ABSTRACT

Title of Document: **MOLECULAR CHARACTERIZATION OF INTERACTIONS BETWEEN TMV REPLICASE PROTEIN AND AUXIN RESPONSIVE PROTEINS: IMPLICATIONS IN DISEASE DEVELOPMENT**

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Tobacco Mosaic Virus and *Arabidopsis thaliana* serve as ideal model systems to study the molecular aspects of virus – host interactions. Using this system, an interaction between the helicase domain within TMV replicase protein and an auxin responsive protein, IAA26 was identified. IAA26 is a member of the Aux/IAA family of transcription factors that function as repressors in signaling pathways controlled by the phytohormone auxin. Characterization of the interaction was carried out utilizing a helicase mutant defective in its interaction with IAA26 and by creating transgenic plants silenced for IAA26 expression. These studies suggest that the interaction was not essential for either viral replication or movement but promoted the development of disease symptoms. Cellular co-localization studies revealed that in TMV infected tissue, the nuclear localization and stability of IAA26 was compromised and the protein was relocalized to distinct cytoplasmic vesicles in association with the viral replicase. In keeping with its role as a transcription factor, the alterations in IAA26 function should

lead to misregulation of downstream auxin responsive genes and this is supported by the fact that ~ 30% of the *Arabidopsis* genes displaying transcriptional alterations to TMV could be linked to the auxin signaling pathway. Aux/IAA family members share significant sequence and functional homology, and an additional interaction screen identified two more *Arabidopsis* Aux/IAA proteins, IAA27 and IAA18 and a putative tomato Aux/IAA protein, *Le*IAA26 that could interact with TMV helicase. The nuclear localization of these three proteins was disrupted by TMV and alterations in *Le*IAA26 levels induced virus infection-like symptoms in tomato. Additionally, transgenic plants over-expressing a proteolysis resistant mutant of IAA26 showed abnormal developmental phenotype, the severity of which was abrogated during TMV infection which blocked nuclear accumulation of the protein. Taken together, these findings suggest that TMV induced disease symptoms can partially be explained by the ability of the virus to disrupt the functioning of interacting Aux/IAA proteins within susceptible hosts. The significance of such interactions is yet to be determined but it appears that they may be advantageous to the virus while infecting tissues that are in a developmentally static stage.

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By

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Dedication

I dedicate this work to my parents.

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I am deeply appreciative of Tam, my fiancée, who experienced with me the ups and downs of graduate life and for giving me the love and space that I needed to proceed in pursuit of my goals. My family has really been the foundation for all my achievements. My parents instilled in me the importance of a good education and I dedicate this thesis to them. I finally thank my younger brother Hari for being my secret cheerleader.

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Abbreviations

TMV - *Tobacco Mosaic Virus*

CP - Coat Protein

MP - Movement Protein

SF1 - Super Family 1

PD - Plasmodesmata

RNA - Ribose Nucleic Acid

TLS - tRNA like structure

RF - Replicative Form

RI - Replicative Intermediate

ER - Endoplasmic Reticulum

VRC - Virus Replication Complex

TOM - Tobamovirus Multiplication

OAS - Origin of Assembly Site

CC - Companion Cells

SE - Sieve Elements

PME - Pectin methyl esterase

HSP - Heat Shock Protein

BMV - *Bromo Mosaic Virus*

DED - DEAD box RNA helicase

eIF3 - eukaryotic Initiation Factor 3

CNV - *Cucumber Necrotic Virus*

PAPK - Plasmodesmata Associated Protein Kinase

TGB - Triple Gene Block

P58IPK - P58 - Inhibitor of Protein Kinase

RdRp - RNA dependent RNA polymerase

HR - Hypersensitive Response

NB-LRR - Nucleotide Binding – Leucine Rich repeats

CC - Coiled-Coiled

TIR - Toll Interleukin Receptor like

ROI - Reactive Oxygen Intermediates

EDS1 - Enhanced Disease Susceptibility1

NDR1 - Non Race specific Disease Resistant

GPI - Glycosyl Phosphatidyl Inositol

PAD4 - Phytoalexin Deficient 4

NPR1 - Non expressor of PR1

NO - Nitric Oxide

CHORD - Cysteine and Hystidine Rich zinc binding Domain

MAPK - Mitogen Activated Protein Kinase

WIPK - Wound Induces protein Kinase

SIPK - Salicylic acid Induced Protein Kinase

PCD - Programmed Cell Death

ATG3, ATG7 - Autophagy3 and Autophagy 7

DCL2 - Dicer Like homolog 2

ATP - Adenosine Tri Phosphate

RISC - RNAi Induced Silencing Complex

PAZ - PIWI-Argonaute-Zwilli

PVX - Potato Virus X

CuMV - *Cucumber Mosaic Virus*

GRAB- Geminivirus RepA Binding

TuMV - *Turnip Mosaic Virus*

PSMV - *Pea Seed Borne Mosaic Virus*

WClMV - *White Clover Mosaic Virus*

TCV - *Turnip Crinkle Virus*

TVCV - *Turnip Vein Clearing Virus*

vid1 - Virus inducible dwarf 1

BCTV - *Beet Curly Top Virus*

IAA - Indole Acetic Acid

PIN - Pin formed

PGP - P Glycoprotein like

SAUR - Small Auxin Upregulated

Aux/IAA - Auxin/Indole Acetic Acid

ARF - Auxin Response Factor

CD- Central Domain

EST - Expressed Sequence Tag

TIR1- transport Inhibitor Response

VIGS - Virus Induced Gene Silencing

PTGS - Post Transcriptional Gene Silencing

TRV - *Tobacco Rattle Virus*

PDS - Phytoene Desaturase

HPV - *Human Papilloma Virus*

DPI - Days Post Infection

DTT - Dithiothretol

SDS - Sodium dodecyl sulphate

PAGE - Polyacrylamide gel electrophoresis

T-DNA - Transferred DNA

WT - Wild-type

Chapter 1

General Introduction

1.1 STATEMENT OF PURPOSE

Viruses are obligate parasites that completely rely on their host for many aspects of their survival. There are approximately 450 species of pathogenic plant viruses and many are responsible for huge losses in crop production and quality in all parts of the world. As a norm, most plant species are resistant to most viruses and but in the rare exception, the virus is able to infect the host and successfully exploit the cellular processes for its own propagation. The fact that viruses have such a narrow host range suggests that they have co-evolved with their hosts and built several intimate and complex interactions with them. In an effort to exercise control over the cell, viruses have also converted some of their proteins into signal interceptors or activators of cellular pathways. Cell-growth and defense pathways are two such networks that are most frequently altered by viruses. Changes in the first pathway are directed towards converting the host into a virus factory while changes in the defense pathway are pre-emptive attempts by the virus to avoid or subvert host surveillance process. The end result of all these alterations is the development of disease symptoms, which are visual markers of the presence of the virus.

The identification of signaling processes and host factors altered during successful viral invasions is important since it will help us uncover the molecular principles underlying compatibility and also aid in the design of strategies for virus control. The

primary objective of this study has been to characterize a previously unknown disease-inducing interaction occurring between *Tobacco Mosaic Virus* and its susceptible hosts. Genomic and cell biological approaches have been used to identify the specific pathway altered by the interaction and the findings provide insight into physiological processes leading to viral pathogenicity and host susceptibility.

1.2 LITERATURE REVIEW

1.2.1 Tobacco mosaic virus

Tobacco Mosaic Virus, the first virus to be isolated more than a century ago, continues to remain one of the predominant model systems used in virology research. Several properties of this system make it extremely useful in addressing fundamental questions in virology. These include a fully characterized genome (Goelet *et al*., 1982), existence of full length infectious cDNA clones (Dawson *et al*., 1986), easy manipulation of the viral genome and extensive information on the 3D virion structure.

TMV is a single-stranded monopartite RNA virus belonging to the Sinbis virus supergroup and is a type member of the genus Tobamovirus (Koonin and Dolja, 1993). It is the classical example of a rod shaped virus (18nm × 300nm) and consists of a single molecule of plus sense RNA embedded within a helical array of identical coat protein subunits. The 6395 nucleotide long viral RNA codes for at least four proteins - two viral replicase proteins, a movement protein and a coat protein (Goelet *et al*., 1982). These coding regions are flanked by the 5' and 3' untranslated regions, both of which are

required for viral replication (Takamatsu *et al.,* 1991, 1992). The 5' end of the genome has a methyl guanosine cap and the 3' terminus folds into a t-RNA like structure (Zimmern, 1975; Pleij *et al*., 1989; Rietveld *et al*., 1984). Like alphaviruses, TMV replicates in the cell cytoplasm within a membrane bound complex (Osman and Buck, 1996).

The 126-kDa replicase protein is synthesized from the full length RNA during co-translational disassembly of the virus and contains a methyl transferase domain thought to play a role in viral RNA capping and a helicase domain that is thought to promote double-stranded RNA unwinding (Dunigan and Zaitlin, 1990; Goregaoker and Culver, 2003). The 183 kDa polymerase protein is formed as a read through protein from a leaky amber codon at the end of the 126-kDa ORF and contains an additional RNA dependent RNA polymerase domain (Pelham, 1978). Both the 126-kDa and 183-kDa proteins are essential for replication. They associate with the endoplastic reticulum and host membrane proteins to form membrane bound replication complexes called 'viroplasms' (Esau and Cronshaw, 1967). The 30-kDa movement protein (MP) and the 17.5-kDa coat protein (CP) are transcribed from sub genomic RNA. The cell to cell movement of the virus is facilitated by the 30-kDa protein (Deom *et al*., 1987). The protein accumulates in the plasmodesmata of host cells and increases permeability thus potentiating short distance movement of viral particles. The coat protein on the other hand is needed for long distance movement of the virus (Ding *et al.*, 1996) and is essential for the proper assembly of the virion (Fig.1.1).

TMV encoded proteins

TMV Replicase Proteins

Several lines of evidence suggest a crucial role for the TMV encoded 126-kDa and the read through 183-kDa proteins in virus replication. Both these proteins have been detected within partially purified, membrane bound TMV RNA polymerase preparations (Young *et al.*, 1986; Osman and Buck, 1996). Antibodies against the 126-kDa protein bound specifically to active TMV replicase (Osman and Buck, 1996) and addition of these antibodies inhibited TMV RNA synthesis within *in vitro* reactions containing membrane bound RNA polymerase (Osman and Buck, 1996). Furthermore, a mutant virus with both the coat protein and movement protein deleted, replicated within tobacco protoplasts, and the time-course for synthesis of viral progeny did not significantly differ from WT-TMV. Taken together, these findings suggest that the 126-kDa and 183-kDa proteins were necessary and sufficient for efficient replication (Meshi *et al.*, 1987).

The TMV replicase proteins share two known domains. The N-terminal domain within these proteins has amino-acid motifs typical of methyl-transferases and carries out virus-specific methyltransferase and guanylyltransferase activities (Dunigan and Zaitlin, 1990; Merits *et al*., 1999). The C –terminal (in case of 126-kDa) or central domain (in case of the 183-kDa replicase) is the helicase domain that functions in unwinding double-stranded RNA intermediates formed during replication. This domain contains six major conserved motifs (I, II, III, IV, V and VI) and based on the extent of amino acid similarity to other known helicases, TMV helicase has been classified as belonging to the SF1 (Super-Family) class.

The different motifs are thought to function co-coordinately in binding an NTP, generally ATP, and using the energy of hydrolysis to unwind dsRNA. Structure-function analysis of members of the SF1 family of helicases have not only helped define specific roles for some of these motifs but also emphasized the close relationship between the conserved motifs and the three-dimensional structures of the enzymatic cores. Mutational analysis of the conserved domains and X ray crystallography studies have revealed that motifs I and II are important for ATP binding, chelation of Mg^{2+} ions and ATP hydrolysis (Walker *et al.*, 1982, Graves-Woodward *et al*., 1997). Domains VI and III link NTP hydrolysis with DNA/RNA binding and unwinding activity, while domains IV and V are necessary for substrate binding (Hall *et al.*, 1999; Lin and Kim, 1999; Schwer and Meszaros, 2000; Tanner and Linder, 2001).

The TMV helicase motif can hydrolyze ATP, bind RNA, and unwind duplexed RNA which suggests that it is a functional helicase (Goregaoker and Culver, 2003). This helicase activity is essential at two stages during replication - unwinding of the duplex RNA intermediate formed during replication and removal of secondary structures within RNA templates so as to enable the efficient translocation of the polymerase.

The N terminus of the 183-kDa protein has amino acids motifs that are characteristic of RNA dependent RNA polymerases (RdRp) and provides the catalytic activity for the synthesis of TMV RNA. While there has been no evidence for polymerase activity *in-vitro*, TMV replicase complexes isolated from infected tissue have been shown to possess template-dependent polymerase activity (Osman and Buck, 1996). Since the 183-kDa replicase protein contains all the domains necessary for replication, it would appear that this protein alone should be sufficient for replication. To test this, Ishikawa *et al.* (1986)

created a mutant virus where the leaky amber stop codon (UAG) was mutated to a tyrosine codon(UAU) such that the virus produced only the 183-kDa protein and while this mutant replicated within protoplasts, its efficiency was less than 20% when compared to the wild-type virus. Furthermore, during later stages of infection, the tyrosine codon reverted back to a stop codon allowing the synthesis of both 126- and 183- kDa proteins and near wild-type levels of replication. Thus it appears that a balanced expression of both the replicase proteins is essential for efficient replication.

30-kDa Movement Protein

The cell-to-cell movement of TMV is an active process wherein the viral nucleic acids are trafficked through intercellular connections as ribonucleoproteins and this process is mediated by a specialized 30-kDa movement protein (MP). The viral genomic nucleic acid is specifically bound by the movement protein and the nucleoprotein complex is targeted to plasmodesmata via interaction with the endoplasmic reticulum and host cell cytoskeleton (Heinlein *et al.*, 1998; Boyko *et al.*, 2000). Once at the plasmodesmata, the viral MP increases the size exclusion limit of the plasmodesmatal channel thereby facilitating the transport of the viral complex into the neighboring cell (Wolf *et al*., 1989 Citovsky *et al.*, 1990, 1992)

TMV MP is a single stranded nucleic acid binding protein and electron microscopic studies have revealed that MP binding unfolds the nucleic acid converting it into thin extended structures that are better suited for transport across the plasmodesmata (Citovsky *et al*., 1992). The estimated 2.5nm nucleic protein complex is still too large to pass through the 1.5nm wide plasmodesmatal opening and movement can therefore be

accomplished only by significant modification of the plasmodesmatal structure. Elegant microinjection experiments revealed that in transgenic plants expressing TMV-MP the size exclusion limit of plasmodesmata was 10 fold higher than control plants (Wolf *et al*., 1989). Taken together with the evidence that in TMV infected tissue, MP was found to localize to the plasmodesmata it appeared that the viral movement protein had the ability to dilate the plasmodesmatal channel (Tomenius *et al.*, 1987; Ding *et al.*, 1992; Waigmann *et al.*, 1994). The MP mediated gating of PD channels is relatively fast (3 to 5 minutes) suggesting that the virus probably utilizes a pathway already present within cells (Waigmann *et al.*, 1994). The movement protein activity appears to be negatively regulated by phosphorylation and TMV mutants carrying amino acids that mimicked phosphorylation were unable to interact with the plasmodesmata and promote cell-to cell movement (Watanabe *et al.*, 1992; Citovsky *et al.*, 1993, Waigmann *et al.*, 2000). Additional support for this hypothesis comes from the identification of plasmodesmata-associated casein kinases that could recognize and phosphorylate the C-terminal residues of TMV MP (Lee *et al.*, 2005).

17-kDa Coat Protein

Being a soil borne pathogen, TMV is highly stable and capable of surviving for extended periods of time in the soil. However, once inside the cell, the virion is rapidly disassembled to initiate infection. This 'stability-switching' ability of the virus is largely attributed to the structural properties of the virus coat protein (CP). The mature virus particle consists of a single genomic RNA compactly coiled in a helix made up of 2130 subunits of the coat protein. Extensive intermolecular interactions between the CP

subunits and between the viral RNA and CP subunits provide structural stability to the mature virion (Bloomer *et al.*, 1978; Namba *et al.*, 1989).

Along with its role in protecting the viral RNA, the coat protein is also required for efficient long distance movement of the virus. CP mutants that were disabled in their ability to assemble resulted in viruses that could move cell to cell but were inefficient in systemic movement (Dawson *et al.*, 1988; Saito *et al.*, 1990). Thus it appears that the ability of CP to assemble into virus particles is essential for efficient phloem loading and long distance transport. Finally, TMV CP has also been identified as the elicitor of gene specific hypersensitive response (HR) in the host plants containing the resistance (R) gene N' (Saito *et al.*, 1989; Culver *et al.*, 1989)

Putative 54-kDa protein

A subgenomic RNA corresponding to the ORF of a 54-kDa protein has been detected in TMV-infected tissue (Sulzinski *et al.*, 1985). This ORF is present within the 3' portion of the 183-kDa gene and contains the RdRp domain. The sub genomic RNA was found to be associated with poly ribosomes in infected tobacco leaves and translation of the RNA gave rise to a 54-kD protein *in vitro*. However the protein has never been detected in infected leaves or protoplasts, is not present within the purified replicase complex and does not appear to be essential for TMV replication.

Untranslated regions on the TMV genome

The coding region within the TMV RNA genome is flanked by the 5' untranslated region (5'- UTR or Ω) and the 3'-UTR, both of which are required for viral replication.

The 5' UTR is a 68 nucleotide G-deficient stretch that acts as a translational enhancer and large deletions within this region abolished replication (Gallie *et al.*, 1987; Takamatsu *et al.*, 1991). Tanguay *et al.* (1996) have shown that the Ω sequence binds a host heat shock protein, HSP101, which ultimately promotes the recruitment of eIF4F leading to efficient translation (Gallie, 2002).

The 3' UTR of TMV is highly structured and contains three conserved pseudoknots followed by a tRNA like structure (TLS) (Rietveld *et al.*, 1984; Pleij and Bosch, 1989; Felden *et al.*, 1996). The 3'UTR serves as the functional equivalent of the polyA tail in that it enhances the stability of the mRNA and promotes translation (Gallie and Walbot, 1990). In keeping with its role in promoting translation, the TLS has been shown to be aminoacylated and in this state, can interact with eukaryotic elongation factor 1A (eEF1A)/GTP with high affinity (Zeenko *et al.*, 2002). The 3' UTR also serves as the promoter for the initiation of minus strand synthesis during virus replication (Takamatsu *et al.*, 1990; Chandrika *et al.*, 2000; Osman *et al.*, 2000)

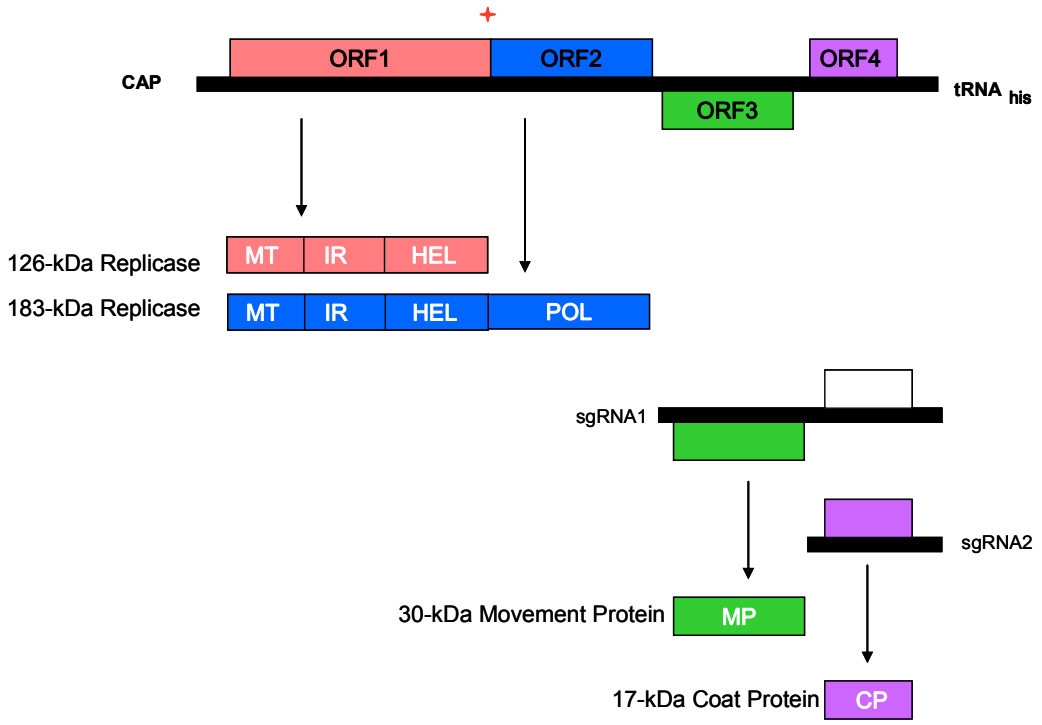


Fig.1.1. TMV Genome organization. Star indicates location of the leaky termination codon. sgRNA represents sub genomic RNA. MT – Methyl transferase, IR – Intervening region, HEL – Helicase, POL – Polymerase.

1.2.2 TMV Life cycle

The virus enters the cell through a mechanical injury on the plant cell wall and plasma membrane. Uncoating of the viral RNA occurs primarily because protein-protein and protein-RNA interactions within the TMV virion are weakened inside the cellular environment. Disassembly is mechanistically driven by the mutual repulsion of carboxylate groups within aminoacids from neighboring subunits of the viral coat protein (Caspar, 1963). In the extracellular milieu, calcium ions and protons stabilize the opposing carboxylate groups, but once inside the cell, the high pH and lower calcium ion concentration in the cytosol triggers the removal of Ca^{2+} ions and protons thereby increasing the repulsion between the opposing groups eventually weakening the virion structure (Namba *et al.*, 1989). The next stage in the disassembly process is dependent on RNA- CP interactions. The 69 nucleotides at the 5' end of the RNA have the weakest interaction with the viral CP and hence disassembly is initiated at this end (Mundry *et al.*, 1991). Removal of the 23 CP subunits at this end exposes the first start codon within the viral RNA; ribosomes now bind to the exposed end and dislodge the CP subunits such that disassembly occurs along with translation of the first open reading frame. This phenomenon of uncoating promoted by ribosome binding to RNA has been termed cotranslational disassembly (Wilson, 1984) (Fig.1.2).

To investigate the disassembly process *in vivo,* Wu *et al.* (1994) utilized a protoplast system where TMV virions were introduced into protoplasts and incubated under conditions in which uncoated RNA was destroyed by nucleases. The protected parts of RNA were then detected by RT-PCR analysis with primers representing varying parts of the viral genome. These studies showed that while 75% of the viral genome was

uncoated within minutes of entry into the cell (presumably by cotranslational disassembly), regions near the 3'end remained coated. Further analysis suggested that disassembly in the 3' to 5' direction occurred but was much slower than the 5' to 3' disassembly and it coincided with the occurrence of progeny negative-strand viral RNA (Wu and Shaw, 1996; 1997). This led to the hypothesis of co-replicational disassembly which proposed that the newly synthesized replicase protein(s) bound to the 3' end of the viral genome to carry out replication thereby promoting the release of bound CP subunits at the 3' end (Wu and Shaw, 1997). Thus it appears that this bidirectional disassembly of the TMV virion is aimed at protecting the viral RNA until it can be accessed for either translation or replication.

Once the replicase proteins have been synthesized, they associate with the 3' end of the viral RNA and direct the assembly of a replication complex which initiates the synthesis of the complementary negative strand. The negative strand serves as a template for both the synthesis of progeny positive strand RNA and sub genomic RNAs for the coat protein and movement protein. During replication, the viral RNA appears to exist in two predominant forms. The replicative-form (RF) RNA consists of genomic-length double stranded RNA while the replicative-intermediate (RI) RNA is partly double-stranded and partly-single stranded in nature. Both these forms have been identified *in vitro* (Watanabe and Okada, 1986; Young and Zaitlin, 1986; Osman and Buck, 1996). Based on the structure of the RI RNA it appears that a single negative strand can serve as a template for the simultaneous production of multiple positive strand RNA.

Like most of the other positive strand virus, TMV replication occurs in close proximity with host derived membranes (Shalla, 1964; Beachy and Zaitlin, 1975; Osman

and Buck, 1996; Reichel and Beachy, 1998). The membranes help not only anchor the replicase complex but also increase the surface area available for replication; compartmentalize the necessary components for optimized replication and likely function to protect viral RNA from host RNA silencing machinery. In TMV infected cells, the replicase proteins have been found to associate with the endoplasmic reticulum (ER) to form membrane bound virus replication complexes (VRCs) (Heinlein *et al.*, 1998, Mas and Beachy, 1999; Figueira *et al.*, 2002). Other studies have shown that in *Arabidopsis*, the replicase protein associates with host integral membrane proteins TOM1 and TOM3 (Tobamovirus multiplication 1 and 3) to anchor itself onto membranes and interestingly the TOM proteins appear to be predominantly present in the tonoplast (Ishikawa *et al.*, 1993; Yamanaka *et al.*, 2000; 2002). Simultaneous deletion of both TOM1 and TOM3 completely abolished virus replication suggesting that these proteins form integral components of the replication complex (Yamanaka *et al.*, 2002).

During the mid-infection stages, the newly synthesized Movement protein associates with viral RNA and replicase protein(s) to enhance the size of VRC's and promote the movement of the ribonucleoprotein complex towards the periphery of the cell. MP interacts with microfilaments to help anchor and stabilize the membrane bound complexes. Alternately it uses microtubules and microfilaments to transport the vesicle-like structures towards the cell periphery for intercellular spread (Heinlein *et al.*, 1998; Mas and Beachy, 1999; Boyko *et al.*, 2002; Liu *et al.*, 2005). As mentioned earlier, cell-to-cell spread occurs by MP mediated dilation of the plasmodesmatal channels which then facilitates the movement of the ribonucleoprotein complex in to the neighbouring cell.

During the late stages of virus infection, the Coat Protein levels peak in the infected cell. These CP subunits interact with each other to form 20S disc-like aggregates which are found to be the predominant form of aggregates formed *in vitro* (Raghavendra *et al.*, 1988; Diaz-Avalos and Caspar, 1998). Virus assembly originates near the internal origin of assembly site (OAS) located between 900 and 1300 nucleotides from the 3' end of the TMV genomic RNA (Zimmern and Wilson, 1976; Zimmern, 1977). The RNA sequence in this region is highly structured and forms 3 characteristic loops. Loop 1 interacts with and inserts into the hole of the 20S disc leading to a conformational change from the double disc to a 'lockwasher-RNA' complex which has a proto-helix like structure (Butler *et al.*, 1977; Schuster *et al.*, 1980; Butler, 1984; Klug, 1999). This serves as the nucleation event and further assembly continues with rapid and cooperative addition of 20 S discs that bind to and incorporate the RNA in the 5' direction (Butler 1984; 1999) Assembly at the 3' end is thought to be much slower and may involve incorporation of single CP subunits or smaller aggregates rather than involvement of discs (Lomonossoff and Butler, 1980).

The next stage in infection is systemic movement of the virus which involves transport from the local site of infection to distal, uninfected parts of the plant. TMV like many other viruses uses the phloem tissue and follows the transport of photo assimilates though unlike solutes, the movement is exclusively symplastic, ie- occurs through the plasmodesmata (Leisner and Turgeon, 1993; Santa Cruz, 1999). The requirement for coat protein for systemic movement suggests that virus assembly occurs prior to phloem loading and whole virions might be transported within the phloem (Dawson *et al.*,1988, Saito *et al.*, 1990). The key step in virus spread is movement of the virion from the

mesophyll cells to the phloem. At the cell-to-cell level, this involves trafficking through the bundle sheath (surrounding the vascular tissue), companion cells (CC) and sieve element (SE). The plasmodesmata between the CC and SE's have a higher size exclusion limit than seen in other cells thus permitting the efficient transport of virus into the sieve elements (Kempers and van Bel, 1997; Santa Cruz, 1999). Once inside the phloem, virus movement appears to be relatively fast with reported values between 1.5 cms/h to 3.5 cms/h (Bennett, 1940; Hull, 2002). The final phase is the egress of the virus from the vasculature to distal, uninfected sink tissue. Using a modified TMV expressing GFP, Cheng *et al.* (2000) have shown that there appears to be functional differences in the mechanism of virus entry and exit with the external phloem functioning in viral import and the internal phloem being used exclusively for export. Pectin methylesterases (PME), that were previously shown to be important for cell-to-cell movement also play a role in viral egress and tobacco plants with reduced PME expression in the vasculature showed a significant delay in viral movement into sink tissue (Chen *et al.*,2000; 2003).

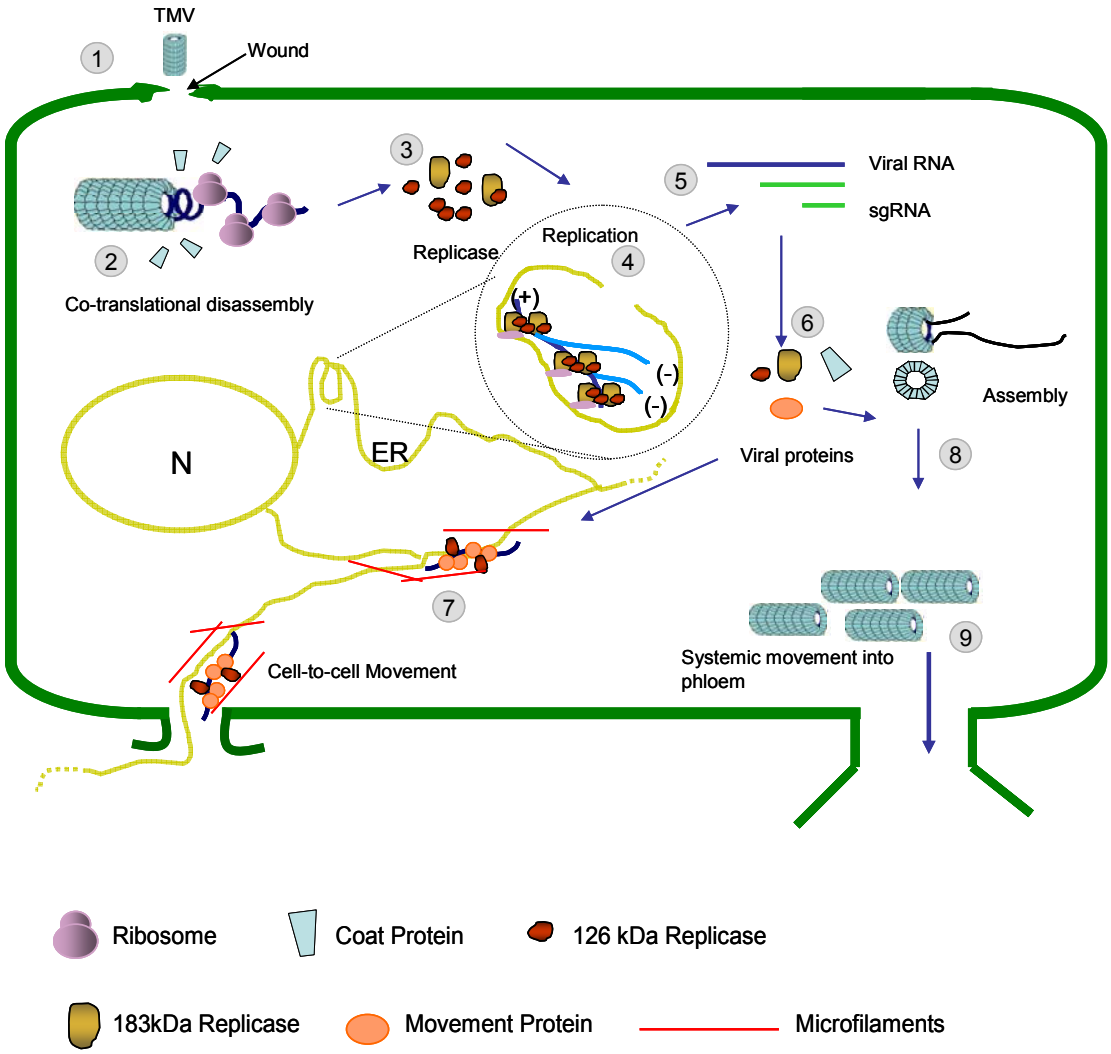


Fig.1.2. TMV Life cycle

1. Virus entry through a wound on the cell wall. 2. Cotranslational disassembly. 3. Synthesis of 126- and 183- kDa Replicase proteins. 4. Replication within membrane associated viral replication complexes. 5. Synthesis of viral full length and subgenomic RNA. 6. Translation of viral proteins. 7. Association of MP with ER, replicase proteins, MF (microfilaments) and plasmodesmata to mediate cell-to-cell movement. 8. Virus assembly. 9. Systemic movement of virion through phloem.

1.2.3 Virus – host interactions

The ability of a plant virus to establish a successful infection depends on its ability to efficiently replicate within the host cell, spread locally, mediate long distance movement and suppress or counteract host defense. A typical plant virus encodes only four to ten proteins and thus for the successful completion of all these processes, the virus has to rely on various components encoded by the host.

On a broad scale, the viral associations with host proteins can be classified as those required for basic compatibility (interactions essential for replication and movement) and those involved in modulating host defense response. Within a susceptible host, the virus is able to establish compatible interactions and successfully replicate and move within the host system. It diverts a substantial amount of plant metabolites for the production of virus specific proteins and nucleic acids and alters host gene expression to create a more favorable environment for infection. This diversion of resources and alterations in host gene expression profile leads to significant changes in plant physiology and development thus leading to the onset of disease. In an incompatible relationship, the virus is unable to replicate within the host cell due to the absence of a key host factor, or the host mounts an active defense response that leads to a hypersensitive response within the site of infection followed by systemic acquired resistance throughout the plant (Baker *et al.*, 1997). This limits both the replication of the virus and tissue damage within the host.

Compatible interactions

Within a susceptible host**,** a virus is able to efficiently usurp the host cellular machinery to selectively promote its own replication and plant viruses have developed multiple mechanisms to ensure this. For example, TMV initiates translation of its replicase proteins by specifically binding a heat shock protein- HSP 101 to its 5' leader sequence which then recruits the translation initiation complex (Gallei 2002). In Potyvirus replication, the VPg protein, which is covalently linked to the 5' end of the viral genome recruits the cap binding, translational initiation factors eIF4E and eIF(iso) 4E(eukaryotic Initiation Factor) and *Arabidopsis* eIF(iso) 4E mutants showed decreased susceptibility to certain potyvirus suggesting a possible role for this association in enhancing viral protein translation (Wittman *et al.*, 1997; Leonard *et al.*, 2000; Duprat *et al.*,2002; Lellis *et al.*,2002). Some host factors can be used for selective translation regulation of viral proteins. Noueiry and co-workers (2000) have shown that Bromo Mosaic virus (BMV) polymerase translation is selectively regulated by a DEAD-box RNA helicase (DED1) in yeast. Additionally, LSM1, a component of the yeast RNA decapping complex regulates the switch between viral RNA translation and replication by recruiting newly synthesized viral RNAs into the replication pool (Noueiry *et al.*, 2003). Finally, many translation factors have been either co-purified with active viral replicase complexes or found to associate with viral RNA. Subunits of eEF3 (eukaryotic Elongation Factor 3) have been found associated with TMV and BMV RdRp (Osman and Buck, 1996; Quadt *et al.*, 1993). Yamaji et al (2006) were able to confirm *in vivo* interaction between tobacco eEF IA and TMV replicase while Zeenko *et al.* (2002) identified an interaction between eIF1A and the 3' untranslated region of TMV RNA.

Plus strand virus replication invariably occurs within membrane enveloped replication complexes and specific viral proteins interact with host membranes to induce the formation of these vesicle-like structures. TMV replicase proteins associate with two transmembrane proteins TOM1 and TOM2 (Tobamovirus multiplication 1 and 2) and these interactions are essential for TMV replication (Yamanaka *et al.*, 2000; Tsujimoto *et al.*, 2002). TMV replication is blocked in *Arabidopsis* with dual mutations in TOM1 and TOM2A (Ishikawa *et al.*, 1993; Yamanaka *et al.*, 2000). Subcellular localization and fractionation studies revealed that TOM proteins were predominantly located within the vacuolar membranes bringing up the possibility that TMV replication complexes may be tethered to the tonoplast. Conversely immunoflourescent microscopy studies have reported the replication to occur on the Endoplastic reticulum with the replicase proteins shown to colocalize with ER (Heinlein *et al.*, 1998; Mas and Beachy, 1999).

Studies on *Bromo Mosaic Virus* have conclusively shown that replication occurs on ER derived spherical invaginations and BMV replicase and helicase-like proteins have been found to colocalize with resident ER luminal proteins (Restrepo-Hartwig and Ahlquist,1996 ;1999). Furthermore the 1a replicase protein can independently interact with the perinuclear ER and stimulate lipid accumulation thus inducing spherule formation (Lee and Ahlquist, 2003). The sensitivity of BMV replication to membrane lipid compostion is highlighted by a study of BMV replication in the yeast mutant *ole1* (Lee *et al.*, 2001). OLE1 encodes an ER resident fatty acid desaturase that is essential for the synthesis of unsaturated fatty acids (UFA) that in turn help maintain the membrane fluidity and plasticity. Unsaturated fatty acid levels appear to be critical for an early step in viral replication since *ole1* mutants are unable to support efficient viral replication.

UFA supplementation studies on *ole1* revealed that BMV replication has a higher requirement for UFA's than that required for general yeast growth and the mutation preferentially depleted UFA levels within the peri-nuclear ER region, the site of virus replication (Lee and Ahlquist, 2001; Lee *et al.*, 2003). These studies suggest that viral replicase complexes may have a higher requirement for membrane fluidity and also highlight the fact that the host membrane is an essential and dynamic component of the viral replicase complex.

Chaperone proteins are another important class of proteins that assist virus replication. Studies on BMV have shown that a yeast homolog of an *E.coli* Dna J protein (that is thought to play a role in protein folding and translocation) is needed for minus strand RNA synthesis (Tomita *et al.*, 2003). *Cucumber necrosis Tombusvirus* (CNV) replication is enhanced by two yeast homologues of HSP70 chaperone protein, SSA1 and SSA2p (Serva and Nagy, 2006). The two proteins showed *in vivo* interaction with the viral replicase protein p33 and the yeast double mutant *ssa1ssa2* showed a dramatic reduction in viral replication. When considered along with the known chaperone properties of HSP70 proteins, it seems plausible that these proteins may assist the assembly of the replicase complex (Nagy and Pogany, 2005; Serva and Nagy, 2006)

An underlying theme for viral cell to cell movement is that most viruses hitch onto the host endomembrane transport system to traffic through the plant. Thus TMV MP and other viral movement proteins have been shown to associate with the ER, microfilaments and microtubules to eventually reach the plasmodesmata (Liu *et al.*, 2005; Ju *et al.*, 2005; Nelson and Citovsky 2005). Once at the PD, the viral movement proteins need to alter the gating capacity of the PD channel to facilitate the passage of the rather large viral

ribonucleoprotein complex. Time course analysis of PD dilation by MP suggests that the process occurs within minutes which lead to the hypothesis that the virus simply adapts the existing plasmodesmatal machinery to promote movement. TMV MP has been shown to bind to calreticulin, a protein that plays a role in calcium signaling and has chaperone activites (Chen *et al.*, 2005). Calreticulin is an ER associated protein but in plants it appears to accumulate in the plasmodesmata. Alterations in the intracellular levels of calreticulin dramatically altered the plasmodesmatal targeting of MP suggesting a functional role for this interaction on virus movement. One possibility mentioned by Chen and associates is that MP binding to calreticulin, a known ER associated protein, might help in translocating the viral complex from ER to PD. Alternately this association might play a role in altering the plasmodesmatal permeability facilitating movement.

TMV MP also interacts with Pectin methylesterase (PME), a cell wall and plasmodesmata associated protein that plays a role in cell wall porosity and turnover and this interaction is speculated to help with cell wall anchoring of the MP protein (Chen *et al.*, 2000; Rhee *et al.*, 2000). TMV MP activity is regulated by post translation modification especially phosphorylation by host protein kinases. Characterization of one such plasmodesmata associated protein kinase (PAPK1) has shown that the kinase has substrate specificity and phosphorylates TMV MP at 3 residues on the C terminal end that have previously been shown to be essential for movement (Lee *et al.*, 2005). PD dilation by viral movement proteins is less well understood but the interaction between the PVX (*Potato Virus X*) TGB2 protein (Triple Gene Block 2) and β 1-3 glucanase, a callose degrading enzyme suggests that one mechanism of modification could involve virus induced callose degradation at the PD (Fridborg *et al.*, 2003).

Viruses have also learnt to recruit host proteins to help inhibit defense response. TMV recruits a plant P58-IPK (P58 Inhibitor of Protein Kinase) which is speculated to negatively regulate a double stranded RNA activated protein kinase. In animals, the latter is activated when the cell senses the presence of dsRNA (which usually indicates the presence of viral replication intermediates). The kinase phosphorylates and inactivates host Initiation Factor (eIF2α) thereby shutting down host (and viral) translation. It is speculated that by recruiting the hosts own inhibitor of ds RNA activated kinase, the virus effectively suppresses an otherwise dangerous defense response (Bilgin *et al*., 2003).

In summary, it is increasingly becoming evident that plant viruses have evolved different mechanisms to manipulate the host machinery so as to be successful within the constraints imposed by the host.

Table 1.1. List of known interactions between TMV and host proteins

Viral Protein/Sequence	Host Protein	Suggested Function	Reference
5' leader sequence	HSP 101	Initiate translation	Gallie, 2002
3' UTR	eIF1A	Replication	Zeenko *et al*., 2002
Replicase	eIF1A	Replication	Yamaji *et al.*, 2006
Replicase	Subunit of eIF3	Replication	Osman and Buck, 1997
Replicase	TOM1 and TOM2A	Membrane anchoring and formation of VRC's	Ishikawa *et al.*, 1993; Yamanaka *et al.*, 2000
Replicase	P58-IPK inhibitor of ds RNA activated kinase	Inhibition of host cell death	Bilgin *et al.*, 2003
Replicase	33K subunit of photosystem II	Unknown	Abbink *et al.*, 2002
Replicase	N gene	Activation of HR	Whitham *et al.*, 1994
MP	Calreticulin	Transport to/into PD	Chen *et al.*, 2005
MP	Pectin methylesterase	Cell wall anchoring/ movement through PD	Chen *et al.*, 2000; Rhee *et al.*, 2000
MP	PD associated protein kinase1	Regulation of MP activity	Lee *et al.*, 2003

Incompatible interactions - Host defense response

For a host, the specific recognition of an invading pathogen and the subsequent response to its presence determines the difference between susceptibility and resistance. A resistant plant can respond to the pathogen in a multitude of ways. These include inducing programmed cell death or hypersensitive response, activation of the RNAi silencing pathway, modification of the cell wall and production of reactive oxygen intermediates.

Hypersensitive response (HR) –

HR is a defense mechanism employed by plants against a variety of pathogens including viruses, bacteria and fungi. It is characterized by the recognition of a specific pathogen encoded compound (called the avirulence factor) by a specific host surveillance protein called Resistance gene product. Once this interaction/ recognition occurs, it triggers a defense response that is characterized by changes in Calcium and ion fluxes, considerable reprogramming of the trascriptome including activation of defense related genes and production of reactive oxygen intermediates which ultimately leads to localized programmed cell death also called the HR response (Nimchuk *et al.*, 2003; Martin *et al.*, 2003). Once initiated, the HR response is highly effective in stopping pathogen infection thereby making the plant resistant.

Recognition of the pathogen

Plants have evolved to have multiple resistance genes (R) to combat pathogens. These genes have been broadly classified into five families based on their structures. Of the five types, the most abundant are the members of NB-LRR family (that contains Nucleotide binding site region and Leucine Rich Repeats) with *Arabidopsis* having 125 members and rice having more than 600 genes and most of them contain characteristic sub-domains (Nimchuk *et al.*, 2003). In addition, these proteins may contain an additional CC (Coiled-Coiled) domain or TIR (Toll Interleukin- Receptor-like) domain. The LRR region has been implicated in protein-protein interaction while the CC or TIR domains may be important for downstream signaling. The NB region has three domains that have the potential to bind ATP and regions which show homology to eukaryotic cell death effectors (Hammond-Kosack and Jones, 1997).

The 'Gene-for-Gene' hypothesis, proposed by Flor suggests that for each avirulence gene product synthesized by the pathogen, the resistant host carries a complementary, single, dominant R gene whose product recognizes the Avr product. During infection, an interaction between these two components induces a defense response.

While many R genes and their corresponding effectors have been identified, direct binding between the two proteins has very rarely been observed. It appears that recognition and signaling occurs by an indirect and complex mechanism involving R protein mediated recognition of perturbations caused by effector proteins. Most effector proteins identified to date possess enzymatic activity that can alter or modify host proteins to help further pathogen infection. The 'Guard Hypothesis' thus suggests that R proteins monitor or safeguard these target proteins and on detecting modifications to

these proteins (thus indirectly monitoring effector activity), activate the defense pathway (van der Biezen and Jones, 1998; Dangl and Jones, 2001). Thus it is possible that the R protein recognizes the complex formed by the effector and 'guardee' protein or conversely, the R protein is constitutively bound to its guardee, but under pathogen attack, the effector protein showshigher affinity to the guardee, thereby disengaging the R protein leading to its activation.

Physiological response following R protein activation

Following pathogen detection, one of the earliest physiological responses seen is a spike in the production of reactive oxygen intermediates (ROI), particularly hydrogen peroxide and superoxide. This oxidative burst can be directly toxic to the pathogen, promotes oxidative cross-linking and 'sealing' of the cell wall and can further activate defense responses. The oxidative burst thus sets up the host cell for the induction of programmed cell death (Levine *et al.*, 1994; Lamb and Dixon, 1997). Hyper sensitive cell death is characterized by the formation of necrotic lesions indicating the collapse of the infected cell that then limits the spread of pathogen infection. Transient Ca^{2+} change regulate or induce formation of ROI. These fluxes also activate kinase cascade which contributes partially to cell death (Ligterink *et al.*, 1997; Romeis *et al.*, 2000)

Once activated, R genes function through multiple pathways. Two of the well studied pathways are the EDS1 (enhanced disease susceptibility)-dependent pathway and NDR1 (non-race specific disease resistance)-dependent pathway (Parker *et al.*, 1996; Century *et al.*, 1997). The EDS1 is a lipase-like protein while NDR1 shows sequence homology to glycosylphosphatidylinositol (GPI) anchored proteins. To date, no biochemical function

has been shown for either protein. The CC-NBS-LRR's predominantly use the NDR1 mediated pathway while EDS1 signaling is used by TIR-NBS-LRR's. EDS1 and its interacting partner PAD4 (phytoalexin deficient 4) acts upstream of the defense signaling molecule Salicylic acid (SA) at an early signaling step (Falk *et al.*, 1999; Feys *et al.*, 2001).

Salicylic acid (SA), a mobile signaling molecule is a potent inducer of defense response and is necessary for both local and systemic resistance in plants (Delaney, 1994; Durner *et al.*, 1997). SA levels increase during pathogen infection and transgenic plants expressing an SA degrading enzyme are more susceptible to pathogens suggesting an important role for this compound in defense (Malamy *et al.*, 1990, Ryals *et al.*, 1996). SA positively regulates the defense responses involved in the timing and occurrence of local lesions. It is also regarded as the important systemic signal that 'immunizes' the entire plant against future infections. This phenomenon called Systemic Acquired Resistance (SAR) involves the activation of many defense genes called Pathogenesis Related (PR) genes that lead to a heightened state of resistance against a wide spectrum of pathogens. SA has a synergistic effect on defense pathways, plays a role in potentiation of H_2O_2 and defense gene induction. SA is also known to induce EDS1 and PAD4 expression thereby acting in a positive feedback loop (Shirasu *et al.*, 1997).

SA mediated gene expression changes are controlled by NPR1 (Non–expressor of PR1). This protein has been shown to be transported into the nucleus following SAR where its physical interaction with the TGA-bZIP (basic Leucine Zipper) family of transcription factors leads to alterations in the activity of the latter (Kinkema *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000). The TGA-bZIP transcription factors bind to

specific sequences within the promoters of PR genes promoting transcription and this binding is inturn controlled by NPR1 (Lebel *et al.*, 1998; Despres *et al.*, 2000). Some of the other transcription factor families activated or induced during defense responses include the WRKY family, the AP2 domain containing bZIP family, the myb family and many zinc finger factors many of which are involved in defense gene activation (Durrant *et al.*, 2000; Maleck *et al.*, 2000; Mysore *et al.*, 2002). Functions of these defense genes include synthesis of salicylic acid and ethylene, production of phytoalexins and anti-microbials, cell-wall lignification, and amplification of HR response.

NO (Nitric Oxide) another important messenger molecule has also been implicated in HR. Nitric oxide synthase activity is elevated during HR and NO activates PR gene expression (Durner *et al.*, 1998). Furthermore NO levels also appear to positively regulate SA levels in infected tissue. The emerging picture of plant defense is that R gene mediated pathogen perception triggers a positive feedback loop involving production of ROI, NO and accumulation of SA. The complex synergistic interaction between these pathways lead to an amplification of the defense signal that then mediates HR (McDowell and Dangl, 2001; Klessig *et al.*, 2000; Nimchuk *et al.*, 2003).

The role of 26S proteasome and the ubiquitin pathway in plant defense signaling were studied following the identification of the *rar1* mutants. RAR1 gene was found to be an important signaling component required for the function of a subset of CC-NBS-LRR and TIR-NBS-LRRs. *Rar1* mutants are compromised in their response to a number of pathogens suggesting a role in non-host resistance response (Muskett *et al.*, 2002; Peart *et al.*, 2002; Liu *et al.*, 2002b). *RAR1* encodes a putative cytosolic protein containing two Cys- and His-rich (CHORD) Zn^{2+} binding domains and interacts with SGT1, a

component of the SCF ubiquitin ligase. This complex inturn promotes ubiquitination of specific proteins that are bound for degradation and target them via the COP9 signalosome into the 26S proteasome. It is speculated that SGT1 and RAR1 interact with each other and promote degradation of possible negative regulators of defense and utilizes the COP9 complex for mediating this degradation (Liu *et al.*, 2002b).

Many R genes conferring resistance to virus have been identified with the first and one of the most well studied one being the N gene (for Necrotic-type response) of *Nicotiana glutinosa* that confers resistance to TMV. N gene containing plants, when infected by the virus, activate HR at the infection site leading to formation of characteristic necrotic lesions and successful restriction of virus infection. N is a member of the TIR-NB-LRR class of R genes and confers resistance to all Tobamoviruses except Ob strain (Whitham *et al.*, 1994; Holmes, 1938). Chimeric TMV-Ob virus was used to identify the helicase domain as the avirulence factor in N gene activation and the non-viral expression of just the C terminal 50kd region of TMV 126 replicase (also called as P50) containing the helicase domain was sufficient to activate HR (Padget *et al.*, 1997; Abbink *et al.*, 1998; Erickson *et al.*, 1999). While direct interaction between R gene products and avirulence gene products has been rarely shown, and many attempts to look for *in vivo* interaction between the N gene product and TMV P50 have failed , Ueda and colleagues have recently detected interaction between the N gene-product and TMV-P50 (Ueda *et al.*, 2006). They used yeast-two-hybrid analysis and *in vitro* pull down assays to show that the TMV helicase domain interacts with the NBS-LRR region of the N protein and presumably this interaction is dependent on the ATP binding and/or ATPase activity of the N gene. Binding of P50 was found to enhance the ATP hydrolysis

by N protein and also inhibits intra-molecular interaction between the TIR-NBS and LRR domains of the N protein. Taken in light of the finding that N protein stability is enhanced by P50 and that P50 promotes oligomerization of the N protein (which is functionally significant), it is possible that in healthy tissue the N gene product is an inactive state. During TMV infection, binding of the helicase domain enhances its ATPase activity leading to a change in structural confirmation, oligomerization and activation of the N gene product (Mestre and Baulcombe, 2006; Ueda *et al.*, 2006).

Once activated the N gene pathway used many of the components in the classical HR pathway. An increase in ROI precedes the production of H_2O_2 and is dependent on Ca^{2+} concentration (Doke and Ohashi, 1988). Mitogen activated protein kinases (MAPK) like WIPK (Wounding-induced protein kinase) and SIPK (Salicylic acid induced protein kinase) are required for the signaling and are thought to activate WRKY1-3 and MYB transcription factors that form the effector arm of signaling (Liu *et al.*, 2004). Silencing RAR1 and EDS1 genes of *N benthamiana* compromises N mediated HR suggesting a role for both these genes in N signaling (Liu *et al.*, 2002). SA production and activation of the SAR pathway has been observed which eventually promote complete resistance to the virus.

As mentioned earlier, once activated, the HR response which manifests itself as rapid and localized cell death at the site of infection. While this PCD is effective in stopping pathogen spread, if unchecked, it can lead to uncontrolled spread of cell death. It appears that autophagy plays a crucial role in limiting the number of cells killed during HR. *N benthmaiana* plants there were silenced for autophagy genes -NbBECLIN1, ATG3 (Autophagy3) and ATG7 (Autophagy 7) showed PCD when infected with TMV, not just

in the infected tissue but also in distal uninfected and systemic tissue (Liu *et al.*, 2005). TMV infection was also enhanced in these plants. Furthermore, in plants showing normal HR response, double membraned vesicles were observed in the systemic tissue confirming the occurance of autophagy during N mediated resistance. It thus appears that autophagy plays a 'pro-survival' role during PCD and probably helps eliminate or control the PCD signals that move out of infected tissue thereby limiting the cell death.

RNA silencing

RNA silencing is an evolutionarily conserved anti viral mechanism that can effectively prevent replication of invading viruses by recognizing and targeting their double-stranded RNA intermediates for degradation (Waterhouse *et al.*, 2001; Baulcombe, 2004). Initially discovered in plants and nematodes, it is now thought to occur throughout the eukaryotic kingdom and forms an important aspect of the hosts' adaptive immune response towards viral pathogens.

Most plant viruses are RNA viruses and produce double stranded RNA intermediates (ds RNA) during their life cycle. In contrast to host messenger RNA, the viral ds RNA is made up of relatively long stretches of complementary RNA strands. These are recognized as foreign and trigger a series of defense responses within the host, the most potent of which is RNAi.

The dsRNA, synthesized either by a viral polymerase or in some cases a host encoded RNA dependent RNA polymerase (RdRp), is recognized and cleaved by a ribonuclease III (RNAse III)-like enzyme called Dicer resulting in the production of 21 to 24 nucleotide duplex molecule calles short-interfering RNAs (si RNA) which form the

central components in the silencing pathway (Hamilton and Baulcombe, 1999; Zamore *et al.*, 2000; Yu *et al.*, 2003). The siRNA's are distinct in that they are represented by both polarities and have two nucleotide 3' overhangs (Elbashir *et al.*, 2001). There are four Dicer-like homologues in *Arabidopsis* and DCL2 appears to be necessary for viral RNA specific siRNA synthesis (Xie *et al.*, 2004). In an ATP dependent step the siRNA's are denatured and one strand is exclusively incorporated into a multi subunit endonuclease called the RNAi Induced Silencing Complex (RISC) (Hammond *et al.*, 2000; Martinez *et al.*, 2002; Pham *et al.*, 2004). The RISC contains a member of the Argonaut (Ago) family of proteins which contain two domains- PAZ (for piwi−argonaute−zwille) and PIWI and bear strong structural similarity to RNase H proteins (Carmell *et al.*, 2002). The PAZ domain recognizes the siRNA duplex, especially the characteristic 3' overhang thereby effectively and exclusively incorporating one strand of the siRNA duplex into the RISC (Lingel *et al.*, 2004). Within the RISC, this siRNA serves as a guide to recognize and bind to the homologous RNA (viral RNA) ultimately leading to RISC mediated degradation of the target RNA via an RNase H like mechanism of cleavage (Elbashir *et al.*, 2001; Hammond *et al.*, 2000, 2001; Zamore *et al.*, 2000; Liu *et al.*, 2004).

One of the key factors that make RNAi such a potent system of host defense is that once initiated, it continues to maintain the silencing effect by relaying a mobile silencing signal throughout the plant. As a result, as the virus moves within the host tissue, those cells which have received the silencing signal are primed to recognize the parasitic viral RNA and activate the degradation pathway thereby effectively eliminating the viral genome. Thus the inherent amplification and maintenance property of RNAi is effective in targeting pathogens that tend to infect, multiply and spread rapidly.

As a counter defense strategy, almost all viruses come equipped with their own silencing suppressors thus ensuring successful systemic infection. These suppressors appear to affect different steps or components in the silencing pathway. For instance, the Potyvirus encoded Hc-Pro, one of the most studied suppressor affects DCL function , the *Tombus virus* P19 suppressor binds to and prevents siRNA incorporation into RISC while the P25 of PVX and 2b protein of CMV (*Cucumber Mosaic Virus*) interfere with the spread of the systemic signal (Dunoyer *et al.*, 2004; Voinnet *et al.*, 2000). Most of these suppressors are proteins with multiple functions and have evolved to develop silencing suppressor activities suggesting co-evolution of the host defense and viral counter-defense pathways. The suppressors are also pathogenecity determinants in that their presence enhances the infectivity and symptoms associated with viruses.

Viral suppressors have traditionally been identified by a 'reversal of silencing assay' where a transgenic plant silenced for a reporter gene (usually Green Fluorescent Protein or GFP) is agro infilterated with the candidate suppressor construct and assayed for the reappearance of the GFP signal. Such assays with TMV and ToMV (*Tomato mosaic Virus*) have identified the 126 kD replicase protein of Tobamovirus as the silencing suppressor (Kubota *et al.*, 2003; Ding *et al.*, 2004). Twenty-one nucleotide siRNA corresponding to the viral RNA were observed in both TMV and ToMV infection and mutations in the 120- kDa replicase protein that attenuated the suppressor activity also reduced symptom development confirming the importance of the suppressor in virulence. While the ToMV suppressor was able to prevent establishment of PTGS in newly developing tissue, it was unable to suppress pre-established silencing which suggests that the suppressor may affect incorporation of siRNA or formation of virus specific RISC.

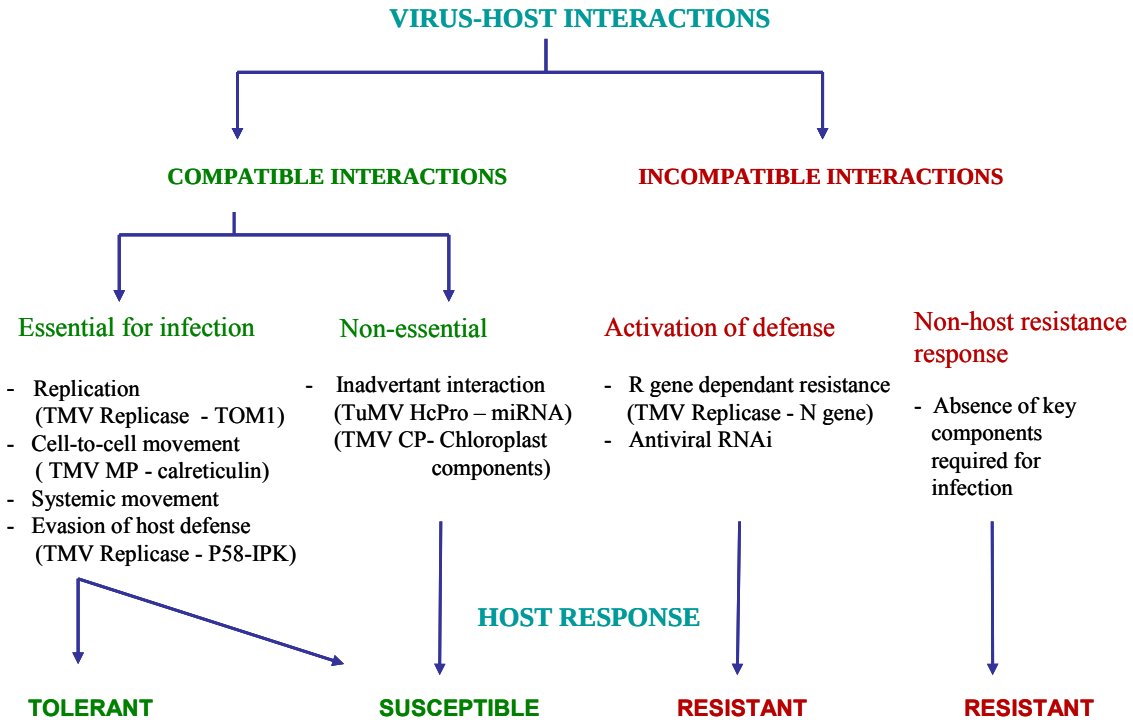


Fig.1.3. Simplified schematic depicting virus-host interactions.

1.2.4 Virus Infections and Symptoms

Virus infected plants display a wide range of symptoms which are dependent on both virus and host and thus serve as important diagnostic features. In many instances, the plant can be stunted with infected leaves showing abnormal colorations like mosaic leaf patterns, ringspot, necrotic spot, leaf blistering, deformation and yellowing. Frequently, the infected leaves may appear curled and in many instances the fruits can display various symptoms leading to a loss in yield and productivity.

Two general models have been used to explain symptom development. The competitive model suggests that plant viruses replicate within the host tissue to such an extent as to usurp a substantial amount of plant metabolite and this diversion in supplies would adversely affect host growth and development. For instance, in TMV infected tobacco plants, viral proteins and genome make up for almost 1% of the fresh weight of the leaf (Hull, 2002). TMV CP accumulation may account for almost half the total protein production in infected cells. Together these factors lead to a dramatic reduction in chloroplast ribosomes and Rubisco levels, have a destructive effect on the photosynthetic apparatus and result in lightened leaf areas characteristic of mosaic symptom. Similarly, TMV-MP mediated changes to plasmodesmata alter the photosynthate assimilate partitioning with reduced carbon allocation to the stem and roots all of which promote to weakening of the whole plant (Lucas *et al.*, 1993; Olesinski *et al.*, 1995)

The non-destructive model is used to describe symptoms ascribed to viruses that show low levels of replication and accumulation. In such instances symptomatology can be ascribed to cytopathic effects of the virus on the cell and virus mediated changes in the

function of key regulatory factors. These include transcription factors, enzymes and hormones (Jameson and Clarke, 2002). These disease inducing interactions broadly occur at two levels. The first level of interactions includes those that alter the host developmental pathways for the benefit of the virus. For instance, Geminiviruses, encode a Rep A protein that interacts with a family of NAC domain proteins-Geminivirus RepA binding proteins (GRAB1 and GRAB2) which are regulators of plant development and senescence (Xie *et al.*, 1999). It is speculated that virus-mediated interference of GRAB protein function might prevent cell differentiation and promote the replication of the DNA virus. The second level of interactions could be thought of as 'accidental' because they do not appear to be critical for the virus but still result in significant changes within the host. In potyviruses, a crucial link between symptom development and alterations in host gene expression has been established based on one such interaction. The silencing suppressor Hc-Pro inhibits silencing by interfering with siRNA metabolism. Based on its mechanism of action, Hc-Pro was found to alter the function of the micro RNA pathway, which shares components of the siRNA pathway. miRNAs act as negative regulators of key transcriptional factors and so in virus infected plants, ectopic expression of these target proteins leads to pleiotrophic developmental defects like loss of leaf polarity, alterations in cell division and loss of reproductive function (Kaschau *et al.*, 2003; Chapman *et al.*, 2004). This suggests that in the case of viruses carrying suppressors of silencing, symptom development may partly occur as a result of inadvertent alterations in miRNA metabolism.

In many instances, virus infection leads to 'host-gene shut off' where viral components can inhibit the functioning of key transcription/translation effectors and

competitively shut off host gene expression. For example, the VPg protein of *Turnip mosaic virus* (TuMV) interacts with host eIFiso4E which probably reduces host gene expression (Leonard *et al.*, 2000). Similarly infection by *Pea seed-borne mosaic Virus* (PSMV) and CMV showed a depletion of a number of host genes within the infection zone and altered gene expression in distant systemic tissue which suggests that viruses have the potential to induce short and long range physiological changes within the host (Wang and Maule, 1995; Havelda and Maule, 2000). Studies like these have not only begun to reveal a molecular basis for symptom induction process during viral infection

1.2.5 Effect of Virus Infection on Phytohormones

Stunting, which is one of the most common symptoms associated with virus infection can be attributed to changes in nutrient uptake, reduction in carbon fixation or alterations in biosynthesis or activity of hormones. Studying the impact of virus infection on hormones has been complicated because of the multiple functional overlaps and complex interplay between different hormones. It has proven to be difficult to measure accurately, the minute changes in hormone levels and furthermore there are multiple levels at which viruses could affect hormone action including synthesis, translocation and site of action. Thus studies aimed at testing the role of hormones during virus infections have more often resulted in contradicting results and provide little understanding of the biochemisty underlying the changes if any. Also, while changes in abscisic acid, gibberellin and ethylene production seem potentially important, there does not appear to be conclusive experimental proof showing a direct correlation between these changes and effect on growth (Fraser and Whenham, 1982). Salicylic acid (SA) and Jasmonic acid (JA), on the

other hand have been conclusively shown to play crucial roles in plant defense (Durrant and Dong, 2004; Takahashi *et al.*, 2004). Elevated levels of SA and JA in infected tissue can be directly attributed to virus infection and coincide with the induction of HR. This process has been discussed in the previous sections

Seminal work by Whenham and colleagues has shown that during TMV infection, cytoplasmic accumulation of free ABA increases in infected plants and influences the development of mosaic symptoms (Whenham *et al.*, 1985; 1986). Cytokinin levels appear to decrease during infection and Clarke *et al.* have shown that in *White clover mosaic virus* (WClMV) infection, there is a marked reduction in cytokinin free bases which corresponds with an increase in virus titer. They speculate that the virus directed reduction in levels of free cytokinin, a potent antioxidant, is needed to promote virus infection (Clarke *et al.*, 1999). Many groups have also reported decrease in Gibberellin (GA) levels and it appears that integrated responses involving GA, ABA and Cytokinins may have a synergistic effect on symptomatology and virulence.

Ethylene appears to be an important factor in the development TMV induced necrotic lesions in N gene containing plants. There is a spike in ethylene production near the time of appearance of lesions and treatment with ethylene inhibitors leads to reduction in lesion size. (Pennazio and Rogero,1990 ; Ohtsubo *et al.*, 1999). There are mixed reports on the role of ethylene in HR, with Kachroo and colleagues (2000) reporting no significant change in *Turnip Crinkle Virus* (TCV) mediated HR development in ethylene mutants while Ohtsubo and coworkers link the ethylene spike during TMV infection to an increase in PR gene transcription and show a positive correlation between ethylene treatment and lesion formation (Kachrro *et al.*, 2000; Ohtsubo *et al.*, 1999). They suggest

a possible role for ethylene in activating the enzymatic activity of chitinases and peroxidases.

Numerous investigations have shown both a general reduction in auxin levels or activity during virus infection as well as increase in cases of infections with severe symptomatology (Fraser and Whenham, 1982 and references therein; Jameson and Clarke, 2002 and references therein). The reduction in auxin levels has been putatively linked to the increase in certain peroxidases which promote auxin catabolism though no experimental data has been provided to link the two factors (Jameson and Clarke, 2002 and references therein). Insights into auxin regulation and virus infection have recently gained momentum with the introduction of *Arabidopsis* as a promising host for some viruses. An *Arabidopsis* mutant showing an increase in symptom development following infection by *Turnip vein clearing virus* (TVCV) was identified by Sheng and co-workers. The healthy *vid1* (virus inducible dwarf1) mutant had a phenotype comparable to wild type, but following infection, the plants showed severe stunting and loss of apical dominance (Sheng *et al.*, 1998). Interestingly, application of exogenous auxin led to an abrogation of the phenotype. The authors speculate that *vid1* mutation might be important for auxin transport and systemic virus infection alters the intercellular transport pathways within the plant thereby severly affecting auxin transport within the *vid1* background. Thus the *vid1* mutant might be enhancing or magnifying the effect of virus mediated disruption in auxin transport. Alterations in auxin levels caused by phloem disruption are also speculated to play a role in Geminivirus infections and subsequent symptom development (Park *et al.*, 2004). The *Arabidopsis* ecotype Sei-O is extremely susceptible to infection by *Beet Curly Top virus* (BCTV), a Geminivirus, with the

induction of callus formation in the inflorescence and leaves. Analysis of infected tissue showed phloem hyperplasia and an increase in auxin induced gene expression within the callus tissue. The authors suggest that the detrimental effects on phloem might lead to accumulation of auxin in specific tissue leading to enhanced cell division and subsequent callus formation.

In spite of all the inroads made, it is quite obvious that the effect of virus infection on the synthesis and action of hormones is extremely complex and does not lend itself to facile analysis. It is still difficult to know if the changes seen in hormonal levels are directed by the virus or simply a consequence of the alterations occurring within the plant. It can be hoped that with the availability of various mutants and with the advent of more sensitive tools for hormone detection, virus-hormone interaction will not remain an under-researched area for long.

1.2.6 Auxin

The phytohormone auxin or Indole Acetic Acid (IAA) elicits a diverse array of cellular responses within the plant cell and plays a major role throughout the development of the plant. At the cellular level, it is needed for cell division, expansion and maintenance of polarity. At the whole plant level, it is thought to regulate responses like embryonic development, tropism, root initiation, leaf patterning, apical dominance and fruit development.

Auxin synthesis and transport

Indole acetic acid (IAA) is the chief biologically active auxin in plants and is synthesized in very young leaves, cotyledons and roots (Ljung *et al.*, 2001; Avsian-Kretchmer *et al.*, 2002). Plants use many pathways to synthesis IAA but it is chiefly a tryprophan dependent route of synthesis. It is present in both the free (active) and conjugated (inactive) forms where the latter is chiefly utilized for transportation, compartmentalization and storage purposes. Auxin is transported between cells through a combination of membrane diffusion and carrier-mediated transport. AUX1, PIN (pin-formed), PGP(P-Glycoportein Like) class of carrier proteins mediate the uptake and efflux of auxin (Parry *et al.*, 2001; Geisler and Murphy, 2006). AUX1, a transmembrane protein, permits influx of auxin while a family of PIN proteins and certain PGP like proteins carry out auxin efflux (Bennett *et al.*, 1996; Luschnig *et al.*, 1998; Muller *et al.*,1998; Kramer and Bennett, 2006). The underlying theme for all the transporters is their asymmetric localization within the cell facilitating polar or directional auxin transport (Galweiler *et al.*, 1998). The localization of these proteins is extremely dynamic and sensitive to external stimuli like gravity and light thus co-ordinating the auxin transport in response to environmental cues (Friml *et al.*, 2002; Peer *et al.*, 2004). Within the plant, an intricate network of these proteins facilitates auxin transport. Thus localized auxin synthesis, metabolism and transport help ensure that appropriate auxin levels are present in cells or tissues at the developmentally appropriate stage.

Auxin signaling

At the molecular level, hundreds of genes have been shown to change their expression in response to auxin (Pufky *et al.*, 2003). The best understood are a specific subset of genes called the primary auxin response genes, so named because of the active induction of these genes within minutes of exposure to auxin. These genes fall into 3 major families - SAUR (Small Auxin Upregulated RNAs), GH3 and *Aux*/IAA (Auxin/Indole Acetic Acid) gene family (McClure *et al.*, 1989; Hagen *et al*., 1984; Walker and Key, 1982). Auxin also indirectly influences the activity of a family of transcription factors called ARF's or Auxin Response Factors.

SAUR trancripts are rapidly induced by auxin, their mRNAs are short lived due to the presence of destabilizing elements in the 3' untranslated regions and so far, their role in auxin signaling is unknown (Sullivan and Green, 1997). GH3-like genes in many instances encode enzymes involved in conjugating IAAs and thus may serve to 'dampen' the auxin signal by conjugating and inactivating IAA (Staswick *et al.*, 2005). The soybean GH3 gene and its promoter has been extensively analysed to identify cis-regulating elements that promote auxin responsiveness. These, elements, called AuxRE (Auxin responsive elements), when stripped down to their basic unit, consists of a hexanucleotide sequence TGTCTC (Liu *et al.*,1994; Hagen and Guilfoyle, 2002). Simple elements are made up of iterations of this unit while composite elements consist of the TGTCTC element lying adjacent to or overlapping with a constitutive element (Ulmasov *et al.*, 1995; Guilfoyle *et al.*, 1998). AuxRE's have been identified in the promoters of all three classes of primary auxin responsive genes and furthermore, synthetic AuxRE's consisting of a palindromic stretch of the TGTCTC elements, when fused to a minimal

promoter can impart auxin responsivness to any downstream reporter gene confirming that the TGTCTC element is sufficient to induce a response to auxin .

Aux/IAA gene family

Of the four auxin responsive gene families, the *Aux*/IAA genes and the ARF genes have been the most extensively studied. Members from Aux/IAA multigene family have been identified in *Arabidopsis*, soybean, tomato, tobacco and pea and based on sequence homology; these genes have also been identified in monocots and gymnosperms. In *Arabidopsis*, 29 Aux/IAA genes have been identified and are referred to as IAA1 to IAA20 and IAA26 to IAA34 (Liscum and Reed, 2002). These genes are predicted to encode short live nuclear proteins, which are thought to function as transcriptional repressors by interacting with and preventing the functioning of ARF's.

Sequence analysis has confirmed that all *Aux*/IAA proteins contain four conserved motifs referred to as domains I, II, III and IV. Domain I and II contain the bipartite nuclear localization motif (Abel *et al*., 1994). Domain II also mediates the rapid auxin dependent turn over of these proteins. Domains III and IV share sequence similarity with the C terminal domains of ARF's and are thought to be involved in homo and hetero dimerization between *Aux*/IAA and ARF proteins (Reed, 2001) (Fig.1.4). The interdomain regions are considered to be either 'filler' loops that promote the correct structural confirmation of the protein or may interact with additional proteins thereby promoting functional specificity. Additional specificity is provided by differences in spatial and temporal expression of these genes and by varying the degree of sensitivity to auxin induction (Abel *et al.*, 1995).

Mutations in several Aux/IAA genes have been identified and shown to result in pleiotrophic effects on the plant, the most common effect being a reduction in auxin response. Most of these mutations were semi-dominant and interestingly, almost all of them mapped to a specific region within Domain II of the protein(s) (Rouse *et al.*, 1998; Nagpal *et al.*, 2000; Reed 2001; Rogg *et al.*, 2001). Biochemical analysis revealed that the mutations enhanced the protein stability, leading to a gain-of-function phenotype which suggested that normal auxin signalling required the rapid degradation of the Aux/IAA proteins (Worley *et al.*, 2000; Gray *et al.*, 2001; Ramos *et al.*, 2001). Aux/IAA loss-of-function mutants have very subtle phenotypes which indicates that Aux/IAA's have redundant and probably compensatory functions.

Auxin responsive factors (ARF)

Auxin Response Factors, a 23 member family in *Arabidopsis*, function as transcription factors by binding to cis acting AuxRE elements present within the promoter elements of Auxin responsive genes and modulating either the activation or the repression of these genes (Reed, 2001; Hagen and Guilfoyle, 2002). ARF1, the founding member of the family was identified by its ability to bind to AuxRE elements within a yeast-one-hybrid system (Ulmasov *et al.*, 1997b). The N terminal region of ARF1 contains a DNA binding domain which appears to be a common domain seen in almost all other ARFs (Ulmasov, *et al.*, 1997b). The central domain (CD) in these proteins is poorly conserved and determines the transcriptional capabilities of the protein. Based on their sequence, they are either activators or repressors with proline rich CD's generally seen in repressors (ARF1) while glutamine/serine/threonine rich ARFs (ARF 5, 6, 7, 8) acting as

transcriptional activators (Ulmasov *et al.*, 1999a). The C terminal ends show striking similarity with domains III and IV of the Aux/IAA proteins and mediate heterotypic interactions with these proteins (Ulmasov *et al.*, 1997a; 1997b) (Fig.1.4).

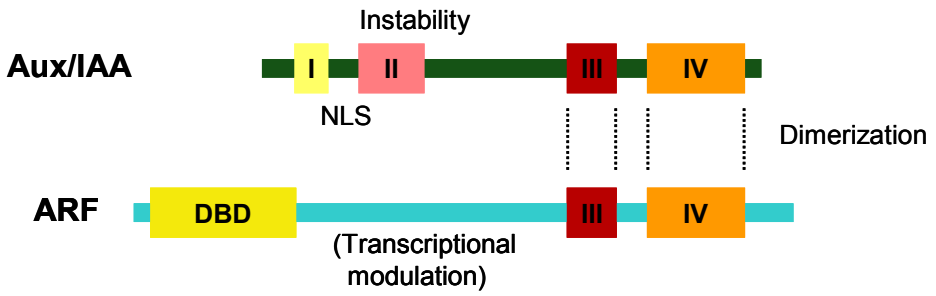


Fig.1.4. Schematic diagrams of Aux/IAA and ARF proteins. Roman numerals indicate individual domains. NLS stands for nuclear localization signal and DBD is the DNA binding domain. (Adapted from Reed, J.W., 2001)

Auxin - mode of action

The current theory for auxin signaling suggests that at basal auxin levels, the Aux/IAA proteins are relatively stable, heterodimerize with ARF's and either prevent them from dimerizing with each other; an interaction necessary for activation of ARF's, or they prevent the latter from binding to the AuxRE present on the Auxin responsive genes (Reed, 2001; Leyser, 2002). The presence of auxin however, promotes the interaction between domain II on the Aux/IAA proteins and the F box protein, TIR1, a member of a specialized E3 ubiquitin ligase complex called the SCF^{TIR1} complex (Gray *et al*., 2001). This then leads to the ubiquitination and targeted degradation of these proteins via the 26S proteasome (Gray *et al.*, 2001; Smalle and Vierstra, 2004).

The ubiquitin mediated proteolysis is a three step process and begins with the energy dependent activation of ubiquitin by the ubiquitin activating enzyme, E1 (Hatfield *et al.*, 2001). Ubiquitin is then passed onto the ubiquitin conjugating enzyme E2, which, acting in concert with the enzyme complex E3, links the ubiquitin to a specific lysine residue within the target protein (Bachmair *et al.*, 2001; Criqui *et al.*, 2002). A multi ubiquitin chain is then extended from the first ubiquitin moiety and substrates marked with the polymer of Ubiquitin are targeted to the multi subunit, ATP dependent protease - the 26S Proteasome. The substrate specificity for this process is dependent on the activities of the E3 ligase and this is reflected by the fact that E3 ligases are very diverse and complex. The SCF protein complex is one such well-studied E3 ubiquitin ligase and has been identified in plants, animals and yeast. It derives its name from 3 of its 4 subunits. Two of these subunits are members of the Cullin and RBX1 (also called ROC1 and HRT1) families and form heterodimers to catalyze the ubiquitin chain formation (Bates and

Vierstra, 1999; Seol *et al*. 1999; Deshais, 1999). A member of the SKP1 protein family mediates the interaction between Cullin/RBX1 dimer and the amino terminus of a corresponding F box protein. Structure analysis seems to suggest that SKP1, Cullin and RBX1 proteins form a common core that then recruits different F box proteins to form functionally distinct E3 ligase complexes (Patton *et al*., 1999; Zheng *et al.*, 2002). The F box proteins are adapter proteins, mediating substrate recognition and are thus the most diverse members in this complex with over 700 F box proteins having been identified in *Arabidopsis* (Gagne *et al.*, 2002). The F box protein TIR1 (Transport inhibitor response1) has been implicated in mediating protein degradation associated with auxin signaling and the SCF^{TIR1} complex was shown to interact with 2 Aux/IAA proteins, IAA7 and IAA17, in an auxin dependent fashion (Gray *et al*., 2001, Zenser *et al.*, 2001).

In a series of elegant experiments, two groups of researchers have shown that the *Arabidopsis* TIR1 is in fact the auxin receptor. Auxin physically binds to TIR1 mediating a conformational change in the SCF^{TIR1} complex which enables recognition of Aux/IAA and subsequent ubiquitination. Auxins effect on TIR1 interaction can be mimicked in a cell free system and introduction of TIR1 into a heterologous, auxin insensitive system, promoted binding with Aux/IAA in the presence of auxin confirming that TIR1 was indeed the auxin receptor (Dharmasiri *et al.*, 2003; Kepinski and Leyser, 2004; Dharamsiri *et al.*, 2005; Kepinski and Leyser, 2005). Domain II of the Aux/IAA is the recognition motif and mutations within this region abolish interaction making the protein stable in the presence or absence of auxin. These findings have thus provided a molecular basis for the gain-of-function phenotype associated with the Aux/IAA domain II mutants.

Degradation of the repressor Aux/IAA proteins releases the ARF's that are then free to bind to specific promoter elements and/or modulate the expression of the downstream auxin responsive genes (Leyser, 2002). Depending on the kind of ARF, downstream genes are either activated or repressed following the auxin stimulus (Fig. 1.5). These genes are eventually responsible for the wide range of auxin mediated physiological responses observed in the plant. The auxin signaling pathway can be considered to be a rapid and short term signaling response because the activation of genes is seen within minutes of auxin exposure. It is also a comparatively short-term response because many Aux/IAA genes are activated leading to a quick accumulation of these proteins and a rapid regulation and dampening of the auxin signal.

On one hand the auxin response appears to be extremely complex because of the sheer number of signaling molecules it affects and because of the variations in the expression patterns of ARF and Aux/IAA genes in different tissue and at different stages of development. On the other hand there is evidence accumulating to show that a select set of players can be attributed to each physiological response seen. For instance, ARF proteins appear to have more limited and specific functions with ARF1 and ARF2 mediating floral senescence while ARF7 and ARF19 controlling leaf expansion and root development (Ellis *et al.*, 2005; Wilmoth *et al.*, 2005). Moreover it appears that specific pairs of Aux/IAA and ARF proteins preferentially bind to each other to generate developmental specificity in the auxin response (Weijers *et al.*, 2005). The challenge ahead is of course to identify these interacting partners and the pathways they alter.

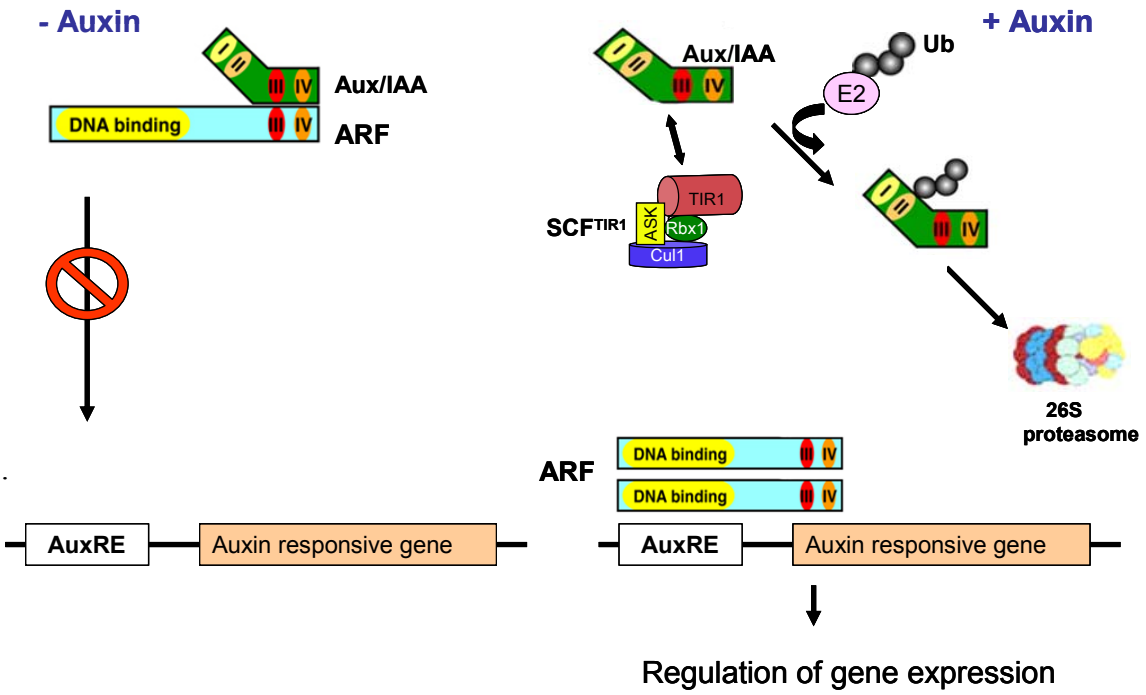


Fig.1.5. Auxin mediated gene regulation. At basal auxin levels, the Aux/IAA proteins act as repressors of ARF proteins. Auxin stimulates the interaction between Aux/IAA and the E3 ubiquitin ligase –SCF^{TIR1} promoting transfer of ubiquitin from the E2 complex to the Aux/IAA protein. This leads to the targeted degradation of Aux/IAA proteins via the 26S proteasome. The ARF proteins bind to the *cis* acting Auxin responsive elements (AuxRE) and modulate the transcription of downstream auxin responsive genes. ASK- *Arabidopsis* Skp protein, Cul1 – *Arabidopsis* Cullin protein, Rbx1 – *Arabidopsis* Ring box protein1, TIR1- F-box containing protein- Transport Inhibitor Response 1.

1.3 RESEARCH OBJECTIVE

Attempts to elucidate the molecular events that lead to disease induction have mainly focused on identifying the role that viral nucleic acids and proteins play in this process and rapid progress has been made in mapping viral determinants of disease development. Unfortunately, much less is known about how the plant responds at the cellular and molecular level to a viral infection and our knowledge of the biology underlying plant susceptibility/tolerance, symptom expression and virus host range is extremely limited.

The work presented in this dissertation is aimed at understanding specific molecular changes occurring within a plant that help mediate susceptibility. Studies were conducted using *Tobacco Mosaic Virus* and *Arabidopsis thaliana* as model systems. *Arabidopis thaliana* ecotype Shahdara is extremely susceptible to TMV infection, showing rapid systemic spread of the virus and producing visible disease symptoms (Dardick *et al*., 2000). Thus the TMV-Shahdara system is ideal to study disease development since it is now possible to individually dissect both virus and host components involved in mediating susceptibility.

A yeast-two-hybrid approach was used to screen for interactions between viral and plant proteins. This screen identified an interaction between the TMV replicase and an *Arabidopsis* Auxin/IAA protein - IAA26. IAA26 is an auxin-induced gene and the protein is a component in the auxin response pathway. Initial studies were aimed at confirming the occurrence of the interaction *in-vivo* and establishing the effect of TMV infection on IAA26. Genomic and cell biology approaches were used to this end and results from these studies indicated a role for the TMV-IAA26 interaction in promoting symptom development. IAA26 is a transcription factor controlling auxin response, and so

my objective was to determine the potential downstream targets of the protein and study how this virus-host interaction leads to disruptions in auxin pathway and host physiology.

Considering the innate sequence similarity and functionally redundancy among Aux/IAA proteins my final goal was to carry out an extensive screen to identify additional Aux/IAA members that associated with TMV in susceptible hosts. Through this work, I have tried to map out an entire disease pathway, starting from the initial virus-host protein interaction to alterations in the transcriptional levels of selected genes and finally the physiological effects of these alterations leading to disease response.

Chapter 2

Interaction of the Tobacco Mosaic Virus Replicase Protein with the Aux/IAA protein PAP1/IAA26 is Associated with Disease Development

2.1 ABSTRACT

Virus infected plants often display developmental abnormalities that include stunting, leaf curling and the loss of apical dominance. In this study, the helicase domain of the *Tobacco mosaic virus* 126/183-kDa replicase protein(s) was found to interact with the *Arabidopsis* Aux/IAA protein IAA26 (also named PAP1) a putative regulator of auxin response genes involved in plant development. To investigate the role of this interaction in symptom display, a TMV mutant defective in the IAA26 interaction was identified. This mutant replicated and moved normally in *Arabidopsis* but induced attenuated developmental symptoms. Additionally, transgenic plants silenced for the accumulation of IAA26 mRNA exhibit virus-like symptoms. In uninfected tissues, ectopically expressed IAA26 accumulated and localized to the nucleus. However, in TMV infected tissues IAA26 failed to accumulate to significant levels and did not localize to the nucleus, suggesting that interaction with the TMV replicase protein disrupts IAA26 localization. The consequences of this interaction would affect IAA26's putative function as a transcriptional regulator of auxin response genes. This is supported by gene

expression data indicating that ~30% of the *Arabidopsis* genes displaying transcriptional alterations in response to TMV contain multiple auxin response promoter elements. Combined, these data indicate that the TMV replicase protein interferes with the plant's auxin response system to induce specific disease symptoms.

2.2 INTRODUCTION

Developmental abnormalities including leaf curling, stunting and the loss of apical dominance are some of the most common and economically important symptoms associated with virus induced plant diseases (Hull, 2002). The majority of these disease symptoms occur only when newly emerged tissue becomes infected, suggesting that interference in developmental processes are a primary cause of disease. Many of these disease responses are likely derived from the ability of specific virus components to interact with and disrupt the function of specific host components. Unfortunately, the host components and pathways through which viruses induce disease remain poorly characterized.

Tobacco mosaic virus (TMV) is the type member of the genus *Tobamovirus* and serves as a model for studying virus-host interactions. TMV is a positive-stranded RNA virus that encodes at least four proteins (Goelet *et al.*, 1982; Fig. 2.1A). The two largest open reading frames (ORF) encode 126- and 183-kDa replicase proteins, the larger produced via read-through of an amber stop codon (Pelham, 1978). Homology studies indicate that the 126-kDa ORF encodes a methyltransferase domain (MT) implicated in

viral RNA capping and a helicase domain (HEL) involved in double-stranded RNA unwinding (Dunigan and Zaitlin, 1990, Goregaoker and Culver, 2001). The read-through portion of the 183-kDa ORF encodes the RNA-dependent RNA-polymerase domain (POL) (Koonin and Dolja, 1993). A 30-kDa protein required for cell-to-cell movement and the 17.5-kDa capsid protein are produced from subgenomic mRNAs (Deom *et al.*, 1987; Hunter *et al.*, 1976;Meshi *et al.*, 1987).

During infection, TMV induces a specific set of disease symptoms in *Arabidopsis thaliana* ecotype Shahdara. These include stunting, necrosis of the inoculated leaf, loss of apical dominance and leaf curling. Numerous changes in specific TMV genes have been identified as conferring either attenuated or severe disease symptoms (Dawson, 1999). For example, amino acid substitutions in the coat protein have been linked to chlorosis while specific mutations in the replicase protein can disrupt its function as an RNA silencing suppressor, resulting in reduced virus accumulations and milder disease symptoms (Banerjee *et al.*, 1995; Culver, 2002; Ding *et al.*, 2004; Kubota *et al.*, 2003). However, the specific molecular mechanisms through which these TMV components affect the disease process remain unidentified. In other virus-host systems, more specific links between virus and host components involved in the display of symptoms have been made. For example, suppression of RNA silencing conferred by the HC-Pro protein of *Turnip mosaic virus* affects the accumulation of specific micro-RNAs involved in the control of plant developmental pathways and results in the display of specific disease symptoms (Kasschau *et al.*, 2003). For geminiviruses, the AL1 replication protein interacts with a host-encoded cell cycle regulator protein to modulate virus tissue

specificities and associated disease symptoms (Kong *et al.*, 2000). Thus, virus induced diseases appear to have multiple causes.

In this study, an interaction between the TMV 126/183-kDa replicase protein(s) and an *Arabidopsis thaliana* auxin/indole-3-acetic acid (Aux/IAA) protein, IAA26, also called Phytochrome Associated Protein 1 (IAA26/PAP1; At3g16500), is described. In general, Aux/IAA proteins, such as IAA26, are thought to function as negative regulators of auxin response factor (ARF) proteins that in turn control the transcriptional activity of primary auxin response genes involved in various aspects of plant development, including cell division, cell expansion, and apical dominance (Reed, 2001;Tiwari *et al.*, 2001). The nuclear localization of Aux/IAA proteins and their ability to heterodimerize with ARF DNA binding proteins support their function as transcription factors (Abel *et al.*, 1994). Furthermore, the stability of Aux/IAA proteins is modulated by the plant hormone auxin, providing a sensitive method for the spatial and temporal control of their function (Tiwari *et al.*, 2001). Interaction with the TMV replicase protein was found to disrupt IAA26 localization and corresponded with the inappropriate expression of auxin-regulated genes and the appearance of disease symptoms. To our knowledge this is the first report to link virus-induced disease symptoms to a disruption in the plant's auxin response system.

2.3 MATERIALS AND METHODS

2.3.1 Two-Hybrid Assays

Five overlapping bait sequences covering the TMV 126/183-kDa ORF(s) were cloned into the yeast bait vector pLexA-NLS as previously described (Goregaoker *et al.*, 2001). An *Arabidopsis* leaf and root cDNA library, CD4-10 - derived from ecotype Nossen and cloned into the yeast prey vector pACT-GAL4 was obtained from the Arabidopsis Biological Resource Center, Columbus, OH. Each of the five TMV 126-183/LexA bait constructs was co-transformed with the *Arabidopsis*/GAL4 prey library into S*accharomyces cerevisiae* strain L40. Transformants displaying bait to prey interactions were selected on -His/-Leu/-Trp/-Ura minimal media at 25^{o}C, a temperature previously shown to promote the interaction of TMV helicase sequences (Goregaoker *et al.*, 2001). Interacting transformants were assayed for β-galactosidase activity on nitrocellulose filter lifts, frozen at -80^{o}C for 5 min, and soaked in a 4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution containing 0.1% Triton X-100 and Z-buffer (1*M* Na_2HPO_4, 40m*M* NaH_2PO_4, 10m*M* KCl, 1m*M* $MgSO_4$, 50m*M* β-mercaptoethanol, pH 7.0). Yeast colonies turning blue within 30 minutes were selected for further analysis.

Interaction positive *Arabidopsis*/GAL4 prey plasmids were shuttled into *Escherichia coli* as described by Ward (1990). Positive interacting *Arabidopsis* clones were retransformed with the interacting TMV 126-183/LexA replicase clone to confirm the maintainace of the interaction as well as with an empty pLexA-NLS clone or one encoding the non-interacting *Arabidopsis* protein ETR1 (Clark *et al.*, 1998) to screen for false interactions. β-galactosidase activity was quantified in liquid culture as previously

described (Goregaoker *et al.*, 2001; Miller, 1972). Positive *Arabidopsis*/GAL4 prey clones were then sequenced for identification. Full-length IAA26 and IAA10 ORFs were obtained by RT-PCR using mRNA derived from leaves of ecotype Shahdara. Full-length ORFs were cloned into the GAL4/prey plasmid and analyzed in yeast for their interaction with TMV HEL/LexA.

2.3.2 IAA26 - Replicase interaction assays

Full-length IAA26 was cloned into the expression vector pTrcHis-A (Invitrogen, Carlsbad, CA) so as to produce an ORF containing an N-terminal hexa-histidine tag. IAA26 protein was then expressed and purified via Ni-affinity columns (Amersham Biosciences, Piscataway, NJ) as previously described (Goregaoker and Culver, 2003). Full-length TMV 126/183 kDa protein was generated by *in vitro* translation. Purified TMV virions, (3 µg) were incubated for 15 min in 0.01M Tris-HCL, pH 8.0, pelleted by centrifugation at 65,000 rpm in a Beckman TLA100.3 rotor for 20min and resuspended in water. Treated virions were added directly to an mRNA-dependent rabbit reticulocyte lysate system (Promega, Madison, WI), containing 250µCi/ml L-[^{35}S]methionine and incubated for 90 min at 30^{o}C. Translation reactions were analyzed by SDS-PAGE via a PhosphoImager and quantified using the program ImageQuant (Molecular Dynamics, Sunnyvale, CA).

For overlay assays purified IAA26 protein was immobilized onto nitrocellulose sheets. Sheets were then blocked for two hrs at 4^{o}C with 5% [w/v] non-fat dry milk in Tris-buffered saline (50mM Tris-HCL, pH 6.8, 200mM NaCL; TBS). Blocked sheets were incubated overnight in TBS containing 5% non-fat dry milk and equivalent levels of ^{35}S-

labelled 126/183-kDa protein, either wild-type or V1087I. Sheets were then washed 3X with TBS, dried and bound 126/183-kDa protein visualized by PhosphoImager.

2.3.3 RNAi Construct and plant transformation

A derivative of the *Agrobacterium* binary transformation vector, pBI121 (Clonetech, Palo Alto, CA), was used to construct a IAA26-specific RNAi silencing vector. Within the pBI121 poly-linker, complementary IAA26 sequences nt 1 - 500 were cloned onto opposite sides of a 102 nt spacer containing an EF1α intron (At5g60390, nt 961 - 1061). Transcription of this construct, derived from the 35S *Cauliflower mosaic virus* promoter, produces a 500 bp IAA26 specific double-stranded RNA. *Agrobacterium* transformations were performed on five week old *Arabidopsis thaliana* Shahdara plants as previously described (Clough and Bent, 1998). Transformants were selected on 1X MS agar containing 30 mg/L of kanamycin. Integration of the IAA26-RNAi construct was confirmed by PCR analysis of leaf extracted genomic DNA (Dellaporta *et al.*, 1983).

Endogenous IAA26 mRNA levels were quantified in both T_0 and T_1 lines by real time-PCR. Total RNA was extracted from the leaves of 4 week old IAA26-RNAi transformed and non-transformed plants using the RNeasy Plant Miniprep kit (Qiagen, Valencia, CA). cDNA was generated from 1μg of isolated RNA pretreated with RQ1 DNase (Promega, Madison, WI) and reverse transcribed in a SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Quantitative Real Time-PCR (QRT-PCR) was preformed using Platinum® qPCR supermix-UDG reagents (Invitrogen, Carlsbad, CA). Each 20μl QRT-PCR reaction

mixture contained 10μM of both labeled LUX™ IAA26 primer (CACGCTTTCATCTGTGAAGAGACTGCG5G) and unlabeled IAA26 primer (TTGCTTACTGCATCCAAATGTCAA) designed using LUX™ designer software (Invitrogen, Carlsbad, CA), ROX reference dye (0.5 μl), cDNA (1.5 μl) and sterile distilled H_2O (4.5 μl). QRT-PCR reactions were performed on an Applied Biosystems (Foster City, CA.) GeneAMP 5700 sequence detection system (50°C for 2 min, 95°C for 10 min for 1 cycle followed by 95°C for 15 sec and 60°C for 1 min for 40 cycles). Relative expression levels of IAA26 were normalized to the housekeeping gene EF1α and fold expression levels determined using the Comparative ΔCt method (Johnson *et al.*, 2000). Expression levels of EF1α were determined as above using 1.5 μl of cDNA and EF1α specific LUX™ labeled (GACTGCCCACACCTCTCACATTGCAG5C) and unlabeled (TCCTTACCAGAACGCCTGTCA) primers.

2.3.4 Identification and construction of a TMV Helicase mutant

TMV-V1087I was previously created by random mutagenesis using hydroxylamine treatment of the TMV helicase/LexA bait construct (Goregaoker *et al.*, 2001). Yeast co-transformations with mutant TMV helicase/LexA and IAA26/GAL4 constructs were done as described above. Stability and expression of HEL-LexA fusion proteins were confirmed by Western immunoblot using anti-LexA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The non-interacting TMV-V1087I helicase coding sequence, *Sac*II (nt 2654) to *BamH*I (nt 3333), was cloned into a similarly digested recombinant full-length infectious TMV clone, pSNC004 (Dawson *et al.*, 1986;

Shivprasad *et al.*, 1990; Turpen *et al.*, 1995). DNA sequencing was carried out to confirm the presence of the mutation within the viral helicase sequence. Infectious RNA transcripts derived from the mutant full-length virus construct were generated *in vitro* as described and used to inoculate leaves of *N. benthamiana* and *A. thaliana*, Shahdara plants (Shivprasad *et al.*, 1990).

2.3.5 Plant growth conditions and virus assay

A. thaliana Shahdara and *N. benthamiana* plants were grown and maintained in growth chambers under a 12-h photoperiod at 24^{o}C. Four week old Shahdara plants and four to five week old *N. benthamiana* plants were used for virus inoculations. Mature rosette leaves of Shahdara plants were dusted with carborundum (Fisher Scientific company, Pittsburgh, PA, USA) and mechanically inoculated with 10μg of purified WT-TMV or TMV-V1087I. The youngest leaves of *N. benthamiana* plants were similarly treated with carborundum and inoculated with 5 μg of WT-TMV and TMV-V1087I. Control plants were similarly dusted with carborundum and mock inoculated with distilled water. Virus accumulation and movement were monitored by Western blot and tissue print immunoblots for the detection of the virus capsid protein as previously described (Dardick *et al.*, 2000). *Arabidopsis* protoplasts were derived from leaf tissues as previously described and inoculated by electroporation with 5 μg of purified viral RNA (Dardisk *et al.*, 2000; Simon *et al.*, 1992). The accumulation of viral RNA in inoculated protoplasts was determined by QRT-PCR using TMV specific LUX™ labeled (CACTCTGGATGCAGCAATCAGGCAGAG5G) and unlabeled

(AGCGGCATAGCACGTATGGA) primers. A QRT-PCR standard curve derived from known amounts of viral RNA was used to determine virus concentration.

For transient expression studies, inoculated Shahdara leaves were used at 12 days post-inoculation and systemic leaves were used at 3 weeks post-inoculation. Inoculated *N. benthamiana* leaves were used 4 to 6 days post-inoculation and systemic leaves were used 7 to 9 days post-inoculation. Viral loads within these tissues were monitored by immunodot blots using TMV coat protein specific antiserum (Dardick *et al.*, 2000).

2.3.6 IAA26 transient expression constructs and assays

The expression vector pCMC1100, containing the *Cauliflower mosaic virus* 35S promoter, served as the parental plasmid for all transient expression constructs (McCabe *et al.*, 1998). The EGFP ORF (Clonetech, Palo Alto CA) was PCR modified to contain a 5' end *BsiWI* site and 3' end *PstI* site and inserted into similarly cut pCMC1100, creating pCMC-GFP. PCR amplification was also used to engineer the IAA26 ORF with a unique 5' end *BspHI* site and a 3' end *BsiWI* site minus the termination codon. The modified IAA26 ORF was cloned into similarly digested pCMC-GFP, placing the IAA26 ORF in frame with GFP and creating pIAA26-GFP. IAA10 was likewise cloned into pCMC-GFP using PCR engineered 5' end *Kpn*I and 3' end *BsiW*I restriction sites, to create pIAA10-GFP.

pCMC-GFP, pIAA26-GFP, and pIAA10-GFP DNA was introduced into *N. benthamiana* and *A. thaliana* Shahdara leaf cells by particle bombardment. The bombardment method was essentially as described by Figueira *et al.* (Figueira *et al.*, 2002). Briefly, 2 μg of plasmid DNA was ethanol precipitated onto 0.5 mg of tungsten

particles (1.3 μm in diameter, Bio-Rad, Hercules, CA). The DNA coated particles were re-suspended in 95% ethanol by sonication in a Brandon 2200 ultrasonic cleanser (Branson Equipment, Shelton, CT). The nucleic acid-tungsten mixture was loaded onto plastic filter screens (Gelman Sciences, Ann Arbor, MI) and dried. The coated screens were mounted into the particle inflow gun (Finer *et al.*, 1992; Takeuchi *et al.*, 1992) and bombarded into leaf tissue using a 50-ms pulse of helium (50psi). Bombarded leaf tissues were incubated for 12 to 16 hrs at room temperature and mounted on glass microscope slides in distilled water under a coverslip. The tissue was imaged for GFP fluorescence using a Zeiss LSM 510 Laser Scanning Confocal Microscope system with 10X NA 0.8 dry and 63X NA 1.2 water immersion lenses (Carl Zeiss Inc., Thonwood, NY). Images were modified in Zeiss LSM Imager Examiner, Version 3.0 and processed for printing in Adobe Photoshop (Grand Prairie, TX).

2.3.7 Auxin leaf treatment and expressions

Rosette leaves were excised from 4 to 5 week old Shahdara plants and vacuum infiltrated with water or water plus 50 μM IAA. Infiltrated leaves were incubated in the same solutions for 90 minutes in the dark and total RNA extracted using the RNeasy Plant Miniprep kit (Qiagen, Valencia, CA). RNA expression levels of At4g38850 (SAUR-AC1), At5g21010, At1g19350, At5g02160 and At3g17790 were quantified via QRT-PCR as described above. Gene specific LUX™ primers (Invitrogen, Carlsbad, CA) were At5g21010, (CACGACGGGTCGCATCAATTCGG and TCGCTATGTGCTTCCCTATACCC); At1g19350, (GACTCGTTCCTCTTCTTCATTCCCGAGC and

TCCTGAGGAAAGGGAAGATTGTG); At5g02160, (CACATTTCACCATCACCGAACAATGG and GCGACAACTACGGAGGAAGAAGA); At3g17790, GACGAATTGTGTATCTTCACCACCTTCGC and ACTAACGGAACCGTCGCTTT); At4g38850, (GACCGAAGAGGATTCATGGCGGC and AAGTATGAAACCGGCACCACAT). Relative expression levels were determined as described above for RNAi analysis.

2.4 RESULTS

2.4.1 The TMV Replicase protein interacts with the Aux/IAA protein IAA26

A yeast two-hybrid approach was used to identify *Arabidopsis* host proteins capable of interacting with TMV replicase sequences (Fields and Song, 1989; Goregaoker *et al.*, 2001). The entire TMV 126/183-kDa open reading frame (ORF) was divided into five overlapping segments covering the MT (a.a. 1-376), HEL (a.a. 814-1211), POL (a.a. 1205-1613) and uncharacterized (a.a. 369-615 and a.a. 589-820) domains (Fig. 2.1B). All five segments were used as "bait" to screen a library of cDNA "prey" constructs derived from *Arabidopsis* leaf and root tissues. Only the "bait" construct covering the TMV HEL domain (a.a. 814-1211) was found to interact with clones from the cDNA library. Specifically, three cDNA clones displayed a strong interaction with the TMV HEL domain as measured by β-galactosidase activity (Fig 2.2A). Sequence analysis of

these clones revealed that all were identical, encoding nearly all (nts 3 to 789) of the 810 nt ORF of *IAA26* (At3g16500), a member of the Aux/IAA family of auxin regulated transcription factors.

Sequence comparisons between the two-hybrid IAA26 cDNA, derived from *Arabidopsis* ecotype Nossen, and PCR amplified cDNA from the ecotype Shahdara as well as EST and genomic sequences available for ecotype Columbia show 100% identity at the protein level. Furthermore, a full-length IAA26 cDNA derived from ecotype Shahdara and cloned into the yeast prey vector also interacted with the TMV helicase domain in manner similar to the original IAA26 library clone (data not shown).

To further assess this interaction an *in vitro* protein-protein assay was used to evaluate the ability of IAA26 to interact with the full-length viral replicase proteins. In this assay, purified histidine tagged IAA26 protein was immobilized on nitrocellulose sheets and used to capture ^{35}S-labeled full-length replicase proteins translated directly from purified virions. Results indicate that IAA26 is capable of binding full-length wild-type TMV replicase (Fig. 2.3).

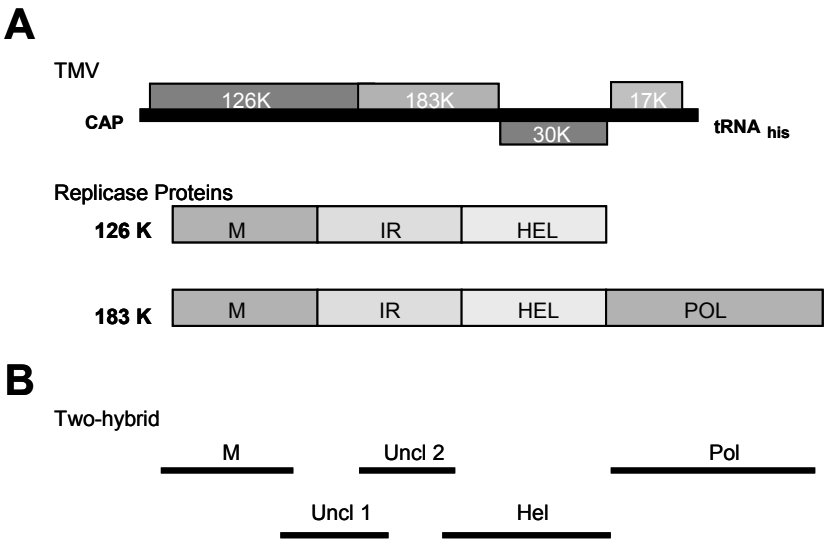


Fig.2.1. TMV genome organization and two-hybrid constructs. (A) Diagrammatic representations of the TMV genome and replicase proteins. (B) Replicase segments used in two-hybrid screens covering the MT- methyltransferase, HEL-helicase, POL-polymerase; and Uncl 1 and 2 - uncharacterized domains.

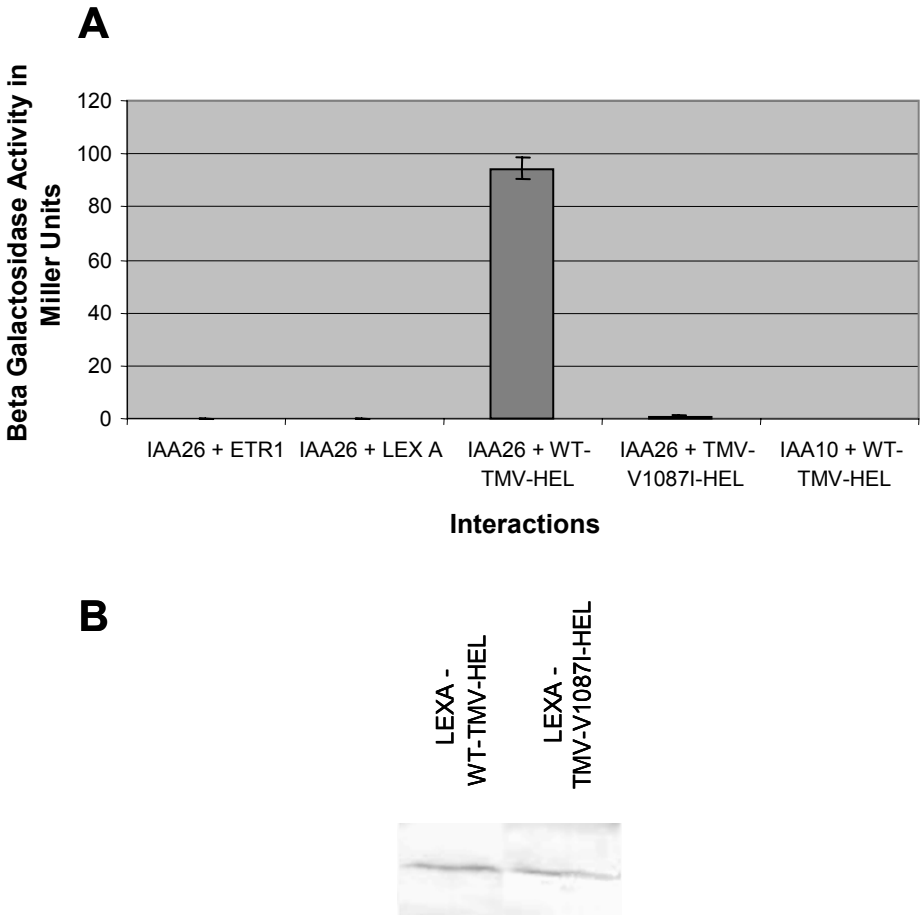


Fig.2.2. TMV helicase interactions. (A) Quantification of β-galactosidase activity between IAA26 and ETR1, a non-interacting control protein; IAA26 and LEXA, the empty vector; IAA26 and WT-TMV-HEL, encoding the helicase domain of the viral replicase; IAA26 and TMV-V1087I-HEL, a mutant TMV helicase; and IAA10, an Aux/IAA family member, with WT-TMV-HEL. (B) Western immunoblot assay comparing the accumulation of WT-TMV-HEL and TMV-V1087I-HEL proteins in yeast.

2.4.2 A TMV Helicase mutation disrupts the IAA26 interaction and confers attenuated disease symptoms

To investigate the role of *IAA26* in virus replication and disease development a series of previously characterized TMV helicase mutations were screened via two-hybrid assay for the ability to interact with IAA26 (Goregaoker *et al.*, 2001). One mutant helicase, containing a V to I substitution at position 1087 within the TMV replicase protein displayed a significantly reduced ability to interact with IAA26 in the two-hybrid system (Fig. 2.2A). Western immunoblots for the detection of the helicase-LexA fusion protein in yeast extracts indicate the 1087 mutation does not affect protein expression or stability (Fig. 2.2B). However, extending the incubation time for the two-hybrid interaction assay from 10 min to 1 hr resulted in detectable levels of β-galactosidase activity between IAA26 and V1087I helicase (data not shown). In addition, the V1087I mutation similarly disrupted the ability of the full-length viral replicase protein to interact with purified IAA26 protein *in vitro* (Fig. 2.3B). These findings suggest that the V1087I mutation greatly reduces the interaction between the TMV HEL domain and IAA26 but does not totally abolish the interaction.

A recombinant TMV virus containing this mutation, TMV-V1087I, was subsequently tested for its ability to replicate and move in both *Arabidopsis* and tobacco (Fig. 2.4A and B). Within Tobacco protoplasts TMV-V1087I was previously shown to replicate at levels similar to the wild-type virus (Goregaoker *et al.*, 2001). Similar levels of replication were also observed in *Arabidopsis* leaf protoplasts infected with either wild-type TMV or TMV-V1087I (Fig. 2.4C). Immunoblots monitoring virus accumulation

and spread demonstrated that TMV-V1087I moves cell-to-cell and systemically at rates and levels similar to wild-type TMV (Fig. 2.4D).

Although the replication and movement of TMV-V1087I was not affected by its inability to interact with IAA26 this recombinant virus consistently induced milder disease symptoms when compared to the wild-type virus. Most significantly, the loss of apical dominance, characteristic of a wild-type TMV infection, was reduced in plants infected with TMV-V1087I (Fig. 2.4E and 2.5C). Plants infected with TMV-V1087I also displayed a near normal rosette patterning when compared to wild-type infected plants. The V1087I mutation thus functions to uncouple the display of specific disease symptoms from virus replication.

The separation of symptom attenuation and virus replication is significant since the TMV replicase also functions as a suppressor of gene-silencing and mutations that disrupt suppressor activity reduce virus accumulation and spread which in turn results in the attenuation of disease symptoms (Ding *et al.*, 2004; Kubota *et al.*, 2003). Additionally, mutations that disrupt replicase suppressor activity all map outside the Tobamovirus helicase domain (Ding *et al.*, 2004; Kubota *et al.*, 2003). Thus, the location of the V1087I mutation within the helicase domain of the viral replicase and the ability TMV-V1087I to replicate and move at levels similar to the wild-type virus indicate that this mutation does not significantly affect the suppressor function of the replicase protein.

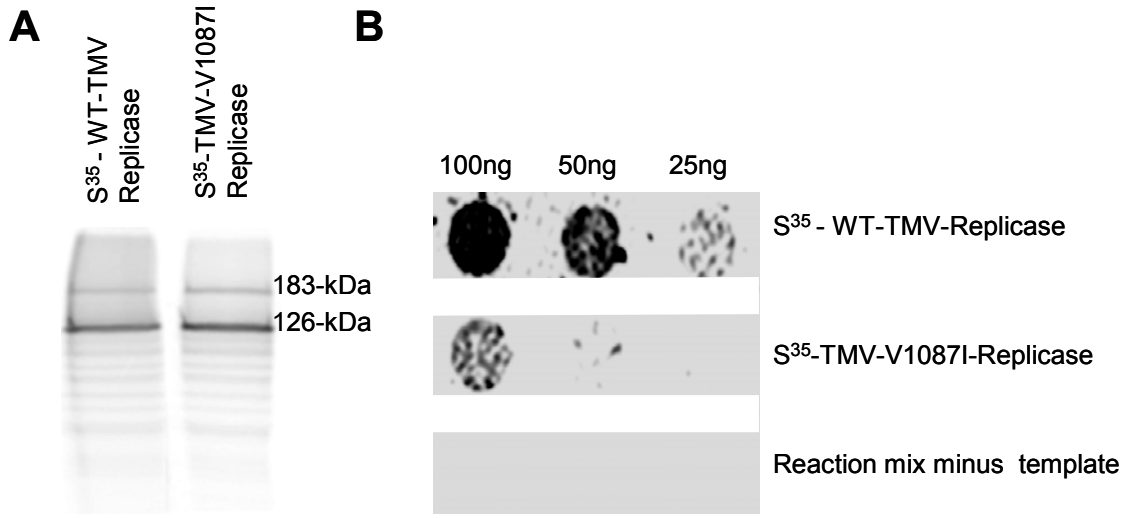
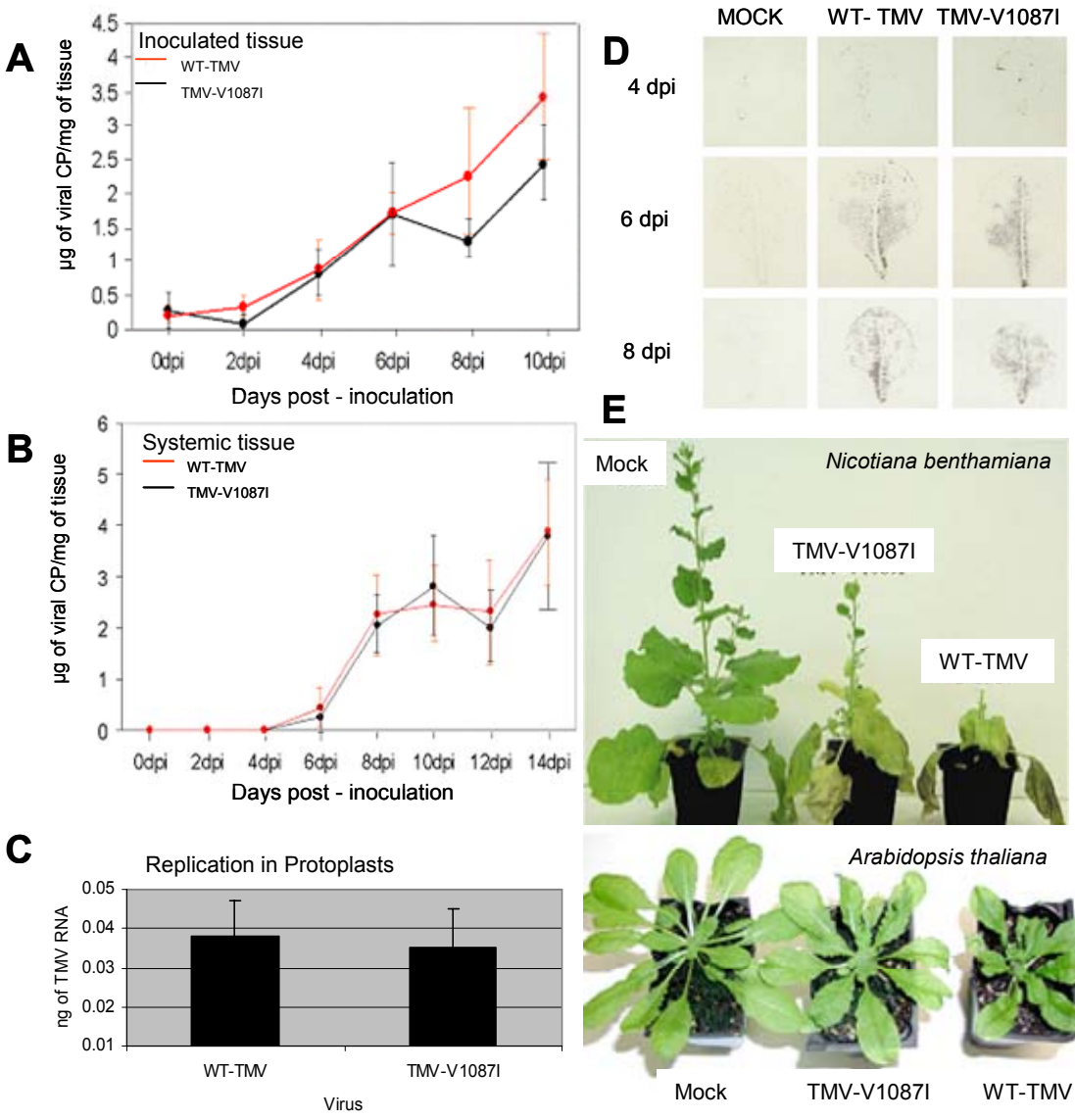


Fig.2.3. Interaction of IAA26 with full-length TMV replicase. (A) ^{35}S labeled 126- and 183-kDa TMV replicase proteins. (B) IAA26 overlay assays using ^{35}S labeled full-length WT-TMV-Replicase or V1087I-replicase proteins. Purified IAA26 was immobilized on nitrocellulose membranes and used to capture radio-labelled full-length replicase proteins translated from purified virions. The in-vitro translation mixture without a template was used as the negative control.

Fig.2.4. Biological characterization of helicase mutant TMV-V1087I. (A) Accumulation of WT-TMV and TMV-V1087I in inoculated *Arabidopsis* leaf tissue. (B) Virus accumulations in systemic leaf tissues. (C) WT-TMV and TMV-V1087I replication in *Arabidopsis* leaf protoplasts. Results from quantitative real-time PCR amplified TMV genomic products were compared against similarly amplified known TMV RNA standards to determine virus concentrations. (D) Tissue print immunoblots showing the cell-to-cell spread of WT-TMV and TMV-V1087I infection foci at 4, 6 and 8 days post-inoculation in *A. thaliana* ecotype Shahdara. (E) Attenuation of disease symptoms caused by TMV helicase mutation V1087I in *Nicotiana benthamiana*, upper panel, or *Arabidopsis thaliana* Shahdara plants. Plants were inoculated with water (mock), TMV-V1087I, or WT-TMV as labeled and photos taken 2 weeks post-inoculation.

(Figs. A,B,D and E are reprinted with permission from Sheetal Golem)


A
Inoculated tissue
WT-TMV
TMV-V1087I
µg of viral CP/mg of tissue
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0dpi
2dpi
4dpi
6dpi
8dpi
10dpi
Days post - inoculation
B
Systemic tissue
WT-TMV
TMV-V1087I
µg of viral CP/mg of tissue
6
5
4
3
2
1
0
0dpi
2dpi
4dpi
6dpi
8dpi
10dpi
12dpi
14dpi
Days post - inoculation
C
Replication in Protoplasts
ng of TMV RNA
0.05
0.04
0.03
0.02
0.01
WT-TMV
TMV-V1087I
Virus
D
MOCK
WT- TMV
TMV-V1087I
4 dpi
6 dpi
8 dpi
E
Mock
Nicotiana benthamiana
TMV-V1087I
WT-TMV
Arabidopsis thaliana
Mock
TMV-V1087I
WT-TMV

2.4.3 Transgenic RNAi plants with reduced accumulations of IAA26 mRNA display virus-like symptoms

To characterize the interaction, I attempted to identify T-DNA knockouts within the At3g16500 gene in an effort to identify a null mutant. One potential SALK line containing a T-DNA insertion within the putative promoter region in At3g16500 was identified in a Col-O background, but quantitative RT-PCR (Reverse-Transcription – Polymerase Chain Reaction) analysis showed no reduction in transcript levels (data not shown).

An RNAi construct was used to transgenically silence the expression of IAA26 mRNA in *Arabidopsis* ecotype Shahdara. This construct produces double-stranded RNA containing nucleotides 1 through 500 of the IAA26 coding sequence. T_O and T_1 plants from six independent transformants were identified as having significantly reduced levels of IAA26 mRNA (Fig. 2.5A). IAA26 RNAi silenced plants were slightly stunted in appearance and displayed a loss in apical dominance as compared to non-transformed control plants. Most notably, IAA26 silenced plants produced multiple shoot apexes, disrupting the rosette patterning of leaves and resulting in the appearance of numerous floral bolts (Fig. 2.5B and C). The phenotype produced by the RNAi suppression of IAA26 is similar to that observed in wild-type TMV infected plants.

RNAi suppressed IAA26 plants also were found to accumulate TMV in both inoculated and systemically infected tissues at levels comparable to non-transformed *Arabidopsis*

plants (data not shown). Thus, reduction in the accumulation of IAA26 does not adversely affect TMV replication or spread.

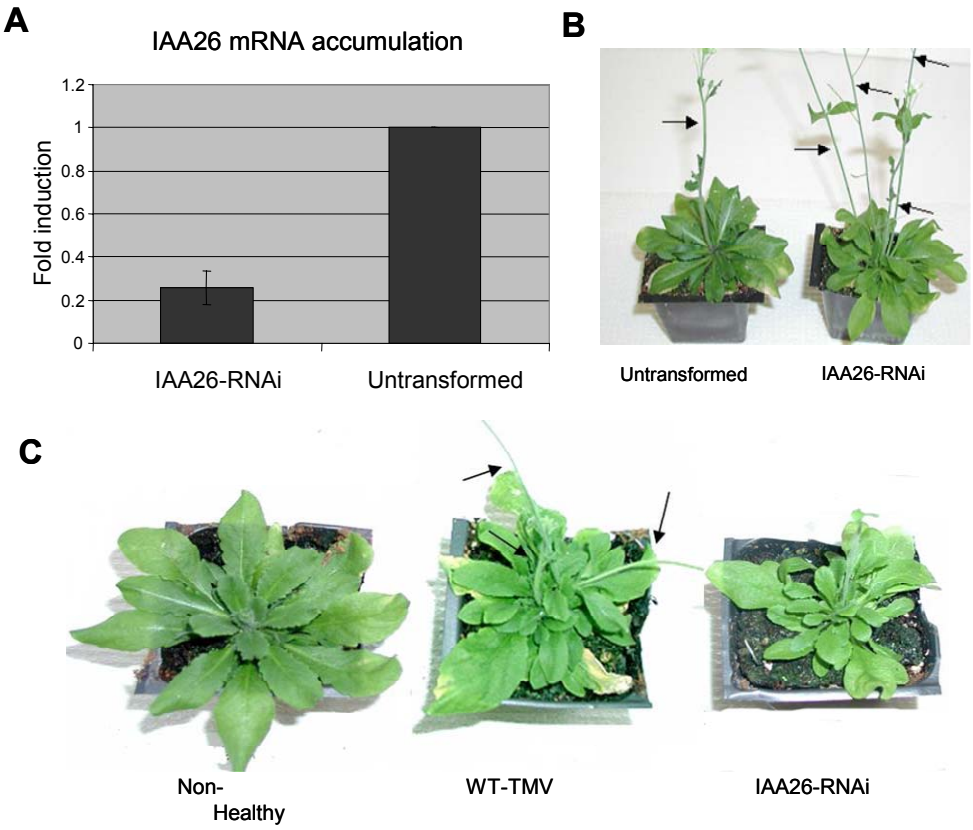


Fig.2.5. Characterization of IAA26 silenced RNAi plants. (A) Quantitative real time PCR analysis of IAA26 mRNA accumulations. Expression levels were normalized to an internal control, EF1α. (B) Photo showing the development of multiple floral bolts in IAA26 silenced plants (arrows). (C) Phenotype comparisons of non-transformed healthy

and WT-TMV infected plants to IAA26 silenced plant. Arrows mark the multiple floral bolts produced in the TMV infected plant.

2.4.4 IAA26 is inhibited in its ability to accumulate and localize to the nucleus in TMV infected cells

To confirm TMV's ability to interfere with IAA26 function *in vivo* a IAA26-GFP fusion construct was transiently expressed, under the control of 35S constitutive promoter, in either mock inoculated, wild-type TMV infected, or TMV-V1087I infected tobacco and *Arabidopsis* leaf tissues. Relative levels of virus infectivity for both wild-type and mutant virus were monitored in these tissues by immuno-dot blot assays (Fig. 2.6A). An unmodified GFP construct also was utilized to demonstrate that transient expression of GFP is not altered within TMV infected tissues (Fig. 2.6A).

Within mock inoculated tissues the IAA26-GFP fusion protein was found to localize predominantly in the nucleus (Fig 2.6A). By comparison, in wild-type TMV infected tissues only a few cells displayed detectable levels of IAA26-GFP fluorescence, indicating a reduction in the stability/accumulation of IAA26-GFP (Fig. 2.7). In addition, localization of IAA26-GFP in TMV infected tissues appeared primarily as faint fluorescent cytoplasmic inclusions that were not nuclear localized (Fig. 2.6A). In contrast, the localization of IAA26-GFP in TMV-V1087I infected tissues showed significantly higher numbers of cells that displayed nuclear localized fluorescence, similar to what is observed in uninfected tissues (Fig. 2.6A and 2.7). However, the number of cells within TMV-V1087I infected tissues that displayed IAA26-GFP fluorescence in the nucleus was half of that observed in uninfected tissues, suggesting

that TMV-V1087I interfered with IAA26-GFP accumulation/localization, but to a lesser degree than wild-type TMV (Fig. 2.7). This finding is consistent with the reduced ability of the V1087I mutant helicase to interact with IAA26.

To further confirm that TMV interference in IAA26 localization was specific to its interaction with the replicase protein a second member of the Aux/IAA family, IAA10 (At1g04100), was cloned and analyzed. IAA10 shares 41% sequence identity with IAA26 but does not interact with the TMV HEL domain in two-hybrid assays (Fig. 2.2A). The expression of IAA10 as a GFP-fusion protein in either TMV infected or mock inoculated tissue resulted in similar accumulations of fluorescence in both the nucleus and cytoplasm (Fig. 2.6B and 2.7). Thus, TMV does not affect the localization of a non-interacting Aux/IAA family member.

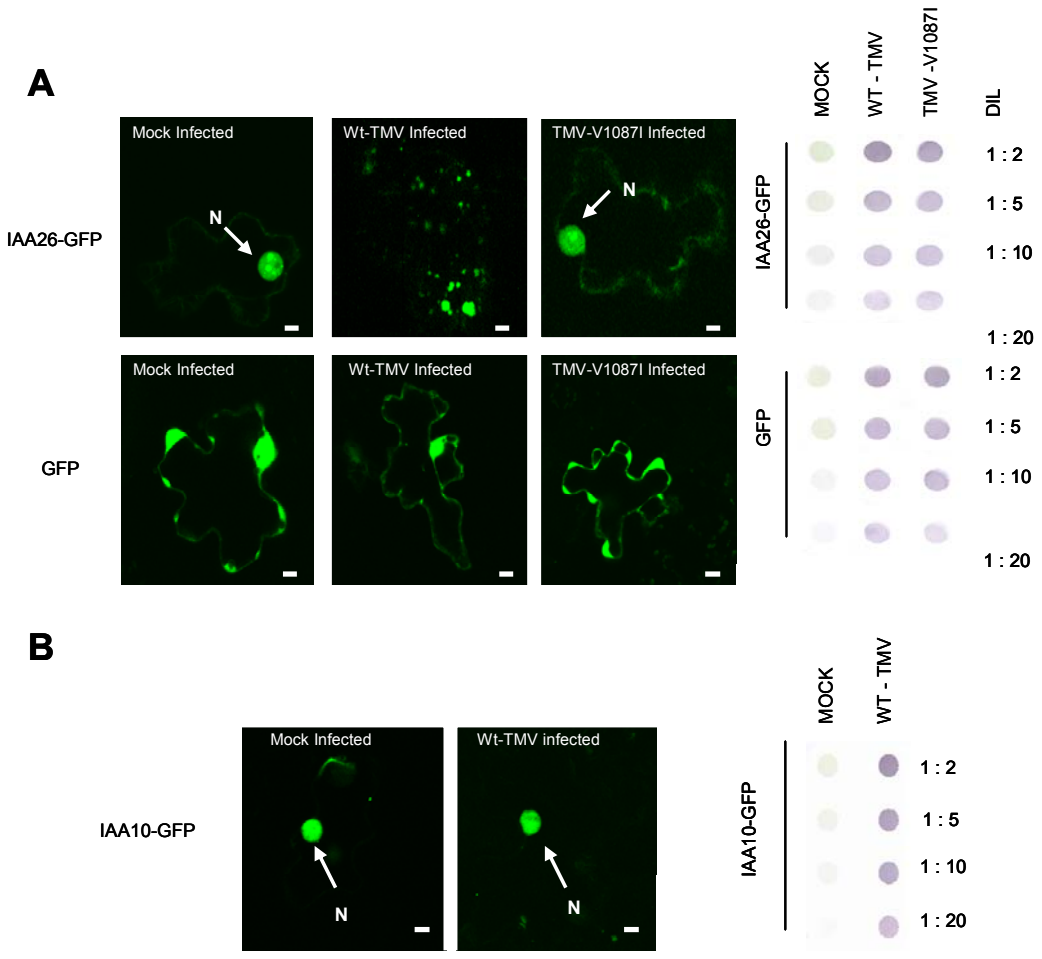


Fig.2.6. Transient expression of IAA26-GFP in *Nicotiana benthamiana* leaf tissues. (A) Fluorescent images of cells expressing IAA26-GFP fusion protein or GFP alone in non-infected (Mock), WT-TMV infected, or TMV-V1087I infected tissue. Bars equal 10µm. Immuno-dot blots showing dilutions (DIL) of leaf tissue homogenate used to monitor

virus levels in IAA26-GFP transformed leaf tissues. (B) Fluorescent cell images of IAA10-GFP in either mock or TMV infected tissue.

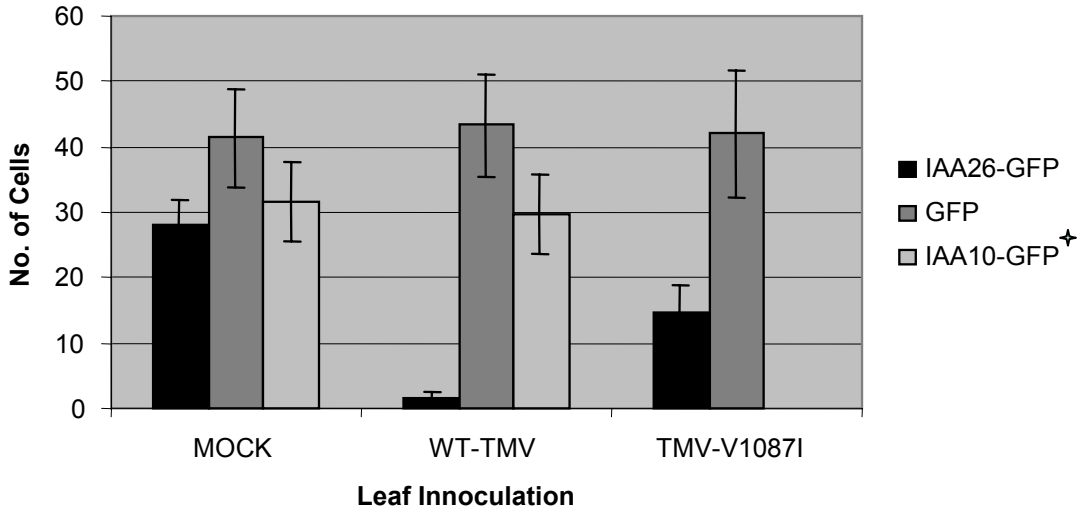


Fig.2.7. Expression and localization totals of GFP, IAA26-GFP and IAA10-GFP constructs in non-infected, WT-TMV infected and TMV-V1087I infected tissues.

Bars represent the number of cells displaying detectable levels of GPF fluorescence within a 15mm^2 leaf area at 16 hrs post-transformation. IAA26-GFP and IAA10-GFP bars represent the number of cells displaying detectable levels of nuclear-localized GFP fluorescence. Cell numbers are averaged from six independent bombardment transformations. ✧ Note, IAA10-GFP was not tested in TMV-V1087I infected tissues.

2.4.5 Transcriptionally altered *Arabidopsis* genes contain Auxin Responsive Elements within their Promoters

Previously performed microarray studies of both inoculated and systemically infected *Arabidopsis* leaf tissues identified 68 genes displaying transcriptional alterations in response to infection by TMV (Golem and Culver, 2003). Microarrays used in these experiments contained cDNAs representing approximately one-third of the *Arabidopsis* genome. An analysis of the 2000 nts immediately upstream of the translational start codon for each of these genes revealed that 20 contained two or more TGTCTC auxin responsive elements (AuxRE). This element is present in the promoters of primary and early auxin response genes that are under the transcriptional control of ARF and Aux/IAA proteins (Higo *et al.*, 1999; Ulmasov *et al.*, 1995). The presence of multiple AuxREs has been correlated with increased alterations in gene expression, both up and down, in response to auxin (Tian *et al.*, 2002; Ulmasov *et al.*, 1999b).

The effect of TMV on the localization of IAA26 suggests that the transcription of specific AuxRE containing genes should be similarly altered in response to either a TMV infection or auxin treatment. To test this possibility, four of the twenty TMV altered AuxRE genes were selected for further studies (Table 2.1). Additionally, SAUR-AC1, a known auxin induced gene that is not affected by TMV infection was used as a positive control (Gil *et al.*, 1994; Golem and Culver, 2003). Upon auxin treatment all four TMV altered AuxRE genes showed reduced levels of transcription with two, At5g02160 and

At3g17790 having reductions of greater than four fold over the control (Table 2.1). Thus, the expression trends for these genes were similar in both auxin treated and TMV infected tissues. Variations in the levels of gene repression observed for these genes may be due to differences between the auxin treatment that presumably affects all Aux/IAA family members and TMV infection that specifically targets IAA26 or other as yet unidentified interacting Aux/IAA proteins. Alternatively, TMV may affect other regulatory pathways that contribute to the transcriptional profile of these genes in a manner not replicated by auxin treatment. The non-synchronous nature of a TMV infection may also impact observed transcriptional levels. However, these data combined suggest that ~30% of the genes displaying transcriptional alterations in response to TMV infection may be linked to an auxin response transcription factor such as IAA26.

Table 2.1. Auxin response of AuxRE containing TMV altered genes.

AFGC gene model ID[a]	Protein name[b]	Number of AuxRE[c]	Fold expression in TMV infection[d]	Fold expression after 50μM IAA trt[e]
At5g02160	Putative protein	3	- 1.9	- 4.1 ± 1.7
At1g19350	Unknown protein	3	- 2.0	- 1.8 ± 1.1
At3g17790	Acid phosphatase type 5	2	- 2.3	- 6.6 ± 4.0
At5g21010	Putative stress protein	3	- 3.5	- 1.5 ± 1.2
At4g38850	SAUR-AC1	1	- 1.1[f]	5.1 ± 2.1

[a] AFGC = Stanford University's *Arabidopsis* Functional Genomics Consortium.

[b] Gene functions based on sequence homologies.

[c] Number of TGTCTC and GAGACA sequences within 2-kbs upstream of the start codon.

[d] cDNA microarray fold expression values averaged from three independent biological replicates of TMV infected tissues. Fold changes at or above 1.6 and at or below -1.9 occur above the 95% confidence interval used to identify transcriptionally altered genes (Golem and Culver, 2003).

[e] QRT-PCR fold expression values and standard error averaged from four independent auxin leaf treatments. Data normalized against the expression levels of EF1-α.

[f] Microarray fold changes for SAUR-AC1 were not above the 95% confidence interval cutoff in TMV infected tissues.

2.5 DISCUSSION

The etiology of disease remains one of the least understood areas of virology. Of particular importance is the identification of host components and pathways that directly interact with or are affected by the infecting virus. In this study, domains from the TMV replicase protein were assessed for their ability to interact with a library of *Arabidopsis* host proteins. The virus replicase protein was selected for this study because it is an essential component of the infection process and previously has been implicated in disease development (Bao *et al.*, 1996). Of the five replicase segments used to screen for interacting *Arabidopsis* proteins only the segment covering the TMV helicase domain yielded putative interacting clones. Of these cDNA clones only those encoding the IAA26 ORF displayed levels of β-galactosidase activity indicative of a strong protein-protein interaction. *In vitro* interaction assays established the ability of the full-length viral replicase protein to interact with IAA26. Additional genetic and localization studies demonstrated a role for this interaction in disrupting the nuclear localization of IAA26. Specifically, the reduced ability of TMV-V1087I to interact with IAA26 corresponded with reduced disruptions in IAA26 localization and attenuated disease symptoms. Correspondingly, RNAi disruption of IAA26 produced a plant phenotype with similarities to the virus induced disease response. Combined these findings indicate that

the interaction of the TMV replicase with IAA26 modulates the display of disease symptoms.

IAA26 encodes a 30-kDa member of the Aux/IAA family of early auxin responsive proteins. IAA26, like other Aux/IAA proteins, contains four conserved domains involved in nuclear localization (domains I and II), protein destabilization (domain II) and dimerization (domains III, and IV) (Liscom and Reed., 2002; Ulmasov *et al.*, 1999a). The current model for auxin signaling suggests that in the absence of auxin, Aux/IAA proteins form heterodimers with ARF proteins and repress their ability to modulate auxin response genes. In the presence of auxin, Aux/IAA proteins dissociate from ARF proteins and are targeted for degradation via the Skp1/Cullin/F-box subunit containing E3 ubiquitin ligase complex, SCF^{TIR1} (Gray *et al.*, 2001; Tiwari *et al.*, 2001; Zenser *et al.*, 2001). *TIR1* encodes the F-box component of this complex and interacts directly with Aux/IAA proteins to promote their ubiquitination and degradation via the proteasome. In the absence of Aux/IAA proteins, ARF proteins function as transcriptional activators/repressors, binding the AuxRE TGTCTC within the promoters of primary auxin response genes (Hagen and Guilfoyle, 2002). During normal plant development the stability of Aux/IAA proteins is regulated by an auxin concentration gradient emanating from the shoot apex. Disrupting the function of Aux/IAA proteins or the genes controlling their stability results in numerous developmental abnormalities, including the loss of apical dominance, alterations in leaf development, and changes in floral promotion (Reed *et al.*, 2001; Rogg *et al.*, 2001).

Based on the current model for auxin signaling, I hypothesize that during a TMV infection, interaction with the viral replicase promotes the destabilization and/or the

inappropriate sequestration of IAA26, thus, interfering with its function. Disruption of IAA26 function directed by the TMV replicase protein would occur independently of the plant's auxin gradient, resulting in the activation of ARFs and alterations in the transcription levels of specific auxin response genes. Consistent with this possibility, a significant portion (~30%) of the transcriptionally altered genes within TMV infected leaf tissues contained multiple AuxRE within their promoter sequences (Golem and Culver, 2003). Furthermore, experimental results indicate that TMV altered AuxRE genes display auxin induced expression trends similar to that observed in TMV infected tissues (Table 2.1). Microarray results also indicate that other genes containing AuxRE promoter sequences, including members of several primary auxin response gene families, such as SAUR (See SAUR-AC1 results in Table 2.1), GH3 as well as other Aux/IAA proteins do not display transcriptional alteration in response to TMV (Golem and Culver, 2003). Thus, the regulation of TMV altered AuxRE genes (Table 2.1) appears specific and not part of a genome wide disruption in auxin sensing. Specificity in the effect of TMV on the auxin response system is further demonstrated by the inability of TMV to alter the localization of IAA10, a non-replicase interacting Aux/IAA family member. Combined these data support a link between TMV altered AuxRE genes and the disruption of IAA26 stability/localization by TMV. TMV altered AuxRE genes are thus candidates for additional studies directed at determining their role in the development of disease symptoms.

The induction of disease symptoms is likely to be complex, involving multiple interactions between host and pathogen components. IAA26 is only one of 29 predicted members of the Aux/IAA family of auxin responsive transcription factors and shares

between ~26% and ~67% sequence homology with the other members. Although IAA10, which shares 41% homology with IAA26, did not interact with the TMV helicase it is possible that other more closely related Aux/IAA members interact in a IAA26-like fashion. In addition, recent studies have determined that several auxin regulatory components, including ARF8, ARF10, and TIR1 are targets for miRNA regulation (Bonnet *et al.*, 2004; Chapman *et al.*, 2004; Kasschau *et al.*, 2003). The ability of viral encoded RNA-silencing suppressors to interfere with the miRNA-directed regulation of such components has also been correlated with the appearance of symptom-like developmental defects (Chapman *et al.*, 2004; Kasschau *et al.*, 2003). Thus, interaction of the TMV replicase with IAA26 likely represent only one avenue through which plant viruses can disrupt the auxin signaling pathway. The ability of TMV V1087I to induce developmental symptoms, albeit reduced in severity, supports a role for other viral processes and interactions in the development of disease symptoms.

Interestingly, the silencing of IAA26 mRNA did not produce a detectable affect on virus replication or movement. Similarly, TMV-V1087I, a helicase mutant with reduced ability to interact with IAA26, replicated and spread at levels similar to the wild-type virus. Thus, the interaction between the TMV HEL domain and IAA26 is not rate limiting for virus function. This type of non-essential interaction may account for the differences between disease and tolerant host responses. Both disease and tolerant hosts show similar levels of susceptibility to a pathogen, however, only the diseased host displays significant damage (Agrios, 1996). In addition, disease and tolerant phenotypes in both host and pathogen backgrounds are heritable characteristics, suggesting the involvement of specific host-pathogen interactions. In fact, virus-induced disease

severity often does not correlate with the ability of an infecting virus to replicate at high levels or spread rapidly within a specific host (Hull, 2002). Therefore, non-essential interactions, such as the one between the TMV replicase and IAA26, may play significant roles in determining disease severity.

Combined, these experiments suggest that the TMV replicase protein disrupts IAA26 function. One possibility is that this interaction destabilizes IAA26 through a ubiquitin-mediated process similar to the auxin-directed degradation of other Aux/IAA proteins. While virus directed protein degradation has not been established as a disease mechanism in plants it has been directly linked to disease development in several animal virus systems (Banks *et al.*, 2003). For example, *Human papillomavirus* (HPV) E6 protein directs the degradation of the cellular tumor-suppressor protein p53 as well as several membrane-associated guanylate kinases, contributing directly to the malignant progression of HPV associated cancers (Thomas *et al.*, 1999). Alternatively, the TMV replicase protein may simply sequester IAA26 protein and prevent its ability to localize to the nucleus. The precise mechanism through which TMV disrupts IAA26 function remains to be determined.

Chapter 3

The Tobacco mosaic virus Replicase Protein Disrupts the Localization and Function of Interacting Aux/IAA proteins

3.1 ABSTRACT

Previously I identified a correlation between the interaction of the *Tobacco mosaic virus* (TMV) 126/183kDa replicase with the auxin response regulator IAA26/PAP1 and the development of disease symptoms (Padmanabhan *et al.,* 2005, J. Virology 79:2549). In this study, the TMV replicase protein is shown to co-localize with IAA26 in the cytoplasm and prevent its accumulation within the nucleus. Furthermore, two additional Aux/IAA family members, IAA27 and IAA18, were found to interact with the TMV replicase and displayed alterations in their cellular localization and/or accumulation that corresponded with their ability to interact with the TMV replicase. In contrast, the localization and accumulation of non-interacting Aux/IAA proteins were unaffected by the presence of the viral replicase. To investigate the effects of the replicase interaction on Aux/IAA function, transgenic plants expressing a proteolysis-resistant IAA26-P108L-GFP protein were created. Transgenic plants accumulating IAA26-P108L-GFP displayed an abnormal developmental phenotype that included severe stunting and leaf epinasty. However, TMV infection blocked the nuclear localization of IAA26-P108L-GFP and

attenuated the developmental phenotype displayed by the transgenic plants. Combined these findings suggest that TMV induced disease symptoms can in part be attributed to the ability of the viral replicase protein to disrupt the localization and subsequent function of interacting Aux/IAA proteins.

3.2 INTRODUCTION

Plant virus infections often disturb host physiology and result in the display of disease symptoms. However, disease severity does not typically correlate with the ability of a virus to replicate or accumulate to high levels, indicating that the diversion of cellular metabolites towards virus synthesis is not a primary factor in the disease process (Hull, 2002). In addition, the induction of specific disease symptoms is often heritable and characteristic for certain virus - host combinations, suggesting that interactions between specific virus and host components are key factors in disease development. Although genetic changes in both viral and host components have been correlated with altered disease phenotypes the mechanisms through which these components function to control disease are not well characterized. A better understanding of these mechanisms represents an important step in the development of new strategies for reducing the economic impact of plant virus diseases.

Tobacco mosaic virus (TMV) is the type member of the genus *Tobamovirus* and functions as a model for the study of virus - host interactions. The TMV 126-kDa and read-through 183-kDa replicase proteins have been linked to the induction of various disease symptoms (Bao *et al.,* 1996; Shintaku *et al.,* 1996). Biochemical

characterizations of the 126-kDa protein have demonstrated guanylyltransferase - like activity that results in the capping of viral RNAs as well as NTPase and RNA-unwinding activity derived from the C-terminal helicase domain (Dunigan and Zaitlin, 1990; Erickson *et al.*,, 1999; Merits *et al.,* 1999; Goregaoker and Culver, 2003). Specificity for 3' RNA binding has been mapped to a 110 amino acid region downstream of the MT domain (Osman and Buck, 2003). Tobamovirus replicase proteins also play a role in virus cell-to-cell movement (Goregaoker *et al.,* 2001; Hirashima and Wantanabe, 2001; Liu *et al.,* 2005) and function in the suppression of gene silencing (Kubota *et al.,* 2003; Ding *et al.,* 2004). Interestingly, Tobamovirus replicase proteins are implicated in several host interactions that affect virus replication and symptom development. These interactions include: association with the RNA binding subunit of eIF-3 (Osman and Buck, 1997); replication dependent association with host membrane proteins (Yamanaka *et al.,* 2000); elicitation of *N* gene-mediated disease resistance (Padgett *et al.,* 1993; Abbink *et al.,* 2001; Erickson *et al.,* 1999); a host cell death response modulated by a plant $P58^{IPK}$-like inhibitor of double-stranded RNA-activated protein kinase (Bilgin *et al.,* 2003); and association with a plant ATPase and a component of the photosystem II oxygen-evolving complex that affects virus accumulation (Abbink *et al.,* 2002). Combined these data indicate that the Tobamovirus replicase plays a multifunctional role in the infection process.

Interestingly, most Tobamovirus replicase interactions have been mapped to the helicase domain, suggesting an importance for this motif in viral pathogenesis. Previously I described an interaction between the auxin/indole acetic acid (Aux/IAA) protein PAP1/IAA26 and the helicase domain of the TMV replicase protein

(Padmanabhan *et al.,* 2005, Chapter 2). IAA26 belongs to a 29-member family of Aux/IAA transcription factors (Liscum and Reed, 2002). Members of this family encode short-lived nuclear proteins that mediate auxin dependent gene expression (Abel *et al.,* 1994; Ulmasov *et al.,* 1997b). Sequence analysis has shown that almost all Aux/IAA proteins have four conserved motifs (Domain I, II, III and IV; Abel *et al.,* 1994). Domain II mediates the rapid auxin dependent turnover of these proteins (Ramos *et al.,* 2001; Zenser *et al.,* 2003). Domains III and IV, located in the C terminal end, mediate protein-protein interactions between Aux/IAA proteins as well as auxin response factor proteins (ARFs) which bind auxin response elements (AuxRE's) in the promoters of auxin responsive genes (Ulmasov *et al.,* 1995 & 1997a; Kim *et al.,* 1997). The current model for auxin signaling suggests that at low auxin concentrations Aux/IAA proteins modulate the activity of interacting ARFs (Ulmasov *et al.,* 1997a; Reviewed by Reed, 2001; Leyser, 2002). At higher auxin concentrations Aux/IAA proteins are targeted for degradation by the SCF^{TIR1} ubiquitin ligase complex, allowing ARF's to function as either activators or repressors of AuxRE containing genes (Gray *et al.,* 2001; Reveiwed by Dharmasiri and Estelle, 2004). Auxin controlled proteolysis of Aux/IAA proteins provides a sensitive method for the control of genes involved in numerous developmental processes including cell expansion and differentiation, tropic responses to light and gravity, organ patterning, vascular development and apical dominance.

Molecular characterization of the IAA26 - TMV replicase interaction suggested that IAA26 function was disrupted in infected tissue resulting in the display of disease symptoms (Padmanabhan *et al.,* 2005, Chapter 2). In this study, I demonstrate that the TMV replicase directly inhibits the nuclear localization of IAA26. Furthermore, two

additional Aux/IAA family members, IAA27/PAP2 and IAA18, also interact with the TMV replicase protein and show changes in localization that correspond to the strength of the replicase interaction. In contrast, the localization of non-interacting Aux/IAA proteins was not affected by the presence of the TMV replicase. TMV infection also reversed stunting and epinasty associated with the abnormal developmental phenotype of transgenic plants that accumulated a proteolysis resistant mutant of IAA26. Interference in the nuclear localization of Aux/IAA proteins thus provides a mechanism for the disruption of auxin mediated plant development and the induction of disease.

3.3 MATERIALS AND METHODS

3.3.1 Aux/IAA cloning and Yeast Two-Hybrid analysis

Total RNA was extracted from *A. thaliana* Shahdara tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was preformed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Carlsbad, CA). Gene-specific primers containing the restrictions sites *BamH*I and *Xho*I were used to amplify all the Aux/IAAs except IAA27 for which *Bgl*II and *Xho*1 were used and the IAA2 primers contained only the *Xho*I restriction sites. The primer sequences are: GAGATCTCATGTCTGTATCTGTAGCA and CTCGAGCTAGTTCCTGCTTCTGCA for IAA27; GGATCCGATGGAGGGTTATTCAAGA and CTCGAGTCATCTTCTCATTTTCTC for IAA18; CTCGAGGATGGCGTACGACGAGAAAGTC and CTCGAGTCATAAGGAAGAGTCTAG for IAA2;

GGATCCAATGCGTGGTGTGTCAGAATTGG and CTCGAG CTAAACAGGGTTGTTTCTTTG for IAA 12; GGATCCGATGGGAGGAGAAAAG and CTCGAG CTATTACTTGCCATGTTT for IAA28; GGATCCGATGGAAGG CGGTCCCGCTAGTGG and CTCGAGTCATAATATCATCTGAGCTTTAC for IAA11; GGATCCGATGGAAAAAGTTGATGTTTATG and CTCGAGTTAAAGACCACCACAACC for IAA4; GGATCCGATTAATTTTGAGGC and CTCGAGTCAACTTCTGTTCTTGCAC for IAA 16 and GGATCCGATGAATGG TTTGCAAGAAG and CTCGAGCTACTTACCTACTCCAGCTCC for IAA10. The resulting PCR fragments were cloned into a pCRII-TOPO (Invitrogen Carlsbad, CA) and subsequently ligated into appropriately digested pACT-GAL4 for yeast two-hybrid analysis. All PCR derived clones were verified by sequencing.

Yeast two-hybrid interaction assays were preformed as previously described (Padmanabhan *et al.,* 2005, Chapter 2). Briefly, the L40 strain of *S. cerevisiae* carrying the pLexA-TMV helicase bait was transformed with pACT-GAL4-Aux/IAA vectors. Quantitative β-galactosidase assays were performed on yeast cultures carrying both the pACT-bait and LexA-prey plasmids gown at 25ºC in -Ura/-Trp/-Leu selection media as described by Miller (1972). Protein accumulation in representative pACT-GAL4 constructs encoding IAA27, IAA18, IAA12, and IAA2 open reading frames were confirmed by Western immunoblot analysis using GAL4 specific antibodies.

3.3.2 Transient expression constructs

The expression vector pCMC1100, containing a polylinker domain flanked by the *Cauliflower mosaic virus* 35S promoter and a polyadenylation signal was used as the

parental plasmid for all transient expression constructs (McCabe, 1988). The construction of pCMC-126-GFP, pCMC-126$^{1-781}$-GFP/pTMV-126ΔHEL-GFP and pCMC-IAA26/PAP1-GFP were previously described (Figueira *et al.,* 2002; Padmanabhan *et al.,* 2005, Chapter 2). PCR based site directed mutagenesis primers (GTGGTGGGTTGGCTTCCGGTTCGT and ACGAACCGGAAGCCAACCCACCAC) were used to create pCMC-IAA26-P108L-GFP. DsRed constructs were prepared by replacing the GFP ORF in pCMC-IAA26-GFP with a PCR modified DsRedII ORF (Clontech, Palo Alto, CA) containing 5' end *BsiW*I and 3' end *Nci*I sites. Full-length IAA2, IAA12, IAA18 and IAA27 cDNAs were obtained as described above and modified by PCR to contain 5' end *Kpn*I and a 3' end *BsiW*I restriction sites. Individually modified Aux/IAA ORF's were ligated into similarly cut pCMC-GUS-GFP (Figueira *et al.,* 2002), replacing the glucuronidase ORF. The GFP ORFs of individual pCMC-Aux/IAA-GFP constructs were replaced by DsRed as described above to create the pCMC-Aux/IAA-DsRed constructs.

3.3.3 Transient expression assays

Transient expression assays were done as described previously (Figueira *et al.,* 2002; Padmanabhan *et al.,* 2005, Chapter 2) using either onion epidermal peels or *Nicotiana benthamiana* leaf tissue. Briefly, a total of 4μg of plasmid DNA was ethanol precipitated onto 0.5 mg of tungsten particles (1.3μm in diameter; Bio-Rad, Hercules, CA). DNA coated particles were resuspended in 95% ethanol by sonication in a Brandon 2200 ultrasonic cleanser (Branson Equipment, Shelton, CT) and loaded onto plastic filter screens (Gelman Sciences, Ann Arbor, MI). Coated screens were dried and mounted into

a particle inflow gun (Finer *et al.,* 1992; Takeuchi *et al.,* 1992) and a 50-ms pulse of helium (50psi) was used to propel the particles into onion monolayers and leaf tissues mounted 2.5 and 3 inches below the filters, respectively. The tissues were incubated for 16 to 20 hrs at room temperature and mounted on glass slides in distilled water under coverslips. A Zeiss LSM510 laser scanning confocal microscope with 10X NA 0.8 dry and 63X NA 1.2 water-immersion lenses (Carl Zeiss Inc.,Thonwood, NY) was used to visualize the samples. Excitation sources were 488nm for GFP and 543nm for DsRed. Images were modified using Zeiss LSM Imager Examiner software, version 3.0 and processed for printing with Adobe Photoshop (Grand Prairie, TX).

3.3.4 Auxin assays

pCMC-IAA26- GFP and pCMC-IAA26-P108L-GFP proteins were transiently expressed in onion epidermal cells as explained above. After 14 hour incubation, cells were mounted onto glass slides in distilled water under cover slips and fluorescent cells examined by confocal microscopy for GFP expression. Approximately 500 μL of 1mM IAA solution was added onto the surface of the onion monolayers and the cells re-visualized for GFP intensity and expression after 60 minutes.

3.3.5 Plant transformation and characterization

A derivative of the *Agrobacterium* binary transformation vector pBI121 (Clonetech, Palo Alto, CA) was used to construct the IAA26 transformation vector. pCMC-IAA26-P108L-GFP was used as a template to PCR amplify the IAA26-P108L-GFP ORF using primers designed to introduce a 5' *Xba*I site

(CTCTAGAATGGAAGGTTGTCCAAGAAAC) and a 3' *Xho*I site (CTCGAGTTACTTGTACAGCTCGTCCATG). The resulting PCR product was digested with *Xba*1 and *Xho*I and inserted downstream of the CaMV 35S promoter within a similarly digested pBI121 vector. The vector was introduced into *Agrobacterium tumefaciens* strain GV3101 for transformation into *A. thaliana* ecotype Shahdara using the floral dip method (Clough and Bent, 1998). Transformed seedlings were selected based on kanamycin resistance. Nine transgenic lines were obtained of which five lines had a severe phenotype and four lines had an intermediate phenotype. T2 seeds from line five, showing an intermediate phenotype were used for phenotypic analysis and TMV inoculation. Protein extractions from Arabidopsis leaves were carried out as described by Osman and Buck (1996). Protein extracts were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose paper (Fisher Scientific Company, Newark, DE). Immunoblot analysis was performed using alkaline phosphatase labeled anti-GFP antibody (Clontech, Palo Alto, CA). TMV infections were confirmed by Western immunoblot analysis using replicase specific antibodies.

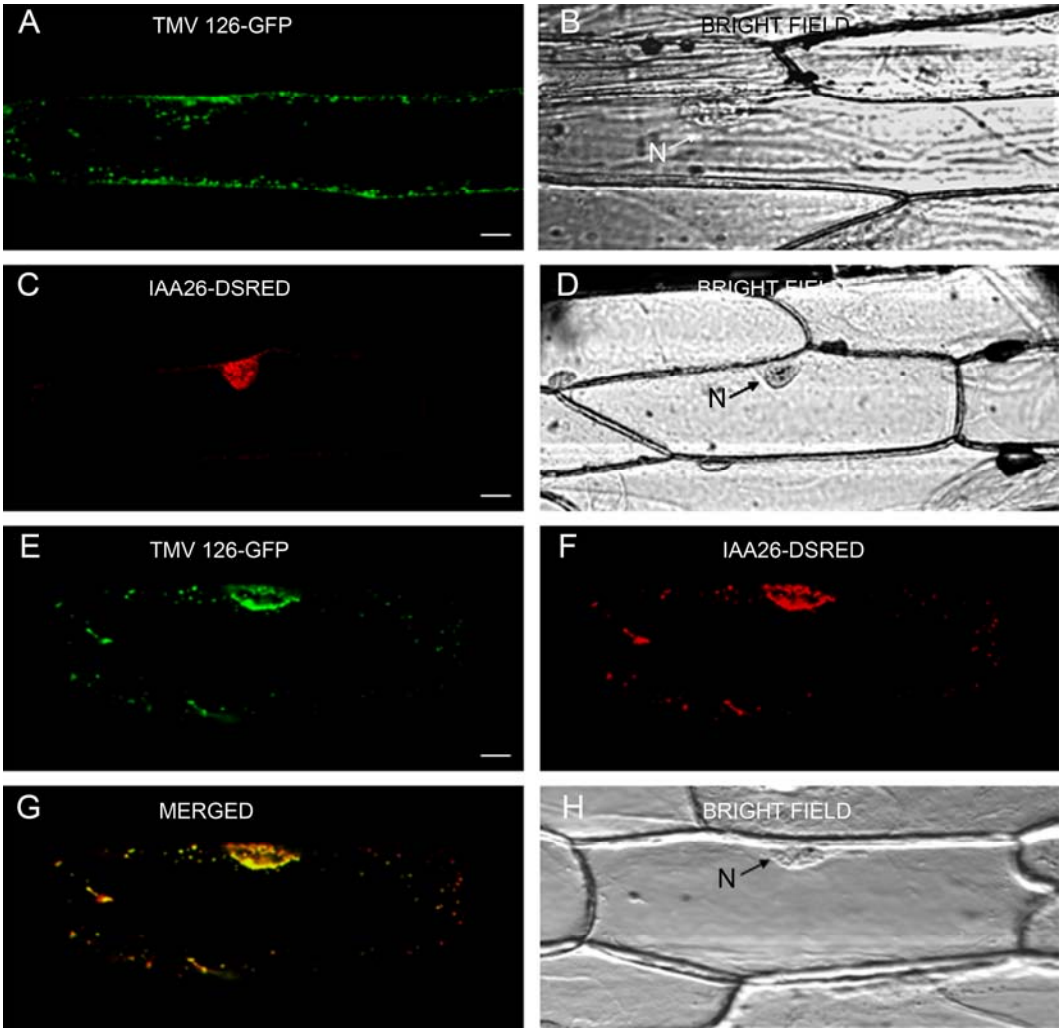
3.4 RESULTS

3.4.1 The TMV 126-kDa Replicase protein disrupts IAA26 nuclear localization

Previously I demonstrated that interaction between the TMV replicase and IAA26 corresponded with altered accumulations of IAA26 in infected tissues (Padmanabhan *et al.,* 2005, Chapter 2). To determine the direct effects of the replicase interaction on

IAA26 localization two transient expression constructs, IAA26-DsRed and TMV126-GFP, were created and co-expressed in onion epidermal cells. When expressed alone IAA26-DsRed localized almost exclusively to the nucleus and TMV126-GFP localized to the cytoplasm forming vesicle-like bodies (Fig. 3.1A - D). These vesicle-like bodies are derived from the endoplasmic reticulum (ER) and are consistent with membrane associated virus replication complexes (VRC) formed during virus replication (Más *et al.,* 1999; Figueira *et al.,* 2002; Liu *et al.,* 2005). When the two fusion constructs were co-expressed, IAA26-DsRed displayed a dramatic shift in localization from the nucleus to the cytoplasm and co-localized almost exclusively with the vesicle-like bodies produced by TMV126-GFP (Fig. 3.1E - H). To examine replicase specificity in the observed alteration in IAA26 localization a TMV126-kDa construct, TMV126ΔHEL-GFP, containing a deletion of the helicase domain of amino acids 782 to 1116 was also analyzed in the co-localization assay. Although TMV126ΔHEL-GFP retained a localization pattern similar to the full-length TMV126-GFP it did not significantly alter the nuclear localization of IAA26-DsRed (Fig. 3.2). Thus, interference in the nuclear localization of IAA26 is dependent upon the presence of the viral helicase domain.

Fig.3.1. Nuclear localization of IAA26 is altered in the presence of the TMV-126kDa replicase protein. Fusion proteins were transiently expressed in onion epidermal cells via particle bombardment and fluorescent images captured 20 hrs post-transformation. For co-expression studies, equal concentrations of expression plasmids were mixed and bombarded together. Nucleus is labeled N. Bar = 20μm. (A) and (B), Fluorescence and bright-field images showing the localization pattern of TMV126-kDa replicase protein fused to GFP. (C) and (D) Fluorescence and bright-field images of cells showing nuclear localization of IAA26 fused to DsRed. (E), (F), (G), and (H), Co-expression of IAA26-DsRed and TMV126-GFP. (E) TMV126-GFP fluorescence. (F) IAA26-DsRed fluorescence. (G) Overlay of fluorescence from (E) and (F). (H) Bright-field image of the co-expressing cell.


A
TMV 126-GFP
B
BRIGHT FIELD
N
C
IAA26-DSRED
D
N
E
TMV 126-GFP
F
IAA26-DSRED
G
MERGED
H
BRIGHT FIELD
N

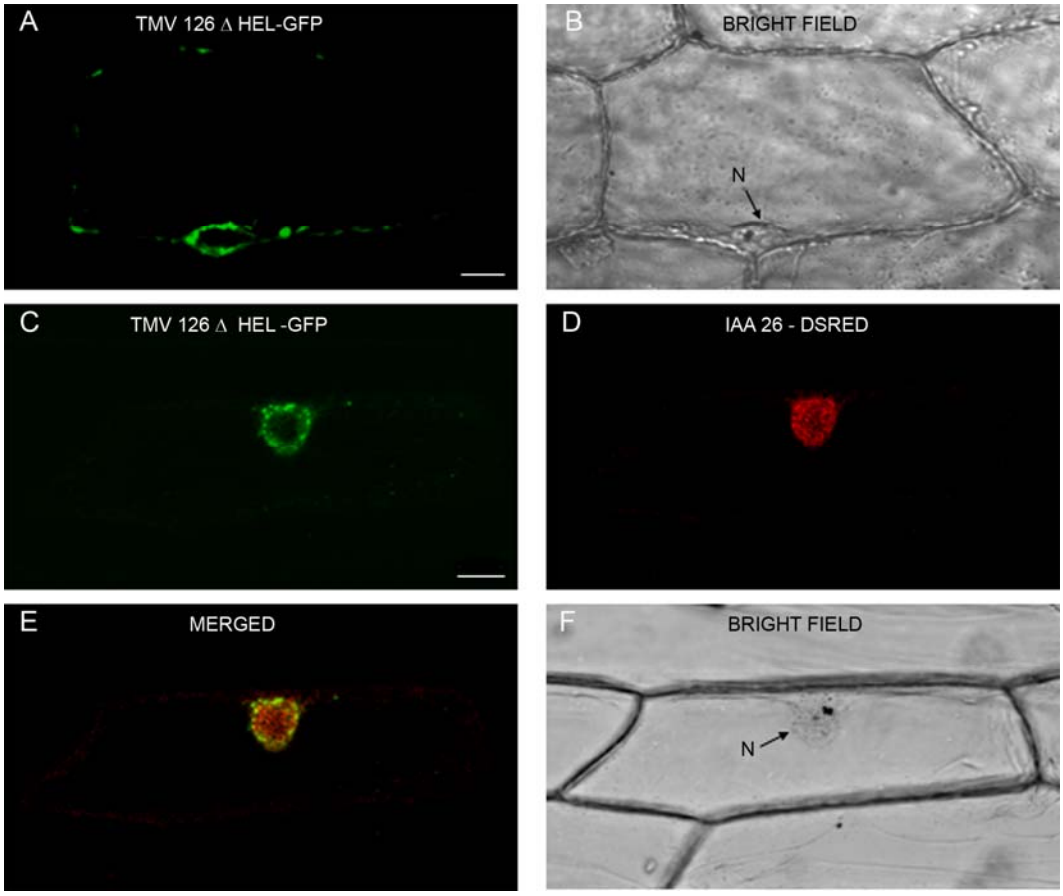


Fig.3.2. Deletion of the helicase domain negates the effect of replicase on IAA26 localization. TMV126-ΔHEL-GFP contains a deletion of amino acids 782 to 1116 required for the replicase - IAA26 interaction. N indicates the location of the nucleus. Images were visualized 20hrs post transformation. Bars = 20μm. (A) and (B) Fluorescence and bright-field confocal images of an onion epidermal cell transiently expressing TMV126-ΔHEL-GFP fusion protein. (C), (D), (E) and (F), Co-expression of TMV126-ΔHEL-GFP and IAA26-DsRed fusion proteins in onion epidermal cells. (C)

TMV126-ΔHEL-GFP and (D) IAA26-DsRed derived fluorescence. (E) Overlay of fluorescence images (C) and (D). (F) Bright field image of the cell.

3.4.2 Additional Aux/IAA members interact with the TMV helicase domain

Since IAA26 belongs to a large family of related proteins the possibility existed that other Aux/IAA members also interacted with the viral replicase. To test this possibility additional Aux/IAA family members sharing varying degrees of homologies with IAA26 were investigated for their ability to interact with the TMV helicase domain (Fig. 3.3). Within the two-hybrid system none of the nine tested Aux/IAA members strongly interacted with the TMV helicase domain at a level similar to that observed for IAA26. However, two Aux/IAA members, IAA27 and IAA18, displayed reduced yet significant interaction levels that were ~47% and ~13% that of IAA26, respectively (Fig. 3.3).

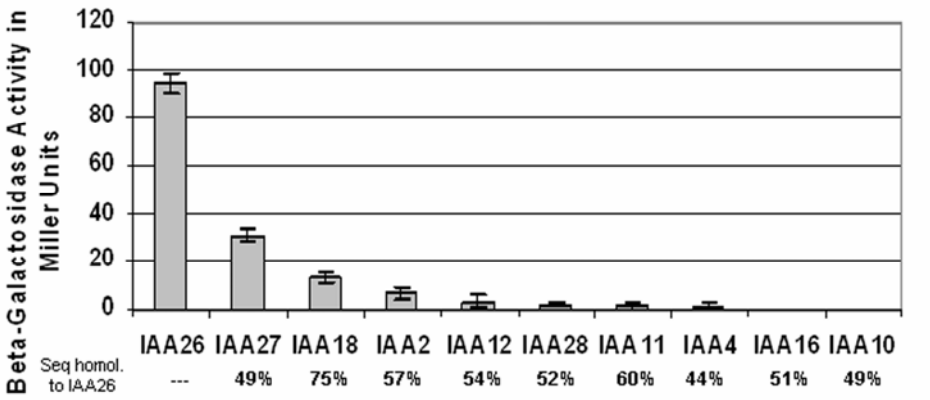


Fig.3.3. TMV replicase interactions with Aux/IAA family members. Quantitative β-galactosidase assays for yeast-two hybrid interactions between individual Aux/IAA members and the TMV helicase domain. Miller unit values for each Aux/IAA represent the mean and standard deviation of three separate assays. The sequence similarity of each Aux/IAA member to IAA26 is listed below the assay results.

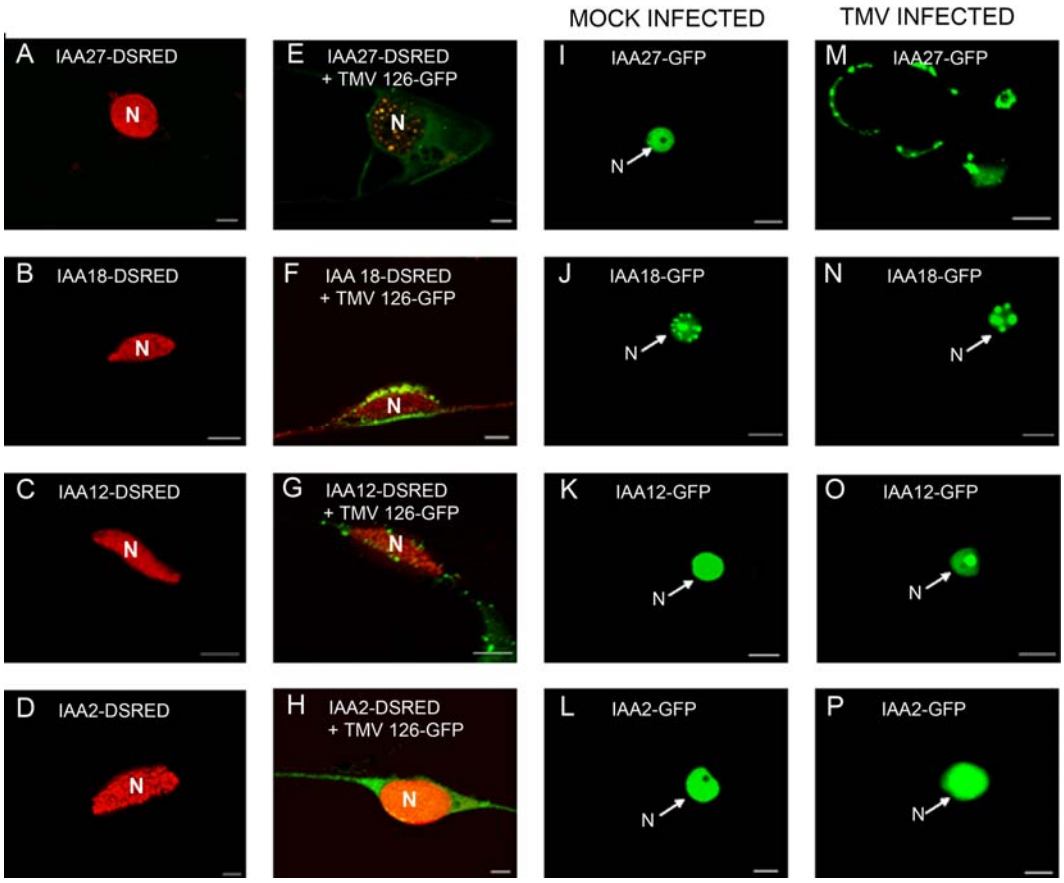
3.4.3 Interaction with the TMV Replicase affects the localization of related Aux/IAA members

To investigate the ability of the TMV replicase to directly affect the localization of other Aux/IAA family members the open reading frames of IAA27, IAA18, IAA12 and IAA2 were fused to DsRed and co-expressed with TMV126-GFP in onion epidermal cells. These Aux/IAA members possess a range of abilities to interact with the TMV helicase domain (Fig. 3.3). In the presence of TMV126-GFP, IAA27-DsRed showed a noticeable change in localization when compared to similar experiments done in the absence of TMV126-GFP (Fig. 3.4A and E). Most significantly, IAA27-DsRed produced only weak accumulations of DsRed fluorescence in the nucleus and the appearance of aggregates that co-localized with TMV126-GFP. In contrast, IAA18-DsRed, IAA12-DsRed and IAA2-DsRed did not display a noticeable change in localization when co-expressed with TMV126-GFP (Fig. 3.4B and F, C and G, D and H).

To further address the biological relevance of the co-localization studies, GFP fusion constructs were also created for IAA27, IAA18, IAA12 and IAA2 and transiently expressed in either mock inoculated or TMV infected leaf tissue (Fig. 3.4). All four Aux/IAA-GFP fusion constructs localized tightly to the nucleus in mock inoculated tissues. However, in TMV infected tissue IAA27-GFP fluorescence appeared predominately as cytoplasmic inclusions (Fig. 3.4I and M). In addition, the total number of cells displaying IAA27-GFP fluorescent in TMV infected tissues was only 23% of the total observed in mock-inoculated tissues (Fig. 3.5). For IAA18-GFP, fluorescence resided primarily in the nucleus, however, the total number of cells displaying IAA18-GFP fluorescence was just 50% of that observed in mock-inoculated tissues (Fig. 3.4J

and N; Fig. 3.5). For non-helicase interacting IAA members, IAA12-GFP and IAA2-GFP, fluorescence appeared exclusively in the nucleus in both mock and TMV infected tissues (Fig. 3.4K and O, L and P). Furthermore, the number of cells displaying IAA12-GFP fluorescence was not significantly different between mock and TMV infected tissues (Fig. 3.5). Collectively, these findings indicate a correlation between the strength of the replicase - Aux/IAA interaction and the ability of the TMV replicase to disrupt Aux/IAA localization.

Fig. 3.4. TMV replicase interacts with and alters the nuclear localization of IAA27 but not IAA18, IAA12 or IAA2. Fusion protein expression was visualized 20 hrs post-transformation in onion epidermal cells and 16 hrs post-transformation in *N. benthamiana* leaf tissue. The nucleus is labeled N. Bars = 10μm. (A), (B), (C) and (D), Confocal images of Aux/IAA proteins fused to DsRed and transiently expressed in onion epidermal cells. (A) IAA27-DsRed. (B) IAA18-DsRed. (C) IAA12-DsRed. (D) IAA2-DsRed. (E), (F), (G) and (H), Confocal images of onion epidermal cells co-expressing Aux/IAA-DsRed proteins with TMV126-GFP. (E) IAA27-DsRed. (F) IAA18-DsRed. (G) IAA12-DsRed. (H) IAA2-DsRed. (I), (J), (K) and (L), Confocal images of Aux/IAA proteins fused to GFP and transiently expressed in *N. benthamiana* leaf tissue mock inoculated with distilled water. (I) IAA27-GFP. (J) IAA18-GFP. (K) IAA12-GFP. (L) IAA2-GFP. (M), (N), (O), and (P), Confocal images of Aux/IAA proteins fused to GFP and transiently expressed in *N. benthamiana* leaf tissue infected with TMV. (M) IAA27-GFP. (N) IAA18-GFP. (O) IAA12-GFP. (P) IAA2-GFP.


MOCK INFECTED
TMV INFECTED
A IAA27-DSRED
N
E IAA27-DSRED
+ TMV 126-GFP
N
I IAA27-GFP
N
M IAA27-GFP
B IAA18-DSRED
N
F IAA 18-DSRED
+ TMV 126-GFP
N
J IAA18-GFP
N
N IAA18-GFP
N
C IAA12-DSRED
N
G IAA12-DSRED
+ TMV 126-GFP
N
K IAA12-GFP
N
O IAA12-GFP
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N
P IAA2-GFP
N

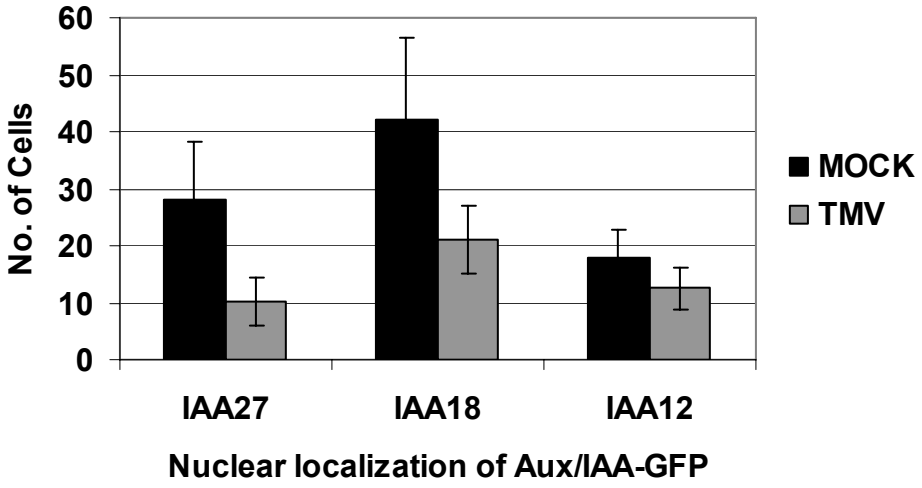


Fig.3.5. Expression and nuclear-localization of IAA27-GFP, IAA18-GFP and IAA12-GFP constructs in non-infected (Mock) and TMV infected (TMV) *N. benthamiana* leaf tissues. Values are the mean number of cells ± standard deviations (error bars) within a 15-mm^2 leaf area visualized 16 hrs post transformation and averaged from five independent bombardment transformations.

3.4.4 Construction of an Auxin resistant IAA26

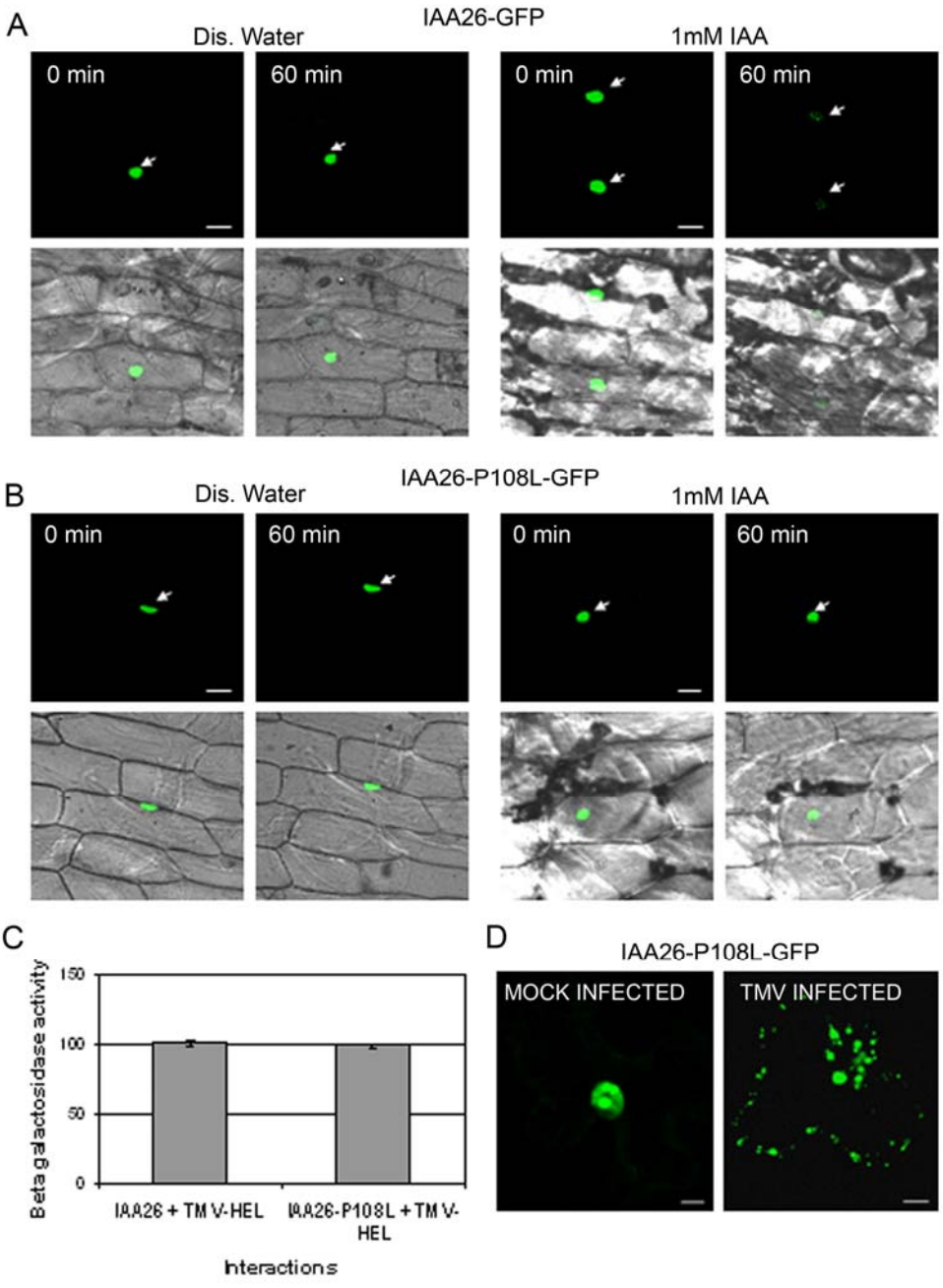
To address the affects of the TMV replicase - Aux/IAA interaction in plants a 35S:IAA26-GFP construct was used to transform *Arabidopsis thaliana* ecotype Shahdara. This ecotype was selected since previous studies had shown that TMV spreads and accumulates at levels that are comparable to other hosts, such as tobacco and tomato (Dardick *et al.,* 2000). I assumed that overexpression of IAA26 would result in developmental abnormalities that could be used to functionally assess the IAA26 - replicase interaction during virus replication. However, transformed plants confirmed by RT-PCR for the accumulation of the transgene mRNA, showed no detectable levels of the IAA26-GFP protein or GFP fluorescence (data not shown). These plants also displayed no significant change in phenotype when compared to non-transformed plants. Similar results have been reported for the overexpression of other wild-type Aux/IAAs (Gray *et al.,* 2001; Rogg *et al.,* 2001; Park *et al.,* 2002). Park *et al.,* (2002) speculated that due to their extremely short half-lives these proteins are unable to accumulate when overexpressed.

To overcome the rapid turnover associated with wild-type Aux/IAA proteins, a P to L substitution was introduced at residue 108 in domain II of IAA26. Similar mutations in other Aux/IAA members have resulted in the enhanced stability and accumulation of the mutant protein even in the presence of excess auxin (Ouellet *et al.,* 2001; Gray *et al.,* 2001; Kepiski and Leyser, 2002; Tian *et al.,* 2003). Stability of the IAA26-P108L-GFP constructs was tested in transient expression assays using onion epidermal cells. Results indicated that in the presence of excess IAA (1mM) IAA26-P108L-GFP remained stable within the nucleus while the wild-type IAA26 degraded rapidly (Fig. 3.6 A and B).

Clearly, the addition of the P108L mutation significantly enhanced auxin resistance and the stability of IAA26.

Two hybrid interaction studies demonstrated that the IAA26-P108L mutation did not significantly affect the interaction of IAA26 with the TMV helicase domain (Fig. 3.6C). In addition, IAA26-P108L-GFP showed markedly different localization patterns in mock and TMV infected tissues. In mock inoculated tissues IAA26-P108L-GFP showed only nuclear localization. In TMV infected tissues IAA26-P108L-GFP was predominately localized within the cytoplasm in small vesicle-like inclusions (Fig. 3.6D). These findings are consistent with results obtained using the unmodified IAA26 protein and indicate that this mutation does not significantly affect the TMV replicase - IAA26 interaction (Padmanabhan *et al.,* 2005, Chapter 2).

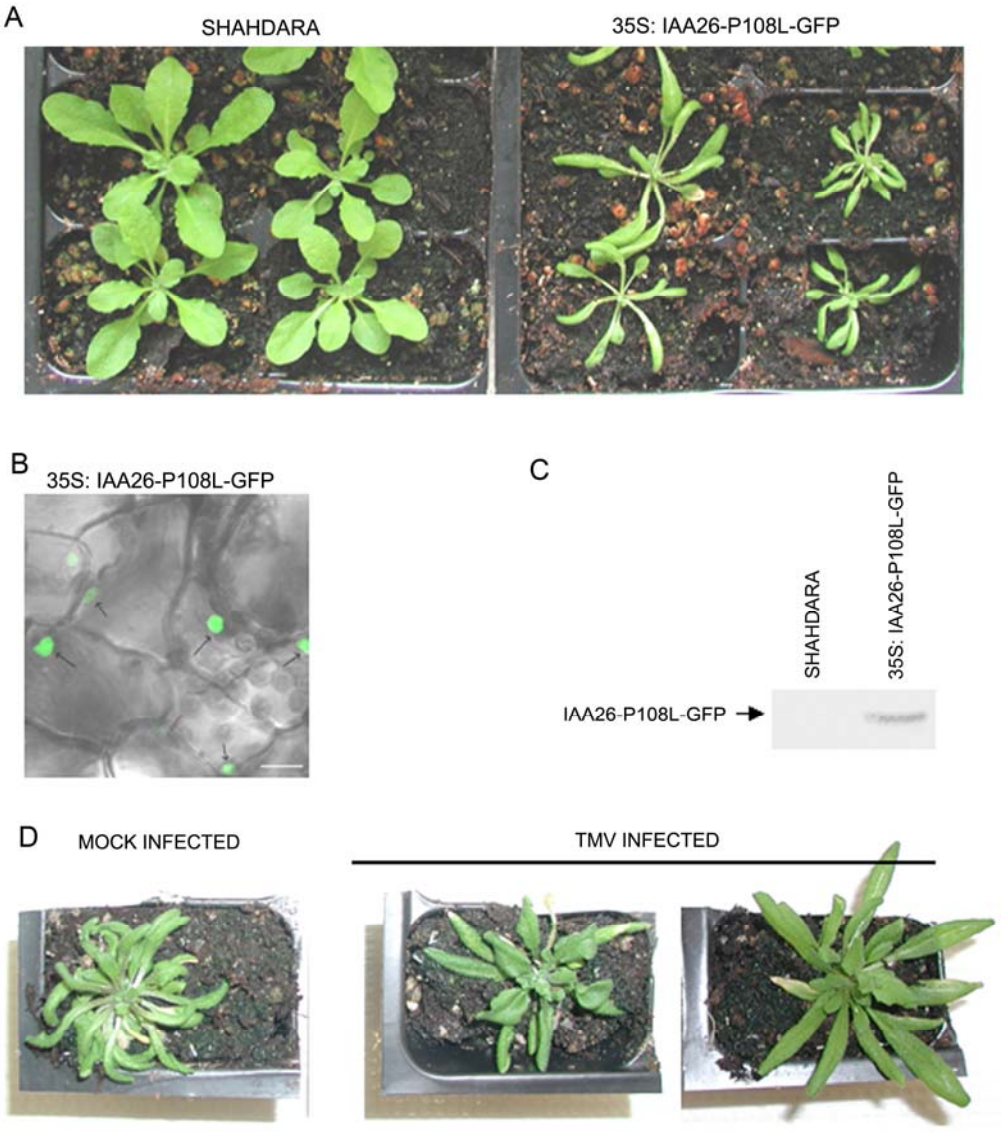
Fig.3.6. IAA26-P108L-GFP is resistant to auxin-mediated degradation but retains its ability to interact with the TMV replicase. (A) IAA26-GFP and (B) IAA26-P108L-GFP. Photos represent fluorescence and light microscopy images of onion epidermal cells expressing the two GFP fusion proteins. Onion cells were treated with water or 1mM IAA and monitored for 1 hr. Bars = 50μm. Arrows indicate the nucleus. (C) Quantitative β-galactosidase activity for yeast-two-hybrid interactions between the TMV helicase and IAA26 or IAA26-P108L. (D) Transient expression and visualization of IAA26-P108L-GFP in mock infected or TMV infected *N. benthamiana* leaf tissue. Images obtained 16 hrs post-transformation. N indicates nucleus. Bar = 10μm.


A
IAA26-GFP
Dis. Water
1mM IAA
0 min
60 min
0 min
60 min
B
IAA26-P108L-GFP
Dis. Water
1mM IAA
0 min
60 min
0 min
60 min
C
Beta galactosidase activity
150
100
50
0
IAA26 + TMV-HEL
IAA26-P108L + TMV-HEL
Interactions
D
IAA26-P108L-GFP
MOCK INFECTED
TMV INFECTED

3.4.5 Interaction with the TMV Replicase affects IAA26 function

A. thaliana ecotype Shahdara transformed with 35S:IAA26-P108L-GFP was generated to test the affects of TMV infection on the function of IAA26. In contrast to the wild-type IAA26-GFP transformed plants, fluorescent microscopy revealed the presence of nuclear localized GFP in young seedlings (Fig. 3.7B). In mature leaf tissues used for virus inoculations, GFP fluorescence was only occasionally observed, however, the presence of the IAA26-P108L-GFP protein was readily detected in these tissues by Western immunoblot analysis (Fig. 3.7C). Similar age dependent differences in GFP fluorescence have previously been accredited to higher levels of chlorophyll interference in older tissue (Zhou *et al.,* 2005). Plants accumulating IAA26-P108L-GFP displayed a severely stunted phenotype along with leaf epinasty (Fig. 3.7A). This phenotype is consistent with that observed in other studies that have utilized auxin resistant Aux/IAA mutants (Gray *et al.,* 2001; Kepiski and Leyser, 2002; Tian *et al.,* 2003). However, when inoculated with TMV, IAA26-P108L-GFP plants showed a marked attenuation in the severity of this phenotype. Most notably, the severe stunting and epinasty in these plants decreased over a period of one to two weeks post-inoculation and was more pronounced in tissues that developed after inoculation (Fig. 3.7D). Decreases in the severity of the IAA26-P108L-GFP phenotype corresponded with the virus's ability to spread and accumulate to high levels within newly developing tissues (Dardick *et al.,* 2002).

Fig.3.7. Transgenic plants overexpressing IAA26-P108L-GFP have an abnormal developmental phenotype that is attenuated during TMV infection. (A) Four-week old untransformed *A. thaliana* ecotype Shahdara and 35S:IAA26-P108L-GFP transformed Shahdara plants. (B) Confocal image of epidermal cells from a two-week old 35S:IAA26-P108L-GFP Shahdara leaf. Arrows indicate IAA26-P108L-GFP derived nuclear florescence. (C) Western immunoblot showing the detection of the IAA26-P108L-GFP fusion protein in transformed 5 week old leaf tissues. (D) Transgenic 35S:IAA26-P108L-GFP plants treated with distilled water (Mock infected) or inoculated with TMV (TMV infected) and photographed 3 weeks post-inoculation.


A
SHAHDARA
35S: IAA26-P108L-GFP
B
35S: IAA26-P108L-GFP
C
SHAHDARA
35S: IAA26-P108L-GFP
IAA26-P108L-GFP
D
MOCK INFECTED
TMV INFECTED

3.5 DISCUSSION

During infection, the TMV replicase proteins promote the development of membrane associated VRCs (Virus replication complexes) (Heinlein *et al.,* 1998; Mas and Beachy, 1999; Liu *et al.,* 2005). VRCs are dynamic entities known to contain a number of host components including ribosomes, cytoskeletal elements and ER, as well as viral RNA, movement and replicase proteins (Beachy and Zaitlin, 1975; Más and Beachy, 1999; Heinlein *et al.,* 1998; Asurmendi *et al.,* 2004; Szecsi *et al.,* 1999). Membrane associated VRCs provide a means to locally concentrate replication components and may function to protect the viral RNA from host defense responses such as RNA silencing. The exact composition and function of these membrane bound complexes is still under investigation but their importance in viral replication is emphasized by the fact that all positive strand RNA viruses utilize such structures for their replication. Previous studies have shown that the TMV 126-kDa replicase protein predominantly associates with the ER, promotes alterations in its morphology and is sufficient to induce the formation of vesicle-like structures (Shalla, 1964; Mathews, 1981; Beachy and Zaitlin, 1975; Reichel and Beachy, 1998; Figueira *et al.,* 2002; Liu *et al.,* 2005). Interestingly, co-localization studies with the TMV 126-kDa replicase protein and interacting IAA26 revealed that the interaction between the two proteins is sufficient to alter the subcellular localization of IAA26 such that it accumulates within the vesicle-like inclusions produced by the 126-kDa protein. Deletion of the interacting helicase domain or use of a non-interacting Aux/IAA protein did not result in co-localization, indicating that the effect of replicase on Aux/IAA localization is dependent on the ability of these proteins to associate. Sequestration of interacting Aux/IAAs within vesicle-like replicase complexes thus provides a mechanism

whereby the virus can directly interfere in the auxin-regulated development of the plant. Combined with previous findings that linked this interaction to the display of disease symptoms (Padmanabhan *et al.,* 2005) it is likely that TMV induced disease symptoms are at least partially derived from the ability of the viral replicase to disrupt the localization of interacting Aux/IAA proteins.

Aux/IAA proteins makeup a conserved family of auxin responsive transcription factors engaged in the regulation of plant development (Liscum and Reed, 2001). Conserved similarities between Aux/IAA family members suggested that the TMV replicase could affect the localization of other Aux/IAA proteins. A two-hybrid screen using Aux/IAA proteins with a range of sequence similarities to IAA26 identified IAA27 and IAA18 as interacting with the TMV helicase domain, albeit at a reduced levels. Localization studies revealed a correlation between the strength of the Aux/IAA - replicase interaction and the level of interference in Aux/IAA localization. Most significantly, the ability of Aux/IAA-GFP fusion proteins to localize to the nucleus in TMV infected tissues versus mock inoculated tissues was less than 5% for IAA26 (previously reported in Padmanabhan *et al.,* 2005), ~23% for IAA27 and ~50% for IAA18. For non-interacting Aux/IAAs, such as IAA12, there was no significant difference in nuclear localization in infected versus mock-inoculated tissues. Co-localization studies also supported this correlation with only the two strongest interactors, IAA26 and IAA27, displaying significant localization changes in the presence of the TMV replicase. The weakly interacting IAA18 appears to be near the threshold needed for the replicase to affect its localization. It was not affected in transient expression assays and only partially altered in infected tissues where replicase accumulations would

be greater. Interestingly, there was no direct correlation between Aux/IAA sequence similarity and the strength of the TMV replicase interaction. For example, IAA18 shares the greatest sequence similarity to IAA26 yet it only weakly interacts with the TMV helicase domain while the less similar IAA27 interacts more strongly (Fig. 3.3). Lack of a correlation between sequence similarity and replicase interaction suggests that the TMV helicase domain interacts with Aux/IAA structural features that are not readily identifiable from the primary sequence. Thus, other Aux/IAA members not tested in this study cannot be excluded as potential targets for replicase interaction. The ability of the TMV replicase protein to alter the localization of multiple Aux/IAA proteins expands the potential impact of the virus on the plant's auxin sensing pathway and the development of disease.

The ability of the TMV replicase protein to alter the localization of Aux/IAA proteins *in vivo* suggests that this interaction could significantly disrupt the normal transcriptional control mediated by interacting Aux/IAA proteins. This possibility is supported by the finding that ~30% of the genes that display transcriptional alterations in response to TMV infection contain two or more AuxRE within their promoters (Padmanabhan *et al.,* 2005). To determine if TMV infection can disrupt the function of an interacting Aux/IAA protein a point mutation in domain II of IAA26 (IAA26-P108L) was created to enhance its resistance to proteolysis and to induce a defined developmental phenotype when over expressed as a transgene. A large body of work had shown that in many Aux/IAA proteins Domain II and its conserved Proline residue form a key structural module that is necessary for auxin mediated interaction with the E3 ubiquitin ligase complex-SCF^{TIR1} ultimately leading to the degradation of the protein via the 26S proteasome (Gray *et al.,*

2001; Kepinski and Leyser 2004, 2005). Several gain-of-function Aux/IAA mutants have a substitution if the conserved proline residue and all these mutants show enhanced stability of the protein in the presence of auxin and a diverse set of auxin response-related phenotypes (Leyser *et al.,* 1996; Rouse *et al.,* 1998; Nagpal *et al.,* 2001; Rogg *et al.,* 2001; Reed 2001). When the conserved proline at position 108 in the IAA26-GFP construct was mutated to leucine, the resultant protein (IAA26-P108L-GFP) was found to be stable even in the presence of high levels of auxin and transgenic plants expressing the protein showed severe stunting, shortened petioles and leaf curling, phenotypes that are associated with altered auxin responses (Leyser *et al.,* 1996; Liscum and Reed 2002). While the point mutation appeared to stabilize IAA26, it did not compromise the interaction of IAA26 with TMV helicase. Based on this finding I speculate that interaction with TMV replicase would reduce the accumulation of IAA26-P108L-GFP in the nucleus thereby lessening the severity of the observed phenotype. This was indeed found to be the case with infected plants showing a reduction in the severity of the phenotype with noticeable decreases in stunting and leaf curling. The change in phenotype was mainly observed in tissues that developed after inoculation and was consistent with the pattern of TMV spread in *A. thaliana* ecotype Shahdara (Dardick *et al.,* 2000). The ability of a TMV infection to attenuate the abnormal developmental phenotype induced by the overexpression and accumulation of IAA26-P108L indicates that even thought the mutant protein is more stable, its interaction with replicase prevents it from reaching its appropriate site of action thereby negating its dominant effect. Furthermore, the replicase levels produced during infection are sufficient to affect the function of interacting Aux/IAA proteins. This is further evidence that the interaction

between the TMV replicase and Aux/IAA proteins can affect auxin mediated pathways.

Virus disruption in plant hormone regulation has previously been attributed to the development of disease symptoms (Hull, 2002; Jameson and Clarke, 2002). Clearly, the effect of TMV replicase on Aux/IAA localization represents one direct mechanism through which this virus can influence hormone controlled plant development. However, virus disruption of hormone regulation and symptom development is likely to be more complex, involving multiple virus and host components. For instance, studies on viral silencing suppressors have shown that viruses can indirectly affect the auxin response pathway by interfering with miRNAs involved in the regulation of certain auxin response factors (Kasschau *et al.,* 2003; Chapman *et al.,* 2004). In another study, interaction between the P2 protein of *Rice dwarf virus* and host *ent*-kaurene oxidases are correlated with the reduced biosynthesis of gibberellins and altered disease symptoms (Zhu *et al.,* 2005). Additionally, while this is the first time that a TMV encoded protein has been shown to alter the subcellular localization of a host protein, it appears that this mechanism for disrupting host function also is utilized by other viruses. Specifically, the coat protein of *Turnip Crinkle Virus* can effectively block the nuclear localization of an Arabidopsis transcription factor, TIP, involved in the induction of the hypersensitive response (Ren *et al.,* 2005). Similarly, the P19 protein of *Tomato Bushy Stunt Virus* interacts with and alters the nuclear localization of a family of plant ALY proteins thought to play a role in RNA transport (Uhrig *et al.,* 2004). Thus, virus directed alterations in host protein localization might represent a common mechanism that affects multiple virus-mediated host responses.

One remaining question is whether the virus gains anything from altering the localization of interacting Aux/IAA proteins. It is possible that disrupting the function of specific Aux/IAA proteins alters host metabolism to provide a more hospitable environment for virus replication and spread. For example, degradation of specific cellular proteins by herpes simplex virus-1 results in more cells being committed to productive infections, particularly at low doses of input virus (Banks *et al.,* 2003). However, partial loss of the TMV - IAA26 interaction, by a helicase mutation, did not affect overall levels of virus replication and accumulation in whole leaf tissues, suggesting that this interaction does not significantly affect viral functions (Padmanabhan *et al.,* 2005). The simplest explanation is that interactions with specific Aux/IAA proteins are inconsequential and independent of virus function. Though such interactions do not appear to be rate limiting for virus replication and spread they can define the difference between a sensitive and tolerant host. Consistent with this possibility is the finding that many host systems can support virus replication and spread and yet display few if any phenotypic effects, indicating that the virus interferes little with normal host physiology (Hull, 2002). The fact that TMV alters the localization and subsequent function of interacting Aux/IAA proteins suggests that this type of interaction plays a key role in defining host sensitivity.

Chapter 4

Putative Aux/IAA protein LeIAA26 interacts with the Tobacco Mosaic Virus Helicase domain to mediate symptom development in Tomato.

4.1 ABSTRACT

The *Arabidopsis* Aux/IAA proteins IAA26, IAA27 and IAA18 have previously been shown to interact with the *Tobacco Mosaic Virus* replicase protein and mediate disease development (Padmanabhan *et al*., 2005; 2006). This study details the identification, cloning and characterization of a Tomato Aux/IAA protein *Le*IAA26 and its role in TMV mediated symptom development. The gene was identified based on its significant sequence similarity with *AtIAA26*. Within the yeast-two-hybrid system, *Le*IAA26 was found to interact with the TMV helicase domain and in TMV infected cells the nuclear localization of *Le*IAA26 was significantly disrupted with the protein appearing within cytoplasmic vesicle-like structures. A VIGS mediated approach was used to knock down the expression of *LeIAA26* transcripts in tomato and *LeIAA26* silenced plants showed significant stunting and leaf curling, a phenotype similar to the symptoms associated with TMV infection. Additionally, the characterization of *At*IAA26 promoter suggested that this gene is primarily expressed in the phloem tissue which is known to be the main route of TMV vascular movement. Taken together with previous data presented in this

document, it appears that TMV mediated disruptions in Aux/IAA protein function may be an evolutionarily conserved mechanism of inducing symptoms in susceptible tissue.

4.2 INTRODUCTION

Tobacco Mosaic Virus is known to infect more that 150 types of herbaceous plants with many hosts belonging to the Solanaceae family which includes economically important plants like tobacco, tomato, potato, egg-plants and peppers. In TMV infected tomato plants, the foliage shows mosaic or mottled symptoms with areas that appear light and dark green. The leaves are curled, have a fern-like appearance with sharply pointed edges with leaf yellowing seen during heavy infections. If infection occurred during the very early stages of plant growth, these plants show a reduction in fruit set with uneven ripening and blemishes seen on the fruit surface. In many instances the entire plant is dwarfed.

Previous studies have shown that in *Arabidopsis*, TMV mediated symptom development partly occurs due to disruptions in the auxin response pathway (Chapter 2 and 3; Padmanabhan *et al.*, 2005, 2006). The Aux/IAA proteins are a large family of auxin responsive transcription factors and have been identified in a number of plants including pea, soybean, *Medicago truncatula*, *Arabidopsis*, tobacco, tomato, maize, rice and cotton (Oeller and Theologis, 1995; Reed, 2001). Phylogenetic analysis and the discovery of a functional Aux/IAA gene in the bryophyte Physcomitrella suggests that this is an ancient family and dates back to the time of evolution of land plants (Remington *et al.*, 2004). All Aux/IAA proteins characterized from the different species

contain the four conserved domains, are auxin responsive and of the ones tested; all appear to be nuclear localized. Furthermore, based on sequence identity, similarities in physical properties and expression analysis, it appears that functional homologues of each Aux/IAA protein exist among different species (Abel *et al.*, 1995).

In tomatoes, more that 11 Aux/IAA genes have been identified with many of them expressed during fruit development (Nebenfuhr *et al.*, 2000; Balbi and Lomax, 2003). *Le*IAA9 is by far one of the best characterized Aux/IAA proteins in tomato and was identified from a tomato fruit cDNA library. It controls leaf patterning, fruit set and development and modulates apical development. Tomato plants silenced for the *Le*IAA9 transcript have pleiotrophic phenotypes consistent with its multiple functions. These include multiple organ fusions, precocious fruit-set and parthenocarpy, development of simple leaves instead of the compound leaves and reduced apical dominance (Wang *et al.*, 2005).

While identification of *Le*Aux/IAA genes has been relatively easy, the functional characterization of these genes has proven to be more difficult. Functional analysis of plant genes has generally involved the use of *Agrobacterium*-mediated genetic transformation leading to either over-expression or downregulation of the desired gene followed by phenotypic, physiological and biochemical characterization of these modified plants. While this process works very well for some plants like *Arabidopsis*, in others systems like Tomato this process entails complex and time consuming procedures, laborious screens and usually suffers from very low efficiency of transformation. In such systems, VIGS (Virus induced gene silencing) has proven to be a promising and effective method for turning down gene expression without the need for genetic transformation.

VIGS exploits the plants inherent RNAi mechanism that has evolved mainly as an anti-viral defense mechanism (Ratcliff *et al.*, 1997; Al-Kaff *et al.*, 1998). This method involves the use of a recombinant virus carrying a partial sequence of the gene that is to be down-regulated. Infection of susceptible plants with this virus and subsequent viral replication activates the PTGS system which effectively degrades the viral RNA as well as the endogenous host mRNA (Baulcombe, 1999; Ruiz *et al.*, 1998). TRV (*Tobacco Rattle Virus*) has been successfully used as a vector to activate VIGS (Ratcliff *et al.*, 2001; Liu *et al.*, 2002a, b). The virus has a broad host species spectrum and causes mild symptoms while effectively invading all tissues including the meristem. VIGS has been successfully used to silence genes in many plants including *N.benthamiana*, Tomato, Poppy and *Arabidopsis* (Liu *et al.*, 2002a, b; Fu *et al.*, 2005; Hileman *et al.*, 2005; Burch-Smith *et al.*, 2006). In recent years VIGS has considerably accelerated the functional characterization of genes in tomato and has the potential to be used as a tool to analyze *Le*Aux/IAA proteins.

Given that TMV alters the functioning of Aux/IAA proteins in *Arabidopsis*, the next question that would logically follow is whether *Le*Aux/IAA proteins also are targeted by TMV. And if so, would this interaction promote symptom development in tomato? Many of the symptoms associated with TMV infection in tomato are reminiscent of physiological changes that can be attributed to auxin mediated effects like changes in leaf morphology and stunting. If TMV does indeed alter a tomato Aux/IAA protein then this would strengthen the evidence for an evolutionarily conserved mechanism that links symptom development to changes in auxin response proteins.

Unlike *Arabidopsis* where the entire genome has been sequenced, most of the genetic information for tomato comes from a large, publicly available EST (Expressed Sequence Tags) database that has been generated by the TIGR (The Institute of Genomic Research) Gene Indices program with support from the National Science Foundation Plant Genome Program (http://www.tigr.org/tdb/tgi/lgi) (Quackenbush *et al.*, 2001; Van der Hoeven *et al.*, 2002). This database is quickly becoming the main data source for gene identification, comparison and functional analysis.

In this chapter, I detail the identification and cloning of a tomato Aux/IAA gene from the tomato EST library that shows considerable sequence homology to *AtIAA26*. This protein was found to interact with the TMV helicase domain within a yeast-two-hybrid screen and the nuclear localization of the putative *Le*IAA26 was considerably altered in TMV infected cells. Furthermore, the phenotype of VIGS silenced *LeIAA26* tomato plants was similar to TMV infected plants. Taken together with similar findings in *Arabidopsis*, it appears that TMV mediated changes in Aux/IAA protein function may be a conserved and wide spread mechanism of promoting symptom development in susceptible plant. Additionally, the spatial and temporal expression analysis of *At*IAA26 revealed that the gene is highly expressed in vascular tissue and was moderately up-regulated by auxin but did not show significant changes during TMV infection.

4.3 MATERIALS AND METHODS

4.3.1 Plant growth conditions and Virus infections

Tomato (*Solanum Lycopersicum*.cv *Bonny Best*), *N.benthamiana* and *A. thaliana* ecotype Shahdara plants were grown under standard growth chamber conditions (12-hr daylight, 24°C). Four-week-old *A. thaliana* ecotype Shahdara P_{IAA26}:GUS plants, 4- to 5-week-old *N. benthamiana* plants and 5-week old tomato plants were used for virus inoculations. The youngest leaves of *N. benthamiana* or mature leaves of tomato plants were dusted with carborundum (Fisher Scientific Company, Pittsburgh, PA.) and inoculated with 5 µg of WT-TMV or mock inoculated with distilled water. Mature rosette leaves of *A. thaliana* transgenic P_{IAA26}:GUS plants were dusted with carborundum and mechanically inoculated with 10 µg of purified wild-type TMV

4.3.2 Sequence analysis

Amino acid sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/index.html)

4.3.3 Cloning of *LeIAA26*

Total RNA from 6 week old *Solanum lycopersicon* leaves was isolated using the RNeasy Plant Miniprep kit (Qiagen,Valencia, CA). cDNA was generated from 1 µg of isolated RNA pretreated with RQ1 DNase (Promega,Madison, WI) and reverse transcribed in a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. Forward

(GGATCCATGGAAGGTTATTCACAAAAATGG) and Reverse primers(CTCGAGTTAGGTCAGCTGCTTAGTTG) containing *BamHI* and *XhoI* restriction enzyme sites and spanning the full length sequence of the largest open reading frame within TC184101 sequence were used to amplify an 860bp fragment from two independent cDNA samples. The fragments were inserted into Topo TA vector (Invitrogen, Carlsbad, CA) and sequenced to confirm the sequence integrity of the DNA fragment.

4.3.4 Two-hybrid assays

pCRII-TOPO (Invitrogen, Carlsbad, CA) clones containing the *LeIAA26* sequence with an additional 5' *BamH1* and 3' *Xho1* sites were digested with the said enzymes and subsequently ligated into appropriately digested pACT-GAL4 for yeast two-hybrid analysis. Yeast two-hybrid interaction assays were preformed as previously described (Padmanabhan *et al.* 2005, Chapter 2). Briefly, the L40 strain of *S. cerevisiae* carrying the pLexA-TMV helicase bait or pLexA-ETR1 was transformed with pACT-GAL4-*Le*IAA26 or pACT-GAL4-*At*IAA26 vectors. Quantitative β-galactosidase assays were performed on yeast cultures carrying both the pACT-bait and LexA-prey plasmids grown at 25ºC in -Ura/-Trp/-Leu selection media as described by Miller (1972).

4.3.5 Transient expression construct

The expression vector pCMC1100, containing a polylinker domain flanked by the Cauliflower mosaic virus 35S promoter and a polyadenylation signal was used as the parental plasmid for the construction of LeIAA26-GFP transient expression construct

(McCabe, 1988). Full length LeIAA26 clone was obtained as mentioned above and modified via PCR to contain a 5' KpnI site and a 3'BsiWI site. The modified LeIAA26 sequence was ligated into similarly cut pCMC-GUS-GFP (Figueira et al. 2002), replacing the glucuronidase ORF with LeIAA26 ORF.

4.3.6 Transient Expression Assays

Transient expression assays were done as described previously (Figueira *et al.* 2002; Padmanabhan *et al.* 2005) using either mock inoculated or TMV infected *Nicotiana benthamiana* leaf tissue. Briefly, a total of 4μg of plasmid DNA (pCMC-*Le*IAA26-GFP) was ethanol precipitated onto 0.5 mg of tungsten particles (1.3μm in diameter; Bio-Rad, Hercules, CA). The DNA coated particles were resuspended in 95% ethanol by sonication in a Brandon 2200 ultrasonic cleanser (Branson Equipment, Shelton, CT) and placed on plastic filter screens (Gelman Sciences, Ann Arbor, MI). The filters were dried and mounted into a particle inflow gun (Finer *et al.* 1992; Takeuchi *et al.* 1992) and a 50-ms pulse of helium (50psi) was used to propel the particles into leaf tissues mounted 3 inches below the filters, respectively. The tissues were incubated for 16 to 20 hrs at room temperature, mounted on glass slides in distilled water under coverslips and Zeiss LSM510 laser scanning confocal microscope with 10X NA 0.8 dry and 63X NA 1.2 water-immersion lenses (Carl Zeiss Inc.,Thonwood, NY) was used to visualize the samples. Excitation sources were 488nm for GFP. Images were modified using Zeiss LSM Imager Examiner software, version 3.0 and processed for printing with Adobe Photoshop (Grand Prairie, TX).

4.3.7 Construction of the VIGS construct pTRV2-LeIAA26 and agroinfilteration

The full length ORF of LeIAA26 was PCR modified to contain a 5' *ECoR1* and 3' *KpnI* site and inserted into a similarly digested pTRV2 plasmid. pTRV2-LeIAA26 and pTRV2 were transformed into *Agrobacterium tumefaciens* strain GV3101. pTRV-LePDS and pTRV1 containing *A. tumefaciens* colonies were provided by Dr. Dinesh Kumar S.P. (Yale University) and their construction is detailed by Liu *et al.*, 2002a.

For the VIGS assay, a 5-ml culture of *Agrobacterium* containing individual TRV constructs were grown overnight at 30°C in selection media containing Rifampicin and Kanamycin. The cultures were used to inoculate into a 50-ml LB medium containing antibiotics, 10 mM MES and 20 µM acetosyringone and grown overnight at 30°C. *Agrobacterium* cells were pelleted and resuspended in infiltration media containing 10 mM $MgCl_2$, 10 mM MES, 200 µM acetosyringone. *Agrobacterium* solutions were adjusted to an O.D. of 1.0 and left at room temperature for 3 h. Each pTRV2 containing strain was mixed with equal volume of pTRV1 infiltrated into the cotyledons of tomato seedlings using a needle-less 1 ml syringe.

4.3.8 Tomato RNA isolation and RT-PCR analysis

Total RNA from VIGS silenced and non-silenced leaves was isolated using the RNeasy RNA extraction kit (Qiagen, Valencia, CA). 1 µg of isolated RNA was pretreated with RQ1 DNase (Promega, Madison, WI) and reverse transcribed in a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions to generate cDNA. The *Le*IAA26, RT-PCR primers were

designed to ensure amplification of only the endogenous gene. The F primer bound 560 bp within the ORF of *LeIAA26* (AGCTTATGACAGCTTATCACA) while the R pimer bound to the 3' untranslated region (GTTGGCTCTACATCTTGTTAGCTCA) so as to amplify a 300bp fragment. *LeEFIα* F (GAGATGCACCACGAAGCTCTCC) and R (CATCTTAACCATACCAGCATCAC) primers designed to amplify a 500bp fragment were used as controls to ensure uniformity in cDNA concentrations. 2µl of cDNA was used as template in each 50µl PCR reaction and each PCR cycle consisted of 94°C for 60 sec, 54°C for 30 sec, and 72°C for 1 min. 8µl of PCR sample was extracted at 21,25,29,33 and 35 cycles. PCR samples were resolved on an agarose gel and photographed using AlphaImager (Alpha Innotech, San Leandro, CA).

4.3.9 Construction of P_{IAA26}: GUS and plant transformation

The 2 kb *IAA26* promoter was amplified by PCR from Shahdara genomic DNA using primers 5' ATCGATCTCCTTTTTTAGTTCACTAA and 3' GGATCCTGATCAACCCAAGATTCC. The primers were modified so as to contain a 5' *ClaI* site and a 3'*BamH1* site. The fragment was ligated into the pBI 101.1 vector (Clontech, Palo Alto, CA) upstream of the GUS (Glucuronidase) reporter gene. pBI-P_{IAA26}:*GUS* construct was introduced into the *Agrobacterium* strain GV3101 and then transformed into Shahdara using the floral dip technique (Clough and Bent, 1998). Transformants were screened for kanamycin resistance and T2 transformants were used for GUS expression analysis.

4.3.10 GUS assay

Plant tissue was infilterated in a solution containing 500µl X-Gluc ((5-bromo-4-chloro-3-indolyl p-D-glucuronide) (Gold Biotechnology, St. Louis, MO), 10mM EDTA, 100mM NaH_2P0_4 pH 7, 5mM Potassium ferricyanide , 5mM Potassium ferrocyanide and 0.1% v/v Triton and incubated at 37°C for 12 hrs or until sufficient staining was observed. For the auxin assay, 2 week old seedlings were treated with 50µM IAA solution or distilled water. Seedlings were removed at specified time points and infilterated with the X-gluc solution followed by incubation at 37°C for 1 hr. After clearing in 70% ethanol overnight, tissues were photographed through a dissecting microscope or a compound microscope.

4.4 RESULTS

4.4.1 Identification and cloning of putative *Le*IAA26

The *Arabidopsis* IAA26 protein sequence was used in a tBlastn sequence homology search against the well annotated Tomato EST library (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato) to identify putative homologues of the protein. This analysis identified one tomato EST (TC184101) with significant sequence homology to *AtIAA26* ($E = 2e^{-49}$). Sequence analysis of the EST led to the identification of an 863bp region that would code for the largest open reading frame. The protein encoded by this ORF shared 48% identity (positives = 63%) with *AtIAA26* and the C terminal 150 amino acid region showed 61% identity (positives

=77%) to the C terminal region of *AtIAA26*. Primers designed to amplify the entire ORF were generated and used to clone the ORF from tomato leaf cDNA. Sequence analysis of this 863bp fragment revealed 100% sequence identity to the sequence within the EST. The encoded protein appeared to contain all the conserved Aux/IAA domains and had multiple nuclear localization sequences (Fig 4.1). This sequence was putatively labeled *LeIAA26*.

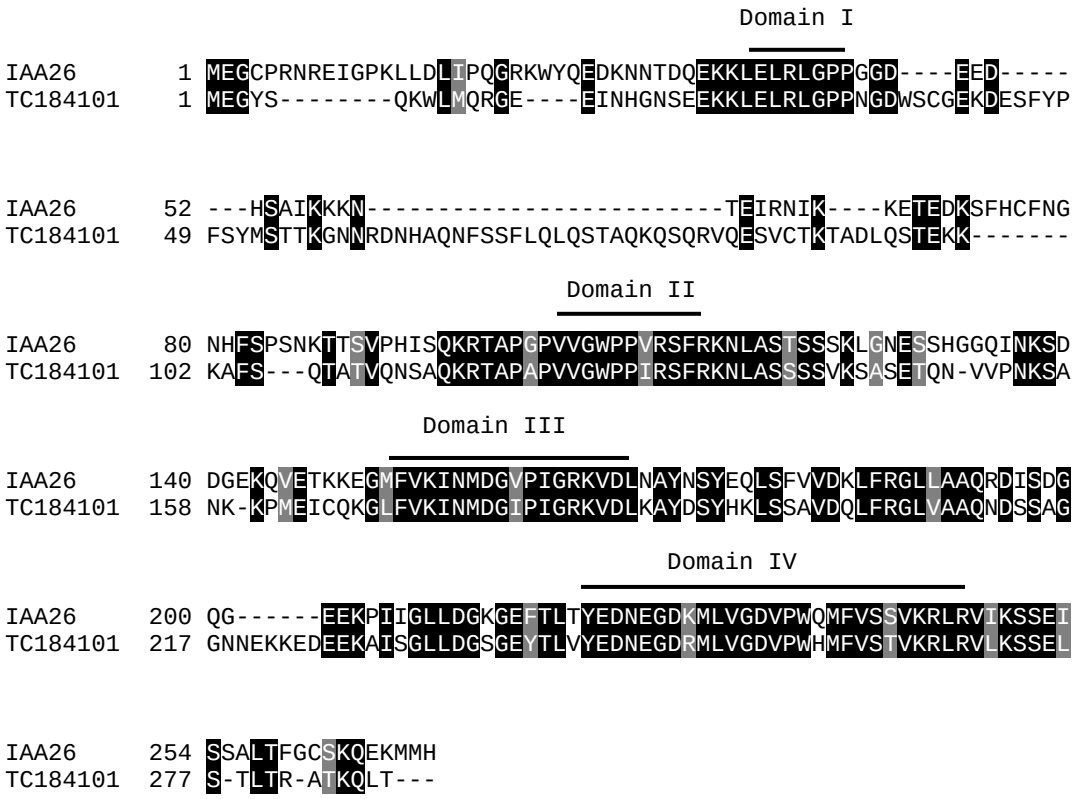


Fig. 4.1. Sequence comparison of *At*IAA26 and TC184101 (*Le*IAA26). Conserved residues are shaded in black, gray shading indicates similar residues. The four conserved domains are underlined.

4.4.2 *Le*IAA26 interacts with TMV Helicase within the Yeast-two-hybrid system

Previous studies have shown that *At*IAA26 interacted with the TMV helicase domain (a.a. 814-1211) within the yeast-two-hybrid system. Similar studies were carried out with *Le*IAA26 where the putative ORF was cloned into the pACT yeast-two-hybrid vector and tested for interaction with TMV helicase within a LexA vector. A pACT construct carrying *At*IAA26 and a LexA construct with AtETR1 (Ethylene receptor 1) were used as positive and negative controls for the assay. Yeast transformed with the bait and prey plasmid(s) were assayed for β-Galactosidase activity grown at 25°C. Within this system, *Le*IAA26 was found to show a siginificant level of interaction with TMV Helicase even though it was three fold weaker than the interaction between *At*IAA26 and TMV-Helicase (Fig 4.2, A and B).

4.4.3 LeIAA26 nuclear localization is disrupted in TMV infected tissue

In keeping with their role as transcription factors, all tested Aux/IAA proteins have been found to localize to the nucleus. Studies with *At*IAA26 have shown that the nuclear localization of this protein is disrupted in TMV infected cells. To confirm the cellular localization of LeIAA26 and to test for TMV mediated alteration, if any, *Le*IAA26 ORF was fused to GFP and transiently expressed in either mock or TMV infected tissue. As seen with other Aux/IAA's, *Le*IAA26 was found to tightly localize to the nucleus. In TMV infected tissue the nuclear localization of *Le*IAA26 was disrupted with the protein appearing to accumulate in distinct cytoplasmic vesicle-like structures (Fig 4.3)

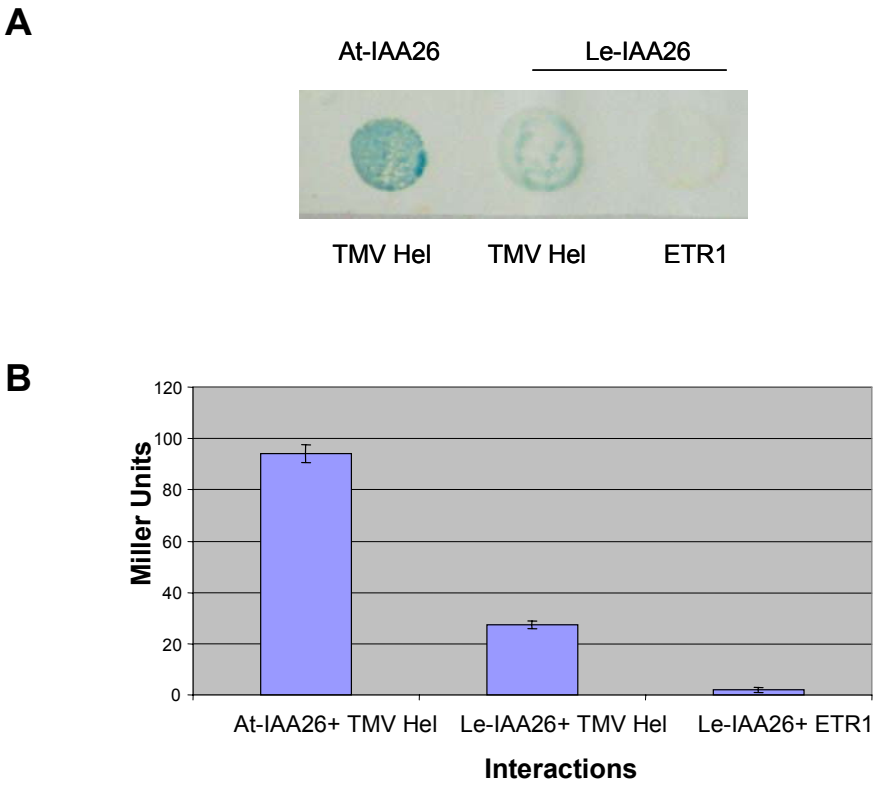


Fig.4.2. TMV helicase interacts with *Le*IAA26. (A) Yeast two hybrid assay showing interaction between the TMV-Hel-Bait protein and *Le*IAA26-Prey protein. *At*IAA26 and ETR1 carrying plasmids were used as positive and negative controls for the assay. (B) Quantification of Beta-galactosidase activity between the interacting proteins

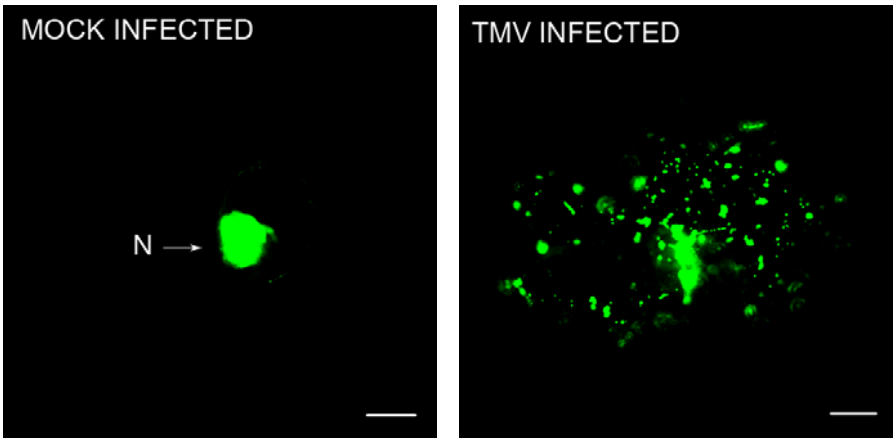


Fig.4.3. TMV alters the nuclear localization of LeIAA26-GFP. Fluorescent images of *N. benthamiana* cells expressing a *Le*IAA26-GFP fusion protein in non-infected (mock infected) or TMV infected cells. N indicates nucleus. Bar = 10μm

4.4.4 Tomato plants silenced for *Le*IAA26 have a phenotype similar to TMV infected plants.

Previous studies have shown that transgenic *Arabidopsis* plants that were downregulated for the *At*IAA26 gene displayed a phenotype that was similar to TMV infected plants. To test if alterations in *Le*IAA26 transcript levels had an effect on the phenotype of the plant, I utilized a TRV activated VIGS approach to induce gene specific silencing. The TRV based VIGS vectors were obtained from the laboratory of Dr. Dinesh-Kumar (Yale University). Their design and construct maps have been detailed by Liu *et al.*, 2002a. In this system, the TRV genome has been segregated, and cloned into two different binary vectors. pTRV-RNA1 plasmid contains the viral RdRp, movement protein and a 16 kd cysteine rich protein. pTRV-RNA2 contains the viral coat protein followed by a multiple cloning site for the insertion of the host gene fragment that is to be silenced. In both cases, the viral genome is under the control of constitutive promoters and terminates in a self-cleaving ribozyme that helps minimize the addition of extraneous nucleotides to the viral genome following transcription. Coinfilteration of *Agrobacterium* containing the two plasmids is necessary to initiate viral replication and activation of PTGS. Using these plasmids, Liu and associates (2002b) have successfully silenced the tomato phytoene desaturase gene (PDS) which is required for carotinoid biosynthesis. pTRV- PDS is now routinely used as a positive control for silencing since it imparts a characteristic photo-bleaching phenotype to the plants.

The full-length open reading frame of *Le*IAA26 was inserted into pTRV2 plasmid that was then co-infilterated with pTRV1 into two-week old tomato seedlings. pTRV-PDS was used as a control to monitor the development of silencing. An empty pTRV2 vector

was used as a negative control so as to identify and ignore any phenotypic changes arising from TRV infection. On an average six plants were syringe infilterated for each vector and the experiment was repeated three times. In the pTRV-PDS infilterated tomato plants, the photo-bleaching effect could be observed as early as two weeks post infilteration and more than 80% of the infilterated plants showed silencing phenotype, albeit at varying levels. The pTRV-*Le*IAA26 infilterated plants appeared to be generally stunted in comparison to pTRV or TRV-PDS silenced plants. The most noticeable difference was in the leaf architecture with the *Le*IAA26 silenced leaves showing significant leaf curling along with alterations in leaf morphology. This phenotype was strikingly similar to the symptoms induced by TMV on Tomato (Fig 4.4 A and B).

VIGS of *Le*IAA26 was confirmed by semi-quantitative RT-PCR (Reverse transcription-Polymerase Chain Reaction). In all plants showing the stunting and leaf curling phenotype, the *Le*IAA26 RNA levels were considerably lower than that seen in either the empty TRV expressing plants or the TRV-PDS silenced plants while the levels of the internal control eF1α were comparable (Fig. 4.4C).

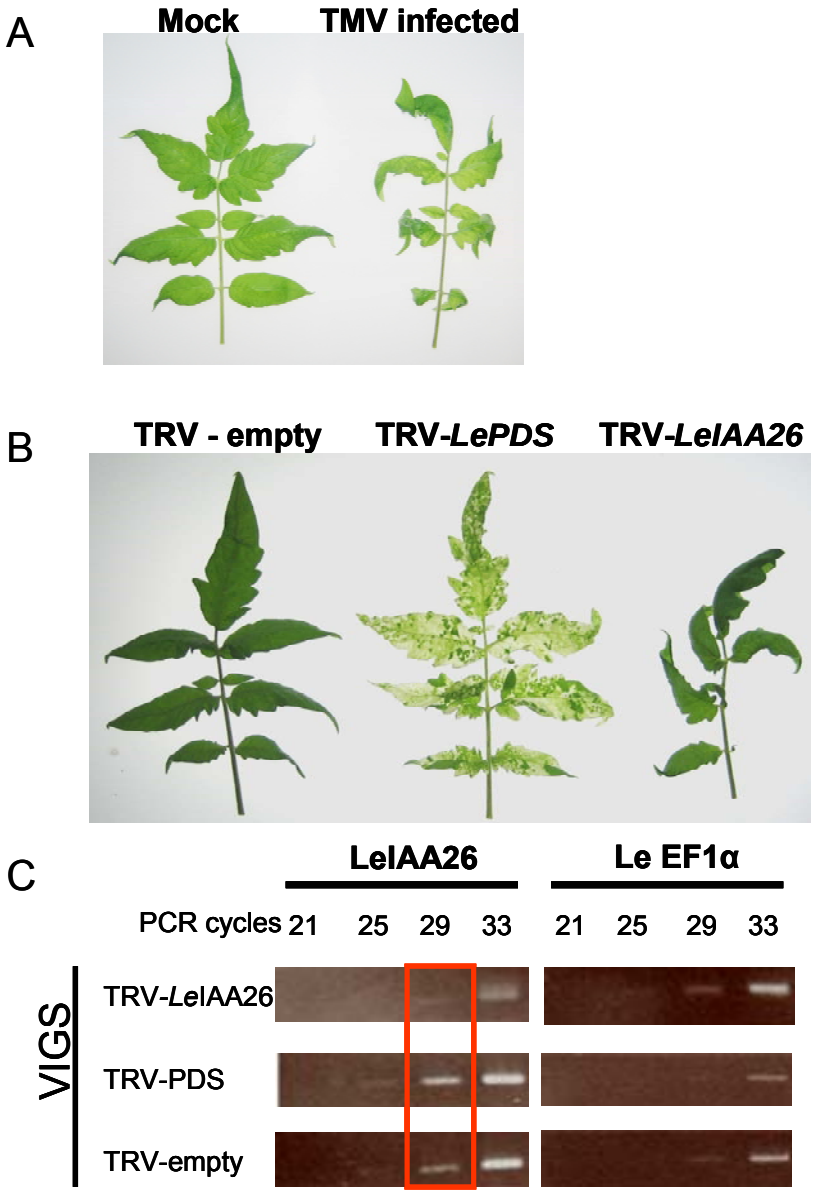


Fig 4.4. Silencing of *Le*IAA26 gene in Tomato and characterization of *Le*IAA26 VIGS plants. (A) Phenotype of healthy and TMV infected leaves of tomato plants 2 weeks post inoculation. (B) Phenotype of leaves of TRV-empty vector, PDS-silenced and *Le*IAA26-silenced plants. (C) qRT-PCR analysis showing levels of *Le*IAA26 and *Le*EF1α transcript in VIGS altered plants.

4.4.5 IAA26 promoter is strongly activated in vascular tissue especially in the phloem

To determine the tissue specific expression of AtIAA26, *Arabidopsis thaliana*, ecotype Shahdara plants were transformed with a construct containing 2kb DNA upstream of the *IAA26* start codon fused to the *Escherichia coli* Beta-glucuronidase gene (*GUS*). More than 20 transgenic lines were obtained. Eight independent lines from the T2 generation were analyzed by histochemical staining for GUS activity and showed similar 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc) staining patterns. IAA26 promoter expression was seen primarily in the vascular tissue and was expressed during all stages of plant growth. GUS activity was observed in the vasculature of the hypocotyl, cotyledon and young leaves (Fig 4.5 A and B), the shoot apical meristem (Fig 4.5C) and in the hypocotyl-root junction (Fig 4.5D). In mature plants, the promoter was expressed in all classes of the leaf veins and within the stem (Fig 4.5 E and G). Analysis of cross sections of the stem revealed strong GUS activity in tissue corresponding to the phloem (Fig 4.5F). When tissue samples were subjected to longer periods of incubation in the GUS substrate (1 hr) GUS expression was observed within the xylem tissue (data not shown). Expression within the root was limited to the sites of lateral root formation (Fig 4.5 B and H) and in the elongation zone of the primary root (Fig 4.5 I). Expression in the mature flowers was seen in the sepal and petal vasculature and the stigma (Fig 4.5J)

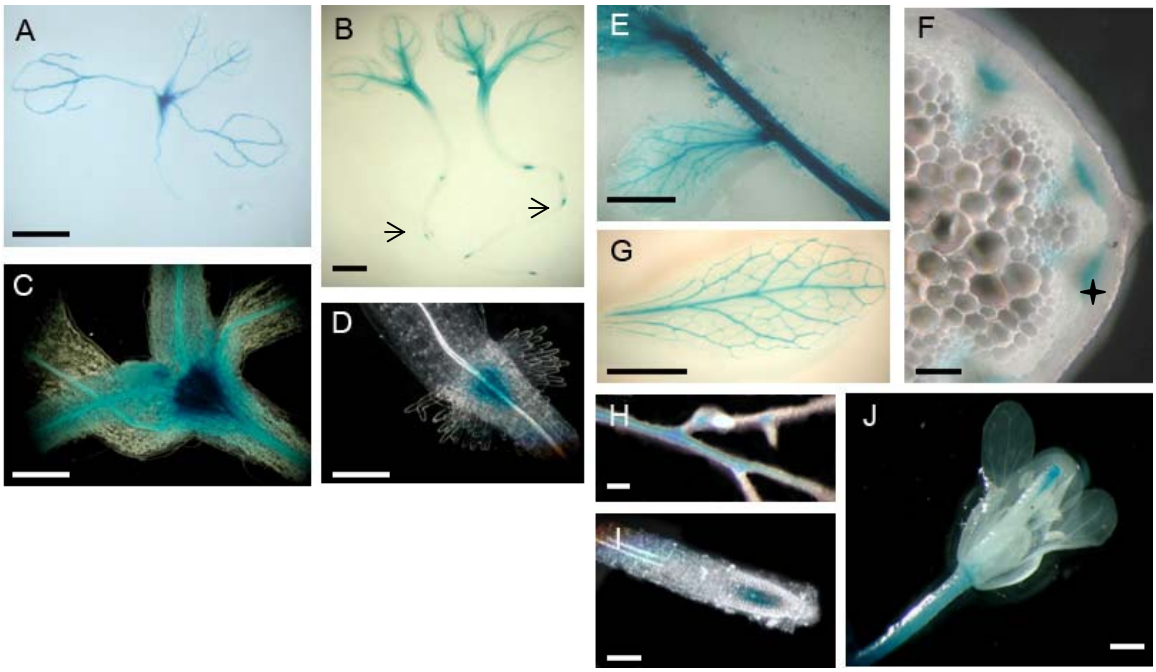


Fig. 4.5. Histochemical analysis of P_{IAA26} : GUS expression . X-gluc staining of light grown seedlings (A) to (D). (A) and (B) GUS expression in the cotyledons, young leaves and roots. Arrowheads head indicate site of lateral root initiation. (C) Shoot meristem (D) Hypocotyl/root junction. (E) Stem (F) Cross section of stem. Star indicates location of phloem (G) Leaf (H) Lateral roots (I) Primary root tip. (J) GUS staining observed in the pedicel and stigma of the flower. Scale in (A, B, C and D) = 0.5mm, (E and G) = 50mm, (F) = 100µm, (H) = 500µm, (I) = 100µm. (J) = 300µm.

4.4.6 P_{IAA26}:GUS expression is moderately enhanced by Auxin but unaltered during TMV infection

Most Aux/IAA genes tested so far show an upregulation in gene expression following treatment with auxin (Tian *et al.*, 2002). To test if IAA26 expression was enhanced in the presence of IAA, seedlings from of one line of P_{IAA26} : GUS were incubated in 50µM IAA solution and the GUS activity tested at .5, 1, 3 and 12 hrs. GUS expression did not show a noticeable increase in any of the early time points when compared to mock treated seedlings (data not shown) but in samples collected 12 hrs post incubation, a moderate increase in GUS gene expression was observed with more cells showing GUS activity in auxin treated tissue when compared to mock (Fig. 4.6).

Characterization of IAA26-TMV replicase interaction has shown that the viral replicase protein(s) inhibit the nuclear localization of IAA26 possibly disrupting its ability to regulate the transcription of downstream genes. To test if this interaction also altered the transcription of IAA26, four week old P_{IAA26} : GUS plants were inoculated with TMV or distilled water (mock) and the GUS activity was assayed at 4 days-post – inoculation (inoculated tissue) and 2 weeks post inoculation (systemic tissue). Cross sections of TMV-infected and mock-infected stem tissue were also tested; three weeks post infection, to detect changes in expression, if any, within vascular tissue. There did not appear to be a significant change in GUS expression in any of the tissues tested.

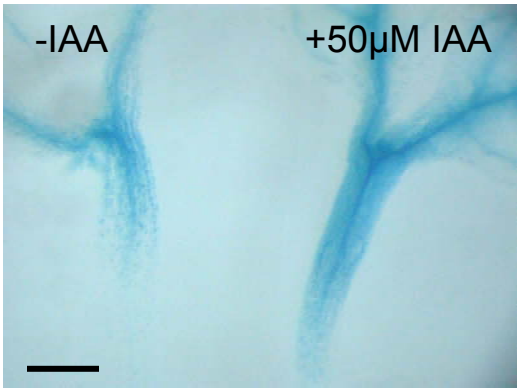


Fig.4.6. Histochemical assay for GUS activity in Auxin treated P_{IAA26}: GUS seedlings. Seedlings were treated with distilled water (-IAA) or 50µM IAA and assayed for GUS activity after 12 hrs. Scale = 750 µm.

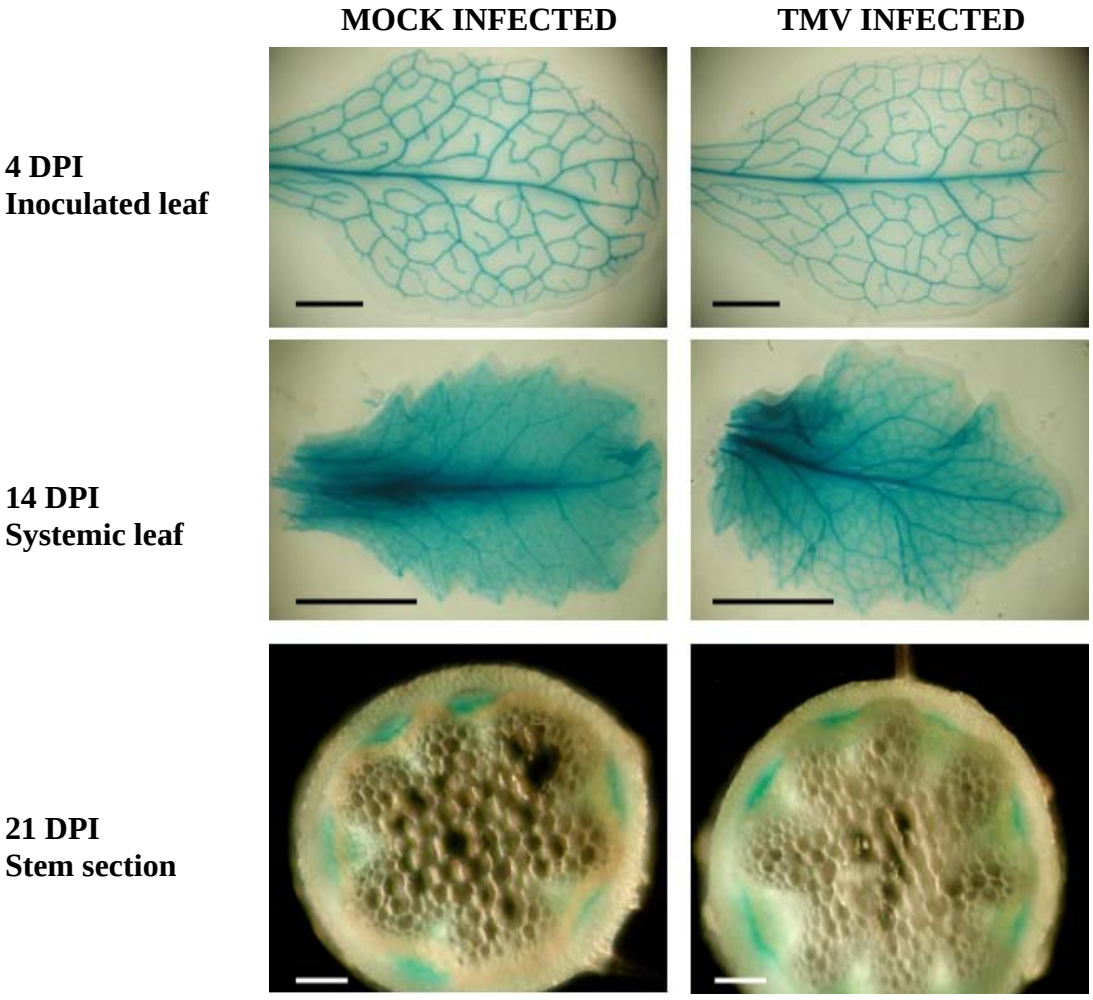


Fig. 4.7. Histochemical analysis of P_{IAA26}: GUS expression during TMV infection.

Scale for leaves = 3mm and stem section = 100μm

4.4 DISCUSSION

This research details the identification and characterization of an interaction between the TMV helicase domain (within the replicase) and a tomato auxin responsive gene which has been putatively labeled LeIAA26. This protein was identified based on the significant sequence similarity it showed with the *Arabidopsis* Aux/IAA protein IAA26. AtIAA26 was previously shown to interact with TMV replicase and this association has been implicated in promoting symptom formation. Additionally *At*IAA27 and *At*IAA18, two more members of the Aux/IAA family were also found to bind to TMV helicase thus bringing up the possibility that the virus may alter the functioning of a small subset of Aux/IAA proteins in susceptible *Arabidopsis*.

Considering the high level of sequence similarity between *AtIAA26* and *LeIAA26* , it is possible that these proteins could be functional homologues. The EST for *LeIAA26* was initially obtained from cDNA library from tomato flower but was also identified in roots and callus tissue (Information obtained from The DF *Lycopersicon esculentum* Gene Index (http://compbio.dfci.harvard.edu). The full length predicted ORF was cloned from a cDNA library obtained from healthy Tomato leaf tissue (var. Bonny Best) confirming that the transcript is also expressed in leaves. Yeast two-hybrid analysis revealed that the full length *Le*IAA26 protein interacted with TMV helicase. Quantitative comparisons between the various TMV Hel- Aux/IAA interactions suggests that the interaction with *Le*IAA26 interaction is two – fold weaker that that seen with *At*IAA26 but comparable to the interaction between *At*IAA27 and higher than that seen with *At*IAA18 (Compare Fig 4.3 and Fig 3.3). Interestingly, there does not appear to be correlation between sequence similarity to *At*IAA26 and strength of the interaction since among the three proteins

tested (*At*IAA27, *At*IAA18 and *Le*IAA26) *At*IAA18 was the most similar to *At*IAA26 but gave the weakest of the interactions.

Using transient expression assays, it has been shown that TMV alters the nuclear localization of AtIAA26 and AtIAA27. When the two proteins were expressed as GFP fusions in TMV infected *N. benthamiana* cells, fluorescence was primarily seen in punctuate, irregular vesicle-like structures distributed across the cell-cytoplasm. In mock infected cells, these proteins exclusively localized to the nucleus. Similar experiments with *Le*IAA67 clearly indicate a similar phenomenon were in healthy tissue, *Le*IAA26-GFP fusion protein is seen only in the nucleus while in TMV infected cells, this localization is disrupted and the protein is seen in cytoplasmic vesicle-like structures. The TMV replicase proteins are known to associate with the endopastic reticulum (ER) and host replication machinery to initiate the formation of virus replication compexes (VRC) which appear as irregular vesicle-like structures (Reichel and Beachy, 1998;Mas and Beachy, 2000; Figueira *et al.*, 2000; Liu *et al.*, 2005). I speculate that the appearance of *Le*IAA26-GFP (and *At*IAA26, and *At*IAA27) as cytoplasmic inclusions in TMV infected tissue might be an indication that these proteins binding to the viral replicase are "trapped" inside the membrane bound VRC's. Previous studies with the *Arabidopsis* IAA proteins also showed that Aux/IAA protein stability was compromised in TMV infected tissue with fewer numbers of TMV-infected cells expressing the Aux/IAA protein. This raises the possibility that the virus may be accelerating protein degradation. Similar quantitative studies could not be carried out with *Le*IAA26-GFP since expression for the fusion protein, even in mock infected tissue was considerably lower that that seen for other proteins and thus was not amenable for quantification.

The functional characterization of the *Le*IAA26-TMV helicase interaction was carried out by knocking-down the expression of *Le*IAA26 transcripts in tomato using VIGS and looking for any developmental or phenotypic differences. *Le*IAA26 silenced plants were stunted in comparison to the control plants and had a phenotype that is similar to the symptoms commonly associated with TMV infection in tomato. In transgenic *Arabidopsis* plants silenced for the expression of *At*IAA26, the phenotype of the plant was reminiscent of the symptoms seen in diseased plants with both showing a loss of apical dominance, leaf curling(minor) and alterations to the symmetrical arrangement of leaves around the central axis (Padmanabhan *et al.*, 2005). The finding that silencing of specific Aux/IAA proteins in tomato and *Arabidopsis* elicits symptom-like phenotypes (in the absence of viral infection) suggests a role for these interactions in modulating disease development. There does not appear to be a complete overlap between the phenotypes of *Le*IAA26 and *At*IAA26 silenced plants but this does not necessarily weaken the hypothesis that the two proteins may be functional homologs. One of the drawbacks of using VIGS is that silencing is not uniform as is confirmed by the PDS silenced plants in which the photobleached phenotype is seen only in patches. Thus it is possible that the phenotype of the VIGS plants is only a milder version of the potential phenotype of *Le*IAA26 silencing in stably transformed plants which may show additional morphological changes. Alternately the two IAA proteins may have evolved to have subtle modifications in their functions. At this point, it can only be speculated that the two proteins are functional homologs since a real confirmation can be carried out only by complementation studies and by comparing tissue specific expression.

The functional diversity of Aux/IAA proteins is partly mediated by the differences in their spatial and temporal expression. For example, IAA3 is expressed in the hypocotyl, cotyledon and expanding leaves, IAA7 is seen in the root and shoot meristem, IAA14 is expressed primarily in the roots and IAA28 is expressed strongly in the roots and inflorescence stems (Fukaki *et al.*, 2002; Rogg *et al.*, 2001; Tian *et al.*,2002). Characterization of transgenic plants carrying a construct made up of the 2kb region upstream of IAA26 fused to the GUS reporter gene (P_{IAA26}: GUS) showed strong expression primarily in the vascular tissue and in leaves it is seen in both minor rand major veins. GUS activity was also observed in the elongation zone of the root and within the lateral root primordia. Auxin is synthesized primarily in young leaves and in the root and it is interesting to note that IAA26 promoter activity was high in these tissues (Ljung *et al.*, 2001). Aux/IAA genes are induced in the presence of auxin and the induction varies from a few minutes to 24 hours for different genes. Treatment with IAA led to a modest increase in the IAA26 promoter activity with more cells expressing the GUS protein in the vascular tissue. This data is consistent with previous microarray information which showed a 1.5 fold increase in IAA26 transcript, 12 hrs after auxin treatment (Goda *et al.*, 2002). Within the vascular tissue, IAA26 was expressed primarily in the phloem cells and this is especially interesting with respect to TMV since the virus has been shown to utilize the phloem cells for long distance movement and systemic infection (Cheng *et al.*, 2000). Using a modified TMV that expresses GFP, Cheng and associates have shown that all veins serve as sources for systemic infection and the virus exclusively uses the phloem (in *N. benthamiana*) for export of the virus from source tissue. The presence of IAA26 in the very cells that are used by the virus for systemic

movement raises the possibility of TMV-IAA26 interaction playing a role in virus transport. Earlier studies characterizing a TMV helicase mutant that showed poor association with IAA26 did not show a significant correlation between the interaction and viral movement. The mutant virus accumulated in host tissue at rates that were comparable to the wild-type virus. In light of the new findings, these studies need to be re-visited and analyzed using more sophisticated techniques to detect subtle differences, if any, in virus movement.

The GUS expression data was compared with microarray data from Genevestigator (www.genevestigator.ethz.ch) which catalogues expression analysis of *Arabidopsis* genes. Genevestigator analysis of *At3g16500* (IAA26) expression suggested that the sites of high expression included callus tissue, hypocotyl, pedicle of flower, stem, node, rosette and cauline leaves and lateral roots all of which paralled data from the GUS expression analysis. Surprisingly this data showed high gene expression within the xylem within leaf tissue. Data from my studies had on the other hand shown stronger expression in the phloem and lower levels in xylem within the stem. I have not carried out cell specific expression within transverse sections of leaves.

TMV infection did not have an apparent effect on IAA26 transcription since I could not detect changes in GUS activity in the inoculated leaves, systemic leaves or in vascular tissue. This result concurs with previous micrarray data from TMV infected Shahdara transcriptome in which IAA26 levels did not appear to be significantly altered (Golem and Culver, 2004). Many Aux/IAA proteins are auto regulated usually in a negative feedback loop and perturbations in their protein levels or activity can directly affect transcript levels (Tian *et al.*, 2003). It appears that IAA26 may not regulate its own

transcription since TMV mediated changes in its localization (and activity) did not seem to have an apparent affect on its transcription. As a caveat, it must be noted that all the tests were carried out in an 'asynchronous infection' environment. Also promoter activity analysis and transcriptional changes during the very early stages of infection, ie- at 12 hrs or 24hrs post infection were not tested. Thus we cannot rule out the possibility that TMV may alter IAA26 transcript levels at a specific developmental stage or in certain tissue types.

The most significant finding of this study is the identification of TMV-Aux/IAA protein interaction in Tomato. With this finding, the TMV replicase has been shown to interact with and alter the cellular localization of Aux/IAA proteins from two hosts. This validates the existence of an evolutionarily conserved mechanism of virus induced alterations in Aux/IAA protein functioning that ultimately manifests as disease symptoms. Symptom development and severity are heritable characteristics which suggest that specific and recurring molecular changes occur within susceptible hosts. Interactions between virus and auxin response proteins may just be one such genetic determinant of symptoms in a broad range of hosts.

CHAPTER 5

Conclusions and Perspectives

Early studies on pathogenic microbes mainly focused on understanding their behavior within controlled laboratory conditions. In the last decade and a half, the focus has shifted to studying microbes in their natural and more complex environment especially in context to their relationship with the host. The very simplicity of viruses suggests the presence of intimate interactions with the host at all stages of their life cycle and the challenge has been to tease out and dissect individual interactions occurring at this level. A detailed knowledge of the mechanisms by which virus induce disease or resistance in the host is important in that it provides an understanding of the evolution of virus-host interactions, gives insights into the cellular processes occurring within plants and provides the basis for engineering resistance.

Successful viral infection produces distinct and recurring symptoms on hosts which suggest that very specific and genetically controlled interactions must occur between the two systems. Previous studies characterizing virus-host interactions on susceptible hosts have mainly used a whole genome approach, such as microarrays, to look for changes in gene expression during virus infection (Golem and Culver, 2003; Whitham *et al.*, 2003). While these studies are important since they provide a global view of the plant transcriptome during infection, it has still proven difficult to pin down the specific inducers of these changes and associate the target genes with the ultimate physiological response. Moreover, genomic analysis is limited to identifying effects occurring during

the later stages of viral infection, after viral proteins have been synthesized. Identifying host factors in susceptible plants that are the primary targets for viral proteins has proven to be quite challenging with only a handful of known interactions that can promote pathogenesis.

In this manuscript, I have attempted to elucidate and dissect a disease-inducing interaction occurring between the *Tobacco Mosaic Virus* and auxin responsive proteins from its susceptible hosts. The work outlined in this dissertation provides some mechanistic insights into how a specific viral protein (TMV replicase) interacts with auxin regulated host transcription factors (Aux/IAA) to alter the functioning of a specific hormone regulated pathway ultimately leading to disease development. The work thus describes a potentially novel pathway for TMV to interfere with growth- and development-related proteins. Along with being one of the few reports to have studied the molecular aspects of symptomatology, this work also improves our understanding of an important class of plant regulatory factors. Finally, while it has always been suspected and speculated that disease development and alterations in hormone signaling are connected, this work provides crucial experimental evidence to substantiate the link.

5.1 Proposed model outlining the functional effect of TMV mediated disruption of Aux/IAA activity

Based on the research detailed in the previous chapters I propose the following model for TMV mediated induction of disease (Fig 5.1). In healthy or uninfected tissue, the Aux/IAA proteins function as negative regulators of their interacting ARFs and help

modulate the expression of the downstream Auxin responsive genes. During TMV infection, the replicase protein, associates with a subset of these Aux/IAA proteins (IAA26, IAA27, IAA18, *Le*IAA26) and either targets the protein for degradation (via an as yet unknown mechanism) or simply prevents its entry into the nucleus. Aux/IAA proteins in general have small half-lives, and thus the trapping of these proteins quickly depletes the concentration inside the nucleus. This would then theoretically free-up the corresponding ARF's that can act as either transcriptional activators or repressors (Ulmasov *et al.*, 1999). The resultant abnormal and auxin-independent regulation of the downstream auxin responsive genes can be partially responsible for some of the disease symptoms seen in infected tissue. Based on this model, both auxin and TMV would have a similar effect on the downstream auxin responsive genes and it would appear that TMV is activating a specific arm of the auxin signaling pathway.

Among the putative auxin responsive genes tested, all transcripts appeared to be down-regulated during TMV infection, a trend that was mirrored by auxin treatment (Chapter 2). This brings up the possibility that the ARF proteins that are controlled by the interacting Aux/IAA's are repressors. There are 22 ARF proteins within *Arabidopsis* and while it has been suggested that Aux/IAA's are partially selective in their association with ARF's, it has proven extremely difficult to identify interacting partners (Weijers *et al.*, 2005). This is mainly due to a certain degree of redundancy that appears to exist among members of both the families (Ellis *et al.*, 2005; Wilmoth *et al.*, 2005). Inspite of this, identification and characterization of the interacting ARF's remain the next crucial step to confirming the hypothesis.

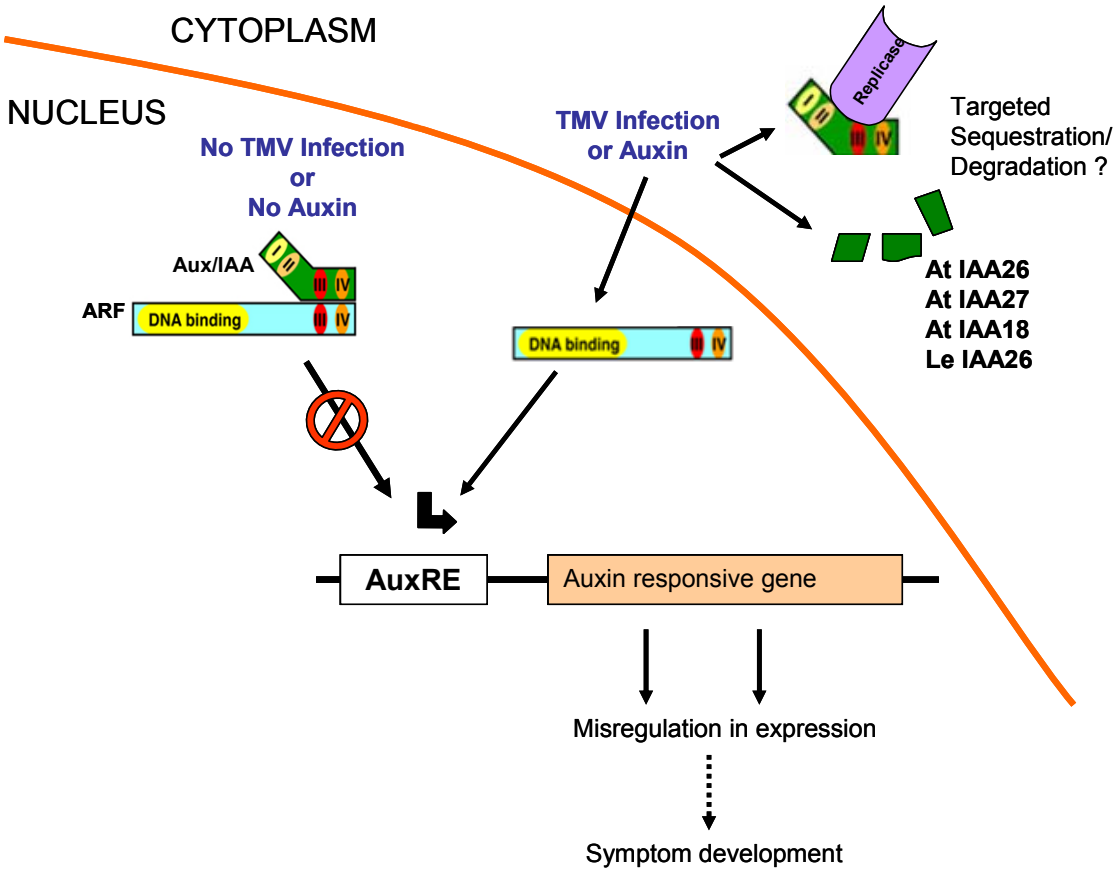


Fig.5.1. Model for TMV mediated induction of symptoms. During TMV infection, viral replicase sequesters or promotes the destabilization of IAA26. This occurs independent of the plants auxin gradient and activates ARF's which alter transcription levels of specific AuxRE containing genes. Misregulation in Auxin responsive gene expression is responsible for some of the disease symptoms

5.2 Functional significance of TMV-Aux/IAA interaction

The identification of multiple Aux/IAA genes that could associate with TMV replicase and the conservation of this interaction in different susceptible hosts suggest that the interaction is evolutionarily significant. Furthermore a preliminary comparison of the helicase domains from multiple members of the Tobamovirus family show that the Valine residue at position 1087 (which was previously shown to be essential for interaction with IAA26) is located just outside the conserved Domain VI and appears to be conserved among 17 of the 19 members (Appendix).

The most important question that arises then is - What does the virus gain from its association with the Aux/IAA proteins? There are a few possible hypotheses that could explain the importance of this association and I outline them in the following sections.

Interaction might alter the cellular environment to make it more favorable for infection

Auxin is known to play an important role in growth promoting processes and it would be advantageous to the virus to stimulate this pathway. Recent research on *Pseudomonas syringae* has shown that auxin enhances bacterial pathogenesis and in fact, one of the plant defense responses involves tuning down the transcripts of TIR1 (the auxin receptor) so as to dampen the auxin response pathway (Navarro *et al.*, 2006). Thus it is possible that TMV's interference of Aux/IAA activity might lead to more host cells being committed to the infection process. There are three pieces of evidence that corroborate this hypothesis.

Firstly, when transgenic plants overexpressing the proteolysis resistant mutant of IAA26 (IAA26-P108L-GFP) were infected with TMV and viral accumulation was compared with that in Wild-type, there appeared to be a consistent decrease in TMV replicase proteins in the transgenic plants. This suggested that accumulation of IAA26 might decrease viral infectivity (Fig. 5.2).

The previous studies comparing TMV and TMV-V1087I showed no change in virus infectivity, but it is to be noted that this analysis was primarily carried out only in young (four-week-old) Shahdara. Aux/IAA proteins are known to be active at specific developmental stages within the plant and it is possible that this interaction is crucial at a another developmental stage within the plant. To this end I have compared virus infectivity in Shahdara at varying stages of their growth and preliminary studies suggest that when the same infectivity assay was repeated in ten-week-old plants there was a marked decrease in TMV-V1087I infectivity (Fig. 5.3). This second piece of evidence suggests that in older and more mature tissue the association between TMV and the IAA26 is clearly advantageous to the virus. Auxin levels are generally much lower in older tissue as a result of which there is a higher accumulation of Aux/IAA proteins and the cells are in a more developmentally static state (Ljung *et al.*, 2001).

After auxin, the second factor controlling Aux/IAA degradation is the levels of TIR1 protein. Auxin binds to TIR1, inducing a conformational change which then facilitates the recognition of Aux/IAA proteins and their degradation (Dharmasiri *et al.*, 2005). Recent work has shown that TIR1 transcript levels are controlled by microRNA, mir393 which is upregulated during stress and pathogen infection (Sunkar and Zhu, 2005; Navarro *et al.*, 2006). The third piece of evidence comes from comparison of TIR1

transcript levels in four-week and ten-week Shahdara leaves which indicated a significant decrease in TIR1 mRNA in the mature rosette leaves (possibly due to increased mir393) (Fig. 5.4).

Thus it would appear that in older leaf tissue, the resultant accumulation of the repressor Aux/IAA proteins (due to the reduction in auxin and TIR1) would slow down the auxin mediated developmental processes within the cell. Such an environment would be less favorable for virus infection and so it would seem plausible that in these tissues, the virus would try to alter the cellular state by mimicking the effect of auxin which is to inhibit repression by Aux/IAA's.

It is one of the generally accepted facts that younger plants are more susceptible to pathogenic attack. Infectivity and symptom development is also enhanced in these plants. The presence of more auxin in younger tissue and the finding that it might have a positive role in pathogenesis might explain this phenomenon.

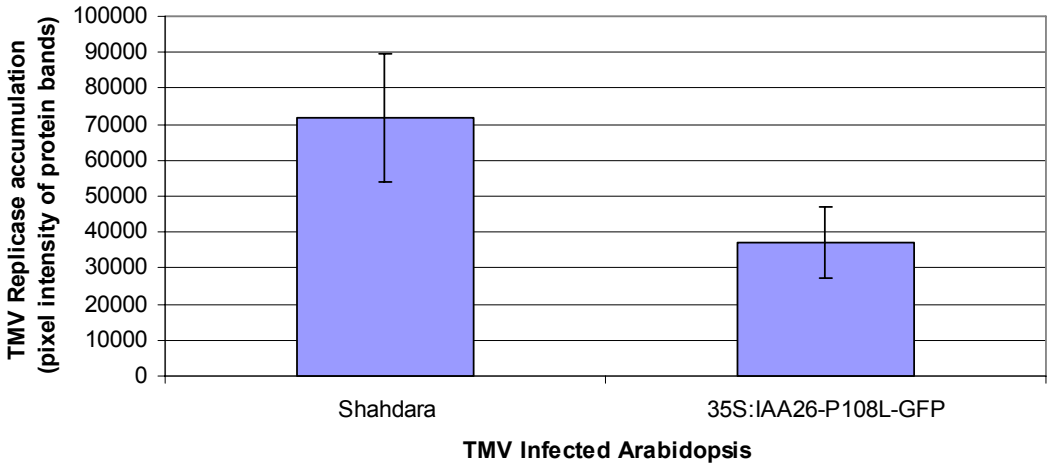


Fig 5.2. TMV infectivity is reduced in plants overexpressing the proteolysis resitant IAA26 protein. Comparison of replicase proteins levels in TMV infected Wild-type and 35S:IAA26-P108L-GFP Shahdara. Four week old Shahdara and 35S:IAA26-P108L-GFP plants were infected with 10 μg WT-TMV and 3 weeks post inoculation, 100mg of leaf tissue was homogenized using a technique modified from Osman and Buck (1996). Proteins from pellet and supernatant fractions were separated by SDS-PAGE and probed for TMV replicase by western-blotting. Band intensities of the replicase protein were quantified using AlfaEase quantification software (Alpha Innotech, San Leandro, CA). Data presented here is quantified from three independent assays.

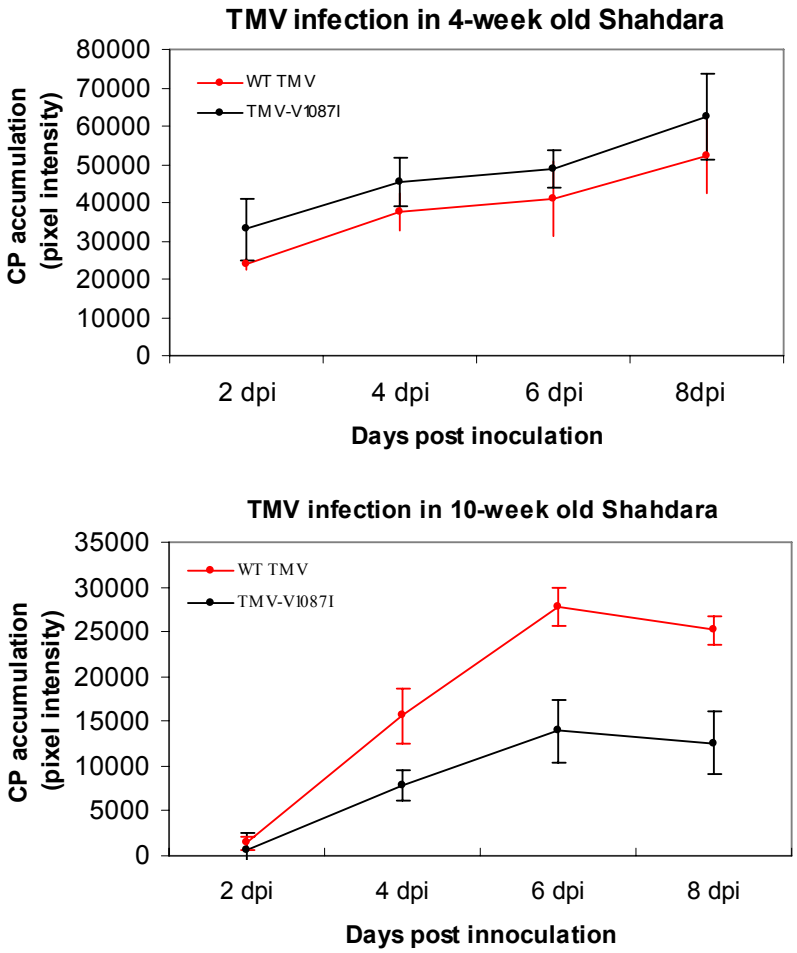


Fig.5.3. TMV-V1087I helicase mutant, disabled in its interaction with IAA26, accumulates to lower levels in older plants. Four-week old and ten-week old Shahdara was inoculated with 10μg of WT-TMV or TMV-V1087I. Leaf discs (1-cm in diameter) from inoculated rosette leaves were harvested at 2, 4, 6 and 8 days post-inoculation. Samples were ground in sample buffer and proteins were extracted, separated and transferred onto nitrocellulose membranes. The blots were probed with anti-CP antibody and CP levels were quantified using AlphaEase quantification software (AlphaInnotech, San Leandro, CA).

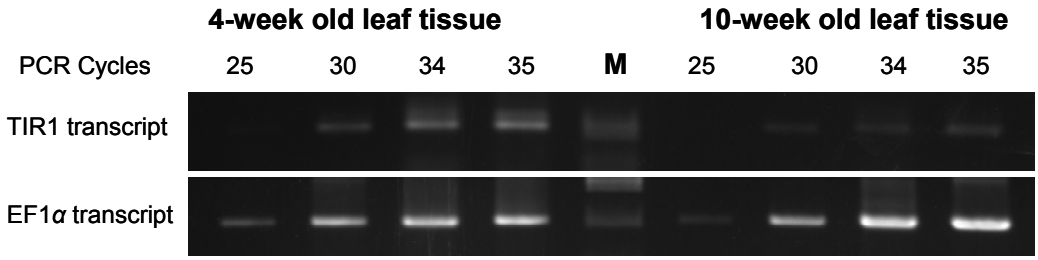


Fig. 5.4. TIR1 trancript levels are reduced in 10-week old Shahdara. Semi quantitiative Real time Polymerase Chain Reaction (RT-PCR) comparing TIR1 transcript levels in four-week old and ten-week old Shahdara leaf tissue. cDNA was prepared from 1µg of total RNA isolated from 100mg leaf tissue. 3µl of cDNA was used in each PCR. EF1α was used as the internal control. M stands for the DNA marker.

Interaction acts as a decoy to prevent self degradation

In an effort to establish successful infection, viruses have developed diverse and multiple strategies to overcome the obstacles set up by the host cell. One of the strategies developed by many animal viruses has been to promote degradation of host proteins, generally those detrimental to their infection. *Human papillomavirus* (HPV) directs the degradation of the tumor suppressor protein P53, *Herpes simplex virus* avoids immune surveillance by ubiquitinating Major histocompatability complex-1 chains and Epstein-Barr Virus promotes degradation of specific kinases which eventually promotes establishment of latent infection (Scheffner *et al.*, 1990; Coscoy *et al.*, 2001; Winberg *et al.*, 2000).

TMV MP has been shown to be ubiquitinated in infected cells and it appears that this is a way to prevent MP from taking over and disabling the ER network in cells (Reichel and Beachy, 2000). Considering the fact that TMV is a target of the 26S proteasome it is possible that the virus could be shunting the Aux/IAA proteins and other as yet unknown cellular proteins into the proteasome as a decoy mechanism so as to slow down degradation of viral proteins. Alternatively, this could be a mechanism to promote degradation of the replicase (where it latches onto proteins that are destined for degradation) and thus a potential mechanism of self regulation.

Interaction defines difference between tolerance and disease

In a broad sense, plant-microbe interactions can be classified into two types- those that play a role in the establishment of infection (consequential interaction) and those that are inconsequential or independent of viral functions. While there does not appear to be an

apparent advantage to plant or the microbe, inconsequential interactions still remain significant since they help define the difference between tolerant and diseased phenotypes. Tolerance is characterized by the ability of the host plant to grow and reproduce even when infected by a pathogen (Agrios, 1996). Such plants show almost no symptoms of infection even while harboring significant amounts of pathogen. Tolerance is a genetic and heritable characteristic which means that specific molecular interactions must be occurring to promote or suppress disease development. The Aux/IAA-Helicase interaction may not be rate limiting to virus replication but instead affects symptom severity. This type of non-rate limiting interaction may thus account for the differences between disease and tolerant host responses.

5.3 TMV Replicase- a multifunctional protein

It is a well documented fact that most viral proteins function in multiple processes. Even among viral proteins it would appear that TMV replicase has taken on a lot of responsibilities. This multidomain protein is needed for replication, cell-to-cell movement, activation of HR, suppression of RNAi and induction of disease. It interacts with a multitude of host factors some of which include a P58 inhibitor of protein kinase that inhibits plant defense, the N gene product which activates HR, a membrane protein (TOM1) that enables tethering and membrane associated replication and activates a beclin protein that induces autophagy (Bilgin *et al*., 2003; Erickson *et al*., 1999; Yamanaka *et al.,* 2000; Liu *et al*., 2005). The findings in this study add to the repertoire of interacting proteins and cement its role in promoting symptom development. Among

all these interactions, the common theme appears to be the involvement of the helicase domain. The yeast-two-hybrid assay mentioned in chapter one was designed to test for interactions between host proteins and the various domains within TMV replicase. All the positive interactions identified involved only the helicase domain. Conventionally helicases are assigned the biochemical function of unwinding double stranded nucleic acids and TMV helicase domain does appear to possess the ability to unwind dsRNA (Goregaoker and Culver, 2002). But it is becoming increasingly evident that TMV helicase is more than a structural RNA-binding protein and also functions as a key adaptor molecule between virus and host cell processes. One of the main challenges ahead is to decipher the mechanistic details that permit the protein to function in diverse processes. Determining and characterizing the atomic-resolution structure of this protein domain would play a big role in advancing this goal.

5.4 Future Goals

The preliminary finding that TMV-V1087I performs poorly in older tissue brings up the exciting possibility that IAA26-TMV association may be advantageous to the virus when infecting older tissue. The more immediate studies will be directed towards testing this hypothesis.

The working hypothesis is that the reduction in auxin concentration and increase in mir393 transcripts (leading to a reduction in TIR1 mRNA) would lead to a concomitant increase in IAA26 protein levels in older tissue. To test this we plan to utilize previously generated IAA26-GFP over-expressing plants. My initial studies with these plants failed

to detect fluorescent proteins and we speculated that the fusion protein was rapidly degraded by the host 26S degradation system. Recent re-testing however has shown that under specific extraction conditions (including urea in extraction buffer) it is possible to detect the proteins through western blotting. The future experiments will utilize these plants to quantitatively test for differences in IAA26-GFP levels in younger and older Shahdara tissue. Assays to test for increase in mir393 levels in older leaf tissue and decreases in TIR1 transcripts will also be carried out. Older tissue will then be infected with the virus and IAA26-GFP levels monitored

If auxin does indeed promote pathogenesis it would be worthwhile to test TMV infection against the backdrop of auxin pathway mutants, the most obvious choice being TIR1 mutant. I have not been able to detect significant difference in TMV infection within a previously characterized TIR1 knock out in a Col-O background (Ruegger *et al.*, 1998) (data not shown). This however does not necessarily negate a role for auxin in TMV infectivity. The TIR1 protein belongs to a four member family of auxin signaling F box proteins (AFB1, 2, 3, TIR1) all of which have been shown to bind auxin and target Aux/IAA's for degradation (Ruegger *et al.*, 1998; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b). Thus the TIR1 knock out is not completely insensitive to auxin signal and this explains the fact that it is phenotypically quite indistinguishable from wild-type Columbia plants (Ruegger *et al.*, 1998). Interestingly, the mir393 targets TIR1 and two of these three AFB proteins (Sunkar and Zhu, 2003; Navarro *et al.*, 2006). Transgenic plants over-expressing mir393 showed significant developmental alterations in line with reduction in the transcript levels of all three target auxin receptors. These plants would be ideal to test for effects of auxin on TMV virulence.

To this end, I have cloned a 170bp fragment of genomic DNA containing mir393 and its flanking regions. The fragment was designed based on the methodology followed by Navarro and associates (2006). The fragment was inserted into an Agrobacterium vector under the control of a constitutive promoter and used to transform Shahdara. I am currently screening for transformants. Once transformants are identified and confirmed, they will be infected with TMV and the infectivity of the virus will be tested. If we observe a decrease in viral load in these plants, it would confirm a positive role for auxin in TMV infectivity.

Putting things in perspective, if we are able to prove that TMV tries to reprogram the cellular environment in older tissue it would prove to be a significant finding. In nature, viruses do not choose the tissue they infect and this might be just one mechanism by which the virus alters the environment it ends up in to make it more suitable for itself.

Earlier on, I had proposed that the virus might induce the degradation of interacting Aux/IAA proteins. I had been unable to confirm this due to the relative unstability of the native IAA26 protein. Now that IAA26-GFP can be detected in transgenic plants, it is possible to test this hypothesis.

Another aspect that needs to be worked on is identifying the targets of IAA26. Previously, I have been able to narrow down the potential Auxin responsive targets of interacting Aux/IAA proteins. The expression of these genes within IAA26 over-expressing plants will be tested to look for variations in their response. One of the more difficult challenges is to identify the interacting ARF(s). One possibility worth looking into would be to screen ARF mutants from SALK knock-out lines and identify ones which have a phenotype mirroring the proteolysis resistant (IAA26-P108L-GFP) plants.

These would serve as potential partners for IAA26 which could then be tested for in-vivo interaction and tissue specific expression.

If this association between the auxin responsive proteins and TMV replicase is important, it would be of interest to test for its occurrence in other viral-host interactions. The first choice of candidates would be members within Tobamovirus like *Tomato Mosaic Virus, Cucumber Green Mottle Mosaic Virus*, *Pepper Mild Mottle Virus* etc.

While, it appears that TMV can alter the Aux/IAA functioning, this may not be the only route of disruption in the auxin response system. Recent research in viral suppressors of silencing has shown that, in an effort to shut down the silencing pathway viral suppressors inadvertently inhibit many miRNAs. This leads to ectopic expression of their target genes and such alterations are partly responsible for induction of symptoms (Kasschau *et al.*, 2003; Chapman *et al.*, 2004; Chellapan *et al.*, 2005). ARF proteins are targets of miRNA mediated regulation and in *Turnip Mosaic Virus* infections, ARF 8, ARF10 and ARF17 are overexpressed due to Hc-Pro mediated inhibition of their corresponding miRNA's (Kasschau *et al.*, 2003). Within TMV, the 126- kDa replicase is the silencing suppressor though it is not yet known if the replicase can inhibit endogenous miRNA pathway (Ding *et al.*, 2004). It would be interesting to see if the replicase interferes with miRNA metabolism as well. Additionally, the virus could alter genes involved in auxin biosynthesis though unfortunately very little is really known about this aspect of virus mediated changes. Thus it is quite possible that there are additional routes through which TMV could perturb the auxin response system.

The finding that the even in tomato plants symptom severity is modulated by associations between viral and auxin responsive proteins shows the evolutionarily

conserved nature of these interactions. In economically important plants, we could then exploit such interactions to develop strategies of plant tolerance/resistance. For instance, we could create transgenic plants over expressing functionally inactive peptides spanning regions within Aux/IAA proteins that could interact with replicase. During infection these peptides could associate with and interfere with the activity of replicase thereby possibly decreasing viral infectivity while not inducing detrimental effects on the plant. It would be also possible to engineer plants to express modified versions of these susceptibility factors ,that do not interact with viral pathogenesis factors, there by reducing the physiological or symptomatic effects of virus infection. Thus a good understanding of the molecular interactions behind symptom development can aid in the development of novel strategies for engineering disease tolerance especially in those hosts where engineering disease resistance is not a viable option.

APPENDIX

Alignment of amino acid sequence within Helicase domains of 19 Tobamoviruses showing conservation of Valine residue at a.a. 1087

Tobamovirus strain	a.a. 1080 ✧ 1113
TMV U1	---SLKYYTV**V**MDPLVSIIRDLEKLSSYLLDMYKVDA
TMV KR	---SLKYYTV**V**MDPLVSIIRDLEKLSSYLLDMYKVDA
TMV-RAK	---SLKYYTV**V**MDPLVSIIRDLEKLSSYLLDMYKVDA
P03586 TMV	---SLKYYTV**V**MDPLVSIIRDLEKLSSYLLDMYKVDA
TOMV	---SLKYYTV**V**MDPLVSIIRDLERVSSYLLDMYKVDA
PPMV	---SIKYYTV**V**LDAVVSVLRDLECVSSYLLDMYKVDV
TMGMV	---SFKYYTV**V**LDPLVQIISDLSSLSSFLLEMYMVEA
TMV OB	---CFKYYTV**V**LDPLVKLVRDLECVSNFLLDVYMVDS
ORSV	---SFKYYCV**V**LDPLVKVCSDLSKVSDFILDMYKVDA
TVCV	---CCKYYTV**V**LDPMVNVISEMEKLSNFLLDMYRVEA
CR-TMV	---RCKYYTV**V**LDPMVNVISEMEKLSNFLLDMYRVEA
RMV-SH	---RCKYYTV**V**LDPMVNVISELGKLSNFLLEMYKVES
CRMV	---RCKYYTV**V**LDPMVNVISELGKLSNFLLEMYKVES
TMV-CG	---RCKYYTV**V**LDPMVNVISEMEKLSNFILDMYKVES
CGMMV	---AMVYYTV**V**FDAVTSIIADVEKVDQSILTMFATTV
CGMMV-W	---AMVYYTV**V**FDAVTSIIADVEKVDQSILTMFATTV
CFMMV	---AMTYYTVTVDPVSCIIADLEKVDQSILSMYASVA
YCGMMV	---TMTYYTVTVDPVSCIIADLEKVDQSILSMYATVA
SHMV	---RFVYYTV**V**PDVVMTTVQKTQCVSNFLLDMYAVA

Star indicates location of Valine residue

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