HOST FACTORS INVOLVED IN VIRAL MOVEMENT THROUGH PLANTS

A Thesis

by

BONNIE L. SEABERG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Herman B. Scholthof
Committee Members,	Karen-Beth G. Scholthof
	Wayne Versaw
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ABSTRACT

Host Factors Involved in Viral Movement Through Plants. (May 2008)
Bonnie L. Seaberg, B.S., California State University, Stanislaus
Chair of Advisory Committee: Dr. Herman B. Scholthof

Tomato bushy stunt virus (TBSV) is a positive-sense single-stranded RNA virus. It encodes five open reading frames (ORFs), including two nested genes, expressing movement-associated proteins. One of these proteins, P22, interacts with a host transcription factor containing a homeodomain leucine-zipper motif, known as HFi22. Similar proteins of this type traffic their RNA from cell-to-cell, suggesting the possiblity that HFi22 is involved in the cell-to-cell movement of TBSV RNA. To further characterize the nature of the interaction between P22 and HFi22 on the cellular level, cellular fractionation experiments were conducted. To investigate the functional role of HFi22 in viral movement I attempted to inactivate its expression using a virus induced gene silencing system with a *Tobacco rattle virus* (TRV) vector. A final objective was based on the notion that different hosts can impact the stability of viruses used to express foreign genes of biotechnological interest. To compare virus stability in different hosts, TBSV expressing the green fluorescent protein (GFP) was inoculated onto various TBSV hosts, and infected leaf tissue was then used as inoculum to be rubbed onto a local lesion host. This technique made it possible to quantify the number of fluorescent foci versus total lesions.

Results obtained for the first objective indicate that P22 and HFi22 co-fractionate in nucleus and membrane-enriched samples. In addition, it was found that HFi22 is largely conserved through a wide variety of plant species but not in lettuce, which was found to be compromised for effective virus spread. Control experiments for the second objective showed that plants were successfully silenced with TRV carrying the phytoene desaturase (PDS) gene resulting in photobleaching, however attempts to silence HFi22 have not yielded conclusive results. The results obtained for the third objective indicate

there is a difference in how efficiently a foreign gene insert is maintained by TBSV in different host plants. In summary, the overall results of this research showed that host factors influence the host-virus interaction but their exact contributions remain elusive.

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CHAPTER I

INTRODUCTION

Tomato bushy stunt virus

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus* in the family *Tombusviridae* (46). The 4.776 kb positive-sense single-stranded RNA genome (Fig. I-1A) is encapsidated by a T=3 icosahedral particle composed of 180 subunits of a 41 kDa capsid protein (CP). The entire particle has a diameter of about 33 nm, and appears granular under an electron microscope due to the arrangement of the coat protein subunits (Fig. I-1B) (18, 46).

TBSV encodes five open reading frames (ORFs) that encode the proteins needed to establish an infection and propagate the virus (Fig. I-1A) (18, 41). The first two ORFs, *p33* and *p92*, are translated directly from the 5'-proximal end of the genomic RNA. The *p92* gene is expressed by read-through from an amber (UAG) stop codon (Fig. I-1A). The resulting proteins, P33 and P92, are 33 kDa and 92 kDa respectively, and compose the virus-encoded replicase (43). TBSV CP is translated from subgenomic RNA 1 (sgRNA1) (18, 34). From sgRNA 2, two nested genes, *p22* and *p19* are translated into a 22 kDa movement protein (P22) and a 19 kDa silencing suppressor (P19), respectively (46). The *p19* gene is expressed as a result of leaky scanning past the sub-optimal start codon of *p22* (39, 41).

TBSV is a soil-borne pathogen; as yet, no biological vector has been identified that facilitates its spread between plants (46). When RNA transcripts of full-length TBSV cDNA are rub-inoculated onto susceptible host plants, infection results (37). TBSV has a wide experimental host range, with more than 120 species from over 20 families experiencing varying degrees of susceptibility ranging from local to systemic infection (46). The host range and host factors required for cell-to-cell movement,

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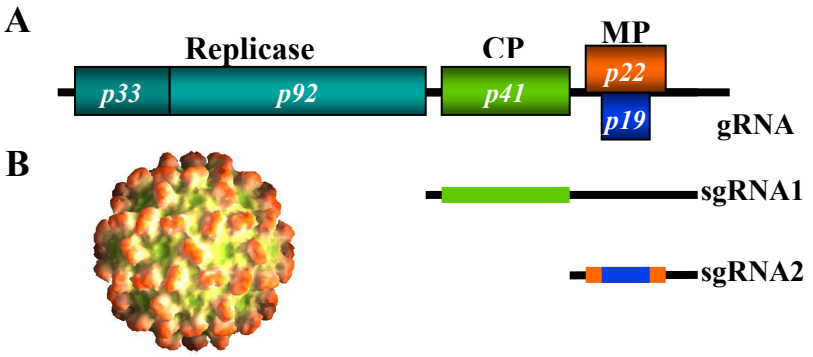

Figure I-1. TBSV genome and particle. (A) Diagram of the 4.8 kb TBSV genome. Each colored box is a different ORF, and the black lines are believed to be untranslated sequences. Protein sizes are given in each box in kDa, and their function is listed above. Shown below are the two subgenomic RNAs from which the CP and movement proteins are translated. (B) Representation of the TBSV T=3 icosohedron composed of 180 capsid protein subunits. The particles are about 33 nm in diameter and appear knobby due to the arrangement of the subunits.

however, have not been established and are a major focus of this thesis. Symptoms vary from host to host, inducing chlorotic or necrotic local lesions, slight to significant mosaic/mottling, and in some host species, extensive necrosis and plant death. TBSV symptoms on tomato plants include curling and crinkling in upper, young leaves and some of the older lower leaves with some tip necrosis, mottling, and possible reduction in fruit yield. Vertical growth is impaired while excessive lateral shoots produce a bushy, stunted phenotype, thus giving the virus its name (46).

Virus Movement

Plant viruses have developed a variety of strategies to move throughout plants (8). These strategies have been classified based on what is needed for the viral nucleic acid to move. Type 1 viruses do not require their CP to move. Instead, they use specialized movement proteins (MP) as their component of transport. For viruses with Type 2 transport, the CP serves as an auxiliary MP. In this way, the MP either actively assists in transport or provides protection for the viral genomic material. Type 3 viruses must include CP as they move as a particle (36). Some Type 3 viruses actually form tubules through the plasmodesmata through which the particles pass (8). Other Type 3 viruses have nucleic acid binding proteins that enlarge the plasmodesmata openings. Regardless of which mechanism a virus uses to move through a plant, host factors and even viral replicases can be involved in trafficking genetic material from cell-to-cell (36). Depending on the host, TBSV is a Type 1 or Type 2 virus.

P22

TBSV-P22 is one of two movement-associated proteins encoded at the 3' terminus of the genome (41). The protein binds viral RNA for transport to and through the plasmodesmata (13) enabling cell-to-cell movement. In previous experiments, eight mutants of p22 were developed by altering one or more amino acids in locations that were thought to be exposed to the surface. Options were limited as it was important to not impair the function of the nested *p19* gene (Fig. I-1A). These eight mutants all

demonstrated the capacity to replicate and produce subgenomic RNA, some better than others, but all efficiently. Five of the mutants produced local lesions and systemic necrosis on susceptible hosts and P22 levels were comparable to those observed for wild type TBSV. Three of the p22 mutants were unable to establish successful infections, yet they replicated in protoplasts with the same efficacy as wild type TBSV. When these movement-deficient mutants were transferred to β-glucoronidase (GUS)-expressing TBSV, GUS expression verified an inability of the mutants to move past the initially inoculated cells (11).

HFi22

The HFi22 protein was first identified from a series of yeast two-hybrid assays performed using bait plasmids expressing the TBSV P22 MP, and prey plasmids from *N. tabacum* cDNA. HFi22 was the only host factor to yield consistent positive results for P22 interaction. To confirm the specificity of this interaction, the host factor was tested against other TBSV proteins and the movement-deficient *p22* mutants, but no interaction was observed in these tests. Thus, of the proteins tested, only wild-type P22 interacted with HFi22. The HFi22-encoding cDNA was rescued from the yeast and transformed into *E. coli*, where it was purified and sequenced (13). There is currently a 1 kb cDNA sequence available (NCBI AAM48290) (Fig. I-2).

Sequence analysis of HFi22 has revealed that it is homologous to plant-specific homeodomain (HD) leucine (Leu)-zipper proteins (13). This family of proteins are transcription factors (TF) with specific characteristics. They have a DNA-binding HD adjacent to a Leu zipper (20). Proteins with this motif activate transcription, and some also traffic their own RNA from cell-to-cell. This provides early support for the idea that host proteins may assist viral RNA in cell-to-cell movement (13).

Preliminary results indicate that when protein extracted from healthy *N. benthamiana* is subjected to western blot analysis and probed with antibodies for HFi22, two bands are seen of approximately 37 and 42 kDa, with the 37 kDa more intense. In plants subjected to stress, sometimes (but not always), the 37 kDa band is not detected,

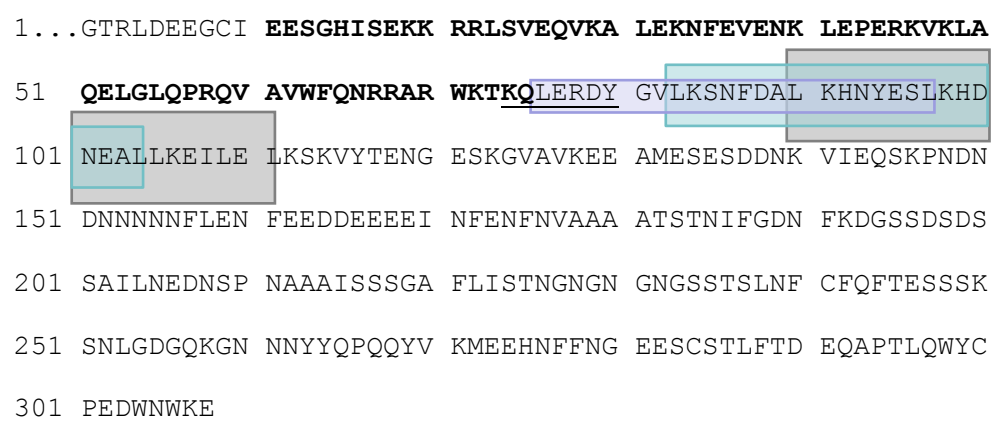

Figure I-2. Amino acid sequence of HFi22 (NCBI AAM48290). **Bold** sequences are associated with Homeobox signature; Underlined sequences represent a Tyr phosphorylation site; The three different sized and colored boxes are three possible Leu zipper regions at aa's 76-97, 83-104, and 90-111. It is predicted that three unidentified amino acids remain between position 1 N-terminus Glycine (G) and the Metianine (M) located upstream.

leaving only the 42 kDa band. In those instances where the lower band is still present, it still becomes less intense than the upper band.

Gene Silencing

When one thinks of an immune system, it is generally members of the animal kingdom, not plants, that come to mind. However, like other multicellular eukaryotic organism that can be parasitized by pathogens, plants have developed their own protection strategies (44). One strategy of primary importance is the ability of the plant to recognize and eliminate foreign RNA. This mechanism has become known as RNA silencing (3, 27). As recently reviewed by Baulcombe (3), silencing falls into three categories: cytoplasmic siRNA silencing, miRNA silencing, and DNA methylation/suppression of transcription. The category most relevant to plant protection from viral infection is cytoplasmic siRNA silencing, a type of post transcriptional gene silencing (PTGS) (3), explained in more detail below (Fig. I-3).

Plant viruses come in a diverse range of shapes, sizes, and composition. Broadly, they can contain single-stranded (ss) or double-stranded (ds) DNA or RNA (21). However, these viruses all have one thing in common: at some point during their replication cycle, they produce RNA transcripts. In turn, many of these transcripts may transiently exist as double-stranded structures, either from complementary dsRNA replicative intermediates or by forming secondary structures. This dsRNA is an ideal target for a type III RNase known as Dicer that cleaves the RNA into double-stranded segments of ~21 nucleotides (nt) in length (Fig. I-3A-B) (3, 28, 45). These 21 nt short interfering RNAs (siRNAs) are loaded onto an RNA-induced silencing complex (RISC) and the second, passenger strand of RNA is degraded, leaving a single strand to serve as a targeting mechanism (Fig. I-3C-D) (23). The loaded RISC uses the bound siRNA to locate any complementary single-stranded RNA with high sequence specificity. When its match is found, RISC cleaves the RNA, rendering it vulnerable to endo- and exo-nucleases (Fig. I-3E-F.) (3, 17). In this way, the plant can specifically degrade viral RNA transcripts using the virus itself as a targeting template (28).

Despite this, viruses are not all at the mercy of plant defenses. Many viruses encode proteins that interfere in various ways with the silencing process, such as the *Potato virus Y* (PVY) HcPro (28, 42). Another example of viral silencing suppressors is the P19 protein of TBSV, which forms a dimer that binds to double-stranded siRNAs and prevents these from being loaded onto RISC, thus interrupting the process (Fig. I-3G) (31, 38).

Viruses as Tools

Gaining an understanding of the natural systems for gene silencing in plants has resulted in a great laboratory tool to research gene function. The ability to induce silencing of specific genes provides a means to study gene knock outs without making transgenic plants. By using virus vectors carrying the desired plant gene it is possible to silence genes and study their effects in the laboratory (37). *Tobacco rattle virus* (TRV) is a commonly used virus gene vector, especially with regards to gene silencing. One gene that is phenotypically useful as a control is the phytoene desaturase (*PDS*) gene, which encodes an enzyme in the carotenoid pathway that maintains green pigment in chloroplasts. By inserting the *PDS* gene into TRV RNA 2, this gene can be silenced and consequently the leaves bleach white (15).

This technique is an example of virus induced gene silencing (VIGS), and is a very useful tool in the laboratory for studying loss-of-function mutants. Using this technique, one only needs a small fragment of the target gene, such as a PCR product, to be inserted into the vector for silencing to occur. In addition, the phenotypic result of silencing can be seen in a short span of time (a few days to just over a week) if the silencing produces a visible change. It can also be used in observing gene families if the gene fragment that induces silencing is part of a highly conserved region. This can also be a drawback if one wishes to study only a single protein, in which case the chosen fragment should come from a unique site in the gene sequence (15).

In addition to silencing, viruses can also be used to express or even overexpress proteins in plants to a variety of purposes, not the least of which includes production of

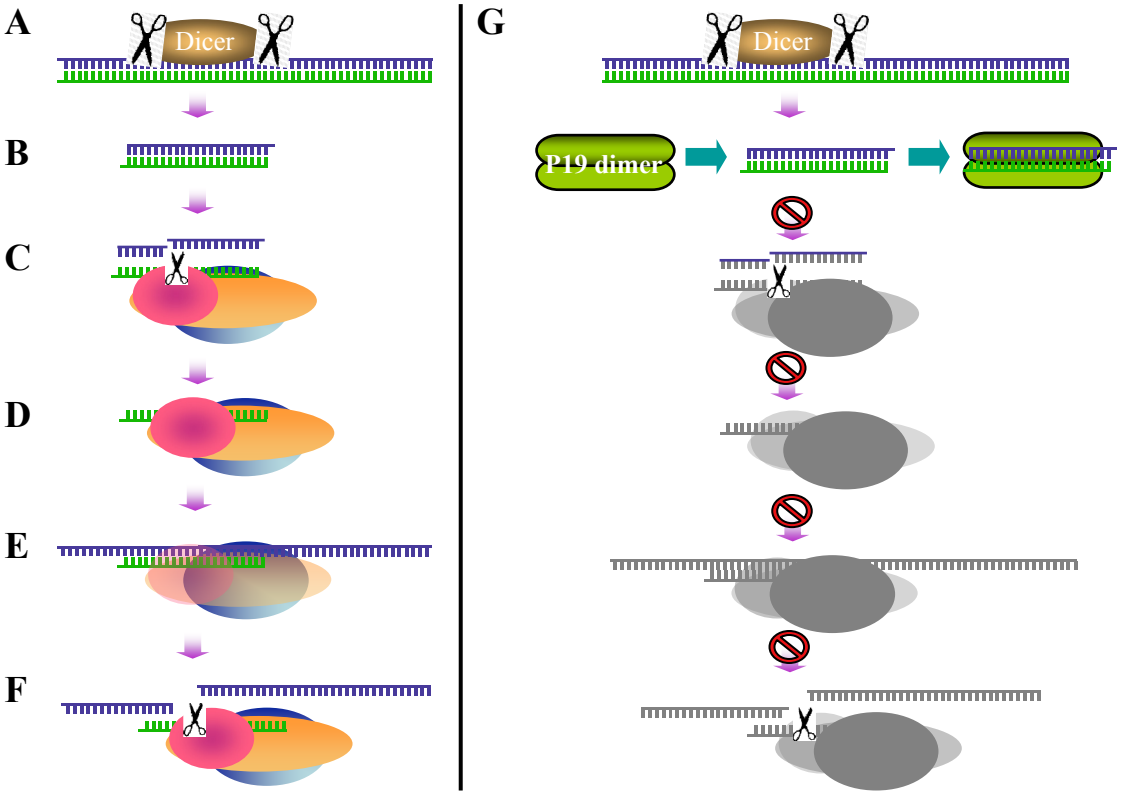

Figure I-3. Proposed model of post transcriptional gene silencing (PTGS). (A) Long dsRNA are recognized by Dicer and cleaved into (B) ~21 nt siRNA duplexes are generated. (C) The siRNA duplex is incorporated into the RISC complex and the passenger strand is cleaved and released. (D) The remaining siRNA strand acts as a guiding unit module for (E) sequence-specific target recognition of ssRNA. (F) Targeted mRNA is cleaved and released. (G) P19 suppresses silencing by binding siRNA prior to RISC activity by forming dimers and binding siRNAs, interrupting the rest of the silencing steps (46). Diagram courtesy of Dr. Rustem Omarov.

antibodies or therapeutic drugs. Proteins expressed by viruses in plants are able to undergo various post-translational modifications commonly found in eukaryotic proteins, making them less likely to cause allergenic reactions (42). In 2006, Giritch *et. al* (16) expressed heavy and light chains of IgG antibodies using *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX) as co-inoculated vectors (16). Viruses have also been used in studies seeking to understand resistance genes, by expressing avirulence genes (42).

When making use of viruses as vectors, it is important to take into account that different hosts seem to have different effects on the stability of viruses. In one example of this, virus mutants with incomplete genomes, such as those with CP deletions, often undergo recombination events that pattern towards regaining the ability to express proteins that probably are selected due to fitness competition. It has been observed that TBSV with partial or complete CP deletions that are inoculated onto plants transgenically expressing TBSV CP, will recombine in the lost segment(s) to achieve functioning CP (5). Even without the presence of wt CP mRNA, TBSV CP mutants have been observed to experience recombination within itself that generates 'more fit' truncated CP that enables the virus to function more optimally. These recombination events seem to take place at different rates in different hosts (14). This is an important factor when inserting a foreign gene into a virus vector; the host and virus type must be considered for the sake of not losing the insert to recombination.

A phenomenon that is seen only in the laboratory thus far is the generation of TBSV-defective interfering (DI) RNAs, which arise as a result of recombination. DIs interfere with virus replication and spread, reducing expression levels. In a study performed by Omarov, *et al.* (29), it was observed that in certain hosts, levels of DI RNAs increase rapidly as TBSV is transferred from plant to plant, while in other hosts these DIs accumulate slowly or not at all (29). Something in these other hosts enables the virus to be more stable in its replication and transcription. This is again an important factor in the context of using the virus as a vector to express a gene, such as *GFP*, where even a small mutation in the insertion can result in a loss of function.

Objectives

The intent of this thesis research was to gain a better understanding of the various aspects of the relationship between TBSV and its hosts with particular attention to virus spread. Viruses are affected by their host environment, and virus-host factor interactions are often crucial to the propagation and spread of the virus. My hypothesis was that the movement of TBSV may be facilitated by the interaction of the movement protein P22 with the host transcription factor HFi22. To test this hypothesis I had two objectives: 1) to study the biochemistry of the HFi22-P22 interaction in *N. benthamiana* and to determine if the presence of HFi22 in other plants correlates with these being hosts for TBSV cell-to-cell movement, and 2) to silence HFi22 using VIGS to observe the impact on viral movement. Since the host environment has such an important impact on viruses, objective 3) was designed to address vector stability by comparing the rate of viral spread and virus vector/foreign gene integrity in different susceptible hosts.

The first objective aimed to establish the co-localization of HFi22 and P22. Previous work determined this interaction occured *in vivo* with a yeast 2-hybrid assay, but it was necessary to determine whether these two proteins interacted *in planta* as this could provide some insight into the nature of their relationship. In addition, it was important to establish how conserved HFi22 is among plant species of TBSV hosts and nonhosts. If HFi22 is a significant factor for a successful TBSV infection, it would stand to reason that it would be present in all TBSV host plants.

For the second objective, it was necessary to establish a successful system to silence HFi22. VIGS was chosen as a rapid and relatively inexpensive alternative to production of a transgenic HFi22 knockout plants. *Arabidopsis* is not a TBSV host, so despite the fact that an HFi22-like protein is present in this commonly used plant, another species such as *N. benthamiana* would have to be transformed, and it is much more time consuming to obtain transgenic lines from this species. It is also unclear how vital HFi22 may be to early plant development; it may not be possible to obtain plants silenced for the host factor. For these reasons, TRV was used as the VIGS vector as it is widely utilized and readily available from commercial sources.

The final objective presented an opportunity to observe firsthand the impact of host environment on virus vector stability. Cowpea (*Vigna unguiculata*) is a local lesion host for TBSV; infection with TBSV-derived vectors results in clear light-colored lesions at the points of individual virus infection. To numerically compare the retention of functional GFP expression from TBSV, cowpea was used for a bioassay. For this, different host plant species were infected with TBSV-GFP then infected tissue was used as inoculum and transferred to cowpea to determine the ratio of white to green fluorescent lesions under UV light.

The results will be discussed in detail in the next three chapters, which address each of the objectives individually.

CHAPTER II

THE RELATIONSHIP BETWEEN HFi22 and P22

Introduction

Host factors are crucial for virus infection, including movement and propagation. Host specificity of viruses is largely affected by the different types of proteins found in given plant species. Enzymes, kinases, transcription factors, chaperones, cytoskeleton proteins, and surface-associated proteins all have been shown to interact with various viruses and their encoded proteins, contributing to factors such as cell-to-cell movement, replication, and long-distance transport (25, 30, 36). For instance, Cheng et al. (10) discovered a host factor 5'-3' exoribonuclease that seems vital for maintaining a stable virus genome as in its absence *Tomato bushy stunt virus* (TBSV) experienced a 10-50x increased rate of RNA recombination (10). However, the exact mechanisms of these interactions are not always known or fully understood. For instance, movement proteins from various virus families including *Tobamovirus*, *Potyvirus*, and *Tombusvirus* have been shown to interact with host transcription-related factors, but the role of these host factors remains elusive (36).

HFi22, the *h*ost *f*actor found to specifically *i*nteract with P*22*, the movement protein of TBSV, was retrieved during a yeast two-hybrid assay experiment and shown to also interact with P22 *in vitro* (13). Based on sequence homology, it was determined to be a type of transcription factor known as a homeodomain (HD) Leucine zipper protein. These proteins can be found conserved throughout different plant species. The major characteristic of the HD Leu zipper proteins is the DNA-binding domain that can be found adjacent to a Leu zipper motif, which often is responsible for mediating protein-dimer formation (20). Some of the HD proteins, as well as other transcription factors, also traffic their own RNA from cell to cell (20, 30), a feature which may be of some significance given the interaction between HFi22 and P22.

P22 is a Type I MP (CP is not required for movement), responsible for cell-to-cell movement and elicitation of defense-related necrotic local lesions in some hosts (36). This protein can be found associated with membranes and has an RNA-binding domain to form ribonucleoprotein complexes (46). In experiments by Chu *et. al* it was demonstrated that movement-defective P22 mutants are still able to elicit host defense responses, yet are unable to move TBSV from cell-to-cell, raising the possibility that the deficiency is the result of a loss of the ability to interact with viral or host factors (11). This was in agreement with a subsequent study showing that P22 interacted with HFi22 using yeast two-hybrid assays, and when movement-defective P22 mutants were used as the bait plasmid, a reduced association with HFi22 was observed (13). Given the features of the two proteins and the results of previous experiments, my hypothesis is that HFi22 and P22 interact either in the nucleus or cytoplasmic membranes (it is known that P22 accumulates in both, [(41), unpublished data]). Since HFi22 is a transcription factor, I postulate that it accumulates to detectable levels in the nucleus, in association with P22. Understanding where these two proteins interact will provide insight to the nature of their interaction. If HFi22 is in the cytoplasm, it may provide some insight into its involvement in movement.

Also, HFi22 yields two bands on a western blot (unpublished results), though the cause of this is unknown. Proteins are frequently subjected to post-translational modifications, such as phosphorylation or glycosylation. It was hypothesized that since the HFi22 protein sequence has a predicted phosphorylation site the upper band may be a post-translational modification product of phosphorylated HFi22. The smaller band might represent unphosphorylated proteins.

The first step was to confirm where HFi22 and p22 localize within the plant. For this, I took advantage of the available stock of antibodies for HFi22 and each TBSV-encoded protein. By infecting *N. benthamiana* with wt-TBSV, infected tissue was collected to perform a series of centrifugations to separate out cytoplasmic material from membrane-bound and nuclear material. These samples were then analyzed with immunoblot assays against HFi22, P22, and Hin19 (a host factor known to be localized

in the nucleus). The goal was to use biochemical fractionation experiments that would enable us to examine where in the cell the two target proteins are localized.

As stated previously, TBSV has a wide host range, but elicits varying degrees of infection in these various hosts. In some species TBSV establishes a robust systemic infection and in others it is restricted to local lesions on the inoculated leaves. It would appear that part of these differences may be associated with how effectively the virus can move in the different plants. If HFi22 is involved in this movement, it would stand to reason that the host protein would be present in the hosts that allow systemic movement, and absent in those that are not susceptible. Therefore experiments were conducted to evaluate the distribution of HFi22 among various plant species, in conjunction with tests to examine these plants for TBSV cell-to-cell movement.

Materials and Methods

SDS/PAGE, immunobloting, and phosphorylation of HFi22

N. benthamiana was inoculated with TBSV virions (stock available in the laboratory). At 7 days post inoculation (dpi), the infected and noninoculated plants were harvested, along with healthy *N. benthamiana*, and the tissue was processed for Western blot analysis. For this,1g of plant tissue was ground in 2 mL 2xSTE+2% SDS (2 mM Tris, 20 mM NaCl, 0.002 mM EDTA, 2% SDS) and solid tissue was strained out through a thin layer of cheesecloth. Subsequently, 5x cracking buffer was added in a 1:1 ratio and the mix was boiled for 5 minutes in a water bath. For phosphorylation assays, prior to adding the cracking buffer to the extracted protein, 20 µL of each sample was mixed with 2 µL of Buffer 3 (New England Biolabs; 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.9 at 25°C) and 2 µL of Calf Intestinal Phosphatase (CIP) and incubated at 37°C for 1 hour. Then 20 µL of 5x cracking buffer was added and the samples were boiled for 5 minutes.

The samples were subjected to SDS-PAGE (40) and immunoblotting for detection of HFi22. For this, 20 µL of each sample was loaded onto SDS-PAGE gels (4 mL 30% acrylamide, 2 mL A/C ddH_2O, 3.8 mL Tris pH 8.8, 100 µL 10% SDS, 100 µL

10% APS, and 4 µL TEMED for the gel; 600 µL 30% Acrylamide, 2.7 mL A/C ddH_2O, 500 µL Tris pH 6.8, 40 µL 10% SDS, 100 µL 10% APS, and 3.2 µL TEMED for the stack) and run at 90V in 1x Running Buffer (24.8 mM Tris, 192 mM glycine, 3.5 mM SDS) for 2-2.5 hours until the blue dye front of the cracking buffer reached the bottom of the gel and partially ran off. Proteins from the gel were transferred to MSI membrane paper in a transfer apparatus run at 260 mA for 1 hour. The membranes were blocked using a solution of 7% non-fat dry milk mixed in 1xTBS/Tween (50 mM Tris, 200 mM NaCl, 0.5% Tween-20) and developed with BCIP and NBT after exposure to HFi22-specific rabbit antiserum (1:2,000 antiserum to 7% milk/TBS/Tween solution) and secondary alkaline phosphotase-conjugated goat antirabbit antibodies (1:2,500 antiserum to 7% milk/TBS/Tween solution).

Nucleus extraction

To examine the possible co-localization point of P22 and HFi22, a nucleus extraction protocol was adapted from Chua *et al.* (12). For this purpose, *N. benthamiana* plants were inoculated with TBSV virions three days apart, then harvested four days after the last inoculation date along with a healthy, uninoculated plant, resulting in one healthy plant, one 4 dpi plant, and one 7 dpi plant. The nucleus extraction experiment was performed at 4ºC by keeping all samples and buffers on ice.

Upper and lower leaf tissue of each plant was harvested and ground in 30 mL of cold Nucleus Isolation Buffer (NIB) (600 mM sucrose, 25 mM tris, 5 mM $MgCl_2$, 40% vol/vol glycerol, 2 mM spermidine, 2.5 mM DTT, 1 tablet of Complete Mini Protease Inhibitor/100 mL solution, bring to 1 L with A/C ddH_2O, adjust pH to 8.0 with HCl and store at 4ºC). Samples were pulverized with a pestle in a mortar until all plant material was "slushy" and tissue was substantially fragmented. This mix was filtered through 6 layers of cheesecloth and excess trapped liquid was carefully squeezed out. On average about 5 mL of liquid was lost, leaving about 25 mL. From this, 500 µL was removed for storage as a Total Protein sample, mixed with 500 µL of 5x cracking buffer, and boiled for 5 minutes.

The remaining filtered liquid suspension was centrifuged at 3 k (Beckman Allegra™ 21R centrifuge; S4180 rotor) for 10 minutes at 4°C. Then 500 µL of the supernatant was removed for storage as a Cytosolic sample, mixed with 500 µL of 5x cracking buffer, and boiled for 5 minutes. The remaining supernatant was discarded. Next, 20 mL of cold Nucleus Isolation washing Buffer (NIwB) (NIB lacking spermidine) was added to the pellet and mixed superficially by gently inverting the tube 4-6 times. This mixed in some of the green layer without disturbing the lower white pellet. This suspension was centrifuged at 3 k for 5 minutes at 4°C. The supernatant was again discarded. Then 20 mL of cold NIwB was added and the pellet was fully resuspended by gently pipetting the mixture. This was centrifuged at 3 k for 10 minutes at 4°C.

A criterion was that all green material needed to be removed. If any green material remained, the previous wash was repeated once more. When the pellet was white, it was resuspend in 1 mL of Nucleus Suspension Buffer (50 mM tris, 5 mM $MgCl_2$, 50% vol/vol glycerol, 2.5 mM DTT, bring to 100 mL with A/C ddH_2O, adjust the pH to 8.0 with HCL, store at 4°C) and transferred to a 1.5 mL eppendorf-type tube. The pellet suspension was centrifuged at 10 k (Beckman Allegra™ 21R centrifuge; F2402H rotor) for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended in 200 µL of 1x TE and 200 µL of 5x cracking buffer to represent the Nucleus/Membrane-enriched sample, then boiled 5 minutes.

Because previous experience had shown detrimental affects were associated with prolonged storage, all western blot analyses for P22, HFi22, and Hin19 were performed on fresh samples before freezing for storage. To obtain a better visualization of the P22 blots, chemiluminescent development was frequently used instead of alkaline phosphatase-mediated detection. Coomassie Brilliant Blue staining was used to visualize equal loading of samples. Samples were run on SDS-PAGE gels as described above, then soaked in Coomassie Stain (50% methanol, 10% acetic acid, 40% A/C ddH_2O, 0.75-1.0g Coomassie Brilliant Blue R-250) overnight (O/N) on a shaker. The gels were destained with Gel Destain (15% methanol, 7% acetic acid) using Kimwipes

to soak the excess dye away for 8-24 hours on a shaker until protein bands were clearly distinguished. The gels were rinsed with water, scanned, and preserved with gel drying film (Promega).

RNA extraction and hybridization

Total RNA was extracted from the upper and lower leaves of the different plants. For this, 0.4 g of leaf tissue were pulverized in a mortar with pestle with 500 µL of 2xSTE+2% SDS and 500 µL phenol/chloroform (Ph/Chl), then another 500 µL Ph/Chl was added and the mix was ground to slurry. This mix was transferred to an RNase-free eppendorf tube and centrifuged at 10k for 15 minutes at 4ºC. The upper aqueous layer was transferred to a clean tube and mixed with 500 µL Ph/Chl, then vortexed and centrifuged at 10k for 10 minutes at 4ºC. This Ph/Chl extraction and centrifugation was repeated 3 more times for each sample. Then the upper aqueous phase was transferred to a new tube and added to an equal volume of 8M LiCl and set at -20ºC for one hour, then centrifuged at 10k for 15 minutes at 4ºC. The supernatant was poured off and the pellet was rinsed with 600 µL of 70% ethanol (EtOH), then spun down for 1 minute at room temperature. The EtOH was poured off and the tube was allowed to air dry for 10 minutes in the fume hood. The final product was resuspended in 98 µL of RNase-free ddH_2O, 1 µL of 10 mM DTT, and 1 µL RNase-inhibitor.

Total RNA (10 µL) was subjected to 1% agarose gel electroporesis and viewed under UV light upon ethidium bromide (EtBr) staining. For northern blot analysis, RNA was transferred to a nitrocellulose membrane by standard capillary transfer with 10x SCC (1.5 M NaCl, 150 mM sodium citrate, pH to 7.0 with HCl). The RNA was bound to the membrane by UV crosslinking. Each side of the membrane was crosslinked twice, turning 90° between each crosslinking. The membrane was soaked in 2x SSPE+1% SDS (20x SSPE – 3 M NaCl, 0.2 M NaH_2PO_4, 26 mM EDTA, pH to 7.4 with 10 M NaOH, dilute to 2x and add 2% SDS) in a 65°C rotator for 3 hours. During this incubation, radioactive TBSV probes were prepared. For this purpose, 3 µL of random primers, 1 µL of TBSV wildtype (wt) plasmid, and 12 µL of A/C ddH2O were mixed

and boiled for 3 minutes then immediately transferred to ice for rapid cooling. Then 2.5 µL EcoPol buffer, 2.5 µL 12 mM dNTP (without dCTP), 2.0 µL ^{32}P-dCTP (10 µCi/µL), and 1 µL Klenow (5,000 U/mL) were added to the primer/plasmid mix and incubated at RT for 1-2 hours. When the membrane incubation was nearing completion, the probe mix was boiled for 3 minutes then transferred to ice for rapid cooling. The probe mix was added to the membrane and buffer and incubated on a rotator for several hours (2-16 as needed) at 65°C. After hybridization, the membrane was rinsed in 2x SSPE+1% SDS, changing the buffer every 15 minutes until the signal strength on a Geiger counter was low enough that film would not be overexposed. Then the membrane was exposed on Kodak X-ray film for 16-24 hours, then developed and scanned.

Host range comparison

Tissue was harvested from various plant species (Table II-1) and processed for western blot analysis using the HFi22 and Hin19 antiserum as described earlier. Pre-immune serum for each protein was used as a control for specificity of the obtained signal and Coomassie staining was used to visualize equal loading of samples as previously described. Further testing was performed with Simpson lettuce when it was found that it lacked a visible HFi22 signal, yet when inoculated with TBSV-GFP there was enough infection to produce green fluorescence (shown in Results). TBSV-GFP transcripts were generated *in vitro* by mixing 1 µL (250-500 ng) of *Sma*I linearized template plasmid, 2.5 µL 5 mM rNTPs, 5 µL 5x transcript buffer (Fermentas, Glen Burnie, MD), 1 µL 0.1 M DTT, 0.5 µL RNasin, 0.5 µL T7 RNA polymerase (20 U/uL), and 14.5 µL A/C ddH2O and incubating at 37C for 1-1.5 hours. Plants were inoculated by mixing 25 µL of transcripts with 125 uL of cold RNA inoculation buffer (50 mM KH_2PO_4, 50 mM Glycine pH 9.0, 1% Bentonite, 1% Celite). To determine if TBSV could systemically infect lettuce, lettuce plants were inoculated with wtTBSV virions, with infected *N. benthamiana* as a control. Tissue was harvested from inoculated and upper leaves 7 dpi and total RNA was extracted. Northern blot analysis was performed using wtTBSV as a probe plasmid.

Table II-1. Plant species tested for conservation of HFi22 and Hin19. In the HFi22 and Hin19 columns, Y indicates at least one band was seen on a western blot analysis that was close to the estimated size of the protein as seen in *N. benthamiana*, while N indicates either no bands were present on the blot or they were of inappropriate size. In the local TBSV column, Y indicates that infection with TBSV-*GFP* results in fluorescent green foci on inoculated leaves, possibly in addition to visible lesions, necrosis, or mottling, N means no fluorescence or evidence of infection was visible, while ND indicates it is not determined if TBSV can locally infect that given host as it hasn't been tested yet. The TBSV-*GFP* data are a compilation of this thesis work and that performed by others in the laboratory. In the systemic TBSV column, Y indicates that infection with TBSV results in systemic TBSV infection, usually demonstrated by symptoms of lesions, necrosis, leaf curling, or mottling, N means TBSV is unable to establish a successful systemic infection, while U/N indicates it is unknown if TBSV can systemically infect that give host as it hasn't been tested yet. The asterisk (*) indicates local lesion hosts.

Plant Host	HFi22	Hin19	Support local TBSV infection	Support systemic TBSV infection
Arabidopsis	Y	Y	N	N
Banana	Y	Y	ND	ND
Bean	Y	Y	Y	N*
Buckwheat	Y	Y	N	N
Cantaloupe	Y	Y	Y	Y
Corn	Y	Y	N	N
Cotton	Y	Y	ND	ND
Cowpea	Y	Y	Y	N*
Cucumber	Y	Y	Y	Y
Geranium	Y	Y	ND	ND
Hibiscus	Y	Y	ND	ND
Lettuce (Simpson)	N	Y	Y	N
Nicotiana	Y	Y	Y	Y
Nicotiana tabacum	Y	Y	Y	N*
Orchid	N	Y	ND	ND
Parsley	Y	Y	ND	ND
Pepper	Y	Y	Y	Y
Petunia	Y	Y	Y	Y
Philodendron	N	Y	ND	ND
Pineapple	N	Y	ND	ND
Pumpkin	Y	Y	Y	Y
Rose	Y	Y	ND	ND
Spiderplant	N	Y	N	N
Spinach	Y	Y	Y	Y
Switchgrass	Y	Y	N	N
Vinca	Y	Y	ND	ND
Zinnia	Y	N	ND	ND

Results

Phosphorylation of HFi22

Healthy *N. tabacum* and *N. benthamiana* plants often yield two HFi22 bands at 37 and 42 kDa (Fig. II-1). The cause of this is still unclear. When CIP-treated and untreated samples of healthy and TBSV-infected *N. benthamiana* protein were subjected to SDS-PAGE gel electrophoresis followed by western blotting with HFi22-specific antiserum, no visible difference was observed between the CIP-treated and -untreated samples. Upper and lower bands remained and were of comparable intensity. The only clear difference was noted when comparing the healthy and the infected samples. For TBSV-infected plants, HFi22 bands appeared lighter in intensity than observed for the healthy plant samples (Fig. II-1), but this was not a consistently reproducible phenomenon. Nevertheless, these results verified that the HFi22 specific antiserum consistently detected this different sized proteins in *N. benthamiana*.

Localization of proteins

For practical purposes, it was necessary to process the plant material for western blot analysis on the same day due to the unstable nature of P22 (unpublished data).To enable processing of all samples at different dpi on the same day, plants were inoculated on different days as described in Materials and Methods. Immunodetection of Hin19, a TBSV P19-interacting host protein that localizes to the nucleus [(7), unpublished data] was used to confirm that the Nucleus/Membrane-enriched pellet indeed contained nuclear material.

Western blot analysis of the different samples of cell fractions revealed that HFi22 was consistently present in both the Total Protein (pre-centrifuging) sample (Fig. II-2A) and the Cytosolic fraction (Fig. II-2F). A relatively weak band was observed for the Nucleus/Membrane-enriched samples in the lower leaves of 7 dpi plants (Fig II-2K). On the other hand, P22 was detectable in the lower leaves of 7 dpi plants in the Total Protein sample (Fig. II-2B and C), absent from the Cytosolic sample (Fig. II-2G and H), but was readily detectible in the Nucleus/Membrane-enriched sample starting in the

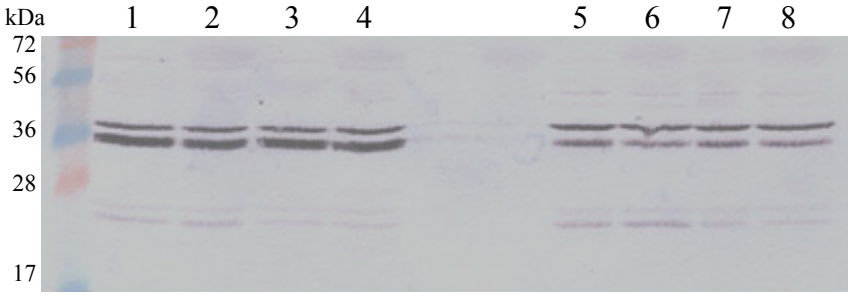

Figure II-1. Western blot of HFi22 digested with CIP. Lanes 1 and 3 are two repetitions of untreated healthy plant protein samples. Lanes 2 and 4 are two repetitions of CIP-digested healthy plant protein samples. Lanes 5 and 7 are two repetitions of untreated 7 dpi TBSV-infected *N. benthamiana* protein samples. Lanes 6 and 8 are two repetitions of CIP-digested 7 dpi TBSV-infected *N. benthamiana* protein samples.

lower leaves of 4 dpi plants (Fig. II-2L and M). Hin19, used as a control for the presence of nuclear protein, was not visible in the Total Protein sample (Fig. II-2D) or in the Cytosolic sample (Fig. II-2I), but was faintly present as a 33 kDa protein in the Nucleus/Membrane-enriched samples in the upper leaves of the healthy plant (Fig. II-2N).

Coomassie staining, used as a loading control, showed fairly consistent loading of the Total Protein (Fig. II-2E) and Cytosolic (Fig. II-2J) samples, but the Nucleus/Membrane enriched sample showed some variation of sample loading (Fig. II-2O). However, since the intensity of the samples in the Total Protein samples is higher than in the Nucleus/Membrane-enriched fractions, it can be concluded that the presence of Hin19, P22, and HFi22 in that sample is due to true fractionation.

Since these results suggest that HFi22 and P22 co-fractionate to some extent, experiments are underway to test if HFi22-P22 can be observed in association with cell walls, where plasmodesmata are retained, or if it is restricted to the nuclear material.

Host range comparison

Tissue was harvested from 27 different plant species and tested for the presence of HFi22 and Hin19 as shown for selected examples in Fig. II-3 and cumulatively summarized in Table II-1. When proteins from *N. benthamiana* and *N. tabacum* are probed with HFi22 antiserum, two bands are seen at approximately 37 and 42 kDa. When other species were tested, the size and features of HFi22-specific bands often differed. For spinach, cowpea, cucumber, pumpkin, cantaloupe, and bean only a single band was detected (Fig. II-3A and G). Other plants such as corn, parsley, and Vinca (not pictured) show multiple bands which, based on the absence of signal with pre-immune serum, are not a result of non-specific binding from other serum elements. In some plants, such as cotton, a fuzzy, broad, and indistinct band was observed, possibly the result of a dual band (such as the ones found in *N. benthamiana*) of such similar size that they slightly overlap one another.

Hin19 was also widely conserved in the tested plant species, as it was found in

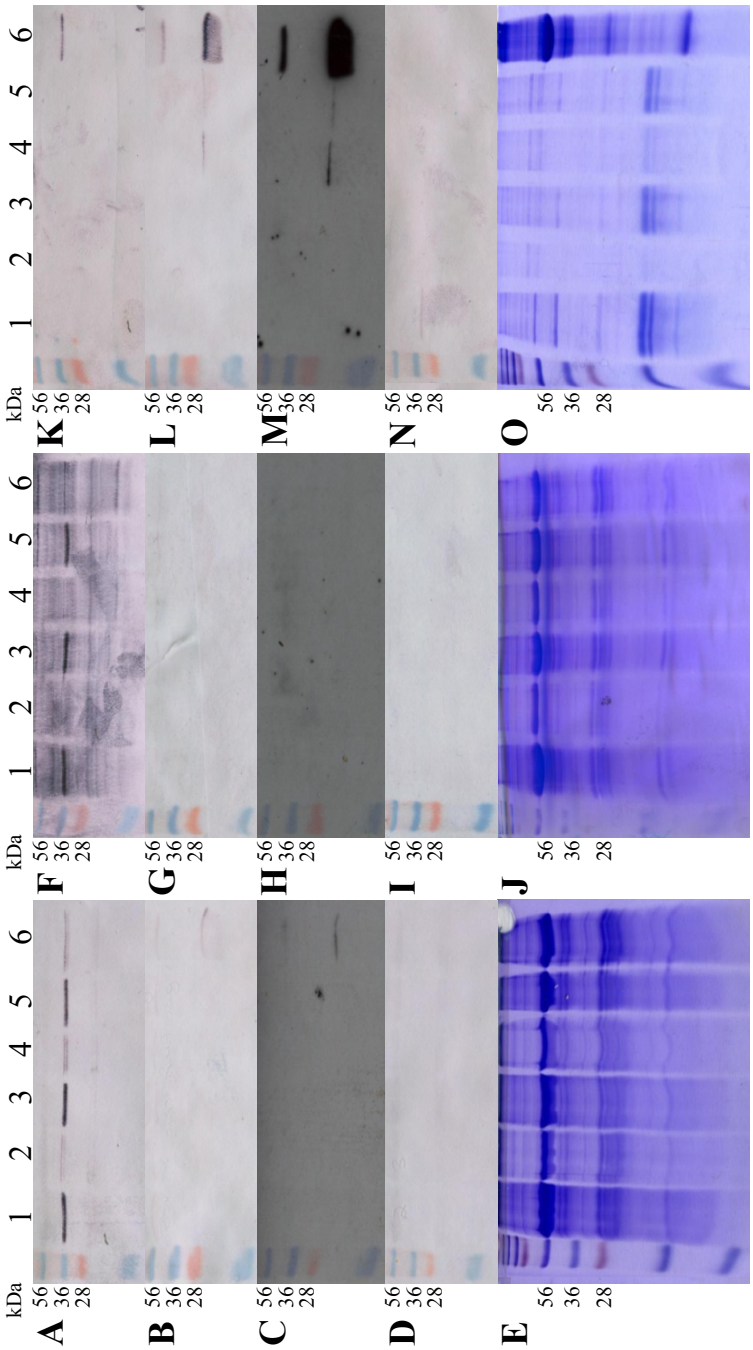

Figure II-2. Immunoblot assay to observe co-localization of HFi22 and P22 upon nucleus extraction. (A-E) Total protein (TP) sample; (F.-J.) Cytosol (C) sample; (K.-O.) Nucleus/membrane enriched (N) sample. (A., F., and K.) Western blot for HFi22; (B., G., and L.) Western blot for P22; (C., H., and M.) Chemiluminescent blot for P22; (D., I., and N.) Western blot for Hin19; (E., J., and O.) Coomassie staining. Lane 1 is healthy plant protein from upper leaves; Lane 2 is healthy plant protein from lower leaves; Lane 3 is 4 dpi TBSV-infected plant protein from upper leaves; Lane 4 is 4 dpi TBSV-infected plant protein from lower leaves; Lane 5 is 7 dpi TBSV-infected plant protein from upper leaves; Lane 6 is 7 dpi TBSV-infected plant protein from lower leaves.

26 of the 27 tested plant species, being absent only in Zinnia (Table II-1). However, unlike HFi22, which was found at a largely consistent size among the different species, the size of Hin19 varied widely from the approximate 32 kDa size seen in *N. benthamiana* (Fig. II-3D and J). The pre-immune blots did show quite a bit of smearing from non-specific serum element binding (Fig. II-3E and K), and may account for some of the patterning seen on the blots.

All of these results indicate that the HFi22, protein is conserved in many species of plants. In the instances where there is a visible size differences in the proteins, there is likely an HFi22 orthologue similar enough that the antibodies can still bind. Some plant species, such as rose, do have an HFi22 signal (Fig. II-3G), yet cannot support TBSV-cherry strain infection (Table II-1). Several of the TBSV non-hosts such as orchid, spiderplant, and philodendron also lack an HFi22 signal.

In an infectivity screen with a TBSV vector in which the coat protein (CP) was replaced with the gene for green fluorescent protein (GFP) (see chapter IV) lettuce was incorporated. This test showed that lettuce could support TBSV cell-to-cell movement. This combination of the lack of HFi22 in Simpson lettuce and its capacity to host TBSV-GFP (Fig. II-4A) initially ran contrary to the hypothesis that there is a correlation between the presence of HFi22 and the ability for TBSV to move. However, localized infection at the point of inoculation is different than full cell-to-cell movement. For instance the GFP foci observed in *N. benthamiana* are larger than in lettuce (Fig. II-4A and B). To determine if wtTBSV could move systemically in lettuce, *N. benthamiana* and lettuce plants were infected with TBSV virions and harvested after 7 days for northern blot analysis. These blots showed that viral RNA was found in both upper and inoculated leaves of *N. benthamiana* after 7 days. However, not only was TBSV RNA absent in lettuce upper leaves, it was no longer present in the inoculated leaves (Fig. II-4C and D), indicating lettuce was unable to support a lasting infection past the first few days.

My results suggest that HFi22 might be involved to promote efficient cell-to-cell movement. Experiments to over-express HFi22 in lettuce by agroinfiltration are in

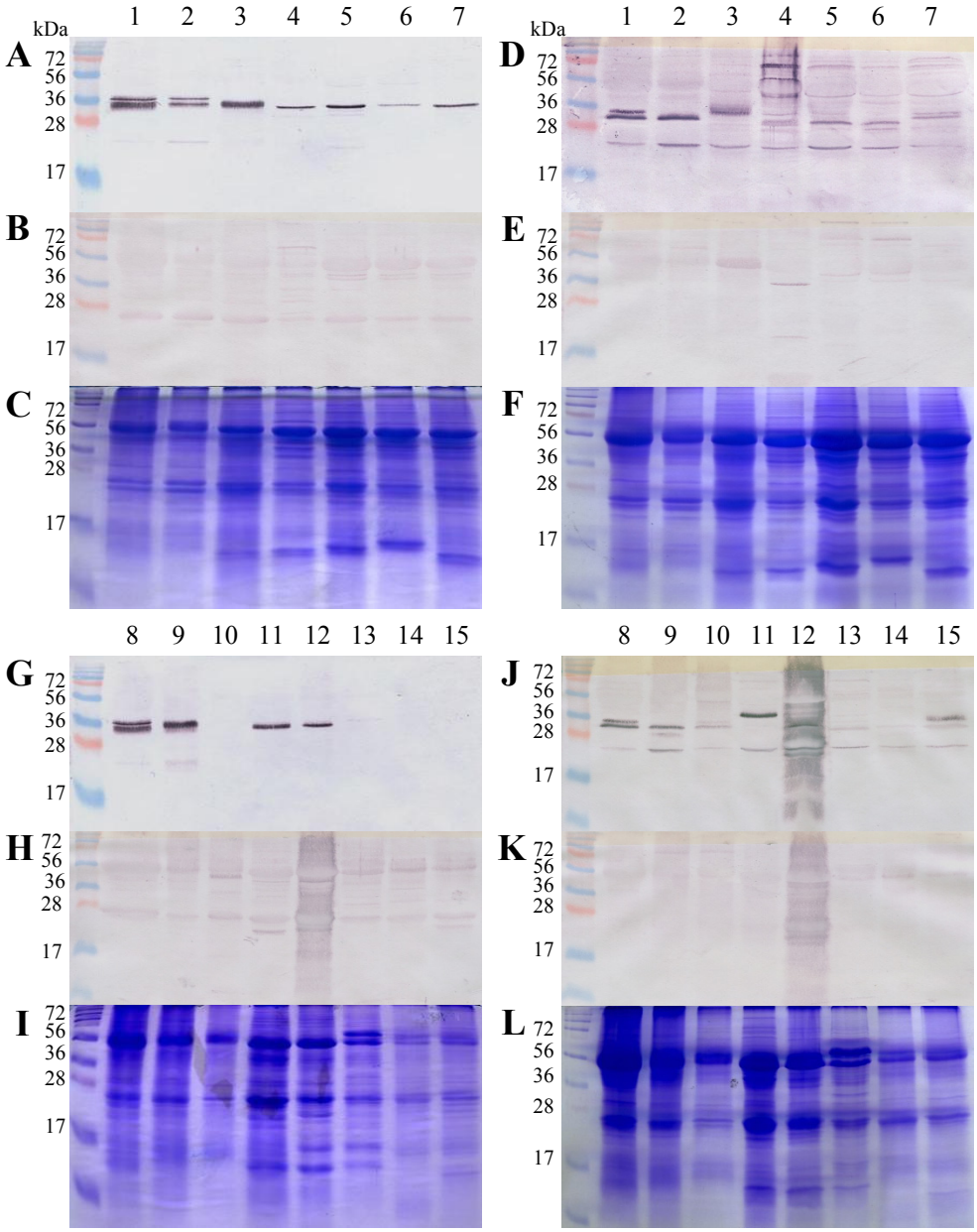

Figure II-3. Western blot analysis for presence of HFi22 and Hin19 in different plants. (A) and (G) are blotted for HFi22, while (B) and (H) use pre-immune serum drawn from the rabbit in which the HFi22 antibodies were later made. (D) and (J) are blotted for Hin19, while (E) and (K) use pre-immune serum drawn from the rabbit in which the Hin19 antibodies were later made. (C), (F), (I), and (L) are corresponding Coomassie stains for loading controls. Lanes 1 and 8 are *N. benthamiana*, lane 2 is *N. tabacum*, lane 3 is *V. unguiculata* (cowpea), lane 4 is *Spinacia oleracea* (Skookum spinach), lane 5 is *Cucumis sativus* (cucumber), lane 6 is *Cucumis melo* (cantaloupe), lane 7 is *Cucurbita maxima* (Big Max pumpkin), lane 9 is *Gossypium hirsutum* (cotton), lane 10 is *Lactuca sativa* (Simpson lettuce), lane 11 is *Phaseolus lunatus* (bean), lane 12 is *Rosa hybrids* (miniature rose), lane 13 is orchid, lane 14 is *Chlorophytum comosum* (spiderplant), and lane 15 is Philodendron erubescens (red-leaf philodendron). Pre-immune serum was used in all cases to observe any nonspecific binding signals resulting from proteins already present in the serum. Not all tested plant species are shown here, but are included in Table II-1.

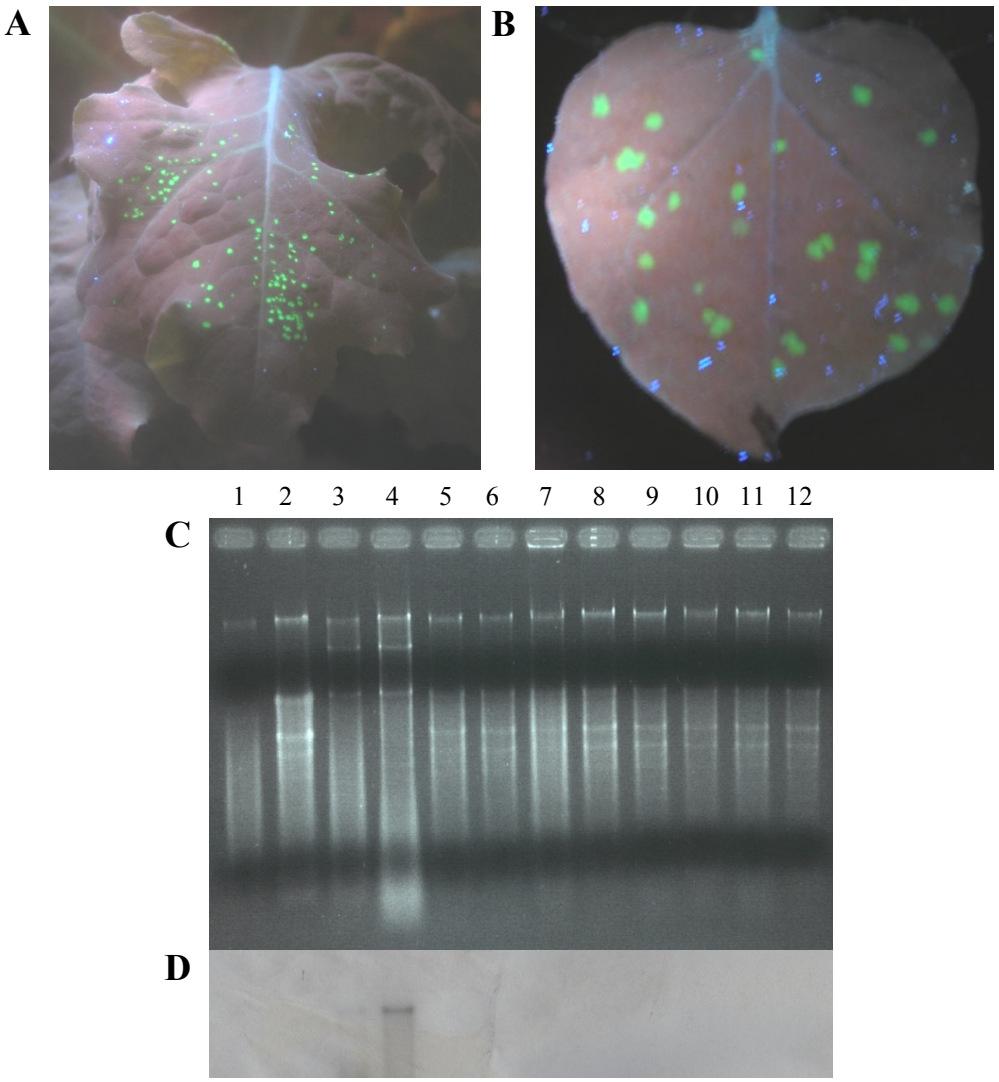

Figure II-4. Infectivity of TBSV on lettuce and *N. benthamiana*. (A) *L. sativa* infected with TBSV-GFP, 3 dpi. (B) *N. benthamiana* infected with TBSV-GFP, 3 dpi. (C) 1% agarose gel of total plant RNA from infected plants stained with EtBr. RNA was extracted 7 days post-inoculation with wtTBSV virions. (D) Northern blot film developed from the gel shown in (B); visible signal is genomic TBSV RNA. Lane 1 is healthy *N. benthamiana mock*-inoculated leaf, lane 2 is healthy *N. benthamiana* upper leaf, lane 3 is TBSV-infected *N. benthamiana* inoculated leaf, lane 4 is TBSV-infected *N. benthamiana* upper leaf, lane 5 is healthy lettuce mock-inoculated leaf, lane 6 is healthy lettuce upper leaf, lane 7 is repetition (rep) 1 of TBSV-infected lettuce inoculated leaf, lane 8 is rep 1 of TBSV-infected lettuce upper leaf, lane 9 is rep 2 of TBSV-infected lettuce inoculated leaf, lane 10 is rep 2 of TBSV-infected lettuce upper leaf, lane 11 is rep 3 of TBSV-infected lettuce inoculated leaf, and lane 12 is rep 3 of TBSV-infected lettuce upper leaf. (A) image courtesy of Yi-Cheng "John" Hsieh.

progress to detect if this positively effects TBSV movement in this host.

Discussion

The results of this study agree with past experiments showing that P22 is associated with cell walls, nuclear material, and membranes, and upon binding RNA it forms ribonucleoprotein complexes with TBSV genomic RNA for cellular transport (11, 13). The nuclear localization of P22 agrees with the presence of a functional nuclear localization signal (T. Rubio and A. O. Jackson, UC-Berkley, unpublished data).

As HFi22 is predicted to be a transcription factor, it was initially expected that this protein would be found primarily in the nucleus. Instead, my results support the notion that HFi22 is a soluble protein found primarily in the cytosol of healthy plants (M. Zhexembekova, unpublished data). P22 and HFi22 co-fractionated in the nucleus/membrane/cell wall enriched samples from infected plants, which supports the *in vitro* and yeast two-hybrid experiments performed by Desvoyes *et al* (13). However, due to the nature of this protocol, it was unclear what specific cellular components contained the two proteins.

It is important to determine to which specific cellular components P22 and HFi22 localize, to define the relevance of their interaction. If P22 and HFi22 co-localize in the membrane or cell wall fractions that the nature of their relationship would be involved in movement, supporting the idea that P22 may take advantage of HFi22 moving between cells. On the other hand, if both proteins co-localize in the nucleus, it could be that their relationship is involved in plant defenses. There are several possibilities that would fit with this scenario. One is that HFi22 recognizes P22 after they interact and activates transcription of defense-related genes. For instance, it is known that P22 elicits a defense response in *N. edwardsonii* (40) and it would be interesting to determine if this reaction involves HFi22. Another possibility is that HFi22 is normally involved in necessary regulatory pathways, and binding by P22 may suppress its normal defense-related function and then facilitate viral infection.

It is unsurprising for a protein such as a HD Leu-zipper TF to be conserved among plant species. If the role this protein plays in plant development or defense is vital, then it makes sense to see either closely related homologous genes or orthologues with similar functions. HFi22 was present in nearly all TBSV host species. There were a few non-host plants, such as rose, that also had an HFi22 band, but support for the hypothesis mostly rests with correlating HFi22 presence in host plants versus its absence in non-hosts, as there are a variety of other factors that can make a plant an unsuitable environment for a virus.

When the conservation of proteins among different plant species was investigated, an unexpected discovery was the absence of HFi22 in Simpson lettuce, in conjunction with the confirmation that lettuce leaves can be infected with TBSV-GFP, resulting in fluorescent green foci on inoculated leaves. However, it was unclear if TBSV was establishing a lasting and mobile infection, as there were no visible lesions, necrosis, chlorosis, or leaf curling symptoms. As it stood, lettuce was the only tested plant that did not initially fit into the correlation between TBSV hosts and HFi22 presence. However, the Northern blot analysis revealed that TBSV infection on lettuce is transient as the viral RNA was not detected at 7 dpi. Thus lettuce does follow the correlation hypothesis, as TBSV cannot establish full cell-to-cell movement in lettuce cells. Because lettuce lacks HFi22 or any closely similar homologue and does not support full TBSV movement, it would provide a good model in which to overexpress HFi22, then infect with TBSV to observe if adding this protein enables TBSV to properly infect and move through this plant.

In conclusion, HFi22 is a soluble cytosolic protein of which a portion co-localizes with P22 to nuclear/membranous components when the plant is infected with TBSV. These findings suggest the P22-HFi22 interaction is of some biological relevance. Likewise, the compromised movement in lettuce in the absence of HFi22 is still in agreement with the original hypothesis that HFi22 is involved in optimum TBSV spread.

CHAPTER III

SILENCING THE HOST FACTOR HFi22

Introduction

The exact relationship between the host protein HFi22 and the cell-to-cell movement protein (MP) P22, encoded by *Tomato bushy stunt virus* (TBSV) is not clear. The host factor was discovered due to its interaction with the MP during a yeast two-hybrid assay (13), and the features of the HD Leu-zipper protein family indicates this protein likely shuttles RNA from cell-to-cell (20). Given these factors, I hypothesize that HFi22 is involved in mediating viral movement of TBSV, either passively or actively. One possible model involves the transport of HFi22 from cell-to-cell through the plasmodesmata. As HFi22 moves, the P22-coated TBSV ribonucleoprotein complex binds to the transcription factor, using it to facilitate cell-to-cell movement. In order to determine the functional importance of HFi22 in the spread of TBSV, the aim was to silence the host factor in *N. benthamiana* using virus induced gene silencing (VIGS) (15, 24). This system would permit measuring the effects of HFi22 depletion on TBSV movement.

To initiate VIGS, *Tobacco rattle virus* (TRV) was selected as the vector to carry the insert for induction of gene silencing of the host factor. TRV is the type member of the *Tobravirus* genus. TRV has a (+)-sense single-stranded bipartite RNA genome that is encapsidated into two rigid cylindrical rod-shaped particles, one large particle for genomic RNA 1 and a smaller one for RNA 2. TRV RNA 1 is about 6.8 kb long (21, 26), encoding 4 open reading frames (ORFs) that express RNA-dependent RNA polymerase (RDRP), a MP, and a methyltransferase/helicase protein. The fourth ORF at the 3' end encodes a protein of unknown function (24). TRV RNA 2 varies among TRV strains. The strain used for this project has an RNA 2 that is about 3.8 kb long (21) and encodes 3 ORFs that express the 23 kDa CP and two proteins used in nematode transmission (24) (Fig. III-1A). TRV can establish an infection with just RNA 1 in the

absence of RNA 2, indicating that the CP and other RNA2 elements are unnecessary for replication and translation (26). Thus virus vector constructs are based on RNA 2 in which the CP ORF is replaced with the needed elements of the created vector (such as a MCS). Constructs with the widely-used *Cauliflower mosaic virus* (CaMV) 35S promotor as well as the various components needed for constructing T-DNA cassettes for agroinfiltration (Fig. III-1B-C) were used for this study. In previous experiments, Liu *et al*. have used a similar TRV silencing construct to silence phytoene desaturase (PDS) and CTR1 (a constitutive triple response 1 kinase gene) to observe and study gene function and loss of function phenotypes (24).

VIGS was chosen as the gene elimination strategy instead of using a transgenic HFi22 knockout plant for several reasons. First of all, there are currently no TBSV host plants with HFi22 gene knockouts available and to generate these in our laboratory would be very time consuming and expensive, and there is no guarantee that this type of KO can be successfully made (*Arabidopsis* is not a host for TBSV (46)). Secondly, it is currently not known what the function of HFi22 is within the plant. That it is a transcription factor makes it possible that HFi22 is important for early developmental processes, and a plant knockout may adversely affect the host and negate the results. Using VIGS instead addresses both of these problems: it is straightforward, fast, and allows the plant to grow prior to experimental depletion of HFi22.

Materials and Methods

Construction of HFi22-silencing TRV vectors

The 1kb HFi22 cDNA (13) was inserted into a TOPO plasmid vector (making 3.5 TOPO/D) using TOPO cloning (Invitrogen, Carlsbad, CA). From here the 1kb segment was transferred into the TRV vector 279 (TRV-279; kindly provided by Dr. Dinesh-Kumar, Yale University) (24) via the Gateway cloning system. However, the full cDNA proved too large for the vector to maintain (data not shown). Subsequently, smaller fragments of the HFi22 cDNA (~250-300 bp) were selected for insertion to achieve silencing. For this, 3.5 TOPO/D was subjected to restriction enzyme digests

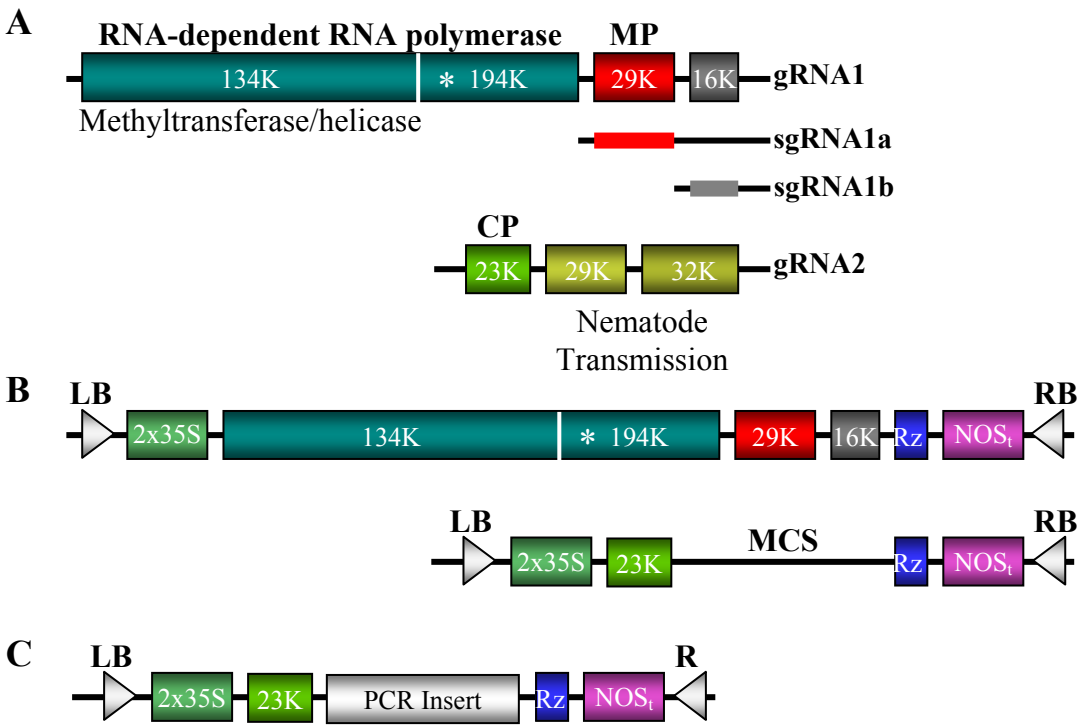

Figure III-1. Genome organization of TRV virus and vector. (A) TRV is a ssRNA virus with a bipartite genome. gRNA1 encodes 4 ORFs. The first translates into either a 134 kDa protein with methyltransferase and helicase domains, or a 194 kDa read-through product with RNA-dependent RNA polymerase function. The second ORF produces a 29 kDa movement protein and the third ORF results in a cystine-rich 16 kDa protein that may be involved in viral gene expression. The fourth ORF is nested in the 16 kDa protein, and it is unclear if a functional protein is translated from this region. The movement protein and 16 kDa protein are each translated from different sgRNAs. gRNA2 encodes three ORFs, the first resulting in a 23 kDa CP. The last two ORFs produce proteins involved in nematode transmission (26). (B) Schematic representation of the TRV vector used for VIGS. A TRV gRNA1 cDNA is inserted between duplicated CaMV 35S promotors (2x35S) and a nopaline synthase terminator (NOS_t). LB and RB refer to the left and right borders of the T-DNA and Rz is a self-cleaving ribozyme (24). (C) Diagram of the TRV gRNA2 used for silencing HFi22. PCR products from the beginning and end of the HFi22 cDNA were inserted into the MCS.

using *Not*I and *Msc*I to remove 700 bp from the 5' end of the cDNA. Then, 1µg/µL plasmid DNA was mixed with 0.5 µL *Not*I, 0.5 µL *Msc*I, 4 µL Buffer 3 (New England Biolabs), and 20 µL ddH_2O and incubated at 37ºC for 3 hours. The linearized fragment was then treated with Klenow polymerase at room temperature for 10 minutes to blunt the cohesive ends, then 1 µL of 12.5 mM dNTPs were added and the mix was incubated for an additional 10 minutes.

To remove all enzymes, the digested DNA was extracted using phenol/chloroform (Ph/Chl). The total volume of sample was brought up to 300 µL using 1xTE (10 mM Tris pH 7.0, 1 mM EDTA), then mixed with 300 µL Ph/Chl and vortexed for 15 seconds. The mix was centrifuged at 10 k for 10 minutes at 4ºC and then the upper phase was transferred to a new tube containing 30 µL of 3 M sodium acetate. To this, 600 µL of cold 95% ethanol was added and mixed by inversion, then placed at -20ºC o/n. The precipitated DNA was centrifuged at 10 k for 10 min at 4ºC and the supernatant was discarded. The pellet was rinsed with 500 µL of cold 70% ethanol and centrifuged at 10k for 1 minute at room temperature. Supernatant was discarded and the product was dried in a "Speed Vac" on high for 1 hour. The remaining pellet of linearized plasmid was resuspended in 10 µL of 1xTE.

The final step in constructing the new plasmid was to re-ligate the linearized TOPO. For this purpose, 7 µL of digested DNA was mixed with 2 µL of fresh T4 DNA ligase (3 U/uL) (Promega, Madison, WI) and brought to 20 µL with A/C ddH_2O. This mix was set at 16ºC overnight and the resulting ligation product (10 µL) was then transformed into 200 µL TOP10 *E. coli* component cells (Invitrogen, Carlsbad, CA) and mixed gently with a pipette. The cells were placed on ice for 30 minutes, followed by heat-shock at 42ºC for 45 seconds. Then the heat-shocked cells were transferred to a glass test tube containing 1 mL luria broth (LB) broth and shaken at 37ºC for 1 hour, then 100 µL of this suspension was spread on an LB plate with Kan^{50} selection. The remainder of the cells were centrifuged and the excess liquid poured off to concentrate the remaining cells. This was resuspended in 100µL LB broth and spread on a second LB plate Kan^{50}.

Individual colonies were picked and DNA minipreps were performed using QIAGEN (Valencia, CA) miniprep protocol. The removal of the 700 bp segment was confirmed by digesting samples of the miniprep DNA with *Pflm*I to linearize the plasmids (500 ng/µL DNA, 0.5 µL *Pflm*I, 2 µL Buffer 3, brought to 10 µL with ddH_2O and incubate at 37ºC for 3 hours). The product was electrophoresed through a 1% agarose gel in comparison to the original 3.5 TOPO/D plasmid digested with *Not*I and *Msc*I to determine if the ligase products is the same size as the original post-digest. One of the plasmids was selected and labeled pBS1-5.

The *HFi22* fragment in pBS1-5 was transferred into TRV-279 using the Gateway cloning system protocol given by Invitrogen. Briefly, the pBS1-5 entry clone and the TRV-279 destination vector were digested with LR Clonase ™ II enzyme in 1x TE buffer at RT for 1 hour. Proteinase K was added to terminate the enzyme activity. This mix was transformed into TOP10 *E. coli* component cells using the previously described heat-shock protocol. DNA was amplified from resulting colonies using a QIAGEN Maxiprep kit, resulting in TRV-pBS1-5. This plasmid was then transformed into *Agrobacterium* using electroporation.

A second TRV vector, TRV-MCS (Fig III-1), lacked the proper recombination sequences to use the Gateway cloning system. As such, it was necessary to revise the procedure for inserting *HFi22* fragments into this vector. Primers were designed with restriction enzyme sites to facilitate the ligation with cohesive ends into TRV-MCS (Fig. III-2B). Using these primers, 200-300 bp fragments from the 5', middle, and 3' end of the *HFi22* cDNA were amplified from a 3.5 pUC plasmid containing the original *HFi22* cDNA (Fig. III-2A). This plasmid was used as a PCR template because it has a different selectable antibiotic resistance marker (Amp^{50}) than the TRV vector, thus eliminating the chance of obtaining transformants that only contained the template.

For PCR, 40 µL ddH_2O was mixed with 5 µL 10x Vent Buffer (New England Biolabs), 1 µL 12.5 mM dNTPs, 1 µL template DNA (concentration 40 ng/µL), 1 µL each of forward and reverse primers (concentration 10 pmol), 1 µL 100 mM $MgSO_4$, and 0.5 µL Vent Polymerase (2,000 U/mL). This mix was subjected to PCR on an Applied

A

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1    gGCACGAGAT TAGATGAAGA AGGATGTATT GAAGAATCTG GTCATATTTC
51   TGAGAAGAAA AGAAGACTAA GTGTTGAACA AGTAAAAGCT TTAGAGAAAA
101  ATTTTGAAGT TGAAAATAAA CTTGAACCTG AAAGGAAAGT GAAGTTGGCT
151  CAAGAACTAG GTTTGCAACC TAGACAAGTT GCTGTTTGGT TTCAAAACAG
201  ACGTGCTCGT TGGAAaacaa agcaattaga gagagattat ggtgttctta
251  aatccaattt tgatgccctc aaacataatt atgaatctct caaacatgac
301  aatgaagctc tcttgaaaga gattcttgag ctgaaatcaa aggtgtatac
351  tgagaatgga gaaagcaaag gtgttgcagt gaaagaagag gctatggagt
401  ctgaaagtga tgacaacaaa gtgattgagc agagcaagcc aaatgataac
451  gacaacaaca ataataattt tcttgaaaat tttgaagaag atgatgaaga
501  agaagaaatc aattttgaga attttaatgt tgctgctgct gctacatcta
551  ccaatatttt tggtgataat ttcaaagatg gatcttcaga tagtgattca
601  agtgcaatct tgaatgaaga taacagtcca aatgctgctg ctatttcttc
651  atctggtgct ttcttgattt caacaaatgg aaatggaaat ggaaatggat
701  cttcaacttc attgaatttt tgcttccaat tcacagaatc aagttcaaaa
751  tcaaatcttg gagatggcca aaagggtaat aataattact accagccaca
801  acagtatgtg aaaatggagg agcataattt tttcaatggt gaagaatctt
851  gcagtactct tttcacagat gaacaagctc ctacacttca atggtactgt
901  cctgaggatt ggaattggaa agaataaagg aaatgtcttt tgggatcaaa
951  tttttgtata gtggctataa gtgcaaacat cagtacagta aatggcctaa
1001 tgggtaaatg tccagatatg aaaaaaaaaa aaaaaaaaa
```

B

5' Forward Primer

5' CACC CGATCG GAATTCGCACGAGATTAGATGAAGAAGG *3'*

TOPO *Pvu*I *Eco*RI

5' Reverse Primer

5' CGATCG GAGCTCTTCCAACGAGCACGTCTGTT *3'*

*Pvu*I *Sac*I

Middle Forward

5' CACC CGATCG GAATTCAGTGATTGAGCAGAGCAAGC *3'*

TOPO *Pvu*I *Eco*RI

Middle Reverse

5' CGATCG GAGCTCCAGCAGCATTTGGACTGTTATC *3'*

*Pvu*I *Sac*I

3' Forward Primer

5' CACC CGATCG GAATTCATCAAATCTGGAGATGGCC *3'*

TOPO *Pvu*I *Eco*RI

3' Reverse Primer

5' CGATCG GAGCTCGCACTTATAGCCACTATACA *3'*

*Pvu*I *Sac*I

Figure III-2. Primers developed from HFi22 cDNA (NCBI AAM48290) for PCR product insertion into a TRV VIGS vector. (A) Nucleotide sequence of *HFi22*. The beginning (5') segment is depicted in **bold**, the middle (Mid) is *italicized*, and the end (3') is underlined. (B) Primer sequences used for PCR amplification. Beginning primers encompass nucleotides 2-215; Middle primers encompass nucleotides 420-640; End primers encompass nucleotides 750-974. RE sites have been added to facilitate insertion of sequences into TRV RNA 2 in the (+) sense orientation. The TOPO clone sequence was added to enable the PCR products to be cloned into TOPO vectors.

Biosystems 2720 Thermal Cycler for the following cycle: 2 minutes at 95ºC, followed by 35 cycles of 95ºC, 55ºC, and 72ºC for a minute each, followed by 10 minutes at 72ºC, and concluded by a holding cycle at 4ºC. Products of the expected/predicted size were confirmed by running a 1% agarose gel.

PCR products were obtained for the beginning (5'), middle (Mid) and end (3') sections and were digested with *Sac*I and *Eco*RI for 2 hours at 37ºC. TRV-MCS was also digested with these enzymes. The PCR fragments were then ligated into TRV-MCS followed by transformation into TOP10 *E. coli* component cells selecting for Kan^{50}. Minipreps were prepared and the resulting DNA and confirmation of the PCR insert was obtained by using the original primers for PCR on the new plasmids. TRV-5' and TRV-3' plasmids were isolated using QIAGEN Maxiprep kit and protocol. TRV-3' plasmids were transformed into *Agrobacterium* (strain C58) to prepare cultures for infiltration, and successful transformation was confirmed by PCR using the original primers that produced the inserts.

Agrobacterium infiltration with TRV constructs

Agrobacterium transformed with the TRV vectors were prepared for infiltration as described in Liu *et. al* (24). For this, the cells were grown to an optical density (OD) of 2.0 in Luria broth with MES buffer (10 mM MES [2-(4-Morpholino)-ethanesulfonic acid], 10 mM $MgCl_2$) and activating by 150 mM acetosyringone (gallacetophenone 3'-4'-dimethyl ether in DMSO). This culture was mixed with *Agrobacterium* containing TRV-RNA1 in a 1:1 ratio and infiltrated into *N. benthamiana* to induce silencing of *HFi22*. For this purpose, a 3 mL syringe was filled with the TRV-RNA 1&2 mixture and pressed gently against the underside of a lower *N. benthamiana* leaf so the liquid culture was pushed into the leaf itself. Successful infiltration was visualized as the leaf surface darkened. Multiple infiltrations were required to fill the leaf. Two leaves per plant were infiltrated.

For this experiment MES buffer-inoculated plants were used as a negative control and TRV-*PDS* infiltrated plants were the positive control (provided by Dr.

Dinesh-Kumar). At 15 days post infiltration, the PDS plants showed clear bleaching of the upper leaves. Tissue from upper leaves corresponding to the *PDS*-silenced plants was processed for immunoblot assay for the presence of HFi22, and Coomassie staining was used as a loading control.

The silencing experiment was approached a bit differently with the TRV-3' vector. To maximize silencing, plants inoculated with *Agrobacterium*-TRV-3' were also co-infiltrated with TRV-PDS. Only one leaf was infiltrated, as previous work with TRV-MCS indicated plants would perish from TRV infection prior to silencing if more than one leaf was infiltrated (data not shown). Five plants were infiltrated with *Agrobacterium*-TRV-3'. Control 1 was an MES buffer-inoculated plant and Control 2 was a TRV-*PDS*-inoculated plant. At 2.5 weeks, 1 g each of upper and lower tissue was harvested and processed as described previously in preparation for Western blot analysis.

The infectivity and viability of the vector construct was also tested. For this, *Agrobacterium* with TRV-279-RNA2 and TRV-MCS (with no foreign inserts) and RNA1 were co-infected into *N. benthamiana* and observed for disease symptoms.

RT-PCR

Total plant RNA was extracted from *Agrobacterium*-infiltrated plants to check transcript levels of *HFi22* with reverse transcription (RT)-PCR. The leaf homogenizing buffer was prepared as follows: 10 mL of Grinding Buffer (0.18 M Tris, 9 mM LiCl, 4.5 mM EDTA, 1% SDS, 0.1% β-Mercatoethanol, pH to 8.2 with HCl), 0.9 mL 2 M NaOAc (pH 4.0), 10 mL phenol, and 2 mL chloroform. Leaves were harvested from upper and lower regions and 0.4 g of tissue was ground in 1.5 mL aliquots of the homogenizing buffer. This mix was centrifuged at 10 k for 6 minutes at 4°C (Beckman Allegra™ 21R centrifuge; F2402H rotor). Supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10 k for 6 minutes at 4°C. The supernatant was extracted with an equal volume of chloroform. Then an equal volume of 4 M LiCl was added to the supernatant and the suspension was incubated at -

20°C o/n. This was centrifuged at 10 k for 30 minutes at 4°C. The pellet was washed with 200 µL of cold 80% EtOH then centrifuged at 10 k for 10 minutes at 4°C. The pellet was dissolved in 100 µL of DEPC treated water. RT-PCR was performed using GE Healthcare's illustra Ready-To-Go™ RT-PCR Beads, with 47 uL A/C ddH2O, 1 uL RNA, and 1 uL each of forward and reverse primers (concentration 100 pmol) (Fig. III-3). Amplification was achieved with an Applied Biosystems 2720 Thermal Cycler PCR machine with the following cycle: 30 minutes at 42ºC, then 5 minutes at 95ºC, followed by 35 cycles of 95ºC, 55ºC, and 72ºC for a minute each, followed by 10 minutes at 72ºC, and concluded by a holding cycle at 4ºC. Products were confirmed by 1% agarose gel electrophoresis and EtBr staining.

Results

Efforts towards HFi22-silencing with TRV-279

Identity between *HFi22* and other homeobox proteins is shared primarily in the 5' end of the gene based on NCBI BLAST results. Therefore, it was decided that specific silencing of HFi22 was most likely when using a 3' region that showed little similarity with other related genes. For this, the HFi22 fragment from pBS1-5 was recombined into TRV-279, and this TRV plasmid was then transformed into *Agrobacterium* (resulting in *Agrobacterium*-TRV-pBS1-5) and used to infiltrate 5 *N. benthamiana* plants along with a healthy and PDS-silenced control. Two leaves were infiltrated as preliminary tests with TRV-279 demonstrated little risk of overdose-related TRV damage during the vector infection.

After 2.5 weeks, clear photobleaching was observed on upper leaves of the control plant infiltrated with *Agrobacterium* containing TRV-*PDS* and tissue was harvested from all plants at that time. Despite the resulting visible effects of silencing in the control plant, evidence of the successful application of the TRV-PDS vector, the western blot assay for HFi22 protein presence suggested that no HFi22 silencing occurred with the TRV-pBS1-5 vector as there was no clear difference in HFi22 band intensity when comparing the healthy and treated plants (Fig III-4). However, since the

3.5 Full Forward Primer
5' CCTGAAAGGAAAGTGAAGTTGG *3'*

3.5 Full Reverse
5' ACTGATGTTTGCACTTATAGCCACT *3'*

PDS Forward Primer
5' CTCACGCCCAACTAAACCAT *3'*

PDS Reverse Primer
5' AGCGTACACACTGAGCAACG *3'*

EIF-4A2 Forward Primer
5' GCAAGAGAATCTTCTTAGGGGTATCTATGC *3'*

EIF-4A2 Reverse Primer
5' GGTGGGAGAAGCTGGAATATGTCATAG *3'*

Figure III-3. Primers for three genes to be amplified with RT-PCR to confirm gene silencing. 3.5 primers encompass nucleotides 127-984 of the 1039 nt HFi22 cDNA (NCBI AAM48290); PDS primers encompass nucleotides 312-1317 of the 1761 nt PDS cDNA from *N. benthamiana* (NCBI DQ469932); EIF-4A2 primers encompass nucleotides 289-783 of the 1596 nt EIF-4A2 cDNA from *Arabidopsis thaliana* (NCBI AK226344).

TRV-*PDS* silencing vector uses a different TRV vector backbone than TRV-279 (24), and considering that previous work in the laboratory with TRV-279-derived constructs were also unsuccessful, this raised questions about the integrity of this vector.

TRV vector viability

To assay the viability of the different vectors (TRV-279 and TRV-MCS), experiments were conducted with vectors not containing inserts and the TRV-*PDS* control. Three weeks after agroinfiltration, the PDS-silenced control showed clear photobleaching (Fig. III-5A), while the healthy control *N. benthamiana* was thriving (Fig. III-5B). However, on the TRV-279-treated plants, very little symptom development could be observed and no leaves showed indication of TRV infection (Fig. III-5C and D). Northern blot analysis also failed to demonstrate any evidence of a TRV infection (data not shown). Because the virus vector was apparently not capable of causing an infection, this was incapacitating its ability to function as a viable vector for VIGS.

When the new TRV-MCS vector was subjected to the same test the results were quite different. Once again the PDS-silenced control was clearly displaying photobleaching due to silencing (Fig. III-5E) while the healthy control showed no symptoms (Fig. III-5F). Most importantly, less than three weeks after inoculation, the TRV-MCS infected plants were clearly symptomatic with TRV infection (Fig. III-5G and H). Based on these results it was decided that this new TRV vector would be used for all future silencing efforts.

Application of TRV-MCS towards silencing of HFi22

Initial agroinfiltration experiments with TRV-3' showed that infection led to necrotic symptoms that prevented monitoring the expected extent of silencing. To reduce symptoms and accelerate onset of silencing, it was decided to continue by co-infiltration of TRV-3' with TRV-*PDS*. For this purpose, eight different clones of *Agrobacterium* with TRV-3' were co-infiltrated with TRV-*PDS* and equal volumes of

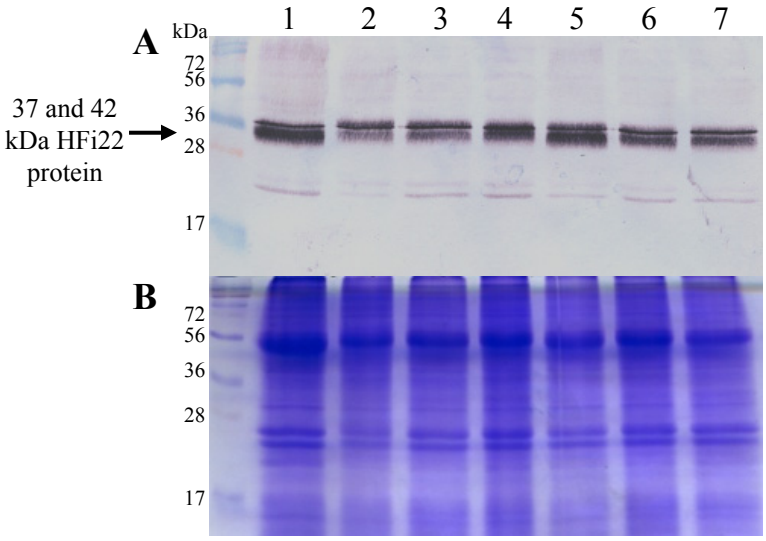

Figure III-4. Silencing assay in *N. benthamiana.* (A) SDS-PAGE followed by Western blotting for detection of HFi22 in N. benthamiana treated with TRV-279-pBS1-5, harvested at 2.5 weeks. Lane 1 is the healthy, MES buffer-inoculated plant. Lane 2 is the PDS-silenced control. Lanes 3-7 are five different samples of plants infiltrated with A*grobacterium* containing TRV-279-pBS1-5 (B) Corresponding Coomassie stain to visualize equal loading of samples.

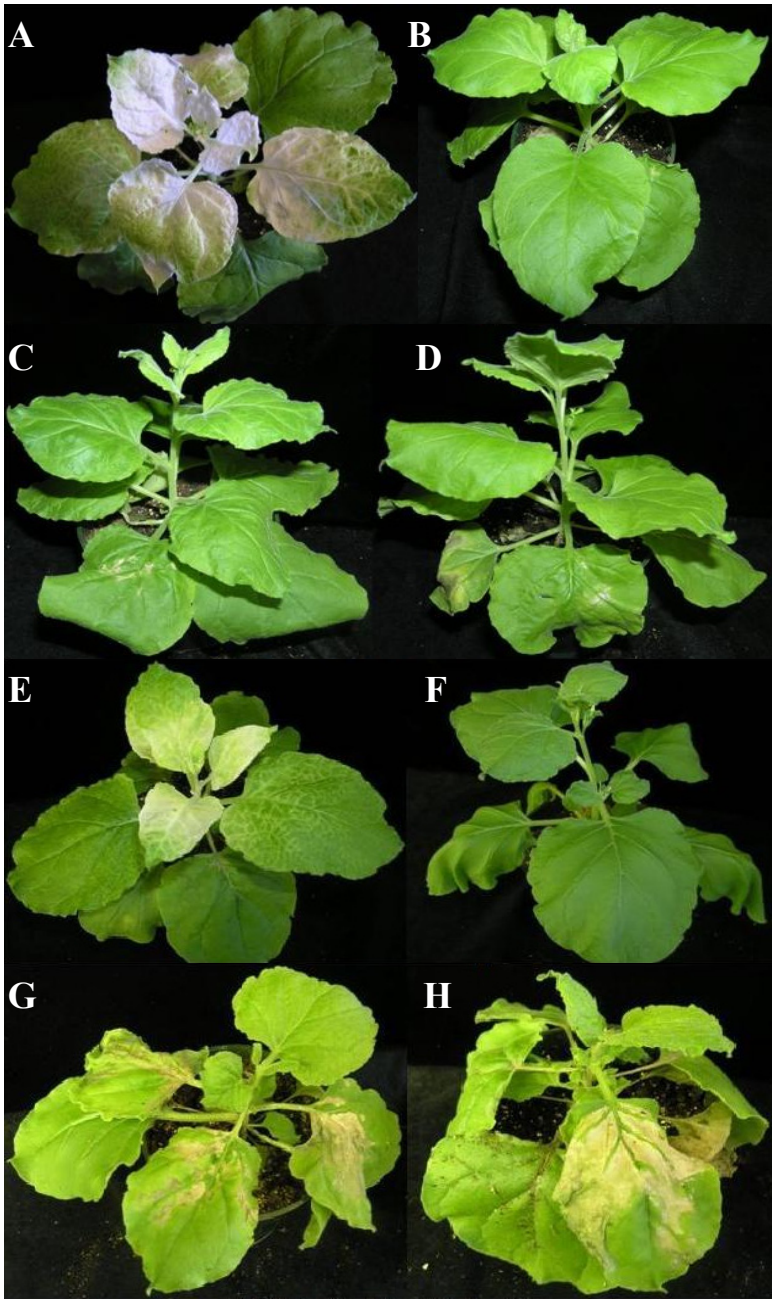

Figure III-5. *N. benthamiana* infected with different TRV vectors to test their viability. (A-D) TRV-279 vector 3 weeks post-inoculation. (E-H) TRV-MCS vector 2.5 weeks post-inoculation. (A) and (E) are controls, infiltrated with *Agrobacterium* containing with TRV-*PDS* to visualize silencing of the phytoene desaturase (*PDS*) gene, visualized by photobleaching. (B) and (F) are controls, inoculated with buffer to show the inoculation process alone does not result in symptoms. (C) and (D) were infiltrated with two different samples of TRV-279, and few symptoms can be seen after three weeks, indicating this vector has limited to no viability. (G) and (H) are infected with two different samples of TRV-MCS, and strong symptoms associated with TRV infection can be seen after 2.5 weeks, indicating this vector is functional.

Agrobacterium-TRV-RNA1 into 1 lower leaf of young *N. benthamiana* plants. At 2.5 weeks post-infiltration most of the plants showed significant photobleaching evidence of PDS-silencing and tissue was harvested from upper and lower leaves for processing. Some plants were suffering from TRV infection more than others, and those with more extensive TRV damage seemed to have less photobleaching (Table III-1). Western blot analysis was performed using the harvested tissue from upper and lower leaves using the HFi22-specific antibodies. No clear difference was visible on the blot when comparing the healthy plant and TRV-3' treated plants (Fig. III-6). Coomassie staining of protein gels indicated loading was consistent between samples.

To detect if the effect of silencing would be detectible for *HFi22* mRNA, RT-PCR was performed. RNA was extracted from the upper leaves of the silenced plants and the two controls, and RT-PCR was performed using primers for the PDS gene and HFi22. Product amplification of both PDS and HFi22 was observed in the healthy control plants (Fig. III-7). Some PDS product could be seen in the PDS silenced plant, but no HFi22 amplified from that sample. Samples 1, 4, 5, 7, and 8 amplified some PDS product, while this was not observed for Samples 2, 3, and 6 (Fig. III-7A). Of samples 5, 7, and 8, some amplified HFi22 product was observed, while 1, 2, 3, 4, and 6 did not contain any visible HFi22 (Fig. III-7B). This suggested that some reduction of HFi22 mRNA may have occurred in some plants, but not consistently.

The experiment was repeated using two sets of plants. The first set was harvested at 2.5 weeks, and the second at 3.5 weeks. Not all of the original 8 *Agrobacterium*-TRV-3' clones were used this second time around. TRV-3'5 Clone 1, Clone 4, and Clone 8 were discarded as they seemed to be less effective than some of the other samples. Western blot analysis for detection of HFi22 was performed with extracts from upper and lower leaf samples. Again, no visible difference in band intensity was evident when comparing the healthy control with the treated plants at 2.5 weeks after infiltration (Fig. III-8A and C) and at 3.5 weeks (Fig. III-8E and G). Coomassie staining of a gel run in parallel indicated loading was not entirely consistent between the wells (Fig. III-8B, D, F, and H). RT-PCR will be performed on the

Table III-1. Visual observations of *N. benthamiana* silenced with TRV-PDS and TRV-3'. Plants were scored based on the amount of tissue damage from TRV infection and the extent of visible photobleaching from PDS silencing. Scale is as follows: — is none; + is mild; ++ is moderate; +++ is extensive.

Plant Sample	TRV infection damage	Photobleaching progress
Buffer-inoculated healthy control	—	—
PDS-inoculated control	—	+++
TRV-3' Clone 1	+++	+
TRV-3' Clone 2	+	+++
TRV-3' Clone 3	++	++
TRV-3' Clone 4	+++	+
TRV-3' Clone 5	+	+++
TRV-3' Clone 6	++	++
TRV-3' Clone 7	+	+++
TRV-3' Clone 8	+++	+

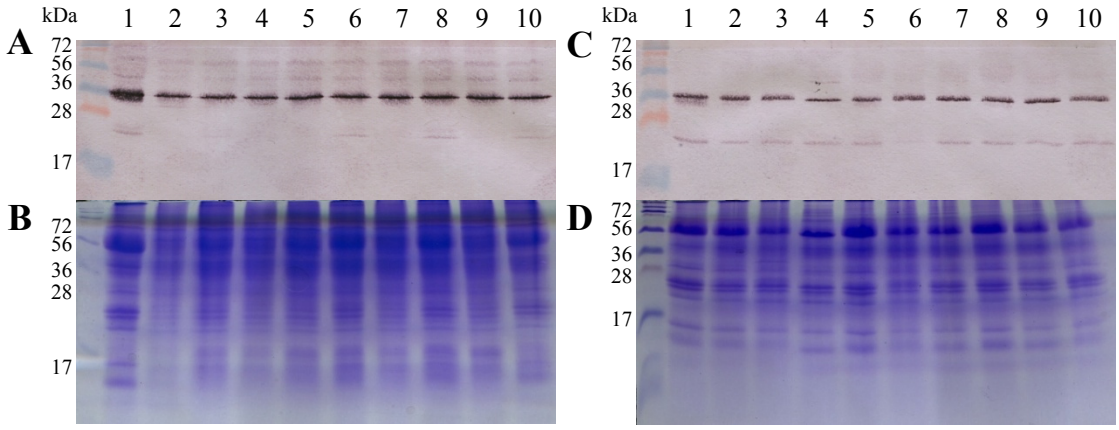

Figure III-6. Western blot analysis of HFi22 from *N. benthamiana* co-infiltrated with TRV-PDS and TRV-3' and harvested at 2.5 weeks. (A) SDS-PAGE followed by Western blotting for detection of HFi22 on samples harvested from the upper leaves of each plant. Lane 1 is the healthy buffer-inoculated control. Lane 2 is the PDS-silenced control. Lanes 3-10 are the 8 different clones of *Agrobacterium*-TRV-3' co-infiltrated with Agrobacterium harboring TRV-PDS. (B) Corresponding Coomassie stain of PAG to visualize equal loading of samples. (C) Samples harvested from the lower leaves of each plant. Lane loading is the same as the upper leaf gel. (D) Corresponding Coomassie stain. The significance of the lower (~20 kDa) band seen on the Western blots is thus far not known.

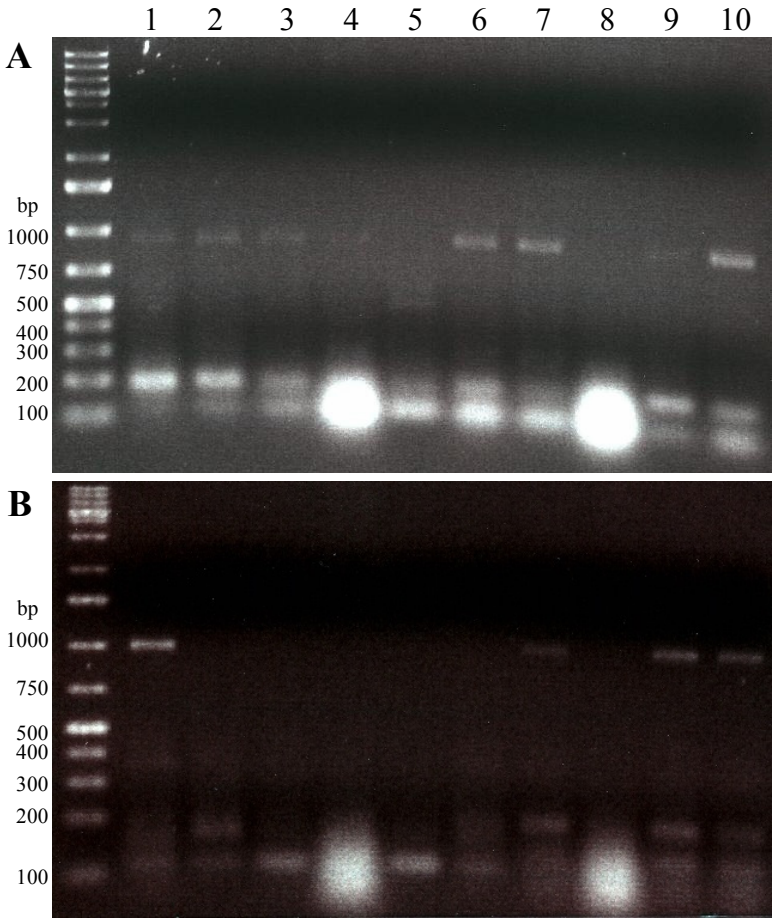

Figure III-7. RT-PCR on *N. benthamiana* tissue to confirm VIGS. (A) RT-PCR with primers that correspond to the PDS gene. (B) RT-PCR with primers that correspond to the HFi22 gene. In both gels, lane 1 is the healthy buffer-inoculated control; lane 2 is the *PDS*-silenced control; lanes 3-10 are plant RNA samples 1-8 of *Agrobacterium*-TRV-3' co-infiltrated with TRV-*PDS*.

extracted RNA, this time adding an internal control using primers for EIF-4A2 to establish loading control (Fig. III-3) (in progress).

Discussion

A result of practical significance was that under conditions used here, available TRV VIGS vectors differed dramatically in their suitability. Thus testing the infectivity of the vectors was significant because if the TRV vector cannot establish a successful infection, it will not produce enough transcripts to activate DICER for siRNA production that is needed for a silencing response. This effectively renders this vector useless for VIGS. Something appeared to be fundamentally wrong with TRV-279 as it caused little infection on *N. benthamiana*. Since no problems were encountered with amplification of the vector carrying the HFi22 insert under selection in *Agrobacterium*, the defect must be related to events that take place at or after agroinfiltration. For instance, it is possible the CaMV 35S promoter is compromised and it no longer yields transcripts. Regardless of the problem, to proceed with the project it was necessary to obtain a new vector. The new TRV-MCS vector proved to be a suitable alternative as it very quickly established infection on *N. benthamiana*. In fact, it was so effective that it became necessary to revisit the infiltration protocol and only infiltrate one leaf, as early attempts at using this vector resulted in lethal infection within 10 days when more than one leaf was infiltrated (data not shown).

While the three separate sets of HFi22 primers, 5', Mid, and 3', each amplified a PCR product, ligation attempts with TRV-MCS and the Mid fragment were unsuccessful. The cause of this is uncertain, as it used the same cohesive-end restriction enzyme sites as the 5' and 3' fragments, which each were inserted into TRV-MCS successfully. Despite the anomaly of the Mid fragment, two useful silencing vectors were obtained with the 5' and 3' inserts. However, TRV-5' was not initially seen as a suitable silencing activator since it would not specifically target HFi22 but also related genes. Thus TRV-3' was used for the duration of the project. Nevertheless, direct conventional cloning of HFi22 PCR products into the TRV-MCS has opened up the

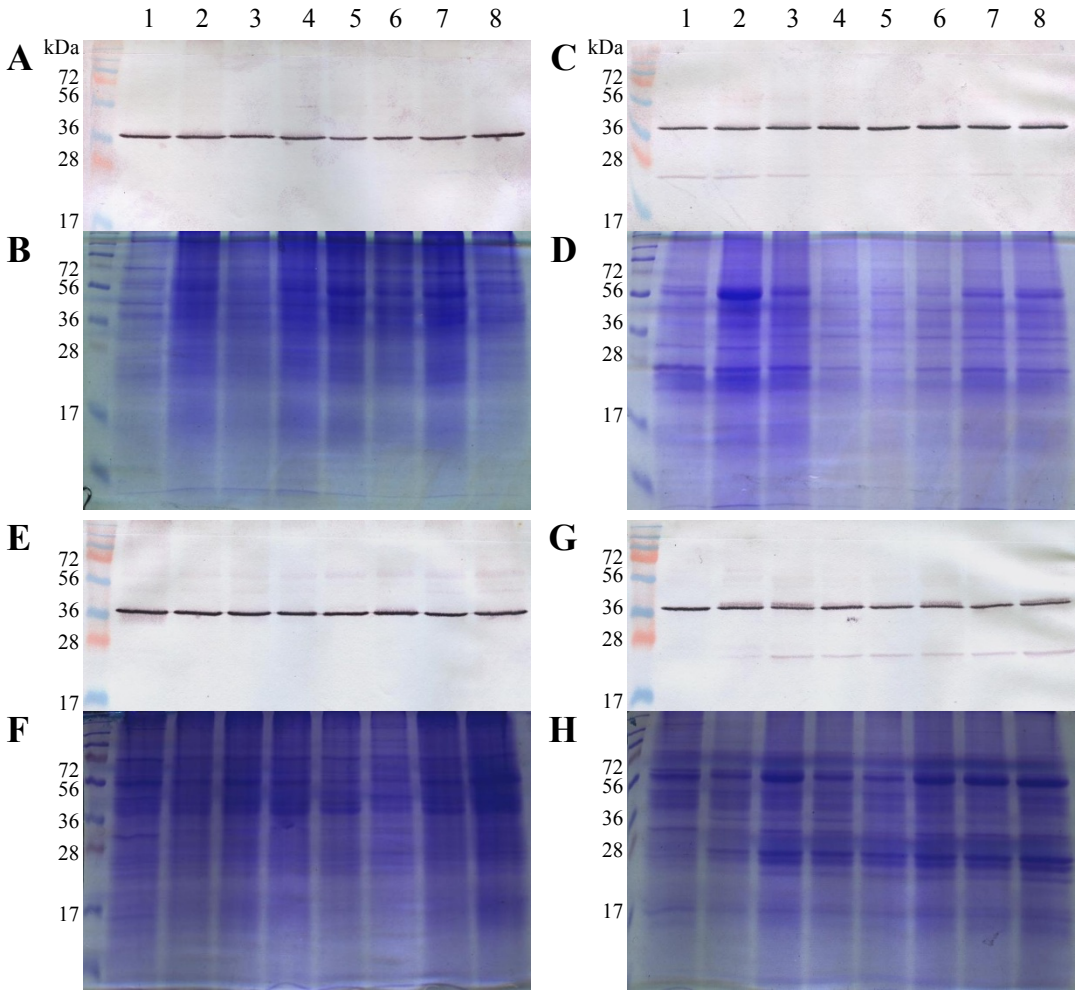

Figure III-8. Western blot analysis for detection of HFi22 in plants co-infiltrated with *Agrobacterium* containing TRV-*PDS* and TRV-3' and harvested at 2.5 and 3.5 weeks. (A) Upper leaf tissue harvested at 2.5 weeks. (B) Coomassie for upper leaf samples. (B) Lower leaf tissue harvested at 2.5 weeks. (D) Coomassie for lower leaf samples. (E) Upper leaf tissue harvested at 3.5 weeks. (F) Coomassie for upper leaf samples. (G) Lower leaf tissue harvested at 3.5 weeks. (H) Coomassie for lower leaf samples. Lane 1 is the buffer-inoculated healthy control, lane 2 is the PDS-silenced control, and lanes 3-8 are six different samples of *Agrobacterium*-TRV-3'/PDS co-infiltrated silenced plants.

opportunity to test different parts of the *HFi22* cDNA sequence for silencing. In addition, while it is hypothesized that the 5' end of the gene could induce silencing on other related proteins, this cannot be confirmed without experimentation. Ideally, whichever of those segments induces optimal silencing would be a candidate for future silencing attempts. This would provide the opportunity to determine if silencing the 5' end causes excessive stress or phenotypically visible problems for the plant, possibly indicating the silencing associated depletions of other needed proteins.

Despite having finally achieved the goal of constructing a viable TRV vector that contains an HFi22 segment, no silencing was evident based on the western blot analysis. Even though the RT-PCR results indicate tentatively that silencing of HFi22 RNA may occur, no internal control has yet been used to confirm amplification of a standard product in each sample as well verifying equal loading. These experiments are currently underway. Assuming the preliminary RT-PCR results were accurate and the transcripts are being subjected to silencing to some degree, the strong presence of the protein on the western blot indicates that the protein may be extremely stable and persistent in the plant tissue. It is also possible that HFi22 is involved in the silencing pathway itself, rendering it unable to be silenced. On the assumption that the protein is highly stable, a second set of plants was added to be harvested at 3.5 weeks to see if extra time would be necessary to see a measureable drop in protein levels. However, it still appeared that the protein levels remained constant.

In conclusion, the experiments show that under the conditions used, the TRV system is effective as a silencer for TRV-PDS. Co-infiltrating this vector with TRV-MCS provides the opportunity to easily monitor silencing progress by visually tracking upper leaf photobleaching, and furthermore this seems to help keep the plant alive presumably by rapidly eliciting a silencing response. Despite the efficacy of this system, silencing of HFi22 is not evident. It may be necessary to use another approach, such as introducing HFi22 into a movement-deficient host like lettuce, and observing how this impacts TBSV infection.

CHAPTER IV

HOST IMPACT ON INTEGRITY OF FOREIGN GENES IN A VIRUS VECTOR

Introduction

Agrobacterium-mediated transformation of foreign genes into plants is not always the optimal approach for studying foreign gene expression. Transient expression in mature plants is often desirable for this purpose because it is relatively fast and does permit high levels of foreign protein expression even if this has negative consequences for the plant. In those instances, using a virus vector to express the foreign gene of interest is a fast and efficient way to achieve the desired results (32, 35, 42).

In essence, there are three strategies to using virus vectors. First, foreign genes can be fused in-frame to existing viral genes to monitor functionality (42). For instance, Heinlein *et al*. (19) fused the gene for green fluorescent protein (GFP) to the gene for the movement protein (MP) of *Tobacco mosaic virus* (TMV) to observe the ways in which MP-genome ribonucleoprotein complexes interact with the plant cytoskeletal network to assist in plasmodesmata targeting (19). An alternative to gene fusion is to replace unnecessary genes that encode proteins that are dispensable for infection of certain plants (42). For example, *Tomato bushy stunt virus* (TBSV) does not require its CP for replication or movement (36), thus it can be replaced with foreign gene inserts (35, 37). In 2006, Giritch *et al*. (16) used a co-inoculation of TMV and *Potato virus X* (PVX) expressing heavy chains (HC) and light chains (LC) of IgG antibodies to assemble full-sized functioning antibodies in plants. The CPs of each vector were replaced with the foreign sequence, and they observed higher expression levels from the vectors without CPs. TMV does not require its CP for movement, but PVX movement is severely retarded without its CP. However, the MP of TMV can compensate for this lack, allowing the PVX vector to move cell-to-cell when co-infected with TMV (16). A third

strategy is to add an additional gene expression cassette. For instance, PVX vectors are commonly used in this manner to express foreign genes (32, 35, 42).

Virus vectors are not perfect expression systems however. There are problems with the stability of the foreign gene inserts, and it appears some of these problems may be related to host environment and size of the insert (2, 14, 29). For instance, in passage experiments with TBSV infecting *N. benthamiana* and pepper, Omarov *et al*. (29) observed TBSV defective interfering RNAs (DI)s accumulated rapidly in *N. benthamiana* but not at all in pepper (29). Since DIs originate from recombination events (9) this implies that these events are host-dependent. Furthermore, Desvoyes *et al*. (14) observed differing rates of recombination in TBSV CP mutants when inoculated on *N. benthamiana* and pepper, whereby less recombination seemed to occur in the latter, as opposed to *N. benthamiana* (14). It was hypothesized that replication in certain hosts may be 'sloppier' than in others, resulting in template hopping that alters the genome (9, 14). As mentioned in Chapter II, host factors have been observed that assist in reducing viral recombination (10). The size of the insert also seems to be a factor, as observed by Avesani *et al.*, where larger inserts seem to be more unstable than smaller ones in PVX-derived vectors (2). Similar effects have been observed in Chapter III, as the full 1 kb cDNA of HFi22 was unable to be stably expressed by the TRV-279 vector.

A common method for actively measuring stability of virus vectors has been the use of reverse transcription (RT) PCR and quantitative (Q) PCR to check for gene expression levels. For instance, Avesani *et al.* (2) made use of Q-RT-PCR was used to measure the stability of PVX vectors expressing foreign genes of varying sizes, comparing the levels of PVX transcripts expressing the various inserts. The recovered products were also sequenced to analyze the extent of recombination *in planta* and to observe any nucleotide changes that occurred (2).

RT-PCR and related techniques followed by sequencing analysis are suitable to examine recombination events at the nucleic acid level. However they are not convenient to quickly screen a wide population of virus vector molecules. Also, from a practical viewpoint, the critical determinant is whether the foreign gene insert is still

expressed at high levels, irrespective of minor silent mutations that have occurred. Therefore it seemed desirable to develop a quick biological assay that would allow for rapidly testing wide virus populations for the ability to maintain foreign gene inserts.

Considering the above information of the effect of the host, and the desirability for an efficient bioassay system, I hypothesize that different host-backgrounds affect the rate of deletion and mutations that affect gene inserts in virus vectors, ultimately impacting the expression of functional foreign genes. Furthermore, I propose that this can be tested using TBSV-GFP (Fig. IV-1) in combination with a straightforward biological inoculation assay exploiting a local lesion host. A local lesion host is a plant that interacts with and recognizes a specific pathogen or pathogen product in such a way that it elicits a hypersensitive response (HR). At a point of pathogen entry, the plant initiates a localized programmed cell death (PCD), killing the infected cell and some of the cells immediately surrounding it. This curtails the ability of the pathogen to spread, restricting it to its point of entry. Visually, this results in the appearance of lesions on the surface of the leaves (1, 22). *Vigna unguiculata* (cowpea) is one such host for TBSV. Infection of cowpea with TBSV-derived gene vectors results in visible white/grey lesions within 4 to 5 days of infection. At the same time, cowpea is also a good host to visualize TBSV-mediated foreign gene expression (37).

The above system offered the novel opportunity to take the percentage of green fluorescent foci caused by infection with TBSV-GFP among the total amount of lesions as an indicator of vector integrity. For this purpose, TBSV-GFP was inoculated on test hosts and after a few days this infected tissue was used as an inoculum on cowpea. The percentage of green foci was determined and used as an indicator of a ratio of the virus population that maintained the foreign gene (GFP) in the originally inoculated test host. This system permitted a comparison of vector integrity when TBSV-GFP was inoculated on different plant species.

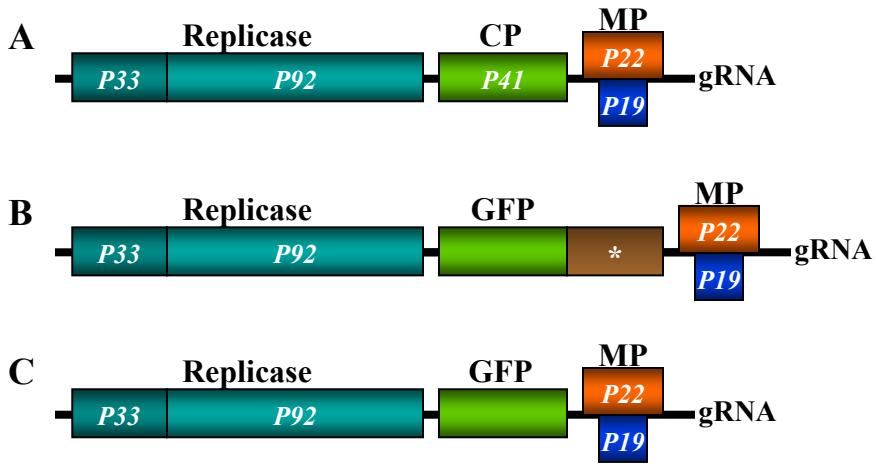

Figure IV-1. TBSV genome and GFP constructs. (A) wtTBSV genome organization. (B) TBSV-*GFP* construct, replacing the majority of the CP with the *GFP* gene (* is the remaining protion of CP RNA sequence). (C) TBSV-RMJ1 construct, devoid of CP RNA sequence. TBSV-RMJ1 is a construct developed by Malika Shamekova in 2007.

Materials and Methods

Plant care and maintenance

Cowpea were planted three seeds to a pot, three pots at a time, every four days to ensure a constant supply of plants. *S. oleracea* (spinach) were planted 5 seeds to a pot, one pot at a time, once a week. *C. sativus* (cucumber), *C. melo* (cantaloupe), and *C. maxima* (pumpkin), were planted 3 seeds to a pot, 1 pot at a time, once a week. Lettuce and *N. benthamiana* seeds were generously sprinkled into single pots, then seedlings were transplanted (about 10-12 days for lettuce and 12-16 days for *N. benthamiana*) into individual pots. All plants were kept on a 16 hour daylight period and housed at approximately room temperature (22-25°C). They were watered on an every-other day schedule or as needed on an individual basis to ensure no drought stress was placed on the plants. Lettuce plants needed to be grown close to their light source, as they would develop in spindly, etiolated growth patterns.

N. benthamiana plants were inoculated at 2.5-3.5 weeks after transplantation while the plants were still young (about 6 leaves present). Lettuce were also inoculated at an early stage when the plant had about 4 larger developing leaves. Spinach were inoculated when they were around 3-4 weeks, so the leaves had enough surface area to provide adequate tissue for later transfer. Cucumber, cantaloupe, and pumpkinwere inoculated at a younger age, when the first leaves were developing above the cotyledons (about 1.5 to 2.5 weeks of age). When cowpea were inoculated with transcripts, they were infected at 2.5-3 weeks of age.

Infection and tissue passage

Infectious clones of TBSV expressing GFP were transcribed from the RMJ1 plasmid construct (Fig. IV-1C) as described in Qiu and Scholthof, 2007 (35) and previously in Chapter II. From this, 25 µL of transcripts were mixed with 125 µL of cold RNA-inoculation buffer (50 mM KH_2PO_4, 50 mM Glycine ph 9.0, 1% Bentonite, 1% Celite). Then, 15-20 µL of the buffer-transcript mix was rub-inoculated onto 1-3 leaves of different test hosts. Plants were placed in dark, moist chambers overnight then

transferred to lighted growth racks placed in the laboratory at room temperature. At three days post inoculation, infected plants were exposed to UV light to observe GFP expression, as described previously (33).

Subsequently, 1g of fluorescing tissue was ground to a fine soup with 4-6 mL of virus-inoculation buffer (0.05M KH_2PO_4, 1.0% Celite, fill to volume with ddH_2O and autoclave; store at 4ºC). Buffer volume varied based on the density of GFP expression in order to dilute the amount of virus material present. This tissue mix was rub-inoculated on the upper leaves of 17-20 day-old cowpea. Best results were obtained if the inoculated leaves were the first sets of true leaves that developed above the cotyledons (Fig IV-2A). The inoculum mix was gently rubbed on these leaves and allowed to remain for 2 minutes, then inoculated leaves were rinsed clean of the inoculation mixture with distilled water. Inoculated cowpea were placed in a dark, moist chamber overnight then moved to room temperature lighted growth racks.

At 4 dpi, when lesions were clearly visible on the leaf surface, the plants were exposed to UV light to observe GFP expression. Green fluorescent foci were counted under UV light, then total lesions were counted. Fig. IV-2B illustrates how lesions were counted to minimize error. To determine the relative amount of gene insert retention, the number of GFP lesions was divided by the total counted lesions. Total counts were averaged for each plant species (Fig. IV-3).

Results

Prior to obtaining the TBSV-RMJ1 construct, an older TBSV-GFP construct that retained part of the CP was used (Fig. IV-1). Results with the original TBSV-GFP were inconsistent; *in vitro* transcription would yield high or low RNA levels using the same linearized plasmid sample as a template, and transcripts that looked of suitable quality upon agarose gel electroporesis would not necessarily yield green fluorescence on *N. benthamiana*. Often the numbers of green foci were extremely low, less than ten per inoculated leaf. It was decided to use the TBSV-RMJ1 construct instead as recent studies in the laboratory showed it to be a superior GFP-expressing vector that replicates

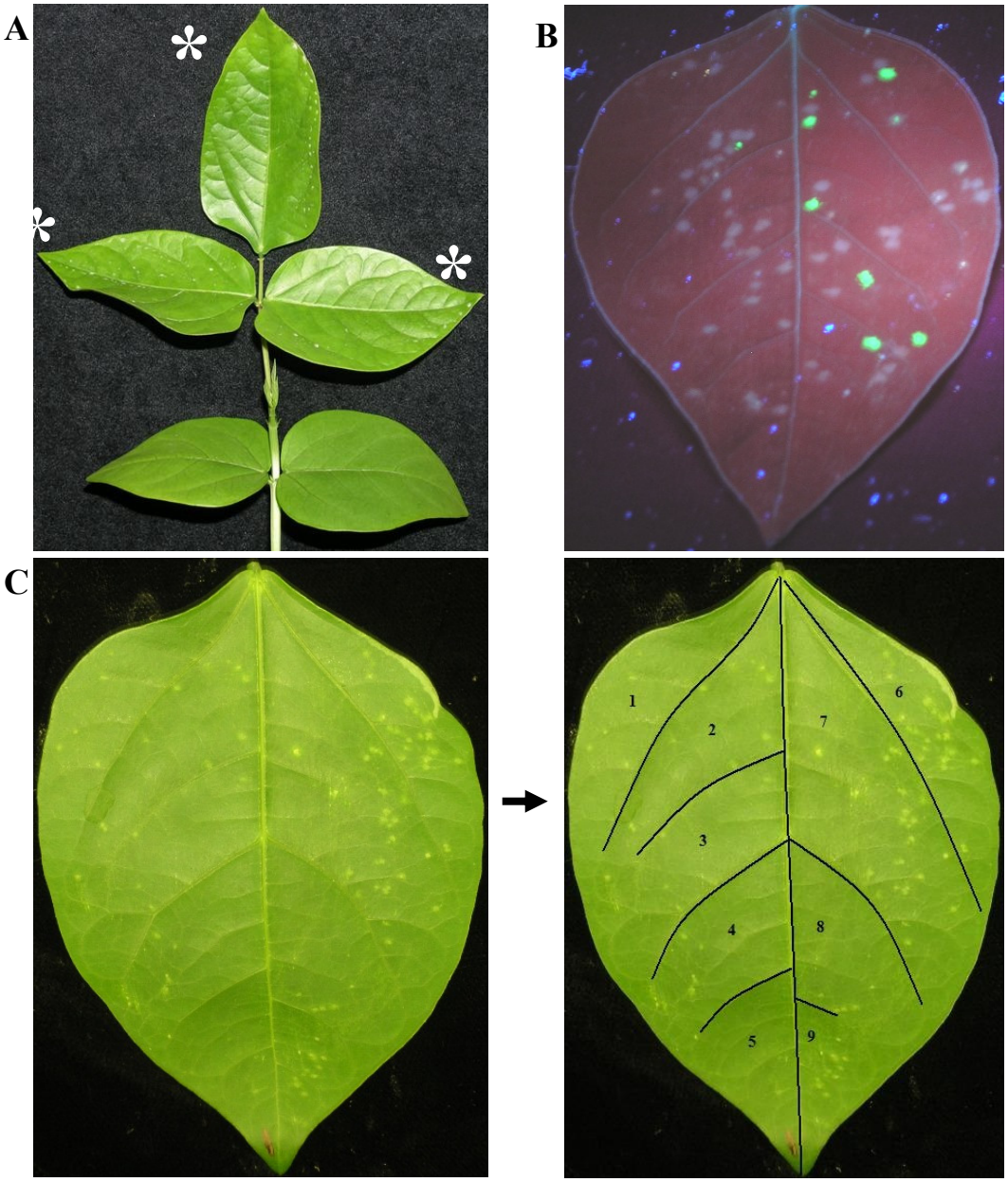

Figure IV-2. Diagram of passage inoculation and lesion counting. (A) An asterisk (*) denotes which cowpea leaves were inoculated. (B) Cowpea leaf 4 dpi with TBSV-RMJ1-infected leaf tissue from spinach. (C) To count lesions, the leaves were divided up into sections using the major visible veins as borders. Lesions within each section were counted then totaled for the final result.

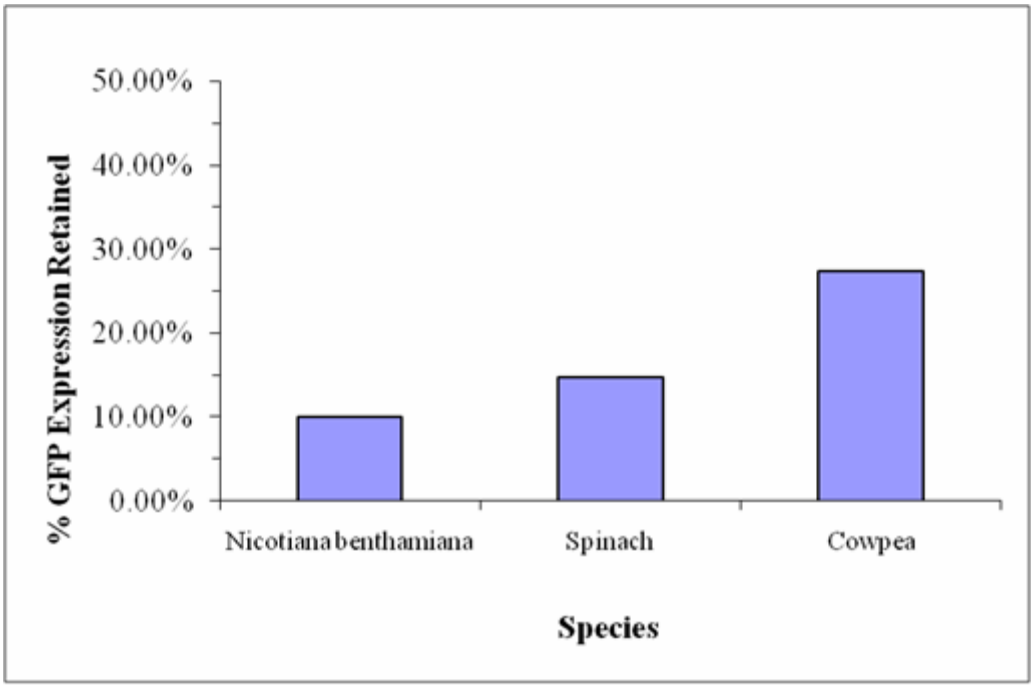

Figure IV-3. Percent GFP retention comparison between hosts. *N. benthamiana* retained 10% GFP expression; spinach retained 15% GFP expression; cowpea retained 27% GFP expression. (See Tables IV-1 thru -3 for complete results.)

readily and gives a strong fluorescent signal *in planta* (Shamekova, unpublished data).

While inoculating different host plant species with RMJ1 transcripts it was observed that some plants were more sensitive to TBSV infection than others. Some species, such as *N. benthamiana*, cowpea, and spinach, were easily rub-inoculated under normal laboratory conditions (room temperature, 16-hour 'daylight' period) using standard procedure of rubbing the upper leaf surface with transcript and Celite buffer. Other plants, such as pepper, tomato, pumpkin, cucumber, and cantaloupe, were somewhat more recalcitrant to inoculation to achieve acceptable levels of infection. For pumpkin, cucumber, and cantaloupe, it was necessary to inoculate the underside of the leaf surface. Pepper and tomato proved more difficult. Extraneous measures were taken to prepare the plant tissue for infection, such as placing plants in the dark and lowering the temperature to 15°C prior to infection. However, these measures have not yet yielded visible TBSV-GFP infection for some species.

Through the course of this study, it was important to establish dilutions of the virus-infected tissue in order to obtain viable results. For instance, 1 g of RMJ1 transcript-infected cowpea on average yielded many more green foci than 1 g of infected *N. benthamana* when transferred to cowpea, and spinach often had even fewer. To obtain counts that were more consistent and comparable, the amount of VIB added to the leaf tissue was adjusted based on the concentration of observed green foci on the infected leaf. This also aided to simplify counting lesions and make it more accurate, as early trials with highly concentrated samples revealed that passage to cowpea resulted in infection so massive that it was impossible to distinguish individual lesions (data not shown).

Infection progression was slightly different for each host inoculated with RMJ1 transcripts. When *N. benthamaina* was inoculated with RMJ1, pinpoint green foci were observed under UV light as early as 36 hours after transcript inoculation. If the infection was permitted to progress those regions of high density green fluorescence could spread and blend with each other, which resulted in mats of green fluorescence. Within three days the green foci could be as large as 2 mm in diameter, and depending on the size of

the leaf there were anywhere from several dozen to several hundred green spots (Fig. IV-4). By 3 to 4 dpi, faint, mottled, chlorotic leasions could be seen on the infected leaves with the naked eye. When this infected tissue was transferred to cowpea, lesion counts revealed RMJ1-infected on *N. benthamiana* has an average functional insert retention at 3 dpi of approximately 10% (Table IV-1).

Spinach was somewhat harder to expose to passage experiments than *N. benthamiana* as there was less tissue to work with and it is not quite as sensitive to TBSV transcript inoculation. Fluorescent foci could be seen at about 48 hours post inoculation as autonomous points of infection, and by 3 dpi foci averaged 1 mm in diameter. The number of foci per leaf ranged from a dozen to just under 100. If infection was allowed to progress, some leaf curling and growth stunting could be observed, though by 3 dpi when tissue was transferred to cowpea, there were little notable visible symptoms. After tissue passage to cowpea, the lesion counts indicated that when RMJ1 is infecting spinach, it has an average functional insert retention at 3 dpi of approximately 15% (Table IV-2).

Because of its high susceptibility to transcript inoculation, cowpea was also used in these experiments. Upon rub-inoculation with TBSV-RMJ1 transcripts, fluorescent green foci could be readily seen by approximately 36 hours. However, green spots on cowpea leaves remained relatively small, ranging from 0.5 to 1 mm in diameter. This is likely a result of cowpea being a local lesion host with limited cell-to-cell movement. However, cowpea is extremely sensitive to TBSV, and one infected leaf could have many hundreds of green spots (Fig. IV-5). Few, if any, white lesions could be seen on transcript-infected cowpea leaves, indicating it is a suitable indicator host for these experiments because inoculation with TBSV expressing *GFP* virtually always gives green foci. Thus, absence of green fluorescence means that a functional *GFP* was absent from the input molecules derived from the tested host. As a local lesion host, cowpea exhibits clear visible lesions at points of virus infection, with some leaf crinkling and limited necrosis that may also be attributed to the inoculation process. When TBSV-RMJ1-infected cowpea tissue was transferred to new cowpea leaves, the lesion counts

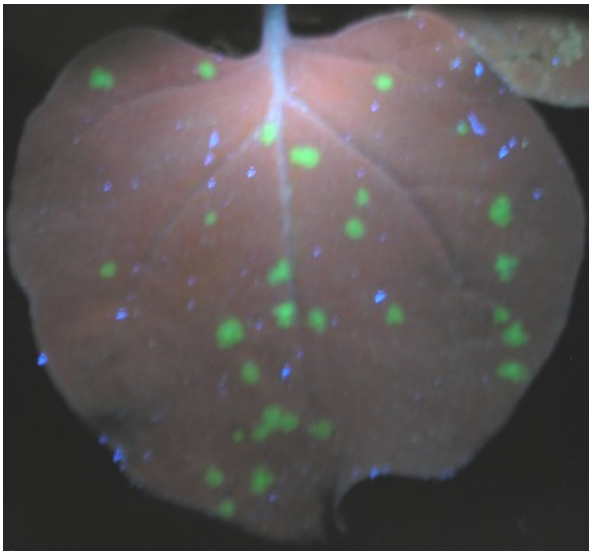

Figure IV-4. TBSV-RMJ1 on *N. benthamiana* 3 dpi. This leaf is an example of a lower concentration of green foci typically seen from an RMJ1 infection.

Table IV-1. GFP foci and total lesion count for *N. benthamiana*. Green fluorescent spots were counted under UV light while total lesions were counted under normal light.

Sample	# GFP	Total	% Retained	
12/26/2007	7	120	5.8%	
12/26/2007	18	262	6.9%	
12/26/2007	14	157	8.9%	
12/26/2007	6	57	10.5%	
12/26/2007	5	51	9.8%	
12/26/2007	16	150	10.7%	
12/26/2007	1	28	3.6%	
12/26/2007	3	41	7.3%	
12/26/2007	2	48	4.2%	
1/4/2008	22	137	16.1%	
1/4/2008	21	132	15.9%	
1/4/2008	11	89	12.4%	
1/4/2008	7	67	10.4%	
1/4/2008	7	79	8.9%	
1/4/2008	4	63	6.3%	
1/4/2008	20	133	15.0%	
1/4/2008	14	104	13.5%	
1/4/2008	9	66	13.6%	
			10%	Average

Table IV-2. GFP foci and total lesion count for spinach. Green fluorescent spots were counted under UV light while total lesions were counted under normal light.

Sample	# GFP	Total	% Retained	
12/26/2007	10	65	15.4%	
12/26/2007	11	65	16.9%	
12/26/2007	10	58	17.2%	
12/26/2007	7	56	12.5%	
12/26/2007	3	35	8.6%	
12/26/2007	5	27	18.5%	
12/26/2007	4	28	14.3%	
			15%	Average

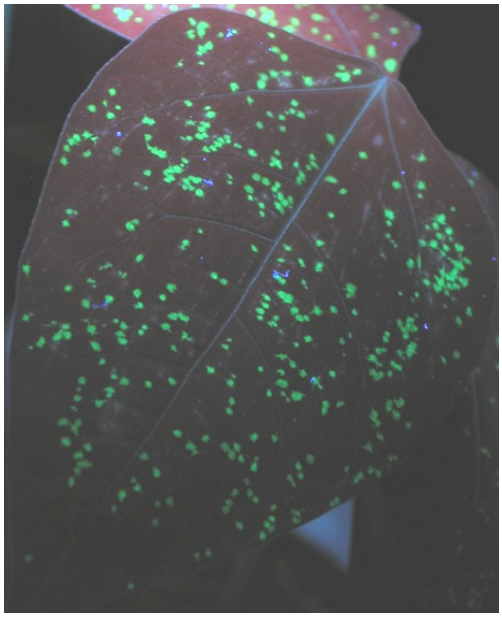

Figure IV-5. Cowpea leaf infected with TBSV-RMJ1 transcripts. Taken at 3 dpi prior to tissue passage. Few non-fluorescing foci are visible, indicating *V. unguiculata* is initially a stable environment for infection.

demonstrate that RMJ1 has an average functional insert retention at 3 dpi of about 27% (Table IV-3).

Not all TBSV hosts were suitable for the passage experiments. Pumpkin, cucumber, and cantaloupe did not yield any lesions, green or otherwise, when passed to cowpea.

Discussion

The use of GFP was a major breakthrough in the field of molecular biology as it provided a way to observe a number of factors without causing harm to the plant. It can be fused to proteins to track their localization, or be included in viral genomes to observe infection progress (4). In the context of this experiment, using TBSV as the vector for GFP expression allowed us to examine a variety of plant species as TBSV has a broad host range (46). This enabled the hypothesis to be tested rather extensively as it was possible to compare the results of plants from several families.

RMJ1, a TBSV-*GFP* construct, was a good vector to use for the integrity studies. *In vitro* production of transcripts resulted in relatively high concentrations of nucleic acid, and it more readily expressed GFP than the previous TBSV-*GFP* construct (Fig. IV-4). Hosts that previously yielded few green foci using the older TBSV-GFP construct instead displayed high expression of fluorescence. It has been observed that the CP gene is a highly recombinative region (14). The original TBSV-*GFP* construct only had a minor portion of the CP gene removed while a major stretch of CP RNA sequence remained (Fig. IV-1). In RMJ1, that region was removed, leaving just the 5' end of the CP upstream of the GFP gene. This may make this construct more stable as the recombinative CP-RNA elements are removed.

It was observed that some hosts, when inoculated with transcripts, yielded many green foci, while some had few and others, none. A blanket cause for this phenomenon would be varying degrees of susceptibility to TBSV transcript inoculation among host plants, whereby the virus can more easily replicate and translate proteins in some, while in others the process is inefficient. It may be that TBSV does not replicate to as high a

Table IV-3. GFP foci and total lesion count for cowpea. Green fluorescent spots were counted under UV light while total lesions were counted under normal light.

Sample	# GFP	Total	% Retained	
12/19/2007	3	8	37.5%	
12/19/2007	2	21	9.5%	
12/19/2007	1	8	12.5%	
12/19/2007	4	20	20.0%	
12/19/2007	5	23	21.7%	
12/19/2007	6	10	60.0%	
1/4/2008	3	8	37.5%	
1/4/2008	5	13	38.5%	
1/4/2008	1	11	9.1%	
			27%	Average

titer in these hosts as in *N. benthamiana*, or the CP contributes to TBSV replication or movement in these plants. It is also possible that there are varying amounts of RNases on the surfaces of leaves from different plant species that may be degrading the transcripts before an infection can be established. Also, in some hosts, such as pumpkin and cucumber, GFP expression is seen as a mat of fluorescence rather than an individual green foci. It is possible this type of expression is seen because as the virus spreads from infected to healthy cells it establishes rapid infection, resulting in translation of viral proteins and GFP in each infected cell and what cumulatively becomes visible is a large area of green fluorescence. This phenomenon may also be related to spread of the virus being limited to lateral movement through the epidermal cells rather than penetrating into the underlying mesophyllic tissue, resulting in a mat of GFP expression rather than individual foci of infection.

Some plant species did not passage well at all, showing few or no lesions on cowpea. There are various possible reasons for this. One, is that some of these plants may release more RNases and other degredating compounds when they are subjected to trauma such as being ground in a mortar. Therefore, by the time the tissue was homogenized for passage, all of the viral RNA was degraded. Without the CP to protect the virions, the viral genome is extremely vulnerable. It is also possible that some of these plants have a more rapid and efficient RNAi response to initial viral infection, eliminating the infection so rapidly that insufficient virus remained to see results after passage. Thus GFP may only be visible during the first few rounds of translation prior to genome degradation.

It was not particularly surprising that *N. benthamiana* had such a low insert retention value given previous studies of virus instability in this host (14, 29). In the experiments performed by Desvoyes *et al.* (14) and Omarov *et al.* (29), TBSV undergoes a higher rate of recombination in *N. benthamiana* than observed for other species. Even though statistical analysis needs to be performed, it is interesting that cowpea had such a relatively high retention rate compared to *N. benthamiana*. This clearly demonstrates that the host has a noticeable impact on the virus. This effect may have to do with

recombination, or may be impacted by viral movement. cowpea does not support full cell-to-cell movement; perhaps some instability in hosts more conducive to movement is brought on by the virus moving into a new cell. This new cell may have received signals from neighboring cells, and has started changing its own internal environment in response, thus the virus is moving into a more hostile environment that may interfere with its stable replication.

An interesting observation noted in this study was the lack of sectioning seen in the local lesion host (Fig. IV-2). Lesions were either green fluorescent, or white; there was no obvious mix or sectioning of expression. Presumably the same cell could be infected by vectors that still express functional GFP mixed with those that had undergone sufficient recombination to knock out (KO) the GFP gene. Why then is there no resulting mixed infection? It is thought that the KO virus is somehow functionally more fit (2, 42), and thus out-competes those still expressing GFP. In addition, with multiple RNA templates of slight variation, recombination is likely occurring between the two viruses, in which case the KO virus would be assisting in recombining out the GFP of the other virus. Thus the numbers obtained in this study determining the retention of functional GFP may be slightly lower than what is actually present, as some of the stable, insert-retaining viruses are getting rapidly out-competed upon infection of cowpea.

The strategy used in this study represents a novel approach that can become a very useful laboratory tool even though technical hurdles need yet to be overcome when incorporating some recalcitrant plant species. Often when viruses are used as gene vectors the aim is to express a functioning protein (16). If the vector-host relationship results in an unstable environment and the virus is recombining parts of the insert, it may be necessary to try to utilize a different host system. Making use of this visual stability aid is a rapid and inexpensive approach to observing stability as from a practical viewpoint it is less relevant what mechanism has changed the virus to inactivate the gene insert (GFP in this case). What really matters is that it can be quickly ascertained if the

protein sequence has been altered enough that it can no longer be stably expressed in a functional form.

Given these numbers obtained in this study (Fig. IV-3), while awaiting statistical analysis, there appears to be a obvious difference between the stability viruses replicating in *N. benthamiana* verses those in cowpea (10% vs 27%), and there are still more hosts to examine. Solanaceous plants are commonly used in biotechnology initialize exploiting the use of virus vectors (32). Based on the data presented here, researchers may want to reconsider testing other plant species if they require stable virus-mediated expression of foreign genes.

CHAPTER V

CONCLUDING REMARKS AND PERSPECTIVES

P22-HFi22 *in planta* Interaction

It has been observed in healthy plants that HFi22 is a soluble cytosolic protein, that co-localizes to the nucleus and/or membranes in the presence of P22 upon TBSV infection. This finding is in agreement with the previous yeast two-hybrid assays and *in vitro* studies that resulted in the original finding of the host factor (13). Of significant note was the absence of HFi22 from Simpson lettuce, which turned out to be a poor host for cell-to-cell movement. This finding supports the hypothesis that HFi22 is involved in movement. However, additional experiments are needed to shed more light on the nature and location of the P22-HFi22 interaction.

Visualizing the co-localization of P22 and HFi22 can be approached from a different angle in the future using fluorescent microscopy. One option would be to construct a TBSV vector expressing Red Fluorescent Protein (RFP) fused to P22 and construct a pKyLx (or similar *Agrobacterium*-based) vector expressing Yellow Fluorescent Protein (YFP) fused to HFi22 that can be introduced into the plant using agroinfiltration. The location of HFi22 can be tracked in a healthy plant, then with the introduction of TBSV-P22-RFP, the localization of both proteins could be observed. Areas where both are fluorescing would indicate a point of co-localization.

To determine if both proteins truly interact *in planta*, the bimolecular fluorescent complementation (BiFC) technique could be used (6). Two different parts of the YFP protein could be fused to constructs expressing P22 and HFi22. When these are introduced into a plant, yellow fluorescence will only occur if the two proteins interact, bringing the parts of the YFP protein in close enough proximity that they yield a bimolecular functioning fluorescent protein.

Several factors will need to be taken into account with the above suggested experiments. The first is that it has been problematic in the past to obtain functional P22

fusion proteins, as the fusion protein interfered with biochemical characteristics of P22 (13). For this strategy to be successful a P22 fusion protein must function as close to its wildtype counterpart as possible. If it does interact with HFi22, steric interference from the fused fluorescent protein must be avoided. This should be feasible since recent studies have indicated that fusing GFP to the N-terminal end of P22 does not entirely abolish its cell-to-cell movement (Rubio and Jackson, unpublished data). A second issue that must be addressed is expressing HFi22 from plasmids. As mentioned previously, the available HFi22 cDNA is incomplete, missing several amino acids at the N-terminus. It is unclear if these amino acids are vital for proper HFi22 folding and function. Previous attempts to express HFi22 from pKyLx vectors have been unsuccessful (see Chapter II), and this may be due to those missing amino acids. It may be necessary to finish sequencing the entire HFi22 gene before this type of experimentation should be pursued.

Silencing HFi22

Currently the results are unclear as to whether or not HFi22 can be silenced using traditional VIGS. Assuming HFi22 can be successfully silenced with VIGS, there are many things that can be considered to continue testing its significance for TBSV infection. Upon establishing a culture of *Agrobacterium*-TRV-HFi22 that successfully silences a plant, the next step would be a time-course experiment to determine how many days after infiltration systemic silencing is established. To do this, a group of ten *N. benthamiana* plants of the same age would be infiltrated with this culture on the same day. Starting two weeks after infiltration, upper and lower leaves from one plant would be harvested for the next ten days, one plant each day. Western blot analysis would be performed on harvested tissue to determine by which day post-infiltration the plant has silenced the host factor, using RT-PCR and QRT-PCR to confirm the removal of HFi22 transcripts. TBSV better infects younger plants. Because of this, it is important to try to obtain silenced plants as young as possible, so the age of the plant does not influence the

rate of virus spread. Thus the earlier TBSV can be introduced the more similar the virus infection process will be to a typical laboratory infection.

Once the proper time frame has been established, the next step is to introduce TBSV into the system. The controls for this experiment would be an unsilenced plant and a PDS-silenced plant. When the silenced plants have reached the previously-established time frame for systemic silencing, they and the control plants would be infected with TBSV using rub-inoculation of transcripts of TBSV-GFP. After inoculation, the spread of the virus would be tracked by UV lamp, observing where the GFP signal appears. By comparing the rate of viral spread in the *HFi22*-silenced plants to the unsilenced control plants, it will be possible to see if an absence of HFi22 has any marked effect. Also, by comparing the rate of viral spread in the *HFi22*-silenced plants to the *PDS*-silenced control plants, it will show the specificity of silencing *HFi22* versus silencing other genes. Silencing PDS without silencing HFi22 should not have any major impact in TBSV spread if HFi22 is the important factor.

Lastly, should silencing prove to have a clear, demonstrative effect on TBSV movement, the final step is to test the specificity of HFi22 in its impact on given species of virus and their movement. To do this, the experiment would be repeated using HFi22-silenced plants, this time infecting these with TMV-GFP and PVX-GFP, again observing to see if these two viruses experience any change in movement rate.

If HFi22 is involved in defense responses, a different host plant can be used in this silencing experiment. When TBSV is inoculated onto *N. edwardsonii*, an HR-like defense response can be seen (40). By silencing HFi22 in this plant and infecting it with TBSV, any change in HR can be observed. Presumably if HFi22 is vital to this pathway, HR should not be elicited, or at least not as strongly as on an unsilenced plant.

Host Impact on Vector Stability

A wide variety of solenaceous plants are available in laboratories around the world. Based on the results from the integrity study, some may be better virus vector hosts than others. This could have quite an impact on future biotechnology research.

However, not everyone has the luxury of working with a virus with as broad a host range as TBSV. Future work with this project should start with continuing to test more TBSV hosts to give as many host options as possible. In addition, it must be confirmed that these results are not affected by the species of virus vector. Therefore this experiment should be repeated using a different species of virus vector that expresses GFP and elicits local lesions on cowpea. Bean is another acceptable local lesion host should cowpea not work as well with a different virus. In this way, results can be compared between different viruses infecting on the same species to see if their foreign gene retention rate is similar.

Another factor to consider is whether a connection exists between host factor activity (such as HFi22) and vector integrity. The interaction of host factors with viral RNA may affect its ability to replicate stably. Another consideration is the possibility that the virus' ability to move within a plant may be contributing to the instability of the foreign gene insert. Yet another possibility is that the gene replaced by the foreign insert may have had more importance than previously recognized. TBSV does not need its CP to move or replicate (36), but the CP may have some stabilization properties that keeps the polymerase on track to prevent template jumping. This could be tested by infecting the same hosts for which insert stabilization values have been found with the same TBSV-RMJ1 transcripts, then co-infecting with a non-virus vector that expresses TBSV-CP and observing any difference in insert retention. The major risk here would be that TBSV would recombine its CP gene back into its genome, as it has been previously observed that the virus has an affinity for recombination with transgenically expressed CP RNA sequences (5).

Overall, it is clear from these various experiments that host-virus interactions that take place during an infection in plants are of extreme importance but yet are very poorly understood. Host factors and environment shape if and how viruses move and impact their replication and expression. In the field of biotechnology these things must be taken into account to make optimal use of both plants and their viruses.

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