CHARACTERIZATION OF THE LETHAL HOST-PATHOGEN INTERACTION BETWEEN TOBACCO MILD GREEN MOSAIC VIRUS AND TROPICAL SODA APPLE

By

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To my Father, who has always inspired the best in me

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LIST OF ABBREVIATIONS

aa	Amino acid(s)
AAB	Association of Applied Biologists
AjMV	Araujia mosaic virus
ATCC	American type culture collection
Avr	Avirulence gene
BLAST	Basic local alignment search tool
BMV	Brome mosaic virus
BSA	Bovine serum albumen
cDNA	Complementary DNA
CFMMV	Cucumber fruit mottle mosaic virus
CGMMV	Cucumber green mottle mosaic virus
CMI	Commonwealth Mycological Institute
CMMoV	Cactus mild mottle virus
CMV	Cucumber mosaic virus
CP	Coat protein
CREC	Citrus Research and Education Center
C-terminal	Carboxy-terminal
cr-TMV	Crucifer-infecting tobamovirus
CTMV-W	Crucifer-infecting tobamovirus, wasabi strain
DPI	Days post-inoculation
dH_2O	Distilled water
DEPC	Diethylpyrocarbonate

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
DSMZ	“Deutsche Sammlung von Mikroorganismen und Zellkulturen” (German Collection of Microorganisms and Cell Cultures)
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent assay
ER	Extreme resistance; endoplasmic reticulum
GFP	Green fluorescent protein
GFPc3	Cycle 3 green fluorescent protein
HLFPV	Hibiscus latent Fort Pierce virus
HLSV	Hibiscus latent Singapore virus
HR	Hypersensitive-response
IFAS	Institue of Food and Agricultural Sciences
kDa	kiloDalton (equivalent to 1,000 atomic mass units)
KGMMV	Kyuri green mottle mosaic virus
LB	Lysogeny broth (a/k/a Luria-Bertani broth); left border
LBA	Lysogeny broth agar (a/k/a Luria-Bertani agar)
LDS	Laemmli dissociation solution
LL	Local lesions

LRR	Leucine-rich repeat
LSHR	Lethal systemic hypersensitive response
MarMV	Maracuja mosaic virus
MP	Movement protein
mRNA	Messenger RNA
NBS	Nucleotide-binding site
NCBI	National Center for Biotechnology Information
NCR	Non-coding region
nt	Nucleotides
N-terminal	Amino terminal
NTLV	Nigerian tobacco latent virus
NTR	Non-translated region
ObPV	Obuda pepper virus
ORF	Open reading frame
ORMV	Oilseed rape mosaic virus
ORSV	Odontoglossum ringspot virus
PaMMV	Paprika mild mottle virus
PBST	Phosphate-buffered saline + Tween 20
PCD	Programmed cell death
PCR	Polymerase chain reaction
PLRV	Potato leafroll virus
PMMoV	Pepper mild mottle virus
PNP	p-nitrophenylphosphate

PR	Pathogenesis-related
PVP-40	Polyvinyl pyrrolidone
PVX	Potato virus X virus
PVY	Potato virus Y virus
R-gene	Resistance gene
RB	Right border
RMV	Ribgrass mosaic virus
RNA	Ribonucleic acid
RT	Reverse transcription; reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
S	Svedberg units
SDS	Sodium dodecyl sulfate
SEL	Size exclusion limit (plasmodesmata)
SFBV	Streptocarpus flower breaking virus
SHMV	Sunn-hemp mosaic virus
SHR	Systemic hypersensitive response
SLL	Single local lesion
SMV	Soybean mosaic virus
SOC	Super optimal catabolite-repression
SPAR	Single primer amplification reaction
ssRNA	Single-stranded RNA
STMV	Satellite tobacco mosaic virus
Taq	*Thermophilus aquaticus*

TBST	Tris-buffered saline + Tween 20
TEV	Tobacco etch virus
TIR	Toll-interleukin-1 receptor
TMGMV	Tobacco mild green mosaic virus
TMGMV-L	Large-type TMGMV
TMGMV-NZ	Nagel-Zettler isolate of TMGMV; PV-0113
TMGMV-S	Small-type TMGMV
TMV	Tobacco mosaic virus
TMV-Cg	Crucifer-infecting tobamovirus, garlic strain
TMV-Ob	Pepper-infecting tobamovirus, Obuda strain
TMV-p	Tobacco mosaic virus, petunia strain
ToMV	Tomato mosaic virus
ToMoV	Tomato mottle virus
TRV	Tobacco rattle virus
TSA	Tropical soda apple
TSAMV	Tropical soda apple mosaic virus
TSWV	Tomato spotted wilt virus
TVCV	Turnip vein clearing virus
USDA-GRIN	U.S. Department of Agriculture Germplasm Resources Information Network
UTR	Untranslated region
UV	Ultraviolet
WPI	Weeks post-inoculation
WT	Wild-type

wtGFP	Wild-type green fluorescent protein
YEP	Yeast-extract and peptone
ZGMMV	Zucchini green mottle mosaic virus

Abstract of Thesis Presented to the Graduate School
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CHARACTERIZATION OF THE LETHAL HOST-PATHOGEN INTERACTION BETWEEN TOBACCO MILD GREEN MOSAIC VIRUS AND TROPICAL SODA APPLE

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Tropical soda apple (*Solanum viarum* Dunal) develops symptoms of lethal systemic hypersensitive response (LSHR) in response to mechanical inoculation with Tobacco mild green mosaic virus (TMGMV; isolate DSMZ PV-0113). Sub-isolates of PV-0113 exhibited no variation in disease phenotype induced on inoculated tropical soda apple (TSA) and *Nicotiana tabacum* cv. "Samsun" (*nn*). Physical properties, host-range analysis, immunodiffusion, enzyme linked immunosorbent assay (ELISA), reverse-transcriptase polymerase chain reaction (RT-PCR), and sequence analysis of the viral coat protein and movement protein genes indicate that PV-0113 is a small type isolate (TMGMV-S). Immunodiffusion using antiserum against Satellite tobacco mosaic virus (STMV) coat protein and analysis of double-stranded RNA from infected plants indicate that STMV is not present in this isolate. Sequences of the coat protein and movement protein genes of PV-0113 were nearly identical to 2 other sequences reported for TMGMV. A nucleotide sequence motif observed in PV-0113 was identical (100% homology) to a motif reported as unique to, and present in, all TMGMV sequences so far examined.

The pathogen and the disease phenotype it causes in TSA were studied to learn more about lethal plant-virus interactions and to explore the possibility of exploiting this interaction to manage TSA. Symptoms of disease in TSA plants infected with PV-0113 appear approximately

5 days post-inoculation as epinasty and local lesions on the inoculated leaves, followed by loss of turgor and abscission of both inoculated and uninoculated leaves at 6 days, leading to complete defoliation of the plant within 30 days. The stem becomes necrotic over a period 3 to 4 weeks post-inoculation as the entire plant dies. Lethal systemic hypersensitive response was not observed in TSA following inoculation with other common tobamoviruses. Inoculation of TSA with a culture of large type TMGMV, containing STMV produced necrotic symptoms that were initially similar to those produced by inoculation with PV-0113, but the plants survived, "recovering" with mosaic symptoms. TSA plants inoculated with TMV are cross-protected against challenge with TMGMV. TSA plants inoculated with the TMV/TMGMV chimera 30B, which expresses the large type TMGMV coat protein, developed mosaic but not necrosis, suggesting that the coat protein of PV-0113 alone is not an elicitor of hypersensitive response in TSA. These results are consistent with the hypothesis that the lethal plant-pathogen interaction between TSA and PV-0113 is highly specific to this virus-host combination.

CHAPTER 1
LITERATURE REVIEW

Host Biology

Tropical soda apple (*Solanum viarum* Dunal), a prickly member of the nightshade family (Solanaceae), has spread from its natural range in South America (S.E. Brazil, N.E. Argentina, Paraguay and Uruguay), to become a problematic weed in Central and North America, as well as Africa, Asia, and the Caribbean (Mullahey et al., 1997; Cuda et al., 2002). An herbaceous perennial, *S. viarum* is 1-2 m high, with alternate, simple, lobed, pubescent leaves covered with sharp prickles perpendicular to the adaxial and abaxial surfaces. The stems also are covered in prickles. White, tomato-like flowers, borne terminally in clusters, develop into green to greenish-white fruit, camouflaged with watermelon-like patterning. These mature to a solid mustard-yellow color, measure about 2-3 cm in diameter, and contain numerous seeds surrounded by a spongy mesocarp. Containing the steroidal glycoalkaloid solasodine, the fruit are mildly poisonous to humans, although it would require about 200 fruit to be lethal. Solasodine has been used in the production of steroid-based medicines; tropical soda apple was once cultivated for this purpose and a body of related literature exists. Production has declined in recent years as better sources of solasodine have been discovered by the pharmaceutical industry (Cuda et al., 2002; Levin et al., 2005).

Tropical soda apple, known in Brazil as "juá bravo" ("angry apple") (Lorenzi, 2000), and hereinafter referred to as *S. viarum* or TSA, is a member of the family Solanaceae; section Acanthophora; subgenus Leptostemonum (Levin et al., 2005). Synonyms for this plant appearing in literature include *Solanum khasianum* var. *chatterjeeanum* Sengupta, *S. chloranthum* DC, and *S. viridiflorum* Schlechtendal (USDA ARS, 2003). *Solanaceae*, the nightshade family, is a diverse, successful family belonging to the Solanales, an order it shares

with the morningglory family, *Convolvulaceae*. Today *Solanaceae* exist on all continents except Antarctica, and in almost every habitat: tropical and temperate, from rainforest to desert (Anonymous, 2002). The family contains many species of great practical and aesthetic benefit to humankind, including the tomato, potato, pepper, and petunia. It also contains many species that are weeds, including the nightshades, horsenettles, and TSA. Some, such as tobacco (*Nicotiana* sp.) and angel's trumpet (*Datura* sp.) might be placed in both categories.

Samples of TSA collected in the United States and subjected to single primer amplification reactions (SPAR) and chloroplast DNA sequencing showed no variation, suggesting that the current infestation is the result of a single introduction, or multiple introductions from a common source (Kreiser et al., 2004). Recent phylogenetic analyses using sequence data from two nuclear and two chloroplast regions place *S. viarum* in a clade along with *S. aculeatissimum*, *S. incarceratum*, and *S. myriacanthum* (Levin et al., 2005, 2006). The close taxonomic relationship between *S. viarum* and *S. aculeatissimum* is also suggested by the original confusion of both species as *S. khasianum*, with TSA later recognized as *S. khasianum* var. *chatterjeeanum* Sengupta. Morphologically, TSA may be distinguished from these relatives by the presence of hairs on the flower ovary (Dr. Lynn Bohs, professor, Department of Biology, University of Utah, Salt Lake City, personal communication).

Tropical soda apple has become established as a noxious weed of pastureland in the United States, especially in the state of Florida. First collected from Glades Co., FL in 1988, (Coile, 1993), TSA rapidly expanded its range from 10,000 hectares in 1990 to infest over half a million in 1995 (Mullahey et al., 1998). While infestation by TSA remains greatest in the state of Florida, it also occurs in Alabama, Arkansas, Georgia, Illinois, Louisiana, Mississippi, North Carolina, Pennsylvania, Puerto Rico, South Carolina, Tennessee, and Texas. Plants reproduce

either by seed or asexually from the roots. In the Northern Hemisphere, TSA blooms from May to September (Ferrell, 2006). Plants growing in Florida may produce 40,000 to 50,000 seeds per individual, about 20% of which remain dormant, usually for one month (Mullahey et al., 1997; Medal et al., 2004). Favorable conditions allow plants to reach maturity in 130 days (Ferrell, 2006). Long prickles on the plant discourage grazing, but the ripe fruits are consumed by cattle, pigs, and wildlife. Animals indiscriminate enough to eat the leaves may find themselves poisoned (Porter et al., 2003). The seeds survive digestion and are disseminated in the animals' droppings (McGovern et al., 1994), causing dense stands of TSA (Figure 1-1, Figure 1-2) to form where cattle congregate (under shade trees, palm hammocks, feeding stations, etc.) that eventually deny access to the cattle. Plants and seed can also be disbursed by movement of contaminated hay and grass seed, generating plants in more urban areas such as lawns, roadsides, etc. (Mullahey et al., 1998). Like many invasives, TSA takes advantage of open niches in disturbed environments, and has been observed spreading into areas disturbed by phosphate mining (Albin, 1994).

Tropical soda apple is causing economic damage, mostly through the loss of grazing land and reduced stocking rates, as well as the expense of control and certification programs. By 1993, TSA infestation had cost the cattle industry in Florida 11 million dollars, reducing Florida beef production by about 1% annually (Mullahey et al., 1998). TSA is on both the Federal and Florida noxious weed lists, and is listed as a Category I invasive species by the Florida Exotic Pest Plant Council, defined as "invasive exotics that are altering native plant communities by displacing native species, changing community structures or ecological functions, or hybridizing with natives" (FLEPPC, 2005). Some states are discussing shipment regulations that would mandate cattle be quarantined until all viable TSA seed has passed through their digestive

system; to quarantine these cattle on a feedlot for two weeks would be costly and result in the cattle suffering increased stress-related illness and weight loss, (Ed Jennings, extension agent, Sumter Co., FL, personal communication). Furthermore, TSA exerts ecological pressure by displacing native species where it occurs in the hammocks and natural areas of Florida. Approximately 10% of the land infested with TSA is wooded with oak/palm hammocks and cypress stands (Cuda et. al., 2002).

Field studies conducted in Florida and Brazil have found at least 45 plant pathogens attacking TSA (Charudattan and DeValerio, 1996). Of these, several viruses of agricultural importance, including Cucumber mosaic virus (CMV), Potato leafroll virus (PLRV), Potato virus Y (PVY), Tobacco etch virus (TEV), Tomato mottle virus (ToMoV), and Tomato mosaic virus (ToMV) have been recovered (McGovern et al., 1994). Additionally, TSA has been infected artificially with Potato virus X (PVX) and Tomato spotted wilt virus (TSWV) (Chagas et al., 1978). TSA supports several crop-damaging insects of economic importance, including tobacco hornworm, tomato hornworm, Colorado potato beetle, tobacco budworm, tomato pinworm, green peach aphid, silverleaf whitefly, soybean looper, and the southern green stinkbug. As a host of these pests and pathogens, TSA may be causing economic damage to Florida vegetable growers (Cuda et al., 2002).

Conventional methods of control for TSA require repeated mowing and applications of a chemical herbicide. Acifluorfen, clopyralid, dicamba, fluroxypyr, picloram, and triclopyr each provided >90% control of TSA seedlings with minimal damage (>10%) to the non-target plant, bahiagrass, observed 145 days after treatment (Akanda et al., 1997). Infested pastures should be mowed at least every 60 days to prevent flowering/fruit set. Remedy (triclopyr) has been the most commonly used treatment for TSA, generally applied at a rate of 1.1 kg/ha, or Roundup

(glyphosate) applied as a 3% solution. None of the conventional herbicide treatments have proven completely satisfactory for control of TSA. Glyphosate causes damage to forage grasses, and triclopyr has little residual activity for controlling TSA seedlings emerging post-application. The cost of applying dicamba at rates necessary for control of TSA is prohibitive (Ferrell et al., 2006). Control is complicated by the need to spot-treat under trees and on hammocks, and must be repeated as new seeds germinate. Complete control by these methods will take 1-3 years, and cost approximately $185 per hectare (Mislevy et al., 1996, 1997; Mullahey et al., 1998; Sturgis and Colvin, 1996).

Aminopyralid, (4-amino-3,6-dichloropyridine-2-carboxylic acid), a chemical developed by Dow Agrosciences, has been shown to provide >96% control of TSA, including emergent TSA, for up to 335 days after application, when applied at the rate of 80 g per hectare. Based on this efficacy and the long residual effect in the soil, aminopyralid has been registered for TSA control (Ferrell et al., 2006; Anonymous, 2007). Recent experimentation has shown that aminopyralid, (marketed as "Milestone" and, in combination with 2,4–D, as "ForeFront R&P"), although well-translocated in most plants, is *not* well translocated within TSA and requires complete and thorough coverage at a rate ≥0.11% to be effective (Ferrell and Sellers, 2007). In contrast, inoculation of a single leaf with a few micrograms of SolviNix, a virus-based biological control agent described in this thesis, is often highly effective.

A tortoise beetle, *Gratiana boliviana* (Chrysomelidae), found associated with TSA in South America, showed promise in controlling the weed by feeding on the leaves, and, after host-specificity studies were certified by USDA-APHIS, was released in Florida for control of TSA in May, 2003 (Cuda et al., 2002; Medal, et al., 2004). However, the field performance of

this beetle has been less than satisfactory (R. Charudattan, professor, Department of Plant Pathology, University of Florida, personal communication).

Pathogen Biology

Few virus-based approaches to biological control of weeds have been proposed before. The non-tobamovirus, Araujia mosaic virus (AjMV), discovered producing disease in the South American *Araujia angustifolia*, was proposed for control of milkweed vine (= strangler vine), *Morrenia odorata* in Florida (Charudattan et al., 1976, 1978), and more recently, for moth plant, *Araujia sericifera*, in New Zealand (Landcare Research, 2003). The mite- and graft-transmissible rose rosette disease has been proposed as a biological control for multiflora rose (*Rosa multiflora*), upon which it causes leaf reddening, shortened petioles, and death (Epstein et al., 1997.) Although a causal agent has yet to be identified, virus-like particles and double-membrane bound inclusion bodies have been observed in tissue symptomatic with rose rosette disease. (Silvestro and Chapman, 2004; Rohozinski et al., 2001). A tobamovirus, "TMV Alke strain," isolated from *Physalis alkekengi*, caused disease when applied to *Solanum carolinense*, a North American species which had been infesting tea plantations in the former Soviet Republic of Georgia. Systemic symptoms produced by the virus were severe, and included mosaic, epinasty, leaf abscission, and "enhanced drought stress" (reported to the West by Izhevsky, 1979). Tobacco mild green mosaic virus (TMGMV) was proposed as a bioherbicide for *Echium plantagineum* in Australia, because systemic infection of *E. plantagineum* by TMGMV was found to cause systemic yellowing and mosaic, increased leaf senescence, and reduced leaf and seed production (Randles, 1986).

Based on this research, a graduate student, M. Pettersen, (Plant Pathology Department, University of Florida) conducted an evaluation of Tobacco mosaic virus (TMV), TMGMV, and ToMV as biocontrol agents against TSA. When mechanically inoculated, all three viruses were

able to systemically infect TSA (unpublished data). TMGMV induced a necrotic response followed by wilting and leading to the eventual death of the plants (Pettersen et al., 2001). TMV and ToMV produced mosaic and mosaic plus mottling symptoms, but did not cause plant death. Subsequently, I tested Pepper mild mottle virus (PMMoV), Sunn-hemp mosaic virus (SHMV), and Tropical soda apple mosaic virus (TSAMV), but none produced systemic, lethal symptoms on TSA.

Many collections of TMGMV have been made by scientists around the world (Table 1-1). In a study sampling gesneriad plants for a latent virus infection (Zettler and Nagel, 1983), an isolate identified as TMGMV was recovered from tissues of the *Columnea* hybrid 'Oneidan' (*C. crassifolia* x *C. allenii*) originating from Ohio (Dr. F.W. Zettler, University of Florida, personal communication). This isolate was propagated in tobacco and deposited as accession PV-0113 at the German Collection of Microorganisms and Cell Cultures (DSMV). It was this culture, reliably lethal to TSA, and casually referred to as the "Nagel-Zettler" or "NZ" strain of TMGMV, that was used in the research described in this thesis.

Tobacco mild green mosaic virus belongs to the genus, *Tobamovirus*. This group includes TMV, the first discovered, and probably most studied, virus in history. It was the first virus to be purified and crystallized (Stanley, 1935), as well as the first virus to be observed by electron microscopy (reviewed by Hull, 2002). TMV was the first RNA virus to have its genome completely sequenced (Goelet et al., 1982).

Tobamoviruses are mechanically transmitted and are not known to be vector-transmitted. Some tobamoviruses may be seed-transmitted, due to autoinoculation when the seedling germinates through the contaminated seed coat (Rast, 1979). Tobamovirus-infected plant debris may allow the virus to survive from planting to planting, but probably does not contribute to

spread (Boubourakas et al., 2004). Nevertheless, research indicates that tobamoviruses are successful and highly ubiquitous. Reports exist, for example, of recovery of TMV and ToMV from forest soils in New York State (Fillhart et al., 1998). Control of these viruses typically relies on proper sanitation and the use of resistant varieties (Agrios, 1997; Letschert et al., 2002).

In addition to PMMoV, TMV, TMGMV, and ToMV, the genus *Tobamovirus* also includes the economically important species Odontoglossum ringspot virus (ORSV) and Cucumber green mottle mosaic virus (CGMMV).

ORSV, PMMoV, TMGMV, TMV, and ToMV are known to occur or have occurred in Florida (Alfieri et al., 1994; Baker and Zettler, 1988; Kucharek et al., 2003; Lamb et al., 2001). Other tobamoviruses detected in Florida include Hibiscus latent Fort Pierce virus (HLFPV) (Kamenova and Adkins, 2004), Maracuja mosaic virus (MarMV) (Alfieri et al., 1994; Song et al., 2006), TSAMV (Adkins et al., 2007.), and an unspecified tobamovirus producing ringspot in *Opuntia* sp. cactus (El-Gholl et al., 1997).

In addition to the subdivision of TMV, new species are continually discovered: Nigerian tobacco latent virus (NTLV), recovered from a mixed infection in Nigerian tobacco in 2003 appears to be somewhat distantly related to more common tobamoviruses. Of these, it most closely resembles TMGMV (Lapido et al., 2003). Recently, two species discovered in Malvaceous hosts: Hibiscus latent Singapore virus (HLSV) (Allen et al., 2005) and HLFPV have been described (Kamenova and Adkins, 2004). Cactus mild mottle virus (CMMoV) (Min et al., 2006) and Streptocarpus flower breaking virus (SFBV) (Heinze et al., 2006) are two more species recently described from infected ornamentals.

Tobamoviruses are positive-sense, single stranded RNA viruses. Virions are hollow tubes, roughly 18 nm in diameter x 300 nm long, composed of approximately 95% protein, and 5%

RNA. Each virion consists of multiple (~2,130), slipper-shaped coat protein (CP) subunits, wound into a right-handed helix around a single, 6.5 Kb-long RNA strand, somewhat like kernels of corn on a cob. Each CP subunit is composed of about 158 amino acids (TMV); about 50% are arranged into α-helixes and 10% into β-structures (Hull, 2002). Every three-nucleotide segment of the RNA strand fits into a groove in each protein subunit (Lewandowski and Dawson, 1999), lending it great resistance to chemical and physical degradation (compared to several other plant viruses). Virions of TMV, for example, remained viable after at least 50 years in storage at room temperature, and are unaffected by RNA-ase, although the naked RNA is unstable and will degrade (Hull, 2002). Association between the CP and the RNA is so strong that they will self-assemble into active viral particles in vitro, with aggregations of CP forming first, that then encapsidate the RNA strand at a specific stem loop, the "origin of assembly." In most tobamoviruses, the origin of assembly is located within the third open reading frame (ORF); when the CP assembles around subgenomic mRNA, the result is the formation of shorter virions. The virions will, however, conveniently disassemble at the extremes of pH (Hull, 2002).

The location of the origin of assembly within the genome may be used to classify tobamoviruses into three subgroups (Fukuda et al., 1981). TMGMV is classified in Fukuda subgroup 1, along with TMV, ToMV, PMMoV, and ORSV. Subgroup 1 is composed of those species in which the origin of assembly is located on ORF 3, and are, with the exception of ORSV, adapted to the *Solanaceae*. In subgroup 2 tobamoviruses, such as CGMMV and SHMV, in which the origin of assembly lies within ORF 4, most commonly infect species of *Cucurbitaceae* or *Fabaceae*. Subgroup 3 is made up of tobamoviruses that primarily infect the *Brassicaceae*, such as Turnip vein clearing virus (TVCV) (Letschert et al., 2002).

The replication strategy and genome organization of TMGMV (Figure 1-3) and other tobamoviruses are comparable to those of the well-studied type species, TMV. The genome of TMV begins with a 5′ 7-methylguanosine cap (an inverted, methylated guanosine subunit). For mRNA to survive enzymatic degradation in a eukaryotic cell, it must carry this cap. Downstream from the cap, a 70 nucleotide (nt) leader, known as the omega (Ω) sequence, competes with host mRNA for ribosomes to greatly enhance translation in both prokaryotic and eukaryotic systems. Since its discovery, this property has been utilized extensively, making it a useful tool in the hands of molecular biologists. The Ω sequence, positioned before the first ORF, is required for tobamovirus replication (Gallie et al., 1987).

ORF 1 codes for a 126 kiloDalton (kDa) protein, with an amber stop codon which allows read-through 5-10% of the time, to create a longer 183 kDa product (Lewandowski and Dawson, 1999; Hori and Watanabe, 2003). A domain closest to the amino-terminal (N-terminal) of these proteins is believed to serve as a methyltransferase, used in forming the 7-methylguanosine cap. A domain near the carboxy-terminal (C-terminal) of the 126 kDa protein is believed to serve as the helicase, unwinding the dsRNA that forms during replication. Further downstream, within the 183 kDa protein is a domain believed to serve as the polymerase for replication. The next ORF encodes an approximately 28 kDa movement protein (MP). Downstream from the MP, the next ORF encodes a 17.5 kDa CP. Downstream from these translated regions, the genome contains more replication machinery, and the RNA coils to form structures called “pseudoknots.” Finally, the 3′-end of the RNA sequence ends in a t-RNA like structure, which itself contains 2 pseudoknots (Lewandowski et al., 1999; Yoon et al., 2006).

Unlike the 126 kDa and 183 kDa replication-associated proteins, the CP and MP are not translated directly, but from subgenomic RNAs. Subgenomic I_1, which begins with a 5' start at

nt 3405, has been isolated *in plantae*. *In vitro*, I_1 is capable of translating a 54 kDa protein, identical in sequence to the C-terminal end of the 183 kDa transcript (Hull, 2002). Subgenomic I_2, serving as the template for translation of MP, spans both ORF 3 and ORF 4. The smallest subgenome produces the CP (Hull, 2002). It is possible that the interaction of subgenomic promoters with viral replicase is responsible for the timing of individual events in the infection process (Dawson, 1992).

Translation of ORF 3 produces the ~28 kDa product in relatively small amounts, compared with the later accumulation of CP. The N-terminus of this MP binds single stranded nucleic acid, while the C-terminus binds to plasmodesmata (Lewandowski et al., 1999). Tobamovirus MP has been shown to associate with viral nucleic acid, and *in plantae* this protein is indeed localized in the plasmodesmata, where it will increase the plasmodesmatal size exclusion limit (SEL) about 10-fold (Heinlein, 2002). Tobamovirus MP is required for efficient cell-to-cell movement through the plant. Differences in host range between closely related tobamoviruses may well be determined by mutations in this region. In the host *Eryngium planum*, for example, TMGMV moves systemically, while TMV does not move out of the inoculated leaf. This feature enables *E. planum* to be used as a "biological sieve" to separate TMGMV from TMV in mixed infections (Johnson, 1947).

The 17.5 kDa CP encoded by ORF 4 is translated, generating subunits that encapsidate the viral mRNA, allowing long-distance movement through the vasculature of the plant. While CP itself is not necessary to establish or maintain infection, mutation or deletion of ORF 4 results in genotypes less effective at movement than the wild-type (Dawson et al., 1988).

Other ORFs observable within the tobamovirus genome code mostly for small proteins of undetermined activity. Morozov described a reading frame (called "ORF-X" or "ORF 6")

beginning at 5669 (5617 in TMGMV) found in some, but not all tobamoviruses, which encodes for a small protein of 33 amino acids (45 amino acids in TMGMV) in length that can be translated *in vitro*. During *in vitro* translation, a larger protein of about 4.8 kDa is also produced (Morozov et al., 1993). The translation product of ORF 6 has been shown to have effects *in plantae*, although its adaptive purpose, if any, remains unknown (Canto et al., 2004).

The approximately 200-nt long 3′ non-translated region (3′ NTR), sometimes called the "untranslated region" (3′ UTR), or the "non-coding region" (3′ NCR), consists of three stem-loops in the RNA known as "pseudoknots" and a tRNA-like 3′ end terminal. Together, these structures help to stabilize the RNA and enhance translation. Proximity to the pseudoknot-containing region of the 3′ NTR affects expression of genomic products, with genes (or transgenes) closest to the 3′ NTR showing higher levels of expression (Shivprasad et al., 1999). The pseudoknot closest to the 3′ end has been shown to be the starting point for synthesis of (−)RNA in TMV, and replication and accumulation of virus was reduced in certain pseudoknot deletion mutants of PMMoV. Both the amino acid (aa) sequence of the replicase protein and the 3-dimensional structure of the pseudoknots seem critical to translation and synthesis of (−)RNA (Yoon et al., 2006).

Wild populations of *Nicotiana glauca*, in southern California harbor a "large-type" variant of TMGMV (historically classified as "TMV-U5"), distinguishable from "small-type" TMGMV ("TMV-U2," TMGMV-S) by a larger 3′ NTR. Large-type TMGMV (TMGMV-L) may be identified by RT-PCR using primers specific to the 3′ end of the virus, or by careful observation of symptom development on the indicator plants: *N. benthamiana*, *N. clevelandii*, and *N. rustica*. TMGMV-L has a 3′ NTR extended by a duplication resulting in six pseudoknots instead of three;

this difference appears to have consequences on disease phenotype and movement in some hosts (Bodaghi et al., 2000).

While the ranges of these two TMGMV subtypes overlap in southern California, and both infect *N. glauca*, mixed infections in this host remain rare (1.6% incidence; Bodaghi et al., 2004). TMGMV-L seems to have a competitive advantage over TMGMV-S, at least in the hosts *N. glauca* and *N. tabacum*; whereas pre-inoculation by either subtype usually provides good cross-protection against the other, once established (5-14 days), TMGMV-L usually displaces TMGMV-S from a host co-inoculated with it (Bodaghi et al., 2004). Why duplication of the 3-pseudoknot segment in the 3' NTR should confer this advantage is not currently understood.

Tobacco mild green mosaic virus is unique in that it is the only tobamovirus found naturally associated with Satellite tobacco mosaic virus (STMV). Satellite plant viruses are very small and completely dependent upon their associated "helper" virus for replication. STMV appears to have a parasitic relationship with TMGMV (the "helper"), reducing accumulation of the helper virus within the host. Discovered in association with TMGMV in wild populations of *N. glauca*, STMV is characterized by 17 nm icosahedral particles containing a 1,059 nt ssRNA genome with two ORFs. ORF 1 codes for a 6.8 kDa protein of unknown function. ORF 2 codes for a CP, which, although about the same size as TMGMV CP (17.5 kDa), is serologically unrelated (reviewed by Hull, 2002). Detected only in samples of TMGMV-infected *N. glauca* from California, STMV has been experimentally propagated with TMV, ToMV, and ORSV, which will serve as helper viruses. No artificially recruited helper virus, however, was nearly as effective as TMGMV in terms of the amount of STMV particles yielded. In contrast, co-infection with STMV significantly reduced the yield of helper virions recovered from inoculated plants. However STMV did not alter the timing or severity of symptom development. Single

local lesion (SLL) isolation may be insufficient to separate this satellite virus from its helper – detectable quantities of STMV reappeared in 3 out of 10 experimental isolates (Valverde et al., 1991).

Because of the close relationship and great similarity between tobamoviruses, the well-studied replication cycle of TMV probably also serves as a good model for that of TMGMV. Infection begins when plant tissue is mechanically inoculated by contact between plants or some other contaminated animate or inanimate object. Although a lone viral particle is theoretically capable of establishing infection, statistics indicate that different regions of a host vary in their susceptibility to infection such that different quantities of virus are required (Roberts, 1964). Even the most susceptible hosts seem to require 50,000 or more virions to initiate a local lesion (LL) (Bawden, 1956). Upon gaining entrance to a cell, virus replication begins with *cotranslational disassembly* as the 5′- end of the virus becomes uncoated within 2-3 minutes, exposing the first ORF for translation (Heinlein, 2002). Once the 126 and 183 kDa proteins are being produced, disassembly proceeds to completion, and a (–) RNA template is synthesized. From these (–) RNA templates, replication of (+) RNA begins, producing both full-length, and shorter, ORF 3 / ORF 4 sub-genomic RNAs (Heinlein, 2002).

Replication in TMV is associated with inclusion bodies *in plantae*; "X-bodies," characteristic of TMV infection, are localized to endoplasmic reticulum (ER) of the host, perhaps to coordinate the process, or to provide some protection from host defenses. X-bodies have been shown to be composed of the aggregated 126 kDa protein (Lapido et al., 2003). Replication of negative strands ceases relatively early, at least, *in vitro*, with translation of MP and CP continuing for some hours afterwards. MP alters the size exclusion limit (SEL) of the host's plasmodesmata, but the resulting slow (μm/h) movement from cell to cell is believed to take

place using a ribonucleoprotein complex rather than by complete virions (Heinlein, 2002). Virion assembly allows movement as fast as 8 cm/h through the phloem (Dawson, 1992; Palukaitus and Zaitlin, 1986).

The mutation rate for RNA viruses is believed to be much higher than in other organisms. Mechanisms that may serve to generate change in such a virus include replication error and recombination (Schneider and Roosinck, 2000). A natural TMV/TMGMV recombinant, "H7," was isolated from herbarium specimens preserved in New South Wales, Australia. Recombination appears to have occurred between nt 1250 and nt 3461 in the genome (Fraile et al., 1997). Studying an 804 nt region of ORF 3 in TMV, Malpica observed that while there was a high rate of mutation, most mutations occurred at nucleotide-binding sites (NBS) and were lethal to the virus progeny (Malpica et al., 2002). When replicating in the permissive host, *N. benthamiana*, TMV was found to have a mutation frequency of 4.3×10^{-4}, which is intermediate between that of Cucumber mosaic virus CMV and Cowpea chlorotic mottle virus (CCMV). The percentage of mutants did not increase or decrease significantly following passage from host to host. None of the mutations studied survived 10 rounds of serial passage, suggesting that they contributed negatively to fitness, and it seems that in the absence of severe bottlenecks, the mutant population reaches a stable level of diversity that remains constant with passage from plant to plant (Schneider and Roosinck, 2000).

Kearney (1999) obtained a mutation rate of 3.1×10^{-4} nt substitutions/base-year while passaging TMV through a variety of plant hosts. Experimental plants from six families with a variety of responses were inoculated with TMV. Population shifts in TMV had been reported following physiological changes in the host, or following transition from one host to another. Kearny (1999), however, found relatively little host-shift, and that mutations that did occur were

predominantly silent (85%), indicating a strong tendency towards conservation of the aa sequence. Of the genomic regions studied, the conserved region of the CP gene, the 3′ region of the MP, and the replicase, the greatest number of nt substitutions was found in the CP sequence. Fewer mutations were found in the replicase domain, and the least number in the MP gene. In another experiment, no changes in a scrutinized region representing 12% of the TMGMV genome were observed after a field population was serially passaged 23 times through *N. tabacum* cv. Samsun. Given the high error rate of viral RNA polymerases, it is surprising that more mutations did not occur. The progeny's need to escape the inoculated leaf is a possible mechanism restricting the spread and proliferation of more divergent tobamovirus genomes within an infected plant (Kearney et al., 1999). This need to conserve the properties of functional viral proteins may reflect a limit of change where back mutations and parallel mutations in the CP of a tobamoviruses are as common as divergent ones (Hull, 2002).

Competition between TMV and TMGMV in *N. glauca* observed in New South Wales, Australia, has been cited as support for a theoretical evolutionary process called "Muller's ratchet" (Muller, 1932), whereby repeated bottlenecks cause asexually reproducing populations to decline until the minimum diversity of fit genotypes required to maintain the population is lost (Fraile et al. 1997). In the case of a tobamovirus, a certain minimum diversity of viral genomes would need to be transferred with each inoculation event to ensure at least one highly competitive genotype gets through to establish a strong beachhead: one well adapted to conditions in the new host environment. As Fraile et al. have argued, TMV appears to have arrived in Australia first. As TMGMV reduced the proportion of TMV in co-infected plants, fewer and fewer TMV virions (and therefore fewer total genotypes), were available to infect the next host. Eventually, passing through a series of bottlenecks and co-infected hosts, the fittest

genotypes were lost, and only less fit genotypes remained in the declining population. The observed number of mutations found in the TMV samples increased, while the population genetics of TMGMV remained unchanged. The end result of this process was the extinction of TMV-type strain in New South Wales *N. glauca* by about 1950 (Fraile et al., 1997; Gibbs, 1999).

Tobacco mild green mosaic virus occurs worldwide, wherever *N. glauca* exists and where *N. tabacum* and the other host species grow. TMGMV can be expected to occur where such plants are cultivated, especially where pruning or vegetative propagation is involved. TMGMV has been found in several members of the Gesneriaceae (Zettler and Nagel, 1983), for example, and the ornamentals *Rhoeo spatheace* (Baker and Zettler, 1988), and *Tabernaemontana divaricata* (Cohen et al., 2001). Tobamoviruses may be recovered from cigarettes and other smoking materials; the practice of smoking has probably contributed to their current widespread distribution (Agrios, 1997). Both TMV and ToMV cause problems for growers of tobacco, peppers, and tomatoes in Florida (Kucharek et al., 2003). Extensive experimentation with TMGMV, however, has shown that it is incapable of infecting tomato *in plantae*, or *in vitro* (protoplasts) (Morishima et al., 2003). In Florida, cultivation of the TMGMV host *N. tabacum* is of historical importance. As of 2004, tobacco was cultivated commercially in Alachua, Baker, Columbia, Gilchrist, Hamilton, Jefferson, Levy, Madison, Sumter, Suwannee, and Union counties, in Florida (Mr. William Brown, Director of the Alachua Co. Extension Service, personal correspondence). Despite the historical significance of tobacco in the state, Florida ranks as one of the smallest producers of tobacco, and tobacco production in the state has been declining rapidly. In 1996, 7,500 acres of tobacco were harvested in Florida, with a cash value of $36.3 million dollars. In 2006, only 1,100 acres were harvested, bringing in $4.3 million dollars (USDA-NASS, 2007.) The accelerated decline in production is largely the result of the

Fair and Equitable Tobacco Reform Act of 2004 (H.R. 4520-105 Title VI – Fair and Equitable Tobacco Reform), which ended the quota and price supports for tobacco and created the $10 billion Tobacco Transition Payment Program, also known as the "Tobacco Buyout" (Anonymous, 2007).

Worldwide, TMGMV shows little overall genetic variation, although differences occur in specific gene sequences between geographically isolated populations. The greater diversity of genotypes found within a geographically isolated population of TMGMV compared with populations worldwide led Fraile (1996) to conclude that there may be an upper limit for TMGMV diversity, which may have been reached in some populations (Fraile et al., 1996, 1997). Examination of century-old herbarium specimens from NSW, Australia has revealed that genetic diversity in TMGMV has not increased over that time (Fraile et al. 1997; Gibbs, 1999).

Early researchers did not distinguish between species of *Tobamovirus*, classifying them, with the best information then available, as strains of TMV. Many synonyms for TMGMV exist, including: "Mild dark-green tobacco mosaic virus," "para-tobacco mosaic virus," "mild strain of tobacco mosaic virus," "South Carolina mild mottling strain of tobacco mosaic virus," "strains U2 and U5 of tobacco mosaic virus" (Siegel and Wildman, 1954), and "green-tomato atypical mosaic virus" (Wetter, 1989). The misleading "green-tomato atypical mosaic virus" refers to the observation that TMGMV (then considered to be a strain of TMV) would *not* infect tomatoes, an *atypical* characteristic when compared to TMV or ToMV. Subsequent experimentation has confirmed that TMGMV is incapable of infecting *Lycopersicon esculentum*, either *in plantae*, or protoplasts (Morishima et al., 2003; my observations).

Wetter (1984) compared various isolates of what was then considered to be TMV from around the world using serological techniques and host-range tests and was able to distinguish

them from TMV-type strain and group them as the species TMGMV. Furthermore, Wetter was able to prove that TMGMV was a species in its own right, and not merely a mutation caused by repeated passage of TMV through various hosts (Wetter, 1984). A good immunogen, TMGMV can be distinguished from other tobamovirus species by immunodiffusion. The 308 nm x 18 nm rod-shaped particles have a sedimentation coefficient of 186 S and an isoelectric point of pH 4.17. TMGMV virions are thermally inactivated in 10 minutes at 85°C. Virus preparations may be evaluated using spectrophotometry: the extinction coefficient for TMGMV at A_{260} = 3.16; a nearly pure preparation should have a ratio: A_{260}/A_{280} = 1.22 (Wetter et al., 1989).

The genome of TMGMV-S is 6355 nt long, as opposed to TMV, which is 6395 nt long. The four essential TMGMV genes range from 480 nt to 3336 nt in size (Table 1-2). Genomic ssRNA is encapsidated in ~2130 subunits, (TMGMV-S) (Wetter, 2004). The specific composition of TMGMV RNA has been determined to be: A=30.9%, C=17.2%, G= 23.8%, U = 28.1% (Knight et al., 1962).

The mass of a single tobamovirus virion may be determined by adding the mass of the RNA genome (5%) to the mass of the sum of the CP subunits (95%).

For TMGMV-S:

- mw ssRNA = (329.2 An + 305.2 Cn + 345.2 Gn + 306.2 Un + 159), where n represents the ratio of each nucleotide to the total, and 159 is the mass of the 5' triphosphate group;
- mw genome = (6355 x 0.309 x 329.2) + (6355 x 0.172 x 305.2) + (6355 x 0.238 x 345.2) + (6355 x 0.281 x 306.2) + 159 = 2.05×10^6;
- mw CP subunits = 17.5 kDa x n Subunits = 17.5×10^3 (2130) = 3.72×10^7 amu;
- mw TMGMV virion = $2.05 \times 10^6 + 3.72 \times 10^7$ amu = 3.93×10^7 amu;
- mass of TMGMV virion in g = 3.93×10^7 amu x $1.66053873 \times 10^{-24}$ amu/g;
- mass of TMGMV virion = 6.53×10^{-17} g.

Using this information, the number of TMGMV-S virions in a given volume of solution can be determined. For example, 5.0×10^{-7} g / 6.53×10^{-17} g = 7.66×10^{9} TMGMV virions would be expected per milliliter of a 0.5 μg/mL purified virus solution. More recently, the technique of electrospray ionization (ESI) mass spectrometry has been used to determine that the mass of preparations of intact TMV virions is between 39×10^{6} and 42×10^{6} Da, which is consistent with the calculated mass of 40.5×10^{6} Da (Fuerstenau et al., 2001).

Host-Pathogen Interaction

While systemic infections by plant viruses are a very common; albeit largely inconspicuous phenomenon, lethal infections appear to be uncommon, and have rarely been reported. Research by Pettersen et al. (2000) established that TMGMV elicits a lethal host response from TSA. Visual observations of the disease process, particularly the development of necrotic local lesions early on, suggested a hypersensitive-response (HR) type mechanism may be involved (Pettersen et al., 2000, 2001).

Lacking the immune system that animals possess, plants are nevertheless capable of defending against invading organisms by a variety of means, both physical and biochemical. One common defense mechanism is the hypersensitive response (HR). Usually localized, HR is a form of programmed cell death (PCD) and occurs in response to specific pathogen-associated molecular patterns (PAMPs). This response is contingent upon recognition of a pathogen-associated molecule, coded for by an avirulence (Avr) gene, by the product of a corresponding host resistance (R) gene. HR is associated with other biochemical plant defenses including the rapid production of reactive oxidative antimicrobial compounds, upregulation of pathogenesis-related (PR) protein genes, and reinforcement of the cell walls (Agrios, 1997). Transcription of PR genes, in particular, is a useful molecular indicator that HR is taking place (Hajimorad et al.,

2005). Other chemical processes associated with PCD include activation of certain proteases, Ca^{++} flux, and membrane exposure of phosphatidylserine (Xu and Roossinck, 2000).

The exact way in which some plant viruses overcome HR and spread systemically is not known. Despite the increasing facility in finding and identifying viral sequences involved in virus-host interactions, the exact mechanisms and processes by which these sequences exert their influence are more difficult to observe and understand. Virus-host interactions may be more complex than simple gene-for-gene interaction. For example, it is known that several variables may allow a virus otherwise confined by HR to spread systemically, including the age of the tissue infected, alteration of the Avr or R gene or genes, inhibition of salicylic acid, inoculation by grafting, and/or extremes of temperature (Culver et al., 1991; Hajimorad et al., 2005).

For convenience, plant hypersensitive responses may be categorized as localized HR, non-lethal systemic HR (SHR), and lethal systemic HR (LSHR). SHR and LSHR can further be qualitatively described in some instances as "trailing HR," a disease process whereby the necrotic areas of the localized HR continue to expand and spread throughout the host tissue progressively from the point of inoculation (Hajimorad et al., 2005). The "trailing HR" phenotype may be seen when certain cultivars of *N. tabacum* are inoculated with TMGMV.

Several hypotheses related to the HR or HR-like disease phenotype observed in the TMGMV-TSA interaction have been proposed. Dr. William Dawson (Eminent scholar, Plant Pathology Department, University of Florida, personal communication) likens the disease process to a "horse race," in which the virus escapes confinement within the LL by rapid replication and/or movement. The progressive necrosis is a HR following the systemic spread of the virus. This hypothesis fits well with the trailing HR observed in certain tobacco species and

cultivars, such as TMGMV on *N. benthamiana*, and may also explain the effects seen on certain cultivars of *C. annuum* following inoculation with TMGMV (Culver et al., 1991).

Culver has proposed a "weak elicitor" hypothesis: that systemic necrosis is at least sometimes the result of a weak interaction between the viral elicitor and a host detection mechanism (Culver and Dawson, 1991; Culver et al., 1991). Mutagenesis of TMV produced several CP mutants capable of producing LL on *N. sylvestris*. The mutants provoking a rapid HR generated small LL successful in confining the virus. Mutants slow to evoke a HR would often escape from the initial lesion to spread systemically, causing necrosis throughout the host. Further experiments with transgenic tobacco plants designed to express CP from these mutants revealed that low concentrations of CP from fast-acting mutants ("strong elicitors") produce a more severe necrotic response than high concentrations of CP from slowly-acting mutants ("weak elicitors").

Many common genes conferring resistance to viruses including the *N* gene, have been determined to code for proteins of the Toll-interleukin-1 receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) variety, where the leucine-rich repeat region (LRR) is thought to recognize PAMPs in a way perhaps analogous to the antibodies of animals, that is, by binding directly to the pathogen-associated molecules. Although a direct interaction has, at the time of this writing, not yet (to the author's knowledge) been observed, it has been established in some pathosystems that variation observed in LRRs is directly related to specificity of pathogen recognition. Based on this knowledge, the "weak elicitor" hypothesis predicts that the binding of the elicitor virus protein to the LRR "sticky tape" of the host R-gene product is weak or inadequate to provoke a strong response (Dinesh-Kumar et al., 2000.)

Another way in which the elicitor may fail to provoke an adequate response from the host, leading to LSHR, is when chemicals needed to trigger the defense-related signal cascade accumulate too slowly to confine the virus, analogous perhaps to water flowing from a faucet to fill a sink with a partially closed drain. Accumulation of proteins necessary for the HR signal cascade (the flow from the faucet) within the cell (the sink) is balanced against destruction of these proteins by the ubiquitin-proteasome system (the drain). If the viral elicitor fails to cause a rapid enough accumulation of the factor(s) that initiate the signal cascade, then the level of that signal fails to rise to the threshold level in the "sink" fast enough to allow HR to occur in time to prevent the virus from escaping (Dr. Wen-Yuan Song, Associate Professor, Plant Pathology Department, University of Florida, Gainesville, personal communication.)

Similar to the "horse race" hypothesis, the "delayed localized HR" hypothesis posits SHR is the result of delayed biochemical events associated with HR, and represents a failure of R-mediated defense response (Dinesh-Kumar et al., 2000). Working with the *N* gene, Dinesh-Kumar observed that mutations in the TIR and NBS, as well as the LRR could produce plants showing a delayed HR phenotype in response to inoculation with TMV, and that some deletion and point mutations to the TIR or NBS would produce plants that responded to TMV inoculation with necrosis, but allowed TMV to move, resulting in SHR. These hosts not capable of mounting an effective defense are described by Dinesh-Kumar as partial loss-of-function mutants (Dinesh-Kumar et al., 2000).

Some evidence exists that host-resistance response is actually two separate phenomena: "extreme resistance" (ER), believed to be an HR-independent response where the virus is halted at the point of infection before visible symptoms or accumulation; and hypersensitive response (HR), where specific biochemical changes take place (e.g., production of PR proteins, etc.),

leading to a necrotic response which usually limits virus spread from the general area of inoculation. In the "two-tiered" hypothesis, ER is believed to operate "independently" of HR, except inasmuch as it is controlled by the same elicitor and R-gene. Supposedly, HR develops only when the ER function is overwhelmed or evaded by the pathogen (Hajimorad et al., 2005). In one hypothesis, ER operates through PCD of the inoculated cells, disrupting viral replication and even degradation of pathogen RNA (Xu and Roossinck, 2000). This seems in contrast with the "micro HR" hypothesis, whereby it is believed that the absence of visible symptoms in highly resistant plants is due to a "micro HR" which limits the infection to a region of 1-3 cells from the point of entry.

Cases of virus-induced lethal systemic hypersensitive response (LSHR)

Although uncommon, examples of plant virus-host interactions resulting in LSHR have been reported, and may serve as models useful in understanding the TMGMV-TSA interaction. In soybean (*Glycine max*), host to Soybean mosaic virus (SMV), the (dominant) resistance gene *Rsv1* provides immunity against virtually all strains of SMV, except for strain G7, which causes a LSHR, and the artificial variant G7d, which causes non-necrotic systemic symptoms. This variation in virulence was mapped to mutations causing 3 significant amino acid substitutions in the P3 region of the virus. Notably, silent mutations had no effect on virulence, supporting the hypothesis that HR or the lack thereof is determined by translated pathogen gene products, rather than nucleic material itself. Curiously, LHSR in the soybean-SMV interaction appears to be delayed by up to 6-8 weeks post infection/inoculation (Hajimorad et al., 2005); a delay similar to, but much longer than, the delay observed in the TSA–TMGMV interaction.

Recently, two sub-isolates of a Japanese strain of Plantago asiatica mosaic virus (PlAMV) obtained from lily were found to have very different disease phenotypes in *N. benthamiana* plants. When inoculated onto *N. benthamiana*, sub-isolate Li1 produced LSHR, while sub-

isolate Li6 produced a latent/asymptomatic infection in this host. When sequenced, these two sub-isolates (PlAMV-Li1 and PlAMV-Li6) were surprisingly similar, sharing a 99.7% identity. Analysis of the genome using chimeras of the two isolates revealed mutation in the viral polymerase to be the source of the variation in pathogenicity (Ozeki et al., 2006).

Certain cultivars of bean (*Phaseolus vulgaris*), develop HR, which is sometimes lethal, in response to inoculation with Bean common mosaic virus (BCMV). As with tobamovirus/host systems, the HR that develops in this pathosystem is variable and influenced by genotype and environment. Depending on the presence or absence of the incompletely dominant allele, *I*, and environmental conditions (i.e., temperature), response to inoculation varies from immunity, to LL, to systemic phloem necrosis (Collmer et al., 2000).

Cucumber mosaic virus is a (+) RNA virus with a broad host range and a tripartite genome that produces two subgenomic RNAs. CMV causes systemic disease in tomato (*L. esculentum*), usually characterized by systemic mosaic. In some infections, CMV is accompanied by satellite RNAs (satRNAs), ranging in size from 332-405 nt in length, capable of modifying the symptomatology. In particular, one satellite, D4-sat RNA, (335 nt), causes an often lethal systemic necrosis in tomato, which develops late in the infection period and does not coincide with systemic movement of the virus. This necrotic symptom appears to be separate from chlorosis induced or enhanced by satellite RNA, and not all satRNAs will cause this symptom. Furthermore, this symptom is host specific; in tobacco D4-satRNA acts to attenuate CMV symptoms (Xu and Roosinck, 2000). Using a PVX vector system, Taliansky (1998) introduced satRNA into *Lycopersicon* cells without the CMV helper and observed that only negative-sense D4-sat RNA produced the delayed necrotic symptoms. Normally, the satRNA would enter the cell packaged in the CMV virions as the positive-sense form. CMV genomic RNA was not

required. Taliansky was able to identify a specific stem and "hairpin loop" in the 5′ region of the (-) satRNA (the 3′ region of the plus sense), required for necrogenic activity. Conversion of the stem-loop portion of the 5′ RNA changed satRNA (-) B5-sat from a non-necrotic virulence to a necrotic one. It is worth noting that very high levels of ds satRNA are recovered from extracts of plants infected with CMV and satRNA, and that unlike many other plant satellite viruses, the ratio of (+):(-) CMV satRNA may be as low as 2:1 (Taliansky et al., 1998; Xu and Roosinck, 2000).

Xu and Roosinck (2000) established that the cause of the necrosis, observed first in the vascular cells around the area of the second node below the meristem, is indeed the result of PCD. The PCD begins in phloem or cambium cells and spreads to adjacent infected cells. The authors suggest that timing indicates the involvement of cell developmental processes in initiating tomato cell death. At 9 – 10 days post-inoculation (DPI), the first visible signs of necrosis appeared in the prevascular cells close to the meristem, and occasionally in phloem or cambium cells on one side of the stem. Necrosis then spread to nearby cells, including those in the leaf petiole and adjacent tissues below the node. Supporting earlier observations, accumulation of (+)satRNA in the apices of infected plants was observed to increase rapidly after inoculation, but rapid accumulation of (−) satRNA did not occur until such time as necrosis was observed.

Another satRNA of CMV causes fruit necrosis. Necrosis produced by the CMV-tomato interaction is not well understood, but thought to be the result of incomplete vascularization in the fruit stalk. Fruit necrosis also sometimes occurs when watermelon (*Citrullus lanatus*) is infected by CGMMV. First reported from Macedonia and Thessaly (Greece), starting in 1999 and 2000, symptoms of the CGMMV-induced disease range from chlorotic leaf spots to pedicel

necrosis and degeneration of the pulp of the fruit. Symptoms were observed on grafted watermellons only (Bourbourakas et al., 2004), suggesting that mode of transmission may have an effect on HR.

Cases of gene-for-gene interactions between tobamoviruses and Solanaceae

Several gene-for-gene interactions between Solanaceae and tobamoviruses have been studied and characterized. Examination of these interactions may lead to insight as to how the TMGMV-TSA interaction occurs.

It was discovered that *N. glutinosa* plants displayed a resistance to tobamovirus infection, marked by the development of LL. The gene responsible for this phenotype, N, was introgressed into varieties of *N. tabacum* by F.O. Holmes, who demonstrated that the inheritance pattern of this resistance was Mendelian (Holmes, 1938). Plants heterozygous, as well as homozygous, for *N* will exhibit cell death at the site of infection, but the virus particles are confined to the region of the cell death (Hull, 2002).

It is known that the 126 kDa product of the TMV ORF 1 will produce HR necrosis in tobacco homozygous for the *N* resistance gene. C-terminal fragments (HEL & MOREHEL) also induced this response, though expression of the complete 183 kDa sequence did not. Of particular interest is the finding that transient expression of the shorter fragment, HEL, caused symptoms that were delayed in development by 11 days (Abbink et al., 1998).

Nicotiana sylvestris plants exhibit a response similar to that observed in tobacco plants which have the N-gene when challenged with most tobamoviruses, but not TMV. The elicitor in this interaction is the viral CP, and it has been shown that certain aa substitutions in the CP will abolish the response. The *N′* gene has been successfully (re)incorporated into at least one cultivar of tobacco "Bright Yellow." It has been established that the HR is elicited by the CP of tobamoviruses such as ToMV, but not TMV proper, although mutants of TMV capable of

eliciting a response from this gene have been created (Culver et al., 1991; Saito et al., 1987; Pfitzner and Pfitzner, 1992). Interestingly, the pedigree of *N. tabacum*, which includes *N. sylvestris* and *N. tomentosiformis*, indicates *N. tabacum* should contain *N′* in its genome. Expression of *N′* however, for some reason, is usually suppressed, leading to susceptibility in most tobacco cultivars. A spontaneous mutant of *N. tabacum* cv. Samsun (*nn*), "Samsun *EN*," does in fact appear to express the *N′* phenotype, as does the *N. tabacum* cv. "Java" —these are presumably back-mutations (Pfitzner and Pfitzner, 1992).

Three genes: Tm1, Tm2, and $Tm2^2$, conferring resistance to tobamoviruses have been discovered in tomatoes (genus *Lycopersicon*). They are designated "Tm," and tomato breeders organize the tobamoviruses they are challenged with (mostly TMV and ToMV) into "Pelham groups," based on their ability to overcome these alleles. Pelham 0 strains are unable to infect plants with any of these genes, P1 strains are capable of overcoming the resistance conferred by Tm1, P2 strains are capable of overcoming the resistance conferred by Tm2, and P1.2 strains can overcome Tm1, Tm2, and $Tm2^2$ resistance (Hollings and Huttinga, 1976).

Tm1 probably originated in *L. hirsutum*, and the resistance it provides has been shown to involve the 126 kDa and/or 183 kDa proteins. Tm1 appears to cause inhibition of the virus to replicate, rather than HR (Hull, 2002). It is relatively easily overcome, with just two amino acid substitutions being sufficient (Meshi et al., 1988; Pelham, 1966). Tm2, from *L. peruvianum*, and $Tm2^2$, from *L. chilense* (Mueller, 2004) are elicited by tobamovirus movement proteins. $Tm2^2$ is particularly durable: the viral mutants capable of overcoming it seem to have low virulence. Furthermore, LL development in resistant plants is microscopic, suggesting rapid recognition of the parasite (Lanfermeijer et al., 2003). Over the years, resistance from these plants has been painstakingly introgressed into lines of *L. esculentum*.

Resistance genes in peppers (genus *Capsicum*) are designated "L," followed by a number in superscript, in order of increasing effectiveness. All *L* genes are elicited by tobamovirus CP, and initiate HR, characterized by LL and leaf abscission that usually confines the virus to the site of infection (Holmes, 1937). Amino acid sequencing of the *L* genes reveal them to be approximately 60% identical to one another (Gilardi et al., 2004). Breeders often designate (advertise) tobamovirus resistance in peppers by pathotype, based on the virus's ability to overcome resistance conferred by the *L* genes. Pathotype 0 (P_0) viruses (i.e., TMV, ToMV) are able to systemically infect only those peppers lacking resistance (host genotype = ll), P_1 viruses (i.e., TMGMV) overcome L^1 resistance, $P_{1,2}$ viruses (e.g., PMMoV-J, PMMoV-S) overcome L^1 and L^2 resistance, $P_{1,2,3}$ viruses (e.g., PMMoV-I, PMMoV-Ij) overcome L^1, L^2, and L^3 resistance (Gilardi et al., 2004; Tsuda et al., 1998).

In studying the natural history of any given gene for disease resistance, it is important to remember that a resistance gene isolated from one member or accession of a species may not be present in all members of that species, or even a common allele within the population. The study of the *L* genes in the genus *Capsicum* is complicated not only by the early lack of differentiation between tobamoviruses (many common tobamoviruses were initially described as strains of TMV), but by the various controversial application of species epithets within the *Capsicum* genus. Although Linnaeus distinguished between *Capsicum annuum* and *C. frutescens* forms, the name *C. frutescens* was later adopted to describe all cultivated peppers within the United States, while *C. annuum* was used to describe all cultivated peppers by the rest of the world. Later research involving plant breeding ultimately justified Linnaeus' separation, with hybridization between *C. annuum* and *C. frutescens* possible, but difficult. The bell peppers of

commerce, and virtually all other peppers cultivated in the United States are properly *Capsicum annuum*, except Tabasco, which is *C. frutescens* (Smith and Heiser, 1951).

In the early part of the 20th Century, Holmes (1937) observed that certain varieties of garden pepper (*Capsicum* sp.) responded to tobamovirus inoculation with either systemic symptoms (mosaic/mottling) or with confining or non-confining necrotic resistance responses, Although at the time, the taxonomies of both genera, *Capsicum* and *Tobamovirus*, were poorly understood, Holmes' work established the presence of the resistance allele "*L*" and laid the foundation for other researchers to better define it. Holmes reported a qualitative difference in the response between plants carrying two allelic *L* genes (Table 1-3):

- *l* – No LL, systemic mottling/mosaic, no recovery.
- L^i – yellowish or largish LL, abscission, usually followed by recovery.
- *L* – distinct necrotic LL, abscission, followed by recovery.

Heterozygotes between L^i and *l* responded to inoculation with LL, followed by death.

Through cross-breeding experiments, Holmes determined that *L* and L^i are allelic, (or very nearly so; Holmes, 1937). Holmes called the allele from Long Red Cayenne the "imperfect localization" gene, or L^i, because the lesions formed are large and indistinct, and because occasionally systemic infection results without obvious symptoms. This is most likely the R-gene now represented as L^1, since he notes distinct lesions in Tabasco (*C. frutescens*). Holmes showed that L^i is partially dominant over the recessive condition, *l*, and *L* is dominant over L^i. Holmes reports L^i to be present in at least some members of Long Red Cayenne (homozygous) Anaheim Chili, Magnum Dulce, Red Cluster, Ruby King, Sweet Meat Glory, and Sweet Mountain (Holmes, 1937). Holmes reports *L* to be present in at least some specimens of Tabasco. He reports the heterozygous recessive condition, *ll* (no resistance), in the following varieties/cultivars: California Wonder, Celestial Chinese Giant, Coral Gem Bouquet, Early

Giant, Giant Crimson, Golden Dawn, Hungarian, Large Bell, Baby Bell, Oshkosh, Pimiento, Red Cherry, Spanish Monstrous, Upright Sweet Salad, and World Beater. It would be tempting to speculate that a pattern of inheritance similar to the *Capsicum*-TMV interaction might be responsible for the TSA-tobamovirus interaction; however, since all of the TSA tested thus far have consistently exhibited LSHR in response to inoculation with TMGMV, the probability that all of these plants (tested in the field or grown from field-collected seeds), should be heterozygous seems highly unlikely.

R-gene L^1 is derived from *Capsicum annuum*, the common pepper (Hull, 2002), and is the least effective of these resistances. TMGMV can escape localization in these plants, leave the inoculated leaf, and cause severe systemic symptoms, including necrosis. This particular interaction is of special interest, because the HR shown by *C. annuum* appears similar to that shown by TSA, and may eventually yield clues as to the nature and mechanics of the response. L^2 resistance occurs in *C. frutescens*, commonly known as Tabasco, and was one of the first viral resistance genes described. It confers resistance to most tobamoviruses, except PMMoV (de la Cruz et al., 1997). L^3 resistance is found in *C. chinense*, a species which includes the Habanero and scotch bonnet types. L^3 provides resistance against most tobamoviruses except for certain strains of PMMoV (Gilardi et al., 1998). More recently, L^4 resistance, discovered in a wild species of pepper, *C. chacoense* (USDA GRIN accession PI 260435), has been shown to be elicited by the CP of all tobamoviruses for which peppers are a natural host, including every strain of PMMoV. While the exact mechanism for this interaction remains unknown, it appears that the gene product of L^4 perhaps recognizes a broader structural configuration within the CP elicitor, relative to the other alleles (Gilardi et al., 2004).

In considering the *L*-gene resistances in pepper, it should be remembered that *Capsicum annuum*, *C. frutescens*, and *C. chinense* bear many resemblances to each other and are assumed by some to be re-domestications of the same ancestral wild species. In addition, the plants can all be hybridized with some degree of difficulty, so gene transfer is possible, and even probable as development and improvement continues by breeders. Thus, when evaluating for resistance, it cannot be assumed that just because a *Capsicum* has been classified a particular species, exhibits resistance, that the resistance allele is the one originally reported from that population (Pickersgill, 1988). Nor can it be assumed that *all* members of a species will carry a resistance allele as evidenced by the presence of L^1 in only *some* cultivars of *C. annuum* (Holmes, 1937).

Many varieties of eggplant, *Solanum melongena*, exhibit HR when exposed to tobamovirus infection. Differences between varieties may be the result of the diverse genetic background, (*aethiopicum* line, *anguivi* line, *melongena* line). The HR response was investigated by Dardick and Culver and discovered to be elicited by the CPs of several tobamoviruses, including TMGMV (U2) (Dardick and Culver, 1997).

It has been observed that various cultivars of *Phaseolus vulgaris,* especially "Pinto," will produce LL when inoculated with TMV, but not TMGMV. This phenomenon is significantly influenced by the growing conditions, but the speed and convenience of generating bean seedlings has made it a useful tool in plant virology (Zaitlin and Israel, 1975).

Petunia X *hybrida*, a commercially important hybrid ornamental from S. America develops HR in response to inoculation with certain tobamoviruses. TMGMV will elicit HR on at least some petunia species and hybrids under some conditions. PMMoV strain J produces LL on *Petunia* X *hybrida*, then mosaic, while PMMoV-Ij produces mosaic only (Tsuda et al., 1998). ToMV also causes the formation of LL (Alexandre et al., 2000).

Table 1-1. Strains/isolates of TMGMV

Isolate	Locality	Reference	Accession #
DSMZ – PV0110	France, Corsica	Wetter	
DSMZ – PV112		Bald, J.G.	
DSMZ – PV0113, ("TMGMV-NZ")	USA, Ohio	Zettler and Nagel, 1983	EF469769
DSMZ – PV0115		Wetter	
DSMZ – PV0116		Wetter	
DSMZ – PV0117		Wetter	
DSMZ – PV0118		Wetter	
DSMZ – PV0119		Wetter	
DSMZ – PV0120	Italy	Wetter	
DSMZ – PV0122		Wetter	
DSMZ – PV0124	Italy	Marte	
DSMZ – PV0228	Japan	Nejidat, et al 1991	
ATCC – PV226		McKinney, H.H., 1929	
ATCC – PV228		McKinney, H.H., 1952	
ATCC – PV585		Valverde, R.A. et al., 1991	
ATCC – PV586		Valverde, R.A. et al., 1991	
ATCC – PV635		Solis and García-Arenal, 1990	
TMGMV-J	Japan	Morishima et al., 2003.	AB078435
TMGMV-U2*		Solis and García-Arenal, 1990.	M34077
TMGMV-U5*	USA, California	Dodds, J.A., 1998.	
"TMV – U"	Japan	Nagai et al. 1987	

ATCC = "American Type Culture Collection", DSMZ = "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (German Collection of Microorganisms and Cell Cultures)

Table 1-2. Size of TMGMV genes and gene products

Reading frame	Gene (nt)	Product (kDa)
ORF1	3336	126
ORF2	4830	183
ORF 3	771	28.5
ORF 4	480	17.5

Table 1-3. Relationship between genotype and disease phenotype in *Capsicum* sp. peppers.

Variety	Genotype (Holmes)	Genotype (modern)	Phenotype TMV (Holmes)	Phenotype TMGMV (Elliott)
Anaheim Chili	L^iL^i	L^1L^1	LL ("imperfect"), SYS	LL, SYS
Hungarian	*ll*	*ll*	SYS (mosaic/mottling)	SYS only
Tabasco	*LL*	L^2L^2	LL (distinct) only	LL only

Response of *Capsicum* sp. cultivars to inoculation with TMV (Holmes, F.O. 1937) and TMGMV (Mark S. Elliott, unpublished data)

Figure 1-1. TSA colonizing pastureland [Credit: Dr. Raghavan Charudattan, University of Florida].

A   B

Figure 1-2. TSA infestations A) TSA infesting pastureland [Credit: FL Division of Plant Industry] B) TSA infesting wooded area [Credit: Dr. Julio Medal, University of Florida].

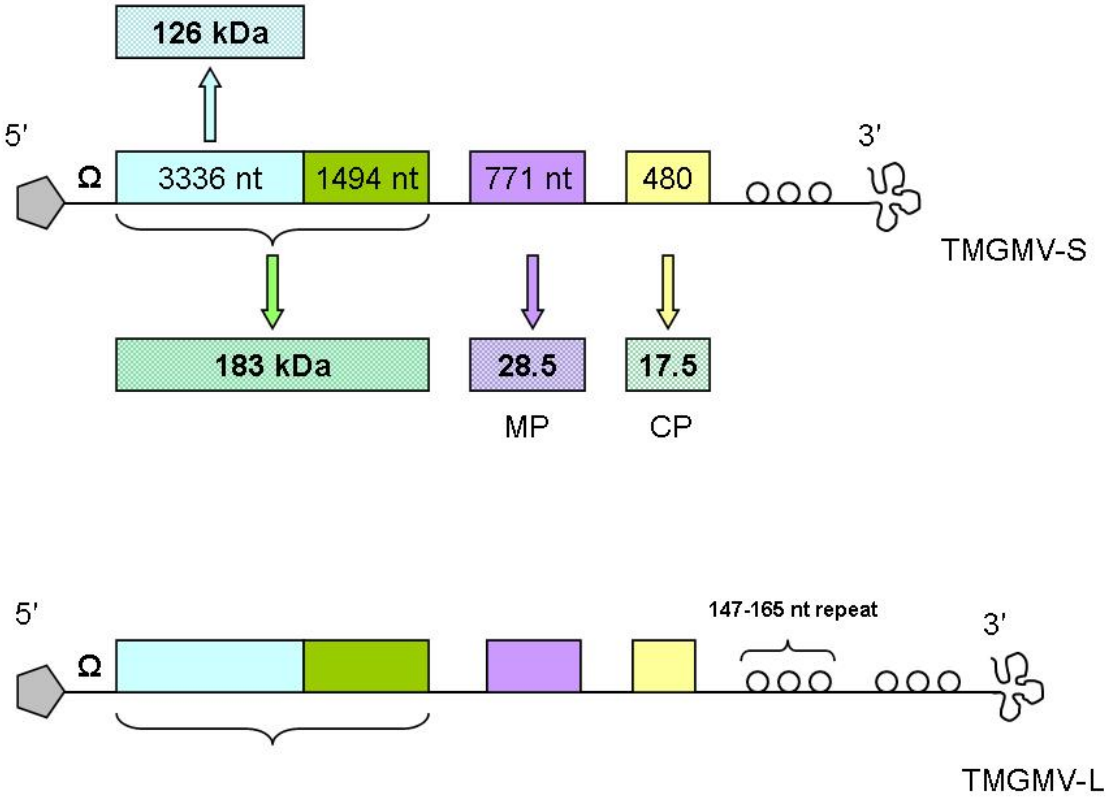


Figure 1-3. Genome organization map of Tobacco mild green mosaic virus (TMGMV; not to scale). Genome is capped 5′ with 7-methylguanosine (shaded pentagon). Omega (Ω) leader sequence is followed by open reading frames (ORF's) 1,2, 3 and 4 that are directly translated (arrows) into replication-associated (126 kDa, 183 kDa) proteins, and subgenomically translated into movement (MP) and coat (CP) proteins. Circles represent RNA pseudoknots (this region is duplicated in TMGMV-L), and are followed by the 3′ tRNA-like structure.

CHAPTER 2
IDENTIFICATION OF COLUMNEA ISOLATE PV-0113

Introduction

Viruses, nearly impossible to isolate as discrete entities, may be described as "quasi-species," with multiple, related genomes serving as a source of genetic variability allowing the population to adapt to changing environmental conditions (Hull, 2002). Techniques such as single local lesion (SLL) isolation have been used to restrict the variability of the virus population in the hopes of obtaining single-genotype isolates. Tobacco mild green mosaic virus (TMGMV) exists as at least two distinct sub-types, distinguished by duplication of the pseudoknot-containing region of the genomic 3′ non-translated region (3′ NTR) (Bodaghi et al., 2000). Small-type TMGMV (TMGMV-S), referred to in older literature as the "U2" strain of TMV, appears to co-exist in nature alongside large-type TMGMV (TMGMV-L) or "U5," although mixed infections of the two are rare. These two subtypes appear to compete within the hosts *Nicotiana glauca* and *N. tabacum* cv. "Xanthi," usually driving one subtype or the other to extinction. Mixed infections sometimes develop in plants initially inoculated with TMGMV-S, especially when challenged only a short time afterwards (Bodaghi et al., 2004). The effect of a mixed infection of TMGMV-S and TMGMV-L on TSA has not been reported. Furthermore, three species of tobacco were identified which display different symptoms that are distinct and diagnostic following inoculation with TMGMV-L or TMGMV-S. Finally, wild populations of TMGMV in *N. glauca* sometimes are associated with Satellite tobacco mosaic virus (STMV). Satellite viruses, including STMV, may attenuate or modify expression of the helper virus' disease phenotype, at least in certain hosts (Dodds, 1998). The presence or absence of STMV in PV-0113 isolate is a concern, since SLL isolation may be inadequate to remove STMV from a TMGMV culture (Valverde et al., 1991). It is possible that any STMV present could affect the

symptomatology of the TMGMV-TSA plant-pathogen interaction. Techniques to detect and identify STMV include serology and double-stranded RNA (dsRNA) extraction and analysis.

In the case of the *Columnea* isolate of TMGMV, PV-0113, there are three sorts of contamination that might have an influence on the disease process or otherwise influence the outcome of experiments using that culture. First, a culture might contain multiple virus species, a possibility that may be assessed by inoculating a range of differential host plants, serology, or reverse transcriptase-polymerase chain reaction (RT-PCR). Next, there is the possibility that multiple genotypes within a species might interact to cause, enhance, or inhibit the tropical soda apple (TSA)-killing effect of TMGMV PV-0113. Then, there is the possibility that satellite RNA or a satellite virus may be associated with the isolate. The objective of this chapter is to confirm the identification and purity of the TSA-killing *Columnea* isolate DSMZ PV-0113 as TMGMV-S ("TMV U2") by host range determination, ELISA, immunodiffusion, and diagnostic RT-PCR methods.

Methods used to determine plant viral infection include physical, *in plantae*, serological and nucleic acid-based techniques. Physical techniques, such as observing stained viral inclusions using light microscopy and direct observation of virus particles under the electron microscope, may reveal virus morphology and provide clues as to the virus family or genus. Other methods are used for identification to species. *In plantae* techniques, such as the use of indicator plants and host-range studies, involve inoculation of living plants with the viral pathogen. Techniques based on serology use antibodies to detect viruses, and include enzyme linked immunosorbent assay (ELISA), immunodiffusion, immunoblotting, and fluorescent antibody microscopy. Molecular biology techniques based on analysis of extracted nucleic acids,

such as RT-PCR, dsRNA analysis, Northern blotting and Southern blotting are also useful in accurately and precisely identifying plant virus infections (Agrios, 1997).

Disease phenotype and host susceptibility to a virus infection can be highly variable under diverse growing conditions. Furthermore, mixed infections of viruses are not uncommon in nature, and sometimes the virulence of one virus can be enhanced or inhibited by another. Tobacco leaves infected with Potato virus X (PVX), for example, develop more local lesions (LL) when inoculated with Tobacco mosaic virus (TMV) than when infected alone. This increase is associated with a severe veinal necrosis not seen in singly infected plants. (Hull, 2002). Tomato mosaic virus (ToMV) also interacts synergistically with PVX, causing a severe necrotic "streak" symptom in tomatoes that often leads to the death of the plant. Infection by Potato virus Y (PVY) in tobacco plants can increase the concentration of PVX in the leaves tenfold, and in potato the two viruses synergize, producing a destructive "rugose mosaic" symptom. Likewise, infection by a less virulent virus, or strain of virus, may "cross-protect" a host plant against a more virulent one, greatly reducing disease severity. In addition to modifying the visible symptoms, infection by one virus may have other significant effects on the biology of a co-infecting species; for example, a virus normally incapable of movement in a particular host may spread systemically (Hull, 2002). Brome mosaic virus (BMV), for example, gains the ability to systemically infect tomato when inoculated along with TMV (Carrington et al., 1996).

One of the most venerable approaches adopted by plant pathologists, the use of differential host range and indicator plants, requires time and greenhouse space, but is relatively simple and straightforward. In addition to the time and space requirements, drawbacks include the need for careful vigilance to avoid and identify potential mixed infections. Despite these limitations, host range testing may be more discriminating than certain other techniques, allowing one to

distinguish strains of a virus that serological methods cannot (Hull, 2002). Plants selected for host range should respond distinctively to infection by different strains and/or species of virus, allowing the creation of a logical table to distinguish between them. One criterion for host selection is the use of a Plant Disease Index (Alfieri et al., 1994) or another reference for the locality from which the infected source material originated. There is little sense in beginning a study by testing for a species not known to occur within the geographic area, or in a host it is not known to infect.

Tobamoviruses, as a group, are said to have a wide host range (Gibbs, 1977; 1986). TMGMV is known to infect species in the Apiaceae, Apocynaceae, Boraginaceae, Chenopodiaceae, Commelinaceae, Gesneriaceae, and Solanaceae (Wetter, 1989; Randles, 1986; Cohen, et al., 2001). *Chenopodium quinoa*, *Datura stramonium*, *Eryngium planum*, *Lycopersicon esculentum*, and various species and cultivars of *Nicotiana* have been used for diagnosis of various tobamoviruses (Wetter, 1989). Unlike TMV and ToMV, TMGMV will not infect tomato (Morishima et al., 2003). TMGMV can be separated from mixed infections with Pepper mild mottle virus (PMMoV) or TMV by inoculating *E. planum*, which the TMGMV will infect systemically, though the infection may become symptomless (Johnson, 1947; Wetter et al., 1984). Conversely, TMV may be separated from TMGMV using *Nicotiana sylvestris* as a host, and various species of *Capsicum* (*C. chinense*, *C. chacoense*) may be used to isolate PMMoV (Gilardi et al., 1998, 2004).

Serological techniques involve the use of antibodies produced by laboratory animals immunized with purified virus. Immunodiffusion detects viruses and hints at relationships between viruses using the property of immunoprecipitation which occurs when antibodies specific to a plant virus encounter and bind to sites on viral coat proteins (antigens), forming a

visible matrix within the gel medium (Purcifull and Batchelor, 1977). Animal-derived antibodies may also be used for ELISA, where antibodies chemically conjugated to an indicator are used to highlight or reveal antigens bound (either directly or indirectly) to the surface of wells in a polystyrene microtiter plate. The plates can then be analyzed using a spectrophotometer, specially adapted for this purpose.

In PCR, a process popularized by Kary Mullis, the enzyme Taq polymerase is used in conjuction with multiple heating and cooling cycles to multiply a template DNA strand into as many as a billion copies, enabling highly sensitive detection and identification of DNA sequences. With a properly designed primer set, PCR results are usually quite specific (Metzker and Caskey, 2001). To detect RNA viruses, such as tobamoviruses, the enzyme reverse transcriptase (RT) may be employed to create a cDNA template from which the PCR reaction can proceed. This may either be performed separately beforehand, or in the same tube as the PCR reaction (Sambrook and Russell, 2001). A series of primers has been designed that could be used to selectively amplify 750-800 bp fragments of different tobamovirus species, which, when subjected to restriction enzyme analysis provide identification of the specific strain or pathotype (Letschert et al., 2002).

Materials and Methods

Plants of *Arachis hypogaea* cv. "Virginia Jumbo," *Capsicum annuum* X "Camelot," *Capsicum annuum* cv. "Jalepeño M," *Capsicum frutescens* cv. "Tabasco," *Cucumis sativus* cv. "Poinsett," *Cucurbita pepo* cv. "Early Straightneck," *Datura stramonium***,** *Eryngium planum, Lycopersicon esculentum* cvs. "Beefsteak" and "Rutgers," *Nicotiana benthamiana***,** *N. glutinosa***,** *N. langsdorfii, N. rustica***,** *N. sylvestris, N. tabacum* cv. "Samsun"(both *nn* and *NN* genotypes), *Phaseolus vulgaris* cv. "Pinto," *Vigna unguiculata* cv. "Cream," and *Zinnia elegans* were grown from seed sown in Metromix potting medium in 4" (10.16 cm) clay pots. Upon attaining a

convenient size (4-6 weeks), plants were inoculated with extracted tobacco sap containing virus provided by F.W. Zettler as TMGMV, and hereinafter referred to as TMGMV PV-0113 (Zettler and Nagel, 1983).

Sap extract from TMGMV PV-0113 infected tobacco, previously maintained frozen at -20°C, was diluted 1:10 in 20 mM $NaHPO_4$/ Na_2PO_4 inoculation buffer (pH 7.2). Diluted sap was applied to both surfaces of each of three carborundum-dusted leaves of each host-range plant by rubbing with sterile cotton cheesecloth pads. For ease of comparison, one specimen of each species of indicator host was inoculated with buffer only and allowed to mature alongside the treated specimen. Host-range plants were observed daily for development of symptoms.

Four common, *Solanceae*-infecting tobamoviruses, TMV, TMGMV, ToMV (kindly provided by Mark S. Elliott, Plant Pathology Department, University of Florida/IFAS, Gainesville), and PMMoV (kindly provided by Dr. Carlye Baker, Florida Division of Plant Industry, Gainesville), were propagated in their Commonwealth Mycological Institute (CMI)-recommended propagation hosts (Zaitlin, 1975; Wetter, 1989; Hollings and Huttinga, 1976; Wetter and Conti, 1988), and inoculated onto various host-range plants, including *Datura stramonium*, *Lycopersicon esculentum* cv. "Rutgers," and *Nicotiana sylvestris*. A good "propagation host" accumulates virus rapidly and allows for easy purification of the virus under study. A good "maintenance host" is long-lived, compact in habit, and tends to resist infection by virus species other than the one under study.

Polyclonal antibodies to sodium dodecyl sulfate (SDS)-dissociated PV-0113, derived from a rabbit antiserum prepared by Zettler and Nagel (1983), and kindly provided by M. S. Elliott were used to detect TMGMV PV-0113 and explore its serological relationship with other known tobamoviruses. Immunodiffusion was carried out in the manner of Purcifull and Batchelor,

1977. Briefly, 3.0 g of tissue, collected 3 weeks post-inoculation (WPI) from tobacco plants inoculated with TMGMV PV-0113, were triturated in a sterilized mortar under 3.0 mL of distilled H_2O (dH_2O) to create a 1:1 homogenate, which was filtered through a double layer of sterilized, dH_2O-saturated cheesecloth. Two 0.75-mL portions of the filtrate were placed in separate plastic Falcon culture tubes. To the first portion, 0.75 mL of dH_2O was added, and to the second, 0.75 mL of a 3% solution of SDS in dH_2O was added. The samples were mixed by inversion and used to load the immunodiffusion plates. A control for the serological tests was tissue extracts from uninoculated tobacco plants.

Wells punched in chilled immunodiffusion media plates containing 0.8% noble agar, 1.0% NaN_3, and 0.5% SDS, were loaded with sample and incubated in a moist plastic chamber at 27 ^oC for 24-48 hours or until precipitate bands were clearly developed and discernable to the naked eye. Samples were also loaded into non-SDS plates containing 0.7% noble agar, 0.85% NaCl, 0.03% NaN_3, and 50 mM Tris, pH 7.5. When necessary, plates were cleared using activated charcoal (15 g/100 ml H_2O) and photographed using a light box and a Nikon Coolpix 5700 digital camera.

Polyclonal antibodies from rabbit to SDS-dissociated PV-0113 were also used in ELISA, which gave strong positive results for most symptomatic (and some asymptomatic) host-range plants tested. Although the rabbit anti-TMGMV serum used in these experiments was prepared to purified, SDS-dissociated PV-0113 itself, reciprocal homologous reactions between PV-0113 and "TMV U2" and antisera prepared to them have been reported (Zettler and Nagel, 1983). Briefly, tissue samples were collected from both inoculated and mock-inoculated control host-range plants. Approximately 0.3 g of frozen-thawed tissue from each sample was processed through a rotary sap-extractor using 3 mL of extraction buffer containing 1x phosphate buffered

saline + Tween-20 (PBST), 0.2% bovine serum albumen (BSA), and 10 mM Na_2SO_3, adjusted to pH 9.6. One hundred (100) µl of 1:10 extract from each sample was loaded directly into 3 wells of a 96-well ELISA plate and incubated at 37° C for 1 hour. Plates were washed 3 times with 1x PBST with 3 minute soaks in PBST between washes. The sample and control wells were then loaded with rabbit anti-TMGMV serum cross-absorbed to healthy *N. tabacum* tissue and incubated at 37 °C for 1 hour. Rabbit anti-TMV and rabbit anti-ToMV sera were also used on some plates for comparison (data not shown). Rabbit antisera were diluted in conjugate buffer containing 1x PBST, 0.2 % BSA, 2 % PVP-40, and pH adjusted to 7.4. Following incubation, wells were again washed 3 times for 3 min with 1x PBST. The washed wells were loaded with a solution of goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:30,000 in conjugate buffer and again incubated at 37 °C for 1 hour and washed 3 times with PBST. After incubation and washing, plates were loaded with a freshly-prepared 1 mg/ml solution of p-nitrophenylphosphate (PNP) in a substrate buffer containing 0.92 mM diethanolamine, 0.02 % sodium azide, pH 9.8., and incubated in the dark for 15 minutes, or until a yellow reaction color was visible to the naked eye. Absorbance at 405 nm was read at 5, 15, and 30 minutes. Samples with average absorbance readings 3 times the background (negative control readings) were assessed positive.

Presence of TMGMV RNA was confirmed using RT-PCR with the diagnostic primers (Fraile et al., 1996) `5'-ATGCAGCTTCCATTTTGGCAG-3', and 5'-GGTAAGTTAACGCTTTGGCTTG-3'` (kindly provided by Kris Beckham, Plant Pathology Department, University of Florida/IFAS, Gainesville). Briefly, total RNA was extracted from a SLL isolate using Trizol extraction reagent as follows: a leaf disk (~0.2 g) was excised using the lid of a sterile 1.5 mL micro-centrifuge tube and homogenized using a sterile, RNA-ase-free

Kontes micropestle and Trizol reagent. The homogenate was extracted with 0.2 mL $CHCl_3$ and centrifuged at 12,000 x *g* for 10 minutes. The aqueous (upper) layer was removed, and RNA precipitated using ½ volume ice-cold isopropanol. The RNA pellet was washed once with 75% ethanol, air dried under vacuum (aspirator), and suspended in 25 µL diethylpyrocarbonate (DEPC)-treated water. RT was carried out using the Titan One-Tube kit (Roche). Each reaction contained 250 µM deoxynucleoside triphosphate (dNTP), 1x RT-PCR buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1 µL AMV RT/Taq polymerase solution, and 2-5 µL of diluted (1:10, 1:100) or undiluted template RNA, prepared as described above, for a total volume of 50 µL per reaction. RT-PCR was carried out in a Biometra thermocycler using sterile mineral oil to prevent evaporation. The program used consisted of : 45°C for 30 minutes, 94°C for 3 minutes, and 35 repetitions of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute. After 35 repetitions, PCR-generated fragments were allowed to extend for 5 minutes at 75°C, then cooled to 4°C and held for electrophoretic analysis.

Samples of PMMoV, TMV, ToMV, TMGMV PV-0113, American Type Culture Collection (ATCC) TMGMV accession PV-586, and two strains of TMGMV from Lake Alfred, FL, provided as "U2" (TMGMV-LAU2), and "U5" (TMGMV-LAU5), along with two unidentified virus infections in TSA were inoculated onto the CMI-recommended propagation hosts. At 3 WPI apical tissue was harvested, and total RNA was extracted from the tissue samples using Trizol extraction reagent as described for PV-0113. Extracted template RNA was adjusted to approximately 1-5µg/µL in molecular biology grade (MBG) H_2O, and diluted further 1:10 and 1:100. To 2-5 µL of each template RNA were added 1 µL reverse primer (Tob-Uni_1), 1 µL 10mM dNTP, and 12 µL MBG H_2O. This mixture was heated to 65°C for 5 minutes and then chilled to 4°C briefly before adding 4 µL 5X first-strand buffer, 2 µL 0.1 M DTT and 1 µL

RN-ase OUT enzyme (40U). Samples were then heated to 37 °C for 2 minutes before addition of the reverse transcriptase (RT).

First-strand synthesis was carried out at 37°C using 0.5 µL Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 1 hour, after which the enzyme was inactivated by heating for 15 minutes at 70°C. PCR amplification was carried out using an Invitrogen kit, with a mixture (for each reaction) of 16 µL MBG H_2O, 2.5 µL 10X reaction buffer, 0.9 µL 50 mM $MgCl_2$, 2.5 µL 2 mM dNTP, 0.125 µL of Taq polymerase, and 1 µL of each specific primer (Table 2-1). Programmed PCR was carried out as: 94°C for 5 minutes, and 25 repetitions of 94°C for 1 minute, 55°C for 45 seconds, and 72°C for 1 minute. After 25 repetitions, the PCR-generated fragments were allowed to extend for 5 minutes at 72°C, then cooled to 4°C and held at that temperature until electrophoresis on 0.9% agarose.

Results

Symptoms caused by this isolate on the propagation host *N. tabacum* cv. Samsun (*nn*) were generally mild and included a greenish mosaic symptom frequently coupled with leaf pleating, and flower color-break (Figure 2-1). On the maintenance host, *E. planum*, TMGMV PV-0113 often produced a yellowish spotting or flecking (Figure 2-2).

On several plant species used in the host range (Table 2-2), TMGMV PV-0113, produced symptoms including but not limited to chlorosis, mosaic, necrosis confined to LL, necrosis not confined to LL, stunting, leaf and fruit abscission, and fruit lesions. When compared to host range data reported in CMI/AAB bulletins (Zaitlin, 1975; Hollings and Huttinga, 1976; Wetter and Conti, 1988; Delgado-Sanchez and Grogan, 1970; Purcifull and Hiebert, 1982; Francki et al., 1979), and Plant Viruses Online (Brunt et al., 1996), results obtained from the host-range study were consistent with the previous identification of TMGMV, and ruled out other common tobamovirus (TMV, ToMV, PMMoV) and non-tobamovirus (CMV, TEV, PVY) contaminants.

Results of SDS/non-SDS immunodiffusion using the common tobamoviruses TMV and ToMV for comparison were consistent with the host-range diagnosis of TMGMV for the *Columnea* (PV-0113) isolate. Rabbit antiserum prepared to SDS-dissociated TMGMV PV-0113 (Zettler and Nagel, 1983) detected the virus in both SDS (Figure 2-3) and non-SDS (Figure 2-4) immunodiffusion media. TMGMV PV-0113 did not react with rabbit anti-ToMV serum (Figure 2-5), and reacted to rabbit anti-TMV only weakly (Figure 2-6). ELISA using rabbit antiserum to PV-0113 yielded data consistent with the immunodiffusion data and an identification of TMGMV (Table 2-3). Antisera to TMV and ToMV produced a weak reaction but did not consistently produce a reaction exceeding the cutoff.

The diagnostic primers for TMGMV consistently amplified RNA extract from infected tissue samples, producing single bands of the expected size for TMGMV (Figure 2-7).

In the initial run of the experiment using the tobamovirus analysis primers (Letschert et al., 2002) every tobamovirus isolate produced a positive PCR product using the universal tobamovirus primer. In addition, every TMGMV isolate except the culture obtained from the Citrus Research and Education Center (CREC), Lake Alfred, as "U2" tested positive using the primers designed to be specific to TMGMV. The "U2" culture obtained from Lake Alfred tested positive using the universal tobamovirus primer and the primer specific to TMV. The TMV isolate behaved as expected, with clear positives from only the universal and TMV-specific primers. The rest of the cultures processed under this system produced nonspecific product bands when analyzed on agarose. Isolates TMGMV PV-586, ToMV, and PMMoV also developed bands in the reactions using the "TMV-specific" primer set. There was a positive result for the TMGMV PV-0113 culture using the ToMV-specific primer, and also the PMMoV primer, although this latter reaction resulted in a product of incorrect size. The culture obtained

from Lake Alfred as "U5" tested positive using the ToMV primer set, as did the PMMoV culture. The ToMV virus culture itself behaved more or less as expected – although there were bands generated by the TMGMV and TMV primer sets, these were not within the size range acceptable as positive. The PMMoV culture obtained from Dr. Carlye Baker (Florida Division of Plant Industry, Gainesville) tested positive using all primer sets EXCEPT the PMMoV-specific primer set. Raising the annealing temperature to 65°C resulted in no product. In an attempt to adapt or optimize this system to work with the virus cultures under study, a two-factor experiment using cDNA prepared from TMV was devised using cDNA dilution and temperature as the independent variables.

The primers will amplify product at 50°C, but may produce non-specific products. It would be important to check the size of the PCR products when annealing at this temperature. Lower annealing temperatures appear to increase sensitivity, but also produce more non-specific products. Products of sizes outside the range of 600-1000 bp should be ignored. First-strand DNA may be diluted 100 fold at lower annealing temperatures, but not at higher temperatures. The optimum temperature for this system will be somewhere between 50°C and 60°C. No reaction was seen; even with the universal tobamovirus primer set and undiluted cDNA above 60°C, indicating that the system is not useful at these temperatures.

Discussion

Collectively, the results of these tests indicate that the TMGMV PV-0113 culture contained TMGMV-S and was unlikely to contain any other virus reported to infect TSA. Compared with tobamoviruses listed in the Florida Plant Disease Index (Alfieri et al., 1994), and viruses recovered locally from TSA (McGovern et al., 1994), or artificially transmitted to it (Chagas et al., 1978), symptoms observed on inoculated host-range plants fit best with the symptoms on these plants caused by TMGMV, but not well with other candidates. In the course

of this study it was determined that the common tobamoviruses, TMV, TMGMV, ToMV, and PMMoV could be distinguished from one another by symptoms elicited in just three kinds of differential indicator plants: *D. stramonium*, *L. esculentum*, and *N. sylvestris* (Table 2-2).

The serological tests ELISA and immunodiffusion supported the host-range determination. Rabbit antiserum prepared to SDS-dissociated PV-0113 reacted with virus recovered from tissue, indicating that the coat protein (CP) is of the isolate TMGMV and not one of the other common tobamoviruses, ToMV or TMV type-strain.

Reverse transcriptase-PCR using various primers amplified a product of size and quantity consistent with TMGMV. A series of species-specific primers (Letschert et al., 2002), proved useful for sensitive tobamovirus detection, but not for discrimination/identification under the conditions used. The optimal temperature for the TMV-specific primer set was determined to be $50°C < y < 60°C$.

Table 2-1. Species-specific primers used in RT-PCR.

Name	5′ → 3′ Sequence	Nt position
Tob-Uni_1	ATT TAA GTG GAS GGA AAA VCA CT	6283 -- 6260
Tob-Uni_2	GTY GTT GAT GAG TTC RTG GA	5479 -- 5498
TMV	CGG TCA GTG CCG AAC AAG AA	5609 -- 5589
ToMV	CGG AAG GCC TAA ACC AAA AAG	5618 -- 5597
PMMoV	GGG TTT GAA TAA GGA AGG GAA GC	5617 -- 5595
TMGMV	AAR TAA ATA AYA GTG GTA AGA AGG G	5590 -- 5565

Species-specific tobamovirus primers proposed by Letschert et al., 2002.

Table 2-2. Experimental host range and symptoms caused by TMGMV PV-0113.

Family	Species	Common name	Symptoms
Apiaceae	*Eryngium planum*		NS/YF
Asteraceae	*Zinnia elegans*		NS/NS
Cucurbitaceae	*Cucumis sativus* cv. Poinsett	Cucumber	NS/NS
	Cucurbita pepo cv. Early straightneck	Squash	NS/NS
Fabaceae	*Arachis hypogaea* cv. Viginia jumbo	Peanut	NS/NS
	Phaseolus vulgaris cv. Pinto	Pinto Bean	NS/NS
	Vigna unguiculata cv. "Cream"	Cowpea	NS/NS
Solanaceae	*Capsicum annuum* X Camelot	Bell pepper	Ab, Ch, LL/N, PD
	Capsicum annuum cv. Jalepeño M	Jalepeño pepper	LL/N, PD
	Capsicum frutescens cv. Tabasco	Tabasco pepper	Ab, LL/NS
	Datura stramonium	Jimsonweed	LL/NS
	Lycopersicon esculentum cv. Beefsteak	Tomato	NS/NS
	L. esculentum cv. Rutgers	Tomato	NS/NS
	Nicotiana benthamiana		Ch, N/N, W, PD
	N. clevelandii		Ch/Ch, M, SP
	N. glutinosa		LL/NS
	N. langsdorfii		LL/NS
	N. rustica	Wild tobacco	LL/Ch, N, PD
	N. tabacum cv. Samsun (*nn*)	Tobacco	NS/FM
	N. tabacum cv. Samsun (*NN*)	Tobacco	LL/NS
	N. sylvestris		LL/NS

Symptoms on inoculated leaves and upper uninoculated leaves indicated to left and right of slash, respectively: Ab = abscission, Ch = chlorosis, FM = faint mosaic, LL = local lesions, M = mosaic, Mo = mottling, N = necrosis, NS = no symptoms, PD = plant death, SP = shoot proliferation, W = wilting, YF = yellow flecking or speckling (not always visible).

Table 2-3. ELISA using cross-absorbed antiserum.

Antibody dilution	Healthy-sap (*N. tabacum*)	TMGMV PV-0113	% difference
	$\mathbf{A_{405}}$	$\mathbf{A_{405}}$	
1:2500	0.058	0.282	490
1:2500 X-abs	0.053	0.218	410
1:5000	0.044	0.183	420
1:5000 X-abs	0.037	0.182	490

A.   B

C   D

Figure 2-1. Symptoms of TMGMV on *N. tabacum* cv. "Samsun" (*nn*) A) Mild green mosaic and pleating on leaves B) Close-up of symptoms on a leaf C) Flowers of an un-inoculated plant D) Flower break symptoms on a TMGMV PV-0113 inoculated plant.

 A  B

Figure 2-2. *Eryngium planum* A) Symptomless (mock-inoculated) *E. planum* B) Yellow fleck symptoms on TMGMV PV-0113–inoculated *E. planum.*

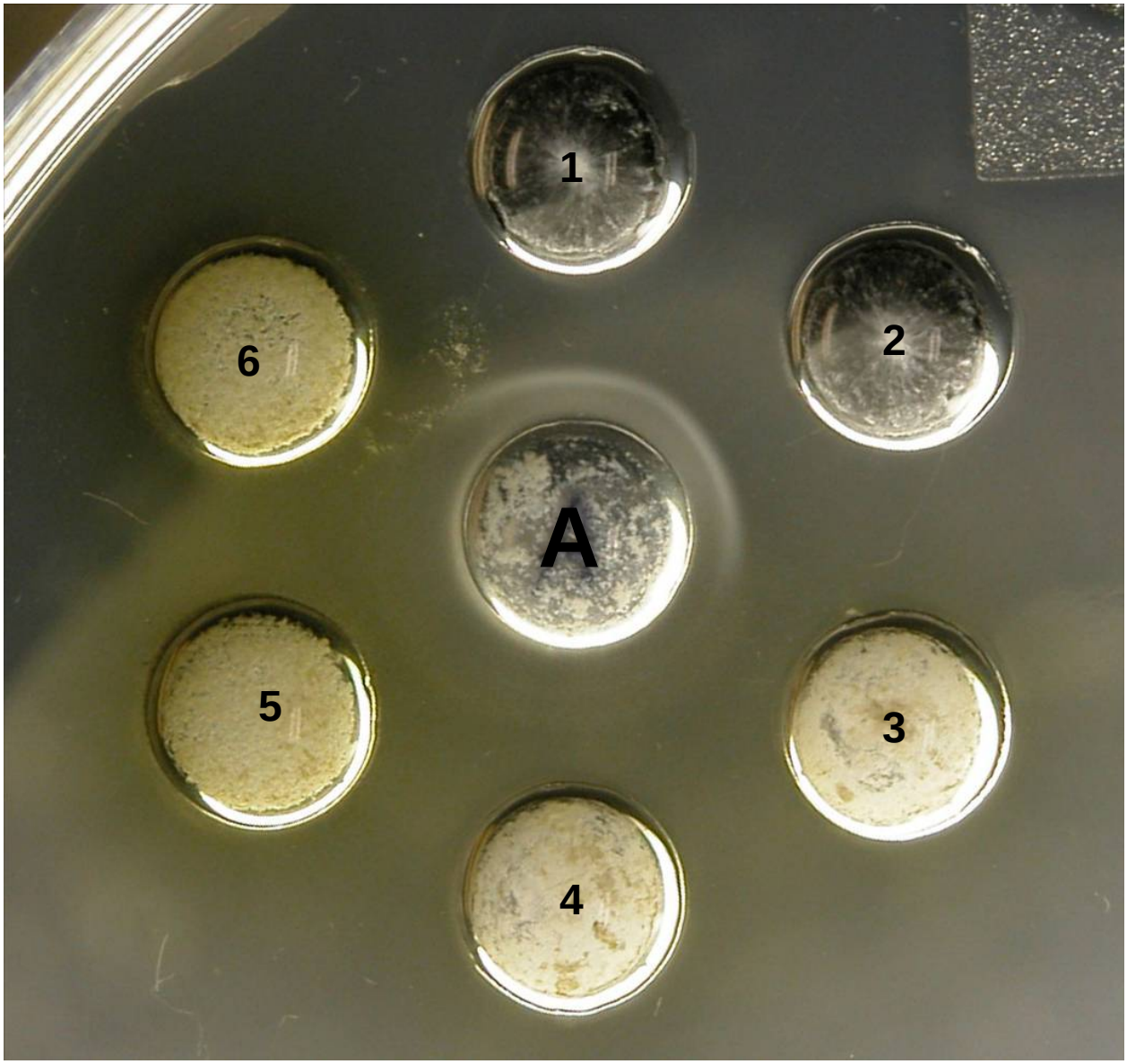


Figure 2-3. TMGMV PV-0113 reacts with rabbit anti-TMGMV serum in SDS media. 1,2 = TMGMV PV-0113; 3,4 = ToMV; 5,6 = TMV; A – rabbit anti-TMGMV serum.

Figure 2-4. TMGMV-PV-0113 reacts with rabbit anti-TMGMV serum in non-SDS media. 1,2 = TMV, 3,4 = TMGMV PV-0113, 5,6 = healthy tobacco sap (no antigen); B = rabbit anti-TMGMV serum.

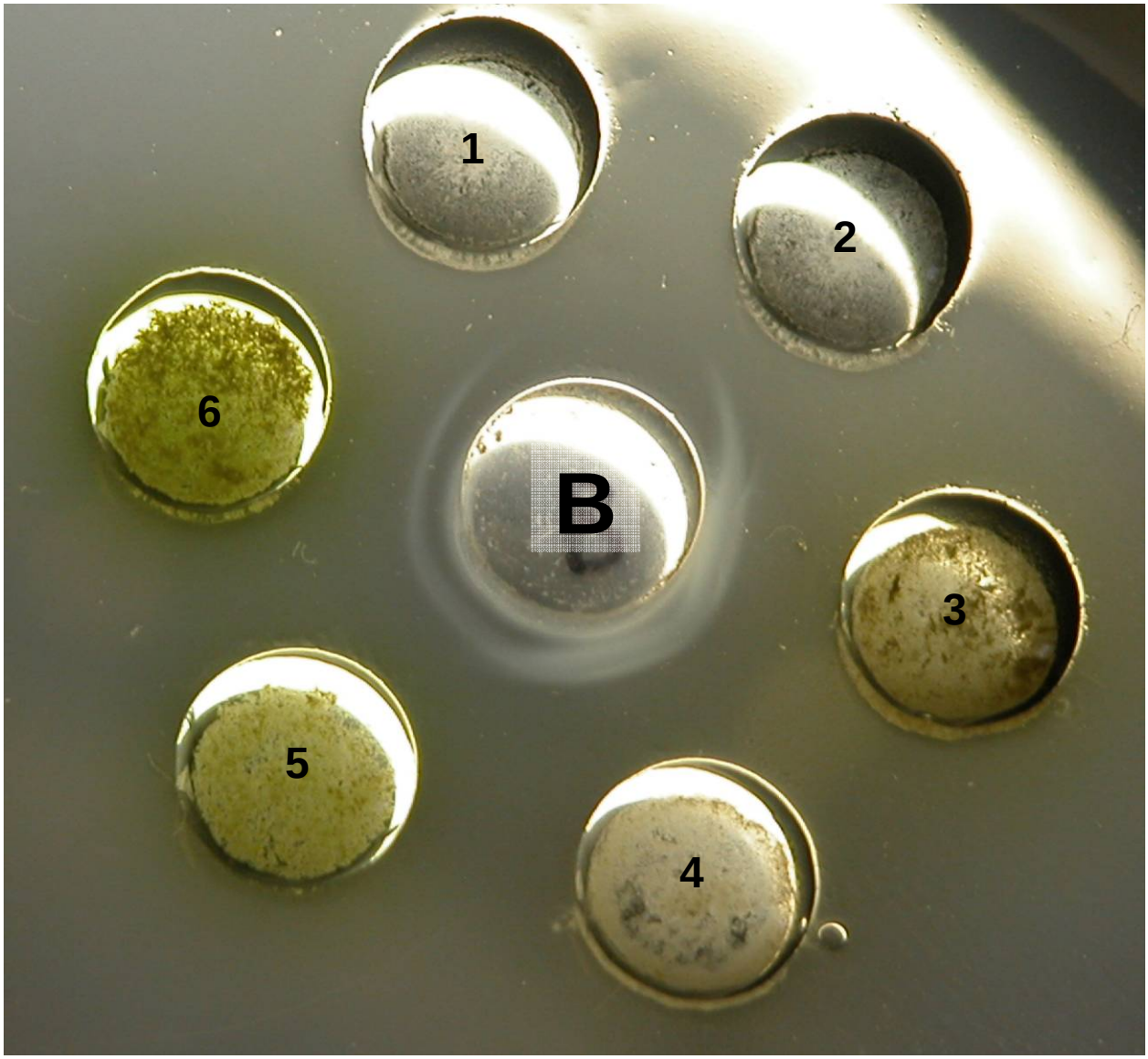


Figure 2-5. TMGMV PV-0113 does not react with rabbit anti-ToMV serum in SDS media. 1,2 = TMGMV PV-0113; 3,4 = ToMV; 5,6 = TMV; B = rabbit anti-ToMV serum.

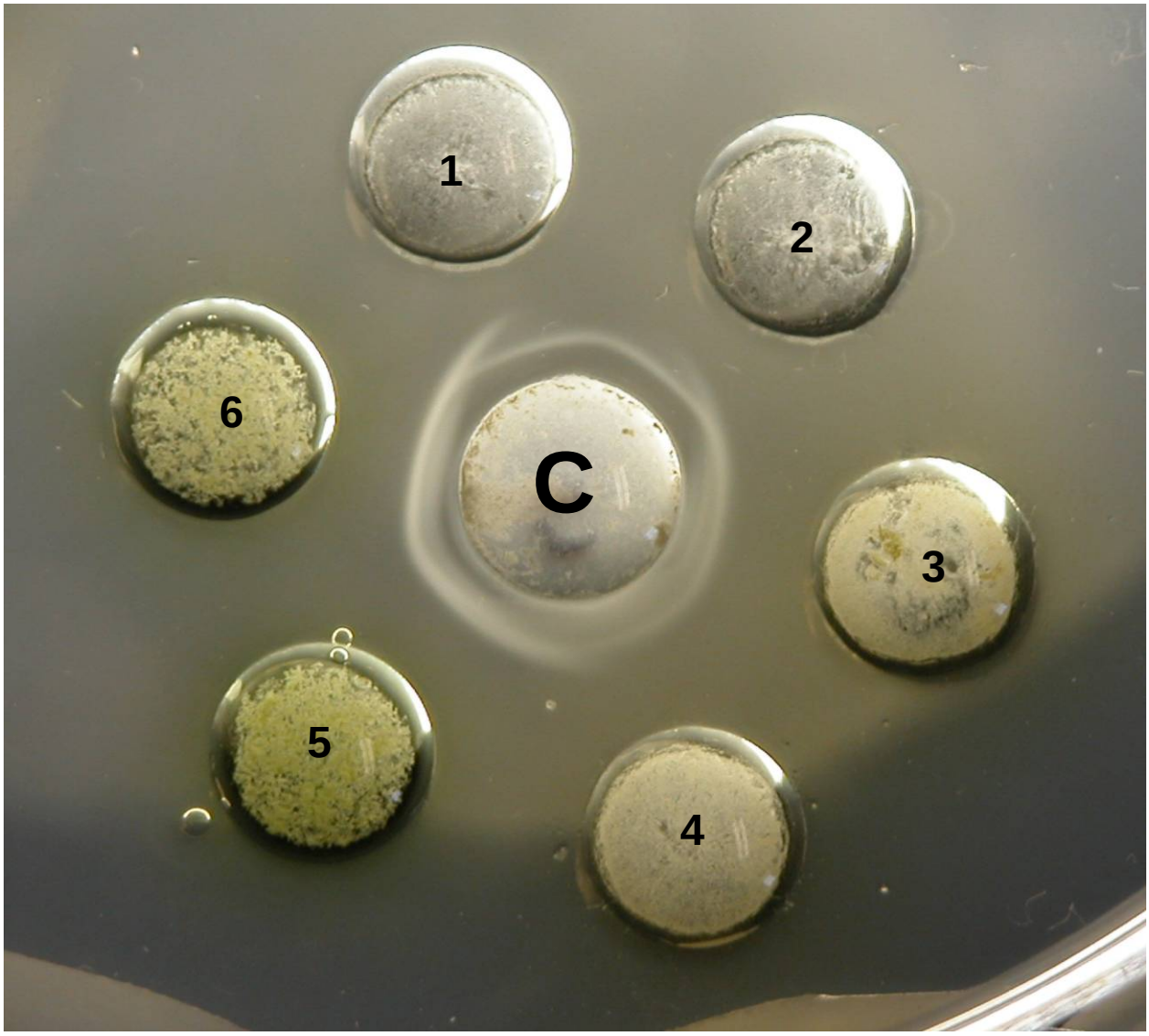


Figure 2-6. TMGMV PV-0113 reacts weakly with rabbit anti-TMV serum in SDS media. 1,2 = TMGMV PV-0113; 3,4 = ToMV; 5,6 = TMV; B = rabbit anti-TMV serum.

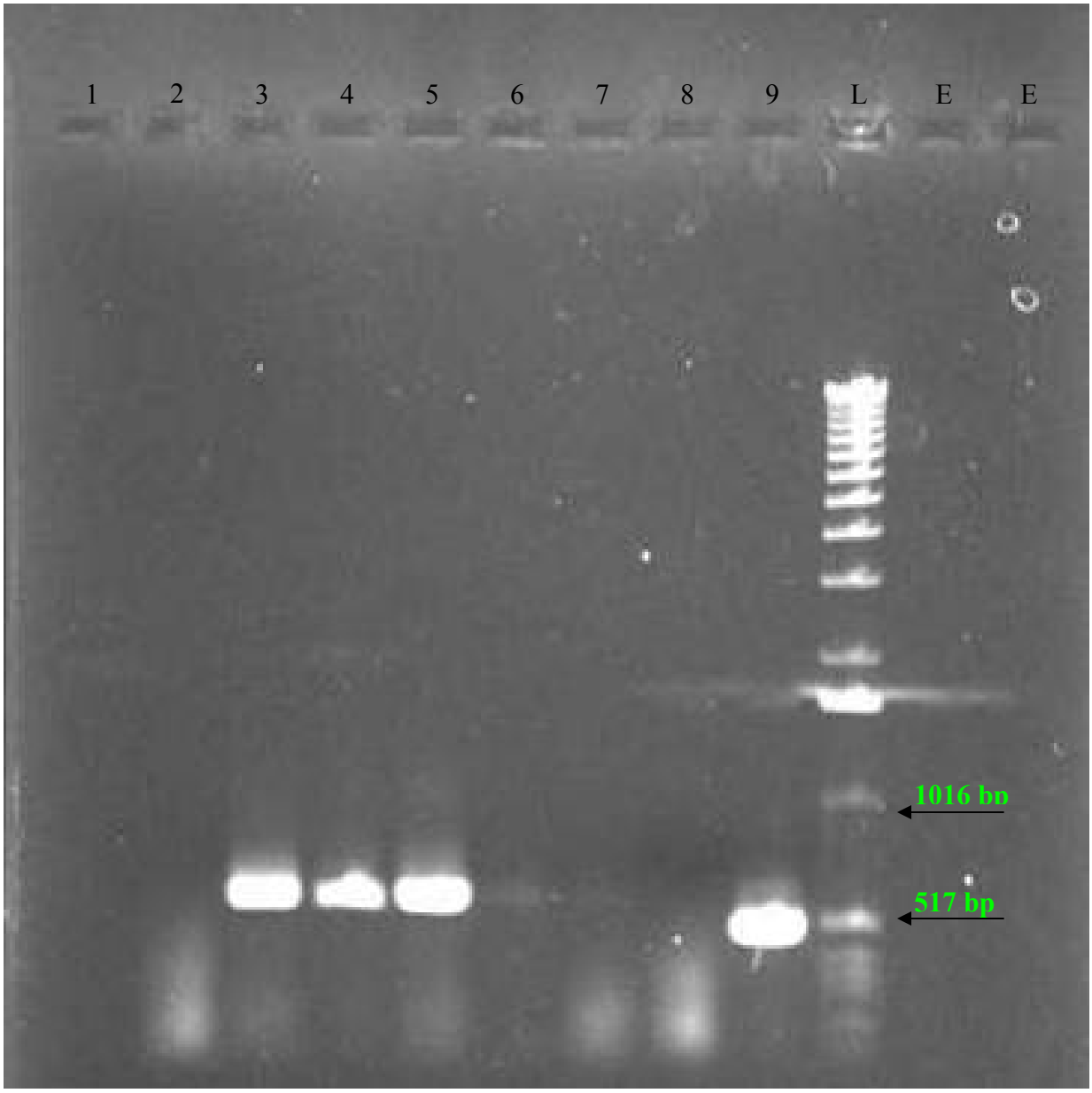


Figure 2-7. RT-PCR products of TMGMV PV-0113 and TMV, amplified with diagnostic primers. 1 = H_2O only, 2 = negative control (no template), 3,4,5 = TMGMV PV-0113 from production house, 6,7,8 = SLL inoculated plants (asymptomatic), 9 = TMV (using Manjunath TMV primer set), L = 1 kB Invitrogen ladder, E = empty lane

TMV Primer 1 (423): 5′ ATG TCT TAC AGT ATC ACT AC 3′
TMV Primer 2 (424): 5′ TCA AGT TGC AGG ACC AGA G 3′
Source: Chandrika Manjunath

TMGMV Primer 1 (400): 5′ ATG CAG CTT CCA TTT TGG CAG 3′
TMGMV Primer 2 (397): 5′ GGT AAG TTA ACG CTT TGG CTT G 3′
Source: Virology (1996) 223: 148-155.

CHAPTER 3
COLUMNEA ISOLATE PV-0113: SUBTYPE, SATELLITE, AND DISEASE PROGRESS

Introduction

Tobacco mild green mosaic virus (TMGMV) has been found naturally associated with Satellite tobacco mosaic virus (STMV) in California. Satellite viruses, including STMV have been known to attenuate or otherwise modify the expression of the helper virus' disease phenotype in some hosts (Dodds, 1998). Single local lesion (SLL) passage may be inadequate to remove STMV from a TMGMV culture (Valverde et al., 1991); the presence or absence of STMV in the *Columnea* isolate, TMGMV PV-0113, is therefore a concern. It is possible that the presence of STMV could affect symptomatology of the TMGMV-TSA interaction. Direct methods for detecting/observing STMV infection include centrifugation and dsRNA extraction, where the dsRNA extract will reveal a band of 1059 bp when subjected to electrophoresis on agarose or polyacrylamide.

The task of detecting contaminants is complicated by the tendency for the heritable material of viruses to change during replication, giving rise to new genotypes or "strains." The countless multitudes of virions in every host constitute a "quasi-species" —a collection of related genomes undergoing constant pressures of mutation, competition, and selection. The quasi-species status of a virus population helps ensure survival by maintaining a pool of genotypes such that at least some members may survive and proliferate should conditions change. Should a favorable mutation arise, the distribution of genotypes within the quasi-species will shift to accommodate it (Hull, 2002).

One method for reducing genotypic variability in a plant virus culture is isolation from a single local lesion (SLL). Although at least 50,000 virus particles are said to be required to initiate infection (Roberts, 1964), individual local lesions (LL) may be excised in order to restrict

the culture to the genotype of the founder populations at the infection foci. Subsequent repeated inoculations onto hypersensitive hosts cause the gene pool to become increasingly restricted.

The two subtypes of Tobacco mild green mosaic virus, TMGMV-S and TMGMV-L, are believed to compete with each other in *Nicotiana glauca* and *N. tabacum* cv. "Xanthi," usually resulting in the extinction of one subtype or the other within the host. Mixed infections in plants first inoculated with TMGMV-S are possible, at least for a limited time period after the primary infection (Bodaghi et al., 2004). The effect of a mixed infection of TMGMV-S and TMGMV-L on tropical soda apple (TSA) has not been reported, nor attempted.

Some tobacco species have been reported to express differential symptoms following inoculation with TMGMV-L or TMGMV-S (Bodaghi et al., 2000). *Nicotiana benthamiana* developed wilting 5 days after inoculation with TMGMV-L, but not until 7 days following inoculation with TMGMV-S. Death occurred in TMGMV-L inoculated plants by day 10, but not until 14 days post-inoculation (DPI) in TMGMV-S inoculated plants. Small-type TMGMV-inoculated *N. clevelandii* plants were systemically necrotic by 15 DPI, whereas large-type TMGMV-inoculated plants survived with shoot proliferation and rugosity. *Nicotiana rustica* plants developed LL in response to inoculation with either sub-type, but only those inoculated with TMGMV-L developed systemic infection. *Nicotiana rustica* inoculated with TMGMV-L also developed larger LL (Bodaghi et al., 2000).

The objective of this study/chapter was to first determine to which subtype TMGMV PV-0113 belongs, based on the reaction of inoculated differential indicator plants and by RT-PCR. The second objective is to assess the presence/absence of STMV in this isolate by subjecting extracted dsRNA was subjected to electrophoresis in agarose and polyacrylamide. Immunodiffusion using rabbit antiserum prepared to STMV was also used to detect STMV. To

better characterize and quantify the disease process produced in TSA by TMGMV PV-0113, a rating scale based on relatively unambiguous symptoms was devised.

Materials and Methods

Three-week old tobacco plants (*N. tabacum*; cv. Samsun; genotype *nn*), were inoculated with TMGMV PV-0113, which is known to produce lethal disease in TSA. After symptoms (leaf pleating, mild green mosaic) developed (2 to 4 weeks post-inoculation; WPI), leaf tissue was harvested and used to inoculate *Eryngium planum* (Apiaceae). Symptoms of viral infection (yellow streaks and flecks) became apparent after several weeks, and newly emergent leaves were harvested. *Eryngium* tissue was triturated in 20 mM $NaHPO_4/Na_2PO_4$ inoculation buffer (pH 7.2) and applied to the hypersensitive host, *N. tabacum* cv. Samsun (*NN*). LL appeared within 72 to 96 hours; select individual lesions were harvested using a narrow-blade scalpel, with care being exercised to avoid including excess leaf tissue. Each piece of excised leaf tissue (1-2 mm^2) was homogenized in 0.3 mL of 20 mM $NaHPO_4/Na_2PO_4$ inoculation buffer (pH 7.2) in a 1.5 mL micro-centrifuge tube using a Kontes micro-pestle. Additionally, 2-3-week-old tobacco plants (both *nn* and *NN* genotype) were dusted with carborundum and inoculated by pestle (e.g. simply rubbing the upper surface of the leaf with the pestle.) After formation, LL were harvested by the aforementioned method and inoculated onto *N. tabacum* cv. Samsun (*nn*). Symptoms developed after a time, and apical tissue from these isolated plants was harvested.

All isolates were tested simultaneously under conditions as identical as possible. Three- to 4- week-old TSA and 3- to 4-week-old tobacco (*N. tabacum* cv. "Samsun;" genotype *nn*) plants were inoculated with the SLL isolates and maintained in a greenhouse, at temperatures between 16°C and 28°C. The day length was approximately10 hours, supplemented by artificial lighting (fluorescent bulbs) for a 15-hour total photoperiod. Plants in this study were fertilized weekly

with a 280 ppm solution of Peter's 20-20-20 soluble plant food. The plants were observed carefully for potential differences in symptom development.

To determine the subtype of TMGMV PV-0113, the differential indicator species *N. benthamiana*, *N. clevelandii*, and *N. rustica* were grown and inoculated as previously described in Chapter 2.

Primers to the 3′ NTR were designed to clone the region for analysis and further research. The forward primer:

5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCTTATACAATCAACTC 3′ was designed to begin annealing to the template from the start of ORF 4, (nt 5666-5687), and had a Tm of 66.2°C. The reverse primer:

5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTGGGCCGCTACCSGCGGTTA 3′ was designed to anneal nt 6355, to nt 6336, with a Tm of 73.3°C. This primer included the degenerate base, S to allow for G/C dimorphism in the 3' NTR occurring in some strains of TMGMV (Bodaghi et al., 2000).

To determine whether or not STMV was present in the TMGMV PV-0113 culture three cultures were propagated in tobacco and dsRNA extracts prepared from the infected tissue: TMGMV PV-586 (a culture of TMGMV-L containing STMV), TMGMV PV-0113 (TMGMV PV0113, the culture being evaluated), and CMV (Cucumber mosaic virus, a tripartite non-tobamovirus). CMV was propagated in *N. glutinosa* to prevent tobamovirus contamination, and the two TMGMV cultures were propagated in *N. tabacum* cv. Samsun (*nn*). Tissue harvested from apical regions 3 WPI was used in a modified Morris and Dodds dsRNA extraction procedure (Morris and Dodds, 1979), as follows:

Seven grams of each infected tissue sample was quick-frozen under liquid N_2, triturated in a chilled sterile mortar, and added to beakers containing a cold (4°C) mixture of 14 mL 2x STE buffer (pH 6.8), 20 mL equilibrated phenol, 2 mL 10% SDS, and 14 mg of bentonite. The mixture was held at 4° C while stirring for 30 minutes, after which the mixtures were centrifuged 15 minutes at 8,000 rpm in phenol-resistant, NaOH-treated sterile (Oakridge) centrifuge tubes. The aqueous phase was then collected, the volume adjusted to 20 mL with 1x STE buffer (pH 6.8), and 3.8 mL 95% ethanol was added, along with 1.0 to 1.5 g Whatman CF-11 charged cellulose powder, under continued stirring at 4°C.

One hour later, the mixtures were poured into columns prepared from 12 mL disposable syringes and baked glass wool, using a solution of 15.0 to 16.5 % ethanol in 1x STE to wash the cellulose until colorless. Samples were then pressed dry using the syringe pistons and dsRNA eluted with 14 mL of 1x STE. To the eluent, 1/10 vol. 3M NaAc (pH = 5.5) and 2.5 vol. 95% ethanol were added, and the samples incubated overnight at - 20°C. The samples were then centrifuged at 9,000 rpm (9,750 x g) for 30 minutes at 4°C to recover the precipitated RNA, which was washed once with 70% ethanol, dried under vacuum, and dissolved in 50 to 100 µL of sterile, distilled, deionized water.

This procedure was repeated using different concentrations of ethanol in the wash step until samples were obtained that produced relatively distinct dsRNA bands on non-denaturing agarose and polyacrylamide gels. To reduce background contamination, samples were then digested at 37°C for 1 hour using 4 to 6 units of DNAase I per 100 µg of nucleic acid extracted, after which samples were heated to 75° C for 10 min in order to inactivate the enzyme.

The presence/absence of STMV was also assessed using immunodiffusion on non-SDS media, (containing 0.7% noble agar, 0.85% NaCl, 0.03% NaN_3, and 5 mM Tris, pH 7.5). Sixty

(60) µL of undiluted rabbit anti-STMV serum (kindly provided by Deborah M. Mathews; University of California, Riverside) was loaded into the central well of each system. Sixty (60) µL of the antigens TMGMV PV-586 (ATCC accession deposited as TMGMV-L + STMV), TMGMV PV-0113, and sap from uninoculated tobacco (Hsap) were loaded into the surrounding wells.

Results

There was little variability in the disease symptoms produced by the SLL isolates in tobacco, with all isolates producing symptoms typical of TMGMV infection: leaf pleating, faint green mosaic, and flower-breaking. Likewise, all isolates retained the ability to infect and kill TSA, and the rate of disease progress was much the same for each isolate (data not shown).

The timing of disease onset produced by each isolate inoculated onto TSA was consistent; all plants inoculated in the first and second repetitions developed symptoms 4 DPI. Symptom development in two isolates used in the third repetition was delayed 1 and 3 days, respectively. Progress of the disease in TSA produced by TMGMV PV-0113 was also rather consistent. The first observable symptom, epinasty, was not as unambiguous as other symptoms, being easily confused with wilting from natural leaf movement in response to time of day, temperature stress, and wilting from other causes). However, data collected indicate that plants developing epinasty did so within 5 DPI.

Two types of hypersensitive response (HR) were observed: discrete LL, and patchy, more diffuse necrosis. Discrete LL often took the form of star-like spots, with a central lesion having minute necrotic veins extending outward from the focus. Usually beginning at 5 DPI, local lesions/necrotic patches were nearly always present by 9 DPI, although sometimes plant collapse made observation difficult. Epinasty and LL/necrotic patches were nearly always immediately followed by permanent apical wilting, resulting in collapse of the plant. The expression of

wilting was modified by the age, size, and condition of the plant. Young, small, and rapidly-growing plants (usually ≤ 3 weeks) would often completely lose turgor and collapse, while in older, larger, and slowly-growing plants (usually > 3 weeks), the wilting was confined to the young tissue around the apical regions. There was some variability in the onset of this symptom between the sets of single local lesion (SLL) isolates; however, this stage of the disease had usually occurred by 12 DPI

Perhaps the most variable symptom observed in the greenhouse was leaf abscission. This symptom was not always present and time of onset was variable, occurring between 8 and 21 DPI. When it occurred, especially in more mature specimens, the leaf abscission symptom would often progress to complete defoliation of the plant.

Disease progress in TMGMV-infected greenhouse-grown TSA inevitably culminated in death. Despite the consistent mortality, some plants, though completely defoliated and mostly necrotized, retained living (green) stem tissue for more than a month. Based on general observations of TMGMV-infected TSA, a scale (Table 3-1) was devised to measure the disease progress, and indexed to photographs (Figure 3-1) for comparison.

Symptoms produced by TMGMV PV-0113 on the experimental indicator hosts *N. benthamiana* and *N. clevelandii* were generally consistent with the results reported for small-type TMGMV (TMGMV-S, "U2") (Bodaghi et al., 2000), while symptoms produced by TMGMV PV-0113 on *N. rustica* were variable, but similar to results reported for large-type TMGMV (TMGMV-L, "U5") (Bodaghi et al., 2000). Timing of death in the host *N. benthamiana* was variable, but always later than 10 days post-inoculation. Infection in *N. rustica* by the culture provided as "U2" was not confined to LL, but became systemic.

Symptoms produced on *N. clevelandii* displayed characteristics of both TMGMV-L and TMGMV-S infection as reported in literature (Bodaghi et al., 2000). Although the plants often developed systemic necrosis, this was preceded in some cases by leaf deformation and shoot proliferation, a symptom reported for TMGMV-L-infected *N. clevelandii*. Symptoms of TMGMV PV-0113 on *N. rustica* varied between experimental sets, but LL were always present.

Reverse transcriptase-PCR using primers specific to the 3′ NTR of TMGMV generated a product of different size depending upon the culture analyzed (Figure 3-2). A culture obtained from CREC, Lake Alfred, as TMGMV "U2" did not generate any product. A culture obtained from CREC, Lake Alfred, as TMGMV "U5," and TMGMV PV-586, known to contain TMGMV-L, both produced products visibly larger than that generated by TMGMV PV-0113.

Relatively impure dsRNA was obtained from TMGMV-infected tobacco leaf tissue using the extraction method employed. Content of dsRNA relative to other nucleic acid materials was highly enriched by the procedure, but contaminants were not completely eliminated. Electrophoresis on agarose and polyacrylamide allowed visualization of subgenomic RNA bands. Digestion using DNA-ase I helped to reduce background created by contaminating host DNA molecules. Nucleic acid bands of a size consistent with STMV could be visualized when dsRNA extract from TMGMV+STMV-infected tissue was analyzed on both polyacrylamide (Figure 3-3) and agarose (Figure 3-4) gels. Satellite tobacco mosaic virus dsRNA was present in quantity and easy to observe in TMGMV culture PV-586 (which is reported to contain it; Anonymous, 2007). It was not observed in any of the extracts from TMGMV PV-0113. Rabbit anti-STMV serum reacted (forming precipitin) to the STMV-containing ATCC accession PV-586, but not to TMGMV PV-0113 or healthy tobacco (Figure 3-5).

Discussion

The disease phenotypes of isolates within each series appeared similar. All SLL isolates tested demonstrated the ability to kill TSA; symptoms on both tobacco and TSA appeared identical. Based on these observations it seems unlikely that TMGMV PV-0113 is a mixture of viruses, but is instead, a quasi-species, with all contributing genotypes retaining the ability to kill TSA.

While data from the use of inoculated indicator hosts was not conclusive, the results were usually consistent with symptoms reported in literature. Greenhouse-grown, uninoculated *N. clevelandii* plants have been observed with thickened, waxy, and distorted leaves similar to the effect seen on some TMGMV-inoculated ones. Perhaps, also, subtle differences in the genotype of virus, host, or growing conditions used resulted in these anomalies. Nevertheless, gel electrophoresis of RT-PCR product corresponding to the 3′ end of the virus indicated that the smaller fragment produced by TMGMV PV-0113 corresponds with TMGMV-S and not the larger fragment produced by TMGMV-L.

While all isolates used in the subtype host-range determination by Bodaghi, Yassi, and Dodds (2000) were subcultured using SLL isolation, nearly all of the cultures initially contained STMV, which has been shown to modify disease phenotype in some hosts (Bodaghi et al., 2000). Since TMGMV PV-0113 does not contain STMV, it is possible that the reaction of these hosts to TMGMV-S and TMGMV-L cultures lacking STMV will be different from those reported in the study (Bodaghi, et al., 2000). The figures may be misleading, because the criteria for rating changed over time, upon realization that plants could recover. At the time data was recorded, plants were rated dead when leaves were necrotic, or the plant completely, or nearly completely defoliated, or after collapse had occurred.

Rabbit anti-STMV serum reacted (forming precipitin) to the STMV-containing ATCC accession PV-586, but not to TMGMV PV-0113 or healthy tobacco. These results, combined with the absence of visible STMV dsRNA in extracts, support the conclusion that TMGMV PV-0113 is a pure culture of TMGMV-S and does not contain STMV.

Table 3-1. Scale for rating disease progress in TMGMV-infected TSA.

	Mature Plant	Immature Plant
0	**Healthy**, no disease symptoms	**Healthy**, no disease symptoms
1	**Local lesions** and/or necrotic spots	**Local lesions** and/or necrotic spots
2	Permanent apical **wilting**, leaf abscission	Permanent **wilting**
3	Complete **defoliation**, green stem	**Collapse**, complete loss of turgor
4	**Dying**, stem ≥50% necrotic	**Dying**, plant ≥50% necrotic
5	**Dead**, stem 100% necrotic	**Dead**, plant 100% necrotic

A   B


C  D


E  F

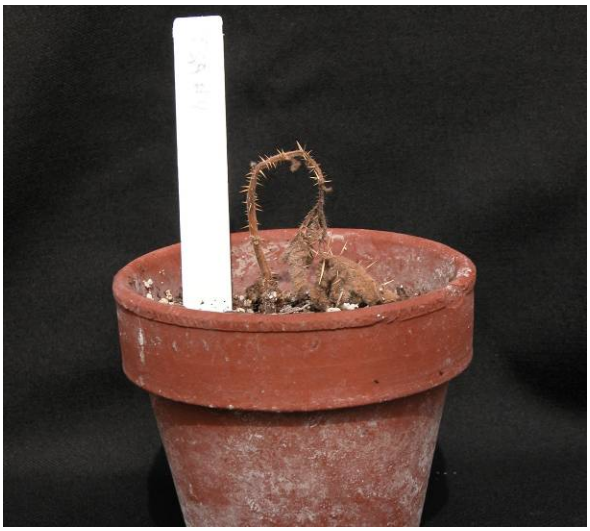
G   H

Figure 3-1. Disease progress in TSA plants inoculated with TMGMV PV-0113. A) "0," 3 week old TSA immediately prior to inoculation. B) "1," TSA developing local lesions (LL) and epinasty 5 DPI. C) Close-up of LL seen through abaxial surface of leaf, 5 DPI D) "2," permanent wilting beginning at shoot apices on TSA plant 7 DPI E) TSA plant 11 DPI F) "3," green stem fully defoliated/leaves necrotic on TSA plant 12 DPI G) "4," necrosis spreading along stem on TSA plant 28 DPI H) "5," Dead (all visible tissue completely necrotic) TSA plant 37 DPI (Photographs, except for F and G, are of the same plant.)

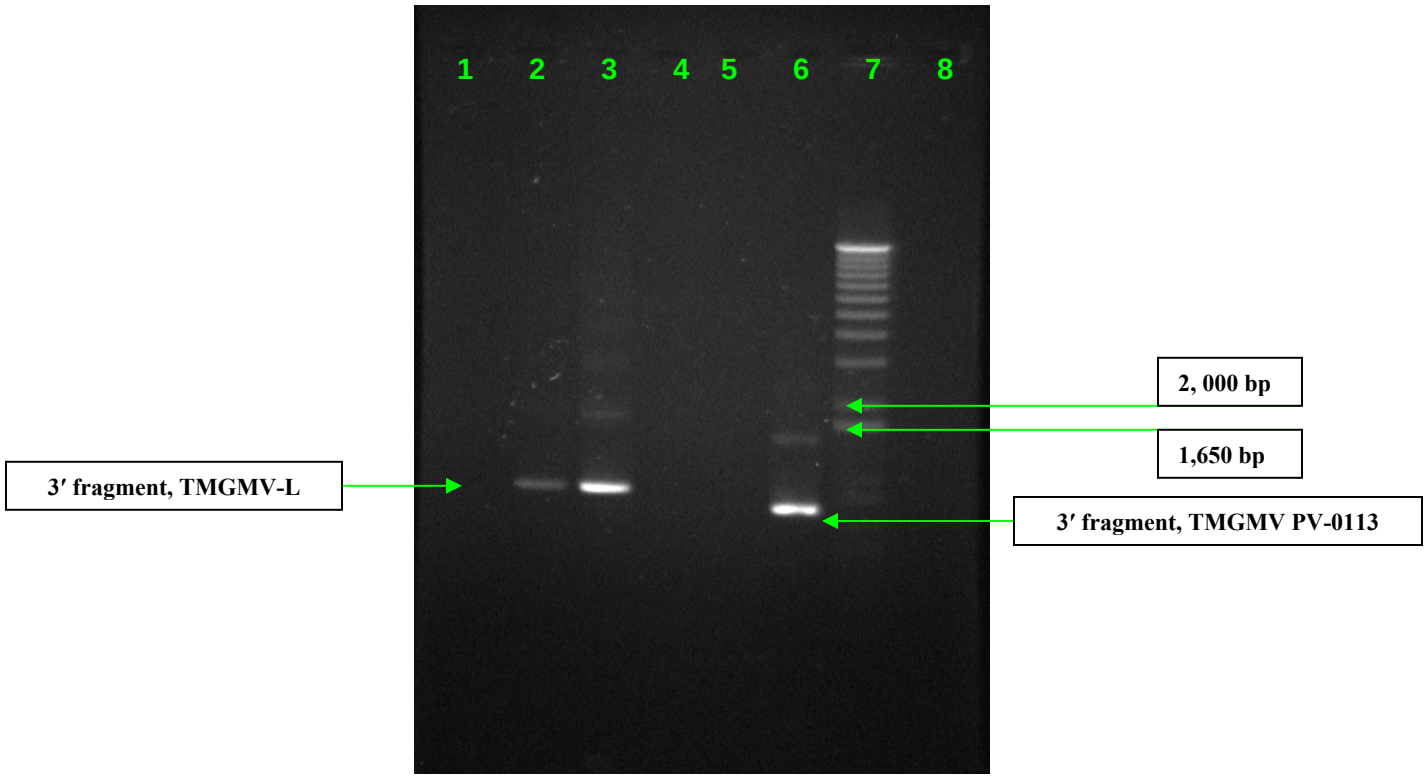


Figure 3-2. Agarose gel analysis of RT-PCR products. 1 = blank, 2 = TMGMV-LAU5, 3 = TMGMV-PV-586 (U5+STMV), 4 = TMGMV-LAU2 (No. 1), 5 = TMGMV-LAU2 (No. 2), 6 = TMGMV PV-0113, 7 = 1 kB ladder, 8 = empty.

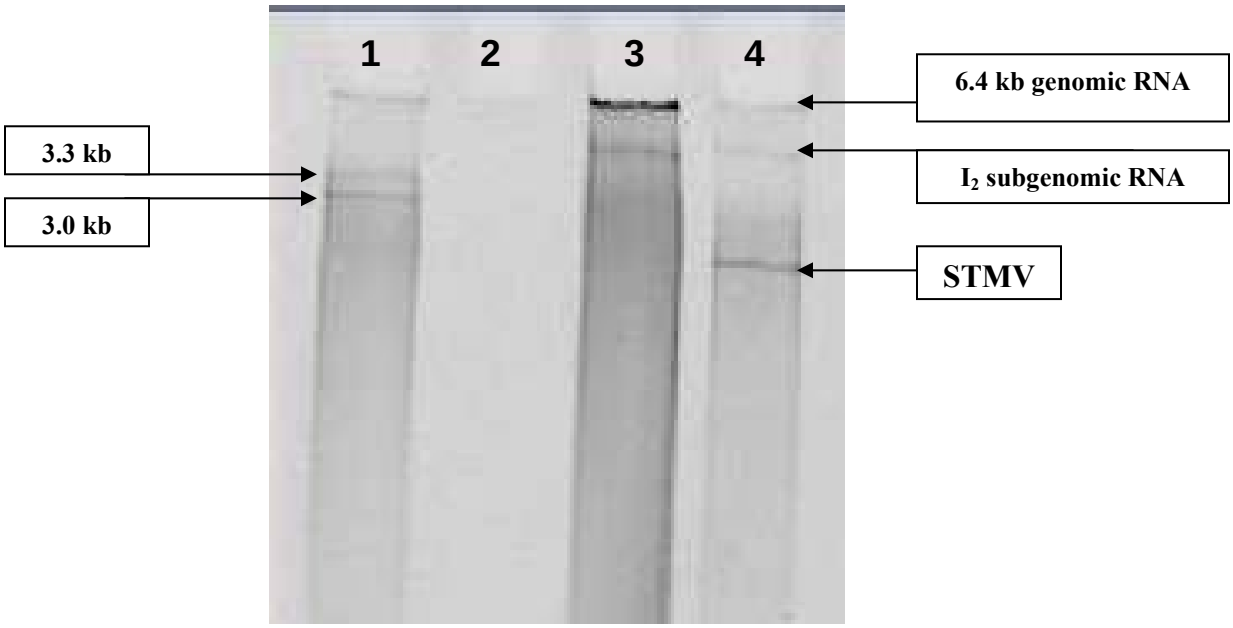


Figure 3-3. Comparison of dsRNA extracted from TMGMV (PV-0113) with that of TMGMV (PV-0586) demonstrating the absence of satellite TMV in isolate TMGMV (PV-0113) resolved in a polyacrylamide gel. Lane: 1 = CMV, 2 = blank (H_2O + EtBr), 3 = TMGMV PV-0113, 4 = TMGMV-PV-586 (TMGMV-L + STMV).

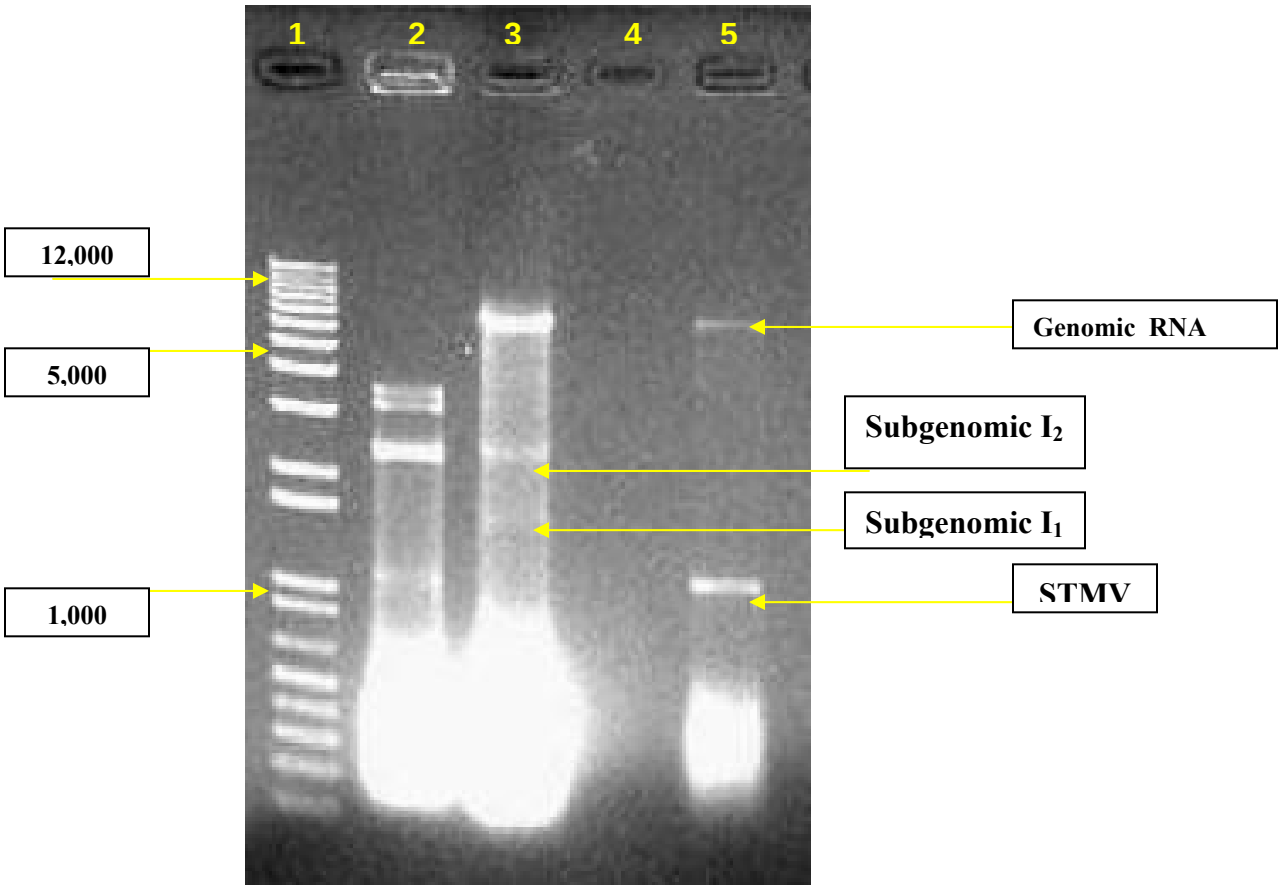


Figure 3-4. Comparison of dsRNA extracted from CMV, TMGMV (PV-0113), and TMGMV-(PV-586) demonstrating the absence of satellite TMV in isolate TMGMV (PV-0113) resolved in an agarose gel. 1 = 1 kB Invitrogen plus ladder, 2 = CMV, 3 = TMGMV PV-0113, 4 = blank, 5 = TMGMV-PV-586.

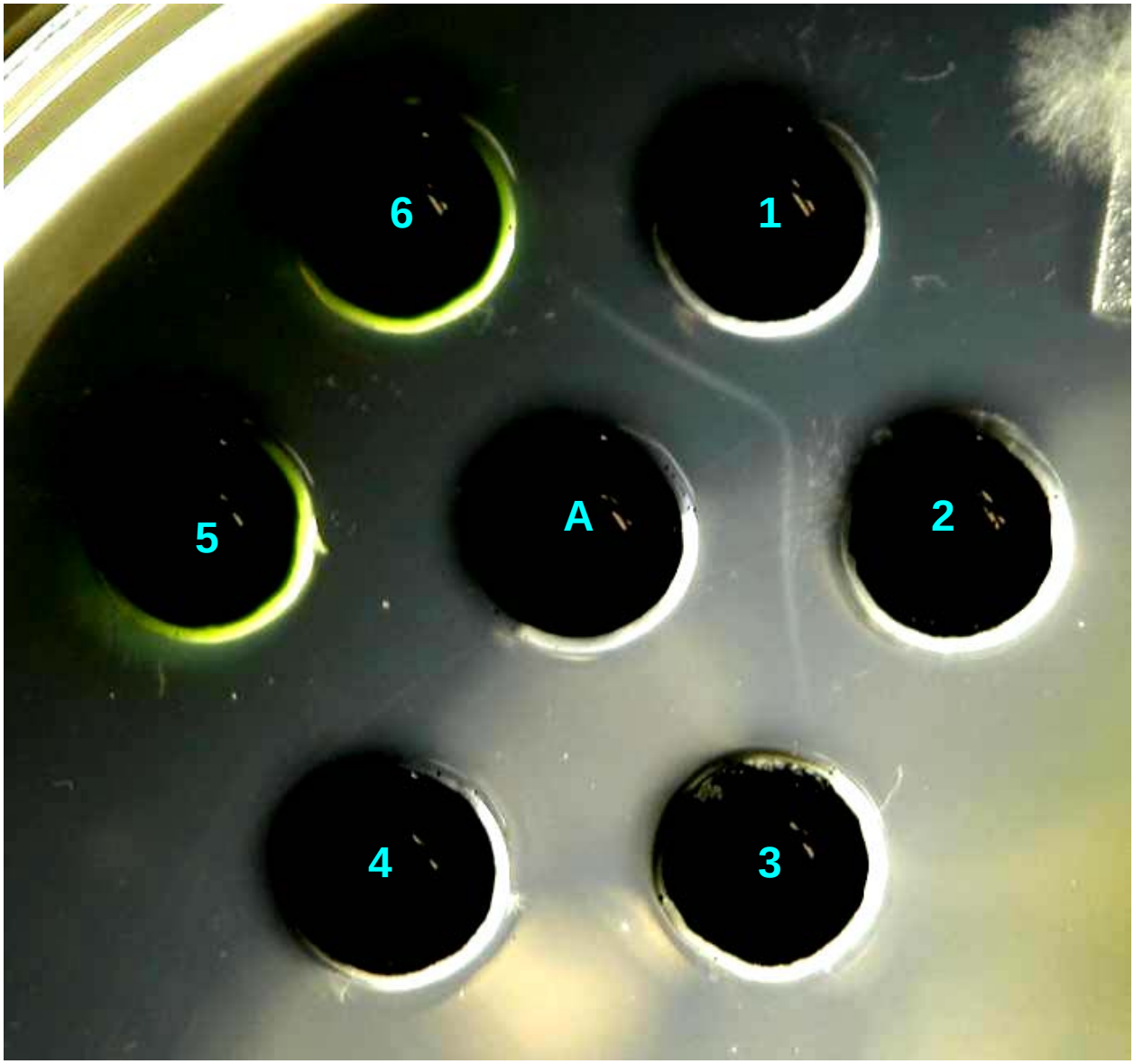


Figure 3-5. Imuunodiffusion analysis with rabbit anti-STMV serum showing no detectable reaction with TMGMV PV-0113 culture. 1,2 = ATCC PV0586; 3,4 = TMGMV PV-0113; 5,6 = Healthy tobacco sap.

CHAPTER 4
DISEASE PHENOTYPE AND COMPARISON OF PV-0113 WITH OTHER CULTURES

Introduction

Since the 1927 collection of TMGMV described as "mild dark-green mosaic strain of TMV" from Gran Canaria in the Canary Islands, (McKinney, 1929), the species has been recovered from several locations, including Australia, France, Germany, Israel, Italy, Japan, Taiwan, and the United States (Bodaghi et al., 2000; Cohen et al., 2001;. Conti and Marte 1983; Li and Chang, 2005; McKinney, 1952; Morishima et al., 2003; Parrella et al., 2006; Wetter, 1989). Within the United States, samples of TMGMV have been obtained from nurseries or outdoor sources in Florida, Georgia, Ohio, South Carolina, Wisconsin, and elsewhere (Baker and Zettler, 1988; Bodaghi et al., 2000; McKinney, 1952; Wetter, 1989; Zettler and Nagel, 1983). Zettler and Nagel (1983) sampled TMGMV (then called TMV U-2) from cultivated gesneriads obtained from growers in California, Connecticut, Florida, and Ohio. The strain isolated from an Ohio sample of *Columnea* was archived as DSMZ accession PV 0113. Reference cultures of TMGMV exist in the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen, the German Resource Center for Biological Material (DSMZ).

Despite functional limitations on change for tobamovirus genes (Fraile et al., 1996.) enough genotypic variation exists in the virus to influence expression of disease phenotype in different hosts (Bodaghi et al., 2000.). For example, some isolates of TMGMV cause lethal systemic hypersensitive response (LSHR) in *Nicotiana benthamiana* (Bodaghi et al., 2000), while an isolate from Taiwan identified as TMGMV caused systemic mosaic symptoms on this host (Li and Chang, 2005). As mentioned previously (chapters 1 and 2; Introduction) , TMGMV

exists in both large type (TMGMV-L) and small type (TMGMV-S) varieties. Infections also sometimes contain Satellite tobacco mosaic virus (STMV).

That different strains, or, at least, subtypes of TMGMV are capable of producing different symptoms on different hosts was established by Bodaghi et al. (2000.), who identified three *Nicotiana* species in which disease progress and/or character varied enough that these species could serve as indicators capable of distinguishing TMGMV-L from TMGMV-S. *Nicotiana benthamiana* expressed delayed symptom development when inoculated with TMGMV-S, relative to TMGMV-L; *N. clevelandii* was killed by TMGMV-S, but not TMGMV-L, and *N. rustica* was systemically infected by TMGMV-L but not TMGMV-S (Bodaghi et al., 2000).

Although overall *yield* of TMGMV-S from infected plants was higher (~2x) than from those infected with TMGMV-L (Bodaghi et al., 2000), experiments have shown that TMGMV-L competes strongly with TMGMV-S preventing it from becoming established, suggesting that the duplication in the 3′ pseudoknot-containing region may confer some competitive advantage, such as faster replication, gene expression, or movement (Bodaghi et al., 2004).

It is known that the pseudoknot-containing region of the 3′ NTR binds cellular proteins, and seems to interact with the Ω leader sequence to enhance translation (Leathers et al., 1993). Duplication of this region in TMGMV-L might improve competition for host resources. Based on observations that proximity to the 3′ NTR affects levels of gene expression, it was discovered that transgene expression may be enhanced by placing a copy of the pseudoknot-containing region immediately downstream (Shivprasad et al., 1999).

STMV does not modify visible symptoms in the hosts *N. tabacum* or *N. glauca*, but in *Capsicum annuum* symptoms produced by the helper virus are modified, and in this host accumulation of the helper virus is severely reduced (Dodds, 1998). Furthermore, the genome of

STMV is itself variable, with at least 14 unique clones; one infectious isolate survived despite a 71-nt deletion (Mathews and Dodds, 1998).

An example of the effect a satellite virus might have can be found in the systemic necrosis induced in tomato (*Lycopersicon esculentum*) by the negative sense (-) strand of the CMV-associated D satellite RNA (D-sat RNA). Different satellite RNAs associated with CMV cause different effects within a particular host, and may add to or detract from the virulence of the helper virus. Thus, while D-sat RNA induces a lethal systemic necrosis, WL1-sat RNA attenuates CMV disease symptoms, and B-sat RNA induces chlorosis. Furthermore, these modifications are host-dependent: the tomato-lethal D-sat RNA attenuates CMV symptoms in tobacco (Taliansky et al., 1998; Xu and Roosinck, 2000).

When this study was undertaken, it was not known whether TMGMV PV-0113 is the only strain of TMGMV that kills TSA or whether all or some isolates are equally lethal. In order to determine this, and the possible differences in disease phenotype, the two cultures of TMGMV, TMGMV-S and TMGMV-L were compared on TSA and other species.

Materials and Methods

To characterize TMGMV disease on TSA, inoculation trials were conducted using plants propagated under various temperature and lighting conditions (greenhouse, outdoor, indoor, and growth chamber). The following plant species were tested in various experiments: *Capsicum annuum*, *Eryngium planum*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, *N. sylvestris*, and *Solanum viarum* (TSA). All were grown from seeds and inoculated at 2-4-weeks post-emergence. These trials were repeated with other available isolates of TMGMV-S and TMGMV-L, including an ATCC accession, PV-586 – a culture of TMGMV-L reported to contain STMV. In addition to TSA, indicator species used by Bodaghi et al. (2000) were

included in some experiments, as well as *C. annuum*, in the hope that one of the isolates might prove virulent to TSA, but not to the commercially important *C. annuum*.

Two cultures of TMGMV, one each of "U2" (TMGMV-S) and "U5" (TMGMV-L) were obtained from the Plant Pathology Department, University of Florida, Gainesville and the Citrus Research and Education Center (CREC) in Lake Alfred, Florida. The presence or absence of STMV in these cultures was uncertain.

Plants of TSA, *N. benthamiana*, and *N. sylvestris* were inoculated by Dr. Dennis J. Lewandowski in Lake Alfred, with *in vitro* transcripts of the chimeric tobamoviruses 30-B (Figure 4-1), and 30-B GFPc3, as well as sap from fluorescing tissue of 30-B GFPc3-infected plants (Shivprasad et al., 1999). Half of the treated plants were maintained at the CREC in Lake Alfred, while half were returned to Gainesville and maintained in a growth chamber for observation, both in visible light, and under a long-wave UV lamp (λ= 366nm).

Growth chambers were set to maintain a maximum daytime temperature of 25°C, a minimum nighttime temperature of 20°C, and a photoperiod of 16 hours. Under these conditions, 3-week-old TSA seedlings were germinated in peat pellets and inoculated with "U5" and "U2" cultures of TMGMV obtained from the CREC, as well as the TMV/TMGMV chimera 30-B.

Sets of 3-week-old TSA and *N. sylvestris* plants maintained under 12 hour lighting in an "Apopka Room" in Gainesville were inoculated with infectious sap containing the chimeric construct 30B as a follow-up to the experiments conducted in Lake Alfred.

To evaluate the effect of STMV on TSA, 1-month-old TSA plants maintained in a quarantine greenhouse at the Florida Division of Plant Industry (DPI) in Gainesville were inoculated using sterile cotton gauze applicator pads saturated with reconstituted, dried tissue

obtained from the ATCC as PV-586 (the STMV-containing culture of TMGMV-L), and maintained in a cool, sealed quarantine greenhouse at the DPI in Gainesville, Florida, under ~14 hours of daylight and observed daily for symptom development. Control plants, mock-inoculated with buffer only, and plants inoculated with infectious tobacco sap containing PV-0113, were maintained in a different greenhouse. Plants were fertilized weekly with 280 ppm Peter's 20-20-20 fertilizer and observed daily for symptom development. The experimental setup at the DPI was then expanded to include the cultures obtained from Lake Alfred, PV-0113, and an assortment of experimental hosts. Due to limited greenhouse space, plants were started in trays of peat pots, and watered and fertilized using an "ebb and flow" method.

Seeds of *Capsicum annuum* X "Camelot," *N. benthamiana*, *N. clevelandii*, *N. rustica*, and TSA were started in Ferry Morse brand "Quick and Easy Peat Pellets" and germinated in a cool greenhouse. At 3 weeks of age, when the seedlings were of a size suitable for inoculation, they were moved to the quarantine greenhouse at the DPI in Gainesville where three leaves per plant were inoculated with virus tissue homogenized in ice-cold 20 mM $Na_2PO_4/NaHPO_4$ inoculation buffer and applied using a sterile cotton swab. Plants were fertilized by drenching (soaking and then draining) with approx. Treatments consisted of inoculation with PV-0113, LA "U2," LA "U5," and PV-586. Plants were also mock-inoculated with $Na_2PO_4/NaHPO_4$ buffer only as a control. Plants were maintained with biweekly drenching of 280 ppm solution of Peter's 20-20-20 fertilizer and observed daily for symptom development. Day length was decreasing over the course of this experiment.

Cultures "U2" and "U5" obtained from the CREC in Lake Alfred were inoculated onto *Eryngium planum* in an attempt to remove potential contaminants, and tissue extract from these

Eryngium plants was used to inoculate the diagnostic indicator plants *Nicotiana sylvestris*, *N. rustica*, and *N. glutinosa*.

Results

Transcripts of 30-B GFPc3 produced no symptoms on TSA distinguishable from wounding. Infectious sap containing 30-B GFPc3 produced systemic symptoms on TSA, including GFP expression visible under UV light starting 4 days post-inoculation. Transcripts were infectious to *Nicotiana benthamiana*, producing systemic symptoms, and, in the 30-B GFPc treatment, fluorescent patches appeared as soon as 3 days post-inoculation (DPI). Similar results on *N. benthamiana* were obtained with the infectious sap. *Nicotiana sylvestris* inoculated with the 30-B transcript developed LL, but were otherwise asymptomatic. Plants maintained at the CREC in Lake Alfred responded in a similar, if not identical manner as those in Gainesville (Dr. Dennis J. Lewandowski, formerly at CREC, Lake Alfred, personal communication). In subsequent experiments conducted under Apopka Room conditions, TSA responded with systemic symptoms to inoculation with 30B, and *N. sylvestris* responded with numerous LL to inoculation with 30B.

In the growth chamber, the large type isolate from Lake Alfred (LA-U5) produced symptoms similar to PV-0113 (epinasty, wilting, necrosis), while the "U2" culture from Lake Alfred (LA-U2) gave mixed results, and no symptoms were observed in the second repetition of this experiment. The TMV/TMGMV chimera, 30B, caused systemic symptoms on TSA without necrosis (Figure 4-2). *Eryngium planum* responded to inoculation with both small type (PV-0113) and large type (LA-U5) isolates with yellow flecking and/or speckling.

A variety of symptoms were produced on TSA by the STMV-containing culture TMGMV PV-586, including abscission, chlorosis, epinasty, mosaic, necrosis, veinal necrosis, and wilting.

Except for the veinal necrosis symptom, disease progress began normally, starting 6-8 DPI, and proceeded to about stage 3 or 4, with complete defoliation of the plant, after which there was new growth, mostly from the axial buds, which displayed a distinct chlorosis/mosaic symptom. Results on the differential indicator hosts *N. clevelandii* and *N. rustica* were generally consistent with those reported for TMGMV-L. *Nicotiana benthamiana* inoculated with PV-586 did not die before 18 DPI, although necrosis was observed as early as day nine (Table 4-1).

Isolates PV-586 and PV-0113 produced epinasty on TSA. Leaf abscission was seen with the PV-0113 culture, but not with the STMV-containing isolate. Leaf abscission was uncommon. Perhaps the earliest, most striking difference in symptom development on TSA plants inoculated with TMGMV-L-STMV was the appearance of a network of small necrotic threads or veins across the leaf. This symptom was concurrent with, but appeared different from, the development of LL.

Generalized necrotic symptoms developed on both PV-0113- and PV-586- inoculated TSA plants at about the same time. No obvious differences were observed at this point. Chlorosis developed in the new growth emerging from the PV-586 inoculated plants only. Wilting produced by both STMV (+) and STMV (-) cultures of TMGMV occurred simultaneously, and tended to affect the apical portions of the plant first. The mosaic symptom was observed on TMGMV-L-STMV inoculated plants only, and was distinct in appearance from the mosaic produced by TMV, ToMV, SHMV, or 30B. The plant inoculated with PV-0113 eventually died. TSA plants inoculated with PV-586 initially appeared to be dying, but recovered with the aforementioned mosaic symptoms (Figure 4-3).

In addition to cultures of TMGMV, TSA plants were inoculated with TMV, ToMV, PMMoV, and SHMV (Figure 4-4). All of these tobamoviruses were able to infect TSA systemically, but none produced LL or necrosis on TSA.

Plants maintained in the DPI quarantine greenhouse developed symptoms, on average, between 5 and 9 DPI. While PV-0113 reliably killed TSA in the DPI greenhouse environment, the other cultures of TMGMV did not. The ATCC culture PV-586 caused disease symptoms resembling those of PV-0113, but inoculated plants did not ultimately die. Epinasty was seen in several inoculated plants in all treatment groups in the DPI greenhouse, but not in the mock-inoculated, buffer-only control group. LL and other necrotic symptoms developed reliably only on the PV-0113 inoculated plants. Some LL and necrosis were seen in the PV-586 and on the TMGMV-L culture from CREC, Lake Alfred treatment groups, but not on the TMGMV-S culture from Lake Alfred, or the negative control group.

Permanent wilting was observed only in those TSA plants inoculated with PV-0113, and in some plants inoculated with PV-586. The other treatments did not produce this symptom on TSA. Mosaic symptoms were eventually observed in plants inoculated with cultures of TMGMV except PV-0113. While this was expected in the PV-586 inoculated plants, the occurrence of mosaic in the other groups was a cause for concern, possibly indicating contamination with another virus. Ultimately, all plants inoculated with PV-0113 died, but not plants inoculated with other viruses, except some plants inoculated with PV-586. Other plants survived in either symptomless or symptomatic condition. The presence of other unusual symptoms such as mosaic, leaf deformation, chlorosis, etc. was limited to treatment groups containing cultures other than PV-0113.

Tissue from *E. planum* inoculated with "U2" obtained from the CREC, Lake Alfred produced no immediate symptoms on *N. sylvestris*, *N. rustica*, or *N. glutinosa* plants. Inoculated *N. sylvestris* plants eventually developed systemic symptoms. Tissue from *E. planum* previously inoculated with "U5" obtained from the CREC, Lake Alfred caused LL on *N. sylvestris*, *N. rustica*, and *N. glutinosa*. *Nicotiana rustica* plants inoculated with this culture went on to develop a more severe necrosis and other systemic symptoms, consistent with the results reported for TMGMV-L.

Discussion

In trials conducted in a growth chamber, isolates of TMGMV from CREC, Lake Alfred identified as "U5" produced local lesions (LL) in the majority of the inoculated TSA. The duplicated sequences in 3′ region of this subtype, which distinguishes this type from the "U2" type, do not apparently prevent the HR. As recovery was unanticipated, these plants were discarded before the disease process had run its course. Failure of the Lake Alfred culture provided as "U2" to produce consistent symptoms may be due to contamination, inadequate inoculation, or nonconductive plant growth conditions (i.e. peat pots, limited space, lack of fertilizer, etc).

Large-type TMGMV that appeared to kill immature TSA plants under growth chamber conditions were discarded soon after symptoms (collapse/wilting/defoliation) occurred because previously, necrotic disease had invariably proved lethal. It remains uncertain whether the duplicated pseudoknot region or the presence of STMV is responsible for survival/recovery of these inoculated plants. One possible experiment would be one in which the response of TSA to isolates of TMGMV-S/TMGMV-L not containing STMV were compared with response to isolates that do contain STMV.

None of the plants inoculated with 30B virions or 30B transcripts developed LL, but most developed systemic mosaic instead. Since both TMGMV-L and 30B contain the open reading frame ORF 4 expressing TMGMV-L coat protein (CP), it stands to reason that TMGMV-L CP accumulates in both the LL and non-LL displaying plants, suggesting that some elicitor other than CP was responsible for the HR component of the lethal response seen in the TSA. *Nicotiana sylvestris* inoculated with transcripts developed very few (0-2) local lesions per leaf, suggesting that in vitro transcripts are not very infectious compared with intact virus.

The recovery of plants infected with PV-586 was unexpected, which underscores the importance of preserving inoculated plants until necrosis has progressed to the full extent. While the CREC, Lake Alfred isolate "U2" produced symptoms in tobacco, indicating that virus is present, most TSA plants inoculated with "U2" remained symptomless, suggesting possible contamination or replacement by some other virus. The indicator plant data, along with difficulties encountered in PCR-amplifying RNA extract from this culture, further raise the possibility that this culture is not TMGMV. Systemic symptoms observed on *N. sylvestris* suggest replacement by TMV type-strain. Other possibilities are that it is a genetically distinct strain of TMGMV, or that it contains STMV or some satellite RNA that modifies its effects on the indicator hosts.

Early symptoms observed in TSA inoculated with PV-586 such as LL, wilting, necrosis – followed by partial recovery, introduce the possibility that the TSA-killing effect is independent of HR, or at least incompletely dependent upon it. If STMV is responsible for the survival and recovery of the plants, the mechanism of this action is unclear.

Ultimately, necrosis progressed to death of all plants inoculated with TMGMV PV-0113, but not in plants inoculated with other isolates of TMGMV or the TMV/TMGMV chimera 30B,

with the exception of a few plants inoculated with PV-586. These results were unexpected; at this stage of the research, however, a rating scale had not yet been devised. In the first trial, it was not known that defoliated TSA could recover, so plants may have been incorrectly recorded as dead and discarded. It is also possible that the mortality in the PV-586 group may be the result of using immature plants. Perhaps immature plants die before the STMV has a chance to counteract the effect. The absence of symptoms in the mock-inoculated, control group suggests that cross-contamination, if it had happened, occurred prior to inoculation.

Peat pots are not the best medium for growing plants for use with this pathosystem. They were used in this case, because limited space was available in the greenhouse and the growth chamber, and because such a method is often used with success for other applications. In particular, *N. benthamiana* is not suited for use in peat pots.

Rate of growth has a big influence on disease phenotype. Even when available space is limited, it is better to give experimental plants plenty of room to grow and expand during the research period, than it is to allow plants to become pot-bound or cease active development, even if this means working with a reduced number of replicates per trial.

Epinasty appears to develop frequently when TSA plants are inoculated with tobamoviruses in general (my observations). The involvement of epinasty in the TSA-killing response may be coincidental.

Although some variability in host response was observed, the culture labeled "U5" obtained from the CREC produced effects consistent with those reported in literature for TMGMV-L. The hosts *Eryngium planum* and *N. rustica* provide a method for identifying and purifying TMGMV-L. One future experiment should be to determine if STMV can be eliminated from an STMV-containing culture using *E. planum* or some other biological sieve.

Cultures of TMGMV-S and TMGMV-L used in subsequent research should come from genotypically and/or geographically defined populations, (*not* PV-0113), and of known STMV (+)/ (-) status.

Table 4-1. Symptoms on various differential/indicator hosts by TMGMV PV-0113 (TMGMV-S) and TMGMV PV-586 (TMGMV-L + STMV).

Host	TMGMV PV-0113	TMGMV PV-586
Nicotiana benthamiana	Death	Death
Nicotiana clevelandii	Chlorosis, Mosaic, Shoot proliferation	Chlorosis, Mosaic
Nicotiana glutinosa	Local lesions	Local lesions
Nicotiana rustica	Local lesions, Death	Local lesions
Nicotiana tabacum (*nn*)	Mosaic	Mosaic

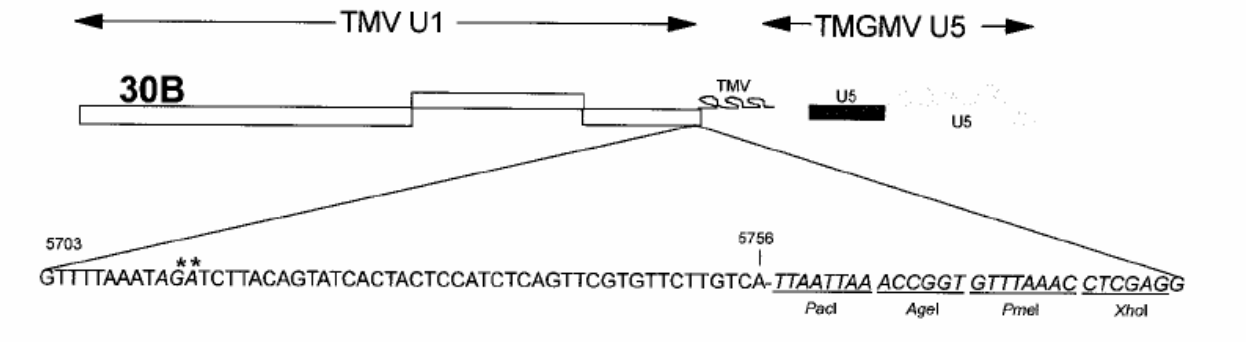


Figure 4-1. Genetic map of TMV/TMGMV chimera 30 B.

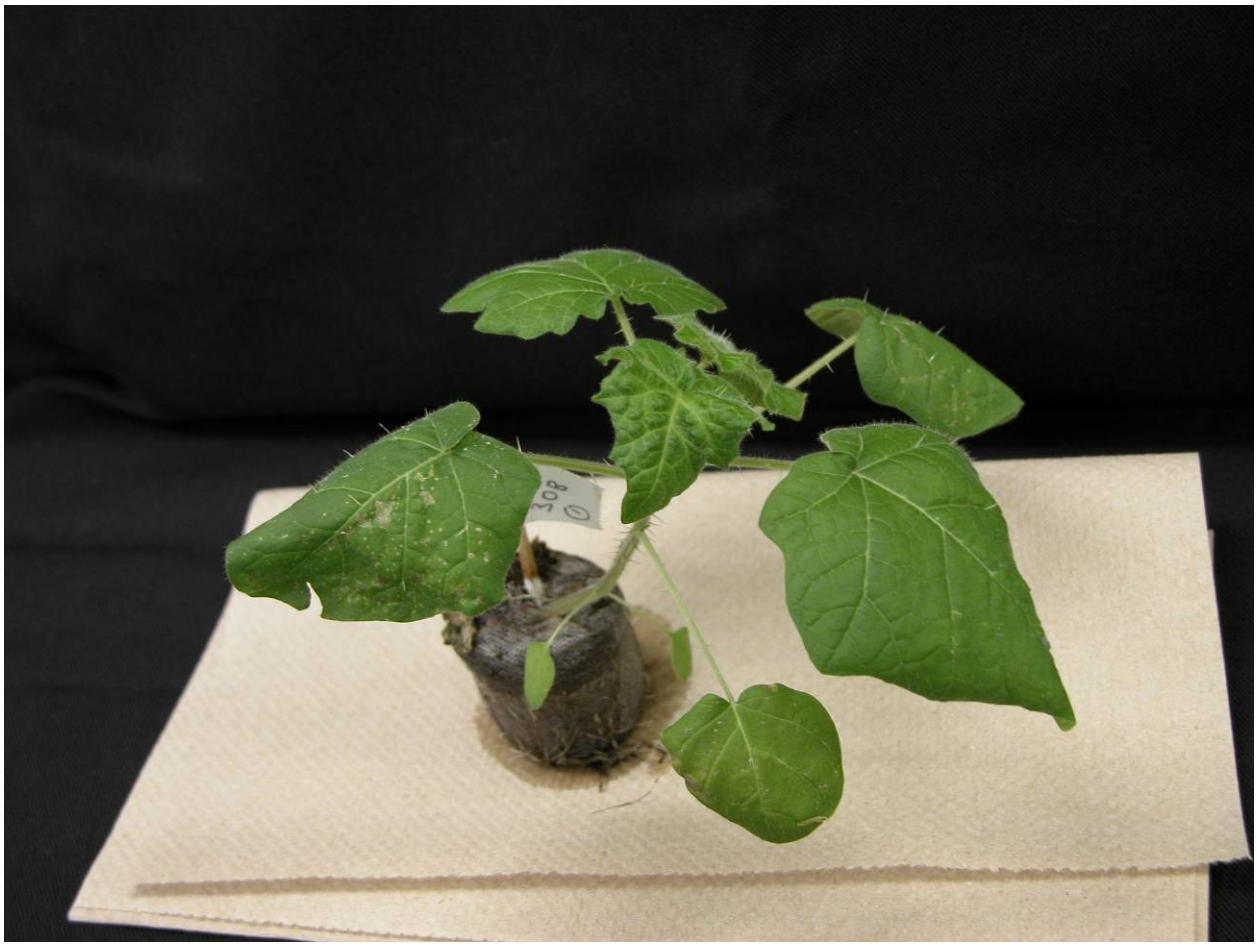
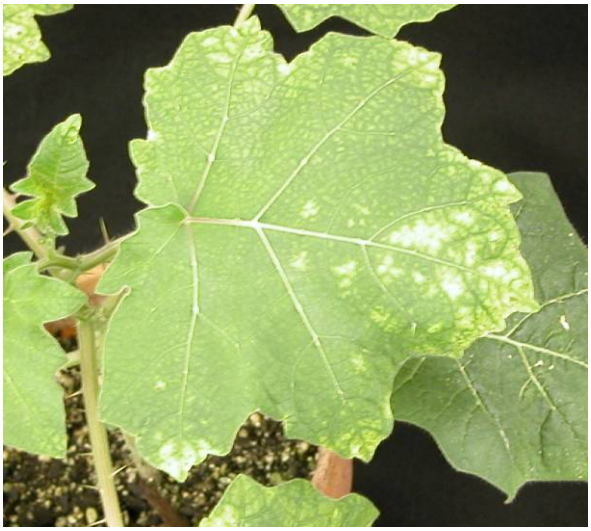


Figure 4-2. Typical symptoms produced in TSA seedling by 30-B TMV/TMGMV chimera.

Figure 4-3. TMGMV PV-586 inoculated TSA recovering with mosaic after dieback.

A.   B

C **D**

Figure 4-4. Photographs of symptoms by various tobamoviruses on TSA. A) TMV, B) ToMV, C) SHMV, D) PMMoV.

CHAPTER 5
CROSS-PROTECTION EFFECTS

Introduction

McKinney (1929) observed that the symptoms of Tobacco mosaic virus (TMV) infection could often be moderated by pre-infection with a less virulent strain. Since that time, researchers have tried to ameliorate the effects of tobamovirus infection by inoculating susceptible crops with mild strains of the virus (Rast, 1979). Cross-protection has been used as a strategy for managing tobamovirus and non-tobamovirus diseases that reduce yield in tomato, pepper, and citrus crops (Yoon, et al., 2006). Conjecture about cross-protection can be segregated into hypotheses that (1) attribute cross-protection to direct competition for host resources, such as replication sites or translation machinery; (2) propose inhibition of one virus by the coat protein (CP) of the other, perhaps disrupting the timing of the infection cycle; and (3) relate cross-protection to nucleic acid interference, perhaps through genetic recombination or activation of gene silencing. These hypotheses are not mutually exclusive: indeed, more than one mechanism may be involved in cross-protection (Aguilar et al., 2000).

In studies of cross-protection between mutants of Pepper mild mottle virus (PMMoV), it was observed that cross-protection resulted from infection by some mutants, despite low accumulation of virus *in plantae* (Yoon et al., 2006). The authors speculated, "host response to cross-protection may prevail throughout the plant without propagation of viral components" (Yoon et al., 2006). Aguilar's studies of cross protection between TMGMV and Oilseed rape mosaic virus (ORMV) in *Nicotiana tabacum* and *Arabidopsis thaliana* provide evidence for host involvement in cross-protection and support for the idea that different mechanisms operate in different situations. When used as the primary inoculum, both TMGMV and ORMV appeared able to prevent systemic infection by the challenging virus. Results in tobacco seemed consistent

with a direct competition hypothesis, but interpreting the results in *Arabidopsis* proved more difficult. Interestingly, TMGMV prevented systemic spread and accumulation of ORMV in *A. thaliana*, even though TMGMV symptoms were latent, and the virus very slow to move and accumulate in this host (Aguilar et al., 2000). This may suggest gene silencing or some other active process within the host.

Tropical soda apple (TSA) seeds collected from fields in the area around Immokalee (Hendry Co.), Florida (2000), developed into plants displaying mosaic-type symptoms when raised in a greenhouse. Plants infected with the contaminant virus failed to die when challenged by inoculation with TMGMV. Subsequent investigation proved the contaminating virus to be TMV. The objective of this study was to examine the effects of potential cross-protection against TMGMV in TSA by an unknown virus, a tobamovirus and a non-tobamovirus.

Materials and Methods

Seeds collected from TSA fruit harvested in the Immokalee area in Hendry Co., Florida in 2000, were planted in Metromix potting medium, and allowed to germinate, and develop in a greenhouse. A portion of the collected seed was surface-sterilized prior to planting, by soaking the seeds for 30-minutes in 1 M HCl, followed by rinsing and a 30-minute soak in a concentrated trisodium phosphate (TSP) solution (400g TSP/ 3.78 L H_2O). The seeds were then rinsed and dried. To verify that the infection was seed-borne, and to determine if the virus was carried internally, or limited to the seed coat, several treated seeds were planted at the same time as the untreated seeds; the rest were saved for use in future studies.

Tissue from TSA symptomatic of a virus contaminant, designated C-1, was used to inoculate healthy TSA plants. When these plants developed mosaic symptoms, they were challenged by inoculation with TMGMV PV-0113. Experimental trials consisted of nine systemically infected plants inoculated with TMGMV PV-0113 and one positive control plant

inoculated with TMGMV PV-0113 only and one negative control plant inoculated with buffer only. The identical experiment was conducted except only eight plants were used per treatment and C-1 and TMGMV PV-0113 were inoculated on these plants simultaneously. The plants were maintained and observed under greenhouse or screen-house (outdoor) conditions. The differential indicator, (*Phaseolus vulgaris* cv. "Pinto"), which develops local lesions (LL) in response to inoculation with TMV, but not TMGMV, was also inoculated with C-1.

One-month-old TSA plants were inoculated with TMV and observed until symptoms developed uniformly on all inoculated plants (approx 3 weeks). Plants were then inoculated with infected plant tissue triturated in 20 mM Na_2PO_4/ $NaHPO_4$ buffer, pH 7.2. Three leaves per plant were inoculated manually, by rubbing carborundum-dusted leaves with inoculum-soaked cheesecloth pads. Plants were inoculated concurrently and sequentially with TMGMV PV-0113 and TMV. TSA plants were also mock-inoculated with buffer as a negative control, TMV only, and TMGMV PV-0113 only. Plants were maintained in a greenhouse, fertilized weekly with 280 ppm Peter's 20-20-20 soluble fertilizer, and observed daily for symptom development.

Examining the possibility that a non-tobamovirus might also cross-protect TSA from TMGMV PV-0113 induced lethality, 3-week-old TSA plants were inoculated with Cucumber mosaic virus (CMV). Once symptoms developed uniformly on all inoculated plants (approx 5 weeks), the plants were then challenge inoculated with PV-0113-infected plant tissue triturated in 20 mM Na_2PO_4/ $NaHPO_4$ buffer, pH 7.2 (except for a negative control set, which was mock-inoculated, using buffer only). Three leaves per plant were inoculated manually, by rubbing the carborundum-dusted leaves with cheesecloth pads. Treatments consisted of inoculation with sap extract from a common strain of CMV obtained courtesy of Dr. Carlye Baker (Division of Plant Industry, DPI) in Gainesville, Florida and propagated in *Nicotiana glutinosa*. Plants were mock-

inoculated with buffer as a negative control. Plants were maintained in a cool greenhouse, fertilized weekly with 280 ppm Peter's 20-20-20 soluble fertilizer, and observed daily for symptom development.

Results

Symptomatic tissue harvested from TSA and tobacco infected with C-1 produced results consistent with TMV infection on the differential indicator (*Phaseolus vulgaris* cv. "Pinto") and other differential host-range plants. Immunodifffusion, ELISA, and RT-PCR also identified C-1 as TMV-type strain (data not shown). No symptomatic or infected plants were found among the seedlings grown from HCl/TSP-treated seeds. TSA plants inoculated with C-1 became chlorotic and developed epinasty when challenged with TMGMV, but did not die. Instead, a distinct mosaic pattern continued to appear on the newly emergent leaves, along with chlorosis that followed the leaf veins to create a branching appearance. In most trials, TSA symptomatically infected with type-strain TMV did not die when challenge-inoculated with TMGMV PV-0113, except in one study where the treated plants died, after lagging behind the positive control plant in symptom development by about 72 hours.

TSA plants inoculated with CMV developed symptoms including vein-clearing and mosaic with frequent chlorotic islands. All TSA plants inoculated with CMV and subsequently challenged with TMGMV PV-0113 developed local lesions, wilting, and necrosis, followed by death (Table 5-1).

Discussion

Seed transmission of tobamoviruses, when it occurs (e.g. PMMoV in *Capsicum* sp.) is believed to be due to the presence or persistence of virus on the seed coat, from which it infects the seedling (auto-inoculation as it emerges during germination). Multiple diagnostic techniques

(host-range, ELISA, RT-PCR) confirmed the contaminating virus associated with the Immokalee seeds to be Tobacco mosaic virus (TMV).

TMV appears to cross-protect against TMGMV, while the non-tobamovirus CMV does not appear to cross-protect. Studies conducted with Tomato mosaic virus (ToMV), and TSA mosaic virus (TSAMV) indicate that these viruses also cross-protect TSA from TMGMV PV-0113 lethality (Mark S. Elliott, Senior biological scientist, University of Florida, personal communication). Although the exact reasons for cross-protection remain unknown, the relationship between the viruses that do cross-protect, sharing similar genomic nucleotide and CP amino acid sequences, seem consistent with both CP interference and gene silencing hypotheses.

Since TMV appears to cross-protect against TMGMV-induced lethality, additional studies of cross protection should be conducted under controlled temperature and lighting conditions. The anomalous decline and death of TSA plants previously inoculated with TMV after inoculation with TMGMV in one study is interesting, and consistent with the multiple mode of action hypothesis.

Table 5-1. Cross-protection study

Inoculum	Symptoms
Buffer only	NS
CMV	E, M, VC
TMV	M
TMGMV (PV-0113)	E, LL, W, N, D
TMGMV (PV-0113) + CMV	E, LL, W, N, D/M, VC, E, LL, W, N, D
TMGMV (PV-0113) + TMV	E, Chl/M

CMV = Cucumber mosaic virus, TMGMV = Tobacco mild green mosaic virus, TMV = Tobacco mosaic virus. Symptoms: Chl = chlorosis, D = death, E = epinasty, LL = local lesions, M = mosaic, N = necrosis, VC = vein clearing, W = wilting. For challenge-inoculations, data presented to the left of the slash is the result of concurrent co-inoculation; data to the right of the slash is the result of sequential inoculation.

CHAPTER 6
TMGMV PV-0113: PURIFICATION, LYOPHILIZATION, AND RNA EXTRACTION

Introduction

Although sap extract from plants infected with the TMGMV, PV-0113 isolate is reliably lethal to tropical soda apple, for in-depth characterization, the virus should be purified. A non-enveloped virus with the stability of TMGMV may be extracted, purified, freeze-dried (lyophilized) —even crystallized while retaining pathogenicity (Yordanova, et al., 2002). Although tobamoviruses in herbarium specimens remain infectious for several decades, (Gibbs, 1999; Hull, 2002), possibly centuries, extracted virus will begin to degrade over time, even if kept in a purified and lyophilized state. Various protectants such as sorbitol and dextran may be used to extend the life of tobamovirus preparations (Yordanova, et al., 2002).

For molecular characterization of TMGMV, genomic RNA must be extracted. As part of the replication cycle, the fragile genomic RNA of (+)ssRNA viruses forms stable double-stranded RNA structures (dsRNAs). These more stable dsRNA's are generally indicative of infection, although dsRNAs of an unknown origin ("cryptic RNA") are also known to occur in plants (Valverde, 1990). Various methods exist for viral RNA extraction from plant tissue, although it is generally easier to extract total RNA than dsRNA only. One approach to isolating dsRNA utilizes selective binding of nucleic acid to charged cellulose followed by washing and elution at different ethanol concentrations (Morris and Dodds, 1979). Total RNA may be adsorbed to cellulose in a column at a particular ethanol concentration, the column washed with ethanol, and then the different ssRNA types released as the ethanol concentration is lowered. The dsRNA may be eluted (Morris and Dodds, 1979). The concentration of ethanol is critical, and must be adapted to each individual batch of cellulose (Dr. Jane Polston, Professor, University of Florida, Plant Pathology Department, Gainesville, personal communication).

Materials and Methods

To purify TMGMV PV-0113 for use in further research, two methodologies were followed: a typical preparation (Virus Prep-D), and a simplified, ("quickie") purification (TMGMV prep-Q). Healthy, 3-week-old tobacco plants (*Nicotiana tabacum* cv. Samsun, genotype *nn*) were inoculated manually with a 1:10 suspension of TMGMV PV-0113 in 20 mM Na_2PO_4/ $NaHPO_4$ buffer (pH 7.4) and maintained in a greenhouse. The plants were fertilized weekly with 800 ppm Peter's 20-20-20 soluble fertilizer. By 3 weeks post-inoculation, systemic symptoms (green mosaic, leaf pleating) had developed, and leaves of various ages from the plants were harvested. One hundred grams (100 g) of symptomatic leaf tissue was homogenized in a Waring blender at 4°C with 200 mL cold (4°C) 200 mM K_2PO_4/ $KHPO_4$ buffer (pH 7.5) and 0.125 M Na_2SO_3 (15.76 g). One hundred milliliters (100 mL) of cold chloroform ($CHCl_3$) was then added, and the mixture further homogenized under a fume hood for 2-3 minutes. The homogenate was centrifuged at 2500 rpm (1,000 x *g*) in a Sorvall centrifuge for 10 minutes. The aqueous supernatant was filtered through moist cheesecloth and re-centrifuged at 12,500 rpm (18,000 x *g*) for 10 minutes. The clarified supernatant was again filtered though moist cheesecloth and to this was added 8% w/v polyethylene glycol (PEG), 0.5 M NaCl, and 1% Triton X-100. The liquid was stirred for 1 hour at 4°C and then centrifuged (under refrigeration) at 10,000 rpm (12,000 x *g*) for 10 minutes. The supernatant was discarded and the pellet resuspended in 50 mL of phosphate buffer. The sample was again centrifuged at 10,000 rpm (12,000 x *g*) for 10 minutes; this time the supernatant was retained and combined with 8% (w/v) PEG and 500 mM NaCl. The solution was stirred for 1 hour at 4°C and centrifuged at 10,000 rpm (12,000 x *g*) for 10 minutes. The white pellet was re-suspended in 8.0 mL of 20 mM HEPES buffer (pH 7.5) to yield a virus suspension that was faintly bluish, visibly opalescent, and

birefringent when viewed across polarized light. Purification was halted at this step. For the "quickie" purification, the step of filtration through cheesecloth and the chloroform treatment was omitted.

Purified virus adjusted to 1 optical density (1 OD) at A_{260} by dilution in Na_2PO_4/ $NaHPO_4$ inoculation buffer was aliquotted into 500-µL units before drying under vacuum using a Dura-Dry condenser module (manufactured by FTS Systems, Inc.). Some lyophilized virus was resuspended in sterile, distilled, de-ionized water and used to inoculate plants. The rest was stored on the shelf at room temperature. Viral preparations "D" and "Q" were evaluated using a Bio-Rad SmartSpec 3000 spectrophotometer and assayed for infectivity by inoculation onto the local lesion host, *N. tabacum* cv. "Samsun" (*NN*) and the systemic host, TSA. Infectivity was determined by counting the number of LL produced by different preparations in comparison with a freshly purified virus preparation.

Total RNA for RT-PCR from newly emergent leaves was prepared using the Trizol reagent (Invitrogen). Leaf disks of symptomatic tissue of tobacco (*N. tabacum* cv. Samsun *nn*) inoculated 3 weeks prior were removed using the lid of the microcentrifuge tube and homogenized with 200 µL Trizol with a DEPC or NaOH treated Kontes micro-pestle. An additional 550 µL of Trizol was added to the homogenate which was then incubated for 5 minutes at room temperature before adding 200 µL of chloroform. The mixture was held at room temperature for an additional 8-10 minutes, after which it was centrifuged at 12,000 x *g* for 10 minutes at 4°C. The aqueous (top) phase was transferred to a new microcentrifuge tube, 0.375 mL of isopropanol was added to precipitate the RNA, the contents mixed by inversion, and held at room temperature for 10 minutes before another centrifugation at 12,000 x *g* for 10 minutes at 4°C. The supernatant was then carefully removed and the RNA pellet washed with 1

mL of 75% RN-ase free ethanol. The supernatant was removed and a 15-second pulse spin, followed by a 6-minute spin-down was applied to remove any remaining supernatant before allowing samples to dry under air-flow (hood) for 10 minutes. Prepared RNA was dissolved in 30 µL molecular biology grade (MBG) water. Resuspended samples were incubated for 10 minutes at 55-60°C before use or storage at -80°C.

Total RNA was extracted from 100 µL purified virus by addition of 100 µL RNA-dissociating solution containing 20 mM Tris, 2% SDS, and 2 mM Na_2EDTA (pH 9.0), along with 10 µL protease K (Invitrogen). After incubation for 10 minutes at room temperature, the solution was combined with 400 µL ice-cold solvent mixture (200 µL adjusted phenol, 200 µL 24:1 chloroform-isoamyl alcohol) and agitated gently for 2 minutes. This virus-solvent mixture was centrifuged at 14,000 rpm (16,000 x *g*) for 5 minutes and the aqueous layer recovered. Three (3) volumes of 100% ethanol and 0.1 volumes (20µL) of 3M NaAc (pH 5.3) were added to the aqueous layer and held overnight at -80°C. The mixture was then centrifuged 14,000 rpm (16,000 x *g*) for 30 minutes and the supernatant discarded. Precipitated RNA was then washed with 500 µL 70% EtOH and re-precipitated at 14,000 rpm (16,000 x *g*) for 5 minutes. The supernatant was carefully removed and the RNA dried under a low vacuum (aspirator) and resuspended in 50 µL MBG H_2O. Unused preparation was stored immediately at -80°C for later use.

Cultures of Tobacco mild green mosaic virus (TMGMV PV-0113, PV-586, LA-U5) were propagated in *Nicotiana tabacum* cv. Samsun (*nn*) as described above. Cucumber mosaic virus was propagated in *N. glutinosa*. Tissues from both plants were harvested 3 weeks post-inoculation, quick-frozen in liquid N_2, and stored at -80°C until use.

Seven grams of each infected tissue sample were frozen under liquid N_2 and triturated in a sterile mortar and added to beakers containing a cold (4°C) mixture of 14 mL 2X STE buffer (pH 6.8), 20 mL equilibrated phenol, 2 mL 10% SDS, and 14 mg of bentonite. The mixture was held at 4°C while stirring for 30 minutes, after which the mixtures were centrifuged 15 minutes at 8,000 rpm in phenol-resistant, NaOH-treated sterile (Oakridge) centrifuge tubes. The aqueous phase was collected, the volume adjusted to 20 mL with 1X STE buffer (pH 6.8), and 3.8 mL 95% ethanol added, along with 1.0 to 1.5 g Whatman CF-11 charged cellulose powder, under continuous stirring at 4°C. One hour later, the mixtures were poured into columns prepared from 12 mL disposable syringes and baked glass wool, using a solution of 15.0 to 16.5% ethanol in 1X STE to wash the cellulose until it became colorless. Samples were then pressed dry using the syringe pistons and the dsRNA eluted with 14 mL of 1X STE. To the eluent, 3M sodium acetate (pH 5.5; 0.1% v/v) and 95% ethanol (2.5 % v/v) were added, and the samples incubated overnight at -20°C. The samples were then centrifuged at 9,000 rpm (9,750 x *g*) for 30 minutes at 4°C to recover the precipitated RNA, which was washed once with 70% ethanol, dried under vacuum, and dissolved in 50 to 100 µL of sterile, distilled, deionized H_2O. This procedure was repeated using different concentrations of ethanol in the wash step until samples were obtained that produced relatively distinct dsRNA bands when separated on agarose and polyacrylamide gels. All nondisposable autoclavable materials used in extraction were made DN-ase and RN-ase free either by baking (glass) at 200°C for 8 h or soaking (plastic) in 100 mM NaOH for 1 h, then rinsing with RN-ase free H_2O, and autoclaving to inactivate the enzymes.

Results

The $A_{260/280}$ was 2.85/2.40 (= 1.19) for preparation "D" and 2.79/2.36 (= 1.18) for preparation "Q;" the reported A_{260}/A_{280} ratio for TMGMV is 1.22 (Wetter, 1986). Significant yield of virus for both preparations was approximately 0.9 mg per gram of fresh leaf tissue

processed or 6.9 mg per gram of dry tissue, based on the extinction coefficient for TMGMV reported in literature of 3.16 at A_{260}. Fresh tobacco tissue is 87% water (Mark S. Elliott, University of Florida, Plant Pathology Department, Gainesville, unpublished). Preparation "D" was slightly bluish, while preparation "Q" was yellowish or cream-colored and milky. Preparation "Q" produced many more local lesions (LL) on Samsun *NN* tobacco at a high concentration (~320 µg/mL), but at lower concentrations (≤ 0.3 µg/mL) it gave approximately the same number of LL as preparation. "D" (Figure 6-1). Freshly prepared virus had a higher biological activity than either reconstituted lyophilized virus or a suspension in inoculation buffer stored in the refrigerator for 14 months.

Virus reconstituted from a lyophilized state was sometimes transparent or turbid and/or had aggregates. A "transparent" reconstituted sample of preparation "D" had the $A_{260}/_{280}$ value of 2.87/2.34, for a ratio of 1.23, which is very close to the reported value for pure TMGMV (Wetter, 1986). Other virus preparations had $A_{260}/_{280}$ ratios of 1.39, 1.37, and 1.31. TSA plants inoculated with PV-0113 purified and stored for 14 months at 4°C developed symptoms and succumbed to infection.

Discussion

Both types of virus purification methods produced similar yields of TMGMV per gram of leaf tissue. Of the two preparations, visually preparation "D" was the most pure. However, the absorbance values (2.85/2.40) and the ratio (A260/280 = 1.19) for preparation "D" and preparation "Q" (2.79/2.36; A260/280 = 1.18) indicate a yield of 0.9 mg virus per g of leaf tissue, which is relatively low for a tobamovirus. The values (2.87/2.34) indicate a successful resuspension of the virus to the former concentration, so effects from dilution or concentration are probably not a factor. Interestingly, the less-purified "quickie" preparation produced more LL than prep "D" on Samsun *NN* tobacco at 1:10 dilution from 1 OD in buffer.

Virus stored refrigerated in Na_2PO_4/ $NaHPO_4$ inoculation buffer remained infective even after 1 year (data not shown). Also surprisingly, the lyophilization procedure used appears to have reduced the viability of the virus inoculum. Perhaps the virus used in any future lyophilization should be suspended in sterile distilled water instead of buffer solution to preclude possible denaturation by the buffer salts as the buffer concentrates. The reasons for the cloudiness/turbidity observed in some lyophilized samples upon reconstitution remain unknown. The reconstituted preparations proved infective despite their abnormal appearance.

The ribonucleic acid (RNA) obtained from leaf tissue using Trizol was as useful as RNA prepared from purified virus for producing cDNA for use in RT-PCR. The appearance of dsRNA extracted using the modified Morris and Dodds protocol (Morris and Dodds, 1979), following electrophoresis on agarose and polyacrylamide gels, was consistent with patterns observed from TMGMV extracts recorded in the literature. An ethanol concentration of 15.5% was critical to successful dsRNA extraction using the available charged cellulose.

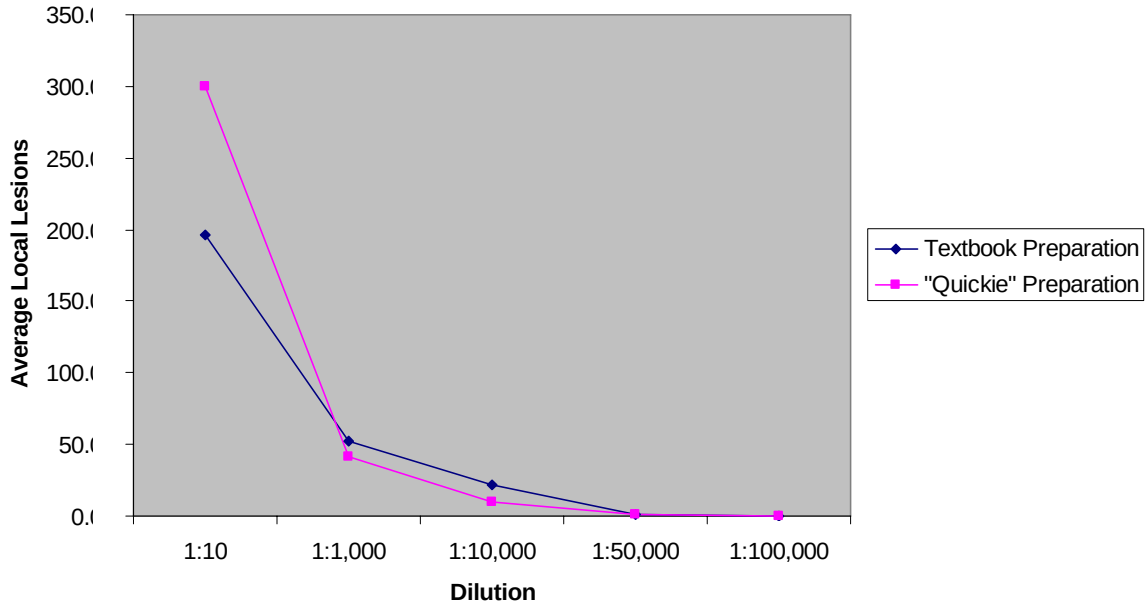


Figure 6-1. Comparison of the activity of two preparations of TMGMV PV-0113.

CHAPTER 7
DILUTION ENDPOINT AND BIOLOGICAL ACTIVITY OF PV-0113

Introduction

It is necessary to establish an optimum inoculum concentration of TMGMV that will elicit the lethal response in tropical soda apple (TSA). The point at which a serial dilution by a virus fails to elicit a particular biological activity, which in the case of the TMGMV-TSA system is local lesions and plant death, is called the "dilution endpoint." To determine the dilution endpoint it is necessary to start with a known amount or concentration of the virus. To quantify a virus present in a viral preparation, several physical, chemical, serological, and biological methods could be used. Physical methods include counting individual virions under an electron microscope or weighing dried virus preparations. Recently, a physical technique involving mass spectrometry of intact virus particles has become available (Fuerstenau et al., 2001). These techniques are often highly laborious and time consuming, limiting their application beyond a modest scale. Chemical microanalysis of viral components, such as nitrogen, may be used, but suffers from the same drawback. Serological methods, such as antigen-antibody precipitation, are relatively sensitive and have been used, but require pure virus preparations to generate antiserum. Also, animal care requirements make production of polyclonal antiserum laborious and/or expensive (Ball, 1964). Biological assays may be qualitative as well as quantitative, and because they depend upon the intrinsic property of viruses to infect and replicate, can provide the most sensitive means available for detecting viruses. Mere infection of a host plant, however, tells nothing more than that there was sufficient virus present to establish infection; inoculation with that amount or a hundred thousand times more might have the same result.

In 1929 F.O. Holmes discovered that Tobacco mosaic virus (TMV) produces countable necrotic lesions when inoculated onto *Nicotiana glutinosa* plants, and that the number of lesions

depended on the virus content of the inoculum. This discovery allowed plant viruses to be assayed by counting the number of local lesions (LL) in a manner similar to Koch's method of counting bacterial colonies on agar plates. However, the LL assay, like all infectivity tests cannot provide an absolute measure of the amount of virus, but may, if carefully planned and conducted, indicate the relative amounts of virus contained in inocula compared simultaneously, in the same study (Bawden, 1956). LL assays suffer from the high number of difficult-to-control variables. Different LL-producing hosts could vary in their sensitivity to inoculum from species to species, cultivar to cultivar, even from plant to plant. Adjacent leaves on a host plant could vary more than different halves of the same leaf. The age of the plant, as well as the age of the leaf on the plant influence the number of LL produced (Bawden, 1956). Various environmental conditions have been shown to influence the formation of LL on a host plant. Holding plants in darkness or low light intensity for 24 hours prior to inoculation tends to increase the number of LL that develop. Variation in temperature may affect LL development upon a host, although the number of LL produced may increase or decrease relative to the variation in temperature depending on the specific plant-pathogen interaction involved. Other factors affecting susceptibility include the availability of water and nutrients. Since any inhibition of plant growth will affect LL development, any substances added during inoculation (Bawden, 1956), such as phosphate-based inoculation buffer and particles of carborundum or diatomaceous earth could influence LL formation. Phosphate and abrasives will increase the number of LL that develop, (Roberts, 1964), while any ribonuclease present will inhibit LL formation (Bawden, 1956). Bentonite, which interferes with ribonuclease, will increase the number of LL produced by naked TMV RNA, but not by intact virions (Bawden, 1956). Even the type of mechanical inoculation -- the amount of pressure applied and the number of passes with the applicator will affect the

development of LL, probably because new infection sites are opened and others destroyed. In any experiment attempting to quantify a virus preparation using LL formation the inoculations should be conducted by the same individual (Bawden, 1956). Finally, some kinds of plants, such as *Capsicum* sp., produce substances which inhibit mechanical virus transmission (Paliwal and Narianai, 1965).

When LL development is plotted against virus concentration, curves generated for tobamoviruses tend to have a flat portion at high concentration, a steeper portion where the slope reflects a more-or-less proportional relationship between concentration and LL number, and a flat portion at low concentration. While many viruses produce results like this when diluted, others such as TSWV and TRV produce curves with different characteristics (Bawden, 1956). Comparisons are most sensitive when samples to be tested are in concentrations producing an average 15-30 LL per half-leaf. For LL produced on tobacco by TMV, this more linear portion of the curve usually occurs after purified virus preparations have been diluted to around 1 part virus to 1,000 parts buffer (1:1,000), or about 10^{-6} g purified virus per milliliter. The best discrimination of virus (TMV) content reported was 25-50% using the half-leaf method and 50 *N. glutinosa* plants, and 10% using bean (Bawden, 1956).

The lesion numbers obtained in the LL assays do not translate directly into relative virus content. For each new virus preparation, a new dilution curve needs to be derived, because the slope of the dilution curve is not constant, indeed, it is unpredictable (Bawden 1956). The minimum number of virions required to initiate an infection in any given host remains unknown (Roberts, 1964). Randomization is important, not just between leaves, but between plants in a treatment group. Data obtained from randomized block designs may be subjected to analysis of variance (ANOVA) to determine the significance of differences between the number of lesions

produced by different inocula, provided the inocula do not differ greatly from one another. To plot the data obtained, a methods of transforming data independent of means depend upon the number of lesions obtained. For 10 or more lesions, the equation z = log (x+c) may be used, where x = lesion number and c is a constant obtained by plotting standard deviations against the means and extrapolating the regression line to the abscissa. For less than 10 lesions, $z = \log \frac{1}{2}\left(x + c + \sqrt{x^2 + 2cx}\right)$ may be applied.

To get the most out of a LL assay involving serial dilutions, variation must be controlled as much as possible. It is known that virtually the same number of LL from a given inoculum will form on each half of an opposite leaf, so a randomized half-leaf method is probably the best to use in most circumstances. With the half-leaf method, the finest discrimination between inoculum concentrations possible was 10% on *Phaseolus vulgaris* (bean), and 20% on *N. glutinosa*, using a 10^{-6} g/mL solution of TMV (Roberts, 1964). One method of producing randomized treatment groups is the Latin square, a grid with a series of letters arranged such that no letter is repeated twice within the same row or column. Each treatment with a virus dilution is assigned a letter, and the arrangement of half-leaves is such that no treatment falls upon both halves of the same leaf. The same experimenter must inoculate every half-leaf in the assay. Uniformity may also be increased by removing all leaves not to be inoculated and putting plants in darkness a day before they are inoculated; this will also increase the number of LL formed (Bawden, 1956).

To determine dilution endpoint of TMGMV PV-0113 and to see if the host response to the virus is consistant with that of TMV (flat at low dilution, steeply-sloping in the middle, and flat at high-dilution), dilution experiments were conducted. Purified PV-0113 was also assayed

using LL counts after lyophilization and storage to determine if the virus retains infectivity when subjected to these treatments.

Materials and Methods

Two experimental designs were used to obtain LL assay-dilution endpoint data. In the first design, two separate viral preparations, "D" and "Q," (chapter 6). After dilution with $Na_2PO_4/NaHPO_4$ inoculation buffer to one optical density (1 OD) at A_{260}, the preparations were diluted 1:10, 1:1,000, 1:10,000, 1:50,000, and 1:100,000 from the 1 OD reference point (approximately 320 µg/mL). Leaves on several tobacco (*N. tabacum* cv. Samsun, *NN*, a local lesion host) plants were labeled such that three entire tobacco leaves were inoculated with each virus preparation; three leaves were mock-inoculated with a buffer solution only. The leaves to be inoculated were randomized in such a manner that one leaf from each of three general positions on the tobacco plant was in each treatment. In addition, each leaf in the set was on a separate tobacco plant. One TSA plant was inoculated concurrently with each treatment.

In the second design, half-leaves on tobacco plants were assigned random treatments using the Latin square method (Table 7-1) and inoculated with various dilutions of PV-0113.

Results

In the first experiment, initially the LL did not appear simultaneously, but continued to appear over time (Figure 7-1, Figure 7-2) until about 5 days post-inoculation (DPI), after which spots counted on the leaves were likely not LL's, but attributable to other causes. To account for this effect, the data were divided into "initial" LL, that is, those LL counted on the first day LL were observed (usually by 3 DPI), and "maximum" LL, the total amount of LL that developed on the inoculated leaf over a 9-day period (Figure 7-3, Figure 7-4). Even highly diluted preparations (1:50,000; 6.3×10^{-6} mg/mL) produced LL on tobacco and disease symptoms (epinasty, LL, wilting) on TSA. An average of local lesions produced at each dilution from a

few different experiments produced a dilution endpoint curve characteristic of a tobamovirus when plotted (Figure 7-5). Plants inoculated with inoculum diluted 1:100,000 (3.2×10^{-6} mg/mL) did not develop symptoms and resembled the mock-inoculated plants in appearance. Both D and Q preparations of TMGMV demonstrated an ability to kill TSA when diluted 1:50,000 (6.3×10^{-6} mg/mL; Figure 7-6, Figure 7-7). The LL-forming ability on tobacco correlated with symptom development (epinasty, local lesions, wilting, and systemic necrosis) on TSA at concentrations up to 1:50,000 (6.3×10^{-6} mg/mL), although in at least one trial TSA was not killed at this dilution.

In one experiment, the reconstituted lyophilized preparation "Q," at dilutions of 1:10 (3.2×10^{-2} mg/mL), 1:100 (3.2×10^{-3} mg/mL), 1:1,000 (3.2×10^{-4} mg/mL), 1: 5,000 (6.3×10^{-5}), 1:10,000 (3.2×10^{-5} mg/mL) , and 1:100,000 (3.2×10^{-6} mg/mL), failed to elicit LL except at the highest concentration. However, in a subsequent experiment using reconstituted preparation "Q," LL were observed at all concentrations except 1:100,000. A "D" preparation of PV-0113 stored under refrigeration (Figure 7-8) and reconstituted from lyophilized virus (Figure 7-9) had a dilution endpoint on *Nicotiana tabacum* cv. Samsun (*NN*) at dilutions of 1:25,000 (1.3×10^{-5} mg/mL) and 1:50,000 (6.3×10^{-6} mg/mL), respectively.

Discussion

Purified TMGMV PV-0113 sometimes produced disease in TSA at the remarkably low dilution of 1 part in 50,000 (6.3×10^{-6} mg/mL). In agreement with results reported for TMV, the linear portion of the curve appears at dilutions > 1:1,000; any future experiments should be carefully designed, probably with intervals ≤ 1:1,000 in order to narrow the dilution at which no more LL are formed.

It may well be worth comparing the relative sensitivity of LL produced by different R-gene/elicitor interactions, because the data obtained might prove useful in establishing the

threshold of elicitor required for LL to occur. One problem with this experiment is that the N-gene and N′-gene containing host species are superficially different and therefore may respond differently to the virus due to physical factors rather than biochemical ones (i.e., the HR signal cascade). However, this problem may be overcome using the Samsun *EN′* tobacco (in which the *N′* resistance gene is active) to minimize effects caused by differences in leaf morphology, topology, or other physiological or non-HR related biochemical differences between the species.

Table 7-1. Latin square design used in *N. tabacum* experiments.

	Plant #1		Plant #2		Plant #3		Plant #4	
	L	R	L	R	L	R	L	R
I	E	G	H	F	B	C	D	A
II	D	F	G	E	A	B	C	H
III	H	B	C	A	E	F	G	D
IV	B	D	E	C	G	H	A	F
V	C	E	F	D	H	A	B	G
VI	A	C	D	B	F	G	H	E
VII	F	H	A	G	C	D	E	B
VIII	G	A	B	H	D	E	F	C

Dilutions: A = 1:10, B = 1:100, C = 1:1,000, D = 1:5,000, E = 1:10,000, F = 1:25,000, G = 1: 100,000, H = (-) no virus. Leaf position: L = left half, R = right half.

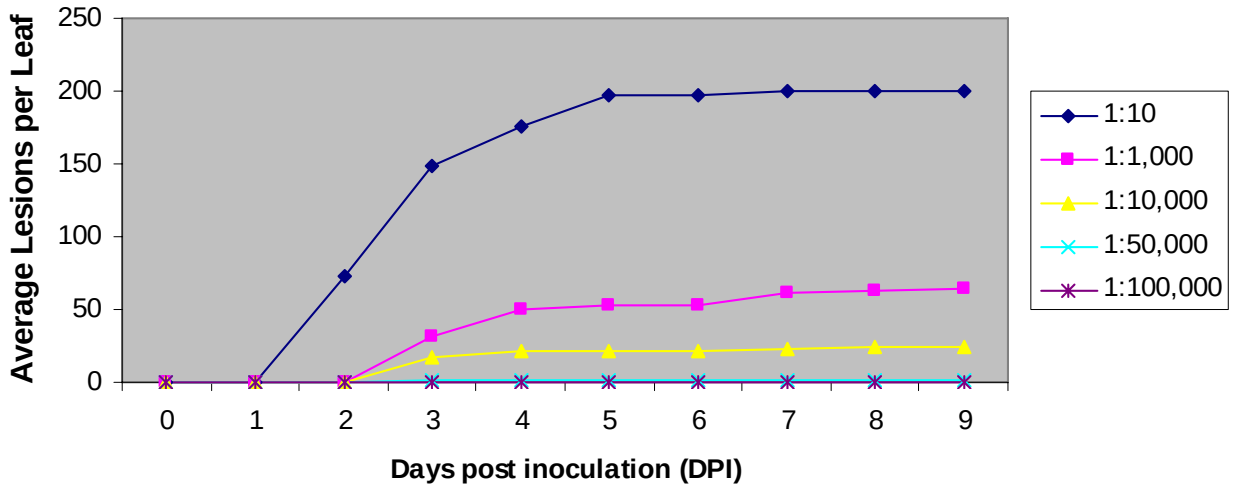


Figure 7-1. Local lesion development over time, Preparation "D."

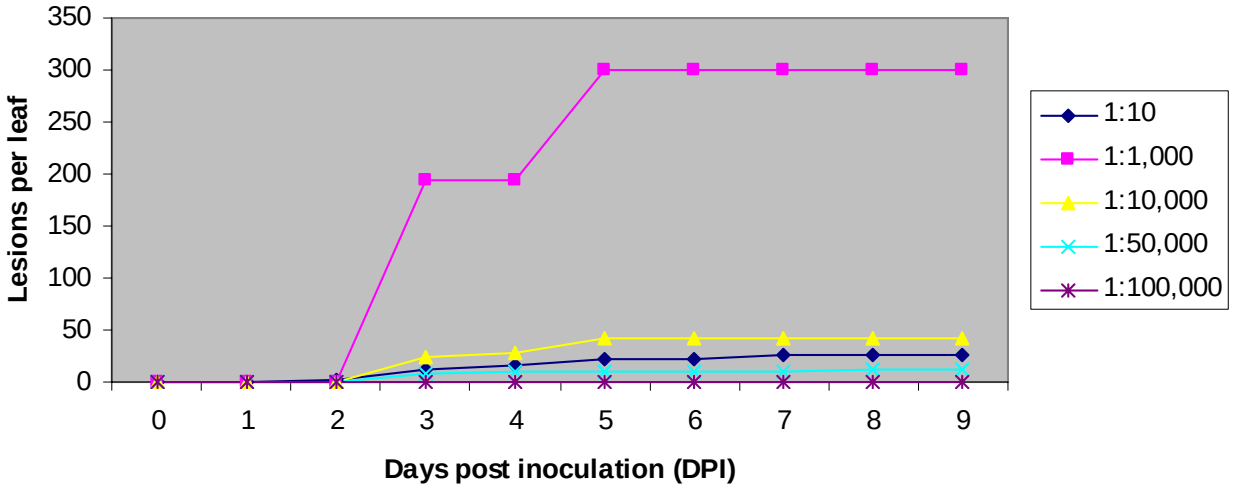


Figure 7-2. Local lesion development over time, Preparation "Q."

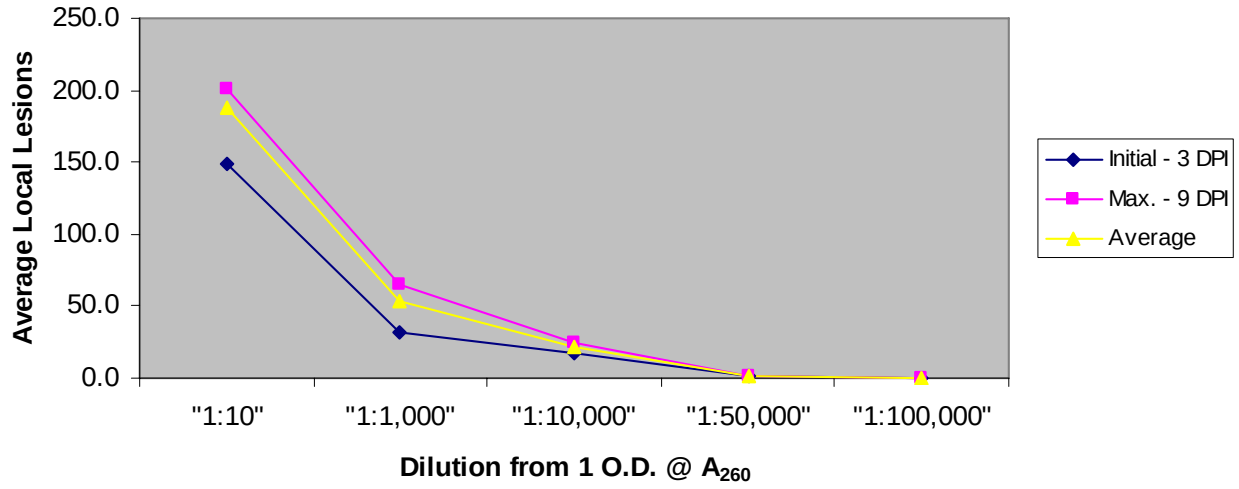


Figure 7-3. Local lesions produced on "Samsun" (*NN*) by TMGMV PV-0113 preparation D; as calculated by taking the average number of LL per half-leaf, divided by the number of half-leaves, x2.

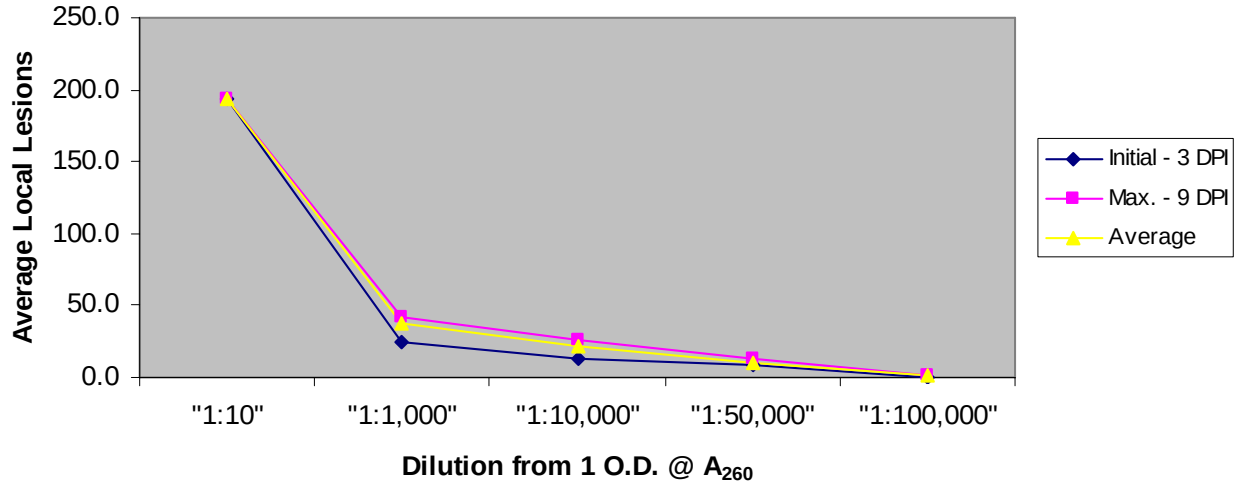


Figure 7-4. Local lesions produced on "Samsun" (*NN*) by TMGMV PV-0113 preparation Q; as calculated by taking the average number of LL per half-leaf, divided by the number of half-leaves, x2.

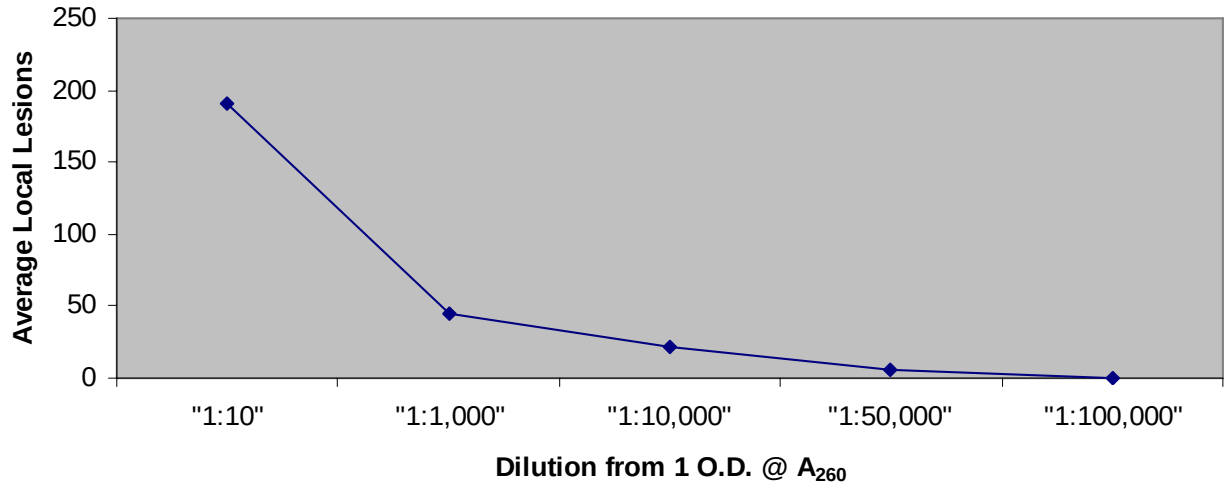


Figure 7-5. Curve formed by average of all local lesions produced on "Samsun" (*NN*) by both D and Q preparations.

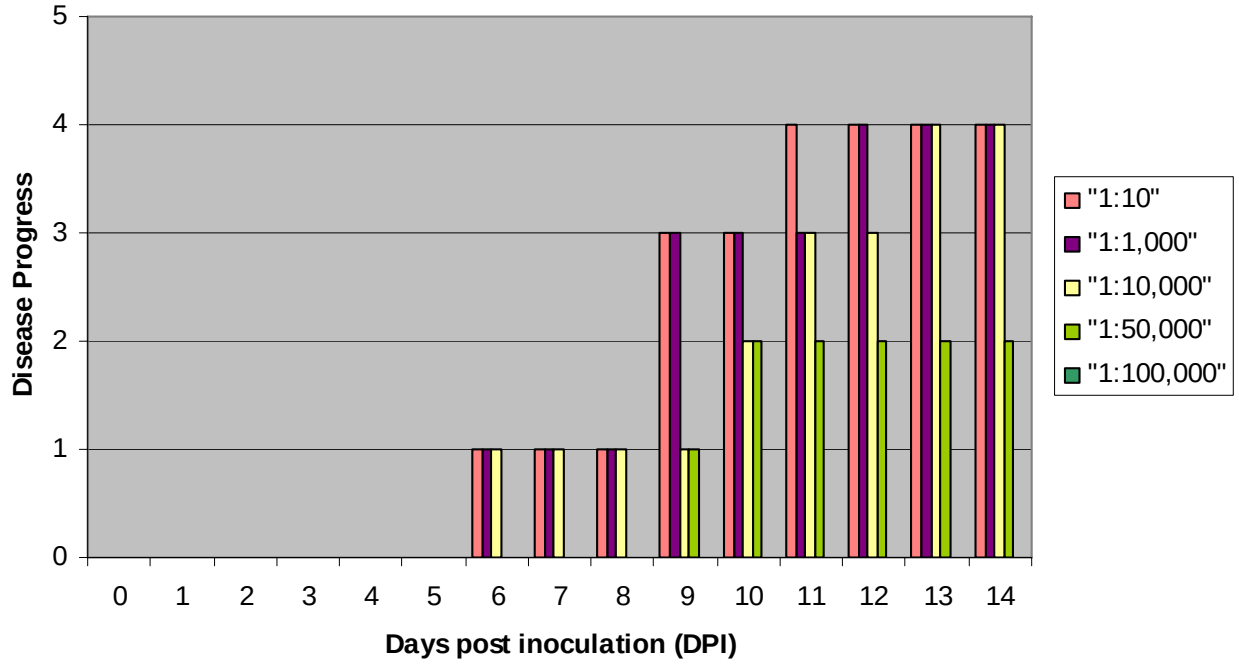


Figure 7-6. Disease progress on TSA (1 plant per dilution) inoculated with TMGMV PV-0113 preparation D.

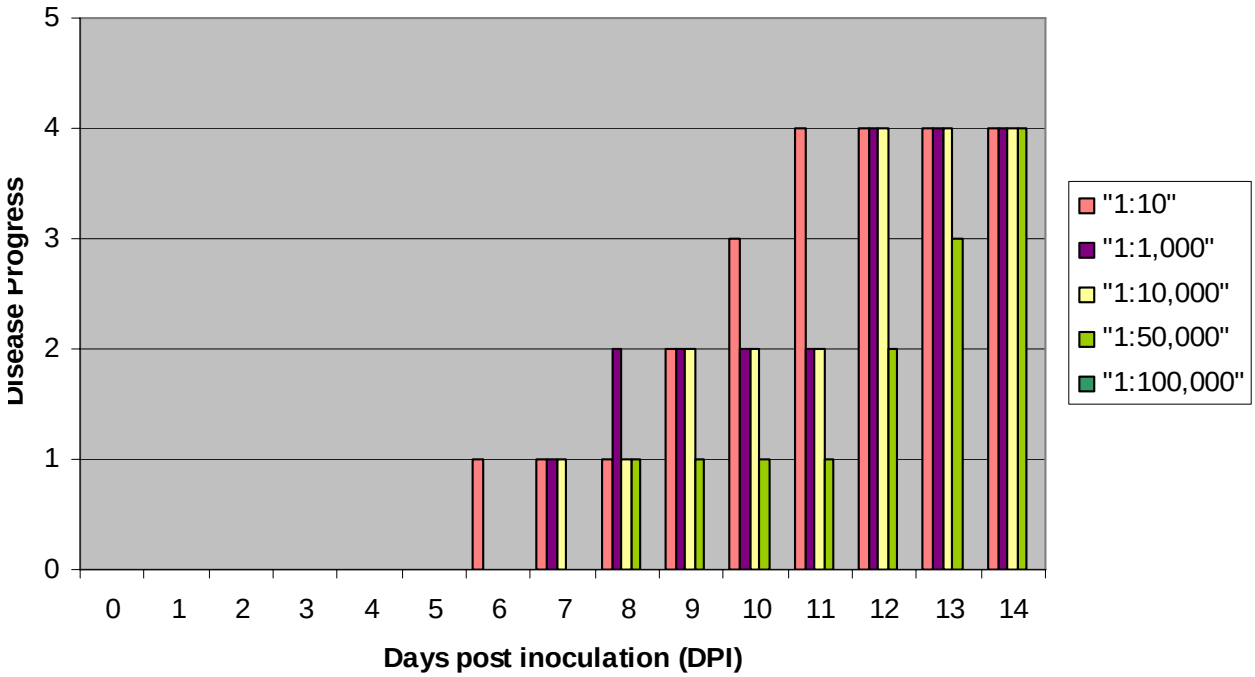


Figure 7-7. Disease progress on TSA (1 plant per dilution) inoculated with TMGMV PV-0113 preparation Q.

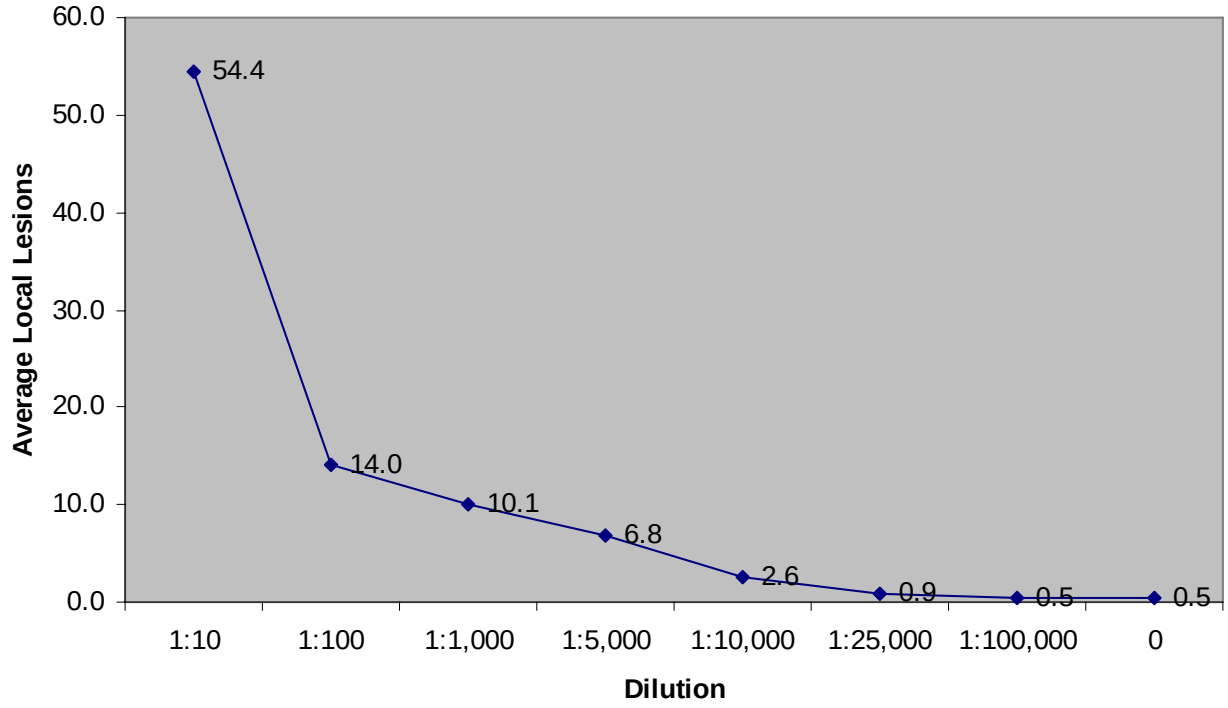


Figure 7-8. Average number of local lesions per half-leaf produced by (refrigerated) preparation "D;" (storage time, four months: 10/03/07-2/04/07).

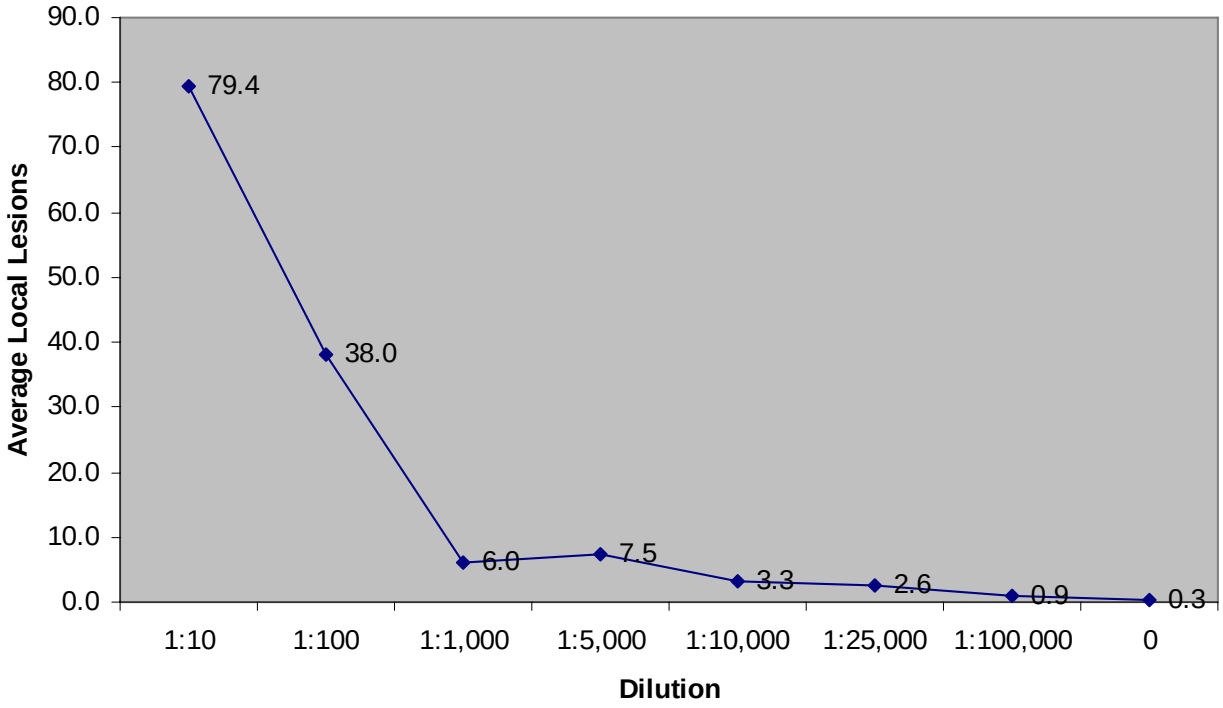


Figure 7-9. Average number of local lesions per half-leaf produced by (lyophilized) preparation "D"; (storage time, four months: 10/03/07-2/04/07).

CHAPTER 8
CLONING OF PV-0113 COAT AND MOVEMENT PROTEIN GENES

Introduction

Since 1982, when TMV became the first RNA virus to have its genome completely sequenced (Goelet et al., 1982; Robinson, 2004), nucleotide sequencing has become standard in characterization of plant viruses, and complete sequences exist for many tobamoviruses. At the time of this writing, complete sequences have been reported for TMV, (including strains cr-TMV, TMV-p, and CTMV-W) (Shimamoto et al., 1998; Yamanaka et al., 1998), and many other tobamoviruses, including Bell pepper mottle virus (BPeMV), Cucumber green mottle mosaic virus (CGMMV), Kyuri green mottle mosaic virus (KGMMV), Maracuja mosaic virus (MarMV), Obuda pepper virus (ObPV/TMV-Ob), Odontoglossum ringspot virus (ORSV), Oilseed rape mosaic virus (ORMV), PMMV, Sunn-hemp mosaic virus (SHMV), TMGMV, ToMV, Turnip vein clearing virus (TVCV), and Zucchini green mottle mosaic virus (ZGMMV). Partial sequences have been determined for many strains of TMV and other tobamoviruses, including Cactus mild mottle virus (CMMoV), Cucumber fruit mottle mosaic virus (CFMMV), Hibiscus latent Fort Pierce virus (HLFPV), Hibiscus latent Singapore virus (HLSV), Nigerian tobacco latent virus (NTLV), Paprika mild mottle virus (PaMMV), Ribgrass mosaic virus (RMV), Streptocarpus flower break virus (SFBV), and even Tropical soda apple mosaic virus (TSAMV) (Rhie et al., 2007; Ryu et al., 2000; Boubourakas et al., 2004; Song et al., 2006; Heinze et al., 2006; Lapido et al., 2003; Alexandre et al., 2000; Gibbs et al., 2004; Min, et al., 2006; Yoon et al., 2001; Yoon et al., 2002; Srinivasan, 2004; Kamenova and Adkins, 2004; Adkins et al., 2007).

At the time of this writing, there are two complete genomic sequences of TMGMV on record: M34077 (Solis and García-Arenal, 1990) and AB 078435 (Okuno et al., 2002), both

archived in the National Center for Biotechnology Information (NCBI) database, and 108 partial ones. Genomic RNA of TMGMV M34077 was 6355 nt in length, while that of TMGMV-J AB 078435 was 6356 nt in length, due to a 5′ variation. TMGMV-J had an initial "GT" sequence while TMGMV had "G." Alignment of the two sequences revealed 223 nucleotide variations or gaps; most notably a 3-nt gap in the TMGMV (M34077) sequence at TMGMV-J position 4069-4071, and a corresponding 3-nt gap in TMGMV-J (AB 078435) at TMGMV position 4770-4772. Search and alignment using the NCBI Basic Local Alignment Search Tool (BLAST) verified an overall sequence identity between the two sequences of 96 percent.

In a 2004 publication, A. Gibbs reported that the phenotypically similar quasi-species such as tobamoviruses were better identified by short nucleotide motifs than traditional nucleototide signatures, because in tobamoviruses, traditional nucleotide signatures are very short and scattered throughout the genome. In a set of 10 sample sequences of TMV and 8 sample sequences of the extremely similar tobamovirus, ToMV, 16.3% of the nucleotides in the sequences identified an isolate as belonging to one group or another; however 1 out of 7 nucleotide *positions* were sufficient to distinguish each isolate of ToMV from TMV. Naming the distinguishing patterns in sequence variation "nucleotide combination motifs," Gibbs chose the TMV 4404-4450 segment because 29 of the 47 nucleotides showed no variability between the many tobamovirus sequences analyzed and because the region so far has proven to match well only within sequences reported for other tobamoviruses for which, so far as is known, it appears unique. Additionally, 10^9 combinations can be obtained from 18 variables, assuming independence and ignoring possible functional constraints on the RNA. This makes it unlikely that new tobamovirus species discovered will be misclassified using this motif. The universal

tobamovirus motif corresponding to Tobacco mosaic virus (TMV) genomic nt positions 4404-4450 reported is:

3′ GG__A_GT_AC_AC_TT_AT_GG_AA_AC__T_AT_AT_GC__C_TG 5′.

In the genome of Tobacco mild green mosaic virus (TMGMV), the Gibbs motif begins at nt 4392 for isolate AB 078435 and at nt 4388 for isolate M34077, within the region bounded by the 3-nt deletions. The motif sequence itself, however, is identical, for both sequences:

3′ GGTGATGTGACTACTTTCATCGGCAATACTGTTATAATAGCAGCTTG 5′.

Since the production of a TMV-containing plasmid (clone) capable of producing infectious RNA transcripts (Dawson et al., 1986), several such infectious tobamovirus clones have been made, including TMV-Ob (Padgett and Beachy, 1993), TMGMV (Morishima et al., 2003), ToMV (Hori and Watanabe, 2003; Weber et al., 1992), ORSV (Yu and Wong, 1998), MarMV (Song et al., 2006), KGMMV (Yoon et al., 2001), and ZGMMV (Yoon et al., 2002).

In 2003, an infectious transcribable clone of a Japanese isolate of TMGMV was created. The virus, TMGMV-J, isolated from peppers in Kochi Prefecture, Japan, was sequenced, and the *in-vitro* transcribable clone, pTGKJW created from it proved infectious. Infectivity was enhanced by encapsidation with purified TMGMV CP. The MP gene of this clone was then used to create a chimeric ToMV that would not infect tomato (Morishima et al., 2003).

Chimeras prepared from tobamoviruses, especially the TMV/TMGMV-L hybrid 30-B, continue to be used as tools to investigate phenomena in molecular biology, and to express proteins of interest (Table 8-1). Peptides produced using tobamovirus constructs include drugs (Takamatsu et al., 1990; Kumagai et al., 1993), vaccines (Yusibov et al., 2002; Szabó et al., 2004; Fujiyama et al., 2006), antibodies (Giritch et al., 2006), and biodegradable pesticides

(Borovsky et al., 2006). The objectives of the work presented in this chapter was to obtain sequences of the TMGMV PV-0113 isolate to compare with other TMGMV and tobamovirus sequences on record, and to obtain and amplify the gene sequences to be used for transient expression work described later (chapter 9) in this thesis.

Materials and Methods

Tobacco mild green mosaic virus genomic RNA was reverse-transcribed and amplified using primers created to the 3rd and 4th open reading frames (ORF), as well as a segment containing the 4404-4450 tobamovirus motif (Gibbs et al., 2004), and purified for use in the Gateway BP recombination reaction in conjunction with the proprietary cloning vector, pDONR221 to transform chemically competent *Esherichia coli.* The bacteria were cultured on lysogeny broth agar (LBA) containing 50 μg kanamycin per mL as a selective agent. Transformants were selected and cultured in lysogeny broth (LB) containing kanamycin (50 μg/mL) and plasmids extracted from them using a Qiagen miniprep kit. The extracted plasmids were then sequenced, and the sequences compared to TMGMV in the NCBI "BLAST" database.

Primers incorporating Gateway-specific modified phage-derived insertion sequences *att*B1 and *att*B2 were designed with the help of pDRAW32 (Olesen, 2003), to each ORF of the TMGMV genome, using the sequence M34077 published by Solis and García-Arenal (1990), and the sequence AB 078435 recovered by Okuno (NCBI BLAST database), as a guide. Oligonucleotides for ORFs 3 and 4, the movement protein and the coat protein, respectively, were synthesized by Integrated DNA Technologies, Inc.

- **ORF 1/2 F:** 5•[GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT C] ATG GCA CAC ATA CAA TCT A 3•
- **ORF 1 R:** 3•[GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC] CTA TCT AC(T/C) ACC TGC TTC 5•

- **ORF 2 R:** 3•[GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC] CTA ACA GCC ATT TAA AAA 5•
- **ORF 3 F:** 5•[GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT C] ATG GCT GTT AGT CTC 3•
- **ORF 3 R:** 3•[GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC] (T/C)TA AAA CGT ACT CGA TGA 5•
- **ORF 4 F:** 5•[GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT C] ATG CCT TAT ACA ATC AAC TC 3•
- **ORF 4 R:** 3•[GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC] CTA AGT AGC YGG AGT TGT 5•

(Brackets indicate Gateway recombination sequences).

Primers described as specific to TMGMV (Gibbs et al., 2004) were supplied by Integrated DNA Technologies, Inc. for cloning and sequencing of a segment of ORF 2 containing the Gibbs' tobamovirus identification motif. Universal Tobamovirus Primer 2, **5•** TTBGCYTCRAARTTCCA **3•**, used for first-strand synthesis, anneals to nt positions 4572–4588 in the TMV genome, and Tobamovirus Primer 3, **5•** CARACXATWGTBTAYCA **3•** anneals to nt positions 4034–4050 in the TMV genome (Gibbs et al., 2004).

First strand cDNA synthesis was accomplished using Invitrogen M-MLV RT or the improved version, SuperScriptIII. Briefly, genomic RNA to be reverse-transcribed was diluted to <500 ng and 2 pmol (1-2 µL) primers added, along with 1 µL 10 mM deoxynucleoside triphosphates (dNTPs). The mixture was adjusted to a volume of 13 µL with RN-ase-free water and incubated in a 65°C water bath for 5 minutes. The mixture was then transferred to ice for 1 minute before pulse centrifugation to bring the contents to the bottom of the tube. To the incubated mixture was added 4 µL 5x First-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 µL 0.1 M dithiotreitol DTT, 1 µL RN-ase inhibitor (40 U/µL), and 1-2 µL SuperScript III (200 U/µL). The reaction mixture was then mixed by pipette and allowed to

incubate at 55°C for 45 minutes. Samples were then heated to 70°C for 15 minutes to quench the reaction, after which 1 μL RNase H was added and the reaction mixture incubated at 37°C for 20 minutes. Template DNA thus prepared was used immediately for PCR or frozen at -20°C for future use. Template DNA was obtained using "ordinary" M-MLV RT by following a similar protocol.

PCR was carried out with the cDNA obtained from RT. For each sample, 3 reactions were conducted, using cDNA (undiluted, 1:10, 1:100) in PCR reaction tubes with 24 μL PCR mix (2.5 μL 10x PCR buffer, 0.9 μL 50 mM $MgCl_2$, 2.5 μL 2 mM dNTP, 16 μL MBG H_2O), 1 μL forward primer, 1 μL reverse primer, and 0.125 μL Taq DNA polymerase. Reaction mixtures were overlaid with mineral oil (when required by the thermocycler), and put through a program including 35 cycles of heating and cooling.

Products obtained from PCR were purified for use in Invitrogen's Gateway BP recombination reaction using polyethylene glycol (PEG) as follows. Fifty microliters (50 μL) PCR product was combined with 150 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 100 μL of PEG solution (30% PEG 8000 w/v, 30 mM $MgCl_2$), vortexed briefly, and centrifuged at 10,000 g for 15 minutes. Supernatant was removed and the pellet re-suspended in 50 μL of TE buffer. Quality of the purified sample (free from dimers) was verified by electrophoresis on 0.9% agarose. Purified PCR-generated fragments were ligated into the commercially available cloning vector pDONR221 (Figure 8-1).

Concentration of DNA in each PCR sample was estimated using agarose electrophoresis against a standard ladder and UV spectrophotometry. To 100 femtomoles (100 fmol) of each PCR sample (19 μL of ORF 3, 12 μL of ORF 4), 2 μL of pDONR (150 ng/μL), and 4 μL of rxn buffer were added. Two microliters (2 μL) of the plasmid pEXP7-tet (50 ng/μL) was used in one

reaction as the positive control for recombination. To each prepared reaction, 2 μL of freshly-thawed BP Clonase enzyme was added, the reaction mixture vortexed twice for exactly 2 seconds, and the samples allowed to incubate overnight at 25°C. Afterwards, recombination was quenched by addition of 2 μL of Proteinase K (2 μg/μL) to each sample and 10 minutes' incubation at 37°C. Plasmids obtained from this reaction were used to transform *E. coli* DH5α and stored frozen at -20°C.

The (standard) heat-shock method was utilized for transformation of *E. coli*. Briefly, frozen competent (Invitrogen DH5α) cells were thawed on wet ice and 50 μL placed into sterile 1.5 mL polypropylene tubes for each transformation. One microliter (1 μL) of ligated pDONR221 was added and mixed very gently by inversion. The ampicillin-resistant plasmid, pUC19 was utilized as a transformation control. Tubes were then incubated on ice for 30 minutes. Heat shock was induced by placing the tubes in a 42° C water bath for 30 seconds and immediately chilling them on ice with addition of 450 μL room temperature super optimal catabolite-repression (SOC) medium. Tubes were then capped and placed on a 37° C incubating shaker set for 200 rpm agitation and 37°C for 1 hour, before spreading onto pre-warmed selective media plates. LBA plates laced with kanamycin (50 μg/μL) were used for selection of PCR/pDONR recombinants, while the recombination control, pEXP7-tet, was assayed on tetracycline (20 μg/μL) and carbenicillin (100 μg/μL) was used for selection of pUC19.

Colonies were screened for inserts using colony PCR. Colonies to be analyzed were sampled using a sterile toothpick and transferred to 50 μL of extraction buffer (1% Triton X-100, 20 mM Tris pH 8.0, 2 mM EDTA) in a 1.5 mL microcentrifuge tube with lid lock and immersed in boiling water for 5 minutes. Upon cooling, 2-5 μL of the bacterial extract was amplified using PCR as described above.

Colonies identified as containing sequences of interest were cultured in 3.0 mL LB containing kanamycin (50 μg/μL) for selection. After 16-18 hours incubation with agitation at 37°C, cultures were centrifuged at setting 8.5 for 15 minutes in a TJ-6 Beckman centrifuge. Supernatant was discarded, and the pellets resuspended in 250 μL of P1 buffer (supplied in kit) before transfer to a microcentrifuge tube. Two hundred and fifty microliters (250 μL) of P2 buffer (supplied in kit) were then added. After mixing by inversion, 350 μL of N3 buffer (supplied in kit) was added, and the samples mixed again by inversion, and centrifuged for 10 minutes in a tabletop centrifuge at maximum speed. The supernatant was then removed by micropipette to the center of a QIAprep column, which was centrifuged for 30-60 seconds at maximum speed, and washed with 750 μL PE buffer (supplied in kit) before elution of DNA with 30 μL molecular biology grade water.

For applications in which plasmid quality was not critical, extraction was accomplished using chloroform/octanol. Bacteria containing plasmids to be extracted were inoculated into 4.5 mL LB containing appropriate antibiotic(s) for selection and incubated at 37°C for 16-18 hours with agitation. Four (4) mL of culture was centrifuged in the TJ-6 centrifuge at setting 8.5 for 15 min, after which, supernatant was discarded and the pellet re-suspended in 350 μL P1 buffer (50 mM Tris HCl, pH 8.0; 10 mM EDTA; 100 μg/mL RNA-ase A), and transferred to a 1.5 mL tube. Then, 400 μL freshly-prepared P2 buffer (~200 mM NaOH [1 pellet in 9 mL distilled, deionized water]; 9 mL sterile distilled, deionized water; 1 mL 10% SDS) was added and the suspension mixed by inversion. The mixture was allowed to incubate at RT for 5 minutes before 400 μL P3 buffer (3.0 M potassium acetate, pH 4.8) was added, and the volume mixed again by inversion, then placed on ice. After 15 minutes, the mixture was removed from ice and spun at maximum speed in a tabletop centrifuge for 15 minutes. The pellet was discarded and 900 μL of

supernatant transferred into a 2.0 mL microcentrifuge tube, to which 1 volume isopropanol was then added. After a 15-30 minute spin-down at the maximum setting, pellets were washed with 70% nuclease-free ethanol and vacuum (aspirator) dried for 7 minutes. Then, the DNA pellet was resuspended in 200 µL molecular biology grade water and extracted with octanol : chloroform (1:24). Following centrifugation, the aqueous phase was collected, and the DNA precipitated using 0.09 volumes of 3 M sodium acetate, and 2.5 volumes ethanol. Following a 20-minute centrifugation at 4°C (maximum-speed), the pellet was washed with 70% ethanol as described previously, vacuum dried, and resuspended in 25 µL molecular biology grade water.

Gateway-compatible gene constructs pKYWG2 and pKYWG2D, suitable for expression *in plantae* (Karimi et al., 2002), were obtained from the Interuniversity Institute for Biotechnology (VIB) in Ghent, Flanders, Belgium. Constructs were delivered *in vivo*, in the form of bacterial stab cultures (i.e. a special strain of *E. coli* resistant to the effects of ccdB) and cultured in LB containing 50 µg per mL spectinomycin as a selective agent. Plasmids harvested from the cultures, were recombined with the pDONR221 clones.

Sequences of interest (PV-0113 ORF 3 and ORF 4) were ligated into VIB plant expression vectors pKYWG2D and pKYWG2 (Figure 8-2), designated "E-1" and "E-2," respectively, using Gateway LR Clonase catalyzed recombination. Briefly, 100 – 300 ng purified pDONR containing PV-0113 sequences (entry clone) were combined with 300 ng of the expression vector and 4 µL 5X LR Clonase reaction buffer in a 1.5-mL tube, and the volume adjusted to 16 µL with TE buffer. The plasmid pENTR-gus (50 ng/µL) was used as a positive control. Four (4) µL freshly-thawed LR Clonase was added to each reaction and briefly vortexed twice. Samples were then allowed to incubate for 1-18 hours at 25°C. After incubation, the reaction was

quenched by 2 μL Proteinase K added to each tube, and a 10 minute incubation at 37°C. Reaction mixtures obtained were used to transform *E. coli* DH5α as described above.

Transformed *E. coli* were selected on LBA plates containing 50 μg per mL spectinomycin. Selected transformants were screened using PCR as described above, cultured in LB containing spectinomycin (50 μg/mL), and the plasmids extracted by the Qiagen method.

Subsequent transformations, screenings, extractions, and purifications were carried out as previously described. Sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) sequencing lab using Perkin Elmer/Applied Biosystems automated sequencing. Nucleotide alignment was accomplished using ClustalW (Chenna et al., 2003; Thompson et al., 1997; Thompson et al., 1994), using default parameters, and Multalin (Corpet et al., 1988), using the DNA symbol comparison table and default settings. Multalin processing typically used the DNA symbol comparison table. Sequences were translated using the "Translate" tool of the ExPASy proteomics server (Gasteiger et al., 2003), and pDRAW32 (Olesen, 2003). Amino acid sequences were aligned using Multalin with the Blosum symbol comparison table and default settings.

Results

Comparison of the ORF 3 nucleotide sequences of TMGMV PV-0113 with TMGMV M34077 (Solis and García-Arenal, 1990) and TMGMV AB078435 (Okuno et al., 2002), using Multalin (Figure 8-3) revealed 13 nt variations between PV-0113 and M34077, nine of which were translationally important (Figure 8-4). Between PV-0113 and AB078435 there were 17 nt substitutions, but only six of these were translationally important. Overall, there was an ~98% identity between the ORF 3 of PV-0113, M34077, and AB078435. Translated, the relationship

between the sequences is more difficult to interpret. Interestingly, TMGMV PV-0113 and AB078435 share a 5-aa sequence spanning aa 209-213 different from that of M34077.

Alignment of ORF 4, the coat protein domain of PV-0113 with M34077 revealed 2 nt variations, neither of which was translationally important, while 10 nt substitutions were discovered relative to AB078435 (Figure 8-5), only 1 of which was translationally important (Figure 8-6). Overall, there was a >99% identity between the ORF 4 of PV-0113 and M34077, and a 98% identity between the ORF 4 of PV-0113 and AB078435.

The nucleotide sequence from TMGMV PV-0113 corresponding to nt 4404-4405 of type-strain TMV matched the predicted Gibbs' motif for the species, TMGMV (Figure 8-7).

Discussion

Since the experiments described herein were completed, a full-length clone of TMGMV PV-0113 has been obtained and sequenced (Dr. Ernest Hiebert, Professor, University of Florida, Gainesville, personal communication). The nucleotide sequence of this clone (Genbank EF 469769) is highly similar to archived TMGMV sequences. The quasi-species nature of viruses will allow variability between individual clones; indeed, a certain amount of variability is expected within any species. Variability is, additionally, a liability of the sequencing procedure itself. In parallel with nature, mistakes sometimes occur in the course of reading thousands of sequence reactions: the same genetic material will have an occasional nucleotide mis-read after multiple reactions have been performed (Dr. E. Hiebert, personal communication). Automated sequencing technology has improved considerably over the years; however, potential errors in archived sequences may persist.

The Gibbs 4404-4450 tobamovirus specific motif appears to be a good indicator for TMGMV. The TMGMV strain PV-0113, which shows small differences between previously analyzed strains was 100% homologous for the entire 47 nt motif-containing segment. It would

be interesting to analyze the tobamovirus-specific motif of tobamoviruses [e.g. Cactus mild mottle virus (CMMoV) and Tropical soda apple mosaic virus (TSAMV)] discovered since the publication of Gibbs' work, and test the prediction that combinatorial probability ensures correct identification and classification using the motif.

The extremely high degree of identity between TMGMV PV-0113 and the archived strains of TMGMV means that separation of particular TMGMV strains using serology may well be impossible. A better method would be to look for rather variable regions within the genome and to find a highly variable "motif," perhaps based on Gibbs' findings. In particular, variability at the 3′ end of the virus between the TMGMV-L and TMGMV-S might be exploited. Used in combination with a TMGMV PV-0113-specific primer set, primers designed to a highly variable region could possibly distinguish closely-related strains of TMGMV.

Table 8-1. Useful products produced by using transgenic tobamoviruses.

Reference	Peptide	Application
Takamatsu et al., 1990.	Leu-enkephalin	Experimental painkiller
Kumagai et al., 1993.	α-Trichosanthin	HIV/AIDS treatment
Yusibov et al., 2002.	Rabies peptide	Inexpensive rabies vaccine
Szabó et al., 2004.	Amyloid-β protein	Alzheimer's research
Borovsky et al., 2006.	Aedes oostatic factor	Biodegradable larvacide
Fujiyama et al., 2006.	Poliovirus peptide	Inexpensive polio vaccine
Giritch et al., 2006.	IgG	Antibodies

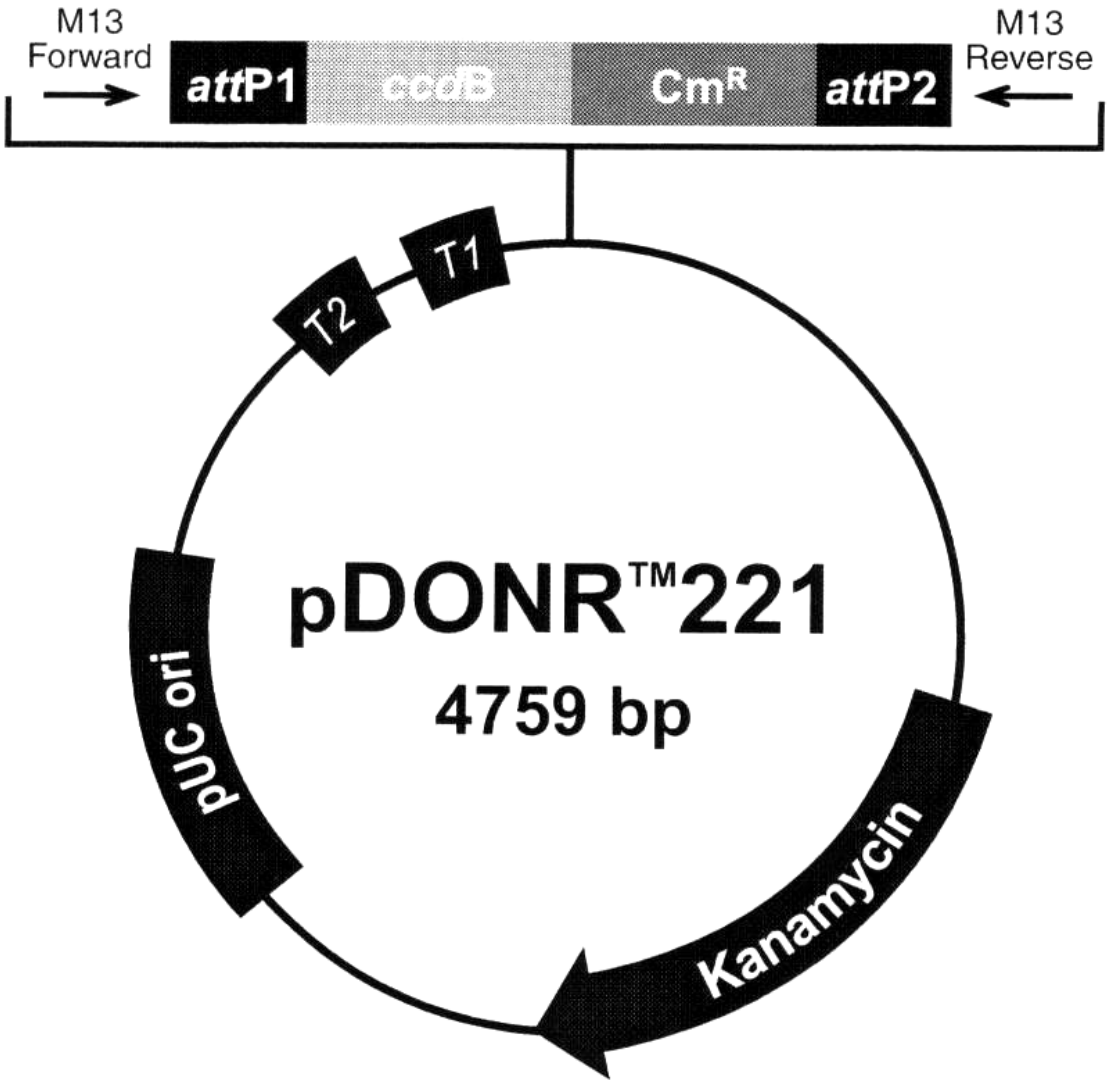


Figure 8-1. Map of the proprietary cloning vector, pDONR221 (Invitrogen Manual). attP1, attP2 = Gateway enzyme-mediate recombination sites; ccdB = gyrase inhibitor for negative selection; CmR = chloramphenicol resistance gene for selection.

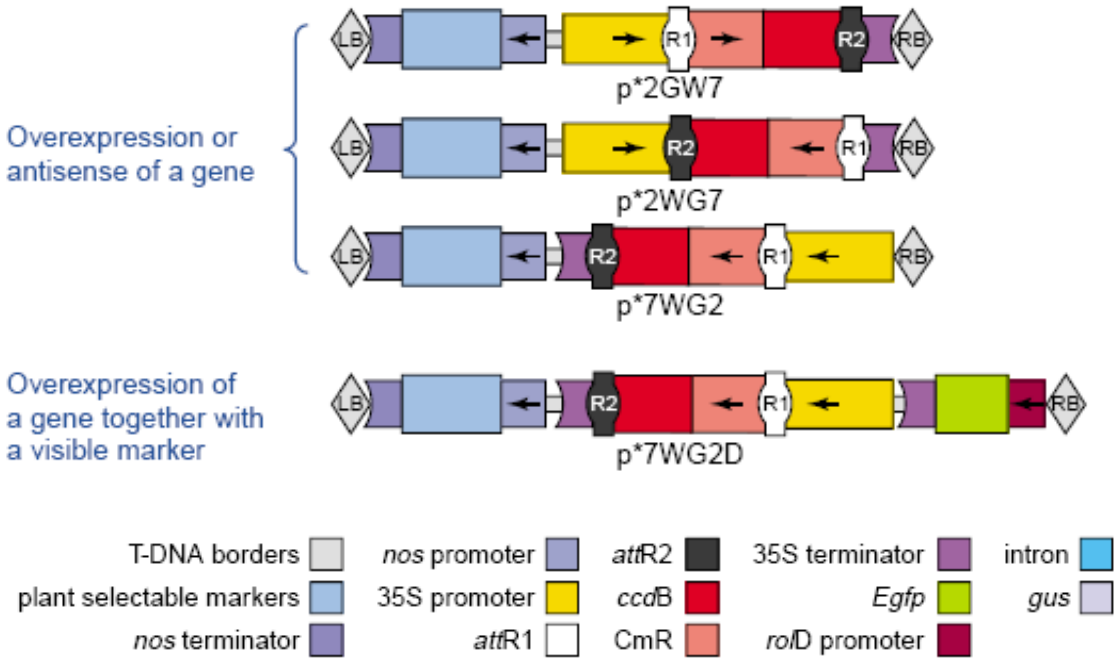


Figure 8-2. Constructs used in TMGMV PV-0113 research (Karimi et al., 2002).

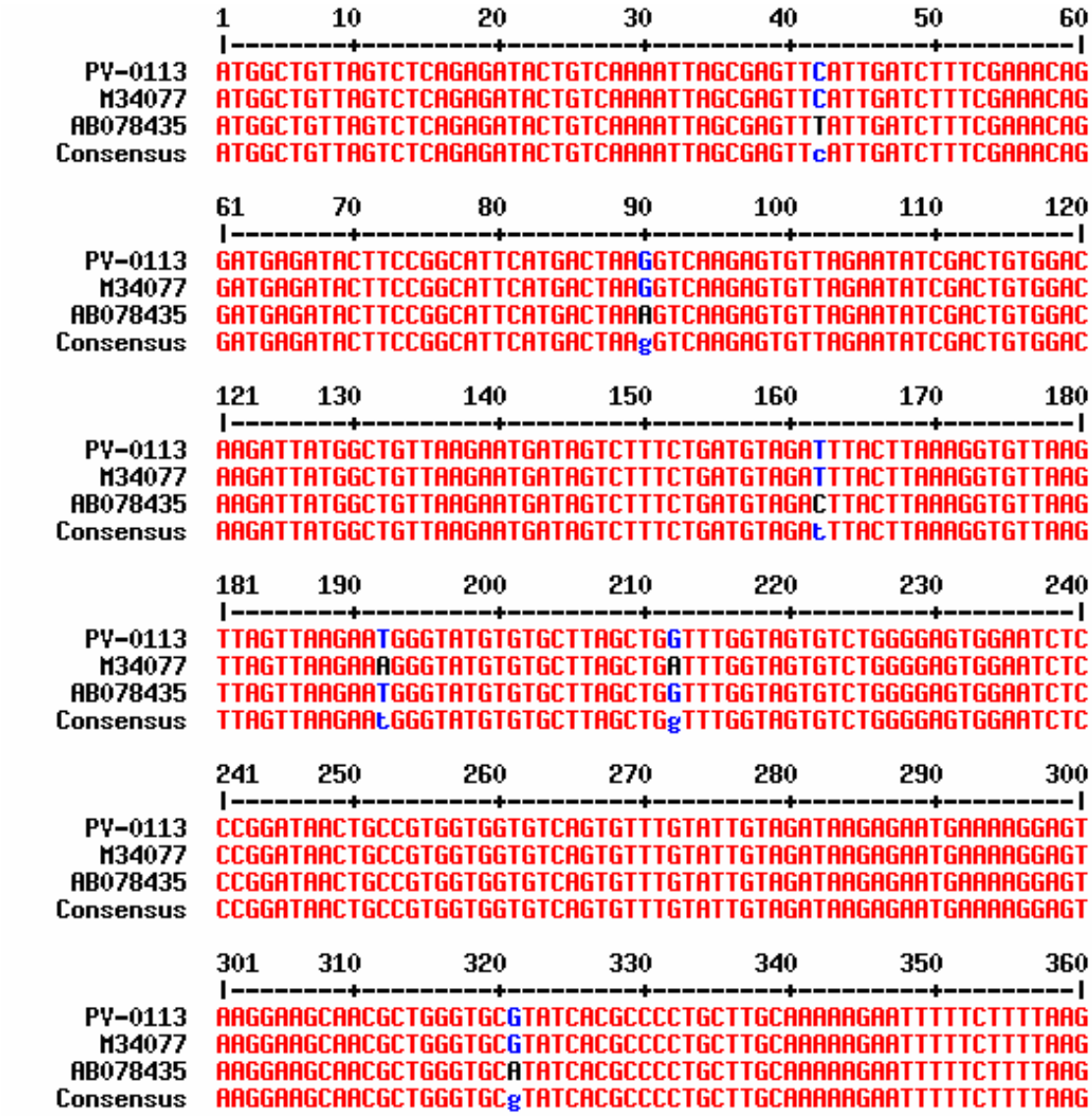


Figure 8-3. Nucleotide alignment of TMGMV PV-0113 ORF 3 with those of GenBank TMGMV sequences M34077 and AB078435.

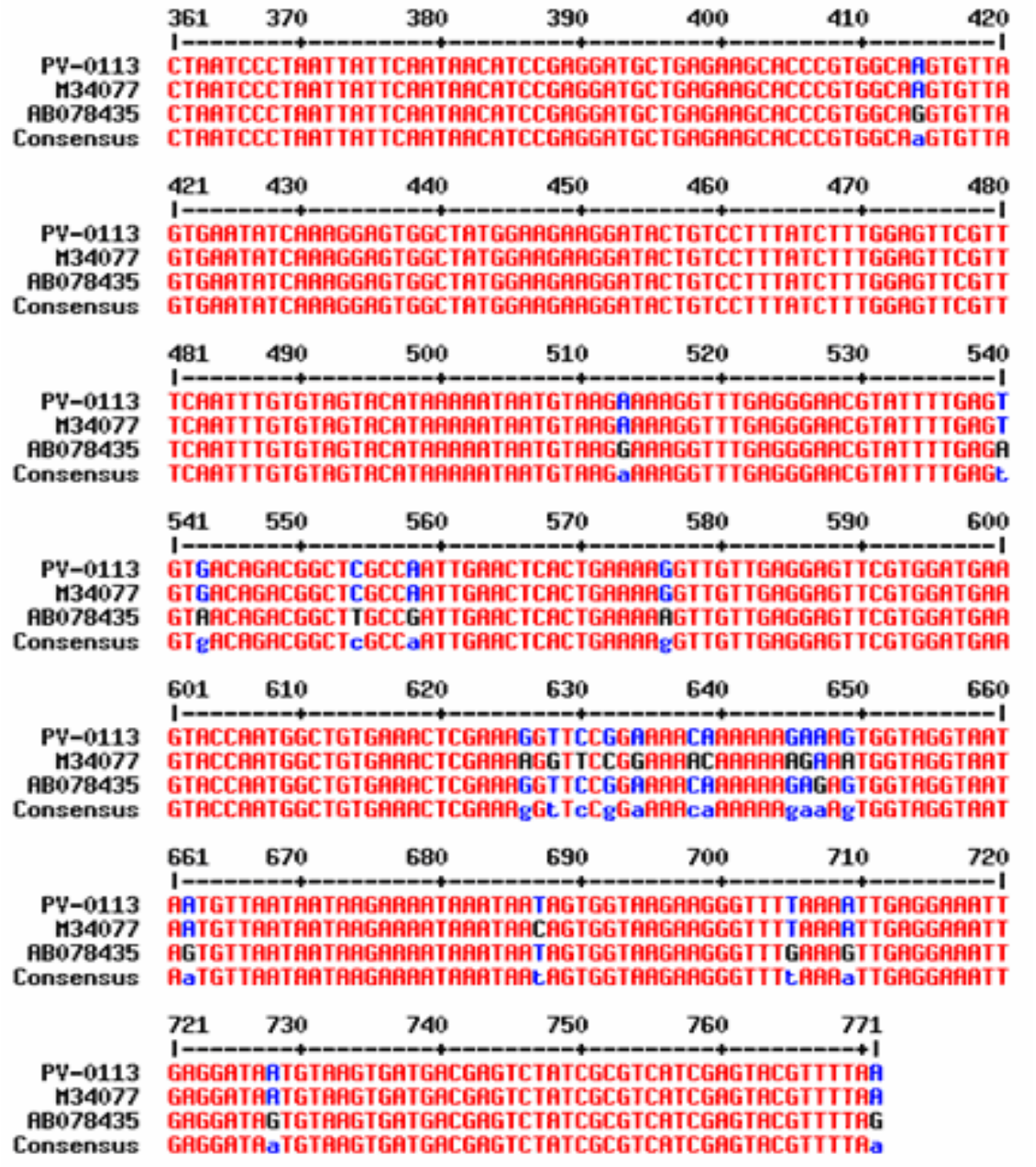


Figure 8-3. Nucleotide alignment of TMGMV PV-0113 ORF 3 with those of GenBank TMGMV sequences M34077 and AB078435 (continued).

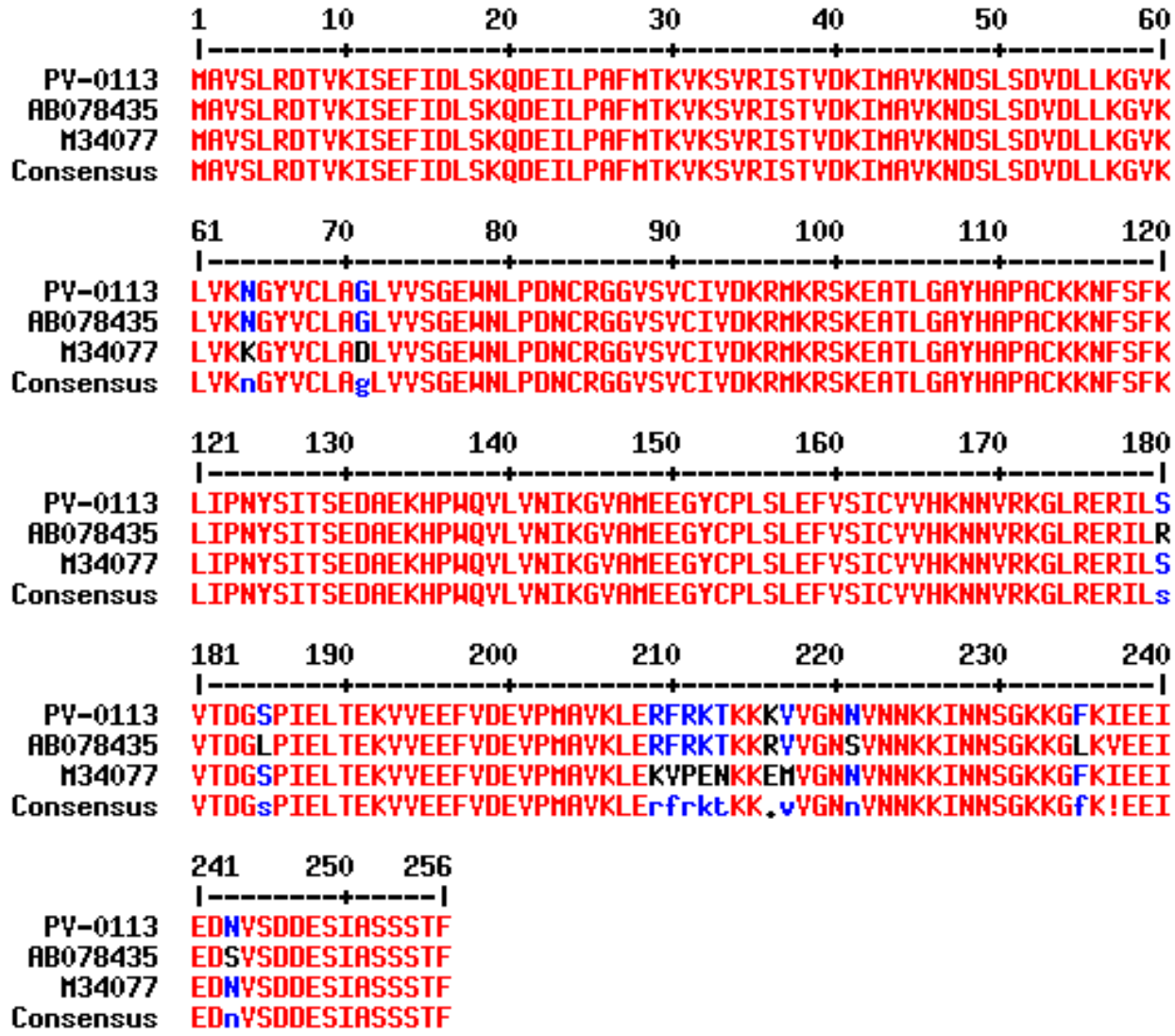


Figure 8-4. Amino acid alignment of PV-0113 MP with those of (translated) GenBank TMGMV sequences M34077 and AB078435.

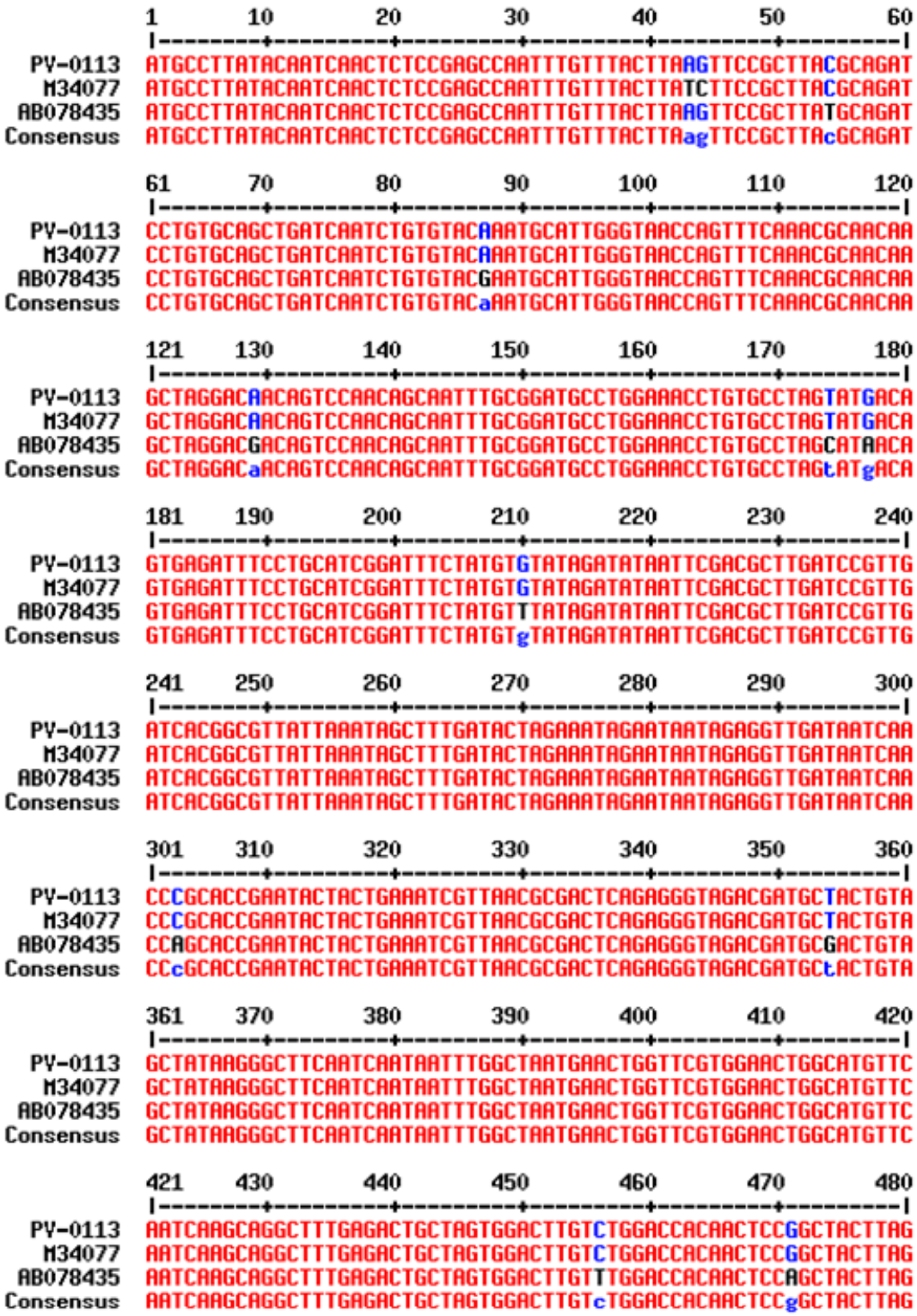


Figure 8-5. Nucleotide alignment of PV-0113 ORF 4 with those of GenBank TMGMV sequences M34077 and AB078435.

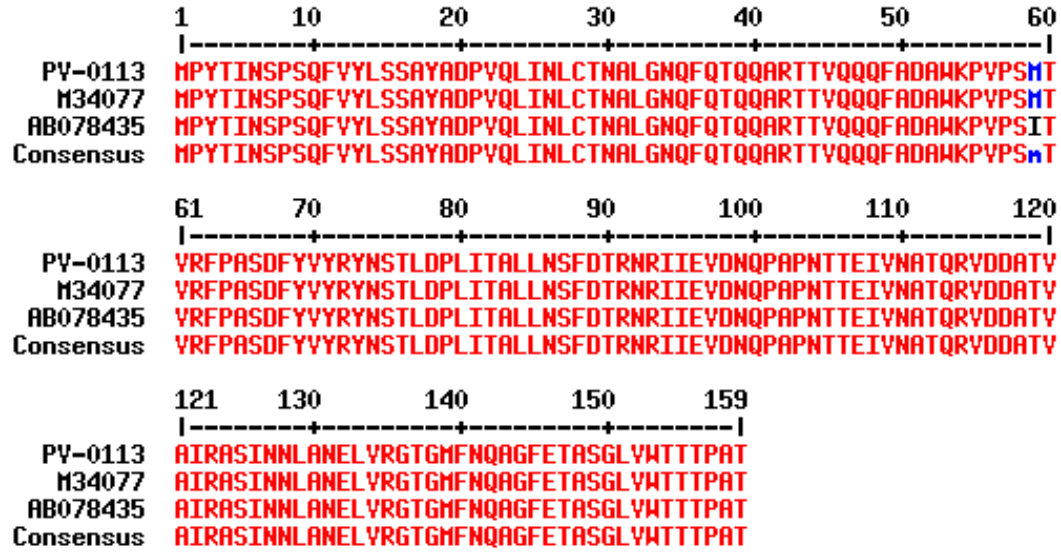


Figure 8-6. Amino acid alignment of TMGMV PV-0113 CP with those of (translated) GenBank TMGMV sequences M34077 and AB078435.

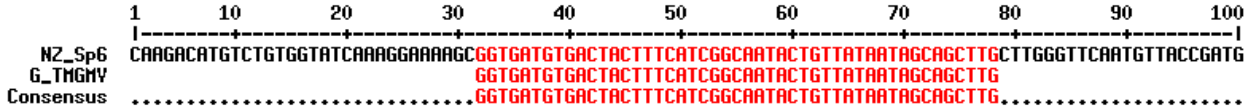


Figure 8-7. Isolate PV-0113 contains the Gibbs TMGMV specific motif.

CHAPTER 9
TRANSIENT EXPRESSION OF PV-0113 COAT PROTEIN

Introduction

To identify the gene or genes responsible for the lethal HR in the TSA-TMGMV system, transient expression systems were tested. Transient expression, in this case the expression of a single viral gene *in planta*, without the intact virus, may be accomplished by various means, including inoculation with a chimeric virus or virus transcript and by plasmids (vectors) delivered using *Agrobacterium tumefaciens* or with the aid of projectiles. Sometimes, two methods may be combined: a plasmid capable of transcribing chimeric virus RNA *in planta* may be introduced into the host where proteins are then expressed.

With a full-length clone in hand, one potentially appealing route is to create a chimeric virus by swapping heterologous regions of two or more closely related viruses. Successful chimeras may enable the identification of the function or phenotype of the heterologous sequence gene.

During transient expression of a foreign protein, one potential issue that arises is the way in which the protein interacts with host cellular machinery. The protein might not, for example, accumulate to high concentrations within the cell. It might inhibit or interfere with cellular metabolism in some way. It might be outright toxic. Strategies employing hybrid vectors help to overcome these concerns, because the rapid expression of viral proteins theoretically would allow a large quantity to accumulate before triggering toxicity to plants (Hori and Watanabe, 2003).

In general, tobamovirus vectors have been designed to work with certain well-studied and laboratory-friendly species such as tobacco that are easily inoculated and infected and susceptible to a wide range of pathogens. One such example is the tender, highly "permissive"

host, *Nicotiana benthamiana*. Often, the ability of the tobamovirus vectors to replicate and express foreign genes seems limited to these hosts (Hori and Watanabe, 2003). For tobamoviruses, it has been determined that more coat protein (CP) is translated than any other gene product. While short peptides have been obtained by fusing the gene of interest directly to the CP/3′ NTR of the tobamovirus, difficulties have been reported in attempting expression of larger proteins such as the marker gene green fluorescent protein (GFP). Success with virus transcripts may be enhanced by *in vitro* encapsidation of the transcripts with viral CP, forming more durable and infectious particles (Hori and Watanabe, 2003).

Chimeras incorporating Tobacco mild green mosaic virus (TMGMV) (Table 9-1) are usually constructed by incorporating TMGMV genes into a modified TMV or ToMV genome. For example, chimeric tobamovirus constructs capable of multiplying and infecting solanaceous plants and protoplasts were created from ToMV and the Japanese isolate, TMGMV-J. The chimera, TocJ, which expressed TMGMV-J CP, was created with the ToMV CP start codon that was disabled using primer-forced nucleotide substitution, and by inserting the TMGMV CP sequence, and the GFP variant G3 (Hori and Watanabe, 2003).

Another early TMV/TMGMV chimera, V23, was used to investigate the relationship between virus movement and the phenotype of local lesions. The product of a movement protein-defective TMV (V1), from which the 5′ end of ORF 3 was removed and ORF 3 of TMGMV (ATCC PV-0228) was inserted, it behaved much like TMV, moving systemically in the TMGMV non-host *Lycopersicon esculentum* (Nejidat et al., 1991).

The TMV/TMGMV-L chimera 30B, which incorporates a duplicated 3′ NTR to enhance transgene expression, suffers from recombination arising *in planta* which results in progeny that have lost the transgene but remain less vigorous than wild-type (WT) tobamoviruses.

Interestingly, GFP-containing vectors bearing TMGMV-L ORF 4 and 3′ NTR sequences produced much more of the transgenic product, GFP, than heterologous sequences from any other tobamovirus species hybridized (Shivprasad et al., 1999).

A direct method for delivery of genes into a cell is the biolisitic or "gene gun" approach. Originally, devices were designed using 0.22 blanks to propel a disk containing DNA-coated particle towards an abrupt stop, hurling the particles towards the plant target and embedding them in the tissue. This crude design was effective enough, but has been improved by the use of compressed helium and a vacuum, so that a finer degree of control over the velocity of the pellets (and therefore the depth of their penetration) can be achieved. Generally, gold particles are preferred over tungsten for their uniformity and chemical inertness (Sawant et al., 2000).

Gene delivery using particle bombardment has been carried out for some time. Transient expression (phytochrome, chloramphenicol acetyl transferase) in cereals (*Hordeum* and *Oryza*) and other plants using the particle gun delivery method was achieved by the end of the 1980's (Bruce et al. 1989.) Morikawa et al. (1989.) used projectiles to effect transient expression (luciferase, GUS) in cultured tobacco and eggplant tissues. The delivery device used pressurized N_2 to drive a Teflon projectile coated with gold particles towards an abrupt stop which hurled the particles into the tissue. From these crude but effective "gene guns" a device called a "particle inflow gun" (PIG) was developed (Finer et al., 1992), using pressurized helium against a partial vacuum to fling DNA coated particles adhering to a screen grid towards the target, rather than to accelerate them using a carrier projectile, as previous models had. Later, Gray et al. (1994) described a simplified design using tungsten particles for delivery. Though relatively inexpensive, it was nonetheless effective, producing transient expression of GUS in cantaloupe cotyledons.

Using a Finer-type gun (Finer et al., 1992), PIG gun, and gold particles, Romano (Romano et al., 2001) describes transient expression (β-galacuronidase, luciferase), and transformation (kanamycin resistance) in potato tissues. It was noted that tissue and cell damage occurs from the process and so the researcher must balance depth of penetration against the number of stable transformants developed. It was discovered that internode tissues were the most tolerant of the mechanical insult from bombardment. The process was further improved when it was determined that calcium-spermidine was superior to calcium phosphate for use in the preparation of the DNA-coated projectiles (Aragao et al., 1993). Another improvement was the determination that baking the projectiles at a high temperature before precipitation would overcome agglomeration, which makes standardization difficult and damages the host tissue. Vortexing, and the use of glycerol and polyethylene glycol (PEG) are also employed to help reduce agglomeration (Sawant et al., 2000).

The objective of the studies reported in this chapter is to determine whether the coat protein (CP) might elicit the observed hypersensitive response (HR) of TMGMV PV-0113 in tropical soda apple (TSA), ectopic expression was attempted using a TMV/TMGMV chimera. Plasmid vectors for *in planta* transient expression of TMGMV PV-0113 CP and movement protein (MP) were constructed and attempts were made to deliver these using biolistic and *A. tumefaciens* delivery systems.

Materials and Methods

The chimeric tobamoviruses 30B (Figure 9-1) and 30B-GFPc3 were kindly provided by Dr. Shailaja Shivprasad, formerly at the Citrus Research and Education Center (CREC), University of Florida/IFAS, Lake Alfred, and the master sequence of 30B was kindly provided by Dr. Dennis Lewandowski, also formerly of the CREC. 30B and 30B-GFPc3 were inoculated onto plants of *Nicotiana benthamiana*, *N. sylvestris*, *N. tabacum*, and *Solanum viarum* (tropical

soda apple; TSA). TSA plants to be inoculated with TMGMV strain "U2" (LAU2) and TMGMV strain "U5" (LAU5) both obtained from Dr. Dennis Lewandowski, were grown in peat pellets and maintained in a growth chamber, (25°C day, 20°C night; 16-hour photoperiod). Plants of TSA and *N. benthamiana* inoculated with 30B in frozen tobacco tissue, provided by Dr. Dennis Lewandowski, and TSA inoculated with TMGMV PV-0113 preserved 1:1 in frozen tobacco sap extract, provided by M. S. Elliott (Senior Biologist, Plant Pathology Department, University of Florida), or with or with TMV preserved 1:1 in frozen tobacco sap extract, and provided by M. S. Elliott, were grown in clay pots and maintained in an Apopka growth room. After inoculation, plants were observed daily for symptom development under both visible (fluorescent) and UV light. UV illumination was initially provided by laboratory model UV illuminators (λ= 356 nm, 366 nm), but further experimentation demonstrated that an ordinary blacklight fluorescent lamp illuminated areas in which GFPc3 was present just as vibrantly.

After symptoms became visible, affected plant tissues were harvested and extracted using Laemmli dissociation solution (LDS) (Laemmli, 1970). Separation was accomplished on a polyacrylamide gel composed of a 5.6% stacker and a 10% separator. Samples loaded included 15µL extract of symptomatic tissue from TSA inoculated with 30-B, LAU5, LAU2, PV-0113, and TMV. 15µL of tissue extract from uninoculated TSA and 4µL BioRad Precision Plus All-blue protein standard (10 kDa-250 kDa; cat. #161-0373) were also loaded.

Electrophoretic separation was done at 125 V and the proteins transferred to a nitrocellulose membrane under Laemmli running buffer (Laemmli, 1970), at 100 V, (current = 0.16 amp at start and 0.20 amp at finish). After electroblotting, the membrane was blocked [to cover open nitrocellulose] with a 5% solution of evaporated milk, washed 3 times in Tris-buffered saline solution with Tween 20 (TBST), and incubated for 1 hour with primary antibody

(rabbit anti-TMGMV; rabbit #995). The membrane was then reblocked using sap from healthy tissue (1:100 in 0.1 M $NaHPO_4/Na_2PO_4$; pH 7.0), washed 3 more times with TBST, and incubated for 1-2 hours with goat anti-rabbit IgG conjugated to alkaline phosphatase. Both primary and secondary antibodies were diluted 1:1 with blocking solution before use. The final concentration of secondary, conjugated antibody was 1:60,000. One (1) drop of sap from healthy TSA was added to 40 mL total primary antibody and blocker (20 mL total used per blot). Finally, the membrane was washed twice more in TBST and once with substrate buffer for 5 minutes with agitation. The protein blot was developed by exposure to Promega Western Blue stabilized substrate in darkness until good contrast was obtained (~5-10 minutes). Development was halted by a rinse with distilled water.

The expression constructs used, pK7WG2 and pK7WG2D, are based on the spectinomycin-resistant pPZP 200 series of binary vectors (Karimi et al., 2002). They carry an origin of replication capable of functioning in both *Escherichia* (~200 copies/cell) and *Agrobacterium* (3-5 copies/cell). The pPZP vectors are small (~6.7 Kb) compared to other binary vectors, and incorporate a multiple cloning site (MCS) for convenient engineering of larger constructs (Hajdukiewicz et al., 1994). Binary vectors created from pK7WG2 and a control marker (pE1-gus), TMGMV PV-0113 ORF3 (pE1-A2) and TMGMV PV-0113 ORF4 (pE1-D2), as described previously, were incorporated into disarmed *Agrobacterium* strain ABI [Ti plasmid pMP90RK; Gm^R, Km^R] (Koncz and Schell, 1986), (kindly provided by Dr. H. Klee, Eminent scholar, University of Florida/IFAS, Horticultural Sciences Department, Gainesville), using electroporation.

Cells were cultured on D1 medium (Kado and Heskett, 1970), before being transferred to yeast-extract peptone (YEP) medium (2 g Bacto-peptone, 1 g yeast extract, 1 g NaCl; pH 7.2).

Five (5) mL of YEP broth, containing 50 µg per mL kanamycin sulfate was inoculated with *Agrobacterium tumefaciens*, disarmed strain ABI, and cultured overnight at 28°C with 200 rpm agitation. Two (2) mL of this culture was then transferred to 200 mL YEP and incubated at 28°C, 250 rpm 4-5 hours, or until the cell concentration reached A_{600} = 0.3 OD. Cultures were centrifuged at 5,000 rpm for 10 minutes in a chilled rotor. The supernatant was discarded and the pellet resuspended in 20 mL of cold (4°C), filter-sterilized 1 mM HEPES buffer (1mM HEPES, 1 mM KOH, pH to 7.0). Cells were recentrifuged and resuspended twice. After a final centrifugation, the cells were resuspended in 2 mL of ice-cold (0°C) filter-sterilized 10% glycerol. Forty (40) µL aliquots of cells were quick-frozen in liquid N_2 and stored at -80°C. The remaining electrocompetent cells were electroporated.

Plasmid DNA (1 µg/40 µL cells) was introduced, and the cultures incubated on wet ice for 2 min. The DNA-bacteria mix was then transferred to a chilled electroporation cuvette and pulsed at 25 µF, 2.5 kV, 400 Ω using a BioRad Gene Pulser Xcell programmable electroporation system. To the electro-shocked bacteria, 1 mL super-optimal catabolite-repression broth (SOC) was immediately added, and the culture incubated at 28°C for 4-6 hours. One hundred (100) µL of each transformation mix were then cultured on YEP agar laced with 50 µg per mL kanamycin and 100 µg per mL spectinomycin for chromosomal and Ti plasmid selection.

Colonies identified as carrying sequences of interest (as determined by colony PCR) were selected for agro-infiltration. Each colony selected was inoculated to 5 mL YEP containing 50 µL per mL kanamycin and 100 µg per mL spectinomycin and cultured for 24 hours at 28°C. One half (0.5) mL of this culture were used to inoculate 200 mL of kanamycin and spectinomycin laced-YEP containing 10 mM MES (pH 5.85) to neutralize metabolites and 20 µM acetosyringone which was dissolved in dimethyl sulfoxide (DMSO) and filter-sterilized to

induce vir gene expression. This culture was then incubated at 28°C overnight with agitation before 40 mL aliquots were removed and centrifuged at 5,000 rpm for 15 minutes in sterile Oakridge tubes. The malodorous pellet was suspended in 35 mL of MES buffer (10 mM MES, pH 5.85 and 10 mM $MgCl_2$). The cells were centrifuged again 6,000 rpm for 15 min and resuspended in 35 mL induction buffer (10 mM MES, pH 5.85; 10 mM $MgCl_2$, 150 μM acetosyringone.) Resuspended cells were incubated at room temperature for 4 hours and then diluted with induction buffer to ~1 O.D. at A_{600} and infiltrated directly into the underside of the leaves. Treatments consisted of *Agrobacterium* carrying pE1_A2 and pE1_D2 alone, and in combination. Cotyledons and primary leaves were preferred for infiltration. Infiltrated plants were maintained in a warm greenhouse.

For certain experiments, expression vectors were combined with AGL0 carrying "gene silencing suppressors" (various viral genes involved in overcoming RNAi-based host cell defenses). These vectors, carrying either gene silencing suppressor p19 (Voinnet et al., 2003; Win and Kamoun, 2004), or 2b (Mayers et al., 2000), in plasmid construct pCASS, were prepared for infiltration in the same manner, and mixed with the PV-0113 ORF3/ORF4-carrying vectors, 1:1 (v/v), immediately prior to infiltration. Another gene silencing suppressor used was already present in the transgenic host, *Nicotiana tabacum* cv. Xanthi [+HCPro] (kindly provided by Dr. Jane Polston, Professor, University of Florida, Plant Pathology Department, Gainesville). Plants infiltrated for each treatment consisted of TSA (5), *Nicotiana tabacum* cv. 'Xanthi' [+HCPro] (3), *Nicotiana sylvestris* (1), and *N. tabacum* cv 'Samsun' [*nn*] (1). Additionally, AGL0 carrying the marker gene, green fluorescent protein (wtGFP) in the binary construct pCASS, was used as a positive control.

AGL1 and AGL0 are "hypervirulent," "disarmed" strains of *Agrobacterium*, derived from EHA101, which carries the engineered "super virulent" Ti-plasmid pTiBo542 (i.e., the "super virulent" plasmid was disarmed, enabling high transformation efficiency). It also contains a mutated *recA* gene, so that recombination events which might disrupt the plasmids are kept to a minimum. Since this strain of *Agrobacterium* carries carbenicillin resistance (Cb^R) for selection, but not spectinomycin resistance (Sp^R; Lazo et al., 1991), it was chosen for use with the Sm^R/Sp^R -containing binary vectors pK7WG2 and pK7WG2D.

After infiltration, plants in treatment groups incorporating the eGFP or wtGFP were observed daily for symptoms under both visible (daylight, fluorescent) and UV (366 nm, blacklight) light. Tissue from pE1-D2 inoculated *N. sylvestris*, *N. tabacum* Xanthi [HCPro] and TSA was harvested 24, 72, and 120 hours post-inoculation, triturated under Laemlli dissociation buffer, and frozen at -20° C in preparation for Western blotting. Western blotting was carried out as described previously in this materials and methods section, except that the tissue extracts used were from the agro-infiltrated *N. sylvestris*, *N. tabacum*.

To investigate sensitivity of TSA to agro-inoculation, *A. tumefaciens* strain ABI, carrying Ti plasmid pMP90RK, was cultured overnight in 5 mL of YEP broth, containing 50 μg per mL kanamycin sulfate (Virulent *A. tumefaciens* strain AT-3, grown in YEP broth without antibiotic, was used as a control.) Five hundred (500 μL) of this culture was used to inoculate a 200 mL flask of YEP containing 10 mM MES (pH of MES adjusted to 5.85), and 20 μM acetosyringone. This flask was incubated at 28°C with agitation (200 rpm) overnight. The overnight cultures were centrifuged in a refrigerated Sorvall centrifuge at 5,000 rpm for 15 minutes. Pellets were resuspended in 175 mL infiltration buffer (10 mM MES, (pH 5.85); 10 mM $MgCl_2$), and recentrifuged at 6,000 rpm for 15 minutes. Supernatant was discarded and the pellets were re-

suspended in 175 mL of the same buffer, with the addition of 150 μM acetosyringone to induce vir-gene expression. Cultures thus prepared were allowed to incubate at RT for 3-5 hours prior to agro-infiltration.

Plants of *N. sylvestris*, *N. tabacum* cv. "Samsun" (*nn*), *N. tabacum* cv. Xanthi [HCPro], and TSA, 3-4 weeks old, were infiltrated with *Agrobacterium* suspension four times in three half-leaves per plant. Additionally, each plant was inoculated by inserting an *Agrobacterium* suspension-coated toothpick into the stem. In TSA, this was accomplished by piercing downward at an axial leaf node along the stem.

Plants of *N. benthamiana*, *N. sylvestris*, and TSA were grown in Metro-mix potting mix, in 6.35 cm square plastic pots until the plants had attained suitable size (~3 weeks) for inoculation. The plants were then used for biolistic infiltration. In subsequent experiments, plants were grown in expanded peat pellets, in a growth chamber (25°C day, 20°C night; 16 hour photoperiod), and subjected to a 24-hour period of darkness prior to bombardment to enhance sensitivity to the infiltrated material.

The particle inflow gun (PIG) was similar to the design described by Gray et al., (1994). Essentially, it is a welded steel box with a thick Plexiglas door fitted with a thick gasket. The nozzle delivering the pressurized helium was located in the top and the vacuum outlet located in the side. The solenoid regulating the helium delivery was triggered by manually pressing an electronic switch.

Five protocols for projectile-mediated transient expression/transformation were examined, and a new protocol was adapted to the available facilities. Gold particles (1.5 – 3.0 μm) were baked in an oven overnight and stored in a desiccator before use. Ten (10) mg of gold particles prepared in this manner were suspended in NaCl/Tris (150 mM NaCl, 60 mM Tris) and

approximately 20 μg of plasmid vector added. To this suspension, 100 μL of 100 mM spermidine, 100 μL of 100 mM PEG, and 100 μL of 2.5 M $CaCl_2$ were added, and the whole suspension was incubated for 10 minutes at room temperature. Particles were pulse-spun in a tabletop centrifuge and the supernatant discarded. Particles were washed twice with 100% ethanol, and then resuspended in 200 μL of absolute ethanol. Three (3) μL of DNA/gold particle suspension per "shot" were evaporated onto the filter screen and used to bombard the plants. One to three (1-3) shots per plant were applied, under a vacuum of ~25 psi.

Transient expression vectors were extracted using the Eppendorf miniprep kit from clones pE1-A2 (pKYWG2D + NZ TMGMV ORF3) and pE1-D2 (p pKYWG2D + NZ ORF4) in *Escherichia coli*, and clones pCASS [+2b] and pCASS [+wtGFP] in *A. tumefaciens* strain AGL0. *Escherichia coli*., strain DH5α, was cultured overnight at 37°C in lysogeny broth (LB) containing 50 μg per mL kanamycin. *Agrobacterium tumefaciens*, strain AGL0 was cultured 20 hours in YEP broth containing 100 μg per mL ampicillin at 25°C.

Extracted plasmids were adjusted to an estimated concentration (agarose gel estimation) of 0.1 μg per μL. Ten (10) μL of this solution was added to 10 μL of freshly-vortexed 50 mg 0.7-μm tungsten metal particles suspended in a sterile 50% solution of glycerol and distilled, DNase and RNase-free water. The projectile samples were incubated at room temperature for 1 minute, before the plasmid DNA was precipitated onto the particles by the addition of 10 μL of 1.25 M $CaNO_3$, pH 10.5. The suspension was then mixed by inversion, brief vortexing, and incubated at room temperature for 3 minutes. Samples were then centrifuged at 13,000 rpm in a tabletop centrifuge, and 2/3 (20 μL) of the supernatant was removed from each sample and discarded.

Samples (including a "tungsten only" negative control consisting of only tungsten prepared as above (glycerol, $CaNO_3$) without plasmid DNA) were then immediately loaded into a Bim-

Laboratory inoculation device, (kindly provided by Dr. Avihai Ilan, Bio-Oz Technologies, Kibutz Yad-Mordechai, Israel) and injected (5 µL per shot, pressure = 3 bar, distance ≤1 cm) into the leaves of *N. benthamiana*, *N. sylvestris*, and TSA (Table 9-2). The Bim-Lab is a device that utilizes compressed air to efficiently deliver small volumes of virus inoculum or DNA into intact plants or plant tissue (Anonymous, 2005). Following inoculation/infiltration, plants were returned to a cool greenhouse and observed visually, both under ambient lighting and a blacklight UV source. After 9 days, infiltrated tissue was collected and a portion extracted with LDS before freezing for future experimentation.

Epidermal peels of construct and virus-infiltrated plants were mounted in 10% glycerol, sealed with clear nail polish, and viewed under an epifluorescent microscope using a GFP-optimized filter set (kindly provided by Dr. Jeff Rollins, Associate Professor, Plant Pathology Department, University of Florida).

Results

Alignment of TMGMV PV-0113 ORF4 with the 30B CP nt sequence using Multalin (Corpet, 1988), revealed four nucleotide variations (Figure 9-2), of which one was translationally important (Figure 9-3).

TSA plants inoculated with LAU5, developed local lesions and either collapsed, developed leaf abscission, or, in a few instances, survived with mosaic symptoms. TSA plants inoculated with LAU2 either developed no symptoms, epinasty, local lesions, or mosaic, but did not succumb. TSA plants inoculated with PV-0113 consistently developed local lesions, leaf abscission, and wilting, followed by complete defoliation or collapse. TSA plants inoculated with 30B developed mosaic (Figure 9-4), but not local lesions, leaf abscission, or necrosis. TSA plants inoculated with TMV developed mosaic with a different appearance, but not local lesions, leaf abscission, or necrosis. *Nicotiana sylvestris* plants inoculated with PV-0113 developed local

lesions (Figure 9-5), while *N. sylvestris* plants inoculated with TMV did not develop local lesions, but newly emergent leaves developed mosaic and leaf distortion (Figure 9-6).

Western blotting (Figure 9-8) revealed tobamovirus CP accumulation in plants inoculated with LAU2, LAU5, PV-0113, TMV, and 30B. Good transient expression of wtGFP from agroinfiltration was accomplished with pCASS[+wtGFP] in AGL0 on *N. benthamiana* plants only. Fluorescence for wtGFP was seen both with and without the use of gene silencing suppressors, however gene silencing suppressors, particularly 2b, clearly enhanced expression (as measured by observed brightness) and persistence.

Tropical soda apple plants inoculated with 30B GFPc3 infected tissue developed green fluorescent patches in the area of inoculation (Figure 9-9) that expanded, but usually not beyond the inoculated leaf. Eventually, 30B GFPc3 inoculated TSA plants developed systemic mosaic symptoms similar to those seen in 30B inoculated plants. Furthermore a chimeric tobamovirus provided by Dr. Shailaja Shivprasad identified as TTU2T-GFP, containing the 5′ NTR, replicase, and movement genes of TMV, the CP subgenomic promoter and the CP of TMGMV-S, and the 3′ NTR of TMV produced systemic symptoms only on inoculated TSA plants, but not HR/lethal systemic hypersensitive response (LSHR; data not shown). *Nicotiana sylvestris* plants inoculated with 30B developed local lesions with much the same appearance (Figure 9-7) as those induced by PV-0113.

As with natural GFP (excitation = 395-475 nm, emission = 510), GFPc3 can be visualized under a blacklight (Figure 9-10), while eGFP, the red-shifted (488/509 nm) variant employed in the Gateway-compatible expression vectors, must be observed under the epifluorescent microscope with a 485/20 excitation filter and a 530/25 emission filter (Bio-Tek, 2004;

Olympus, 2004). Fluorescence was observed under the microscope in both GFPc3-inoculated *N. benthamiana* (Figure 9-11) and TSA (Figure 9-12).

TSA plants inoculated with the chimeric construct 30B GFP expressed GFP, but did not develop delayed HR, and went on to develop a mosaic after a period of time. Expression of GFP did not coincide with the mosaic, suggesting that the systemic virus consisted mostly of recombinants.

No GFP-related fluorescence was observed on any TSA infiltrated with *A. tumefaciens*. Fluorescence was observed on all (5/5) *N. benthamiana* and 1/3 *N. sylvestris* plants infiltrated with AGL0. This fluorescence was enhanced (much brighter) in the plants co-infiltrated 2b, where the period of expression was extended from 2 to 4 days post-inoculation (DPI) to nearly a month.

Some *N. sylvestris* plants infiltrated with *A. tumefaciens* strain ABI carrying expression construct pE1-D2 developed necrosis throughout the infiltrated leaf panels (Figure 9-13). No CP was detected in any of the *Agrobacterium*-infiltrated TSA or tobacco tissues by Western blotting (data not shown).

Wild-type, pathogenic *A. tumefaciens* caused gall formation and other effects (i.e. adventitious root formation, persistence of chlorophyll in senescent leaves) on inoculated *N. sylvestris* and *N. tabacum* but not on TSA. On TSA, *A. tumefaciens* caused chlorosis, necrosis, or leaf abscission, but not gall formation.

Bombardment with gold particles caused considerable trauma to the leaves: at pressures and distances required to infiltrate TSA, *N. benthamiana* leaves were destroyed. Under the microscope, gold particles were observed embedded in sectioned host tissues (data not shown).

Tissue bombarded by uncoated control particles exhibited yellow-green fluorescence similar to that observed in treatment groups (data not shown).

Discussion

Based on the host response to inoculation with TMGMV PV-0113 and to 30B, it appears that TMGMV CP alone is not the elicitor of the HR-like response in the disease process observed in the TSA. Both PV-0113 and 30 B elicit local lesions typical of HR in the CP-sensitive host, *N. sylvestris*, and the sequences of the CP of both PV-0113 and 30B are nearly identical. Culture LAU5 also consistently produces necrotic symptoms on TSA, but not lethality. This culture was later demonstrated to contain STMV by serological methods (data not shown). While PV-0113 produced local lesions in *N. sylvestris* and lethal response in TSA, TMV moves systemically through both hosts without causing necrosis or lethality. The chimera 30B, expressing TMGMV-L CP only, behaves similarly to TMV: moving systemically through TSA without causing necrosis. Serology (Western blotting) demonstrates a substantial accumulation of CP in 30B-infected TSA plants. Taken together, these data indicate that TMGMV CP is not responsible for the disease process produced by PV-0113 in TSA. Unlikely, but possible scenarios nullifying this hypothesis include: 1) something about 30B may cause a difference in timing of the infection cycle disrupting elicitor recognition (perhaps it is unavailable for recognition by host detection following aggregation); and 2) tobamovirus CP may form a complex with other TMGMV proteins *in planta*. This protein complex may itself be what is recognized by the elicitor.

The limited development of green fluorescence on TSA plants inoculated with 30B GFPc3-infected sap are consistent with the formation of recombinants in tobamovirus vectors reported previously (Shivprasad et al., 1999; Dawson et al., 1989).

While expression of wtGFP was clearly enhanced by the presence of the gene silencing suppressors, the system appears optimized to function only in *N. benthamiana*. Only 1 out of 3 infiltrated *N. sylvestris* plants responded with fluorescence. Additionally, while both *N. sylvestris* and *N. tabacum* responded to inoculation with pathogenic wild type strains of *A. tumefaciens* with galls and other symptoms, TSA was immune. Infiltration and injection of TSA with wild type *A. tumefaciens* cultures produced no gall formation, or other effects (e.g., persistent green areas on senescent leaves) seen after infiltration, injection, and toothpick-inoculations on tobacco species (*N. sylvestris* and *N. tabacum*). This suggests that the strains of *A. tumefaciens* used are not efficiently transforming TSA, and the procedure must be optimized until reliable transformation/transient expression in TSA with a strain of *Agrobacterium* can be achieved. It appears that different strains of *A. tumefaciens* work better with different hosts in binary vector expression systems (Dr. Greg Martin, Professor, Plant Pathology Department, Cornell University, NY). A strain of *Agrobacterium* pathogenic to TSA should be identified before future attempts at agroinfiltration are attempted. There exists a report of hairy root culture of *Solanum aculeatissimum,* a species closely related to TSA (Jacob and Malpathak, 2004). Future work with *Agrobacteria* should follow the procedures outlined in this paper and the strain of *A. rhizogenes* used should be considered.

Cotyledons and primary TSA leaves were easiest to infiltrate. Growth-chamber-grown plants tended to have fewer and more flexible prickles than greenhouse grown plants. Finally, greenhouse-grown TSA plants infiltrated with 10 mM $MgSO_4$ buffer solution showed tissue collapse and bleaching in the infiltrated areas, necessitating a switch to 10 mM $MgCl_2$ buffer.

Transient expression of CP by pE1-D2 should be verified: The vector should be agro-infiltrated into an CP insensitive, yet permissive host such as *Arabidopsis thaliana* along with a

gene-silencing suppressor such as 2b, and possibly wtGFP to measure timing of expression, harvested, and tested using ELISA or Western blotting. Western blotting appears useful to demonstrate transgenic CP expression, but perhaps ELISA would work equally well as a protein detection method. For the other gene products, some other method of demonstrating transgene expression will be required, unless antiserum specific to the TMGMV 126 and 183 kDa proteins and MP can be obtained. The visible synergy between gene silencing suppressor 2b and GFP suggest that revealing co-infiltration experiments may be possible (i.e., wtGFP and 2b might be infiltrated along with vectors carrying PV-0113 ORF3 and ORF4, compared to wtGFP and 2b along with the vector carrying an unrelated insert). Transient expression of MP by pE1-A2 will be more difficult to establish without MP-specific antiserum, but could perhaps be achieved using a dye of the right molecular weight, or by inoculating a small region of an infiltrated leaf panel on the host *Eryngium planum* with 30B GFPc3 and then looking for systemic movement of the virus.

To summarize, when transient expression in an undomesticated host such as TSA is desired, expression of construct must first be demonstrated in a permissive host such as *N. benthamiana*, then strains of *A. tumefaciens* must be screened against the (less-permissive) host plant to find a strain that will infect it.

Using a "gene gun" had some drawbacks. Plants to be tested had to be produced in peat pots, to which not all species were adaptable. Plants in 2" pots literally had soil blown out of pots by the treatment, contaminating the entire field. Furthermore, it was difficult to calibrate, there needs to be a variety of distances and/or gas pressures are required to achieve correct depth of tissue penetration. Particle bombardment necessarily causes trauma to the host tissue. Density of tissue varies from plant type to plant type. *Nicotiana benthamiana* tissue is so

succulent that it is actually destroyed at pressure/vacuum settings required to embed particles in TSA. It would be best to use the cotyledons and heart-shaped primary leaves of TSA for any future experiments requiring biolistic infiltration.

Evidence of successful particle delivery was observed under the microscope – gold particles were seen embedded in host tissues (data not shown). It stands to reason, therefore, that particles carrying DNA were getting into the cells. Extensive tissue trauma, however, may obscure the marker gene -- tissue bombarded by uncoated control particles also exhibited yellow-green fluorescence (data not shown). Detection of eGFP, unlike GFPc3, requires special epifluorescent filter set. Perhaps the GUS-transformed VIB vector (E1-gus) could be used for calibration, as the blue pigment will be easier to visualize. Phenolic compounds released during wounding, particularly in TSA appear to fluoresce under the UV microscope, potentially obscuring eGFP. If projectile delivery is to be used in the future, it would be better to use β-galacuronidase as a marker, or perhaps a different color fluorescent protein such as blue or cyan.

In conclusion, while all three methods (chimeric viruses, agroinfiltration, and particle bombardment) have the potential to express individual PV-0113 proteins *in planta*, they differ in their ease of application. The chimeric virus approach seems the easiest to apply, once the virus has been created. Agroinfiltration using binary vectors requires some optimization to the host, but is also facile. Particle bombardment is a relatively crude method that requires calibration and damages the plant tissue. It is perhaps better suited to transformations than transient expression.

Table 9-1. Tobacco mild green mosaic virus-containing tobamovirus chimeras.

Identifier	Component Viruses	Author
Toc-J	ToMV/TMGMV-J	Hori and Watanabe, 2003.
Toc-J/GFP	ToMV/TMGMV-J/GFP	Hori and Watanabe, 2003.
30B	TMV/TMGMV-L	Shivprasad, et al. 1999.
30B-GFP(C3)	TMV/TMGMV-L/GFP(C3)	Shivprasad, et al. 1999.
V23	TMV/TMGMV-S	Nejidat, et al. 1990.

Table 9-2. Bim-Lab-inoculated treatment plants.

Plasmid construct (in ABI, AGL0)	Infiltrated Host
pCASS [+2b, +GFP]	
	Nicotiana benthamiana (small)
	N. benthamiana (large)
	N. sylvestris
pE1-A2 (PV-0113 ORF3)	
	Nicotiana benthamiana
	N. sylvestris
	Solanum viarum (small TSA)
	S. viarum (large TSA)
pE1-D2 (PV-0113 ORF4)	
	Nicotiana benthamiana
	N. sylvestris
	Solanum viarum (small TSA)
	S. viarum (large TSA)

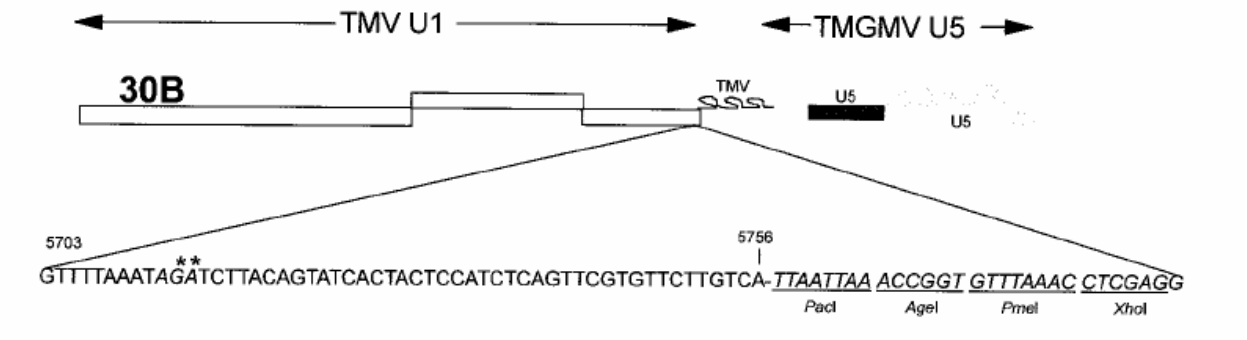


Figure 9-1. Map of TMV/TMGMV chimera, 30B (Shivprasad et al., 1999).

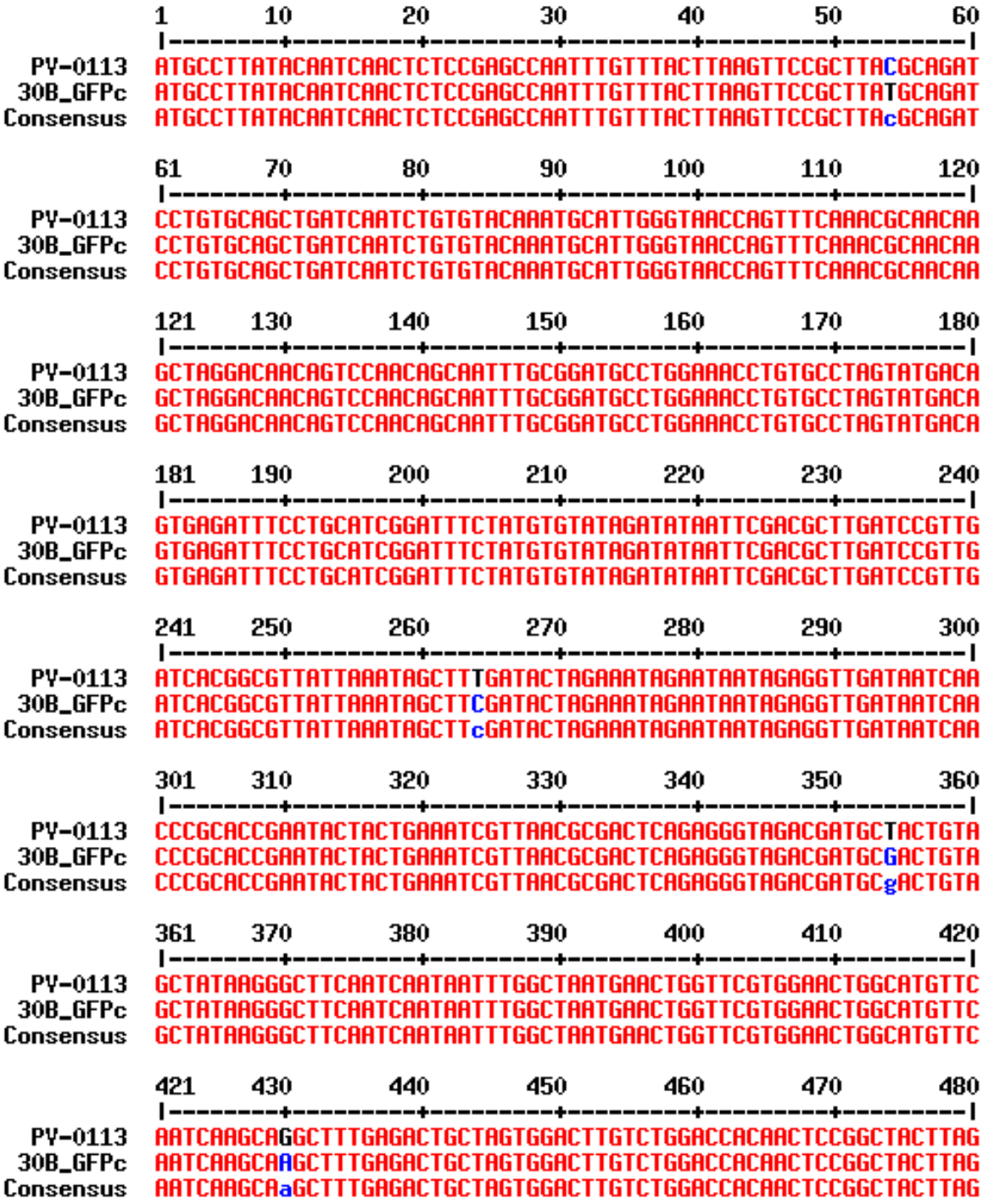


Figure 9-2. Nucleotide alignment between 30B CP domain and PV-0113 ORF4.

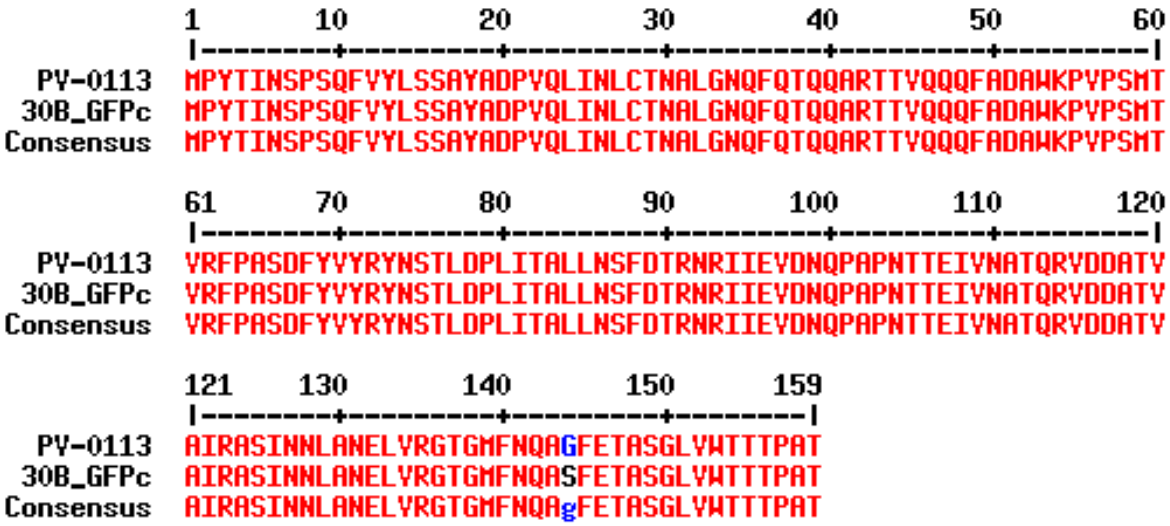


Figure 9-3. Amino acid alignment between 30B CP and PV-0113 CP.

Figure 9-4. Symptoms on TSA leaf following inoculation with the 30B construct.

Figure 9-5. Local lesions on *N. sylvestris* following inoculation with TMGMV PV-0113.

Figure 9-6. Symptoms (mosaic, leaf distortion) on *Nicotiana sylvestris*, following inoculation with TMV.

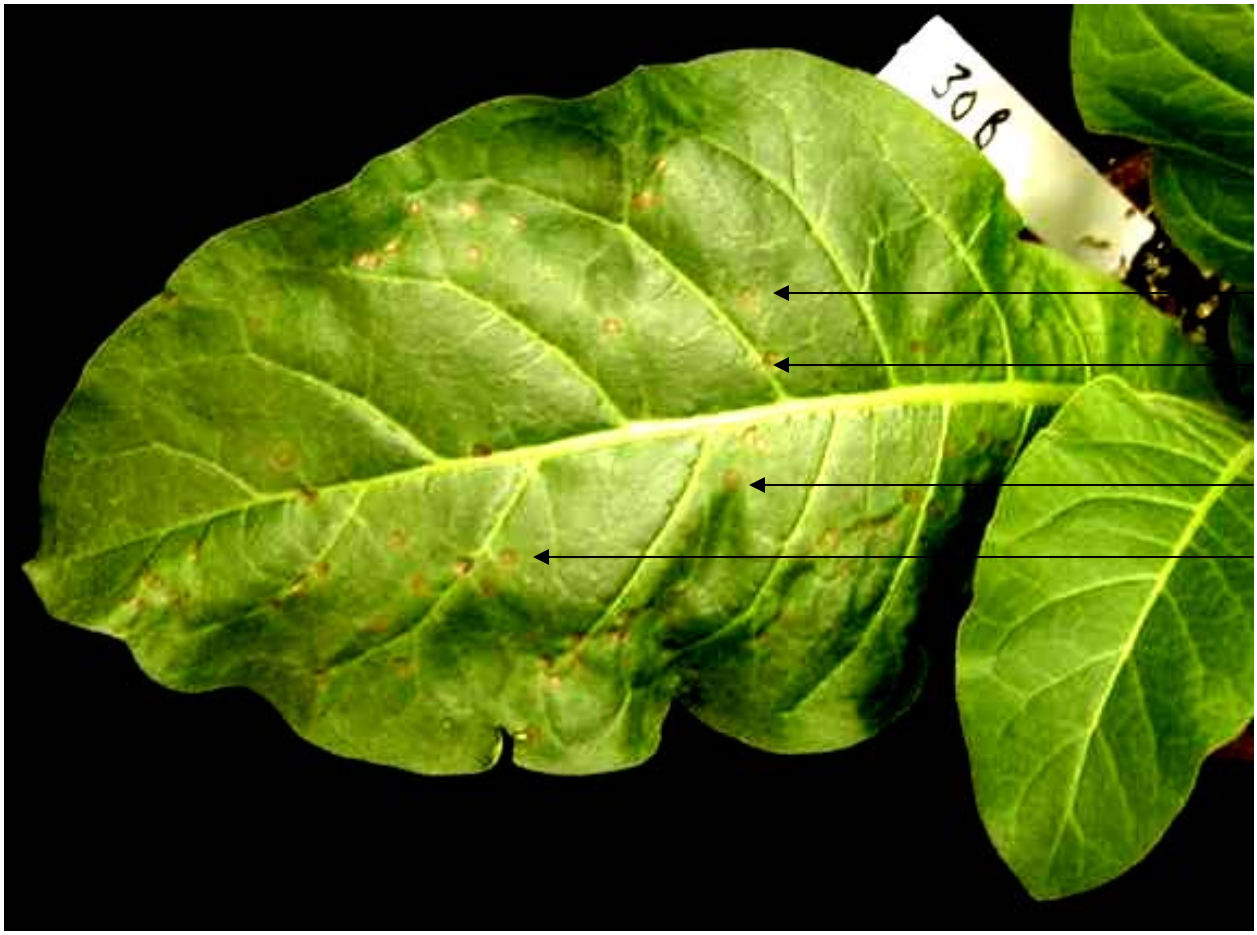


Figure 9-7. Local lesions on *Nicotiana sylvestris* following inoculation with the 30B construct.

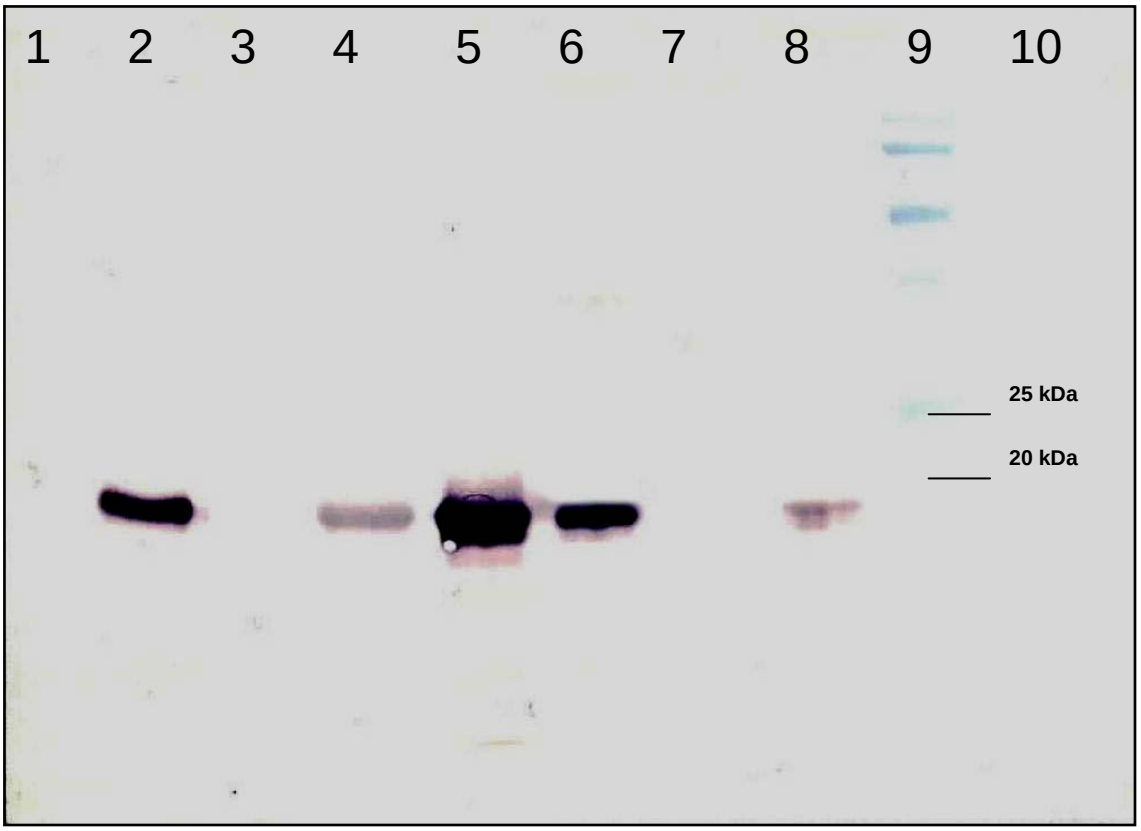


Figure 9-8. Western blot showing CP accumulation in TSA. 1) Empty lane, 2) 30B, 3) water blank, 4) LAU5, 5) LAU2, 6) TMGMV PV-0113, 7) healthy tissue, 8) TMV, 9) protein standard ladder, and 10) empty lane.

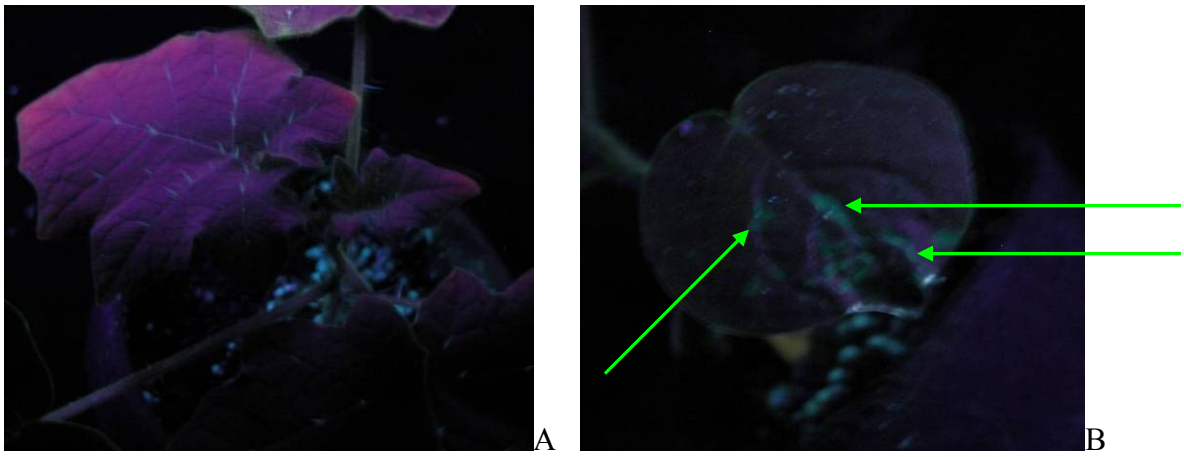


Figure 9-9. *Solanum viarum* inoculated with 30B GFPc3. A) Uninoculated (healthy) TSA viewed under UV light (glowing objects in soil are perlite particles). B) TSA inoculated with 30B-GFPc3 showing fluorescent areas.

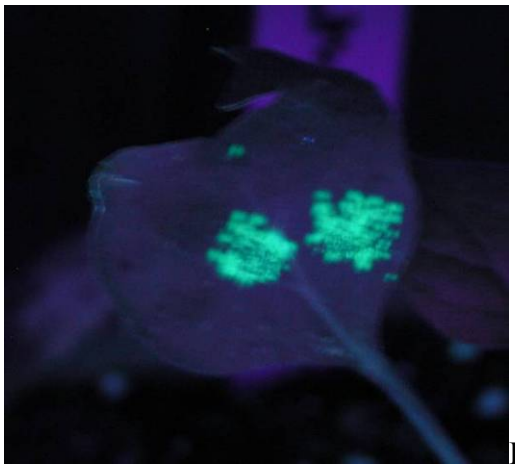

A

B

Figure 9-10. *Nicotiana benthamiana* inoculated with sap containing infectious 30B GFPc3. A) Viewed under fluorescent light and B) under UV illumination.

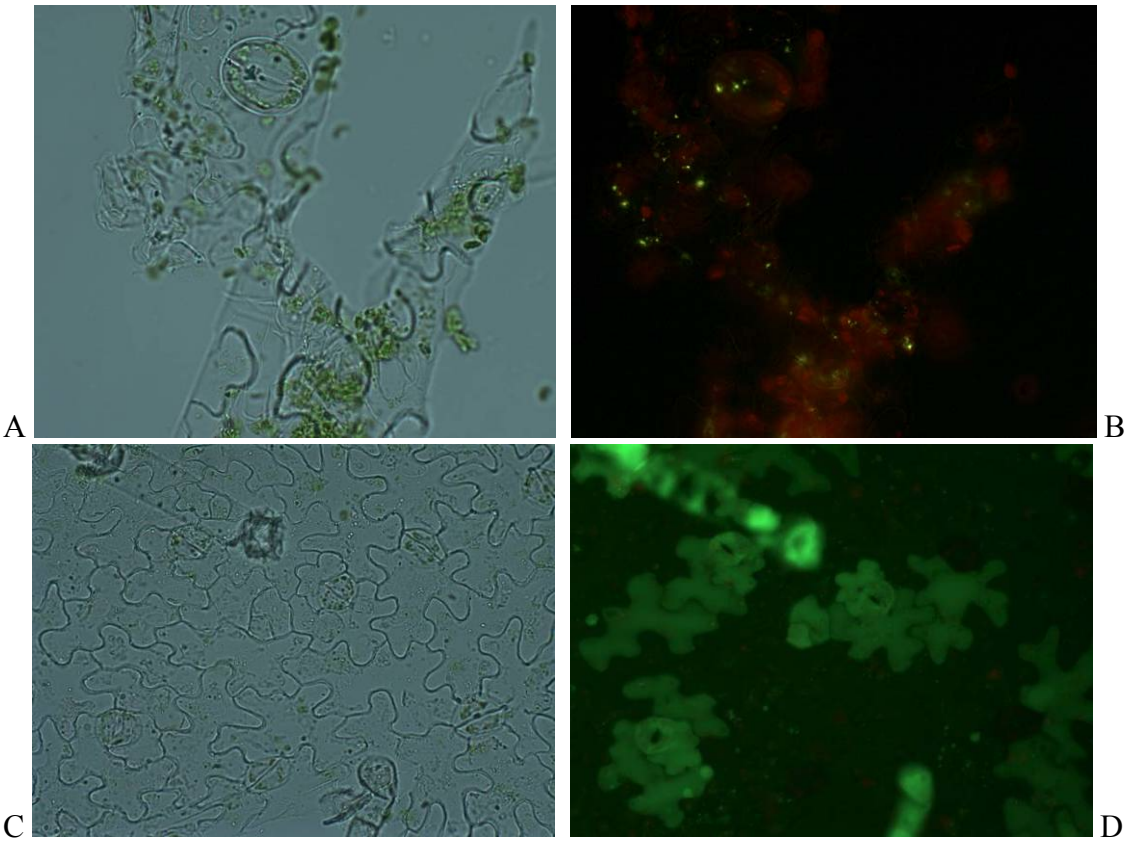


Figure 9-11. Epifluorescent microscopy of TMV/TMGMV chimera 30B-GFPc3 in *Nicotiana benthamiana*. A) Healthy tissue viewed under visible light B) Healthy tissue viewed under UV light C) Inoculated tissue under visible light D) Inoculated tissue under UV light.

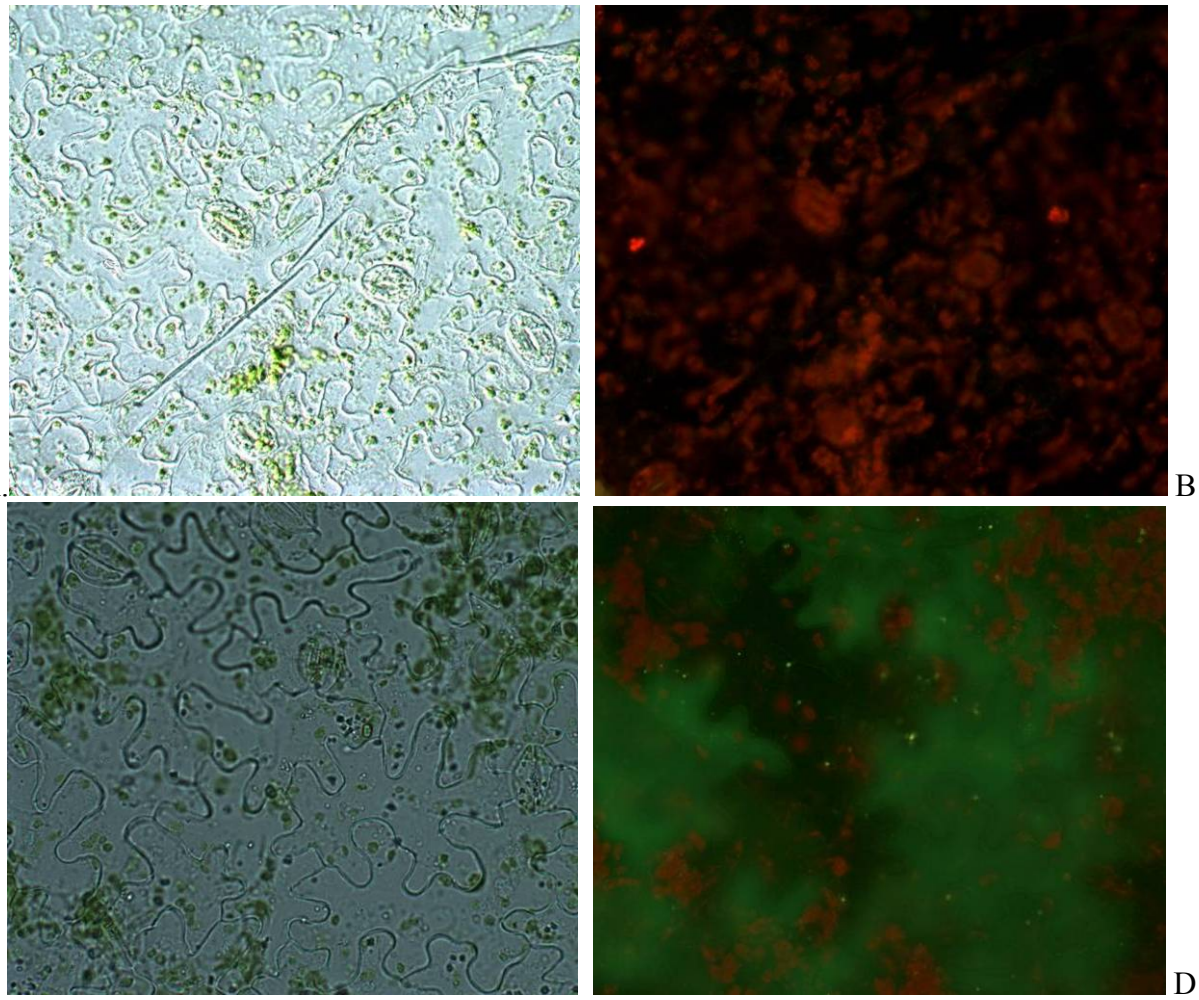
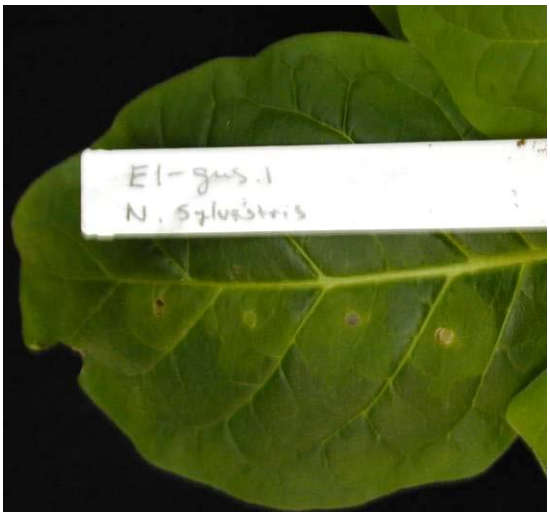
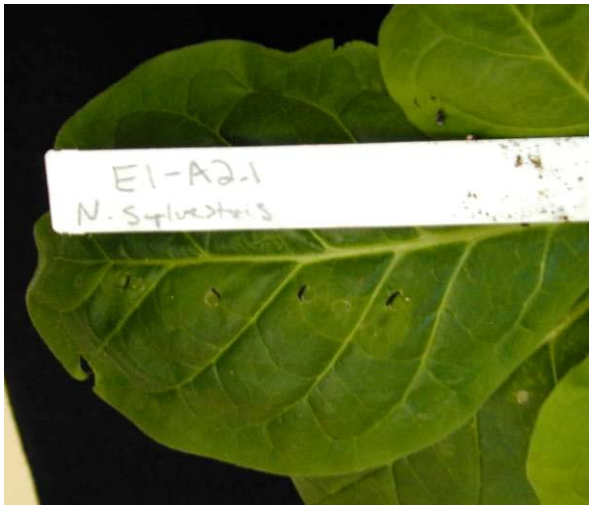


Figure 9-12. Epifluorescent microscopy of TMV/TMGMV chimera 30B-GFPc3 in TSA. A) Healthy tissue viewed under visible light. B) Healthy tissue viewed under UV light. C) Inoculated tissue under visible light. D) Inoculated tissue under UV light.

A 



B

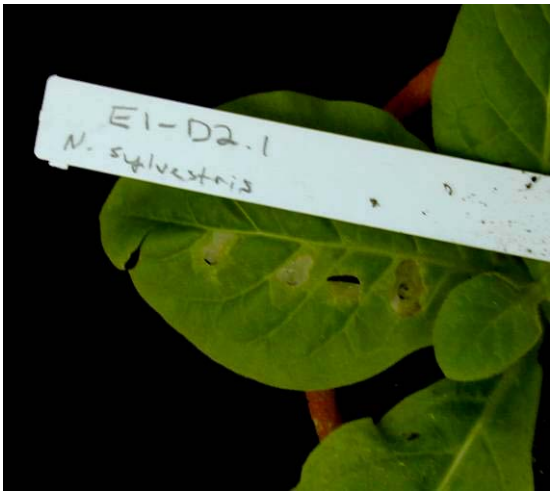
C 

Figure 9-13. Necrotic areas on *N. sylvestris* in response to infiltration with pE1-D2. A) ABI [pE1-gus] (β-galacuronidase). B) ABI [pE1-A2] (PV-0113 MP), C) ABI [pE1-D2] (PV-0113 CP).

CHAPTER 10
GENERAL DISCUSSION

Tropical soda apple (TSA) remains a major weed problem in the Southeastern United States and in many countries around the world. Outside of Florida, TSA is expected to continue to spread into more temperate areas. The plants regenerate from the roots after a hard frost (Mullahey et al., 1998), and, indeed the range of TSA in South America includes areas that are not strictly tropical, but subtropical. Recent studies have shown that the promising new herbicide, aminopyralid, (marketed as "Milestone," and, in combination with 2-4 D, as "Forefront"), which is well-translocated in most weeds, is not well translocated within TSA and requires complete and thorough coverage at a rate ≥0.11% to be effective (Ferrell and Sellers, 2007). In contrast, inoculation of a single leaf with a few micrograms of the bioherbicide Tobacco mild green mosaic virus (TMGMV) is usually effective. The bioherbicide activity is then amplified until the host TSA is dead.

As presented in this thesis, the *Columnea* isolate PV-0113 is the small-type TMGMV [TMGMV-S (based on the size of the 3′ end of the genomic RNA)], and does not appear to contain Satellite tobacco mosaic virus (STMV). TMGMV PV-0113 appears to be a relatively stable quasi-species, without pathogenic variants or contaminants. Open reading frames (ORFs) 3 (movement) and 4 (coat protein) of PV-0113 are highly similar to the corresponding ORF sequences reported for other TMGMV strains. However, mutations resulting in a single amino acid substitution in tobamovirus proteins have been demonstrated to attenuate necrotic host response (Lewandowski and Dawson, 1993; Hamamoto et al., 1997; Hagiwara et al., 2002; Divéki et al., 2004). Not all cultures of TMGMV are lethal to TSA. TSA plants inoculated with (ATCC PV-586), which is reported to contain large-type TMGMV (TMGMV-L) and STMV, exhibited hypersensitive response (HR)-like symptoms but recovered with systemic symptoms.

Because of the unusual number of variables (for a tobamovirus) applicable to TMGMV, each culture used in experimentation must be carefully evaluated before use to determine whether it is large or small-type TMGMV, and whether or not STMV is present. Spurious and inconsistent results in experiments using cultures obtained from the CREC in Lake Alfred, Florida are likely related. In the case of "U2," contamination or replacement by another tobamovirus and, in the case of "U5" to the presence of STMV (which may have been minor in the initial sample but accumulated to higher levels in inoculated plants) may have occurred.

A simplified protocol for purification of TMGMV appears about as effective as the standard one. There is a reduction of pathogenicity upon lypohilization and storage. There is also a reduction of pathogenicity in unlyophilized virus stored over a long period of time. Virus held in storage (above 0°C) retains enough potency to produce local lesions (LL) and kill TSA for at least several months. TSA is highly sensitive to purified PV-0113 which displays a dilution curve typical for a tobamovirus.

Cross-protection may prove an obstacle to development of PV-0113 as a bioherbicide. While the mechanism for cross-protection is not well understood, it is possible that several tobamoviruses will cross-protect TSA against TMGMV. The possibility that STMV, as well as TMV both prevent lethality through some cross-protection mechanism is noteworthy. STMV CP is quite dissimilar from that of TMV and TMGMV, making simple competition between CPs unlikely. TMGMV and STMV share common elements in the 3′ end, including multiple pseudoknots and two nearly identical 40-50 base domains (Dodds, 1998). It is possible that the mechanism of cross-protection depends on these elements. Although STMV does not appear to interfere with the TMGMV-elicited HR in the TSA, it is not known whether the subsequent recovery of the plants inoculated with PV-586 is due to the presence of STMV or is a

characteristic of TMGMV-L. To determine this, an STMV-free culture of TMGMV-L will be required. Another option would be to introduce STMV into a subculture of PV-0113 and inoculate TSA to observe any changes in disease progress.

As an obligate parasite, it is not advantageous for TMGMV to kill its host. Unless related to some function enhancing transmission, (e.g. encephalitis-related behavioral changes seen in rabies), death of the host reduces the reproductive fitness of the virus. As horrific as the effects of a mortal disease such as Ebola may appear to us, they must ultimately be viewed as failure of the infectious agent to negotiate a more commensal relationship with its host. As an example of such a failed host-pathogen interaction, the TSA-TMGMV disease process may serve as a good model to investigate what is probably lethal systemic HR. Indeed, perhaps a better question might be: "if a virus that causes local lesions on a host goes on to produce systemic mosaic (e.g., TMGMV PV-586 on TSA), why does the entire plant not become necrotic?" or, put in another way, "Why is the "trailing HR" form of lethal systemic HR not the norm?" Either the HR in these cases must be initiated by some protein only transiently present during the virus life cycle (in this hypothesis, you would expect not to see it in R-genes responding to CP, which accumulates) OR successful viruses have some mechanism (HR-silencing?) by which they shut down the host defense mechanisms.

Further study of the interaction between TSA and TMGMV PV-0113 might also reveal more about R-gene genetics and evolution. While it seems plausible, even probable that there is a gene-for-gene interaction occurring between TSA and TMGMV, it is also possible that multiple genes are involved, or multiple host factors. Replication, movement, host-range, and symptomatology are all under the control of the virus genome. It appears likely that sequences in the tobamovirus genome must perform multiple functions to co-ordinate such events (Dawson et

al., 1988). Recent phylogenetic work examining both nuclear and chloroplast DNA has grouped TSA (*Solanum viarum*) in a clade with *S. aculeatissimum*, *S. myriacanthum*, and *S. incarceratum* (Levin et al., 2005, 2006). A study of the reaction of these species to inoculation with PV-0113 might prove fruitful if a host showing no resistance (systemic infection with no HR) or localization (LL only) can be hybridized with TSA and the reaction of the offspring analyzed. In addition, it appears that TSA has been successfully hybridized with the eggplant, *S. melongena*, (Daunay, 1999), members of which may respond to PV-0113 inoculation with either LL or symptomless systemic infection (unpublished data).

Indeed, there is evidence to suggest that heterozygosity of resistance alleles contributes to LSHR, at least in some interactions. Holmes (1937), describes the response of *Capsicum* species to inoculation with a tobamovirus, (most likely TMV), non-resistant (susceptible) hosts developed systemic symptoms, while resistant hosts developed LL, usually confining the virus to the inoculated leaf. When hybridized, the F_1 offspring showed a severe systemic necrosis resulting in the death of the plant. Holmes went on to demonstrate the resistance allele was inherited in a Mendellian fashion (Holmes, 1937).

Similarly, the *I* gene of *Phaseolus vulgaris* may produce extreme resistance (no visible symptoms), hypersensitive resistance (LL), or systemic vascular necrosis in response to Bean common mosaic virus (BCMV), depending upon gene dosage and environmental conditions. Plants heterozygous for *I* often develop a necrosis of the phloem when inoculated with BCMV and grown at a high temperature (Collmer et al., 2000).

The effect of heterozygosity on disease phenotype might be related to lower expression of the R-gene product. In the "sink hypothesis" translation of a protein is balanced with catabolism. That is, the constant breakdown of proteins within the cell may require a certain equilibrium

between the R-gene product, the pathogen elicitor, and the complex between them at which programmed cell death (PCD) can occur in time to confine the pathogen to the site of infection.

The prospect of using chimeric tobamovirus vectors for elucidating the elicitor of HR in TSA remains attractive, especially following the development of a full-length clone of PV-0113, by Dr. Ernest Hiebert (GenBank accession EF469769). In addition to determining the elicitor of HR, such chimeras might be useful, along with natural TMGMV, in examining the physiology of the response. Aside from various marker genes, virus progress and accumulation might be visualized by terminal deoxyribonucleotidyl transferase – mediated deoxyuridine triphosphate nick and labeling (TUNEL) assay or the possibly easier agarose electrophoresis of total DNA to look for laddering (Xu and Roossinck, 2000).

Since binary vectors incorporating ORF3 (ex. pE1-A2) have already been created, it might be productive to re-evaluate these, especially if an agroinfection system efficient at transforming TSA could be established. While there are different ways to deliver genetic material to plants to get transient expression for analysis of the host-pathogen interaction, some approaches are better suited than others. Biolistic infiltration is messy and cumbersome. Agroinfiltration shows potential, but must be optimized to work with TSA. The chimeric virus approach, while requiring technical sophistication, is probably the most elegant.

While the discovery of TMGMV PV-0113 mortality on TSA is a "lucky" find (Drs. Raghavan Charudattan and Ernest Hiebert, professors, Plant Pathology Department, University of Florida, personal communications) perhaps virus-induced lethality is not so uncommon, just merely unnoticed. When inoculated mechanically, *Nicotiana tabacum* (tobacco) is known to support several species of *Tobamovirus* it is not normally associated with, so it may not be

unreasonable to expect an "exotic" tobamovirus to produce disease in, or even lethal systemic HR in a problematic solanaceous weed.

Several relatives of TSA are listed as noxious weeds. In addition to TSA, two other Solanum species, wetland nightshade (*S. tampicense*) and turkey berry (*S. torvum*) are of concern in Florida (Cuda et al., 2002). To screen these and other weedy/invasive species of Solanum using tobamoviruses to see if any exhibit a similar lethal systemic HR might prove useful, especially in a situation in which much applied science —the same applied science knowledge accumulated in working with PV-0113— could be brought to bear with a similar tobamovirus, preferably a tobamovirus of limited or no economic importance (e.g., Frangipani mosaic virus, FrMV). Candidate tobamoviruses are continually being discovered. A new tobamovirus, Tropical soda apple mosaic virus (TSAMV), was recently discovered associated with TSA in Florida (Adkins et al., 2007).

After the large investment of time and research into methods of isolating, screening, producing, processing, purifying, applying and evaluating the TMGMV tobamovirus, it seems reasonable to proceed to evaluation of other tobamovirus species as biological controls against other solanaceous pests. The best candidates for evaluation would probably be those of minor or no economic importance, or those for application in regions in which economically or environmentally important host species do not occur.

Although reasons for the tendency for host susceptibility to plant viruses to run very roughly along family lines remain unknown, it is worth noting that new tobamoviruses such as TSAMV and Streptocarpus flower breaking virus (SFBV) (Heinze et al., 2006) continue to be discovered, possibly explaining both susceptibility in (e.g., the gesneriads) and resistance in (e.g., *Gomphrena*) species that would otherwise appear to be "erratic" hosts. It is tempting to

speculate that a tobamovirus geographically removed, or infecting a more distantly related host might be more likely to result in a maladaptive host response leading to mortality, based on that hypothesis.

In the future, it may be possible, using site-directed mutagenesis or other methods to create tobamoviruses tailor-made for use as a bioherbicide against a particular host. Such use would, however, require a thorough understanding of the host and perhaps modifications (e.g., knockout of CP) that would prevent their escape into the environment.

Based on the current state of knowledge, TMGMV seems to be an unusually complex species for a tobamovirus. With both large and small type variants being maintained in the wild, along with STMV (+) and STMV (–) infections, the virus may have more evolutionary options than other tobamovirus species.

CHAPTER 11
SUMMARY AND CONCLUSIONS

The objective of this research, to discover the way in which lethal plant-virus interactions occur in the tropical soda apple (TSA)-Tobacco mild green mosaic virus (TMGMV) PV-0113 interaction, in the hope of harnessing this phenomenon to control other invasive plants with a species-specific biological control approach, remains unfulfilled. Several avenues for investigation are now apparent, however, including: physiological studies using techniques designed to detect and localize programmed cell death (PCD).

My work, however, has laid the foundation for the exploration of the interaction between TSA and TMGMV, the accurate identification of the host and pathogen, is not as simple as it would seem. The identity of the pathogen PV-0113, which was once classified as a strain of Tobacco mosaic virus (TMV) (Siegel and Wildman, 1954), is now known to be a small-type isolate of the species TMGMV (chapter 2).

Sub-isolates of PV-0113 showed no variation in disease phenotype or in the progression of disease development in the hosts TSA and *Nicotiana tabacum* cv. "Samsun" (*nn*). Disease progress on TSA typically began around 5 days post-inoculation with symptoms such as epinasty and local lesions on the inoculated leaves, followed by wilting of the apical regions or collapse, and necrosis or abscission of both inoculated and uninoculated leaves. Eventually, the stems of PV-0113 inoculated plants become necrotic and the entire plant dies (chapter 3).

Reverse-transcriptase PCR has confirmed that the PV-0113 is a small-type isolate of TMGMV, and dsRNA extraction and immunodiffusion using rabbit antiserum has confirmed that STMV is not present in this isolate (chapter 3).

At least one culture of TMGMV, PV-586, a large type isolate containing STMV, produced symptoms on TSA that initially were similar to those produced by TMGMV PV-0113, but TSA

plants inoculated with TMGMV PV-586 recovered with mosaic symptoms. No other species of tobamovirus besides TMGMV tested thus far has been found that causes death of the host, TSA (chapter 4).

A strain of TMV discovered as a greenhouse contaminant, C-1, cross-protects TSA plants against TMGMV PV-0113 induced lethality. The non-tobamovirus, Cucumber mosaic virus (CMV), does not cross-protect TSA plants from TMGMV PV-0113, and plants die following the typical death phenotype (chapter 5).

TMGMV PV-0113 could be conveniently purified and the purified preparations had physical properties consistent with those of purified TMGMV (chapter 6).

The dilution curve of TMGMV (flat at high concentration, steeply sloping at moderate concentration, and flat at low concentration) is consistent with the dilution curve of a tobamovirus. Disease in TSA is produced by TMGMV PV-0113 even at the dilution of 1:50,000 from 1 optical density at an absorbance of 260 nm (chapter 7).

Coat protein (CP) and movement protein (MP) genes cloned from the genome of TMGMV PV-0113 were sequenced and found to be highly similar to sequences of TMGMV on record with the National Center for Biotechnology Information (NCBI). A nucleotide motif known to be common to tobamoviruses and characteristic of the species TMGMV was found to be identical in isolate PV-0113 (chapter 8).

TSA plants inoculated with the chimeric construct 30B, which expresses TMGMV coat protein, developed systemic mosaic symptoms, but not necrosis or death, indicating that TMGMV CP alone is not an elicitor of hypersensitive response (HR) in TSA (chapter 9).

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BIOGRAPHICAL SKETCH

Jonathan Robert Horrell was born to Robert and Cynthia Horrell in Fort Lauderdale, Florida. He lived and attended school in Lake Worth, Florida, until 1983. From 1983 until 1989 he lived various places in southwestern New York State, thereafter returning to Lake Worth where he attended high school at Lake Worth Community High School.

Upon graduating high school, Jonathan traveled the country alone, stopping to work occasionally as a handyman or farm laborer. Returning to Lake Worth, he then worked in advertising before deciding to pursue a higher education at the University of Florida.

Working his way through college, Jonathan earned a Bachelor of Science in Plant Pathology in 2002. At the encouragement of his advisor, Dr. Francis William Zettler, Jonathan decided to pursue further education in graduate school, under the mentorship of Dr. Raghavan Charudattan, who kindly provided him with an assistantship.

Applying himself to many projects, his primary research goal has been the investigation of the host-parasite interaction between tropical soda apple and Tobacco mild green mosaic virus, and the identification of the predicted gene-for-gene interaction that takes place.