiRNAs) are the most abundant, post-TuMV infection, indicating DCL4 is the main progenitor of the RNA silencing signal in regards to pathogen attack (Garcia-Ruiz et al., 2010). In the absence or compromised function of DCL4, 22-nt and 24-nt vsiRNAs produced by DCL2 and DCL3, respectively, become more abundant (Garcia-Ruiz et al., 2010). DCL4 and DCL2 act redundantly to recruit antiviral RNA-induced silencing complex (RISC) to defend against plant viruses (Mossiard and Voinnet, 2006). In the absence or compromised activity of DCL2 and DCL4, 24-nt vsiRNAs produced by DCL3 become the main progenitor of the RNA silencing signal (Garcia-Ruiz et al., 2010).

RNA silencing is a plant mechanism which regulates endogenous genes and defends against pathogens. One or more RNA-dependent RNA-polymerase (RDR) paralogs, including A. thaliana RDR6 and RDR1, may strengthen primary silencing responses by producing dsRNA
from viral template, conditioning antiviral immunity in non-infected tissues (Deleris et al., 2006). Therefore, RNA silencing can be thought of as an antiviral defense mechanism.

1.4 Viral Suppressors of RNA Silencing

Systemic viral infection requires the virus to move from cell to cell and over long distances by exploiting and modifying pre-existing pathways (Carrington et al., 1996). Pathogenesis provides numerous opportunities for viral proteins and nucleic acids to interact with and influence the activity of host proteins and nucleic acids. It is relatively easy to identify genes with altered expression, deciphering how and why the changes are initiated remains a major challenge (Whitham et al., 2006). However, plants possess a multi-tier immune system designed to recognize virus replication, dsRNA, transcription of inverted repeat sequences, convergent transcription, and transposons collectively known as RNA silencing. To counter this defense mechanism, viruses encode RNA silencing suppressor proteins that interfere with RNA silencing (Csorba et al., 2009).

1.4.1 Cucumber mosaic virus 2b

*Cucumber mosaic virus* (CMV) is translated from its tri-partite genome, RNAs 1 – 3. The RNA silencing suppressor of CMV, the 2b protein, is translated from RNA 4A, a sub-genomic RNA transcribed from the 3’ half of RNA 2 (Ding et al., 1994). Approximately one year later, Ding and associates (1995a) confirmed that CMV genome and satellite RNA replication occurs independent of 2b activity. Subsequently, 2b has been reported as having a role in virus movement and virulence (Soards et al. 2002, Shi et al. 2002, Brigneti et al. 1998, Lucy et al. 2000).

Building on their previous results, Ding and associates (2007) investigated CMV accumulation and siRNA pattern formation in several RNA silencing defective *A. thaliana*
mutants in conjunction with CMV strains containing varying levels of 2b suppressor activity. CMV-Δ2b, lacks RNA silencing suppressor activity and is unable to systemically infect wild-type Arabidopsis or induce a disease phenotype (Ding et al., 1995a). Interestingly, CMV-Δ2b accumulation in dcl mutants is strikingly similar to the accumulation of wild-type CMV, the parental/virulent strain, in wild-type A. thaliana plants (Ding et al., 2007). This result suggests that viral symptoms are uncoupled from the activity of the 2b silencing suppressor.

Ding and associates (2007) performed several RNA blot experiments to analyze patterns of CMV-Δ2b accumulation in dcl mutants. It was hypothesized that CMV-Δ2b could be rescued in RNA silencing defective mutants lacking the machinery necessary to initiate anti-CMV defenses. In contrast to preliminary experiments, Ding and associates (2007) reported differential accumulation of 21-, 22-, and 24-nt siRNAs, when comparing CMV and CMV-Δ2b infection. Further analysis revealed CMV and CMV-Δ2b infection were indistinguishable in dcl 2-1 dcl 4-2 and dcl 2-1 dcl 3-1 dcl 4-2 (Ding et al., 2007). These results demonstrate dcl activity is hierarchal in function with DCL 4 and DCL 2 being the main targets of 2b (Ding et al., 2007).

Goto and associates (2007) utilized another attenuated CMV strain, CM95, characterized by mild symptoms and a spontaneous revertant derivative, CM95R, characterized by severe symptoms. These two strains differ by one amino acid (position 46) and could be used to analyze the mechanism of 2b suppression of PTGS. CM95 weakly bound siRNAs, while CM95R could bind siRNAs and long dsRNAs (Goto et al., 2007). The differences in 2b siRNA-binding affinity were attributed to the single amino acid change (C46R; Goto et al., 2007). Subsequently, a causal relationship between symptomology and siRNA binding was demonstrated.
1.4.2 Tomato bushy stunt virus p19

Tomato bushy stunt virus (TBSV), a tombusvirus, encodes a 19-kDa protein (p19) that is a well-characterized suppressor of RNA silencing. Functions correlated with p19 activity include systemic spread and virulence (Chu et al., 2000). In an effort to discern if these functions are related to the same domain of p19, several mutants were generated. pT19/75-78 contains glycine for arginine substitutions at residues 75 and 87, delaying symptomology in spinach without affecting accumulation in Nicotiana benthamiana or Nicotiana clevelandii (Chu et al., 2000). pT19/43 contains an R43W substitution and is characterized as producing an intermediate disease phenotype when compared to wild-type TBSV (Chu et al., 2000). Further analysis revealed impaired siRNA binding affinity, clearly demonstrating uncoupled virus accumulation and symptomology (Turina et al., 2003; Omarov et al., 2006).

1.4.3 HC-Pro (modified from Publication 2 intro)

TuMV contains a RNA silencing suppressor, HC-Pro, which interferes with pathways that depend on negative regulation by miRNAs, triggering developmental defects associated with infection (Kasschau et al., 2003). Helper component protease, HC-Pro, is a multifunctional protein of potyviruses, with three distinct regions: N-terminal, central, and C-terminal (Maia et al., 1996, Urcuqui-Inchima et al., 2001). The current study focused on the central region of HC-Pro, specifically the FRNK box motif, as it relates to symptomology. The FRNK box motif is located at amino acid position 179 – 182 or 181 – 184 in ZYMV or TuMV, respectively.

Previously, it was reported that a naturally occurring ZYMV variant, ZYMV-HC(-), which contains a mutation in the PTK motif of HC-Pro, was poorly aphid transmissible and cause mild symptoms (Huet et al., 1994). In the same report, two other mutations were described in HC-Pro suggesting that one or both of them might contribute to symptomology. Another group
which was more interested in the mechanism(s) of symptomology than transmissibility used those finding as the basis of a site-directed mutagenesis experiment. In a first-of-its-kind report, Gal-On (2000) demonstrated that an amino acid change, R to I, within the FRNK box of HC-Pro attenuated symptoms without affecting virus accumulation. The FRNK motif was also shown to be highly conserved among the potyviruses. In the same study, it was found that substituting Asp to Gly at position 148 had no effect on symptom expression (Gal-On 2000). The second mutation, located within the PTK motif, was not studied any further because its importance in aphid transmissibility was previously demonstrated (Huet et al., 1994). These results established a role for potyvirus HC-Pro in eliciting symptoms in a susceptible host plant, and pointed to the FRNK box as an important functional domain.

Although HC-Pro is a multi-functional protein, the FRNK to FINK mutation solely affected symptom expression. An explanation centered on protein-protein interaction was offered in an attempt to characterize this biological phenomenon. It was hypothesized that this protein-protein interaction, which induces disease symptoms, may have been adversely affected by the mutation (Gal-On 2000). Additionally, replacing a basic amino acid, Arg, with a hydrophobic amino acid, Ile, may have adversely affected motif polarity (Gal-On 2000). Even though it was hypothesized that HC-Pro interacts with a putative host factor, no candidates were identified.

Several years later, putative host factors were discovered in the form of small RNAs (sRNAs; Lakatos et al., 2007; Merai et al., 2006). Following the discovery that HC-Pro binds sRNAs, it was postulated that the similarity between virus symptoms and sRNA pathway mutant phenotypes was caused by a disruption of shared components (Shiboleth et al., 2007). Using a severe and mild ZYMV strain, ZYMV\textsuperscript{FRNK} and ZYMV\textsuperscript{FINK}, squash (C. pepo L. cv. Ma’ayan) were bombarded and tissue was collected at 5, 7, and 14 days post-inoculation (dpi). At 5 dpi,
both strains caused vein clearing, but ZYMV<sup>FRNK</sup> induced symptoms that progressed to leaf deformation, dwarfing, and mosaic patterning by 14dpi (Shiboleth et al., 2007). In contrast, ZYMV<sup>FINK</sup>-infected plants looked very similar to healthy plants with subtle vein clearing (Shiboleth et al., 2007). To ensure attenuated symptomology associated with the mild ZYMV strain was not an artifact of HC-Pro concentration or titer levels, western blotting was performed. As previously reported, ZYMV<sup>FRNK</sup> and ZYMV<sup>FINK</sup> virus concentrations are indistinguishable at 5 dpi, but ZYMV<sup>FINK</sup> accumulates to lower levels than the severe strain at 7 and 14 dpi (Shibboleth et al., 2007). Subsequently, gene expression data for several highly conserved microRNAs (miRNAs) and their complementary strands (miRNA*<sup>s</sup>) were quantified pre-ZYMV infection and 5 dpi via a sRNA-specific microarray. Generally speaking, miRNA and miRNA* levels were elevated in comparison to healthy plants (Shiboleth et al., 2007). However, miRNA and miRNA* accumulation was higher in ZYMV<sup>FRNK</sup> infected plants than ZYMV<sup>FINK</sup>-infected plants. This result suggested that symptomology and virus accumulation had been uncoupled. Additionally, the data suggested ZYMV<sup>FINK</sup> sRNA binding affinity was affected. An electromobility shift assay (EMSA) confirmed the HC-Pro of the mild ZYMV strain had a reduced affinity for miRNA/miRNA* duplexes. Interestingly, RNA silencing suppressor activity is unaffected by the Arg to Ile mutation. This result is consistent with the idea that symptomology and virus accumulation had been uncoupled, and that RNA silencing suppressor activity and miRNA binding are coupled (Shiboleth et al., 2007). Thus, symptomology can be interrupted as a function of HC-Pro sRNA binding affinity and the developmental stage of the host plant (Shiboleth et al. 2007).

Wu and associates (2010) offered evidence of their own supporting the theory that HC-Pro sRNA binding affinity has a direct effect on leaf-shape development and pathogenicity.
Using a transgenic approach, *A. thaliana* plants were transformed with a binary vector containing a double 35S promoter driving ZYMV P1/HC-Pro expression. Several independent lines with relatively equal transgene expression were selected per construct for visual and molecular analysis. Mutations in amino acids 180, 205, and 396 were evaluated for sRNA binding and symptomology. It was found that single mutations at these residues as well as the F<sub>205</sub>L/E<sub>396</sub>N double mutation cause attenuated symptoms of leaf curling, leaf lobing, and weak serration (Wu et al., 2010). Double mutations R<sub>180</sub>I/F<sub>205</sub>L and R<sub>180</sub>I/E<sub>396</sub>N induced slight leaf serration and lobing (Wu et al. 2010). The triple mutation R<sub>180</sub>I/F<sub>205</sub>L/E<sub>396</sub>N transformants grew normally like their non-transformed counterparts (Wu et al. 2010). These phenotypes were also analogous to results obtained using severe and equivalent mutant ZYMV strains in *C. pepo* L. var. zucchini.

### 1.4.3 TuMV-AS9-GFP

In a landmark paper, Garcia-Ruiz and associates (2010) successfully demonstrated uncoupling of TuMV RNA silencing suppressor activity from virus-derived short interfering RNA (vsiRNA) accumulation. This was accomplished utilizing several RNA silencing defective *A. thaliana* mutants in conjunction with TuMV strains containing varying levels of suppressor activity. Additionally, small RNA libraries were generated and analyzed for patterns correlating to *dcl* activity.

TuMV-AS9-GFP contains a mutationally inactivated HC-Pro, replicates poorly, and is unable to systemically infect *Nicotiana benthamiana* (Garcia-Ruiz et al., 2010). Alanine-scanning mutations, the technology used to generate TuMV-AS9-GFP, was borrowed from another potyvirus model system, *Tobacco etch virus* (Kasschau et al., 1997). Subsequently, it was revealed TuMV-AS9-GFP could successfully infect a plant at the systemic level if co-expressed with p19 (Garcia-Ruiz et al., 2010). Taken together, the results suggested TuMV-AS9-
GFP was unable to systemically infect plants due to HC-Pro loss-of-silencing suppressor activity.

To ensure TuMV-AS9-GFP HC-Pro loss-of-function was the root cause of poor infectivity, an experiment was setup comparing viral accumulation in wild-type Arabidopsis and *dcl 2-1/3-1/4-2*, a RNA silencing defective mutant. Although TuMV-AS9-GFP failed to infect wild-type plants, infectivity in *dcl 2-1/3-1/4-2* was reminiscent of a TuMV-GFP infection in wild-type plants (Garica-Ruiz et al., 2010). The results supported the idea that RNA silencing activity in wild-type Arabidopsis is sufficient to defend against a pathogen lacking RNA silencing suppressor activity, but the same virus can systemically infect plants lacking RNA silencing activity.

Based on preliminary data, Garcia-Ruiz and associates (2010) proposed that TuMV-AS9-GFP replication is dependent on the activity of endogenous host silencing machinery. In order to develop a detailed view of TuMV infection in Arabidopsis at the sRNA level, a deep-sequencing study was performed utilizing plants with varying levels of RNA silencing activity. Ultimately, it was demonstrated that systemic TuMV infection is dependent on *dcl4* in cauline leaves and *dcl2* in inflorescence (Garcia-Ruiz et al., 2010). Interestingly, *dcl3*-dependent 24-nt siRNAs had no apparent effect on pathogenesis (Garcia-Ruiz et al., 2010). Taken together, successful TuMV infection is dependent on the function of RNA silencing suppressors and the hierarchal function of endogenous host RNA silencing activity.

1.5 Effects of TuMV on host gene expression

Previously, our lab completed a microarray study analyzing TuMV–infected *A. thaliana* tissue (Yang et al., 2007). Several suites of genes were found to be significantly induced or suppressed. Genes related to cell wall function, hormone biosynthesis, chloroplast function, and
sulfur uptake and utilization were down-regulated. These genes have functions consistent with those expected to be involved in symptom development (e.g. abnormal vegetative and reproductive tissue development, stunted growth, chlorosis, enhanced symptom severity). It was proposed that the altered regulation of these genes may eventually be linked to sRNA pathways disrupted by TuMV infection. The literature and previous studies by our lab suggests symptomology may be partially explained by interference with developmental pathways that share components with the RNA silencing pathway.

1.6 Conclusion and Dissertation Organization

Utilizing natural and engineered viruses is an accepted approach to studying plant-virus interactions as it relates to symptomology. The majority of the research topics were generated by deciphering where short-comings in the literature existed. Specifically, how TuMV HC-Pro sRNA binding affinity affects expression of genes correlated with disease phenotypes and studying debilitated viruses in a variety of RNA silencing deficient *A. thaliana* plants. Taken as a whole, the research presented in this dissertation addressed the susceptibility of *A. thaliana* to TuMV.

The dissertation consists of three journal articles preceded by a General Introduction and followed by a General Conclusion. Each article is formatted according to the requirements of the journal. Chapter 2, “Down regulation of cell wall function and hormone biosynthesis genes in response to *Turnip mosaic virus* infection involves an unique RNA silencing pathway”, was conducted to monitor genes implicated in symptomology in various RNA silencing pathway mutant backgrounds. I hypothesized that an in vitro approach, in conjunction with an in silico study would reveal the mechanism TuMV utilizes to regulate sRNA expression, post-infection. Whitham lab member, Yeunsook Lee, graduate student in Bioinformatics and Computational
Biology at Iowa State University assisted me with the in silico study with data I downloaded from the short read archives at the National Center for Biotechnology Information. Chapter 3, “Turnip mosaic virus pathogenesis mediated by its RNA silencing suppressor, HC-Pro, requires the conserved FRNK box and a flanking neutral, non-polar amino acid”, focused on severe, moderate, and weak TuMV strains, versus A. thaliana response to pathogen challenge. I hypothesized that TuMV HC-Pro FRNK box mutants that differed in their ability to infect plants affected the function host sRNA in graduated steps. I also postulated that these mutants might allow me to uncouple developmental abnormalities associated with disease progression and accumulation of the virus itself as was observed for ZYMV. Unsurprisingly, some miRNAs implicated in leaf biogenesis and their targets were up-regulated in our study. Moderate and weak TuMV infectious clones were synthesized by a former Whitham lab technician, Valerie Torney. RNA silencing suppressor experiments were performed by a former Whitham lab graduate student, Tyrell Carr. Chapter 4, “VIGS constructs targeting hormone pathway genes in SDS-resistant soybean produce foliar symptoms when inoculated with F. virguliforme filtrate”, was a collaboration focused on identifying genes involved in resistance to soybean sudden death syndrome using a virus-induced gene silencing approach. I combined my passion for plant pathology and molecular techniques to explore a topic unrelated to potyviruses. Chapter 5 discusses general conclusions from my research tenure and future directions. Brian Anthony Campbell was the primary investigator for this work under the supervision of Dr. Steven A. Whitham and is the first author of chapters 2 and 3, and the co-first author of chapter 4.

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CHAPTER 2

DOWN REGULATION OF CELL WALL FUNCTION AND HORMONE BIOSYNTHESIS GENES IN RESPONSE TO TURNIP MOSAIC VIRUS INFECTION INVOLVES AN UNIQUE RNA SILENCING PATHWAY

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A manuscript in preparation to Molecular Plant Microbe Interaction

Author contributions: B.A.C., C. Y., and S.A.W. designed experiments, C.Y. generated over-expression lines and preliminary data, Y.L. performed in silico analysis on deep-sequencing data, B.A.C. characterized TuMV infection in dcl mutants, performed gene expression analyses and all qPCR experiments, and co-wrote the paper with C.Y.

B.A.C., C.Y., Y.L., and S.A.W. are from the Department of Plant Pathology and Microbiology at Iowa State University

Abstract

Turnip mosaic virus (TuMV) strain UK1 usually causes severely stunted growth in Arabidopsis thaliana plants (Columbia-0 ecotype). The molecular basis of this stunted growth phenotype, which is caused by a variety of plant viruses, remains poorly understood. Previous
reports show that suites of genes with functions associated with cell wall extensibility and growth are down regulated during viral infection. These genes encode proteins that are predicted or known to possess the following biochemical functions: pectin methylesterases, xyloglucan endotransglucosylase/hydrolase, expansins, and the brassinolide biosynthesis enzyme DWARF1 (DWF1). In order to test the functions of these genes in the stunted growth phenotype, CaMV 35S over-expression lines were generated. However, when over-expression lines were infected with TuMV, they all showed stunted growth phenotypes similar to TuMV-infected wild type plants. Analysis of mRNA expression demonstrated that the transcript levels of these genes were still down regulated as they were in wild type plants infected by TuMV. The activity of the 35S promoter was not reduced by TuMV infection, implying that cell wall function and hormone biosynthesis genes are down regulated by a post-transcriptional mechanism. Down-regulation of these genes in response to TuMV was perturbed in dcl1, dcl3, and rdr1/rdr2/rdr6 silencing mutants. Using a publicly available deep-sequencing dataset, small RNAs (sRNAs) associated with these genes were quantified. The data suggest the sRNAs associated with the down-regulated genes of interest are in low abundance and their levels inversely correlate with expression of their targets. In addition, 15 sRNAs were identified that share sequence identity with the A. thaliana and TuMV genomes. GAL2, a gene involved in leaf morphogenesis and positive regulation of flower development was also found to be down-regulated in a manner similar to the cell wall and hormone biosynthesis genes. These data suggest that TuMV infection activates an RNA silencing mechanism that can target endogenous host genes.

Introduction

A typical consequence of successful virus infection is onset of disease symptoms. Upon viral invasion, viruses and plants interact by a variety of offensive, defensive, and counter-
defensive strategies (Carrington and Whitham, 1998; Maule et al., 2002; Lecellier and Voinnet, 2004; Whitham and Wang, 2004; Voinnet, 2005). The balance of those interactions determines the level of systemic infection, which is ultimately manifested by the appearance of specific symptoms. The degree of symptom severity can be affected by many factors, such as growth conditions, host growth stage, and host genotype among others. The severity of symptoms is not necessarily correlated to inoculum titer implying that disease in most cases is the result of specific interactions and not an accumulation of general distress.

Typical symptoms caused by positive-stranded RNA viruses on *A. thaliana* include chlorosis, compact rosette, mosaic and curled leaves, stunting, and sterility. We previously reported that a group of genes involved in cell wall extensibility and hormone biosynthesis are down regulated during viral infection, including PME (pectin methylesterases), XTH (xyloglucan endotransglycosylase/hydrolase), expansins and brassinolide biosynthesis enzyme DARWF1 (DWF1) (Yang et al., 2007). XTH, PME and expansin all are engaged in the function of plant cell wall expansion. XTH activity is often correlated with growth rate. XTHs loosen primary cell walls and enable cell expansion by catalyzing the transfer of xyloglucan which functions as polymers that cross link cellulose microfibrils, or they may also catalyze hydrolysis to breakdown the xyloglucan-cellulose network (Rose et al., 2002). PMEs catalyze the demethylesterification of cell wall polygalacturonans and are necessary in developmental processes such as stem elongation (Micheli, 2001). Expansins have been accepted as key regulators of cell wall expansion during plant growth, and it has been implicated in biological processes associated with cell wall breakdown or softening (Li et al., 2003). DWF1 catalyzes the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. The *dwf1* mutant of *A. thaliana* has reduced synthesis of bioactive brassinosteroid, causing dwarfism
(Choe et al., 1999). Thus, there is a significant amount of literature indicating that coordinated down regulation of these genes may contribute to the stunted growth phenotype of TuMV-infected plants.

Extensive work has been undertaken to investigate how viral components interfere with host processes to achieve infection (Maule et al., 2002; Lecellier and Voinnet, 2004; Whitham and Wang, 2004; Biemelt and Sonnewald, 2006; Kachroo et al., 2006; Robaglia and Caranta, 2006). However, deciphering the biological principles behind virus-induced symptoms from the plant perspective has been less studied. Currently, the identities of host components required for infection and involved in the development of disease symptoms remain poorly understood.

Maule et al. (2002) proposed a model that host gene expression is regulated spatially in relation to the direction of virus replication and movement. Accompanying the active replication of viruses, the mRNA accumulation of different suites of host genes becomes induced or suppressed. Indeed, microarray analyses have shown that diverse viruses induce the expression of common sets of genes in compatible host plants and the altered expression of these gene sets have distinct patterns of temporal regulation (Whitham et al., 2003; Whitham et al., 2006). These reports lead to the idea that viral components interfere with host signaling networks and progressively cause physiological changes and developmental abnormalities, which culminate in the appearance of specific symptoms and disease. Thus, investigation of pathways through which viruses induce disease has been a key aspect of understanding viral symptomatology.

Inhibition of small RNA generation and function is a mechanism by which viruses manipulate the host in order to achieve infection and to cause abnormal plant growth (Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004). Short interfering RNAs (sRNAs) and microRNAs (miRNAs) both can mediate RNA interference (RNAi) that is involved in regulating
development, genome stability, and responses to biotic and abiotic stresses. In addition, RNAi has a protective function through the degradation of viral RNA species. This antiviral response is mediated through the production of virus-derived sRNA (Voinnet, 2001). As a counter-defensive strategy viruses encode various RNAi suppressors. A few examples include P1-HC-Pro of Turnip mosaic virus (TuMV), the P38 protein of Turnip crinkle virus (TCV), and the P19 protein of Tomato bushy stunt virus (TBSV) (Llave et al., 2000; Qu and Morris, 2002; Thomas et al., 2003). These multifunctional viral proteins can cause dramatic effects on plant growth and development, because their roles in protecting viruses from degradation coincidently interfere with either the processing or functions of endogenous miRNAs (Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004).

RNAi pathways are triggered by double-stranded RNAs (dsRNAs) which are cleaved by Dicer-like proteins (DCL), an RNase III enzyme, to produce sRNAs or miRNAs. These 21-25 nucleotide small RNAs are then incorporated into Argonaute (AGO) in which they direct the cleavage of single-stranded RNAs complementary to small RNAs. RNA-dependent RNA polymerase (RDRP) is also required in some cases to initiate and amplify the primary silencing target. In A. thaliana, ten AGOs, four DCLs and three functional RDRs have been identified (Dalmay et al., 2000; Morel et al., 2002; Schauer et al., 2002). Accumulating evidence supports the view that small RNA pathways in A. thaliana comprise a complex network (Vaucheret, 2006).

In this study, we addressed the potential functions of cell wall and hormone biosynthesis genes in virus-infected plants and mechanisms by which their expression is regulated. Over-expression of EXP10 (At1g26770), PME3 (At3g14310), XTH6 (At5g65730), and DWF1 (At3g19820) under control of the constitutive Cauliflower mosaic virus (CaMV) 35S promoter
did not alter the symptoms, and more interestingly, did not prevent the decreased expression of the genes of interest. Further analysis of the endogenous expression of these genes in a collection of RNA silencing mutants demonstrated a RNA degradation mechanism dependent upon DCL1, DCL3, and RDR1/RDR2/RDR6. Analysis of small RNA sequencing data from TuMV-infected Arabidopsis plants (Garcia-Ruiz et al., 2010) identified sRNAs associated with these genes, and expression of genes associated with sRNAs were quantified. The data suggested the sRNAs associated with the down-regulated genes of interest are low in abundance and are negatively correlated with expression of mRNA targets. These results demonstrate that a previously uncharacterized virus-induced RNAi mechanism may be involved in the repression of some host genes in response to viral infection.

Results

**Transgenic *A. thaliana* lines that over express PME3, XTH6, EXP10 and DWF1**

Previously we reported that the mRNA transcript abundance of a suite of cell wall modification genes and a brassinolide biosynthetic gene, including *EXP10* (At1g26770), *PME3* (At3g14310), *XTH6* (At5g65730), and *DWF1* (At3g19820) was reduced during TuMV infection. To begin investigating the functions of *PME3, XTH6, EXP10* and *DWF1* in the TuMV induced disease symptoms, we fused the full-length open reading frame of each gene with the constitutive CaMV 35S promoter and over expressed each of these genes in the Columbia-0 (Col-0) *A. thaliana* ecotype (Fig. 1A). Five to ten independent transgenic lines were generated for each construct. The growth and development of all 35S::PME3, 35S::XTH6 and 35S::EXP10 transformants were similar to wild type, but there were noticeable differences between 35S::DWF1 over-expression lines and Col-0. The leaves and stems of 35S::DWF1 transgenic plants were thicker than wild type plants (not shown). We
used semi-quantitative RT-PCR and quantitative PCR to compare the transcript levels of each of the transgenes to their normal levels in wild type plants. Transgenic lines expressing a much higher RNA level were identified for all of the genes. As shown in Figure 1B, four T2 lines from 35S::EXP10 construct had much higher EXP10 transcript level compared to Col-0. Similar results were observed for three T2 lines from 35S::PME3 and two T2 lines from 35S::DWF1. Of the four 35S::XTH6 T2 lines tested, three lines had elevated levels of XTH6 transcripts, but the fourth was not different from control plants.

**Over expression of PME3, XTH6 and EXP10 did not alter the disease symptoms caused by TuMV**

To test whether over expression of selected cell wall genes could alter the stunted growth symptoms caused by TuMV, four 35S::EXP10, two 35S::XTH6, and two 35S::PME3 lines were challenged with TuMV. Infection of the Col-0 ecotype with TuMV typically causes a severely stunted phenotype characterized by the decreased growth of the flowering bolts of infected plants is compared to the bolts of non-infected plants. In this experiment, we compared the bolt length of mock and TuMV-infected transgenic lines (mock vs TuMV) towild-type A. thaliana (mock vs TuMV) to analyze whether the stunted growth phenotype was affected in the over-expression lines. Plants from the 35S::EXP10 transgenic lines had shorter bolts beginning from 5 dpi compared to wild type plants but both types of plants grew with a similar rate to wild type (Fig. 2A). However, starting at 10 dpi, the TuMV infected wild type and 35S::EXP10 plants grew at a reduced rate compared to non-infected plants. The 35S::PME3 and 35S::XTH6 plants had longer average bolt lengths than wild type plants at 0 dpi. The bolts of wild type and transgenic plants grew at a similar rate in all the lines until 6 dpi, when bolt growth became dramatically reduced
in both wild type and transgenic plants (Fig. 2A). Thus, over expression of any of the three cell wall genes did not compensate for the stunted growth of infected plants.

The inability of over expression of these genes to prevent the stunted growth phenotype could be explained by a few alternative hypotheses. The expression of these genes might be down regulated even though they are under control of the constitutive 35S promoter, which is not known to have reduced activity in virus-infected plants. Since we determined that the transcript levels of each gene were increased in transgenic lines, we were not concerned about transgene-mediated PTGS. In addition, many studies have shown that PTGS of 35S promoter fusions to reporter genes such as GUS and GFP can be reversed by potyvirus infection (Kasschau and Carrington, 1998). A trivial explanation could also be that the down-regulation of these genes has no role in the stunted growth phenotype.

In order to determine the expression levels of the PME3, XTH6, and EXP10 in virus-infected transgenic plants, we used semi-quantitative RT-PCR and quantitative RT-PCR (qRT-PCR). The mRNA transcript levels of both mock- and TuMV-infected plants from each transgenic line compared to wild type. Surprisingly, dramatic decreases of the target gene mRNA transcript were observed for nearly all of the lines of transgenic plants infected by TuMV (Fig. 2B and Fig. 3). For example, the transcript level of PME3 was reduced similarly in the TuMV-infected wild type and 35S::PME3 T2-4 plants. The 35S::PME3 T2-3 plants were not as dramatic, but a strong decrease was also demonstrated. Similar results were also observed in 35S::XTH6 T2-1 and T2-10 plants, 35S::EXP10 T2-1 and T2-5 plants, and 35S::DWF1 T2-3 plants.

To exclude the possibility that TuMV specifically reduces the activity of the CaMV 35S promoter, we analyzed the activity of CaMV 35S::GUS reporter fusion in response to TuMV
infection by a GUS fluorometric assay. As shown in Fig. 4, there is no decrease in GUS activity of TuMV-infected tissue compared to mock-infected tissue.

**Cell wall function and hormone biosynthesis genes are down-regulated in WT and dcl 2/4 plants, but remain stable in dcl 2/3/4 Arabidopsis**

RNA silencing is a conserved mechanism of post transcriptional gene regulation among eukaryotic organisms. To investigate the whether an RNA silencing pathway is involved in the down regulation of the mRNA of the selected cell wall genes, we selected two DICER-LIKE mutant lines of *A. thaliana, dcl2-1/dcl4-1* and *dcl2-1/dcl3-1/dcl4-1* for analysis of down-regulated genes following TuMV infection. These mutants were selected, because they have defects in the major anti-viral *DCL* genes (Xie et al. 2004, Xie et al. 2005). The transcript levels of the four target genes were determined by qRT-PCR in infected versus mock-infected plants. To ensure the abnormal leaf morphology and stunted growth phenotype associated with TuMV infection was induced by the virus, tissue samples were harvested and tested for the presence of TuMV by RT-PCR. Virus accumulation was detected at similar levels in all genotypes tested at 10 dpi but was not detected in non-inoculated control samples (Fig. 5). Subsequently, qRT-PCR was performed on systemically infected tissue. *DWF1* expression was reduced approximately 1.8- and 1.7-fold in wild type and *dcl 2-1/4-2* plants, respectively, but no significant change were observed in *dcl 2-1/3-1/4-2* plants (Fig. 6A). Similar to *DWF1*, expression of *EXP10* was reduced approximately 1.5- and 1.3-fold in wild type and *dcl 2-1/4-2* plants, respectively, but no significant change was observed in *dcl 2-1/3-1/4-2* plants (Fig. 6B). *PME3* expression was also attenuated in wild type and *dcl 2-1/4-2* plants, approximately 1.2- and 1.4-fold, respectively (Fig. 6C). *XTH6* expression was diminished approximately 2.4- and 1.7-fold in wild type and *dcl 2-1/4-2* plants, respectively (Fig. 6D). In summary, we found *DWF1, EXP10, PME3*, and *XTH6*...
were down-regulated in wild-type plants and a silencing defective mutant, *dcl2-1/4-2*, yet remained stable in silencing defective mutant *dcl 2-1/3-1/4-2* (Fig. 6). The results suggest that the down-regulation of cell wall function and hormone biosynthesis genes requires *DCL3*.

**Genes involved with an RNAi mechanism are required for the decreased expression of cell wall and hormone biosynthesis genes**

To further investigate the RNA silencing pathway associated with the down regulation of the mRNA of the selected cell wall genes, we repeated experiments and added additional *A. thaliana* mutants with loss of function mutations in *DICER-LIKE (DCL)* and *RNA DEPENDENT RNA POLYMERASE (RDR)* genes. The transcript levels of the four target genes were estimated by semi-quantitative RT-PCR in infected versus mock-infected plants. In the *dcl1-7* single mutant and the *dcl2-1/dcl3-1/dcl4-1* and *rdr1-1/rdr2-1/rdr6-15* triple mutants, *PME3* mRNA was only slightly reduced, which was in contrast to the dramatic decrease in Col-0 wild-type plants (Fig. 7A). This result indicated that *DCL1* in combination with other *DICER* and *RDR* genes were necessary for the decreased expression of *PME3*.

To further delineate the role of the specific *DCL* and *RDR* genes required for down regulation of *PME3*, its expression was measured in the *dcl2-1/dcl3-1*, *dcl2-1/dcl4-1* and *dcl3-1/dcl4-1* double mutants (Fig. 7B). As in wild type plants, *PME3* mRNA was dramatically reduced in *dcl2-1/dcl4-1*, but it was only slightly reduced in *dcl2-1/dcl3-1* and *dcl3-1/dcl4-1* indicating the repression of *PME3* was *DCL3* dependent. The *rdr1-1, rdr2-1*, and *rdr6-15* single mutants were also tested for their effects on *PME3* mRNA accumulation. A decrease in *PME3* mRNA level was observed for all three of the mutants, but not to the same extent as in wild type. This result indicated that *RDR1, RDR2*, and *RDR6* have partially redundant functions in decreased accumulation of *PME3* mRNA in response to TuMV infection.
To determine whether these effects applied to other genes that were down regulated in response to TuMV infection, the expression of XTH6, EXP10, and DWF1 was also analyzed in the three DCL double mutants and the three RdRPs single mutants (Fig. 7B). Similar results were obtained demonstrating that these four genes are down regulated by TuMV through the same pathway involving DCL1, DCL3, and RDR1/RDR2/RDR6.

TuMV and down-regulated genes of interest share no significant nucleotide sequence similarity

The role of RNA silencing genes in the down-regulation of suites of Arabidopsis genes suggests that decreased expression is mediated by small RNAs in the 21-24 nt size range. One potential source of small RNAs (sRNAs) is the TuMV genome, which is targeted by DCL2, DCL3, and DCL4 during infection (Garcia-Ruiz et al. 2010). If these small RNAs had sufficient sequence identity to the down-regulated host genes, then they could mediate their decreased expression. To test the possibility that small RNAs of TuMV origin shared sequence identity with host genes, we used CLUSTALW (Thompson et al. 1994) and BLAST (Altschul et al. 1990) to perform pairwise alignments between the target genes and the complete viral genome or 100 bp windows over the viral genome. Our results demonstrate that PME3, XTH6, EXP10, and DWF1 share no significant regions of sequence identity with the TuMV genome (Fig. 8. and data not shown). These results indicate that if small RNAs are involved in regulating expression of the selected genes, then they are not derived from TuMV.

sRNAs associated with cell wall function and hormone biosynthesis genes are up-regulated in wild-type plants, remain stable in silencing defective mutants post-infection

Next, we investigated if sRNAs with sequence similarity to the genes of interest could be produced in non-infected and infected plants from an endogenous source. The public availability
of the deep sequencing data from Garcia-Ruiz et al. (2010) allowed sRNAs of host and viral origin to be identified from systemically infected tissue. The same data set was also analyzed for sRNAs derived from cell wall function and hormone biosynthesis genes. Expression of PME3-derived sRNAs increased approximately 2.6-fold in wild type plants, while remaining relatively stable in dcl 2-1/3-1/4-2, a silencing defective mutant. Similar trends were observed for EXP10-, XTH6-, and DWF1-derived sRNAs. Fold changes of 3.0, 1.3, and 1.6 were observed, respectively. The data suggest that these sRNAs are inversely correlated with the expression of their targets in wild-type plants, while remaining relatively stable in silencing defective mutant, dcl 2-1/3-1/4-2, in response to TuMV challenge (Fig. 9). As a control, sRNAs associated with BGL2 were quantified because the mRNA transcripts of this gene are typically induced by TuMV infection (Yang et al. 2007). Therefore, sRNAs derived from BGL2 are not expected to also be induced. In line with this expectation, sRNAs associated with BGL2 remained stable post-TuMV infection, indicating BGL2 mRNAs are not post-transcriptionally regulated by sRNAs (Fig. 10). These data indicate that sRNAs with sequence identity to the cell wall function and hormone biosynthesis genes are produced from an endogenous source at 10 dpi. Due to their low abundance, we were not able to quantify these sRNAs by other methods such as Northern blot or qRT-PCR.

**Unique A. thaliana & TuMV sRNAs correspond to 15 genes**

While analysis of the cell wall and hormone biosynthesis genes identified sRNAs of endogenous origin, we also found 15 unique sRNAs corresponding to both the A. thaliana and TuMV genome (Table 2). Five target genes, FAAH (AT5G64440), GAL2 (AT5G083701), GLR2.6 (AT5G11180), NTT1 (AT1G80300), and TRX2 (AT5G39950), were further explored because annotation suggested possible roles in TuMV pathogenesis and/or symptomology.
GAL2, involved in leaf morphogenesis and positive regulation of flower development, was found to be down-regulated post-TuMV infection here and in microarray data from a previous study (Yang et al., 2007; Fig. 11). These results further validate our in silico experiments and reinforce the idea that a virus induced RNA silencing mechanism may be involved for the repression of specific suites of host genes, post-TuMV challenge.

Discussion

Suites of Arabidopsis genes related to cell wall function (PME3, XTH6, EXP10) and hormone biosynthesis (DWF1) were down-regulated post-TuMV infection (Yang et al., 2007). Down-regulation of these genes has been postulated to contribute to the abnormal leaf morphology and stunted growth phenotype associated with infection. In order to test this idea, over-expression lines were generated to further characterize their functions. Surprisingly, the cell wall function and hormone biosynthesis genes, PME3, XTH6, EXP10, and DWF1 remained down-regulated post-TuMV infection despite being expressed under control of the 35S promoter. These results coupled with the demonstration that the Cauliflower mosaic virus (CaMV) 35S promoter is not down-regulated post-TuMV infection suggested a post-transcriptional gene silencing (PTGS) mechanism underlying the down-regulation of the cell wall and hormone biosynthesis genes.

Analysis of a collection of RNAi mutants demonstrated that down-regulation of these genes is dependent on DCL1 and DCL3, RDR1, RDR2, and RDR6. These findings identify an RNA silencing pathway that is induced by TuMV infection and targets specific endogenous genes for down regulation.

The in silico analysis of sRNAs, identified sRNAs associated with cell wall and hormone biosynthesis, suggesting a possible role in negatively regulating target genes. Although qRT-PCR experiments designed to monitor the expression of all genes (Table 1) were successful,
detecting and quantifying sRNAs associated with these transcripts was largely unsuccessful. Fortunately, a deep-sequencing dataset quantifying sRNAs, became publicly available (Garcia-Ruiz et al., 2010). Garcia-Ruiz et al. (2010) reasoned using wild-type virus-encoded silencing suppressors could mask the effect of wild-type plant silencing factors. Therefore, using different combinations of TuMV mutants with debilitated HC-Pro function and A. thaliana RNA silencing deficient mutants could provide a detailed view of the sRNAs formed during TuMV infection. While they were interested in sRNAs of TuMV origin, we were able to mine their dataset to identify endogenous sRNAs associated with the down-regulated genes of interest. After normalizing the data, we observed that these sRNAs are not abundant, which explains the negative sRNA blotting results (data not shown). We determined there are sRNAs corresponding to EXP10, PME3, XTH6, and DWFL that are induced in response to TuMV at 10 dpi. Small RNAs associated with an induced gene, BGL2, were quantified as an in silico control and did not change significantly as expected. The diminished target gene expression coupled with the increase in sRNAs associated with the down-regulated genes of interest suggested negative regulation by the sRNAs.

In A. thaliana, four specialized DCL proteins have been identified (Brodersen and Voinnet, 2006; Vaucheret, 2006). Coordinated or hierarchical actions of DCL proteins have been demonstrated to be required for antiviral defense and virus-induced silencing of host genes (Deleris et al., 2006; Moissiard and Voinnet, 2006). In the process of antiviral defense, DCL4 is the primary sensor that generates 21-nt sRNAs and activity of DCL2 is only manifested when DCL4 is suppressed. A combined action of both proteins is required for complete anti-viral defense. In the case of anti-viral silencing directed toward CaMV, DCL2, DCL3, and DCL4
efficiently produced 22-, 24-, and 21-nt vsRNAs, respectively and DCL1 plays a facilitating role for accumulation of these species of viral sRNAs (Moissiard and Voinnet, 2006).

Among four specialized DCLs, DCL3 is the only one functioning in the nucleolus and it mediates the nuclear dsRNA cleavage to generate 24-nt sRNAs that guide RNA-directed DNA methylation (RDDM) (Chan et al., 2004; Xie et al., 2004). DNA methylation then results in heterochromatin formation or transcriptional repression of transposon and DNA repeats. In the nucleus, short RNAs or even dsRNA could guide methylation of complementary DNA. It may directly guide the chromodomain of chromomethylase (CMT) to DNA sequence, or base pair with one homologous DNA strand, and leave another single-stranded DNA to form an unusual bulge, which can attract a de novo DNA methyltransferase (DNMT) (Matzke et al., 2001; Chan et al., 2004). Possible mechanisms for the down-regulation of cell wall function and hormone biosynthesis genes involving DCL3 include transcriptional gene silencing (TGS). Transcriptional gene silencing (TGS) is associated with RNA-directed DNA methylation and characterized by chromatin modifications that silence transcription (Verdel et al., 2009). DCL1 functions in cleavage of the intergenic/intronic fold-back precursors encoded by MIR genes to generate 21 nt miRNAs (Bartel, 2004). At this time, the mechanism which DCL1 and DCL3 work collectively to down-regulate the expression of AtEXP10, AtPME3, AtXTH6, and AtDWF1 is unclear.

RDRP functions in initiation and maintenance of silencing responses by producing dsRNA from viral/transgene templates or amplifying mobile silencing signals. Involvement of the RDR 1, RDR2 and RDR6 suggests that the RNA silencing signal is specifically amplified for these genes. As to a specific role of each RDRP, RDR1 and RDR6 are thought to be involved in VIGS and PTGS. RDR6 is also required for DCL4-depedent trans-acting sRNA (tasRNAs)
production (Gasciolli et al., 2005). RDR2 is required for the generation of all endogenous sRNA analyzed and its exact role is not yet clearly understood (Wassenegger and Krczal, 2006).

Regarding the results presented here, one hypothesis is that DCL1 mediates the primary cleavage from endogenous source of dsRNA induced by TuMV. Then RDRs specifically amplify the small RNA signal for those genes, and then DCL3 is involved in the subsequent steps of RNA cleavage. Analyzing the species of sRNA produced in this process and mutants of other components of silencing machinery, such as the required AGOs, will be necessary to further explore this pathway.

**Material & Methods**

**Plasmid construction**

The full-length open reading frames, *EXP10* (At1g26770), *PME3* (At3g14310), *XTH6* (At5g65730), and *DWARF1* (At3g19820) were amplified from *A. thaliana* first-strand cDNA with HiFi Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) using the oligonucleotide primers listed in Table 1. The PCR products were TOPO-cloned into the pCRII vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced to verify the accuracy of the DNA fragment. For expression in *A. thaliana*, the open reading frames of *EXP10* (At1g26770) and *DWF1* (At3g19820) were cloned into the BglII and PmlI site of pCambia 3301 under control of the 35S promoter, and the open reading frames for *PME3* (At3g14310) and *XTH6* (At5g65730) were cloned into XbaI and XhoI sites of binary vector PBI111L under control of the CaMV 35S promoter.

**Analysis of next-generation sequencing data**

The sequencing data can be found in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE20197 (Garcia-Ruiz et al.
All reads from GSE20197 were blasted against genes of interest from Yang et al., 2007 (e-value cutoff ≤ 1). Using Microsoft Excel, a table containing the average expression level was quantified after calculating Reads per Kilobase Mapped (RPKM).

**Plant Materials**

CaMV35S::GUS over-expression transgenic plants were kindly provided by Xiaomin Yu from Dr. Eve Wu tele’s lab. The *dcl* and *rdr* mutant lines were kindly provided by Dr. James Carrington’s lab (Deleris et al., 2006).

*Arabidopsis thaliana* plants were grown from seed in (10-cm) pots. Seedlings were thinned five days after sprouting and grown under long-day conditions (16 h light, 8 h dark) in a growth chamber at 22°C. All constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 and transformation of the *A. thaliana* Columbia-0 ecotype was conducted as described (Clough and Bent, 1998). T1 seeds from the 35S::*PME3* and 35S::*XTH6* construct transformations were screened on MS plates with 100 mg/L kanamycin. T1 seeds from 35S::*EXP10*, 35S::*DWF1*, and the BGL2::*PME3* construct transformations were screened with diluted herbicide (1:1000 v/v) Finale (AgrEvo USA, Wilmington, DE, USA). Five to ten T1 seedlings from each construct that survived selection were transferred to individual pots for self fertilization and production of T2 seeds. Rub-inoculation was performed when plants were approximately 7-8 weeks old.

**Rub Inoculation**

TuMV-GFP inoculation was completed according to Yang et al. (2007). Briefly, *A. thaliana* plants were grown for approximately 3 weeks in 16 h of light at 22°C until plants began to bolt. Three to four rosette leaves from each plant were dusted with Carborundum and rub.
inoculated with TuMV-GFP diluted in 20 mM phosphate buffer (pH 7.2, 1:5, wt/vol) using a cotton-stick applicator. Control plants were dusted with Carborundum and three to four rosette leaves from each plant were mock inoculated with a cotton-stick applicator that was soaked in phosphate buffer spiked with uninfected Arabidopsis leaf sap. At the times indicated in each experiment, systemic tissue samples were harvested from three plants per treatment per genotype under UV illumination, pooled, frozen in liquid nitrogen, and stored at -80°C. Three independent biological replicates were performed unless indicated otherwise.

**RNA Extraction**

Total RNA was extracted from systemic rosette leaves, stems, and reproductive tissue according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen). Samples were treated with RNase-free DNase (TURBO DNA free™ kit, Ambion) before downstream applications.

**Semi-Quantitative RT-PCR**

First strand cDNA was synthesized using 1 μg of total RNA, oligo d(T24) primer and Superscript III reverse transcriptase (Invitrogen). Primers used for semi-quantitative RT-PCR of PME3 and ACTIN8 are shown in Table 1. PCR reactions for PME3 were conducted using the following thermal cycling profile: 95 °C, 30 second; 55 °C, 30 second; and 72 °C, 2 minutes for 27 cycles. PCR reactions for ACTIN8 were conducted using the following thermal cycling profile: 95 °C, 30 second; 58 °C, 30 second; and 72 °C, 60 second for 27 cycles.

**Quantitative RT-PCR**

Five micrograms of DNase-treated total RNA was used as template for first strand synthesis and performed according to the manufacturer’s instructions (SuperScript™ III First Strand Synthesis, Invitrogen). Afterwards, samples were diluted 1:20 and 1:4 for genes of interest and controls, respectively. Quantitative PCR was performed using a PerfeCTa SYBR
Green Fast Mix for iQ (Quanta BioSciences, Gaithersburg, MD, USA). Primers used for qPCR are shown in Table 1. Conditions are as follows – 3 min at 95°C followed by 45 PCR cycles: 15 sec at 95 °C, 1 min at primer T_M, which was followed by melt curve analysis to confirm presence of single product free of primer dimer contamination.

**GUS histochemical and fluorometric assay**

GUS activity was visualized by immersion of dissected leaf in 50 mM sodium phosphate (pH 7.2), 0.5 mM potassium ferri- and ferro-cyanide, 2 mM X-gluc, and 0.05% Triton X-100 under vacuum for 10 min, followed by incubation at 37 °C overnight.

The fluorogenic reaction was performed in 40mM NaH2PO4 pH7.0, 10mM EDTA pH8.0, 0.1% Triton X-100, 3.4 mM sodium lauryl sarcosine, 8.3 mM β-mercaptoethanol, 1.9 mM 4-MUG substrate buffer with a reaction volume of 50 μl. The reaction was incubated at 37°C for 30 minute, and then terminated with the addition of 88 mM Na2CO3 to 1.5 ml total. Fluorescence was measured on a Hoefer DyNA Quant 200 spectrofluorimeter (Amersham, Uppsala, Sweden).

**Acknowledgements**

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**References**


Table 1. Sequence and annealing temperature of primer pairs used for semi-quantitative RT-PCR and qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→ 3’)</th>
<th>Annealing Temp (°C)</th>
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<tr>
<td>ACTIN8qF</td>
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</tr>
<tr>
<td>ACTIN8qR</td>
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Table 2. Target genes of 15 unique sRNAs derived from *A. thaliana* and TuMV genome

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Fig. 1. RT-PCR analysis of transgenic over-expression lines.

A, Cloning scheme of the four over-expression constructs. B, Semi-quantitative RT-PCR was used to determine relative levels of each gene in leaf tissue of wild type and over-expression lines. In this and other figures, Actin8 was utilized as a control gene, which does not respond to viral infection (Yang et al 2007).
**B**

![Gene Expression Images](image)

**Fig. 2.** Over expression of cell wall genes does not inhibit the stunted growth caused by TuMV.

**A.** Bolt growth in transgenic lines over expressing cell wall genes as compared to Col-0 wild type plants. a, b, c, and d, four independent lines of transgenic 35S::*EXP10*. e and f, two independent lines of transgenic 35S::*XTH6*. g and h, two independent lines of transgenic 35S::*PME3*. Bolt length is the average value from 5 to 6 plants. **B.** Relative expression levels of each gene in fluorescence tissue from wild type and over-expression lines in response to TuMV infection by semi-quantitative RT-PCR at 14 dpi.
**Fig. 3.** Expression of cell wall function gene, *PME3*, 10 days post *Turnip mosaic virus* (TuMV) infection. The error bars represent the standard error of the mean for each arithmetic mean (n = 6). Star indicates a statistical difference between treatments per genotype per gene of interest by Student’s t-test (P ≤ 0.05).
Fig. 4. Activities of CaMV35S promoter in response to TuMV comparing to mock treatment. Using CaMV 35S::GUS reporter fusion, the activity of 35S promoter was determined using a fluorogenic GUS activity assay.
Fig. 5. A. Wild-type and RNA silencing defective *Arabidopsis thaliana* plants under UV illumination. B. Reverse-transcriptase polymerase chain reaction of TuMV Coat protein region. *A. thaliana ACTIN 8* was used as a positive control to ensure equal loading, because its expression is unaltered by TuMV infection.
A

![DWF1](image)

**Fold Change**

- Genotype: Col 0
- Genotype: dcl 2-1/4-2
- Genotype: dcl 2-1/3-1/4-2

**Genotype**
**PME3**

Flod Change

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Significant difference indicated by ★
Fig. 6. Fold change in expression of selected down-regulated genes, 10 days post *Turnip mosaic virus* (TuMV) infection. **A**, *PME3*. **B**, *EXP10*. **C**, *PME3*. **D**, *XTH6*. The error bars represent the standard error of the mean for each arithmetic mean (n = 9). Star indicates a statistical difference between treatments per genotype per gene of interest by student’s t-test (P ≤ 0.05).
Fig. 7. RNA silencing genes are involved in the down-regulation of *EXP10*, *PME3*, *XTH6*, and *DWF1*.

A, RT-PCR results of the comparison of *PME3* mRNA transcript levels in Col-0 and *dcl1-7*, *dcl2-1/3-1/4-1* and *rdr1-1/2-1/6-15* mutants in response to TuMV. B, RT-PCR results showing the relative expression level of four genes in three DCL double mutants and three RDR single mutants.
Fig. 8. TuMV, cell wall function and hormone biosynthesis genes share no significant nucleotide sequence similarity. Nucleotide similarity was determined using the pairwise alignment function within VectorNTI 11. TuMV secondary structure was determined using MFOLD software.
Fig. 9. Small RNAs associated with cell wall and hormone biosynthesis genes are up-regulated, 10 days post Turnip mosaic virus (TuMV) infection. The average expression level was quantified after calculating Reads per Kilobase Mapped (RPKM) for data set GSE20197, derived from Garcia-Ruiz et al. 2010. The error bars represent the standard error of the mean for each arithmetic mean (n = 3). Star indicates a statistical difference between treatments per genotype per sRNA associated with gene of interest by Student’s t-test (P ≤ 0.05).
**Fig. 10.** sRNAs associated with BGL2 remain stable, 10 days post *Turnip mosaic virus* (TuMV) infection. The average expression level was quantified after calculating Reads per Kilobase Mapped (RPKM) for data set GSE20197, derived from Garcia-Ruiz et al. (2010). The error bars represent the standard error of the mean for each arithmetic mean (n = 3). Star indicates a statistical difference between treatments per genotype per sRNA associated with gene of interest by Student’s t-test (P ≤ 0.05).
Fig. 11. GAL2; AT5G083701, is down-regulated 10 days post Turnip mosaic virus (TuMV) infection. The error bars represent the standard error of the mean for each arithmetic mean (n = 9). Star indicates a statistical difference between treatments per genotype per gene of interest by Student’s t-test (P ≤ 0.05).
CHAPTER 3

TURNIP MOSAIC VIRUS PATHOGENESIS MEDIATED BY ITS RNA SILENCING SUPPRESSOR, HC-PRO, REQUIRES THE CONSERVED FRNK BOX AND A FLANKING NEUTRAL, NON-POLAR AMINO ACID

Brian A. Campbell, Tyrell Carr, Valerie Torney, and Steven A. Whitham

A manuscript in preparation for submission to Molecular Plant Microbe Interaction

Author contributions: B.A.C., T.C., V.T., and S.A.W. designed experiments, V.T. generated TuMV mutants and HC-Pro mutants and performed N. benthamiana bombardments, T.C. generated PVX expressing wild-type and mutant HC-Pros and co-wrote the paper, B.A.C. performed qPCR and A. thaliana rub inoculations and A. thaliana bombardments and LHCB4 western blot and co-wrote the paper. B.A.C., T.C., V.T., and S.A.W. are from the Department of Plant Pathology and Microbiology at Iowa State University

Abstract

A major determinant of symptom expression in potyvirus-infected plants is the viral-encoded helper-component proteinase (HC-Pro). Previous studies have shown that potyvirus HC-Pro mutants that attenuated symptom expression were impaired in virus accumulation or movement. However, a mutation in the highly conserved FRNK box of Zucchini yellow mosaic potyvirus (ZYMV) HC-Pro was found that uncoupled HC-Pro’s role in symptom expression from its role in virus accumulation and movement. Interestingly, when the same mutation was introduced into Turnip mosaic virus (TuMV) HC-Pro, it impaired viral accumulation and
symptom expression. Because ZYMV and TuMV HC-Pro mutants differ in amino acids flanking the FRNK box, this prompted us to examine whether these amino acids conditioned the different phenotypes. To investigate this possibility, additional TuMV mutants were generated and tested for infectivity and pathogenesis and corresponding HC-Pro constructs were tested for RNA silencing suppression activity. We have found that TuMV pathogenesis and RNA silencing suppressor activity require the FRNK sequence and the flanking neutral, non-polar amino acid isoleucine. The FRNKI box was also required for increased symptom development in plants infected with PVX expressing HC-Pro. Hence, the FRNKI box of TuMV HC-Pro in RNA silencing suppressor activity is correlated with viral pathogenicity.

**Introduction**

Helper component protease, HC-Pro, is a multifunctional protein of potyviruses with three distinct domains: N-terminal, central, and C-terminal (Maia et al., 1996, Urcuqui-Inchima et al., 2001). The central domain has been implicated in RNA silencing suppressor function and binding to small RNAs (sRNAs). The conserved FRNK box motif of *Zucchini yellow mosaic virus* (ZYMV) at amino acid position 179 – 182 was shown to be involved in silencing suppressor activity and sRNA binding. It was reported that a naturally occurring ZYMV variant, ZYMV-HC(-), which contains an isoleucine (Ile; I) for arginine (Arg; R) substitution, attenuated symptoms without affecting virus accumulation (Gal-On 2000).

Although HC-Pro is a multi-functional protein, the FRNK to FINK mutation solely affected symptom expression. Gal-On (2000) proposed that protein-protein interactions were disrupted that affected the virulence of ZYMV. It was hypothesized that this protein-protein interaction, which induces disease symptoms, may have been adversely affected by the mutation (Gal-On 2000). Additionally, replacing a basic amino acid, Arg, with a hydrophobic amino acid,
Ile, may have adversely affected motif polarity (Gal-On 2000). Although it was hypothesized that HC-Pro interacts with a putative host factor, no candidates were identified.

Subsequently, Shiboleth et al. (2007) showed that HC-Pro binds sRNAs and that mutations in the FRNK box motif affected their binding (Shiboleth et al., 2007). Wild type HC-Pro\textsuperscript{FRNK} and mutant HC-Pro\textsuperscript{FINK} were shown to differentially bind highly conserved micro RNAs (miRNAs). This differential binding affected the accumulation of the conserved miRNAs and their complementary strands (miRNA*s) during ZYMV infection. The results suggested that differences in symptomology of the wild type and mutant ZYMV strains were due to their interaction with regulatory miRNAs. In addition, these ZYMV isolates were able to accumulate to similar levels as symptoms were induced suggesting that virus accumulation and symptomology had been uncoupled (Shiboleth et al., 2007). Interestingly, ZYMV HC-Pro\textsuperscript{FINK} retained its ability to function as an RNA silencing suppressor despite the weaker binding of miRNA/miRNA* duplexes (Shiboleth et al., 2007).

The TuMV-Arabidopsis pathosystem has been developed as a model for studying host-potyvirus interactions. TuMV HC-Pro contains the conserved FRNK box motif in the central domain and TuMV HC-Pro functions as a strong RNA silencing suppressor (Garcia-Ruiz et al., 2010). This system is suitable for studying the genetic requirements for viral infection and symptomology. Because of the potential to uncouple symptoms from virus accumulations, we chose to study the role of the FRNK box motif in TuMV as a first step towards using it as a probe to investigate genetic requirements for symptomology. In our study, we found that TuMV\textsuperscript{FINK} was poorly infectious and did not induce any visible symptoms in plant species examined due to deficient HC-Pro\textsuperscript{FINK} RSS activity as previously suggested by Shiboleth and associates (2007). This poorly infectious phenotype was also
observed among other TuMV clones carrying mutations in the FRNK box. By replacing a neutral amino acid flanking the FRNK box with a positive charged amino acid, we found that TuMV was able to move systemically but the symptoms became very mild. Interestingly, viral accumulation was not observed in stems, veins and newly emerging leaves. Taken together, full TuMV pathogenesis mediated by HC-Pro RSS activity not only requires the FRNK box but a flanking neutral amino acid.

**Results**

**TuMV infectivity of *A. thaliana* and *N. benthamiana* likely requires an intact FRNKI sequence in HC-Pro.**

To determine if the FRNK box of TuMV HC-Pro (Fig. 1A) was required for TuMV pathogenesis, we generated the viral mutant TuMV$^{\text{FINK}}$ containing the same point mutation that was shown to uncouple ZYMV HC-Pro’s roles in viral accumulation and symptom expression in cucurbits (Shiboleth et al., 2007). p35STuMV-GFP plasmids encoding full-length TuMV$^{\text{FRNK}}$ or TuMV$^{\text{FINK}}$ were introduced by particle bombardment into 3 week-old leaves of *A. thaliana* and *N. benthamiana* and GFP fluorescence was monitored daily. At 11 or 15 days after inoculation (DAI), multiple plants were photographed under UV light to detect GFP fluorescence. In *A. thaliana*, wild type TuMV$^{\text{FRNK}}$ was highly infectious in rosette leaves and systemic tissues and it induced severe symptoms. In contrast, TuMV$^{\text{FINK}}$ infection foci were barely visible in inoculated leaves and there were no symptoms (Fig. 2A). This result suggested that the effects of the FRNK to FINK mutation were deleterious to TuMV as compared to this mutation in ZYMV.

Because ZYMV HC-Pro encodes an arginine (R$_{183}$) flanking the FRNK box (FRNKR) (Shiboleth et al., 2007), we hypothesized that the poor infectivity by TuMV$^{\text{FINK}}$ (UK1 isolate) was due to a loss of charge at this position because the wild type FRNK box of TuMV HC-Pro is
flanked by isoleucine (I_{185}) (Fig 1B). The amino acids, isoleucine and arginine are neutral-hydrophobic and positive-basic, respectively. Accordingly, TuMV^{FRNKR} and TuMV^{FINKR} mutants (p35STuMV-GFP) were generated and bombarded in A. thaliana. The TuMV^{FRNKR} and TuMV^{FINKR} differed from each other and from wild type TuMV^{FRNKI} in infectivity. TuMV^{FRNKR} was detected in inflorescence tissues but not stems (UV light), and TuMV^{FINKR} infection was indistinguishable from TuMV^{FINKI} infection (Fig. 2A). Mild symptoms developed in A. thaliana in response to TuMV^{FRNKR} infection as compared to the severe symptoms of TuMV^{FRNKI}. When these TuMV constructs were inoculated onto N. benthamiana plants, similar phenotypes were observed (Fig. 2B). In particular, GFP fluorescence of TuMV^{FRNKR} was not detected in stems, veins or newly emerging leaves and it only induced mild symptoms. Other mutants tested in this study, including TuMV^{ARNKI}, TuMV^{FRNAI} and TuMV^{FRNEI} were not infectious (data not shown).

Collectively, these studies show that TuMV infectivity of A. thaliana and N. benthamiana strictly requires an intact FRNKI sequence, unlike ZYMV.

**The FRNKI sequence is likely required for increased symptom development in N. benthamiana infected with Potato virus X (PVX) expressing HC-Pro**

Recombinant PVX expressing the wild type HC-Pro has been shown to increase PVX pathogenicity in N. benthamiana (Pruss et al., 1997; Brigneti et al., 1998; Gonzalez-Jara et al., 2005). Using an infectious PVX binary vector, GFP::HC-Pro or HC-Pro constructs were generated and transformed into Agrobacterium. The bottom-most leaves of N. benthamiana plants were infiltrated and the development of systemic symptoms was monitored daily over four weeks. At 7 days post infiltration (DPI), symptom development in the systemic most leaves of plants infected with PVX, PVX-GFP::HC-Pro^{FRNKI} or PVX-GFP::HC-Pro^{FRNKR} but not PVXGFP::HC-Pro^{FINKR} were clearly visible (Fig. 3A). In contrast to the newly emerging leaves
of plants infected with PVX or PXV-GFP::HC-Pro\textsuperscript{FRNKI}, increased symptom severity characterized by epinasty of the newest leaves was consistently observed in response to PXV-GFP::HC-Pro\textsuperscript{FRNKI}.

Differences in the amount and pattern of PVX accumulation was readily observed for the viral constructs carrying mutant wild type HC-Pro fused with GFP. Mutant and wild-type constructs were consistently observed in systemic leaves, but only GFP::HC-Pro\textsuperscript{FRNKI} was readily detected (UV light) in new plant growth (Fig. 3A). By 10 DPI, only plants infected with PXV-GFP::HC-Pro\textsuperscript{FRNKI} displayed severe necrosis in the systemic leaves unlike PVX-GFP::HC-Pro mutant or PVX empty vector-infected plants (Fig. 3B). In our study, PVX-GFP::HC-Pro\textsuperscript{FINKI} was not tested because results from the TuMV studies revealed that both the FINKI and FINKR mutants compromised infectivity and symptom development. With regard to symptom development in plants infected with PVX expressing wild type or mutant HC-Pro, PVX-HC-Pro\textsuperscript{FRNKI} induced more symptoms compared to any of the PVX-HC-Pro mutants at 10 DPI (Fig. 3C). As with PVX-GFP::HC-Pro\textsuperscript{FINKI} infected plants, PVXGFP::HC-Pro\textsuperscript{FINKI} infected plants eventually died, but this did not occur in the other infected plants during the observation period (data not shown). Thus, it appears that differences among GFP::HC-Pro / HC-Pro wild-type and mutants in mediating symptom development are independent from the activities of other TuMV proteins and possibly the result of altered RSS activities.

**Accumulation and RSS activity of wild type and mutant HC-Pros in N. benthamiana**

To investigate the accumulation and RSS activities of various mutations and the FRNKI box, each HC-Pro or GFP::HC-Pro was placed under the control of the 35S promoter, transformed into Agrobacterium, and infiltrated into N. benthamiana leaves, which were monitored daily for GFP fluorescence for two weeks. At 3 DPI, GFP fluorescence was
consistently detected in all infiltrated leaves. GFP::HC-Pro\textsuperscript{FRNKI} fluorescence was always much more intense compared to the GFP::HC-Pro mutants (Fig. 4A). Among the GFP::HC-Pro mutants, the GFP::HC-Pro\textsuperscript{FRNKR} fluorescence was similar to wild type but very weak for the other mutants. Protein blot assays showed that the accumulation of each GFP::HC-Pro construct correlated with its fluorescence profiles (Fig. 4B). We also expressed HC-Pro\textsuperscript{FRNKI} and HC-Pro\textsuperscript{FINKI} fused to a different tag and observed a reduction in HC-Pro\textsuperscript{FINKI} demonstrating that reduced expression was unrelated to the GFP fusion tag (data not shown).

To determine if differences among GFP::HC-Pro wild-type and mutants was due to mRNA or protein stability, we monitored mRNA accumulation of GFP::HC-Pros and HC-Pros at 3 DPI (Fig. 4C). The mRNAs of GFP::HC-Pro\textsuperscript{FRNKI} and HC-Pro\textsuperscript{FRNKI} accumulated at higher levels compared to the GFP::HC-Pro mutant mRNAs implying that the mRNA stability of the mutant GFP-HC-Pro and HC-Pro constructs was compromised. To test if reduced mRNA stability was due to loss of RSS activity, we transiently co-expressed P19, the tombusvirus RSS (Qiu et al., 2002) or HC-Pro\textsuperscript{FRNKI} along with wild-type and mutant GFP::HC-Pro in \textit{N. benthamiana} leaves. In all leaves examined at 3 DPI, co-expression of P19 or HC-Pro\textsuperscript{FRNKI} increased fluorescence and protein accumulation of GFP::HC-Pro protein similar to GFP::HC-Pro\textsuperscript{FRNKI}, but an increase was not observed in response to \textit{Agrobacterium} transformed with the empty vector (Fig. 4A, 4D and data not shown). A slight increase in GFP::HC-Pro\textsuperscript{FINKI} and GFP::HC-Pro\textsuperscript{FINKR} was also detected when co-expressed with HC-Pro\textsuperscript{FRNKR} (data not shown).

Because both P19 and HC-Pro\textsuperscript{FRNKI} caused increased expression of GFP::HC-Pro mutants, it is clear that the stability of HC-Pro mRNA requires its RSS activity and thus the FRNKI box. Further confirmation that GFP::HC-Pro / HC-Pro wild-type and mutant RSS activities differed was revealed when they were transiently expressed in transgenic 16c \textit{N.}}
*benthamiana* expressing a *GFP* transgene (Ruiz et al., 1998). Because 16c plants actively express GFP, then any *GFP*-like RNA sequence introduced will trigger RNA silencing against the *GFP* transgene and against itself. As expected, GFP::HC-Pro\textsuperscript{FRNKI} and GFP::HCPro\textsuperscript{FRNKR} fluorescence and protein expression was detected well above background 16c GFP expression whereas GFP::HC-Pro\textsuperscript{FINKI} was not (Fig. 5). Thus, TuMV HC-Pro likely requires the FRNKI box for effective RSS activity.

**Characterization of TuMV HC-Pro mutants in wild-type and silencing defective *A. thaliana***

HC-Pro has been shown to be a key protein that induces symptoms in virus-infected plants (Kasschau et al., 2003). Because of this, we were interested in understanding how these mutant viruses behaved in wild type *A. thaliana* plants and in *dcl* mutants defective in antiviral silencing. Wild-type *A. thaliana* plants were challenged with TuMV UK1 strain and attenuated mutants derived from the same strain via site-mutagenesis. Plants infected with a severe strain of TuMV-GFP expressed GFP in bombarded leaves and all newly emerging tissue (Fig. 6A; bottom-left panel). Furthermore, these plants were severely stunted when compared to their mock-infected counterparts. Conversely, plants infected with TuMV\textsuperscript{FRNKR}, a moderate strain of TuMV-GFP, expressed GFP in bombarded rosette leaves, portions of the bolt, some cauline leaves, and most floral tissue (Fig. 6A; bottom-middle panel). It should be noted that plants infected with the moderate strain exhibited stunting, but were taller than TuMV-GFP infected plants; GFP expression was also less intense. As expected, plants infected with TuMV\textsuperscript{FINKI}, a weak strain of TuMV-GFP, were asymptomatic (Fig 6A; bottom-right panel). In wild-type *A. thaliana*, the FRNKI to FRNKR mutation attenuated GFP expression, GFP location, degree of stunting, and transcript accumulation. Additionally, the FRNKI to FINKI mutation is deleterious in terms of symptomology.
To further characterize plant responses to the attenuated TuMV-GFP strains, RNA silencing defective *dcl 2-1/4-2 A. thaliana* plants were inoculated with TuMV\textsubscript{FRNK}, TuMV\textsubscript{FRNKR}, and TuMV\textsubscript{FINK} using particle bombardment. Surprisingly, the moderate and severe strains were indistinguishable in terms of GFP expression, GFP location, and symptomology (Fig 6B; bottom-left and bottom-middle panel). TuMV\textsubscript{FINK}-infected plants displayed severe stunting when compared to wild-type virus-infected plants, but GFP fluorescence was less than the other two viruses (Fig 6B; bottom-right panel). In RNA silencing defective *dcl 2-1/4-2 A. thaliana* plants, the FRNKI to FRNKR mutation had no effect on pathogenesis at the whole plant level. Furthermore, the FRNKI to FINKI mutation was also deleterious in the *dcl 2-1/4-2* background in terms of symptomology.

DCL3 has been shown to have weak antiviral functions, and so we also inoculated RNA silencing defective *dcl 2-1/3-1/4-2 A. thaliana* by particle bombardment with wild-type and attenuated viruses. We expected a severe virus infection phenotype, independent of virus treatment because the RNA silencing machinery is debilitated in this background. Surprisingly, a height and GFP fluorescence gradient phenotype was observed when comparing the severe, moderate, and weak virus strains (Fig. 6C; bottom-left, bottom-middle, and bottom-right panel). TuMV\textsubscript{FINKI} produced abnormal leaf morphology and stunting, while GFP fluorescence was less intense (Fig 6C; bottom-right panel). In RNA silencing defective *dcl 2-1/3-1/4-2 A. thaliana*, the FRNKI to FRNKR mutation attenuated GFP expression, GFP location, and stunting. Surprisingly, the FRNKI to FINKI mutation was also deleterious in the *dcl 2-1/3-1/4-2* background in terms of fluorescence, but not stunting. These data show that TuMV can cause severe symptoms in anti-viral silencing defective *A. thaliana* plants despite the lack of a functional HC-Pro.
ARGONAUTE1 and PHAVOLUTA are induced by TuMV-GFP in wild-type Arabidopsis thaliana. AUXIN RESPONSE FACTOR8 and REVOLUTA remain constant.

Transgenic Arabidopsis plants expressing P1/HC-Pro, a potyviral silencing suppressor, phenocopy developmental defects characterized in the ago1 background (Kasschau et al., 2003). It was hypothesized that these developmental defects were the consequence of perturbed miRNA pathways, and it has been reported that expression of genes involved in RNA silencing such as ARGONAUTE1 (AGO1) are induced in response to pathogen challenge (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006; Varallyay et al., 2010). Interestingly, AGO1 is regulated in part by a miRNA (Garcia-Ruiz et al., 2010). Recent studies have shown that HC-Pro miRNA binding affinity and symptomology are correlated (Shibolet et al., 2007, Wu et al., 2010), and that HC-Pro sRNA binding causes sRNAs and their targets to hyper-accumulate (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006; Varallyay et al., 2010). This deregulation is the result of HC-Pro binding to miRNA duplexes and facilitating their stabilization, hence preventing their incorporation into RNA induced silencing complexes (RISCs). In our study, we utilized three infectious TuMV clones with varying levels of sRNA binding affinity and virulence. Similar to other confirmed reports, we hypothesized that TuMV HC-Pro binds miR168. Additionally, we hypothesized this binding could be characterized visually by leaf deformation phenotypes and molecularly by monitoring gene expression. Wild type and silencing defective A. thaliana plants were bombarded with the three infectious clones of TuMV and systemically infected tissue harvested 10 dpi. In agreement with our hypothesis and the literature, we observed enhanced accumulation of AGO1 mRNA in wild-type A. thaliana plants, post-pathogen challenge (Fig. 7A).
Although several other genes have been implicated in leaf morphogenesis and/or disease-induced deformation, we also chose to monitor *AUXIN RESPONSE FACTOR 8* (*ARF8*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) expression because they have functions consistent with those expected to be involved in symptomology, and they are also regulated by miRNAs (Wu et al., 2006, McConnell et al., 2001; Prigge et al., 2005). Previously, it was reported that ZYMV up-regulates miR159, miR166, miR167, and miR168 in squash, five days post-infection (Shiboleth et al., 2007). *ARF8* is targeted by miR167 (Wu et al., 2006). Recently, ectopic *ARF8* expression was demonstrated to be the root cause of severe developmental abnormalities in transgenic *Arabidopsis* constitutively expressing RSSs (Jay et al., 2011). Based on these reports, we hypothesized TuMV HC-Pro binds miR167. Furthermore, miR167/miR167* stabilization would allow deregulation of *ARF8* expression, resulting in enhanced accumulation of *ARF8* and developmental abnormalities. To our surprise, *ARF8* expression was not induced or repressed by the presence of TuMV-GFP (data not shown).

miR166 targets PHV and REV, two well-characterized proteins belonging to the Class III homeodomain-leucine zipper (HD-Zip III) family. Members of this family are involved in leaf biogenesis (McConnell et al., 2001; Prigge et al., 2005). Previously, it was reported that miR166, a regulator of shoot apical meristem development and leaf polarity (Liu et al., 2009, Yao et al., 2009), hyper-accumulates in the presence of ZYMV (Shiboleth et al., 2007). The literature suggests this misregulation is caused by HC-Pro miRNA pathway interference, specifically HC-Pro stabilizing miR166/miR166* duplexes. To test our hypothesis, we monitored the expression the *PHV* and *REV*. We reasoned that misregulation of miR166 would cause misregulation of *PHV* and *REV*, similar to miR168-AGO1 homeostasis (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006; Varallyay et al., 2010). We found *PHV* hyper-accumulates in wild-type
Arabidopsis in the presence of TuMV-GFP (Fig 7B). Similar to ARF8, REV expression was not induced or repressed by TuMV-GFP (data not shown).

**miR168, miR166, and their complementary strands hyper-accumulate in the presence of TuMV-GFP**

Because we observed hyper-accumulation of AGO1 (Fig. 7A) and PHV (Fig. 7B), we investigated the effects of TuMV infection on miR168/miR168* and miR166/miR166*, which regulate AGO1 and PHV, respectively. Wild type Col-0 and dcl2-1/dcl4-1 mutants were inoculated with wild type TuMV and the TuMV<sub>FRNKR</sub> and TuMV<sub>FINK</sub> mutants, so that the effects of the mutant HC-Pros on miRNA accumulation could be monitored.

Similar to AGO1 expression data, the accumulation of both miR168 and miR168* increased in response to TuMV-GFP at 10dpi (Fig.8A and 8B). TuMV<sub>FRNKR</sub> induced miR168 to the same level as the parental strain (Fig. 8A). Conversely, TuMV<sub>FINK</sub> did not significantly affect miR168* accumulation (Fig. 8B). The data offers strong evidence that HC-Pro sequesters the miR168/miR168* duplex, thereby preventing its degradation and disrupting the miRNA-mediated regulation of AGO1.

We also monitored the accumulation of miR166 and its complementary strand miR166* following TuMV infection. We reasoned that hyper-accumulation of PHV could also be the result of HC-Pro stabilizing miR166/miR166* duplexes. We found that miR166 and miR166* hyper-accumulated in response to TuMV-GFP at 10dpi (Fig. 8C and 8D). Unlike miR168/miR168* duplex, TuMV<sub>FRNKR</sub> had no effect on miR166 or its complementary strand (Fig. 8C and 8D).
Discussion

Virus Accumulation, RNA Silencing Suppressor Activity, and Symptomology

In the recent past, there has been a renewed interest in uncoupling plant virus disease progression and plant virus accumulation in host plants. Several labs have successfully accomplished this feat in a variety of plant-pathogen systems (Diaz-Pendon et al., 2007; Garcia-Ruiz et al., 2010; Goto et al., 2007; Omarov et al., 2006; Shibolet et al., 2007; Wu et al., 2010). We were particularly interested in mutating the highly conserved FRNK motif of HC-Pro to FINK, which was shown to uncouple symptoms from virus accumulation in ZYMV (Shibolet et al., 2007). Such a mutation would provide a powerful tool to further investigate mechanisms underlying potyvirus-induced symptoms in the model host, *A. thaliana*. Several infectious TuMV clones were characterized, and it was discovered that an intact FRNKI box is necessary for efficient viral infection and severe symptom development in wild type *N. benthamiana* and *A. thaliana* (Fig. 2 and 3). In addition to viral infection, the RSS activity was investigated in those HC-Pro mutants and the FINKI mutation abolished RSS activity (Fig. 4 and 5). This result was again in contrast to the FINK mutation in ZYMV HC-Pro, which did not disrupt the RSS activity but did alter binding to miRNAs. Taken the together, the results suggest that TuMV HC-Pro does not tolerate mutations in the FRNK motif as well as the HC-Pro of ZYMV.

We were able to find some parallel results that stand in agreement with the role of the FRNK motif in small with ZYMV. For example, HC-Pro interferes with miRNA pathways by directly binding double-stranded miRNA/miRNA* duplexes (Shibolet et al., 2007). In agreement with the literature, this interference can be visualized by monitoring mRNA expression of the miRNA target genes for hyper-accumulation (Csorba et al., 2007; Havelda et al., 2008; Shibolet et al., 2007; Varallyay et al., 2010; Wu et al., 2010; Zhang et al., 2006). We
monitored the expression of AGO1, ARF8, PHV, and REV, targets of miR168, miR167, miR166, and miR166, respectively. 10 dpi, we observed enhanced accumulation of AGO1 and PHV, suggesting their role in pathogenesis (Fig. 7). Varallyay and associates (2010) have suggested AGO1 induction is a hallmark of host defenses and miR168 is a hallmark of viral counter-defenses. To test whether miR168/miR168* and miR166/miR166* duplexes hyper-accumulate in the presence of TuMV, we monitored their expression in our pathosystem. Similar to AGO1 expression data, the accumulation of both miR168 and miR168* increased in response to TuMV-GFP at 10dpi (Fig. 8A and 8B).

The FRNK box of ZYMV HC-Pro has been proposed as a binding site for viral siRNAs and host miRNAs because mutations such as FINK reduce the affinity of ZYMV HC-Pro for smRNA species (Shiboleth et al., 2007; Wu et al., 2010). Reduced affinity of ZYMVFINK HC-Pro for miRNAs correlated with attenuated plant symptoms induced during infection. At 5 dpi, both ZYMVFINK and ZYMVFRNK caused vein clearing, but ZYMVFRNK-induced symptoms progressed to leaf deformation, dwarfing and mosaic patterning by 14dpi (Shiboleth et al., 2007). Generally, miRNA and miRNA* levels were elevated in comparison to healthy plants (Shiboleth et al., 2007). However, miRNA and miRNA* accumulation was higher in ZYMVFRNK-infected plants than ZYMVFINK-infected plants. To ensure attenuated symptomology associated with the mild ZYMV strain was not an artifact of HC-Pro concentration or titer levels, western blotting was performed. As previously reported, ZYMVFRNK and ZYMVFINK virus concentrations were indistinguishable at 5 dpi, but ZYMVFINK accumulates to lower levels that the severe strain at 7 and 14 dpi (Shibboleth et al., 2007). Additionally, the data suggested ZYMVFINK sRNA binding affinity was affected. An electromobility shift assay (EMSA) confirmed the mild ZYMV strain had a reduced affinity for miRNA/miRNA* duplexes. Interestingly, RSS activity was unaffected.
by the Arg to Ile mutation. Thus, symptomology can be interrupted as a function of HC-Pro sRNA binding affinity and the developmental stage of the host plant (Shiboleth et al. 2007).

Wu and associates (2010) offered evidence of their own supporting the theory HC-Pro sRNA binding affinity has a direct effect on leaf-shape, development, and pathogenicity. Using a transgenic approach, A. *thaliana* plants were transformed with a binary vector containing a double 35S promoter driving ZYMV P1/HC-Pro expression. Several independent lines with relatively equal transgene expression were selected per construct for visual and molecular analysis. Amino acids 180, 205, and 396 were evaluated for sRNA binding and symptomology. It was found that single mutations to these residues as well as the F<sub>205</sub>L/E<sub>396</sub>N double mutation cause attenuated symptoms of leaf curling, leaf lobing, and weak serration (Wu et al., 2010). Double mutations R<sub>180</sub>I/F<sub>205</sub>L and R<sub>180</sub>I/E<sub>396</sub>N induced slight leaf serration and lobing (Wu et al. 2010). Transformants expressing the triple mutation R<sub>180</sub>I/F<sub>205</sub>L/E<sub>396</sub>N grew normally like their non-transformed controls (Wu et al. 2010). These phenotypes were also analogous to results obtained using severe and equivalent mutant ZYMV strains in *Cucurbita pepo* L. var. zucchini (Wu et al. 2010).

To further explore the uncoupling of symptomology and virus accumulation, Wu and associates (2010) monitored the expression of several microRNAs (miRNAs) related to leaf development and their targets. sRNA northern blot data demonstrated hyper-accumulation of miR159, miR159*, miR160, miR160*, miR164, miR164*, miR165, miR165*, miR168, miR168*, and their targets AGO1 (miR168), ARF10 (miR160), and PHV (miR165) in transgenic *Arabidopsis*, suggesting abolition of miRNA pathway suppression (Wu et al., 2010). Interestingly, this result was phenocopied by single mutation R<sub>180</sub>I with mutations at residue 205.
or 396 providing a synergistic effect (Wu et al., 2010). Taken together, the results reiterated the importance ZYMV amino acid residue 180 in symptom development and suppressor activity.

**Effects of FRNK box mutations in TuMV HC-Pro RSS activity**

In contrast to the FRNK to FINK mutation in ZYMV, we found that TuMV\(^{\text{FINKI}}\) and TuMV\(^{\text{FRNKI}}\) HC-Pro mutants were poorly infectious, did not induce symptoms in *A. thaliana* and *N. benthamiana* (Fig. 2), and that they had no detectable RSS activity (Fig. 5). However, TuMV\(^{\text{FRNKI}}\) accumulated in systemic leaves but it was not detected in stems, veins or in emerging leaves compared to TuMV\(^{\text{FRNKI}}\) (Fig. 2), and it retained partial RSS activity (Fig 5). As discussed above, wild-type ZYMV and wild-type TuMV contain a FRNKKR sequence and FRNKI sequence, respectively (Shiboleth et al., 2007). From a limited search of TuMV isolates to identify any FRNKI box derivations, two isolates TuMV CAR39 (gi 124507407) and TuMV CDN 1 (gi 33504662) were found that contained a FRNKV sequence (Fig. 1A). Both isoleucine and valine are neutral and hydrophobic, suggesting that these properties might be essential for general TuMV pathogenesis. Other factors that are apparently required for TuMV pathogenesis are amino acid charge and/or size in the FRNK box, because additional TuMV mutants generated like TuMV\(^{\text{ARNKI}}\), TuMV\(^{\text{FRAKNI}}\) and TuMV\(^{\text{FRNEI}}\) were not infectious (data not shown). Based on these findings, TuMV infectivity in *A. thaliana* and *N. benthamiana* mediated by HC-Pro probably requires the FRNK and a flanking neutral, nonpolar amino acid, in our case the FRNKI sequence.

Expression of GFP::HC-Pro / HC-Pro wild-type and mutants by PVX in *N. benthamiana* suggested that differences among TuMV wild-type and mutant infected plants was due to HC-Pro RSS activity (Fig. 3). It has been established that wild-type HC-Pro is a strong symptom determinant (Brigneti et al., 1998; Kasschau et al., 2003; Gonzalez-Jara et al., 2005; Lim et al.,
In our study, we found that only PVX-GFP::HC-Pro$^{\text{FRNKI}}$ and PVX-HC-Pro$^{\text{FRNKI}}$ induced severe symptoms eventually leading to plant death unlike PVX expressing HC-Pro$^{\text{FINK}}$ mutants and PVX empty vector control. Because *N. benthamiana* plants displayed similar symptoms in our TuMV and PVX studies expressing GFP::HC-Pro or HC-Pro wild-type and mutants, the logical explanation was that *GFP::HC-Pro / HC-Pro* mutant mRNAs were targets of RNA silencing.

We confirmed that *GFP::HC-Pro* and *HC-Pro* mutant mRNAs were likely targets of RNA silencing (Fig. 4C) by co-expressing P19 or HC-Pro$^{\text{FRNKI}}$ with wild-type and mutant GFP::HC-Pros in *N. benthamiana* leaves (Fig. 4A, 4D, and data not shown). Both P19 and HC-Pro$^{\text{FRNKI}}$ caused increased fluorescence and protein accumulation of GFP::HC-Pro mutants similar to GFP::HC-Pro$^{\text{FRNKI}}$. Our results extend what others have reported, in that point mutations or insertions that abolish HC-Pro RSS activity allow the *HC-Pro* mRNA to be targeted for RNA silencing (Kasschau and Carrington, 2001; Varrelmann et al., 2007). Thus, it appears that a region of RNA encoding HC-Pro is itself an efficient RNA silencing target. To directly test for suppressor activity, we expressed GFP::HC-Pro wild-type and mutants in transgenic *N. benthamiana* expressing a GFP (Ruiz et al., 1998) and demonstrated that GFP::HC-Pro$^{\text{FRNKI}}$ possesses the strongest RSS activity followed by GFP::HC-Pro$^{\text{FRNKR}}$ (Fig 5). Based on this finding, TuMV HC-Pro RSS activity requires the intact FRNKI box. The observation of the FRNKI box or a similar FRNKV box in other TuMV isolates suggests that the neutral, non-polar amino acid is necessary at this position for RSS activity.

The differences between the RSS activities of GFP::HC-Pro$^{\text{FRNKI}}$ and GFP::HC-Pro$^{\text{FRNKR}}$ are interesting. GFP::HC-Pro$^{\text{FRNKR}}$ has limited RSS activity, yet it is effective enough to protect TuMV from RNA silencing and mediate systemic movement and induction of relatively mild
symptoms in plants. Some possible explanations are that GFP::HCPro\textsuperscript{FRNKR} / HC-Pro\textsuperscript{FRNKR} has reduced yet significant binding with viral siRNAs and host miRNAs. Alternatively, GFP::HC-Pro\textsuperscript{FRNKR} / HC-Pro\textsuperscript{FRNKR} might be unable to interact (or has weak interaction) with a host protein that is involved in the antiviral pathway or mediates symptom expression. Several studies have shown that HC-Pro interacts with a number of host proteins but none to date are known to be required for potyvirus pathogenesis (Anandalakshmi et al., 2000; Guo et al., 2003; Ballut et al., 2005; Jin et al., 2007).

**Pathogenesis of potyvirus mutants containing altered FRNK motif of HC-Pro**

In ZYMV and TuMV, the HC-Pro FRNK box motif is located at the amino acid position 179 – 182 and TuMV 181 – 184, respectively. In addition, ZYMV HC-Pro and TuMV HC-Pro differ in amino acid sequences, especially near the FRNK box (Fig. 1; Shiboleth et al., 2007). Thus, it is possible that TuMV HC-Pro mutants have altered structures that impair their RSS activities compared to TuMV HC-Pro wild-type, ZYMV wild-type and ZYMV HC-Pro mutants. Because both *A. thaliana* and *N. benthamiana* are hosts for TuMV, it is likely that TuMV FRNKI box mutants would display the same phenotypes in other host plants. Therefore our results suggest that the conserved FRNK box of TuMV HC-Pro is likely required for pathogenesis and its full RSS activity is dependent on a flanking neutral, non-polar amino acid. It should be noted that pathogenesis of TEV on tobacco requires an intact FRNK box, because a FRN to RPA mutation delayed TEV systemic movement and reduced TEV amplification in protoplasts (Cronin et al., 1995).

Direct comparisons of the ZYMV and TuMV results are somewhat confounded by the differences in the two pathosystems. With respect to inoculation methodology and virus kinetics, *C. pepo* plants were inoculated at 5 days old (Shiboleth et al., 2007) versus 21 day old *A.*
thaliana plants in our study. By 5 dpi, ZYMV systemically spread to newly emerging tissue, as denoted by vein clearing (Shibolet et al. 2007). Under our growth conditions, TuMV-GFP infection foci are just becoming visible at 5 dpi (Yang et al. 2007), and the virus typically requires approximately 7 days to spread systemically in A. thaliana Col-0 ecotype (Yang et al. 2007). On the other hand, differences between ZYMV^{FRNK} and ZYMV^{FINK} are readily apparent by 7 dpi (Shibolet et al., 2007). We have harvested systemically-infected tissue at 7, 10, and 15 dpi. At 7 dpi, our gene expression data is inconsistent (data not shown), but those effects are mitigated at later sampling time points. In this study, 10 dpi was chosen because we previously demonstrated a set of sRNAs negatively regulate their targets post-TuMV infection (Campbell et al., unpublished).

**TuMV^{FXYNNKX} mutants produce phenotypic gradients in the dcl 2/3/4 triple mutant**

Because the HC-Pro FINK mutation abolished RSS activity and TuMV-GFP pathogenicity, we reasoned that *dcl* mutants represented an alternative strategy to understanding the potential role of the FRNK motif in pathogenicity. To further characterize the attenuated TuMV-GFP strains, RNA silencing defective A. thaliana mutants were inoculated. It was expected that all viruses would cause severe symptoms in a *dcl 2-1/3-1/4-2* background due to a lack of antiviral RNA silencing. Intriguingly, all plants exhibited severe symptoms but GFP fluorescence became less apparent when comparing the infections and transcript levels remained relatively constant. Taken together, the data suggests virus accumulation, visual phenotype, and molecular phenotype have been uncoupled in this *dcl* triple mutant. These data further suggest that the RSS and miRNA binding activities mediated by the HC-Pros of potyviruses are not necessarily required for induction of symptoms.
We observed hyper-accumulation of miR166, miR167*, miR168* and their targets, PHV, ARF8, and AGO1, respectively, in the dcl2/4 plants in response to the wild-type TuMV$^{FRNK}$ (Fig 6 and 7). In contrast, hyperaccumulation of these miRNAs, miRNA*, and target mRNAs were not observed in response to the TuMV$^{FINK}$ virus. These results suggest that wild type TuMV HC-Pro is able to sequester some regulatory miRNAs resulting in the increased abundance of the miRNAs and their miRNA*s. We also tested accumulation of ARF8 mRNA targeted by miRNA167, because it was recently reported that misregulation of ARF8 may be responsible for TuMV-induced leaf deformation (Jay et al., 2011). Interestingly, we did not observe altered expression of ARF8 suggesting that ARF8 is not misregulated by TuMV under all conditions. We also found that the accumulation of miRNAs, miRNA*s, and target mRNAs tested were not significantly misregulated in dcl2/4 mutants in response to TuMV$^{FRNK}$ and TuMV$^{FINK}$ mutant in spite of that it causes symptoms similar to TuMV$^{FRNK}$. These results suggest two things. The first is that the HC-Pro of TuMV$^{FRNK}$ or TuMV$^{FINK}$ must have reduced binding of miRNA duplexes, because the levels of the miRNAs were not significantly altered. Secondly, the symptoms induced by TuMV in A. thaliana are not necessarily dependent on the ability of HC-Pro to misregulate the expression of key regulatory genes. Successful potyvirus infection is a highly dynamic process which involves misregulation of leaf morphology genes and plant host defenses, but misregulation may not always be the cause of viral symptomology. Thus, there must be other factors or interactions that underlie symptomology. Further work needs to be done to understand the molecular basis of how plant viruses interact with and target host genes to cause plant disease.
Materials and Methods

Full-length TuMV and GFP::HC-Pro / HC-Pro expressing plasmids

The HC-Pro FRNKi (TTT-CGC-AAC-AAG-ATT) box mutations, FINKi (TTT-ATT-AAC-AAG-ATT), FINKr (TTT-ATT-AAC-AAG-AGA) and FRNKR (TTT-CGC-AAC-AAG-AGA) were introduced into p5PK-TuMCS and p35STuMV-GFP, which were generously provided by James Carrington (Oregon State University). Both plasmids are derived from the TuMV UK1 isolate. These mutations were generated in the p5PK-TuMCS intermediate clone by site-directed mutagenesis using the GeneTailorTM system (Invitrogen, Carlsbad, CA, USA). The forward primers corresponding to each mutation listed above are as follows: FINKiF 5'-GAAGGGTTTCACTGAAAGTCTTTATTAAACAAGATTTCCAG-3', FINKrF 5'-GAAGGGTTTCACTGAAAGTCTTTATTAAACAAGAGATCCAG-3' and FRNKrF 5'-GAAGGGTTTCACTGAAAGTCTTTCCGAACCAAGAGATCCAG-3'. The reverse primer used was FRNKrR 5'-GGACTTCAGTGAACCCTTCTCAATGTTTCTC-3' (the region of overlap with the forward primers is underlined). After sequencing to confirm the mutations, p5PKTuMCS was digested with Smal / KpnI or NcoI / KpnI and the fragment was ligated in p35STuMV-GFP cut with the same restriction enzymes. It should be noted that p5PKTuMCS and p35STuMV-GFP carry the GFP transgene inserted between the P1 and HC-Pro polypeptides. To generate binary vectors expressing the GFP::HC-Pro or HC-Pro transgenes under control of the CaMV 35S promoter, these sequences were PCR amplified with forward and reverse primers incorporating the NcoI and SacI restriction sites, respectively. In the primer sequences shown, the NcoI and SacI restriction sites are underlined, start codon is shown in bold and the stop codon is in bold italics: NcoGFP::HC-ProF 5'-CCATGGCTAGTAAAGGAGAAGAACTTTCAC-3', NcoHC-ProF 5'-CCATGGCTAGTGCAGCGGGAGCCAACTTCTGG-3' and SacGFP::HC-ProR / HC-
ProR 5'-GAGCTCTCATCCAACGCGGTAGTGGTTTCAAGC-3'. The resulting PCR products were first cloned in pCR2.1TOPO® (Invitrogen, Carlsbad, CA, USA). Using the same primer sets described above to introduce FRNKI box mutations in full-length TuMV-GFP, site-directed mutagenesis was performed on pCR2.1TOPO-GFP::HC-Pro / HC-Pro plasmids. Digested fragments were then ligated into pRTL2 cut with the NcoI and SacI, and afterward pRTL2 plasmids were digested with PstI to obtain the 35S promoter-GFP::HC-Pro / HC-Pro-nos terminator cassette that was then ligated into pCAMBIA3300 cut with PstI. The resulting pCAMBIA3300-GFP::HC-Pro / HC-Pro plasmids were transformed in Agrobacterum tumefaciens strain GV3101 by electroporation.

Plant Materials and rub inoculation

Arabidopsis thaliana plants were germinated from seed in (10-cm) pots. Seedlings were thinned five days after sprouting and grown under short-day conditions (10 h light, 14 h dark) in a growth chamber at 22°C. Rub-inoculation was performed when plants were approximately three to four weeks old. Three to four rosette leaves from each plant were dusted with Carborundum and rub inoculated with TuMV-GFP diluted in 20 mM phosphate buffer (pH 7.2, 1:5, wt/vol) using a cotton-stick applicator. Control plants were dusted with Carborundum and three to four rosette leaves from each plant were mock inoculated with a cotton-stick applicator that was soaked in phosphate buffer spiked with uninfected Arabidopsis sap. At the times indicated for each experiment, systemic tissue samples were harvested from three plants per treatment per genotype under UV illumination, pooled, frozen in liquid nitrogen, and stored at -80°C. Three independent biological replicates were performed.
Particle Bombardment

Twenty-one day old plants were bombarded with infectious clones of TuMV containing a wild-type or mutant HC-Pro sequence, p35S::TuMV-GFP-FXNKX. Seventy-five μl of 1μm gold particles were added to a premix composed of 15 μg plasmid, 300 μl 50% glycerol, 150 μl 2.5M CaCl₂, and 120 μl 0.1M spermidine. DNA-coated gold particles were centrifuged, washed with 420 μl 70% isopropanol, 420 μl 100% isopropanol, and resuspended in 150 μl 100% isopropanol. Six μl of solution was pipetted on the middle of a macro-carrier and used to bombard the medial portion of the plant using a PDS 1000/Helium biolistic gun (BioRad, Hercules, CA, USA) and 1100psi rupture disks. Afterwards, each plant was sprayed with distilled water and placed under a clear plastic hood overnight to recover. Six plants were bombarded per construct per genotype. At 10 to 15 dpi, a long-wave UV lamp was used to detect GFP fluorescence. Systemically infected tissue was collected and stored at -80°C. Three independent biological replicates were performed.

Potato virus X expression of HC-Pro in N. benthamiana

The *Potato virus X* (PVX) binary vector (Liu et al., 2002) was used to express wild-type or mutant GFP::HC-Pro / HC-Pro transgenes in *N. benthamiana*. Full-length sequences were amplified from pCAMBIA3300 encoding wild-type or mutant GFP::HC-Pro / HC-Pro transgenes using the following primer sets incorporating the Gateway® attB sequences (underlined), start codon AUG (bold) and stop codon (bold italics). GFP::HC-ProF 5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGG-GTAGTAAAGGAGAACTT-3', HC-ProF 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGG-GTAGTAAAGGAGAACTT-3', HC-ProF 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGG-GTAGTAAAGGAGAACTT-3', HC-ProR 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGG-GTAGTAAAGGAGAACTT-3', HC-ProR 5'-

GGGGACCACTTTGTACAAGAAAAGCAGGCTTCACCATGG-GTAGTAAAGGAGAACTT-3', HC-ProR 5'-

GFP
alone was also amplified using the primers, GFPF: 5’-GGGGACAAGTTTGTACAAA-
AAAGCAGGCTTCACCATGGGTAGGAGAACTTT-3’ and GFPR 5’-GGGGACC-
ACTTTGTACAAGAAGCTGGGTATTTGTATAGCTCATCCAT-3’. The resulting PCR
products were recombined into pDONR207 (entry vector) and then PVX by Gateway®
recombinase (Invitrogen, Carlsbad, CA, USA). PVX constructs transformed into A. tumefaciens
strain GV3101 were then cultured in 5 ml LB media overnight at 30 °C, inoculated in 50 ml LB
media (supplemented with 10 mM MgCl₂, 10 mM MES and 20 μM acetosyringone) and cultured
overnight at 30°C. GV3101 cells were pelleted by centrifugation and resuspended in infiltration
buffer (10 mM MgCl₂, 10 mM MES and 200 μM acetosyringone), adjusted to the OD₆₀₀ of 1.0
and incubated for 8 – 12 hours at room temperature. The bottom-most leaves of 21-day old N.
benthamiana plants were infiltrated and plants were photographed 7 and 10 dpi under white light
for symptom development or UV light for GFP fluorescence. For each construct, 5 plants were
infiltrated and experiments were repeated twice.

**Transient expression of HC-Pro and P19 in N. benthamiana**

GFP::HC-Pro / HC-Pro wild-type and mutants in pCAMBIA3300, pCAMBIA3300 empty vector
and P19 encoded by pCB301-p19 (generously provided by Herman B. Scholthof (Texas A&M
University)) were transformed into A. tumefaciens strain GV3101 and used in transient
expression assays in N. benthamiana as described above. Fully expanded leaves of 21-day or 28-
day old N. benthamiana plants were infiltrated either with Agrobacterium expressing GFP::HC-
Pro / HC-Pro constructs or co-infiltrated with GFP::HC-Pro constructs and P19 or HC-Pro
constructs mixed at a 1 to 1 ratio. For each construct, 4 leaves were infiltrated and experiments
were repeated at least twice. At 3 dpi, leaves were photographed under UV light for GFP
fluorescence and/or infiltrated zones were dissected and placed in 50 ml conical tubes for storage at −80°C for later use.

**Protein isolation and immunoblot analysis**

Total soluble proteins were extracted with Tris-buffered saline (TBS; 50 mM Tris-HCl, 300 mM, NaCl, and 5 mM EDTA at pH 7.4) containing the protease inhibitors leupeptin (5 μg/ml), aprotinin (5 μg/ml), and PMSF (100 μM). Protein was quantified in extracts using the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). For detection of the GFP::HC-Pro (~77 kDa: GFP = ~27 kDa and HC-Pro = ~50 kDa) or P19 (19 kDa) in infiltrated leaves, 15 μg of total protein in sample loading buffer (0.5 M Tris-HCl (pH 6.8), 50% glycerol, 10% SDS, 0.5% bromophenol blue, and 5% β-mercaptoethanol) was separated by polyacrylamide gel electrophoresis (PAGE) using 10% or 15% polyacrylamide in the presence of SDS running buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 1% SDS), and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Protein transfer efficiency and equal loading was estimated by staining the membranes with Ponceau S solution (0.1% wt/vol in 5% acetic acid vol/vol). For immunoblot assay, membranes were incubated in 1X PBS – 5% non-fat milk blocking buffer containing 1% Tween-20 for 2 hours then incubated for 4 hours at room temperature with mouse monoclonal GFP antibody (Roche, Indianapolis, IN, USA) at 1:3,000 or rabbit polyclonal P19 antibody (provided by HB Scholthof) at 1:5,000 in blocking buffer. Membranes were washed three times with 1X PBS – 1% Tween-20 and then incubated with mouse anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) or anti-rabbit conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) at 1:20,000 in blocking solution for 1 hour at room temperature. The membrane blot images were developed using an enhanced chemiluminescence system (ECL, Amersham, Piscataway, NJ, USA) according to manufacturer’s instruction.
RNA Extraction

Total RNA was extracted from reproductive tissue according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen). Samples were treated with RNase-free DNase (TURBO DNA free™ kit, Ambion, Grand Island, NY, USA) before downstream applications.

qRT-PCR

qRT-PCR was performed using methods described in Hewezi et al (2008). Briefly, total RNA was extracted from systemic rosette leaves, stems, and reproductive tissue according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen). Samples were treated with RNase-free DNase (Turbo DNA free, Ambion) before qPCR. 5 μg of DNase-free total RNA was polyadenylated according to the manufacturer’s instructions (Poly (A) Tailing Kit, Ambion). Subsequently, first strand synthesis was performed according to the manufacturer’s instructions (SuperScript™ III First Strand Synthesis, Invitrogen). Afterwards, samples were diluted 1:20 and 1:4 for genes of interest and controls, respectively. Genes of interest were amplified with 10 μM gene-specific primers (Table 1) and quantitative PCR performed using a PerfeCTa SYBR Green Fast Mix for iQ (Quanta BioSciences, Gaithersburg, MD, USA). Conditions are as follows – Initial denaturation: 3 min at 95ºC; 45 PCR cycles: 15 sec at 95 ºC, 1 min at primer T_M; Dissociation stage: 10 sec at 55 ºC, repeat 80 times, increase temp 0.5 ºC each repeat.

Acknowledgements

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References


proteins in antiviral defense and small interfering RNA biogenesis during Turnip Mosaic Virus infection. Plant Cell 22, 481 – 496.


Table 1. Sequence and annealing temperature of primer pairs used for qPCR

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<tr>
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<th>Annealing Temp (°C)</th>
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<td>ACTIN8qR</td>
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Table 2. sRNAs associated with genes of interest

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Fig. 1. Alignment of amino acids of the central domain of potyvirus HC-Pros that includes the highly conserved FRNK box. The first amino acid of each HC-Pro region is indicated. (A) Turnip mosaic virus UK1 isolate gi 25013650, CAR39 isolate gi 124507407, CDN 1 isolate gi 33504662 (TuMV), Bean common mosaic necrosis virus gi 25013913 (BCMVNV), Bean common mosaic virus gi 25013490 (BCMV), Bean yellow mosaic virus gi 25013500 (BYMV), Beet mosaic virus gi 40254034 (BtMV), Chilli veinal mottle virus gi 45004657 (ChiVMV), Clover
yellow vein virus gi 25013510 (CIYVV), Cocksfoot streak virus gi 25014039 (CSV), Cowpea aphid-borne mosaic virus gi 25013520 (CABMV), Daphne virus Y gi 96980663 (DVY), Dasheen mosaic virus gi 25013784 (DsMV), East Asian passiflora virus gi 85539888 (EAPV), Japanese yam mosaic virus gi 25013883 (JYMV), Johnsongrass mosaic virus gi 25013809 (JGMV), Konjak mosaic virus gi 90093254 (KoMV), Leek yellow stripe virus gi 25013893 (LYSV), Lettuce mosaic virus gi 25013530 (LMV), Lily mottle virus gi 39163617 (LMoV), Maize dwarf mosaic virus gi 25013540 (MDMV), Onion yellow dwarf virus gi 32493289 (OYDV), Papaya leaf distortion mosaic potyvirus gi 32493279 (PLDMV), Papaya ringspot virus gi 25013550 (PRSV), Pea seed-borne mosaic virus gi 25013560 (PSbMV), Peanut mottle virus gi 25013833 (PeMoV), Pepper mottle virus gi 25013570 (PepMoV), Peru tomato mosaic virus gi 28519942 (PTV), PPV gi 25013580 (PPV), Potato virus A gi 25013590 (PVA), Potato virus V gi 25013850 (PVV), PVY gi 25013600 (PVY), Scallion mosaic virus gi 25013997 (SVY), Shallot yellow stripe virus gi 76803358 (SYSV), Sorghum mosaic virus gi 25013823 (SrMV), Soybean mosaic virus gi 25013610 (SMV), Sugarcane mosaic virus gi 25013620 (SCMV), Sweet potato feathery mottle virus gi 25013774 (SPFMV), Thunberg fritillary virus gi 68989219 (TFV), Tobacco etch virus gi 25013634 (TEV), Tobacco vein mottling virus gi 25013639 (TVMV), Watermelon mosaic virus gi 51949948 (WMV), Wild potato mosaic virus gi 25141239 (WPMV), Wisteria vein mosaic virus gi 116723233 (WVMV), and Yam mosaic virus gi 48249198 (YMV), Zucchini yellow mosaic virus gi 118566318 (ZYMV). (B) TuMV UK1 and ZYMV. Conserved amino acids in the FRNK box region are highlighted and a C-terminal flanking amino acid is boxed.
Fig. 2. Infectivity of TuMV-GFP expressing the wild-type or mutant FRNKI box in (A) *A. thaliana* and (B) *N. benthamiana*. Three-week-old plants were particle bombarded with p35S::TuMV wild-type or mutant plasmids and photographed (A) 15 DAI or (B) 11 DAI under UV light. (A) Panels: (i) TuMV\textsuperscript{FRNKI} – left and TuMV\textsuperscript{FRNKR} – right; (ii) TuMV\textsuperscript{FINKI} – left and TuMV\textsuperscript{FINKR} – right. (B) Panels: (i) TuMV\textsuperscript{FRNKI} – top and TuMV\textsuperscript{FRNKR} – bottom; (ii) TuMV\textsuperscript{FINKI} – top and TuMV\textsuperscript{FRNKR} – bottom; (iii) TuMV\textsuperscript{FRNKI} – left and TuMV\textsuperscript{FRNKR} – right; (iv) TuMV\textsuperscript{FRNKI} –...
– close up of infected leaf from (iii) left yellow box and (v) TuMV\textsuperscript{FRNK}

– close up of infected leaf from (iii) right yellow box.
Fig. 3.
Fig. 3. Symptom development in *N. benthamiana* plants infected with PVX or PVX-GFP::HC-Pro and PVX-HC-Pro constructs carrying wild type or mutant HC-Pros (A) 7 DAI and (B, C) 10 DAI. (A) Plants infiltrated with *Agrobacterium* carrying (i) PVX, (ii) no-insert; (iii, iv) PVX-GFP::HC-Pro FRNK1; (v, vi) PVX-GFP::HC-Pro FINKR or (vii, viii) PVX-GFP::HC-Pro FRNKR. Plants were photographed under white light (i, ii, iii, v and vii) and under UV light (iv, vi and viii). (B) Plants infiltrated with *Agrobacterium* carrying (i) no insert, (ii) PVX; (iii) PVX-GFP::HC-Pro FRNK1; (iv) PVX-GFP::HC-Pro FINKR or (v) PVX-GFP::HC-Pro FRNKR. (C) Plants infiltrated with *Agrobacterium* carrying (i) no insert, (ii) PVX; (iii) PVX-HC-Pro FRNK1; (iv) PVX-HC-Pro FINKR or (v) PVX-HC-Pro FRNKR. Arrows point to systemic most leaves displaying symptoms.
Fig. 4. Characterization of GFP fluorescence, protein accumulation, and mRNA expression of wild type and mutant GFP::HC-Pros and HC-Pros in *N. benthamiana*. (A) GFP fluorescence under UV light: Panels (i, v, ix) GFP::HC-Pro^{FRNKI}; (ii, vi, x) GFP::HC-Pro^{FINKI}; (iii, vii, xi) GFP::HC-Pro^{FRNKR} and (iv, viii, xii) GFP::HC-Pro^{FINKR}. GFP::HC-Pro alone: Panels i-vi. GFP::HC-Pro + P19: Panels v-viii. GFP::HC-Pro + HC-Pro^{FRNKI}: Panels ix-xii. (B) Protein blot of GFP::HC-Pro wild-type and mutants. (C) RT-PCR analysis of *GFP::HC-Pro / HC-Pro* wild-
type and mutant mRNAs. (D) Protein blot of GFP::HC-Pro wild-type and mutants co-expressed with P19. Protein blot of GFP::HC-Pro wild-type and mutants co-expressed with HC-Pro\textsuperscript{FRNKI} is not shown. GFP::HC-Pro protein was detected by immunoblot assay using a monoclonal antibody to GFP.
Fig. 5. GFP fluorescence in transgenic 16c *N. benthamiana* plants expressing *GFP* infiltrated with in wild type and mutant GFP::HC-Pros (A) GFP::HC-Pro fluorescence. Panels: (i) GFP::HC-Pro\textsuperscript{FRNKI} (ii) GFP::HC-Pro\textsuperscript{FINKI} and (iii) GFP::HC-Pro\textsuperscript{FRNKR}. (B) Protein blot assay of GFP::HC-Pro wild-type and mutant accumulation. GFP is used as a loading control because it is constitutively expressed. GFP::HC-Pro protein and constitutively expressed GFP was detected by immunoblot assay using a monoclonal antibody to GFP.
White Light

\[ \text{dcl2/4, FRNKI, 10dpi} \]

\[ \text{dcl2/4, FRNKI, 10dpi} \]

\[ \text{dcl2/4, FINKI, 10dpi} \]

UV Light

\[ \text{dcl2/4, FRNKI, 10dpi} \]

\[ \text{dcl2/4, FRNKI, 10dpi} \]

\[ \text{dcl2/4, FINKI, 10dpi} \]
Fig. 6. Characterization of TuMV HC-Pro mutants in wild-type and silencing defective
Arabidopsis thaliana. (A) In the wild-type Col-0 background, attenuated viruses display height and GFP fluorescence gradient compared to WT virus. (B) In the dcl2 dcl4 background, moderate TuMV$^{\text{FRNKR}}$ is indistinguishable from severe, wild-type TuMV$^{\text{FRNKI}}$. The weak TuMV$^{\text{FINKI}}$ displays abnormal leaf morphology, stunting, and faint GFP fluorescence. (C) In the dcl2 dcl3 dcl4 background, moderate TuMV$^{\text{FRNKR}}$ virus displays height and GFP fluorescence gradient compared to wild-type TuMV$^{\text{FRNKI}}$. The weak TuMV$^{\text{FINKI}}$ displays abnormal leaf morphology, stunting, and faint GFP fluorescence.
Starting Quantity

Genotype

Col 0   dcl 2/4

Mock
TuMV-GFP
TuMV-FRNKR-GFP
TuMV-FINKI-GFP
Fig. 7. Leaf morphogenesis hyper-accumulate in wild-type and dcl 2-1 dcl4-2 background, while remaining stable in silencing defective mutant, dcl 2-1 dcl3-1 dcl4-2 Arabidopsis, 10 days post TuMV infection. (A) AGO1. (B) PHV. The error bars represent the standard error of the mean for each arithmetic mean (n = 9). Star indicates a statistical difference between treatments per genotype per gene of interest by Student’s t-test (P ≤ 0.05).
A

### miR168

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<td>TuMV-FINKI-GFP</td>
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- miR168 expression levels are shown for different genotypes.
- The graph compares the expression levels among Col 0 and dcl 2/4 genotypes.
- The y-axis represents starting quantity.
miR168*

Starting Quantity

Genotype

Col 0          dcl 2/4

Mock          TuMV-GFP
TuMV-FRNKR-GFP
TuMV-FINKI-GFP
Starting Quantity

miR166

Col 0  dcl 2/4

Genotype

- Mock
- TuMV-GFP
- TuMV-FRNKR-GFP
- TuMV-FINKI-GFP
Fig. 8. miRNAs and miRNA*’s hyper-accumulate in wild-type and dcl 2-1 dcl4-2 background, while remaining stable in silencing defective mutant, dcl 2-1 dcl3-1 dcl4-2 Arabidopsis, 10 days post TuMV infection. (A) mir168 (targets AGO1). (B) miR168*. (C) miR166 (targets PHV). (D) miR166*. The error bars represent the standard error of the mean for each arithmetic mean (n = 9). Star indicates a statistical difference between treatments per genotype per gene of interest by Student’s t-test (P ≤ 0.05).
CHAPTER 4

VIGS CONSTRUCTS TARGETING HORMONE PATHWAY GENES IN SDS-RESISTANT SOYBEAN PRODUCE FOLIAR SYMPTOMS WHEN EXPOSED TO

F. VIRGULIFORME CULTURE FILTRATE

Vijitha K. Silva, Brian A. Campbell, Michelle A. Graham, Chunling Yang, Jaime Dittman, John H. Hill, Steven A. Whitham, and Leonor Leandro

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Author contributions: B.A.C., V.K.S, M.A.G., C.Y., J.D., J.H.H., S.A.W. and L.L. designed experiments, V.K.S. generated plant material and performed SDS toxin inoculations, phenotyping, and harvested plant tissue, C.Y. generated VIGS constructs and performed particle bombardments, J.D. generated VIGS constructs and performed particle bombardments, B.A.C. performed gene expression analyses and wrote the paper. B.A.C., V.K.S., C.Y., J.D., S.A.W, and L.L. are from the Department of Plant Pathology and Microbiology at Iowa State University

Abstract

Fusarium virguliforme, a soil-borne fungal pathogen and causal agent of soybean sudden death syndrome (SDS). SDS is characterized by root rot, interveinal chlorosis, leaf necrosis, defoliation, and pod abortion. Previous studies have demonstrated F. virguliforme culture filtrate induces foliar SDS symptoms, suggesting the presence of at least one translocating toxin. This collaboration focused on identifying genes involved in resistance to SDS using a virus-induced gene silencing (VIGS) approach. Visual phenotypic data indicated that VIGS constructs targeting
intracellular membrane-bound Ca^{2+}-independent phospholipase A2 (iPLA_{2}) and ethylene response factor (ERF1) interfered with host defense mechanisms, post-filtrate inoculation, resulting in foliar symptoms in SDS-resistant soybean cultivar MN1606SP. The loss of resistance to the culture filtrate suggests that these two genes have critical roles in defense against this nectrophic fungus. Subsequently, we analyzed iPLA_{2} and ERF1 gene expression to confirm their involvement in SDS resistance. The VIGS constructs caused reduced expression of iPLA_{2} and ERF1 confirming their involvement in resistance to SDS.

**Introduction**

Sudden death syndrome (SDS) of soybean is caused by *Fusarium virguliforme*, a soil-borne fungal pathogen (Roy et al., 1989; Rupe, 1989). SDS is characterized by root rot and foliar symptoms including interveinal chlorosis, leaf necrosis, defoliation, and pod abortion (Roy et al., 1989; Rupe, 1989). Several studies showed that *F. virguliforme* could only be cultured from root tissue suggesting that foliar symptoms are due in part to translocating toxins (Ji et al., 2006; Jin et al., 1996; Li et al., 1999; Roy et al., 1989; Rupe 1989).

Brar and associates (2011) successfully cloned a *F. virguliforme* toxin gene, *FvTox1*. *FvTox1* produces foliar SDS-like symptoms in soybeans exposed to culture filtrate in the presence of light. Presumably, *FvTox1* is one of at least two phytotoxins that ultimately cause programmed cell death (PCD) and symptom development in soybean (Ji et al., 2006, Jin et al., 1996). *FvTox1* is a 13.5 kDa protein that is encoded by a single-copy gene, and it contains a chloroplast transit peptide (cTP; Brar et al., 2011). The purified *FvTox1* protein causes foliar SDS symptoms in the presence of light when it is translocated from roots to leaves supporting the idea that the cause of foliar symptoms is due to one or more mobile toxins (Brar et al., 2011). To further investigate the importance of *FvTox1* in SDS, the next step was producing an
antibody specific to FvTox1. Using a small chain variable fragment of FvTox1, Brar and associates (2012) successfully identified Anti-FvTox1-1 and Anti-FvTox1-2 which interact with different binding affinities to the antigenic site of FvTox1. Transgenic soybean expressing Anti-FvTox1-1 displayed enhanced tolerance to the phytotoxin (Brar and Bhattacharyya, 2012). Taken together, these studies demonstrate that FvTox1 is a major pathogenicity factor (Brar and Bhattacharyya, 2012).

Virus-induced gene silencing (VIGS) manipulates endogenous plant defenses to knock-down genes of interest. A recent review discussing the advantages, limitations and possible solutions, as well as future directions of VIGS in plant biology is available (Senthil-Kumar and Mysore, 2011). A DNA-based Bean pod mottle virus (BPMV) VIGS vector was chosen for this study due to its high-throughput potential and ability to silence genes in soybean shoot and root systems (Zhang et al., 2010). Using a reverse genetics approach, BPMV VIGS constructs were designed targeting hormone pathway genes implicated in defense signaling to determine their potential involvement in resistance to SDS (Table 1). These studies demonstrated two genes previously identified in a screen and microarray study, respectively, affect the response of the SDS-resistant soybean cultivar MN1606SP when silenced (Graham, personal communication, Radwan et al., 2011).

Results

Selection of candidate genes affecting soybean resistance to SDS foliar symptoms

One gene (Glyma07g38890) encodes an intracellular membrane-bound Ca\(^{2+}\)-independent phospholipase A2 (iPLA\(_2\); Phytozome). Its homeolog, Glyma17g01850, is 90.1% similar at the protein level (Phytozome). iPLA\(_2\) is 76.9% similar to, AtPLAI (AT1G61850), which is involved in basal jasmonic acid (JA) production and resistance to the necrotrophic fungus, Botrytis
cinerea, in Arabidopsis (Phytozome; Yang et al., 2007). A recent review discusses various other phospholipase pathways that are activated as a plant defense in response to pathogen challenge (Canonne et al., 2011).

An Affymetrix microarray analysis was conducted on F. virguliforme-infected root tissue at the onset of foliar SDS and several transcripts were identified as having differential gene expression (Radwan et al., 2011). Glyma02g00870, annotated in their study as ethylene response factor 1 (ERF1), was found to be up-regulated in response to pathogen challenge (Radwan et al., 2011). Glyma02g00870 was also found to be up-regulated in the SDS–susceptible and –resistant genotypes, when the microarray approach was validated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR; Radwan et al., 2011). Radwan and associates (2011) also listed four other genes, Glyma10g00980, Glyma20g34570, Glyma10g33060, and Glyma03g31940, as ERF1. Using gene model information, protein similarity to Glyma02g00870 was 62.1%, 67.5%, 64.0%, and 53.7%, respectively (www.phytozome.org).

**VIGS constructs designed targeting hormone pathway genes**

VIGS construct 3D07 was designed to knock-down Glyma07g38890 and Glyma17g01850 with the objective of determining iPLA₂ involvement in resistance to SDS (Fig. 1). VIGS construct 2D08-3 was designed to knock-down Glyma02g00870, Glyma10g00980, Glyma20g34570, Glyma10g33060, and Glyma03g31940 with the objective of determining ERF1 involvement in resistance to SDS (Fig. 2).

**Foliar symptoms develop after exposure to SDS filtrate when jasmonic acid and ethylene pathway genes are down-regulated by VIGS in an SDS–resistant cultivar.**

As stated above, the roots of SDS–resistant cultivar, MN1606SP, were exposed to F. virguliforme culture filtrate with the objective of targeting hormone pathway genes to verify
their connection to SDS resistance (Huang and Hartman, 1998; Fig. 3). Thirty days post infection (dpi), tissue was harvested and gene expression data was collected via qRT-PCR. Gene expression data was analyzed with Relative Expression Software Tool (REST) 2009 to determine up- and down-regulation of target gene expression.

The two genes identified in this study, iPLA₂ and ERF1, have one and four homologs, respectively. Our group was also interested in determining if VIGS constructs can down-regulate homologs, and if there was a nucleic acid identity threshold. In order to answer this question for iPLA₂, the 3’ end of Glyma07g38890 and Glyma17g01850 consensus sequence was inserted into the BPMV VIGS vector and targeted for silencing (Table 2 and 3). There was enough dissimilarity between Glyma07g38890 and Glyma17g01850 in other portions of these genes to facilitate the synthesis of two sets of qPCR primers that uniquely amplified each homolog. We found VIGS construct 3D07 significantly down-regulated iPLA₂ and its homolog (Fig. 1 and 4).

Next, we aligned the 3’ end of ERF1 and its four homologs to further discern if a lower threshold limit exists for VIGS. As previously stated, ERF1 is 62.1%, 67.5%, 64.0%, and 53.7% similar to its four homologs at the protein level. While we were able to design consensus sequence primers targeting all five genes, there was not enough diversity in sequence to design primers targeting each homolog. Nevertheless, we found that VIGS construct 2D08-3 down-regulated ERF1 (Fig. 2 and 5). Visual phenotypic data suggested the VIGS constructs interfered with MN1606SP defense mechanisms, resulting in foliar SDS. Taken together, these VIGS experiments suggest that jasmonic acid and ethylene pathway genes, iPLA₂ and ERF1 are involved in resistance to SDS.
Silencing does not occur consistently in all experiments

SDS symptom severity was evaluated on the third trifoliate of SDS-resistant and SDS-susceptible soybean cultivars every 3 days, post culture filtrate assay (Fig. 3). VIGS construct 3D07 significantly increased symptomology in the first four replicates of plants visually scored. Replicates three and four were chosen for qPCR analysis as confirmation that down-regulation of the target gene, iPLA2, induced a loss-of-function phenotype correlated with SDS susceptibility (Fig. 1, 3, and 4). However, the 3D07 phenotype from the fifth replicate looked very similar to the empty vector and mock control controls (Fig. 6). Two additional replicates yielded comparable phenotypes. At this point our group was faced with a poignant question, should silencing be confirmed in every replicate of the experiment? Replicate 7 was chosen for qPCR analysis and a lack of down-regulation by construct 3D07 was discovered (Fig. 7). Intriguingly, the third trifoliate on some plants were asymptomatic, while the fourth trifoliate was indicative of SDS foliar symptom development (data not shown). QRT-PCR data for asymptomatic leaves showed that the target gene was not down-regulated while in symptomatic leaves the target gene was down-regulated (Fig. 7). Furthermore, when results from the third, fourth, and seventh replicate were combined, iPLA2 was not significantly down-regulated by VIGS construct 3D07 (data not shown). Taken together, these findings show that there can be inconsistent results between experiments, which is not unexpected in VIGS experiments. However, in those replicates where the altered phenotype was observed, there was a perfect correlation with silencing of the target gene.
Discussion

**BPMV VIGS combined with a-F. virguliforme culture filtrate assay identifies soybean genes involved in foliar resistance to SDS**

Seeds from SDS-resistant and SDS-susceptible plants were grown for approximately 11 – 12 days in a controlled environment. Unifoliate leaves with were bombarded with VIGS constructs at the VC stage and given 2 weeks to recover. Post-recovery, plants were uprooted and placed in cell-free *F. virguliforme* culture filtrate and the third trifoliate was evaluated every 3 days for for SDS foliar symptom development (Fig. 3). Using this novel screening assay, our group successfully demonstrated two genes from jasmonic acid and ethylene hormone pathways, which when down-regulated via VIGS, led to significantly increased SDS foliar symptom severity post-filtrate assay (Table 1).

**The Role of Phospholipase A During Pathogen Attack**

In our study, the roots of SDS–resistant cultivars were exposed to *F. virguliforme* culture filtrate. Shortly thereafter, foliar SDS symptoms developed when expression of iPLA₂ was knocked down, breaking resistance to SDS foliar symptoms (Fig. 1). This shows iPLA₂ has a role in conferring resistance to this nectrophic fungus.

iPLA₂ and AtPLAI are 76.9% similar at the protein level (www.phytozome.org). AtPLAI hydrolyzes phospholipids including, but not limited to phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid (PA), and phosphatidylserine to release fatty acids at the sn-1 an sn-2 positions (Yang et al., 2007). PA, phospholipase D (PLD), and phospholipase C (PLC) are well documented in response to plant defense signaling (Canonne et al., 2011). Nitric oxide (NO) has been shown to generate PA via activation of the PLC/diacylglycerol kinase (DGK) pathway in response to pathogen-associated molecular pattern
Nodulation factor (NF) and another PAMP, flg22, were shown to stimulate PA production via PLC/PLD and PLC, respectively (Serna-Sanz et al., 2007). This finding provided evidence that fungal symbiosis and fungal pathogen defense utilize overlapping, but distinct pathways. *Oryza sativa* PLDβ1 has been proposed as a negative regulator of plant defense signaling and disease resistance (Yamaguchi et al., 2009). de Torres Zabela et al. (2002) demonstrated differential expression of PLD isoforms in response to virulent bacteria, avirulent bacteria, mechanical injury, and defense-related hormone application.

Researchers have confirmed PLD functions in a cascade with PDK1 (3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE1) and OXI1 (OXIDATIVE SIGNAL INDUCIBLE1), using beneficial fungi (Camehl et al., 2011). SUPPRESSOR OF AVRBST-ELICITED RESISTANCE (SOBER1), has phospholipase activity and confers resistance to *Pseudomonas syringae* pv *tomato* DC300 expressing its analogous type III effector (Cunnac et al., 2007). *AtPlA* is a negative regulator of AtMYB30-mediated plant defense responses (Froidure et al., 2010). *AtPlAI* is involved in basal jasmonic acid (JA) production and resistance to *Botrytis cinerea* (Yang et al., 2007). PLA has also been reported to have a critical role in plant reproduction (Kim et al., 2011).

**The Role of Ethylene Response Factors During Pathogen Attack**

ERF1 is a member of a subfamily belonging to the AP2 transcription factor (TF) family, which is regulated by hormones and pathogen challenge (Gutterson and Reuber 2004). ERF1 is well documented in the plant pathogen interactions literature as being up-regulated in response to necrotrophic fungi (Anderson et al., 2011; Berrocal-Lobo and Molina, 2007; Berrocal-Lobo and Molina, 2004; Berrocal-Lobo et al., 2002; Radwan et al., 2011). Constitutive expression of ERF1 in transgenic *Arabidopsis* confers resistance to *B. cinerea, Plectosphaerella cucumerina, F.*
oxysporum sp. conglutinans, and *F. oxysporum* f. sp. *lycopersici*, but decreased tolerance to *Pseudomonas syringae* pv *tomato* DC3000 (Berrocal-Lobo and Molina, 2007; Berrocal-Lobo et al., 2002; Berrocal-Lobo, 2004). ERF1 functions as a downstream component of ethylene (ET) and JA pathways in ET/JA–dependent defense signaling networks, activating pathogenesis-related 4 (PR4) and plant defensin 1.2 (PDF1.2) (Berrocal-Lobo and Molina, 2007; van Loon et al., 2006). In *Nicotiana tabacum* cv *bairhong*, transgenic tobacco plants overexpressing *Gossypium barbadense* ERF1 had increased resistance to *Pseudomonas syringae* pv *tabaci* (Qin et al., 2006). In *Medicago truncatula*, ERF1 is up-regulated in response to fungal necrotroph *Rhizoctonia solani* (Anderson et al., 2010). Overexpression of ERF1 in *M. truncatula* roots increases resistance to *R. solani* and *Phytophthora medicaginis*, but not root knot nematode (Anderson et al., 2010). In *Bupleurum kaoi*, a medicinal plant, ERF1 is induced by methyl jasmonate (MeJA) and overexpression increases resistance to *B. cinerea* (Liu et al., 2011). Overexpression of BkERF1 in transgenic Arabidopsis increased resistance to *B. cinerea* (Liu et al., 2011). The majority of tobacco and *Arabidopsis* ET-insensitive mutants tested displayed susceptibility to several necrotizing pathogens, highlighting the importance of necrotrophic pathogen defense mechanisms (Geraats et al., 2003; Geraats et al., 2002).

In our study, the roots of SDS–resistant cultivars were challenged with *F. virguliforme*–filtrate. Subsequently, foliar SDS symptoms developed when ERF1 was knocked down, breaking resistance to the translocated toxin(s) (Fig. 2). Taken together, ERF1 has a role in response to several necrotrophic fungi. ERF1 attenuation generally confers susceptibility to necrotrophic fungi, ERF1 overexpression generally confers resistance to necrotrophic fungi, and ERF1 is part of an ET–dependent defense pathway.
Future Directions

Currently, our group is focused on testing our BPMV-VIGS-filtrate assay on other resistant varieties such as Forrest, Hartwig, Jack, and AR 10 SDS. Broader implications of breaking SDS resistance in other cultivars would bolster our results; iPLA2 and ERF1 have conserved roles during pathogen attack, conferring resistance to SDS foliar symptoms, when challenged. Additionally, we are still actively screening for VIGS constructs which increase SDS severity when exposed to culture filtrate, post-bombardment.

Materials and Methods

Plant Material

All *Glycine max* plants used in this study were MN1606SP, a SDS resistant cultivar, or AG2403, a SDS susceptible cultivar. MN1606SP, a maturity group 1.6 cultivar, was developed by the University of Minnesota (Malvick and Bussey 2008). AG2403, a maturity group 2.4 cultivar, is from Asgrow, and it is a Roundup Ready® soybean harboring the Rps1\(^k\) gene, which confers multi-race Phytophthora root rot resistance. AG2403 is also described as a product with excellent tolerance to brown stem rot. Both cultivars were grown in soil in a growth chamber under long-day light conditions, 16 h light and 8 h dark.

*Fusarium virguliforme* Culture filtrate Assay

Seedlings were bombarded with VIGS constructs at the VC stage, once unifoliates formed. Afterwards, plants were incubated under long-day light conditions, 16 h light at 22°C and 8 h dark at 22°C, for fourteen days. Subsequently, plants were uprooted and placed in either a cell-free *F. virguliforme* culture filtrate (+ toxin) or an autoclaved culture filtrate (- toxin) under long-day light conditions, 16 h light at 24°C and 8 h dark at 17°C. Foliar severity was evaluated on the 3\(^{rd}\) trifoliate every 3 days.
**VIGS Construct Particle Bombardment**

Twenty-five microliters of 1 μm gold particles were added to a premix composed of 3 μg BPMV RNA 1, 3 μg BPMV RNA 2, 50 μl 50% glycerol, 25 μl 2.5M CaCl₂, and 10 μl 0.1M spermidine. DNA-coated gold particles were centrifuged, washed with 70 μl 70% isopropanol, 70 μl 100% isopropanol, and resuspended in 25 μl 100% isopropanol. Six μl of solution was pipetted on the middle of a macro-carrier and used to bombard the medial portion of the plant using a PDS 1000/Helium biolistic gun (BioRad, Hercules, CA, USA) and 1100psi rupture disks. Afterwards, each plant was sprayed with distilled water and placed under a clear plastic hood overnight to recover. At least 3 plants were bombarded per construct. At 30 dpi, tissue was collected and stored at -80°C for qRT-PCR.

**qRT-PCR**

qRT-PCR was performed using methods described in Hewezi et al. (2008). Briefly, total RNA was extracted from leaf (3rd trifoliate) tissue according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen). Five micrograms of total RNA were treated with RNAse-free DNase (Turbo DNA free, Ambion) before qRT-PCR. Following purification, first strand synthesis was performed according to the manufacturer’s instructions (SuperScript™ III First Strand Synthesis, Invitrogen). Afterwards, samples were diluted 1:20 and 1:4 for genes of interest and controls, respectively. Genes of interest were amplified with 10 μM gene-specific primers (Table 2) and quantitative PCR was performed using the PerfeCTa SYBR Green Fast Mix for iQ (Quanta BioSciences, Gaithersburg, MD, USA). Conditions were as follows – Initial denaturation: 3 min at 95°C; 45 PCR cycles: 15 sec at 95°C, 1 min at primer TM; Dissociation stage: 10 sec at 55°C, repeat 80 times, increase temp 0.5°C each repeat.
QRT-PCR data analysis

REST 2009 is a computer program developed by M. Pfaffl and QIAGEN, to determine up and down regulation of genes from quantitative PCR experiments (Pfaffl 2001; Pfaffl et al., 2002; Vandesompele et al., 2002). Four or two biological replicates and 10,000 iterations were used to determine the expression of iPLA2 or ERF1, respectively.

References


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Ethylene-insensitive tobacco shows differentially altered susceptibility to different pathogens. Phytopath 93, 813 – 821.


Pfaffl, M.W., Horgan, G.W., Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30, e36


Table 1. Genes targeted for VIGS and qRT-PCR

<table>
<thead>
<tr>
<th>Construct</th>
<th>Category</th>
<th>Description</th>
<th>Locus Name</th>
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<tr>
<td>Control</td>
<td></td>
<td>Elongation factor 1 beta/delta chain (EF-1)</td>
<td>Glyma02g44460</td>
</tr>
<tr>
<td>3D07</td>
<td>Target Gene</td>
<td>Intracellular membrane-bound Ca(^{2+}) independent phospholipase A2 (iPLA(_2))</td>
<td>Glyma07g38890</td>
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<td></td>
<td></td>
<td>Intracellular membrane-bound Ca(^{2+}) independent phospholipase A2 (iPLA(_2))</td>
<td>Glyma17g01850</td>
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<tr>
<td>2D08-3</td>
<td>Target Gene</td>
<td>ethylene-responsive transcription factor 1B-like (ERF1)</td>
<td>Glyma02g00870</td>
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Table 2. Sequence used for virus-induced gene silencing constructs

<table>
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<tr>
<td>3D07</td>
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| 2D08-3    | Ggatccaacctctcatctttcccccttcaacgaaaaacgacccggaagagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgacccggaagagatgtctctctacggcatgatcagtcataccagaaaaagagatcatcaagagaagaggtgaaactcagaaaaacgac
Table 3. Sequence and annealing temperature of primer pairs used for qRT-PCR

<table>
<thead>
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<th>Primer</th>
<th>Sequence (5’→ 3’)</th>
<th>Annealing Temp (°C)</th>
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<tr>
<td>CGCSP1F</td>
<td>AGCATCCAAACTGGTGTTTCTG</td>
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<tr>
<td>CGCSP1R</td>
<td>CAACTGTCAGCCTGTCCTCA</td>
<td>60.02</td>
</tr>
<tr>
<td>3D07PP1F</td>
<td>TTGGAAGAGACCTTCGGAGA</td>
<td>59.92</td>
</tr>
<tr>
<td>3D07PP1R</td>
<td>ACGACGAGGACGAAGAAGA</td>
<td>59.99</td>
</tr>
<tr>
<td>3D07hPP1F</td>
<td>CGGGTTTCTTAGGCTATTTGG</td>
<td>58.72</td>
</tr>
<tr>
<td>3D07hPP1R</td>
<td>TGCAAGAGAAGGAGGTGTCGAA</td>
<td>59.57</td>
</tr>
<tr>
<td>2D08-3PP1F</td>
<td>TCGTTTTTCCCTAATATGTAATGTGA</td>
<td>60.03</td>
</tr>
<tr>
<td>2D08-3PP1R</td>
<td>CTGATATTCCTTTGATGCTCATCTTT</td>
<td>59.17</td>
</tr>
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Fig. 1. Glyma07g38890 and Glyma17g01850 are down-regulated by VIGS construct 3D07. The error bars represent the standard error of the mean for each arithmetic mean (n = 4). Star indicates a statistical difference between treatments per gene of interest by Student’s t-test (P ≤ 0.05).
Fig. 2. Glyma02g00870 is down-regulated by VIGS construct 2D08-3. The error bars represent the standard error of the mean for each arithmetic mean (n = 2).
**Fig. 3.** *Fusarium virguliforme* culture filtrate assay, coupled with virus-induced gene silencing identifies soybean genes involved in foliar resistance to SDS. Foliar severity was evaluated on the 3rd trifoliate every 3 days.
Fig. 4. VIGS construct 3D7-1 down regulates iPLA$_2$, increasing SDS symptom severity when exposed to culture filtrate.
**Fig. 5.** VIGS construct 2D08-3 down-regulates ERF1, increasing SDS severity when exposed to culture filtrate.
Fig. 6. Lack of down-regulation by construct 3D07 may explain inconsistent results between experiments. SDS symptom severity visual data and qRT-PCR results from experiment 7 are shown.
Fig. 7. Differences in down-regulation by construct 3D07 between symptomatic and asymptomatic tissues. The error bars represent the standard error of the mean for each arithmetic mean (n = 2).
CHAPTER 5. CONCLUSIONS AND FUTURE WORK

1.1 Overview

My dissertation was based on analyzing the transcripts and small RNA’s (sRNAs) of genes correlated with *Turnip mosaic virus* (TuMV) disease symptomology, quantifying their expression in wild-type and RNA silencing defective *Arabidopsis thaliana* plants, and characterizing various TuMV viruses lacking RNA silencing suppressor activity. Chapter 1 introduced potyviruses, RNA silencing (host defenses mechanisms), and viral suppressors of RNA silencing (viral counter-defense mechanisms). In Chapter 2, I collaborated with two additional lab members to investigate gene regulation and signaling pathways that may contribute to symptomology. In Chapter 3, two infectious clones of TuMV characterized by attenuated symptoms were bombarded onto wild-type and RNA silencing defective *A. thaliana* plants and viral RNA silencing suppressor activity and *ARGONAUTE1 (AGO1)*, *AUXIN RESPONSE FACTOR 8 (ARF8)*, *PHAVOLUTA (PHV)*, and *REVOLUTA (REV)*, host regulators of leaf biogenesis, were monitored.

1.2 Virus-induced RNA Silencing Pathway study

In Chapter 2, I analyzed the expression of transcripts and sRNAs of genes hypothesized to be correlated with the stunted growth phenotype in *A. thaliana* plants infected with TuMV. Yang and associates (2007) previously demonstrated genes related to cell wall function and hormone biosynthesis are down-regulated post-pathogen challenge. Subsequently, several independent transgenic over-expression lines were developed and infected with TuMV-GFP. Peculiarly, the same genes were still down-regulated in their respective over-expression counterparts, post-TuMV infection. The results strongly suggested a post-transcriptional gene silencing mechanism may be the underlying cause of transcript accumulation abolition.
Additional analysis revealed results were dependent on dicer-like 1 (dcl 1), dicer-like 3 (dcl 3), RNA dependent RNA polymerase 1 (rdr 1), RNA dependent RNA polymerase 2 (rdr 2), and RNA dependent RNA polymerase 6 (rdr 6), indicating TuMV may alter their expression via an RNA silencing pathway targeting endogenous genes related to cell wall function and hormone biosynthesis.

To further explore the hypothesis that TuMV stunted growth disease symptoms are the outcome of mis-regulating RNA silencing pathway targets, gene expression was monitored in wild-type and silencing defective A. thaliana plants, 10 days after infections (dai). I observed down-regulation of genes correlated with stunted growth in wild-type and silencing defective dcl 2-1/4-2 plants. Gene expression remained stable in dcl 2-1/3-1/4-2, a RNA silencing defective mutant characterized by an inability to generate 21-,22-, or 24-nt siRNAs. I hypothesized TuMV bound siRNAs corresponding to cell wall function and hormone biosynthesis, resulting in their misregulation and the appearance of disease symptoms (Fig. 1). I collaborated with Yeun Sook Lee, a bioinformatics student in the lab to analyze a deep-sequencing dataset produced by Garcia-Ruiz and associates (2010) who demonstrated successful pathogenesis requires a functional viral suppressor of RNA silencing (VSR) and viruses lacking a functional VSR can be genetically rescued in plants lacking anti-viral activity (Garcia-Ruiz et al., 2010). Ultimately, I found siRNAs associated with cell wall function and hormone biosynthesis were up-regulated, during TuMV infection, which was indicative of a negative regulation mechanism. Taken together, our data suggests the genes chosen for study are negatively regulated by the siRNAs which target them. Additionally, siRNAs generated during TuMV replication were found to match sequence derived from Arabidopsis and TuMV genomes (Fig. 1). However, the mechanism that TuMV utilizes to perturb their homeostasis is unclear (Fig. 1). We propose
TuMV efficiently sequesters these siRNAs, thereby blocking their incorporation into RNA-induced silencing complex (RISC) machinery (Fig. 1). Furthermore, this misregulation of siRNAs associated with cell wall function and hormone biosynthesis genes possibly leads to hypermethylation of their target mRNAs and down-regulation (Fig. 1). Down-regulation of cell wall function and hormone biosynthesis genes was previously hypothesized to be correlated with the stunted growth phenotype associated with TuMV infection (Fig. 1).

1.3 TuMV HC-Pro FRNKI box study

In Chapter 3, I investigated HC-Pro’s role in TuMV infection and symptom development in *A. thaliana* using a genetic loss-of-function approach. Previously, it was demonstrated that HC-Pro can bind siRNAs and miRNA/miRNA* duplexes (Lakatos et al., 2006). Transgenic *Arabidopsis* plants expressing P1/HC-Pro, a potyviral silencing suppressor, phenocopy developmental defects characterized in the *ago1* background (Kasschau et al., 2003). It was hypothesized that these developmental defects were the consequence of perturbed miRNA pathways. Several years later, reports began to emerge documenting the induction of *AGO1* in response to pathogen challenge (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006; Varallyay et al., 2010). In the years to come, two groups demonstrated a correlation between potyviral infection, RNA silencing suppressor activity, misregulation of *AGO1, ARF8, PHV*, and *REV*, and symptomology (Shiboleth et al., 2007, Wu et al., 2010). A third group determined TuMV infection is dependent on the function of RNA silencing suppressors and the hierarchal function of endogenous host RNA silencing activity (Garcia-Ruiz et al., 2010). It became evident that utilizing natural and engineered viruses is an accepted approach to studying plant-pathogen interactions as it relates to symptomology.
After characterizing two infectious clones of TuMV predicted to have reduced virulence, it was determined we successfully uncoupled RNA silencing suppressor activity of HC-Pro from disease symptom progression (Campbell et al., unpublished). Although RNA silencing suppressor activity was found to be dependent on a non-polar residue flanking the FRNK box, other mechanisms related to RNA silencing may exist (Anandalakshmi et al, 2000; Lakatos et al., 2006). My results indicated AGO1 and PHV hyper-accumulate in the presence of TuMV, while ARF8 and REV remain stable (Campbell et al., unpublished). In agreement with the literature, HC-Pro is most likely binding miR168/miR168* and miR166/miR166* duplexes, thereby protecting them from degradation, resulting in deregulation of the miRNA pathway and shoot apical meristem development/leaf polarity, respectively (Fig. 2). Similar to the model proposed by Shiboleth and associates (2007), we propose mutations in HC-Pro affect its miRNA binding affinity (Fig. 2). Our data suggest that TuMV<sup>FRNK</sup> and TuMV<sup>FINKI</sup> inefficiently bind endogenous miRNA duplexes, leading to their incorporation in host RNA silencing machinery. Ultimately, misregulation of miR168/miR168* and miR166/miR166* duplexes leads to hyper-accumulation of AGO1 and PHV, respectively. However, we expected increased transcript accumulation of ARF8 and REV. Recently, misregulation of ARF8 was purported to be the major cause for the developmental defects exhibited in P1/HC-Pro transgenic plants and during TuMV infection (Jay et al., 2011). These discrepancies underscore the ambiguity of ARF8 function during TuMV infection.

1.4 VIGS constructs study

In Chapter 4, VIGS was used in conjunction with a <i>F. virguliforme</i> culture filtrate assay to screen candidate genes for correlation with SDS symptom severity. I analyzed the expression of two genes previously identified in a screen and microarray study, respectively, that affect the
response of the SDS-resistant soybean cultivar MN1606SP when silenced. Ultimately, our group found literature which supported our hypothesis, iPLA\textsubscript{2} and ERF1 have a role in conferring resistance to this nectrophic fungus. Broader implications of testing our novel system in other SDS-resistant cultivars would bolster our results; iPLA\textsubscript{2} and ERF1 have conserved roles during pathogen attack, conferring resistance to SDS foliar symptoms, when challenged. In conclusion, using previous studies to validate and optimize our system facilitates high-throughput screening for additional VIGS constructs which increase SDS severity when exposed to culture filtrate, post-bombardment.

1.5 Future Directions

It may be worthwhile to check the methylation status of the genes examined in Chapter 2. Although DCL3 has been shown to be indispensible during successful pathogenesis (Garcia-Ruiz et al., 2010), our data indicate a role for DCL3 in the maintenance of cell wall function and hormone biosynthesis genes. DCL3 siRNA products guide heterochromatin formation and transcriptional repression of transposons and DNA repeats (Mossiard and Voinnet, 2006). These siRNAs function through AGO4/PolIV complexes to direct DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) dependent RNA-directed DNA methylation at cytosine positions in a CNN context (Cao and Jacobsen, 2002). I hypothesize dsRNAs produced during TuMV replication target both TuMV and \textit{A. thaliana} genomes. The resulting misregulation of endogenous genes appears to promote a successful systemic infection, but is mutually exclusive from the known role of DCL3 during pathogen attack.

Future experiments to provide a higher resolution of the mechanism regulating \textit{ARF8} and \textit{REV} involve bombarding additional RNA silencing defective (AGO1, NRPD1A, HEN1) \textit{A. thaliana} mutants with our TuMV collection and monitoring gene expression. Information
gleaned from this study could provide additional insight into the step which TuMV interferes with host gene expression. Our study suggests this step is located downstream of DCL3 activity but upstream of RDR1, RDR2, and RDR6. HC-Pro, the multi-functional protein of potyviruses, has been demonstrated to concurrently enhance and repress the expression of suites of genes related to defense and organogenesis, respectively. The next logical step in the battle against plant viruses is discernment regarding whether these changes -or lack thereof- are coincidental versus a novel mechanism to facilitate infection.

1.6 Acknowledgments

At this time we would also like to thank all former and current Whitham lab members for assistance in troubleshooting and critically reviewing these findings.

1.7 References


Fig. 1. Proposed model of HC-Pro interaction with host RNA silencing machinery and siRNA products leading to symptomology and repression of cell wall function and hormone biosynthesis genes.
Fig. 2. Proposed model of HC-Pro interaction with host RNA silencing machinery and miRNA/miRNA* duplexes leading to symptom development and hyper-accumulation of key host leaf biogenesis genes.
ACKNOWLEDGEMENTS

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