

ABSTRACT

Title of Document: CHARACTERIZATION OF THE TMV REPLICASE PROTEINS: LOCALIZATION AND INTERACTIONS WITH RAB GDI PROTEINS

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Tobacco mosaic virus (TMV) is a model positive-strand RNA virus. TMV encodes two replicase proteins, both of which contain methyltransferase and helicase domains; the 183 kDa protein contains an additional RNA-dependent RNA polymerase domain. Using this virus, virus-host interactions important in the initial establishment of infection and formation of replicase complexes were investigated. Specifically, on the virus side, replicase proteins were examined for regions that may contribute to its localization to the endoplasmic reticulum (ER) during TMV infection. An ER localization domain was identified in a region between amino acids 599 and 701. Alanine substitutions were introduced into this region and examined for their effects on the virus. Several possible hypotheses are discussed as to how this domain may function during infection.

Concerning the host, an interaction with a host protein, a Rab GDP Dissociation Inhibitor (Rab GDI), was examined. This interaction occurred with tomato and

tobacco Rab GDIs as well as with the originally identified *Arabidopsis thaliana* Rab GDI (*AtGDI2*). Silencing of Rab GDI transcripts enhanced the number of infection sites in TMV:GFP-infected plants, but did not alter viral movement or overall accumulation, indicating a possible role in initial establishment of infection. Rab GDI-silenced *Nicotiana benthamiana* plants showed cellular morphologies similar to those of TMV-infected cells. Moreover, TMV infection results in Rab GDI proteins localizing to structures associated with viral replication. Taken together these data indicate a role for Rab GDI proteins in the initial establishment of infection. Two models of how Rab GDI proteins may contribute to TMV infection are discussed.

These studies examine parts of the viral life cycle that are not very well understood, in particular the initiation and establishment of infection. Although vesicle trafficking has been shown to be important for several different pathogens, this is the first time that a Rab GDI protein has been identified as participating in viral replication. Understanding initiation of infection and susceptibility of a host to a pathogen are vital to elucidating pathogen-host interactions and developing disease resistance strategies.

CHARACTERIZATION OF THE TMV REPLICASE PROTEINS:
LOCALIZATION AND INTERACTIONS WITH RAB GDI PROTEINS

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Dedication

I would like to dedicate this work to my husband, John, who has been with me throughout my research career and without whom I would have given up long ago.

Thank you for love, support, and proofreading.

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I would like to thank Dr. Shunyuan Xiao for the pictures of trypan blue stained *LeGDI*-silenced tomato tissue. Thank you also to Dr. Dinesh-Kumar for the *Tobacco rattle virus* vectors. Thanks go to both Shannon Dean and Liz Royston for critical reading of this document. Thank you, also, to my committee members who have been critical, helpful and encouraging over the past few years. I would also like to thank my advisor, Jim Culver, who has always been available when I need him and always seems to come up with new ideas and experiments.

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Abbreviations

aa	amino acid
AVR	avirulence gene
bp	base pair
BMV	<i>Brome mosaic virus</i>
CMV	<i>Cucumber mosaic virus</i>
CP	coat protein
CP-MR	coat protein-mediated resistance
cv	cultivar
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
GAP	guanosine activator protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanosine exchange factor
GFP	Green Fluorescent Protein
eGFP	enhanced GFP (a GFP variant)
GTP	guanosine triphosphate
GUS	β -glucoronidase
HA	hemagglutinin
HSP	heat shock protein
Ipk	inhibitor of protein kinase
IR	intervening region

MP	movement protein
NLS	nuclear localization signal
nt	nucleotide
ORF	open reading frame
PR	pathogenesis-related
pv	pathovar
PVX	<i>Potato virus X</i>
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
SA	salicylic acid
SAR	systemic acquired resistance
SEL	size exclusion limit
siRNAs	small interfering RNAs
SNARE	soluble n-ethylmaleimide-sensitive factor adaptor protein receptor
TMV	<i>Tobacco mosaic virus</i>
ToMV	<i>Tomato mosaic virus</i>
TRV	<i>Tobacco rattle virus</i>
UTR	untranslated region
VIR	virulence gene
wt	wild type
SF	superfamilies

Chapter 1: General Introduction

The most exciting phrase to hear in science, the one that heralds the most discoveries, is not “Eureka!” but “That's funny...” – Isaac Asimov

Rationale and significance

Humans have been both fearing and using viruses since we developed agrarian lifestyles and became dependent on cultivated animals and plants for survival. Understanding of viruses and other pathogens has become essential to our continued survival on a planet with decreasing arable land and an increasing human population. Interactions between a virus and its host determine whether a virus can establish an infection, whether the host is able to detect and defend against the virus, whether symptoms will develop, and what their severity will be. These interactions determine the life and death of the host and the future propagation of the virus. It is in the balance of the virus infecting the cell and usurping but not killing the host that we learn more about the virus and the host alike.

There are approximately 450 species of plant viruses that cause symptoms, which can range from the highly-valued petal colorations of *Tulip breaking virus* to the devastating outbreaks of cassava mosaic geminiviruses, which caused the loss of 19 million tons of cassava at a cost of \$1.9 million in the 1990s [1]. (+)-strand RNA viruses are the largest subgroup, making up about 75% of all plant viruses.

The goal of this study is to use a model virus, *Tobacco mosaic virus* (TMV), to examine interactions between a virus and its host that are important in establishing and

maintaining an infection. Studies focus on developing an understanding of how the replicase proteins interact with the viral host—in localization, host cell membrane rearrangements, and alterations to host cell functions. Domains within the replicase proteins responsible for localization during infection are identified and shown to function outside the context of the virus. Furthermore, changes to the host cell's endomembrane system during infection are established. An interaction with an essential host protein, a Rab GDP Dissociation Inhibitor, is characterized and shown to be an essential part of initial establishment of viral infection. Results from these studies should better elucidate mechanisms of establishing and maintaining an efficient infection in a host, in addition to examining the role of membrane biology in infection, which is an emerging field in virus biology.

Tobacco mosaic virus

TMV is a model (+)-strand RNA virus that encodes two replicase proteins (126 kDa and 183 kDa) as well as a coat protein and a movement protein [2, 3]. TMV infection can result in stunting, leaf curling, necrosis, chlorosis, ringspots, yellowing, and plant death. TMV infection may cause severe crop losses in species such as *Lycopersicon esculentum* (tomato) and *Nicotiana tabacum* (tobacco), causing significant problems for farmers and greenhouse growers. As with many replicase proteins, the TMV 126 kDa and 183 kDa proteins have methyltransferase, helicase, and RNA binding activities. The 183 kDa protein contains an extra RNA-dependent RNA-polymerase domain and is produced from a read-through of an amber stop codon ([4]; Figure 1).

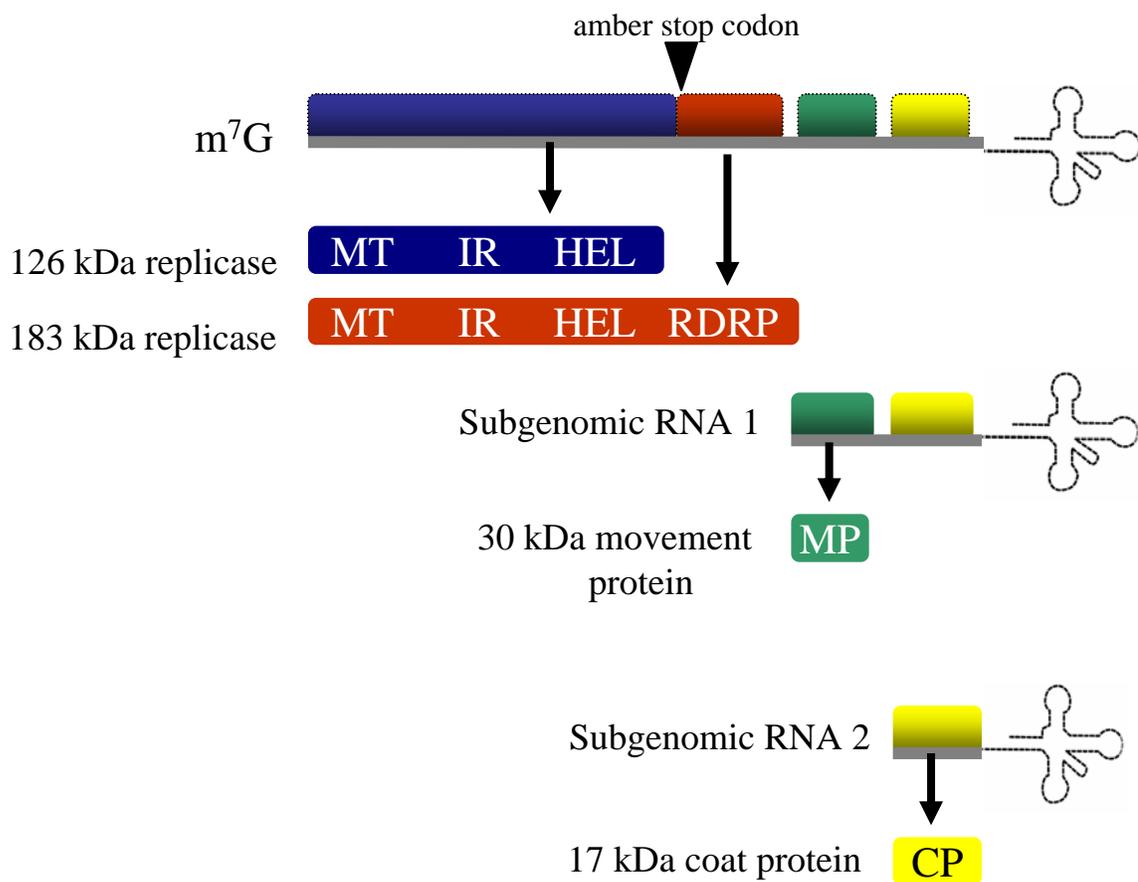


Figure 1. Genome organization of TMV. TMV is a (+)-strand RNA virus that encodes at least four proteins. The genome has a 5' methyl cap and a 3' tRNA-like end. The two replicase proteins are translated first from genomic RNA and both contain methyltransferase (MT), helicase (HEL), and an intervening region (IR). The 183 kDa protein contains an additional RNA-dependent RNA-polymerase domain (RDRP) which is produced from the readthrough of an amber stop codon (UAG). This readthrough occurs about 10% of the time and is influenced by upstream and downstream sequences [5, 6]. The movement and coat proteins are translated later in infection from subgenomic RNAs and share the same 3' tRNA-like UTR with the genomic RNA.

Although these activities have been demonstrated *in vitro*, new research suggests that these proteins may have a greater role in the viral life cycle, such as in viral movement and in suppressing host defense mechanisms [7, 8].

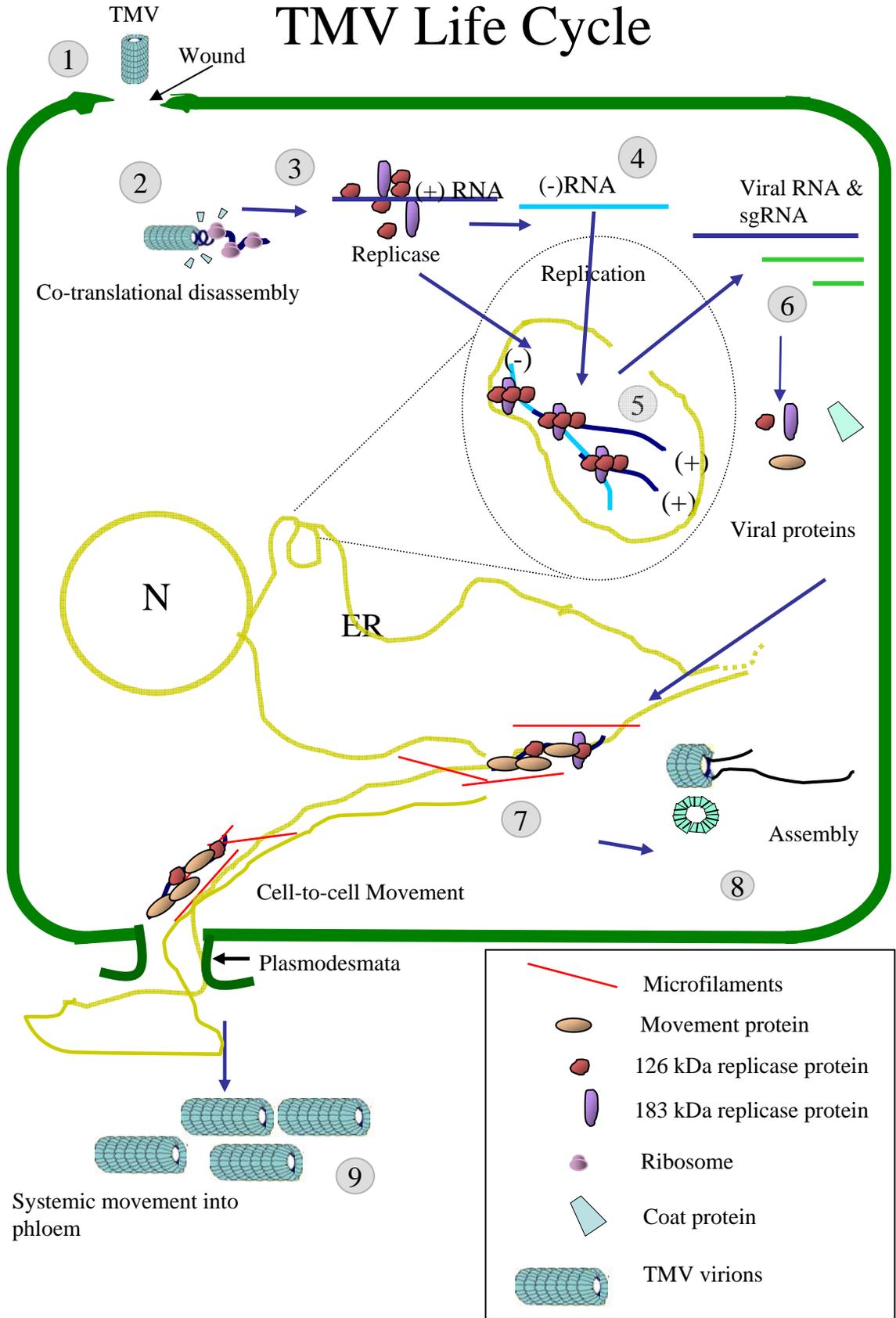
Viral life cycle

In addition to TMV's four known proteins, there are two other predicted open reading frames (ORFs) that may encode a 54 kDa protein and a 4.8 kDa protein, although these putative proteins have never been found in infected tissue [9, 10]. The RNA has a 5' methyl cap and a 3' tRNA-like end. The virion is a rigid, rod-like structure 300nm x 18nm in size, with 2130 coat protein subunits surrounding one genomic RNA molecule [11]. The virus enters the host cell via mechanical wounding (Figure 2) [12]. Once inside the cell, the change in pH and Ca^{2+} concentration causes the coat protein subunits to begin to disassociate [13]. Free ribosomes in the cytoplasm of the cell attach to the 5' untranslated region (UTR) of the viral RNA and undergo cotranslational disassembly, in which translation of the RNA facilitates disassociation of the coat protein from the RNA [14, 15].

The first proteins made are the replicase proteins. The 126 kDa protein is produced in tenfold greater amounts than the 183 kDa protein within the cell, due to the leaky amber stop codon [5]. (+)-strand RNA is transcribed to (-)-strand RNA and, from the (-) strand, genomic RNA and subgenomic (+)-strand RNA are produced. It is unknown whether negative-strand synthesis occurs in the cytoplasm of the cell or in other replication structures, possibly in association with biological membranes. The

Figure 2. The TMV life cycle. Adapted from [16]. *Tobacco mosaic virus* enters a host cell through mechanical wounding (1) Wounding of the plant cell allows for entry of TMV virions. (2) After entering the cell, the change in pH and calcium ion concentration causes the coat protein subunits to repel each other and exposes the 5' end of the genomic RNA. Ribosomes bind to the 5' end and displace the coat protein subunits via a process termed cotranslational disassembly. (3) Replicase proteins are translated from genomic RNA. (4) Replicase proteins transcribe (-)-strand RNA from genomic RNA, possibly in the cytoplasm. (5) Viral (-)-strand RNA and replicase proteins form replication complexes that are most likely associated with the host cell's endoplasmic reticulum. The model shown here, based on work by Paul Alhquist's group on *Brome mosaic virus*, depicts replicase complexes forming invaginations in the ER [17]. Positive-strand RNA, both genomic and subgenomic, would be transcribed and released into the cytoplasm. However, the exact mechanism of the formation of replicase complexes and their association with the ER is unknown. (6) Coat and movement proteins are translated from those subgenomic mRNAs in the cytoplasm. (7) Viral RNA and the protein complex moves cell-to-cell via cytoskeletal elements through the plasmodesmata. The movement protein allows for transfer of viral RNA and proteins through the plasmodesmata. (8) The viral RNA and coat protein assemble into mature virions. (9) The coat protein is required for long-distance movement through the phloem, where virions travel with the photosynthate to roots and developing meristematic tissue.

TMV Life Cycle



subgenomic RNA leads to production of the movement protein and the coat protein [18]. (-)-strand synthesis ceases six to eight hours post-inoculation (hpi) in tobacco protoplasts while (+)-strand synthesis continues for 16-18 hpi, suggesting that multiple (+)-strand RNAs are transcribed from one (-)-strand RNA [19].

The movement protein is required for cell-to-cell movement while the coat protein is required for long-distance movement through the plant phloem [20]. How the virus enters the phloem from companion cells is not well understood. After the virus enters the plant phloem, it travels as virions with the photosynthate to the newly developing meristematic tissue. At the cellular level, TMV replication occurs in close association with viroplasm in the cytoplasm. Both replicase proteins can be found in these viroplasms, as well as polyribosomes, tubule-like structures and endoplasmic reticulum (ER)-derived membranes [21, 22].

Movement protein

The movement protein (MP) is one of the most studied proteins of TMV and has been exploited to understand basic cellular mechanisms involved in cell-to-cell communication [23]. The MP is produced from its own subgenomic RNA and is produced later in infection than the replicase proteins. It is not required for replication, but is essential for virus spread, and has long been thought to be a key protein in localization and movement of viral RNA [23]. It is an integral membrane protein. The N- and C-terminal ends of the protein protrude from the same side of the membrane, presumably into the cytoplasm, but this has not been directly demonstrated. Two hydrophobic domains and 70 amino acids

protrude into the other side of the membrane [23, 24]. There is evidence that the C-terminal portions of adjacent MPs can associate with each other and dimerize into a structure that is now resistant to trypsin digestion [24]. This conformation leaves the N-terminal region free to associate with other proteins or viral RNA [25]. Which membrane the MP may be associating with has not been yet been shown, but may possibly be the ER based on localization of replication complexes [26].

During TMV infection, the MP can be seen in several areas in the cell, depending on the time course of the infection. At early stages of infection (4-6 hpi), the MP and viral RNA colocalize around the host cell's nucleus and in small aggregates near the plasma membrane. Later in infection, the MP can be seen associated with ER strands then large ER-derived aggregates [26]. Based on morphological changes to the ER and work done on another member of the alphavirus superfamily (*Brome mosaic virus*; BMV) the ER has long been accepted at the site of replication for TMV [21, 22, 27]. Interestingly, late in infection, the MP is no longer seen associated with ER-derived aggregates, but more predominantly with small aggregates in the cell membrane and cell wall which are indicative of plasmodesmata [26].

During infection, the MP has been shown to be phosphorylated and ubiquitinated [23, 28]. What roles these modifications play in viral infection remains unclear. In the presence of MP, TMV RNA forms linear strands, possibly forcing the viral RNA into a form that allows it to travel more easily in the plasmodesmata [29]. During infection, the TMV MP can associate with microtubules [30, 31] and viral RNA [32], lending support

to the idea that the MP may play an important role in the distribution of viral RNA within a cell and within a host. Additionally, BY-2 protoplasts (made from BY-2 tobacco suspension cells) late in TMV infection form finger-like projections from the plasma membrane that contain both viral RNA and MP, possibly indicating a role of MP and microtubules in cell-to-cell transport [26].

The MP of TMV has been shown to interact with two other host proteins, a resident cell wall protein, pectin methylesterase, and the Ca^{2+} signaling molecule, calreticulin [33, 34]. When pectin methylesterase expression was reduced, systemic TMV movement was severely delayed, indicating a possible role in viral long distance movement through the phloem [35]. Calreticulin was localized to plasmodesmata and disrupted both TMV MP localization and TMV cell-to-cell movement in over-expression transgenic plants, indicating a possible role in MP plasmodesmatal localization and TMV infection [34].

One of the interesting and most exploited aspects of the TMV MP is its ability to associate with plasmodesmata. In both TMV infection and MP-GFP-expressing transgenic *Nicotiana benthamiana* plants, the MP can be seen associated with plasmodesmata. In the presence of the MP, the size exclusion limit (SEL) as tested by microinjected dye, was increased 10-15-fold [36]. Interestingly, when this same experiment was repeated using a less harsh method of microinjection—ionophoresis—instead of increasing the SEL of cells, it decreased it [37]. The exact method of viral RNA and protein trafficking through the plasmodesmata and the nature of possible alterations to the plasmodesmata remain topics of active investigation.

Coat protein

The coat protein (CP) of TMV is also produced from a subgenomic RNA and expressed after the replicase proteins. The CP is not required for replication [38] or cell-to-cell spread of the virus, but is required for long-distance movement through the phloem, and helps to protect mature virions in the environment [11, 39]. TMV virions are extremely stable and have been known to persist in dead plants in the soil, in contaminated seeds, and even in infected tobacco leaves in cigars and cigarettes for years [40].

There are approximately 2130 CP subunits per virion, which assemble with both C- and N-termini exposed to the outside of the virion, while an interior channel is formed around viral RNA. In the host cell, the CP exists as several aggregates—a 20S form, a 4S form, and smaller aggregates of monomers, dimers, and trimers. There has been some controversy over the form of the 20S aggregate, as to whether it is a flat disk or in a helical form [11, 41]. During virion assembly, the RNA origin of assembly, near the 3' end, associates with the 20S aggregate and initiates assembly [42]. Once bound to the RNA, the 20S aggregate has been shown to be in the helix form, in what has been termed a lock washer formation [41, 43]. The virion assembles in both the 3' and 5' direction; assembly in the 5' direction is rapid and consists of subsequent additions of 20S aggregates, while assembly in the 3' direction is much slower and is thought to include incorporation of smaller CP aggregates [42].

Disassembly of the virion is triggered by both a change in Ca^{2+} concentration and a change in pH. Between adjacent CP subunits on the virion, negatively charged

carboxylate groups repulse each other in the absence of positively-charged ions [44, 45]. These interactions are stabilized by Ca^{2+} ions and protons outside of the cell, but which are sequestered out of the cytoplasm in a host cell. The absence of these ions causes the subunits to repulse each other, but not sufficiently to disassemble the virion. Rather, the weakest interaction with the viral RNA is at the 5' RNA end and, during disassembly, this end becomes exposed to free ribosomes in the cytoplasm. The ribosomes bind and as they progress along the RNA, they push off the CP subunits. This process has been called cotranslational disassembly [13]. It has also been suggested that once the replicase proteins, the first to be translated, are present, they bind and assist in disassembly in the 3' to 5' direction [15].

During the late 1980's a phenomenon known as coat protein-mediated resistance (CP-MR) was discovered. Tobacco plants stably transformed with the TMV CP were resistant to subsequent TMV infection [46]. Work focused on whether the mechanism was RNA- or protein-mediated. Earlier work from Jim Culver's group and recent work from Roger Beachy's group has shown a correlation between the ability of the CP to assemble into virions independent of RNA binding and the ability to protect against TMV infection [47-49]. Based on mutation studies, the more able the CP is in assembling into virions, the greater the cross-protection becomes. Asurmendi et al. suggest that the mechanism of CP-MR may be in creating such an excess of CP that can bind TMV RNA that disassembly becomes energetically unfavorable [48].

During infection, the CP alters the formation of viral replication complexes, both in their composition and in their size during infection [50]. While CP is not required for cell-to-cell movement, it contributes to it and is involved in symptom development [38]. Studies have shown that CP accumulation in chloroplasts correlates with chlorotic symptoms [51].

Replicase proteins

The replicase proteins are the first viral proteins translated during infection and are multi-domain, multi-function proteins [11]. They contain regions for methyltransferase and helicase activities; only the 183 kDa protein contains an extra RNA-dependent RNA-polymerase domain. Both proteins display methyltransferase-derived guanylyltransferase-like activity that results in the capping of viral RNA. RNA polymerase activities have been shown from purified replication complexes that are template-dependent and template-specific [52].

Viral helicases have been divided into three superfamilies (SFs) based on seven conserved motifs (I, Ia, II, III, IV, V, VI). The TMV helicase belongs to helicase SF1, which is also known as the alphavirus-like supergroup [53, 54]. SF1 helicases contain all but the Ia and IV domains. Based on several mutational studies and x-ray crystallography, all seven helicase domains are involved to varying degrees in ATP binding and hydrolysis. The first two motifs, I and II are involved in NTP-binding and Mg⁺ ion chelation [53, 55]. Motifs Ia and III are the least characterized and have not been assigned a potential function. Motif V has been shown to have nucleic acid binding activity [55] and motif VI has been shown to have ATPase activity [53]. The TMV

helicase domain has been shown to unwind duplexed RNA and displays NTPase activity as well as RNA binding activity *in vitro* [56]. The helicase domain probably acts in two different steps of replication: the first is to unwind RNA duplexes that may form during RNA synthesis; the second is to unwind RNA secondary structure on (-)-strand that may inhibit (+)-RNA synthesis [54].

Both replicase proteins have been shown to be required for efficient replication. When the amber stop codon in TMV is mutated to a hard stop codon, producing only the 126 kDa protein, the virus is unable to replicate. When the amber stop codon is mutated to a tyrosine codon, producing only the 183 kDa protein, the virus replicates to 20% of wild-type levels with protein production reduced and delayed, indicating that the 126 kDa protein is required for efficient viral replication [57]. Since the 183 kDa protein is able to cap viral RNAs without the 126 kDa protein and has all three functional domains, it suggests that there may be additional roles for the 126 kDa protein other than replication of genomic RNA.

The 126 kDa protein is produced in tenfold excess over the 183 kDa protein, but in replication complex purification, the two proteins are found in equal proportions [58]. Recent work has indicated that the replicase proteins may assemble into large-molecular-weight complexes such as hexamers *in vitro*, but it is unknown how replicase complexes assemble *in vivo* [56]. It is also unknown why the 126 kDa protein is produced in such excess compared to the 183 kDa protein, since some viruses, such as *Potato virus X* (PVX), produce only one replicase protein, which is the equivalent of the 183 kDa

protein [59]. One possible explanation has been provided by work indicating that the 126 kDa protein is a suppressor of gene silencing [8]. Recent work has shown that the replicase proteins can bind small RNA duplexes of about 19-25 nucleotides in size, which are typically produced as part of a host defense response against viral pathogens [60].

Putative proteins

There are two putative ORFs that may encode a 54 kDa protein and a 4.8 kDa protein, although these putative proteins have never been seen in infected tissue [9, 10]. The 54 kDa protein has a possible ORF in the readthrough portion of the replicase ORF and has been expressed *in vivo*. When this protein was expressed via transgenic *Nicotiana tabacum* plants, the transformed plants were resistant to TMV infection [10].

The ORF for the putative 4.8 kDa protein lies between the movement and coat protein ORFs. When the 4.8 kDa protein is expressed via either PVX or *Tobacco rattle virus* (TRV) expression vectors, it localizes to the plasmodesmata and seems to enhance virulence when later infected with wt TMV [9].

Untranslated regions (UTRs)

The UTRs of the TMV genome and subgenomic RNAs have been shown to be essential in replication and translation of the virus; both UTRs have been shown to be independent translational enhancers. The UTRs both act in a similar way to host cell 3' poly A tail and 5' methyl cap and provide template specificity for the replication complex [61].

The 3' terminus contains a tRNA-like structure that contains pseudoknots, unlike a true tRNA, and can be aminoacylated by histidine aminoacyl-tRNA synthetases and adenylated by tRNA nucleotidyltransferases. After aminoacylation, the tRNA-like end can bind elongation factors more efficiently [54]. Interestingly, in chimera experiments, it was shown that TMV could replicate RNA containing the 3' terminal region of a closely related virus, BMV, albeit less efficiently, but BMV could not replicate RNAs with the TMV 3' terminal region [62]. However, BMV could replicate RNAs with 3' terminal regions from *Cucumber mosaic virus* (CMV). Taken together, these data suggest that replicase complexes may recognize general 3' terminal structures, but there are other determinates of template specificity [54].

The 5' terminus of TMV contains a 7-methylguanosine cap and several highly conserved CAA repeats that serve as translational enhancers [61]. Large deletions (nucleotides 9-47 or 25-71) within this region result in replication reduced to below detectable levels. Additional deletions showed that only a deletion of nucleotides 2-8 was required to abolish replication [63]. The heat shock protein (HSP) HSP101 has been shown to interact with, and be required for, the 5' UTR-mediated translational enhancement. Additionally, the eukaryotic initiation factor eIF4F was specifically required for 5' UTR translational enhancement [61].

Host-protein interactions

As with all (+)-strand RNA viruses, TMV requires host membranes to replicate. Other related viruses also utilize and cause significant changes in the host's membranes. For example, another member of the Alphavirus superfamily, *Semliki forest virus*, requires

cholesterol in host membranes for infection [64]. BMV, another member of the Alphavirus superfamily, replicates on the ER, has been seen in small, spherical invaginations in the membrane, and is also sensitive to changes in cellular cholesterol content [65]. Purification and functional assays of the TMV replicase proteins have indicated that template-dependent transcription is much more efficient while in association with a membrane than not, indicating that host membranes are required for efficient replication [52]. TMV has been shown to associate with the host cell's ER and replicate in close association with the organelle, but more work is needed to examine the structures that are formed [21, 66]. It has been suggested that viral movement occurs through intact replicase complexes that traffic together from cell-to-cell [67].

Host proteins have been shown in complex with TMV replicase proteins; one specific host protein found is the EF1- α protein [68]. EF1- α assists ribosomes in binding new aminoacyl tRNAs. Also, translation initiation factor eIF3 was shown to copurify with replicase complexes [69]. Interestingly, it has been shown that the 126 kDa replicase protein interacts with microfilaments, which may provide a mechanism of replication complex movement [70]. Another host protein, TOM1 (tobamovirus multiplication protein), was identified through a yeast two-hybrid interaction and has seven transmembrane domains, although its normal cellular function is unknown. When TOM1 and the related protein Tom-3 (also identified in a similar screen) were knocked out, TMV was no longer able to replicate in the host to detectable levels [71]. TOM1 is thought to act as a membrane tether for the replicase complex. An auxin-responsive protein, PAP1/IAA26, was identified in another yeast two-hybrid screen and was shown

to interact with the 126/183 kDa helicase domain and modulate symptom development, but TMV was still able to replicate and move at a rate close to wild-type levels when the interaction was severely reduced [72]. Recent evidence suggests that the PAP1/IAA26 degradation in infected older tissues may help to make the cells more active and a better host for the virus [73].

The helicase domain also interacts with the host protein, P58^{ipk} (an inhibitor of dsRNA-activated protein kinase), in *Arabidopsis* and *Nicotiana benthamiana* plants. P58^{ipk} inhibits double-stranded RNA-mediated defense responses in animals, but does not appear to have this function in plants. Intriguingly, in P58^{ipk}-silenced or knocked-down plants, TMV infection causes host plant death. This interaction appears to be a virulence factor that allows the virus to replicate without triggering host death in a wild-type infection [74].

Abbink et al. found that the replicase proteins interact with two other proteins: a 33 kDa component of the oxygen-evolving complex of photosystem II and a member of the AAA family (ATPases associated with various activities). The authors found that when they silenced the 33 kDa protein, it resulted in a ten fold increase in TMV accumulation, while silencing the AAA protein resulted in a two-fold reduction in TMV accumulation [75].

The function of these proteins during infection is not well understood, but CP accumulation in chloroplasts has been associated with chlorosis and symptom development during infection [11].

The helicase domain has also been shown to be important in eliciting a defense response in tobacco. The region between nucleotides 2082 and 3418, which includes the helicase domain and upstream sequences, produces a 50 kDa protein (p50) that was shown to be the elicitor in TMV of N-gene-mediated defense response in tobacco [76]. The N-gene is a defense gene isolated from tobacco that elicits a strong hypersensitive type response to TMV infection. The hypersensitive response and other defense responses are discussed in greater detail in the following sections. A direct protein-protein interaction was found between the helicase domain and the N gene which was ATP-dependent, and work is continuing in other labs to identify proteins that interact with both the N gene and p50 [77, 78].

TMV-host interactions			
Viral protein	Host protein	Host protein function	Citation
126 kDa replicase	eIF3	Translation initiation factor	[69]
126 kDa replicase	EF1- α	Translation elongation factor	[68]
Helicase domain; replicase proteins	N gene	Defense gene against TMV; elicits HR	[76]
Helicase domain; replicase proteins	P58ipk	Inhibitor of dsRNA-activated protein kinase	[74]
126 kDa replicase	Actin (microfilaments)	Cytoskeleton	[70]
126 kDa replicase	TOM1, TOM2a	Unknown	[79]
126 kDa replicase	PAP1/IAA26	Auxin responsive factor	[72]
126 kDa replicase; helicase	33k subunit of oxygen-evolving complex	Part of photosystem II; water-splitting enzyme	[75]
126 kDa replicase; helicase	GenBank #AF26837	AAA ATPase	[75]
5' UTR	HSP101	Heat shock protein	[61]
Movement protein	Microtubules	Cytoskeleton	[32]
Movement protein	Plasmodesmal-associated protein kinase (PAPK)	casein kinase I family	[80]
Movement protein	Pectinmethylesterase	Cell wall; plant growth and development	[33]

Table 1. Known proteins that interact with *Tobacco mosaic virus*.

Pathogens and vesicle trafficking

Vesicle trafficking has increasingly become an area of active research for plant pathologists in the past several years. One of the first discoveries to spark more interest in vesicle trafficking was the finding that members of the superfamily of N-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE) polypeptides limit entry by powdery mildew in both monocots and dicots. Later work showed that syntaxin proteins, or t-SNAREs, are involved in disease resistance. The syntaxin proteins, AtSYP122 and AtSYP132, are phosphorylated in response to bacterial elicitors of host defense responses and have been implicated in exocytosis of a subset of pathogenesis-related (PR) defense proteins in *N. benthamiana* [81]. The syntaxin AtSYP121, when mutated, showed enhanced invasion by grass powdery mildew fungus, *Bumeria graminis* [82]. AtSNAP-33, another syntaxin, was shown to increase in expression when infected with bacterial pathogens, *Plectosporium tabacinum*, *Peronospora parasitica*, and *Pseudomonas syringae* pv tomato. This up-regulation was simultaneous with increased expression of pathogenesis-related protein 1 (PR1) [83]. Also, the rice small GTPase, *rgp1*, conferred resistance to TMV when expressed in tobacco plants, and has been implicated in the Hypersensitive Response [84]. The groundnut rosette virus movement complex (composed of viral movement proteins, viral RNA, and possibly host and viral proteins) has been found to associate with a Rab-like protein, and *Cauliflower mosaic virus* movement protein interacts with a Rab acceptor homologue [85].

Vesicle trafficking has also been shown to be important in animal defense responses as well as plants; Arf1 GTPase, which is an essential GTPase in vesicle trafficking, has been

shown to interact with polio proteins 3A and 3CD. Its abundance increases four fold during infection and becomes mostly membrane-bound [86]. For the enveloped viruses—*Human immunodeficiency virus type I*, Ebola, Marburg, and measles—Rab 9 which is involved in Golgi trafficking, is essential for replication [87]. Interestingly, the intracellular pathogen, *Legionella pneumophila*, produces a protein, DrrA, which acts both as a Rab guanosine exchange factor (GEF) and Rab guanosine dissociation factor (GDF) to recruit and activate Rab 1 to ER-derived vesicles [88].

Rab GDP Dissociation Inhibitors

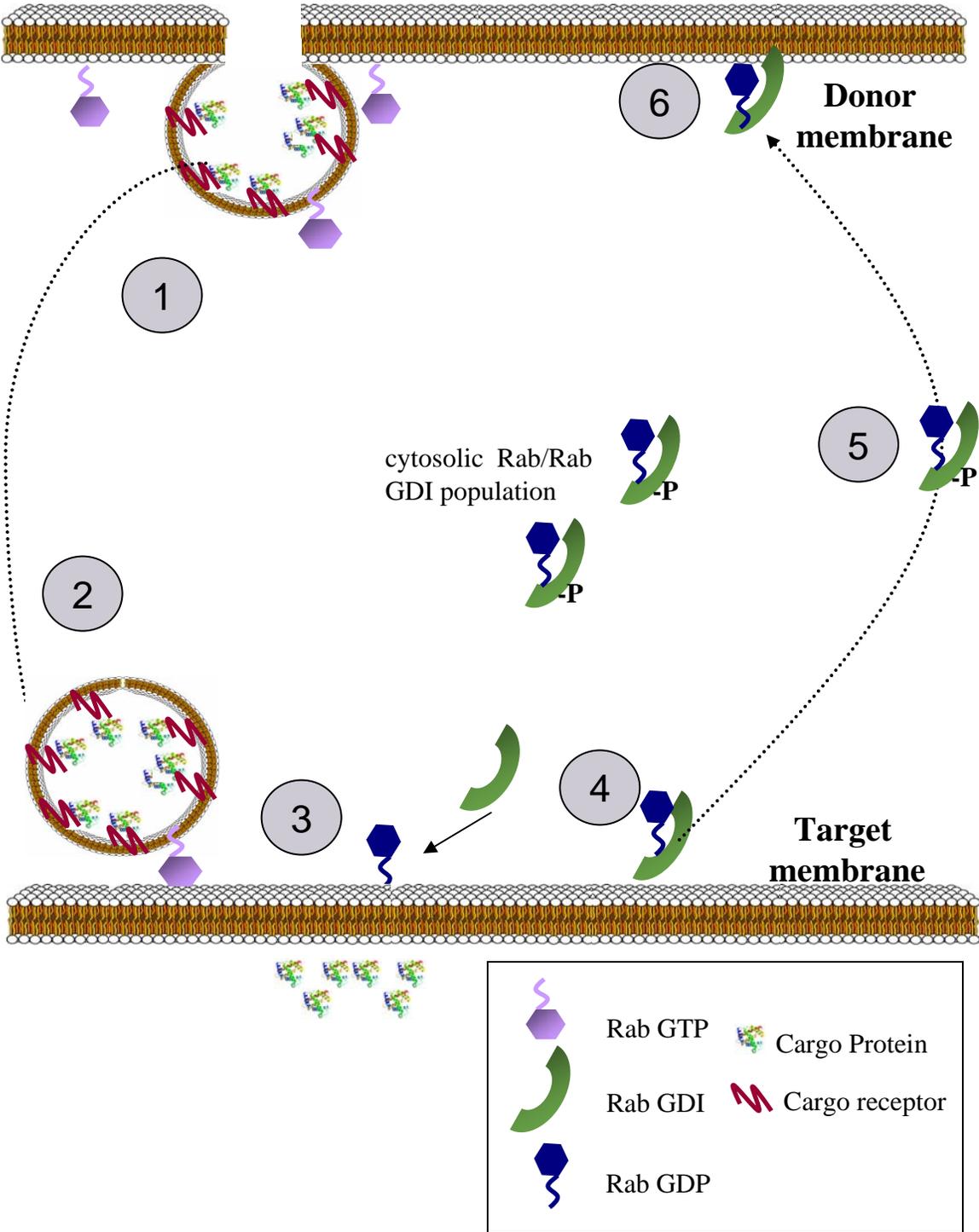
Rab GDP Dissociation Inhibitors (GDIs) regulate the activity of Rab proteins. Rab proteins belong to the family of Ras-like small GTPases and are essential regulators of vesicle trafficking within a cell. Rab proteins play a role in everything from secretion and endocytosis to signal transduction and development. Since they were first discovered in the 1980s, they have been shown to be important regulators of vesicle budding, cargo recruitment, and vesicle tethering and fusion in transport. Rabs have also been implicated in organizing functionally distinct subdomains within membranes, defining organelle identity, and functioning in mitogenesis [89, 90].

Rab proteins form the largest in the family of Ras-like small GTPases, with 11 proteins in yeast, over 60 different Rab proteins in humans, and 57 identified in *Arabidopsis thaliana* [91, 92]. Many of these proteins are specific to certain cell types. Rab proteins are peripheral membrane proteins due to two geranylgeranyl groups added post-translationally, and spend the majority of their time oscillating from the inactive GDP-bound form to the active GTP-bound form. Interestingly, defects in Rab prenylation in

mammals cause the degenerative disease choroideremia, which eventually results in complete blindness [91]. When activated on the donor membrane, Rab proteins will help to recruit cargo proteins and assist in vesicle formation [93]. During trafficking, Rabs may participate by interacting with motor proteins and later assist in vesicle docking and fusion [94]. During fusion, the Rab proteins with the help of Guanine Nucleotide Exchange Factors (GEFs) will hydrolyze GTP to make GDP. Once in their GDP-bound state in the target membrane, they become a target for another set of proteins, Rab GDIs. Rab GDIs only have a high affinity for Rab proteins in their GDP-bound state. Rab GDI proteins stabilize the highly hydrophobic geranylgeranyl groups and allow Rab protein-mediated removal from membranes [93]. Rab GDIs seem to have very little specificity for particular Rab proteins; there are many fewer types of Rab GDIs (two in Arabidopsis) than Rabs (57 in Arabidopsis), and Rab GDIs have been shown to bind to and extract several different Rabs [95]. Once Rab GDIs have bound Rab proteins and are not membrane-associated, they become phosphorylated, possibly to prevent rebinding with the target membrane [96]. Rab GDIs deliver the Rab proteins back to the donor membrane where GDI displacement factors (GDFs) catalyze the dissociation of a Rab from its Rab GDI and promote recruitment onto membranes (Figure 3). Interestingly, Sivars et al. showed that in a mammalian system, Yip3 selectively acted as a GDF, selectively

Figure 3. The role of Rab GDI in vesicle trafficking. During normal vesicle trafficking, Rab GTP proteins assist with the formation of vesicles (1) and may help in movement via motor proteins to the target membrane (2). Once at the target membrane, the Rab GTP is hydrolyzed to GDP (3). In its GDP form, Rab proteins now become a target of Rab GDI proteins, which bind Rab GDP, extract them from membranes, and keep them in the inactive, GDP form (4). Rab GDIs are phosphorylated after binding to their target Rabs, possibly to keep from reassociating with the membrane target membrane. Interactions with other proteins, such as guanosine exchange factors (GEF) and guanosine activating proteins (GAP), may assist Rab GDP proteins in attaching to the correct donor membrane (5). Once in the correct donor membrane, GEF proteins exchange GDP for GTP and the Rab protein is able to start the cycle anew (6).

Role of Rab GDI in vesicle trafficking



displacing Rab9 and Rab5 from their Rab GDI. It is possible that GDFs help to add specificity to Rab protein localization to promote vesicle formation [97]. After Rab proteins dissociate from their GDI, guanosine exchange factors (GEFs) exchange GDP for GTP and activate the Rab proteins again to start the cycle of vesicle formation and transport. This Rab-GEF interaction may give additional specificity for Rab protein localization since each GEF is specific for a particular Rab; however, seldom are GEFs themselves membrane proteins, rather requiring recruitment by other proteins [98]. Interestingly, Rab GDIs, themselves seem to be regulated by chaperone proteins; Sakisaka et al. report isolating Hsp90 from complexes containing Rab- α GDI in rat synaptosomes and suggest a role in regulating Rab- α GDI [99].

Plant defense responses

Plants have developed ways of defending themselves from colonization by pathogens, some of which are derived from ancient defense mechanisms that persist in such evolutionarily distant organisms as humans and fruit flies. The first step in a defense response to pathogens is recognition of the pathogen. The plant can then respond using several different mechanisms, such as a hypersensitive response (HR), programmed cell death (PCD), activation of viral-induced gene silencing (VIGS), activating systemic acquired resistance (SAR), and general physiological mechanisms, including thickening of the cell wall, closing of plasmodesmata, and release of reactive oxygen species (ROS).

There are two major hypotheses for how plants detect pathogens. The first is the gene-for-gene hypothesis, which argues that for each pathogen that the host mounts a defense response, some pathogen-derived virulence (AVR) gene product interacts with a specific

receptor or other host resistance (R) protein. This interaction causes various physiological responses (discussed below) to try to contain or stop the infection [100]. The alternate hypothesis, or guard hypothesis, is that certain host proteins may be targets for pathogens or may be essential for pathogens. These host proteins are called “guards” whereby when altered, they or a target protein can trigger a defense reaction [101]. Since very few AVR proteins have been shown to interact directly with R proteins, current data suggest the role for other proteins or “guard” proteins.

Resistance (R) genes

R-genes in both monocots and dicots have a strong similarity in their basic structure. The most common type of R gene produces an intracellular protein with a nucleotide-binding leucine rich repeat (NB-LRR) with a variable N-terminal region. Other types of R proteins include a serine/threonine protein kinase group, and a transmembrane protein with an extracellular LRR and an intracellular C-terminal domain that may contain a kinase. The NB-LRR proteins contain an additional leucine zipper domain or a toll-like interleukin-1-like (TIR) domain. The Pto protein from tomato falls into its own category with no membrane-spanning region, but just contains a protein kinase domain. A few of the more recently discovered R proteins such as Arabidopsis RPW-8 (coil-coil domain and membrane domain) and HM1 (toxin reductase) of maize do not fall into any of the above categories, but are unique.

Responses to R gene activation

One of the first responses to R gene activation is an increase in cytosolic Ca²⁺ activation which results in the phosphorylation of NADPH oxidase, leading to the production of

reactive oxygen species (ROS) such as O_2^- and H_2O_2 . This oxidative burst leads to the cross-linking of cell walls, the triggering of the cell death pathway, and the production of defense-related genes. This cellular response that results in cell death and necrotic lesions is known as a hypersensitive response (HR) [102, 103].

The increased Ca^{2+} concentration and ROS can also activate a signaling cascade that activates salicylic acid (SA). SA is involved in disease resistance both at the site of infection and in SAR. SAR is a broad-spectrum resistance to pathogens that is seen in the parts of the plant that have not been inoculated with the pathogen. SA results in the up-regulation of PR proteins and promotion of ROS production, both of which help to establish a strong defense response to pathogens. It has long been thought that SA acts as a signal in SAR to the rest of the plant and triggers the systemic resistance [104]. Recent evidence suggests this is not the case, but rather SA is converted to methylsalicylate in un-inoculated tissues, giving methylsalicylate the role of a mobile SAR signal [105]. Based on work with DIR1, a lipid transfer-like protein, and SFD1, which is involved in lipid metabolism, there is also a lipid based molecule that also acts with or upstream of the methylsalicylate signal [106, 107].

Viral induced gene silencing (VIGS)

Viral induced gene silencing (VIGS), or post-transcriptional gene silencing, is a general and specific defense response against viral pathogens. This mechanism is widely conserved with pathways discovered in humans, *Caenorhabditis elegans*, and fungi. During VIGS, dsRNA structures trigger a response by the RNase, Dicer, which cuts dsRNA into 21-25 nt segments. These small RNA segments are called small interfering

RNAs (siRNAs). These RNAs are then incorporated into a multi-protein RNA-induced silencing complex (RISC). The RISC complex uses the siRNA to target viral RNAs for degradation. An additional mobile silencing signal is produced, which results in systemic resistance to the inoculating virus. This silencing signal has not yet been identified, but due to the continued resistance specificity for the inoculating virus, the siRNAs must still be involved to direct degradative enzymes to the viral RNA [108].

Gene silencing has been shown to be an important defense target of viruses. Numerous viruses, including TMV (see below), encode proteins that act as suppressors of gene silencing. The suppressors act in several different steps of VIGS. P1/HC-Pro protein of potyviruses was one of the first silencing suppressors discovered and experiments suggest that it affects the assembly, or targeting, of the RISC complex. The p19 protein of tombusviruses have been shown to directly interact with siRNAs and possibly prevent their incorporation into the RISC complex. The 2b protein of cucumoviruses prevents the systemic spread of RNA silencing as does p25 of *Potato virus X* (PVX). The coat protein of carmoviruses acts to interfere with the processing of dsRNA, and large complex viruses such as Citrus tristeza virus appear to encode several suppressors of gene silencing that act in different steps of the pathway [109]. The 126 kDa replicase protein of TMV has been shown to bind siRNAs and prevent their incorporation into the RISC complex [60].

Defense responses to TMV

The N gene is a NB-LRR R protein that is specifically activated in response to TMV infection. The helicase domain of TMV replicase proteins is the elicitor of N gene

activation. The N gene from *Nicotiana glutinosa* confers resistance to all known tobamoviruses except Ob [76, 110]. In the absence of the N gene, the virus spreads throughout the plant, causing the classic dark green-light green mosaic pattern. However, in the presence of the N gene, the hypersensitive response (HR) is triggered within 48 hours of infection and the virus is restricted to the region immediately surrounding the necrotic lesion [76]. In the presence of the N gene, systemic acquired resistance (SAR) is also triggered via salicylic acid (SA). When TMV-infected tissue was exposed to exogenous SA, TMV was restricted to inoculated tissues, which was not seen when the same assay was used with *Cucumber mosaic virus* (CMV) [111]. Additionally, the restriction of TMV movement was a mechanism independent of plasmodesmal restriction. siRNAs are produced during TMV infection, though as mentioned above, it was recently shown that TMV replicase proteins bind duplexed 21-25 nt RNAs and can possibly prevent their incorporation into the RISC complex [60].

Research objectives

The primary objective of this work was to develop an understanding of the role of virus-host interactions during infection specifically using the TMV replicase proteins. My first objective was to determine what domain, or domains, within the replicase proteins might be responsible for their localization within the host cell during infection. The replicase proteins contain an N-terminal nuclear localization signal (NLS), but viral replication occurs in close association with the host's ER [112]. A domain in the intervening region (IR) of the replicase proteins was identified that was able to localize the replicase proteins to the ER. This localization was conferred to another protein when it was fused to the C-terminal end. Point mutations were created to determine if an established intramolecular

interaction within replicase proteins might also contribute to localization and viral replication, but no direct correlation between localization and replication could be established by the point mutations.

My second objective was to investigate an interaction between a host protein Rab GDI and the replication proteins. This interaction was confirmed with tobacco, Arabidopsis, and tomato Rab GDI proteins and was shown to be important in establishing viral infection in a host and may provide a mechanism for host protein synthesis shutdown which is characteristic of many viral infections. The replicase proteins were shown to interact with a Rab GDI *in vivo* and to alter its expression and localization. Work towards my second objective has also established changes to a host cell's endomembrane system during infection, some of which can be mimicked by knocking-down Rab GDI protein expression. This work is currently being prepared for publication.

Chapter 2: Characterization of a domain required for ER localization of TMV replicase proteins

Abstract

Tobacco mosaic virus is a (+)-strand RNA virus and the type member of the tobamovirus family. While the TMV replicase proteins contain a functional N-terminal nuclear localization signal, replication occurs in close association with membranes derived from the endoplasmic reticulum (ER) [26]. A possible ER localization motif between amino acids 388 and 781 was previously identified within the replicase proteins [112]. The following studies narrow and characterize this region involved in the replicase proteins' localization.

A region between amino acids 599 and 701 was found to be necessary and sufficient for ER localization of the replicase proteins. Addition of this domain to the β -glucuronidase (GUS) protein allows for localization outside of the nucleus in transient expression assays. To characterize the effects of this ER localization domain on virus infection, nine mutations were introduced into this region that changed the amino acid translated to alanine. These mutants were tested for localization with transient expression assays, for an intramolecular interaction via a yeast two-hybrid assay, for replication in protoplast assays, and for replication and movement in local lesion assays.

All of the mutants showed a decrease in intramolecular interaction and several mutants show a reduction in replication and movement; however, only V696A lost its localization

outside the nucleus. This change in localization does not correlate with an effect on viral replication and movement. In contrast, the L605A mutant shows a severe decrease in replication and movement, but very little alteration in protein localization. Several possible mechanisms for this localization domain are discussed; further work is necessary to characterize fully the function of this domain.

Introduction

Replicase complexes, or “viral factories,” are composed of host proteins, viral proteins, viral RNA, and typically host membranes. Formation of these complexes and the nature of their localization are poorly understood because biological membranes and replicase proteins have proven difficult to manipulate. The role of membranes has become a topic of interest as evidence has shown that lipids and membrane proteins are important in replicase complex formation [17, 65, 113]. For (+)-strand RNA viruses, biological membranes are required for efficient replication; the mechanism of replicase complex association and formation represents an avenue to characterize viral replication and develop possible targets for disease resistance.

TMV is a model (+)-strand RNA virus and the type member of the tobamovirus family. The genome encodes two replicase proteins, a 126 kDa protein and a 183 kDa protein. Both proteins contain methyltransferase and helicase regions, while the 183 kDa protein contains an additional RNA-dependent RNA polymerase domain. Both proteins also contain an intervening region (IR) of unknown function between the methyltransferase and helicase domains. The TMV replicase proteins have been shown to be important in

not only genome duplication but cell-to-cell movement [114], cytoskeletal association [70], and as suppressors of gene silencing [8].

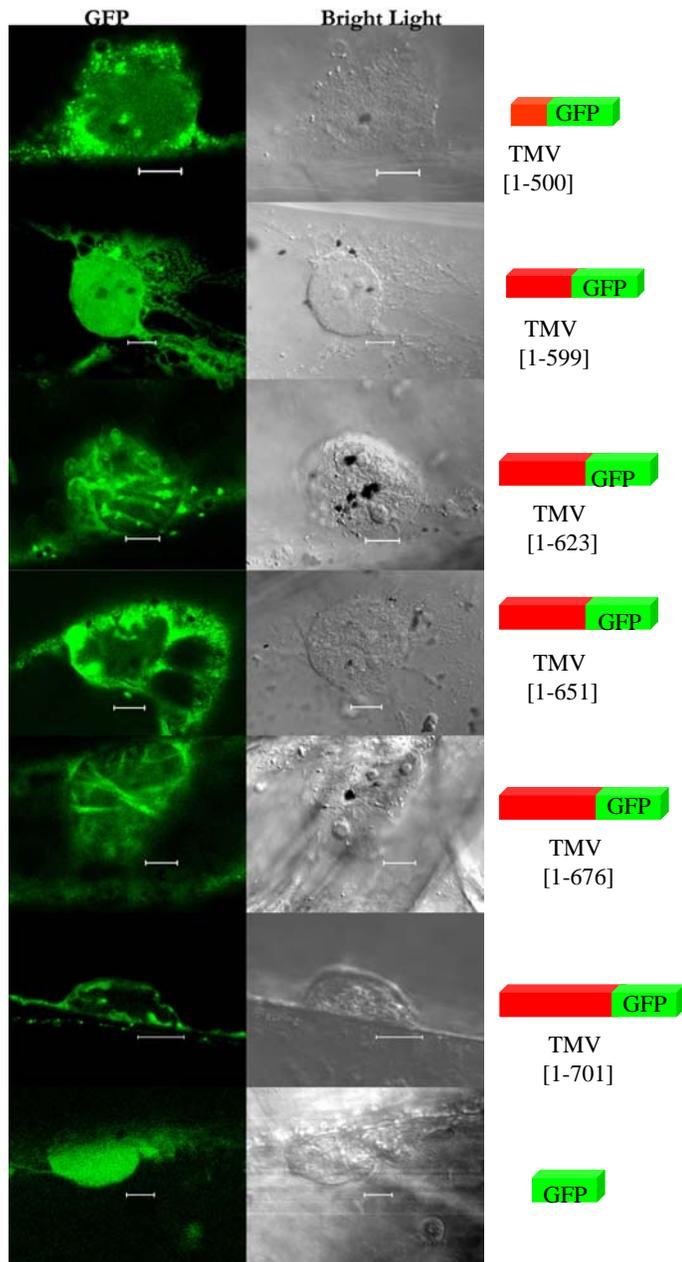
During TMV infection, endoplasmic reticulum (ER)-derived membranes have been shown to be involved in and severely modified during replication. Positive-strand viral RNA, (-)-strand viral RNA, and replicase proteins have been shown to associate with ER derived membranes [26]. Late in infection, large structures known as viroplasms, or x-bodies, are seen that contain host proteins, viral proteins, and viral RNA [21]. The replicase proteins have been shown to localize to the endoplasmic reticulum of host cells independently of the rest of the virus, but also to contain a functional N-terminal nuclear localization signal (NLS). Initial deletion experiments have identified a possible ER localization motif in the IR region [112]. The following study further narrows the ER localization domain and examines the effect this domain has on replication, localization, an intramolecular interaction, and viral movement.

Results

Identification of an ER localization domain

To identify this ER localization motif more closely, a series of sequential deletions of this region were created and transiently expressed in onion epidermal cells via gene bombardment. The protein localization switches from outside the nucleus to localizing inside the nucleus in the deletions between aa 599 and 701 (Figure 4). Further deletions of this region were made, but were inconclusive in their localization (deletions 623, 651 and 676). When all of these amino acids (599-701) were deleted

Figure 4. Identification of sequences important in ER localization. C-terminal Green Fluorescent Protein fusion constructs were made using the pCMC1100 vector [112] which contains a single 35S promoter and a NOS terminator sequence. Fusion proteins were transiently expressed in onion epidermal cells via gene bombardment and observed 12-16 hours post-bombardment. Images were visualized using a Zeiss LSM510 laser scanning confocal microscope system with a 63x NA 1.2 water-immersion lens (Carl Zeiss Inc., Thornwood, NY). Enhanced GFP (eGFP), a GFP variant, constructs were excited at 488 nm and emissions were collected at 543 nm. Bars indicate 10 μ m. Shown are representative images of several independent experiments. Onion cell nuclei are shown with bright-light images on the right and the corresponding emissions at 543 nm on the left. To the far right are diagrams of the constructs used in those images. Numbers indicate the amino acid number deleted from the 126 kDa replicase protein. Sequential deletions from the 126 kDa protein were made to identify a possible ER localization domain. Note that the localization changes from the 599 to the 701 construct.



from the full-length 126 kDa replicase protein, localization changed from small aggregates which were previously shown to associate with the ER [112], to a dispersed localization inside the nucleus (Figure 5a). When this same domain was added to the β -glucuronidase (GUS) protein with the TMV NLS at the N-terminus, the protein was retained outside of the nucleus (Figure 5b).

Creation of mutations

To determine the mechanism of the newly narrowed ER localization domain, nine point mutations were introduced into this region of the replicase proteins. One double mutant was created, for a total of ten mutants. Amino acids 604, 605, 606, 607, 619, 696, 697, 699 were mutated based on strong similarity to other related tobamoviruses with similar host ranges (Figure 6 and 7). Amino acid 650 was mutated because it lacked strong similarity to related tobamoviruses. A tenth mutant had mutations at both amino acids 696 and 699. All mutations converted the original amino acid to alanine, either by single or double point mutations.

Local lesion assays

To determine the biological effect of these point mutations, they were introduced into viral cDNA. Infectious RNA was transcribed and rub-inoculated onto a local lesion host, *Nicotiana tabacum* cv xanthi NN. This cultivar contains a functioning N gene and produces a hypersensitive response at the site of infection. The size of the lesions produced is indicative of replication and movement of the virus. Local lesion assays are similar to plaque assays in mammalian cell lines. Half of the leaf was inoculated with infectious RNA from a mutant virus and the other half was inoculated

Figure 5. The ER localization signal is required for 126kDa localization and acts dominantly over the NLS. C-terminal Green Fluorescent Protein fusion constructs were made as previously described in Figure 4. Fusion proteins were transiently expressed in onion epidermal cells and imaged 12-16 hours post-bombardment. Bars indicate 10 μ m.

a) Full-length 126 kDa replicase protein fused to eGFP as well as the 126 kDa protein with an internal deletion fused to GFP. The region deleted (amino acids 599-701) corresponds to the range identified by Figure 4. The wt protein localizes to small aggregates outside the nucleus while in the absence of the 102-amino-acid sequence, the full-length construct localizes to the nucleus.

b) Fusion constructs were made to determine if the ER localization domain could act dominantly over the NLS. GUS, GUS with the NLS from TMV, and GUS with the NLS and the putative ER localization domain were transiently expressed. In the presence of the NLS, the fusion protein localizes to the nucleus while with the addition of the ER localization domain the fusion protein stays outside the nucleus.

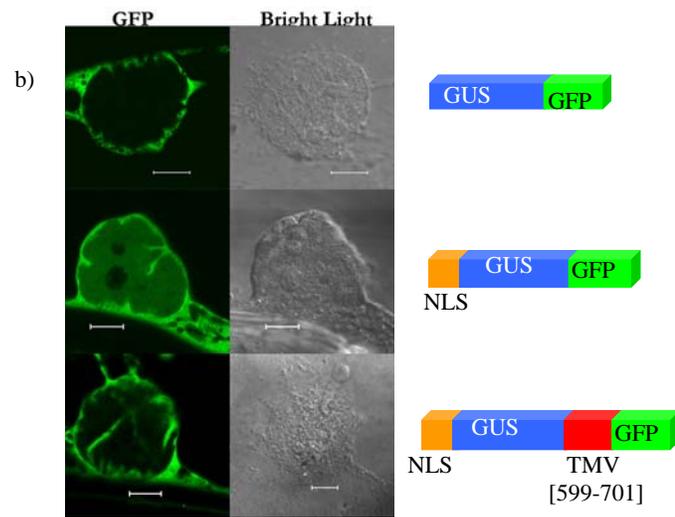
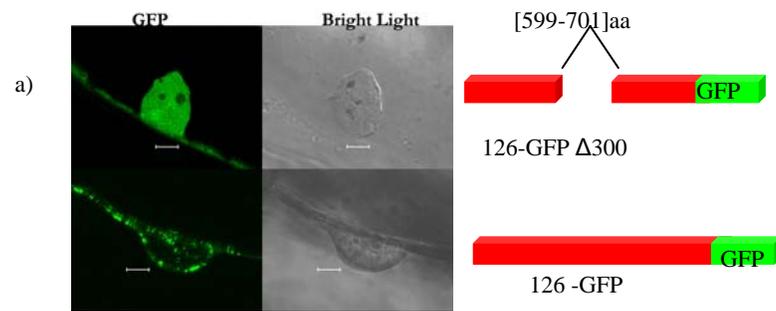


Figure 6. Alignment of replicase protein intervening regions. Shown is an alignment of the tobamovirus replicase proteins in the region between the methyltransferase and helicase domains. Abbreviations: TGMMV, *Tobacco green mild mottle virus*; ToMV, *Tomato mosaic virus*; TMV, *Tobacco mosaic virus*; PeMMV, *Pepper mild mosaic virus*; PaMMV, *Paprika mild mosaic virus*. Residues shown in white with a black background are identical. Residues shown in black with a grey background are similar. Boxes indicate amino acids chosen to mutate. Numbers indicate the amino acid numbers that correspond to the TMV replicase protein and are positioned directly above the amino acid.

		604	605	606	607													619				
TGMMV	E	N	R	S	G	L	T	L	T	F	D	K	P	T	E	E	N	V	A	K	A	L
ToMV	S	N	E	S	G	L	T	L	T	F	E	Q	P	T	E	A	N	V	A	L	A	L
TMV	S	N	E	S	G	L	T	L	T	F	E	R	P	T	E	A	N	V	A	L	A	L
PeMMV	S	N	E	S	G	L	T	L	T	F	E	R	P	T	E	A	N	V	A	L	A	L
PaMMV	-	N	E	S	G	V	T	L	P	F	K	E	P	T	E	G	N	I	A	E	A	L
ORSV	-	N	E	-	-	L	T	L	P	F	N	N	P	T	P	E	A	L	S	D	A	L

TGMMV	K	S	T	-	A	S	E	A	V	V	R	L	E	P	T	S	D	E	V	M	V	N
ToMV	Q	D	S	-	E	K	A	S	D	G	A	L	V	V	T	S	R	D	V	E	E	P
TMV	Q	D	Q	-	E	K	A	S	E	G	A	L	V	V	T	S	R	E	V	E	E	P
PeMMV	Q	P	T	-	T	S	K	E	E	G	A	L	K	I	V	S	S	D	V	G	E	S
PaMMV	K	P	N	-	G	R	D	E	V	L	T	L	E	N	V	T	-	E	N	T	D	L
ORSV	S	P	L	-	P	K	D	L	D	M	R	-	-	-	-	-	-	-	-	-	-	-

						650															
TGMMV	K	F	-	S	I	A	E	R	G	K	L	P	V	C	A	E	-	-	-	-	-
ToMV	S	I	K	G	S	M	A	R	G	E	L	Q	L	A	G	L	-	-	-	-	-
TMV	S	M	K	G	S	M	A	R	G	E	L	Q	L	A	G	L	-	-	-	-	-
PeMMV	S	I	K	E	V	V	A	R	S	E	I	S	M	L	G	L	-	-	-	-	-
PaMMV	T	C	K	S	M	V	S	S	G	S	L	P	L	C	G	I	-	-	-	-	-
ORSV	-	F	-	S	L	L	-	-	-	K	L	S	T	C	A	P	F	P	S	V	K

TGMMV	-	-	S	H	G	L	T	N	A	N	-	L	-	-	-	-	E	H	Q	E	L
ToMV	-	-	S	G	D	V	P	E	S	S	Y	T	-	-	-	-	R	S	E	E	I
TMV	-	-	A	G	D	H	P	E	S	S	Y	S	-	-	-	-	K	N	E	E	I
PeMMV	-	-	T	G	D	T	V	S	D	E	F	Q	-	-	-	-	R	S	T	E	I
PaMMV	-	-	S	G	E	I	A	C	D	T	F	V	-	-	-	-	R	N	E	E	I
ORSV	L	D	S	G	L	L	P	K	Q	S	Y	G	D	E	R	Q	F	E	S	Q	S

TGMMV	E	S	L	N	D	F	H	K	A	C	V	D	S	V	I	T	K	Q	M	A	S	V
ToMV	E	S	L	E	Q	F	H	M	A	T	A	S	S	L	I	H	K	Q	M	C	S	I
TMV	E	S	L	E	Q	F	H	M	A	T	A	D	S	L	I	R	K	Q	M	S	S	I
PeMMV	E	S	L	Q	Q	F	H	M	V	S	T	E	T	I	I	R	K	Q	M	H	A	M
PaMMV	N	S	L	E	E	Y	H	M	L	A	A	E	S	V	I	N	N	R	M	A	S	I
ORSV	V	S	V	S	D	F	H	L	K	S	V	E	S	V	K	I	K	S	M	S	S	A

	696	697	699																		
TGMMV	V	Y	T	G	S	L	K	V	Q	Q	M	K	N	Y	V	D	S				
ToMV	V	Y	T	G	P	L	K	V	Q	Q	M	K	N	F	I	-	-				
TMV	V	Y	T	G	P	I	-	-	-	-	-	-	-	-	-	-	-				
PeMMV	V	Y	T	G	P	L	K	-	-	-	-	-	-	-	-	-	-				
PaMMV	V	Y	S	G	P	L	-	-	-	-	-	-	-	-	-	-	-				
ORSV	V	Y	T	G	P	L	K	V	Q	Q	M	-	-	-	-	-	-				

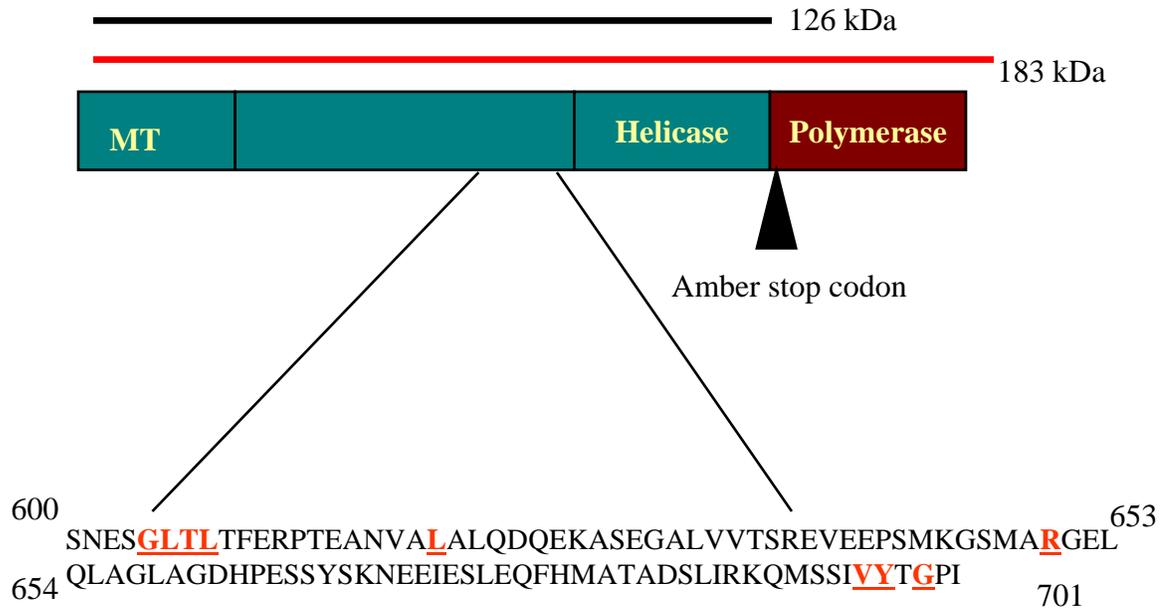


Figure 7. Location of mutation in putative ER retention domain. Shown is the location and amino acid sequence of amino acids 600 to 701 of the replicase protein. The region lies in the intervening region (IR) which is directly upstream of the helicase domain. Nine point mutations were made by PCR-mediated sited directed mutagenesis. All amino acids were mutated to alanine and were chosen based on conserved regions of tobamoviruses with similar host ranges. Amino acids highlighted in red text are those that were mutated to alanine.

with wt infectious RNA. In Figure 8a, the images of the lesion assays are shown and are quantified in Figure 8b. The diameter of each lesion was measured and compared to wt infectious RNA inoculated on the same leaf.

Mutations at amino acids 605, 607, 619, 650, 696, and 697 exhibited lesions similar to that of wt viral RNA. Mutations at 604, 606, 699, and 696/699 showed decreased lesion size, indicating either an effect on either movement or replication. Note that the mutation at amino acid 650 was chosen because it was predicted not to have a strong effect on the virus, based on lack of sequence similarity to other tobamoviruses. Results confirmed that the mutant moves and replicates similarly to wt.

Replication assays

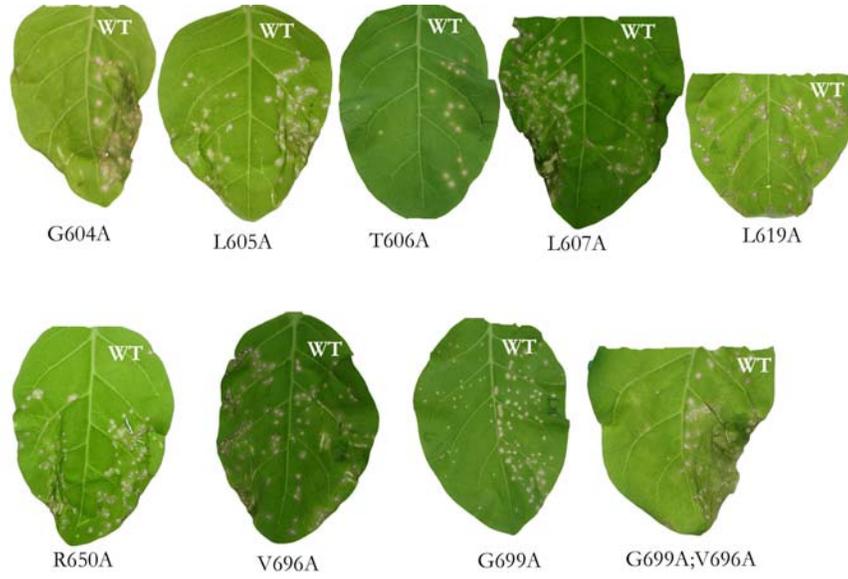
To determine the effect of these mutations solely on replication, a protoplast replication system was employed. *N. tabacum* cv xanthi suspension cells were used to make protoplasts and electroporated with wt or mutant infectious viral RNA. The protoplasts were collected about 20 hours after electroporation and RNA was extracted. The RNA was analyzed using a Northern blot with cDNA derived from viral RNA labeled with ³²P. The band intensities were measured and compared to wild type for each blot. The percentage of wt replication was averaged for all blots and each mutation was tested at least twice (Figure 9).

Figure 8. Local lesion assays. *Nicotiana tabacum* cv xanthi NN was used as a local lesion host to examine infectivity and cell-to-cell movement. Size of lesions indicates relative levels of replication and spread of the virus. Half-leaf assays were used so that each mutant could be compared to the wt virus on the same leaf.

a) Half-leaf assays were performed, inoculating one half of the leaf with RNA transcribed from a plasmid containing the viral wt cDNA. The other half of the leaf was inoculated with RNA transcribed from cDNA containing the indicated mutation. WT indicates wild-type RNA inoculation. The brown-to-translucent spots are lesions; the numbers indicate the mutation tested. The lower right leaf had two point mutations.

b) The average difference between wild-type TMV lesions and mutant lesions on the same leaf. The diameter of each lesion was measured in centimeters and recorded, then averaged for each leaf. Zero indicates that the lesions were the same size as wild type. Negative numbers indicate that the mutant virus had lesions smaller than wild type, while positive numbers indicate lesion sizes larger than wild type. Error bars indicate the range of these averages over several experiments.

a)



b)

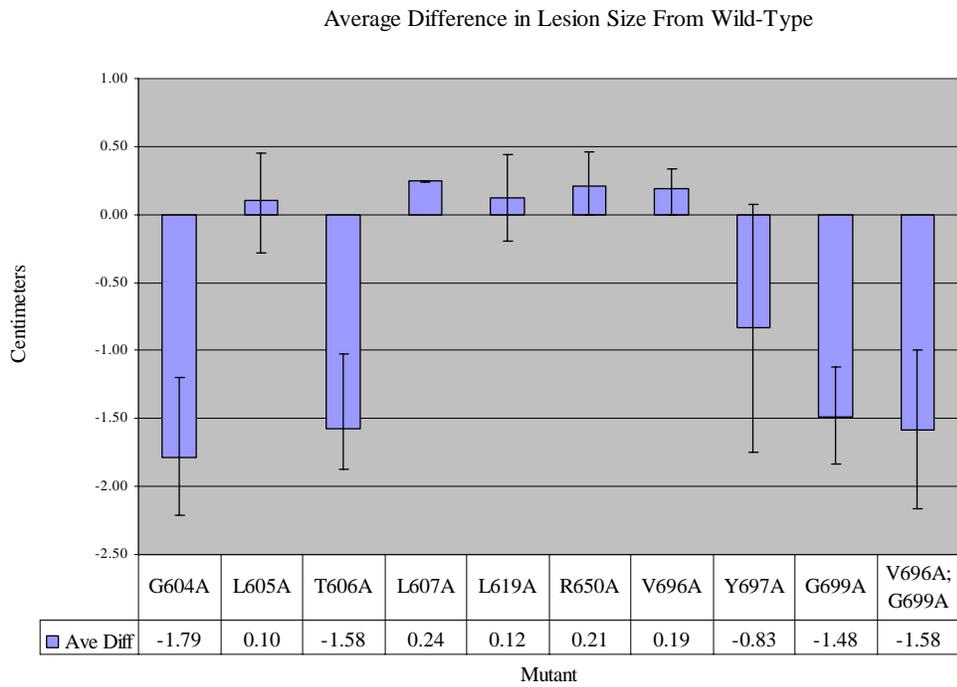
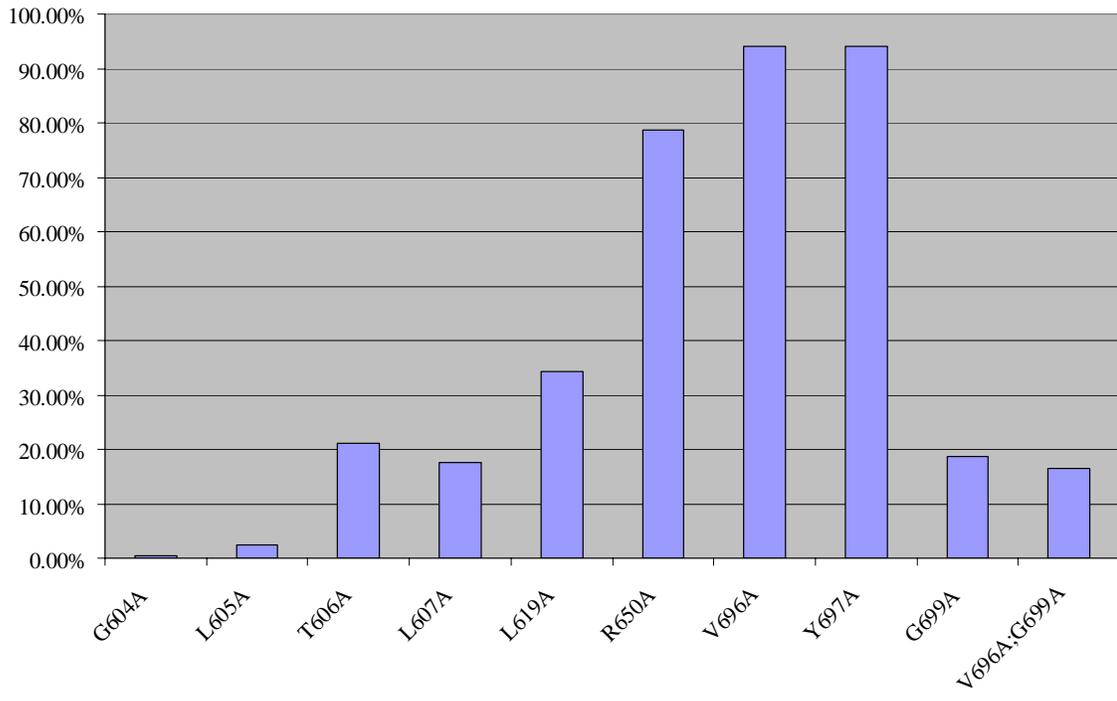


Figure 9. Viral replication assays. cDNA clones of either wt or mutant viral RNA were transcribed using T7 RNA polymerase to produce infectious RNA. The viral RNA was electroporated into protoplasts derived from *N. tabacum* cv xanthi suspension cells. Protoplasts were incubated for about 20 hours post-electroporation and collected for RNA extraction. Whole-cell RNA extract was loaded onto a formaldehyde denaturing agarose gel and electrophoresed. Northern blots were performed to determine viral RNA concentration. The blots were probed with dCTP γ 32P-labeled viral cDNA encoding the coat protein and upstream sequences.

Graph of mutant viral accumulation from Northern blot analysis is shown. Bands were quantified using ImageJ software (National Institutes of Health). Each band was measured and the background subtracted, then compared to the wild-type control for that blot. Data represent an average of at least two independent assays and are displayed in the same order as previous figures for comparison.

Average viral RNA accumulation as a percentage of wild type



Interestingly, R650A, which was mutated because it was part of a region within this domain with lower sequence similarity to other tobamoviruses, replicated similarly to V696A and Y697A, which were chosen in regions of high similarity. Other mutants replicated to 50% or less of wild type and the double mutant replicated to levels equal or just below the G699A single mutant. Replication for G604A was not detected above background levels.

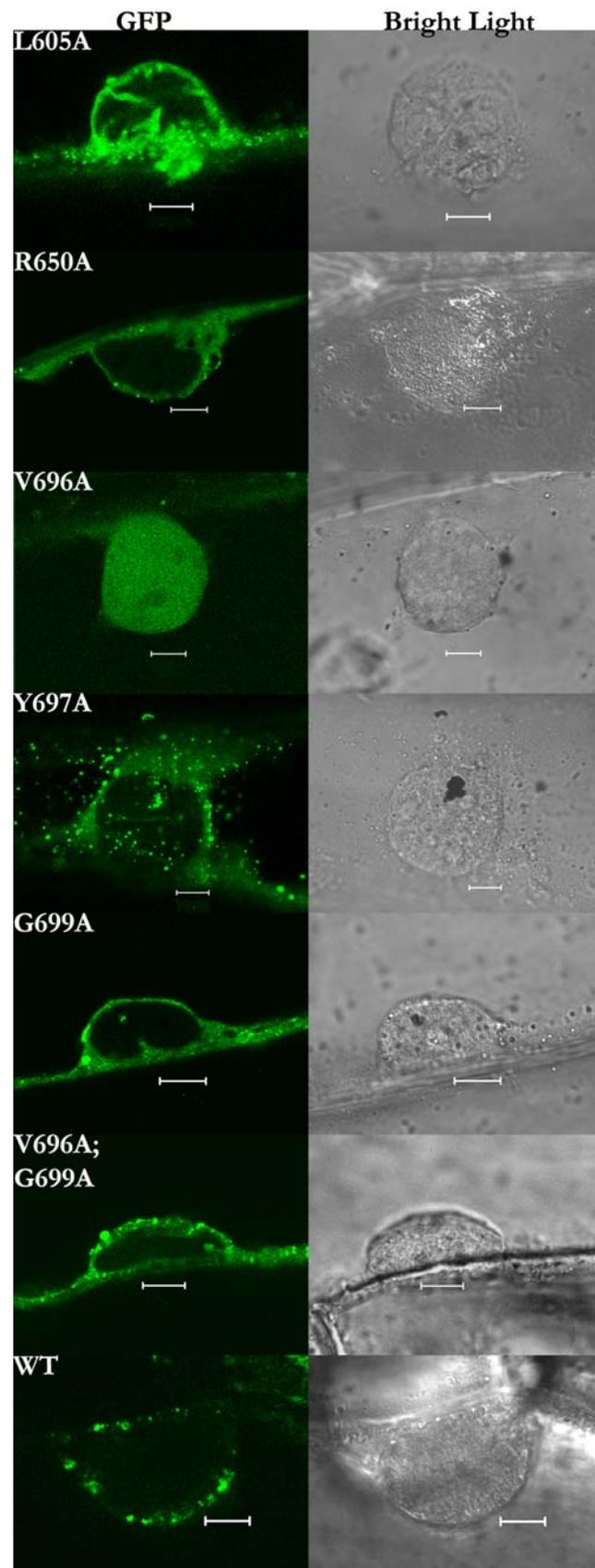
Localization

To understand more fully how the ER localization domain functions in protein localization, the full length 126 kDa replicase protein was expressed either as wild-type or mutant, and transiently expressed in onion epidermal cells via gene bombardment (Figure 10). The wt replicase protein forms small aggregates that appear around the periphery of the nucleus in an area previously identified as ER [112]. In the V696A mutant, ER localization is entirely lost and the protein is seen inside the nucleus. For mutants L605A, R650A, G699A, and V696A;G699A, the amount of aggregated protein decreased and the protein still localizes outside of the nucleus. With mutation 697, images looked very similar to wt replicase protein. For mutation G604A, no expression was detected.

Contributions of an intramolecular interaction

Since the 126 kDa replicase protein forms aggregates when transiently expressed and this association may be important in replicase formation and movement, a helicase-helicase interaction was investigated [7, 112, 115]. Previously, an intramolecular interaction was identified between two different, overlapping regions of the replicase proteins (aa 549-868 and aa 814-1211; Figure 11a). This interaction may contribute to forming dimer or high-order oligomers of the replicase proteins during infection [115]. Aggregate formation seemed to be impaired in the localization assays for certain mutants, suggesting that this interaction may

Figure 10. Localization of mutant replicase proteins in onion epidermal cells. The full-length 126 kDa replicase protein, either mutant or wild type (WT), was transiently expressed with a C-terminal eGFP tag. C-terminal Green Fluorescent Protein (GFP) fusion constructs were made using the pCMC1100 vector [112] which contains a single 35S promoter and NOS terminator sequence. Fusion proteins were transiently expressed in onion epidermal cells via gene bombardment and observed 12-16 hours post-bombardment. Images were visualized as previously described. Shown are representative images of several independent experiments. Bars indicate 10 μ m. Seen in the left column are images of onion cell nuclei and the fusion proteins tagged with eGFP. The right column is the bright-light image of the same cell. The numbers indicate the location of the mutation in the IR region of the replicase protein; WT indicates wild type. Note that in WT cells, the replicase protein aggregates at the periphery of the nucleus. This localization is changed with the V696A mutant, which localizes inside the cell nucleus. Mutations L605A, G699A, and R650A, although localizing outside the nucleus, show a lack of the aggregates seen with the WT protein.



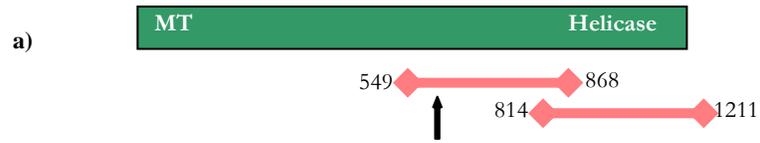
contribute to that aggregation and possible localization. The same yeast two-hybrid system used to identify this interaction was used, with the same overlapping regions used as “bait” and “prey.” The same nine mutations were introduced into the region between amino acids 549 and 868 (Figure 11a) and expressed in the yeast two-hybrid system. Quantitative β -galactosidase assays were performed at 25° C, the temperature at which the host plants are grown.

All of the mutations except T606A in the ER localization domain reduced the strength of the intramolecular interaction below that of the wild-type viral sequence (Figure 11b). Mutations at amino acids 697, 699;696, and 699 were severely reduced in their interactions with the “prey” and the double mutation was barely detectable above the negative control, which, interestingly, was greater than either of the two individual mutations. The T606A mutation in the local lesion assay, showed a decrease in replication and movement; in this assay, it shows only a minor decrease in its intramolecular interaction from wt. It is possible that changes in the IR and helicase regions that destroy the ability to dimerize or form higher-order structures are not directly correlated with replication and movement. In addition, although all mutations decreased the interaction, regions of the ER localization motif that were more C-terminal and closer to the overlapping region in this assay seemed to have a greater affect than other mutations.

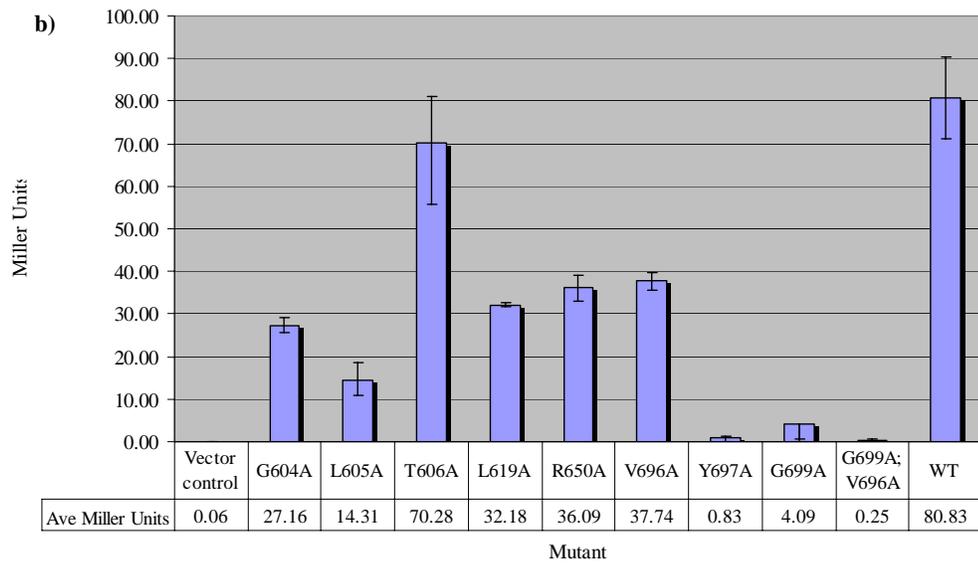
Figure 11. Disruption of an intracellular interaction. An intracellular interaction between the helicase domain and upstream sequences has been reported [115] and implicated as being important in cell-to-cell movement [7]. Based on that information, we examined whether these point mutations may have disrupted this interaction.

a) Diagram of the 126 kDa protein showing the regions used to evaluate the intracellular interaction using a yeast two-hybrid system. Numbers indicate amino acid, and the arrow indicates the area of the protein where the point mutations occur.

b) Graph of yeast two-hybrid interaction data measured using a quantitative β -galactosidase assay and reported in Miller units. Sequences between amino acids 814 and 1211 were used as “bait” and were cloned into the pLexA yeast vector. Sequences between amino acids 549 and 868 were used as “prey” and cloned into the pGAD (Clontech) yeast vector. The assay was done at 25° C, the temperature at which the normal hosts grow. The negative control was an empty pLexA vector without a TMV sequence. WT indicates the “bait” was the wild-type TMV sequence.



Average Quantitative Beta-galactosidase Assay



Methods and materials

Transient expression assays: Either full-length or altered proteins (as indicated by figures 4 and 5) were expressed with a C-terminal eGFP (Clontech) fusion using the CMC 1100 expression vector as described in [112]. Expression and visualization of constructs in onion epidermal peels were as described in with the following modifications: 1 cm thick sections of white onion (*Allium cepa*) epidermal tissue were cut from the bulb and shot with DNA precipitated on tungsten particles. The whole section was incubated overnight at room temperature in a moist environment to prevent dehydration.

Confocal imaging: Bombarded onion epidermal tissue was carefully peeled from the lower layers of tissue. Epidermal tissue was mounted using water and imaged using a Zeiss LSM510 laser scanning confocal microscope system with 10x NA 0.8 dry and 63x NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thornwood, NY). Excitation sources were 488 nm for GFP. Images were modified in Zeiss LSM Imager Examiner and processed for printing in Adobe Photoshop CS (Grand Prairie, TX).

Introduction of point mutations: Single- or double-point mutations were introduced into the intervening region of replicase proteins using PCR-based mutagenesis and cloned back into the cDNA using preexisting Stu I and Sac II restriction endonuclease sites. The TMV expression construct was derived from the wt TMV cDNA construct of [116].

Replication assays: Assays were based on [117] with the following modifications.

Nicotiana tabacum cv xanthi suspension cells were maintained at 32 °C in suspension culture media and subcultured every seven days. Protoplasts were isolated using the following enzyme solution: 0.75g Cellulysin, 0.075g Pectinase, 0.05g Macerase dissolved in a 50 mL solution of 0.5M D-mannitol, 10 mM MES (C₆H₁₃NO₄S), and 10 mM CaCl₂ (pH 5.6) and filter-sterilized.

There were 7x10⁶ cells per electroporation with 22µl of viral RNA from a T7 *in vitro* transcription reaction. The cells and reaction mixture were electroporated with 250 volts, 125 microFaraday, and 100 ohms in 0.4 cm gap cuvettes. Cells were incubated at 25°C in the dark for 20 hours and collected for Northern blot analysis.

Xanthi suspension cell culture media: Murashige and Skoog Basal Salt with Minimal Organics Medium supplemented with the following per liter of media: 30g sucrose, 1g MES, 1.5 mM KH₂PO₄, 200 µg 2,4-Dichlorophenoxy acetic acid (C₈H₅Cl₂NaO₃ · H₂O), 100 µg Kinetin (C₁₀H₉N₅O), 1ml 1000x Gamborg's Vitamin Solution, with a pH adjusted with KOH to 5.8.

Northern blot: Protoplast samples were collected and RNA was extracted using the Qiagen Plant RNeasy protocol. The Northern blot was performed as per [118]. Viral RNA isolated from virions was used as an RNA ladder. cDNA encoding the TMV coat protein and upstream sequences (bp 5460-6394) was used to create a labeled probe using the Prime-a-Gene Labeling Kit (Promega) with dCTP³²P. Probe labeling was performed

as per manufacturer protocols. Samples were run on a 1% agarose/formaldehyde gel, blotted overnight, and probed the next day.

Yeast two-hybrid: *Saccharomyces cerevisiae* strain L40 was transformed with both pGAD (Clontech) containing the “prey” and pLexA containing the “bait” construct. Filter lift assay: Yeast transformed with both bait and prey constructs were grown on appropriately selective yeast minimal media plates at 30 °C. A nitrocellulose membrane was used in the filter lift. The yeast was incubated at 30° C in the presence of X-gal for one hour.

Quantitative assay: Yeast containing both bait and prey constructs were grown in liquid media to an OD at 600nm of 0.6. One mL of culture was pelleted and resuspended in 100 µL of Z buffer. Added to the culture were 10 µL of 0.1% SDS, 20µL of chloroform, and 200 µL of ONPG (ortho-Nitrophenyl-β-galactoside). Samples were incubated for 30 minutes at 25° C. The reaction was stopped by adding 500µL 1M Na₂CO₃. The OD was measured at both 420 nm and 550 nm and Miller units were calculated.

X-gal solution: 5 µL Triton X 100, 100 µL X-gal (5-methyl-4-chloro-3-indoly-B-D-galactosidase) (20mg/mL), 5 mL Z-buffer

Z buffer (1L): 16.1g Na₂HPO₄, 5.5g NaH₂PO₄, 0.7g KCl, 0.25g MgSO₄ 2.7 mL β-mercaptoethanol

Local lesion assay: An *in vitro* T7 transcription reaction with either wild type or mutant TMV cDNA templates (as described above) were mixed with 50 μ L FES and rub-inoculated onto *Nicotiana tabacum* cv xanthi NN. Each inoculated leaf was rub-inoculated half with wt TMV RNA and half with mutant TMV RNA. Lesions were measured and imaged six days post-inoculation.

FES: 0.1M glycine ($\text{HO}_2\text{CCH}_2\text{NH}_2$), 0.6 M potassium phosphate (KH_2PO_4), 1% sodium phosphate (NaH_2PO_4), 1% macaloid, 1% cealite, pH 8.5-9.0

Virus strains: The U1 strain of TMV was used for all assays and is referred to as the wild type sequence. The strain is maintained in tobacco and all cDNA clones are originally based off work from [116].

Discussion

Replicase complex formation is a poorly understood but essential step in the life of a virus. For another member of the alphavirus superfamily, BMV, the replicase proteins form and shape invaginations in the ER [119]. Replicase proteins play an important role in replication complex formation and host protein and ribosome recruitment. For TMV, both replication proteins and membranes are required for efficient replication.

Localization plays an important role in viral replication and protection from host defenses, suggesting that this 102-amino-acid region of the replicase protein, previously implicated in localization, is important in replication and replicase complex formation.

As previously stated, efforts to narrow this localization domain identified the region between amino acids 599 and 701 to be necessary and sufficient to retain the replicase protein outside the nucleus of plant cells and to retain another tested protein outside as well. Mutations were introduced into this region to reveal its mechanism of action and biological relevance. These studies are summarized in Table 2. Mutations at both ends of this region (G604A, L605A, G699A, and V696A; G699A) were the most affected in replication, movement, and protein localization. Interestingly, the V696A mutation seemed to have no effect on replication and movement, but was severely altered in its cellular localization. The fusion protein localized to the nucleus and no longer formed aggregates as with the wt protein, suggesting that helicase-helicase interaction may be important in localization.

Mutant	β-gal Assay	Replication Assay	Local Lesion Assay	Localization
WT	+++	+++	+++	Cytoplasmic; aggregates
G604A	++	-	+	No expression
L605A	+	-	+++	Cytoplasmic
T606A	+++	+	+	No data
L607A	No data	+	+++	No data
L619A	++	++	+++	No data
R650A	++	+++	+++	Nuclear and Cytoplasmic
V696A	++	+++	+++	Nuclear
Y697A	-	+++	++	Cytoplasmic; aggregates
G699A	+	+	+	Cytoplasmic
V696A;G699A	-	+	+	Cytoplasmic

Table 2. Summary of data from mutational analysis.

For each assay, shown are relative levels as measured. For the β-galactosidase assay, the levels are based on Miller units. For the replication assay, levels are based on relative levels of viral RNA as measured by ImageJ software (National Institutes of Health). For the local lesion assay, levels are measured by average diameter of lesions in centimeters. Localization was determined to be nuclear or cytoplasmic based on transient expression assays in onion epidermal cells.

Based on replication, GFP expression and local lesion assays, the G604A mutation seems to impair the expression of the replicase proteins. The R650A mutation, which was created because it lacked similarity with related viruses with similar host ranges, performed close to wt in most assays except localization.

For many of the mutant viruses created, replication was severely reduced due to these mutations. This was predicted because this region contributed to ER localization of the protein, which is important for viral replication. Surprisingly, this impairment in replication did not always correlate to alteration in local lesion assays or even protein localization.

There are several possible explanations for these conflicting results. Discrepancies in replication levels and local lesion assays may be due to reversions of the mutant virus back to wild type or some other compensatory mutation. Inoculated transcripts were not later screened for the viral RNA that was being incorporated into virions. Also, in the case of protein localization, by expressing the replicase protein outside the context of the virus, observed results may not be truly indicative of the processes occurring during a viral infection. This may explain the discrepancies between replication, movement, and localization. In addition, the mechanism by which the replicase proteins localize to the ER has not been directly demonstrated. The proteins do not contain a membrane-spanning region, a region of hydrophobic side groups, or fatty acid modifications that would allow it to anchor directly to membranes. Although the TMV movement protein has been shown to be membrane-spanning, no direct interaction between the movement

protein and replicase proteins has been found. Therefore, an interaction with a host protein is a very plausible mechanism for localization. A host protein, *AtTOM1* from *Arabidopsis thaliana* and related proteins have been shown to be membrane bound, essential to viral replication, and may possibly represent a membrane anchor that the replicase proteins utilize during infection. However, efforts to replicate the interaction between the full-length 126 kDa replicase protein and *AtTOM1* using a yeast two-hybrid system was unsuccessful. Also, the ER localization domain, when expressed outside the context of the replicase proteins, localized to cellular membranes without the assistance of TOM1, which made it impossible to determine the effect of these mutations on the TOM1 interaction (data not shown).

Another possible explanation is that the ER localization domain could be in a part of the replicase protein that is exposed to the cytoplasm, and would then allow for direct binding with a host protein or set of host proteins. Mutations to this region would have varying effects based on its interface with the host protein, which could produce incomplete localization and loss of aggregation. The effect on replication and movement would also be mixed depending on how important replicase localization is in secondary infection sites. Additionally, there are both cytosolic and membrane bound populations of 126 kDa replicase proteins found during infection. A mutation that alters a host protein interaction may change the ratio of cytosolic to membrane bound protein and thus alter replication and spread. Also, replicase proteins and their interactions with the movement protein have been shown to be important in viral movement [7]. A mutation that affects

replication may or may not affect interactions with the movement protein and viral spread.

Alternatively, the ER localization domain may be part of an internal region of the protein that does not directly interact with any host protein, but contributes to a particular tertiary structure that makes it available to bind to a host protein or subset of proteins. Thus, mutations in this region may not have a direct correlation to localization, replication and movement. Additionally, the intracellular interaction, although shown *in vitro*, has not been shown *in vivo* to be the active complex [115]. Replicase extraction assays have shown that the replicase proteins are found in a 1:1 ratio, but the structure of this complex has not been demonstrated [52]. The protein aggregation of the 126 kDa protein may not be biologically active and may not be a good measure of how the mutants affect the virus.

The ER localization motif may not directly contribute to replication, but may act to enhance replication or increase the half-life of the protein. When the ER localization domain was deleted from the 126 kDa protein and expressed as a GFP fusion protein, the fusion protein proved very difficult to express in the gene bombardment system. When this domain was fused to a protein that is rapidly degraded by the host cell, Pap1/IAA26, protein expression increased as measured visually in the gene bombardment system. However, there was no significant increase in protein accumulation, as measured by Western blot in an *Agrobacterium tumefaciens* infiltration system (not shown).

No crystal structure has been determined for the TMV replicase proteins because of the difficulty of their purification. Therefore, these data may support both models for the mechanism for the identified ER localization domain. What host proteins may be involved in replicase localization also remains an important question. More work on both these fronts is necessary to characterize more fully the localization domain and what role the intervening region of the replicase proteins plays in protein function.

Chapter 3: Identification and characterization of an interaction between Rab GDP Dissociation Inhibitor (Rab GDI) proteins and the *Tobacco mosaic virus* 126 kDa replicase protein

Abstract

The *Tobacco mosaic virus* (TMV) is a (+)-strand RNA virus that encodes a 126 kDa replicase protein with a C-terminal helicase domain that has been shown to be important in virus-host interactions. To determine host factors involved in TMV replication, this domain and upstream sequences were used in a yeast two-hybrid screen against an *Arabidopsis thaliana* cDNA library. Ten proteins were identified by this screen; one, the Rab GDP Dissociation Inhibitor *AtGDI2*, was chosen for further study because of its potential role in vesicle trafficking.

As with all (+)-strand RNA viruses, TMV replicates in close association with host membranes during infection, forming vesicle-like viral replication complexes. Rab GDI proteins regulate the activity of Rab proteins, which are essential in the docking and transport of vesicles. Full-length Rab GDI proteins from *Nicotiana tabacum* (GDI1), *Nicotiana benthamiana* (*NbGDI*), *Lycopersicon esculentum* (TC162880), and *Arabidopsis thaliana* (*AtGDI2*) were shown to interact with the helicase domain of the 126 kDa TMV replicase protein in a yeast two-hybrid assay. This interaction was confirmed using an *in vivo* pull-down assay with infiltration of *AtGDI2* with a C-terminal HA epitope tag (derived from the influenza hemagglutinin protein) in TMV-infected *N. benthamiana* tissue.

Using the *Tobacco rattle virus* VIGS system, Rab GDIs were knocked down in *N. benthamiana* and in *L. esculentum* cultivars Tiny Tim and Pilgrim. Both tomato varieties showed necrotic regions in silenced tissue, but there was no visible phenotype in *N. benthamiana*. When inoculated with the GFP-expressing virus TMV:GFP, *NbGDI*-silenced tissue showed an increase in the number of infection sites when compared to control plants. Additionally, in *NbGDI*-silenced plants, the vacuolar membrane showed a phenotype similar to those seen in TMV-infected tissue. Taken together, these data indicate a role for Rab GDI proteins in TMV infection.

Introduction

Viruses are obligate parasites and, as such, are dependent on their host for energy and machinery required for continued propagation. Virus-host interactions affect every part of a viral life cycle, from the virus usurping the host cell's machinery to the triggering of defense responses. *Tobacco mosaic virus* (TMV) is a (+)-strand RNA virus and the type member of the tobamovirus superfamily. TMV encodes two replicase proteins (126 kDa and 183 kDa), one movement protein, and one coat protein[2, 3]. A yeast two-hybrid screen of an Arabidopsis cDNA library identified an interaction between the TMV 126 kDa replicase protein and a Rab GDP Dissociation Inhibitor (Rab GDI), *AtGDI2* [120].

Rab GDI proteins regulate the activity of Rab proteins, which are essential components of vesicle trafficking within their host cells. Rab proteins, in combination with other proteins, direct vesicles from their donor to their target membrane, where cargo proteins are deposited. Rabs have been shown to be important regulators of vesicle budding, cargo recruitment, and vesicle tethering and fusion in transport [89, 90]. Rab proteins are

members of the family of small GTPases. Rab GDI proteins bind to Rab proteins in their inactive, GDP bound state and return them to their donor membrane for use in further vesicle trafficking (Figure 3).

TMV replicates in close association with the host cell's endoplasmic reticulum (ER) and requires membranes for replication [26]. Because Rab GDIs play such an essential role in vesicle trafficking and because TMV requires membranes for replication, the interaction between *AtGDI2* and the TMV replicase proteins were further investigated and characterized.

Results

Rab GDIs interact with the TMV 126 kDa protein in vitro and in vivo.

AtGDI2 of *Arabidopsis thaliana* was originally identified from a cDNA library screen in a yeast two-hybrid assay using the TMV replicase protein's helicase domain and upstream sequences [bp 814 to 1211] as bait. To confirm this interaction and determine if it is unique to *Arabidopsis*, Rab GDIs were amplified from other TMV hosts:

Lycopersicon esculentum (tomato; TIGR gene index TC162880), *Nicotiana tabacum* (Turkish tobacco, *NtGDI1*), *Nicotiana benthamiana*, and *Arabidopsis thaliana* (*AtGDI2*) ecotype shahdara. The *N. benthamiana* Rab GDI has not been previously identified and will be called *NbGDI* henceforth. The Rab GDIs were sequenced (the alignment can be seen in Figure 12). The four Rab GDI proteins are greater than 81% identical and 89% similar at the amino acid level.

Because these proteins are so highly similar, we reasoned that they might represent a conserved viral-host interaction. The Rab GDI proteins identified were tested in a yeast two-hybrid assay. All of these Rab GDI proteins interact with the TMV helicase domain in this assay; *AtGDI2* shows the strongest interaction (Figure 13). Pull-down assays were performed to test whether this interaction occurs *in vivo* as well as *in vitro*. Using an *Agrobacterium tumefaciens* infiltration system, HA:*AtGDI2* was transiently expressed in TMV-inoculated tissues, two days post-inoculation; samples were collected at four days post-inoculation. In these samples, anti-HA antibodies were able to pull-down complexes containing both 126 kDa replicase protein and HA:*AtGDI2*, indicating that these two proteins interact during TMV infection (Figure 14).

Silencing of NbGDI enhances TMV infection

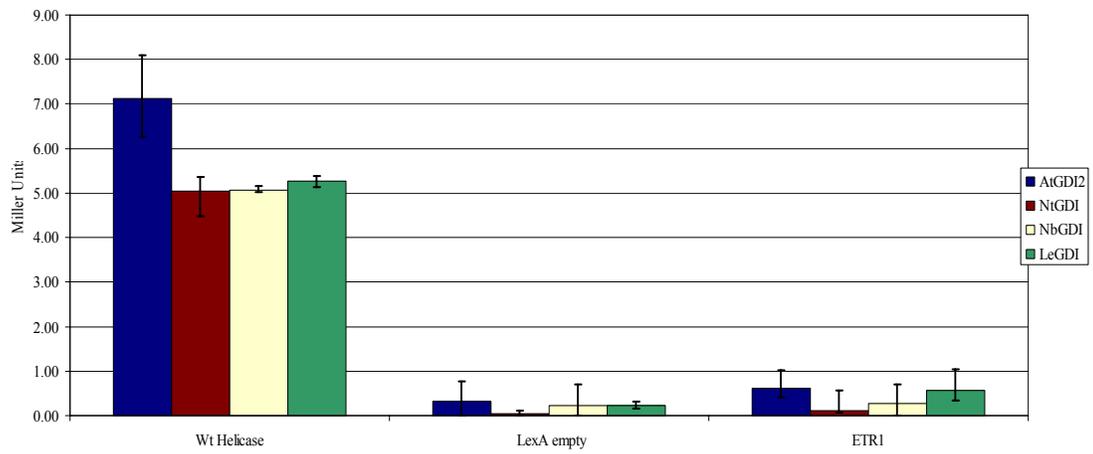
To understand how Rab GDIs are involved in TMV infection, *NbGDI* was silenced using a *Tobacco rattle virus* (TRV) gene silencing system. TRV produces mild symptoms in tobacco and tomato, and this system takes advantage of viral induced gene silencing (VIGS) to knock down expression of endogenous genes. Plants were inoculated with constructs containing either *NbGDI*, Phytoene desaturase (PDS), which is an early enzyme of the carotenoid biosynthetic pathway, or TRV itself. PDS was used as a visual indicator of successful silencing since it produces a white, bleached phenotype. Silencing was confirmed with RT-PCR (Figure 15c). When *NbGDI*-silenced plants were infected with TMV expressing eGFP (TMV:GFP), there was about a three-fold increase in the number of infection foci per inoculated leaf when compared to the TRV vector control. Using a paired, two-tailed t-test, this difference in the number of infection foci was found to be statistically significant (Figure 15b). These results indicate that not only do Rab

<i>NtGDI</i>	1	MDEEYDVIVLGTGLKECILSGLLSVDGLKVLHMDRNDYYGGESTSLNVLQWLKR	
<i>NbGDI</i>	1	MDEEYDVIVLGTGLKECILSGLLSVDGLKVLHMDRNDYYGGESTSLNVLQWLKR	
<i>LeGDI</i>	1	MDEEYDVIVLGTGLKECILSGLLSVDGLKVLHMDRNDYYGGESTSLNVLQWLKR	
<i>AtGDI2</i>	1	MDEEYEVIVLGTGLKECILSGLLSVDGVKVLHMDRNDYYGGESTSLNVLNQLWKK	
<i>NtGDI</i>	55	FRGSDKPPAELGSSRD FNVDMI PKF IMANGALVRVLIHTDVTKYLYFKAVDGSF	
<i>NbGDI</i>	55	FRGSDKPPAELGSSRD FNVDMI PKF IMANGALVRVLIHTDVTKYLYFKAVDGSF	
<i>LeGDI</i>	55	FKGS DKPPAELGSSRD YNVDMI PKF IMANGALVRVLIHTDVTKYLYFKAVDGSF	
<i>AtGDI2</i>	55	FRGE EKAPEHLGASRD YNVDMM PKF M MGNGLVRTL IHTDVTKYLSFKAVDGSY	
<i>NtGDI</i>	109	VYNKGKVKVPATDMEALKSPLMGIFEKRRARKFFIYVQDYNESDPKTHEGMDL	
<i>NbGDI</i>	109	VYNKGKVKVPATDMEALKSPLMGIFEKRRARKFFIYVQDYNESDPKTHEGMDL	
<i>LeGDI</i>	109	VYNKGKVKVPATDMEALKSPLMGIFEKRRARKFFIYVQDYKESDPKTHEGMDL	
<i>AtGDI2</i>	109	V FVKGKVQKVPATPMEALKSSLMGIFEKRRAGKFFS FVQ EYDEKDPKTHDGM DL	
<i>NtGDI</i>	163	TRVTTRELI AKYGLDDNTVDF IGHALALHRDDRYLDEPAQDTV KRMKLYAESLA	
<i>NbGDI</i>	163	TRVTTRELI AKYGLDDNTVDF IGHALALHRDDRYLDEPAQDTV KRMKLYAESLA	
<i>LeGDI</i>	163	TKVTTRELI AKYGLDDNTVDF IGHALALHRDDRYLDEPA LDTV KRMKLYAESLA	
<i>AtGDI2</i>	163	TRVTTKELI AKYGLD GNT I DFI GHAVALHTNDQHL DQPAFD TVMRM KLYAESLA	
<i>NtGDI</i>	217	RFQGGSPYIYPLYGLGELPQAFARLSAVYGGTYMLNKPECKVEFDEEGKVCGVT	
<i>NbGDI</i>	217	RFQGGSPYIYPLYGLGELPQAFARLSAVYGGTYMLNKPECKVEFDEEGKVCGVT	
<i>LeGDI</i>	217	RFQGGSPYIYPLYGLGELPQAFARLSAVYGGTYMLNKPECKVEF D AEGKVCGVT	
<i>AtGDI2</i>	217	RFQGT SPYIYPLYGLGELPQAFARLSAVYGGTYMLNKPECKVEFDEGGKVI I GVT	
<i>NtGDI</i>	271	SEGETAKCKKVVCDPSYLPNKVRKVGKVARAIAIMSHPI PNTNDSHSVQI I LPQ	
<i>NbGDI</i>	271	SEGETAKCKKVVCDPSYLP SKVRKVGKVARAIAIMSHPI PNTNDSHSVQI I LPQ	
<i>LeGDI</i>	271	SEGETAKCKKVVCDPSYLP SKVRKVS KVARAIAIMSHPI PNTS E SHSVQI I LPQ	
<i>AtGDI2</i>	271	SEGETAKCKK I VCDPSYLPNKVRK I GRVARAIAIMSHPI PNTNDSHSVQVI I LPQ	
<i>NtGDI</i>	325	KQLGRKSDMYLFCCSYTHNVAPKGKFI AFVSTEAETDNPES ELKQGVNLLGPVD	
<i>NbGDI</i>	325	KQLGRKSDMYLFCCSYTHNVAPKGKFI AFVSTEAETDNPES ELQGVNLLGPVD	
<i>LeGDI</i>	325	KQLGRKSDMYLFCCSYTHNVAPKGKFI AFVSTEAETDNPENELKPGV SLLGPVD	
<i>AtGDI2</i>	325	KQLARKSDMYV FCCSY SHNVAPKGKFI AFVST D AETDNPQ TELKPGTDLLGPVD	
<i>NtGDI</i>	319	EIFYENYDRSEPVNEPSLDKCFI STSYDATTQFESTVDDVLNLYTKITGKVL DL	
<i>NbGDI</i>	319	EIFYETYDRSEPVNEPSLDNCFI STSYDATTHFESTVDDVLNLYTKITGKVL DL	
<i>LeGDI</i>	319	EIVYETYDRSEPVNE C TLDNCFV STSYDATTHFESTVDDVLNLYTKITGKVL DL	
<i>AtGDI2</i>	319	EIF FDMYDRYEPVNEPE L D NCFI STSYDATTHFET TVADV L NMYTLITGKQL DL	
<i>NtGDI</i>	333	NVDLSAASAAEE	444
<i>NbGDI</i>	333	NVDLSAASAAEE	444
<i>LeGDI</i>	333	NVDLSAASAAEE	444
<i>AtGDI2</i>	333	SV DLSAASAAEE	444

Figure 12. Multiple sequence alignment of Rab GDI proteins. *Nicotiana tabacum*, *NtGDI* (TC3480), *Lycopersicon esculentum*, *LeGDI* (TC162880), *Arabidopsis thaliana*, *AtGDI2* (At3g59920), *Nicotiana benthamiana*, *NbGDI* sequence obtained from sequenced *N. benthamiana* cDNA. Identical amino acids are shown with a black background and similar residues are shown with yellow text on a grey background.

Figure 13. Rab GDI proteins interact with the helicase domain of the 126 kDa replicase protein in a yeast two-hybrid assay. Shown is a graph of a quantitative β -galactosidase assay. All four Rab GDI proteins were expressed using full-length cDNA derived from *Lycopersicon esculentum* cv pilgrim, *Arabidopsis thaliana* ecotype shahdara, *Nicotiana tabacum* cv xanthi suspension cells or *Nicotiana benthamiana*. In all four assays, the Rab GDI protein was used as “prey” while either the wt helicase domain or empty pLexA vector was used as “bait.” The helicase domain expressed is composed of aa 814 to 1211 of the 126 TMV replicase protein. The full-length replicase protein was not used due to its poor solubility and expression in yeast and bacterial systems (data not shown). The empty vector or the pLexA vector containing the ETR1 open reading frame were used as negative controls. ETR1 is an ethylene receptor and is used here as a non-interacting protein. Yeast were grown to an OD₆₀₀ of 0.6 at 25°C and incubated with a solution of X-gluc (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide) for one hour. OD was then taken at 420nm and 550nm to determine Miller units. Each bar represents the average of three reactions; error bars indicate the range. All Rab GDI proteins interact above the negative controls (Lex A empty and ETR1).

Quantitative β -Galactosidase Assay



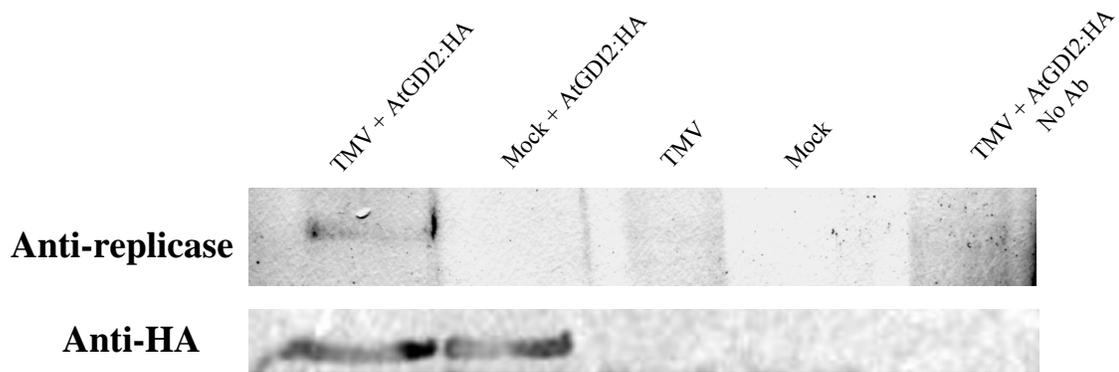


Figure 14. Co-immunoprecipitation of AtGDI2 and 126 kDa replicase protein in leaf tissue. Shown is a co-immunoprecipitation of the *AtGDI2* protein and the 126 kDa replicase protein. *N. benthamiana* tissue was inoculated with TMV and infiltrated at two days post-inoculation with pBIN expressing *AtGDI2* with an epitope tag from the influenza hemagglutinin protein. Samples were taken at four days-post-inoculation. The precipitation was performed using Agarose A bead and anti-HA antibodies. Washed samples are shown above in Western blots with primary antibodies as indicated. The replicase protein can be seen as a distinct band only in the sample with both proteins and the precipitating antibody. The anti-HA blot is shown here to indicate expression of the *AtGDI2* protein in the samples.

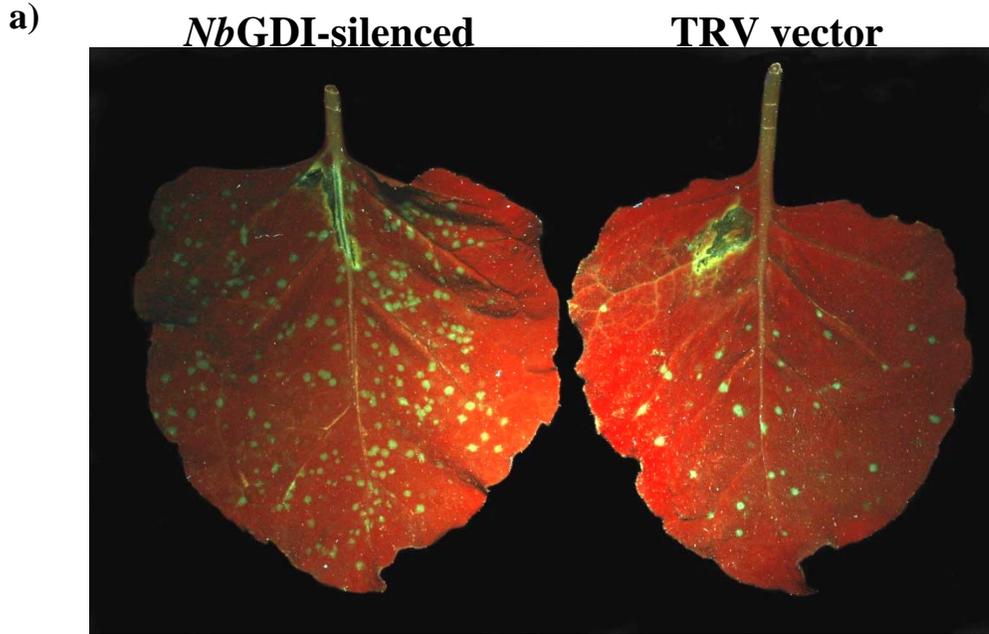
Figure 15. Silencing of *NbGDI* enhances TMV:GFP infection. *N. benthamiana* plants were inoculated with wt TRV or TRV-containing sequences from either *NbGDI* or *Phytoene desaturase* (PDS). PDS is used as a visual marker of successful silencing as it produces a white, bleached phenotype. Plants were then inoculated with TMV:GFP virions (20 μ L of 0.1 mg/mL per leaf). Foci were counted six days post-inoculation. Silencing was confirmed using semi-quantitative RT-PCR.

a) Silenced *N. benthamiana* tissue seven days post-inoculation with TMV:GFP taken using a long wavelength UV lamp and a Tiffen™ 58 mm Yellow2 wavelength filter on a Cannon EOSD60 digital camera with a tripod.

b) Descriptive statistics of the number and size of TMV:GFP lesions seen six days post-inoculation. Data represents two independent experiments with six leaves per experiment. The size of the foci were measured for 40 lesions in the same experiment. T-test values were calculated based on a two-tailed, equal variance, un-paired t-test. Based on the calculated T-value, there was a significant difference between the number of foci on the TRV vector control leaves and the *NbGDI*-silenced leaves; there was not a significant difference in lesion size.

c) Seen is a 1% agarose gel of RT-PCR products from Rab GDI-silenced, or wild-type, uninfected tobacco plants. Samples were taken at 27, 30, and 33 cycles of the PCR machine. eEIF1A primers were used to determine total RNA used. RNA was extracted using the Trizol™ reagent (Invitrogen) and cDNA was created using the SuperScript™ First-Strand Synthesis System (Invitrogen). One microgram of RNA for each sample was used to create the cDNA. Primers were used to amplify either a 300 bp fragment of eEIF1A cDNA or part of the Rab GDI cDNA (bp 300-700).

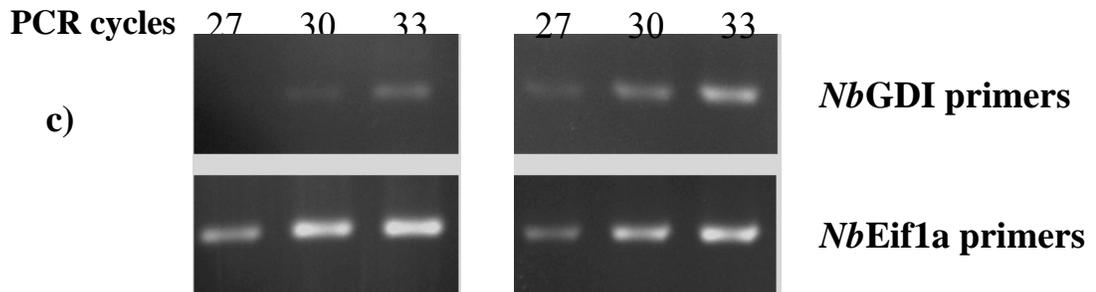
TMV:GFP 7 dpi



b)

	<i>NbGDI</i>	TRV
Mean count of foci per leaf	56.67	19.25
Standard deviation	27.18	8.23
P(T<=t)	0.00015	

	<i>NbGDI</i>	TRV
Mean size of foci (cm)	0.887	0.896
Standard deviation	0.200	0.209
P(T<=t)	0.46021	



GDI proteins interact with the replicase proteins during infection, but also they modulate the susceptibility of the cell to initial infection. In contrast, there was no statistically significant increase in the size of the infection sites (Figure 15a and b). Interestingly, total wt TMV accumulation in inoculated leaves was the same between *NbGDI*-silenced and TRV vector control plants (Figure 16). The absence of change in TMV accumulation and size of local lesions indicates that the viral movement (cell-to-cell and systemic) is not affected by the absence of Rab GDI proteins.

Silencing of LeGDI induces cell death

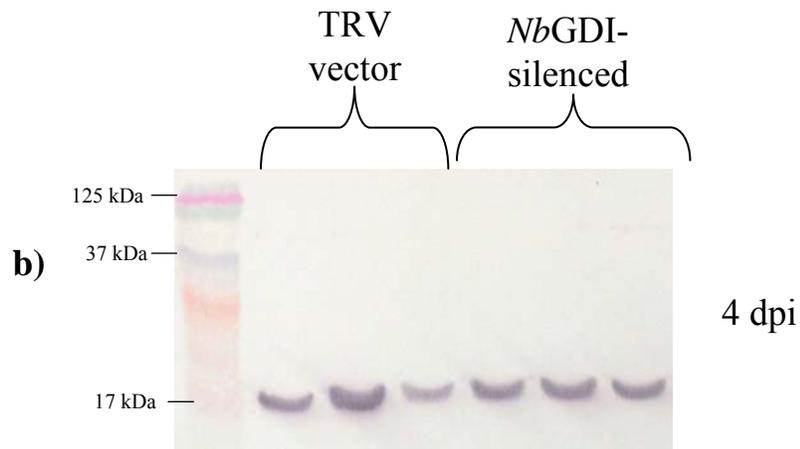
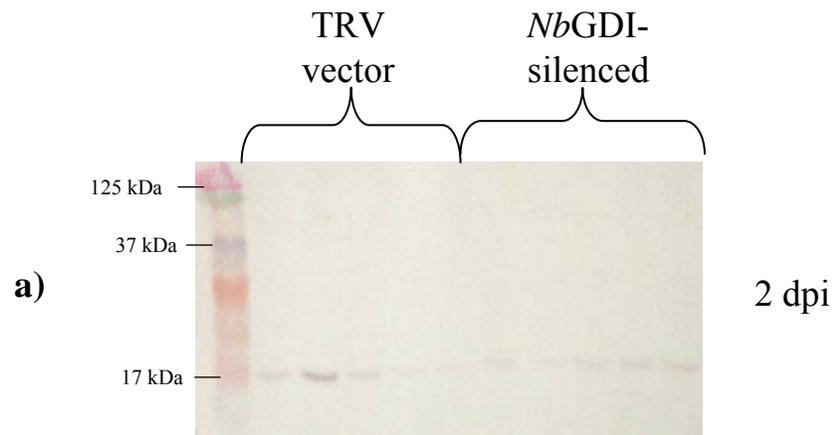
To determine if similar effects are seen in other TMV hosts, tomato plants, *L. esculentum* cv Tiny Tim and cv Pilgrim were silenced for *LeGDI*. Unlike *NbGDI*-silenced tobacco plants, a phenotype was seen on silenced leaves (Figure 17b). Small necrotic lesions appeared on the leaves. Trypan blue staining confirmed that these regions were composed of dead cells (Figure 17a). Since TMV:GFP does not maintain consistent GFP expression in tomato plants, wt TMV was used to test for an effect on TMV infection. There was no difference in total viral accumulation in silenced, inoculated tissues (Figure 18). Because Rab GDI proteins are so similar to each other, silencing one Rab GDI gene may also silence all the other Rab GDI genes as well. *LeGDI* is highly similar (greater than 95%) to another tomato protein (TC174162), possibly another Rab GDI protein. The cell death seen here may be indicative of both Rab GDIs being silenced, which tomato plants may be less tolerant of than *N. benthamiana* plants. Similarly, when Salk t-DNA knock-out line of the two Arabidopsis Rab GDIs (*AtGDI1* and *AtGDI2*) were crossed, no seed was ever recovered from three different attempts, confirming that these proteins are essential

Figure 16. No clear difference in TMV coat protein accumulation in *Nb*GDI-silenced

***Nicotiana benthamiana* tissues.** *Nb*GDI-silenced and TRV vector control tobacco leaf tissues were manually inoculated with 20µl of 0.01mg/mL wt TMV. Inoculated tissues were collected two and four days post-inoculation. The tissue was ground in phosphate buffer (pH 7.0). Total protein concentration was determined using a Bradford assay. Five micrograms of protein were loaded for each sample onto an SDS polyacrylamide gel and blotted with anti-CP antibody.

Shown are blots of the same plants at two **(a)** and four days post inoculation **(b)**. The bands were measured and the average for each blot is seen in **(c)**. These data are representative of at least three independent samples and each set of data showed no distinct difference in coat protein accumulation between the two groups, indicating that affect of Rab GDI silencing are only on initial establishment of infection rather than maintenance or movement of infection.

Coat protein accumulation in Rab GDI-silenced *N. benthamiana* tissue



c) Average coat protein levels in inoculated, silenced *N. benthamiana* leaf tissue

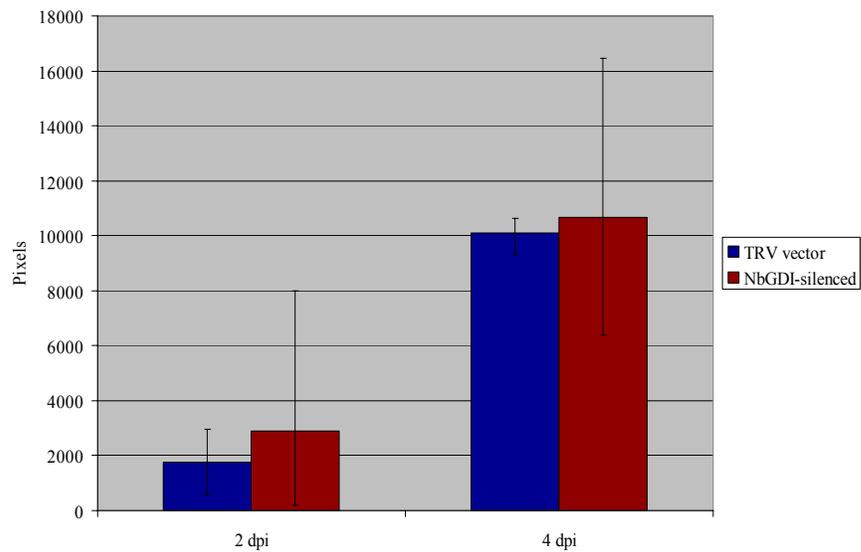


Figure 17. *L. esculentum* tissue silenced for Rab GDI (TC 162880) exhibits a cell death phenotype. *L. esculentum* plants were silenced using the Tobacco rattle virus (TRV) gene silencing system. The system takes advantage of Viral Induced Gene Silencing (VIGS) to target host mRNAs for destruction by expressing a small (300-500 bp) piece of target cDNA from RNA 2 of the TRV.

a) Sections of *L. esculentum* cv Tiny Tim silenced for Rab GDI show trypan blue staining.

Sections of TRV-inoculated or Rab GDI-silenced *L. esculentum* cv Tiny Tim were stained with trypan blue and then destained with chloral hydrate. Positive staining indicates dead cells, as seen in the Rab GDI silenced tissue. Each bar indicates 100 μ m.

b) Phytoene desaturase (PDS)-silenced, Rab GDI (TC162880)-silenced, or empty TRV

inoculated *L. esculentum* plants cv Pilgrim and cv Tiny Tim. PDS is an early enzyme of the carotenoid biosynthetic pathway that when silenced, produces a white, bleached phenotype, visible in the leaf in the middle of each picture. Because PDS produces such a visible phenotype, it was used to verify that the silencing system is working in the tomato plants. Plants were infiltrated with *Agrobacterium tumefaciens* carrying the TRV RNAs, and observed here 3.5 weeks post-infiltration. Necrotic lesions were observed on the leaves of the Rab GDI-silenced plants, visible in the leaf in right side of the picture.

c) RT-PCR of silenced *L. esculentum* cv Tiny Tim and cv Pilgrim. Seen is a 1% agarose gel of

RT-PCR products from PDS-silenced, Rab GDI-silenced, or wild-type, uninfected tomato plants. There are comparable levels of eEF1A product among the three samples, with slightly lower levels in the Rab GDI-silenced plant. RNA was extracted using the Trizol™ reagent (Invitrogen) and cDNA was created using the SuperScript™ First-Strand Synthesis System (Invitrogen). One microgram of RNA for each sample was used to create the cDNA. PCR products are seen here

after 35 cycles in a PCR machine. Primers were used to amplify either a 300 bp fragment of eEF1A cDNA or part of the Rab GDI cDNA (bp 300-700).

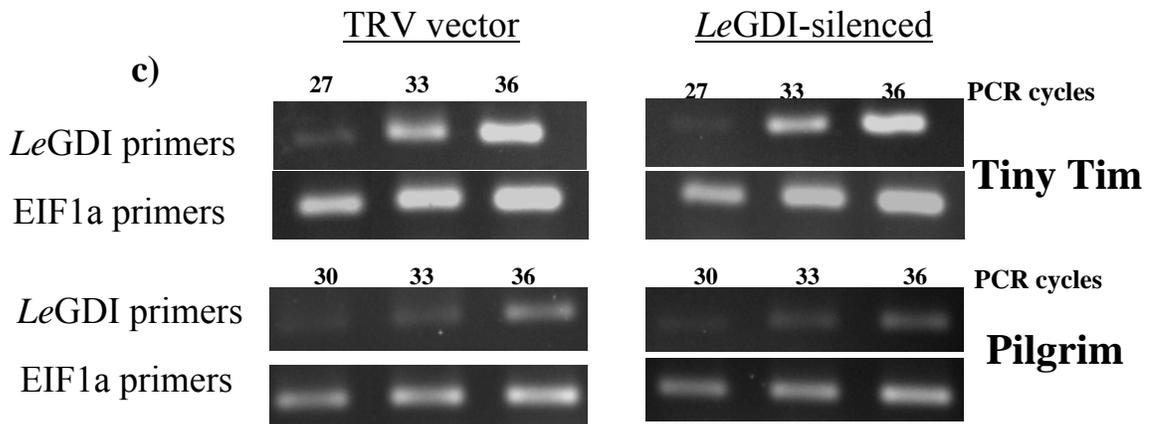
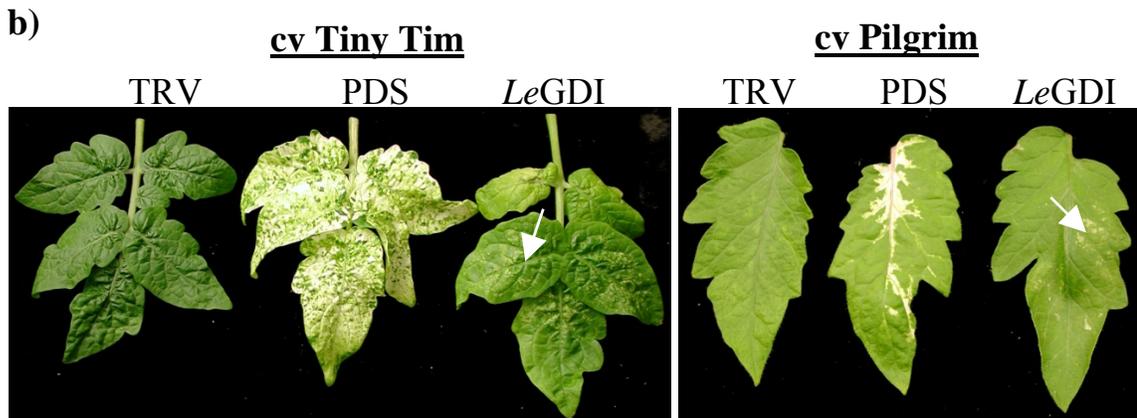
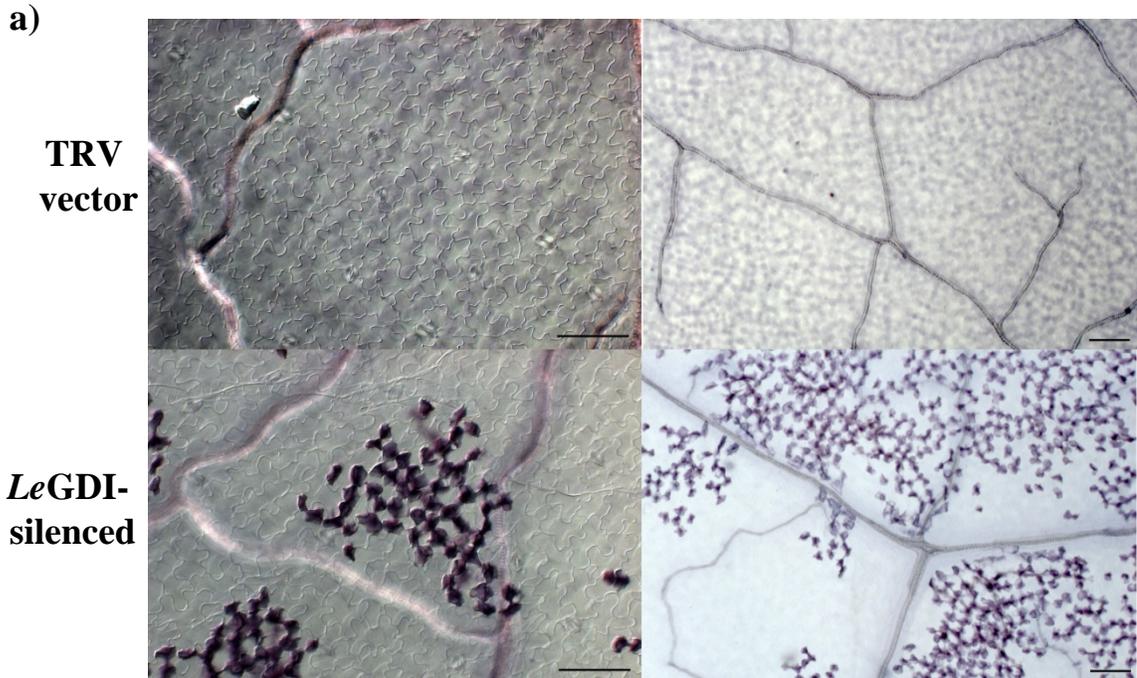
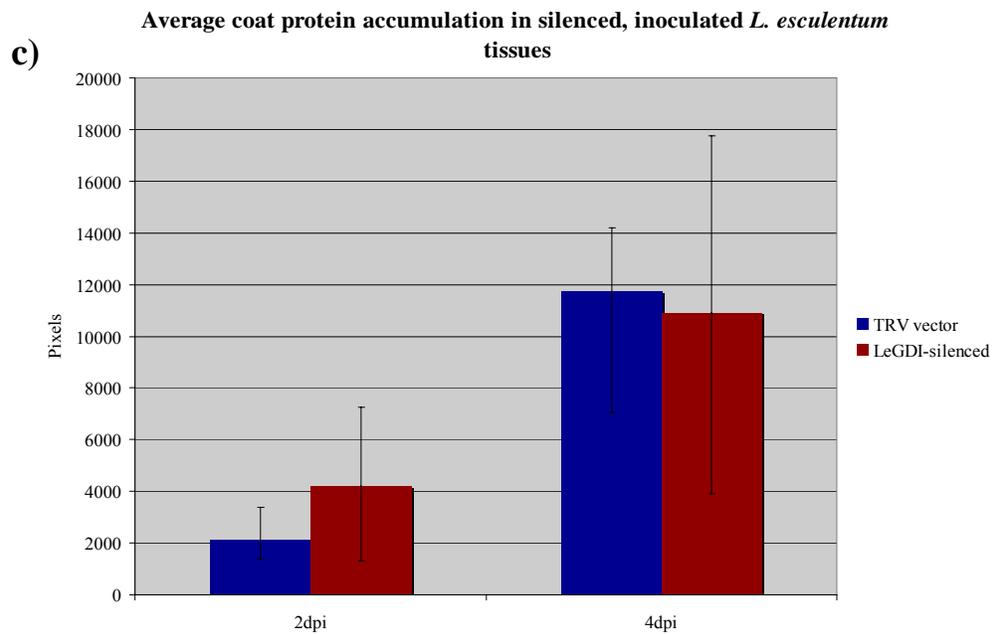
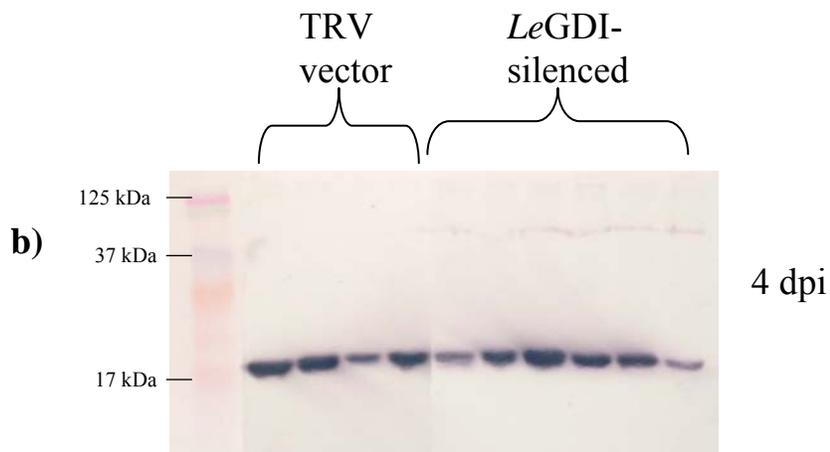
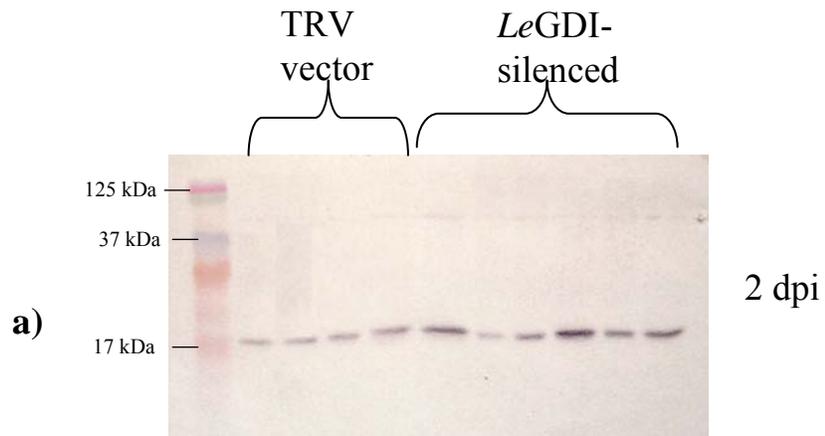


Figure 18. No clear difference in TMV Coat protein accumulation in *LeGDI*-silenced *L. esculentum* tissues. *LeGDI*-silenced and TRV vector control tomato leaf tissues were manually inoculated with 20µl of 0.5mg/mL wt TMV. Inoculated tissues were collected two and four days post-inoculation. The tissue was ground in phosphate buffer (pH 7.0). Total protein concentration was determined using a Bradford assay. Five micrograms of protein was loaded for each sample onto an SDS polyacrylamide gel, blotted, and detected with anti-CP antibody. Shown are blots of the same plants at two (**a**) and four days post-inoculation (**b**). The bands were measured and the average for each blot is seen in (**c**). These data are representative of at least three independent samples. Each set of data showed no distinct difference in coat protein accumulation between the two groups of plants.

Coat protein accumulation in Rab GDI-silenced *L. esculentum* tissue



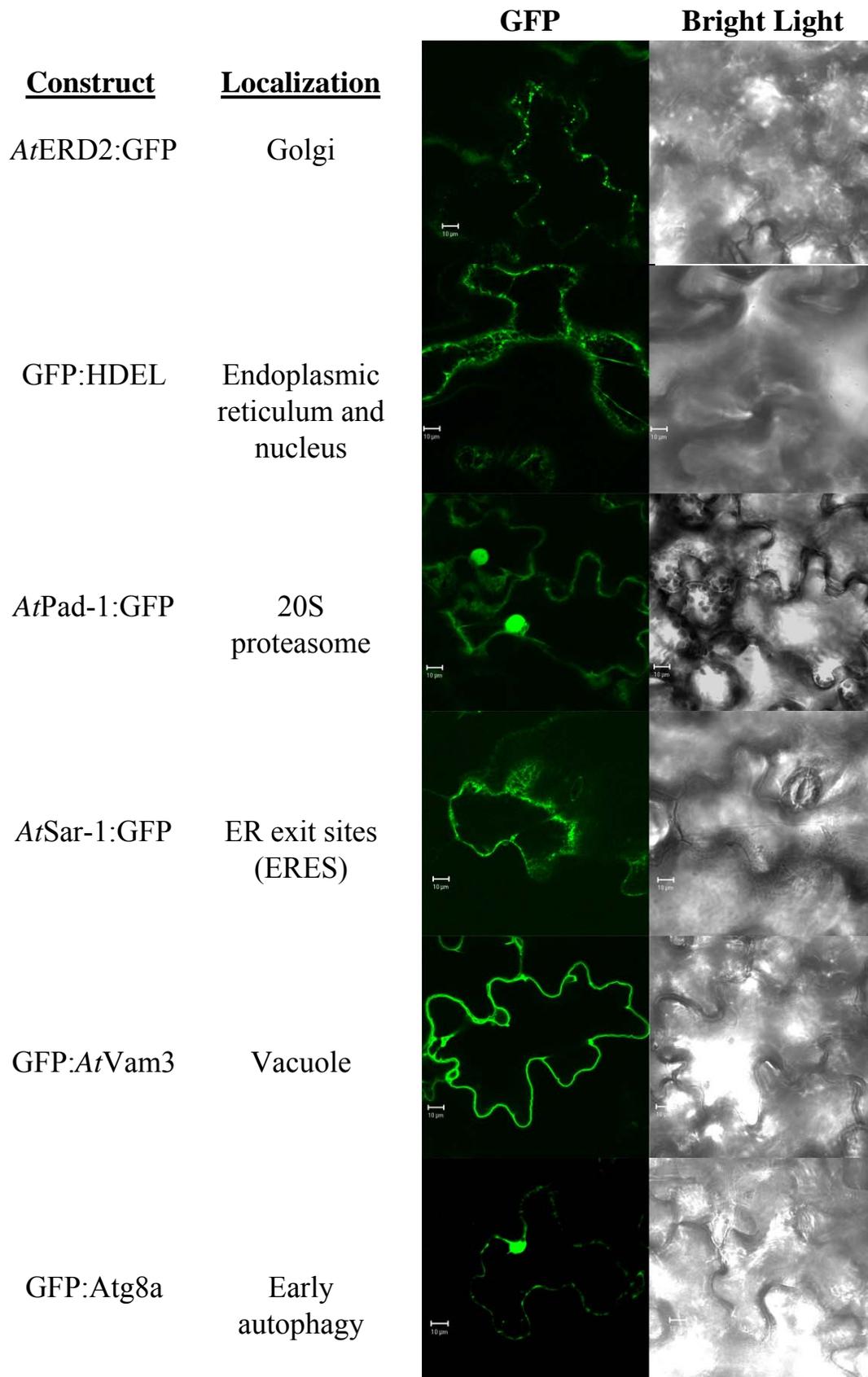
and their absence may be lethal. The absence of a change in viral accumulation in the *LeGDI*-silenced tomato plants may be due to cells actively silencing Rab GDIs, and then dying. Since protein samples are equilibrated on total protein levels, silenced, dead cells would have no effect on viral accumulation as seen by a Western blot.

Silencing NbGDI produces endomembrane changes similar to TMV infection

Since Rab GDI proteins regulate the activity of essential proteins in vesicle trafficking and because TMV replicates on and alters host membranes, changes to the endomembrane system were examined in Rab GDI-silenced and TMV infected cells. To monitor changes to the endomembrane system, several membrane marker proteins were chosen based on their localization and expressed as GFP fusion proteins (see Appendix I for more information on these proteins). Each protein targets a different intracellular membrane and has been previously characterized. Transgenic plants constitutively expressing the marker protein from a 35S promoter were created and stably transformed lines were selected based on GFP expression. As depicted in Figure 19, the proteins localize to parts of the cell as previously characterized, although the GFP:HDEL localized to the nucleus of the cell as well as the ER, possibly due to the small size of the protein (~26 kDa).

Each of these transgenic plants was examined for changes due to TMV infection and *NbGDI* silencing. During TMV infection, there were clear changes to the vacuolar membrane and the endoplasmic reticulum. Plants were inoculated with a TMV:dsRed virus that expresses dsRed (a red fluorescent marker protein) from a CP promoter. On the leaves that have been inoculated with the TMV:dsRed virus, infection

Figure 19. Marker proteins developed for intracellular organelles. Seen on the right are confocal microscopy images of GFP fusion proteins transiently expressed in *N. benthamiana* leaf epidermal tissue. Each fusion protein was expressed from the pBI121 vector with a single 35S promoter. Leaf tissue was infiltrated with *Agrobacterium tumefaciens* carrying the modified pBI121 vector and images were monitored 40-46 hours later. GFP is expressed as either N- or C-terminal fusions, as indicated to the left of each image. In the middle-left column is the organelle to which each protein has been shown to localize. The middle-right column is the GFP fusion proteins and the right column is the same cells imaged using bright light microscopy. Bars indicate 10 μm .



foci develop. These foci are areas where a one or two cells are initially infected with the virus. The virus establishes an infection and then moves cell-to-cell towards neighboring cells from those initial cells in a circle-like pattern. What develops is an area of infection where the cells in the center have been infected by the virus for a longer time than those on the periphery, which is in this case marked by the dsRed express protein (Figure 20a). Cells in the middle of the foci at six to ten days post-inoculation are shown in Figures 20 and 22. Previously, it has been shown that TMV replication occurs in close association with the host cell's ER and alters the morphology of ER [26, 121]. Late in infection, large bodies known as x-bodies appear, composed of TMV RNA, proteins, host proteins, and ER-derived membranes [21]. Therefore, the changes to the GFP:HDEL transgenic plants are consistent with previous reports. Large bodies (average 13 μm), sometimes larger than the cell nucleus (average 11 μm) and composed of ER membrane, were seen within the cells (Figures 20b, c, and d). Also, cells that had been infected later and were on the outer edge of the infection foci exhibited small GFP:HDEL punctates at the periphery of the cell, which is consistent with the literature describing plasmodesmata (Figure 21) [67]. Since plasmodesmata are the main route of virus to travel from one cell to another, as had been suggested by previous work, the alterations seen to the ER are possibly to allow viral cell-to-cell spread [26, 66, 67, 122].

Changes to the vacuole during TMV infection have not been previously described. In mock-infected cells, an invaginated vacuolar membrane is seen with the GFP:*AtVam3* construct, which is consistent with previous reports of plant vacuoles (Figures 19, 22b, and 22d) [123]. The fusion protein is also seen surrounding small mobile spheres,

Figure 20. The ER is altered by TMV infection.

a) TMV:dsRed infection foci. Shown is a single TMV infection area in *N. benthamiana* leaf epidermal tissue. Cells were imaged using the 10x dry lens and the 543nm and 488nm wavelengths to image GFP fusion proteins and dsRed express. The image is combined from dsRed, GFP, and bright light images. The dsRed is being expressed from the TMV genome inoculated into a transgenic *N. benthamiana* plant expressing GFP:HDEL constitutively. The box indicates where in the foci the images in **b)** were taken; the closer to the middle of the area, the older the TMV infection.

b, c, and d) Infected GFP:HDEL plants from center of infection area. Shown are three images of cells taken from the center of an infection area as indicated by **a)**. Arrows indicate formations that only appear in infected plants and are reminiscent of previously described x-bodies [21, 121]. N indicates the nucleus of the cell and the bar indicates 10 μ m.

HDEL-GFP
TMV-infected 5dpi

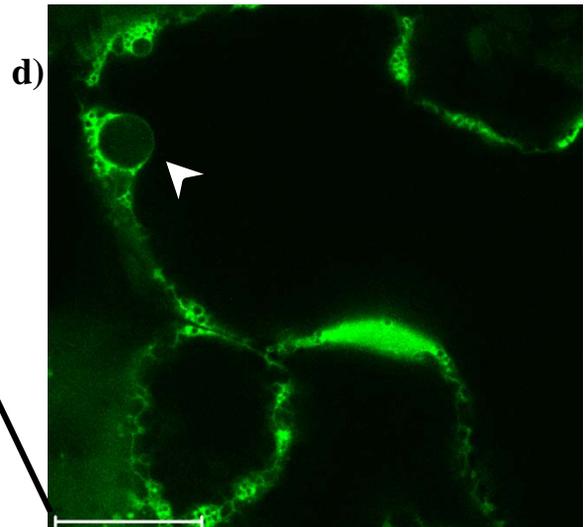
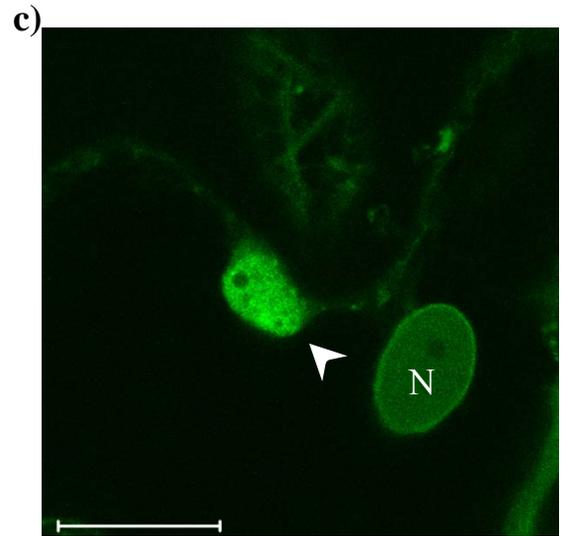
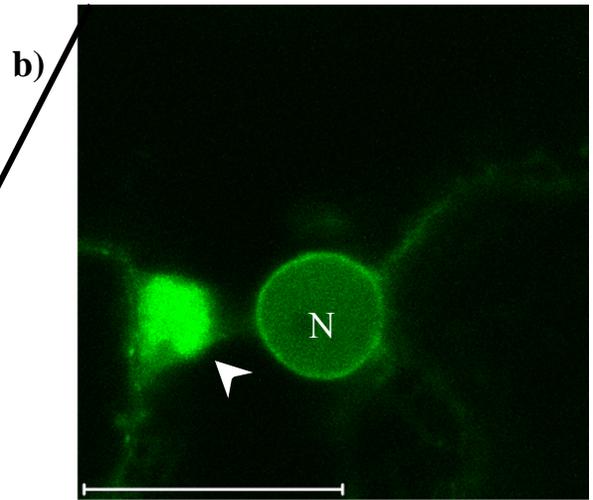
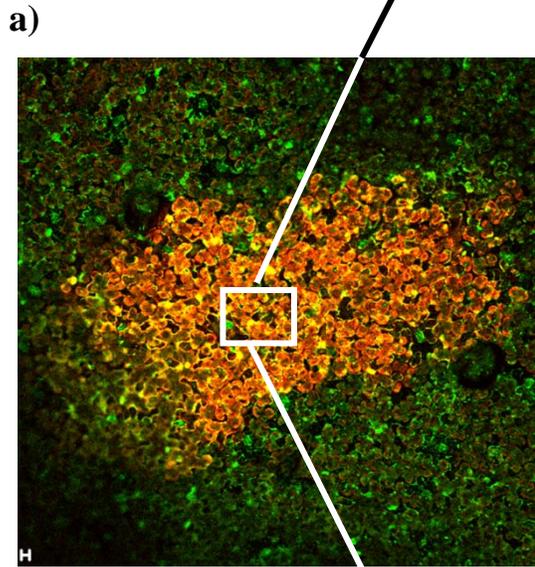
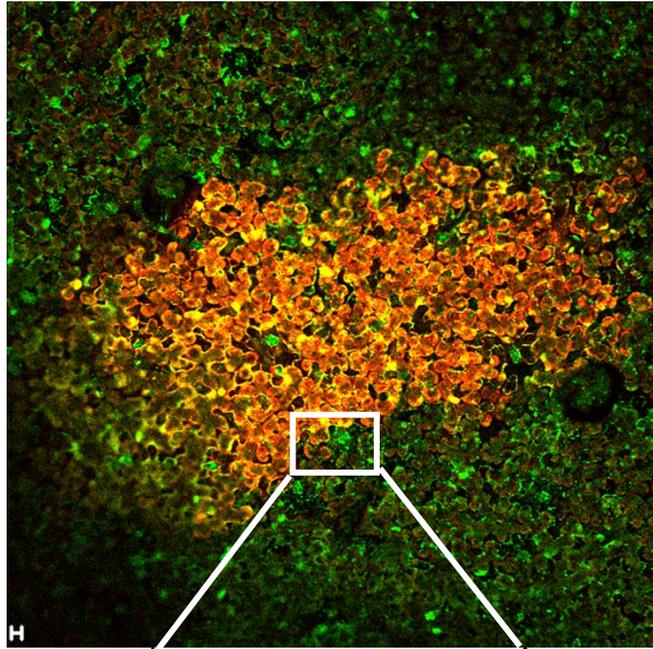


Figure 21. ER vesicles appear at the cell periphery during initial stages of infection. At the top is an image of a TMV:dsRed area in GFP:HDEL *N. benthamiana* plants. The image is combined from dsRed, GFP, and bright light images. The box indicates where the lower image was in relation to the foci. Since the cell is at the periphery of the infected area, the infection is at an earlier stage than those seen in the previous figure. The lower image depicts the GFP:HDEL protein in small aggregates, possibly vesicles, at the periphery of the imaged cell. The paired aggregates at the edge of the cell are similar to images identified as labeling the plasmodesmata of the cell [124].

HDEL-GFP
TMV-infected 5dpi

a)



b)

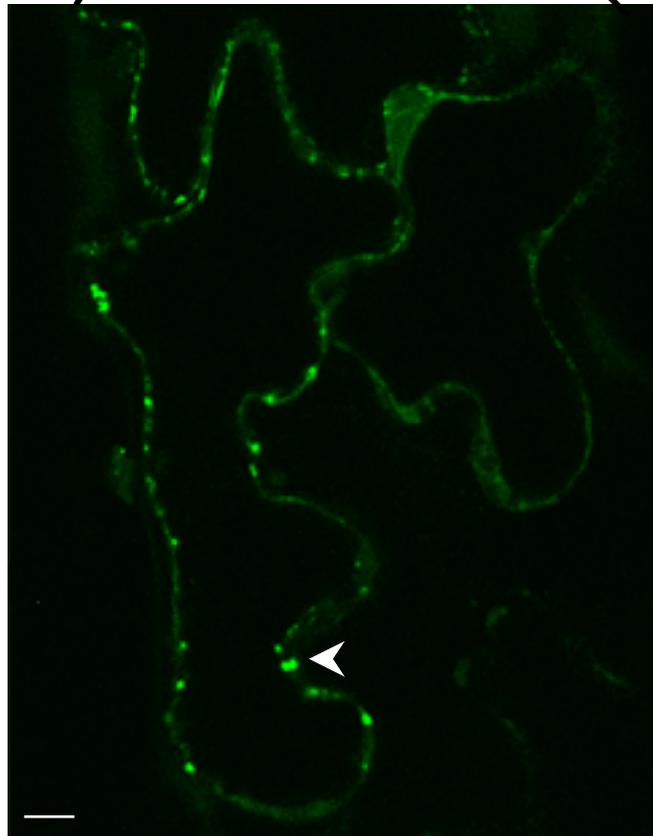
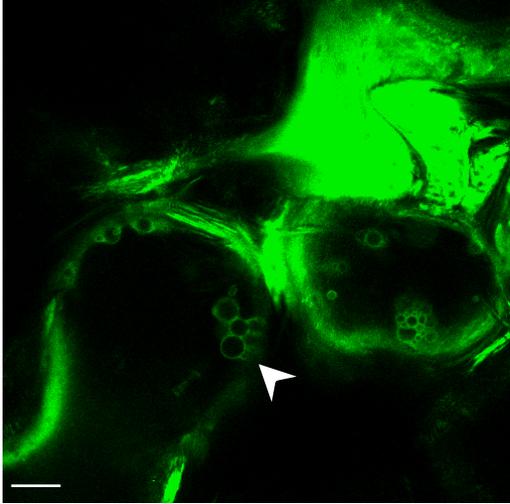


Figure 22. TMV infection alters vacuolar morphology. Seen are confocal images of TMV:dsRed or mock infected GFP:AtVam-3 transgenic *N. benthamiana* plants. Cells were imaged as previously described.

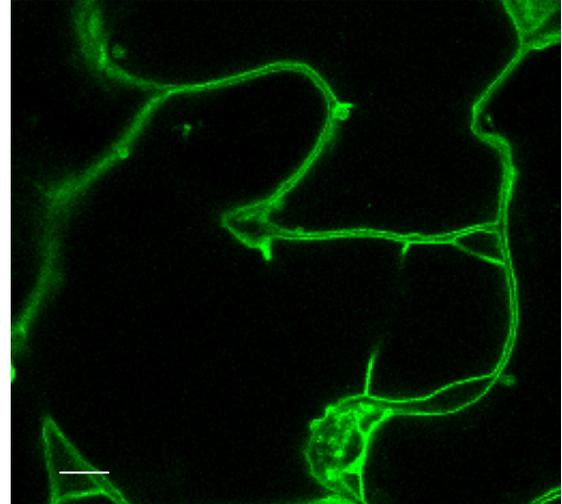
a) and **c)** are TMV-infected plants. AtVam-3 localizes to the vacuolar membrane, or tonoplast of the cell. Arrows indicate aggregates of spheres with vacuolar membrane that, although present in both mock (**b** and **d**) and TMV-infected (**a** and **c**) tissue, appears to aggregate only in TMV-infected tissue. The vacuolar membrane has not been previously shown to be altered during TMV infection. In **e)** an image of a TMV:dsRed infection area is shown with a box to indicate the location of the images taken. Bars indicate 10 μm .

Vacuolar Marker (GFP:*AtVam3*)

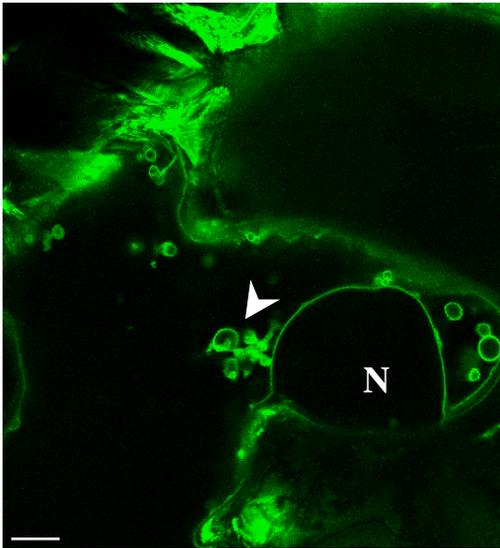
a) **TMV-infected**



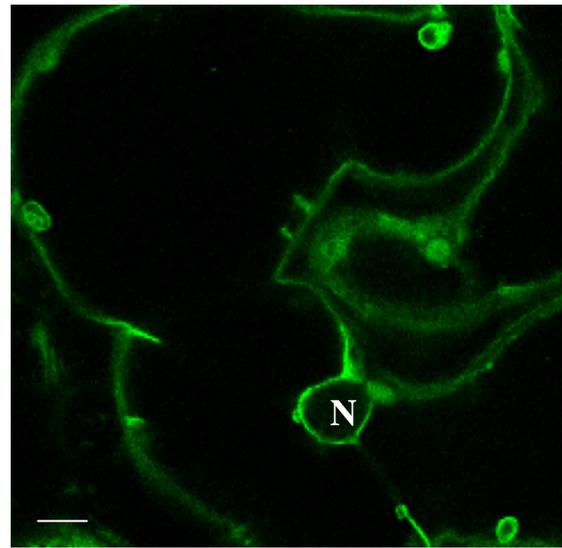
b) **Mock- inoculated**



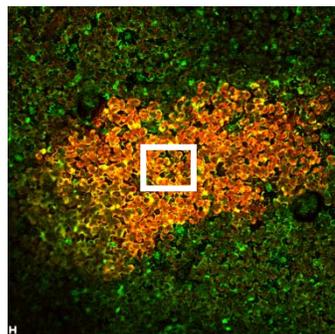
c)



d)



e)



possibly vesicles. During TMV infection, the small vesicle-like bodies appear to accumulate to a greater extent than in mock-infected cells (Figure 22). Preliminary data of three independent sets of confocal images indicates that there is about a four-fold increase in the number of vesicle-like structures in TMV- versus mock-infected cells.

Interestingly, when *NbGDI* was silenced in these marker plants, there was no noticeable effect on either the GFP:HDEL or the Sar-1:GFP transgenic plants which target the ER (data not shown). However, in the GFP:*AtVam3* plants there was about a two-fold increase in vesicle-like body accumulation based on preliminary data of five different sets of confocal images, which is similar to what was seen with the TMV-infected plants, indicating a possible role for Rab GDI proteins in membrane rearrangement during TMV infection (Figures 22 and 23).

TMV infection alters NbGDI:GFP expression and localization

Since the absence of Rab GDI proteins seems to induce morphological changes to the cell similar to TMV infection, the next question was whether TMV affects Rab GDI proteins, possibly reducing their levels. To directly determine the effects of TMV on Rab GDIs during infection, *NbGDI:GFP* transgenic plants were created. *NbGDI:GFP* was expressed from a double 35S promoter with a NOS terminator. During TMV infection, *NbGDI:GFP* expression was altered as compared to the mock-inoculated plant, when measured by confocal microscopy and by Western blot (Figure 24). Reduction of Rab GDI proteins during infection is consistent with the previous observation that silencing of this protein produced changes similar to infection.

Figure 23. *NbGDI*-silenced plants have a vacuolar morphology similar to TMV infection.

a) Shown are confocal images of transgenic GFP:*AtVam-3* *N. benthamiana* plants. The cells were imaged as previously described. Shown are TMV-or mock-infected cells on the bottom and *NbGDI*-silenced or TRV vector on the top. Both the *NbGDI*-silenced and the TMV infected cells show aggregation of vacuolar membrane spheres, possibly vesicles (white arrows), which are absent in the mock and vector controls. Bars indicate 10 μ m.

b) Seen are samples from a semiquantitative RT-PCR of GFP:*AtVam-3* tissue either silenced for *NbGDI* or with the TRV vector alone. PCR reactions were performed with 1 μ g of cDNA from leaf tissue and samples were taken at several cycles. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and imaged with AlphaImager transilluminator and camera (Alpha Innotech Corp.). Primers for *ef1A* were used as a control for total RNA concentration. Levels of *NbGDI* were reduced in *NbGDI* silenced tissue as compared to the vector control.

Vacuolar Marker (GFP:*AtVam3*)

a)

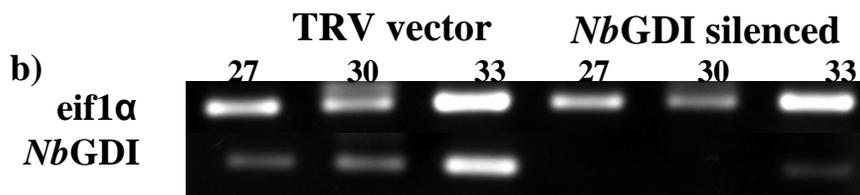
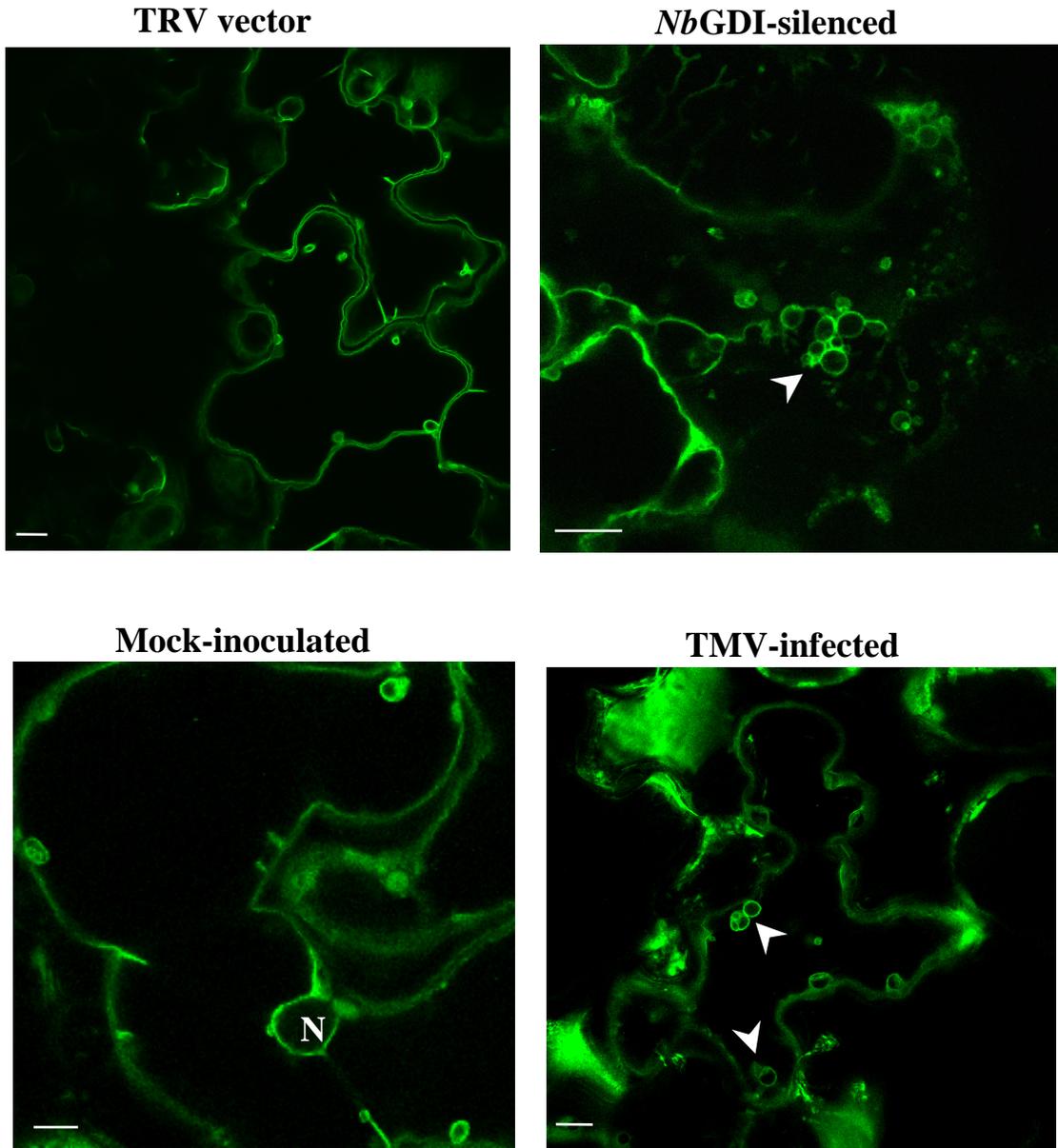


Figure 24. *AtGDI2*:GFP is altered by TMV infection.

a) Confocal images of transgenic *AtGDI2*:GFP *N. benthamiana* plants show altered

expression in infected cells. Cells were imaged as previously described using a 10x dry lens.

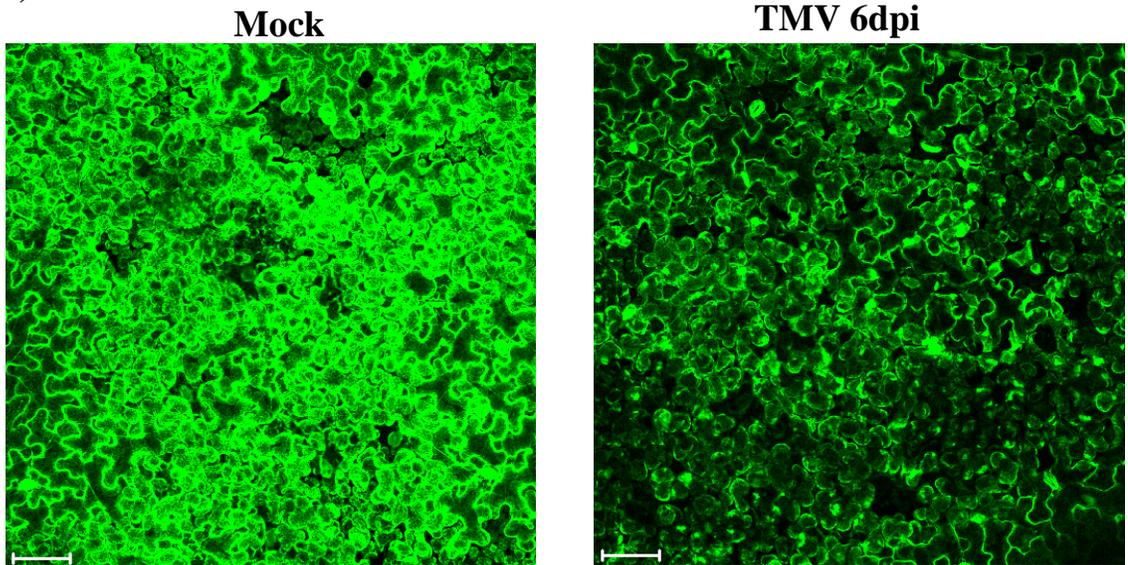
AtGDI2:GFP plants were rub inoculated with either 20 μ l of 0.1 mg/mL of TMV:dsRed or 20 μ l phosphate buffer (pH 7.0). On the left is mock-inoculated tissue and on the right is TMV:dsRed-inoculated tissue six days post-inoculation. The dsRed is not shown here to visualize the change in *AtGDI2*:GFP expression. The same detection settings were used in both images to show the changes in expression.

b) Western blot of wt TMV-infected transgenic *AtGDI2*:GFP *N. benthamiana* plants. Tissue

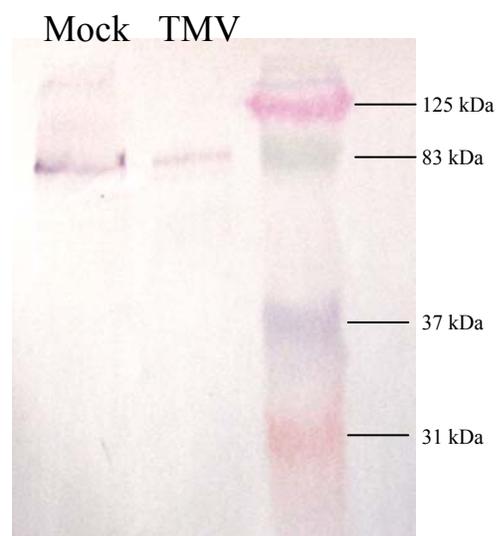
was collected at six days post-inoculation, ground in Laemmli loading buffer (1mg tissue/1 μ L buffer), and loaded onto a polyacrylamide gel. The gel was blotted onto nitrocellulose; bands were detected using anti-GFP antibodies. The far right lane is the protein ladder (Biorad). Protein sizes are indicated to the right of the ladder. Note that protein *AtGDI2*:GFP protein accumulation is lower in infected tissues.

***AtGDI2*:GFP is altered by TMV infection.**

a)

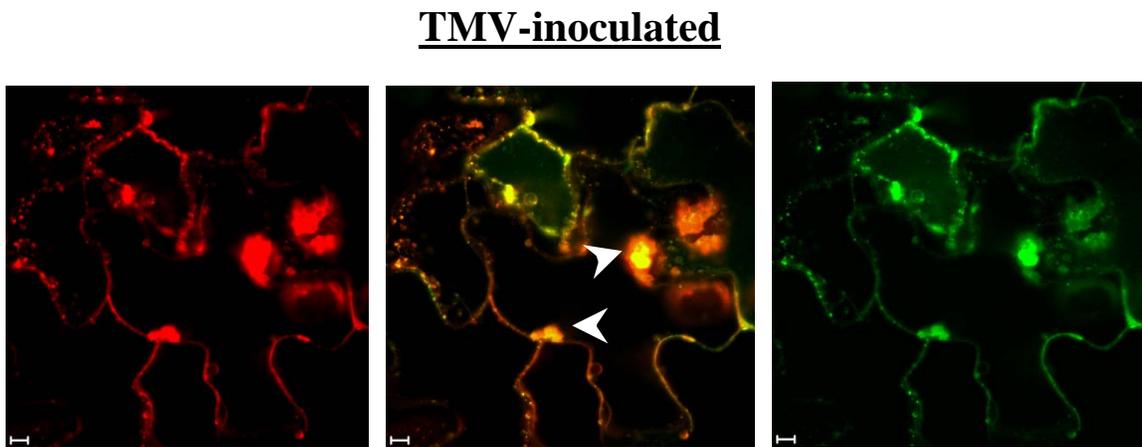
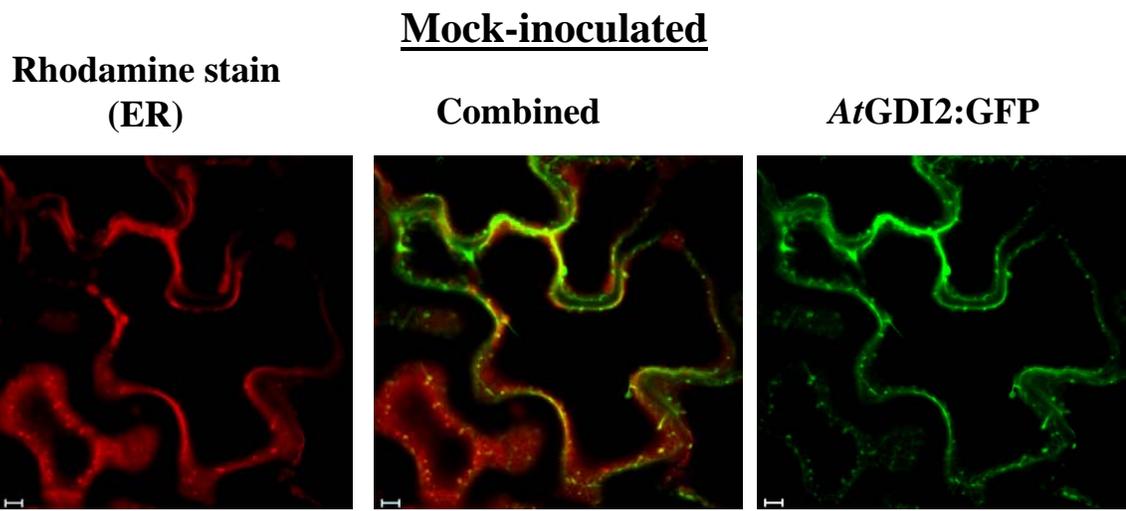


b)



To characterize further how *NbGDI:GFP* might be altered during TMV infection, further confocal microscopy was performed to determine if protein localization is also altered (Figure 25). Since initial experiments on TMV-infected tissue showed *NbGDI:GFP* localizing to large aggregate bodies similar to what was observed for the ER, *NbGDI:GFP* TMV- and mock-inoculated samples were stained with a rhodamine ER stain (rhodamine B hexyl ester). In mock-infected cells, there is incomplete colocalization between *NbGDI:GFP* and the ER stain. *NbGDI:GFP* appears around small vesicle-like structures and at the periphery of the cell. Rab GDI proteins are involved in vesicle trafficking throughout the host cell, not just with the ER, so this localization is consistent with its function. In infected cells, large bodies form which contain *NbGDI:GFP* and fluoresces with the ER stain. As seen with the TMV-infected GFP:HDEL plants, these bodies are similar to x-bodies that have been previously described, indicating a possible role for Rab GDIs in replication or replicase complex formation [21, 121].

Figure 25. TMV infection alters *AtGDI2* localization. Shown are confocal images of mock- and TMV-infected transgenic *AtGDI2*:GFP *N. benthamiana* plants. Images were taken ten days post-inoculation. The two TMV-infected images were from two independent samples. The samples were stained with rhodamine B hexyl ester by vacuum infiltration of a 1µg/mL solution; rhodamine B hexyl ester localizes to the ER and mitochondria of cells. The confocal images were taken as previously described. The left column shows the samples that were excited with a 543nm laser. The right column shows samples excited with a 488 nm laser and the middle column is the combined images. The top row of images is of mock-infected cells. Note that there is partial colocalization of the ER stain and the *AtGDI2*:GFP protein. The lower two rows are of TMV-infected cells. White arrows indicate formations not seen in mock-infected cells and which shows both the ER stain and the presence of the *AtGDI2*:GFP protein. As seen in the GFP:HDEL transgenic plants, these formations occur only in infected cells and are reminiscent of x-bodies which have been previously reported [21, 121].



Methods and materials

Yeast two-hybrid: *Saccharomyces cerevisiae* strain L40 was transformed with both pGAD (Clontech) containing the “prey” and pLexA containing the “bait” construct.

Quantitative assay: Yeast containing both bait and prey constructs were grown in liquid media to an OD₆₀₀ of 0.6. One mL of culture was pelleted and resuspended in 100µL of Z-buffer. Added to the culture was 10µL of 0.1% SDS, 20µL of chloroform, and 200µL of ONPG (ortho-nitrophenyl-β-galactoside). Samples were incubated for 30 minutes at 25° C. The reaction was stopped by adding 500µL 1M Na₂CO₃. The OD was measured at both 420 nm and 550 nm and Miller units were calculated. X-gal solution: 5µL Triton X 100, 100 µL X-gal (5-methyl-4-chloro-3-indoly-B-D-galactosidase) (20mg/mL), 5mL Z-buffer; Z buffer (1L): 16.1g Na₂HPO₄, 5.5g NaH₂PO₄, 0.7g KCl, 0.25g MgSO₄, 2.7 mL β-mercaptoethanol

RT-PCRs: RNA was extracted as per manufacturer’s protocols using the Trizol reagent. One microgram of RNA was pretreated with RQ1 DNase (Promega) and then reverse-transcribed using the SuperScript® First-Strand Synthesis System (Invitrogen). Four microliters of the reaction were used for PCR analysis. For semiquantitative analysis, a 50-µL PCR reaction was used to amplify fragments of the gene as indicated. Four microliters of sample were removed at the end of 27, 30, and 34 cycles. These cycles were used based on previous semiquantitative PCRs, which used a range of between 18 and 36 cycles. Samples were visualized via agarose gel electrophoresis and photographed using AlphaImager (Alpha Innotech). For a list of primer sequences used to amplify gene segments, please refer to Appendix I.

Pull-down assays: Fifty-mL cultures of *A. tumefaciens* containing individual pBIN vector alone, or the 126 kDa helicase with an HA tag, or *AtGDI2* with a GFP or HA tag were grown overnight at 30°C in LB media containing antibiotics, 10 mM MES and 20 µM acetosyringone. Agrobacterium cells were pelleted and resuspended in infiltration media (10 mM MgCl₂, 10 mM MES, 200 µM acetosyringone) at an O.D. of 0.5. For coinfiltration, equal volumes of Agrobacterium cultures were used and infiltrated into three-week-old *N. benthamiana* seedlings. For infiltration of infected tissue, *N. benthamiana* plants at two days post-inoculation were infiltrated with Agrobacterium culture containing pBIN *AtGDI2*:GFP on inoculated leaves. Leaf samples were taken 40-48 hours post-infiltration.

Leaf samples were ground in extraction buffer on ice using a mortar and pestle. Samples were then centrifuged at full speed for ten minutes. Supernatant was removed. One mL of supernatant was placed in a 1.5mL tube and preincubated with 30 µL of pre-washed Agarose A beads. Samples were incubated with beads, shaking at 4° C for 2-3 hours. Samples were centrifuged at 4,000 G for one minute and the supernatant was removed and placed in new 1.5 mL tube. The sample was incubated with or without the appropriate antibody, shaking at 4° C overnight. The next day, beads were collected by centrifugation at 4,000 G for one minute and the supernatant was discarded. The samples were washed twice with wash buffer and resuspended in Laemmli sample buffer for Western blot analysis.

Wash buffer: 25mM Tris (pH 7.5), 250mM NaCl, 2mM EDTA, 0.05% Triton x-100, 1mM Phenylmethanesulfonyl fluoride (C₇H₇FO₂S).

Extraction buffer: 50mM Tris (pH 8.0), 150mM NaCl, 0.5% Triton x-100, 0.2% β-mercaptoethanol, 5% glycerol, 10 μL/mL protease inhibitor cocktail (Sigma-Aldrich), 1mM Phenylmethanesulfonyl fluoride (C₇H₇FO₂S).

Imaging of whole leaves: Whole leaves were removed from the plant and imaged using a long wavelength UV lamp and a Tiffen™ 58mm Yellow2 wavelength filter on a Canon EOSD60 digital camera with a tripod. Images were taken ten days post-inoculation with TMV:GFP.

Confocal imaging: Epidermal tissue was mounted using water and imaged using a Zeiss LSM510 laser scanning confocal microscope system with 10x NA 0.8 dry and 63x NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thornwood, NY). Excitation sources were 488 nm for GFP and 543nm for dsRed. Images were modified in Zeiss LSM Imager Examiner and processed for printing in Adobe Photoshop (Grand Prairie, TX).

Trypan blue staining: Tomato leaf tissue was placed in a 20mL capped glass tube. Three milliliters of trypan blue solution (0.67 mg/mL trypan blue in 1:1:1:1:6 (water:glycerol:lactic acid:phenol:ethanol; vol:vol)) were added. Samples were incubated in boiling water for two minutes. After cooling, the trypan blue solution was removed and replaced with 5mL of chloral hydrate (1 kg /400 mL of water) and destained for 24 hours.

The solution was then replaced with fresh chloral hydrate and destained for an additional one to two days. Cells were imaged using a light microscope attached to a digital camera.

TMV infection: Leaves from either Rab GDI-silenced plants or TRV vector control plants were dusted with carborundum and mechanically inoculated with 20 μ L of 0.1 mg/mL wt TMV for tomato or 20 μ L of 0.05 mg/mL wt TMV for tobacco plants. For TMV:GFP, 20 μ L of 0.5 mg/mL of purified virus was used for manual inoculation. Each virus assay sample represented leaf tissue taken from two independently inoculated leaves. Samples were ground either in 0.1 M Sodium phosphate buffer (pH 7.0) or directly in Laemmli sample buffer.

Coat protein Western blots: Protein levels loaded onto denaturing PAGE gels were equalized by determining total protein levels via Bradford assay. Five micrograms of protein were loaded per well and were separated by PAGE. Polyacrylamide gels were blotted to nitrocellulose membranes. Blotted proteins were probed with anti-TMV-CP antibody as previously described. TMV CP levels were quantified using ImageJ software (National Institutes of Health).

Marker proteins: Marker proteins were created as either C-terminal or N-terminal fusion proteins as indicated (Appendix I) and cloned into the pBI121 binary expression vector. pBI 121 was modified to replace the β -glucuronidase gene with a short linker sequence that contained several restriction endonuclease sites (Appendix I). pBI 121 contains a single 35S promoter and NOS terminator.

Transgenic tobacco plants: Protocol to make transgenic plants was adapted from [125]. *Agrobacterium tumefaciens*, strain GV 3101, carrying the modified pBI 121 plasmid with the marker protein was grown up in a 5mL culture of LB containing 25µg/mL gentamycin, 50µg/mL rifampicin, and 50µg/mL kanamycin. Surface-sterilized leaf tissue was incubated with the *Agrobacterium* and incubated on regeneration plates. Two days later, the leaf tissue was transferred to new plates containing 400µg/mL cefotaxime. Two days later the leaf tissue was transferred to new plates containing 400µg/mL cefotaxime and 50µg/mL kanamycin. Leaf tissue was transferred to new plates with 400µg/mL cefotaxime and 50µg/mL kanamycin and monitored for callus formation. After calluses were formed and had established at least two small leaves, the calluses were cut from the original leaf tissue and moved into magenta boxes containing rooting media. Once roots had been established, the plants were removed from the rooting media and placed in soil. Plants were maintained in planter flats with domes to increase humidity for two weeks and gradually introduced to the humidity of the growth chambers. All chambers were maintained at 23°C with a 12-hour photoperiod at 55% humidity.

Regeneration media: Murashige and Skoog Basal Salt Mixture (1x), 1.16mg/mL 6-benzylaminopurine (C₁₂H₁₁N₅), 0.1mg/mL 3-indoleacetic acid (C₁₀H₉NO₂), 30g/L sucrose, Gamborg's Vitamin Solution (1x), and 8g/L agar (pH 6.0).

Rooting media: Murashige and Skoog Basal Salt Mixture (1/2x), 10g/L sucrose, 0.2mg/mL 3-indoleacetic acid (C₁₀H₉NO₂), 8g/L agar (pH 5.7).

Virus strains: The U1 strain of TMV was used for all assays and is referred to as the wild type sequence. The strain is maintained in tobacco and all cDNA clones are originally based on work from [116]. Inoculation of wt virus was performed with purified virions; 20 μ L of 0.05mg/mL was inoculated onto tomato leaf tissue and 20 μ L of 0.005 mg/mL was inoculated onto in *N. benthamiana* tissue.

TMV:GFP and TMV:dsRed: GFP or dsRed express was expressed from the TMV30B plasmid with the fluorophore PCR-modified to contain Pac-1 and Xho-1 enzyme sites. The plasmids were transcribed using a T7 RNA polymerase reaction and inoculated into *N. benthamiana* plants. The plants were monitored for fluorescence and later virions were purified as previously described [126]. Infection with these viruses was performed via rub inoculation of 20 μ L of 0.5mg/mL of purified virions.

Tobacco rattle virus (TRV) system: The *Tobacco rattle virus* VIGS vectors (pTRV1 and pTRV2) were kindly provided by Dr. S. P. Dinesh-Kumar, Yale University. The *LeGDI* ORF, *NbGDI* ORF, *NtGDI* ORF, and *AtGDI2* ORF were PCR-modified to contain 5' EcoR1 and 3'KpnI sites, inserted into a similarly-digested pTRV2 plasmid and transformed into *A. tumefaciens* strain GV3101. For VIGS assays, 50 mL cultures of *A. tumefaciens* containing individual TRV vectors were grown overnight at 30°C in LB media containing antibiotics, 10mM MES and 20 μ M acetosyringone. Agrobacterium cells were pelleted and resuspended in infiltration media (10 mM MgCl₂, 10 mM MES, 200 μ M acetosyringone) at an OD₆₀₀ of 0.5. *A. tumefaciens* containing pTRV2-Rab GDI or the unmodified empty pTRV2 vector were mixed with an equal volume of cells

containing the pTRV1 vector and syringe-infiltrated into the cotyledons of two-week-old tomato seedlings or two-week-old *N. benthamiana* seedlings.

Discussion

Interactions between a virus and its host can determine the establishment and course of an infection. Here, I have investigated one particular interaction between *AtGDI2* and the 126 kDa replicase protein. The two proteins interact *in vitro* in a yeast two-hybrid assay and *in vivo* in infected tissue. This interaction is not unique to Arabidopsis, but is also present in other TMV hosts, such as tobacco and tomato. Using the TRV-based gene silencing system, silencing of *N. benthamiana* Rab GDI resulted in an enhancement in the number of TMV infection sites, but no corresponding increase in viral spread and accumulation. Because TMV:GFP is used as a visual marker of infection areas and spread, it is important to note that because this virus carries the extra GFP gene on its genome, it requires a higher concentration of virions to infect plants and moves slower in systemic leaves than wt TMV. This means that TMV:GFP is an impaired virus compared to wt. It is possible that the increase in infection sites on *NbGDI*-silenced plants makes a more noticeable difference with impaired viruses. However, the data still suggest that Rab GDI proteins play a role in TMV infection in initial establishment of infection sites, but not in viral spread. This effect is unique in that most host proteins identified affect multiple stages of infection, not just the initial establishment of infection.

In tomato plants, on the other hand, silencing of *LeGDI* resulted in necrotic lesions.

Because Rab GDI transcripts have such a high similarity to each other, silencing of one Rab GDI may silence all of the Rab GDI transcripts in a host. The necrotic lesions seen in tomatoes may be a result of silencing multiple Rab GDI genes, which tomato plants may be less tolerant of than tobacco plants. Studies using GFP- or dsRed-expressing TMV

could not be done in tomato plants because the virus very quickly reverts to wt and loses its fluorescent marker. However, wt virus accumulation in *LeGDI*-silenced plants was the same as the vector control. One possible reason that there was no effect on TMV accumulation is that the silenced cells had already gone through cell death and thus there was no effect on the virus.

Because Rab GDI proteins are essential to vesicle trafficking and because TMV replicates in close association with biological membranes, marker proteins were created both to examine the effects of TMV on the host cell's endomembrane system and to determine the effect that Rab GDI proteins have on that same system. Predictably, TMV alters ER morphology during infection, producing large structures that consist of ER-derived membranes, possibly indicative of x-bodies. Also, ER-derived vesicles appeared at the periphery of the cell earlier in infection, which is indicative of plasmodesmata. A cell's ER traverses the plasmodesmata and connects neighboring cells. TMV is known to travel cell-to-cell via the plasmodesmata and the TMV movement protein has been shown to interact with components of the plasmodesmata [23]. Therefore, changes to the ER near plasmodesmata may be indicative of a role for the ER in TMV movement.

Significantly, the vacuolar membrane was affected by TMV infection, which has not been previously reported. In infected cells the vacuole, which is very active with vesicle-like structures moving throughout the cell, appears deficient in the fusion of these vesicles to their target membranes, which leads to the accumulation of these vesicle-like structures. There was no effect on the Golgi, which is surprising since work has shown that the Golgi contributes to ER remodeling and is physically in contact with the ER at

times [127]. There was also no apparent effect on the proteasome, nor on the early autophagy marker.

When *NbGDI* was silenced in these marker plants, there was no apparent effect on the ER, which is significantly altered during TMV infection; however, in *NbGDI*-silenced GFP:*AtVam3* plants, the vacuole exhibited a similar phenotype to TMV infection. In both cases, vesicle-like bodies accumulated and appeared to be impaired in their ability to fuse to target membranes. Additionally, TMV infection alters *AtGDI2*:GFP expression and localization. Protein expression is reduced in TMV-infected versus mock-infected cells. Late in infection, *AtGDI2*:GFP is seen in large bodies that colocalize with the rhodamine ER dye and are similar to the same structures seen in the GFP:HDEL-infected tissue. These structures are most likely viral replication complexes or their precursors that have been previously described. Because *AtGDI2*:GFP localizes to structures during infection that are most likely the sites of viral replication, and because silencing of this transcript alters the endomembrane system in a similar way to that seen in TMV infection, Rab GDI proteins may be playing a role in endomembrane rearrangement during infection and possibly in replication or replicase complex formation.

Taken together, these data suggest a role for Rab GDI proteins in the establishment and possibly the cytological effects of TMV infection. Very few host proteins have been found, and none that interact with TMV, that only affect initial stages of infection. There are several proteins that are required for either replication or movement of TMV, but very little work has been done to understand what makes a particular cell more susceptible

than another to infection, even in the same host. Therefore, this interaction represents a unique opportunity to examine how viruses establish an infection and what makes one cell more susceptible than another. Below are two possible models for how this interaction may play a role in virus infection. In both models, TMV interacts with Rab GDI proteins to create an environment that is more favorable for the establishment of infection.

First, the replicase proteins may be interacting with Rab GDI proteins to induce structures that are advantageous to TMV replication. Membranes are required for TMV replication and host membranes are altered during TMV infection. Also, a host protein, TOM1, is a transmembrane protein that localizes to the vacuole and interacts with the TMV replicase proteins [128, 129]. TOM-1 and its homologue, TOM-3, are required for TMV infection [71]. However, since TMV replicates in close association with the ER, there has been no evidence to indicate how a vacuolar protein contributes to replication in the ER. These vacuolar structures formed during infection, and *NbGDI* silencing may allow the TMV replicase proteins to interact with TOM-1 at a point before it reaches its final destination in the vacuole as it progresses through the secretory pathway.

Another possibility is that the TMV replicase proteins may be interacting with Rab GDI proteins to slow down host protein synthesis. Reduction of host protein synthesis during viral infection is a common strategy of viruses, including TMV [130]. Reduction of host protein synthesis diminishes the production of defense proteins and reduces competition for host protein synthesis components, such as ribosomes and tRNAs. Because Rab GDIs

regulate vesicle trafficking, without them, Rab proteins would not be returned to their donor membrane, slowing down the entire secretory pathway. If the replicase proteins compete with host proteins (such as Rabs) for binding to Rab GDIs, there would be a significant reduction, but not elimination of Rab GDIs for use in the secretory pathway. This reduced pool of Rab GDI proteins would slow down the secretory pathway, including the production of many defense proteins, but not induce cell death, which could result from ER stress. The silencing of Rab GDIs through the VIGS system may reduce their protein to a level much lower than what is seen during TMV infection and tomato plants may be more sensitive to a loss in Rab GDIs than *N. benthamiana* plants, which results in necrotic lesions. Therefore, interaction with Rab GDI proteins may provide a way for the virus to out-compete the host for host cell components, slow down defense protein production, and avoid inducing cell death.

Whatever the effect of the Rab GDI-replicase interaction, it is clear that it affects susceptibility to infection; however, many questions remain. Silencing of Rab GDI genes enhances TMV infection, which is different from most proteins that interact with TMV. This effect of Rab GDI silencing is quite curious. If turning this gene off enhances infection, why does the virus not do so to a greater extent? If Rab GDI proteins are so well conserved, is there a difference in this interaction with non-host species of TMV? If this interaction is important in susceptibility, could it help determine resistant versus susceptible hosts? The models presented above are not mutually exclusive and further work is necessary to identify and understand more completely the mechanism behind Rab GDIs' involvement in establishment of TMV infection. Taken together, these data

indicate that this interaction represents an important part of how a virus can initially establish an infection in the first cells, change the host to be advantageous for replication, and possibly provide a competitive advantage to the virus. Exactly why some cells or some hosts are more susceptible to infection remains an area of active research and an important target for disease resistance strategies.

Chapter 4: Summary and Future Directions

There are several steps to a viral infection: 1) entry, 2) disassembly, 3) translation and replication, 4) viral assembly, and 5) viral movement out of the cell. For the positive-strand RNA virus TMV, entry is through mechanical wounding and changes in ion concentrations cause disassembly of the viral RNA from the coat protein subunits [11]. It has been shown that the replicase proteins are the first proteins translated and that efficient viral replication requires biological membranes [52]. The ER of the host cell becomes severely altered during the course of TMV infection [21, 121]. The infection process in an initial cell, and that of subsequent cells, is different with different constraints [67].

There has been significant work devoted to understanding TMV viral entry and disassembly. However, how a single, or even a few, uncoated viral RNA molecules form into a replication complex, and how TMV takes advantage of biological membranes, are not understood. It is a big step from a few naked RNA molecules to a fully functioning viral replication complex.

When leaf tissue is manually inoculated with TMV, not all cells inoculated will become infected. We do not fully understand why one cell is more susceptible than another to infection, even on the same plant. Understanding the initial establishment of infection and the question of susceptibility of a host cell to infection are integral parts to characterizing any viral infection and may represent an essential path to developing disease resistance strategies. This study strives to begin to answer a few of those questions.

In the first part of this study, TMV was altered in order to understand more fully how the replicase proteins localize to particular parts of the host cell during infection, and how that may contribute to viral infection. In the second part of this study, the host was manipulated in order to understand the role of a host protein involved in establishing TMV infection.

Replicase proteins and their localization

If a virus does not have the correct proteins and nucleic acids in the correct place at the correct time during infection, its ability to establish an infection and evade host defense systems can be severely compromised. It has been established that TMV replicates in close association with the ER, but its localization to the ER is not well understood. The current study identified a region essential in TMV localization (an ER localization domain) and examined several properties of the replicase proteins to understand what effect their localization has on viral infection. Three models have emerged from this work: 1) the ER localization domain identified in these studies may lie in an area of the replicase protein that is involved in interactions with host proteins; 2) the ER localization domain may not directly contribute to replication, but instead may act to enhance replication or increase the half-life of the replicase protein; 3) the ER localization domain may be part of an internal region of the replicase protein that does not directly interact with any host protein, but contributes to a favorable tertiary structure.

One way to determine if the ER localization domain is involved in host protein binding would be to investigate interactions with known host proteins. TOM1 is the best-known candidate host protein to investigate, and the ER localization domain overlaps with a

region originally identified to bind to TOM1. Work has already been done to study this interaction in the context of a yeast two-hybrid system. Since both TOM1 and the replicase proteins may localize to membranes, a yeast two-hybrid system, which relies on nuclear translocation, is not the best system to study this protein-protein interaction. Instead, a split yellow fluorescent protein (YFP) system, or bimolecular fluorescent complementation assay (BMFC), would allow for *in vivo* detection of an interaction and allow for both proteins to localize normally [131]. In this system, the C-terminal and N-terminal portions of YFP are fused, one to either protein, and coexpressed in the same cell. When the two portions of YFP are in close proximity to each other, the YFP emits light when excited. Each of the previously described mutations could be tested for decreased interaction with TOM1.

Also, in this study, Rab GDI proteins were determined to be important for establishment of initial infection. The same split YFP system could be used to determine if alterations to the ER localization domain alter these host-protein interactions. If a loss of interaction between the replicase and either host protein corresponds to decreased replication or movement, it would support the hypothesis that the ER localization domain acts by interacting with host proteins. To this end, a set of split YFP expression vectors has been developed with multiple cloning sites to allow for both N- and C-terminal fusions of either part of the YFP to both proteins tested. Cloning for these constructs is ongoing.

To determine if the ER localization domain acts to increase half-life or decrease degradation of the replicase proteins, proteins with different half-lives within the cell

could be expressed as GFP fusion proteins with the ER localization domain fused in between the GFP tag and the protein. If expression levels increase with the addition of the ER localization domain, it may be acting as a translational enhancer. To determine if the half-life of the protein is affected, the same GFP fusion proteins could be expressed in transgenic plants and half-lives determined.

Because of the difficulty of expressing and purifying the TMV replicase proteins, there is no resolved structure for the proteins. Therefore, determining if the ER localization domain is part of an internal region of the replicase proteins is problematic. Problems arose in these studies because, although point mutations to alanine are a proven way to test the function of those amino acids in the context of the protein or to test the function of the domain, without structural data, it is harder to predict which amino acids to mutate and how. Mutating amino acids to change their charge or polarity might have produced data that better clarifies the mechanism of function for the ER localization domain.

In the future, more careful consideration of what charges or polarities would be most appropriate to investigate the function of this ER localization domain would be a more effective strategy. Also, sequencing mutant viruses after they have infected the plants would yield better insight into those mutants by determining possible compensatory mutations or reversions. In all of the localization assays, the transient expression in onion epidermal cells provided good visual data, but there were not high enough expression levels to confirm the constructs using a Western blot. Another method of transient expression might be better for confirming that the fusion proteins were still attached to

the GFP tag. However, the replicase protein is difficult to express in any system outside of the virus, so it is unclear if methods such as *Agrobacterium* infiltration would yield better results.

There are many questions left to be answered about how the ER localization domain acts and how that affects TMV replication, infection, and subsequent symptom development. Replicase proteins are complex proteins with multiple domains that seem to function at several different stages during infection, from silencing suppression to membrane remodeling and cell-to-cell movement. Previous work on *Tomato mosaic virus* has even shown that two different populations of replicase proteins may exist within a host cell—a membrane-bound and a cytosolic population. Altering the ratio of these populations impairs viral replication [132]. The current studies have attempted to dissect the function of the replicase proteins in the context of the whole virus. As more data are gathered about the structure of the replicase proteins, more accurate predictions can be made as to the role of this domain and, consequently, better-targeted modifications can be made.

Characterization of a virus-host interaction: Rab GDI proteins and the TMV replicase

On the host side, Rab GDI proteins were examined for their role in TMV infection. An interaction between the TMV replicase proteins and Rab GDIs was established. Yeast two-hybrid data indicate that this interaction is not unique to *Arabidopsis thaliana* but also occurs in tobacco and tomato species. A model of this interaction can be seen in Figure 26.

Rab GDI proteins are essential regulators of vesicle trafficking (Figure 3) and when their transcripts are silenced in tomato plants, necrotic lesions form. In tobacco, silencing Rab GDI enhances TMV infection sites compared to the control, but there is no change in viral accumulation or movement, indicating that Rab GDI may be affecting TMV infection only at an early stage. Rab GDI-silenced cells showed a cellular phenotype similar to that of TMV-infected cells. Also, during infection, Rab GDI proteins localize to ER-derived structures similar to those described as sites of viral accumulation (x-bodies), indicating a possible role in viral replication or replicase complex formation [21, 122]. Taken together, these data suggest that TMV may alter Rab GDI proteins to make the host cell a better environment for TMV infection. There are several hypotheses, which are not mutually exclusive, as to how Rab GDI proteins may play a role during infection: 1) The virus may bind to Rab GDI proteins and alter host membranes to be more favorable for replication, and possibly use Rab GDIs to help localize to membranes (Figure 27); 2) binding to Rab

Figure 26. Model of 126 kDa replicase protein's interaction with Rab GDIs during infection.

Seen is a modification of Figure 3 to show how the 126 kDa replicase protein might be interacting with the Rab GDI proteins. Instead of binding to Rab (GDP) proteins to ferry them back to their donor membranes, Rab GDI proteins bind the 126 kDa protein. The implications of this interaction are discussed in the text; however, in all models there is a decrease in the cytosolic Rab (GDP) population and an increase in the membrane-bound Rab (GDP) population.

Model of 126 kDa replicase protein's interaction with Rab GDIs during infection

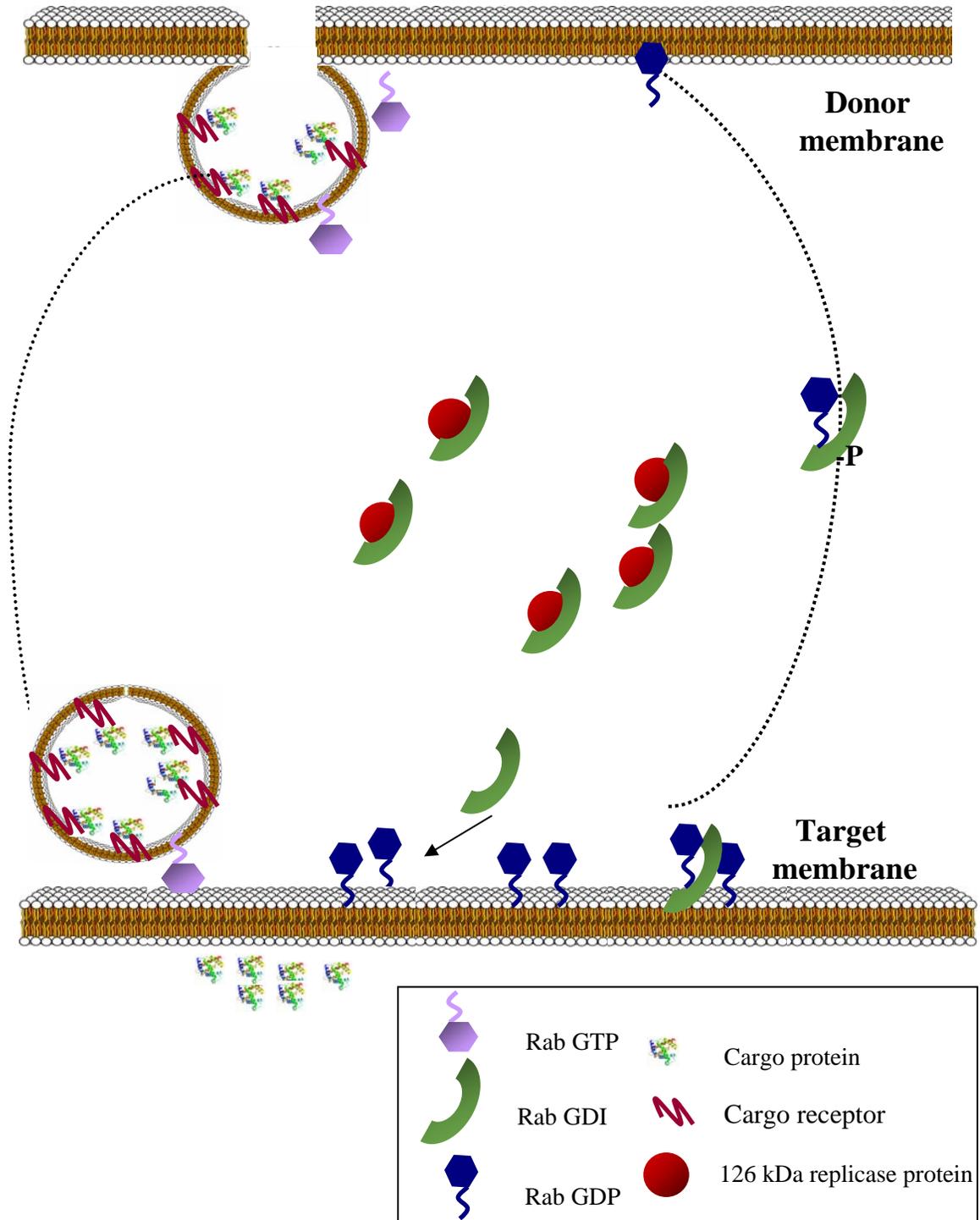
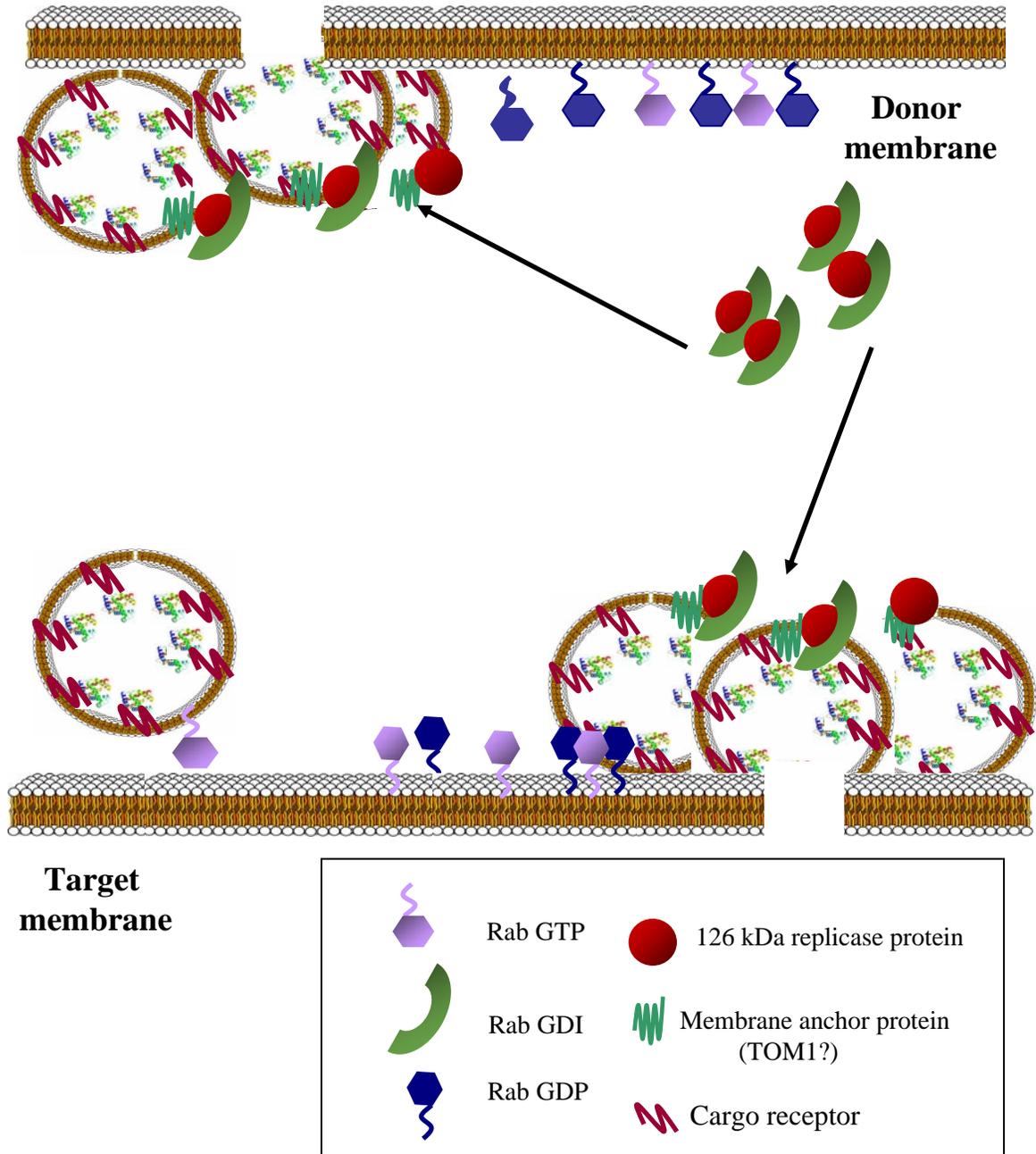


Figure 27. Model of Rab GDI involvement in host cell membrane rearrangement during TMV infection. Shown is a model of how Rab GDI proteins may be involved in membrane rearrangement during TMV infection. The 126 replicase protein (red spheres) binds to Rab GDI proteins (green arcs) and prevents them from recycling Rab GDP proteins (blue octagons). There is a build-up of Rab proteins in host membranes and Rab proteins may activate (purple octagon) in the incorrect membrane. The decrease in available Rab GTP proteins in the correct membrane may result in membrane structures that do not fully form or that form without a correct Rab protein. These membrane formations would allow an increase in surface area for TMV replication and might allow the TMV replicase proteins to bind to a host membrane anchor protein such as TOM1 (green curved line). Viral replication complexes would then be able to form on membranes. Also, the Rab GDI proteins may be providing a way for the TMV replicase proteins to travel to host membranes.

Model of Rab GDI involvement in host cell membrane rearrangement during TMV infection



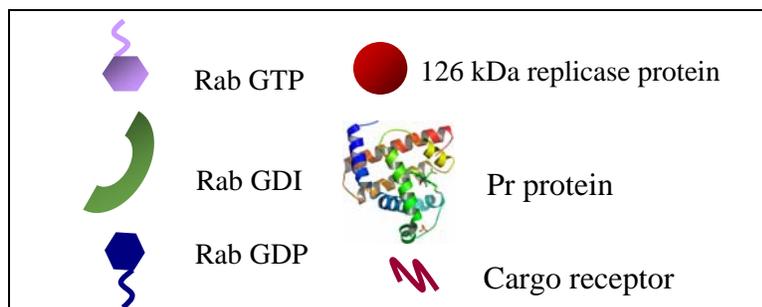
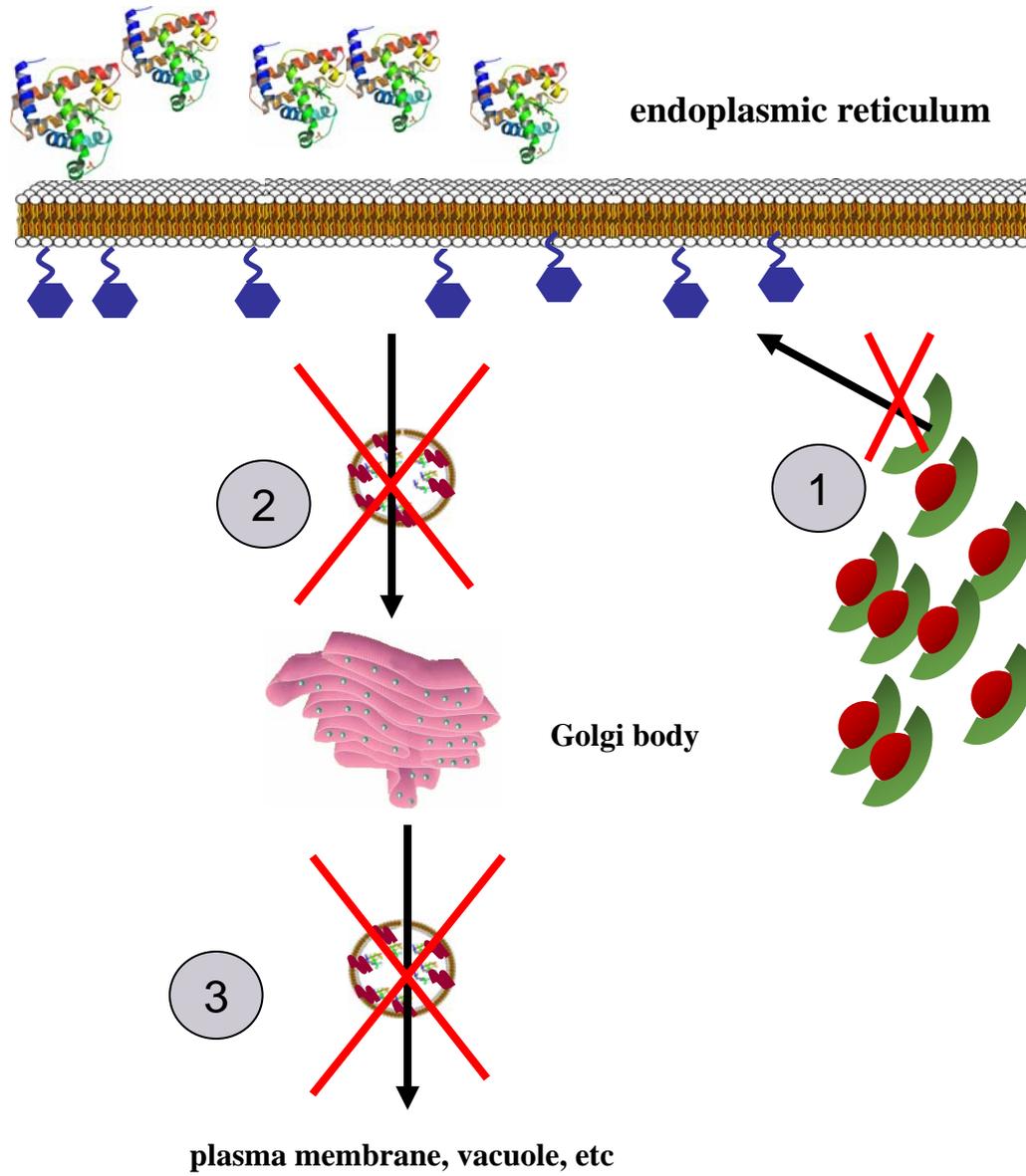
GDI proteins decreases host protein synthesis, including that of defense genes, and improves the virus' competition for host components (Figure 28).

The changes seen to the tonoplast during TMV infection and *Nb*GDI silencing, as well as the colocalization of *At*GDI2:GFP with ER-derived membrane structures in the cell during TMV infection, indicate a possible role of Rab GDI proteins in TMV-induced membrane alterations. TMV may bind Rab GDI proteins to increase vesiculation and possibly allow more surface area for viral replication on membranes. Also, the increased vesiculation may allow the replicase proteins to bind to the host protein TOM1 during infection (Figure 27). Work is ongoing to determine if there is an increase in total vacuolar membrane during TMV infection by isolation and quantification of GFP:*At*Vam3 plant membranes. One way to determine if this vacuolar phenotype allows for an interaction of TOM1 and the replicase proteins would be to create TOM1:GFP- and TOM1:dsRed-expressing *N. benthamiana* plants. These plants could be either infected with TMV or silenced for Rab GDI and probed with fluorescently conjugated anti-replicase antibodies. If these proteins interact at the site of active replication in infected and Rab GDI-silenced tissue, it would provide an as-yet-missing connection between these two proteins.

For a closely related tobamovirus, *Tomato mosaic virus*, the situation is similar. There are two populations of replicase proteins: the membrane-bound population, which participates in replication, and the cytoplasmic population [132]. It is possible that Rab GDI proteins help to modulate membrane-bound versus cytoplasmic populations

Figure 28. Possible effects of reduced Rab GDI availability on the host's endocytic pathway. Shown are some possible effects of reduced available Rab GDI within the host cell. **1)** TMV replicase proteins bind to Rab GDI proteins and prevent them from recycling Rab proteins to their donor membrane (Figure 3 depicts the normal function of Rab GDI proteins). A lack of active Rab proteins in the correct membrane would significantly reduce formation of vesicles and movement of proteins at several different stages in the endocytic pathway, such as ER to Golgi **(2)**, and Golgi to other organelles **(3)** [133]. The slowdown of the endocytic pathway would cause a reduction in host protein production, including that of pathogenesis-related (PR) proteins. The reduction in host synthesis would give TMV a competitive advantage in using host components such as ribosomes and tRNAs. Also, the reduction in PR protein production would reduce defense responses to the virus [134].

Possible effects of reduced Rab GDI availability on the host's endocytic pathway



of replicase proteins. However, when *At*GDI2:GFP plants were infected with wt TMV, there seemed to be no difference in symptoms, either systemically or locally, and no change in coat protein accumulation. It is possible, though, that these effects on membrane-bound versus cytoplasmic populations are only essential in initial infection. One way to test this possibility would be to evaluate tissue from TMV-infected TRV or *Nb*GDI-silenced inoculated leaves for changes in membrane versus cytoplasmic populations of 126 kDa replicase proteins. If Rab GDI proteins are modulating cytoplasmic versus membrane-bound replicase proteins, there should be a difference in the relative replicase protein levels in the different cellular fractions between the TMV-infected *Nb*GDI-silenced and TRV control plants.

An alternative hypothesis is that the TMV replicase proteins may be binding to and possibly degrading Rab GDI proteins, which would slow host protein synthesis, including that of host defense proteins [134]. The replicase proteins may compete with host proteins for binding of Rab GDIs, which would reduce, but not eliminate, the pool of available Rab GDI proteins to fulfill their normal function. This reduction would slow protein synthesis by reducing the amount of Rab GDI proteins available, which in turn would reduce the amount of active Rab proteins in the correct membrane, and thus slow down the endocytic pathway (Figure 28). Increasing viral protein synthesis and decreasing host protein synthesis is a common tactic that many viruses, including TMV, are known to employ [130].

Many defense genes contain post-translational modifications that require progression through the ER and Golgi or exocytosis to be excreted [82, 134]. To test this hypothesis, two Rab proteins, *AtRab6* and *AtRab8*, have been cloned from *A. thaliana* ecotype shahdara cDNA and placed in an *Agrobacterium* binary vector. Work is ongoing to express these proteins in plant tissue via *Agrobacterium tumefaciens* infiltration. There should be two populations of Rab proteins—those bound to membranes, either in the target or donor membrane, and those in the cytoplasm, bound to Rab GDI proteins (Figure 3). A reduction in Rab GDI proteins should result in an increase in membrane-bound Rabs and a decrease in the cytoplasmic population of Rab proteins. TMV-infected, mock-infected, *NbGDI*-silenced, and TRV control plants will be examined for relative levels of *AtRab6* and *AtRab8* in cytosolic and membrane portions of the cell. Similar changes in Rab populations in both the *NbGDI*-silenced and TMV-infected cells would indicate that Rab populations, and possibly the endocytic pathway, are affected by a loss of Rab GDI proteins.

During the course of these studies, *AtGDI2*:GFP has been expressed via gene bombardment, *Agrobacterium* infiltration, and transgenic plants. In all three cases, there was an effect due to viral infection, but it was only when expressed in the context of a transgenic plant that the uninfected localization was close to what might be predicted based on its function and studies of other related proteins. The reason for the difference between expression from the host genome vs. plasmids is unclear, but for future studies, transgenic plants seem to be the best way to examine over-expression of this protein.

These studies have identified and characterized an interaction between a host protein and the TMV replicase proteins. This interaction is unique in that silencing of a host gene enhances infection rather than reducing it. The mechanism of how this interaction functions during infection is still being investigated and more work is needed.

Nonetheless, these data indicate that this interaction is important in an area in which very little work has been done—initial establishment of infection. Little is known as to why any particular cell is more susceptible than another cell, even in the same host. It is possible that the intricate interactions that lead to a successful infection are even more complicated than they appear. Therefore, these studies have begun to characterize the how and why of susceptibility in a given host. Specifically, these studies have provided insight into how the replicase proteins may produce some of the cytological morphologies seen during infection, how TMV RNAs may be out-competing the host mRNAs, and one possible way TMV might be escaping host defense mechanisms.

The interactions between pathogen and host are a complex, elaborate process; the host tries to detect and restrict the virus, and the virus attempts to take over parts of the cellular machinery without killing the cell, while eluding host defense responses. The more we understand how the virus is able to use the host cell, the more we come to understand how the host itself works. These studies have examined both the virus and the host, allowing us a glimpse at how these two entities interact with and compete with each other for limited resources.

Pathogen-host interactions remain a large proverbial black box. We have much left to discover about why certain interactions are favorable and how exactly complexes form and traffic in the host. These studies have attempted to address just a few of those questions, and have left many more questions left to be tested. As we face a world where diseases threaten both life and livelihood, answers to these questions may help us better succeed in such a world.

Appendix

Protein	Function	Localization	TAIR index	Citation
GFP:HDEL	ER signal tag	ER and nucleus		[135]
GFP: <i>AtVam3</i>	t-SNARE	Tonoplast (vacuole)	AT5G46860	[123]
<i>AtSar-1</i> :GFP	ARF-like GTPase family member	ER exit sites (ERES)	AT1G56330	[127]
<i>AtPad-1</i> :GFP	20S proteasome alpha subunit D1	20S proteasome complex	AT3G51260	[136]
<i>AtERD2</i> :GFP	KDEL receptor	Golgi	AT1G29330	[127, 137]
GFP: <i>Atg8a</i>	Early component of autophagy complex	Cytoplasm and autophagic bodies	AT4G21980	[138]

A1. Table of marker proteins created. Host proteins were RT-PCR amplified from *Arabidopsis thaliana* ecotype shahdara cDNA and expressed as a GFP fusion protein as indicated. Marker proteins were created to examine endomembrane changes during infection and silencing of Rab GDI proteins. Gene function, localization, and index as well as citation are indicated for each construct.

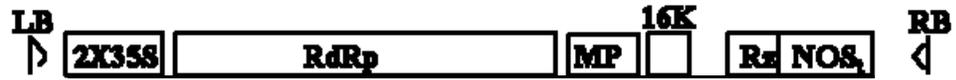
Protein	Host	TAIR/TIGR gene index
<i>AtGDI2</i>	<i>Arabidopsis thaliana</i> ecotype shahdara	AT3G59920
<i>LeGDI</i>	<i>Lycopersicon esculentum</i> cv Pilgrim	TC162880
<i>NbGDI</i>	<i>Nicotiana benthamiana</i>	To be submitted
<i>NtGDI</i>	<i>Nicotiana tabacum</i> cv xanthi	TC3480

A2. Table of Rab GDI proteins. Listed are the Rab GDI proteins, their host organism and their gene index. Indices starting with TC refer to TIGR plant gene indices while indices starting with AT indicate *Arabidopsis thaliana* gene location as per the TAIR database.

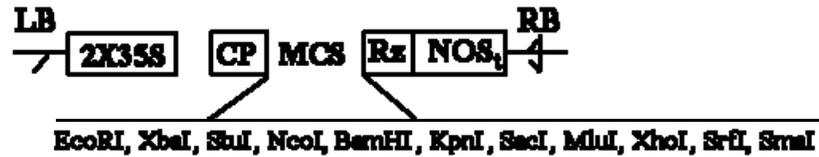
Gene	Salk tDNA stock number	Location of tDNA insertion
<i>AtGDI1</i> (At2g44100)	117972C	within 300 bases of the 5' end
<i>AtGDI2</i> (At3g59920)	064619	inside an annotated exon

A3. Table of Salk tDNA insertion seed stocks crossed. Stocks were crossed three times without producing viable seeds. The genotype of each stock was verified via RT-PCR, and crossed plants were homozygous for the tDNA insertion.

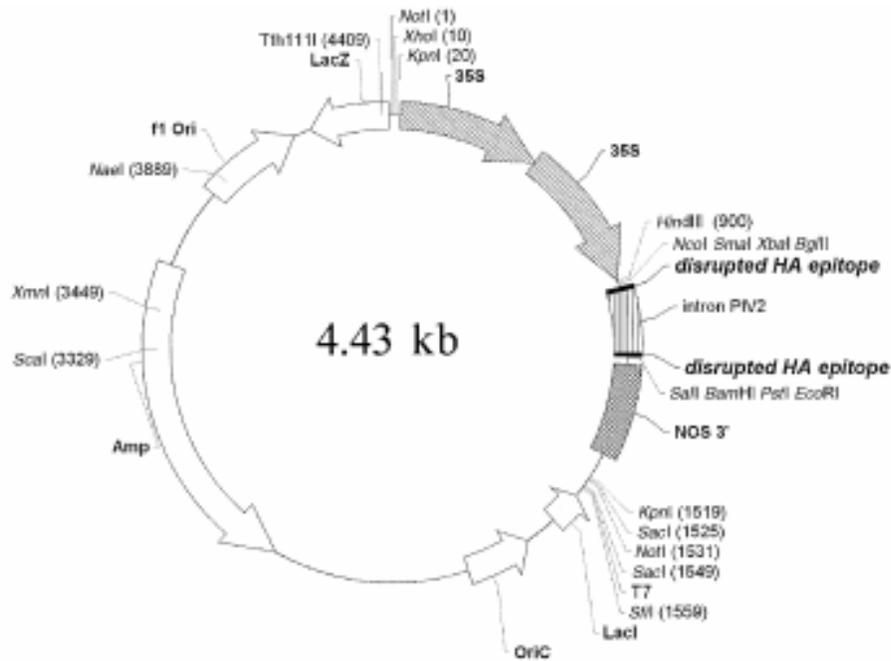
pTRV1



pTRV2



A4. *Tobacco rattle virus* (TRV) Viral Induced Gene Silencing system (VIGS) . Above is a diagram of the TRV vectors used in the gene silencing of Rab GDI proteins. TRV cDNA clones were placed between the duplicated CaMV 35S promoter (2x35S) and the nopaline synthase terminator (NOST) in a T-DNA vector. LB and RB refer to left and right borders of T-DNA. RZ, self-cleaving ribozyme. MCS, multiple cloning site. Both T-DNAs were placed in binary vectors and were maintained in *Escherichia coli*. The plasmids were expressed from *Agrobacterium tumefaciens* in the host plant. The gene accession number for TRV1 is AF406990 and TRV2 is AF406991 [139]. TRV system was a gift from Dr. Dinesh-Kumar.



A5. *Agrobacterium tumefaciens* binary cloning vector information. The above image of the pPily cloning vector (accession number AY720433) that contains an intron in frame to ensure only expression in the infiltrated plant and not in the bacterial vector [140]. The entire expression cassette including the gene of interest was cut out of pPily using the restriction endonuclease, *KpnI*, and inserted into the binary vector pBIN 19 plus (accession number X77672) [141]. Both vectors were a gift from Dr. Shunyuan Xiao, University of Maryland Biotechnology Institute.

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