ABSTRACT

Title of Document: FUNCTIONS OF THE TOBACCO MOSAIC VIRUS HELICASE DOMAIN: REGULATING FORMATION OF THE VIRUS REPLICATION COMPLEX AND ALTERING THE ACTIVITY OF A HOST-ENCODED TRANSCRIPTION FACTOR

Xiao Wang
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Directed By: Dr. James N. Culver
Center for Biosystems Research

Tobacco mosaic virus (TMV)-encoded 126-kDa and 183-kDa replicases are multidomain and multifunctional proteins. The helicase domain shared by both replicases has been shown to perform multiple tasks during the virus life cycle. *In vitro* structural and functional analyses demonstrated that monomers and dimers of the TMV helicase domain were the active forms for ATP hydrolysis. However, self-interaction of the helicase polypeptides resulted in the formation of higher-order structures that likely serve as structural scaffolds for the assembly of virus replication complexes (VRCs). Mutagenesis studies of the TMV helicase motifs showed that conserved amino acid residues played important roles in protein ATPase and/or RNA binding activities. A close correlation between ATPase activity of the helicase

domain and assembly of wild-type VRC-like vesicles by the 126-kDa replicase further suggests that ATPase activity of the TMV helicase domain may modulate proper VRC assembly.

In addition to helicase self-interaction, a novel virus-host interaction involving ATAF2, a NAC domain transcription factor was identified. Members within the NAC domain family are involved in plant developmental processes and stress/defense responses. In this study, transgenic plants overexpressing *ATAF2* showed a strong developmental phenotype. Inoculation of TMV in these transgenic plants resulted in reduced virus accumulations. Additionally, transcriptional induction of *ATAF2* occurred in response to TMV infection and salicylic acid treatment. Combined, these results suggest that ATAF2 is involved in a host defense response. One interesting finding was that in susceptible hosts, virus-directed induction of *ATAF2* and *PR1*, a well-defined pathogenesis-related (*PR*) marker gene for host defense system, occurred only in locally-infected but not in systemically-infected tissues. Dynamic changes in the expression of host defense genes suggest that viruses have evolved certain mechanisms to actively modulate host gene expression. Interaction between the TMV helicase domain and ATAF2 may provide one way to suppress the ATAF2-mediated host defense signaling pathway.

Combined these studies investigated the importance of the TMV helicase domain in VRC formation and in manipulating the host defense system. The results confirmed the functional versatility of the TMV helicase domain in establishing a successful virus life cycle.

FUNCTIONS OF THE TOBACCO MOSAIC VIRUS HELICASE DOMAIN: REGULATING FORMATION OF THE VIRUS REPLICATION COMPLEX AND ALTERING THE ACTIVITY OF A HOST-ENCODED TRANSCRIPTION FACTOR

By

Xiao Wang

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Advisory Committee:
Dr. James Culver, Chair
Dr. Jonathan Dinman
Dr. Douglas Julin
Dr. Zvi Kelman
Dr. David Straney

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Abbreviations

Abbreviation	Full name/description
35S	Promoter from the *Cauliflower mosaic virus* that makes a 35S RNA transcript
ABA	Abscisic acid
AMV	*Alfalfa mosaic virus*
At	*Arabidopsis thaliana* (arabidopsis)
Aux/IAA	Auxin/indole-3-acetic-acid
BMV	*Bromo mosaic virus*
bZIP	Basic domain-leucine zipper
CaMV	*Cauliflower mosaic virus*
CGMMV	*Cucumber green mottle mosaic tobamovirus*
CMV	*Cucumber mosaic virus*
CP	Coat protein
CPMV	*Cowpea mosaic virus*
CREST	Chimeric repressor silencing technology
CUC	Cup-shaped cotyledon
DPI	Days post inoculation
DTT	Dithiothretol
eIF3	Eukaryotic initiation factor 3
EAR	ERF-associated amphiphilic repression
eEF1A	Eukaryotic elongation factor 1A
ER	Endoplasmic reticulum

ERF	Ethylene-responsive element binding factor
FHV	*Flock house virus*
GFP	Green fluorescent protein
GRAB	Geminivirus RepA binding
GUS	Beta-glucuronidase
HA	Peptide sequence from influenza hemagglutinin protein (epitope tag)
HCV	*Hepatitis C virus*
HR	Hypersensitive response
HRT	Hypersensitive response to TCV
HSP	Heat shock protein
IAA	Indole-3-acetic acid
JA	Jasmonic acid
MeJA	Methyl jasmonate
MP	Movement protein
NAC	NAM, ATAF, and CUC transcription factors
NAM	No apical meristem
NLS	Nucleus localization signal
Nt	*Nicotiana tabacum* (tobacco)
OA	Origin of assembly
ORF	Open reading frame
Os	*Orzya sativa* (rice)
P. syringae	*Pseudomonas syringae*

PAGE	Polyacrylamide gel electrophoresis
PAPK	Plasmodesmata associated protein kinase
PCD	Programmed cell death
PD	Plasmodesmata
PME	Pectin methylesterase
PR	Pathogenesis-related
PTGS	Post transcriptional gene silencing
PVX	*Potato virus X*
RdRp	RNA-dependent RNA polymerase
REn	Replication enhancer
RF	Replicative form
RI	Replicative intermediate
RNP	Ribonucleoprotein
Rx	R protein conferring resistance to potato virus X
SA	Salicylic acid
SAM	Shoot apical meristem
SAR	Systemic acquired resistance
SEL	Size exclusion limit
SDS	Sodium dodecyl sulphate
SF	Super family
SFV	*Semliki forest virus*
Sl	*Solanum lycopersicum* (tomato)
TAR	Transcription activation region

TCV	*Turnip crinkle virus*
T-DNA	Transferred DNA
TEV	*Tobacco etch virus*
TLS	tRNA-like structure
TIP	TCV-interacting protein
TMV	*Tobacco mosaic virus*
ToMV	*Tomato mosaic virus*
TOM	Tobamovirus multiplication
TRV	*Tobacco rattle virus*
TYMV	*Turnip yellow mosaic virus*
UPD	Upstream pseudoknot domain
UTR	Untranslated region
VIGS	Virus-induced gene silencing
VRC	Virus replication complex
WDV	*Wheat dwarf geminivirus*
WT	Wild-type

Chapter 1: General Introduction

1.1 Statement of Purpose

Positive-stranded RNA viruses encompass over one-third of all virus genera and include numerous pathogens that cause disease in plants, animals and humans. Within plant systems, virus infections cause significant damage to economically important crops (Matthews, 1991). Developing better strategies for controlling viral infection requires a better understanding of how viruses utilize host systems to promote their own replication cycles. The goal of this study is to utilize *Tobacco mosaic virus* (TMV) as a model system to analyze the process of virus replication complex (VRC) assembly in addition to the possible ways viruses have developed to evade host defense system.

Despite different genome organizations, all positive-stranded RNA viruses form membrane-associated VRCs in host cells and share common replication mechanisms. Thus, identifying components of the TMV VRCs and their structures are critical to expanding our knowledge of virus replication. The TMV replicase protein has been shown to be a major component in the formation of membrane-associated VRCs. Additionally, the helicase domain within the replicase proteins is capable of self-interacting, and disruption of the interaction abolishes viral replication. This suggests that helicase-helicase interaction plays an important role in directing the formation of the whole VRC. In this study, structural, biochemical, and cell biology-related approaches have been used to characterize the structure-function relationship

of the TMV helicase domain and the role of this domain in modulating the assembly of the VRCs.

Upon virus infection, a susceptible host generally develops localized and systemic disease symptoms that mostly associate with alterations of host gene expression, disruption of plant hormone metabolism, and modifications of the host proteins (for review, see Culver and Padmanabhan, 2007). The ability of a virus that encodes limited amount of genetic information capable of inducing extensive host responses shows that the virus has evolved complicated mechanisms of communication with its host. Characterizing specific virus-host interactions that contribute to a successful viral invasion provides a better view on how viruses utilize and modify the cellular resources to suit their own needs and on how the host reacts to cope with the intrusion. In this study, a novel stress-inducible plant transcription factor is identified to interact with the helicase domain of the TMV replicase proteins and further characterization of this interaction using genomic and cell biology-oriented approaches shows that this interaction plays an important regulatory role on suppressing host defense systems. Insights gained from this study add to our knowledge of the molecular network of virus-host interactions underlying viral pathogenesis. Ultimately this information will be useful in the development of new anti-viral therapies.

1.2 Literature Review

1.2.1 Tobacco mosaic virus (TMV)

Over a century ago, a causative agent of tobacco mosaic disease was found capable of passing through filters that are impermeable to bacteria. This filterable agent described by M. W. Beijerinck as a *contagium vivum fluidum* is now well known as *Tobacco mosaic virus* (TMV) (Beijerinck, 1898). Being the first identified virus in history, TMV has led pioneering research on various aspects of biology, including structural biology, genetics, biochemistry, cellular biology, biotechnology, immunology, and plant pathology.

TMV is the type species of the genus Tobamovirus and a member of the alphavirus-like superfamily of positive-strand RNA viruses. TMV infects tobacco and other members in the *Solanaceae* family. A vector is not required for TMV to spread from plant to plant. The virus is transmitted by physical contact and mechanical wounding of the leaves. Disease symptoms associated with TMV infection include stunting, necrosis, leaf curling and a characteristic mosaic pattern of intermingled light and dark green patches on leaf tissue.

TMV particles are rigid rods approximately 300 nm in length and 18 nm in diameter. Each particle consists of a single-stranded viral RNA surrounded by 2130 coat protein subunits. The monopartite genome of TMV is approximately 6.4 kb and contains an mRNA-like 5'-cap structure (7-methylguanosine triphosphate, m^7Gppp) and an aminoacylated tRNA-like 3'-terminal structure (Rietveld *et al.*, 1984; Zimmern, 1975). TMV RNA genome contains four open reading frames (ORFs) which encode a 126-kDa and a 183-kDa replicase protein, a 30-kDa movement protein

(MP) and a 17.5-kDa coat protein (CP) (Fig. 1.1) (Goelet *et al.*, 1982). Both the 126-kDa and the 183-kDa protein are translated from genomic RNA with the same 5'-proximal initiation codon and are required for efficient viral RNA replication (Ishikawa *et al.*, 1986; Lewandowski and Dawson, 2000). Both MP and CP are translated from subgenomic (sg) RNAs derived from the 3'-ends of the viral RNAs and are dispensable for viral replication. The MP is involved in virus cell-to-cell movement (Deom *et al.*, 1987; Meshi *et al.*, 1987) and the CP is essential for assembly of virions which are required for systemic long-distance movement (Dawson *et al.*, 1988; Ding *et al.*, 1996; Saito *et al.*, 1990).

TMV-encoded proteins

126- and 183-kDa replicase proteins

TMV 126- and 183-kDa replicases are multi-domain and multi-functional proteins. The 183-kDa protein is produced by translational read-through of an amber stop codon of the 126-kDa ORF. Both 126- and 183-kDa proteins contain an N-terminal methyltransferase domain (MT), an intervening region (IR) and a helicase-like domain (HEL) (Fig. 1.1). The read-through portion of the 183-kDa protein contains a motif typical of RNA-dependent RNA polymerase (POL) activity (Fig. 1.1) (Pelham, 1978). The MT domain of the replicase proteins possesses virus-specific methyltransferase and guanylyltransferase activities and is responsible for viral RNA capping, which protects the genomic RNAs from cellular 5' exonucleases and is also involved in cap-dependent translation (Dunigan and Zaitlin, 1990). The HEL domain has been shown to able to unwind double-stranded RNAs *in vitro*, thus playing an

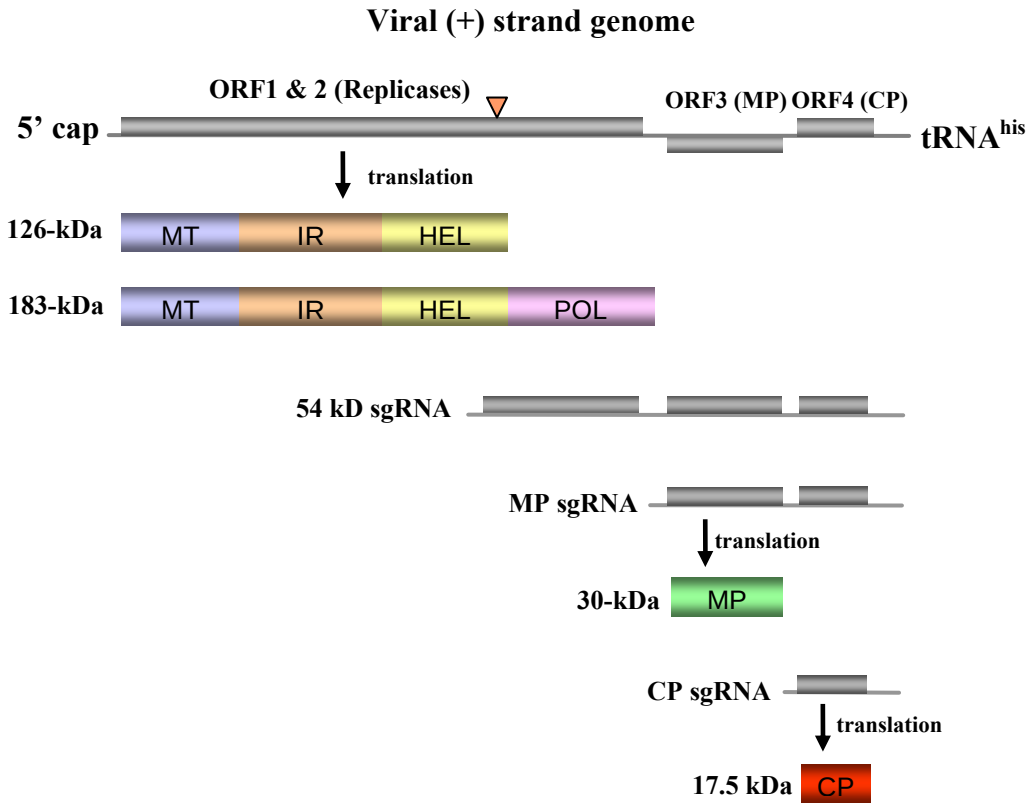


Fig. 1.1. TMV genome organization and expression strategies. Single stranded RNA genome of TMV with highly structured elements in the 5' and 3' UTRs is shown on top. Two replicase proteins are produced directly from the genomic RNA. The triangle indicates the position of the leaky amber stop codon at the end of the TMV ORF1. MP and CP are translated from subgenomic mRNAs. Both genomic and subgenomic RNAs are illustrated by gray boxes. Green and red boxes represent TMV MP and CP, respectively. Domains within the replicase proteins are shown in purple (MT), orange (IR), yellow (HEL), and pink (POL). MT, methyltransferase; IR, intervening region; HEL, helicase; POL, polymerase; MP, movement protein; CP, coat protein.

important role in virus replication (Goregaoker and Culver, 2003). Studies on the TMV HEL suggest that it plays multiple roles in mediating virus infection and host responses. The structural and functional complexity of this domain and its involvement in the virus life cycle will be discussed in detail in the following section. The exact function of IR is not well characterized. Individual amino acid substitutions within the IR region alter the disease symptoms displayed in systemically infected leaves, suggesting a role for the IR region in symptom modulation (Bao *et al.*, 1996).

In TMV-infected plant tissues, the 126-kDa protein is expressed ten times more abundantly than the 183-kDa protein (Lewandowski and Dawson, 2000; Watanabe *et al*., 1999). The reason for the presence of excess 126-kDa proteins is not clear yet. When the amber stop codon of the 126-kDa protein is altered to code for tyrosine, only the 183-kDa replicase protein is produced and viral replication efficiency is dramatically reduced ten fold, suggesting a regulatory role for the excess 126-kDa proteins in TMV replication (Ishikawa *et al.*, 1986; Lewandowski and Dawson, 2000). Both the 126- and 183-kDa proteins can be detected from membrane-associated complexes isolated from TMV-infected plant tissues (Watanabe *et al*., 1999). These crude membrane complexes have been shown to synthesize both TMV genomic and subgenomic RNAs in a template-dependent manner *in vitro*. RNA synthesis activities are greatly inhibited by pre-incubation with antibodies against various portions of the 126-kDa or the POL domain of the 183-kDa proteins, suggesting that both replicase proteins are actively involved in virus replication (Osman and Buck, 1996). *Brome mosaic virus* (BMV) 1a protein, which is analogous to the TMV 126-kDa protein, recruits viral RNA templates for replication by binding

to the tRNA-like structure at the 3' terminus (Chen *et al.*, 2001). Similar to BMV 1a protein, the TMV 126-kDa protein is shown to bind to the viral RNA 3' terminal region (TR) in an *in vitro* cross-linking study, whereas the 183-kDa fails to do so (Osman and Buck, 2003). This research suggests that the 126-kDa replicase may function as a bridge connecting the catalytic domain (POL) of the 183-kDa protein to the viral RNA template.

Besides the replication activities, the 126- and 183-kDa replicases were shown to be involved in the virus cell-to-cell movement (Hirashima and Watanabe, 2001; Hirashima and Watanabe, 2003). This result, together with co-localization of the 126-kDa protein and MP to the ER membrane, indicated that TMV replicases and MP are functionally linked. Wu and Shaw (1997) demonstrated that the virus particles containing mutant viral RNAs defective in producing the 126- and 183-kDa replicases failed to disassemble in the 3'-to-5' direction, suggesting the involvement of the replicase proteins in viral particle disassembly. Furthermore, the 126-kDa protein of TMV has been shown to serve as a suppressor of the host post-transcriptional gene silencing (PTGS) defense system (Ding *et al.*, 2004).

TMV movement protein (MP)

Most plant viruses encode one or more nonstructural MPs that facilitate the transport of progeny viral genomes through plasmodesmata (PD), intercellular channels consisting of extended plasma membrane and ER. TMV MP is the most extensively studied protein involved in viral cell-to-cell movement. In general, PD are specialized narrow channels that only allow passage of small molecules, such as

water, nutrients, and metabolites (Lucas and Wolf, 1993). Virus MP, however, has the ability to increase the size exclusion limit (SEL) of PD (Wolf *et al.*, 1989). The exact mechanism on how MP triggers the PD channel dilation is not clear. Evidence including MP localizing to PD in infected leaf tissues (Atkins *et al.*, 1991; Ding *et al.*, 1992) and the greater SEL of PD measured from transgenic plants constitutively expressing TMV MP (Wolf *et al.*, 1989) suggest that MP-directed modification of PD is involved in the intercellular trafficking of viral RNAs. An *in vitro* study showed that TMV MP is capable of binding to single-stranded RNA to form viral RNA-protein (vRNP) complexes (Citovsky *et al.*, 1990; Citovsky *et al.*, 1992). Visualization of these vRNP complexes with electron microscopy revealed unfolded and elongated tubular-like structures, which are compatible in size for the MP-modified PD (Citovsky *et al.*, 1992). Other than facilitating vRNA transport intercellularly, TMV MP also actively recruits host components to mediate translocation of vRNAs intracellularly, from replication site to PD. A double-labeling fluorescence microscopy study has shown that MP co-aligns with microtubules (MTs) and disruption of MTs affects distribution of MP in protoplasts (Heinlein *et al.*, 1995; Heinlein *et al.*, 1998). TMV MP was also observed to bind to actin *in vitro* (McLean *et al.*, 1995). Association of MP with cellular MTs and microfilaments suggests the involvement of cytoskeleton structures in MP-mediated viral RNA transport. A host cell wall protein, pectin methylesterase (PME) has also been identified to interact with TMV MP (Chen *et al.*, 2000; Dorokhov *et al.*, 1999). Since disruption of PME-MP binding results in the inactivation of TMV cell-to-cell spread, this host protein may play an important role in directing and anchoring the vRNP to PD or it may be

involved in MP-dependent modification of the PD (Chen *et al.*, 2000). Taken together, MP-associated virus cell-to-cell movement through plasmodesmata is a highly coordinated process that requires participation of both virus and host components.

During the course of infection, MP accumulates in the early stages of infection, whereas in late stages, the amount of MP decreases (Lehto *et al.*, 1990a). Addition of the 26S proteosome inhibitors into the TMV-infected protoplasts leads to the accumulation of ubiquitinylated MP (Reichel and Beachy, 2000). The depletion of MP at late stages of infection may help to recycle the host membrane system, such as ER, and elements of cytoskeleton to promote a complete virus life cycle. MP is also observed to be phosphorylated *in vitro* by a cell-wall protein kinase (Citovsky *et al.*, 1993) and *in vivo* by a plasmodesmal-associated protein kinase (PAPK) (Lee *et al.*, 2005). The exact function of MP phosphorylation is unknown and controversial. Mutational analysis implies the involvement of MP phosphorylation in controlling protein stability (Kawakami *et al.*, 1999). However, other studies suggest it affecting the SEL of PD (Waigmann *et al.*, 2000). Moreover, phosphorylation of MP *in vitro* abrogates the ability of the protein to repress translation of vRNA, suggesting a regulatory role for MP phosphorylation in controlling viral life cycle (Karpova *et al.*, 1999).

TMV coat protein (CP)

TMV CP is a structural protein translated from a subgenomic mRNA. CP subunits interact with each other extensively to form a cylindrical helical structure that

functions as a protective shell for the viral genomic RNA. For a century, TMV CP has served as a model system to study macromolecular self-assembly and protein-RNA interactions. Using an X-ray diffraction method the structure of TMV CP has been resolved at 2.9Å resolution as a four-helix bundle with two pairs of alpha-helices connected by a loop structure (Namba *et al.*, 1989). Depending on pH and ionic strength in solution, TMV CP aggregates into different forms, including a 4S trimer/pentamer/monomer mixture, a 20S bilayer disk with 17 CP molecules in each layer, and a virus-like helical aggregate (Durham *et al.*, 1971; Schuster *et al.*, 1980). At pH 7.0 and 20ºC, 4S and 20S proteins exist in an equilibrium mixture in solution and both are thought to contribute to virion assembly. Formation of 20S aggregates is more important due to the requirement for disks to initiate virus assembly. In addition, a recent study suggests the involvement of 20S aggregates in CP-mediated resistance (CP-MR) (Asurmendi *et al.*, 2007).

CP-MR was first described as a phenomenon that transgenic plants expressing self-assembling CP conferred certain levels of resistance against infection by TMV and other related Tobamoviruses (Abel *et al.*, 1986). It is observed that accumulation of CP but not CP non-translated mRNA confers resistance (Powell *et al.*, 1989). Furthermore, CP-MR is effective against infection by TMV, but not TMV-RNA, indicating that presence of CP interferes with proper disassembly of challenging viral particles, thus inhibiting virus replication and spreading (Nelson *et al.*, 1987; Register and Beachy, 1988).

Although TMV CP does not play a role in virus cell-to-cell movement, it is indispensable for virus long-distance movement through phloem, the plant's vascular

tissues. Mutant viruses defective in CP assembly have no effect on TMV cell-to-cell spreading, but fail to move systemically indicating that proper assembly of TMV is required for viral RNA trafficking over long-distance (Dawson *et al.*, 1988). A study on another closely related Tobamovirus, *Cucumber green mottle mosaic tobamovirus* (CGMMV), showed that only packaged virions but not free RNAs could be detected in infected phloem (Simon-Buela and Garcia-Arenal, 1999). This result confirms the role of CP assembly in viral long-distance movement and also suggests that no replication activities occur inside the phloem during the whole transport process. However, the exact mechanism on how CP promotes virus movement via phloem remains unknown.

Putative 54-kDa and 4.8-kDa proteins

Another subgenomic RNA, termed I_1 RNA, corresponding to the polymerase domain can be detected from TMV-infected tobacco tissues and it contains an ORF encoding for a putative 54-kDa protein (Fig. 1.1) (Sulzinski *et al.*, 1985). Despite the fact that the expression of this protein has never been detected *in vivo*, double-stranded RNA with a size corresponding to the duplex I_1 subgenomic RNA has been observed (Palukaitis *et al.*, 1983; Zelcer *et al.*, 1981). In addition, plants expressing the 54-kDa plus-sense gene, but not its antisense RNA, exhibit complete resistance to TMV infection, suggesting that this gene or maybe its encoded putative protein negatively interferes with the virus accumulation (Golemboski *et al.*, 1990).

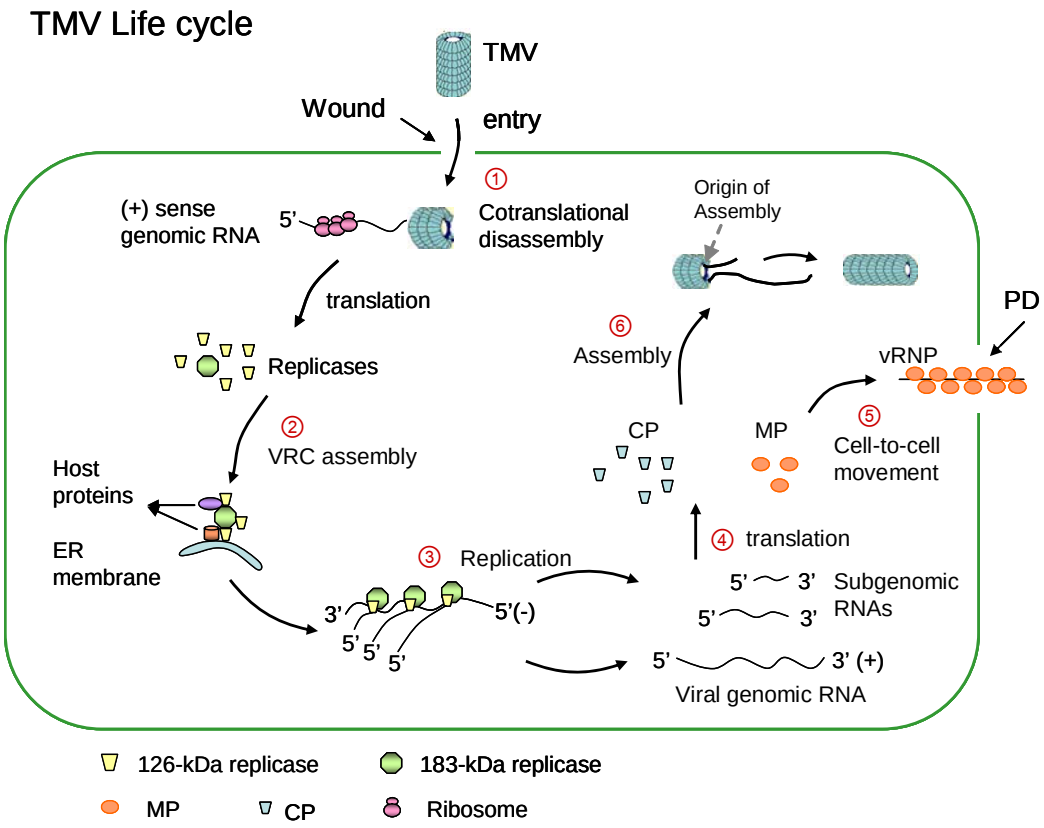
A sixth ORF encoding a 4.8kDa protein has been recently described to be present in the genome of TMV (Canto *et al.*, 2004). This small protein overlaps the C

terminus of the MP and the N terminus of the CP. Elimination of ORF6 by changing its AUG start codon to ACG resulted in reduced virulence of the virus (Canto *et al.*, 2004). In addition, *in vitro* expressed 4.8-kDa protein is shown to bind strongly to cellular translation factor eEF1A (Morozov *et al.*, 1993). Whether eEF1A association has an effect on the pathogenicity of the 4.8-kDa protein remains unknown.

TMV virus life cycle

The viral life cycle starts when the virus particle enters the host cells through wounds (Fig. 1.2). Although TMV particles are highly stable in the environment, an efficient uncoating process takes place in the host cytoplasm, where concentrations of calcium ions and protons are relatively low. Depletion of these positively charged ions from carboxyl-carboxylate and carboxyl-phosphate pairs between adjacent CP subunits and between CP and viral RNA results in electrostatic repulsive forces that trigger the destabilization of the virus particles. It is noted that the interaction between CP subunits and genomic RNA at the 5' end is relatively weaker due to lack of guanine residues in the 69-nucleotide 5'-leader sequence. When treating TMV particles in a cellular-like mild alkali or detergent condition, the first 200 nucleotides at the 5' end of the genome is exposed, suggesting that the TMV *in vivo* disassembly starts from the 5' end of TMV genome (Mundry *et al.*, 1991). Uncoating of the 5'-leader sequence releases the first start codon of the 126- and 183-kDa protein. Cellular ribosomes recognize the start codon and initiate translation of the messenger-sense genomic RNA. Simultaneously, ribosome binding to and scanning through the

Fig. 1.2. TMV life cycle. The first stage of the infection cycle involves viral entry into the plant through wounds followed by the release of plus-sense genomic RNA via a cotranslational disassembly mechanism (1). Translation of the genomic RNA produces 126- and 183-kDa replicase proteins important for the assembly of the ER-associated VRCs (2), where virus replication occurs (3). Replication process involves the synthesis of minus-sense genomic RNA, which then serves as a template for the synthesis of plus-sense genomic RNA and subgenomic RNAs, specific messenger RNAs for translation of the viral structural proteins. Translation of subgenomic RNAs produces viral MPs and CPs (4). MPs bind to viral genomic RNA and direct the formed RNP to the neighboring cells via plasmodesmata (PD) (5). CP subunits encapsidate the viral plus-sense genomic RNA and the new virions are released or transport through phloem to systemic tissues (6).


TMV Life cycle
TMV
Wound
entry
(+) sense
genomic RNA
5'
① Cotranslational
disassembly
Origin of
Assembly
translation
Replicases
PD
⑥
Assembly
vRNP
② VRC assembly
CP
MP
⑤
Cell-to-cell
movement
Host
proteins
ER
membrane
④ translation
③ Replication
5' 3' Subgenomic
RNAs
3'
5'(-)
5'
5' 3' (+)
Viral genomic RNA
126-kDa replicase
183-kDa replicase
MP
CP
Ribosome

viral RNA dislodges more CP subunits at a 5'-to-3' direction. This translation-associated disassembly process is referred as a cotranslational disassembly (Wilson, 1984). The TMV 5'-to-3' disassembly stops when ribosomes reach the termination codons of the 126- and 183-kDa proteins. The newly released replicases are capable of binding to the 3' end tRNA-like structure (TLS) of viral RNA and initiating uncoating of the remaining CP subunits in a 3'-to-5' direction (Wu and Shaw, 1996; Wu and Shaw, 1997). The 3'-to-5' disassembly is coupled with the synthesis of the negative-strand genomic RNA, thus the process is called coreplicational disassembly. The bidirectional disassembly is beneficial to the viruses in that viral RNAs remain protected by CP subunits until they become involved in the replication process.

The next stage of the life cycle is the replication of TMV viral RNAs that includes synthesis of minus-strand genomic-length RNA, which further acts as a template for producing more plus-strand genomic RNAs and subgenomic RNAs (Fig 1.2; for review, see Buck, 1999). Synthesis of minus-strand genomic RNA ceases a few hours after inoculation, whereas plus-strand synthesis continues throughout the entire time of virus infection (Ishikawa *et al.*, 1991). Similar to other positive-stranded RNA viruses, an asymmetric production of TMV genomic RNAs is observed with a hundred fold excess of plus-strand over minus-strand RNAs being produced (Kielland-Brandt, 1974). Two replicative structures, the replicative form (RF) and the replicative intermediate (RI) have been detected in TMV-infected plants (Jackson *et al.*, 1971; Nilsson-Tillgren, 1970) and in an *in vitro* RNA synthesis reaction using TMV-derived RdRp complexes (Osman and Buck, 1996). RF is a duplex genomic-length plus- and minus-strand RNA molecule and RI is a multistranded structure

composed of several nascent plus-strand RNAs complementary to one single minus-strand RNA. The presence of both RF and RI forms of viral RNAs may suggest different mechanisms of synthesizing minus- and plus-strand RNAs. For TMV, both the 5'- and the 3'-end untranslated regions are involved in virus replication. The 5' 69-nucleotide untranslated region (UTR), defined as "Ω" sequence, has been shown to serve as a translational enhancer (Gallie *et al.*, 1987). Takamatsu *et al.* (1991) further demonstrated that a small deletion (nt 2-8) in this region was enough to abolish virus replication. Precisely how this untranslated region affects virus replication remains unknown. The TMV 3' untranslated region containing a TLS and three upstream pseudoknot domains (UPDs) has been demonstrated to act as a *cis*-acting element for virus replication. Deletion or disruption of one pseudoknot structure just upstream of the TLS completely abolished synthesis of viral genomic RNAs, suggesting an important regulatory role of this region in virus replication (Takamatsu *et al.*, 1990). Synthesis of subgenomic RNA is initiated by replicase proteins binding to the internal promoters on negative-strand RNA template. MP is expressed at the early stage of infection but CP is expressed late and it is believed that the internal promoters control the timing of protein expression. It was shown that expression of MP under the control of the CP subgenomic promoter led to late expression (Lehto *et al.*, 1990b).

To establish a successful infection, the newly synthesized genomic RNAs need to be transported from the virus entry site to other parts of a host plant. The TMV MP mediates virus local movement and has been identified to contain both the nucleic acid- and NTP-binding domains (Citovsky *et al.*, 1990). Li and Palukaitis (1996) suggested that the MP-mediated transportation of vRNP is energy-dependent requiring

GTP hydrolysis. The last stage of the cycle involves the assembly of viral particles and the transportation of the progeny virions to uninfected systemic tissues. Encapsidation of the TMV viral RNA starts at a unique sequence of nucleotides (nts 5444-5518) called origin of assembly (OA) that forms a hairpin configuration (Fig. 1.2) (Zimmern, 1977). Insertion of the hairpin-loop OA into the central hole of the initiating 20S CP disk stabilizes the nucleoprotein complex and allows the elongation proceed. It is proposed that the elongation process occurs in both directions by sequentially adding 20S disks to the 5' longer tail and the 4S A protein to the 3' smaller tail (Lebeurier *et al.*, 1977). Consistent with this assumption, the elongation process along the shorter RNA tail has been observed to occur much slower than assembly of 5'longer tail.

1.2.2 Virus Replication Complexes (VRCs)

Although diverse in morphology, all known positive-strand RNA viruses of plants and animals form membrane-associated VRCs that play an essential role for viral genome replication and transcription (for reviews, see Buck, 1996; van der Heijden and Bol, 2002). Formation of membrane-associated VRCs is beneficial to viruses in multiple ways, including increasing the available surface area for RNA synthesis, concentrating the viral RNAs and viral proteins by creating a membrane compartment, and supplying a less hostile environment for replication. In a membrane floatation assay of *Hepatitis C virus* (HCV)-infected cell culture, RNA synthesis can only be detected in the membrane-bound fractions containing viral nonstructural proteins, but not in the soluble fractions, suggesting the membrane-bound complex is

an active form for enzymatic activity (El-Hage and Luo, 2003). Depletion of membranes from tobacco protoplasts leads to failure of the synthesis of *Tomato mosaic virus* (ToMV) related RNAs even though the translation of the viral 130- and 180-kDa replicase proteins is not affected (Komoda *et al.*, 2007). This study suggests that the membranes are not necessary for viral protein translation, but are required in initiation of ToMV RNA synthesis.

For different viruses, distinct intracellular membranes are involved in assembling VRCs, such as ER membrane for TMV, BMV, and *Tobacco etch virus* (TEV) (Más and Beachy, 1999; Restrepo-Hartwig and Ahlquist, 1999; Restrepo-Hartwig and Ahlquist, 1996; Schaad *et al.*, 1997); mitochondrial membrane for *Flock house virus* (FHV) (Miller *et al.*, 2001); chloroplast outer membrane for *Turnip yellow mosaic virus* (TYMV) (Prod'homme *et al.*, 2001); vacuolar membrane for *Alfalfa mosaic virus* (AMV) (Van Der Heijden *et al.*, 2001); and the lysosomal or endosomal membranes for *Semliki forest virus* (SFV) (Kujala *et al.*, 2001). For polioviruses, the entire secretory apparatus including ER, trans-Golgi, and lysosome is involved in formation of VRCs (Schlegel *et al.*, 1996). Although some viruses utilize the same host membrane system for VRC assembly, the underlying mechanisms are quite different. For example, both TEV and *Cowpea mosaic virus* (CPMV)-induced membrane vesicles have proven to originate from the ER. TEV is able to alter the preexisting cortical ER network for assembly of its own VRCs (Schaad *et al.*, 1997). CPMV, on the other hand, requires the proliferation of the new ER membranes (Carette *et al.*, 2000). The diversity of virus-induced membrane association indicates that viruses have evolved specific ways to assemble VRCs in the host. Interestingly,

Schwartz *et al*. (2002) have shown that the VRCs formed by BMV reassemble the replicative cores of retrovirus and dsRNA virus, suggesting that viruses from diverse families are possibly evolutionarily related.

Upon infection by TMV, dramatic morphological changes of the ER membrane are observed in transgenic *Nicotiana benthamiana* plants expressing GFP in the ER (Reichel and Beachy, 1998). At early stages of infection, the tubular ER network collapses into aggregated inclusion bodies, which convert back to tubular like structures at the late stage of infection (Reichel and Beachy, 1998). It is interesting to note that TMV-associated morphological changes of the ER resemble the changes of the ER induced by TEV, suggesting that one strategy for VRC assembly can be applied by different virus species (Schaad *et al.*, 1997). TMV viral RNAs, when labeled with fluor-RNA probe, are observed to co-localize with an ER resident protein, Bip, indicating that VRCs assembled on the ER membrane serve as actual "viral factories" (Más and Beachy, 1999). Detection of the RdRp activities from the membrane-bound TMV replicase complexes, but not the non-membrane-bound replicases, confirms the functional importance of cellular membranes in TMV virus replication (Hagiwara et al., 2003; Nishikiori et al., 2006). In addition to the ER membrane, increasing evidence suggest that the cytoskeleton elements, such as microtubules (MTs) and microfilaments (MFs), are associated with TMV-induced VRCs (Heinlein *et al.*, 1998; Liu *et al.*, 2005; Más and Beachy, 1999). These elements are postulated to facilitate the intracellular trafficking of newly synthesized viral RNAs (Heinlein *et al.*, 1998; Liu *et al.*, 2005; Más and Beachy, 1999). Using

confocal laser scanning microscopy, it was observed that TMV could spread intracellularly and intercellularly as intact VRCs (Kawakami *et al.*, 2004).

Involvement of viral components in VRC formation

Although little is known about the mechanisms responsible for virus-directed VRC assembly, virus-encoded proteins are recognized to play key roles in modulating VRC formation. A subset of nonstructural viral proteins, when expressed in isolation, has been shown to induce membrane vesiculation similar to those formed in virus-infected cells. Examples include the BMV 1a protein (Schwartz *et al.*, 2002), the TEV 6 kDa protein (Schaad *et al.*, 1997), and the poliovirus 2BC protein (Cho *et al.*, 1994; Suhy *et al.*, 2000). Of these nonstructural viral proteins studied, many have been reported to contain hydrophobic transmembrane domains (Schaad *et al.*, 1997; Schmidt-Mende *et al.*, 2001; Towner *et al.*, 1996). Deletion of the hydrophobic domain from the TEV 6 kDa protein disrupted its membrane-binding activity and further affected membrane vesiculation in the host (Schaad *et al.*, 1997).

Association of viral proteins with cellular membranes leads to a range of membrane morphologies in host cells. For instance, the poliovirus 3A protein is able to dramatically induce the swelling of the ER membrane (Doedens *et al.*, 1997) and the expression of the BMV 1a protein alone leads to membrane invagination to form spherules (Schwartz *et al.*, 2002; Schwartz *et al.*, 2004). It is noteworthy that although some viral proteins do not associate with the host membrane directly, they play a regulatory role in VRC formation. Schwartz *et al*. (2004) showed that overexpression

of BMV $2a^{pol}$, a 1a interacting protein, led to membrane rearrangement from vesicle spherules to large stacks of double-membrane layers.

Expression of the 126-kDa replicase protein in the presence or absence of other viral components, has been shown to associate with the ER membrane (dos Reis Figueira *et al.*, 2002) and form irregular shaped cytoplasmic bodies (Liu *et al.*, 2005). Isolation of membrane-bound complexes from TMV infected tissues has been achieved by a sucrose density gradient centrifugation (Osman and Buck, 1996) or an immunoaffinity purification using antibodies against domains within the replicase proteins (Watanabe *et al*., 1999). In both methods, 126- and 183-kDa proteins are present within the membrane-bound complexes yielding RdRp activity, indicating that both replicase proteins are major components of the VRC (Osman and Buck, 1996; Watanabe *et al.*, 1999). Although TMV MP is not essential for virus replication, co-localization studies suggest that MP is also associated with VRCs (Heinlein *et al.*, 1998; Liu *et al.*, 2005; Más and Beachy, 1999). A recent study identified two major hydrophobic transmembrane domains of MP and suggested a possible role of MP in anchoring viral replicase proteins to the ER membrane (Fujiki *et al.*, 2006). The involvement of TMV CP in VRC formation is unknown. Transgenic plants expressing a mutant CP affects the MP accumulation, which further inhibits the formation of VRCs (Asurmendi *et al.*, 2004). This result suggests that CP may play a regulatory role in the assembly of TMV VRCs in the host via its effect on MP expression.

Involvement of host proteins in VRC formation

Although host proteins have long been recognized to perform essential functions in virus replication, their roles in assembly of active VRCs are poorly understood. Since host membranes are required in replication of positive strand RNA viruses, it is reasonable to speculate that integral membrane proteins would participate in the formation of membrane-associated VRCs. A SNARE-like protein, hVAP-A has been shown to interact with two HCV nonstructural proteins, NS5A (Tu *et al.*, 1999) and NS5B (Shi *et al.*, 2002). The interaction between NS5A and hVAP-A is postulated to be involved in targeting the viral proteins to the membrane during assembly of replicase complexes (Tu *et al.*, 1999). Similarly, two host membrane integral proteins, TOM1 and its homolog TOM3, have been identified to be necessary for efficient replication of Tobamoviruses in Arabidopsis (Yamanaka *et al.*, 2002; Yamanaka *et al.*, 2000). TOM1 interacts with the helicase domain of the TMV replicases in a yeast SOS recruitment system (Yamanaka *et al.*, 2002; Yamanaka *et al.*, 2000) and can be co-fractionated with the membrane-bound replicases (Hagiwara *et al.*, 2003), suggesting that TOM proteins may function as tethers to recruit the TMV replicase proteins to specific membranes. The assembly of poliovirus VRCs is mediated by the COPII proteins, which induce vesicles in uninfected cells on the ER for protein transport (Rust *et al.*, 2001). In addition, several lines of evidence suggest that cellular protein kinases may play a regulatory role on the formation of VRCs. *Cucumber mosaic virus* (CMV)-encoded 1a and 2a proteins interact with each other and disruption of the interaction by 2a phosphorylation inhibits new replication complex formation (Kim *et al.*, 2002). Overall, combined results indicate that host

proteins are actively involved in VRC assembly. However, the molecular mechanism by which they function in VRC structural and functional activities remains for the most part, unknown.

1.2.3 Helicases

Helicases are ubiquitous proteins found in almost all living organisms, including eukaryotes, yeast, bacteria, and virus. In general, they are molecular motors that function in unwinding double stranded nucleic acid by disrupting the hydrogen bonds between base pairs using the energy of NTP hydrolysis. Helicases can be grouped into RNA helicase or DNA helicase based on their substrate specificity. DNA helicases are essential in DNA replication, recombination, transcription, and repairing. RNA helicases modulate the RNA structures and are involved in all aspects of RNA metabolism, such as transcription (Walstrom *et al.*, 1997), translation initiation (Chuang *et al.*, 1997; Rozen *et al.*, 1990), RNA splicing (Raghunathan and Guthrie, 1998; Staley and Guthrie, 1999), ribosome assembly (Nicol and Fuller-Pace, 1995), RNA editing (Missel *et al.*, 1997), and RNA degradation (Py *et al.*, 1996). Although helicases are involved in various cellular processes and have different substrate specificities, there is high conservation within their sequences, suggesting that they are in a close evolutionary relationship. Based on their amino acid sequence similarities, helicases can be classified into three distinct superfamilies (SF1, SF2, and SF3) (Gorbalenya *et al.*, 1989; Gorbalenya *et al.*, 1990). Both superfamilies 1 and 2 encompass a large number of helicases and each contain seven conserved motifs (designated I, Ia, II, III, IV, V, and VI) (Fig. 1.3). Except for motif III and IV, other motifs are quite similar in sequences and arrangements suggesting that they may share

similar structural and functional activities. Helicase SF2 can be further classified into three subfamilies based on variations in motif II (DEAD, DEAH, and DexH). Members within superfamily 3 (SF3) are putative helicases found in DNA and RNA viruses. Only three characteristic motifs are identified in SF3 helicases (Fig. 1.3).

Crystal structural analyses on members within SF1 (*E. coli* Rep and *Bacillus stearothermophilus* PcrA) (Korolev *et al.*, 1997; Subramanya *et al.*, 1996) and SF2 (HCV NS3) (Kim *et al.*, 1998; Yao *et al.*, 1997) reveal that helicase proteins share a very similar tertiary structure. Their helicase motifs are especially arranged in a similar spatial pattern by clustering together at the interface between domains to form both the nucleotide and the nucleic acid binding pockets (for review, see Caruthers and McKay, 2002). The structural conservation of the helicase motifs suggests their functional significance. Motif I and II (also known as Walker A and Walker B) are well identified and involved in NTP binding, Mg^{2+} chelating, and NTP hydrolysis. Both motifs are found to be conserved within all three helicase superfamilies. Mutations in either of the motifs abolish the NTP hydrolysis activity as well as the helicase activity of the proteins (Gross and Shuman, 1995; Pause and Sonenberg, 1992). Motifs Ia and IV are the least known helicase motifs within helicase SF1 and SF2 and no specific function has been determined yet. Motif III shows some level of divergence in its sequences between SF1 and SF2. A mutagenesis study of PcrA helicase, a DNA helicase within SF1, exhibits conserved residues within motif III that affect both ATP hydrolysis and single-stranded DNA binding (Dillingham *et al.*, 1999). This result suggests a potential role of helicase motif III in mediating the ATP-

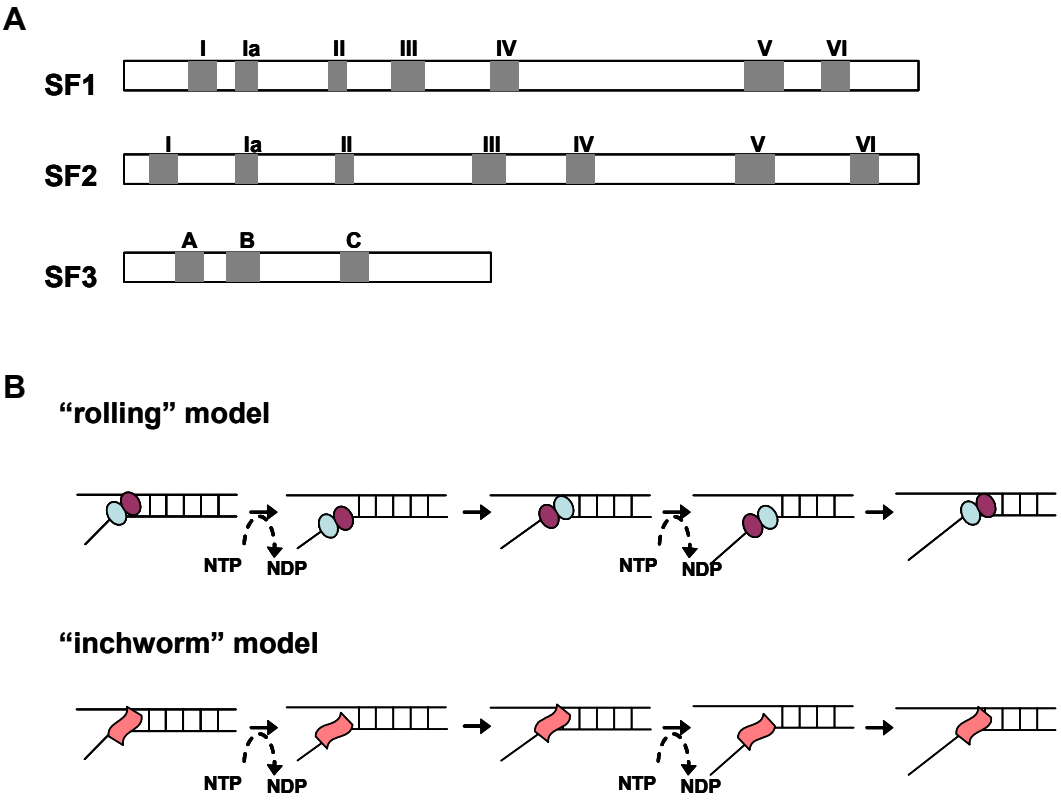


Fig. 1.3. Schematic diagrams showing conserved helicase motifs and proposed models for the helicase unwinding activity. (A) The helicase SF1 and SF2 enzymes contain seven conserved motifs and members of the SF3 contain three motifs. Gray boxes represent well conserved helicase motifs. The motif sizes and the relative positions are approximate. (B) "Active rolling" versus "inchworm" models for the helicase activity. The rolling model describes that helicase subunits which are colored differently bind alternatively to double-stranded and single-stranded nucleic acids. In the inchworm model, the monomeric enzyme contains two binding sites that simultaneously bind to single-stranded and double-stranded nucleic acids. In both models, NTP hydrolysis is coupled to duplex destabilization and unwinding. The step sizes of enzyme translocation are not shown.

dependent DNA-binding activity. However, whether motif III within SF2 helicases plays a similar role needs to be determined. To date, the role of motif V in nucleic acid unwinding is controversy. One structural analysis shows that residues in motif V of Rep make direct contacts with the bound nucleic acid (Korolev *et al.*, 1997). However, point mutations within motif V of the cylindrical inclusion (CI) RNA helicase from *Plum pox potyvirus* (PPV) disrupt only ATP hydrolysis, but have no effect on RNA binding activity (Fernández *et al.*, 1997). Helicase motif VI contains a group of basic residues, thus is predicted to be involved in nucleic acid binding. Mutations within this motif show reduced activity in nucleic acid binding, as well as defects in NTP hydrolysis. A high-resolution structural analysis of PcrA (Velankar *et al*., 1999) together with a mutagenesis study of eIF4A, a RNA helicase within helicase SF2 (Pause *et al*., 1993) reveal that one residue, arginine, within motif VI interacts with the nucleotide. Upon binding to the nucleotide, the protein undergoes a structural conformational change, which is suggested to play a role in transducing the energy into the separation of the nucleic acid. Overall, all the conserved helicase motifs play important roles in enzymatic activities. The structural analyses further suggest that they most likely function in a coordinated way, rather than being seven independent functional domains.

It has been reported that some helicase proteins function as monomers, and the others assemble into oligomers, including dimers or ring-like hexamers (for review, see Patel and Picha, 2000). Some proteins may require accessory proteins for their helicase activity, such as helicase eIF4A needs eIF4B for stabilizing the eIF-4A/mRNA complex (Méthot *et al.*, 1994; Rozen *et al.*, 1990). Currently, two models

have been proposed to describe the helicase mechanism, the "inchworm" and the "active rolling" models (Fig. 1.3B) (for reviews, see Bird *et al.*, 1998; Hall and Matson, 1999). The active rolling model requires the protein to be at least a dimeric form. Two protein subunits bind alternatively to double-stranded and single-stranded nucleic acids and the exchange of the substrates for each subunit relies on nucleotide binding and hydrolysis. The inchworm model, however, applies to both monomeric and oligomeric forms of the protein. The model works when the protein slides along the duplex nucleic acid by simultaneously binding to both duplex and single-stranded nucleic acid. Both models support the substantial conformational changes of the protein in response to a cycle of NTP binding, hydrolysis, and product release and both rely on the NTP hydrolysis in separating the duplex nucleic acids. More recently, helicases have been shown to be able to displace proteins form their bound nucleic acid, which certainly broaden our views in understanding the functional versatility of these molecular motors (Byrd and Raney, 2004; Fairman *et al.*, 2004).

Viral RNA helicases

Virus-encoded RNA helicases have been detected in double-stranded DNA viruses as well as in some positive-stranded RNA viruses. During virus infection, helicases play an essential role in unwinding double stranded templates or removing secondary structures from the RNA template. *In vitro* biochemical studies show that there is no absolute RNA sequence requirement for viral RNA helicases, but a 3'-end single stranded extension is needed for initiating unwinding process. Several well characterized viral helicases, including NS3 helicase of HCV (Levin and Patel, 1999),

and the simian virus-encoded large T antigen (Smelkova and Borowiec, 1997), have been shown to be active as oligomers. The vaccinia virus NPH-II RNA helicase, on the other hand, functions in a monomeric form *in vitro* (Shuman, 1992). Similar to cellular helicases, virus-encoded helicases within helicase SF2 contain seven signature motifs. However, two motifs, Ia and IV are missing in members within viral RNA helicases SF1 (for review, see Kadaré and Haenni, 1997). For positive stranded RNA viruses, helicase activity has been described from SF2 members, such as the NS3 of HCV (Gwack *et al.*, 1996; Tai *et al.*, 1996), and the CI of PPV (Fernández *et al.*, 1997; Laín *et al.*, 1990). Information on helicase activity from SF1 helicases is rather limited. BMV-encoded 1a, a close homolog to TMV 126-kDa protein, contains a helicase domain at the C-terminal region. Mutations within 1a helicase motifs disrupt virus replication (Wang *et al.*, 2005). It is interesting to note that, though none of the mutations affect 1a associating with ER membrane and recruiting 2a polymerase to the membrane, some mutations within motif IV or VI disrupt formation of membrane-associated VRCs, suggesting another unidentified, yet biological significant role of viral helicases in the host cell (Wang *et al.*, 2005).

The TMV RNA helicase belongs to helicase SF1 and contain five major conserved motifs (I, II, III, V, and VI). The helicase domain shared by the 126-kDa and 183-kDa replicase proteins performs multiple functions within the host cell, therefore plays an important role in the viral life cycle. An *in vitro* analysis on TMV helicase polypeptide has demonstrated its ATPase activity and double-stranded RNA unwinding activity (Goregaoker and Culver, 2003). Yet, the importance of each helicase motif in protein structure formation, enzyme activity, and viral replication has

not been established in detail. Interactions between TMV helicases and host proteins have been identified and shown to contribute to the formation of an active membrane-associated replicase complex (Hagiwara *et al.*, 2003) as well as implicated in a host disease development (Padmanabhan *et al.*, 2005). The TMV helicase is also able to trigger hypersensitive response (HR) in *N* gene-containing host plants (Erickson *et al.*, 1999). In addition, when the helicase domain of TMV-U1 strain was replaced with the same domain of TMV-R strain, which has a different host range, the resulting chimeric virus was defective in cell-to-cell movement, suggesting that the helicase domain may have a yet unknown role in cell-to-cell movement (Hirashima and Watanabe, 2001).

1.2.4 Virus-Host Interactions in Plants

As an important group of plant pathogens, viruses have long been used to identify the molecular and physiological basis for a host cell reacting to pathogens. A susceptible host generally develops localized and systemic disease symptoms upon virus infection. By contrast, in a resistant plant, viruses induce a rapid, programmed cell death (PCD) at the infection site which further limits the replication and spreading of the viruses. Such a localized defense response is well-known as the hypersensitive response (HR). In both cases, interactions between viruses and hosts are key determinants for the host responses and are present throughout the whole virus life cycle. Progress in the study of virus-host interactions has led to the identification of the transcriptional changes and proteomic modifications of host factors in infected tissues (Geri *et al.*, 1999; Golem and Culver, 2003; Whitham *et al.*, 2003). Yet the

complexity of virus-host interactions has been illustrated (for reviews, see Culver and Padmanabhan, 2007; Nelson and Citovsky, 2005). Active investigations of any specific individual virus-host interactions certainly add our knowledge in understanding the host response mechanisms.

Virus-host interactions in host defense system

Much is known about the plant *R* genes which confer the HR response to the virus. *R* gene products (receptors) recognize the specific ligands (elicitors) derived from the corresponding pathogen and in turn initiate the host defense signaling pathway. Originally, a gene-for-gene concept was proposed to explain *R*-gene mediated host defense. More recently, growing evidence suggests that the HR response is not merely a direct interaction between receptors and elicitors. A 'Guard hypothesis' has been proposed that implies the R protein, considered as a 'Guard', indirectly interacts with the pathogen-derived elicitors (Avr determinants) through a third host factor, called 'Guardee' (Dangl and Jones, 2001; Van der Biezen and Jones, 1998). Guardee proteins undergo a structural change when interacting with pathogen elicitors and guard proteins can perceive this change and further induce the rapid HR response. Several R proteins that confer the resistance to virus infection have been identified. A cellular protein Rx is capable of eliciting the HR response in the presence of the CP of *Potato virus X* (PVX) (Bendahmane *et al.*, 1999). Similarly, HRT (hypersensitive response to TCV) protein reacts to the CP of TCV (Ren *et al.*, 2000). Interestingly, TCV CP also interacts with a host transcription factor, TCV-interacting protein (TIP) and the interaction plays a role in HR response to TCV,

suggesting TIP might serve as a guardee for the *HRT*-mediated host defense (Ren *et al.*, 2000). Tobacco *N* gene, which confers resistance to TMV infection, has been identified (Whitham *et al.*, 1994). The viral protein p50 that contains the helicase domain of the 126-kDa replicase is demonstrated to be an elicitor in the *N*-gene mediated HR response upon TMV infection (Abbink *et al.*, 1998; Erickson *et al.*, 1999). Recently, Ueda *et al.* (2006) demonstrated that a direct interaction between p50 and the N-factor was enough to trigger the HR in the host.

Subsequent to localized HR response, the plant also develops an enhanced resistance in systemic uninfected tissues, which is referred as a systemic acquired resistance (SAR). SAR is normally associated with the increased levels of salicylic acid (SA) and the expression of a subset of defense genes, called pathogenesis-related (PR) genes. Several key signal components, such as *NPR1*-encoded protein, have been identified as involved in SAR signaling cascades against bacterial and fungal infections (Bowling *et al.*, 1994; Delaney *et al.*, 1995). However, whether they participate in a similar signal transduction pathway against virus infection needs to be determined.

Recently, RNA-mediated host defense systems have gained lots of attention as potent virus defense mechanisms. The most prominent is RNA silencing. Viral dsRNAs formed as replication intermediates or viral-RNA secondary hairpin structures can be cleaved by the Dicer-like nuclease into short interfering RNAs (siRNAs) approximately 21-25 nucleotide (nt) in length (Hamilton and Baulcombe, 1999). siRNAs then direct sequence-specific RNA degradation by recruiting the complementary viral genomic RNAs into the RISC complex for cleavage. It is

interesting to note that siRNAs can transport through phloem to systemic tissues, suggesting that siRNAs may serve as systemic silencing signals as well (Yoo *et al.*, 2004). Plant viruses, on the other hand, have developed certain strategies to counteract RNA-mediated host defense, e.g. encoding a suppressors of gene silencing (for reviews, see Li and Ding, 2001; Vance and Vaucheret, 2001). Numerous studies have shown that mutant viruses lacking functional suppressors fail to accumulate to high levels and induce disease symptoms (Qiu *et al.*, 2002; Voinnet *et al.*, 2000; Voinnet *et al.*, 1999). Clearly, viruses act as both inducers and targets in the RNA silencing pathway, during which extensive virus-host interactions occur in a host specific manner.

Virus-host interactions in disease development

Using yeast as an alternative host, host proteins that are implicated in viral translation and replication have been identified (Kushner *et al.*, 2003; Panavas *et al.*, 2005). A yeast factor Lsm1, a component within the yeast RNA decapping complex, has been demonstrated to act as a molecular switch by recruiting viral RNAs from translation to replication (Noueiry *et al.*, 2003). YDJ1, another yeast protein related to *Escherichia coli* DnaJ chaperone, when mutated, appears to affect BMV genome replication by blocking the initiation of negative strand RNA synthesis (Tomita *et al.*, 2003). Although the yeast system gives rise to a high-throughput result in identifying host factors involved in virus infection, the interactions between these host proteins with virus and their *in vivo* significance need to be further evaluated.

Host proteins involved in TMV virus replication have been extensively studied (Table 1.1). A tobacco HSP101 homolog of heat shock proteins is found to be a TMV Ω sequence-binding protein and their interaction is required for translational enhancement of Ω-containing mRNAs (Wells *et al.*, 1998). Translation elongation factor (eEF1A) interacts with the pseudoknot structure upstream of TLS in the 3'-UTR of TMV RNA genome (Zeenko *et al.*, 2002) and with 126- and 183-kDa replicase proteins (Yamaji *et al.*, 2006), indicating the involvement of eEF1A in the TMV-associated protein synthesis. Multiple host proteins have been copurified with the membrane-associated complexes from TMV-infected tissues (Osman and Buck, 1997; Watanabe *et al.*, 1999). One of them has been identified as the GCD10 protein, the RNA-binding subunit of the host translation initiation factor eIF3 (Osman and Buck, 1997). A yeast two-hybrid analysis shows that GCD10 interacts with the TMV replicase proteins by binding to their methyltransferase domain (Taylor and Carr, 2000). Addition of antibody against the GCD10 protein inhibits the RdRp activity of membrane-associated complexes, suggesting the involvement of GCD10 in virus replication (Osman and Buck, 1997).

Interactions between virus and host have been shown to play a role in disease development. Bilgin *et al*. (2003) showed that the helicase domain of the replicase protein interacted with a plant $P58^{IPK}$, which when silenced, makes the plants more susceptible to TMV infection, indicating the involvement of $P58^{IPK}$ in virulence. A subset of Aux/IAA transcription regulators have also been shown to interact with the TMV replicase protein with the interactions altering the localization of the Aux/IAA

Table 1.1 An overview of interactions between TMV viral components and host-encoded proteins.

Activity	Host protein	viral component	Identification method	Specific function	References
Host defense	Tobacco N factor	p50	Yeast two-hybrid	Mediating HR response	Ueda *et al.*, 2006
genome translation and replication	Tobacco HSP101	Ω sequence in the 5'-UTR	Luciferase assay	Translational enhancement	Wells *et al.*, 1998
	eEF1A	Pseudoknot in the 3'-UTR	UV cross-linking	TMV-associated protein synthesis	Zeenko *et al.*, 2002
		Replicase	Immunoprecipitation		Yamaji *et al.*, 2006
	TOM 1 & 3	Replicase	Yeast two-hybrid	Formation of VRCs, virus replication	Yamanaka *et al.*, 2002
	GCD-10	Replicase	Immunoprecipitation	Initiation of RNA synthesis	Osman and Buck, 1997
Viral movement	PME	MP	Immunorecognition	Viral RNA cell-to-cell movement	Chen *et al.*, 2000
	MPB2C	MP	Yeast SOS system	Directing MP to the MT site for degradation	Kragler *et al.*, 2003
	Calreticulin	MP	Affinity chromatography	Removing MP from the ER for degradation	Chen *et al.*, 2005
	Tobacco PAPK	MP	*In vitro* phosphorylation assay	MP phosphorylation	Lee *et al.*, 2005
Disease symptoms	$P58^{IPK}$	Replicases	Yeast two-hybrid, *In vivo* pull-down	Host cell death response	Bilgin *et al.*, 2003
	IAA26	Replicase	Yeast two-hybrid	Development of disease symptoms	Padmanabhan *et al.*, 2005

proteins from the nucleus to the cytoplasm (Padmanabhan *et al.*, 2005; Padmanabhan *et al.*, 2006). It is noted that virus-Aux/IAA interactions reprogram the auxin signaling pathway, resulting in enhanced disease symptoms in tissues that are less productive, suggesting a specific role of this interaction in alteration of host physiology (Padmanabhan *et al.*, 2008). Overall, given the fact that the small TMV genome encodes only few proteins, it is conceivable that recruitment of host proteins, which are present in a relatively rich pool, is an efficient strategy used for virus to complement its life cycle.

1.2.5 NAC Domain Proteins

In plant systems, families of transcription factors play important regulatory roles in plant developmental processes and stress/defense responses (for review, see Singh *et al*., 2002). NAC (petunia NAM, and Arabidopsis ATAF1, ATAF2, and CUC2) domain proteins are newly identified plant specific transcription factors and members within this family contain a well-conserved N-terminal NAC domain and a variable C-terminal transcription activation region (TAR). Sequence analysis reveals five subdomains (A, B, C, D, and E) within the NAC domain and a putative nuclear localization signal (NLS) located within subdomain C and D (Kikuchi *et al.*, 2000; Ooka *et al.*, 2003). Despite the high divergence, the C-terminal domains of several NAC proteins have been reported to activate transcription in yeast (Duval *et al.*, 2002; Hegedus *et al.*, 2003; Xie *et al.*, 2000). Stretches of serine/threonine, acdic, or proline/glutamine residues have been detected in the C-terminal TAR domain and are postulated to play a role in transcription activation (Hegedus *et al*., 2003). Using the

conserved NAC domain as a guide, 75 and 105 NAC proteins are predicted in *Orzya sativa* (rice) and *Arabidopsis thaliana*, respectively (Ooka *et al.*, 2003). Although a large number of proteins comprise this family of transcription factors, only a small portion of the NAC proteins has been characterized to date. The identified NAC proteins are shown to be involved in various aspects of plant biology (For review, see Olsen *et al.*, 2005).

Recently, the structure of the NAC domain from ANAC has been resolved by X-ray crystallography (Ernst *et al.*, 2004). It is illustrated that the NAC domain consists of a central antiparallel β-sheet flanked with two α-helices. Although the structure of NAC domain does not resemble any known DNA-binding motifs, the NAC domain surface contains a cluster of positive residues, suggesting a novel fold for the DNA binding activity (Ernst *et al.*, 2004). Xie *et al.* (2000) have shown that the NAC protein NAC1 is able to self-interact in yeast. Structural analysis revealed salt bridges and hydrophobic interactions between NAC domains, which in accordance with the observed self-interacting activity of the NAC proteins, suggest that the NAC domain mediates protein dimerization (Ernst *et al.*, 2004).

NAC proteins in plant developmental processes

Twelve years ago, the first two NAC genes, *NO APICAL MERISTEM* (*NAM*) and *CUP-SHAPED COTYLEDON* (*CUC2*) were described by mutational approaches. The loss-of-function *nam* mutant in petunia showed strong embryonic defects in the development of shoot apical meristem (SAM) (Souer *et al.*, 1996). A similar phenotype was observed in Arabidopsis *cuc1* and *cuc2* double mutant lines, which

also developed fused cotyledons (Aida *et al.*, 1997). Both *nam* and *cuc2* mutant plants display abnormal floral organs, indicating that these two genes are involved in both embryonic and postembryonic development. Based on the significant amino acid homology of NAM and CUC2 within their N-terminal domain with Arabidopsis ATAF1 and ATAF2, Aide *et al*. (1997) defined this new protein family as the NAC domain family. Since no previously known amino acid motif has been detected in members of this family, NAC proteins constitute a novel class of proteins in plants. Characterization of other NAC proteins in plant development has followed. For example, Arabidopsis *CUC1* has been shown to promote the SAM formation (Hibara *et al.*, 2003; Takada *et al.*, 2001); *CUC3* is involved in the establishment of the cotyledon boundary and the shoot meristem (Vroemen *et al.*, 2003); and *NAP*, which is expressed mainly at the base of the inflorescence meristem, is shown to be activated by *APETAL3* and *PISTILLATA*, two genes essential in differentiating floral organs (Sablowski and Meyerowitz, 1998). Another development-related *NAC* gene, *NAC1*, is induced by auxin and promotes lateral root development (Xie *et al.*, 2000).

NAC proteins in plant defense and stress responses

Plants have evolved an elaborate system in regulating their gene expression in response to the biotic and/or abiotic stresses. In most cases, gene expression is governed by several large families of transcription factors, including well-characterized ERF, bZIP, and WRKY (for review, see Singh *et al.*, 2002). More recently, the NAC domain family is emerging as a new class of transcription factors involving in the defense/stress responses. Expression of *Brassica napus* NAC

(*BnNAC*) genes has been shown to be induced in response to mechanical wounding, herbivore attack, and fungal infection (Hegedus *et al.*, 2003). Tomoto (*S. lycopersicum*) *SlNAC1* was induced upon infection by *Tomato leaf curl virus* (TLCV) (Selth *et al.*, 2005). Delessert *et al.* (2005) demonstrated that *ATAF2* was rapidly induced at the wound site in Arabidopsis. A similar result was seen in Potato (*Solanum tuberosum*) where *StNAC*, a close relative to the *ATAF2* gene, was shown to be wound-inducible (Collinge and Boller, 2001). These results suggest that NAC proteins share a common yet unidentified function that is conserved in different plant species in reaction to the defense/stress response. Enhanced drought tolerance has been observed in transgenic Arabidopsis overexpressing *NAC* genes, *ANAC019*, *ANAC055*, or *ANAC072* (Tran *et al.*, 2004), and in transgenic rice overexpressing *SNAC1* (*STRESS-RESPONSIVE NAC 1*) (Hu *et al.*, 2006). Overexpression of *ATAF2* in Arabidopsis, however, results in higher susceptibility to fungus *Fusarium oxysporum* (Delessert *et al.*, 2005). Interestingly, expression of *ANAC072*, also described as *RD26* (*RESPONSIVE TO DESICCATION 26*) is up-regulated upon abscisic acid (ABA) treatment (Fujita *et al.*, 2004). In contrast, *ATAF2* expression is not affected by ABA, but is induced by methyl jasmonate (MeJA) and SA (Delessert *et al.*, 2005). SA, ABA, and MeJA have been well recognized as defense signals involved in distinct signal transduction pathway. Therefore, the different reactions of *NAC* genes to ABA treatment suggest that though structurally similar, NAC proteins have evolved into different defense pathways, which at certain level might explain the presence of a high abundance of different *NAC* genes in plant.

Being a class of transcription factors, the target genes of NAC proteins have largely remained a mystery. A microarray analysis showed that a high proportion of *PR* genes were down-regulated or up-regulated in *ATAF2* overexpression or knockout transgenic plants, respectively (Delessert *et al.*, 2005). Further studies monitoring the expression profile of downstream genes in response to the stress or pathogen-inducible NAC expression will be necessary in order to better understand the NAC gene-induced stress and defense pathway.

NAC proteins involved in virus infection

Several NAC proteins have been characterized on the basis of their interactions with virus-encoded proteins. Wheat GRAB1 and GRAB2 (for Geminivirus RepA-binding) proteins interact with *Wheat dwarf geminivirus* (WDV) RepA protein (Xie *et al.*, 1999). Mapping of the interaction region to the N-terminal well-conserved NAC domain suggests that NAC proteins might play a common regulatory role in the virus life cycle. Although the direct role of GRAB-RepA interactions on virus life cycle is unknown, over-expression of GRAB proteins was shown to severely impair virus replication in cultured cells (Xie *et al.*, 1999). Another Geminivirus protein, replication enhancer (REn), was shown to interact with tomato SlNAC1 in a yeast two-hybrid system (Selth *et al.*, 2005). Interestingly, rather than inhibiting viral DNA replication, *SlNAC1* over-expression enhances viral DNA accumulation in a transient replication assay (Selth *et al.*, 2005). The different roles of NAC proteins in geminivirus replication suggest the functional diversity of NAC proteins in response to the virus infection. Different from GRABs and

SlNAC1, which interact with viral nonstructural proteins, NAC protein TIP (for TCV-interacting protein) is capable of binding to the TCV coat protein (CP) and disruption of this interaction correlates with the inability of the virus to induce HR in TCV-resistant plant, indicating that TIP is involved in a specific TCV resistance response pathway (Ren *et al.*, 2000). It is conclusive that NAC proteins actively participate in various aspects of the viral life cycle. Yet the functions of NAC proteins in virus replication and resistance remain unclear. Thus, additional identification of NAC proteins implicated in the viral life cycle will certainly help clarifying the functional complexities of this transcription factor family.

1.3 Research Objectives

Helicases are generally considered as molecular motors that function in unwinding double-stranded nucleic acids. Despite their enzymatic activities, a growing body of evidence has suggested that virus-encoded helicases play multiple roles in completing a viral life cycle. The primary objective in this work is to identify the importance of the TMV helicase domain in relation to viral replication and pathogenesis.

My first objective in this dissertation was focused on elucidating the structural and functional relationship of the TMV helicase domain, analyzing the biochemical properties of individual well-conserved helicase motifs, and finally determining the biological significance of the helicase domain in mediating the formation of the membrane-bound VRCs in the host.

Using a yeast two-hybrid approach, an interaction between TMV helicase domain and a host-encoded NAC protein, ATAF2, has been identified. Members within the NAC-domain family are plant-specific transcription factors that are involved in plant development processes and host defense/stress responses. The second objective of this study was to characterize the effects of viral helicase interacting with ATAF2 on modification of ATAF2 expression, suppression of host defense systems, and accumulation of virus in susceptible hosts.

Chapter 2: Structural and functional characterization of the helicase domain within the *Tobacco mosaic virus* 126- and 183-kDa replicase proteins

2.1 Abstract

Tobacco mosaic virus (TMV)-encoded 126-kDa and 183-kDa replicases are major components in membrane-bound virus replication complexes (VRCs). The helicase domain shared by the 126-kDa and 183-kDa replicases plays a key role in virus replication. In this study, purified TMV helicase polypeptides formed dimers and higher ordered structures *in vitro*, suggesting a self-interaction between replicase proteins. Biochemical analysis showed both monomeric and dimeric forms of the helicase proteins possessed ATPase activity, but not the higher ordered structures. To further characterize the biochemical activities of the TMV helicase, point mutations were introduced into four conserved helicase motifs; I and II (NTP binding and hydrolysis motifs), V (RNA binding motif), and VI. Of these motifs, only motif V retained ATPase activity when mutated, while mutations within motif I and V significantly reduced helicase RNA-binding activities. Individually each mutation disrupted virus replication. *In vivo* expression of the 126-kDa protein showed that both wild-type and motif V mutant replicases assembled into VRC-like inclusion bodies, while the mutants with the defective ATPase activities (motifs I, II, and VI) formed much larger irregular aggregates in host cells. Taken together, these studies suggest that the active forms of the TMV helicase polypeptides reside predominantly

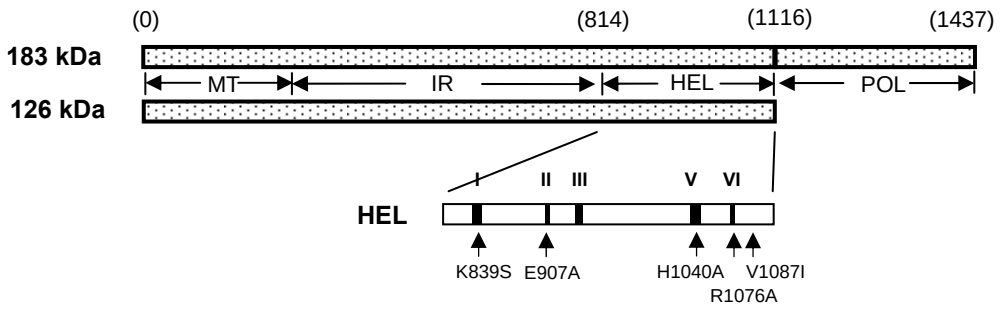
within monomers and dimers and that formation of wild-type VRC-like vesicles is correlated to the ability of the helicase domain to hydrolyze ATP.

2.2 Introduction

Positive-strand RNA viruses, including those infecting plants and animals, form membrane-associated virus replication complexes (VRCs) within host cells (Egger *et al.*, 2000; Lyle *et al.*, 2002; Schaad *et al.*, 1997; Schwartz *et al.*, 2002). These complexes serve as sub-modules within the host cell, supporting viral genome replication and translation. Discerning the structural assembly and dynamic processes involved in VRC formation will improve our understanding of the viral life cycle. In the past several years, numerous studies have indicated that viral protein-protein interactions, viral-host protein interactions and the recruitment of host cellular membranes all play important roles in VRC assembly (for reviews, see Ahlquist *et al.*, 2003; Buck, 1999; Strauss and Strauss, 1994; van der Heijden and Bol, 2002). However, detailed information on the initiation of VRC assembly, potential intermediate states, and the dynamic processes that drive VRC assembly, remain for the most part, unknown.

Similar to other positive-strand RNA viruses, *Tobacco mosaic virus* (TMV), the type member of the genus Tobamovirus, replicates in close association with ER-derived host membranes (Heinlein *et al.*, 1998; Más and Beachy, 1999). The genome of TMV encodes two nonstructural 126-kDa and 183-kDa replicase proteins (Goelet *et al*., 1982). Both 126-kDa and 183-kDa proteins possess an N-terminal methyltransferase (MT) domain and a helicase (HEL) domain at the C-terminus of the 126-kDa (Fig. 2.1A). The read-through portion of the 183kDa contains an RNA-

A



B

Motif	Conserved motifs within viral helicase SF1*	TMV helicase motifs	aa position	mutations
I	bxGxPGxGKS/Tx2b	LVDGVPGCG**K**T KEILS	830-845	K839S
II	b3Deb	LFID**E**GL	903-909	E907A
III	b4GDx2Q	AYVYGDT**Q**Q	928-936	Q935A**
V	Tbx3QGxTbx2Vxb2	TV**H**EVQGETYS DVSLV	1038-1053	H1040A
VI	bVAuTR	LVALS**R**	1071-1076	R1076A

* Kadare and Haenni. 1997. *J. Virol*. 71:2583-2590

** Q935A mutant forms inclusion bodies in *E. coli* cells and was not included in subsequent studies.

Fig. 2.1. Point mutagenesis within the conserved TMV helicase motifs. (A) Schematic representation of TMV 126-kDa & 183-kDa replicases and their shared helicase domain. Numbers refer to amino acid residue positions. Black boxes represent five conserved helicase motifs. The point mutations within the helicase motifs were shown by arrows. MT, methyltransferase; HEL, helicase; POL, polymerase. (B) Comparison of TMV helicase motifs with other viral helicases within helicase superfamily-1. The single letter b, u, x code for hydrophobic residue, aliphatic residue and any residue, respectively. The underlined amino acids within the TMV helicase motifs represent the mutated residues in this study. The resulting mutations are shown at the right.

dependent RNA polymerase domain (RdRp) (Fig. 2.1A). An *in vitro* immunoprecipitation assay has shown that both 126-kDa and 183-kDa replicase proteins are major components of membrane-associated VRCs (Osman and Buck, 1997). TMV also encodes a movement protein (MP) and a capsid protein (CP) derived from subgenomic RNAs (Goelet *et al*., 1982). Both MP and CP are not required for virus replication.

The VRCs formed during TMV infection are intricate structures, containing viral RNAs, 126- and 183-kDa replicase proteins, viral MPs, host proteins and the host ER membrane system (Heinlein *et al.*, 1998; Liu *et al.*, 2005; Más and Beachy, 1999). TMV derived VRCs also associate with cellular microtubules (MTs) and microfilaments (MFs), potentially facilitating the intracellular trafficking of viral RNA (Heinlein *et al.*, 1998; Liu *et al.*, 2005; Más and Beachy, 1999). Identification and characterization of protein-protein interactions within the VRCs is a first step to elucidating the contents and formation of these replication complexes. Immunoprecipitation experiments using antibodies against different replicase domains have detected a 126-/183-kDa heterodimer from crude membrane-associated complexes isolated from TMV-infected plant tissues (Watanabe *et al.*, 1999). Interactions between viral and host proteins occur intensively during many aspects of the infection process (for review, see Buck, 1999). Examples of the host proteins involved in TMV genome replication and VRC formation include a GCD-10 subunit of the host translation initiation factor eIF3, which is found associated with membrane-bound TMV replicase complexes capable of synthesizing both plus- and minus- strand viral RNAs (Osman and Buck, 1997), and two membrane integral

proteins, TOM1 and TOM3, which are required for efficient replication in Arabidopsis (Yamanaka *et al.*, 2002; Yamanaka *et al.*, 2000). How all these components coordinate with each other and with the host membrane system to achieve a successful viral life cycle is still unclear. Expression of the 126-kDa itself is sufficient to direct the assembly of ER-associated complexes in host cells in the absence of other viral components, suggesting that the 126-kDa plays a key role in recruiting and organizing viral and host components within the VRCs (dos Reis Figueira *et al.*, 2002; Liu *et al.*, 2005). A study also shows that the size of the VRCs is modulated by the 126-kDa replicase protein (Liu *et al.*, 2005). Similarly, Brome mosaic virus (BMV) encoded 1a protein, an ortholog of the 126-kDa protein, is capable of recruiting viral RNA and viral protein 2a to membrane-bound replication complexes, suggesting a similar function for the TMV 126-kDa protein (Chen *et al.*, 2001; Schwartz *et al.*, 2002; Wang *et al.*, 2005).

Using the yeast two-hybrid system, a self-interaction within the helicase domain of the TMV replicase has been identified (Goregaoker and Culver, 2003; Goregaoker *et al.*, 2001). Disruption of this interaction by mutagenesis affects virus replication in protoplasts (Goregaoker *et al.*, 2001). Helicase domains have been widely reported to be encoded by many RNA viruses (for review, see Kadaré and Haenni, 1997). RNA virus-encoded helicases generally function in unwinding RNA duplexes formed during virus replication and removing secondary structures from RNA templates by using the energy of NTP hydrolysis. Helicases can be classified into three distinct superfamilies (SF1, SF2, and SF3) defined by their amino acid sequence similarity (Gorbalenya *et al.*, 1989; Gorbalenya *et al.*, 1990). The TMV RNA helicase belongs

to SF1 superfamily and contains five conserved motifs (I, II, III, V, and VI) (Fig. 2.1A & B). The TMV helicase polypeptide expressed *in vitro* forms ring-like higher order structures (Goregaoker and Culver, 2003). However whether this higher-ordered helicase-helicase interaction is functionally important in VRC formation remains to be addressed.

In this study, I show that the TMV helicase domain self-interacts both *in vivo* and *in vitro*. The interaction led to the protein to form a dimer and a higher ordered structure. However, ATPase activity resided predominantly within the monomeric and dimeric forms. To further characterize the structure-function relationship of the helicase domain, various point mutations within the well-conserved helicase motifs (I, II, V, VI) were made. Further biochemical analysis and cellular biology approaches showed that mutant helicases maintained their ability to self-associate, yet disruption of the ATPase activity correlated to the inability of the 126-kDa protein to form wild-type VRC-like vesicles in host cells, suggesting an essential role for the helicase ATPase activity in the formation of functional VRCs.

2.3 Materials and Methods

Virus

Plasmid pTMV007 carrying the full-length cDNA of the TMV U1 strain under the control of T7 promoter was used in this study.

Site-directed mutagenesis

A PCR-based site-directed mutagenesis method was used to create point mutations within the TMV helicase domain (aa 814 to 1116) on vector pTMV007.

The final constructs were created as p007-E907A, p007-H1040A, and p007-R1076A with a point mutation from glutamic acid, histidine, and arginine to alanine, respectively. Mutant p007-K839S was kindly supplied from Dr Dawson's group, University of Florida (Lewandowski and Dawson, 2000). Mutant p007-V1087I was derived from a yeast TMV pLexA-helicase construct which was produced by a random mutagenesis method as described previously (Goregaoker *et al.*, 2001). All constructs were sequenced to verify the mutations. These full-length TMV wild-type and mutant cDNA clones were then used as parental templates to make other plasmid constructs.

Plasmid construction

For gene expression of the helicase polypeptide in a bacterial system, the C-terminal portion of TMV 126-kDa replicase, encompassing the yeast two-hybrid interaction region (aa 549-1116), was amplified by PCR using primers with added *KpnI* and *XhoI* restriction sites. The digested product was ligated into similarly digested pTrcHisA (Invitrogen, Carlsbad, Calif.) to create pTrcHis-HEL with a hexa-histidine tag at the amino terminus. All the mutations were similarly introduced into the same vector for gene expression.

Production of the His-tagged helicase for transient expression in plant cells was achieved by introducing the wild-type helicase segment (aa 549 to 1116) into the polylinker region of a plant expression vector pBI121 (Clonetech, Palo Alto, Calif.) to create pBI-6xHis-HEL with a hexa-histidine tag at the amino terminus. Production of the HA-tagged wild-type and mutant helicase constructs were done by cloning the segments of helicase polypeptides (aa 549 to 1116) into another binary vector pBin19

resulting pBin-HA-HEL (or mutants) with a HA-epitope at the N-terminus. Both pBI-6xHis-HEL (wild-type) and pBIN-HA-HEL (wild-type or mutants) were transformed into *Agrobacterium tumefaciens* strain GV3101 separately using a freeze-thaw method (Holsters *et al*., 1978).

An expression vector harboring full-length wild-type 126-kDa fused to enhanced green fluorescent protein (eGFP) ORF has been described previously (dos Reis Figueira *et al.*, 2002). Mutant pCMC-126-GFP carrying point mutations within the helicase domains were created by inserting the full-length 126-kDa ORFs from the respective p007 mutant clones into the *NcoI* and *BsiWI* sites of pCMC-126-GFP. Another expression vector, pAVA, was used to express the 126-kDa protein in protoplast (von Arnim *et al.*, 1998). pAVA-126-GFP and its mutant constructs were created by inserting the PCR-generated 126-kDa-eGFP fusions, which have *NcoI* and *PstI* sites at both ends, into the pAVA-polylinker region at the *NcoI* and *NsiI* sites.

Expression and purification of the TMV helicase and mutant polypeptides

To purify 6x histidine-tagged TMV helicase and mutant polypeptides, *Escherichia coli* BL21 codon (+) cells harboring pTrcHisA-HEL (or mutants) were grown at 37 ºC in Luria-Bertani medium in addition with 100 µg/ml of ampicillin and 50 µg/ml of chloramphenicol until the OD_{600} reached 0.5. The culture was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM followed by continuous shaking at 16 ºC overnight. The cells were harvested and resuspended in lysis buffer containing 10 mM Tris, pH 8.0, 10% glycerol, 500 mM NaCl, and 1 mg/ml lysozyme. The cell lysate was incubated at 4 ºC for 1h and further disrupted by pulsed sonication (0.5s pulse followed by 0.5s pause)

for 3 minutes. The resulting protein extract was centrifuged at 12,000 rpm for 20 min, and the supernatant was applied to a 1 ml Ni-NTA affinity column (Amersham biosciences, Piscataway, N.J.). The column was washed with 10 column volumes of lysis buffer followed by 5 column volumes of wash buffer (lysis buffer plus 20 mM imidazole). The protein was eluted in elution buffer (lysis buffer plus 150 mM of imidazole). The concentration of the eluted protein was determined using a standard Bradford assay (BioRad, Hercules, Calif.). The purified products were flash-frozen in liquid nitrogen and saved at -80 ºC.

Gel filtration chromatography

The purified TMV helicase and mutant polypeptides (~100 µg each) were incubated in buffer S containing 10 mM Tris, pH 7.0, 10% glycerol, 150 mM NaCl at room temperature or 4°C. Samples taken at different time points were then loaded onto gel filtration superdex 200 HR 10/30 columns (Amersham Biosciences, Piscataway, N.J.) which were pre-equilibrated with 1.5X bed volumes of buffer S. Columns were run at 4°C with a flow rate of 0.2 ml/min. Fractions (250 µl each) were collected.

ATPase assay

The ATPase activity of TMV wild-type and mutant helicase polypeptides was examined by mixing 0.5 µCi of [γ-^{32}P]ATP (NEN, Boston, Mass.) with purified proteins (20 ng or 100 ng) in a buffer containing 20 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 40 µM ATP for 30 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 0.1M and 25% of the reaction mixtures were spotted on the polyethyleneimine-cellulose thin layer chromatography (TLC)

plates (Fisher, Malvern, PA). The released phosphate was separated from ATP in developing buffer containing 1 M formic acid and 0.5 M LiCl and further visualized by Phosphor Imager (Molecular Dynamics, Sunnyvale, Calif.).

Nitrocellulose filter binding assay for RNA binding

Purified TMV wild-type and mutant helicase polypeptides (0 μg, 10 μg, 20 μg, 40 μg, 80 μg, and 160 μg) were immobilized on a pre-wetted nitrocellulose membrane using a 96-well dot-blot apparatus (Minifold I, Schleicher & Schuell). The membrane was incubated with 5 nM of a ^{32}P-end labeled 40-mer RNA (5'-UUUGUUUGUUUGUUUGCCAUCGGGUGCCUGGCCGCAGCGG-3') (2×10^6 cpm) overnight in buffer RN (25 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, and 1X Denhardt's solution). After washing the membrane four times in buffer RN, the membrane was air dried and exposed to X-ray film. The results were further analyzed using AlphaImager software (Alpha Innotech Corp., San Leandro, Calif.).

Co-immunoprecipitation

Agrobacterium cultures carrying 6xHis- and HA-tagged helicase polypeptides were mixed at the same OD_{600} value and co-infiltrated into *N. benthamiana* plant leaves. Forty-eight hours post agro-infiltration, the infiltrated plant tissues were ground in liquid nitrogen and homogenized in two volumes of extraction buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.5% TritonX-100, 0.2% 2-mercaptoethanol, 5% glycerol, PMSF, proteinase inhibitor cocktail). Ground tissues were pelleted by centrifugation for 10 min at 15,000 rpm. The supernatant (1 ml) was incubated with 5 μl of anti-polyHis antibody (Sigma, St. Louis, MO) at 4°C overnight with gentle

shaking followed by the addition of 30 µl of protein A agarose (Invitrogen, Carlsbad, Calif.) for additional 3 h. The agarose-immune complexes were then centrifuged and washed three times in 1 ml wash buffer (25 mM Tris-HCl at pH 7.5, 250 mM NaCl, 2 mM EDTA, 0.05% TritonX-100, 1 mM PMSF). After the last wash, the precipitated protein complexes were resuspended in 2X sample buffer and analyzed by SDS-PAGE and western–blotting with the antibody against the HA epitope (Roche, Indianapolis, IN).

Transient expression of TMV helicase domain in plant containing the *N* gene

Agrobacteirum carrying pBIN-HA-HEL (wild-type or mutants), pBIN-GFP, or pBin was grown at 30 ºC overnight. After centrifugation, the culture was resuspended in infiltration medium (10 mM MES, pH 5.7, 10mM $MgCl_2$, 150 µM acetosyringone) at OD_{600} of 0.5. The suspension was then infiltrated into *N. tabacum* cv. Xanthi NN leaves using a syringe. For each construct, several square centimeters of leaf tissues were covered. The hypersensitive response (HR) was observed at four days post-inoculation.

Local lesion assay

Full-length RNA transcripts of TMV wild-type and mutants were generated by *in vitro* transcription using TMV007 and 007-mutants as templates. RNA transcripts of each recombinant virus were mixed with equal volumes of FES buffer (0.1 M Glycine, 0.06 M potassium phosphate, 1% sodium phosphate, 1% macaloid, 1% celite, pH 8.5-9.0) and rub-inoculated on half of each *N. tabacum* cv. Xanthi NN leaf and the wild-type RNA transcript was inoculated on the other half as a control. Five days post-inoculation (dpi), lesions were recorded.

Protoplast replication assay

Tobacco protoplasts prepared from *N. tabacum* cv. Xanthi suspension cells (6 × 10^6 cells per rxn) were transfected with TMV wild-type or mutant RNA transcripts via electroporation (125 V and 300 uF). After being cultured in AOKI growth media (0.5 M D-mannitol, 10 mM MES, 10 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4 \cdot 7H_2O$, 1 M KI, 0.01 M $CuSO_4$, and 3% sucrose) for 20 h at room temperature, the protoplasts were harvested and total RNA was extracted followed by a standard northern blot assay (Sambrook *et al.*, 1989). ^{32}P-labelled probe was made by using a *NcoI/KpnI* digested TMV007 fragment corresponding to TMV nts 5460 to 6394 and a Prime-a-Gene Labeling System (Promega, Madison, W.I.) as described by manufacturer's protocol.

Transient expression of TMV 126-kDa replicase

A particle bombardment method was used to express TMV 126-kDa wild-type and mutant replicases in *N. benthamiana* epidermal cells. Briefly, plasmid DNA (pCMC-126-GFP or 126-mutants-GFP) was coated onto tungsten particles (1.3 μM in diameter; Bio-Rad, Hercules, CA) in ethanol. The DNA-coated particles then were dried on plastic filters and bombarded into leaf tissues using a gene gun apparatus (Bio-Rad, Hercules, Calif.). After incubating the bombarded tissues for 12 to 14 h at room temperature, GFP fluorescence was visualized using a confocal microscope.

Expression of TMV 126-kDa replicase in protoplasts was done by introducing the 126-kDa constructs (plasmid pAVA-126-GFP or pAVA-126-mutants-GFP) into *N. tabacum* cv. Xanthi suspension cells (50 μg per 6 × 10^6 cells) via electroporation at

300 V and 600 uF. The protoplasts were then cultured in AOKI growth media for 48 h at room temperature and GFP fluorescence was visualized via confocal microscopy.

Confocal microscopy

A Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss Inc., Thornwood, N.Y.) was used to visualize fluorescent images. EGFP fluorescence was excited by using a 488-nm argon laser beam and emission was captured at between 500 and 560 nm. The images were taken under a 63× 1.2-numerical-aperture water immersion lenses and further analyzed with Zeiss LSM Imager Examiner software, version 3.0, and optimized with Adobe Photoshop 6.0. Diameters of 126-kDa complexes were measured. For each wild-type and mutant 126kDa construct, 30 to 45 complexes were measured.

Electron microscopy

Purified TMV helicase polypeptides were applied on carbon-coated copper grids and stained with 1% uranyl acetate. The grids were then dried and visualized under the Zeiss EM 10CA transmission electron microscope (TEM).

For thin-section electron microscopy, *Agrobacterium*-infiltrated tissue (~ $1mm^3$) were fixed in 2% glutaraldehyde and followed by embedding in Spur's resin as described previously (Hepler, 1980). Thin sections (between 60-90 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate (5 min). The thin sections were then visulalized under the TEM.

2.4 Results

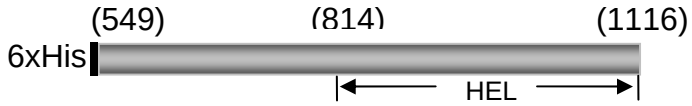
Self-association of the TMV helicase domain results in a dimer and a higher ordered structure

In a previous yeast two-hybrid assay, the TMV helicase domain within the 126-/183-kDa replicases was shown to self-interact (Goregaoker *et al.*, 2001). The interacting region covers the C-terminus of the intervening region (IR) and the entire helicase domain (HEL). To further characterize the self-interaction of the TMV helicase domain, the whole interacting region, spanning from amino acid 519 to 1116 was subcloned into a bacterial expression vector, pTricHisA, to create a six-histidine tag at the N terminus. The resulting construct was expressed in *E. coli* cells under an optimized induction condition at 16ºC overnight and purified by affinity chromatography in a high salt, high pH buffer condition (500 mM NaCl, pH 8.0). The expression yield and the purified product was analyzed using SDS-PAGE and was confirmed to be at least 95% pure via gel filtration chromatography (Fig 2.2B and 2.2C). Under these purification conditions, the TMV helicase domain accumulated to high levels as a soluble monomer (Fig 2.2C).

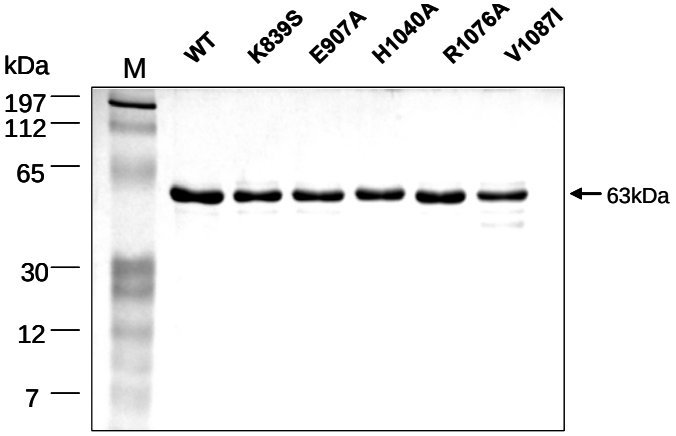
The TMV 126- and 183-kDa replicase proteins have been suggested to interact with each other to form a heterodimer (Watanabe *et al*., 1999). To test whether the helicase domain is involved in the replicase protein-protein interaction, the purified helicase polypeptide was incubated in a buffer close to the physiological condition (150 mM NaCl, pH 7.0). The incubated products were then tested for their oligomeric formation using gel filtration chromatography. After 2 hours incubation at room temperature, a peak with a molecular mass equivalent to a dimeric form was

Fig. 2.2. *In vitro* expression and purification of TMV wild-type and mutant helicase polypeptides. (A) Schematic representing the construction of 6xHis-tagged helicase or mutant polypeptide. The number refers to amino acid position. (B) The purified products (~ 15 μg each) were resolved in a 10% SDS-PAGE gel and stained with Coomassie blue. (C) Gel filtration chromatography suggests the monomeric formation of wild-type helicase polypeptide in buffer containing 500 mM NaCl, pH 8.0. Molecular weight markers, thyroglobulin (670 kDa), gamma globulin (158 kDa), Ovalbumin (44 kDa), and myoglobin (17 kDa) are indicated.

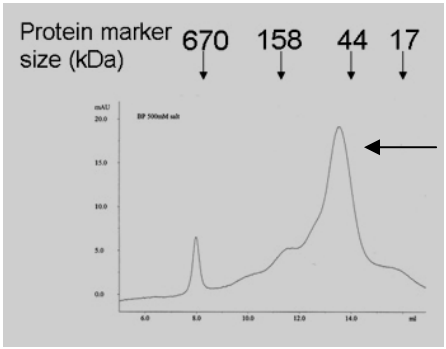
A.



B.



C.



Monomer, 63-kDa

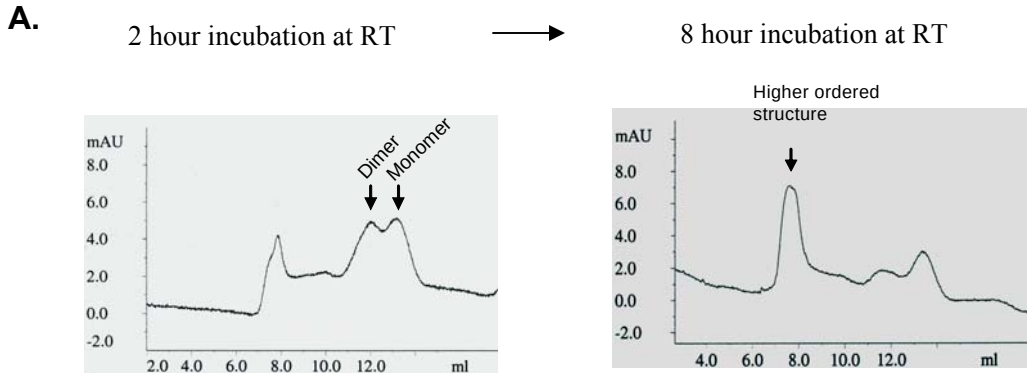
observed (Fig 2.3A). Prolonged incubation resulted in appearance of higher molecular-weight aggregates (Fig 2.3A). The presence of the TMV helicase protein within both the dimeric and the higher-ordered structure forms was confirmed with SDS-PAGE and Commassie blue staining (Fig 2.3B). Electron microscopy studies of fractions from the higher molecular-weight peaks supported the presence of the aggregate as helical-like structures (Fig 2.4). To identify the effect of nucleotide binding on protein oligomerization, AMPPNP, a nonhydrolyzable ATP analog, was added into the TMV helicase solution and no obvious effects were detected on formation of the helicase dimer and the higher-order structure (data not shown).

ATPase activity of the TMV helicase domain resides predominantly within the monomeric and dimeric forms

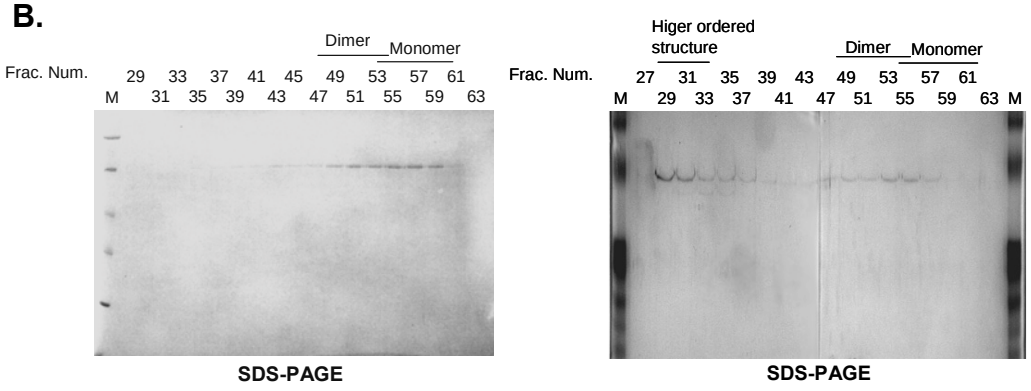
TMV helicase has been shown to possess ATPase activity and double-stranded RNA unwinding activity (Goregaoker and Culver, 2003). To investigate the active form of the TMV helicase domain, different conformations of the TMV helicase, including monomers, dimers and the higher order structures, were assayed for their ability to hydrolyze ATP. Results demonstrated that the monomer and the dimer forms of TMV helicase could hydrolyze ATP efficiently, while the higher-order aggregates showed little detectable ATPase activity (Fig. 2.3C). Addition of single-stranded RNA did not enhance the ATPase activity of any of the aggregate states (data not shown). This finding indicates that the ATPase activity of the TMV helicase domain is conferred by a monomer/dimer form and suggests that these helicase proteins may represent the active enzymatic form for virus replication.

Fig. 2.3. Active forms of TMV helicase polypeptide reside mainly within monomer and dimer forms. (A) Gel filtration chromatography showing that TMV helicase polypeptide forms a dimer (with a molecular mass equivalent to ~126-kDa) in buffer containing 150 mM NaCl, pH 7.0. Prolonged incubation resulted in formation of a higher ordered structure. Black arrows point to different oligomeric forms of the TMV helicase polypeptide. (B) SDS-PAGE showing the presence of the TMV helicase polypeptide in different structural forms. Fractions (50 μl each) collected from the gel filtration chromatography were resolved on 10% SDS-PAGE. The gels were stained with Commassie blue. (C) Both monomer and dimer forms of TMV helicase polypeptides are active in ATPase activity. The higher ordered structures have little detectable ATPase activity. Monomer, dimer or aggregate fractions (~ 5 ng) collected from gel filtration column were incubated with 0.5 μCi of [γ-^{32}P]ATP at room temperature for 30 min. The aliquot of reaction mixture was spotted onto thin-layer chromatography (TLC) plates and released Pi was visualized via Phosphor Imager.

A.


2 hour incubation at RT
8 hour incubation at RT
Higher ordered structure
mAU
8.0
6.0
4.0
2.0
0.0
-2.0
Dimer
Monomer
2.0 4.0 6.0 8.0 10.0 12.0 ml
4.0 6.0 8.0 10.0 12.0 ml

B.


Frac. Num.
M 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63
Dimer
Monomer
SDS-PAGE
Frac. Num.
M 27 29 31 33 35 37 39 41 43 47 49 51 53 55 57 59 61 63 M
Higer ordered structure
Dimer
Monomer
SDS-PAGE

C.


Frac. Num.
– 35 37 39 41 43 45 47 49 51 53 55 57 59 61
Dimer
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Pi
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ATPase activity
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Higher ordered structure
Dimer
Monomer
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ATPase activity

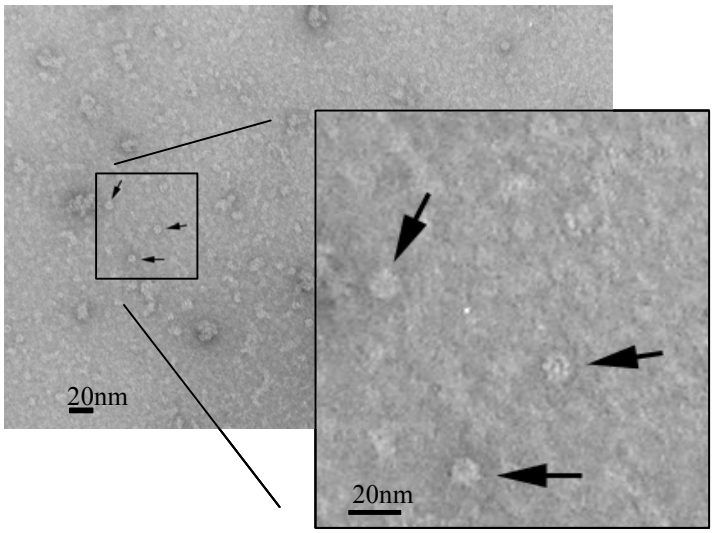


Fig. 2.4. An electron micrograph showing the higher-order structure formation of the TMV helicase polypeptides. Black arrows point to the helical-like higher ordered structures form.

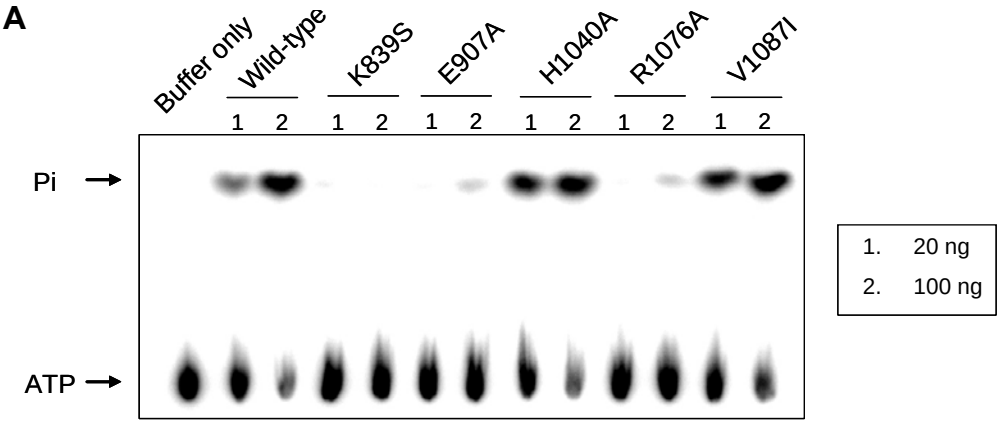
Site-directed mutagenesis of conserved residues within helicase motifs

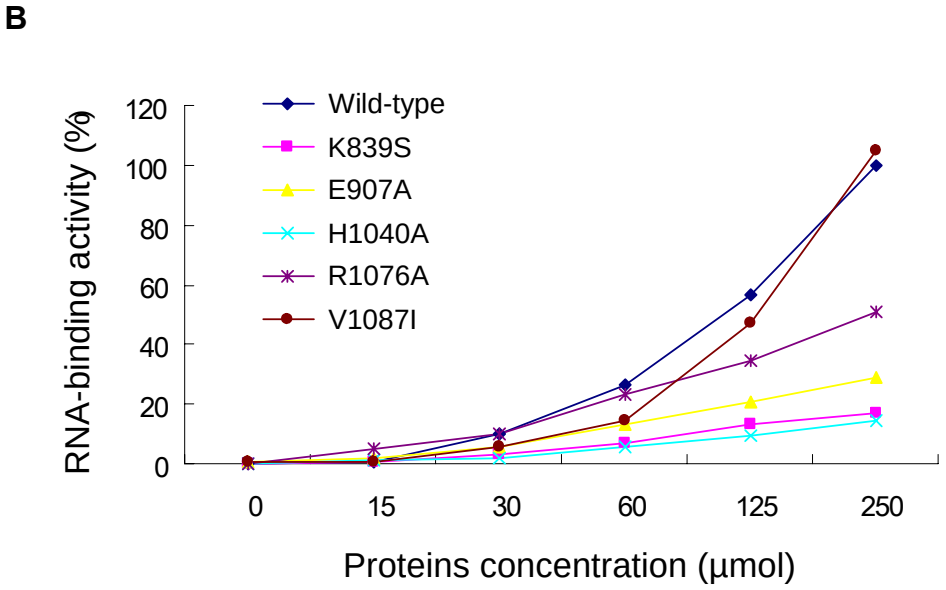
Based on their sequence similarities to other helicases with known functions, the TMV helicase domain is found to contain five conserved motifs (Fig. 2.1). To investigate the effect of each conserved helicase motif on helicase activities, individual mutations were made in all five motifs, including K839S (motif I), E907A (II), Q935A (III), H1040A (V), and R1076A (VI) (Fig. 2.1A and 2.1B). Another mutant with a mutation outside of helicase motifs (V1087I) was used in this study as a control. The majority of mutations created in this study were selected due to their conservation in SF1 helicases encoded by RNA viruses (Fig. 2.1) and are suggested to play a structure/function role (for review, see Caruthers and McKay, 2002). Mutant proteins were similarly expressed in the bacterial expression system. Except the mutant Q935A, which produced low solubility of the protein in *E. coli* cells, the other mutant proteins were expressed in high concentrations as a soluble form as shown in SDS-PAGE gel (Fig. 2.2B).

Biochemical characterization of the TMV helicase mutants

To test the effect of helicase mutations on ATPase activity, the purified wild-type and mutant helicase polypeptides were assayed for their ability to hydrolyze ATP. TMV helicase motif I and II are known to be NTP binding and hydrolysis motifs. As expected, mutations within motif I (K839S) and II (E907A) impaired the ATPase activity of the protein (Fig 2.5A). The motif VI mutant R1076A also displayed a severe defect in hydrolyzing ATP. Of all the mutant proteins tested, only H1040A, a mutation in motif V, and V1087I retained ATPase activity similar to that

Fig. 2.5. Biochemical activities of TMV wild-type and mutant helicase polypeptides. (A) ATPase assay showing that motif V mutant, H1040A and mutant V1087I retained ATP hydrolysis activity. Each reaction contains 0.5μCi of [γ-^{32}P]ATP and 20ng (lane 1) or 100ng (lane 2) of purified proteins. The first lane contains no protein and serves as a control. (B) RNA-binding activities of TMV helicase mutants. A nitrocellulose filter binding assay was carried out by incubating purified wild-type and mutant helicase polypeptides (as monomers) at various concentrations (0, 15, 30, 60, 125, 250 μmol) with 5nM of 5' ^{32}P-labelled RNA oligonucleotide (2×10^6cpm) at room temperature overnight. The binding activity with wild-type helicase polypeptide at the protein concentration of 250 μmol was defined as 100%. Mutations within motif I (K839S), II (E907A), V (H1040A), and VI (R1076A) all affected the helicase RNA binding activity, showing only 18%, 25%, 15%, and 50% of wt activity, respectively.


A
Buffer only
Wild-type
K839S
E907A
H1040A
R1076A
V1087I
1 2 1 2 1 2 1 2 1 2 1 2
Pi
ATP
1. 20 ng
2. 100 ng


B
RNA-binding activity (%)
120
100
80
60
40
20
0
Wild-type
K839S
E907A
H1040A
R1076A
V1087I
0
15
30
60
125
250
Proteins concentration (μmol)

of wild-type helicase (Fig. 2.5A). These data indicate that TMV helicase motif I, II, and VI are actively involved in ATPase activity.

To investigate the influences of the well-conserved motifs on helicase RNA-binding activity, a nitrocellulose filter binding assay was used. Wild-type and mutant helicases at various concentrations (0, 15, 30, 60, 125, 250 μmol) were loaded onto a nitrocellulose filter followed by incubation with a 5nM radio-labeled 40-mer RNA oligonucleotide. As shown in Fig. 2.5B, mutations within motif I (K839S), II (E907A), and V (H1040A) significantly reduced the helicase RNA binding activity, showing only 18%, 25%, and 15% of wt activity, respectively. Motif VI (R1076A) mutant also showed weakened RNA binding activity (50%). In contrast, the V1087I mutant control had no effect on the helicase RNA-binding activity. These results suggest that TMV helicase motifs I, II, and V all contribute to specific interactions with oligonucleotides.

Effects of point mutations within the helicase motifs on virus infectivity

Since both ATPase and RNA binding activities are essential for helicase activity, all mutations tested within the well-conserved motifs are postulated to affect virus replication. To confirm this, a local lesion assay was used to examine the infectivity and movement of recombinant virus containing each of the helicase mutations. *N. tabacum* cv. Xanthi NN contains the *N* gene which confers resistance to TMV. The TMV infection on this plant results in formation of lesions due to the cell-death at the site of the infection and the size of the lesions correlates to the virus's ability to replicate and move from cell-to-cell. *In vitro*-generated wild-type

and mutant viral RNA transcripts were rub-inoculated on plant tissues and formation of lesions was recorded five days post-inoculation (dpi). As expected, lesions were observed from wild-type and V1087I mutant-inoculated leaves and all the mutations within the helicase motifs blocked the formation of local lesion, suggesting an essential role of these conserved motifs in completion of a viral life cycle (Fig. 2.6A). In addition, RNA transcripts of recombinant viruses were utilized to transfect tobacco protoplast to test whether the mutation specifically affects virus replication. The production of progeny viral genomic RNAs was monitored by northern blot. Similar to the results observed from the local lesion assay, all mutations within helicase motifs disrupted virus replication in the host cell (Fig 2.6B). Wild-type like mutant V1087I, which was shown to possess both ATPase activity and RNA binding activity, retained the ability to replicate in protoplast (Fig. 2.6B).

Effects of point mutations within the helicase motifs on inducing HR responses

TMV helicase domain induces a resistant hypersensitive response (HR) in *N* gene containing tobacco plants (Erickson *et al*., 1999). The HR is a host defense response associated with formation of necrotic lesions at the site of infection that further prevent the pathogen from spreading. To test whether any mutations within the helicase motifs disrupt helicase-induced HR, a binary vector, pBin19, was used and modified to make the constructs expressing wild-type or mutant helicase polypeptide with a hemagglutinin (HA) tag at the N-terminus. *Agrobacterium tumefaciens*-mediated expression of both wild-type and mutant helicases resulted in

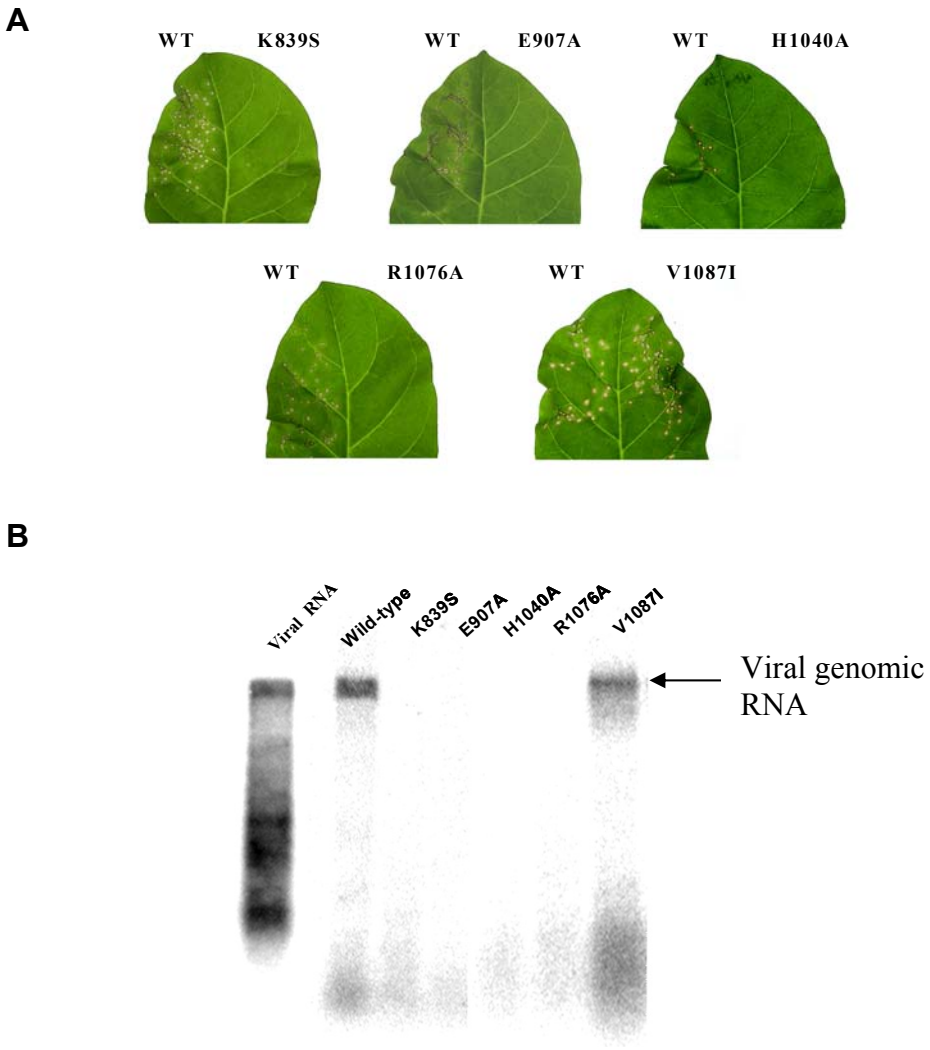


Fig. 2.6. Effects of the point mutations within the TMV helicase motifs on viral replication and infectivity. (A) Formation of necrotic local lesions on *N. tabacum* cv. Xanthi NN leaves inoculated with wild-type (left half of each leaf) and mutant TMV transcripts (right half of each leaf). Lesions were observed four days post inoculation. (B) Northern blot analysis of total RNAs extracted from wild-type and mutant TMV-infected protoplasts. Accumulation of genomic RNA was measured with the probe complementary to the 3' end of the TMV genomic RNA.

HR-like cell death as shown in Fig. 2.7. The timing on appearance of the HR phenotype is comparable among all the tested constructs, suggesting none of the mutations affected the ability of helicase to induce resistance in plants. In contrast, pBin vector only and the pBin -GFP construct failed to activate HR in plant (Fig 2.7).

Effects of point mutations within the helicase motifs on helicase self-interaction

As shown previously, the wild-type TMV helicase domain can self-associate and such protein-protein interactions positively correlate with viral replication activity in protoplasts (Goregaoker *et al*., 2001). To further investigate the effects of mutations on helicase protein-protein interaction *in vivo*, an immunoprecipitation assay was conducted by co-expressing a 6x-His tagged wild-type helicase and a HA-tagged wild-type or mutant helicase in the same *N. benthamiana* plants using an *Agrobacterium*–mediated transient expression method. The Agro-infiltrated tissues were collected and precipitated using anti-polyHistidine antibody followed by probing with antibody against anti-HA epitope. The data obtained from the study confirmed the occurrence of a helicase protein-protein interaction within the biological system (Fig. 2.8). In addition, none of the mutations within the helicase motifs affected the helicase-helicase interaction (Fig. 2.8)

The ATPase activity of the 126-/183-kDa helicase domain regulates the assembly of the VRCs

In this study, I have determined the ability of each recombinant mutant virus to replicate in host cells (Fig. 2.6). For positive-stranded RNA viruses, formation of

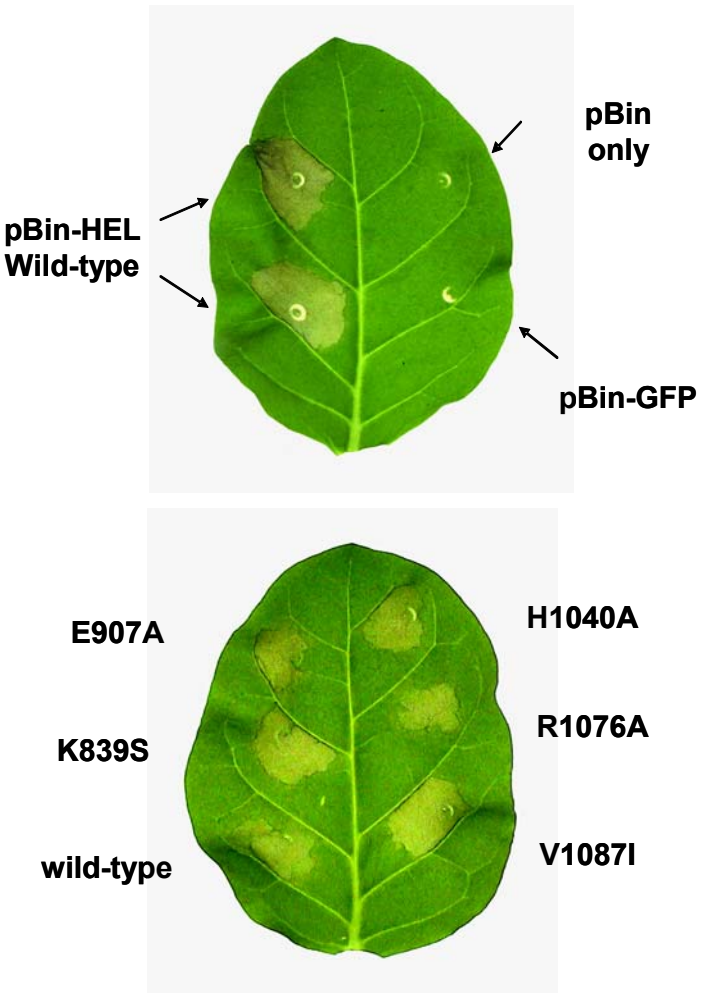


Fig. 2.7. Mutations within the TMV helicase motifs have no effect on the protein inducing HR on *N* gene containing tobacco plants. An *Agrobacterium*-based transient expression of wild-type *(left and i on right)* or mutant *(ii to vi on right)* helicase polypeptides all induced HR in *N. tabacum* cv. Xanthi NN. Vector only (pBin) and GFP ORF *(left)* were used as controls. The pictures were taken 3 days post infiltration.

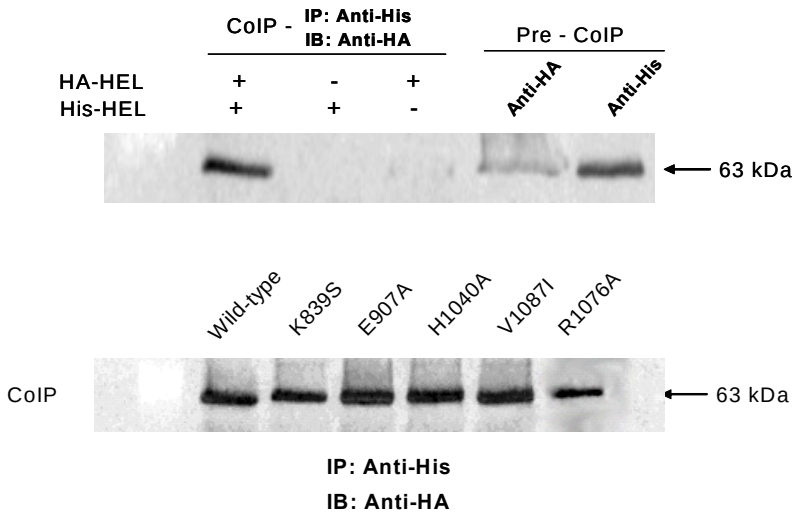
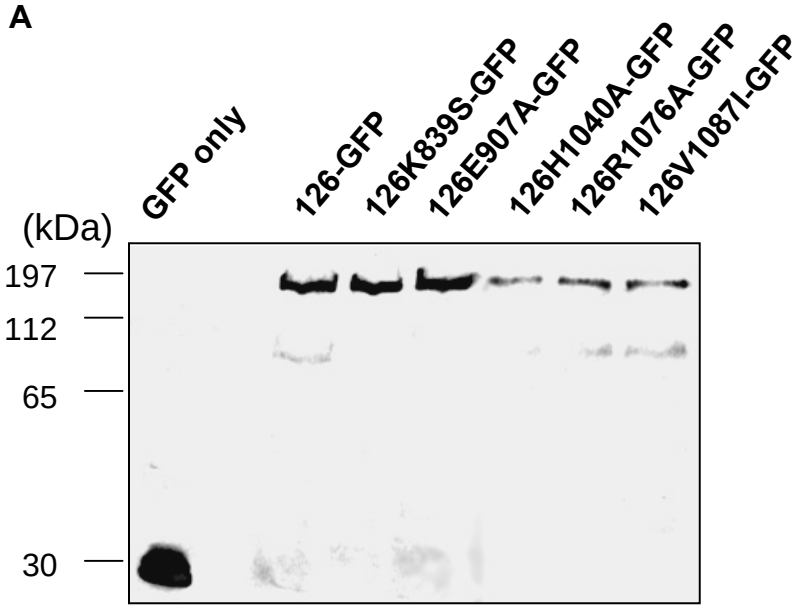


Fig. 2.8. Mutations within the TMV helicase motifs have no effect on helicase self-interaction. Co-immunoprecipitation of wild-type (*top panel*) and mutant (*bottom panel*) helicase polypeptides. 6xHis-tagged wild-type helicase and HA-tagged wild-type or mutant helicases were co-infiltrated into *N. benthamiana* leaves. After 48 hours, the ground tissues were immunoprecipitated with anti-polyHis antibody and probed with anti-HA antibody.

membrane associated VRCs is essential for viral replication. In a transient expression system, the 126-kDa replicase protein has been shown to be able to form vesicle-like ER inclusions, suggesting a role of the 126-kDa replicase in inducing the formation of membrane-associated VRCs (dos Reis Figueira *et al*., 2002, Liu *et al*., 2005). The size of the VRCs is also modulated by the 126-kDa replicase protein (Liu *et al*., 2005). To evaluate the significance of each helicase motif in forming membrane-associated vesicle, each mutation was engineered into a full-length 126-kDa-GFP construct and transiently expressed in *N. benthamiana* leaves via agro-infiltration. Expression of full-length 126-kDa replicase and its mutants was confirmed by western blot using anti-GFP antibody (Fig. 2.9). Confocal fluorescent analysis further demonstrated that vesicles formed by the constructs fell into two groups. The first group contained mutants H1040A and V1087I, which formed inclusion bodies with their sizes similar to that of wild-type 126-kDa replicase (Fig 2.9 and Table 2.1). The second group included mutants K839S, E907A, and R1076A, which formed much larger aggregates (Fig 2.9 and Table 2.1). Formation of distinct sizes of vesicles in the host was further confirmed by expressing 126-kDa wild-type or mutant replicase in *N. benthamiana* epidermal cells by particle bombardment or in tobacco protoplast by electroporation (Fig. 2.10). Both methods supported the results obtained from agro-infiltration, suggesting a role for the helicase motifs in modulating the size of vesicles formed by the 126-kDa replicase. To further examine the detailed structural conformation of the cytosolic vesicles induced by the 126-kDa protein and its mutants, thin sections prepared from *N. benthamiana* leaf tissues expressing pBin/126-GFP or pBin/126K839S-GFP constructs were analyzed by EM.

A



B

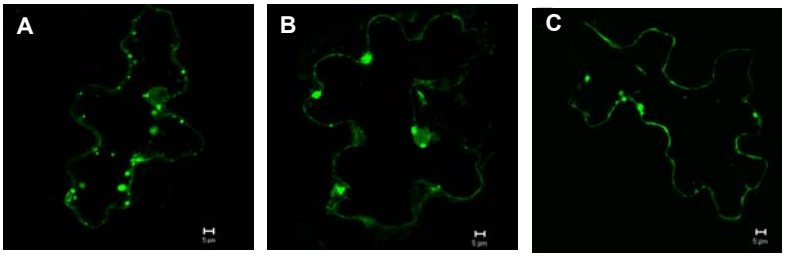
Diameters of VRC-like inclusion bodies (μm)* in *N. benthamiana* 48 hours post agro-infiltration	
126-GFP	**3.34 ± 0.37**
126K839S-GFP	**10.98 ± 1.49**
126E907A-GFP	**7.82 ± 0.84**
126H1040A-GFP	**4.09 ± 0.72**
126R1076A-GFP	**8.30 ± 2.25**
126V1087I-GFP	**4.17 ± 0.68**

* The calculated mean and standard deviation of the data are presented

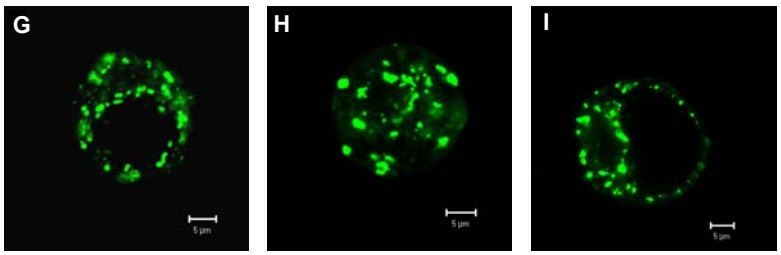
Fig. 2.9. Expression of TMV 126-kDa and mutant replicase proteins in *N. benthamiana* leaf tissues via agro-infiltration. (A) Western blotting confirming the expression of 126-kDa replicase and its mutants. The first lane is GFP control. (B) Sizes of vesicles formed by 126-kDa and mutant replicase proteins in *N. benthamiana* 48 hours post agro-infiltration. The numbers were obtained from the calculated mean and standard deviation of 30-50 vesicles of each replicase protein.

Fig. 2.10. Transient expression of TMV 126-kDa and mutant replicase proteins in *N. benthamiana* leaf tissues (A - F) and in *N. tabacum* cv. Xanthi suspension cells (G - L). Expression plasmids were particle bombarded into *N. benthamiana* leaves or transformed into suspension cells via electrophoresis. Confocal images were taken 14 to 16 hours post bombardment and 48 hours post transformation, respectively. Vesicles are observed from wild-type 126-GFP (A and G); 126-K839S-GFP (B and H); 126-E907A-GFP (C and I); 126-H1040A-GFP (D and J); 126-R1076A-GFP (E and K); and 126-V1087I-GFP (F and L).

Group I: Wild-type like vesicles



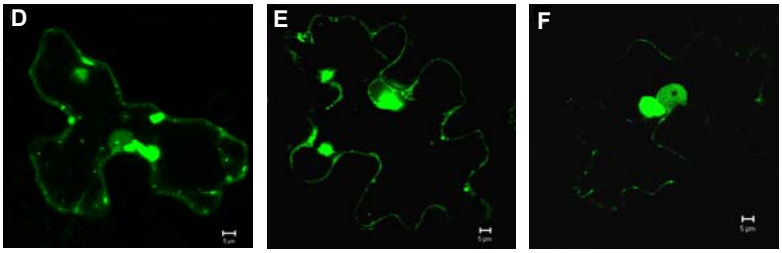
Nicotiana benthamiana



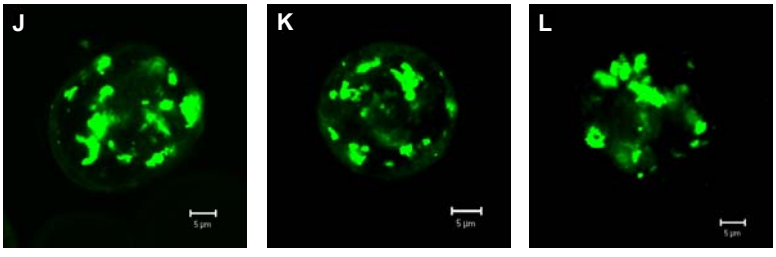
Protoplasts

126-GFP **126H1040A-GFP** **126V1087I-GFP**

Group II:Larger aggregate



Nicotiana benthamiana



Protoplasts

126K839S-GFP **126E907A-GFP** **126R1076A-GFP**

Table 2.1 Formation of VRC-like vesicles in host cells correlates to the ATPase activity of the TMV helicase domain.

	Diameters of VRC-like inclusion bodies (μm)*		ATPase activity	RNA binding activity
	N. benthamiana	Protoplasts		
Group I: Wild-type like vesicles				
126-GFP	**1.78 ± 0.26**	**1.01 ± 0.13**	+	+++
126H1040A-GFP	**2.68 ± 0.25**	**1.12 ± 0.18**	+	**-**
126V1087I-GFP	**1.93 ± 0.48**	**0.98 ± 0.16**	+	+++
Group II: Larger aggregate				
126K839S-GFP	**4.01 ± 1.15**	**2.03 ± 0.31**	-	**-**
126E907A-GFP	**4.90 ± 1.45**	**1.72 ± 0.21**	**-**	+
126R1076A-GFP	**5.56 ± 0.65**	**2.05 ± 0.35**	**-**	++

* Forty-five to fifty vesicles of each replicase protein were measured to obtain the calculated mean and standard deviation.

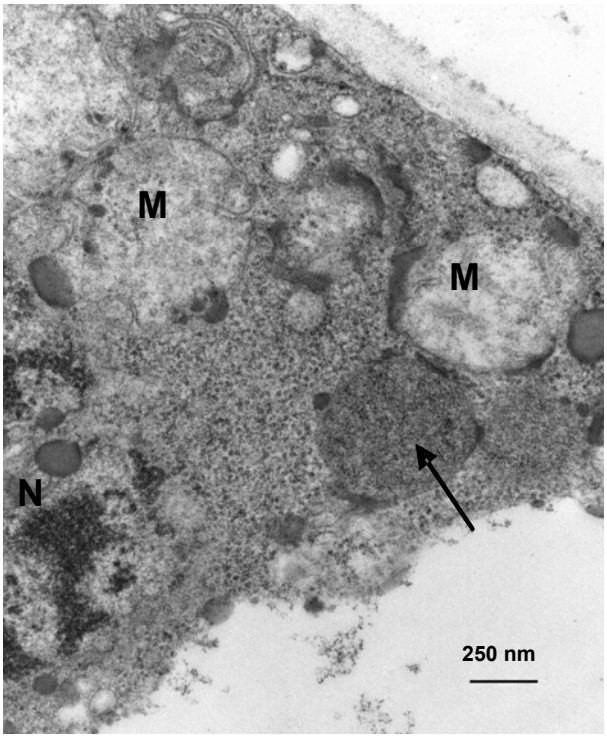
Consistent with the confocal data, the vesicles formed by 126-kDa protein were small and less dense, while the mutant 126K839S-kDa replicase formed much larger densed vesicles in the cytosol (Fig. 2.11). In contrast, similar vesicles were not detected in control plants expressing pBin vector only (data not shown). Interestingly, the vesicles formed by both proteins were observed to contain tubular like structures, which were also seen in "X-bodies" of TMV-infected plants (Hills *et al.*, 1987), suggesting a function of the 126-kDa replicase in promoting VRC formation.

It is interesting to note that there is a correlation between the biochemical activities of the TMV helicase domain and the assembly of 126-kDa vesicles (Table 2.1). Proteins possessing ATPase activities produced wild-type-like vesicles (wild-type, H1040A, and V1087I). In contrast, members producing larger aggregates (K839S, E907A, and R1076A) all failed in their ability to hydrolyze ATP (Fig 2.5A, Table 2.1). Such a correlation was not found on the protein's ability to bind to RNA (Fig. 2.5B, Table 2.1). The combined results suggest that ATPase activity, but not the RNA binding activity of the 126-kDa helicase domain may play a role in regulating the assembly of wild-type-like VRCs.

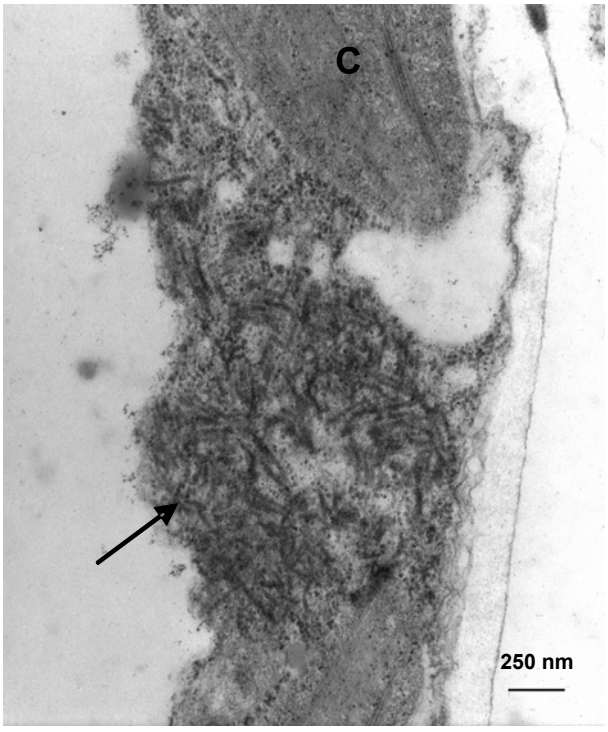
2.5 Discussion

Virus-encoded helicases have been demonstrated to play important roles in the viral life cycle (Gu *et al.*, 2000; Ivanov *et al.*, 2004; Wang *et al.*, 2005). Structural analysis showed that the signature motifs of the helicases are crucial for protein enzymatic activity and protein-protein interactions (for review, see Caruthers and McKay 2002). The helicase domain within the 126- and 183-kDa replicase proteins

Fig. 2.11. Electron micrographs of vesicles formed by 126-kDa and its mutant protein in *N. benthamiana*. The thin section was stained with lead citrate and uranyl acetate. The black arrows point to the 126-kDa induced small light vesicle and the 126K839S-kDa induced large dense vesicle, respectively. C, Chloroplast; M, mitochondria; N, nucleus.



126-GFP



126K839S-GFP

has been identified to perform multiple functions within the host (Abbink *et al*., 1998; Hagiwara *et al*., 2003; Padmanabhan *et al*., 2005). Yet the structure-function relationship of the TMV helicase domain remains at most unknown. In this study, individual mutations within four helicase motifs I, II, V, and VI were tested for their effects on protein structural conformation, biochemical properties, and the biological relevance in the formation of VRCs.

Biochemical activities of the TMV helicase polypeptide

The TMV helicase domain is a member of helicase SF1. Structural analyses of other helicases within helicase SF1 have shown that the active form of the enzyme is most likely a monomer (for review, see Singleton *et al.*, 2007)). Two general models, termed "inchworm" and "active rolling", have been described for the mechanisms of helicase activities. The "active rolling" model requires at least a dimeric form of the protein. The "inchworm" model, however, can be applied to either monomeric or oligomeric forms of the protein. In this study, both the monomers and the dimers of the TMV helicase polypeptides were shown to possess ATPase activity (Fig. 2.3C), which would favor the "inchworm" model. However, becaue the ATPase assay was performed with a relatively low concentration of the protein (~ 1.5 μM), it is possible that a dimer-monomer exchange occurs and the active ATPase activity comes from the monomeric but not the dimeric form. Thus, these findings cannot be used to identify which model, "active rolling" or "inchworm", is likely to be used by the TMV helicase. Further studies including crystal structure determination and a functional helicase assay on different forms of

the TMV helicase polypeptides will be necessary to identify the active form of the TMV helicase.

Viral helicases within SF1 contain five major motifs (For review, see Kadaré and Haenni, 1997). Helicase motifs I and II (also known as Walker A and B) are well known as NTP binding and hydrolysis motifs (Hall and Matson, 1999; Kadaré and Haenni, 1997). The failure of TMV helicase motif I (K839S) and II (E907A) mutants to hydrolyze ATP confirmed their activities (Fig. 2.5A). K839S mutation also dramatically interfered with RNA binding activity (Fig. 2.5B), indicating this residue may also play a role in transducing energy for RNA binding. The residues within motif V of other helicases have been shown to be involved in nucleic acid binding, primarily through interacting with the sugar-phosphate backbone (Hall and Matson, 1999). The mutant created in this study, H1040A, retained ATPase activity, but failed in binding RNA (Fig. 2.5). Structural tudies on helicase motif VI show that arginine residue contacts with the gamma pohophate of the nucleotide and functions in mediating the conformational change of the protein when binding to NTP (Hall and Matson, 1999). Further structure-function analysis suggests that helicase motif VI is involved in the transition of the NTPase activity to nucleic acid binding (Hall and Matson, 1999). Consistent with these results, the mutation from arginine to alanine (R1076A) within TMV helicase motif VI abolished the ATPase activity of the TMV helicase polypeptide and showed a reduced ability to bind RNA (Fig. 2.5). Combined, all individual mutations, when introduced into the infectious wild-type cDNA, disrupted the virus's ability to replicate in the host (Fig. 2.6), indicating that the biochemical properties of each single motif are indispensable for virus infectivity.

Viral protein-protein interactions in promoting VRC formation

Formation of membrane-bound VRCs is essential for replication of positive-strand RNA viruses. Viral protein-protein interactions have been shown to play important roles in promoting VRC assembly (Kaiser *et al.*, 2006). BMV-encoded 1a, a close homologue of 126-kDa protein, interacts with itself (O'Reilly *et al.*, 1995) and with 2a, a polymerase-like protein (O'Reilly *et al.*, 1997) and the interactions are necessary for formation of VRCs. Similarly, both CCMV and CMV-encoded 1a proteins self-interact in a yeast two-hybrid system (O'Reilly *et al.*, 1997). A previous immunoaffinity analysis demonstrated an interaction between TMV 126- and 183-kDa replicase proteins (Watanabe *et al.*, 1999). The interacting region has been mapped to be within the helicase domain in a yeast two-hybrid system (Goregaoker *et al.*, 2001). In this study, *in vitro* purified TMV helicase polypeptide was able to self-associate to form a dimer and a higher ordered structure as shown by gel filtration chromatography (Fig. 2.3) and electron microscopy (EM) (Fig. 2.4). The self-interaction of TMV helicase and its oligomerization thus may be important in forming an active enzyme complex by dictating intermolecular interactions between replicase proteins and directing the assembly of the VRC. The observations that the TMV 126-kDa replicase was able to form VRC-like vesicles in host cells further suggest a crucial role for the 126-kDa protein in assembly of membrane-associated VRCs (Fig. 2.9, 2.10). The protein-protein interactions within the helicase domain of the TMV replicases may provide a structural basis necessary for VRC formation.

ATPase activity of the TMV helicase plays a role in regulating VRC formation

To investigate the biological significance of the TMV helicase motifs, individual mutations within the helicase motifs (K839S, E907A, H1040A, R1076A) and outside of the helicase motifs (V1087I) were tested for their effects on protein-protein interaction and VRC formation. The *in vivo* pull-down assay showed that none of the mutations affected the ability of the TMV helicase polypeptide to self-associate (Fig. 2.7A). Additionally, all the tested 126-kDa mutants were able to form vesicles in host (Fig. 2.9 and 2.10), suggesting that the enzymatic activity of the helicase signature motifs are not necessary in initiating VRC assembly. For any wild-type or mutant 126-kDa replicase protein tested in this study, the vesicles formed in protoplasts were observed to be smaller than those in *N. benthamiana* plants. There are two possible explanations for the size difference. First, protoplasts are derived from *N. tabacum* cv. Xanthi, a separate species from *N. benthamiana*, and may have different fundamental biochemical and biophysical properties that affect formation of the 126-kDa vesicles. Second, lack of certain skeleton structure on protoplasts may have an effect on the assembly of higher-ordered replicase structures, resulting in smaller vesicles in the host cells.

Based on sizes, the VRC-like vesicles formed by 126-kDa protein and its mutants can be grouped into two categories: wild-type like vesicles and larger aggregates (Fig. 2.9 and 2.10). Interestingly, the mutants that resulted in larger vesicles were all unable to hydrolyze ATP (Fig. 2.5 and 2.9). In contrast, replicases that formed wild-type vesicles retained ATPase activity (Fig. 2.5 and 2.9), indicating that ATP hydrolysis may regulate proper VRC assembly. For TMV, an appropriate range of VRC sizes has been shown to be prerequisite for virus replication (Liu *et al.,*

2005). In their study, 126-kDa mutants with mutations within the IR region formed smaller vesicles in host (Liu *et al*., 2005). This result together with our data here suggests that different domains of the 126-kDa replicase may have distinct effects in VRC assembly. A mutational analysis on the helicase motifs of BMV 1a protein also demonstrated that helicase mutants affect the size of VRCs (Wang *et al*., 2005). Especially a mutation within BMV helicase motif III resulted in larger VRC-like spherules than that of wild type (Wang *et al*., 2005). In this study, other helicase motifs, including I, II, and VI, were shown to affect VRC formation (Fig. 2.8 and 2.9). One interesting mutant observed here is H1040A with a mutation within the helicase motif V. Although this mutant retained ATPase activity (Fig. 2.5A) and formed wild-type like inclusion bodies (Fig 2.8, 2.9 and Table 2.1), the mutant helicase polypeptide failed to bind RNA (Fig. 2.5B) and had no replication activity in the corresponding mutant virus (Fig. 2.6). This observation indicates that the recruitment of RNA template by the TMV helicase is not necessary for the proper assembly of VRCs. However, RNA binding activity is required for synthesis of viral genomic RNAs.

Monomer/dimer vs. higher ordered structure – why two forms?

In TMV-infected plant cells, the 126-kDa protein is produced in a larger amount than the 183-kDa protein (Lewandowski and Dawson, 2000; Watanabe *et al*., 1999). Although not required for RNA synthesis, the 126-kDa protein increases the replication efficiency ten times more, suggesting that the excessive 126-kDa replicases perform other important, yet unidentified functions (Ishikawa *et al*., 1986;

Lewandowski and Dawson, 2000). In this study, *in vitro* structural analysis shows that the TMV helicase polypeptide possesses two forms: a higher ordered structure and a monomer/dimer mixture. I speculate that both forms co-exist within the TMV-infected host cell. The higher ordered aggregates may represent a nonenzymatic form of the 126-kDa protein that acts as a structural platform necessary for assembling of membrane-associated VRCs. The presence of such a non-active replicase complex might explain the necessity of excess 126-kDa proteins produced during TMV infection. Furthermore, it is shown in this study that ATPase activity modulates the TMV replicase protein forming VRC-like vesicles. Possibly, the ATPase activity of the replicase monomers/dimers plays a regulatory role in maintaining the structural conformation of the protein, which further prevents it from aggregation. Other than viral replication activities, TMV replicase proteins have been shown to perform multiple functions during virus infection, such as cell-to-cell movement (Hirashima and Watanabe, 2001; Hirashima and Watanabe, 2003), virus disassembly (Wu and Shaw, 1997), induction of HR in host (Abbink *et al*., 2001; Erickson *et al*., 1999) and suppression of host RNA silencing (Ding *et al*., 2004). Distinct forms of viral replicase as shown in this work might partially explain its functional complexity during the process of virus life cycle. The higher ordered structures of the TMV replicase may be involved in constructing ER-associated VRCs and in viral intracellular movement. The monomeric/dimeric forms of the protein, on the other hand, are active in enzymatic activities and possibly involved in suppression of RNA silencing. Exactly what structural forms of the TMV replicase are involved in these different functions needs to be further determined.

Chapter 3: Interaction between the *Tobacco mosaic virus* replicase and a NAC domain transcription factor modulates virus accumulation

3.1 Abstract

NAC-domain proteins represent a large family of transcription factors associated with plant developmental processes, senescence and defense. Using a yeast two-hybrid system, interactions between the helicase domain of the *Tobacco mosaic virus* (TMV) replicase and an Arabidopsis NAC protein, ATAF2, and its tomato homologue were identified. The interaction between ATAF2 and the TMV replicase was further confirmed *in vivo* using an immuno-pull-down assay. Transient expression of ATAF2-GFP fusion in healthy plant tissues showed that the protein localized exclusively in the nucleus. Upon TMV infection, however, the accumulation of ATAF2 in the nucleus was dramatically reduced, suggesting that interaction with the TMV replicase affects ATAF2 function. To further characterize this interaction, transgenic Arabidopsis over-expressing ATAF2-GFP were created. The resulting plants developed a distinct phenotype similar to symptoms associated with TMV infection. TMV infection within *ATAF2* over-expressing plants resulted in a substantial decrease in virus accumulation when compared to wild-type non-transformed plants. In contrast, knock-out of *ATAF2* in Arabidopsis and silencing of *ATAF2* homologue in tomato showed no effect on virus accumulation. These results suggest that ATAF2-TMV interaction has a regulatory role in virus infection. Using a GUS reporter gene, the transcriptional level of ATAF2 was found to increase upon

TMV infection with GUS activities corresponding to virus accumulation and spread. However, such an induction did not occur in systemically infected tissues even though the virus titer was higher, indicating that expression of *ATAF2* is additionally modulated during the TMV infection cycle. Since *ATAF2* is a stress associated host gene that is responsive to salicylic acid (SA), I propose that virus-directed induction of *ATAF2* is involved in a SA-dependent host defense response and viral replicase protein interacting with ATAF2 suppresses this host defense resulting in virus accumulation.

3.2 Introduction

The consequences for virus infections vary depending on the extensive interactions between the virus and its host. For a susceptible host, virus infection results in specific disease symptoms that are generally associated with alterations in the normal functions of cellular proteins. A microarray analysis on RNA virus-infected Arabidopsis revealed common sets of host genes being induced, of which 10% were identified as host transcription factors (Whitham *et al.*, 2003). Many of these transcription factors have been recognized as members of important gene families that regulate a number of cellular processes involved in host stress/defense responses (for review, see Singh *et al.*, 2002).

A plant-specific transcription family, the NAC domain protein family, was identified a decade ago. Members within this family contain a highly conserved N-terminal NAC domain and a divergent C-terminal transcription activation region (TAR). The well-conserved N-terminal domain can be divided into five subdomains

(A to E) (Kikuchi *et al*., 2000). Subdomian D and E were shown as a DNA-binding domain (Duval *et al*., 2002). Several lines of evidence indicate that NAC genes are induced by various biotic and abiotic stresses suggesting they are involved in host stress/defense responses (Collinge and Boller, 2001; Hegedus *et al*., 2003). Furthermore, four NAC proteins are known to interact with viral proteins and the interactions play important roles in virus pathogenesis. GRAB1 and GRAB2 (for Geminivirus RepA binding) proteins were isolated by their interactions with a geminivirus RepA protein in a yeast two-hybrid system. GRAB protein expression was shown to interfere with geminivirus DNA replication in cultured cells (Xie *et al*., 1999). Arabidopsis NAC protein TIP (for TCV-interacting protein) interacts with the *Turnip crinkle virus* (TCV) CP and the interaction is involved in a host resistance response (Ren *et al*., 2000). Interaction between Tomato SlNAC1 (for *S. lycopersicum* NAC1) and the geminiviral replication accessory protein was also detected and found to enhance virus replication (Selth *et al*., 2005). Different effects of NAC proteins on virus accumulation indicate the functional diversity of this transcription factor family.

Tobacco mosaic virus (TMV) contains a single-stranded positive-sense RNA genome that encodes four proteins, two replicase proteins, a movement protein (MP), and a coat protein (CP). The TMV-interacting host proteins have been extensively studied (For reviews, see Boevink and Oparka, 2005; van der Heijden and Bol, 2002). TMV replicases, specifically, interact with various host factors and these interactions are involved in different viral activities, including elicitation of *N* gene-mediated resistance (Abbink *et al*., 2001; Erickson *et al*., 1999), formation of virus replication

complexes (VRCs) (Hagiwara *et al*., 2003; Yamanaka *et al*., 2000), replication of viral genomic RNAs (Osman and Buck, 1997), and inhibiting host defenses (Bilgin *et al.*, 2003). A yeast two-hybrid approach using individual TMV replicase fragments as "bait" to screen the Arabidopsis cDNA library has revealed a group of host proteins that interact with the virus (unpublished data). One interaction involving members of Aux/IAA proteins of auxin-mediated transcription regulators resulted in plant developmental disease symptoms (Padmanabhan *et al*., 2005). In addition, a cDNA microarray analysis was performed to study the changes of host gene expression in response to TMV infection (Golem and Culver, 2003). Both methods are useful in that they provide high-throughput results in identifying host genes and their gene products involved in TMV infection. However, studying specific virus-host interactions and specific genes that contribute to a successful viral invasion and/or host defense will shed light on a better understanding of the host cellular responses against TMV, ultimately adding our knowledge to develop anti-viral strategies.

In this study, yeast two-hybrid and *in vivo* pull-down assays identified an interaction between ATAF2, a NAC domain transcription factor, and the TMV 126-kDa replicase. The interaction was found to affect ATAF2 accumulation in the nucleus. SlNAC1, a tomato ATAF2 homologue, was also found to interact with the TMV helicase domain in yeast, suggesting that this TMV replicase-ATAF2 interaction is conserved among TMV hosts. In addition, *ATAF2* over-expression lines showed strong developmental abnormalities and resulted in a substantial decrease in virus accumulation. In response to TMV infection, expression of *ATAF2*

gene was up-regulated locally, but not systemically. Taken together, these results suggest that the interaction between ATAF2 and the TMV replicase plays a role in regulating virus infection in susceptible plants.

3.3 Materials and Methods

Plant materials and virus inoculation

A. thaliana ecotype Shahdara and tomato (*Solanum lycopersicon* cv. Pilgrim) plants were grown as described (Padmanabhan *et al.* 2005). All transgenic lines used in this study were derived from Arabidopsis ecotype Shahdara. *ATAF2* T-DNA insertion lines (Salk_015750 and Salk_136355) purchased from TAIR (The Arabidopsis Information Resource) are under Col-0 background.

Leaves of four-week old Arabidopsis ecotype Shahdara were dusted with carborundum (Fisher Scientific Company, Pittsburgh, PA.) and inoculated with 0.1mg/ml purified virus using a cotton swab. After inoculation, the plants were maintained in a high moisture condition overnight and then transferred to the regular growth condition. Controls were mock-inoculated with water.

Yeast Two-hybrid and β-galactosidase Assay

The full-length *ATAF2* gene was produced by RT-PCR using the total RNA extracts prepared from four-week-old Shahdara leaves. The amplified fragments were modified to contain a 5' *BamHI* and a 3' *XhoI* site and cloned into the *BamHI/XhoI* sits of pGAD10, producing pGAD-ATAF2. The bait construct was produced by introducing the helicase domain (aa. 814-1116) of the TMV replicase into the plasmid plexA-NLS to generate the plexA-HEL, which contains the in-frame

fusion of TMV helicase and LexA DNA binding domain. Both pGAD10-ATAF2 and pLexA-HEL constructs were transformed into a yeast strain L40 using a standard lithium acetate transformation method. The transformed yeast cells were then grown on a minimal medium lacking uracil, tryptophan, and leucine. The resulting colonies were assayed quantitatively for β-galactosidase activity in the presence of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described (Goregaoker ***et. al***., 2001).

For tomato ATAF2 homologue, the complete SlNAC1 ORF was PCR-amplified to contain a 5'-*BamHI* site and a 3'-*XhoI* site. The PCR fragment was then ligated into a similarly digested pACT vector to create pACT-SlNAC1. The construct pACT-SlNAC1 was transformed into a yeast strain L40 containing pLexA-HEL. The interaction between SlNAC1 and the TMV helicase domain was similarly examined using a β-galactosidase assay as described previously (Goregaoker ***et. al***., 2001).

ATAF2 transient expression assay

To obtain ATAF2-GFP fusion construct, the entire coding region of ATAF2 was PCR amplified with a 5' *KpnI* site and a 3' *BsiWI* site. The amplified fragment was cloned into plasmid pCMC-126-GFP (dos Reis Figueira *et al*., 2002) by replacing the 126-kDa replicase ORF with ATAF2. The designated plasmid, pCMC-ATAF2-GFP contains a single CaMV 35S promoter and the napaline synthase polyadenylation signal. Transient expression of ATAF2-GFP in *N. benthamiana* epidermal cells was achieved using a particle bombardment method. For each shot, 1~2 μg of plasmid DNA was coated with 1.3 μM tungsten particles and bombarded into leaf tissues using the method described previously (Padmanabhan *et al*., 2005). The bombarded tissues were incubated at room temperature for 12 to 14 h and

visualized under a LSM510 laser scanning confocal microscope with 10X NA 0.8 dry and 63X NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thonwood, N.Y.). Images were further analyzed with Zeiss LSM Imager Examiner software, version 3.0.

Constructs for plant transformation

Using pCMC-ATAF2-GFP as a template, a DNA fragment corresponding to ATAF2-GFP was PCR amplified using primers designed to contain a 5'-*KpnI* site and a 3'-*PstI* site. The amplified fragment was cloned downstream of CaMV 35S promoter in a standard binary transformation vector, pBI121 (Clonetech, Palo Alto, CA). The produced construct, designated as pBI-ATAF2-GFP, was further transformed into *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw method (Holsters *et al.*, 1978).

To make *ATAF2 Promoter::GUS* fusion construct, a 2-kb DNA fragment upstream of the *ATAF2* coding region was PCR-amplified from Shahdara genomic DNA with a 5' *PstI* site and a 3' *BamHI* site. The modified PCR fragment was ligated to the β-glucuronidase (GUS) coding sequence and a nopaline synthase (NOS) 3' ploy (A)-sequence in pBI101.1 vector (Clontech, Palo Alto, CA). The resulting construct, pBI-*pATAF::GUS*, was similarly transformed into *A. tumefaciens* GV3101.

Generation of transgenic lines

Both ATAF2-GFP over-expression lines and P_{ATAF2}*::GUS* transgenic lines were obtained using the floral dip method described previously (Clough and Bent, 1998). Positive transformants were selected on solid Murashige and Skoog medium supplemented with 50 μg/ml kanamycin. T1 transformants overexpressing ATAF2

were used for virus infection and T2 P_{ATAF2}*::GUS* transformants were used for GUS activity.

GUS assay

Histochemical staining for GUS activity was performed using X-Gluc (5-bromo-4-chloro-3-indolyl p-D-glucuronide) (Gold Biotechnology, St. Louis, MO) as a substrate. Plant tissues were vacuum-infiltrated in X-Gluc reaction buffer containing 500 µl X-Gluc, 10 mM EDTA, 100 mM NaH_2P0_4 pH 7, 5 mM Potassium ferricyanide, 5 mM Potassium ferrocyanide and 0.1% v/v Triton and incubated at 37°C overnight. After 2-3 time washes in 70% ethanol, the stained tissues were kept in 50% ethanol until being photographed.

***In vivo* pull-down assay**

ATAF2-GFP transgenic Arabidopsis plants were inoculated with 0.1 mg/ml of wild-type TMV virus using a cotton swab. Fourteen days post inoculation (dpi), 0.5 gram of systemically infected leaf tissues were collected and homogenized in extraction buffer containing 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.5% TritonX-100, 0.2% 2-mercaptoethanol, 5% glycerol, PMSF, and proteinase inhibitor cocktail (Sigma, St. Louis, MO). The ground tissues were pelleted by centrifugation for 10 min at 15,000 rpm. The supernatant (1 ml) was incubated with 5 µl of anti-polyGFP antibody (Sigma, St. Louis, MO) at 4°C overnight with gentle shaking followed by adding 30 µl of protein A agarose (Invitrogen, Carlsbad, CA) to the protein complex and incubating for additional 3 h. The immune complexes were then centrifuged and washed three times in 1 ml wash buffer (25 mM Tris-HCl at pH 7.5, 250 mM NaCl, 2 mM EDTA, 0.05% TritonX-100, 1 mM PMSF). After the last

wash, the precipitated protein complex was resuspended in 2X sample buffer and analyzed by SDS-PAGE and western–blotting with the polyclonal antibody raised against replicase proteins.

Isolation of ATAF2 homologue in Tomato

The tomato ATAF2 homologue was identified using a BLAST search program within the TIGR Tomato Gene Index database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi). The closest match to the ATAF2 query sequence revealed a tomato cDNA clone (TC171280), namely SlNAC1. The largest ORF within SlNAC1 sequence was further amplified by RT-PCR using tomato cDNA mixture and cloned into the TA-cloning site of the PCRII vector (Invitrogen, Carlsbad, CA). The resulting TA-SlNAC1 clone was sequenced for confirmation.

Construction of gene silencing vector and VIGS

The *Tobacco rattle virus* (TRV)-based VIGS vectors pTRV1 and pTRV2 were kindly provided by Dr. Dinesh-Kumar's lab at Yale University, New Haven, CT. Two SlNAC1 cDNA fragments, corresponding to nucleotides 1-304 and 597-906, were separately amplified by PCR with primers containing *BamHI/XhoI*, and *BamHI/SalI* restriction sites, respectively. The PCR products were then cloned into pTRV2 vector digested with *BamHI* and *XhoI* to create pTRV2-SlNAC1-N and p pTRV2-SlNAC1-C. Both constructs were separately introduced into *A. tumefaciens* GV3101 using a standard transformation method (Holsters *et al.*, 1978). *Agrobacterium* containing a pTRV2-PDS construct was obtained from Dr. Dinesh-Kumar.

Agrobacteria containing pTRV1 and pTRV2 or its derivatives were coinfiltrated into cotyledons and lower leaves of 3-week-old tomato plants using a 1ml needleless syringe. For the VIGS assay, *Agrobacteria* transformed with pTRV vector only and pTRV-PDS, which contains a fragment of the gene encoding phytoene desaturase were used as a negative control and a positive control, respectively.

Analysis of TMV virus accumulation

TMV-infected tissues were collected and homogenized in ESB buffer. 5 µg of total proteins, determined with the Bradford assay (BioRad, Hercules, Calif.) was fractionated in a 12% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was probed with a polyclonal rabbit antiserum raised against TMV CP followed by an alkaline phosphatase-conjugated secondary anti-rabbit immunoglobulin G (Sigma, St. Louis, MO). The detection was achieved using NBT (nitro BT; Fisher Scientific) and BCIP (5-bromo-4-chloro-3-indolyl phosphate; Fisher Scientific) as substrates. The CP levels on the blot were further quantified using AlphaImage software (Alpha Innotech Corp., San Leandro, Calif.).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described previously (Liu *et al*., 2002). Briefly, Total RNA from Arabidopsis and tomato was extracted using the RNeasy RNA extraction kit (Qiagen, Valencia, CA). 1 µg of total RNA was reverse transcribed using a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) and the resulting cDNA was further used as a template for PCR reaction. During the successive PCR cycles (27, 30, 33, and 36), five-microliter aliquots were collected

and further separated on an agarose gel. The elongation factor (eEF1A) served as an internal control for RNA quantity in RT-PCR.

3.4 Results

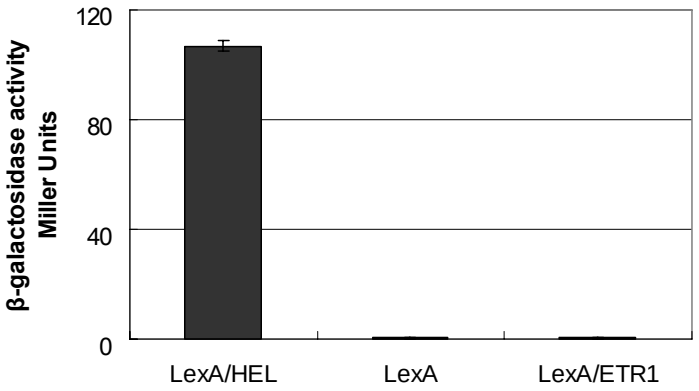
Interactions between the TMV replicase and NAC proteins within ATAF2 subfamily

A yeast two-hybrid screen has previously been performed by a senior graduate student Sameer Goregaoker to identify the host proteins interacting with the TMV replicase protein. The helicase domain of the TMV replicase was fused to the LexA DNA-binding domain to serve as "bait" and the whole *A. thaliana* cDNA library was screened. One positive transformant that showed high ß-galactosidase reporter activity in yeast was found to contain a single open reading frame encoding a plant transcription factor, ATAF2 (Fig 3.1A). The plasmid was recovered and retransformed into yeast along with the TMV helicase bait construct and the interaction was further confirmed.

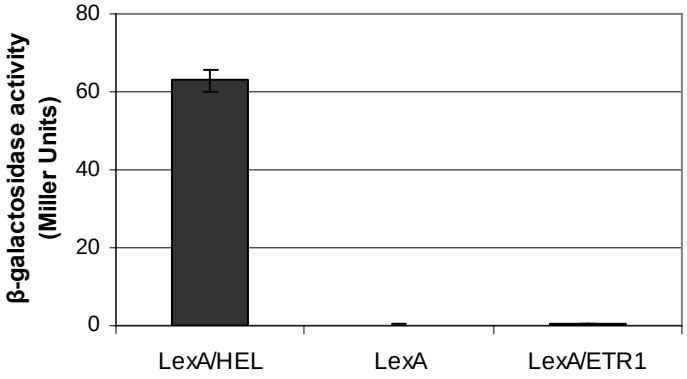
ATAF2 belongs to the NAC domain protein family. Members within this family participate in diverse plant biological roles, including development, senescence, and viral pathogenesis. The *ATAF2* gene encodes a 283-amino-acid protein with a calculated molecular mass of 32 kDa. To further determine whether ATAF2 interacting with the TMV replicase in plant cells, an immuno-pull down assay was employed using transgenic plants overexpressing ATAF2-GFP. The TMV infected tissues were immuno-precipitated with anti-GFP antibody and the pull-down complex was examined by western blot using antibody against the TMV replicase protein.

Fig. 3.1. Arabidopsis ATAF2 and its tomato homologue, SlNAC1, interact with the TMV replicase. (A). ß-Gal assay showing the interactions between the helicase domain of the TMV replicase and NAC domain proteins from both Arabidopsis and tomato in yeast. Both ATAF2 and SlNAC1 were expressed as a fusion with the GAL4 activation domain and the helicase domain was fused to the DNA binding domain of the LexA. Three independent transformants were analyzed for β-galactosidase activity produced by activation of the *lacZ* reporter by protein/protein interactions. The empty LexA vector and the LexA containing ETR1 opening reading frame were used as negative controls. (B) Co-immunoprecipitation assay confirming the interaction between ATAF2-GFP and the 126-kDa replicase. ATAF2-GFP transgenic plants were infected with the wild-type virus. The infected tissues were precipitated with anti-GFP antibody and probed with anti-replicase antibody.

A

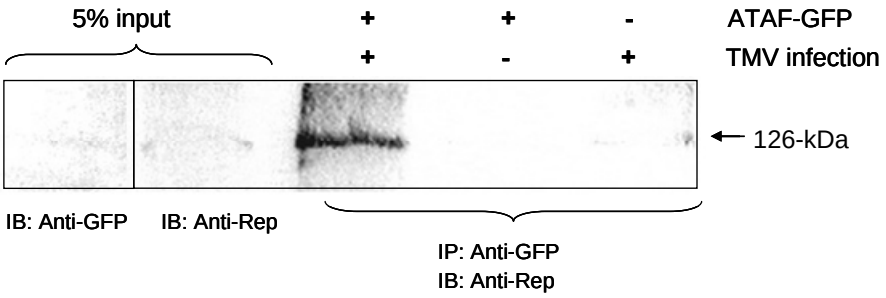


pACT/ATAF2



pACT/SlNAC1

B



The result confirmed the interaction between ATAF2 and the TMV replicase *in vivo* (Fig 3.1B).

Based on amino acid sequences, NAC proteins can be classified into several subfamilies (Ooka *et al*., 2003) and members within ATAF2 subfamily can be found in various plant species (Appendix A.1) (Selth *et al*., 2005). To find out whether this ATAF2-TMV infection is also conserved in plant system, a Tomato Gene Index database was blast searched against ATAF2 amino acid sequence. It revealed that the closest ATAF2 relative in tomato is gene *SlNAC1*, which encodes a 301-amino-acid protein. The conserved NAC domains between ATAF2 and SlNAC1 showed 85% identity and 92% similarity (Appendix A.2). Tomato SlNAC1 ORF was similarly introduced into the yeast two-hybrid system and the interaction between SlNAC1 and the TMV helicase domain was also detected (Fig. 3.1A).

ATAF2 accumulation is disrupted upon TMV infection

Like other members within the NAC domain family, ATAF2 was found to contain a nuclear localization signal (NLS) (PRDRKYP) in the subdomain C by using a PSORT II program (http://bioweb.pasteur.fr/seqanal/interfaces/psort2.html). To further investigate whether ATAF2 expression and nuclear localization is affected by TMV infection, a construct containing an ATAF2-GFP fusion under the control of the CaMV 35S promoter was produced and transiently expressed in either mock- or TMV-infected tissues. As expected, ATAF2 localized exclusively in the nucleus in mock-inoculated tissues (Fig. 3.2A). In TMV-infected tissues, however, the accumulation of ATAF2 was dramatically reduced to an undetectable level (Fig. 3.2).

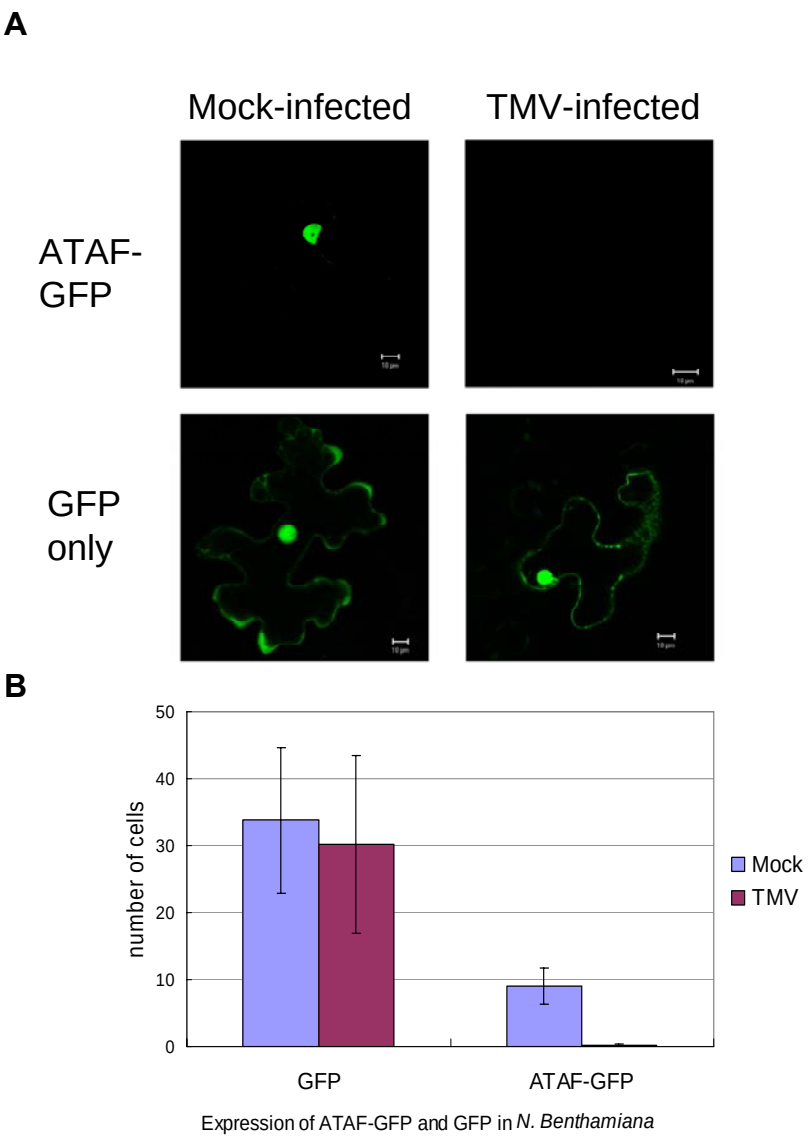


Fig. 3.2. Transient expression of ATAF2-GFP in *N. benthamiana* epidermal cells. TMV infection affects the accumulation of ATAF2-GFP as shown by fluorescent images (A) and by numbers of cells expressing detectable fluorescent signals (B). Expression plasmids containing either ATAF2-GFP or GFP were particle bombarded into *N. benthamiana* leaves. Fluorescent images were taken 14 to 16 hours post bombardment. Cell numbers were averaged from 10 independent bombardment experiments.

In contrast, no obvious changes were observed for the expression of GFP in either mock- or TMV-infected tissues (Fig 3.2). This result suggests that the interaction between ATAF2 and the TMV replicase alters the ATAF2 localization and accumulation in the host. Whether expression of ATAF2 is inhibited or ATAF2 is directed by the viral protein for degradation is not known.

ATAF2 induces a developmental phenotype in transgenic Arabidopsis

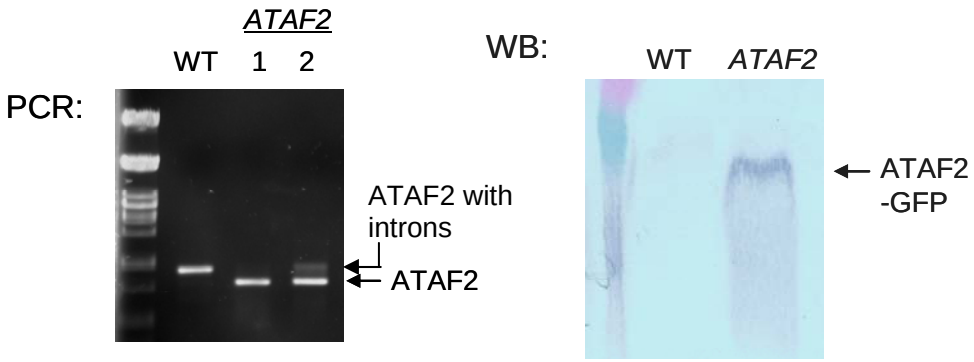
To characterize the function of ATAF2 in Arabidopsis, the full-length ATAF2 ORF with a GFP fusion was cloned into an *Agrobacterium* binary vector under the control of the CaMV 35S promoter. The construct was further introduced into Arabidopsis ecotype Shahdara. Transgenic lines overexpressing ATAF2-GFP showed a developmental phenotype that included stunting of the plants, lack of apical dominance, curled or cup-shaped leaves, poorly developed inflorescence, and small siliques (Fig. 3.3). All successful transgenic lines were confirmed by PCR and western-blot analyses (Fig. 3.3B).

ATAF2 over-expression inhibits virus accumulation

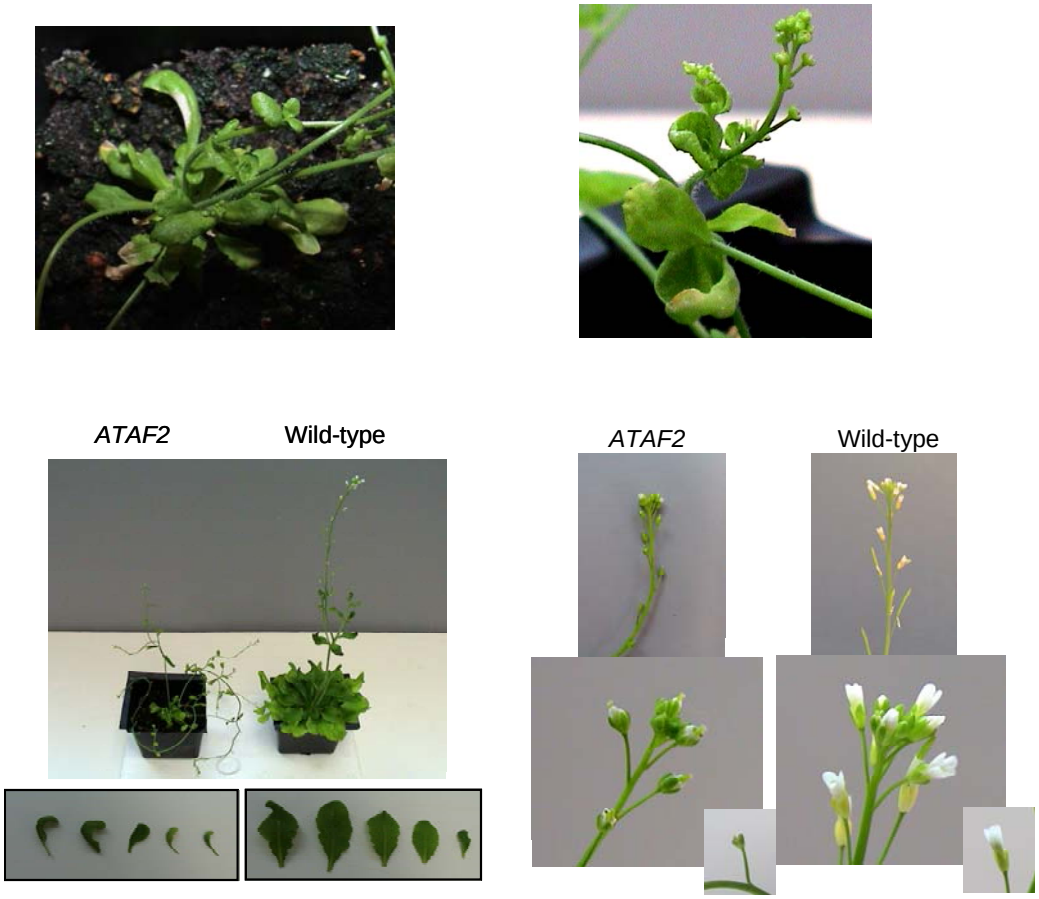
To determine the effect of ATAF2 protein in relation to virus infection, accumulation of TMV coat protein (CP) in three independent *ATAF2-GFP* transgenic lines was analyzed. In this study, The F1 progeny of ATAF2 transgenic lines were used. The same amount of purified TMV virions was rub-inoculated onto either *ATAF2-GFP* transgenic or wild-type non-transformed plants. At various time points (2, 4, and 6 dpi), the inoculated tissues were collected and proteins were extracted for

Fig. 3.3. Over-expression of ATAF2-GFP in Arabidopsis. (A). Successful transgenic lines were confirmed by PCR analysis using ATAF2 specific primers and Western blot using anti-GFP antibody. ATAF2 genomic DNA in wild-type Arabidopsis plants contains two introns (nucleotide 279-367 and 649-739). Amplification of the entire exon and intron region from wild-type Arabidopsis resulted in a longer PCR product than that from *ATAF2* overexpression lines. (B) ATAF2-GFP transgenic lines showed strong developmental phenotype. Photos were taken when the plants were 6 week old. Observed phenotype includes severely stunted plants lacking shoot apical dominance; curled or cup-shaped leaves; and poorly developed inflorescence leading to small siliques.

A


ATAF2
WT 1 2
PCR:
ATAF2 with introns
ATAF2
WB:
WT ATAF2
ATAF2 -GFP

B


ATAF2
Wild-type
ATAF2
Wild-type

analysis by SDS-PAGE and Western-blot. Accumulation of CP in transgenic lines was greatly reduced when compared to wild-type control plants (Fig. 3.4). Especially at 6 dpi, virus accumulation in *ATAF2-GFP* transgenic lines was found to be one-sixth that in wild-type non-transformed plants (Fig. 3.4). The reduced accumulation of virus in *ATAF2* over-expression plants suggest that ATAF2 plays a role in host defense to virus infection.

ATAF2 knockouts have no effects on virus accumulation

Next, the effects of *ATAF2* knockouts on TMV CP accumulation were assessed. Two *ATAF2* T-DNA insertion knockout lines, Salk_015750 with an intron insertion and Salk_136355 with an exon insertion, were obtained from TAIR. Both knockout lines were confirmed by RT-PCR and both exhibited no obvious developmental phenotype distinct from that of wild-type plants (Fig. 3.5). In addition, there was no significant difference in TMV accumulation between *ATAF2* knockout lines and wild-type plants (Fig. 3.5).

More recently, VIGS (for virus-induced gene silencing) has proven to be an efficient way to shut down host gene expression. Double-stranded RNA molecules formed during virus replication trigger a host silencing response through a homology-dependent RNA degradation mechanism. Therefore, host gene fragments, when introduced into the viral genome, will direct the host defense system to degrade the corresponding endogenous host mRNA, resulting in a down-regulation of host gene expression. Liu *et al*. (2002) have efficiently utilized a TRV- based VIGS system to

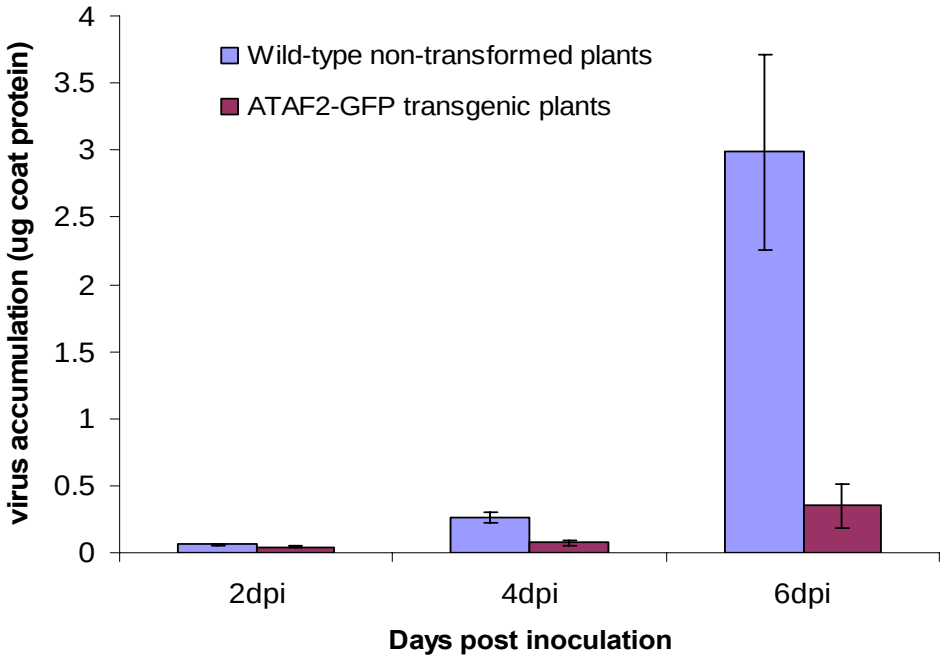


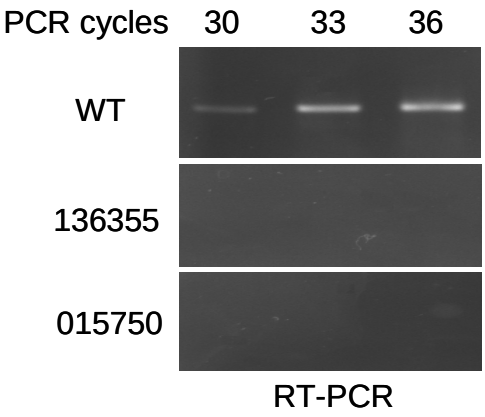
Fig. 3.4. Over-expression of ATAF2 inhibits TMV accumulation. TMV accumulation in ATAF2-GFP transgenic and wild-type non-transformed plants is determined by measuring the TMV CP content in the inoculated tissues at 2, 4, and 6 days post-inoculation (dpi). Bars represent the levels of coat protein (CP) accumulated in inoculated tissues as determined by Western immunoblot. The measurement of CP levels were performed using three independent *ATAF2* over-expression lines. The test was repeated twice.

Fig. 3.5. *ATAF2* knockouts have no effect on virus accumulation. (A) Two independent *ATAF2* T-DNA knockout lines (Salk_015750 and Salk_136355) obtained from TAIR show no obvious phenotype in comparison to wild-type plants. Photo was taken when the plants were 6 week old. (B) Both knockout lines are confirmed by RT-PCR amplifying the full-length *ATAF2* gene. (C) Comparison of virus accumulation in two *ATAF2* knockout lines and in wild-type plants. TMV accumulation in *ATAF2*-knockout and wild-type plants is determined by measuring the TMV CP content in the inoculated tissues at 2, 4, and 6 days post-inoculation (dpi). The same amount of total proteins (5 μg) collected from the infected tissues were fractionated in a 12% SDS-PAGE followed by a western blot analysis using antibody against TMV CP. No difference of CP levels is detected between wild-type and *ATAF2* T-DNA knock-out lines.

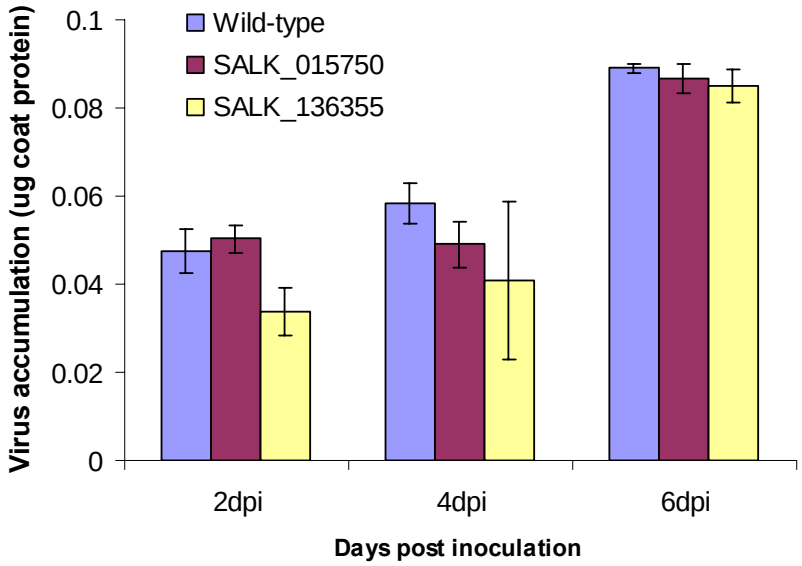
A


Wt
136355
015750

B


PCR cycles
30
33
36
WT
136355
015750
RT-PCR

C


Virus accumulation (ug coat protein)
0.1
0.08
0.06
0.04
0.02
0
Wild-type
SALK_015750
SALK_136355
2dpi
4dpi
6dpi
Days post inoculation

shut down the host gene expression in tomato. Using a similar loss-of-function approach, DNA fragments from tomato *SlNAC1* gene were introduced into TRV2 VIGS vector. All NAC proteins contain a highly conserved N- terminal NAC domain and a variable C-terminal TAR region. VIGS targeting at the conserved N-terminal domain of *SlNAC1* presumably disrupts accumulation of similar *NAC* genes in tomato. In contrast, introduction of the C-terminal region to the VIGS system will only down-regulate the specific *SlNAC1* gene itself. The effectiveness of VIGS was analyzed by a semi-quantitative RT-PCR as shown in Fig. 3.6B. The results showed that silencing *SlNAC1* only did not affect plant growth in comparison to the control plants possibly due to the redundancy of *NAC* genes in plants (Fig. 3.6A). However, targeting at the conserved NAC domain for gene degradation led to a strong developmental phenotype (Fig. 3.6A). This result onfirmed the involvement of NAC proteins in plant developmental process. The *SlNAC1* silenced plants were inoculated with TMV virions. Interestingly, although silencing *NAC* genes resulted in a strong developmental phenotype in tomato, there was no obvious difference in virus accumulation between *SlNAC1*-silenced and control plants (Fig. 3.6C).

ATAF2 is induced in response to TMV infection

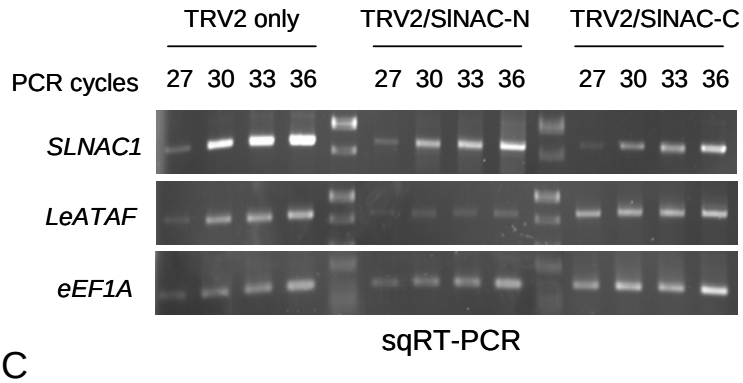
In order to determine the expression of ATAF2 in response to virus infection, the promoter region of *ATAF2* (2000 bp) was fused to the GUS reporter gene and the construct was further transferred to Shahdara using an *Agrobacterium*-mediated transformation. After kanamycin selection, T2 generation from three transgenic lines was examined for GUS activity. *ATAF2* promoter activity was detectable in

Fig. 3.6. Silencing of *SlNAC1* in tomato has no effect on TMV accumulation. (A) *SlNAC1*-silenced plants targeting at the N-terminal conserved NAC domain (TRV2/SlNAC-N) showed strong developmental defects. VIGS targeting at the *SlNAC1* specific TAR region (TRV2/SlNAC-C) showed no phenotype. Silencing phytoene desaturase (*PDS)* gene led to a characteristic photobleaching phenotype on tomato leaves indicative the effectiveness of VIGS system. (B) RT-PCR analysis of *S.lycopersicon* cv. Pilgrim *SlNAC1*-silenced and TRV2 control plants. *LeATAF2* is another member of *NAC* gene family in tomato. The 300-bp of specific C-terminal TAR regions from both *SlNAC1* and *leATAF* were RT-PCR amplified to analyze the efficacy of VIGS. Down-regulation of both *SlNAC1* and *leATAF2* was observed in *SlNAC1*-silenced plants targeting at the conserved NAC domain. VIGS targeting at the *SlNAC1* specific TAR region resulted in down-regulation of *SlNAC1* gene only. Tomato *eEF1A* was used as an internal control. (C) Both *SlNAC1*–silenced plants and TRV2 control plants were rub-inoculated with TMV and infected tissues were collected at three time points (2 dpi, 4 dpi, and 6 dpi). The same amount of total proteins (5 μg) extracted from infected tissues was fractionated in a 12% SDS-PAGE followed by a western blot analysis using antibody against TMV CP. No significant difference in virus accumulation was observed in both *SlNAC1*-silenced plants in compared to control plants.

A

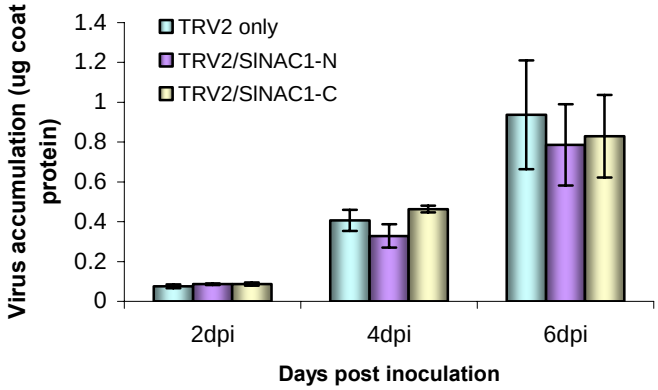

TRV2 only
TRV2/PDS
TRV2/SlNAC-N
TRV2/SlNAC-C

B


TRV2 only
TRV2/SlNAC-N
TRV2/SlNAC-C
PCR cycles
27 30 33 36
27 30 33 36
27 30 33 36
SLNAC1
LeATAF
eEF1A

sqRT-PCR

C


Virus accumulation (ug coat protein)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TRV2 only
TRV2/SlNAC1-N
TRV2/SlNAC1-C
2dpi
4dpi
6dpi
Days post inoculation

seedlings, young plants, leaves, root vascular cylinder, and floral organs (Fig 3.7). No activity was observed in root tips (Fig 3.7).

To test *ATAF2* expression in response to virus infection, four-week-old transgenic plants were inoculated with purified virions, and the *ATAF2* expression was carefully monitored at different time points. Induction of GUS activity was observed in TMV-inoculated tissues (Fig. 3.8) and activation of GUS activity was very similar to the patterns of virus accumulation and spreading determined by tissue print immunoblot using antiserum against TMV CP (Fig. 3.8). However, mock-inoculated tissues which were similarly dusted with carborundum did not show increased GUS activity (Fig. 3.8), suggesting that expression of *ATAF2* in inoculated tissues was specifically induced by the virus.

In a susceptible host, TMV can spread through vascular tissues to uninoculated tissues. The ability of the virus to move systemically determines the viral pathogenicity. In this study, we also examined the ATAF2 promoter activity in systemically infected tissues. Although the virus accumulated in systemic tissues at a level comparable to that in the inoculated tissues, the increased activity of ATAF2 promoter was not observed (Fig. 3.8B). The induction of *ATAF2* in TMV-inoculated tissues, but not in systemic-infected tissues was further confirmed by semi-quantitative RT-PCR (Fig. 3.9). These results suggest that *ATAF2* expression is modulated by TMV at the different stages of virus life cycle.

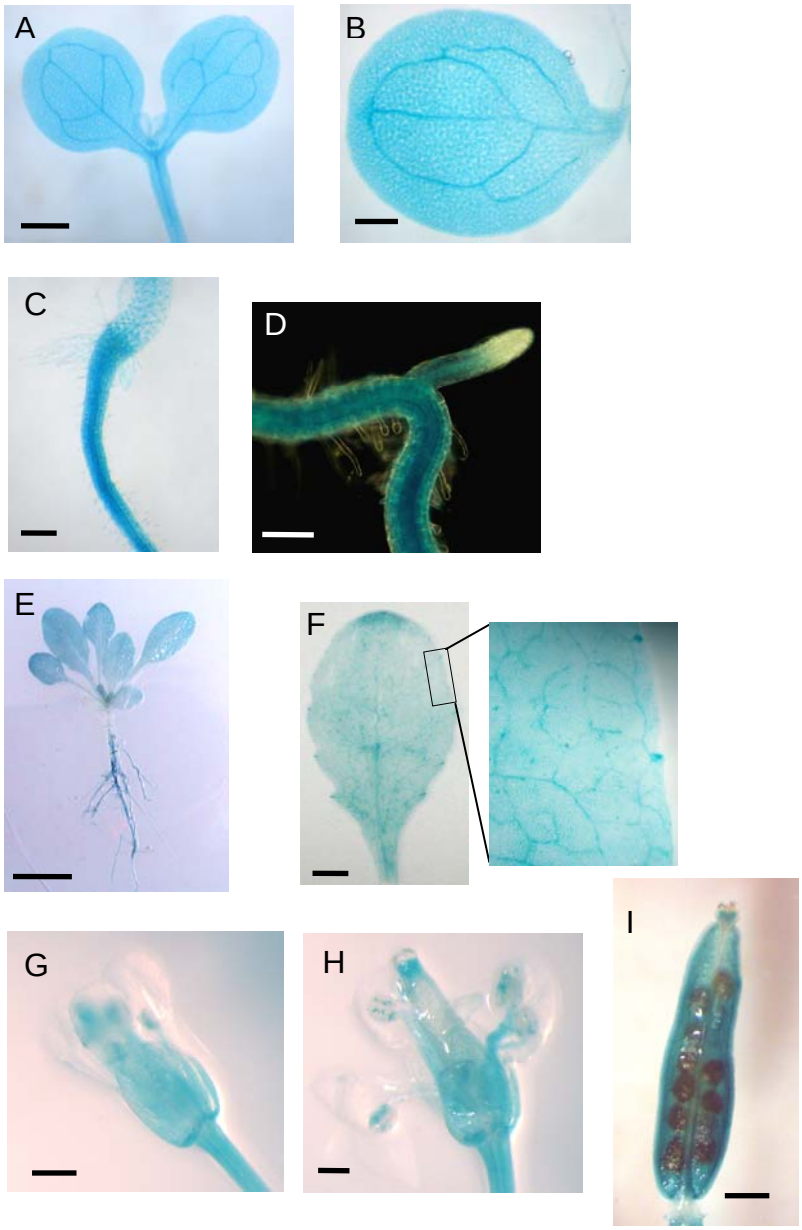
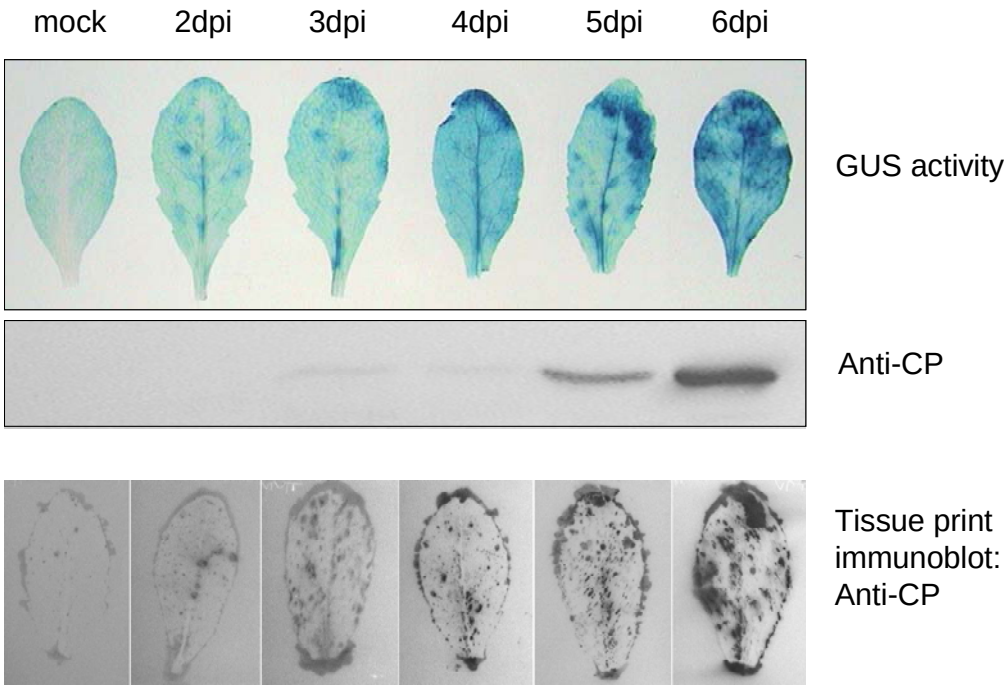


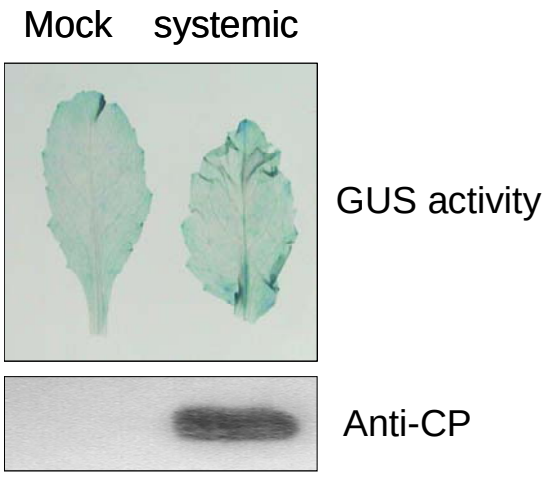
Fig. 3.7. Histochemical analysis of GUS activity in transgenic Arabidopsis expressing *pATAF2::GUS* fusion constructs. Shown are *ATAF2* promoter activity in cotyledons (A and B), root and Hypocotyl/root junction (C), root central cylinder (D), 2-week-old young plant (E), leaf and hydathodes (F), sepals (G), filaments and pollen grain (H), and silique (I). GUS activity is not detected in root tips (D), and petals (G and H) and less in root epidermal cells. Bars in (A, B, C, E, G, H, and I) = 0.5 mm, (D) = 0.2 mm, (F) = 5mm.

Fig. 3.8. *ATAF2* is induced in locally inoculated, but not in systemically infected tissues. (A) Induction pattern of *ATAF2* expression in TMV-inoculated tissues is similar to the pattern of virus accumulation and spreading. Mock- or TMV-inoculated tissues collected at different time points (2, 3, 4, 5, and 6 dpi) were analyzed on their GUS activity and TMV accumulation. After histochemical staining for GUS activity, individual leaf was analyzed for its CP content by Western immunoblot. To monitor the pattern of TMV accumulation and spreading, a tissue print immunoblot method was employed. (B) Induction of GUS activity was not observed in systemically infected tissues. The systemic tissues were analyzed at 14 days post inoculation.

A


mock
2dpi
3dpi
4dpi
5dpi
6dpi
GUS activity
Anti-CP
Tissue print
immunoblot:
Anti-CP

B


Mock
systemic
GUS activity
Anti-CP

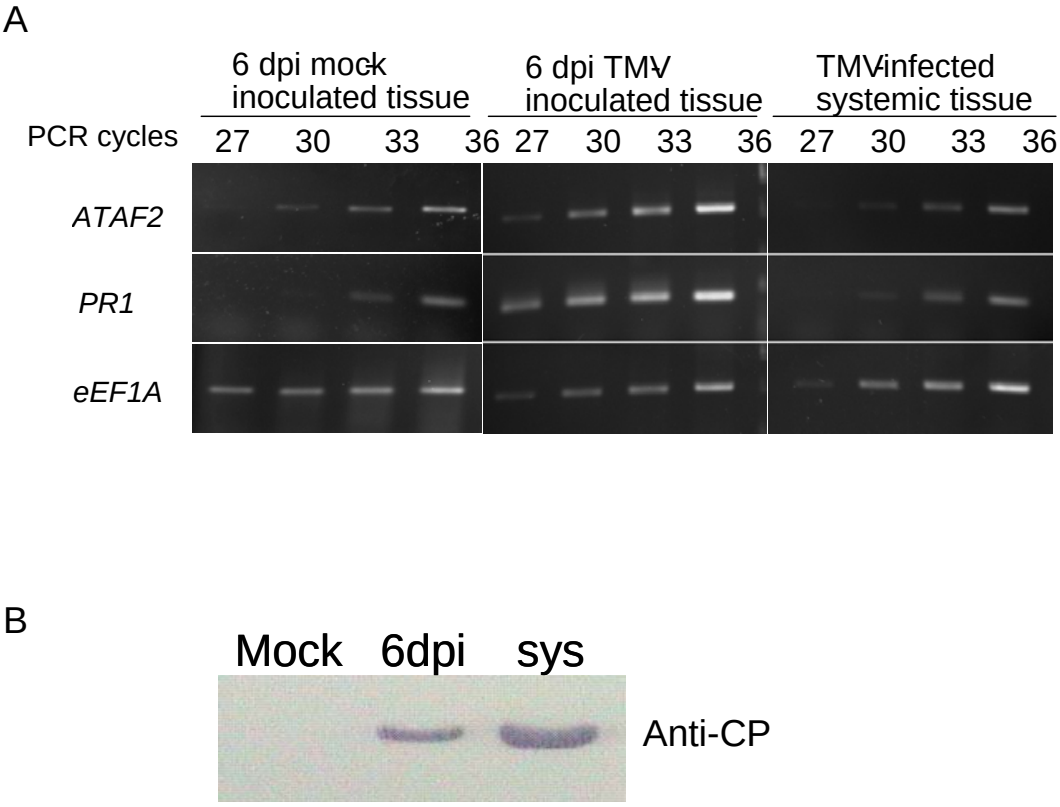


Fig. 3.9. A correlation between *ATAF2* and *PR1* expression in inoculated and systemic-infected tissues. (A) Comparison of ATAF2 and PR1 transcriptional levels in 6 dpi inoculated tissues and systemic tissues using a semi-quantitative RT-PCR. 1 µg of total RNA was employed for RT-PCR and total of 36 PCR cycles were used. eEF1A was used as an internal control. (B) Western blot to confirm the TMV accumulation within 6-dpi inoculated and systemic tissues.

Altered expression level of *ATAF2* correlates to expression of *PR1*, a host defense gene

NAC domain proteins have been shown to be involved in host defense system (Collinge and Boller, 2001; Hegedus *et al*., 2003). A microarray analysis demonstrated that *ATAF2* over-expression repressed *PR* gene expression (Delessert *et al*., 2005). Expression of *PR* genes is tightly correlated with the host defense responses, including the immediate hypersensitive response (HR) and the systemic acquired resistance (SAR). To investigate whether *PR* gene expression is related to altered *ATAF2* expression during TMV infection, an RT-PCR approach was performed to compare both *ATAF2* and *PR1* gene levels in TMV locally and systemically infected tissues. The data collected here showed that although viruses accumulated more in systemic tissues, the transcription levels of both *ATAF2* and *PR1* are higher in inoculated tissues (6 dpi) (Fig 3.9). Since *PR1* is one of the key players in host defense system, the correlation of *ATAF2* to *PR1* expression suggests that ATAF2 is also involved in host defense responses. In addition, *ATAF2* was observed to be induced by salicylic acid (SA) treatment (Fig. 3.10), further implicating involvement of *ATAF* in host defense responses.

3.5 Discussion

All plant viruses contain a small genome when compared to their host. Thus developing various strategies, including maximum use of host cellular machinery and active suppression of host defense systems, are necessary for viruses to achieve a successful infection. Virus-host interactions have been recognized to play

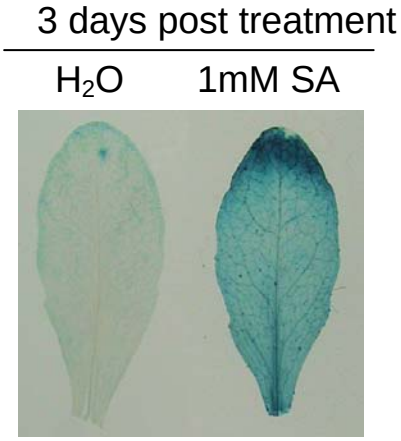


Fig. 3.10. Salicylic acid induces *ATAF2* expression. 4-week-old *ATAF2 protmoter::GUS* transgenic plants were watered with either 1mM SA or water. GUS activity was monitored three days post SA treatment.

important roles in virus pathogenesis. The TMV 126-kDa replicase protein is required for virus replication and is involved in several protein-protein interactions. In this study, novel interactions between the TMV replicase protein and NAC proteins of host transcription factor are characterized. The possible involvement of NAC proteins in host defense signaling pathway is also proposed.

Roles of ATAF2 in plant development and defense responses

Being one of the earliest discovered members within the NAC domain family, ATAF2 was originally identified by its ability to alleviate the repressed activity of the CaMV 35S promoter in yeast (Xie *et al*., 1999, cited as personal communication). Overexpression of *ATAF2* driven by the CaMV 35S promoter in Arabidopsis ecotype Shahdara gave rise to strong developmental phenotype that included stunting of the plants, curled and cup-shaped leaves, and underdeveloped floral organs (Fig. 3.3). Similar phenotypes were observed when introducing six different *Brassica napu*s *bnNAC* genes into *A. thaliana* (Hegedus *et al*., 2003). Interestingly, *ATAF2* overexpression in Arabidopsis ecotype Ler resulted in a distinct phenotype with the plant showing increased leave size and biomass (Delessert *et al*., 2005). Different effects of NAC proteins on plant developmental processes suggest that although they share a conserved N-terminal domain, members within this transcription family function in different ways. Consistent with a previous study, two *ATAF2* T-DNA homozygous knockout lines (Salk_136355 and Salk_015750) showed wild-type like phenotype (Fig. 3.5A) (Delessert *et al*., 2005). Since 105 members of *NAC* genes have been detected in Arabidopsis genome (Ooka *et al*., 2003), it is most likely that

lack of apparent phenotypes in *ATAF2* knock-out plants is due to redundancy of NAC gene family. A similar result was observed in tomato that when *SlNAC1* gene itself was targeted for degradation, plants showed wild-type like phenotype (Fig. 3.6). However, down-regulation of all genes that share the conserved NAC domain resulted in developmental defects (Fig. 3.6).

Previous studies have demonstrated interactions between NAC proteins and viral proteins from *Wheat dwarf virus* (WDV) (Xie *et al*., 1999), *Tomato leaf curl virus* (TLCV) (Selth *et al*., 2005), and TCV (Ren *et al*., 2000). In this study, a NAC protein ATAF2 was identified to interact with the TMV replicase protein (Fig. 3.1). Over-expression of ATAF2 resulted in reduced virus accumulation, suggesting a role of ATAF2 in basal resistance to TMV infection. Interestingly, a previous study showed that overexpression of ATAF2 led to increased susceptibility to the *Fusarium oxysporum* fungal infection (Delesser *et al*., 2005). Fungal infection is generally associated with toxin secretion and plant cell death for the purpose of nutrient uptake. In contrast to that, being an obligate parasite, a viruse requires a living host for completing its replication cycles. Therefore, different pathogens including viruses and fungi trigger distinct host defense responses. Combined findings suggest that ATAF2 may act as a key regulator that differentially modulates host physiology and defense responses against viral and fungal infections. As a transcriptional factor, it is possible that ATAF2 modulates different sets of downstream genes that result in different host physiological conditions that are conducive for fungi but unfavorable for viruses. When dealing with the same pathogen, NAC proteins may also act in a different way. Geminivirus replication has been shown to be enhanced by expression

of tomato SlNAC1 (Selth *et al*., 2005), but inhibited by GRAB proteins (Xie *et al*., 1999). Taken together, different roles for NAC proteins in pathogen infection suggest a functional diversity for these transcription regulators.

TMV-modulated ATAF2 expression in susceptible plants

A previous study has shown that ATAF2 is rapidly induced by stress, including wounding and *P. syringae* infection (Delesser *et al*., 2005). In this study, TMV inoculation resulted in a rapid induction of GUS activity in transgenic plants expressing *ATAF2 promoter::GUS* fusion (Fig. 3.7). Such an activation of ATAF2 promoter activity was not observed in mock-inoculated tissues, suggesting the ATAF2 induction is very specific for TMV. Interestingly, induction of ATAF2 was observed to be restricted to locally inoculated tissues even though the virus accumulates to greater levels in systemic-infected tissues. This result indicates that expression of ATAF2 was modulated during the course of virus infection. The NAC domain proteins have been shown to play a role in basal resistance to biotic stress (Collinge and Boller, 2001; Hegedus *et al*., 2003). Results in this study suggest that in a susceptible plant, basal resistance of NAC proteins can be modulated by TMV.

A previous microarray analysis has linked *ATAF2* over-expression to the repression of pathogenesis-related *PR* genes in Arabidopsis ecotype Ler (Delessert *et al*., 2005). *PR* genes are recognized to be involved in the host defense system that are generally associated with the localized hypersensitive response (HR) and the development of systemic acquired resistance (SAR). Salicylic acid (SA) has long been recognized as a critical signal molecule that mediates the activation of defense-

related genes in both local and systemic resistance responses. SA treatment has been shown to inhibit TMV replication in tobacco mesophyll protoplasts and whole plants (Murphy and Carr, 2002). In this study, expression of *ATAF2* was observed to be induced upon SA treatment (Fig. 3.10), implicating an involvement of *ATAF2* in the SA signaling pathway. In addition, induction of *ATAF2* locally but not systemically correlated to the expression of *PR1* gene (Fig. 3.9). Combined results suggest that TMV-directed *ATAF2* expression is involved in the host defense pathway in a SA-dependent manner.

A model for the function of ATAF2-TMV replicase interaction in virus infection

The battle between virus and its host involves extensive interactions and alterations of host gene expression. The observations that *ATAF2* can be induced by abiotic and biotic stresses indicate that expression of *ATAF2* is a general stress/defense response in plant. In this study, the interaction between the viral replicase protein and ATAF2 is shown to play a role in suppressing basal defenses in a susceptible host. In the proposed model (Fig. 3.11), TMV infection rapidly induces ATAF2 expression at the initial infection site. Being a transcription regulator, early induction of ATAF2 initiates a series of transcription events that results in the activation of a defined set of defense-related genes, such as *PR1*. Accumulation of ATAF2 and other defense molecules further confers host basal resistance to viral infection. For a susceptible host, the suppression of host basal defenses is a key step for viral pathogenesis. I propose that the interaction between viral replicase proteins and ATAF2 disrupts the ATAF2 function, resulting in the down-regulation of

ATAF2-mediated signaling transduction pathway. In TMV-infected plant tissues, 126-kDa replicase accumulates ten times more than the183-kDa replicase (Lewandowski and Dawson, 2000). The finding that TMV replicase interacts with ATAF2 as well as with other host proteins might explain the function of excess 126-kDa replicase in modulating host defense systems or other aspects of the viral life cycle. Consequently, expression of host defense-related genes is altered and the virus is able to establish a systemic infection.

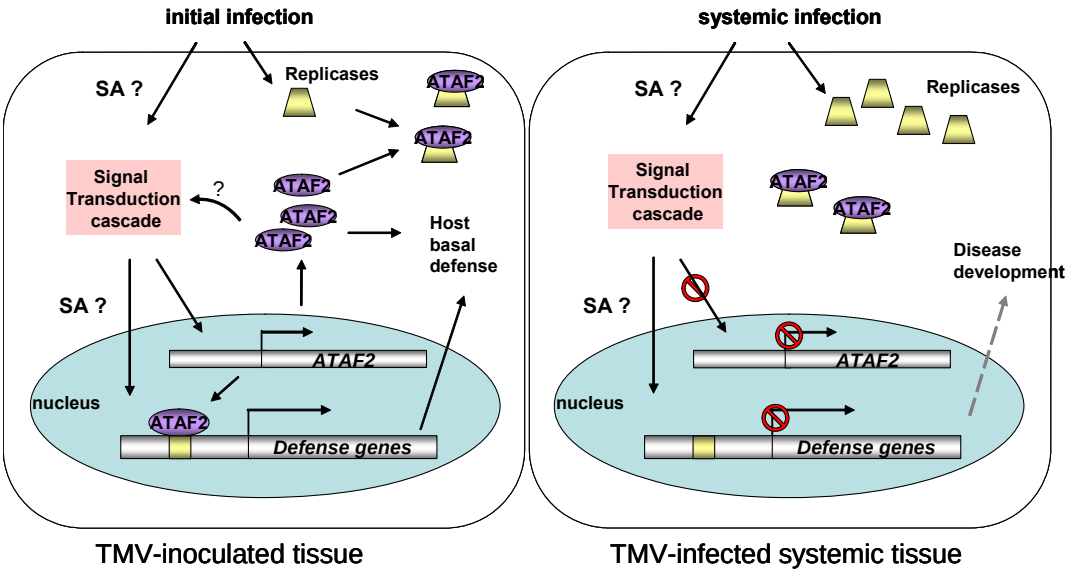


Fig. 3.11. A model illustrating the ATAF2-mediated signaling transduction pathway. In TMV-inoculated tissues, initial infection rapidly induces *ATAF2* expression which further activates a series of host defense genes, including *PR* genes. Both ATAF2 and PR proteins confer basal resistance to virus infection. In systemic-infected tissues, however, interaction between the replicase proteins and ATAF2 disrupts the ATAF2-mediated signaling pathway, resulting in the down-regulation of host defense genes and the subsequent development of disease symptoms.

Chapter 4: Conclusions and Perspectives

For the past century, plant virologists have made considerable progress in understanding the fundamentals of viral activities, such as viral genetics, replication, movement, and pathogenesis. However, due to the multi-functional nature of viral proteins and the complexicity of virus-host interactions, it is still not clear how viruses direct the assembly of their replication complexes and what mechanisms viruses have evolved to evade host defense systems. Similar to many other closely related members within the alphavirus-like superfamily, TMV encodes two multidomain replicases each containing a helicase domain. RNA viruses-encoded helicases have been considered important in viral replication because of their NTPase and RNA unwinding activities. However, a growing body of evidence suggests that virus-encoded helicases are involved in viral activities other than replication during the viral life cycle. In this study, I have successfully expanded the available knowledge on the biological significance of the TMV helicase domain in promoting a successful viral replication cycle. The interaction that I have examined between the TMV helicase domain and ATAF2, a host transcription factor, further illuminated the complex interactions between the virus and its host.

The goal of my first project was directed at identifying TMV helicase domain intermolecular protein-proteins interactions, defining biochemical properties of the conserved helicase motifs, and analyzing the biological relevance of the helicase motifs in forming replication complexes. Virus-encoded helicases have been shown to be actively involved in viral protein-protein interactions. The helicase domains within BMV-encoded 1a and AMV-encoded P1 interact with their respective RdRp

2a and P2 and these interactions are required for virus replication (Kao and Ahlquist, 1992; Van Der Heijden *et al.*, 2001). Despite extensive studies on virus protein-protein interactions, there is still limited information about the higher-order structures formed by replicase interactions. My data indicates a self-interaction of the TMV helicase domain as observed both *in vivo* and *in vitro*. The helicase-helicase interaction is postulated to serve as a structural platform for assembling virus replication complexes (VRCs). Positive stranded RNA viruses generally assemble their VRCs on the host membrane. Poliovirus viral protein 2c, a membrane-associated protein with RNA-dependent ATPase activity, is capable of inducing host membrane vesiculation when expressed by itself (Aldabe and Carrasco, 1995). Similarly, the expression of BMV 1a, a helicase domain containing viral protein, leads to the formation of membrane-bound spherules (Schwartz *et al.*, 2002). Both membrane-associated structures are considered sites for virus replication, suggesting that viral proteins play an important role in directing the formation of VRCs in host cells. However, the underlying mechanism as to how viral proteins regulate the VRC assembly has not been fully characterized. The correlation between the ATPase activity of the TMV helicase domain and formation of active VRC-like vesicles indicates that the helicase domain is a key player in regulating VRC formation. This is the first time that the ATPase activity of a viral helicase is shown to have a regulatory role during VRC assembly. This research therefore not only broadens our views of the functional activities of the TMV helicase domain, but also suggests a possible mechanism for the assembly of VRCs of other related viruses.

Further studies for this project should be directed toward obtaining an in-depth understanding of the intermediate states of VRCs. To do so, resolving the three dimensional structure of the helicase polypeptides, a key organizer for TMV VRC assembly would be critical. Detailed structural conformation information on the TMV helicase polypeptide would provide insight into how the helicase polypeptides inter-connect to form higher-order structures. By comparing the detailed structural conformations of wild-type and mutant helicase polypeptides, the important residues related to their biochemical activities can be assessed. Furthermore, a mutational analysis of the TMV helicase domain should be conducted based on the structural model. Combined, such information would provide a more complete understanding of the structural and functional relationship of this important protein in virus replication. The tools and methods I have developed in this study, such as protein expression and purification, will serve as key factors in this effort.

To establish a successful infection, not only is it necessary for viruses to assemble virus replication factories in the host cell, but viruses must also develop strategies to evade host defense systems. It has been recognized that plants utilize multiple signaling pathways to control defense responses. Several families of transcription factors, including bZIP, WRKY, and ERF have been reported to be associated with host basal defenses (for review, see Singh *et al.*, 2002). For example, a WRKY-type transcription factor was shown to be induced by TMV infection in resistant tobacco plants (Yoda *et al.*, 2002). A NAC domain family of transcription factors was identified only a decade ago and our knowledge of this gene family on host defenses has only just begun. In this dissertation, a NAC protein, ATAF2, was

identified as a possible regulator of host defenses. Expression of ATAF2 is observed to be rapidly induced in response to TMV infection. It remains unknown how the *ATAF2* gene expression is triggered. Signaling molecules, such as SA, ethylene, and jasmonic acid, have been widely recognized as important messagers in activating host defense genes (Reymond and Farmer, 1998). The observation that ATAF2 expression is induced upon SA treatment suggests that ATAF2 may be involved in the SA-mediated host defense pathway.

Representatives of bZIP and WRKY transcription families have been shown to bind to *cis*-acting elements of defense-related genes and to regulate their expression (Eulgem *et al.*, 1999; Jakoby *et al.*, 2002). Examples of these *cis*-acting elements include G, C, W, and *as-1* boxes. Similar to other transcription factor families, DNA-binding motifs are detected within the well-conserved NAC domain, suggesting that NAC proteins may bind to a similar set of DNA elements (Duval *et al*., 2002). Xie *et al*. (2000) showed that NAC1 bound to a region of the CaMV 35S promoter containing the *as-1* activation element. Several BnNAC proteins were further characterized and shown to trans-activate a minimal CaMV 35S promoter containing the *as-1* element (Hegedus *et al*., 2003). Although there is no direct evidence showing that the activation of defense-related genes is regulated by the ATAF2 transcription factor, a correlation between *ATAF2* and *PR1* expression in locally and systemically infected tissues suggests that both genes are transcriptionally connected. Consistent with this hypothesis, *PR1* genes from both Arabidopsis and tobacco have been found to contain an *as-1* related element in their promoter region (Lebel *et al.*, 1998; Strompen *et al.*, 1998). In addition, phosphorylation and protein modification

have been shown to play a role in activating latent transcription factors required for defense gene activation. WRKY transcription factors act downstream of the SIPK/WIPK (MAP kinases) cascade when the cascade is activated by various stresses including wounding and pathogen infection (Kim and Zhang, 2004). Detection of an area rich in serine/threonine residues within the ATAF2 C-terminal TAR region implies that the phosphorylation of ATAF2 may represent an alternative method to induce downstream defense response genes (Hegedus *et al*., 2003). Altogether, it is postulated that ATAF2 possesses a regulatory role that functions in activating host defense responses against viral infection.

In addition to identifying sequence elements of target genes, a large-scale transcriptome analysis has related the expression of host defense genes to *ATAF2* (Delessert *et al*., 2005). In contrast to my observation, they show that *PR1* was repressed in *ATAF2* over-expressed plants. Considering different Arabidopsis host systems used in these two studies (Ler in their study and Shahdara in this work), it is possible that ATAF2 functions differentially in activating or repressing defense-related genes in different host systems. Furthermore, *ATAF2* overexpression inhibits TMV infection in the Arabidopsis ecotype Shahdara. Overexpression of *ATAF2* in Arabidopsis Ler, however, conferred a higher susceptibility to the soil-borne fungal pathogen *Fusarium oxysporum* (Delessert *et al*., 2005). Again, opposite responses to pathogen invasions suggest that ATAF2-mediated basal host defenses likely include multiple signals and functions that ultimately regulate the defense responses.

The interaction between the NAC protein and the TMV replicase represents an important finding. Previous studies have shown several members of the NAC protein

family are involved in the host defense systems against viral infection. For example, GRAB1 and GRAB2 were identified to interact with *Wheat dwarf geminivirus* (WDV) RepA protein and their overexpression inhibited viral replication in cell culture (Xie *et al*., 1999). Interaction between the NAC protein TIP and the coat protein of TCV was shown to be involved in the induction of the hypersensitive response, suggesting that TIP plays a role in the host defense response pathway (Ren *et al*., 2000). The ability of the TMV helicase domain to interact with both Arabidopsis ATAF2 and its tomato homologue, SlNAC1, indicates that this specific virus-host interaction is conserved in plants. Interactions between viral proteins and NAC domain proteins suggest a major role of NAC domain proteins in viral pathogenesis.

In a susceptible host, the molecular events that follow virus systemic movement are not yet understood. In my model, ATAF2 is an important component in regulating the transcription of host defense genes in systemic tissues. Despite high accumulation of virus in systemically infected tissues, expression of *ATAF2* and *PR1* was observed to reduce to a level similar to that in uninfected cells. This result indicates that during the course of viral infection, viruses are capable of modulating the expression of host defense genes, even though it remains unclear what signal triggers the transcriptional reprogramming of host defense genes in systemically responding tissues. A number of WRKY genes has been shown to contain *cis*-acting elements in their promoter region, suggesting a self-regulation of WRKY genes in host defense systems (Dong *et al.*, 2003; Eulgem *et al.*, 1999). It is possible that *ATAF2* expression undergoes a similar self-regulation pathway. I suspect that

interacting with the TMV replicase disrupts the nuclear localization and function of ATAF2, affecting its feedback mechanism on transcriptional regulation. Depletion of *ATAF2* further results in downregulation of downstream defense-related genes. Alternatively, miRNA-mediated gene expression may be involved in *ATAF2* expression in systemic tissues. A bioinformatic analysis has predicted that most of the miRNA targets are transcription factors (Rhoades *et al.*, 2002). Expression of *NAC* genes, including *NAC1*, *CUC1* and *CUC2*, has been shown to be post-transciptionally regulated by miRNAs (Laufs *et al.*, 2004; Mallory *et al.*, 2004). Thus miRNA-directed degradation of *ATAF2* possibly plays a role in controlling expression of host defense genes in systemic tissues. However, whether this hypothesis is true needs to be evaluated.

Much of our knowledge about host defenses counts on the resistance (*R*) gene-mediated hypersensitive response (HR), which is often associated with the induction of a set of pathogenesis-related (*PR*) genes. However, the expression of host defense-related genes, including *PR* genes, in a virus susceptible host suggests that both compatible and incompatible virus-host interactions share a common defense signaling pathway. A transcriptome analysis has revealed that there exist extensive overlaps on host gene expression in both systemic acquired resistance (SAR) and the plant basal defense (Maleck *et al.*, 2000). Similarly, a set of genes is found to respond to biotic and abiotic stress in a similar manner (Narusaka *et al.*, 2004). Overall, it is likely that some signaling pathways are common in different plant defense systems, thus identifying specific ATAF2-mediated signaling pathways may provide important insight into understanding the general mechanisms of plant defense

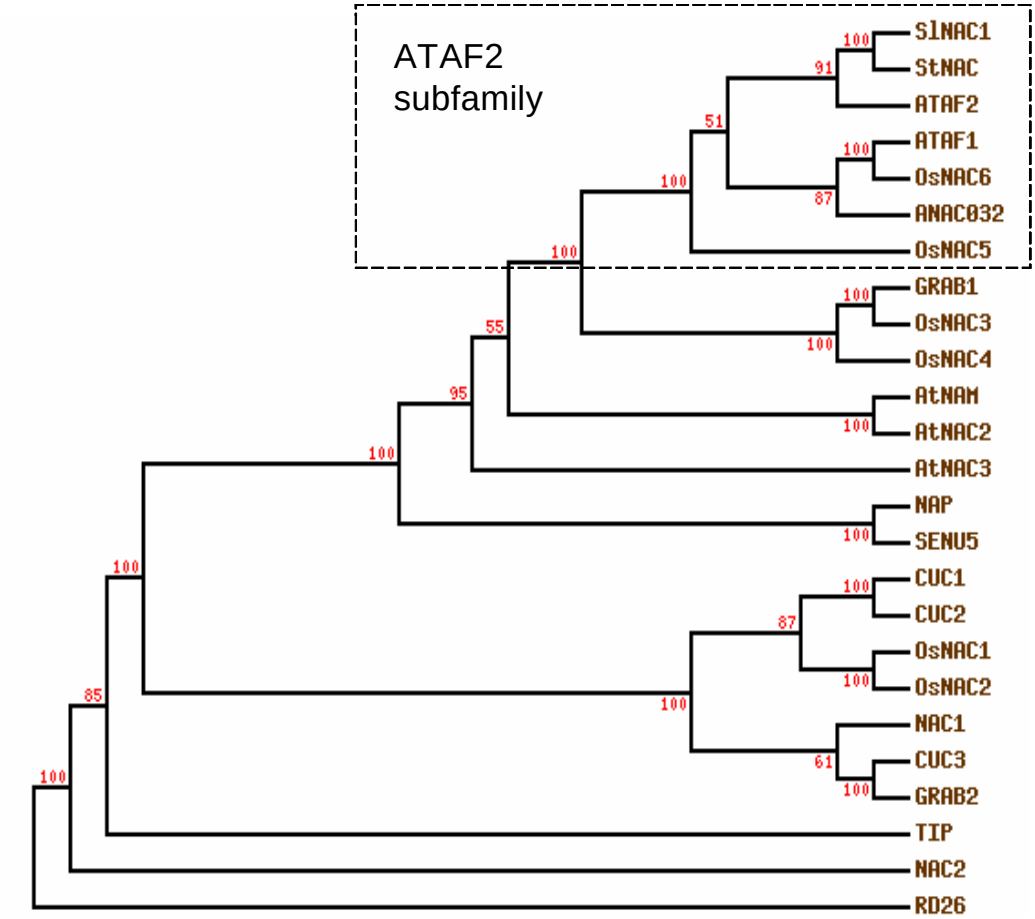
responses. Furthermore, a growing body of evidence suggests that there is an overlap between the gene expression triggered by pathogen inoculation and other physiological processes, such as senescence (Quirino *et al.*, 1999). NAC proteins have been shown to be involved in plant developmental processes (Aida *et al*., 1997; Hegudus *et al*., Souer *et al*., 1996). Overexpression of *ATAF2* resulted in a strong developmental phenotype similar to the symptoms caused by TMV infection. These results suggest that there might also be overlaps between the development-associated signaling network and that activated by pathogen infection.

Due to the high genetic redundancy for transcription factors, *ATAF2* knockout lines were not informative in revealing ATAF2 functional activities. More recently, two studies have successfully utilized a repression domain of the EAR (ERF-associated amphiphilic repression) motif to suppress the expression of specific NAC genes in the presence of other redundant transcription factors (Fujita *et al*., 2004; Hiratsu *et al*., 2003). This technique, known as CRES-T (Chimeric Repressor Silencing Technology), makes a translational fusion of a target gene with an EAR repressor motif, SRDX, with the fusion proteins actively suppressing the function of the target gene. Using this approach, Arabidopsis transgenic lines overproducing an ATAF2-SRDX fusion protein can be constructed. The resulting transgenic plants should silence a suite of downstream target genes, thus representing repression lines for ATAF2. I would expect that in contrast to *ATAF2* overexpression lines, *ATAF2* repression lines would show enhanced susceptibility to TMV infection. Furthermore, within *ATAF2* repressed plants, changes in the *PR* gene expression upon TMV

infection can be monitored. Combined these studies should allow us to have an improved understanding of ATAF2 function in regulating the host defense response.

In conclusion, studies presented in this dissertation provided new insight into the functional activities of virus-encoded helicases. The molecular characterization of the TMV helicase domain indicated that it is involved in VRC assembly and is essential for virus replication. Furthermore, the TMV helicase acts as a virulence determinant through interaction with specific NAC domain family members. The knowledge obtained from these studies would help us to develop efficient antiviral strategies by targeting at the viral helicase domain and ultimately to achieve our final goal at controlling viral infection.

Appendices



Appendix 1. A phylogenetic tree showing the relationship between ATAF2 and other NAC domain proteins. Both ATAF2 and SlNAC1 belong to ATAF2 subfamily. Alignment of NAC domain protein sequence was conducted using the ClustalW2 program. The phylogenetic tree was drawn using a GeneBee program at http://www.genebee.msu.ru/services/phtree_reduced.html.

Appendix 2. Sequence alignments of Arabdopsis ATAF2 and tomato SlNAC1 and LeATAF2. Black areas represent identical residues. Gray areas represent similar residues.

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