Genetic and molecular investigation of compatible plant-virus interactions

Tyrell Carr
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Plant Pathology Commons, and the Virology Commons

Recommended Citation
Carr, Tyrell, "Genetic and molecular investigation of compatible plant-virus interactions" (2007). Retrospective Theses and Dissertations. 15853.
https://lib.dr.iastate.edu/rtd/15853

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Genetic and molecular investigation of compatible plant-virus interactions

by

Tyrell Carr

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:
Steven A. Whitham, Major Professor
W. Allen Miller
Adam Bogdanove
Coralie Lashbrook
Erik Vollbrecht

Iowa State University

Ames, Iowa

2007

Copyright © Tyrell Carr, 2007. All rights reserved.
DEDICATION

This Dissertation is dedicated to the Carr, Swithwick and Hudson Families and the Biology and Honors Program Faculty and Staff at Elizabeth City State University during my tenure as an undergraduate biology major from 1997 - 2001. I especially dedicate this dissertation to the memory of my Mother, Angeline Smithwick Carr and Grandmother Rosanna Smithwick
# TABLE OF CONTENTS

## CHAPTER 1. INTRODUCTION

- Overview                                                   1
- Viral Genome Expression                                   1
- Tobamovirus and Potyvirus Movement                       2
- Host Protein Essential in Tobamovirus and Potyvirus Infections 5
- Antiviral Defense in Susceptible Plant Cells – RNA Silencing 11
- Virus-Induced Symptoms                                    20
- Future Research                                           23
- Conclusion and Dissertation Outline                       23
- References                                                24

## CHAPTER 2. TOBAMOVIRUS INFECTION IS INDEPENDENT OF *HSP101*

- mRNA INDUCTION AND PROTEIN EXPRESSION                      58
  - Abstract                                                 58
  - Introduction                                             59
  - Materials and Methods                                    60
  - Results                                                  66
  - Discussion                                               72
  - Acknowledgements                                         75
  - References                                               76

## CHAPTER 3. DIFFERENTIAL EXPRESSION OF *A. THALIANA HSP70* GENES AND SELECT *HEAT STRESS TRANSCRIPTION FACTOR* GENES IN RESPONSE TO VIRUS INFECTIONS

- 90
  - Abstract                                                 90
  - Introduction                                             91
  - Materials and Methods                                    93
  - Results                                                  96
  - Discussion                                               98
  - Acknowledgements                                         100
  - References                                               101

## CHAPTER 4. *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

- 112
  - Abstract                                                 112
  - Introduction                                             113
  - Materials and Methods                                    116
CHAPTER 1. INTRODUCTION

Overview

Viral infections of agricultural and commercial plants can cause significant economic losses when yield, quality or physical appearance is affected. To date, various cultural and biotechnological strategies are in practice to prevent the spread of pathogenic viruses and restrict their abilities to cause disease in plants (Gonsalves, 2002; Hull, 2002). Despite these practices, there are many plant viruses that still cause significant problems world-wide, especially in developing countries. The underlying principle that governs virus infections is the genetics of host-virus interactions. Both host factors and viral factors play major roles in plant virus infections (Whitham and Wang, 2004; Carr and Whitham, 2007). Identifying essential plant genes that mediate virus pathogenesis has been a challenge, whereas numerous viral genes have been characterized. Although much is known about the required roles of many viral genes in plant viral pathogenesis, detailed analyses are lacking on how the proteins encoded by these genes mediate processes necessary for systemic infections.

Genetic and molecular techniques can identify plant genes that are usurped by viral proteins in order to regulate pathogenesis. *Arabidopsis thaliana* is a model system that can be exploited to identify host genes involved in plant responses to virus infections (Golem and Culver, 2003; Whitham et al., 2003; Huang et al., 2005; Carr and Whitham, 2007; Yang et al., 2007). The *A. thaliana* genome has been sequenced, numerous loss-of-function mutants are available, microarrays are available and it is a host for many viruses (Table 1). *Nicotiana benthamiana* is another popular model system for virus research, especially for virus movement and symptom expression studies. Many viruses that infect *A. thaliana*, as well as many others that do not, infect *N. benthamiana*. While its genome has not been sequenced,
expressed sequence tags (ESTs) and various techniques are available to silence gene expression. The overall focus of this research is to study the susceptibility of *A. thaliana* and *N. benthamiana* to virus infections. This introduction provides an overview of plant viruses and virus-related processes and discusses specific host responses that favor systemic infections in susceptible plants.

**Viral Genome Expression**

Viral genomes consist of either double-stranded (ds) or single-stranded (ss) DNA or RNA. Positive (+) sense ssRNA viruses comprise the largest group of plant viruses. The genomes of some (+) sense ssRNA viruses are similar to cellular mRNAs in that they contain a 5' - 7mGpppN-cap and 3' - poly(A). Examples of such viruses are the members of the genus *Potexvirus* (*i.e.* Potato virus X, PVX) (Huisman et al., 1988). RNAs of the potyviruses (*i.e.* Tobacco etch virus, TEV) lack a 5' - cap but possess a 3' - poly(A) tail (Shukla et al., 1994), whereas the tobamoviruses (*i.e.* Tobacco mosaic virus, TMV) possess a 5' - cap but no 3' - poly(A) tail (Gallie and Walbot, 1990). Strikingly, *Luteoviridae* (*i.e.* Barley yellow dwarf virus, BYDV) and *Tombusviridae* (*i.e.* Tobacco bushy stunt virus, TBSV) members lack both a 5' - cap and 3' - poly(A) tail (Miller and White, 2006).

*Potyviridae* family members possess a protein virally linked to their RNA genome (VPg) at its 5' - end, whereas tobamoviruses have a pseudoknot at their 3' - end. A number of strategies are used by (+) ssRNA viruses to express their genomes in plant cells (Miller and Koev, 2000; Thivierge et al., 2005; Dreher and Miller, 2006; Kneller et al., 2006; Miller and White, 2006). Because my research utilizes tobamoviruses and potyviruses, their general expression strategies are presented below.
Tobamovirus Expression. TMV (U1 strain) encodes at least four opening reading frames (ORF) that are translated from the genome and from sub-genomic (sg) RNAs (sgRNAs) (Goelet et al., 1982; Hunter et al., 1983; Zaitlin, 1999) (Fig. 1A). The two 5' ORFs encode 126 kDa and 183 kDa replication proteins that are directly translated from the genomic RNA. The smaller protein is translated from ORF1 and the larger protein is translated from ORF1 and ORF2 as a read-through product due to leaky stop codon termination. Both proteins have methyltransferase and helicase domains while the 183 kDa protein has the RNA dependent RNA polymerase (RdRp, replicase) domain. The major cellular site for TMV replication is in viroplasms that are derived from the endoplasmic reticulum (ER) (Mas and Beachy, 1999). Efficient genome replication by the replicase is mediated by promoter sequences and structures in the 5' and 3' untranslated regions (UTR) (Takamatsu et al., 1990; Takamatsu et al., 1991; Osman et al., 2000). The replicase of TMV is also essential for expressing two downstream ORFs in the genomic RNA. ORF 3 and ORF 4 encode the movement protein (MP) and coat protein (CP), respectively, that are translated from sgRNAs (Grdzelishvili et al., 2000). Several mechanisms have been proposed for sgRNA synthesis but only two occur in plant viruses: internal initiation and premature termination (Miller and Koev, 2000; Miller and White, 2006).

In internal initiation, the replicase initiates (+) sgRNA synthesis internally on (-) viral RNAs through promoter sequences (Miller et al., 1985; French and Ahlquist, 1988; Marsh et al., 1988; Miller and Koev, 2000). Premature termination requires that the replicase be disrupted during (-) strand synthesis. This results in aborted (-) viral RNAs that serve as templates for translatable (+) sgRNAs (Sit et al., 1998; Miller and Koev, 2000; Miller and White, 2006). TMV sgRNAs likely employ the internal initiation method because sgRNA
promoters have been mapped (Grdzelishvili et al., 2000). For these TMV sgRNAs, only the CP sgRNA is believed to be capped at its 5' end (Guilley et al., 1979; Hunter et al., 1983; Joshi et al., 1983). In the case of MP sgRNA, cap-independent translation is probably mediated by an internal ribosome entry site (IRES) in its 5' UTR (Skulachev et al., 1999). An IRES is also present in the crucifer-infecting TMV (crTMV) MP and CP sgRNAs, where it may be required for translation (Ivanov et al., 1997; Skulachev et al., 1999; Dorokhov et al., 2006). Collectively, findings presented here indicate that tobamoviruses use different strategies to express downstream ORFs encoded by sgRNAs. Host proteins required for TMV gene expression will be discussed later, as will be the importance of the TMV 5' UTR translational element referred to as the Omega (Ω) leader.

**Potyvirus Gene Expression.** Similar to TMV, replication of the potyvirus *Tobacco etch virus* (TEV) genomic RNA relies on structures and sequences in its 3' end that are recognized by its viral-encoded replicase (Mahajan et al., 1996; Haldeman-Cahill et al., 1998; Teycheney et al., 2000). At least three potyviral proteins have known or putative roles in regulation. They are termed nuclear inclusion ‘b’ (NIb), nuclear inclusion ‘a’ (NIa) and cylindrical inclusion (CI), have known or putative roles in replication. NIb provides replicase activity (Domier et al., 1987). NIa interacts with NIb which is thought to direct NIb to replication complexes (Schaad et al., 1996; Li et al., 1997; Daros et al., 1999). CI possesses RNA helicase activity (Fernandez et al., 1995). Other potyviral proteins like 6K1 and 6K2 have been associated with replication (Riechmann et al., 1992) but further studies are needed. As to NIb and NIa, different potyviral proteins interact with these proteins (Merits et al., 1999; Guo et al., 2001; Kang et al., 2004). The replication site for potyviruses may be the ER
because the 6K2 protein is involved in recruiting the replication complex to this site (Schaad et al., 1997).

Unlike TMV, potyviruses do not require sgRNAs because their genomic RNA is expressed from a single ORF (Fig. 1B). This long ORF is translated into a polyprotein that is cleaved by viral-encoded, auto-catalytic proteinases into ten functionally divergent proteins (Allison et al., 1985; Adams et al., 2005) (Table 2). As mentioned above, the potyviral genomic RNA is not capped at its 5' end but is covalently linked to VPg that binds translation-related protein complexes (Leonard et al., 2000; Leonard et al., 2004; Khan et al., 2006; Michon et al., 2006; Miyoshi et al., 2006; Beauchemin et al., 2007). Such interactions may indicate that VPg can mediate cap-independent translation of the potyviral genomic RNA. However, the TEV 5' UTR that naturally embeds an IRES recruited translation-related proteins and mediated efficient translation of model mRNAs in the absence of VPg (Gallie, 2001; Zeenko and Gallie, 2005; Ray et al., 2006). Thus, VPg might be required for cap-independent translation as viral RNAs are being released from virions. Alternatively, VPg might work synergistically with the 5' UTR IRES. Other examples of potyviruses that have a 5' UTR IRES include Potato virus Y (PVY) (Levis and Astier-Manifacier, 1993) and Turnip mosaic virus (TuMV) (Basso et al., 1994).

**Tobamovirus and Potyvirus Movement**

Plant viruses achieve systemic infection through intracellular, intercellular and long-distance spreading mechanisms (Ueki and Citovsky, 2007). Detailed analyses of viral movement have been limited to a few viruses like TMV. Regardless of virus type and genera, a common feature of intercellular movement is movement through plasmodesmata (PD) (Scholthof, 2005). Passage through PD requires MP or MP-like proteins that increase the size
exclusion limit (SEL), a process termed gating. Interactions between MP or MP-like proteins and cell wall proteins will be discussed later. Unlike MPs from comoviruses (i.e. *Cowpea mosaic virus*, CPMV) and other viruses that form tubules in PD, MPs from tobamoviruses and potyviruses do not appear to form these structures (Ritzenthaler and Hofmann, 2007). The host mechanisms that transport RNA viruses as intact virions (encapsidated) or viral ribonucleoprotein (vRNP) complexes to the PD are not fully understood.

**Tobamovirus Movement.** TMV is transported intracellularly and intercellularly in the form of vRNP complexes containing MP and genomic RNA, possibly as a replicating complex (Kawakami et al., 2004). Its MP interacts with the cytoskeletal, actin and microtubules (MT), as well as the ER of plant cells (Heinlein et al., 1995; McLean et al., 1995; Heinlein et al., 1998; Reichel and Beachy, 1998). The importance of these interactions is unclear because studies have led to conflicting results. Inhibitor and TMV mutant studies support a required role for MT in TMV intracellular and intercellular movement (Heinlein et al., 1995; Boyko et al., 2000; Boyko et al., 2000; Seemanpillai et al., 2006; Boyko et al., 2007). In constrast, other studies support a requirement for actin and the cortical ER in TMV transport (Gillespie et al., 2002; Kawakami et al., 2004; Wright et al., 2007). Conflicting results might be reconciled by observations that MT inhibitors used in TMV studies were not fully effective because intact MT were detected (Seemanpillai et al., 2006). Furthermore, the α-tubulin marker tagged with GFP used to detect intact MT in the presence or absence of inhibitors was not reliable. This suggested that intact MT remaining after inhibitor treatment was sufficient for TMV movement. Thus, TMV movement might depend on MTs, actin and the ER under certain conditions. In fact, targeting of *Potato leafroll virus* (PLRV, polerovirus) MP to PD employed actin and ER networks, but not MT (Vogel et al., 2007).
However, MTs were required to target PLRV MP for degradation. A similar mechanism involving MTs in 26S proteasome degradation of TMV MP has been proposed (Gillespie et al., 2002). To further add complexity, TMV MP interfered *in vitro* with kinesin-transport activities on MTs (Ashby et al., 2006). Clearly, the prospective roles of MTs, actin and the ER in TMV movement are far from clear.

**Potyvirus Movement.** It is not known, whether potyviruses move intercellularly as intact virions or as CP-requiring vRNP (Dolja et al., 1994; Dolja et al., 1995). Unlike TMV that encodes a single MP, the potyvirus *Bean common mosaic necrosis virus* (BCMNV) encodes at least two MP-like proteins. These proteins, the CP and helper component-proteinase (HC-Pro) have been shown to increase PD SEL (Rojas et al., 1997). CI proteins of some potyviruses also have a role in viral movement (Rodriguez-Cerezo et al., 1997; Carrington et al., 1998; Roberts et al., 1998). While, CI proteins have been shown to interact with PD and CP, there is no evidence indicating that this interaction increases PD SEL. There is some evidence of MT involvement in potyvirus targeting to PD. This is implied because potyviral VPgs interact with the cap-binding protein eIF4E and/or eIF(iso)4E that binds eIF4G and/or eIF(iso)4G that associate with MT (Bokros et al., 1995; Hugdahl et al., 1995; Lellis et al., 2002; Grzela et al., 2006; Khan et al., 2006; Miyoshi et al., 2006; Nicaise et al., 2007). If potyviruses are indeed associated with MT, it could be used as a host strategy to target viral proteins like VPg for degradation as suggested for PLRV MP. The role of VPg and eIF4E and/or eIF(iso)4E in potyvirus infection will be discussed later.

Long-distance movement of most plant viruses from inoculated leaves to systemic leaves is through phloem by bulk flow (Leisner and Turgeon, 1993; Santa Cruz, 1999; Scholthof, 2005). Both TMV and TEV move through non-vascular and vascular tissues
A typical invasion route for TMV and TEV infection starts in epidermal cells then moves into mesophyll cells, the bundle sheath and then phloem cells to infect distant leaves. Phloem loading of encapsidated tobamoviruses and potyviruses is through both minor and major veins (Ding et al., 1998; Cheng et al., 2000; Rajamaki and Valkonen, 2003). Yet, it appears phloem unloading of viruses is only through major veins (Roberts et al., 1997). It should be noted that not all viruses move through the same cell or vein types, especially in inoculated leaves. Furthermore, cell-to-cell and long-distance movement is complex and involves an array of interacting host and viral proteins (Whitham and Wang, 2004; Ueki and Citovsky, 2007).

**Host Proteins Essential in Tobamovirus and Potyvirus Infections**

Several host proteins or genetic loci have been identified that are essential for plant viral infections (Whitham and Wang, 2004; Carr and Whitham, 2007; Ueki and Citovsky, 2007). Candidate host proteins have also been identified based on selective criteria including, their interactions with viral nucleic acids or viral proteins. A few of these host proteins pertaining to tobamovirus and potyvirus infections will be discussed here. In particular, host proteins that mediate (or have suggestive roles) in viral translation, replication or movement will be discussed.

**Host Proteins Involved Tobamovirus Infection.** In a protein binding assay, the HSP101 heat shock protein from wheat germ and tobacco bound the TMV 5' \( \Omega \) leader poly(CAA) sequence (Tanguay and Gallie, 1996; Wells et al., 1998). This sequence is essential for *in vitro* translational efficiency of TMV and likely other tobamoviruses (Gallie and Walbot, 1992). Using model mRNAs fused to the TMV \( \Omega \) leader, tobacco HSP101
enhanced translation up to 50-fold compared to mRNAs lacking the Ω leader (Wells et al., 1998). Tobacco HSP101 enhancement of translation recruited eIF4F consisting of the cap-binding protein, eIF4E and the scaffold protein, eIF4G (Gallie, 2002). A functional role for HSP101 in tobamovirus pathogenesis was not reported.

Four *A. thaliana*-encoded proteins referred to as TOM (tobamovirus multiplication) proteins mediate TMV-Cg (crucifer-infesting) replication (Yamanaka et al., 2000; Yamanaka et al., 2002; Tsujimoto et al., 2003). Three of these proteins, TOM1, TOM2A and TOM3 encode membrane-associated proteins. TOM1 and TOM3 homologues interact with the helicase domain of TMV replicase protein, whereas TOM2A interacts with itself and TOM1. Thus it appears that TOM1, TOM2A and TOM3 are required for TMV replication on the tonoplast (Hagiwara et al., 2003). TOM1 and TOM3 are both required for TMV replication in tobacco has been reported (Asano et al., 2005). In *N. benthamiana*, TOM1 is also required for TMV replication but it was not reported if TOM3 is required (Chen et al., 2007). In the Chen et al. (2007) study, homologous *TOM1* mRNA was silenced in *N. benthamiana*. Because TOM1 and TOM3 are homologous in *A. thaliana* and tobacco, it is likely that any *N. benthamiana* *TOM3* mRNA present was also silenced. The function of TOM2B in TMV infection remains to be determined.

Interacellular and systemic movement of TMV in tobacco requires a pectin methylesterase (PME) that interacts with TMV MP (Chen et al., 2000; Chen and Citovsky, 2003). Other non-tobamovirus MPs interact with PME. PMEs catalyze the demethylesterification of cell wall polygalacturonans that constitute structural components of pectin and are essential in plant development (Pelloux et al., 2007). A role for PME in TMV intercellular movement was demonstrated by deleting the potential PME binding region in
TMV MP, amino acids 130 – 135 (Chen et al., 2000). Delayed TMV systemic movement was shown in antisense PME tobacco plants. The exact function of PMEs in TMV infections is not fully understood. Recently it was reported that a transiently expressed PME enhanced RNA silencing against TMV in *N. benthamiana* (Dorokhov et al., 2006). This would suggest that PMEs might have different roles in TMV infections. An overview of RNA silencing will be presented later.

**Host Proteins Involved in Potyvirus Infection.** The cap-binding protein eIF4E and/or eIF(iso)4E is recruited by VPg for successful potyvirus infections (Duprat et al., 2002; Lellis et al., 2002; Nicaise et al., 2003; Sato et al., 2005; Ruffel et al., 2006; Nicaise et al., 2007). In *A. thaliana*, eIF(iso)4E is required for several potyvirus infections including TEV, TuMV, *Plum pox virus* (PPV) and *Lettuce mosaic virus* (LMV) (Duprat et al., 2002; Lellis et al., 2002; Sato et al., 2005; Decroocq et al., 2006). Conversely, LMV requires eIF4E instead of eIF(iso)4E in lettuce (Nicaise et al., 2003) as does *Clover yellow vein virus* (CIYVV) in *A. thaliana*. This would suggest that potyviral VPg selection of eIF4E and/or eIF(iso)4E is virus specific. Interactions between VPg and eIF4E and/or eIF(iso)4E might be essential for potyvirus translation, replication and/or intercellular movement.

A recessive mutation in the *Pisum sativum* (pea) *eIF(iso)4E* gene conferred resistance to *Pea seed borne mosaic virus* (PSbMV, potyvirus) demonstrating that it was required for replication and movement activities (Gao et al., 2004). An argument could be made that eIF(iso)4E was needed to interact with eIF(iso)4G and/or eIF4G for virus interaction with MTs as previously discussed. Selective recruitment of eIF4G and eIF(iso)4G by potyviruses through eIF4E and eIF(iso)4E is also evident in *A. thaliana* (Nicaise et al., 2007). For example CIYVV, PPV and TuMV requires eIF4G, eIF4(iso)4G1, and both eIF4(iso)4G1 and
eIF4(iso)4G2, respectively. Thus it appears that eIF4E and eIF4G interactions are isoform-dependent. Why PPV and TuMV have different eIF4(iso)4G requirements in *A. thaliana* remains to be determined.

Several pea proteins interact with the VPg of PSbMV in a Y2H system (Dunoyer et al., 2004). One strongly interacting protein identified as PVIPp (*Potyvirus* VPg-interacting protein from pea) was similar to two cysteine-rich *A. thaliana* proteins (At5g48160, PVIP1 and At3g07780, PVIP2). Y2H analysis was also performed to detect interactions between VPgs of several *A. thaliana* infecting potyvirus and these two proteins. Deletion analysis of TuMV VPg determined regions necessary for VPg-PVIP interactions. These interactions required amino acids 1-16 and 42-66 in the N-terminal region of TuMV VPg. *A. thaliana* RNAi (RNA interference) lines with reduced expression of PVIP1 or PVIP2 showed restricted movement of TuMV (wild-type) when compared to wild-type plants. TuMV levels were reduced in the inoculated leaves of the mutants, indicating that intercellular movement was restricted.

Similar results were also observed in *N. benthamiana* after inoculation with TuMV containing a mutation in VPg that blocked its interaction with PVIP-related proteins. However, when the TuMV VPg mutant was delivered by agroinfiltration, reduction in TuMV levels was systemic instead of localized. Collectively, the *A. thaliana* and supporting *N. benthamiana* data suggest that VPg interactions with specific cysteine-rich proteins are necessary for the movement of TuMV and possibly other potyviruses.

**Antiviral Defense in Susceptible Plant Cells – RNA Silencing**

General antiviral mechanisms in plant that target and eliminate viral RNAs (Crum et al., 1988; Bilgin et al., 2003; Wang and Metzlaff, 2005). The most studied mechanism is the
RNA silencing pathway that centers on the coordinates the actions of host proteins and viral-derived small RNAs (Wang and Metzlaff, 2005; Waterhouse and Fusaro, 2006). Interchangeable terms for this pathway include RNAi, post-transcriptional gene silencing (PTGS), virus-induced gene silencing (VIGS) and virus-induced RNA silencing (VIRS). Viral initiators of VIRS are double-stranded (ds) RNAs (Tenllado and Diaz-Ruiz, 2001) and structured single-stranded (ss) RNAs (Molnar et al., 2005). In plant cells, viral dsRNAs accumulate as genomic material (Nuss and Dall, 1990), replication intermediates (Buck, 1999), complementary overlapping ssRNAs (Vanitharani et al., 2005) or substrates generated by the host to propagate VIRS (Ahlquist, 2002). Because DNA viruses do not require a dsRNA step, the likely culprits in DNA-VIRS are structured viral RNAs and complementary overlapping viral RNAs (Vanitharani et al., 2005; Moissiard and Voinnet, 2006).

**Dicer-Like (DCL) Proteins.** A major class of plant proteins required for VIRS initiation and other RNA silencing pathways are DCL proteins that belong to the ribonuclease (RNase) III family (Mallory and Vaucheret, 2006; Waterhouse and Fusaro, 2006). DCL proteins recognize structured viral and host RNAs and cleave them into ~21-24 nucleotide (nt) small RNA duplexes with a 2 nt overhang at the 3' ends. These small RNAs, in return, direct cleavage of complementary transcripts to negatively regulate gene expression (Bonnet et al., 2006). In *A. thaliana*, there are four DCL proteins DCL1, DCL2, DCL3 and DCL4 that generate 21 nt, 22 nt, 24 nt and 21 nt small RNAs, respectively (Gasciolli et al., 2005; Bonnet et al., 2006; Waterhouse and Fusaro, 2006). Virus-derived small RNAs are referred to as small interfering RNAs (siRNAs), whereas small RNAs regulating host gene expression are named based on origin and function (Bonnet et al., 2006). The roles of DCL proteins and siRNAs in VIRS have been investigated using different RNA and DNA viruses.
DCL4 appears to be the major protein that initiates VIRS against RNA viruses, like *Cucumber mosaic virus* (CMV, cucumovirus) and *Turnip crinkle virus* (TCV, carmovirus) (Blevins et al., 2006; Deleris et al., 2006; Diaz-Pendon et al., 2007). DCL2 is functionally redundant to DCL4 in defense, thus in the absence of DCL4 and DCL2, RNA-VIRS is severely impaired. The function of DCL3 is unclear in RNA-VIRS although it can mediate defense in a partially active DCL4 genetic background (Diaz-Pendon et al., 2007). Nonetheless, DCL3 is required by the host to produce repeat associated siRNAs (ra-siRNAs) derived from endogenous repeated genes (Xie et al., 2004).

A role for DCL1 in RNA-VIRS has not been established. However, active DCL1 is functionally redundant to DCL2, DCL3 and DCL4 in DNA-VIRS involving *Cauliflower mosaic virus* (CaMV, caulimovirus) and *Cabbage leaf curl virus* (CaLCuV, begomovirus) (Blevins et al., 2006; Moissiard and Voinnet, 2006). Initially, DCL1 was shown to produce 21 nt small RNAs called microRNAs (miRNAs) from host-encoded long stem-looped transcripts (Park et al., 2002; Kurihara and Watanabe, 2004). DCL1 also produces 21 nt natural antisense siRNAs (nat-siRNAs) from overlapping host transcripts (Allen et al., 2005; Borsani et al., 2005). In host cells, miRNAs and nat-siRNAs direct cleavage of host transcripts to negatively regulate gene expression. The likely explanation as to why DCL1 is active in DNA-VIRS might be linked to structures in nuclear viral RNAs that bear resemblance to precursor miRNAs (Blevins et al., 2006; Moissiard and Voinnet, 2006). Because DCL1 localizes in the nucleus, this seems highly plausible (Papp et al., 2003). Additional studies of DCL1 function in DNA-VIRS may determine why DCL proteins are functionally redundant.
RNA-Induced Silencing Complex (RISC). Negative regulation of viral and host gene expression by small RNAs in plants is complex and involves various host proteins (Vaucheret, 2006). Essential in this process is RISC comprised of at least a small RNA and host protein (Tang, 2005). A major RISC protein conserved among several organisms is Argonaute (AGO) (Carmell et al., 2002). In *A. thaliana* there are ten AGO genes, but only AGO1, AGO4, AGO6 and AGO7 have been assigned functions (Vazquez, 2006; Zheng et al., 2007). AGO1 binds miRNAs and certain siRNAs to cleave host transcripts (Baumberger and Baulcombe, 2005). AGO1 binding of viral siRNAs was not detected when first assayed but was ultimately shown to bind viral siRNAs (Zhang et al., 2006).

AGO4 and AGO6 are necessary for the accumulation of heterochromatic siRNAs that guide RNA directed DNA methylation (RdDM: transcriptional gene silencing, TGS) of host genes and transgenes (Zheng et al., 2007). AGO7, on the other hand, is required for the accumulation of some trans-acting siRNAs (ta-siRNAs) that regulate leaf polarity (Adenot et al., 2006; Fahlgren et al., 2006). Thus, it appears AGO proteins regulate specific functions. Further studies are needed to examine the potential roles of AGO1 or other AGO proteins in VIRS.

Functional analyses of RISC-related proteins in plant VIRS are lacking, however, RISC-like complexes (50 kDa – 650 kDa) with ribonuclease activity induced by virus infections have been detected (Omarov et al., 2007; Pantaleo et al., 2007). Small RNAs eluted from these protein complexes consisted of viral siRNAs and miRNAs. Whether individual complexes contain homogenous or heterogeneous small RNAs and AGO proteins remains to be determined. Studies on the assembly of proteins and small RNAs in these complexes are also needed. In terms of small RNAs, studies in *Drosophila* have shown
duplex RNAs are loaded into RISC but only one strand is used as the guide RNA and the passenger strand is degraded by an AGO protein (Matranga et al., 2005; Tang, 2005). The role of plant AGO proteins in degrading the passenger strand of siRNAs is unknown.

**Silencing Signal.** A requirement in plant VIRS is the propagation of a silencing signal that restricts virus infection of non-inoculated leaves and blocks meristematic tissue invasion (Foster et al., 2002; Qu et al., 2005; Schwach et al., 2005; Xie and Guo, 2006; Hohn et al., 2007). The mobile VIRS signals are viral siRNAs that apparently move from cell-to-cell through plasmodesmata (Ruiz-Medrano et al., 2004). Long distance movement of the mobile VIRS signal is evidently through phloem (Yoo et al., 2004). Different sized mobile siRNAs might be required for local and long-distance silencing (Hamilton et al., 2002) but in a *Cucumber yellows virus* (*CuYV*, closterovirus, RNA virus)-infected *Cucurbita maxima* (pumpkin), ~21 nt viral siRNAs were detected in phloem sap (Yoo et al., 2004).

The plant mobile signal is amplified by host encoded RNA dependent RNA polymerases (RdRps or RDRs) (Wassenegger and Krczal, 2006). More than one *RDR* gene is present in *Nicotiana* species and *A. thaliana*, with the latter having six genes (Wassenegger and Krczal, 2006). RDR1, RDR2 and RDR6 have been assigned roles in RNA silencing. RDR1 and RDR6 are both required for VIRS as shown in different plant species (Xie et al., 2001; Yu et al., 2003; Yang et al., 2004; Qu et al., 2005; Schwach et al., 2005), unlike RDR2 that is required for RdDM (Xie et al., 2004). In RDR1-deficient plants and RDR6-deficient plants increased susceptibility to virus infection are exhibited compared to wild-type plants. For example, in RDR1-deficient *N. tabacum* (tobacco), PVX was systemically infectious but in wild-type tobacco it is restricted to inoculated leaves (Xie et al., 2001). In RDR6-deficient
plants, PVX and TMV invasion of meristematic tissues was observed (Qu et al., 2005; Schwach et al., 2005).

Grafting experiments using wild-type rootstocks and RDR6-deficient scions revealed that RDR6 was required for scion cells to perceive a systemic silencing signal (Schwach et al., 2005). In the reverse, RDR6-deficient rootstocks did not prevent the systemic silencing signal from reaching wild-type scions. Thus, RDR1 may have a role in producing the systemic VIRS signal. Additional support for this hypothesis is based on findings that RDR1-deficient plants accumulated lower levels of viral siRNAs in response to a CMV mutant, CMVΔ2b compared to wild-type plants (Diaz-Pendon et al., 2007). Further studies are necessary to confirm the role of RDR1 or possibly other proteins in producing the systemic VIRS signal.

Other RNA silencing-related proteins have been examined for a role in VIRS (Hohn et al., 2007). An example is HEN1 (methyltransferase HUA Enhancer 1), that deposits a methyl group on the 3' end of miRNAs and siRNAs for stability (Li et al., 2005; Yu et al., 2005; Yang et al., 2006). In response to virus infection, HEN1-deficient plants are more susceptible compared to wild-type plants (Boutet et al., 2003). Another RNA silencing-related protein in plants is RNA polymerase IV (RNAP IV) (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Zhang et al., 2007). The main role of RNAP IV has been assigned to small RNA metabolism in TGS but a role in VIRS has not been established.

**Suppression of VIRS.** Many viruses suppress VIRS through viral-encoded silencing suppressor proteins (Li and Ding, 2006). First discovered among plant viruses, these proteins not only suppress VIRS but other forms of RNA silencing like RdDM (Anandalakshmi et al.,
1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Voinnet et al., 1999; Guo and Ding, 2002; Qu and Morris, 2005; Hohn et al., 2007). Several plant viral suppressors have been identified but only a few have been studied in detail. Viral suppressors that are discussed here are those encoded by RNA viruses (Fig. 2). However, DNA viruses also encode silencing suppressors (Hohn et al., 2007).

The tombusvirus suppressor P19 from *Carnation Italian ringspot virus* (CIRV) and *Tomato bushy stunt virus* (TBSV) was the first to be shown to bind viral siRNAs (Vargason et al., 2003; Ye et al., 2003). Binding of viral siRNAs by P19 suggests that it suppresses VIRS by preventing viral siRNA incorporation into RISC. A similar action is proposed for other plant viral suppressors including TEV HC-Pro and for TMV replicase protein (Ye and Patel, 2005; Lakatos et al., 2006; Merai et al., 2006; Csorba et al., 2007; Kurihara et al., 2007). In addition, inhibition of 3' end methylation of viral siRNAs has been detected in response to TEV HC-Pro (Ebmardt et al., 2005) and TMV and *Oilseed rape mosaic virus* (ORMV) replicase (Vogler et al., 2007) protein expression. Viral siRNAs that are not methylated are potentially unstable; thus their function in VIRS is compromised. TCV suppressor, CP (P38) seemingly interferes with the processing of viral dsRNAs into siRNAs (Qu et al., 2003). P38 may suppress the activities of DCL4 and DCL2 thereby blocking VIRS (Deleris et al., 2006). CMV suppressor, 2b binds AGO1 to block small RNA guided cleavage of RNA transcripts (Zhang et al., 2006). The polerovirus suppressor P0 from *Beet western yellows virus* also binds AGO1 and targets it for degradation (Baumberger et al., 2007; Bortolamiol et al., 2007). Collectively, plant viruses have evolved a variety of strategies to suppress VIRS.
Interestingly, some plant viral suppressors are not required for local viral infection but for systemic infection. This was shown using a *Cymbidium ringspot virus* (CymRSV, tombusvirus) P19 mutant and the CMVΔ2b mutant, which are deficient in suppressor activity (Havelda et al., 2003; Diaz-Pendon et al., 2007). Compared to their corresponding wild-type viruses, mutant viruses accumulated at similar levels in inoculated leaves but not in systemic leaves. These and other findings indicate the general role of viral suppressors is to prevent the spread of the silencing signal ahead of viruses (Guo and Ding, 2002). It should be noted that viral suppressors like P19 and HC-Pro also bind miRNAs (Chapman et al., 2004; Lakatos et al., 2006).

Although viral suppressors are essential for virus infection, they appear unable to reverse established VIRS (active RISC) in systemic non-inoculated leaves (Lakatos et al., 2006). This was shown when HC-Pro, P19 and P21 (*Beet yellows virus*, BYV, closterovirus) were transiently expressed in non-inoculated leaves with active RISC targeting a reporter gene fused to CymRSV sequence. Mobile silencing signals were derived from a CymRSV P19 mutant that replicated only in lower inoculated leaves. These results confirm the role of viral suppressors in preventing the spread of virus-derived siRNAs to inhibit the formation of active RISC.

**Salicylic Acid (SA) Role in VIRS.** SA is a signaling molecule required for plant resistance to a number of pathogens including viruses (Shah, 2003; Durrant and Dong, 2004; Robert-Seilanianz et al., 2007). The first report of a link between SA and VIRS was shown in CMVΔ2b – tobacco studies (Ji and Ding, 2001). As expected, CMVΔ2b only infected inoculated leaves whereas it was unable to systemically infect tobacco. In contrast, transgenic tobacco expressing the bacterial salicylate hydroxylase (NahG) that degrades SA supported
CMVΔ2b systemic infection indicating SA was required to mediate VIRS. Similar to CMVΔ2b, wild-type PPV is restricted to inoculated leaves of tobacco (Saenz et al., 2002) but systemically infects tobacco expressing NahG (Alamillo et al., 2006). PPV HC-Pro can suppress RNA silencing (Tenllado et al., 2003; Alamillo et al., 2006), but it is not known why it is not sufficient for PPV systemic infection of tobacco. However, tobacco expressing TEV HC-Pro and non-transgenic *N. benthamiana* support systemic PPV infection (Saenz et al., 2002; Alamillo et al., 2006). Compared to NahG tobacco and TEV HC-Pro tobacco, enhanced PPV systemic infection was detected in NahG X HC-Pro tobacco (Alamillo et al., 2006). These results indicate that SA enhances VIRS in tobacco and HC-Pro encoded by some potyviruses is able to overcome this response.

The function of the role of SA in VIRS is unclear. However, it has been shown that SA treatment induces resistance to viruses by restricting virus replication and movement (Naylor et al., 1998; Murphy and Carr, 2002; Wong et al., 2002; Mayers et al., 2005). To date, only RDR1 is induced by SA and viruses in tobacco (Xie et al., 2001), *A. thaliana* (Yu et al., 2003), and *N. benthamiana* (Yang et al., 2004). As previously discussed, RDR1 might be required for production of the mobile silencing signal because RDR1-deficient tobacco supported systemic infection of PVX that is normally restricted to inoculated leaves. Additional support for this hypothesis can be drawn from studies in *A. thaliana* and *N. benthamiana*.

In RDR1-deficient *A. thaliana*, TMV-Cg and *Tobacco rattle virus* (TRV, tobravirus) was detected at higher levels in inoculated and systemic leaves compared to wild-type plants (Yu et al., 2003). Both TMV-Cg and TRV systemically infect wild-type *A. thaliana*. RDR1 in *N. benthamiana*, unlike other plant species, is naturally truncated and has been linked to
increased hypersusceptibility to tobamoviruses (Yang et al., 2004). This was concluded after transgenic *N. benthamiana* expressing RDR1 from *Medicago truncatula* accumulated reduced levels of tobamoviruses and displayed reduced symptoms. It would appear CMVΔ2b and PPV systemic infection of NahG tobacco was due to non-SA-inducible RDR1. Then again, other potential suppressed SA-inducible or -dependent VIRS-related genes in NahG tobacco might have been responsible for CMVΔ2b systemic infection. Recently it was shown that RDR1-deficient *A. thaliana* did not support CMVΔ2b systemic infection in spite of reduced viral siRNAs (Diaz-Pendon et al., 2007). Thus it is likely, suppression of other host genes maybe necessary for CMVΔ2b to systemically infect *A. thaliana*. Additional studies are needed to better understand the role of SA in VIRS.

**Virus-Induced Symptoms**

The onset of symptom development in virus-infected plants is frequently a result of viral – host factor interactions (Kasschau et al., 2003; Chellappan et al., 2005; Culver and Padmanabhan, 2007). For instance, transgenic *A. thaliana* plant expressing silencing suppressors HC-Pro, p19 and 2b share phenotypic similarities with virus-infected *A. thaliana*, like stunted growth and deformed leaves (Kasschau et al., 2003; Chen et al., 2004; Dunoyer et al., 2004; Chellappan et al., 2005). This is due in part to silencing suppressor interference with miRNA regulation of host genes. Several plant miRNAs are essential to plant development, including those that regulate auxin signaling, miR160 and miR167 (Liscum and Reed, 2002; Park et al., 2002; Rhoades et al., 2002) and gibberellin signaling, miR171 (Silverstone et al., 1998; Pysh et al., 1999; Llave et al., 2002). Targets of these miRNAs are auxin-responsive transcription factor (ARF) mRNAs and scarecrow-like transcription factor (SCL) mRNAs that encode protein regulators of auxin signaling and gibberellin signaling,
respectively. Inhibition of mRNA cleavage by their respective miRNAs was detected in TEV HC-Pro transgenic and TEV-infected non-transgenic *A. thaliana* (Kasschau et al., 2003).

Although mRNA cleavage was blocked in HC-Pro plants, increased accumulation of several miRNAs was detected. It was subsequently shown that *DCL1* mRNA was also up-regulated in HC-Pro *A. thaliana* explaining why miRNAs levels were high (Xie et al., 2003). Under wild-type conditions, *DCL1* mRNA accumulates at low levels due to negative regulation by miR162. Thus, altered miRNA function leading to altered plant development might be a general outcome of viral suppressor activity. Interestingly, a transgenic line of tobacco expressing TMV MP and a CP variant (MP X CP) also accumulated increased levels miRNAs and developed abnormally, although MP and CP do not function as viral suppressors (Bazzini et al., 2007). This would suggest that some non-viral suppressor proteins that induce symptom expression in plants might also inhibit miRNA regulation of host genes.

Viral proteins also interact with host proteins that regulate hormonal and developmental signaling pathways (Culver and Padmanabhan, 2007). Two of these interactions involving auxin (Aux) and gibberellin (GA) signaling pathways will be discussed in response to TMV and *Rice dwarf virus* (RDV, phytoreovirus) infections, respectively. Using a Y2H approach, the helicase domain of TMV replicase protein interacted with the Aux/IAA protein PAP1/IAA26 of *A. thaliana*, but a helicase mutation, V1087I disrupted this interaction (Padmanabhan et al., 2005). Aux/IAA proteins generally function as regulators of auxin-responsive gene expression by binding ARF proteins in the absence of auxin (Liscum and Reed, 2002). In mock-infected and TMV$^{V1087I}$-infected leaves,
PAP1/IAA26 localized in the nucleus, whereas in TMV-infected leaves it had cytoplasmic localization.

Whole plant infection of *A. thaliana* (ecotype Shahdara) and *N. benthamiana* resulted in mild symptom development in response to TMV$^{V1087I}$ compared to TMV. This strongly suggested that PAP1/IAA226 relocation was responsible for symptom differences among TMV$^{V1087I}$- and TMV-infected plants. Support for this hypothesis was provided by transcriptional analysis of TMV-infected *A. thaliana*: ~30% of genes containing multiple auxin-responsive promoter elements had expression that was altered. Other Aux/IAA proteins interact with TMV replicase protein and have their localization altered including IAA18 and IAA27 (Padmanabhan et al., 2006). To date, IAA18 and IAA27 role in TMV-induced symptom development in plants has not been explored. Furthermore it is not known if TMV replicase protein targets Aux/IAA proteins in general as a strategy to suppress host defense responses. Down-regulation of auxin signaling has recently been shown to restrict *Pseudomonas syringae* growth in *A. thaliana* (Navarro et al., 2006).

In *Oryza sativa* (rice) plants, the RDV encoded outer capsid protein P2 interacted with *ent*-kaurene oxidase (Zhu et al., 2005), a key enzyme in GA biosynthesis (Helliwell et al., 1999). GA1, the major active component in rice tissues was significantly reduced in RDV-infected rice. Similar to observations in *ent*-kaurene oxidase rice mutants (Sakamoto et al., 2004), RDV-infected rice plants are stunted in growth. Treatment of RDV-infected rice with GA3 restored a non-dwarfed phenotype (Zhu et al. 2005). Although it is not known why RDV P2 targets rice *ent*-kaurene oxidase, *ent*-kaurene oxidase may participate in phytoalexin biosynthesis (Itoh et al., 2004). Phytoalexins are defense compounds that are active in plants...
against bacterial and fungal pathogens but their role in response to viruses is poorly understood (Hammerschmidt, 1999).

**Future Research**

Future studies are needed to investigate why host proteins involved in plant development are targeted by viral proteins. In addition, studies are needed to examine in detail how viruses alter host gene expression resulting in symptom development in infected plants. Virus-induced symptoms like stunted plant growth are likely caused in part by altered expression of cell wall related-genes. This is evident in response to TuMV and RDV, because several cell wall related-genes were down-regulated in *A. thaliana* (Yang et al., 2007) and rice (Shimizu et al., 2007), respectively.

**Conclusion and Dissertation Outline**

Presented here are only a few examples of the various strategies used by plant viruses to express their genomes and infect susceptible plants. The overall focus of this dissertation research is to study mechanisms of susceptibility of *A. thaliana* and *N. benthamiana* to virus infections. Research topics were developed based on some of the material presented here, in particular the role of plant proteins in viral infections and why viruses induce symptom expressions in plants. Three chapters summarizing results from these studies in *A. thaliana* and *N. benthamiana* will be presented. In Chapter 2, expression of the *A. thaliana* HSP100 gene family and the role of HSP101 during ORMV infection will be addressed. HSP101’s role in *N. benthamiana* in response to TMV will also be discussed. Chapter 3 summarizes the expression profiles of the *A. thaliana* HSP70 gene family and *A. thaliana* heat shock transcription factors (HSFs) in response to ORMV and CMV. In Chapter 4, the effects of TuMV mutants on *A. thaliana* and *N. benthamiana* symptom development will be discussed.
Specifically, the role of a conserved motif in TuMV HC-Pro will be addressed. The final chapter in this dissertation addresses future experiments that are need to better understand the topics presented.

References


Culver JN, Padmanabhan MS (2007) Virus-Induced Disease: Altering host physiology one interaction at a time. Annu Rev Phytopathol 45: 221-243


Diaz-Pendon JA, Li F, Li WX, Ding SW (2007) Suppression of Antiviral Silencing by Cucumber Mosaic Virus 2b Protein in Arabidopsis Is Associated with Drastically Reduced Accumulation of Three Classes of Viral Small Interfering RNAs. Plant Cell 19: 2053-2063


Dorokhov YL, Ivanov PA, Komarova TV, Skulachev MV, Atabekov JG (2006) An internal ribosome entry site located upstream of the crucifer-infecting tobamovirus coat protein (CP) gene can be used for CP synthesis in vivo. J Gen Virol 87: 2693-2697


Gallie DR, Walbot V (1990) RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells. Genes Dev 4: 1149-1157


that microtubules are dispensable for the cell-to-cell movement of tobacco mosaic virus. Plant Cell 14: 1207-1222


Hugdahl JD, Bokros CL, Morejohn LC (1995) End-to-end annealing of plant microtubules by the p86 subunit of eukaryotic initiation factor-(iso)4F. Plant Cell 7: 2129-2138


Mas P, Beachy RN (1999) Replication of tobacco mosaic virus on endoplasmic reticulum and role of the cytoskeleton and virus movement protein in intracellular distribution of viral RNA. J Cell Biol 147: 945-958


Rajamaki ML, Valkonen JP (2003) Localization of a potyvirus and the viral genome-linked protein in wild potato leaves at an early stage of systemic infection. Mol Plant Microbe Interact 16: 25-34


Tanguay RL, Gallie DR (1996) Isolation and characterization of the 102-kilodalton RNA-binding protein that binds to the 5' and 3' translational enhancers of tobacco mosaic virus RNA. J Biol Chem 271: 14316-14322


Table 1. Viruses that infect *Arabidopsis* (Carr and Whitham, 2007).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genus: Begomovirus (ssDNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Cabbage leaf curl virus (CLCV)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Genus: Bromovirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Brome mosaic virus (BMV)</td>
<td>M1</td>
</tr>
<tr>
<td>Cow pea chlorotic mottle virus (CCMV)</td>
<td>M1</td>
</tr>
<tr>
<td>Spring beauty latent virus (SBLV)</td>
<td>ATCC PV-369</td>
</tr>
<tr>
<td><strong>Genus: Carmovirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Turnip crinkle virus (TCV)</td>
<td>M</td>
</tr>
<tr>
<td><strong>Genus: Caulimovirus (dsDNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Cauliflower mosaic virus (CaMV)</td>
<td>CM4-184, CM1841, W260</td>
</tr>
<tr>
<td><strong>Genus: Curtovirus (ssDNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Beet curly top virus (BCTV)</td>
<td>CFH, Logan</td>
</tr>
<tr>
<td><strong>Genus: Cucumovirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Cucumber mosaic virus (CMV)</td>
<td>O, Y</td>
</tr>
<tr>
<td><strong>Genus: Nepovirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Tobacco ringspot virus (TRSV)</td>
<td>Grape, Bud blight, and uncharacterized field isolate</td>
</tr>
<tr>
<td><strong>Genus: Polerovirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Beet western yellows virus (BWYV)</td>
<td>N/A</td>
</tr>
<tr>
<td>Cucurbit aphid-borne yellows virus (CABYV)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Genus: Potexvirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Potato virus X (PVX)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Genus: Potyvirus ((+)+ ssRNA)</strong></td>
<td></td>
</tr>
<tr>
<td>Lettuce mosaic virus (LMV)</td>
<td>AF199, E, 0</td>
</tr>
<tr>
<td>Plum pox virus (PPV)</td>
<td>NAT, R, PS, Soc, EA</td>
</tr>
<tr>
<td>Potato virus Y (PVY)</td>
<td>3</td>
</tr>
<tr>
<td>Tobacco etch virus (TEV)</td>
<td>HAT, Madison, ST1</td>
</tr>
<tr>
<td>Tobacco vein mottling virus (TVMV)</td>
<td>N/A</td>
</tr>
<tr>
<td>Virus Name</td>
<td>Genus</td>
</tr>
<tr>
<td>-------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Turnip mosaic virus (TuMV)</td>
<td>Genus: Tobamovirus</td>
</tr>
<tr>
<td></td>
<td>((+) ssRNA)</td>
</tr>
<tr>
<td>Oilseed rape mosaic virus (ORMV)</td>
<td></td>
</tr>
<tr>
<td>Tobacco mosaic virus (TMV)</td>
<td></td>
</tr>
<tr>
<td>Turnip vein clearing virus (TVCV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genus: Tobravirus</td>
</tr>
<tr>
<td></td>
<td>((+) ssRNA)</td>
</tr>
<tr>
<td>Tobacco rattle virus (TRV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genus: Tospovirus</td>
</tr>
<tr>
<td></td>
<td>((-) &amp; (+/-) RNA)</td>
</tr>
<tr>
<td>Tomato spotted wilt virus (TSWV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genus: Tymovirus</td>
</tr>
<tr>
<td></td>
<td>((+) ssRNA)</td>
</tr>
<tr>
<td>Turnip yellow mosaic virus (TYMV)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Potyviral Proteins (Adams et al., 2005).

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>Known or Suggested Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1, Protein 1</td>
<td>proteinase, symptom induction</td>
</tr>
<tr>
<td>HC-Pro, Helper component-proteinase</td>
<td>proteinase, symptom induction, RNA silencing, suppressor, movement, aphid transmission</td>
</tr>
<tr>
<td>P3, Protein 3</td>
<td>infectivity, symptom induction</td>
</tr>
<tr>
<td>6K1, 6 kDa 1</td>
<td>membrane anchor protein for replication</td>
</tr>
<tr>
<td>CI, Cylindrical inclusion</td>
<td>ATPase, RNA helicase</td>
</tr>
<tr>
<td>6K2, 6 kDa 2</td>
<td>membrane anchor protein for replication</td>
</tr>
<tr>
<td>VPG, viral-linked protein to genome</td>
<td>Replication, translation, movement</td>
</tr>
<tr>
<td>Nla-Pro, nuclear inclusion “a” - proteinase</td>
<td>major proteinase</td>
</tr>
<tr>
<td>Nb, nuclear inclusion “b”</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>CP, coat protein</td>
<td>movement and aphid transmission</td>
</tr>
</tbody>
</table>
Fig. 1. Schematic representation of the tobamovirus and potyvirus genomes. A. Tombamovirus (+) ssRNA genome. The 126 kDa replicase and the 183 replicase read-through product are translated from the genomic RNA. The movement protein (MP) and the coat protein (CP) are translated from subgenomic RNAs. B. Potyvirus (+) ssRNA genome. A single long polyprotein is translated from the genomic RNA. The polyprotein is cleaved into 10 smaller proteins by the viral encoded proteases, P1, HC-Pro and NIa-Pro. See Table 2 for protein descriptions and functions.
Fig. 2. General overview of RNA virus-induced RNA silencing (VIRS) in plants and the function of select viral suppressors of RNA silencing. 1. Dicer-like (DCL) proteins, for example DCL4 and DCL2, cleave double-stranded (ds) viral RNAs into 21 or 22 nucleotide (nt) small interfering RNA (siRNA) duplexes with a 2 nt overhangs at the 3' end, respectively. 2. The guide strand of the siRNA duplex is then incorporated into the RNA induced silencing complex (RISC) that contains an Agronaute (AGO) protein. 3. RISC is then guided to complementary viral RNA transcripts by the siRNA for AGO to cleave transcripts. P38, the coat protein of *Turnip crinkle virus* (TCV) suppresses VIGS by inhibiting siRNA accumulation by interfering with DCL cleavage activity, its binding to dsRNA, or by targeting it for degradation. P19 from tombusviruses, HC-Pro from
potyviruses and *Tobacco mosaic virus* (TMV) replicase protein all have binding affinity for siRNAs and likely suppress VIRS by preventing siRNA programming of RISC. P19 binds siRNAs as a dimer, but it is not known if HC-Pro or replicase protein dimerization is required for binding. The (?) symbol denotes that the suppressor’s precise function or requirements is not known. Both 2b from *Cucumber mosaic virus* (CMV) and P0 from *Beet western yellows virus* (BWYV) binds AGO1 presumably to suppress VIRS. 2b inhibits AGO1 cleavage of RNA transcripts, whereas P0 targets AGO1 for degradation. It is unclear if AGO1, AGO1 bound to the guide siRNA or both are targets of P0. The circle marked with X denotes degradation.
CHAPTER 2. TOBAMOVIRUS INFECTION IS INDEPENDENT OF HSP101 mRNA INDUCTION AND PROTEIN EXPRESSION

Tyrell Carr, Yongzeng Wang, Zhonglian Huang, Joanne M. Yeakley, Jian-Bing Fan, and Steven A. Whitham


Author contributions: T.C. and S.A.W. designed experiments, T.C., Z.H., J.M.Y. J.-B.F. and S.A.W. analyzed microarray data, Y.W. generated inducible promoter constructs and corresponding transgenic plants, T.C. performed majority of experiments and wrote the paper, T.C., Y.W., Z.H. and S.A.W. are from the Department of Plant Pathology at Iowa State University and J.M.W. and J.-B.F. are from Illumina, Incorporated, San Diego, CA.

Abstract

Heat shock protein 101 (HSP101) has been implicated in tobamovirus infections by virtue of its ability to enhance translation of mRNAs possessing the 5′ Ω - leader of Tobacco mosaic virus (TMV). Enhanced translation is mediated by HSP101 binding to a CAA-repeat motif in TMV Ω leader. CAA repeat sequences are present in the 5′ leaders of other tobamoviruses including Oilseed rape mosaic virus (ORMV), which infects Arabidopsis thaliana. HSP101 is one of eight HSP100 gene family members encoded by the A. thaliana genome, and of these, HSP101 and HSP98.7 are predicted to encode proteins localized to the cytoplasm where they could potentially interact with TMV RNA. Analysis of the expression of the HSP100s showed that only HSP101 mRNA transcripts were induced significantly by ORMV in A. thaliana. The induction of HSP101 mRNA was also correlated with an increase in its protein levels and was independent of defense-related signaling pathways involving salicylic acid, jasmonic acid, or ethylene. A. thaliana mutants lacking HSP101, HSP98.7, or both supported wild-type levels of ORMV replication and movement. Similar results were
obtained for TMV infection in *Nicotiana benthamiana* plants silenced for *HSP101*, demonstrating that HSP101 is not necessary for efficient tobamovirus infection.

**Introduction**

Increased expression of genes encoding heat shock proteins (HSPs) is a common response of plant and animal hosts to diverse viruses (Aranda et al., 1996; Escaler et al., 2000; Glotzer et al., 2000; Whitham et al., 2003; Aparicio et al., 2005). In plants, this response was initially characterized by the induction of HSP70 at sites of active virus replication (Aranda et al., 1996). In *A. thaliana*, several positive-strand RNA viruses elicit the accumulation of mRNA transcripts of *HSP17.4, HSP17.6A, HSP23.6, HSP70*, and *HSP83* (Whitham et al., 2003). In addition, *A. thaliana HSP101* appeared to be induced effectively by the tobamoviruses ORMV and *Turnip vein clearing virus* (TVCV).

The increased expression of *HSP101* mRNA in response to tobamovirus infection in *A. thaliana* is interesting in the light of studies that show HSP101 orthologs from other plants bind to a CAA-rich sequence in the 5′ leader of the TMV strain U1 genomic RNA (Tanguay and Gallie, 1996). The 5′ leader of TMV is referred to as the Omega (Ω) leader, and its interaction with HSP101 enhances translation in vitro (Wells et al., 1998; Gallie, 2002). In particular, tobacco HSP101 was shown to enhance the translation of mRNA transcripts fused to the TMV Ω leader (Wells et al., 1998) and an endogenous plant mRNA also containing CAA repeats (Ling et al., 2000). These observations suggest that increased expression of *HSP101* could potentially benefit tobamovirus infection.

In *A. thaliana*, the *HSP100* gene family consists of at least eight genes including *HSP101* (Agarwal et al., 2001), which is more closely related to its orthologs from tobacco
(NtHSP101; AF083343), wheat (TaHSP101; AF083344), and yeast (ScHSP104; M67479) than to its paralogs in A. thaliana (Carr and Whitham, unpublished). Among the A. thaliana HSP100 gene family, only HSP101 and HSP98.7 encode for cytoplasmic proteins (Agarwal et al., 2001) making them the most likely candidates for interacting with viral RNAs or proteins. A key role for HSP101 in heat tolerance in A. thaliana seedlings has been established through analysis of loss-of-function mutants (Hong and Vierling, 2001). The function of HSP101 in thermotolerance is probably mediated by its chaperone activity. As for HSP98.7, there is currently little information about its function in A. thaliana. Other HSP100s that are targeted to the chloroplast have been reported to be necessary for chloroplast functions (Constan et al., 2004; Park and Rodermel, 2004). Thus, HSP100 family members have various functions in host growth and development under normal and stress conditions. However, their roles in plant-pathogen interactions have not been investigated in detail.

Of particular interest to us was the role of HSP101 in successful tobamovirus infections. To address this topic, we used the model plant systems A. thaliana and N. benthamiana to investigate HSP101 expression and its function in tobamovirus infections.

Materials and Methods

Preparation of virus inoculum. ORMV inoculum was prepared from sap isolated from virus-infected Nicotiana tabacum cv. SR1 (nn genotype) by grinding leaves in 20 mM phosphate buffer (pH 7.2) or from purified virions (Chapman, 1998). Sap inoculum of TMV strain U1 tagged with GFP (TMV-GFP) was prepared from N. benthamiana leaves that
became infected after infiltration with *Agrobacterium tumefaciens* strain GV2260 harboring a full length infectious cDNA clone (Jin et al., 2002).

**Growth of Arabidopsis thaliana HSP101 and HSP98.7 mutants and ORMV inoculation.** Mutants of *A. thaliana* HSP101 (hot1-3), HSP98.7 (hsp98.7), and the hot1-3/hsp98.7 double mutant were kindly provided by Dr. E. Vierling (University of Arizona). The hot1-3 mutant was derived from the Columbia (Col-6) ecotype, and the hsp98.7 mutant was derived from the Wassilewskija (Ws-2) ecotype (Hong and Vierling, 2001). Wild-type and mutant plants were grown with a 14 h photoperiod at 22°C until 21 or 28 days of age. Four leaves on each of three plants were labeled with a Sharpie™, dusted with carborundum, and then rub inoculated with 10 μL of ORMV virions that were diluted to 0.1 μg/μl in 20 mM phosphate buffer (pH 7.2). Control plants were mock inoculated with the phosphate buffer alone. In all experiments, inoculated or systemic leaves were immediately frozen in liquid nitrogen and stored at –80°C. In each experiment, viral disease symptoms were monitored in a parallel set of plants up to 14 days after inoculation (DAI).

**RNA gel blot analysis of ORMV viral RNA accumulation.** Plant RNA was isolated using a modified TRIZOL method (38% saturated phenol, pH 4.3, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% glycerol) (Chomczynski and Sacchi, 1987; Huang et al., 2005). Total RNA (5 μg) was treated with glyoxal, electrophoresed in 1% agarose gels, and then transferred to nylon membranes (Zeta-probe GT, Bio-Rad, Hercules, CA, USA). The blots were hybridized with an ORMV coat protein (CP) gene-specific probe, stripped, and hybridized with an 18S rRNA probe. The ORMV CP probe was amplified using the primers ORMVCPF 5’-TCACCACATGGTTTACAACATCAC-
GAGCTCG-3′ and ORMVCP 5′-CAGCTCAGACTATGTAGCTGGCGCAGTAGCC-3′. The 18S rRNA probe was amplified from *A. thaliana* DNA using the primers 18SF 5′-GACAGACTGAGAGCTCTTTCTTGA-3′ and 18SR 5′-ACGTAAGCTTAGTACAGGCTGAG-3′. The PCR products were labeled with $\alpha^{32}$P-dCTP using the Prime-a-Gene labeling system (Promega, Madison, WI, USA). Northern blot hybridizations were performed in Rapid-Hyb buffer according to the manufacturer’s protocol (Amersham, Piscataway, NJ, USA). Radioactive signals were detected by phosphorimager, and then quantified using Image Quant version 5.2 (Molecular Dynamics, Sunnyvale, CA, USA).

**Gene expression measurements by the DNA-mediated annealing, selection, extension, and ligation (DASL™) assay and fiber optic bead array matrices.** Gene expression analysis of the *HSP100* gene family members in *A. thaliana* wild type Col-0 and defense-related signaling mutants was performed using microarray data generated in our previous studies (Huang et al., 2005). The normalized expression data were reported as “average signal” values. Statistical significance of the values were analyzed using a linear mixed effects statistical model (Huang et al., 2005).

**Inducible expression of ORMV CP and analysis of HSP101 gene expression by QRT-PCR.** The estradiol-inducible pER8 binary vector (Zuo et al., 2000) was modified by introducing the Ω leader sequence of ORMV and a HA tag sequence into the multiple cloning site. The three oligomers used for the modification were: 5′-ATCACTCGAGGTTTATTTTTGTGCAACAAACAAACAAATTACAAATAACAACA AAACAAATACAAACAAACAAACAC-3′ (*XhoI* underlined); 5′-TGGCGCGGCGGGCCCA-GCGCTGTAAATTAGTTGTGGTTTGTATTGTTTTGTTG-3′ (*AscI*, *ApaI*, *Eco47*).
III, and PacI underlined); and 5'-GGACTAGTTAGGCTAGCGTAATCCGGAAC-
ATCGTATGGGTACATTGGCGCGCCGGCCC-3' (SpeI, AseI, and ApaI underlined). The
PCR products were digested with XhoI and SpeI and cloned into pER8 to replace original
multiple cloning sites. The final clone was designated pXVEΩHA (Fig. 2A). ORMV CP and
GFP::GUS genes were cloned into pXVEΩHA at the PacI and AseI sites, and transgenic
Col-0 plants were made by floral-dip transformation with A. tumefaciens strain GV3101
(Clough and Bent, 1998). Estradiol induction conditions were optimized using a homozygous
GFP::GUS line. Homozygous seeds were germinated on 1/2X Murashige-Skoog (MS)
medium, and eight-day old seedlings were transferred to 1/2X MS medium containing 20 μM
17-β-estradiol (Sigma, St. Louis, MO, USA) plus 0.1% DMSO or to 1/2X MS medium
containing only 0.1% DMSO. At 32 hours after transfer, seedlings were snap-frozen in liquid
nitrogen, and total RNA was extracted from the seedlings using RNAqueous (Ambion,
Austin, TX, USA). Accumulation of HSP101 mRNA and 18S rRNA transcripts was
measured by quantitative reverse transcriptase-PCR (QRT-PCR) as described by (Cooper,
2001; Whitham et al., 2003) using primers HSP101F 5′-AGGGAAGCTTGATCCTGTGA-3′
and HSP101R 5′-CCTGGCTCTCCAATAAGCAC-3′; and 18SF 5′-GACAGACTGAGAGC-
TCTTTCTTGTA-3′, and 18SR 5′-ACG TAGCTAGTTAGCAGGCTGAG-3′, respectively.

**Virus-induced gene silencing (VIGS) of HSP101 in N. benthamiana.** VIGS was performed
using a Tobacco rattle virus (TRV) vector (Dinesh-Kumar et al., 2003) containing a 406 bp
fragment of the N. benthamiana HSP101 gene. This HSP101 fragment was amplified with
the following primers: HSP101F 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCG-
TACCTTCTCAACACCGG-3′ and HSP101R 5′-GGGGACCACTTTGTACAAGAA-
AGCTGGGTGCTAAATGGCTGTCACCACG-3’. The resulting PCR fragments were introduced into TRV RNA2 by Gateway® recombination (Invitrogen, Carlsbad, CA, USA) to make the TRV-HSP101 clone. The TRV RNA2 vector and TRV-HSP101 were each transformed into *A. tumefaciens* strain GV2260 for co-infiltration into the leaves of 21-day old *N. benthamiana* plants together with GV2260 containing TRV RNA1 (Dinesh-Kumar et al. 2003). The age of *N. benthamiana* plants and the positions of leaves suitable for efficient VIGS with the TRV system was determined using a TRV vector carrying a segment of the *phytoene desaturase* (*pds*) gene that causes photobleaching as a visible marker (Liu et al., 2002). Silencing of *HSP101* was verified at 14 days post infiltration (DPI) by removing the two leaves directly above those that were infiltrated and subjecting them to heat shock at 42 °C for 2 hours. Immunoblot analysis was performed as described below to demonstrate that HSP101 did not accumulate in selected tissues of silenced plants.

The *HSP101*-silenced and control *N. benthamiana* plants were rub-inoculated with TMV-GFP sap at 14 DPI onto two systemic leaves (opposite of each other) that were directly above the infiltrated leaves. Accumulation and spread of TMV-GFP was monitored daily by visualizing GFP fluorescence under long-wave UV light. Leaves were sampled at 3, 7 and 10 DAI for virus accumulation assays.

**Isolation of total protein from plant tissues.** Total soluble proteins were extracted with 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 HSP101 in mock-inoculated and ORMV-infected leaves, 30
μg of total protein were precipitated with 20% trichloracetic acid and resuspended in sample loading buffer (0.5 M Tris-HCl (pH 6.8), 50% glycerol, 10% SDS, 0.5% bromophenol blue, and 5% β-mercaptoethanol). For detection of viral CP, 10 μg of total protein were used without precipitation.

**Immunoblot analysis.** Protein extracts were separated by polyacrylamide gel electrophoresis (PAGE) using 8% 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 polyvinylidene difluoride (PVDF) 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, the membranes were incubated in blocking buffer (5% non-fat milk in 20 mM Tris base, pH 7.6, 137 mM sodium chloride) for 1 hour, then incubated for 3 hours at room temperature with antisera of *A. thaliana* HSP101 at 1:1000 (Hong and Vierling, 2000), wheat HSP101 at 1:2000 (Wells et al., 1998), ORMV CP at 1:4000 (generated and characterized in our laboratory), or TMV CP at 1:1000 dilution (ATCC, PVAS-961, Manassas, VA, USA), respectively in blocking buffer with 1% Tween-20. Next, the membranes were incubated with donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:20 000 dilution; Amersham, Piscataway, NJ, USA) 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.
Results

Expression of *A. thaliana HSP100* gene family members in response to ORMV. Previous results suggested that *HSP101* mRNA is induced during tobamovirus infection in *A. thaliana* (Whitham et al., 2003). Because *HSP101* is one of eight members of a multigene family in *A. thaliana*, it was necessary to further examine the specificity of its induction in response to viral infection. To accomplish this objective, we profiled the expression of the *A. thaliana HSP100* family in response to ORMV infections by analyzing gene expression data that was previously generated by Huang et al. (2005). In brief, fully expanded rosette leaves of Col-0 plants had been inoculated with ORMV or mock treated with phosphate buffer alone in three independent biological replications. Total RNA was extracted from inoculated leaves that were harvested at 2 and 5 DAI, and infection was confirmed by detection of ORMV viral RNAs in inoculated leaves using RNA gel blot and qRT-PCR analyses. Subsequently, total RNA samples were labeled by the DASL™ assay (Fan et al., 2004; Shou et al., 2004), and hybridized to custom fiber-optic array matrices representing 388 unique *A. thaliana* genes (Huang et al., 2005).

The average signals of seven of the eight *HSP100* genes in the ORMV-infected samples were divided by their average signals in the mock-inoculated samples to produce an average signal ratio (fold change) for each gene (Fig. 1A). *HSP92.7 (At4g14670)* was excluded from Fig. 1A, because its signal was not significantly different from the background in all samples indicating it was not expressed. Among the *HSP100* genes that produced a significant signal, *HSP101 (At1g74310)* mRNA was induced in infected rosette leaves at 5 DAI (*p < 0.05*). In contrast, the abundance of the mRNA transcripts of the other *HSP100* genes was similar in virus-infected and mock-inoculated samples, because their
average signal ratios were approximately 1. These results demonstrated that of the HSP100 family members HSP101 mRNA was significantly induced by ORMV infection.

Accumulation of A. thaliana HSP101 protein was induced by ORMV infection. Because HSP101 mRNA was induced by ORMV infection, we then examined whether this correlated with an increase in the accumulation of HSP101 protein in inoculated rosette leaves of A. thaliana Col-0 plants collected at 1, 2, 3, and 4 DAI (Fig. 1B). In each lane, 30 μg of total protein were loaded for separation by SDS-PAGE and detection of HSP101 by immunoblot assay. HSP101 accumulation was increased at least from 1 to 3 DAI in ORMV-infected leaves, whereas, it remained undetectable in mock-inoculated leaves (Fig. 1B). Increased accumulation of HSP101 was reproducible in numerous replications of this experiment and was also found to occur in response to another tobamovirus, TVCV. The abundance of HSP101 protein was correlated with the accumulation of its mRNA transcripts over the 4-day time course (Fig. 1C) indicating that HSP101 expression in response to ORMV infection is transcriptionally regulated.

ORMV CP induces HSP101 mRNA. TMV CP was previously implicated as an elicitor of virus-induced HSP gene expression in tobacco (Jockusch et al., 2001), however, this function for TMV CP was not uncoupled from other TMV proteins or viral replication. In our pathosystem, we tested if ORMV CP alone was sufficient to induce A. thaliana HSP101 mRNA expression. The ORMV CP was expressed under control of an estradiol-inducible promoter in transgenic Col-0 plants (XVE; (Zuo et al., 2000). The binary plasmid carrying the promoter was modified for efficient translation and detection of expressed target proteins (Fig. 2A). Homozygous transgenic A. thaliana lines were generated that contained either the
empty pXVEΩHA vector (XVE) that expresses an HA tag, or versions that express the GFP-GUS fusion gene (GG) or the ORMV CP in the presence of estradiol. Analysis of HSP101 mRNA expression by QRT-PCR in seedlings harvested at 32 h after estradiol induction showed that HSP101 mRNA was significantly induced 5-6 fold ($p < 0.05$) in ORMV CP transgenic plants compared to non-induced or vector control plants (Fig. 2B). The accumulation of HSP101 mRNA accumulation in the GG line was not significantly different from the Col-0 and XVE plants at the $p < 0.05$ threshold.

**ORMV-induced expression of HSP101 mRNA is not dependent on salicylic acid.** Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are key signaling molecules that regulate defense responses to pathogens in *A. thaliana* and other plants (Kunkel and Brooks, 2002). SA is required for the increased expression of a suite of defense-related genes that are upregulated during ORMV infection in susceptible Col-0 plants (Huang et al., 2005). Other studies suggest that there are links between these signaling pathways and expression of heat shock genes in biotic and abiotic stress responses (Cronje and Bornman, 1999; Clarke et al., 2004; Larkindale et al., 2005). Therefore, we tested whether defense-related signaling pathways mediate the induction of HSP101 mRNA transcripts in response to ORMV infection. Expression of HSP101 was analyzed in our previous data set that included wild-type Col-0 plants as well as mutants defective in SA (*pad4-1, eds5-1, npr1-1*, and *NahG*), JA (*jar1-1*), and/or ET (*ein2-1*) signaling pathways that had been mock inoculated or infected with ORMV (Huang et al., 2005). As shown in Fig. 3, accumulation of HSP101 mRNA transcripts was significantly greater ($p < 0.05$) in response to ORMV infection in all wild type and mutant genotypes tested when compared to mock-inoculated plants. These results suggest that induction of HSP101 gene expression is independent of SA-, JA-, and/or ET-
mediated signaling pathways that regulate expression of defense-related genes in compatible and incompatible interactions.

**Analysis of ORMV infection in HSP101 and HSP98.7 mutants.** Based on published observations that HSP101 enhances the translation of model mRNAs containing the TMV leader, HSP101 is a chaperone that could assist in proper folding of viral proteins, and our findings that *HSP101* mRNA and protein expression can be induced by tobamovirus infection, we next investigated whether HSP101 is necessary for tobamovirus infection in *A. thaliana*. TMV strain U1 does not infect the Col-0 ecotype of *A. thaliana* efficiently in our hands. Therefore, we elected to use ORMV in HSP101 functional studies. The leader sequence of TMV strain U1 is not identical to those of ORMV or other tobamoviruses which efficiently infect Col-0 plants, but importantly, the leader sequences are all rich in poly(CAA) sequence motifs (Fig. 4). The poly(CAA) sequence that has been implicated in HSP101 binding in the TMV leaders is identified in Fig. 4 (Tanguay and Gallie, 1996), and ORMV, TVCV, and TMV strain Cg, also have an additional stretch of poly(CAA) sequences toward the 3′ end of their leader sequences. Interestingly, the leader of a plant mRNA that is rich in poly(CAA) sequence motifs can also enhance translation of model mRNA substrates in a HSP101-dependent fashion (Ling et al., 2000).

To investigate the question of whether HSP101 has a significant role in ORMV infection in *A. thaliana*, null mutants of *HSP101* (*hot1-3*) and *HSP98.7* (*hsp98.7*) and a *hot1-3/hsp98.7* double mutant were analyzed for accumulation and systemic movement of ORMV. These two HSP100s were selected, because they are predicted to be cytoplasmic and their expression is detectable in virus-infected plants. *hot1-3* and *hsp98.7* are null mutants as a result of T-DNA insertions (Hong and Vierling, 2001). We expected that if HSP101 and/or
HSP98.7 facilitate ORMV infection, then virus accumulation would be reduced in the mutants compared to wild-type plants.

Fully expanded rosette leaves of mutant and wild-type plants were inoculated with ORMV and analyzed over a 4-day time-course in two independent replications. The accumulation of ORMV genomic and subgenomic RNAs was not reduced in hot1-3, hsp98.7, or hot1-3/hsp98.7 double mutants when compared to wild-types Col-0 and Ws-2 at 2 and 4 DAI (Fig. 5A and 5B). Similar results were observed for the accumulation of the ORMV CP in the mutants (data not shown). The apparent increase in ORMV genomic RNA in hot1-3 leaves at 2 DAI (Fig. 5A) was not reproducible in other replications of this experiment. In summary, the accumulation of ORMV RNA species and CP were similar to wild-type in this panel of mutants suggesting that it was independent of HSP101 and HSP98.7.

We reasoned that the effects of the HSP101 and/or HSP98.7 mutants might not be detectable in inoculated leaves, but perhaps could result in delayed systemic movement. Therefore, we investigated whether accumulation of ORMV in systemic tissues was affected in the hot1-3, and hot1-3/hsp98.7 mutants. Inoculated rosette leaves and systemic floral tissues were collected at 8 DAI. Analysis of ORMV CP accumulation in collected tissues showed that levels of ORMV CP were similar in the mutants and wild-type controls in inoculated and systemic tissues (Fig. 5C). These results indicated that the loss of cytoplasmic HSP100 proteins (HSP101 and HSP98.7) did not inhibit the accumulation of ORMV in local and systemic tissues in A. thaliana under these experimental conditions.

Silencing of HSP101 in N. benthamiana does not inhibit TMV replication and movement. The unexpected results from the A. thaliana–ORMV pathosystem could be due
to sequence differences in the leaders of TMV-U1 and ORMV and/or the necessity of *A. thaliana* HSP101 to interact with the ORMV leader. Because TMV strain U1 did not infect the Col-0 ecotype of *A. thaliana* in our hands, we alternatively used the *N. benthamiana*-TMV pathosystem to further investigate if HSP101 plays a role in promoting virus infection. *N. benthamiana* was selected because it is a good host for TMV infection, the HSP101 protein is nearly identical to its tobacco ortholog, and virus-induced gene silencing (VIGS) with a *Tobacco rattle virus* (TRV) vector is very efficient (Dinesh-Kumar et al. 2003).

In order to silence *N. benthamiana* HSP101, a 406 bp fragment of the gene was amplified and cloned into the TRV RNA2 vector to make the TRV-HSP101 construct. Sequence analysis demonstrated that this fragment of *N. benthamiana* HSP101 was 99% identical to *N. tabacum* HSP101 (data not shown). VIGS of *N. benthamiana* HSP101 was induced by co-infiltrating *A. tumefaciens* strains harboring TRV RNA1 and TRV-HSP101, and the efficiency of HSP101 silencing was tested at 14 DP1. The leaves immediately above those that were infiltrated were removed and subject to heat shock at 42 °C for 2 hours followed by 4 and 12 hr recovery periods at room temperature. HSP101 protein was not found in the heat-shocked leaves of silenced plants, but it was abundant in corresponding leaves of non-silenced control plants, demonstrating that expression of HSP101 was effectively silenced (Fig. 6A). In three independent repetitions of this experiment each using 3-5 plants, the corresponding leaves of HSP101-silenced (TRV-HSP101) and non-silenced control plants (TRV) were inoculated with TMV-GFP. GFP fluorescence was observed daily under illumination with ultra-violet light in both silenced and non-silenced control leaves. Leaves were photographed at 7 DAI to demonstrate that the numbers and sizes of TMV-GFP
infection foci were similar in silenced and non-silenced plants (Fig. 6B). Furthermore, the accumulation of TMV CP was similar in the inoculated leaves of silenced and non-silenced controls at 3 and 7 DAI (Fig. 6C and data not shown). Virus disease symptoms characterized by wilting of the systemic leaves developed simultaneously in silenced and non-silenced plants infected with TMV-GFP (Fig. 6D). This result indicated that there was no delay in TMV-GFP systemic movement or reduction in pathogenicity in HSP101-silenced versus non-silenced plants. In summary, TMV-GFP accumulation in HSP101-silenced N. benthamiana plants coincided with the results obtained from the A. thaliana-ORMV pathosystem and further indicated that HSP101 is not essential in mediating tobamovirus infections under our experimental conditions.

Discussion

A variety of heat shock genes are induced in response to plant virus infections and many have been postulated to have roles in viral infections. Here the expression and functions of the members of the HSP100 gene family in A. thaliana were investigated in response to viral infection. The data demonstrated that the accumulation of only HSP101 mRNA and protein was induced in response to ORMV infection. Some of the other genes are expressed to significant levels in A. thaliana leaves, but their mRNA transcript levels are not altered significantly in response to ORMV. Increased expression of HSP101 was not dependent on replicating virus, because the ORMV CP alone was sufficient to induce its expression. It is possible that the ability of ORMV CP to induce HSP101 is parallel to the role of the adenovirus Gam1 protein in inducing HSPs and as such is a specific elicitor of HSP expression (Glotzer et al., 2000). A second possibility is that accumulation of ORMV CP
triggers a general response to protein accumulation in the cytoplasm as has been demonstrated recently for other viral proteins (Aparicio et al., 2005). This possibility would be most consistent with conclusions from previous studies that suggested mutants of TMV CP that readily aggregate are effective inducers of HSP expression (Jockusch et al., 2001).

Induction of \textit{HSP101} mRNA in response to ORMV infection was not suppressed in mutants defective in the SA-, JA-, or ET-dependent signaling pathways tested. Despite a number of interactions between signaling pathways controlling the expression of defense-related and heat shock genes, this observation suggests that the induction of \textit{HSP101} in response to ORMV occurs by an as yet uncharacterized mechanism that is distinct from well-characterized defense and heat shock responses (Aranda et al., 1999; Whitham et al., 2003). It has been shown that \textit{HSP101} and \textit{HSP17.6} are not dependent on SA signaling to activate thermotolerance in \textit{A. thaliana} (Clarke et al., 2004). However, some HSPs such as HSP17.6 can be induced by SA treatment, whereas HSP101 cannot. Clarke et al. (2004) also reported that expression of \textit{PR-1} was induced in Col-0 plants by heat-shock treatment. Our results demonstrate that expression of HSP101 in compatible interactions is not coupled to expression of pathogenesis-related (\textit{PR}) genes, such \textit{PR-1}. Expression of \textit{PR-1} is strongly dependent on SA-mediated signaling in response to pathogen attack, and its induction in compatible host-virus interactions is completely dependent on SA (Huang et al., 2005). Expression of \textit{HSP101} was induced in ORMV-infected \textit{NahG} plants, which demonstrated that SA was not required. Thus, \textit{PR-1} and \textit{HSP101} are regulated by independent mechanisms during compatible host-virus interactions.
A particularly intriguing possibility is that viruses might exploit the induction of HSP genes to enhance their infections as has been shown in a few specific cases (Sullivan and Pipas, 2001). For example, Gam1 an avian adenovirus-encoded protein, induces the expression of HSPs including HSP70 and HSP40 (Glotzer et al., 2000). Gam1 deletion mutants do not induce these HSPs and are defective in replication, but heat shock complements the Gam1 deficiency by providing HSP70 and HSP40 needed for replication. A yeast HSP40 chaperone, a DnaJ homolog, was also shown to be functionally important in the replication of *Brome mosaic virus* in yeast cells (Tomita et al., 2003). Together, these studies suggested that virus-induced HSPs can have beneficial effects on virus infection. Further support of this theory derives from viruses that carry their own homologs of cellular heat shock proteins. For example, plant clostrviruses, encode an hsp70h gene that has roles in virus assembly and movement (Alzhanova et al., 2001; Prokhnevsky et al., 2002).

Here, we hypothesized that induction of HSP101 could be beneficial to tobamovirus infection in *A. thaliana*. This hypothesis was based on the increased expression of mRNA and protein during ORMV infection, and the multifunctional nature of HSP101, which could conceivably benefit virus accumulation through its role as a chaperone (Hong and Vierling, 2001; Hong et al., 2003) or by participating in translation (Tanguay and Gallie, 1996; Wells et al., 1998; Gallie, 2002). HSP101 from tobacco and wheat function as RNA binding proteins by interacting with CAA rich sequences in the Ω leader of TMV, the type member of the *Tobamovirus* genus (Tanguay and Gallie, 1996; Wells et al., 1998; Gallie, 2002). This RNA binding activity has been shown to enhance translation of reporter genes fused to the Ω leader through an eIF4G dependent mechanism (Wells et al., 1998; Gallie, 2002).
The requirement for HSP101 in tobamovirus accumulation and movement was investigated using null mutations in \textit{HSP101} (\textit{hot1-3}) and \textit{HSP98.7} (\textit{hsp98.7}). The accumulation and movement of ORMV in the \textit{hot1-3, hsp98.7, and hot1-3/hsp98.7} double mutants demonstrated that cytoplasmic \textit{HSP100} homologs were not needed to establish ORMV infection in \textit{A. thaliana}. Because there are differences in HSP101 from tobacco and \textit{A. thaliana} as well as in the leader sequence of TMV-U1 and ORMV, the conclusions derived from ORMV may not necessarily apply to TMV. To investigate this idea, we silenced \textit{HSP101} in \textit{N. benthamiana} and challenged plants with TMV-GFP. TMV-GFP accumulation and pathogenesis were similar in the silenced and non-silenced plants, indicating that HSP101 did not affect local or systemic accumulation of the virus. Collectively our results indicate that tobamovirus infection is independent of HSP101 functions under the conditions that we tested.

\textbf{Acknowledgments}

We thank E. Vierling (University of Arizona) for the \textit{HSP101} and \textit{HSP98.7} mutants and \textit{A. thaliana} HSP101 antisera, D. Gallie (University of California – Riverside) for wheat HSP101 antisera, S.P. Dinesh-Kumar (Yale University) for the TRV VIGS vector, and B. Baker (University of California, Berkeley) for the TMV-GFP infectious clone. We thank T. Crosbie and Monsanto Company for providing fellowship support to T.C. during the course of this work. This work was supported by the USDA-NRI (grant no. 02-35319-12566 to S.A.W.), the Iowa State University Plant Sciences Institute, and by the Hatch Act and State of Iowa Funds.
References


Tanguay RL, Gallie DR (1996) Isolation and characterization of the 102-kilodalton RNA-binding protein that binds to the 5' and 3' translational enhancers of tobacco mosaic virus RNA. J Biol Chem 271: 14316-14322


Fig. 1. Expression of *A. thaliana* HSP101 mRNA and protein in response to ORMV. (A) Expression of HSP101 mRNA was induced in response to ORMV treatment. HSP101 (*At1g74310*), HSP98.7 (*At2g25140*), HSP105.7 (*At3g48870*), HSP108.9 (*At5g15450*), HSP103.4 (*At5g50920*), HSP103 (*At5g51070*), HSP108.7 (*At5g57710*). The average signal ratio was determined by dividing the average signal of the virus-infected sample by the mock-inoculated sample. A ratio of approximately 1 indicates no change in expression. Average signals were taken from Huang et al. (2005). (B) Induction of HSP101 protein by
ORMV. In each lane, 30 μg of total protein were loaded and separated by SDS-PAGE. HSP101 was detected by immunoblot assay in ORMV-infected (I) and mock-inoculated (M) rosette leaves at 1, 2, 3, and 4 days after inoculation (DAI). As a positive control, HSP101 was detected in protein extracts from leaves that were heat shocked (HS) for 2 hrs at 37ºC. (C) *HSP101* mRNA transcript levels derived from Whitham et al. (2003) were graphed for ORMV-infected (I) and mock-inoculated (M) rosette leaves at 1, 2, and 4 DAI.
Fig. 2. ORMV CP is sufficient to induce the expression of \textit{HSP101} mRNA. (A) A modified pXVE estradiol-inducible promoter system was used to express viral and reporter proteins (Zuo et al., 2000). (B) RNA was extracted from samples collected at 32 hrs after treatment with 20 \( \mu \text{M} \) 17-\( \beta \)-estradiol. QRT-PCR was used to assay the levels of \textit{HSP101} mRNA in Col-0 wild-type and transgenic plants expressing genes under control of the estradiol-inducible promoter. \textit{HSP101} mRNA levels were normalized to 18S rRNA and then plotted as the ratio of estradiol-induced/non-induced. Vertical lines represent the standard deviation of two biological replicates. Col-0, Columbia-0 ecotype; wild-type; XVE, vector used; CP, coat protein; GG, GFP::GUS.
Fig. 3. Expression of *A. thaliana* *HSP101* after mock inoculation or ORMV infection in defense signaling mutants. In this experiment, Col-0 (wild type Columbia-0), *ein2* (*ethylene insensitive 2*), *jar1* (*jasmonic acid resistant 1*), *eds5* (*enhanced disease susceptibility 5*), *npr1* (*no expression of pathogenesis related genes 1*), *NahG* (*salicylic acid hydroxylase*), and *pad4* (*phytoalexin deficient 4*) plants were inoculated with ORMV or mock-inoculated. Inoculated leaf tissue was harvested at 5 days after inoculation (DAI) for RNA extraction and gene expression assay by fiber optic bead array. The average signal of *HSP101* in response to mock or ORMV treatment at 5 DAI were taken from Huang et al. (2005) and graphed here. Vertical bars represent the standard deviation of three biological replicates.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TATTTTACAACAAATTAC</td>
<td>CAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTTATTGTGCAACAAACAACAACAAGAACTTTCAATAACAACAAAAAATAAACTAACAACACAACAACAACAACAACAACAACAACAACATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTTATTGTGCAACAAACAACAACAAGAACTTTCAATAACAACAAAAAATAAACTAACAACACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTTATTGTGCAACAAACAACAACAAGAACTTTCAATAACAACAAAAAATAAACTAACAACACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTTATTGTGCAACAAACAACAACAAGAACTTTCAATAACAACAAAAAATAAACTAACAACACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Alignment of the 5′ Ω - leader of TMV-U1 (M24955, TMV-Cg (D38444), ORMV (NC_004422) and TVCV (NC_001873). Sequences were aligned using the Clustal W program (Thompson et al., 1997). The line segment indicates the position of poly(CAA) sequences in TMV-U1 that bind to HSP101 (Tanguay and Gallie, 1996).
Fig. 5.
Fig. 5. ORMV accumulation in inoculated leaves and systemic tissues of *hot1-3* and *hsp98.7* mutants and wild-type *Col-0* and *Ws-2* plants. (A) and (B) ORMV genomic and coat protein (CP) subgenomic RNAs were detected at 2 (A) and 4 (B) days after inoculation (DAI) by RNA gel blot analysis with a CP probe. Relative accumulation of ORMV RNA species was normalized to *18S* rRNA and graphed. Vertical lines represent the standard deviation of two biological replicates. (C) Systemic accumulation of ORMV CP in *Col-0*, *hot1-3*, and *hsp98.7* plants at 8 DAI. In each lane, 10 μg of total protein were separated by SDS-PAGE and immunoblot assay was used to detect ORMV CP in inoculated rosette leaves and non-inoculated floral tissues of *Col-0*, *Ws-2*, *hot1-3*, and *hot1-3/hsp98.7* plants. For the loading controls, membranes were first stained with Ponceau S to detect total proteins, and the region corresponding in size to rubisco is shown.
Fig. 6. TMV-GFP accumulation and pathogenesis in *HSP101*-silenced *N. benthamiana* plants. A 406 bp fragment of *N. benthamiana HSP101* was cloned into the TRVRNA2 vector to make the TRV-HSP101 construct. (A) Confirmation of silencing of HSP101 expression. *N. benthamiana* leaves were infected with TRVRNA1/TRVRNA2 (TRV) or TRVRNA1/TRV-HSP101 (TRV-HSP101). At 14 days after infiltration, systemic leaves were heat shocked at 42 °C for 2 hrs and allowed to recover for 4 hrs or 12 hrs. Total protein was extracted, quantified, and 10 μg of were loaded and separated by SDS-PAGE, and immunoblot assay was used to determine levels of HSP101 expression in non-silenced (NS) leaves and silenced (S) leaves. For the loading controls, membranes were first stained with Ponceau S to detect total proteins, and the region corresponding in size to rubisco is shown.
(B) TMV-GFP infection foci at 7 DAI in *HSP101*-silenced and control plants. (C) Accumulation of TMV CP at 7 DAI in inoculated leaves in silenced and non-silenced plants. In each lane, 10 μg of total protein were loaded and separated by SDS-PAGE, and TMV CP was detected by immunoblot assay using a TMV CP antiserum (ATCC, PVAS-961). The loading controls are as described for panel (A) above. (D) Symptoms of *HSP101*-silenced and control plants at 7 days after TMV-GFP inoculation.
CHAPTER 3. DIFFERENTIAL EXPRESSION OF A. THALIANA HSP70 GENES AND SELECT HEAT STRESS TRANSCRIPTION FACTOR GENES IN RESPONSE TO VIRUS INFECTIONS

Tyrell Carr, Yongzeng Wang and Steven A. Whitham

A manuscript in preparation for submission to Virology Journal

Author contributions: T.C. and S.A.W. designed experiments, T.C. and S.A.W. analyzed microarray data, Y.W. generated inducible promoter constructs and corresponding transgenic plants, T.C. performed majority of experiments and wrote the paper and T.C., Y.W. and S.A.W. are from the Department of Plant Pathology at Iowa State University

Abstract

Members of the major gene families of heat shock proteins (HSPs) including HSP70 are induced in plants in response to viral infections. The HSP70 family consists of at least 14 members in the model dicot plant, Arabidopsis thaliana. In order to obtain a comprehensive view of how each HSP70 family member and select heat stress transcription factors (HSFs) are affected by viral infection, a microarray-based assay was used to investigate the expression of each in parallel. Wild type A. thaliana plants and a set of mutants with defects in defense-related signaling were mock-infected or infected with either Cucumber mosaic virus (CMV) or Oilseed rape mosaic virus (ORMV) and expression of HSP70 genes and HSFs were assayed. These analyses demonstrated that specific HSP70 genes namely, HSP70, HSC70-2, BiP2, and BiP3 were significantly up-regulated during viral infections, and their induction did not require the defense-related signaling pathways involving salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). We also found that both CMV and ORMV infections induced HSFA2 mRNA, a heat-inducible HSF. Finally, inducible expression of ORMV coat protein (CP) was shown to increase HSP70 mRNA but not other viral or non-
viral proteins tested. Results presented here suggest that different viruses commonly induce heat- or ER-stress related genes.

**Introduction**

In plants and other organisms, HSP70 family members play important roles in protein folding (Forreiter et al., 1997; Lee and Vierling, 2000), protein complex assembly and disassembly (Liu et al., 2007) and protein trafficking across membranes (Zhang and Glaser, 2002; Vojta et al., 2007). In addition, plant HSP70s may traffic non-cell autonomous proteins through plasmodesmata, because an intercellular class has been discovered (Aoki et al., 2002). A potential role for plant HSP70s in promoting virus infections in susceptible plants has also been implied based on the following reasons. First, in response to different plant viruses like potyviruses and tobamoviruses, *HSP70* mRNAs accumulate to higher levels (Aranda et al., 1996; Escaler et al., 2000; Whitham et al., 2003; Aparicio et al., 2005). Second, the replicase protein, p33 of *Cucumber necrosis tombusvirus* (CNV) was found to interact with yeast HSP70s to presumably enhance replication of a *Tomato bushy stunt tombusvirus* (TBSV) replicon in yeast (Serva and Nagy, 2006). Third, a HSP70 homolog (HSP70h) encoded among members of the Closteroviridae family of plant viruses (Dolja et al., 2006), is required for *Beet yellows closterovirus* (BYV) virion assembly and intercellular movement (Peremyslov et al., 1999; Prokhnevsky et al., 2002). Fourth, several animal viruses require HSP70s to facilitate viral processes in host cells (Mayer, 2005).

*A. thaliana* encodes at least 14 genes that comprise the *HSP70* family (Sung et al., 2001). Five of these genes that are predicted to encode cytoplasmic proteins, *HSP70*, *HSP70B* and the *heat shock cognate 70 (HSC70)* genes *HSC70-1*, *HSC70-2*, and *HSC70-3* were previously examined in response to virus infection by Aparicio et al. (2005). Among
these genes, *HSP70*, *HSC70-1*, *HSC70-2*, and *HSC70-3* were induced by both *Turnip crinkle carmovirus* (TCV) and *Turnip mosaic potyvirus* (TuMV) infections. Although *HSP70B* was not induced by virus infection, it was induced by heat stress along with the other four genes. To further investigate *HSP70* expression, Aparicio et al. (2005) tested the affects of viruses, viral proteins and plant proteins on the expression of β-glucuronidase (GUS) fused to the *A. thaliana* *HSP70* (pHSP70::GUS) in *Nicotiana benthamiana*. All viruses tested including TCV and TuMV in addition to viral proteins and plant proteins were found to induce pHSP70::GUS expression. Based on these findings, it was concluded that plant virus induction of *HSP70s* reflects a general response to viral protein accumulation in the cytosol. However, this conclusion does not rule out a potential role for plant HSP70s in promoting viral infection.

Presently, it is not known how *HSP70* or other *HSP* genes are regulated during viral infections. In eukaryotes, heat stress induced *HSPs* are regulated by a conserved network involving HSFs that bind the *HSP* promoter heat stress element (HSE) sequence 5'-AGAAnnTTCT-3' (Pelham, 1982; von Koskull-Doring et al., 2007). However, HSEs do not appear to be sufficient to mediate heat shock gene expression in response to viral infection. For example, *HSP70* mRNA is strongly induced in pea plants in response to pea seed-borne mosaic potyvirus (PSbMV), but *HSFA* mRNA is not (Aranda et al., 1999). Both of these genes possess HSEs in their promoters, and thus, their differential expression suggests that HSEs are not involved in virus-induced *HSP* gene expression. A similar conclusion was made by Whitham et al. (2003) regarding the expression of heat shock mRNAs in Arabidopsis in response to viral infection.
In this current study, we examined all 14 known members of the *A. thaliana HSP70* gene family in response to CMV or ORMV infection in the wild-type ecotype Columbia-0 (Col-0) and SA, JA, and/or ET defense signaling mutants. Expression of all *HSP70* family members in response to virus infection was of interest because only those predicted to encode cytosolic proteins were previously studied (Aparicio et al., 2005). In *A. thaliana*, HSP70s localize to the cytoplasm, chloroplast, mitochondrion or endoplasmic reticulum (ER). Thus profiling different classes of plant *HSP70s* in particular those encoding non-cytoplasmic proteins was necessary to determine the specificity or generality of their induction by viruses.

Defense signaling mutants were selected to investigate whether *HSP70s* induced by viruses are reflective of a defense-related response requiring SA, JA and/or ET signaling pathways. This question is of interest because a *N. benthamiana* cytosolic HSP70 was reported to be essential for resistance to a bacterial pathogen (Kanzaki et al., 2003). Although we are studying compatible plant-virus interactions that allow systemic virus infections, basal defense pathways are induced in virus-infected plants (Huang et al., 2005). In our study we also profiled two *A. thaliana HSFs* previously studied for their roles in regulating the heat stress-induced pathway to determine if they were virus-inducible. In *A. thaliana*, their are at least 21 *HSFs* (von Koskull-Doring et al., 2007). To gain further insight into *HSP70* induction, an inducible promoter system was used to express viral proteins or non-viral proteins of interest to identify specific viral elicitors.

**Materials and Methods**

**Preparation of virions.** CMV strain Y (referred to here as CMV) and ORMV were propagated in *Nicotiana tabacum* cv. Xanthi nc (*NN* genotype) and *N. tabacum* cv. SR1 (*nn* genotype), respectively. Inoculum was prepared from sap isolated from virus-infected
tobacco or as purified virions as previously described (Chapman, 1998; Roossinck and White, 1998; Huang et al., 2005).

**A. thaliana growth and virus inoculation.** Wild-type and SA (*pad4-1, eds5-1, npr1-1,* and *NahG*), JA (*jar1-1*), and/or ET (*ein2-1*) signaling mutant plants were grown as previously described (Huang et al., 2005). In brief, four leaves of 21 day-old plants were dusted with carborundum, and rub-inoculated with 10 μL of CMV or ORMV virions that were diluted in 20 mM phosphate buffer (pH 7.2). Control plants were mock inoculated with phosphate buffer alone. In all experiments, harvested plant tissues collected at 2 and 5 days after inoculation (DAI) were immediately frozen in liquid nitrogen and stored at –80°C.

**RNA isolation for HSP70 and HSF gene expression studies.** Total RNA was isolated using a modified TRIZOL method (38% saturated phenol (pH 4.3), 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% glycerol) (Chomczynski and Sacchi, 1987; Huang et al., 2005).

**Gene expression measurements by the DNA-mediated annealing, selection, extension, and ligation (DASL™) assay and fiber optic bead array matrices.** Experimental design, infection of wild type *A. thaliana* Col-0 plants and defense-related signaling mutants and statistical analysis of microarray data used for the study HSP70 gene family and select HSFs were described in detail in Huang et al. (2005). In that study only data collected from virus-infected plants 5 DAI are presented.

**Inducible expression of viral genes and analysis of HSP70 expression by QRT-PCR.** A estradiol-inducible pER8 binary vector (Zuo et al., 2000) that was modified by introducing the Ω leader sequence of ORMV and a HA tag sequence into the multiple cloning site (Carr
et al., 2006) was used to express viral and non-viral proteins in this study. Genes encoding CMV movement protein (MP), CMV coat protein (CP), ORMV CP and GFP::GUS (GG) were cloned as previously described (Carr et al. 2006). Transgenic Col-0 plants were made by floral transformation with Agrobacterium tumefaciens (Clough and Bent, 1998). Conditions for plant growth, estradiol induction and RNA extraction are as previously described (Carr et al., 2006). Accumulation of HSP70 and 18S rRNA transcripts was quantified by QRT-PCR (Cooper, 2001; Whitham et al., 2003) using the following primers, HSP70-F 5′-TGGCCCAAAGATTGAAGAAG-3′, HSP70-R 5′-TCACACACCAGTTTCAGAGTGA-3′, 18S-F 5′GACAGACTGAGAGCTCTTTCTTGA-3′, and 18S-R5′ ACGTAGCTAGTTAGCAGGCT-GAG-3′.

**Isolation of total protein from plant tissues and immunoblot analysis.** Total soluble proteins from transgenic plants were extracted with sample loading buffer (0.5 M Tris-HCl (pH 6.8), 50% glycerol, 10% SDS, 0.5% bromophenol blue, and 5% β-mercaptoethanol). For detection of expressed HA, 20 μl of total protein was resolved on a 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 (Bio-Rad, Hercules, CA, USA). Membranes were blocked, washed and incubated as previously described (Carr et al. 2006). HA was detected using mouse monoclonal HA antibody (Sigma, St. Louis, MO, USA) at 1:3000 and rabbit anti-mouse IgG (Sigma, St. Louis, MO, USA) at 1:6000 followed by chemiluminescence according to manufacturer’s instruction.
Results

Expression of *A. thaliana* HSP70 gene family members in response to CMV or ORMV in wild-type Col-0 and SA, JA and/or ET signaling deficient mutants. Previously we reported that *A. thaliana* HSP70 (At3g12580) was induced in response to CMV and ORMV infection (Whitham et al., 2003). To gain a comprehensive understanding of CMV and ORMV induction of HSP70s, all 14 known genes comprising the *A. thaliana* HSP70 family were profiled in the wild-type Col-0 ecotype and SA (pad4-1, eds5-1, npr1-1, and NahG), JA (jar1-1), and/or ET (ein2-1) signaling mutants. Data from wild-type Col-0, revealed that HSP70 (At3g12580) was induced in response to CMV and ORMV (*p = 0.05* and *p < 0.05* respectively) at 5 DAI (Fig. 1). In addition to HSP70, HSC70-2 (At5g02490) and BiP-3 (At1g09080) were also induced (*p < 0.05*) in response to both viruses at 5 DAI (Fig. 1). BiP-2 (At5g42020) was also induced by ORMV (*p = 0.05*) but not by CMV. Both BiP-2 and BiP-3 are HSP70 family members that encode ER luminal binding proteins (BiPs) that respond to ER stress (Gething, 1999; Noh et al., 2003). From our analysis of HSP70 family gene expression profiles, in particular HSP70 mRNA accumulation, its expression in the different signaling mutants infected with CMV and ORMV at 5 DAI (Fig. 2.), correlated with virus-infected Col-0 wild-type plants. Therefore, we conclude that HSP70 family members induced upon virus infection are SA, JA and ET independent. It should be noted that CMV or ORMV infection was not compromised in sampled leaves of defense signaling mutants (Huang et al., 2005).

ORMV CP induces HSP70 mRNA. Tobacco mosaic tobamovirus (TMV) CP was previously implicated as an inducer of HSP70 (Jockusch et al., 2001). However, this function for CP was not uncoupled from the other viral proteins or viral replication. In order to test if
CMV CP or ORMV CP alone was sufficient to induce *HSP70* mRNA. CMV CP, ORMV CP, CMV movement protein (MP) or GFP::GUS were expressed under the control of a modified estradiol-inducible promoter pXVEΩHA vector (Carr et al., 2006) (Fig. 3A) in transgenic *A. thaliana*. For each construct, two independent transgenic lines were used and two replicates of the experiment were performed. Vector alone transgenic plants and non-transgenic Col-0 plants were used as controls. Seedlings were grown in the absence of 17-β-estradiol until 8 days after germination at which time half the plants were transferred to fresh media and the other half to media containing 20 μM 17-β-estradiol. RNA and protein was extracted from seedlings collected at 32 hours after transfer. QRT-PCR was used to assay *HSP70* mRNA expression (Fig. 3B) and protein expression for each transgene was confirmed by immunoblot assay using the HA tag (Fig. 3C). In our hands, *HSP70* mRNA was induced only in response to induced ORMV CP expression; however, it varied among replicates and was not significant when compared to other proteins and the controls. Increased *HSP70* mRNA accumulation in response to ORMV CP might in part be due to ORMV CP high expression.

**Expression profiles of two *A. thaliana* HSFs in response to virus infection.** As previously mentioned, the heat stress response pathway in eukaryotes is regulated by HSFs that bind *HSE* regions in *HSP* genes, but it is not known how these genes are regulated during virus infections. The expression profiles of *A. thaliana HSFA2* (*At2g26150*) and *A. thaliana HSF3* (*HSFA1B*) (*At5g16820*) were monitored in mock-, CMV-, and ORMV-infected Col-0 wild-type plants and signaling defense mutants. Of these, *HSFA2* was induced in response to CMV and ORMV (p = 0.075 and p < 0.05, respectively) when compared to mock-infected samples at 5 DAI and was not dependent on SA, JA and/or ET signaling pathways (Fig. 4A). *HSF3* was not induced in our studies in response to either virus (Fig. 4B).
Discussion

In response to different stimuli such as cold, drought, heat, salt, or water stress, HSP70s are differentially induced as a mechanism to restore cellular homeostasis (Alvim et al., 2001; Sung and Guy, 2003; Cho and Hong, 2006; Zhang and Guy, 2006). Likewise, different plant viruses also induce HSP70 mRNAs and presumably their encoded proteins, but it is not fully understood why this occurs (Aranda et al., 1996; Escaler et al., 2000; Whitham et al., 2003; Aparicio et al., 2005). From the study by Aparicio et al. (2005), four HSP70s predicted to encode cytosolic proteins were up-regulated by plant virus infection reflecting a general response to viral protein accumulation in the cytosol. However, this does not rule out a potential role for HSP70s in promoting viral infections, because in yeast, HSP70s was shown to interact with the replicase protein, p33 of CNV to presumably enhance replication of a TBSV replicon (Nagy and Pogany, 2006). This suggests that HSP70s might be required for viral protein function and/or complex formation in plants. Given that BYV encodes a HSP70 homolog that is required for virion assembly and movement (Peremyslov et al., 1999; Prokhnevsky et al., 2002), it is possible that plant viruses that do not encoded HSP70 homologs may require plant HSP70s for similar functions.

To better understand the relationship between virus infection and HSP70 mRNA accumulation, we profiled the HSP70 gene family in A. thaliana wild-type and SA, JA and/or ET defense signaling mutants. From our analysis of CMV- or ORMV-infected wild-type A. thaliana, only two HSP70s predicted to encode cytosolic proteins, HSP70 and HSC70-2 were up-regulated at 5 DAI (Fig. 1) unlike the studies by Aparicio et al. (2005). In spite of this, our findings do not conflict with the conclusions of Aparicio et al. (2005) because different viruses were used, and it is possible that viruses differentially induce HSP70s. BiP-2 and
BiP-3 that encode ER chaperone proteins, were significantly induced by ORMV whereas CMV only significantly induced BiP-3 (Fig. 1.). Induction of BiP-3 by both CMV and ORMV infection might be reflective of an ER stress response associated with viral protein accumulation.

This is mentioned because BiP-3 protein (BiP-L) was shown to be up-regulated in A. thaliana seedlings treated with the antibiotic tunicamycin (Noh et al., 2003), a potent inducer of the unfolded protein response (UPR) that blocks N-linked protein glycosylation (Mahoney and Duksin, 1979). Because newly synthesized proteins are folded in the ER (Stevens and Argon, 1999), increased amounts of unfolded viral proteins during infection might activate BiP-3 for viral protein folding and/or to stabilize ER stress. Considering that BiP-3 mRNA induction is stronger in response to ORMV than CMV might imply that ORMV infection triggers more ER stress. It was previously shown that TMV infection of N. benthamiana induced dramatic morphological changes in the ER by converting it from a tubular form into large aggregates (Reichel and Beachy, 1998). Because both TMV and ORMV are tobamoviruses, ORMV might be inducing similar changes in A. thaliana ER.

The observation that expression of HSP70 genes did not significantly differ between wild-type plants and SA, JA and/or ET signaling defective mutants infected with CMV or ORMV (Fig. 2) demonstrates that these signaling pathways are dispensable for induction of HSP70s. To identify potential viral inducers of HSP70 mRNA expression, we expressed CMV CP, ORMV CP, CMV MP or GFP::GUS under the control of an estradiol-inducible promoter (Zuo et al., 2000) (Fig. 3A). Results from these studies showed that only ORMV CP induced HSP70 mRNA, but this induction was not significant due to variability between
experimental replicates (Fig. 3B). These results correlate with our previous reported data, showing that ORMV CP significantly induced $HSP101$ mRNA (Carr et al., 2006).

It would appear that our results contradict Aparicio et al. (2005) who showed pHSP70::GUS was induced by both viral and non-viral proteins. Major differences between our approach and their approach include, first their use of a constitutive promoter to express proteins of interest, second a transient method to expression proteins interest in $N. benthamiana$ and third a sampling time of 3 days after co-infiltration. In our study, all experiments were carried out in transgenic plants transformed with inducible promoter constructs and sampling was performed at 32 hours after estradiol induction.

Interestingly, we found the $A. thaliana$ HSF, HSFA2 but not HSF3 was induced by ORMV and CMV infection (Fig 4.). HSFA2 is the strongest induced member of the $A. thaliana$ HSF gene family in response to heat stress conditions (Busch et al., 2005). Over-expression of HSFA2 in non-heat stressed $A. thaliana$ has been shown to induce several HSP mRNAs including $HSP70$ (Nishizawa et al., 2006). Although HSF3 was not induced by CMV or ORMV, it is a regulator in the heat-stress induced pathway (Prandl et al., 1998; Schoffl et al., 1998; Panchuk et al., 2002). Therefore, further experiments are needed to determine if CMV and ORMV induction of HSFA2 and/or HSFs not tested in this study regulate $HSP70$ expression during virus infection. Based on our findings we conclude overall that $HSP70$ family members and select HSFs are differentially regulated by CMV and ORMV and virus induction of BiP-2 and BiP-3 is reflective of an ER stress response.

Acknowledgements

We thank T. Crosbie and Monsanto Company for providing fellowship support to T.C. during the course of this work. This work was supported by the USDA-NRI (grant no. 02-
35319-12566 to S.A.W.), the Iowa State University Plant Sciences Institute, and by the Hatch Act and State of Iowa Funds.

References


Fig. 1. Expression of the *A. thaliana* HSP70 gene family in response to CMV and ORMV. For each gene and treatment, the average signal was taken from Huang et al. (2005) and graphed as a ratio of virus treatment verses mock treatment at 5 DAI. A ratio at or near 1 indicated that expression was not altered by virus infection (dashed line). Vertical bars represent the standard deviation of three biological replicates. *BiP3* (*At1g09080*), *HSP70b* (*At1g16030*), *HSP70t-2* (*At2g32120*), *HSC70-3* (*At3g09440*), *HSP70* (*At3g12580*), *cpHSC70-1* (*At4g24280*), *mtHSC70-1* (*At4g37910*), *HSC70-2* (*At5g02490*), *HSC70-1* (*At5g02500*), *mtHSC70-2* (*At5g09590*), *BiP2* (*At5g42020*), and *cpHSC70-2* (*At5g49910*). Not included are *HSP70t-1* (*At1g56410*) and *BiP-1* (*At5g28540*) due to their lows signals.
**Fig. 2.** Expression of *A. thaliana* HSP70 in mutants defective in SA, JA, and ET signaling after mock inoculation, CMV or ORMV infection. The average signals of *HSP70* in response to each treatment at 5 DAI were taken from Huang et al. (2005) and graphed. Vertical lines represent the standard deviation of three biological replicates.
Fig. 3. ORMV CP induces the expression of *HSP70* mRNA. (A) A modified pXVE estradiol-inducible promoter system was used to express viral and non-viral proteins (Zuo et
al., 2000). (B) *HSP70* mRNA in Col-0 wild-type and transgenic plants expressing genes under control of the estradiol-inducible promoter at 32 hrs. *HSP70* mRNA levels were normalized to 18S rRNA and then plotted as the ratio of estradiol-induced/non-induced. Vertical lines represent the standard deviation of four biological replicates. (C) Protein expression from viral transgenes after 32 hrs on media containing no estradiol (-) or 20 μM estradiol (+). WT, wild-type; XVE, vector used; CP, coat protein; MP, movement protein; GG, GFP::GUS. Not shown is GG expression that was detected by GUS staining assay.
Fig. 4. Expression of *A. thaliana* (A) *HSFA2* and (B) *HSF3* in wild-type Col-0 and mutants defective in SA, JA, and ET signaling after mock inoculation, CMV or ORMV infection. The average signal of *HSFA2* and *HSF3* in response to each treatment at 5 DAI were taken from Huang et al. (2005) and graphed. Vertical lines represent the standard deviation of three biological replicates.
CHAPTER 4. 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

Tyrell Carr, Valerie Torney and Steven A. Whitham

A manuscript in preparation for submission to Virus Research

Author contributions: T.C., V.T. and S.A.W. designed experiments, V.T. generated TuMV mutants and HC-Pro mutants and performed bombardments, T.C. generated PVX expressing wild type and mutant HC-Pros, performed majority of the experiments and wrote the paper and T.C., V.T. and S.A.W. are from the Department of Plant Pathology 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 potyvirus (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**

Virus-induced RNA silencing (VIRSI) is a conserved antiviral strategy activated in plants by structured viral RNAs that prompt their own degradation (Ding and Voinnet, 2007). Plant enzymes required for VIRSI include Dicer-like (DCL), RNA-directed RNA polymerase (RDR) and Argonaute (AGO) proteins. DCL proteins initially recognize structured viral RNAs and cleave them into ~21-24 nucleotide (nt) small RNA duplexes with 2 nt overhangs at their 3’ ends, which are referred to as viral small interfering RNAs (siRNAs) (Bonnet et al., 2006; Waterhouse and Fusaro, 2006). These viral siRNAs in turn direct cleavage of complementary viral transcripts through RNA-induced RNA silencing complexes (RISCs) comprised of an AGO protein that possesses RNA slicer activity to negatively regulate viral gene expression (Baumberger and Baulcombe, 2005; Bonnet et al., 2006; Zhang et al., 2006). RDR proteins are essential in amplifying the VIRSI signal that protects adjacent and/or distant cells from advancing virus infection (Hohn et al., 2007).

Plant viruses escape VIRSI through the functions of their various RNA silencing suppressor (RSS) proteins (Deleris et al., 2006; Lakatos et al., 2006; Li and Ding, 2006; Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007). A plant viral RSS of much interest is the helper component-proteinase (HC-Pro) encoded by members of the genus *Potyvirus* that likely suppresses VIRSI by multiple mechanisms to promote infection (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Ebhardt...
et al., 2005; Lakatos et al., 2006; Shibolet et al., 2007). One mechanism in particular is the binding of viral siRNAs that leads to discontinued programming of RISCs (Lakatos et al., 2006; Shibolet et al., 2007). Additionally, HC-Pro binds plant-encoded microRNAs (miRNAs) and inhibits miRNA-directed mRNA cleavage (Kasschau et al., 2003; Lakatos et al., 2006; Shibolet et al., 2007). Thus, HC-Pro’s general ability to interfere with small RNA (smRNA) metabolism illuminates how virus accumulation and symptom expression can be coupled in potyvirus-infected plants.

The probable point of contact with smRNAs is the highly conserved FRNK box positioned in the central domain of HC-Pro (Shibolet et al., 2007). In brief, Shibolet et al. (2007) reported that wild type HC-Pro$^{\text{FRNK}}$ of *Zucchini yellow mosaic virus* (ZYMV) has a strong affinity for smRNAs unlike the HC-Pro$^{\text{FINK}}$ and HC-Pro$^{\text{FRNA}}$ mutants. Furthermore, ZYMV$^{\text{FRNK}}$ but not ZYMV$^{\text{FINK}}$ or ZYMV$^{\text{FRNA}}$ induced severe symptoms in cucurbits but mutant viruses did induce mild symptoms (Gal-On, 2000; Shibolet et al., 2007). During early stages of systemic infection, ZYMV$^{\text{FINK}}$ and ZYMV$^{\text{FRNA}}$ levels were similar to ZYMV$^{\text{FRNK}}$ but declined afterward (Shibolet et al., 2007), implying that programmed RISCs were abundant due to weak viral siRNA binding by HC-Pro$^{\text{FINK}}$ and HC-Pro$^{\text{FRNA}}$. Hence, positive charged amino acids in the FRNK box are critical for prolonged suppression of VIRS and interference with miRNA-directed mRNA degradation in ZYMV-infected cucurbits. Conversely, the FINK and FRNA mutant box can temporarily uncouple ZYMV accumulation and ZYMV-induced symptom expression.

Interestingly, when Shibolet et al. (2007) tested RSS activities of HC-Pro$^{\text{FINK}}$ mutants from *Potato virus Y* (PVY), *Tobacco etch virus* (TEV) and *Turnip mosaic virus* (TuMV), they were found to be inactive, suggesting that HC-Pro$^{\text{FINK}}$-mediated uncoupling of
potyvirus accumulation and induced plant symptom expression might be restricted to ZYMV. Among the ZYMV, PVY, TEV and TuMV HC-Pros, the FRNK box is located at the amino acid position 179 – 182, 179 – 182, 182 – 185 and 181 – 184, respectively. To further investigate the role of the FRNK box of TuMV HC-Pro in virus accumulation, symptom expression and RSS activity, viral and HC-Pro FRNK box mutants were generated and tested in *Arabidopsis thaliana* and/or *Nicotiana benthamiana*. Both plant species are systemically susceptible to TuMV and display stunted growth and altered leaf morphology due in part to HC-Pro expression (Sanchez et al., 1998; Kasschau et al., 2003; Dunoyer et al., 2004). Consistent with other potyviruses, TuMV is a flexuous rod-like particle comprised of coat protein (CP) subunits that encapsidate a ~10-kd single-stranded RNA of (+) polarity that is covalently attached to a viral protein at its 5' end and polyadenylated at its 3' end (Shukla et al., 1994; Urcuqui-Inchima et al., 2001). Its genome consists of a single long open reading frame that is translated into a large polyprotein cleaved into 10 smaller proteins by three virus-encoded proteinases including HC-Pro (Adams et al., 2005).

We found that TuMV$^{\text{FINK}}$ was poorly infectious and did not induce any visible symptoms in plant species examined due to deficient HC-Pro$^{\text{FINK}}$ RSS activity as previously suggested by Shibolet et al. (2007). This poorly infectious phenotype was also observed among other rTuMV 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.
**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 GeneTailor™ system (Invitrogen, Carlsbad, CA, USA). The forward primers corresponding to each mutation listed above are as follows: FINKIF 5'-GAAGGGTTCACTGAAGTCTTTATTAACAAGATTTCCAG-3', FINKRF 5'-GAAGGGTGTTCACTGAAGTCTTTTCGCAACAAGATCCCAG-3' and FRNKR 5'-GAAGGGTGTTCACTGAAGTCTTTTCGCAACAAGATCCCAG-3'. The reverse primer used was FRNKIR 5'-GGACTTCAGTGAACCCTTCTCAATGTTCTC-3' (the region of overlap with the forward primers is underlined). After sequencing to confirm the mutations, p5PK-TuMCS was digested with SmaI / KpnI or NcoI / KpnI and the fragment was ligated in p35STuMV-GFP cut with the same restriction enzymes. It should be noted that p5PK-TuMCS 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-Pro 5'-CCATGGCTAGTAAGGGAGAAGAACTTTTCAC-3', NcoHC-ProF 5'-CCATGGCTAGTGCAAGCAGGAGCCAACT-TCTGG-3' and SacGFP::HC-ProR / HC-ProR 5'-GAGCTCTCATCCACCGCGGTAGTGTTTCAAGC-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 Agrobacterium tumefaciens strain GV3101 by electroporation.

**Growth of plants and TuMV bombardment.** A. thaliana and N. benthamiana were grown in a growth chamber or growth room set at a 14 h photoperiod at 22 °C. Twenty-one or 28 day-old plants were bombarded with p35S::TuMV-GFP carrying wild-type or mutant HC-Pro sequences described above. Five μg of plasmid was mixed with 50 μl of 1 μm gold particles, 100 μl of 50% glycerol, 50 μl of 2.5 M CaCl₂ and 20 μl of 0.1 M spermidine. DNA-coated gold particles were centrifuged and washed in 70% and 100% isopropanol then resuspended in 60 μl of 100% isopropanol. Afterward, 6 μl of this solution was placed on a macro-carrier and bombarded onto two fully expanded leaves using the BioRad PDS 1000/Helium biolistic gun (Bio-Rad, Hercules, CA, USA) and 1100 psi rupture disks. Clear plastic hoods were placed over plants overnight to allow recovery. For each construct, 3 plants were bombarded and experiments were repeated at least twice. All bombardments
were performed at the Iowa State University Plant Transformation Facility. For detection of GFP fluorescence at 15 and 11 days after inoculation (DAI) in *A. thaliana* and *N. benthamiana*, respectively, a long-wave UV lamp was used.

**Potato virus X expression of HC-Pro in N. benthamiana.** The *Potato virus X* (PVX) binary vector, p254 generously provided by S.P. Dinesh-Kumar (Yale University) 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'-GGGGACAAGTTTGTACAAAAAAGCAGGTTCACCAGGCTTCACCATGGGTAGTAAAGGAGAACTT-3', HC-ProF 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCAGGCTTCACCATGGGTAGTGCAGCGGGAGCC-3' and GFP::HC-ProR / HC-ProR 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTATCCACGCGGTAGTGT-3'.

GFP alone was also amplified using the primers, GFPF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCAGGCTTCACCATGGGTAGTAAAGGAGAACTT-3' and GFPR 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTATCCACGCGGTAGTGT-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 acitosyringone) 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 OD$_{600}$ of 1.0 and incubated for 8 – 12 hours at room temperature. The bottom most leaves of 21-old day N. benthamiana plants were infiltrated and plants were photographed 7 and 10 days post infiltration (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 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 isolation and RT-PCR analysis.** Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified for cDNA synthesis to detect *GFP::HC-Pro / HC-Pro* mRNA expression and 18S RNA expression as an internal control from infiltrated zones of *N. benthamiana* leaves. The following primer sets were used to detect *GFP::HC-Pro / HC-Pro* and 18S expression HC-ProF: 5'-GGATCCATGAGTGCAGCGGGACCCCTTCTGG-3', HC-ProR: 5'-TCTAGATCATCAACCACGGTAGTGTTCGAGAGGAGCAGCAACGAGGAGCGAGGC-3', 18SF 5'-GACAGACTGAGCTCTTTTTTGA-3' and 18SR 5'-ACGTAGCTAGTTAGCAGGAC-
TGAG-3'. Amplification of PCR products for *HC-Pro* and *18S* were assayed at 25 and 20 cycles, respectively in two independent experiments.

**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<sup>FINK</sup> containing the same point mutation that was shown to uncouple ZYMV HC-Pro role in viral accumulation and symptom expression in cucurbits (Shiboleth et al., 2007). p35STuMV-GFP plasmids encoding full-length TuMV<sup>FRNK</sup> or TuMV<sup>FINK</sup> 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<sup>FRNK</sup> was highly infectious in rosette leaves and systemic tissues and it induced severe symptoms, whereas, TuMV<sup>FINK</sup> 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<sub>183</sub>) flanking the FRNK box (FRNKR) (Shiboleth et al., 2007), we hypothesized that the poor infectivity by TuMV<sup>FINK</sup> (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<sub>185</sub>) (Fig 1B). The amino acids, isoleucine and arginine are neutral-hydrophobic and positive-basic, respectively. Accordingly, TuMV<sup>FRNKR</sup> and TuMV<sup>FINKR</sup> mutants (p35STuMV-GFP) were generated and bombarded in *A. thaliana*. The TuMV<sup>FRNKR</sup> and TuMV<sup>FINKR</sup> differed from each other and from wild type TuMV<sup>FRNKi</sup> in
infectivity. TuMV\textsuperscript{FRNKR} was detected in inflorescence tissues but not stems (UV light), and TuMV\textsuperscript{FINKI} infection was indistinguishable from TuMV\textsuperscript{FINKI} infection (Fig. 2A). Mild symptoms developed in \textit{A. thaliana} in response to TuMV\textsuperscript{FRNKR} infection as compared to the severe symptoms of TuMV\textsuperscript{FRNKI}. When these TuMV constructs were inoculated onto \textit{N. benthamiana} plants similar phenotypes were observed (Fig. 2B). In particular, GFP fluorescence of TuMV\textsuperscript{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\textsuperscript{ARNKI}, TuMV\textsuperscript{FRNAI} and TuMV\textsuperscript{FRNEI} were not infectious (data not shown). Collectively, these studies show that TuMV infectivity of \textit{A. thaliana} and \textit{N. benthamiana} requires an intact FRNKI sequence.

The FRNKI sequence is likely required for increased symptom development in \textit{N. benthamiana} infected with \textit{Potato virus X} (PVX) expressing HC-Pro. Recombinant PVX expressing the wild type HC-Pro has been shown to increase PVX pathogenicity in \textit{N. benthamiana} (Pruss et al., 1997; Brigneti et al., 1998; Gonzalez-Jara et al., 2005). Using an infectious PVX binary vector, \textit{GFP::HC-Pro} or \textit{HC-Pro} constructs were generated and transformed into \textit{Agrobacterium}. The bottom most leaves of \textit{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\textsuperscript{FRNKI} or PVX-GFP::HC-Pro\textsuperscript{FRNKR} but not PVX-GFP::HC-Pro\textsuperscript{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{FRNI}. 
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, PVX-GFP::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 in a pCAMBIA binary vector, 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::HC-Pro\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.

**Discussion**

The FRNK box is a highly conserved amino acid motif among potyvirus-encoded HC-Pros including TuMV HC-Pro (Shibolet et al., 2007) (Fig. 1). The FRNK box of ZYMV HC-Pro has recently been proposed as a potential binding site for viral siRNAs and host miRNAs because mutations such as FINK reduce the affinity of ZYMV HC-Pro for smRNA species (Shibolet et al., 2007). Reduced affinity of ZYMV\textsuperscript{FINK} HC-Pro for miRNAs correlated with attenuated plant symptoms induced during infection. However, ZYMV\textsuperscript{FINK} HC-Pro mutants were still able to suppress RNA silencing enabling ZYMV replication and movement similar to wild type virus over the first five days after infection (Shibolet et al., 2007). Thus, mutations in the FRNK box of ZYMV HC-Pro uncouple viral accumulation and plant symptom expression early in infection.

In contrast to the FRNK to FINK mutation in ZYMV, we found that TuMV\textsuperscript{FINKI} and TuMV\textsuperscript{FINKR} 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\textsuperscript{FRNKR} accumulated in systemic leaves but it was not detected in stems, veins or in emerging leaves compared to TuMV\textsuperscript{FRNKI} (Fig. 2), and it retained partial RSS activity (Fig 5). As discussed above, wild-type ZYMV and wild-type TuMV contain a FRNKR 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 is amino acid charge and/or size in the FRNK box because additional TuMV mutants generated like TuMV$^{\text{ARNKI}}$, TuMV$^{\text{FRNAI}}$ 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, non-polar amino acid, in our case the FRNKI sequence.

Using PVX to express GFP::HC-Pro / HC-Pro wild-type and mutants in N. benthamiana, it was 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 alone is a strong elicitor of plant symptom development (Brigneti et al., 1998; Kasschau et al., 2003; Gonzalez-Jara et al., 2005; Lim et al., 2007). 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 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-Pors in N. benthamiana leaves (Fig. 4A, 4D, and data not shown). Both P19 and
HC-Pro\textsuperscript{FRNKI} caused increased fluorescence and protein accumulation of GFP::HC-Pro mutants similar to GFP::HC-Pro\textsuperscript{FRNKI}. Our results extend what others have reported, in that point mutations or insertions in \textit{HC-Pro} mRNA target it for RNA silencing (Kasschau and Carrington, 2001; Varrelmann et al., 2007). Thus, it appears that a region of RNA encoding HC-Pro is itself a target for RNA silencing and that HC-Pro’s RSS activity is needed to disrupt this silencing.

To directly test for suppressor activity, we expressed GFP::HC-Pro wild-type and mutants in transgenic \textit{N. benthamiana} expressing a GFP (Ruiz et al., 1998) and demonstrated that GFP::HC-Pro\textsuperscript{FRNKI} possesses the strongest RSS activity followed by GFP::HC-Pro\textsuperscript{FRNKR} (Fig 5). Based on this finding, TuMV HC-Pro RSS activity requires the FRNKI box in our study. The observation of a similar FRNKV box in the HC-Pro of at least two 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\textsuperscript{FRNKI} and GFP::HC-Pro\textsuperscript{FRNKR} are interesting. GFP::HC-Pro\textsuperscript{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::HC-Pro\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).
Differences in ZYMV and TuMV mutant pathogenesis might also be explained by the position of the FRNK box. In ZYMV HC-Pro and TuMV HC-Pro it 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 (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 FRNK 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). Thus, our findings coupled with previous studies implicate a role for the conserved FRNK box of HC-Pro in mediating potyvirus infections.

**Acknowledgements**

We thank James Carrington for p5PK-TuMCS and p35STuMV-GFP, S.P. Dinesh-Kumar for PVX p254 plasmid, and Herman B. Scholthof for pCB301-p19 and P19 antibody. We also thank Yongzeng Wang and Al Eggenberger who provided technical support for TuMV bombardment studies and Kan Wang and Bronwyn Frame from the Iowa State University Plant Transformation Facility for providing equipment. During the course of this work T.C. was supported by an Iowa State University George Washington Carver Doctoral Fellowship. This research was supported by the United States – Israel Binational Agricultural Research
and Development (BARD) Fund (US-3623-04) and the USDA-NRI (02-35319-12566), and by the Hatch Act and State of Iowa Funds.

References


Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130: 413-426


Fig. 1. Alignment of an amino acid region 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 (BCMNV), Bean common mosaic virus gi 25013490 (BCMV), Bean yellow mosaic virus gi
25013500 (BYMV), Beet mosaic virus gi 40254030 (BtMV), Chilli veinal mottle virus gi 45004657 (ChiVMV), Clover yellow vein virus gi 25013510 (CYVV), 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*FRNKI* – left and TuMV*FRNKR* – right; (ii) TuMV*FINKI* –
left and $\text{TuMV}^{\text{FINKR}}$ – right. (B) Panels: (i) $\text{TuMV}^{\text{FRNKI}}$ – top and $\text{TuMV}^{\text{FRNKI}}$ – bottom; (ii) $\text{TuMV}^{\text{FINKI}}$ – top and $\text{TuMV}^{\text{FRNKR}}$ – bottom; (iii) $\text{TuMV}^{\text{FRNKI}}$ – left and $\text{TuMV}^{\text{FRNKR}}$ – right; (iv) $\text{TuMV}^{\text{FRNKI}}$ – close up of infected leaf from (iii) left yellow box and (v) $\text{TuMV}^{\text{FRNKR}}$ – 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-sert; (iii, iv) PVX-GFP::HC-Pro$^{FRNKI}$; (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$^{FRNKI}$; (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$^{FRNKI}$; (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^{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<sup>FRNKI</sup> (ii) GFP::HC-Pro<sup>FINKI</sup> and (iii) GFP::HC-Pro<sup>FRNKI</sup>. (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.
CHAPTER 5. CONCLUSION

Overview

In this dissertation, I presented results on the genetics and molecular biology of compatible plant-virus interactions. Chapter 1 provided a detailed overview of previous research on compatible plant-virus interactions and the many remaining questions that need to be answered. In Chapters 2 and 3, I investigated the induction of genes encoding heat shock proteins (HSP) by different viruses in plants and in Chapter 4, I investigated the role the conserved FRNK box in the potyvirus encoded helper-component proteinases (HC-Pro) in mediating RNA silencing suppressor (RSS) activity.

**HSP101 study.** As discussed in Chapter 2, I examined HSP101’s role in tobamovirus infection in *A. thaliana* and *N. benthamiana* using a genetic loss-of-function approach. HSP101 was previously shown to bind the poly(CAA) region of the *Tobacco mosaic virus* (TMV) 5' Ω leader (Gallie and Walbot, 1992; Tanguay and Gallie, 1996). A role for HSP101 in TMV pathogenesis was speculated but never reported. However, it was shown to enhance translation of model mRNAs fused to the Ω leader *in vitro* (Wells et al., 1998; Gallie, 2002). The tobamoviruses tested in *A. thaliana* and *N. benthamiana* were *Oilseed rape mosaic virus* (ORMV) and TMV, respectively. ORMV was selected because it was shown to induce *HSP101* mRNA in *A. thaliana* (Whitham et al., 2003) and it contains a poly(CAA) rich region in the 5' Ω leader.

From my results, it was found that tobamovirus infection was independent of *HSP101* mRNA induction and protein expression in both pathosystems (Carr et al., 2006). However, these results do not rule out a role for HSP101 in tobamovirus infection because only two pathosystems were examined. Future experiments that need to be performed include
repeating all genetic experiments using diluted tobamoviruses and determining if *A. thaliana* HSP101 and *N. benthamiana* HSP101 bind the ORMV and TMV Ω leader. By performing experiments using diluted tobamoviruses, a role for HSP101 in establishing infection when the virus is inoculated at low titers might be revealed. If *A. thaliana* HSP101 and *N. benthamiana* HSP101 indeed bind the ORMV and TMV Ω leader and enhance translation of model mRNAs fused to the Ω leader, it might imply that HSP101 has a conditional role in tobamovirus infections. My hypothesis is that HSP101 may be involved in establishing initial tobamovirus infection when virus titer is very low by direct binding to the Ω leader to enhance viral translation. An approach to test this hypothesis is to measure and compare virus accumulation in protoplasts of wild-type and hsp101 loss-of-mutants.

**HSP70s and HSFs study.** Other HSPs have been suggested to help facilitate virus infection in plants like HSP70 since its mRNA is induced by several viruses (Escalar et al., 2000; Whitham et al., 2003; Aparicio et al., 2005). Interestingly, a HSP70 homolog (HSP70h) encoded by *Beet yellows closterovirus* (BYV) is required for virion assembly and intercellular movement in plants (Peremyslov et al., 1999; Prokhnevsky et al., 2002). Thus, plant HSP70s might function as molecular chaperones in viral protein folding and/or viral transport. As discussed in Chapter 3, I investigated the expression profiles of the *A. thaliana* HSP70 gene family consisting of 14 members in response to *Cucumber mosaic virus* (CMV) and ORMV infection since past studies only examined a few of these genes during virus infection. In addition, I also profiled, two genes encoding heat shock transcription factors (HSFs) to determine if they were induced by ORMV and/or CMV to better understand the regulation of virus-induced HSP70 genes and other HSP genes.
Among HSP70 genes, both ORMV and CMV induced HSP70 (At3g12580), HSC70-2 (At5g02490) and BiP-3 (At1g09080). Induction of BiP-3 was interesting since it encodes a protein that functions as an ER stress chaperone (Noh et al., 2003). Thus induction of BiP-3 by ORMV and CMV might indicate an ER stress response. Previously, it has been shown that TMV infection of N. benthamiana induced dramatic morphological changes in the ER by converting it from a tubular form into large aggregates likely to facilitate virus replication (Reichel and Beachy, 1998). Alternatively, BiP-3 induction and subsequently its protein expression might be essential for viral protein folding and/or preventing viral protein aggregation since protein synthesis occurs in the ER. Therefore, future experiments are needed to examine the role, if any, of BiP-3 in ORMV and CMV infection of A. thaliana using BiP-3 loss-of-function mutants. My hypothesis is that BiP-3 has a role in preventing viral protein aggregation to promote infection.

Regulation of virus-induced HSP gene expression like HSP70 in A. thaliana might in part involve HSF proteins, because ORMV and CMV induced HSFA2 (At2g26150) expression. To investigate a role for HSFs in virus-induced HSP gene expression, additional HSF genes need to be profiled in response to virus infection and A. thaliana HSFA2 loss-of-function mutants infected with ORMV or CMV need to be examined for HSP gene induction. My hypothesis is that HSFA2 regulates HSP70 and HSP101 induction during virus infection (ORMV) because its over-expression up-regulates these genes (Nishizawa et al., 2006).

**TuMV HC-Pro FRNK1 box study.** The RSS activity of HC-Pro is required for potyviruses to systemically infect plants and produce symptoms in plants (Kasschau and Carrington, 2001; Kasschau et al., 2003; Shibolet et al., 2007). HC-Pro likely suppresses
RNA silencing through a variety of mechanisms among which viral siRNA and host miRNA binding has been shown (Lakatos et al., 2006; Shiboleth et al., 2007). Shiboleth et al. (2007) recently reported that the highly conserved HC-Pro FRNK box of Zucchini yellow mosaic virus (ZYMV) is a probable point of viral siRNA and host miRNA binding. ZYMV HC-Pro FRNK box mutants were shown to have reduced affinity for small RNAs that correlated with attenuated symptoms induced by ZYMVFINKR in cucurbits. Compared to ZYMVFRNKR, ZYMVFINKR induced mild symptoms were not due to reduced virus accumulation during early infection, implying that HC-ProFINKR uncoupled ZYMV accumulation and induced symptom expression. To examine if this phenotype mediated ZYMV HC-ProFINKR is conserved, Turnip mosaic virus (TuMV) mutants were generated. As discussed in Chapter 4, the TuMV FRNK box is also flanked by isoleucine (FRNKI), which is required for TuMV HC-Pro’s RSS activity.

All TuMV mutants tested in A. thaliana and N. benthamiana differed from TuMVFRNKI infectivity and symptom expression. In brief, TuMVFINKI and TuMVFINKR were poorly infectious and TuMVFRNKR did not infect stems, veins or emerging leaves. This suggested that HC-Pro mutants were compromised in RSS activity, which was confirmed in my PVX expression studies and my transgene expression in the absence or presence of wild-type RSS proteins in N. benthamiana. Based on these results the FRNKI box of TuMV HC-Pro is required for RSS activity in mediating infectivity and induced symptom expression. Key experiments to be performed in the future are to examine TuMV mutant accumulation in A. thaliana loss-of-function mutants for Dicer-like proteins and Argonaute proteins. Detailed descriptions of these proteins are reviewed in Chapter 1. My hypothesis is that the A. thaliana triple DCL2-DCL3-DCL4 loss-of-function mutant and the triple RDR1-RDR2-RDR6
will support TuMV mutant infection and induced plant symptoms. In these studies, double
and single mutants will also be tested. Future experiments are needed to examine if TuMV
HC-Pro wild-type and mutants bind smRNAs to confirm that the FRNK box is the point of
contact and if a neutral-hydrophobic property encoded by isoleucine in the FRNKI box of
TuMV HC-Pro is required. To test this idea, the FRNKI box of TuMV could be mutated to
FRNKV, since two TuMV isolates are known to encode this version of this sequence motif.
Other possible amino acids that can be tested are alanine (A), leucine (L) and phenylalanine
(F) that also have neutral-hydrophobic properties. My hypothesis is that TuMV HC-Pro
FRNKI box indeed binds smRNAs and that different neutral-hydrophobic amino acids can
substitute for isoleucine to maintain RSS activity.

**Impact of Dissertation Study**

All three studies presented in my dissertation have contributed new knowledge in
understanding the genetics and molecular biology of compatible plant-virus interactions. The
HSP101 – tobamovirus study filled in the gap on the function of HSP101 in tobamovirus
pathogenesis, since it was only speculated in publications. Based on my findings HSP101
induction by tobamoviruses is not essential for infection. The *HSP70* gene family – virus
study provided evidence that certain HSP70 proteins might have a role in stress responses
like in the ER. In addition, I provided evidence that HSFs might regulate virus-induced *HSP*
gene expression in *A. thaliana*. The TuMV HC-Pro – FRNKI box study confirms that the
conserved FRNK box is required for TuMV pathogenesis and provides new insights into a
key amino acid and possibly its biochemical property in HC-Pro mediated viral pathogenesis.
Acknowledgements

I would like to thank all former and current members of the Whitham, Hill, Miller and Bassham Laboratories from 2001 – 2007 for support and constructive criticism. I would like to especially thank Phan Quang Nguyen and Jacquelyn Jackson for scientific discussions and essential materials.

References


gene silencing, is required for small RNA binding and mediates symptom

**Tanguay RL, Gallie DR** (1996) Isolation and characterization of the 102-kilodalton RNA-
binding protein that binds to the 5' and 3' translational enhancers of tobacco mosaic
virus RNA. J Biol Chem **271:** 14316-14322

**Wells DR, Tanguay RL, Le H, Gallie DR** (1998) HSP101 functions as a specific
translational regulatory protein whose activity is regulated by nutrient status. Genes
Dev **12:** 3236-3251

Diverse RNA viruses elicit the expression of common sets of genes in susceptible
Arabidopsis thaliana plants. Plant J **33:** 271-283